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Metabolic Effects of Omega-3 Long-Chain Polyunsaturated Fatty Acids

Benjamin B. Albert

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine, The University of Auckland, 2016.
“Let food be thy medicine, and medicine be thy food”

Hippocrates 460-370 BC
Abstract

Obesity and its associated cardiometabolic disorders are among the world’s greatest health problems. However, non-surgical weight-loss treatments are ineffective. Treatments that can reduce cardiometabolic risk in the overweight and obese are urgently needed. Further, as the offspring of obese and/or diabetic women are programmed to become obese and insulin resistant with age, interventions in pregnancy could have substantial long-term impacts on public health. Supplemental omega-3 fatty acids appear promising as they modulate important metabolic pathways and are insulin-sensitising in rats.

The central hypothesis in this thesis is that long chain omega-3 polyunsaturated fatty acids, can modify the effects of gene-environment interactions to improve aspects of glucose and lipid metabolism. Four predictions were tested 1) that an n-3 PUFA supplement would be insulin sensitising in overweight men and 2) that an n-3 PUFA supplement used in a model of insulin resistant pregnancy would have metabolic benefits to the offspring 3) that fish oils sold at retail would have excessive oxidation, and 4) that an oxidised fish oil supplement would not have benefits to the offspring when used during pregnancy.

I used a randomised controlled crossover trial to investigate the metabolic effects of krill/salmon blended oil on insulin sensitivity in overweight men. At baseline, higher n-3 PUFA levels were associated with greater insulin sensitivity. However, supplementation with the marine oil led to reduced insulin sensitivity, suggesting increased cardiometabolic risk.

In an animal model of insulin-resistant pregnancy (maternal high-fat diet supplementation with unoxidized fish oil prevented the development of insulin resistance in the adult offspring.

I found that fish oil products purchased at retail were substantially oxidised and did not meet labelled n-3 PUFA content. I then assessed the effects of oxidised fish oil supplementation during rat pregnancy, and found a substantial increase in neonatal mortality and persistent maternal insulin resistance.

I have shown that oxidation changes the metabolic effects of n-3 PUFA rich supplements. It is possible that there are negative consequences to consuming oxidised fish oil during human pregnancy, and unrecognised oxidation may have confounded the n-3 PUFA clinical trial literature. Whether n-3 PUFAs have insulin-sensitising effects in humans remains unresolved.
Acknowledgements

I would like to express my special thanks to Sir Graeme and Ngaire Douglas and the Douglas Charitable Trust, the Royal Australasian College of Physicians, the benefactor of the Odlin Fellowship, and the Australasian Paediatric Endocrine Care (Pfizer) grants for their generous funding of this research program. I also thank Seadragon Ltd. for provision of freshly extracted frozen fish oil. 51 busy but generous men gave a substantial amount of their time, blood and hair to participate in the clinical trial, for which I am extremely grateful.

I would like to thank my supervisors and advisors, Prof Wayne Cutfield, Prof Paul Hofman, Prof David Cameron-Smith, Prof Mark Vickers and Dr José Derraik, for sharing their time, insights and encouragement at all stages of my research. In particular Wayne Cutfield and Paul Hofman have been most generous, enabling wonderful opportunities, pushing me to the edge of my ambition and giving me just enough rope. I am grateful for José Derraik’s relentlessly optimistic outlook, his statistical support and assistance with writing. I also acknowledge the important contribution of Prof Manohar Garg, who performed all gas chromatographic analyses. I thank Clint Gray, Clare Reynolds and Rachna Patel for substantial technical assistance, Janene Biggs and Christine Brennan for their nursing and organisational contributions during the clinical trial, and Eric Thorstenson, Chris Keven, Greg Smith, James Markworth and Sergey Tumanov for assistance with laboratory work. Yannan Liang provided additional statistical advice. I thank the fellows I have worked alongside, Martin de Bock, Natasha Heather, Ahila Ayyavoo, Valentina Chiavaroli, Sumudu Seniveratne and Tim Savage, for comradery and sharing their ideas and stories.

I would also like to thank my clinical colleagues, A/Prof Craig Jefferies, Prof Al Gunn, Dr Fran Mouat and Dr Philippa Carter who trained me as a paediatric endocrinologist during the period of my PhD research, and made allowances that enabled me to do both.

I am grateful to my family. In particular, I thank my friend and brother Nick, for his enthusiasm and interest. I remember my mother, who incited my desire to know how everything works, and I thank my father for answering all the questions that generated. Most importantly, I thank my wife Olivia for her love and understanding, and for the many sacrifices that she has made to allow me to pursue a career in science.
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<tbody>
<tr>
<td>8-OH-2dG</td>
<td>8-hydroxy-2-deoxy-guanosine</td>
</tr>
<tr>
<td>α-MSH</td>
<td>α-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AV</td>
<td>anisidine value</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AGEs</td>
<td>advanced glycation end-products</td>
</tr>
<tr>
<td>AIR</td>
<td>acute insulin response</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
</tr>
<tr>
<td>AMP-kinase</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>Apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine amino-transferase</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AST</td>
<td>aspartate amino-transferase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BBI</td>
<td>Bang’s blinding index</td>
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<tr>
<td>BP</td>
<td>blood pressure</td>
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<tr>
<td>BW</td>
<td>birth weight</td>
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<tr>
<td>CIMT</td>
<td>carotid intima-media thickness</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
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<tr>
<td>CoA</td>
<td>cofactor A</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CPT-1</td>
<td>carnitine palmitoyltransferase-1</td>
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<tr>
<td>CRP</td>
<td>c-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>DEXA</td>
<td>dual x-ray absorptiometry</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
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<tr>
<td>DI</td>
<td>disposition index</td>
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<tr>
<td>DOHaD</td>
<td>developmental origins of health and disease</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DPA</td>
<td>docosapentaenoic acid</td>
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<tr>
<td>EFSA</td>
<td>European food safety authority</td>
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<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMAS</td>
<td>European Menopause and Andropause Society</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
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<tr>
<td>ET-1</td>
<td>endothelin-1</td>
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<tr>
<td>FAHFs</td>
<td>fatty acid esters of hydroxy-fatty acids</td>
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<td>FFAs</td>
<td>free fatty acids</td>
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<tr>
<td>FSIVGTT</td>
<td>frequently sampled intravenous glucose tolerance test</td>
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<tr>
<td>GIP</td>
<td>gastric inhibitory polypeptide</td>
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<tr>
<td>GLP-1</td>
<td>glucagon like peptide-1</td>
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<tr>
<td>GLUT</td>
<td>glucose transporter</td>
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<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
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<tr>
<td>GPR</td>
<td>g-protein coupled receptor</td>
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<tr>
<td>GSIS</td>
<td>glucose stimulated insulin secretion</td>
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<tr>
<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>GWG</td>
<td>gestational weight gain</td>
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HbA1c  haemoglobin-a1c
HDL  high density lipoprotein
HEC  hyperinsulinaemia euglycaemic clamp
HF  high fat
HFD  high fat diet
HGP  hepatic glucose production
HHE  4-hydroxy-trans-2-hexenal
HNE  4-hydroxy-trans-2-nonenal
HNF-4α  hepatocyte nuclear factor-4α
HOI  highest tertile of omega-3 index
HOMA-β  homeostasis model assessment-β-cell function
HOMA-IR  homeostasis model assessment-insulin resistance
HPA  hypothalamic pituitary adrenal axis
hsCRP  highly sensitive c-reactive protein (assay)
ICAM  intracellular adhesion molecule
IDL  intermediate density lipoprotein
IGF  insulin-like growth factor
IGT  impaired glucose tolerance
IL  interleukin
IPAQ  international physical activity questionnaire
IQ  intelligence quotient
IR  insulin resistance
IRS  insulin receptor substrate
ISI  insulin sensitivity index
IV  intravenous
IVGTT  intravenous glucose tolerance test
KS oil  krill salmon oil
LBW  low birth weight
LDL  low density lipoprotein
LA  linoleic acid
LDH  lactate dehydrogenase
LGA  large for gestational age
LOI  lowest 2 tertiles of omega-3 index
MAP  mitogen-activated protein
MCP-1  macrophage chemotactic protein-1
MI  myocardial infarction
MRI  magnetic resonance imaging
mTOR  mammalian target of rapamycin
MHO  metabolically healthy obese
MONW  metabolically obese normal weight
MPAC  mean parental age at childbirth
n-3  omega-3
n-6  omega-6
NAFLD  non-alcoholic fatty liver disease
NASH  non-alcoholic steatohepatitis
NFκB  nuclear factor kappa B
NO  nitric oxide
NZDep2006  New Zealand deprivation index 2006
OECD  Organisation for Economic Co-operation and Development
OGIS  oral glucose insulin sensitivity
OGTT  oral glucose tolerance test
OXF0  oxidised fish oil
PAI-1  plasminogen activator inhibitor-1
PI(3)K  phosphoinositide 3-kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PUFAs</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PV</td>
<td>peroxide value</td>
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<tr>
<td>QUICKI</td>
<td>quantitative insulin sensitivity check index</td>
</tr>
<tr>
<td>RCT</td>
<td>randomised controlled trial</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RRP</td>
<td>recommended retail price</td>
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<tr>
<td>SGA</td>
<td>small for gestational age</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<td>sterol regulatory element binding protein 1-c</td>
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<td>SNS</td>
<td>sympathetic nervous system</td>
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<td>T1DM</td>
<td>type 1 diabetes mellitus</td>
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<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
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<tr>
<td>TLR-4</td>
<td>toll-like receptor-4</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>Totox</td>
<td>total oxidation</td>
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<tr>
<td>UCP-2</td>
<td>uncoupling protein-2</td>
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<tr>
<td>UnFO</td>
<td>unoxidised fish oil</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>VAT</td>
<td>visceral adipose tissue</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular adhesion molecule</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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</table>
Chapter 4.6. Oxidation of Marine Omega-3 Supplements and human health.
BBA wrote the commentary with input of WSC, DC-S and PLH

Chapter 7. Fish oil supplements in New Zealand are highly oxidised and do not meet label content of n-3 PUFA
BBA, WSC, PLH, DC-S and JGBD conceived and designed the study. BBA performed chemical analyses with assistance from ST and SGVB and compiled the data. MLG took responsibility for GC analysis. JGBD carried out the statistical analyses and compiled the results. BBA, JGBD and WSC wrote the manuscript with input from all other authors.

Chapter 8. Higher omega-3 index is associated with increased insulin sensitivity and more favourable metabolic profile in middle-aged overweight men.
BBA, WSC, PLH, DC-S and JGBD conceived and designed the study. BBA, CMB and JBB recruited and performed the tests, collected and compiled the data. MLG took responsibility for the red blood cell fatty acid analysis. GCS was responsible for the active GLP-1 analysis. JGBD carried out the statistical analyses. BBA, JGBD and WSC wrote the manuscript with input from all other authors.
Chapter 9. Supplementation with a blend of krill and salmon oil is associated with increased metabolic risk in overweight men.

BBA, WSC, PLH, DC-S, and JGBD conceived and designed the study. BBA, CMB, and JBB recruited and performed the tests, collected and compiled the data. MLG took responsibility for the red blood cell fatty acid analysis. JGBD carried out the statistical analyses. BBA and JGBD wrote the manuscript with input from all other authors.

Chapter 10. Oxidised fish oil in rat pregnancy causes high newborn mortality and increases maternal insulin resistance.

BBA, MHV, DC-S, PLH, and WSC conception and design of research; BBA, MHV, CG, CMR, and SAS performed experiments; BBA, JGBD, PAL, and MLG analysed data; BBA, MHV, CG, CMR, SAS, JGBD, PAL, MLG, DC-S, PLH, and WSC interpreted results of experiments; BBA and JGBD prepared figures; BBA, JGBD, and WSC drafted manuscript; BBA, MHV, CG, CMR, SAS, JGBD, PAL, MLG, DC-S, PLH, and WSC edited and revised manuscript; BBA, MHV, CG, CMR, SAS, JGBD, PAL, MLG, DC-S, PLH, and WSC approved final version of manuscript.

Chapter 11. Fish oil supplementation to rats fed a high-fat diet during pregnancy prevents the development of impaired insulin sensitivity in male adult offspring.

BBA, CG, CMR and SAS carried out the animal experiments. MLG was responsible for analysis of the fish oil by gas chromatography. BBA carried out laboratory analysis. BBA and JGBD compiled the data and carried out the statistical analyses. BBA and JGBD wrote the manuscript with input from all other authors.

Chapter 14. Marine oils and health revisited: a highly complex intervention, with a confusing, and potentially confounded trial literature.

BBA wrote this commentary with input of WSC, DC-S, PLH, JGBD and MLG.

Chapter 16. Among overweight middle-aged men, first-borns have lower insulin sensitivity than second-borns.

BBA, WSC, MdB, PLH and JGBD conceived and designed the study. BBA, MdB, CB and JB recruited and performed the tests. BBA and MdB collected and compiled the data. JGBD carried out the statistical analyses. BBA, JGBD and WSC wrote the manuscript with input from other authors.
Chapter 17. Increasing parental age at childbirth is associated with greater insulin sensitivity and more favourable metabolic profile in overweight adult male offspring.

BBA, WSC, MdB, PLH and JGBD conceived and designed the study. BBA, MdB, CB and JB recruited and performed the tests. BBA and MdB collected and compiled the data. JGBD carried out the statistical analyses. BBA, JGBD and WSC wrote the manuscript with input from other authors.


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Chapter 4.6 is based on the following publication:

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<th>Nature of contribution by PhD candidate</th>
<th>The outline of this review was conceived by myself with Professor Cutfield, I performed the literature review and wrote the manuscript.</th>
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</thead>
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<tr>
<td>Extent of contribution by PhD candidate (%)</td>
<td>90</td>
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**CO-AUTHORS**

<table>
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<th>Nature of Contribution</th>
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<tbody>
<tr>
<td>Professor David Cameron-Smith</td>
<td>Assisted with writing of the manuscript</td>
</tr>
<tr>
<td>Professor Paul Hofman</td>
<td>Assisted with writing of the manuscript</td>
</tr>
<tr>
<td>Professor Wayne Cutfield</td>
<td>Conceived of this commentary, and assisted with writing the manuscript</td>
</tr>
</tbody>
</table>

**Certification by Co-Authors**

The undersigned hereby certify that:
 Saúde the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
 Saúde the candidate wrote all or the majority of the text.

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Chapter 7 is based on the following publication:

Nature of contribution by PhD candidate: I conceived of the study, planned it purchased the products and carried out the analysis of markers of oxidation, with assistance from coauthors I wrote the manuscript.

Extent of contribution by PhD candidate (%) 90

**CO-AUTHORS**

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<tr>
<td>Professor David Cameron-Smith</td>
<td>Assisted with the writing of the manuscript</td>
</tr>
<tr>
<td>and Professor Paul Hofman</td>
<td></td>
</tr>
<tr>
<td>Professor Wayne Cutfield</td>
<td>Supervised this study, with a major role in its conception, planning and writing</td>
</tr>
<tr>
<td>Professor Manohar Garg</td>
<td>Performed analysis of fatty acid content using gas chromatography</td>
</tr>
<tr>
<td>Dr. José Derral</td>
<td>Performed the statistical analysis and assisted with writing the manuscript</td>
</tr>
<tr>
<td>Associate Professor Silas Vilas-Boas</td>
<td>Assisted in the conception and planning of this trial</td>
</tr>
<tr>
<td>Sergey Tumanov</td>
<td>Assisted in the planning of this trial and with learning appropriate the laboratory techniques</td>
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**Chapter 8 is based on the following publication:**

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<th>I conceived of the study, planned it and carried it out. With assistance from coauthors I decided to publish the baseline finding separately, and wrote the manuscript.</th>
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<tr>
<td>Professor David Cameron-Smith and Professor Paul Hofman</td>
<td>Assisted with the conception of the study and writing of the manuscript</td>
</tr>
<tr>
<td>Professor Wayne Cutfield</td>
<td>Conceived of the clinical trial, assisted with planning, supervision and writing</td>
</tr>
<tr>
<td>Professor Manohar Garg</td>
<td>Performed analysis of red cell phospholipid using gas chromatography</td>
</tr>
<tr>
<td>Dr. José Derrall</td>
<td>Performed the statistical analysis and assisted with writing the manuscript</td>
</tr>
<tr>
<td>Christine Brennan and Janene Biggs</td>
<td>Provided valuable organisational and clinical/technical support during both clinical trials</td>
</tr>
<tr>
<td>Dr. Greg Smith</td>
<td>Carried out assays of GLP-1 in plasma</td>
</tr>
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</table>

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<td>Janene Biggs</td>
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Chapter 9 is based on the following publication:

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**Chapter 10 is based on the following publication:**

Oxidised fish oil in rat pregnancy causes high newborn mortality and increases maternal insulin resistance

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<td>I conceived the potential for n-3 PUFAs to have a harmful effect, or lack of effect in pregnancy, and designed and carried out the trial with assistance from co-authors. I wrote the manuscript with input from co-authors.</td>
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<td>Professor David Cameron-Smith and Professor Paul Hofman</td>
<td>Assisted with writing the manuscript</td>
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<tr>
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<td>Held a major role in planning the study, assisted with animal manipulations and writing the manuscript</td>
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<tr>
<td>Dr Clint Gray, Dr Clare Reynolds, and Stephanie Sagović</td>
<td>Assisted with animal manipulations and collecting samples and measurements and writing the manuscript</td>
</tr>
<tr>
<td>Professor Wayne Cutfield</td>
<td>Assisted with the conception of this trial and writing the manuscript</td>
</tr>
<tr>
<td>Dr Josiah Derraik</td>
<td>Carried out the statistical analysis and assisted with writing the manuscript</td>
</tr>
<tr>
<td>Professor Manohar Garg</td>
<td>Measured n-3 PUFA composition of the trial oils and assisted with writing the manuscript</td>
</tr>
<tr>
<td>Professor Paul Lewandowski</td>
<td>Measured markers of redox status and assisted with writing the manuscript</td>
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**Chapter 11 is based on the following publication which is under review:**

Fish oil supplementation to rats fed a high-fat diet during pregnancy prevents the development of impaired insulin sensitivity in male offspring

| Nature of contribution by PhD candidate | **I conceived of the potential for n-3 PUFAs to prevent programming, and designed and carried out the trial with assistance from coauthors. I wrote the manuscript with input from coauthors.** |
| Extent of contribution by PhD candidate (%) | 70 |

## CO-AUTHORS

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<td>I was invited to submit a commentary into a special issue of this journal. I conceived the arguments in this commentary and wrote the manuscript</td>
<td>90</td>
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Introduction
Chapter 1. The obesity epidemic and strategies for preventing cardiometabolic disease

1.1. Obesity a new problem for an old species

The human species has seen a change in life course and environment that is unprecedented in other species. Evolved as hunter-gatherers of the African savannah, we find ourselves in an industrialised, sedentary and food rich world, with hygiene, sanitation and increasingly effective health treatments. As a result life expectancy for a new born child has increased dramatically, from an estimate in the 20’s in prehistory and the middle ages, around 38 by 1750 (Northern Europe), 48 in 1900 (US) and 78.5 in 2010 (US). However, while the health of nations has clearly increased there has also been a dramatic increase in the incidence of obesity and overweight worldwide and this has been predicted to halt further increases of life expectancy during the 21st century.

The rise of obesity is primarily due to an increasingly obesogenic environment characterised by greater availability of foods high in energy and low in nutrition, in combination with the adoption of a more sedentary lifestyle. As the rate of environmental change has far outpaced the slow process of evolution our previously physically active hunter-gatherer species finds itself genetically mismatched to its environment. “Thrifty genes”, genetic variants that improved survival when physical energy expenditure was high, food availability was intermittent, and the diet consisted of lean meat and unrefined plant material are obesogenic in contemporary life, where near limitless calories are available in energy dense, low nutrient foods. Such maladaptations include preference for energy dense foods, rich in sugar and fat, and the efficient storage of excess energy in adipose tissue. As obesity has never been a normal part of the human life course, we would not expect adaptations to prevent its negative effects on health.

In addition to the external environment, it is now well established that the intrauterine environment has long term consequences for metabolic health and adiposity of the offspring. In particular, babies born to obese or diabetic women are at increased risk. As obesity has increased in women of child-bearing age, this effect serves to perpetuate obesity in the next generation.

Aside from societal changes in diet and physical activity, and the special role of maternal obesity there are many other aspects that influence metabolism and obesity, such as genetic variants, gut hormones, bowel flora,
Introduction

knowledge about healthy lifestyles, socioeconomic status, enjoyment of exercise and psychological factors. These are important but are not the main cause of the obesity epidemic.

1.2. Obesity is a global problem

Obesity is a disease state characterised by excess body fat, that is associated with diabetes and cardiovascular disease. It is usually defined by body mass index, calculated from height and weight (BMI = weight/[height]²; kg/m²), a definition that has limitations (Section 2.3.1). In adults a BMI between 25-30 kg/m² is considered overweight, while a BMI ≥ 30 indicates obesity. However, these cut-offs are based on health outcome data of Europeans, and underestimate risk in some ethnicities such as South Asians.

There has been an upwards shift across the spectrum of body mass index globally. The largest, most comprehensive systematic review, showed that over a 33 year period the age-standardised global prevalence of overweight or obesity (body mass index (BMI) ≥25) in males rose from 29% to 37% (females; 30% to 38%). There are now more than 500 million obese (BMI ≥30) individuals globally, and the rise affects all age groups including children. In developing countries the prevalence of obesity is 50% lower, but is steadily rising and there are now 7 countries where more than half the male or female adult population is obese. In NZ the majority of men and women are now overweight or obese (Men: 43% overweight, 28% obese; Women: 30% overweight, 30% obese) with rates similar to Australia and the US. Maori and Pacific People are overrepresented (48% and 68% respectively are obese), as are people of lower socioeconomic status (independent of ethnicity).

Obesity increases the risk of many important non-communicable diseases such as osteoarthritis, back pain, obstructive sleep apnoea and obesity-hypoventilation syndrome, which are largely due to the mechanical effects of increased weight. However the greatest morbidity, occurs through the effects of obesity on glucose and lipid metabolism; effects that are mediated by a pathological reduction in sensitivity to the peptide hormone insulin (insulin resistance). Insulin resistance underlies the components of the metabolic syndrome: dyslipidaemia, hypertension, dysglycaemia, and thus the key pathophysiological process underlying cardiovascular disease, and the complications of type 2 diabetes (T2DM). It is insulin resistance and the metabolic syndrome, not body mass index per se that increases the risk of cardiovascular disease in the obese.
Introduction

1.3. Approaches for preventing disease in the overweight and obese

There are several potential approaches to reduce the morbidity and mortality caused by obesity. These can be divided into those focussed on preventing obesity, those focussed on achieving weight-loss in the obese, and those aimed at preventing (or treating) metabolic disease in the obese.

1.3.1 Preventing Obesity

There are two major avenues to preventing obesity; those that focus on behavioural change through education about a healthy lifestyle, and higher level regulatory approaches to reducing the obesogenicity of the environment. To date, health promotion about obesity has primarily focussed on education about eating and exercise, whether in schools, media or the doctor’s office\textsuperscript{32,33}. It is evident that this has not been especially effective, and there are calls for the use of regulatory approaches\textsuperscript{32}. Such regulation would include controlling the price, advertising or packaging of foods to impel healthier choices (such as the sugar tax to be introduced into the UK\textsuperscript{34}), creating public exercise venues such as parks or pools or designing cities that better permit walking or cycling. Lastly, as maternal obesity during pregnancy can lead to obesity and metabolic disease in the offspring\textsuperscript{20}, interventions that improve the health of women of fertile age, could help to prevent obesity in later generations.

1.3.2 Aiding the obese to lose weight

Similar to the approaches for prevention of obesity, education and regulation could aid the obese to lose weight. In addition, specific treatments could be offered such as provision of dietetic advice, psychological support, enrolment in exercise programs, use of medications or nutraceuticals, or bariatric surgery. When considering weight-loss treatments a 5% or greater weight-loss is usually considered clinically meaningful\textsuperscript{35,36}, as it leads to significant metabolic improvement\textsuperscript{37,38}. This typically equates to 4-6kg in the weight range of participants of typical medical studies, but a greater mass in the severely obese.

Lifestyle advice and support programs

Weight-loss programs based on increasing exercise, and reducing dietary energy or both, appear to be moderately effective, leading to weight of loss of between 5 and 10kg\textsuperscript{39,41}. However 50% of lost weight is typically regained within 1-2 years. Further, programs that are effective are intensive with sufficient supervision to allow quantification of exercise\textsuperscript{41}. Simply providing advice, is ineffective\textsuperscript{41}. 
Introduction

It is likely that the efficacy of these interventions is overestimated because of the Hawthorne effect: the improvements seen when participants know they are being observed as part of a study. This effect has been clearly demonstrated in settings where behaviour change is sought. In clinical practice, only a small proportion of patients advised to modify their diet and physical activity, achieve long term weight-loss. For example, only one in six overweight or obese Americans surveyed had achieved and maintained a 10% weight-loss for 12 months at any time in their lives. Weight-loss, particularly due to caloric restriction, is countered by powerful compensatory responses that resist a reduction in fat mass, leading to increased hunger, decreased satiety and the seeking out of energy dense, sweet and fatty food. Thus some degree of “study like” supervision may be necessary to aid the patient to resist these factors, and make lifestyle advice effective. Advice with such support underlies the “green prescription”, but although it increases exercise, it has no effect on BMI. Thus lifestyle programs and advice, even with good support, appears to be only modestly effective and no effect can be expected in the unmotivated.

Medications for weight-loss

Treatment with medications and nutraceuticals appear seductive. A drug or micronutrient that could induce weight-loss, without changing lifestyle, would be transformative. The public’s desire for such a treatment is clear; in an American survey, 39% of those that had attempted to lose weight, had used a non-prescription weight-loss pill while the market for obesity pharmaceuticals was 359 million dollars in 2011.

Currently available medications that induce weight-loss include orlistat, lorcaserin and a combination of phentermine and topirimate which lead to weight-loss in excess of placebo of 3%, 3% and 9% of body weight respectively when given in addition to lifestyle interventions. Metformin, an insulin sensitising agent used in T2DM leads to modest weight-loss (2%) . Liraglutide is a GLP-1 agonist that must be injected daily. In meta-analysis it lead to a 3% greater weight-loss than control over 20 weeks, and 6% over 2 years. However, many patients have little or no benefit from these drugs; 30-60% of patients fail to lose 5% of their body weight.

Obesity medications have important adverse effects, which limit their tolerability. For example orlistat causes fat malabsorption, with significant gastrointestinal adverse effects including faecal incontinence (7%). These are so troublesome that less than 10% of patients continue this drug for a year. Further, when drugs are used by large populations, uncommon but severe adverse effects emerge. Two moderately effective anti-obesity drugs have been withdrawn; sibutramine increased the rate of nonfatal myocardial infarction and stroke, while...
rimonabant induced psychiatric disease\textsuperscript{56}. The results of a post-marketing study of lorcaserin are not yet available\textsuperscript{57}, while topirimate is teratogenic\textsuperscript{58} and if it was widely used in women of childbearing age, some unplanned pregnancies would be affected.

Most anti-obesity medications are expensive, so that wide use in the obese population would put a substantial financial burden on public health services and private medical insurance. Further, where patients must pay personally, this excludes people of lower socio-economic status that are over-represented in the obese population. Given these issues it is doubtful that the obesity epidemic can be adequately treated with medications.

\textit{Nutraceuticals for weight-loss}

Nutritional products that are intended to have beneficial health effects, such as supplements and herbal treatments are highly acceptable to the population and widely used\textsuperscript{59}. However, in a systematic review of the 12 most studied weight-loss supplements, none were both safe and effective\textsuperscript{60}. Supplements containing ephedrine, have been linked to severe cerebrovascular and cardiovascular effects including death\textsuperscript{61,62}. A recent systematic review concluded that a range of herbal drugs, were effective at causing weight-loss\textsuperscript{63}. However, these conclusions were unreasonable as weight-loss was trivial or not different from placebo, and the severe adverse effects of ephedrine were ignored\textsuperscript{63}. Current evidence suggests no natural or herbal supplement is both safe and effective when used to aid weight-loss.

\textit{Bariatric Surgery}

Bariatric surgery encompasses procedures that modify the gut to restrict the capacity of the stomach, divert the flow of chyme (gut contents), or both. These techniques were conceived with the intention to cause early satiety (restrictive procedures), or malabsorption (divertive procedures), however these are not the mechanisms by which bariatric surgery has its effect\textsuperscript{45}. Divertive procedures such as roux-en-Y gastric bypass appear to work primarily by reducing the transit time of food into the small bowel, with subsequent effects on the secretion of gut hormones, the circulation of bile salts and the bowel microbiota\textsuperscript{45}. Restrictive procedures such as gastric banding appear to work by increasing intraluminal pressure, which is detected through the vagus nerve\textsuperscript{45}. Procedures such as the sleeve gastrectomy, may have a combination of these effects. Importantly, unlike calorie restrictive diets, which are accompanied by powerful effects on appetite, with craving of high energy food, bariatric surgery is associated with reduced appetite\textsuperscript{45}.
Introduction

Bariatric surgery is extraordinarily effective in the severely obese (BMI>40kg/m$^2$). Systematic reviews of observational studies (n=158000) and RCTs (n=3385) indicate that mean weight-loss is greater than 50% of excess body weight, reducing BMI by >11kg/m$^2$, with greater weight-loss after diversionary procedures$^{64,65}$. Weight-loss is maintained 10-15 years after$^{66}$.

Bariatric surgery also has substantial metabolic benefits, reducing blood pressure (BP), fasting glucose and insulin, HOMA-IR (an index of insulin resistance), LDL-cholesterol and triglycerides, and increasing HDL-cholesterol$^{67}$. In the largest and most recent meta-analysis, diabetes, hypertension and dyslipidaemia resolved in 92%, 75% and 76% respectively$^{65}$. Where diabetes does not resolve, glycaemic control is improved$^{68}$. This leads to a greater than 50% reduction in myocardial infarction, stroke and overall mortality$^{69}$. Gastric bypass is more effective than banding for resolution of diabetes (95% vs 74%), hypertension (81% vs 54%) and dyslipidaemia (80% vs 40%)$^{65}$.

There is little head to head data comparing bariatric surgery to non-surgical treatment, but no medication, nutraceutical or exercise program has comparable results. In a study of mild obesity, bariatric surgery had greater effects on weight-loss and metabolism than a comprehensive medical program including low calorie diet, orlistat, encouragement and exercise advice$^{70}$. This indicates that surgery is more effective than the best non-surgical approach. Bariatric surgery is cost-effective$^{71}$, and while there are risks, with complications in 17% and reoperation in 5%, mortality is low (<1%), so that the benefits far outweigh the risks of severe obesity$^{65}$.

Can the obesity epidemic be treated surgically?

Bariatric surgery is clearly superior to medical and lifestyle intervention, but this treatment cannot be used to address obesity at a population level. The obese population is enormous, affecting 1 in 3 adults globally$^{3}$ and public health systems lack the capacity to provide surgery to all who may benefit. Currently, less than 1% of eligible patients in Canada, the US and the UK receive a bariatric procedure, and in Canada, waiting lists average 5 years$^{71}$. Further ethnic minorities and people of lower socioeconomic status are less likely to receive surgery$^{71}$. Lastly, even if surgery was available, many people would refuse it, have unjustifiably high intraoperative risk or could be predicted in advance to fail.
Introduction

1.3.3 Improving metabolic health in the overweight and obese

As there is no effective weight-loss treatment for obesity that can be applied at a population level, there remains a need for acceptable treatments that can improve the cardio-metabolic health of people who are overweight or obese. Such treatments could potentially include medications or nutraceuticals, and would be complementary to approaches such as education and regulatory change. The central hypothesis of this thesis is that supplemental omega-3 polyunsaturated fatty acids (n-3 PUFAs) may improve metabolic health in the overweight or obese by increasing insulin sensitivity.
Introduction

Chapter 2. Insulin resistance: the key pathological process behind a global epidemic of obesity related non-communicable disease

The chapter is a comprehensive description of the role of insulin in glucose and lipid metabolism, the development of insulin resistance and the processes which link insulin resistance to the major components of the metabolic syndrome. Together this indicates the crucial role of adipose tissue in the metabolic effects of obesity, and the potential for supplemental omega-3 polyunsaturated fatty acids (n-3 PUFAs) to ameliorate metabolic dysfunction in the obese.

2.1. Obesity causes metabolic dysfunction

The metabolic syndrome is the association of dyslipidaemia, hypertension, dysglycaemia, abdominal adiposity, hyperuricaemia, dysovulation (polycystic ovarian syndrome) and a proinflammatory and prothrombotic state, which is predominantly seen in those who have abdominal obesity. While various diagnostic criteria have been proposed, the most recent, multi-organisational consensus statement defines metabolic syndrome when a subject has three out of five criteria: elevated waist circumference, elevated triglycerides, reduced HDL-cholesterol, elevated BP and elevated fasting glucose. There is debate over whether a diagnosis of metabolic syndrome is clinically useful or carries greater cardiovascular risk than the diagnosis of its individual components. Nevertheless, metabolic syndrome is a useful term, because each of these components have a common underlying cause; pathological reduction of the sensitivity of metabolic tissues to the peptide hormone insulin: insulin resistance. In addition, insulin resistance appears to have a role in the development of some cancers which are more common in the obese. This raises the possibility that interventions that improve insulin sensitivity might reduce the risk of T2DM, cardiovascular disease and malignancy in the overweight and obese. Such interventions could have an important impact on the global burden of non-communicable disease.

2.2. The physiological role of insulin

The plasma glucose concentration is tightly regulated in health because of the acute and chronic impairments of function that occur with hypo- and hyper-glycaemia. Glucose homeostasis must be maintained in the face of major perturbations such as consumption of carbohydrate rich meals, fasting, illness,
stress and exercise. Insulin, a polypeptide secreted by β-cells in the islets of Langerhans of the pancreas is the primary hormone that regulates plasma glucose, but it also affects a wide range of physiological systems, including cell growth and division, appetite, lipid metabolism, vascular function and BP. Thus the derangement of insulin function that occurs in obesity has wide negative consequences.

2.2.1 Regulation of circulating energy carrying molecules in the fed and starved state

Following a meal, there is absorption of glucose, amino acids and some fats (medium chain triglycerides) from the gut into the portal and then systemic circulation. Most fats are incorporated into chylomicrons, which pass through the lymphatic system to enter the systemic circulation through the lymphatic duct. Excess glucose and fats must be taken up into tissues responsible for their storage. The primary roles of insulin are to induce the uptake of glucose into liver, muscle and adipose tissue. This glucose is then used as a substrate for glycogen production in liver and muscle, and also de novo fatty acid synthesis by liver. In adipose tissue it is converted to glycerol which is necessary for the production of triglycerides.

In the starved state, insulin secretion is suppressed; glucagon (and in hypoglycaemia, catecholamines) induce the release of glucose (and ketone bodies) from the liver, while catecholamines also increase the rate of free fatty acid release from adipocytes. The effects of insulin are modulated by many hormones, including growth hormone, cortisol, sex steroids and catecholamines which reduce the effectiveness of insulin.

2.2.2 Regulation of Insulin Secretion

Insulin secretion is tightly controlled by the β-cells’ glucose sensing mechanism which transduces the extracellular glucose concentration into biophysical changes in the cell which trigger insulin secretion. This primary mechanism is then modulated by other metabolites, hormones and medications.

The glucose sensor – linking extracellular glucose to insulin secretion

When the plasma glucose is less than 5mmol/l, it does not stimulate secretion of insulin. However concentrations greater than 5mmol/l lead to graded increases in insulin secretion. Glucose enters the β-cell passively through the GLUT-2 membrane channel which is constitutively expressed and has a high capacity for glucose transport. This rapidly equalises extracellular and intracellular glucose concentration. Glucose is metabolised to produce ATP, thus the ratio of ATP and ADP scales with the glucose concentration. The
resting membrane potential of the β-cell is approximately -70mv and is maintained by the ATP sensitive potassium channel, (a tetramer of kir6.2, and SUR1 subunits). Kir6.2 is open when the ATP concentration is low, but closes in response to an increase in the ATP/ADP ratio\textsuperscript{101}. Thus an increase in glucose concentration, leads to closure of the kir6.2 channel and depolarisation of the β-cell\textsuperscript{101}. This opens voltage dependent calcium channels in the cell membrane, and calcium influx triggers exocytosis of preformed insulin\textsuperscript{101}.

**Modulators of insulin secretion**

Many factors modulate insulin secretion including dietary protein and fat, circulating fatty acids and gut hormones. Medications\textsuperscript{104-106} and genetic mutations\textsuperscript{107-110} that disrupt the glucose sensor have more dramatic effects. Any cause of impaired insulin action (insulin resistance), will also lead to an increase in insulin secretion, but this is discussed separately.

The incretins are hormones secreted by enteroendocrine cells of the gut that bind to transmembrane receptors of the β-cell increasing glucose stimulated insulin secretion (GSIS)\textsuperscript{111,112}. Gastrointestinal insulinogenic peptide (GIP) is produced by K cells in the duodenum and proximal jejunum\textsuperscript{113} in response to detecting absorption of glucose and fat\textsuperscript{114}. Glucagon like peptide-1 (GLP1) is secreted by L cells of the terminal ileum\textsuperscript{113} predominantly in response to detection of glucose, fat and essential amino acids\textsuperscript{114}. These hormones increase GSIS enabling rapid increases of insulin secretion in response to the imminent absorption of nutrients\textsuperscript{111,112,115}. They also increase satiety\textsuperscript{116} and slow gastric emptying\textsuperscript{117}.

Insulin secretion increases in response to protein consumption\textsuperscript{118}. This is partly mediated by incretins\textsuperscript{115}, but the amino acid leucine also has a direct effect on the β-cell. Leucine increases activity of the enzyme glutamate dehydrogenase, which shuttles glutamate into the citric acid cycle, increasing the ATP/ADP ratio, which increases insulin secretion\textsuperscript{119}.

Dietary fats increase insulin secretion through two mechanisms, both of which rely on G-protein coupled cell receptors that are activated by fatty acids. GPR-40 is activated by a range of unsaturated fatty acids including n-3 and n-6 PUFAs, oleic acid and less strongly to saturated fatty acids\textsuperscript{120}. GPR-120 is more specific, as it is primarily activated by n-3 PUFAs\textsuperscript{121}. These receptors form part of the nutrient detecting mechanism of gut enteroendocrine cells; activation of either receptor increases GLP-1 secretion\textsuperscript{122,123}, while GPR-40 also increases GIP release\textsuperscript{123}. These incretins increase GSIS. GPR-40 and GPR-120 are also expressed by β-cells and activation triggers calcium influx, directly increasing insulin secretion\textsuperscript{124-126}. These two processes link fat
Introduction

Consumption and absorption to increased insulin secretion, which increases storage and production of triglyceride, and reduces release of FFAs from adipose tissue.

In summary, insulin secretion is primarily determined by the plasma glucose concentration, but amino acids, fatty acids and gut hormones also act to modulate the insulin response to a meal.

First and Second phase insulin secretion

Insulin secretion is divided into two phases based on the response to fixing the plasma glucose at an elevated level by intravenous administration of glucose (hyperglycaemic clamp). This bypasses the gut and incretin system, demonstrating the response of β-cells response to the direct detection of hyperglycaemia. There is an initial peak of insulin secretion in the first 10 minutes (first phase), followed by a gradual increase of plasma insulin over the next 2 hours (second phase). Loss of the first phase insulin response has been considered the first detectable abnormality in people destined to develop T2DM, however both phases are equally predictive of incident T2DM.

2.2.3 Insulin signalling and the physiological effects of insulin

Insulin is primarily an anabolic hormone of the fed state, facilitating the uptake of glucose into skeletal muscle and adipocytes, its storage as glycogen (muscle and liver) or conversion and storage as triglyceride (adipocytes). It also induces uptake of triglyceride from circulating lipoproteins into adipocytes for storage. Importantly, it inhibits the catabolic metabolism of the starved state, including liver glycogenolysis, gluconeogenesis, and ketone body production, and the release of FFAs from adipocytes. Insulin also has a direct role reducing appetite, through insulin receptors in the hypothalamus. Lastly, insulin has important mitogenic effects, potentiating the effects of growth factors: IGF-1, endothelial growth factor, platelet derived growth factor and vascular endothelial growth factor.

These effects of insulin are mediated through the insulin receptor which belongs to the tyrosine kinase family of transmembrane receptors. Two α-chains act as the receptor for insulin on the extracellular surface, and two β-chains have tyrosine kinase activity on the intracellular surface. At rest the tyrosine kinase is repressed by the α-chain. Binding of insulin causes a conformational change that leads to loss of repression of the tyrosine kinase setting off the intracellular second messenger systems that mediate the effects of insulin.
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There are nine different substrates for the insulin receptor’s tyrosine kinase, including insulin receptor substrates (IRS)-1 through to 4 which are homologous and have complimentary roles\textsuperscript{82,131}. There are differences in the tissue localization and activity of these proteins\textsuperscript{82}. The insulin receptor tyrosine kinase activates the IRS proteins by phosphorylation leading to complex cascades of phosphorylation which ultimately lead to the cellular response to insulin. The enzyme PI(3)K is a crucial part of this cascade as it influences a variety of intracellular signalling molecules\textsuperscript{132} and suppression of this enzyme blocks most of the effects of insulin\textsuperscript{82}.

Insulin signalling is inhibited by serine phosphorylation of the insulin receptor tyrosine kinase and the IRS proteins, which reduces their activity\textsuperscript{82}. The enzymes glycogen synthase kinase-3 and mammalian target of rapamycin (mTOR) are known to have this action\textsuperscript{82}. Importantly, cytokines such as TNF-α\textsuperscript{133}, and the accumulation of intracellular lipid\textsuperscript{134,135} also lead to serine phosphorylation of IRS proteins reducing the intracellular response to insulin binding to its receptor. In addition, protein tyrosine phosphatases may inactivate the insulin receptor and IRS proteins by removing (dephosphorylating) tyrosine residues\textsuperscript{82}. These mechanisms are the major factors that mediate the insulin resistance of obesity\textsuperscript{135}.

**Insulin increases glucose uptake into skeletal muscle and adipose tissue**

A major function of insulin is to regulate glucose uptake into skeletal muscle and adipose tissue\textsuperscript{82}. At rest GLUT-4, the insulin responsive glucose transporter is found in intracellular vesicles which slowly cycle from intracellular stores to the cell membrane by exocytosis. Insulin signalling increases the rate of exocytosis, and decreases the rate of endocytosis\textsuperscript{136}. Thus it increases the number of glucose transporters in the cell membrane, and the rate of passive glucose uptake into these tissues\textsuperscript{82,136}, lowering the plasma glucose. Glucose influx into other cells is not regulated, as other glucose transporters (GLUT-1, GLUT-2, GLUT-3, GLUT-5) are constitutively expressed\textsuperscript{137}. This reflects the constant need for glucose to support cellular metabolism. Although the regulated uptake through GLUT-4 makes up only 15-25\% of uptake\textsuperscript{138}, this is the major regulator of the post-prandial glucose concentration\textsuperscript{82}.

**Insulin increases storage of lipid in adipose tissue and inhibits release of fatty acids**

In addition to increasing glucose uptake into adipose tissue, insulin induces lipid synthetic enzymes which convert glucose to triglyceride, and facilitates uptake of lipid from circulating lipoproteins (through inhibiting post-translational inactivation of lipoprotein lipase, thereby increasing its activity)\textsuperscript{139}. Insulin also inhibits
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lipolysis of triglyceride and release of fatty acids into the circulation. Thus the effect of insulin on adipose tissue is to drive uptake of glucose and triglyceride, where it is stored, and prevent the release of FFAs. Resistance of adipose tissue to insulin, with subsequent inappropriate release of fatty acids, is one of the most important pathological events that connect obesity to metabolic dysfunction, T2DM and CVD.

Insulin directly and indirectly inhibits hepatic glucose production

Insulin has direct and indirect effects on the liver which serve to inhibit gluconeogenesis and glycogenolysis and reduce the release of glucose into circulation. These effects are mediated not only by the insulin receptors of hepatocytes, but also through effects on visceral adipose tissue (VAT); the intra-abdominal fat drained by the portal vein. VAT is relatively resistant to insulin (compared to subcutaneous fat) so that there is a constant release of FFAs into the portal vein. Portal FFAs induce hepatic insulin resistance and stimulate hepatic glucose production. Following a meal, the increased insulin concentration serves to reduce FFAs in the portal vein. It appears that up to 75% of the effect of insulin on hepatic glucose production is mediated by this reduction in portal free fatty acid concentration, leaving a much smaller role for direct effects via hepatic insulin receptors. This underscores the importance of the VAT to metabolic health.

2.3. The pathogenesis of insulin resistance in obesity

The mechanisms linking obesity to metabolic dysfunction are increasingly well understood. Dysfunction of adipose tissue appears to be the cornerstone defect, suggesting that the adipocyte may be the best target for metabolic interventions.

2.3.1 Obesity, but not obesity alone

The role of obesity in the development of diabetes and cardiovascular disease is long recognized and there is no doubt that insulin resistance is correlated with BMI. However, two anomalous groups show the limitations of BMI. Firstly, the metabolically healthy obese (MHO), who have normal metabolic markers and make up 15-45% of the obese population. Secondly, the metabolically obese normal weight (MONW), who have metabolic dysfunction despite normal BMI, and make up 9-24% of the normal weight population.

BMI does not account for body composition and metabolic risk is better correlated with direct measures of adiposity. In addition, a great many other factors also influence metabolic health, including the distribution
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of body fat\textsuperscript{150}, the balance between adipocyte hypertrophy and hyperplasia\textsuperscript{151}, genotype\textsuperscript{152-154}, perinatal factors\textsuperscript{155-157}, physical activity\textsuperscript{158,159}, diet\textsuperscript{160-163}, age\textsuperscript{164}, sex\textsuperscript{165}, and the hormonal milieu\textsuperscript{97-99}.

Distribution of body fat

There are two archetypal fat distributions, android (typical in men); characterised by abdominal adiposity with relatively little fat in the buttocks or limbs, and gynoid (typical in women) where the abdomen is spared with greatest adiposity over the buttocks and thighs\textsuperscript{166}. Multiple lines of evidence indicate that the distribution of body fat is a major factor determining cardiometabolic disease and that the intra-peritoneal (visceral fat) confers the greatest risk\textsuperscript{150,167}:

- In cross-sectional studies, abdominal adiposity (skin fold thickness, waist circumference or waist:hip ratio) is associated with metabolic dysfunction including greater BP\textsuperscript{168-171}, triglycerides\textsuperscript{168-171}, fasting glucose\textsuperscript{168-171} and insulin\textsuperscript{170,171}, and greater glucose and insulin excursions following a glucose load\textsuperscript{170,171}.
- Longitudinal studies\textsuperscript{168,169,172} and a meta-regression\textsuperscript{173} indicate that measures of central adiposity are associated with greater incidence of T2DM\textsuperscript{172}, ischaemic heart disease\textsuperscript{168,169,172} and stroke\textsuperscript{168,172}, independent of BMI\textsuperscript{168,169,172}. Central adiposity is a stronger predictor of CVD than BMI\textsuperscript{174}.
- Following menopause or oophorectomy, females have a redistribution of body fat, increasing central adiposity\textsuperscript{166}. This correlates with the increase in CV and metabolic risk following menopause\textsuperscript{175,176}.
- Most studies using CT or MRI to measure specific fat depots show that it is intra-abdominal, not subcutaneous fat that is independently associated with insulin resistance, glucose intolerance, cardiovascular risk factors\textsuperscript{177-180}, and the long term incidence of T2DM and coronary heart disease\textsuperscript{181,182} (with a few exceptions\textsuperscript{183-185}).
- Subcutaneous liposuction reduces waist circumference but does not affect metabolism\textsuperscript{186}.
- Thiazolidinediones increase subcutaneous fat, yet improve insulin sensitivity and other metabolic markers\textsuperscript{150,187}.
- In Prader-Willi syndrome there is severe obesity, but normal visceral fat mass, and metabolic complications are uncommon\textsuperscript{188}.
- After weight-loss, changes in the visceral fat mass correlate best with improvements in metabolism\textsuperscript{189}.
- Where the intraperitoneal and retroperitoneal fat depots have been compared, the retroperitoneal fat mass has either not been associated with metabolic markers\textsuperscript{185}, or is more weakly associated than the intraperitoneal fat mass\textsuperscript{190}.

Thus there is substantial evidence that the intraperitoneal fat has a special role in metabolic dysfunction.
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2.3.2 Metabolic differences between visceral and subcutaneous adipose tissue

The primary metabolic functions of white adipose tissue are to take up triglycerides and glucose in the fed state and store them as fat, and in the starved state, hydrolyse triglyceride and release FFAs into the circulation as an energy source. Visceral fat has a greater rate of lipolysis and release of FFAs\textsuperscript{167,191}, and is more sensitive to many hormones including, catecholamines\textsuperscript{192,193}, glucocorticoids\textsuperscript{194,195}, testosterone\textsuperscript{196} and growth hormone\textsuperscript{167,197}, but is less sensitive to oestrogen\textsuperscript{198,199}. These differences underlie the central obesity seen in glucocorticoid excess\textsuperscript{194,195} and growth hormone deficiency\textsuperscript{167,197}. In men over 50\textsuperscript{198,200}, and post-menopausal women\textsuperscript{166} there is a redistribution of body fat to VAT due to reducing levels of testosterone and oestrogen.

Adipose tissue is also an endocrine organ\textsuperscript{198,201}. Adipocytes secrete inflammatory cytokines such as interleukin-6 (IL-6) and TNF-α, macrophage chemotactic protein-1 (MCP-1), angiotensinogen, plasminogen activator inhibitor-1 (PAI-1) and the adipokines; adiponectin, leptin and resistin\textsuperscript{201}. Aside from leptin, all of these are secreted in greater quantity by visceral adipose tissue\textsuperscript{198}. Importantly, as visceral fat is drained by the hepatic portal vein, secreted FFAs and adipokines can have a disproportionate effect on the liver compared with other fat depots.

2.3.3 Obesity is associated with adipose tissue inflammation which leads to dysfunction

The metabolic dysfunction of obesity is not simply due to increased visceral adipose tissue mass, but to dysfunction of adipose tissue\textsuperscript{135}. This is illustrated by the very similar metabolic phenotype associated with lipoatrophy (pathologically reduced fat mass)\textsuperscript{202}.

Obesity is associated with low level inflammation of many sites including adipose tissue\textsuperscript{203}. Inflamed adipose tissue is less sensitive to insulin due to the paracrine effects of cytokines\textsuperscript{135} such as TNF-α which disrupts insulin signalling by inducing serine phosphorylation of the IRS-1 protein\textsuperscript{133}. This local insulin resistance leads to a greater rate of lipolysis and release of FFAs into the circulation and secretion of an abnormal adipokine profile\textsuperscript{135}.

Adipocyte hypertrophy leads to local inflammation

The underlying cause of the adipose tissue inflammation appears to be adipocyte hypertrophy and hypoxia. During positive energy balance, adipose tissue increases in size. This can occur in one of two ways 1) hyperplasia; where there is an increase in adipocyte number, as well as connective tissue and blood vessels, or 2) cellular hypertrophy, an increase in the size of adipocytes\textsuperscript{151}. Adipocyte hypertrophy appears is
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Unfavourable as larger adipocytes are associated with greater fasting insulin and triglycerides, greater inflammatory markers (TNF-α, IL-6 and CRP) and are more predictive of T2DM than BMI.

As adipocyte size increases above 140µm in diameter, the distance over which oxygen diffuses efficiently, adipocytes become hypoxic. Hypoxic adipocytes secrete inflammatory cytokines which lead to infiltration with macrophages, local insulin resistance and reduced adiponectin secretion. Importantly, hypoxia inhibits differentiation of preadipocytes to mature cells capable of storing lipid, reinforcing cellular hypertrophy as the mechanism to store excess lipid. Factors that influence whether the response to positive energy balance is adipose hypertrophy or hyperplasia include genetic factors, the hormonal milieu (catecholamines and glucocorticoids inhibit proliferation and differentiation of adipocytes), and medications (thiazolidinediones increase proliferation and differentiation).

Within adipose tissue it is not only the adipocyte that secretes inflammatory cytokines. Pro-inflammatory macrophages infiltrate in response to factors secreted by adipocytes such as macrophage chemotactic protein-1 (MCP-1). Macrophages and preadipocytes secrete a range of inflammatory cytokines, including IL-1β, IL-6, IL-8, PAI-1 and TNF-α.

Saturated fatty acids also contribute to inflammation. Adipocytes and macrophages express toll-like receptor-4 (TLR-4) which is involved in the innate immune response and activates the proinflammatory NF-κB pathway. TLR-4 is activated by bacterial endotoxin, but also saturated fat which is common in the modern diet.

Metabolic roles of the adipokines

Adipocytes secrete a number of metabolically important hormones. Adiponectin is anti-inflammatory, anti-atherogenic and increases oxidation of fat in liver and muscle, which reduces intracellular lipid. It is the only insulin sensitising adipokine and infusion of adiponectin, restores insulin sensitivity in both obesity and lipoatrophy. Adiponectin secretion is reduced from hypertrophied adipocytes and adiponectin levels are lower with abdominal obesity.

Leptin is released in greater amounts by subcutaneous fat and the concentration serves as an indicator of nutritional status as it increases after a meal, reduces during starvation, and is proportional to the adipose tissue
Leptin acts in the hypothalamus to reduce appetite, and increase thermogenesis and oxidation of fat, through increasing sympathetic activity and direct effects in muscle, which reduces intracellular lipid. In obesity leptin is elevated, but there is leptin resistance.

Resistin is secreted in greater amounts by visceral adipose tissue and is not well understood. It is pro-inflammatory, and infusion leads to hepatic insulin resistance and leptin resistance. Secretion is increased with obesity and by inflammatory cytokines.

Other circulating adipokines that may be important in metabolic disease include angiotensinogen, which has a role in the regulation of BP and vascular function and PAI-1, which is involved in thrombosis and fibrinolysis. IL-6 is involved in hepatic and muscular insulin resistance, increases hepatic triglyceride production and up-regulates all levels of the hypothalamic-pituitary-adrenal axis increasing secretion of cortisol, which has unfavourable metabolic effects (previously discussed).

Free fatty acids play a central role in the metabolic dysfunction of obesity

There is substantial evidence pointing to excess plasma FFAs, as the major cause of both hepatic and muscular insulin resistance. Circulating FFAs are increased in obesity and T2DM and they are associated with key aspects of the metabolic syndrome, including hypertension, dyslipidaemia and hyperuricaemia. Lowering FFAs pharmacologically leads to improved insulin sensitivity, while raising fatty acids with a lipid infusion reduces insulin sensitivity in a dose-dependent manner.

Effects of adipose tissue dysfunction on the liver

The effects of adipose tissue dysfunction on the liver are threefold. Firstly, excess FFAs drive hepatic glucose production. Secondly, accumulated intracellular lipid interferes with insulin signalling. Thirdly, the abnormal adipokine profile favours accumulation of intracellular lipid, and inflammation.

Elevated portal FFA stimulate hepatic glucose production (HGP), while suppression of FFA release reduces HGP. Importantly, the reduced HGP following a meal is in large part due to insulin’s anti-lipolytic action in visceral adipose tissue. Chronic increases in FFAs increase HGP, opposing the hepatic effects of insulin. This is due to increasing gluconeogenesis as acetyl CoA (a major metabolite of fatty acids)
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stimulates the rate limiting steps, while fatty acid oxidation supplies the necessary energy and reducing power.

Elevated FFAs also lead to accumulation of lipid within hepatocytes (triglycerides, diacylglycerols and long chain fatty acyl-CoAs). This intracellular lipid inhibits insulin signalling by two related mechanisms: inducing serine phosphorylation of IRS-1 and activation of NF-κB, leading to production of inflammatory cytokines (IL-6 and TNF-α) which also inhibit insulin signalling.

The adipokine profile is also important. Adiponectin decreases hepatic lipid content (through stimulating its β-oxidation), and is anti-inflammatory so that infusion restores insulin sensitivity. In contrast, resistin induces hepatic insulin resistance. Thus the dysfunctional adipokine profile characterised by low adiponectin, and elevated resistin and IL-6 contributes to insulin resistance by increasing hepatic lipid content and inflammation.

In summary, dysfunctional visceral adipocytes release excess FFAs and an unfavourable adipokine profile that leads to hepatic steatosis, inflammation and insulin resistance. This tends to increase HGP and plasma glucose, however provided insulin secretory capacity is sufficient, plasma insulin will increase normalising glucose.

2.3.6 Effects of adipose tissue dysfunction on skeletal muscle

In skeletal muscle, as in the liver, adipose tissue dysfunction leads to insulin resistance through excess FFA release and the unfavourable adipokine profile, which leads to accumulation of intracellular lipid and inflammation.

There is an inverse relationship between muscular insulin sensitivity and both plasma FFA concentration and intramyocellular lipid content. Importantly infusion of FFAs leads to increased intramyocellular lipid (including triglycerides, diacylglycerols (DAGs), fatty acyl-CoAs and ceramides) and reduced insulin sensitivity. Adiponectin reduces intramyocellular lipid by increasing fatty acid oxidation (through AMP kinase) and increasing glucose transport into muscle.
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Inflammation of muscle is also an important mechanism of insulin resistance. Intramyocellular lipid activates NF-κB\textsuperscript{241} and secretion of MCP-1, IL-6 and TNF-α. In response, macrophages infiltrate and secrete cytokines themselves\textsuperscript{243}. Both directly, and through the effects of cytokines, intracellular lipid inhibits insulin signalling (reduces activation of IRS-1 and PI-3-kinase\textsuperscript{244,246}) and inhibits important steps involved in glucose transport, glycogen synthesis and glucose oxidation\textsuperscript{135,229,247}.

Lastly, one of the major effects of insulin is to increase blood flow to muscle, increasing the efficiency of glucose disposal\textsuperscript{89}. This is mediated by insulin binding to receptors on endothelial cells, increasing nitric oxide synthesis\textsuperscript{248}. Endothelial cells also accumulate intracellular lipid and become resistant to insulin\textsuperscript{249}, further reducing glucose uptake into muscle.

The muscular insulin resistance that occurs in the obese is due to chronically elevated FFAs and reduced adiponectin leading to lipid deposition in myocytes and local inflammation. In addition, endothelial insulin resistance effectively reduces the efficiency of muscular glucose uptake.

2.3.7 Summary: Obesity leads to insulin resistance through inflamed and dysfunctional adipose tissue

The metabolic dysfunction that occurs with obesity is the result of inflammation and dysfunction of adipose tissue. This appears to be caused by an unfavourable hypertrophic adipocyte response to positive energy balance and the visceral adipose tissue is of greatest metabolic importance. Adipose tissue dysfunction leads to prolonged and inappropriate release of FFAs into the hepatic portal, and systemic circulations, as well as pro-inflammatory adipokines, and reduced secretion of adiponectin. This leads to accumulation of lipid in hepatocytes, muscle and endothelial cells. Intracellular lipid interferes with insulin signalling both directly, and through triggering inflammation. These processes link obesity to insulin resistance and are also likely to underlie metabolic dysfunction in the metabolically obese normal weight.

In the absence of an effective population based treatment for inducing weight-loss, the treatments most likely to improve metabolic health will be those that redistribute lipid to subcutaneous fat, induce a proliferative adipose response to excess energy consumption, reduce free fatty acid release, and inflammation of fat, liver and muscle.
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2.4. Mechanistic links between insulin resistance and features of the metabolic syndrome

Insulin resistance is associated with an increased plasma insulin concentration. This is because reduced insulin action leads to a rise of plasma glucose which is sensed by the pancreatic β-cell. In response, insulin secretion increases, and the plasma glucose is normalised. Thus if β-cell function is adequate, insulin resistance leads to hyperinsulinaemia with normal glucose tolerance. Aside from T2DM, the disorders of the metabolic syndrome, which include hypertension, dyslipidaemia, hepatic steatosis and metabolic dysovulation are the result of decreased insulin signalling through resistant insulin pathways, excess signalling through non-resistant pathways, or directly due to excessive free fatty acid release by dysfunctional adipose tissue. These mechanisms also mediate other pathological changes in metabolic syndrome including endothelial dysfunction, the prothrombotic state, hyperuricaemia, oxidative stress and systemic inflammation.

2.4.1 Essential hypertension and endothelial dysfunction

A number of observations link hypertension to insulin resistance. For example, hypertension is more common in the obese and those with T2DM, weight-loss and physical activity improve both insulin sensitivity and blood pressure, and hypertensives, (whether obese, or non-obese) have poorer insulin sensitivity than normotensives. Insulin resistance (and hyperinsulinaemia) increase BP through effects on 1) sympathetic nervous system (SNS) activation, 2) renal handling of sodium and 3) the regulation of nitric oxide production by vascular endothelium.

Hyperinsulinaemia activates the SNS

Insulin activates the SNS, probably through insulin receptors in the hypothalamus, that link energy consumption (signified by increased plasma insulin) to SNS mediated thermogenesis. Through this mechanism, hyperinsulinaemia leads to constriction of skeletal muscle vasculature, increasing BP, and elevation of the pulse rate, and plasma noradrenaline.

Hyperinsulinaemia leads to sodium retention

Insulin has a direct effect on the kidney through receptors in the proximal and distal renal tubules, which lead to reduced excretion of sodium, potassium and uric acid. Further, insulin also indirectly reduces sodium excretion by activating the sympathetic nervous system, and subsequently the renin-angiotensin-
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aldosterone system\textsuperscript{244}, which increases aldosterone secretion\textsuperscript{255} (and further increases SNS activity\textsuperscript{256}). Both aldosterone and SNS activity reduce sodium excretion\textsuperscript{255,257}. The control of sodium excretion is the major way in which the extracellular fluid volume\textsuperscript{258}, and long-term BP control\textsuperscript{259}. Importantly, these effects of insulin are not reduced in the context of insulin resistance\textsuperscript{253}.

\textit{Insulin resistance causes endothelial dysfunction with reduced production of nitric oxide}

Insulin has an important role in the distribution of blood flow, and the tone of small blood vessels. It induces nitric oxide synthase in endothelial cells, the enzyme responsible for production of the powerful local vasodilator, nitric oxide (NO)\textsuperscript{260}. NO diffuses from endothelial cells to nearby smooth muscle where it triggers vasodilation. Insulin increases muscular blood flow through this mechanism\textsuperscript{248} which improves the efficiency of glucose disposal\textsuperscript{89} and affects vascular resistance and BP\textsuperscript{261}.

The intracellular signalling pathway in endothelial cells is very similar to the pathway that mediates the effects of insulin in adipose tissue, liver and muscle, involving IRS proteins and the PI3K enzyme\textsuperscript{89,260,262}. Excess FFAs lead to lipid accumulation in endothelial cells, oxidative stress, inflammatory signalling and together these mechanisms interrupt insulin signalling\textsuperscript{89}. Insulin also signals through an alternative pathway (Ras/MAP kinase) in the endothelial cell to increase the release of the potent vasoconstrictor endothelin-1 (ET-1)\textsuperscript{89,263}. As the Ras/MAP kinase pathway does not become resistant, metabolic insulin resistance and hyperinsulinaemia lead to both impaired NO production and increased ET-1\textsuperscript{89}.

This contributes to hypertension, impaired flow mediated dilatation\textsuperscript{264}, and failure of the normal diversion of blood flow to muscle that occurs in response to insulin\textsuperscript{89,249}. Thus endothelial insulin resistance is a major contributor not just to hypertension but also to the impaired glucose disposal that is central to the metabolic dysfunction of obesity\textsuperscript{89}.

\textit{Summary}

Insulin resistance affects BP by 1) inducing sodium retention and expansion of blood volume, 2) sympathetic activation which increases vasomotor tone, and 3) through impaired secretion of NO and increased secretion of ET-1. Together these mechanisms tie together the strong relationship between insulin resistance and hypertension\textsuperscript{29,91,250}. 
Introduction

2.4.2 Dyslipidaemia and Hepatic Steatosis

Metabolic syndrome is associated with an atherogenic lipid profile characterized by hypertriglyceridaemia, elevated very low density lipoprotein (VLDL), the presence of small dense low density lipoprotein (LDL) and reduced high density lipoprotein (HDL)\(^{265,266}\). The central defect appears to be overproduction of triglyceride and apolipoprotein-B (Apo-B), the rate limiting components of VLDL\(^{265-267}\).

To produce VLDL, the liver must synthesise or recycle triglyceride. There are three sources: 1) synthesis from circulating FFAs, 2) uptake from VLDL, IDL or chylomicron remnants, and 3) \textit{de novo} synthesis of fatty acids, which are then esterified into triglyceride\(^{265}\). All three of these processes are affected by the insulin resistant, hyperinsulinaemic state.

Firstly, dysfunctional VAT releases excess FFAs into the portal vein\(^{135}\), 20-30% of which is taken up by hepatocytes\(^{267}\). Portal FFAs directly drive triglyceride production\(^{268,269}\). Secondly, insulin resistance leads to incomplete unloading of LDL and chylomicrons following a meal\(^{265}\) because peripheral lipoprotein lipase, the enzyme that facilitates unloading of these lipid particles is induced by insulin\(^{270}\). Hepatic uptake of triglyceride from these remnant lipoprotein particles provides additional triglyceride for incorporation into VLDL\(^{265}\). This accounts for the exaggerated postprandial hypertriglyceridaemia that is seen in insulin resistance\(^{265}\). Lastly, \textit{de novo} lipogenesis is increased with insulin resistance\(^{265}\) through effects on SREB1-c, a transcription factor that increases expression of enzymes required to synthesise triglyceride\(^{271}\).

Elevated VLDL leads to the other aspects of dyslipidaemia. Through cholesterol ester transfer protein (CETP) which is active in plasma, cholesterol ester in VLDL is exchanged with triglyceride from HDL\(^{265}\). This produces cholesterol deplete LDL, and after these particles become stripped of triglyceride by the liver or peripheries they become small, dense, and more atherogenic\(^{265}\).

The insulin resistant state can lead to hepatic steatosis (abnormal accumulation of triglyceride in the liver)\(^{265,272}\). Apo-B is an essential component of VLDL that is degraded in response to insulin. Hepatocytes with insufficient Apo-B cannot export triglyceride. Interestingly, free fatty acid induced hyperinsulinaemia is required in order to develop hepatic steatosis\(^{124,265}\). This suggests that steatosis develops where the balance of hyperinsulinaemia, circulating FFAs and hepatic insulin resistance lead to excess triglyceride production, with insufficient Apo-B\(^{265}\). Hepatic steatosis is frequently associated with inflammation which may lead to damage (steatohepatitis) and cirrhosis\(^{273}\). This appears to be due to inflammatory cytokines produced by lipid filled hepatocytes\(^{133,237}\), and VAT\(^{273}\).
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### 2.4.3 Platelet function, coagulation and fibrinolysis

Insulin resistance leads to a prothrombotic state through three major effects. Firstly, insulin inhibits platelet aggregation\(^{274}\), by inducing NO production in endothelial cells\(^{275}\). This effect is lost in obesity\(^{274}\), because of reduced NO production (endothelial insulin resistance), and resistance of platelets to the effects of NO\(^{275}\). Secondly, insulin resistance is associated with increased concentrations of procoagulant clotting factors such as tissue factor, factors VII and IX and von Willebrand factor, and fibrinogen\(^{30,276}\). Lastly, insulin resistance is associated with impairment of fibrinolysis as dysfunctional adipocytes and hepatocytes secrete PAI-1, which inhibits the initial steps of fibrinolysis\(^{276}\). Notably, the acute insults related to cardiovascular, cerebrovascular and peripheral vascular diseases result from rupture of atherosclerotic plaque following by the formation of a stable clot.

### 2.4.4 Development of type 2 diabetes mellitus – the importance of insulin secretion

Insulin resistance alone does not generally lead to hyperglycaemia because the initial rise in blood glucose caused by impaired insulin signalling is detected by the β-cells which respond by increasing insulin secretion, and normalising plasma glucose. Unless insulin resistance is extremely severe, for there to be elevation of blood glucose there must be insufficient secretion of insulin.

Diabetes mellitus (DM) is defined by abnormally elevated plasma glucose. Current diagnostic criteria include: fasting blood glucose >7.0mmol/l, random blood glucose >11.1mmol/l (in the presence of symptoms of hyperglycaemia) or glucose >11.1mmol/l following a 75g oral glucose load\(^{277}\). Diabetes can also be diagnosed on the basis of elevated glycosylated haemoglobin, (HbA1c>6.5% or 48mmol/mol) which reflects a long term average blood sugar of approximately 7.6mmol/l\(^{277}\).

DM can occur because of four main reasons: 1) insulin resistance is so severe that maximum insulin secretion by normally functioning β-cells is insufficient (Type A insulin resistance\(^{278}\) or Donahue syndrome\(^{279}\)), 2) dysregulation of insulin secretion causing “underestimate” of the blood glucose and undersecretion of insulin (glucokinase deficiency)\(^{280}\), 3) severely reduced insulin secretion (Type 1 DM, HNF1-α mutation)\(^{281}\) or 4) a combination of insulin resistance and relative insulin deficiency, which is characteristic of T2DM.
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In T2DM acquired impairment of insulin secretion is due to lipotoxicity and later glucotoxicity of the insulin secreting pancreatic β-cells. While FFAs increase insulin secretion through incretin dependent and direct effects on the β-cell, chronically elevated FFAs are associated with desensitisation of β-cells, with reduced insulin secretion for the ambient glucose concentration, and eventual apoptosis of the β-cell, causing a permanent reduction of insulin secretory function.

Evidence from a mouse model of obesity has shown that β-cell desensitisation occurs as a result of chronic signalling through the free fatty acid receptor GPR-40. GPR-40 signalling increases expression of CPT-1 and uncoupling protein-2 (UCP-2); these proteins reduce the efficiency of ATP generation from oxidation of glucose, thereby reducing the amount of insulin secreted for a given glucose concentration. In addition, chronically elevated FFAs may lead to accumulation of lipid metabolites in the β-cell which interfere with the ATP-sensitive potassium channel, UCP-2, the exocytic machinery or modulate gene expression. Thus FFAs appear to have a central role not only in the development of insulin resistance, and the hyperinsulinaemic response, but also in the eventual failure of β-cell function that underlies the metabolic decompensation from an insulin resistant state, to T2DM.

Once insulin secretion is insufficient, the plasma glucose rises. Elevated glucose is toxic to the β-cell and may have synergistic toxic effects in combination with elevated FFAs. Two major mechanisms have been proposed to explain this. Firstly glucotoxicity increases intracellular oxidative stress and advanced glycation end products (AGEs; non-enzymatically glycosylated proteins, lipids and nucleic acids) which reduce transcription of insulin, and eventually induce apoptosis. Secondly, excess glucose may decrease PPAR-α expression, reducing metabolism of intracellular lipid and leading to greater accumulation of lipid and lipotoxicity.

The relative contributions of IR and impaired insulin secretion to the development of T2DM have long been debated. Although modern debate accepts both are important, the primacy of each factor in setting an individual on a path to T2DM is still discussed. It has been argued that T2DM occurs in people who have a genetically determined impairment of insulin secretion. The development of insulin resistance, for example as they become obese, precipitates diabetes. Genome wide association studies (GWAS) have shown that most gene variants that increase the risk of T2DM do affect insulin secretion, through the size of the β-cell mass, or the intracellular pathways that link glucose sensing to insulin secretion. However, there are also acquired impairments of insulin secretion such as lipotoxicity and gene variants that are linked to obesity, visceral fat mass and insulin resistance. Thus the cause of T2DM is complex with both genetic and environmental factors influencing insulin secretion and insulin resistance.
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2.4.5 The link between metabolic dysfunction and cardiovascular disease

Cardiovascular disease is primarily a disease of atherosclerosis, the accumulation of lipid within the wall of blood vessels. The major processes that lead to the development of atherosclerosis from early fatty streaks to enlarged inflamed and weakened plaques that may ultimately rupture and trigger thrombosis are well understood.

Cachofeiro provides a clear summary\textsuperscript{291}. In brief, atherosclerosis begins in areas of the vasculature where flow is turbulent, such as the coronary, carotid and cerebral arteries and the aorta. Turbulence applies shear forces to the vessel walls, causing mild endothelial damage. Damaged endothelium allows entrance of LDL into the vessel wall, where it is oxidised, and initiates an inflammatory process. Monocytes migrate into the vessel wall, take up oxidised LDL and become lipid filled foam cells. This early lesion is called a fatty streak. Over time, the streak enlarges as smooth muscle cells migrate from the media to the intima, proliferate and produce extracellular matrix, making a fibrous cap which protects the plaque from the circulating blood. The plaque is weakened by inflammation, and may eventually rupture. Rupture exposes collagen to the blood, which triggers thrombosis and vessel occlusion. When this occurs in coronary arteries, this leads to myocardial infarction\textsuperscript{292}. Atherosclerosis is also the major cause of peripheral vascular disease\textsuperscript{293}, aortic aneurysm\textsuperscript{294}, and some strokes\textsuperscript{295}.

The insulin resistant state increases cardiovascular risk through enhancing atherogenesis. The major factors involved in atherogenesis, dubbed the “atherosclerotic triad”, are oxidative stress, inflammation and endothelial dysfunction\textsuperscript{291}. The insulin resistant state is associated with all of these factors, through the underlying systemic inflammatory state, adverse changes in adipokine secretion and excess reactive oxygen species. Further insulin resistance is associated with a proatherogenic dyslipidaemia, and in diabetics, hyperglycaemia and AGEs increase oxidative stress further.

Dyslipidaemia

Insulin resistance is characterised by a typical lipid profile characterised by increased VLDL, decreased HDL and formation of small dense LDL particles. This contributes to atherogenesis, through increased deposition of lipid in the vessel wall, and reduced resorption. Small dense LDL particles are important as they pass directly through endothelial fenestrations to enter the subendothelial space\textsuperscript{296}.
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In order to be absorbed into macrophages, LDL must be modified by oxidation or reacting with AGEs\(^\text{297,298}\). Both insulin resistance and hyperglycaemia are associated with excess reactive oxygen species\(^\text{299}\), and chronic hyperglycaemia is also associated with production of AGEs\(^\text{300}\). Once modified, LDL is then taken up by the macrophage scavenger receptor, to form foam cells\(^\text{297,298,301}\).

Hypertension and endothelial dysfunction

Hypertension is a risk factor for cardiovascular disease but it is not clear if it enhances atherosclerosis or simply has a common cause, the insulin resistant pro-inflammatory, pro-oxidative state. At minimum, there is an indirect role, as left ventricular hypertrophy is predictive of cardiovascular events\(^\text{302}\).

As has been introduced, insulin resistance leads to endothelial dysfunction with reduced secretion of NO\(^\text{89,249}\), which contributes to hypertension\(^\text{261}\). NO also has important actions that protect against the development of atherosclerosis, including reducing platelet aggregation, leukocyte adhesion to endothelial cells and infiltration of plaque, proliferation of vascular smooth muscle cells and oxidation of LDL\(^\text{263,303,304}\). Further, insulin resistance is a pro-oxidant state, with increased reactive oxygen species\(^\text{305}\) and this increases the breakdown of nitric oxide\(^\text{306}\).

Systemic inflammation

Insulin resistance is associated with systemic inflammation, due to the release of inflammatory cytokines from fat, liver and muscle\(^\text{133,140,151,203,237,241}\) and this contributes to atherogenesis. CRP is secreted by the liver in response to IL-6\(^\text{291}\) and it has been shown to reduce nitric oxide secretion, and increase expression of cell adhesion molecules, recruitment of monocytes into atherosclerotic lesions, and the production of inflammatory cytokines by foam cells\(^\text{291}\). Higher CRP concentration is associated with weaker plaques that are more prone to rupture\(^\text{291}\), and greater cardiovascular risk\(^\text{307}\).

Adipokines

The balance of adipokines is also important in the development of atherosclerosis particularly adiponectin and leptin. Adiponectin levels are inversely related to central adiposity and insulin sensitivity\(^\text{218}\). Aside from effects on insulin sensitivity, adiponectin has many effects on the processes important in the development of...
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Atherosclerosis. Adiponectin improves endothelial function, promoting NO production and reducing its oxidation\textsuperscript{308-310} and it reduces oxidative stress, protecting the endothelium from injury\textsuperscript{308,309}. Through inhibition of NF-κB, adiponectin reduces endothelial expression of cell adhesion molecules and thereby infiltration of monocytes into the arterial wall\textsuperscript{311}. It reduces uptake of lipid into macrophages, by inducing a more favourable lipid profile (reducing small dense LDL and VLDL triglyceride)\textsuperscript{312} and inhibiting expression of the scavenger receptor\textsuperscript{313}. It also reduces the migration and proliferation of smooth muscle cells,\textsuperscript{301} and inhibits the enzymes responsible for weakening the plaque cap (matrix metalloproteinases)\textsuperscript{314}. Thus low adiponectin levels, contribute to both atherogenesis and plaque rupture.

In contrast, leptin is increased in obesity\textsuperscript{220}, proportionally to the subcutaneous fat mass\textsuperscript{289,301}. Leptin receptors are found on endothelial cells and atherosclerotic lesions\textsuperscript{315}, and leptin induces proliferation and migration of vascular smooth muscle cells and contributes to the prothrombotic state\textsuperscript{301,316}.

**Prothrombotic state**

In the event of plaque rupture, the formation of a stable thrombus that occludes the arterial lumen leads to myocardial infarction\textsuperscript{292}. The prothrombotic state that is associated with insulin resistance\textsuperscript{30,274,276} increases the likelihood of vessel occlusion.

**Summary**

Insulin resistance leads to greater cardiovascular risk through many overlapping mechanisms including dyslipidaemia, endothelial dysfunction, systemic inflammation, oxidative stress, an adverse change in secreted adipokines and the prothrombotic state.

2.4.6 Hyperglycaemia and cardiovascular disease

In those with T2DM there is a consistent association between the degree of hyperglycaemia (fasting and postprandial) and cardiovascular risk\textsuperscript{317,318}, with the postprandial glucose concentration being most predictive\textsuperscript{319}. In fact even below the diabetic range (<11mmol/l) there is a graded association between postprandial glucose and cardiovascular risk\textsuperscript{247,248}. 

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Infusion of glucose to achieve hyperglycaemia, leads to increased reactive oxygen species (ROS)\textsuperscript{299}, which reduce endothelial NO production\textsuperscript{318}, and increase expression of cell adhesion molecules\textsuperscript{320} by activating inflammatory pathways\textsuperscript{321}. This enables circulating monocytes to enter the vascular wall, where they secrete inflammatory cytokines in response to oxidative stress\textsuperscript{303}.

Chronic hyperglycaemia is associated with formation of AGEs, (due to chemical alteration of proteins, lipids and nucleic acids)\textsuperscript{300}. These have a role in the generation of reactive oxygen species and activation of monocytes\textsuperscript{303} and reduce the efficiency of HDL in reverse cholesterol transport\textsuperscript{303}. Further, ROS and AGEs modify circulating LDL particles increasing their atherogenicity\textsuperscript{303}. Hyperglycaemia also contributes to acute thrombotic events through a direct osmotic effect on platelet reactivity\textsuperscript{30}.

Hyperglycaemia does have unfavourable effects on many processes central to atherogenesis and myocardial infarction. However, a recent systematic review of the largest trials, showed only a weak benefit of tight glycaemic control on the rate of (non-fatal) myocardial infarction\textsuperscript{322}. This suggests that while hyperglycaemia has unfavourable cardiovascular effects, cardiovascular risk is primarily mediated by factors related to insulin resistance and hyperinsulinaemia.

2.4.7 Other minor features of metabolic dysfunction

Metabolic dysfunction is associated with elevated uric acid\textsuperscript{323}, as it is a by-product of triglyceride synthesis\textsuperscript{323}, and hyperinsulinaemia reduces its excretion\textsuperscript{253}. Elevated uric acid causes gout\textsuperscript{324} and may contribute to atherogenesis\textsuperscript{325}. Insulin resistance is also a major factor contributing to polycystic ovarian syndrome, which is characterised by excessive androgens, dysovulation and reduced fertility\textsuperscript{7,326}.

2.4.8 Mitogenic effects of insulin: linking obesity to cancer

Obesity is a strong risk factor for a range of cancers\textsuperscript{327}, including colorectal cancer where obesity increases the risk by 41\%, and risk increases further with greater BMI\textsuperscript{328}. This is probably mediated by insulin’s priming effect on cellular proliferation.\textsuperscript{329} All growth factors signal through the Ras-Raf-MAP kinase pathway\textsuperscript{87,329}. This pathway cannot be activated unless the Ras protein is anchored to the cell membrane, and insulin induces this change\textsuperscript{87,329}. Importantly, this effect of insulin is not mediated by the IRS and PI(3)K pathway, so that this pathway does not become resistant with obesity\textsuperscript{87}. Thus hyperinsulinaemia increases the cellular proliferation induced by other growth factors\textsuperscript{87}. 

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This data emphasises that whenever possible the preferred strategies for treating metabolic syndrome and T2DM should be those that improve metabolic insulin sensitivity, and thus reduce the circulating insulin concentration. Treatments that increase the insulin level, including sulphonylureas and exogenous insulin, appear to increase the risk of malignancy.

2.5. Strategies for improving insulin sensitivity

The adverse cardiometabolic effects of obesity stem from dysfunction of inflamed visceral fat characterized by local insulin resistance, inappropriate lipolysis and free fatty acid release, secretion of proinflammatory cytokines and reduced secretion of adiponectin. This leads to insulin resistance of liver and muscle and compensatory hyperinsulinaemia. A combination of reduced insulin signalling through insulin resistant pathways, and excess signalling through non-resistant pathways, largely accounts for the associations of endothelial dysfunction, hypertension, dyslipidaemia, and hepatic steatosis with obesity. Together these and other effects accelerate the rate of atherosclerosis. Further, if insulin secretion is also insufficient then decompensation to T2DM occurs which accelerates atherosclerosis further, and leads to diabetic retinopathy, neuropathy and nephropathy.

Insulin sensitising treatments are most appropriate, and the most elegant target is adipose tissue. Potential insulin sensitising treatments include: 1) medications such as metformin and thiazolidinediones, 2) bariatric surgery, 3) weight-loss and physical training, and 4) modification of the diet.

As has been discussed, lifestyle changes, including dietary change and physical activity regimes are difficult for most of the population to achieve. Medications to induce weight-loss are relatively ineffective and while bariatric surgery induces profound changes in body weight and normalises metabolism it cannot be applied to all who would benefit. Medications or micronutrients that improve insulin sensitivity may be acceptable to patients and practical to apply to the large obese population. Recently, olive leaf extract has been shown to have insulin sensitising properties, although this result requires replication. The long chain n-3 polyunsaturated fatty acids, or chemically similar agonists, represent potentially important insulin sensitising agents.
2.6. Key molecular regulators of inflammation and lipid metabolism may be targeted by supplementation with polyunsaturated fatty acids

A number of important transcription factors, receptors and enzymes have major influence over aspects of physiology which are disordered in the context of insulin resistance, so should be considered as molecular targets for drugs or supplements. These include NF-κB, and its inhibitors, COX-2, PPAR-γ, PPAR-α, SREB1-c, HNF-4α and the G-protein linked receptor GPR-120. All of these are affected by the concentration of specific fatty acids, thus supplementation with fatty acids represents a potentially powerful metabolic treatment.

2.6.1 NF-κB: major regulator of inflammation

NF-κB is a transcription factor that has been called the master regulator of inflammation. It responds to a range of harmful cellular stimuli, including many highly relevant to metabolic disease such as inflammatory cytokines, arachidonic acid derived eicosanoids, oxidative stress, intracellular lipid and toll-like receptors binding to ligands such as saturated fatty acids. When inactive, NF-κB is sequestered in the cytoplasm by specific inhibitors. In response to immunological or inflammatory stimuli, NF-κB dissociates from its inhibitor, translocates to the nucleus and initiates transcription of pro-inflammatory genes. These genes include TNF-α and IL-6, both of which reduce insulin signalling. NF-κB has an important role in inflammation in adipose tissue, myocytes, hepatocytes, and within atherosclerotic plaques. This pathway is inhibited by transcription factors and receptors for fatty acids including PPAR-α, PPAR-γ and GPR-120.

The importance of NF-κB in insulin resistance is illustrated by the insulin sensitising effects of salicylates, which at high dose prevent activation of NF-κB (by inhibiting IKK, the enzyme responsible for degradation of NF-κB inhibitor). Salicylate treatment in rats, reduced or prevented the insulin resistance caused by infusion of FFAs and reversed the insulin resistance caused by high fat or high fructose diets. In humans high dose salicylates reduced the insulin resistance induced by lipid infusion, while in diabetics they improved insulin sensitivity, increased adiponectin, reduced the glucose and fatty acid rises after a mixed meal and lowered HbA1c. Adverse effects preclude the use of high dose salicylates in people with diabetes or insulin resistance but it is clear that inhibiting NF-κB may be useful in the treatment of metabolic disease.
2.6.2 COX-2 produces inflammatory eicosanoids

Cyclooxygenase-2 converts the n-6 PUFA arachidonic acid into inflammatory eicosanoids\textsuperscript{350}. In rats, treatment with a COX-2 inhibitor prevented the deterioration in insulin sensitivity and hepatic steatosis caused by a high fat or high fructose diet\textsuperscript{350,351}. COX-2 inhibition prevented the high fat diet (HFD) induced increase in TNF-α expression\textsuperscript{350}, which is known to impair insulin signalling\textsuperscript{133}. Thus treatments that inhibit COX-2 or reduce the production of inflammatory eicosanoids may be useful in obesity. Importantly, eicosanoids produced from n-3 PUFAs are less inflammatory or anti-inflammatory\textsuperscript{352}. Further, other enzymes that produce inflammatory mediators from n-6 PUFAs, also make an alternative series from n-3 PUFAs including the resolvins and protectins, which resolve inflammation and n-3 endocannabinoids\textsuperscript{352}. Thus n-3 PUFAs, lead to an anti-inflammatory pro-resolving profile of inflammatory mediators.

2.6.3 PPAR-γ: master regulator of adipose tissue

PPAR-γ is the major transcription factor regulating adipose tissue and adipogenesis. In fact knockout of PPAR-γ leads to lipodystrophy\textsuperscript{353}. It is also expressed in a variety of other cell types including hepatocytes, myocytes, macrophages and the pancreatic β-cell\textsuperscript{354}. The major ligands for this receptor are unsaturated fatty acids, in particular the n-3 and n-6 PUFAs, and oleic acid\textsuperscript{355}. In addition, molecules derived from n-3 PUFAs including the eicosanoid protectin-D1 are more potent than the n-3 PUFAs themselves\textsuperscript{356}. PPAR-γ activity increases proliferation and differentiation of preadipocytes to mature adipocytes that are capable of storing lipid and secreting adipokines, thus activity of PPAR-γ increases fat storage capacity\textsuperscript{242}. In addition, because it increases adipocyte number, it leads to a reduction in adipocyte size\textsuperscript{357}. PPAR-γ upregulates enzymes required to extract and store lipid from circulating lipoproteins and reduces release of fatty acids\textsuperscript{242,357,358}. It also upregulates major parts of the insulin signalling pathway including IRS-1, PI(3)K and the GLUT-4 insulin responsive glucose transporter, increasing the sensitivity of adipose tissue to insulin\textsuperscript{354}. Thus PPAR-γ counters the adipose tissue dysfunction associated with obesity, by increasing lipid storage capacity and uptake of glucose and lipid, and reducing the release of FFA.

Inflammation is a major contributor to the pathogenesis of insulin resistance\textsuperscript{134,135} and activation of PPAR-γ reduces adipose inflammation in three ways: 1) it inhibits NF-κB in adipocytes, reducing the secretion of proinflammatory cytokines such as TNF-α\textsuperscript{359}, 2) it increases the secretion of adiponectin\textsuperscript{357,360,361} which is anti-inflammatory\textsuperscript{203}, and 3) activity of PPAR-γ downregulates the activity of proinflammatory M1-macrophages and increases activity of anti-inflammatory M2-macrophages\textsuperscript{354}. In addition to anti-inflammatory effects, the increased secretion of adiponectin is also important because it reduces intracellular lipid in hepatocytes and myocytes which increases insulin sensitivity\textsuperscript{216}. 
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Thiazolidinediones are potent insulin sensitizers used in the treatment of T2DM and their primary effect is through PPAR-γ in adipose tissue\(^{354}\). These drugs also reduce BP, retard progression of atherosclerosis, improve endothelial function and reduce microalbuminuria, through indirect effects of PPAR-γ\(^ {362}\). While thiazolidinediones do have important adverse effects including weight gain\(^ {354}\), fluid retention\(^ {354}\), increased risk of heart failure\(^ {363}\), myocardial infarction (MI)\(^ {364}\) and fracture\(^ {354,365}\), modulation of PPAR-γ is an important avenue for reducing adipose dysfunction, improving insulin sensitivity and reducing cardiovascular risk. As PUFAs are also PPAR-γ agonists with similar effects in tissue culture\(^ {361}\), there is the potential for supplemental PUFAs to have important metabolic benefits through this pathway.

2.6.4 PPAR-α, SREB1-c and HNF-4α regulate the production and oxidation of lipid

PPAR-α is another transcription factor activated by a variety of fatty acids\(^ {333,366}\). However, as eicosanoids are 1-2 orders of magnitude more potent activators of PPAR-α, the concentration of PUFAs (from which eicosanoids are derived) has a greater effect than other fatty acids\(^ {366,367}\). In the liver PPAR-α activation increases fatty acid β-oxidation, and reduces hepatocellular fat content and the release of triglyceride into circulation\(^ {242,333}\). It also inhibits inflammation, (partially via NF-κB in immune cells)\(^ {333,368}\) and inhibits processes important in atherogenesis\(^ {333,368}\).

Fibrates are agonists of PPAR-α and these drugs lower plasma triglycerides\(^ {369}\), increase HDL\(^ {369}\), and reduce markers of systemic inflammation\(^ {370}\) and the incidence of MI\(^ {296,297}\). In rodents, fibrates reduce hepatic steatosis and steatohepatitis\(^ {371,372}\), improve insulin sensitivity\(^ {373,374}\), and reduce adiposity\(^ {373}\) and intramuscular lipid content\(^ {374}\). However, there is only limited evidence for a beneficial effect on hepatic steatosis in humans\(^ {375,376}\). In a small study, a PPAR-α agonist reduced fasting and stimulated insulin levels\(^ {377}\) suggesting improved insulin sensitivity but two studies using the gold standard measure of insulin sensitivity (hyperglycaemic euglycaemic clamp) found no effect on insulin sensitivity or adiponectin\(^ {370,378}\). Thus in humans PPAR-α agonists have an important normalising effect on dyslipidaemia and may reduce systemic inflammation, but appear not to reduce hepatic steatosis and inflammation.

SREB1-c is a transcription factor in the liver that increases expression of enzymes that synthesise fat\(^ {379,380}\). It is inhibited by fatty acids, in particular n-6 and n-3 PUFAs, which reduce the production of triglyceride\(^ {379}\). HNF4-α is another transcription factor in the liver. It is activated by saturated fat and inhibited by PUFAs\(^ {380}\). HNF4-α activity indirectly increases triglyceride production by increasing the metabolism of glucose to pyruvate, which is a substrate for triglyceride production\(^ {367}\).
PUFAs decrease circulating triglycerides by increasing PPAR-α mediated degradation, and by inhibiting production through inhibition of SREB-1c and HNF4-α. While both n-3 and n-6 PUFAs have this effect, n-3 PUFAs are more potent.

2.6.5 GPR-120 mediates anti-inflammatory and insulin sensitising effects

The GPR-120 receptor is a fatty acid sensor with specificity for n-3 PUFAs. It is also activated by the newly identified class of lipids, branched fatty acid esters of hydroxy-fatty acids (FAHFAs), an endogenous ligand. It has an important role in appetite, glucose and lipid metabolism and is expressed by a range of cell types including pancreatic β-cells, gut enteroendocrine cells, hypothalamic neurons, mature adipocytes and proinflammatory adipose tissue macrophages. It inhibits inflammation through NF-κB in multiple tissues and this mediates important insulin sensitising and appetite reducing effects.

The importance of GPR-120 in adipose tissue has been demonstrated by a series of studies including *in vitro* and *in vivo* rodent models, and clinical genetics. Absence of GPR-120 in a mouse model leads to obesity with adipocyte hypertrophy, adipose tissue inflammation hepatic steatosis and insulin resistance; the same metabolic abnormalities seen in human obesity. Recently, two inactivating mutations of GPR-120 were described in humans; both produced an obese phenotype.

In rodents, n-3 PUFAs have clear insulin sensitising effects. Supplementation reverses or prevents insulin resistance whether caused by a high fat, high sucrose or high fructose diet. This effect is primarily mediated by GPR-120 as knockout of this receptor abolished the insulin sensitising effect. Through GPR-120 n-3 PUFAs inhibited TNF-α and toll-like receptor inflammatory pathways, reducing the number of proinflammatory macrophages and increasing the number of anti-inflammatory macrophages. This led to greater glucose uptake into adipose tissue, and improved systemic insulin sensitivity. More recently a selective GPR-120 agonist has also been shown to increase insulin sensitivity.

2.6.6 Conclusions

n-3 PUFAs activate PPAR-α, PPAR-γ, and GPR-120 and inhibit SREB1c and HNF-4α, key receptors and transcription factors involved in glucose and lipid metabolism. Thus supplementation with n-3 PUFAs could have an important role in reducing the metabolic dysfunction in people who are overweight or obese.
Chapter 3. Measurement of insulin sensitivity and insulin secretion

Glucose metabolism is complex and there is no simple way to measure insulin sensitivity. In addition to expensive and labour intensive direct measures, a large number of surrogates have been developed. Surrogates are simpler, cheaper and more acceptable to participants, but though they correlate to gold standard direct measures, they have limitations. These must be considered when designing a metabolic study.

3.1. Definition of insulin sensitivity

Insulin sensitivity is the effectiveness of insulin, to bind to insulin receptors, induce intracellular signalling, and ultimately cause the uptake of glucose into insulin responsive peripheral cells (myocytes and adipocytes), and to inhibit glucose release from hepatocytes. Poorer insulin sensitivity (often termed insulin resistance) implies that a greater insulin concentration would be required in order to have the same effect. Insulin resistance may also refer to pathologically reduced insulin sensitivity, i.e. that associated with disorders such as dyslipidaemia, hypertension and T2DM.

This metabolic definition of insulin sensitivity does not account for the many effects of insulin outside of glucose metabolism, such as effects on endothelial cells, hypothalamic neurons or mitogenic effects. While the effects of insulin on these target cells may also be blunted, this is not typically called insulin resistance, and is not measured in the assessment of insulin sensitivity.

3.2. Measurement of insulin sensitivity – a complex problem

There is no simple marker of insulin sensitivity that can be measured. Plasma insulin and glucose concentrations are related to insulin sensitivity but cannot be interpreted in isolation. This is because of the reciprocal regulation of plasma glucose and insulin. Extracellular glucose concentration regulates insulin release and therefore the plasma insulin concentration. In reciprocal, the plasma insulin concentration regulates glucose uptake into peripheral tissues, and inhibits glucose release from the liver; hence the plasma insulin concentration regulates plasma glucose. To directly measure insulin sensitivity, the insulin concentration must be controlled, so that the rate of glucose uptake can be measured.
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Glucose metabolism changes markedly between the fed and starved states. In the fasting state, insulin secretion is relatively suppressed and the predominant factor determining the glucose concentration is the rate of hepatic glucose production. However, following a meal, insulin secretion increases in response to rising plasma glucose, fatty acids, amino acids, and incretins. The predominant determinant of glucose concentration is the rate of peripheral glucose uptake into muscle and adipose tissue. Thus indices derived solely from measures in the fasting state, are most influenced by hepatic insulin sensitivity, while indices derived after a glucose load better reflect peripheral insulin sensitivity.

Insulin sensitivity is affected by other important contextual factors. There is a circadian rhythm of insulin sensitivity which correlates with the circadian pattern of cortisol and free fatty acid levels. Growth hormone and catecholamines also have a circadian pattern, and reduce insulin sensitivity. In addition, these hormones and glucagon are secreted in response to physiological and psychological stress and have synergistic effects elevating plasma glucose. Lastly, physical activity also increases insulin sensitivity and the effect can last hours. Thus tests of insulin sensitivity usually begin in the morning after an overnight fast, without recent exercise and at a time that the subject is well.

3.3. Direct measurement of insulin sensitivity

Clamp techniques solve the complexity inherent in the reciprocal regulation of insulin and glucose levels, by holding one variable (glucose or insulin) constant and measuring the effect on the other. The hyperinsulinaemic euglycaemic clamp (HEC) is the gold standard method for measuring insulin sensitivity.

During HEC, insulin is infused at a constant rate, to produce a steady supraphysiological plasma insulin concentration. Glucose is infused and the rate adjusted so that the blood glucose is “clamped” at a constant target. At steady state the glucose infusion rate is equal to the glucose disposal rate. Greater insulin sensitivity will lead to greater glucose disposal, thus the steady state glucose infusion rate $M$ is directly related to whole body insulin sensitivity. The insulin sensitivity index (ISI) is calculated as $M/(\text{insulin concentration})$ and to enable comparison between subjects of different body size, ISI can be expressed adjusted for body weight.

This method is highly reproducible (CV 6-10%) and can be modified with 3H labelled glucose to allow measurement of the relative contributions of liver and peripheral tissues (muscle and adipose tissue) to whole body insulin sensitivity. In addition, a range of modified methods allow estimation of muscle glucose transport, skeletal muscle glucose uptake, splanchnic glucose exchange, regional glucose uptake, glycogen
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synthesis, substrate oxidation, lipolysis, protein turnover, insulin induced vasodilatation and sympathetic activation\(^\text{400}\).

Clamp techniques are labour intensive, take many hours, require frequent sampling and are expensive. Thus they are impractical in studies with large numbers of participants such as epidemiological studies, and may be prohibitive even in moderately sized studies. They are likely to be less acceptable to participants, especially if they are done more than once. For this reason surrogate measures of insulin sensitivity are often used in metabolic studies.

3.4. Surrogates of insulin sensitivity

A large number of surrogates have been developed and none are ideal. A useful surrogate should correlate well with the gold standard HEC across a large range of insulin sensitivity, as well as with disease risk, and it should give reliable results on retest. When insulin sensitivity changes, this should be reflected in the surrogate. Lastly, it should be congruent with contemporary understanding of glucose physiology; if not it may be misleading.

Indices of insulin sensitivity are validated by determining the correlation coefficient of the index against HEC or a more reliable index. The most commonly used surrogates are HOMA-IR, QUICKI, Bergman’s minimal model and the Matsuda index. Surrogates can be divided into those measuring the response to an intravenous (IV) or oral glucose load, and those based on fasting insulin and glucose.

3.4.1 Intravenous glucose tolerance test

Bergman’s Minimal Model is a simplified computer model of the relationship between insulin and glucose levels following a single IV glucose injection\(^{392,402-404}\). From the insulin and glucose response measured during a frequently sampled intravenous glucose tolerance test (IVGTT), the insulin sensitivity index can be calculated\(^{402}\).

Following the IV glucose load, there is a burst of insulin secretion within the first 10 minutes, the first phase response, and the plasma glucose peaks and rapidly drops. The model assumes that this drop is entirely due to insulin dependent uptake into muscle and adipose cells (which reflects insulin sensitivity), and insulin independent uptake into other cells. However, part of the reduction is due to equilibration of glucose from
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plasma to the interstitium. This error leads to overestimate of insulin sensitivity$^{402,405}$ and poor correlation between the minimal model and the HEC gold standard ($r=0.44$)$^{406}$.

Modified protocols include a second burst of insulin after equilibration of plasma and interstitial glucose. In the tolbutamide modified method, the sulphonylurea is administered 20 minutes after the glucose infusion starts, inducing endogenous insulin secretion$^{405,407}$. The method gives an estimate of insulin sensitivity mathematically equivalent to HEC and strongly correlated to it ($r=0.87$)$^{402}$. Tolbutamide is no longer available so an alternative protocol uses an insulin infusion at 20 minutes. It underestimates insulin sensitivity because exogenous insulin initially leads to greater systemic than portal insulin concentration, so that there is a period of poorer inhibition of hepatic glucose release, but it is similarly correlated to HEC$^{407}$.

The IVGTT is less expensive and labour intensive than the HEC, and provides a similar estimate of insulin sensitivity, but it remains more labour intensive than other surrogates, requires intravenous access and a large number of blood draws and assays.

3.4.2 Surrogates derived from the oral glucose tolerance test

An oral glucose tolerance test (OGTT) is more similar to a meal. By measuring the insulin and glucose response, a number of surrogate indices of insulin sensitivity can be calculated, most of which utilise both fasting glucose and insulin and their response to the glucose load$^{408-410}$. Thus they incorporate both hepatic (predominantly reflecting in fasting values) and peripheral (predominantly post-prandial) insulin sensitivity$^{410-413}$.

Many surrogates have been developed and compared to HEC. The Matsuda index$^{410}$, has been included in most comparative studies and performs well, making it a reasonable choice. In the studies with >100 participants, the correlation coefficient for Matsuda with HEC was strong; 0.66-0.78$^{410,412,414,415}$. Excluding studies with a high proportion of diabetics, the Matsuda had similar correlation to the Belfiore$^{410,414}$, Stumvoll$^{412,414}$ and OGIS indices$^{416}$, and outperformed the Gutt$^{414}$ and Cederholm$^{410,414}$ indices. The Soonthornpun index ($r=0.87$) may correlate to HEC better than the Matsuda index, but needs independent validation$^{417}$.

Oral indices are probably not appropriate in patients with T2DM. Many factors can influence the glucose and insulin concentrations following a glucose load confounding the estimate of insulin sensitivity, including β-cell function$^{419}$, the rate of gastric emptying$^{419}$ and efficiency of glucose absorption$^{418}$. β-cell function is
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Reduced in impaired glucose tolerance (IGT) and T2DM, and gastric emptying is frequently delayed in T2DM. Consistent with this correlation between OGTT derived indices and HEC is poorer in the studies with the greatest proportion of diabetic subjects, and in one study the diabetic subgroup, demonstrated poorer correlation than in non-diabetics.

Repeatability and sensitivity to change are also important, particularly if participants will be assessed more than once. Oral indices are relatively insensitive, for example a 50% reduction of insulin sensitivity led to a 9% reduction of Matsuda index in simulation. However the Matsuda index is highly repeatable (r=0.89 for repeat measures). Thus where an OGTT is used to estimate insulin sensitivity the Matsuda index is a good choice in non-diabetics.

3.4.3 Fasting indices of insulin sensitivity

Fasting indices of insulin sensitivity are derived from a combination of fasting plasma glucose, insulin or triglycerides. As they use only fasting measures, they predominantly reflect hepatic insulin sensitivity so that defects of peripheral insulin sensitivity may not be detected. The most reported fasting indices are HOMA-IR and QUICKI which are valuable because of their ubiquity.

Glucose is tightly regulated in non-diabetics, even in the presence of insulin resistance, as insulin levels rise to normalise blood glucose. Thus in the fasting state insulin resistance manifests as increased plasma insulin with normal glucose. This means that the variation in fasting indices of insulin sensitivity, is predominantly due to variation in fasting insulin and that the fasting insulin concentration (or 1/insulin) is a reasonable proxy for insulin sensitivity, but fasting glucose is not. However, if there is relative insulin insufficiency, then glucose will rise and 1/insulin will overestimate insulin sensitivity. Thus glucose must also be considered.

The fasting glucose:insulin ratio (G:I) has been claimed to be a useful measure of insulin sensitivity, but it is misleading. In non-diabetics G/I and 1/I are equivalent, but in diabetics, the elevated glucose and inappropriately low insulin raise the G/I ratio erroneously suggesting improved insulin sensitivity. Unsurprisingly, G/I does not correlate with HEC.
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Homeostasis model assessment and QUICKI

The homeostasis model\(^{425}\) is based on the reciprocal physiological relationship between insulin and glucose. In this model the degree to which fasting glucose is elevated, represents impairment of β-cell function (insulin release), and the degree to which fasting insulin is elevated represents insulin resistance. HOMA-IR is the index of insulin resistance derived from the homeostasis model and is calculated from fasting glucose and insulin, or using a computer model\(^{425}\). HOMA-IR rises with increases of fasting glucose or insulin and thus is physiologically appropriate. The quantitative insulin sensitivity check index (QUICKI) is also derived from fasting glucose and insulin\(^{426}\), and though developed independently\(^{426}\) it is equivalent to Log HOMA-IR\(^{392}\). Both QUICKI and Log HOMA-IR have moderately good repeatability (\(r = 0.69\) and \(r = 0.79\) respectively)\(^{427}\).

Comparing fasting indices

A number of other fasting indices have also been developed\(^{414,428}\). The best comparative data comes from two studies\(^{414,428}\), which show that all fasting indices correlate to a similar degree with HEC. Interestingly, the correlation of HOMA-IR to HEC has varied greatly between studies (\(r = 0.23-0.71\))\(^{410-412,414,416,417,428-434}\), suggesting important differences between different patient populations. For example, in Japanese subjects HOMA-IR correlated very poorly with HEC (\(r = 0.23-0.26\))\(^{431,432}\). Nevertheless, if a fasting index is to be used, HOMA-IR is a good choice because of its widespread use.

3.4.4 Fasting versus Oral GTT derived indices

In studies comparing the correlation of indices to HEC, correlation coefficients are slightly lower for fasting indices than for the Matsuda index and other oral GTT derived indices\(^{410-412,414,416,417,431-434}\), with some exceptions\(^{429,430}\). One of these exceptions was a study including a high proportion of diabetics\(^{429}\). In another study HOMA-IR correlated with HEC better in obese than lean subjects, and even more strongly in those with T2DM, possibly because the greater fasting insulin could be measured with better precision\(^{427}\). However, this advantage does not extend to the OGTT derived indices\(^{418}\), thus in studies of insulin sensitivity in diabetics, if HEC or modified IVGTT cannot be used, fasting indices are more appropriate than those based on an OGTT.

3.4.5 Assessing insulin sensitivity in animal models

Animal models are commonly used in studies where insulin sensitivity is an outcome. HEC remains the gold standard method in rodents and non-human primates, however QUICKI and HOMA-IR are considered a
Introduction
reasonable and reliable approximation\textsuperscript{435}. Correlation coefficients for QUICKI and HOMA-IR to HEC is 0.58 and 0.56 respectively in non-human primates\textsuperscript{436}, 0.4-0.75 and 0.41-0.71 respectively in rats\textsuperscript{437,438} and \( r=0.44 \) and 0.45 respectively in mice\textsuperscript{439}. Recently, the use of Matsuda index in rats has been reported\textsuperscript{440,441} and this index has the advantage of integrating fasting and post-prandial data.

3.5. Choosing an appropriate measure of insulin sensitivity

HEC represents the gold standard method of measuring insulin sensitivity and with modification allows many other mechanistic variables to be measured. It is expensive, labour intensive and a significant inconvenience to participants of a study. Thus may be most appropriately used in small studies, designed to identify small effects, or to investigate very specific metabolic effects.

The IVGTT with insulin infusion is less intensive than HEC but more so than an OGTT and only fractionally more robust in non-diabetics. OGTT based indices such as the Matsuda index offer a small advantage over fasting indices, correlating better to HEC, and better reflecting hepatic and peripheral insulin sensitivity, but are more labour intensive and not appropriate in diabetics. Thus in moderately sized studies of non-diabetics where insulin sensitivity is the major focus IVGTT or OGTT are most appropriate. Fasting indices such as HOMA-IR are simple, cheap and of only minor inconvenience to subjects. As the advantage of OGTT over these is small, where insulin sensitivity is not the primary outcome, fasting indices may be preferred\textsuperscript{442}.

3.6. Assessing the adequacy of insulin secretion

When assessing glucose metabolism, the adequacy of insulin secretion should be considered as impaired insulin secretion leads to decompensation from an insulin resistant state to T2DM. Importantly, some interventions, such as n-3 PUFAs may influence both insulin secretion\textsuperscript{122,126} and insulin sensitivity\textsuperscript{121,387-391).

Insulin secretion can be assessed by the hyperglycaemic clamp where a glucose infusion is adjusted to maintain a constant glucose level and the insulin secretory response is measured\textsuperscript{128}, but this is labour intensive.

Insulin secretion is more frequently assessed during a FSIVGTT. The area under the insulin curve above baseline in the first 8 minutes gives the acute insulin response (AIR) which is an index of insulin secretion\textsuperscript{443}. However the AIR cannot be interpreted in isolation because of the hyperbolic relationship between it and
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Insulin sensitivity is where insulin sensitivity is high less insulin is secreted (which is appropriate), conversely insulin resistance is associated with greater insulin secretion. The disposition index (DI) calculated by multiplying the acute insulin response and insulin sensitivity is a constant that reflects the adequacy of insulin secretion in the context of the ambient insulin sensitivity.

The oral disposition index is an analogous measure derived from the oral glucose tolerance test. Following a glucose load the incremental increase of insulin from baseline to 30 minutes is divided by the incremental increase in glucose, to give the insulinogenic index. This is then multiplied by the Matsuda index of insulin sensitivity to give the oral disposition index. This index correlates modestly to the IVGTT disposition index (r = 0.22), but has been shown to be predictive of incident T2DM. Thus it is a useful index of insulin secretion that can be calculated when the Matsuda index is derived from an OGTT.

HOMA-β is an index of insulin secretion derived from fasting values, but as it is not adjusted for insulin sensitivity, it can be misleading. Further, it fails to identify reductions in insulin secretion that are identifiable with the oral disposition index, and it does not correlate with the IV disposition index. Thus when insulin secretion is of interest, the disposition index should be calculated from an IVGTT or OGTT.
Chapter 4. Omega-3 polyunsaturated fatty acids as a nutritional intervention

4.1. Omega-3 and omega-6 polyunsaturated fatty acids: definition, sources and basic metabolism

Omega-3 (n-3) fatty acids are polyunsaturated (they have two or more carbon-carbon double bonds, with the first double bond between the 3rd and 4th carbons from the methyl end of the fatty acid chain, while n-6 indicates it is between the 6th and 7th carbons. As animals lack the necessary enzymes to desaturate carbons in in the 3 or 6 positions, n-3 and n-6 PUFAs cannot be synthesised de novo. Thus α-linolenic acid (ALA, n-3) and linoleic acid (LA, n-6), the shortest fatty acids in their class, are essential in the diet. However, animals can desaturate and elongate these essential fatty acids. This converts ALA to the long chain n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and converts LA to the long chain n-6 PUFA arachidonic acid (AA) (Figure: 1). The majority of consumed ALA is stored in intracellular lipid pools, 30% undergoes β-oxidation for energy production, and only a fraction (0.5%) is incorporated into cell membrane phospholipid.
Introduction

Figure: 1 Elongation/desaturation pathway of n-3 and n-6 PUFAs

Note that both pathways are catalysed by the same enzymes⁴⁴⁹
Introduction

In the modern diet, LA is much more abundant than ALA. The estimated n-6:n-3 ratio is currently 10-50:1\textsuperscript{448,450}, very different to palaeolithic man, where ALA equalled\textsuperscript{450} or exceeded LA consumption\textsuperscript{451}. This leads to a greater intracellular pool of LA which competes for desaturation by the rate limiting enzyme (Δ6-desaturase)\textsuperscript{448}, reducing EPA production\textsuperscript{448,450}. As a result when ALA is consumed, only 0.2% is converted to EPA and 0.05% to DHA\textsuperscript{452}. Even substantial increases in dietary ALA, increase EPA only slightly, with no effect on DHA\textsuperscript{453}. ALA, itself is probably not metabolically important\textsuperscript{448}. Thus for most people, in order to produce a meaningful increase in EPA and DHA, these fatty acids must be included in the diet. As very little EPA or DHA are found in fat stores\textsuperscript{449}, there is a need for continuous consumption.

ALA is found in some grains and nuts including flaxseed, canola and walnuts\textsuperscript{448}, but only traces of EPA and DHA are found in plant material, and small quantities in commonly eaten meats\textsuperscript{454}. Marine sources such as oily fish, krill and calamari are rich in EPA and DHA\textsuperscript{454,455}. Ultimately marine n-3 PUFAs are produced by algae\textsuperscript{455}, and pass through the food chain.

4.2. N-3 PUFA rich supplements are popular

Marine n-3 PUFA supplements, are the most popular (non-vitamin non-mineral) dietary supplements in the United States (US)\textsuperscript{456}. Marketing focussed on the purported cardiovascular, anti-inflammatory and neurocognitive benefits of fish oil led to a meteoric rise in use\textsuperscript{456-458}, such that n-3 supplements are now used by more than a third of the 17% of American adults who take a dietary supplement\textsuperscript{456}. World sales are in excess of a billion US dollars per year, and are projected to increase\textsuperscript{459}. Data from New Zealand is limited but in a sample of older New Zealand men 13% were taking fish oil\textsuperscript{460}, while studies in women who were pregnant or undergoing assisted fertility indicated 14-20% of women were taking fish oil\textsuperscript{461,462}. These data indicate that fish oil supplementation is common in potentially vulnerable populations, as older men have greater than average cardiovascular risk, and in pregnancy, the fetus is at special risk from chemicals that could influence growth or development.

4.3. Marine oils are a highly complex intervention

n-3 PUFAs have many biological effects, and marine oils also contain other fatty acids and lipids and potentially a large range of lipid soluble molecules (products typically claim 30-60% n-3 PUFA by weight). Krill oils may contain a wider range still, as the PUFAs are in the form of phospholipids\textsuperscript{463,464}, which are amphipathic, thus these oils may also contain water soluble molecules.
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The metabolic effects of n-3 PUFAs through the transcription factors PPAR-α, PPAR-γ, SREB1-c and HNF4α, the enzyme COX-2 and GPR-120 have been introduced (Section 2.5.1) and suggest potentially important effects on insulin sensitivity, insulin secretion and the regulation of energy balance. In addition to these potentially important metabolic effects, n-3 PUFAs also have a range of other actions:

Incorporation of EPA and DHA into cell membrane phospholipid increases cell membrane fluidity, dispersing transmembrane enzymes and receptors that are clustered together in rigid parts of the membrane called “lipid rafts”\(^\text{465,466}\). Once dispersed these function less efficiently. This mediates the inhibitory effect of n-3 PUFAs on T lymphocyte activation\(^\text{467}\). As previously discussed, these n-3 PUFAS also inhibit inflammation (and platelet aggregation) through altering the balance of pro- and anti-inflammatory eicosanoids, resolvins, protectins and endocannabinoids\(^\text{450,465,468}\), and through activating GPR-120\(^\text{121}\), PPAR-α\(^\text{333,368}\) and PPAR-γ\(^\text{354,357,359-361}\).

n-3 PUFAs function as antioxidants, as they react with reactive oxygen species\(^\text{469}\). However, as this produces a lipid peroxide radical which itself is highly reactive\(^\text{470}\), this may not be beneficial. Further, as will be discussed n-3 PUFAs may oxidise to lipid peroxides prior to consumption\(^\text{470}\). In one study 200mg/day DHA had an anti-oxidant effect, but 1600mg/day increased oxidative stress\(^\text{471}\). The redox effects of a marine oil may depend on a number of other contextual factors such as dosage, the degree of peroxidation prior to consumption, the concentration of anti-oxidants in the oil (such as tocopherols and astaxanthin) and already in the body, and other factors that influence oxidative stress such as obesity\(^\text{472}\), infection\(^\text{473}\), or hyperglycaemia\(^\text{299}\).

There is increasing evidence that antioxidants do not positively influence the risk of cardiovascular disease\(^\text{474}\), and may even increase mortality\(^\text{475,476}\). Thus it is unlikely that any beneficial effects of n-3 PUFAs are related to its activity as an antioxidant.

Thirdly, DHA is a major structural component of the central nervous system; making up 35% of fatty acids within synaptic membranes\(^\text{477}\). DHA uptake is rapid in late gestation and infancy and deficiency is associated with abnormalities of retinal function\(^\text{477,478}\). Consensus guidelines recommend women consume 200mg/day of DHA during pregnancy\(^\text{479}\). In addition, supplementation with n-3 PUFAs has been investigated as a treatment for a wide range of neurological and psychiatric disorders including psychosis, depression, Alzheimer’s disease, multiple sclerosis, autism and attention deficit disorder\(^\text{480}\).
Introduction
Lastly, like any fat, n-3 PUFAs can undergo β-oxidation to produce energy or be stored in intracellular lipid pools.

The potential effects of n-3 PUFA supplementation are many and varied. To target a similar range of biological effects with pharmaceuticals, one would need to combine a COX-2 inhibitor, a fibrate (PPAR-α agonist), a thiazolidinedione (PPAR-γ agonist), an anti-arrhythmic, and a GPR-120 agonist. But even this complex cocktail of medications would not account for n-3 PUFAs energy content, structural role in the brain, or potential anti- and pro-oxidant effects. Further, marine oils, are not pure n-3 PUFA supplements; they contain other biologically active chemicals and the composition may be different between marine species, in different seasons and in different fishing locations\textsuperscript{481,482}. Thus marine oils constitute a highly complex intervention.

4.4. Is the dietary n-6:n-3 ratio important?

While the dietary n-6:n-3 ratio is higher than in palaeolithic man\textsuperscript{448,450,451}, it is unclear whether reducing this ratio, or simply increasing n-3 PUFAs would be most beneficial. These options are not independent as increasing n-3 PUFAs accomplishes both. However if the n-6:n-3 ratio is important it implies that n-6 PUFAs should be reduced. Simopoulos advocated reducing this ratio to different levels, depending on the indication, for example 5:1 to improve asthma, 4:1 to reduce cardiovascular mortality and 2.5:1 to reduce cellular proliferation in rectal carcinoma\textsuperscript{450}. Whether the ratio is important might depend on the mechanism of the targeted effect. For example, where a direct agonist effect of n-3 PUFAs is required increasing n-3 PUFAs would be sufficient. Insulin sensitivity would be predicted to be such an indication because n-3 PUFAs activate GPR-120\textsuperscript{121}, PPAR-γ\textsuperscript{355}, PPAR-α\textsuperscript{366} and SREB1-c\textsuperscript{379}, and n-6 PUFAs also activate most of these molecular targets\textsuperscript{355,366,379}. Thus reducing n-6 PUFAs might be counterproductive. However, in the treatment of inflammatory disease (outside of adipose tissue), reducing n-6 and increasing n-3 PUFAs, might be more beneficial because of the effects on the production of eicosanoids and other inflammatory mediators. Better understanding of the mechanistic effects of n-3 and n-6 PUFAs may allow for more rational recommendations.

Interestingly, a recently published study showed that replacing saturated fat in the diet with corn oil, rich in n-6 PUFAs increased cardiovascular risk\textsuperscript{483}. This suggests that n-6 PUFAs are not benign.
4.5. Evidence for an insulin sensitising effect of n-3 PUFAs

As has been introduced n-3 PUFAs affect a number of transcription factors and receptors that have important effects on adipose tissue and could counter the metabolic dysfunction of obesity.

4.5.1 Evidence from animal models

Evidence from rodent models of insulin resistance show that insulin resistance can be prevented or reversed by supplementation with fish oil. This is true in high fat\textsuperscript{121,387}, high sucrose\textsuperscript{388-390} and high fructose\textsuperscript{391} models of insulin resistance.

\textit{Adipose tissue}

Supplementation with fish oil, improves key aspects of adipose metabolic function including insulin stimulated glucose uptake\textsuperscript{121,484,485}, the secretion of adipokines (adiponectin and leptin)\textsuperscript{486,487}, and inflammatory cytokines (IL-6 and TNF-\(\alpha\))\textsuperscript{488}. In addition it normalises adipocyte cell size\textsuperscript{389} and reduces adipose inflammation\textsuperscript{121}. The most important mechanism responsible for this effect is mediated by GPR-120\textsuperscript{121,385}, a receptor for n-3 PUFAs, although effects on adiponectin secretion appear to be PPAR-\(\gamma\) dependent\textsuperscript{486}.

\textit{Skeletal Muscle}

N-3 PUFAs prevent and ameliorate the negative consequences of a high saturated fat diet on muscle\textsuperscript{340,489}. A HFD leads to increased TLR-4 expression, and impairment of adiponectin induced fatty acid oxidation, and insulin-stimulated glucose transport\textsuperscript{489}. All these effects were prevented by supplemental n-3 PUFAs\textsuperscript{489}, maintaining muscular insulin sensitivity\textsuperscript{121}. Further, addition of n-3 PUFAs into the diets of animals already consuming a HFD, led to normalised adiponectin induced fatty acid oxidation, increased expression of GLUT-4, and reduced expression of TNF-\(\alpha\) and IL-6\textsuperscript{488}. 
Introduction

Liver

n-3 PUFAs also improve hepatic metabolic disease in rodents. Addition of n-3 PUFAs to a HFD restored hepatic insulin sensitivity and decreased intra-hepatocellular lipid\textsuperscript{121}. As this effect was greatly reduced in GPR-120 knockouts, a receptor not expressed by hepatocytes, the major mechanism is through improved adipose tissue function\textsuperscript{121}. However, a direct PPAR-\(\alpha\) mediated effect of n-3 PUFAs also contributes\textsuperscript{490}.

Non-human primates

Insulin sensitising effects of n-3 PUFAs have also been shown in rhesus macaques, normalising the insulin resistance and hypertriglyceridaemia induced by a high fructose diet\textsuperscript{491}.

Summary – animal models

N-3 PUFAs reverse insulin resistance in multiple models of metabolic dysfunction, including a non-human primate. This is supported by mechanistic evidence that fits well with the current understanding of the pathophysiology of insulin resistance.

4.5.2 Evidence from cross-sectional and longitudinal cohort studies

Fish consumption and diabetes risk

Most epidemiological evidence for an insulin sensitising effect of n-3 PUFAs comes from longitudinal studies that estimate fish, n-3 PUFA consumption or the n-3 content of circulating lipid and assess the later incidence of T2DM. This approach has many limitations, and evidence is mixed.

In a large ecological study across 41 countries, greater per capita fish consumption was associated with lower prevalence of T2DM\textsuperscript{492}. In addition a number of small and large longitudinal studies based on dietary questionnaires have shown greater fish consumption is associated with lower fasting glucose\textsuperscript{493}, 2hr blood glucose (OGTT)\textsuperscript{494}, and reduced incidence of IGT\textsuperscript{495} and T2DM\textsuperscript{496,497}. However, there are conflicting findings. In a study of 116000 Chinese men and women, greater fish consumption reduced diabetes risk only in women, but shellfish reduced risk to both sexes\textsuperscript{496}. In contrast in a cohort of 25000 English men and women, fish
Introduction

consumption reduced risk in both sexes, but shellfish increased it\textsuperscript{497}. In a smaller Dutch cohort, lean fish increased risk of diabetes, while oily fish had no effect\textsuperscript{498}. In two large studies in the US\textsuperscript{499,500}, greater fish consumption was associated with a greater risk of diabetes.

A variation of these analyses uses dietary questionnaires and associates intake with later risk. While n-3 PUFA consumption was shown to be beneficial in Chinese women\textsuperscript{496}, no effect was seen in some cohorts\textsuperscript{496,498,501,502}, with increased risk of diabetes in others\textsuperscript{499,503}.

Cohort studies may be confounded

Three meta-analyses have examined the relationship between fish consumption and diabetes risk\textsuperscript{504-506}; only one suggested that greater consumption of EPA and DHA led to a small reduction in the risk of diabetes\textsuperscript{505}. However, disparate effects are shown in different parts of the world\textsuperscript{505,506} and when eastern and western countries were separated, eastern countries showed a stepwise reduction in diabetes incidence with increasing fish consumption, while western countries saw a stepwise increase in risk\textsuperscript{506}. This suggests that the metabolic effects of fish consumption vary according to location. Such a difference could be mediated by differences in the type of fish consumed, its n-3 PUFA content, methods of cooking, or differences in other chemical compounds in the fish including contaminants.

Oily fish have greater n-3 PUFA content than non-oily fish, but there are also differences depending on fish species, habitat (fresh vs salt water), and the season and geographical location of catch\textsuperscript{481,482,496,507}. Deep frying of fish reduces polyunsaturated fatty acids (due to oxidation), increases the ratio of n-6:n-3 fatty acids and in some species increases total fat\textsuperscript{508}. Further, the unsaturated fats in deep frying oils transform to trans-fatty acids\textsuperscript{509}, which are associated with cardiovascular disease\textsuperscript{510} and inhibit the desaturation/elongation of essential fatty acids\textsuperscript{511}.

A number of other factors may affect the health effects of consuming fish. Methyl-mercury accumulates in fish throughout their life, so is highest in long lived fish. In vitro and in vivo animal models show that low dose methyl-mercury impairs β-cell function and induces apoptosis\textsuperscript{512,513}. Fish consumption also increases selenium levels\textsuperscript{514,515}, and selenium supplementation may increase diabetes risk\textsuperscript{515,516}. Pollutants and toxins may also be important. The serum concentration of persistent organic pollutants is strongly associated with diabetes risk\textsuperscript{517} and freshwater fish, and shellfish may accumulate these more\textsuperscript{518}. Shellfish also have high cholesterol content. Lastly, the n-3 PUFA content of farmed fish depends on the content of the fish feed\textsuperscript{519}. 49
Introduction

Socioeconomic status affects health outcomes including obesity and all aspects of the metabolic syndrome\textsuperscript{520-522}. It could confound studies of fish consumption, due to the relative price of fish, or because more educated people may know more about the health benefits of oily fish. Moreover, people who eat fish regularly may be more concerned about their health, and have other positive behaviours relating to diet or exercise. None of the epidemiological studies have controlled for exercise\textsuperscript{492-500}.

Epidemiological studies rely on dietary questionnaires and assume that fish intake at the time of survey is representative of the long term diet prior to the survey and for up to 20 years after the survey when the incidence of T2DM is estimated. This may not be true. Further, as development of diabetes occurs after many years of obesity, shorter studies may fail to demonstrate a real effect of eating fish. Lastly, if n-3 PUFAs reduce diabetes risk by improving insulin sensitivity, the effect would be greater in insulin resistant groups (e.g. the elderly and obese). Studies not confined to at-risk groups may be underpowered.

In summary, the mixed evidence relating fish consumption to diabetes risk may be due to environmental factors related to eastern and western populations but there are many limitations.

\textit{Circulating n-3 PUFA and risk of T2DM}

Another variation of these studies, measures circulating n-3 PUFAs and relates this to long term diabetes risk. These studies are fewer, and use smaller cohorts. In an elderly US cohort, greater n-3 PUFAs in plasma phospholipid were associated with reduced incidence of diabetes over a 10 year period\textsuperscript{523}. This was not shown in middle-aged adults in Sweden\textsuperscript{524} or the US\textsuperscript{502}, however, n-3 PUFA levels in their participants were low. Thus if there is a threshold effect, above which n-3 PUFAs are insulin sensitising, these results could be explained. These studies may be confounded by the factors previously discussed, and it must also be considered that circulating levels of fatty acids are determined by relatively recent diet over the preceding weeks, and may not be representative long term.

4.5.3 Evidence from cross-sectional studies

Cross-sectional studies enable indices of glucose metabolism to be measured simultaneously with n-3 PUFAs, reducing the confounding introduced by changes in diet or PUFA levels over time. Further, indices of both insulin sensitivity and secretion can be measured.
Introduction

In a study of obese adolescents, there was an inverse relationship between the omega-3 index (%EPA+DHA in red cell phospholipid) and HOMA-IR, showing greater n-3 PUFAs were associated with greater insulin sensitivity.\(^{525}\) In men, greater levels of DHA, were associated with insulin sensitivity whether measured in muscle or serum.\(^{526}\) In a similar study, AA and LA in muscle were associated with greater insulin sensitivity, but a relationship with n-3 PUFAs alone was not found,\(^{527}\) however n-3 PUFA levels in that study were very low.\(^{527}\) A Chinese study showed a positive relationship between EPA and DHA and insulin sensitivity in diabetic and non-diabetic subjects.\(^{528}\)

In summary, cross-sectional studies suggest insulin sensitising effects of n-3 PUFAs. Ultimately, in order to demonstrate any causal effect of fatty acids on insulin sensitivity, interventional studies are required.

4.5.4 Evidence from randomised controlled trials

The problems with studies in participants with T2DM

Non-diabetic obese people, have adipose tissue inflammation,\(^{203}\) abnormal adipokine secretion,\(^{218}\) and accumulation of intracellular lipid in muscle and liver that interferes with insulin signalling.\(^{226}\) Diabetic subjects are likely to have had greater chronicity of adipose inflammation and dysfunction, greater intracellular lipid accumulation, and more severe insulin resistance. In addition relative β-cell failure leads to hyperglycaemia and accumulation of AGEs, which cause oxidative stress and activation of inflammatory pathways through NF-κB. Thus, it seems likely that insulin resistance would be less easily reversed in T2DM than in non-diabetic obesity. Further, standard treatments of diabetes includes medications, dietary modification and exercise, all of which may influence insulin sensitivity or secretion. Meta-analyses in type 2 diabetics have shown no effect of supplemental fish oil on glycaemic control,\(^{529,530}\) but this does not preclude an effect in non-diabetic overweight or obesity. Thus potential insulin sensitising effects of n-3 PUFAs should be examined in people without diabetes.

The meta-analysis of RCTs investigating insulin sensitising effects of n-3 PUFAs is flawed

A meta-analysis collating RCTs investigating the effects of n-3 PUFA supplementation on insulin sensitivity, showed a small insulin sensitising effect in those studies that measured the index of insulin resistance, HOMA-IR, but no effect overall.\(^{531}\) However the included trials were highly heterogeneous, with a low total patient
Introduction

number (n = 618). Of the 11 RCTs, 3 used no control intervention and 3 included participants with diabetes mellitus. One study was in elderly participants receiving total enteral nutrition for a period of at least 6 months. This highly abnormal cohort, had no increase in n-3 PUFAs after supplementation suggesting malabsorption. One study was of supplemental ALA which would be unlikely to affect insulin sensitivity due to poor conversion to LC-PUFAs and storage predominantly outside of lipid membranes and circulating lipid fractions. Lastly, the dose range varied widely (0.138g to 4g/day), and some studies enrolled patients taking medications known to affect insulin sensitivity and insulin secretion.

Thus it is appropriate to consider the RCTs individually.

**RCTs showing metabolic benefits of n-3 PUFA supplementation**

In overweight children, supplementation with n-3 PUFAs (900mg/day) reduced HOMA-IR, TNF-α, and leptin and increased adiponectin, indicating improved insulin sensitivity and adipokine profile. Further, the insulin sensitising effect was synergistic with weight-loss. Similar results were shown in two studies of overweight adults on an energy restrictive diet; addition of 3 oily fish meals a week, or fish oil (1300mg/day of n-3 PUFAs) led to reduced HOMA-IR. In 12 elderly adults, replacement of fish in the diet with oily fish led to an improvement in a direct measure of insulin sensitivity.

Studies in patients with NAFLD indicate insulin sensitising effects; in children DHA supplementation improved insulin sensitivity (Matsuda), and reduced liver fat and plasma triglycerides, while in adults, adding fish oil to an American Heart Association diet improved HOMA-IR.

In three studies, it is likely that n-3 PUFAs had insulin sensitising effects, but fasting tests (insulin, glucose, HOMA-IR and QUICKI) were too insensitive to change. In the first, adding fish oil to lifestyle advice led to greater reductions (than advice alone) of glucose and insulin areas under the curve (OGTT), and increased adiponectin. In the second, weight-loss plus fish oil led to greater improvement of HOMA-IR than weight-loss alone (no effect of fish oil alone). In the third, n-3 PUFAs led to improved endothelial function and adiponectin and reduced TNF-α; factors known to be associated with insulin sensitivity, but there was no effect on QUICKI.

**RCTs where the metabolic effects of n-3 PUFAs were unclear**

In Indian participants randomised to high or moderate n-6 PUFA diets, and then assessed before and after fish oil supplementation, fish oil lead to a large reduction in circulating FFAs (12% and 25% in the moderate and
Introduction

high n-6 PUFA groups respectively\(^{348}\). The difference was not significant, and insulin sensitivity did not differ\(^{348}\), but power may have been insufficient.

In a larger trial of patients with metabolic syndrome the Mediterranean diet led to reduced HOMA-IR in addition to improvements in weight, lipid profile, BP, endothelial function and inflammatory cytokines\(^ {160}\). However, there were many dietary changes in addition to increased n-3 PUFAs and the principle n-3 PUFA was ALA (not EPA or DHA).

In a small study of obese adolescents (14 females and 11 males), supplementation with n-3 PUFAs improved insulin sensitivity (HEC and IVGTT) in females but not males\(^ {549}\). This raises the possibility of sex specific effects on insulin sensitivity, but the numbers of males were small, and in this age group there could have been confounding by differences in pubertal stage.

In a study of overweight male children and adolescents, fish oil was baked into bread\(^ {536}\). No difference was seen in HOMA-IR. However, the majority of participants correctly guessed which intervention they took\(^ {536}\), suggesting detectable differences in the bread. Compliance was estimated by self-report at 90% but this is unlikely as adolescents typically have poor compliance\(^ {550}\), and self-report is unreliable\(^ {536}\).

**RCTs showing absent or unfavourable metabolic effects n-3 PUFAs**

The KANWU study, looked at the effects of n-3 PUFA supplementation in participants also randomised to receive a diet enriched with monounsaturated or saturated fatty acids\(^ {551}\). A moderate dose of n-3 PUFAs was used and insulin sensitivity was measured using the IVGTT, a high quality measure. No effect was seen on insulin sensitivity or insulin secretion. However, as the BMI range of included participants included those in the “normal weight” of 22-25kg/m\(^ 2\), the ability to show improvement may have been reduced. Nevertheless this is an important negative finding that requires replication.

Lastly, a small (n=17/group) parallel RCT in women with polycystic ovarian syndrome, an insulin resistant group, showed that supplementation with fish oil led to reduced insulin sensitivity (Matsuda index)\(^ {552}\).
Introduction

4.5.5 Summary of current evidence for an insulin sensitising effect of n-3 PUFAs

It is clear that in the rat, n-3 PUFAs have a potent insulin sensitising effect. Epidemiological evidence is mixed, and potentially confounded. In the context of an Eastern lifestyle, fish consumption may reduce the incidence of T2DM, suggesting it may increase insulin sensitivity, insulin secretion or both. The RCT evidence is conflicting and there are major differences between studies, making meta-analysis invalid. Many of the studies are very small which both reduces power and increases the risk that positive results are spurious. Many studies rely solely on fasting indices of insulin sensitivity, which are less sensitive and do not assess peripheral insulin sensitivity. Some studies suggest that consuming n-3 PUFAs in fish oil, or oily fish leads to improvements in insulin sensitivity, especially in the context of energy restricted diet or weight-loss. However, the relatively large KANWU trial throws doubt on the insulin sensitising effects of n-3 PUFAs. There remains a need for carefully designed RCTs comparing a marine oil to a suitable control, without other changes in diet or physical activity.
4.6. Oxidation of marine omega-3 supplements and human health

4.6.1 Preface

This chapter consists of a modified version of a manuscript published in the journal *Biomed Research International*[^553].

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4.6.2 Introduction

Marine oils, rich in EPA and DHA are very popular[^456,457,460,461] and are used for a range of indications including cardiovascular protection[^554], improving metabolism[^555], treating inflammatory disease[^352], improving neurodevelopment in early life[^480] and preventing cognitive decline[^480]. However, it is important to understand that unlike most pharmacological and nutraceutical interventions, these oils are highly susceptible to oxidation[^470]. There has been concern about the safety of oxidized fish oil since the 1950s[^556] and although there is evidence that over-the-counter supplements are frequently oxidized[^557-561], this appears to have had no real impact on the requirements for storage and labelling, or on the design of human clinical trials. No human efficacy trials have reported the oxidative state of the trial oil which brings the validity of the results and conclusions of these trials into question. As it is currently unclear to what degree the oxidation of marine oils influences their efficacy (or harm) interpretation of the trial literature is difficult.

4.6.3 Marine oils are chemically unstable

n-3 PUFAs are highly prone to oxidation due to their large number of double bonds[^470,562]. This is because bisallylic carbons, those between two double-bonded carbon atoms, have a low activation energy for hydrogen loss and free radical formation[^470]. Importantly, the long chain n-3 PUFAs have more bisallylic carbons (EPA: 4, DHA: 5) than ALA (2) (Figure 2) or n-6 PUFAS (e.g. AA: 3) while monounsaturated and saturated fatty acids have none. In the presence of various initiators, a lipid radical is formed starting an expansive chain reaction which creates lipid peroxides and more radicals from unoxidised PUFAs[^470]. A complex array of
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different peroxide molecules arise depending on the position of the oxidized carbon. These undergo cis-trans
isomerisation and a shift of double bonds to produce conjugated dienes and trienes which have different
polarity and shape to the original fatty acid\(^\text{470}\). Some oxidation products have specific biological effects, for
example the F3-isoprostanes and F4-neuroprostanes which are formed from EPA and DHA act similarly to
prostaglandins\(^\text{563}\). Thus the primary oxidation products of n-3 PUFAs are chemically different from unoxidised
n-3 PUFAs and may have different biological properties.

Lipid peroxides are unstable and degrade to form malodourous secondary oxidation products\(^\text{564}\) including
aldehydes such as 4-hydroxyhexenal (HHE) and malondialdehyde (MDA)\(^\text{565}\). As the oil oxidizes there is an
initial exponential increase in the concentration of lipid peroxides. These later degrade and the concentration
of secondary oxidation products increase as the lipid peroxides decrease\(^\text{566}\) (Figure 3).

The rate of peroxidation is influenced by light, heat and oxygen concentration, but it occurs in standard room
conditions\(^\text{470}\) and even oil refrigerated (4°C) in the dark may oxidize unacceptably within a month of storage\(^\text{567}\).
Added antioxidants reduce but do not prevent oxidation\(^\text{568}\). The tendency of n-3 PUFAs to oxidize under light
is also influenced by the presence of impurities such as protein or heavy metals and the molecule they are
conjugated to; phospholipids are more prone to oxidation than triglycerides\(^\text{470}\). Because peroxidation is an
accelerating chain reaction, small concentrations of peroxides in the source oil, or exposure to oxidising
conditions during processing could have a large effect on the rate of oxidation. In addition, deodorisation,
and encapsulation involve high temperature which may accelerate secondary oxidation. Together this suggests
that lipid oxidation is highly likely to occur in over-the-counter marine oil supplements which are commonly
kept at room temperature both in retail shops and in the home.

Because it oxidises, oil in an omega-3 supplement may differ substantially from the oil found in fresh fish. As
a result these supplements should be viewed as a complex mix of EPA, DHA, other fatty acids, additives and
an unspecified concentration of potentially toxic lipid peroxides and secondary oxidation products.
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Figure 2: Shape and structure of n-3 PUFAs

Bisallylic carbons are indicated by an arrow; longer chain n-3 PUFAs have a greater number. Modified from Shahidi\textsuperscript{470}(used with permission).

Figure 3: Time course of oxidation of edible oils.

Lipid peroxides, aldehydes/ketones, and total oxidation as indicated by the peroxide, anisidine, and totox values respectively (Modified from Miller\textsuperscript{566}, used with permission)
4.6.4 Oxidation of marine oils can be easily quantified

Measurement of specific lipid oxidation products requires gas-chromatography mass-spectrometry or other advanced techniques which are expensive and require significant technical expertise. However the oxidative status of supplemental oils can be easily estimated using the peroxide value (PV) and anisidine value (AV) assays. While these are nonspecific they are repeatable, simple and cost effective and there are recommended maximum levels for marine oil supplements. The PV is a simple titration enabling quantification of the concentration of peroxide groups in oil, while the AV is a colorimetric test which enables estimation of the concentration of secondary oxidation products. Both measurements are required to estimate total oxidation (Totox = 2xPV+AV) (Figure 3). Recently, a nuclear magnetic resonance based technique for estimating oxidation of fish oil was described, this method produces data that is very closely correlated to the PV, AV and Totox, from a single test, requiring less labour, sample and solvents. However, this test requires further validation.

Many organisations have endorsed maximum recommended levels of oxidation in supplements with some variation. However, the most common limits are set at PV<5, AV<20 and Totox<26, and these limits are in the collaborative draft guideline produced by the Food and Agriculture Organisation of the UK and the World Health Organisation. Unfortunately, due to the paucity of human evidence these are based on palatability; not established effects on human health. There is a need for evidence based guidelines. Studies of oxidised marine oils and specific oxidation products are required. Even without dedicated studies, the knowledge base could be improved significantly if all clinical trials of marine oils, reported the PV and AV so that the results could be interpreted in the context of the oxidative state.

4.6.5 Over-the-counter marine omega-3 supplements are significantly oxidised

The oxidative states of retail oils are not typically labelled. Early studies assessing oxidation of retail purchased marine oil supplements were highly variable, with 11-62% of products exceeding recommended levels. Recently, there has been renewed interest in this problem, with between 31% and 93% of products tested exceeding recommended levels, while Labdoor, a for-profit North American company reported that 28% of products had twice the recommended limit. Preparation makes a difference, oil presented in liquid form is more oxidised than capsules. Thus consuming purchased supplements will frequently lead to consumption of unacceptably oxidized oil and it is highly likely that the marine oil supplements used in many clinical trials have also been significantly oxidized. Understanding the effects of
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oxidized marine oils on health is important both for the large population of consumers and for scientists and clinicians interpreting the medical literature.

4.6.6 The relative efficacy of oxidised marine oils is unknown

n-3 PUFAs have multiple interacting biological effects including acting as a ligand for intracellular and extracellular receptors, competition for metabolism by enzymes, structural roles in the cell membrane and stearic interference with ion channels. For illustration, triglyceride lowering is mediated by interaction with SREBP1-c and PPAR-α. Anti-inflammatory, hypotensive and anti-platelet effects may be mediated by competition with arachidonic acid for synthesis of eicosanoids by COX. Anti-arrhythmic effects are in part due to stearic interference with ion channels. Insulin sensitisation is partly mediated by interaction with PPAR-γ an intracellular transcription factor and binding to GPR-120 on the cell surface. Omega-3 fatty acids also have anti-oxidant effects and influence cell membrane fluidity.

As lipid peroxides have different shape, polarity and reactivity to their parent fatty acid, it is likely that they will have different biological effects, and may lose efficacy. Further, because these mechanisms are diverse, some beneficial effects may be lost but not others. Lipid peroxides may even have their own unique functions.

Surprisingly, there are no clinical trials investigating the effects of oxidation on the efficacy of n-3 PUFA rich oils. However, in a clinical trial of fish oil supplementation with and without vitamin E, triglycerides decreased significantly more in the vitamin E group, and in another study, vitamin E prevented a glucose-raising effect of fish oil. Vitamin E is primarily an antioxidant thus these suggest oxidation of the n-3 PUFAs (either prior to consumption or in vivo), led to negative biological effects. In an in vitro study, unoxidised prescription n-3 PUFAs prevented oxidation of LDL, but fish oil supplements that were oxidised did not. Interestingly, in a study of liver tissue in culture, oxidized EPA inhibited the inflammatory NF-κB pathway. This may be mediated by n-3 derived isoprostanes, as these peroxides inhibit macrophage NF-κB activation in tissue culture and affect vascular and platelet function. While it is not clear whether these effects are important in vivo, they indicate that the biological effects of marine oils change when they are oxidized. Clearly the effect of oxidation on efficacy of marine oils requires further investigation.
Lipid peroxides are absorbed through the gut and incorporated into chylomicrons, LDL, and VLDL. Secondary oxidation products which can be consumed in the oil, or formed from decomposition of lipid peroxides in the gut are also absorbed. Thus it is important to consider the biological effects of these lipid oxidation products.

Lipid peroxides hasten oxidation of other fatty acids to create more lipid peroxides in an expansive chain reaction. In vivo this could potentially lead to lipid membrane peroxidation, cell damage and oxidative stress. Peroxidation of lipid membranes alters membrane fluidity, transport and cellular signalling. Importantly, acute severe lipid and protein peroxidation is the cause of death when despite appropriate treatment, people die from organophosphate poisoning. Chronic lipid peroxidation may be a mechanism in carcinogenesis and in the pathogenesis of Alzheimer’s disease where the secondary oxidation product 4-hydroxynonenal (HNE) appears to have a role in both the formation of neurofibrillary tangles and neurotoxicity. Oxidative stress activates the NF-κB pathway and increases production of pro-inflammatory cytokines. Chronic low grade inflammation and oxidative stress are involved in atherogenesis and inflammation has a major role in the pathogenesis of insulin resistance (Section 2.3.3-2.3.6).

Animal studies provide clear evidence that oxidized lipids are harmful, though typically using higher doses of oil than humans consume or administering oxidation products in non-physiological ways. Chronic feeding of oxidized PUFAs to rats led to growth retardation, intestinal irritation, liver and kidney enlargement, haemolytic anaemia, decreased vitamin E, increased lipid peroxides and inflammatory changes in the liver, cardiomyopathy and potentially malignant colon cell proliferation. A major secondary oxidation product of omega-3 oils is the aldehyde 4-hydroxyhexanal (HHE). HHE injected into the peritoneum causes necrotising peritonitis and when injected intravenously causes liver damage, it is chemically similar to the better studied omega-6 oxidation product HNE which is highly toxic and causes DNA damage.

Oxidation of LDL has a central role in atherogenesis as it is necessary for the accumulation of lipid in vascular smooth muscle cells and macrophages. Because ingested lipid peroxides are transported in LDL they could potentially enhance atherogenesis. This is supported by a study in rabbits where addition of fish oil to a high cholesterol diet led to rapid atherosclerosis. The oil was probably oxidised as it led to oxidative stress. If this accelerated atherosclerosis was due to oxidation of LDL then oxidised marine oils could be atherogenic in humans.
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Consuming marine oil leads to increased plasma and urinary MDA in humans and mice, due to both absorption of peroxidised oil and in vivo oxidation with subsequent degradation of peroxides. This is reduced but not prevented by addition of antioxidants. MDA induces DNA mutation, causes thyroid tumours when fed to rats and skin cancer when applied topically. The little evidence in humans is unclear, however women with breast cancer have higher concentrations of MDA-DNA adducts in their normal breast tissue than controls, consistent with MDA exposure increasing risk.

Only one RCT has examined the effects of oxidised marine oil in humans. Participants consumed a moderately oxidized or an unoxidised fish oil for 7 weeks. No difference was found in markers of in vivo lipid peroxidation (urinary 8-isoFGF2α, plasma HHE and HNE), markers of antioxidant activity, C-reactive protein or liver function tests. Further, there was no increase of circulating oxidised LDL or inflammatory mediators. This suggests oxidized marine n-3 may not be associated with acute oxidative toxicity. However this is only partially reassuring as the study was short, power was limited and there were no assessments of atherosclerosis (such as carotid artery intima-media thickness), markers of DNA damage, or inflammation in key metabolic tissues such as liver, muscle or adipose tissue. Thus the risks of atherosclerosis, DNA damage, malignancy and inflammation especially at tissue level remain undetermined. If low grade, chronic, peroxide, aldehyde or MDA exposure is important in disease it may require long periods of follow up to identify an effect. Some pathological effects may be difficult to detect without invasive methods such as muscle, liver or adipose tissue biopsy.

While lipid peroxidation is involved in the pathogenesis of human disease, the health effects of consuming oxidised lipids are largely unknown. Evidence in animals suggests they may cause organ damage, inflammation, carcinogenesis and accelerated atherosclerosis. These potential deleterious effects should not be ignored, especially when marine oils are often taken by vulnerable populations including pregnant women, and those at increased cardiovascular risk, and for prolonged periods of time.

4.6.8 Could oxidation explain the disappointing literature surrounding n-3 PUFAs and prevention of cardiovascular disease?

Epidemiological studies link fish consumption to lower cardiovascular risk

In the 1970s the reports of Bang and Dyerberg launched scientific interest in n-3 PUFAs. They showed that Greenland Inuits eating a fatty but n-3 PUFA rich diet of almost exclusively seal meat and fish, had lower triglycerides and markedly lower rates of cardiovascular disease and diabetes than Inuits in Denmark consuming a western diet. Subsequently, greater bleeding time was demonstrated and EPA was shown to inhibit platelet aggregation, implicating n-3 PUFAs. Since this time a very large number of studies have
shown fish consumption to be associated with reduced risk of CVD and this is confirmed by systematic reviews. The greatest benefit appears to be increasing consumption to one fatty fish meal per week, as in populations where average intake is greater, this relationship is not demonstrated. The importance of n-3 PUFAs is further suggested by studies that show greater plasma n-3 PUFAs are associated with a lower risk of sudden death.

**RCTs with risk factors or surrogates as outcomes show beneficial effects of fish oil supplementation**

RCTs and meta-analyses have shown supplementation with fish oil or fortified foods leads to favourable changes in a range of cardiovascular risk factors, or surrogates including lipid profile, BP, heart rate, heart rate variability, platelet aggregation, endothelial function, and atherosclerotic plaque stability. Thus considerable evidence from RCTs examining surrogate outcomes, adds to the evidence from epidemiological studies suggesting that consuming oily fish or fish oil will reduce cardiovascular risk.

**RCTs with hard cardiovascular outcomes are conflicting but suggest no overall effect**

In the months after myocardial infarction, fish oil appears to reduce sudden cardiovascular death, probably due to an antiarrhythmic effect. However, the results of primary and secondary prevention trials, where n-3 PUFAs might be expected to modify the progression of atherosclerosis, have been conflicting and recent systematic reviews found no overall effect of marine oil supplementation on the risk of all-cause mortality, cardiac death, sudden death, myocardial infarction or stroke.

**Important differences in the biological effects of oxidised and unoxidised marine oils could explain paradoxical results**

This result is paradoxical, given our current understanding of atherosclerotic disease, a treatment that reduces BP, platelet aggregation, triglyceridaemia, stabilises plaque and reduces platelet aggregation should the risk of myocardial infarction and death. In explaining the conflicting effects of marine oil on health authors have considered many explanations including a true lack of efficacy, obscuration of benefit by other treatments that improve cardiovascular risk such as statins, aspirin and beta-blockers, high background fish consumption and underpowered studies. However, oxidation of the trial oils could also potentially explain these disappointing results. If oxidized oils are less efficacious, or if they cause harm, for example by accelerating atherosclerosis then provided some studies used oxidized supplements, mixed results would be expected.
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Further, as rancid fish would rarely be consumed because of the unpalatable odour, oxidation could explain why epidemiological studies show fish consumption is beneficial, but studies of fish oil are mixed and disappointing. We are currently in danger of concluding that marine n-3 supplements are ineffective in the prevention of cardiovascular disease, before they have been adequately investigated.

4.6.9 Implications of oxidation for interpretation of the literature and future clinical trials?

The oxidation problem is largely unrecognised

To assess the degree to which the importance of oxidation of marine oil is understood, we identified all human clinical trials published in 2012 using Pubmed. Of 107 reports, only one study investigating short term harm reported the oxidative state of the trial oil (previously described)\textsuperscript{613}. This strongly suggests that the instability of marine oil is unrecognized or not considered important.

Implications of the oxidation problem

It is currently impossible to determine how oxidation affects the efficacy or potential harms of marine oil. This makes interpretation of the clinical trial literature problematic. If the oxidative state of marine oils affects efficacy or harm, then physicians should recommend, and consumers select, a supplement with the same oxidative state as the oils used in the clinical trials that showed benefit. This is currently impossible because although over-the-counter-supplements are frequently oxidized\textsuperscript{557-561,582-585}, the oxidative state of trial oils and retail supplements remain unreported.

Future safety and efficacy trials, particularly in humans, should report the oxidative state of the marine oil. This could most easily be done by reporting the peroxide, anisidine and totox values. Even established benefits of marine oil need to be reinvestigated with provision of this information. In parallel there should be a move to labelling marine oil supplements with these same oxidative indices and a production and storage chain that minimises oxidation prior to purchase. Only then can we generalise efficacy and safety trial data to the available omega-3 supplements and provide informed recommendations to patients and consumers.
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Chapter 5. Metabolic programming: the long reach of early life factors into adult health suggests a special window for intervention

In 1989 Barker described the association between low birth weight (LBW) and cardiovascular disease, creating a new paradigm; the developmental origins of health and disease (DOHaD). There is now substantial evidence that early life factors such as fetal nutrition have long term effects on the risk of diabetes and cardiovascular disease. Thus early life represents a window of opportunity where health interventions could have a dramatic impact on global health.

5.1. Fetal undernutrition

Barker and colleagues followed up cohorts in the United Kingdom in which careful records of birth weight and anthropometry had been kept. They observed that LBW was associated with greater BP in childhood and adulthood, greater glucose and insulin excursions after a glucose load, and a greater incidence of impaired glucose tolerance (IGT). In addition LBW was associated with a relative hypercoagulable state (increased fibrinogen, factor VII and PAI-1), and a more atherogenic lipid profile (increased total cholesterol, LDL-cholesterol and Apo-B). This led to a dramatic increase in the prevalence of metabolic syndrome; adults born weighing less than 2.95kg had a 10 times greater risk of metabolic syndrome than those born >4.31kg. Consistent with their observations, data from follow up of famine cohorts show that maternal famine exposure during pregnancy is associated with hypertension, IGT, greater BMI and waist circumference and a more atherogenic lipid profile in the offspring. These important effects were borne out by large systematic reviews which showed that for every 1kg increase in birth weight, the risk of T2DM reduces by 15% and cardiovascular risk by 10-20%. Underscoring the importance of insulin resistance, a large systematic review showed an inverse relationship between birth weight and insulin sensitivity, but an inconsistent relationship with insulin secretion.

Premature delivery is analogous to LBW, as whereas term babies born SGA have been exposed to an adverse in utero environment, premature babies are exposed to an adverse ex utero environment at a similar stage of biological maturity that is often associated with severe illness, physiological stress and malnourishment. Premature delivery is associated with impaired insulin sensitivity, a large increase in the risk of T2DM, and increased BP. Further, twin pregnancy, and hyperemesis gravidarum which also represent early life nutritional insults to the fetus, are associated with adverse metabolic phenotype in childhood.
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5.2. Fetal overnutrition

The rates of overweight and obesity have soared around the world and as a result up to 60% of women of reproductive age from western countries are now overweight or obese. This has led to a high prevalence of gestational diabetes mellitus (GDM), affecting up to 11.6% of pregnancies.

In pregnancy, hormones such as placental growth hormone induce a state of relative insulin resistance such that by late pregnancy, insulin sensitivity is reduced by 40-60%. This adaptive response is associated with reduced ability of insulin to suppress lipolysis, increased circulating FFAs, and increased hepatic glucose production. Thus the insulin resistance of pregnancy appropriately increases the delivery of glucose and fatty acids to the fetus. However, where it is exaggerated this response can lead to fetal overnutrition.

This is easily understood in gestational diabetes where severe (pathological) insulin resistance leads to hyperglycaemia. Maternal hyperglycaemia leads to fetal hyperglycaemia and in response, hyperinsulinaemia. Insulin is an important fetal growth factor, thus the fetus exposed to gestational diabetes develops macrosomia; overgrowth with excess body fat. However, even without gestational diabetes, exposure to maternal obesity or excess gestational weight gain is associated with overgrowth. In fact, the maternal pre-pregnancy insulin sensitivity is the factor most predictive of fetal adiposity. Lipid delivery to the fetus is also important. In women who have gestational diabetes or macrosomic babies, there is elevation of triglycerides and fatty acids, the levels of which correlate with birthweight and indices of adiposity in childhood.

Macrosomia is associated with complications during parturition and in the early neonatal period including shoulder dystocia, skeletal and nerve injuries, asphyxia and hypoglycaemia, which may lead to death or disability. That macrosomia is common, despite risk to the survival and future reproductive success of the infant, indicates that pre-existing maternal insulin resistance, as is common now, is evolutionarily novel. The long term effects of fetal overnutrition are also unfavourable leading to increased adiposity and metabolic risk of the offspring. These long-term effects are not obviously adaptive in an environment of plenty. It has been suggested that they represent a strategy to have earlier puberty and thus a longer period of fertility, but insulin resistance also contributes to infertility.
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Obesity in pregnancy: the effect of pre-pregnancy BMI

There is substantial evidence that maternal pre-pregnancy obesity is associated with macrosomia at birth, greater BMI and adiposity in childhood and obesity in later life\textsuperscript{703,704}. In fact maternal obesity appears to be the strongest\textsuperscript{156,705}, or one of the strongest\textsuperscript{706} factors predicting obesity. In contrast, the effect of paternal adiposity is inconsistent\textsuperscript{707,708}.

This greater adiposity has important metabolic implications. Babies born large for gestational age (LGA) have increased risk of diabetes in adulthood\textsuperscript{16}. In young adults\textsuperscript{709} and children\textsuperscript{704} those born to an obese mother have poorer insulin sensitivity\textsuperscript{704,709}, greater triglycerides\textsuperscript{704} and nearly twice the risk of metabolic syndrome\textsuperscript{17}. Increasing pre-pregnancy BMI is associated with greater adiposity\textsuperscript{157,710}, BMI\textsuperscript{157,710}, systolic BP\textsuperscript{157,710}, leptin\textsuperscript{157,710}, CRP\textsuperscript{710}, IL-6\textsuperscript{710} and reduced insulin sensitivity\textsuperscript{157,710} and adiponectin\textsuperscript{710}. In fact, increasing maternal pre-pregnancy BMI is associated with reduced insulin sensitivity, even within the normal BMI range\textsuperscript{157}. However, maternal pre-pregnancy BMI is not associated with β-cell function\textsuperscript{157,709}, indicating that, like those born SGA, the primary adverse effect of exposure to maternal obesity is impaired insulin sensitivity, not secretion. Thus maternal obesity is associated with an adverse metabolic phenotype in the offspring that includes increased adiposity, insulin resistance, systemic inflammation and adipose dysfunction.

Excess Gestational Weight Gain

Greater gestational weight gain (GWG) is associated with increased risk of an LGA neonate\textsuperscript{696}, greater BMI, adiposity, and prevalence of overweight in childhood\textsuperscript{710-712}, greater BMI and prevalence of obesity in adolescence\textsuperscript{713} and greater waist circumference\textsuperscript{714} and BMI in adulthood\textsuperscript{714,715}; effects that are independent of pre-pregnancy BMI and gestational diabetes. In a prospective study following the offspring of Swedish women for 42 years, greater GWG led to increased BMI that tracked through life, leading to a greater prevalence of obesity in middle-age\textsuperscript{716}. Evidence for associated metabolic dysfunction is limited, but includes reports of greater leptin\textsuperscript{710} and triglycerides\textsuperscript{704}, lower HDL\textsuperscript{704} and trends to greater BP\textsuperscript{711} and poorer insulin sensitivity in childhood\textsuperscript{704}. It appears that the effects of GWG are greater in the pregnancies of women who are under- or normal-weight prior to pregnancy\textsuperscript{715,717}. This may explain a study where metabolic dysfunction present at age 32 was related to pre-pregnancy BMI, but not gestational weight gain\textsuperscript{714}.
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Diabetes in pregnancy

Gestational diabetes mellitus (GDM) like T2DM is characterised by insulin resistance and relatively impaired insulin secretion which leads to hyperglycaemia. Like T2DM, defects of insulin signalling in muscle and adipose tissue underlie insulin resistance. In women that are overweight or obese, the physiological insulin resistance of pregnancy often precipitates GDM. Recent changes to the diagnostic criteria have increased the proportion of women recognised as having GDM, but better reflect the evidence for adverse outcomes.

The offspring of women with GDM or T2DM have greater birth weight (for gestational age) and although differences in BMI are not detectable during infancy, greater weight and BMI trajectory emerge in childhood. BMI velocity accelerates in puberty increasing the rate of overweight in adulthood.

Exposure to maternal GDM or T2DM increases percentage body fat, and both subcutaneous and visceral adipose tissue, with a more centripetal distribution - the distribution most associated with metabolic dysfunction. In fact, the fasting glucose concentration is a strong predictor of infant adiposity. GDM interacts with maternal BMI; among diabetic pregnancies, greater BMI is associated with a stepwise increase in the risk of obesity.

Consistent with the effects on body composition, the offspring of diabetic women are more insulin resistant in childhood and adulthood and have greater incidence of glucose intolerance in childhood, T2DM in adolescence and T2DM and metabolic syndrome in adulthood. Further, young patients with T2DM are more likely than controls to have been exposed to diabetes during pregnancy, and the offspring of women born after a maternal diagnosis of diabetes have a greater rate of T2DM than those born before the diagnosis. Interestingly, during pregnancy the plasma glucose level 2hrs after an oral glucose load is associated with diabetes risk in the offspring even below the diagnostic threshold for diabetes (11.1 mmol/l). There is a strong relationship between the insulin concentration at birth (cord blood) and glucose intolerance in childhood and adolescence emphasising the importance of the hyperinsulinaemic response of the fetus to overnutrition. Maternal diabetes is also associated with other aspects of metabolic dysfunction in the offspring including greater BP, reduced HDL, decreased adiponectin and endothelial dysfunction.
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The importance of hyperglycaemia has recently been questioned. Donovan et al. argued that the increased risk of metabolic disease in the offspring of diabetics reflects maternal obesity, not hyperglycaemia. Maternal BMI is the strongest predictor of offspring BMI, and in one study controlling for maternal BMI eliminated the relationship between GDM and metabolic syndrome in the offspring. Most studies of diabetes in pregnancy have not controlled for maternal BMI. They also argued without specific evidence that the relationship between maternal and offspring BMI is solely due to the postnatal environment, i.e. that there are no metabolic effects of maternal diabetes, and transgenerational transmittance of diabetes is a social phenomenon. This is contradicted by considerable evidence in animal models (Section 5.2.1). Nevertheless, maternal hyperglycaemia is not the only factor linking GDM to the metabolic health of offspring, and may not be the most important.

The importance of lipid delivery in insulin resistant pregnancy

Type 1 diabetes (T1DM) is characterised by severe insulin deficiency which is treated with exogenous insulin. Because there are typically periods where the insulin dose is mismatched to requirement, T1DM is associated with intermittent hyperglycaemia. However, in addition to hyperglycaemia, T2DM and GDM are also characterised by abnormal insulin resistance, with elevated circulating triglycerides and FFAs. The placenta hydrolyses triglyceride to FFAs, which are transported to the fetal circulation, thus maternal insulin resistance, as occurs in maternal obesity or GDM is associated with excess delivery of fatty acids to the fetus. This appears important as in GDM maternal triglyceride and FFA levels correlate with BW, BMI, and weight:hip ratio in childhood. This important difference in lipid delivery between T1DM and GDM, may explain the observation that while many studies have shown maternal T1DM to be associated with metabolic dysfunction in the offspring, when compared with GDM, the effects have been weaker, or absent.

Excess lipid delivery to the fetus also helps to explain the development of macrosomia and metabolic dysfunction in the offspring of obese but non-diabetic women. FFAs increase insulin secretion, and induce insulin resistance in adults, and neonates of obese non-diabetic women have insulin resistance, and hyperinsulinaemia at birth. Because the mitogenic effects of insulin do not become resistant, this is likely to explain overgrowth not only in the context of maternal GDM, but also maternal obesity.

In the last trimester of pregnancy the human fetus, unlike other animals, rapidly accretes fat stores. Given the important effect of FFAs on differentiation and proliferation of adipocytes, exposure to abnormal concentrations of fatty acids in utero might have long term effects on adipose tissue function, contributing to systemic inflammation and insulin resistance.
5.2.1 Animal models

Animal models present major advantages over human studies in the investigation of DOHaD. Firstly, it is possible to use harmful interventions, and test potential treatments to ameliorate their effects. Secondly, it takes relatively little time for small animals to reach adulthood, when the metabolic phenotype can be characterised. Thirdly, animals can be culled for tissue samples. Lastly, the environment is easily controlled. Four types of animal model have been used to investigate the effects of in utero hyperglycaemia or insulin resistance: maternal glucose infusion, maternal insulin deficiency, maternal T2DM and a maternal obesogenic diet.

Maternal Glucose infusion

In two studies, intravenous glucose infusion in late pregnancy led to IGT with mild insulin deficiency in the offspring, suggesting impaired development or function of β-cells. However, this model is unusual as the dams would have had relatively normal insulin sensitivity and insulin secretory capacity. The combination of hyperglycaemia and hyperinsulinaemia, with suppression of lipolysis is unlike T1DM, GDM or T2DM.

Maternal Insulin deficiency

A number of studies have used genetic, or β-cell toxin induced rodent models of insulin deficiency. These models lead to a relative insulin deficiency (as complete deficiency would be fatal), which is unlike T1DM. Relative insulin deficiency is more akin to T2DM and GDM, except that this model is not insulin resistant.

In these studies the effects on weight and body composition of the offspring have been inconsistent. However, multiple studies indicate that the offspring develop insulin resistance, including one using the HEC, where insulin sensitivity was reduced by 74%.

The effect on insulin production is more complex; maternal insulin deficiency has been associated with reduced insulin secretion in the offspring, but in one study, the offspring of rats from dams with mild diabetes had an exaggerated insulin response despite a reduced β-cell mass.
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*MATERNAL T2DM*

In a unique study, normal rat embryos were transferred into the uterus of dams bred to develop T2DM. Exposure to this diabetic environment, was associated with glucose intolerance in adulthood.755

*DiET INDUCTED INSULIN RESISTANCE*

Diets rich in sugar and saturated fat contribute significantly to human obesity and have been shown to induce insulin resistance in rodents. Thus animal models using high fat and/or sugar diets in pregnancy are highly relevant to humans.

In rodents, exposure to a maternal HFD or high fat/high sugar leads to important changes in the regulation of energy balance, with hyperphagia,756-762 preference for high fat food763 and reduced physical activity756,757. This appears to be due to abnormal development of leptin receptor expressing neurons in the hypothalamus (arcuate nucleus), the major regulator of energy balance.761 These neurons detect the energy state through direct detection of glucose, as well as through leptin (reflects fat stores) and insulin (increased in the fed state by all macronutrients) binding to their receptors221,764. In response to increases in glucose, leptin or insulin, expression of neuropeptide Y (which is appetite stimulating) is reduced, and proopiomelanocortin, from which α-MSH is derived (appetite reducing) is increased764. Energy expenditure is also increased766. In the neonatal period there is a surge of endogenously produced leptin, that is important for normal development of the hypothalamic neurons.765 The maternal HFD leads to reduced leptin on day one766 followed by an abnormally large and long leptin surge that leads to abnormal development of the hypothalamus with reduced leptin receptor expression761. Thus a maternal HFD disrupts the energy regulation centre causing leptin resistance.

The hypothalamic-pituitary-adrenal (HPA) axis, links physiological stress to production of the glucocorticoid. Excess glucocorticoid leads to central adiposity and insulin resistance767. A maternal HFD during pregnancy or lactation leads to greater HPA responsiveness and increased corticosterone level in rat offspring768,769. Thus greater activation of the HPA axis may contribute to the adverse body composition and metabolic phenotype.

HFD or high fat/high sugar diet in pregnancy lead to greater body weight757,758,760,761,764,768,770,771 and adiposity757,758,760,762,768,770-772 with reduced muscle mass757,759. Importantly, this is associated with adipocyte hypertrophy and abnormal development of skeletal muscle, with fewer muscle fibres, and increased numbers of adipocytes773,774.
Introduction

At birth, offspring exposed to a maternal HFD have similar or reduced insulin and triglycerides compared with controls, but they develop insulin resistance and impaired fasting glucose or glucose tolerance as they age. These effects are not specific to the HFD, a hypercaloric diet even without changes to the balance of macronutrients also led to increased fasting insulin and leptin, and altered lipid metabolism.

Maternal HFD is also associated with greater BP, triglycerides, total cholesterol, FFAs, CRP (a marker of systemic inflammation) and hepatic fat content. Programmed hypertension may be due to abnormal hypothalamic development induced by the abnormal leptin surge.

An important study showed that the offspring of wild-type dams fed a high fat had a more severe phenotype than those of dams with genetic insulin resistance (heterozygous for a mutant insulin receptor). This may be due to the greater adipose tissue inflammation and dysfunction expected in the wild-type dams fed a HFD.

Differential effects of saturated and polyunsaturated fatty acids

Most studies of HFD in pregnancy have used a diet rich in saturated fat such as lard. Saturated fatty acids increase adipose inflammation and insulin resistance. n-3 PUFAs, are expected to have insulin sensitising effects on dams by activating GPR-120, PPAR-γ, and PPAR-α, thereby increasing adiponectin secretion and reducing adipose tissue inflammation. This in turn is expected to lead to reduced FFA release from adipocytes, reduced hepatic and intramyocellular lipid, and increased peripheral and hepatic insulin sensitivity. Consistent with this, unlike lard based diet, which leads to adverse changes in food preference, food consumption and increased weight and adiposity in the offspring, a fish oil enriched diet did not. Further, while a saturated fat enriched diet reduced physical activity in the offspring, an n-3 PUFA rich diet increased physical activity.

The timing of a maternal HFD

The pre-pregnancy diet appears relatively unimportant as when a HFD is consumed in pregnancy, the phenotype is the same irrespective of whether a HFD was consumed before pregnancy. A recent cross-
Introduction

Fostering study separated the effects of a HFD in pregnancy from lactation. Exposure during either period lead to increased fat mass (%), reduced muscle mass (%), and increased leptin, triglycerides and BP as well as increases in fasting glucose and insulin. Greater effects on fasting glucose and insulin were seen in offspring exposed to HFD in both time periods but elevated corticosterone was only seen where a HFD was consumed in pregnancy. IGT only occurred in the group where HFD was solely consumed in pregnancy. Together these complex results indicate that a maternal HFD during pregnancy and a maternal HFD during lactation both have important effects on the metabolic phenotype of the offspring.

Non-human primates

There are few studies in non-human primates. A maternal HFD leads to changes in fetal life including hypothalamic inflammation with abnormal expression of proopiomelanocortin (suggesting altered regulation of energy balance), and increased liver triglyceride and oxidative stress. The offspring develop greater adiposity, insulin resistance and endothelial dysfunction. Interestingly, the maternal HFD leads to reduced n-3 fatty acids and an increased n-3:n-6 ratio in fetal life. This raises the possibility that reduced n-3 PUFA levels may have a direct role in mediating the adverse effects of the HFD, and that supplementation in the context of a maternal HFD, might reduce these negative effects.

5.3. Preventing transgenerational perpetuation of obesity

If the offspring of women who are obese, are programmed to be obese themselves, obesity will be perpetuated through generations. Further, as our obesogenic environment, leads more women without an epigenetic predisposition to become obese themselves, we may expect ever-increasing rates of obesity. However, the DOHaD paradigm also implies that interventions during early life could have a major impact on the risk of disease through the lifespan.

There is clear evidence from studies of siblings, whose mother had bariatric surgery and weight-loss between their births, that being born after the weight-loss, is associated with lower risk of LGA and childhood obesity, improved insulin sensitivity, more favourable lipid profile and reduced systemic inflammation. Thus, the substantial metabolic benefits of bariatric surgery, translate to beneficial effects on the metabolic phenotype of the offspring. Bariatric surgery is not a realistic option to treat all women who are obese and of reproductive age. However, if insulin sensitivity could be increased during pregnancy, this might prevent or reduce the development of metabolic dysfunction in the offspring. Three simple candidate treatments are exercise, metformin, and n-3 PUFAs.
5.3.1 Exercise

There is some evidence that in obese pregnant women, exercise can improve glucose tolerance and limit gestational weight gain\textsuperscript{785}. However, only one report addresses the effects of maternal exercise on phenotype of the offspring. It showed reduced subcutaneous fat at birth with persisting effects at 5 years of age, but the women were not obese, and metabolism in the offspring was not assessed\textsuperscript{786}. An RCT of exercise in overweight pregnancy, assessing infant metabolism and adiposity is awaited, but preliminary results suggests that adiposity was increased with exercise\textsuperscript{787}.

5.3.2 Metformin

Metformin is an insulin sensitising medication that reduces hepatic glucose production, and increases peripheral glucose uptake\textsuperscript{788}. It is an activator of AMP-activated protein kinase (AMPkinase)\textsuperscript{789}, an enzyme that coordinates the intracellular response to depleted energy. In liver it inhibits lipid and glucose production, while in muscle it increases lipid oxidation and glucose uptake\textsuperscript{789,790}, reducing intramyocellular lipid\textsuperscript{791}. Metformin is frequently used in the pregnancy of women with gestational diabetes or polycystic ovarian syndrome (which is associated with insulin resistance).

In rodents and sheep many of the effects of maternal overnutrition on fetal development and metabolism are mediated by reduced AMPkinase activity in liver\textsuperscript{792} and skeletal muscle\textsuperscript{773,774}. In rodent models, maternal metformin treatment normalised muscle development\textsuperscript{773}, improved peripheral glucose uptake\textsuperscript{773} and reduced systemic inflammation\textsuperscript{793}. However, in a recent study, metformin during pregnancy of mice fed a regular diet led to increased adiposity, IGT and elevated fasting glucose in adulthood\textsuperscript{794}. There was no insulin resistant group for comparison, but this could reflect the delicate balance that maternal insulin sensitivity plays in offspring nutrition. Normal pregnancy is associated with relative insulin resistance that increases nutrient delivery to the fetus. Thus metformin treatment of normal dams, may have led to pathologically increased insulin sensitivity and subsequent fetal undernutrition with its associated metabolic phenotype. If so, insulin sensitising agents should only be considered in women with abnormally increased insulin sensitivity such as occurs in obesity or gestational diabetes.

In human gestational diabetes, where dietary modification is insufficient to control blood glucose, insulin and metformin are the major treatment options. Insulin does not cross the placenta, thus any effects of maternal insulin treatment on the offspring are mediated through maternal factors such as lipid and glucose delivery to the fetus. In contrast, metformin crosses the placenta\textsuperscript{795}, thus could have direct effects on the fetus. In studies of gestational diabetes where insulin and metformin have been compared, there is no difference in their effect.
Introduction

on birth weight\textsuperscript{796}. However, the offspring of mothers treated with metformin have less than half the incidence of neonatal hypoglycaemia\textsuperscript{796}. This suggests that the fetal hyperinsulinaemia and β-cell hyperplasia seen in gestational diabetes is less severe with metformin treatment. However, it is unknown whether this is due to maternal effects (reduced delivery of glucose and lipid to the fetus), or to direct effects on fetal metabolism such as hepatic lipid and glucose synthesis or peripheral glucose disposal. In 2 year old children whose mother was randomised to metformin for treatment of gestational diabetes, there was greater subcutaneous fat, but similar total fat to children whose mother received insulin, suggesting a possible reduction of visceral fat\textsuperscript{797}. However, in a study of women with polycystic ovarian syndrome (which is characterised by insulin resistance), treatment with metformin, compared with placebo, had no effect on body composition or insulin sensitivity of the offspring at 8 years of age\textsuperscript{798}. Thus while metformin has important metabolic effects, in pathways affected by maternal overnutrition, limited human evidence suggests it does not improve the metabolic phenotype of the offspring. Further, the recent result in mice\textsuperscript{794} emphasises the need to be cautious with insulin sensitising agents in women with normal insulin sensitivity.

5.3.3 n-3 PUFAs

Like metformin, n-3 PUFAs have a range of potential biological actions that could affect fetal development. Their actions in the mother include inhibition of lipid synthesis pathways\textsuperscript{379}, activation of lipid oxidation pathways\textsuperscript{367}, reduction of hepatic glucose production (through improved insulin sensitivity)\textsuperscript{121} and anti-inflammatory and adipokine normalising effects in adipose tissue\textsuperscript{361,799}, thus they might reduce the flux of lipid and glucose to the fetus, reducing the tendency to overnutrition. Secondly, they may have direct effects on the development of the fetus. N-3 PUFAs are actively transported across the placenta\textsuperscript{800}, such that the concentration is higher in the fetus than the mother\textsuperscript{801}. Maternal obesity is associated with fetal insulin resistance\textsuperscript{745}, but DHA in cord blood is positively associated with insulin sensitivity\textsuperscript{802}. Thus maternal n-3 PUFA supplementation might directly affect fetal insulin sensitivity and circulating glucose, fatty acids and triglycerides. Further, as n-3 PUFAs modulate many transcription factors including PPAR-α\textsuperscript{367}, PPAR-γ\textsuperscript{355}, SREB1-c\textsuperscript{379}, they could potentially affect gene expression during critical periods of development. In particular, PPAR-γ has a critical role in the development of adipose tissue\textsuperscript{343}, so that effects of n-3 PUFAs during fetal life, could potentially have long term effects on adipose tissue function. It is noteworthy that n-3 PUFAs activate PPAR-γ while metformin downregulates it, thus these two treatments could have different effects on the fetus.

There is little data to test the hypothesis that n-3 PUFAs in the context of insulin resistant pregnancy improve metabolic and body compositional outcomes of the offspring, as no studies have been performed in insulin resistant populations. Within normal weight pregnant women, effects of n-3 PUFA supplementation on BMI and body composition are conflicting\textsuperscript{803}. One study showed reduced BMI in the offspring at 21 months of
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Age\textsuperscript{804}, but others have shown a subtly increased BMI at 2.5 years\textsuperscript{805} and no effect at age 7\textsuperscript{806}. In a study comparing supplementation with n-3 and n-6 PUFAs (without a control), no differences were found in birth weight or other auxological measures\textsuperscript{807}. The longest follow up after prenatal n-3 PUFA supplementation was 19 years and there was no effect on fasting glucose, insulin, HbA1c or BMI\textsuperscript{808}, however this may have been confounded by the high drop-out rate. In a recently reported open label RCT, women were randomised to a diet with a reduced n-6:n-3 ratio\textsuperscript{809}. No effect on body composition was seen at age 5 when compared with standard advice\textsuperscript{809}. However, as in previous studies, the mothers were normal weight, so their offspring were not expected to have programmed development of obesity.

Two studies suggested a relationship between fetal n-3 PUFAs and body composition and metabolism as cord blood n-3 PUFAs were associated with fetal insulin sensitivity\textsuperscript{802} and reduced subcutaneous adiposity at age three\textsuperscript{801}. However, associational studies may be easily confounded, and these effects are not supported by the limited RCT data.

No rodent studies have examined the effect of supplemental n-3 PUFAs in the context of insulin resistant pregnancy. However, a number of studies have suggested positive metabolic effects. Fish oil supplementation in pregnancy is associated with reduced body weight and age-related decline in insulin sensitivity in the adult offspring\textsuperscript{810}. Maternal ALA supplementation led to increased EPA and reduced leptin levels in the offspring\textsuperscript{811}. Conversely, maternal n-3 PUFA deficiency led to elevated BP in the offspring\textsuperscript{812}.

A maternal protein deficient diet programs an adverse phenotype in the offspring and this was partially ameliorated by n-3 PUFAs, which reduced the hypertensive effect\textsuperscript{813}, and prevented elevations of fasting glucose and insulin\textsuperscript{814,815}.

A notable study used streptozotocin to render dams insulin deficient during pregnancy and assessed the effects of supplemental fish oil\textsuperscript{816,817}. The streptozotocin model is somewhat similar to gestational diabetes as there is insufficient insulin signalling, with subsequent inappropriate lipolysis and hepatic glucose production. The offspring were born macrosomic, had increased serum and liver triglyceride, greater post-natal weight gain, and increased fasting glucose and insulin. Enrichment of the maternal and offspring diet with fish oil, led to a milder phenotype. However, it is not possible to distinguish the effects of the n-3 PUFAs in pregnancy, lactation or postnatal life.
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*Summary*

The potential for n-3 PUFA supplementation in pregnancy to improve the metabolic phenotype of the offspring has not been tested in at-risk human pregnancies such as those complicated by obesity or GDM. Given the high prevalence of obesity in fertile aged women, this approach could make a valuable contribution to global health.
6.1. Summary of introduction

I have argued that obesity and the associated non-communicable diseases, especially those related to insulin resistance such as T2DM, cardiovascular disease and some cancers, are among the world’s greatest health problems. Cardiometabolic disease develops with obesity because of dysfunction of adipose tissue that leads to insulin resistance. While weight loss is effective at reducing this dysfunction, no approach to weight-loss is both effective and available for the large population of obese people. A second important contributor to worldwide obesity is the increasing prevalence of obesity in women who are pregnant, as this leads to long term negative effects on body composition and metabolism in the offspring. These effects probably occur as a result of the insulin resistant maternal environment. Long chain n-3 PUFAs have important effects on adipose tissue, so that in rodents and primates they improve insulin sensitivity. They must be consumed as they cannot be synthesised de novo. However, they are chemically fragile, so that they oxidise easily during storage, to distinct chemicals which may be harmful.

6.2. Central hypothesis

Long chain omega-3 polyunsaturated fatty acids, can modify the effects of gene-environment interactions to improve aspects of glucose and lipid metabolism.

6.3. Predictions

- Greater n-3 PUFAs in red cell phospholipid will be associated with greater insulin sensitivity in overweight middle-aged men.

- Supplementation with a marine oil rich in n-3 PUFAs will lead to increased insulin sensitivity in overweight middle-aged men.

- In rat dams fed a high-fat diet during pregnancy, supplementation with a marine oil rich in n-3 PUFAs will prevent long-term impairment of insulin sensitivity in their offspring.

- In rat dams fed a high-fat diet during pregnancy, supplementation with an oxidised marine oil will not have beneficial effects.

- Fish oils purchased at retail in New Zealand will be substantially oxidised and contain less n-3 PUFAs than labelled.
Data Papers

Chapter 7. Fish oil supplements in New Zealand are highly oxidised and do not meet label content of n-3 PUFA

7.1. Preface

This chapter consists of a modified version of a manuscript published in the journal *Scientific Reports*\(^{18}\).

- **Authors:** Benjamin B Albert, José GB Derraik, David Cameron-Smith, Paul L Hofman, Sergey Tumanov, Silas G Villas-Boas, Manohar L Garg, Wayne S Cutfield
- **Journal:** Scientific Reports
- **Year of Publication:** 2015
- **Volume:** 5
- **Page:** 7928
- **Impact Factor when published:** 5.23
7.2. Introduction

Fish oils are among the most popular dietary supplements in the world. In the US, more than a third of the 17.7\% of adults who use dietary supplements take fish oil\textsuperscript{456,457}. Fish oils contain significant quantities of omega-3 long-chain polyunsaturated fatty acids (n-3 PUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are considered to be the metabolically active compounds\textsuperscript{819}. Consumers take fish oil supplements for a variety of reasons, but particularly because of promising effects of lowering inflammation\textsuperscript{352}, improving cognition\textsuperscript{480}, and lowering cardiovascular disease risk\textsuperscript{554}.

n-3 PUFAs are highly prone to oxidation due to the large number of double bonds within the fatty acid chain\textsuperscript{470,562}. As fish oils oxidise, unoxidised fatty acids diminish and are replaced by a complex 'soup' of lipid peroxides and secondary oxidation products (aldehydes and ketones)\textsuperscript{470}. Addition of antioxidants to fish oils reduces but does not prevent oxidation\textsuperscript{568}.

While specific oxidation species are difficult to measure, the degree of oxidation can be described by measuring the peroxide value (PV) and the anisidine value (AV). PV reflects the primary oxidation products (lipid peroxides) while AV reflects secondary oxidation products (aldehydes and ketones). Together, these parameters are used to estimate the total oxidation value (Totox). A number of organisations have recommended maximum levels for these indices\textsuperscript{577,580,820}. However, these industry standards are based on palatability, as there are insufficient data to set standards based on health effects\textsuperscript{579}.

When over-the-counter fish oil supplements have been investigated, the reported frequency of excessive oxidation has been highly variable\textsuperscript{553}. A 2007 survey of retail fish oil supplements in New Zealand showed that 4 of 29 products tested had a Totox value greater than recommended levels, but limited information was provided about specific oxidative markers or study methodology\textsuperscript{584}. In addition, 5 of the 29 products contained less n-3 PUFA than labelled\textsuperscript{584}. Thus, in order to fill an existing knowledge gap, we aimed to comprehensively evaluate the fish oil supplements currently available in the New Zealand market, measuring simple oxidative markers as well as concentrations of n-3 PUFA.
7.3. Methods

All encapsulated fish oil supplements sold at retail or online stores in New Zealand were eligible for inclusion in the study. Marine oils from other sources (such as krill, calamari, or algae, which have important differences in composition) fell outside the scope of this study and were not included. We selected encapsulated oils because this is the most common medium for consumption of fish oil supplements. If there were multiple fish oil supplements available from a particular company, all were included in the study, provided there was a claimed difference in oil composition. One vial of each supplement was purchased for sampling. Where products were found in multiple stores, the first identified vial with a best before date between 12 and 24 months from the time of purchase was selected, otherwise vials 9 to 30 months prior to best before date were eligible.

The recommended retail price and best before date were recorded. Immediately after purchase, oil capsules were removed from their packaging, stored in amber glass jars that were numbered and sealed, and placed inside a cardboard box. The box was then stored at 4°C. Thus, the oil capsules were stored in conditions expected to minimise oxidation. All products were subsequently tested for the oil content, n-3 PUFA composition, PV, and AV. All tests and data analyses were performed by investigators who were blinded to the identity of individual supplements.

Mass of oil content

Capsule oil mass was measured by weighing the oil capsule ($m_1$), piercing and evacuating the oil contents, then washing the halved capsule in hexane. After drying the capsule shell, it was re-weighed ($m_2$). Mass of capsule content was calculated as: $m_1 - m_2$. This procedure was performed in triplicate.

A 12 ml sample of oil was produced by combining the contents of 8–24 capsules (depending on capsule size). From this pooled oil sample, PV, AV, Totox, and fatty acid concentration were measured in triplicate.

PV, AV, and Totox

PV was measured according to the European pharmacopoeia by visual titration of iodine. 2.5g of oil was weighed in a volumetric flask. 50ml of 3:2 (glacial acetic acid:trimethylpentane) was added with 500μl of
saturated potassium iodide solution. The flask was occluded and vigorously shaken for 60 seconds, and 30ml of water added. The yellowed solution was titrated with 0.01N sodium thiosulphate solution until almost colourless, when 500μl of 1% starch solution was added. The resulting solution was titrated until colourless using 0.01N sodium thiosulphate solution. The sodium thiosulphate volume was used to calculate the peroxide value in meq/l according to the formula “PV=[10x(V-V_control)]/m”, where m is the oil mass previously obtained. Based on triplicate measures, the intra- and inter-assay coefficients of variation were 2.6 and 1.8%, respectively.

AV was measured according to the European pharmacopoeia using absorbance spectrophotometry after reaction with p-anisidine. Briefly, 0.2g of oil was weighed into a small vial. 10ml of trimethylpentane was added. The A₁ absorbance was measured at 350nm against a reference solution of trimethylpentane. 1ml of 2.5g/l p-anisidine in acetic acid was added to 5ml of the oil-trimethylpentane solution. After 10 minutes, the A₂ absorbance was measured against a reference solution of 5ml trimethylpentane with 1ml of p-anisidine in acetic acid. AV was calculated from these absorbances and the oil mass (m) previously obtained: "10x[1.2x(A₂–A₁)]/m". Based on triplicate measures, the intra- and inter-assay coefficients of variation were 3.0 and 3.8%, respectively. Totox was subsequently calculated by the formula "(2xPV)+AV".

Markers of oxidation were compared to international guidelines, with recommended values for PV, AV, and Totox of <5, <20, and <26 meq/l, respectively.

**n-3 PUFA composition**

1 ml of fish oil was frozen at -80°C for storage prior to analysis. 50mg of oil samples were weighed out into solvent washed methylation tubes and dissolved in 10ml of toluene. From this 200μl (1.0mg) was taken for trans-methylation followed by gas chromatographic analysis. Briefly, 2 ml of methanol:toluene (4:1 v/v, containing C19:0, 20µg/ml as internal standard) was added to the sample. Acetyl chloride (200µl) was added while vortexing and the contents were heated for 1 hour at 100°C. The tubes were cooled in water (5 minutes) and K₂CO₃ 6% (5ml) added and centrifuged (3000 x g, 5min, 4°C). The upper toluene phase was collected and stored in a gas chromatograph (GC) vial at -20°C for analysis.

Methylated fatty acid samples were analysed by gas chromatography using a fixed carbon-silica column 30m x 0.25mm (DB-225) (J&W Scientific, Folsom, CA, USA). The GC was equipped with a flame ionization detector, auto-sampler and auto-detector. Injector and detector ports were set at 250°C. Oven temperature was
programmed: 170°C for two minutes, increased 10°C/minute up to 190°C where it remained stationary for one minute. Temperature then increased 3°C/minute up to 220°C, which was maintained for a total run time of 30 minutes per sample. A split ratio of 10:1 and an injection volume of 3µl were used. A known fatty acid mixture was used to identify peaks according to retention time and their concentration was determined using a 6890 Series gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) with Chemstations Version A. 04.02 (Chemstations Inc., Houston, TX, USA)\textsuperscript{821}. Each oil sample was analysed in triplicate.

The mass of individual fatty acids in each capsule were calculated in mg/capsule by the formula “specific fatty acid content = specific fatty acid concentration x capsule oil mass”

**Data analyses**

Adjusted values for PV, AV, and Totox were also calculated to account for the higher concentration of n-3 PUFA in some oils. Oils with a higher concentration of n-3 PUFA have more substrate for oxidation, and are therefore expected to have higher levels of oxidation markers. Adjusted values standardised the oxidative indices, reflecting the exposure to oxidised compounds per 300 mg of combined EPA+DHA (the most frequent labelled dose per capsule). This adjustment may provide a better estimate of a consumer’s exposure to oxidation products, as fewer capsules of a more concentrated fish oil would be ingested to achieve a targeted n-3 PUFA intake. The adjusted PV was calculated as: \((PV \times 300)/(EPA+DHA)\), where the concentrations are the labelled content in mg/g of oil. Similarly, the adjusted anisidine value was calculated as: \((AV \times 300)/(EPA+DHA)\). Adjusted Totox was calculated as: \((2 \times \text{AdjPV}) + \text{AdjAV}\). Associations between variables of interest were assessed using simple linear correlations and Spearman's rank correlations. Parameters measured were compared between countries of origin (New Zealand, Australia, and Other) using general linear regression models.

**7.4. Results**

We initially studied 32 individual fish oil supplements available in the New Zealand market (Table 1). An extra four products were later identified and also examined, but did not have their EPA+DHA content measured (Table 1). There was a considerable variation in price among different brands, with the recommended retail price (RRP) per gram of fish oil varying from $0.05 to $0.77 (USD) (Table 1).
The total oil content of individual capsules exceeded 97% of the labelled oil content for all supplements tested (Table 1). However, there was a marked disparity between the label-claimed content of EPA+DHA and the actual capsule content of these fatty acids (Table 1; Figure 4), with supplements containing on average 68% (SD=23%) of the claimed content. Only 3 of the 32 oils tested contained a quantity of EPA+DHA that was equal to or higher than that claimed by the label, with more than two-thirds of supplements tested (22; 69%) containing less than 67% (Table 1; Figure 4). Two supplements contained approximately one third of the label concentrations of EPA+DHA (Table 1; Figure 4).

There was some indication that the more expensive brands had more accurate labelling of EPA+DHA content, as RRP was positively correlated with the ratio of measured to claimed EPA+DHA (ρ=0.55; p=0.001). In addition, despite the majority of fish oils having less n-3 PUFA than claimed, the label EPA+DHA content was well correlated with the actual n-3 PUFA content (ρ=0.71; p<0.0001).

Oxidative markers

There were high levels of oxidation in the fish oils assessed (Table 1), with the vast majority of supplements tested failing to meet recommended levels of oxidation markers (Figure 4). 30/36 (83%) products exceeded the recommended PV, 9/36 (25%) exceeded AV, and 18/36 (50%) exceeded recommended Totox thresholds (Figure 5). Only 3 of 36 oils tested (8%) met all the international recommendations, not exceeding any of these indices (Figure 4). After adjustment for concentration, only 19% (7/36) were within the recommended limits, indicating that the high frequency of excess oxidation was not simply an artefact caused by the concentrated oils.

There were no associations between the time to best-before-date and concentrations of oxidation markers (data not shown). There was a positive correlation between RRP and both PV (ρ=0.41; p=0.013) and Totox (ρ=0.39; p=0.018). However, this association likely reflected increased n-3 concentrations in more expensive products, as there was no association between RRP and content-adjusted PV (ρ=0.34) and Totox (p=0.46).
Table 1: List of retail fish oils tested.

<table>
<thead>
<tr>
<th>Code</th>
<th>Country of origin</th>
<th>RRP (US$)</th>
<th>BBD (days)</th>
<th>Oil mass per capsule (g)</th>
<th>EPA+DHA per capsule (mg)</th>
<th>PV (meq/kg)</th>
<th>AV</th>
<th>Totox</th>
<th>Adj PV (meq/kg)</th>
<th>Adj AV</th>
<th>Adj Totox</th>
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<td>1.064</td>
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<td>100.0</td>
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Adj, adjusted for labelled n-3 PUFA content to reflect the oxidation products per 300 mg combined EPA + DHA; AV, anisidine value; BBD, time to best before date; PV, peroxide value; RRP, recommended retail price in US dollars per gram of fish oil (equivalent to one capsule in most cases); Totox, total oxidation value. Note: the country of origin reflects the country claimed on the product packaging or by a company representative. It reflects the site of encapsulation or packaging; the vast majority of oils were sourced from South American deep sea fish. International guidelines recommend values for PV, AV, and Totox of <5 meq/kg, <20, and <26, respectively. 

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Data Papers

Figure 4: The actual n-3 PUFA content in individual retail fish oil products in relation to the claimed content (dotted line).

n-3 PUFA content = EPA+DHA
Figure 5: Indices of oxidation in retail purchased fish oil.

Dotted line reflects recommended maximum limits. 

Data Papers
Figure 6: The association between missing EPA+DHA and both anisidine value (AV) and Totox.

AV and Totox data have been log-transformed, missing EPA+DHA = (label claim - actual content).

There was an observed association between the missing EPA+DHA (i.e. label claimed minus actual content) and both AV (r=0.45; p=0.011) and Totox (r=0.35; p=0.053) (Figure 6). Missing EPA+DHA content was not associated with PV (r=0.06; p=0.75).

*Country of origin*

Fish oil supplements manufactured in countries other than New Zealand or Australia were considerably more expensive according to the RRP per gram of fish oil. Supplements from other countries cost on average $0.57
in comparison to $0.13 from New Zealand (p<0.0001) and $0.25 from Australia (USD; p<0.0001). However, these international supplements were not associated with better supplement quality. The percentage EPA+DHA content (in proportion to claimed label content) was virtually identical in supplements from the three sources, and there were no differences in levels of any oxidative markers assessed (data not shown).

7.5. Discussion

This study shows that almost all fish oil supplements available in the New Zealand market contain much lower concentrations of long chain n-3 PUFAs than claimed by the product label. Importantly, the majority of fish oils exceeded the recommended indices of oxidative markers.

The discrepancy between actual and labelled n-3 PUFA content is consistent with a study of fish oils sold at retail in South Africa, and an older international survey where products were purchased in Canada, USA and UK; both found that more than half of the marine n-3 PUFA products surveyed contained less than 90% of the n-3 PUFAs claimed. A small study in Ohio showed even greater discrepancy. In contrast, an Australian survey of retail products showed relatively close agreement between the labelled and measured n-3 PUFA content. However, the method used to convert qualitative data to capsule content in that survey did not take into account the glycerol content of the oil, and would therefore overestimate the n-3 PUFA content.

As the labelled content of most fish oil products was inaccurate, it is important to consider how and when the n-3 PUFA content had been measured. The product labels did not indicate the method used to measure n-3 PUFA content, or at what stage of production this occurred. However, it is interesting that 17 products listed the same concentration of n-3 PUFAs (180 mg EPA and 120 mg DHA per gram of oil). Similar concentrations would be expected given that most oils are sourced from oily fish from the west coast of South America. However, such striking uniformity suggests that the companies selling these products have relied on data provided by extractors who supply fish oil to multiple brands. Thus, the labelled composition may not reflect the final n-3 PUFA content of product, which may have changed due to oxidation during transport, encapsulation, packing, and storage. Further, while there is evidence of seasonal variation in the n-3 PUFA content of oily fish, (in one fish species, it varied between 11.3% and 20.8%) the labelled n-3 PUFA content of individual brands does not vary with the best before date (which is related to the date of manufacture). This suggests that the fatty acid content is unlikely to be measured in individual batches of fish oil.
The high levels of oxidation identified in this study are broadly consistent with other surveys, which have shown that 11-62% of fish oil products are oxidised above international recommendations. As oil oxidises, the concentrations of EPA and DHA (the purported active compounds) decrease, suggesting reduced efficacy. In our study, oxidation may at least in part account for the low n-3 PUFA levels observed as there was an association between AV and Totox and the missing n-3 PUFA. Note that it is not surprising that PV was not associated with missing n-3 PUFA as lipid peroxides are initially formed during oxidation but subsequently broken down over time to secondary oxidation products, such that a low PV is consistent with both minimal and severe levels of oxidation. Nonetheless, the relatively weak correlations between these measures suggest that other unknown factors are also important, which could include poor quality control during manufacture.

The health implications of oxidised fish oil consumption remain unclear. There is some evidence that specific n-3 PUFA oxidation products (i.e. resolvins, protectins and n-3 PUFA derived isoprostanes) have a role in mediating the anti-inflammatory effects of n-3 PUFA supplementation. However, it is not clear whether these anti-inflammatory oxidation products are produced in sufficient quantities during capsule storage, to confer a net benefit to consuming oxidised fish oil. In fact, evidence from animal studies show that large doses of oxidised lipids may cause organ toxicity, growth retardation, and accelerated atherosclerosis. Only one relatively short study has compared the effects of oxidised and unoxidised fish oil in humans, observing no evidence of acute oxidative toxicity. However, the effects of long-term exposure to oxidised oils (particularly on markers associated with atherosclerosis) have not been studied. To our knowledge, the oxidative state of fish oils adopted in clinical trials has never been reported, so the possible effects of oxidation on trial outcomes are unknown. It is possible that the conflicting and often disappointing results in clinical trials may have resulted from the use of highly oxidised supplements. The high levels of oxidation shown in this study underline the importance of understanding the effects of oxidised fish oil taken in dietary supplements, particularly for pregnant women, children, and consumers with inflammatory, metabolic, or cardiovascular disease.

Our results also indicate that consumers would be unable to identify unoxidised fish oil supplements. The time until the best before date printed on the packaging had no relationship to the level of oxidation. Cost was also unhelpful, for while there was a correlation between cost and oxidation such that the more expensive supplements had greater oxidation, this relationship was an artefact of concentration (concentrated supplements were more expensive). In addition, supplements from outside Oceania were as oxidised as those manufactured in Australia and New Zealand. Even the two products that could only be purchased after naturopathic consultation had excess oxidation, and though one was labelled with the peroxide value, the true PV exceeded the label by more than four-fold.
It is important to emphasise that all products were well within their best-before dates, with the shortest being 270 days from analysis. The range of best-before dates observed in this study reflects the substantial variability presented on retail shelves. All retailers with a physical store were observed to keep their oil under the same conditions; at room temperature under artificial lighting, (the storage conditions of online only stores could not be observed). As fish oil products were selected based on predefined rules that were applied to all supplements tested, selection bias is unlikely. The major limitation of this study is that each product was only purchased from a single store, so that our study would have been unable to identify variations between batches or stores. Nevertheless, this study represents a comprehensive sample of the fish oil products available to consumers in New Zealand at the time of sampling. As most fish oil products sold globally (including New Zealand) appear to be sourced from deep sea fish from the west coast of South America, the results of our survey are likely to have international relevance.

In this study, we included all identified fish oil products (the most popular form of n-3 PUFA supplement), but not n-3 PUFA supplements produced from other marine sources such as krill. There are important differences in the composition of krill oils, particularly in the incorporation of n-3 PUFA as phospholipids\textsuperscript{830} and the presence of naturally occurring antioxidants such as the pigment astaxanthin\textsuperscript{831}. These differences may affect the propensity of these oils to oxidise\textsuperscript{470}. Thus, the results of our study cannot be generalised to other marine oils that are not sourced from fish, and krill oils should be studied separately.

In summary, the majority of fish oil supplements available for purchase in New Zealand not only have n-3 PUFA contents well below those claimed by labels, but are also considerably oxidised (with PV, AV, and Totox values above recommended levels). The associated health implications are unclear. Future studies should investigate the effect of environmental conditions on oxidation of encapsulated fish oils, particularly regarding the oxidation process when supplements are stored in retail or home environments. Further, clinical trials investigating the health effects of fish oil products should measure and report their peroxide and anisidine values, so that the importance of oxidation to efficacy and harms can be better understood.
Chapter 8. Higher omega-3 index is associated with increased insulin sensitivity and more favourable metabolic profile in middle-aged overweight men

8.1. Preface

This chapter contains a modified version of a manuscript published in the journal *Scientific Reports*.

- **Authors:** Benjamin B. Albert, José GB. Derraik, Christine M. Brennan, Janene B. Biggs, Greg C. Smith, Manohar L. Garg, David Cameron-Smith, Paul L. Hofman, Wayne S. Cutfield
- **Journal:** Scientific Reports
- **Year of Publication:** 2014
- **Volume:** 4
- **Page:** 6697
- **Impact Factor when published:** 5.58
8.2. Introduction

Insulin resistance, defined as a pathological reduction in insulin sensitivity, has an important role in the pathogenesis of essential hypertension, dyslipidaemia, and T2DM. These conditions are components of the metabolic syndrome, and are major risk factors for cardiovascular and cerebrovascular disease, chronic renal failure, and retinopathy. In addition, insulin resistance may be a risk factor for malignancy. As the rates of overweight and obesity continue to rise, insulin resistance is becoming one of society’s most pressing health problems.

Many factors influence insulin sensitivity, including age, adiposity, perinatal factors, and genotype. Lifestyle factors such as diet and physical activity also affect insulin sensitivity, and are particularly important because they are modifiable. Although weight-loss and increasing physical activity improve insulin sensitivity, these goals are difficult to achieve for a large proportion of the population. In contrast, relatively small dietary modifications (such as supplementation with nutraceuticals or increased consumption of fish) are much easier to attain. Thus, if such dietary modifications can improve insulin sensitivity in at-risk groups, it may be possible to lower the incidence of T2DM, the metabolic syndrome, and cardiovascular disease in the general population.

There is increasing evidence suggesting that dietary omega-3 polyunsaturated fatty acids (n-3 PUFA), particularly the long-chain fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in marine oils, may improve insulin sensitivity or reduce the incidence of T2DM. Epidemiological studies have linked higher dietary or plasma n-3 PUFA concentrations with lower risk of diabetes. Rodent studies have also shown that insulin resistance can be reversed by supplementation with fish oil. However, human intervention trials have yielded inconclusive results. In a systematic review that included 11 RCTs and 618 participants, n-3 PUFA supplementation did not influence insulin sensitivity. However, the individual trials were highly heterogeneous, including participants with and without T2DM, utilising a wide range of n-3 PUFA doses, as well as adopting a range of treatment and control oils. In association with weight-loss or caloric restriction supplementation with fish or fish oil increased insulin sensitivity. A study showed that switching to a Mediterranean diet (which includes a lower dietary n-6:n-3 ratio) also increased insulin sensitivity, but it was not possible to isolate the specific effect of n-3 PUFA due to the complexity of dietary interventions. In a simple dietary intervention trial of 12 healthy older adults, changing from non-oily fish to oily fish improved insulin sensitivity. However, in the multicentre KANWU study, addition of fish oil to a high saturated fat or high monounsaturated fat diet did not influence insulin sensitivity.
The relationship between insulin sensitivity and n-3 PUFA (EPA/DHA) concentrations in red blood cells (omega-3 index) has not been previously examined in adulthood. If higher blood n-3 PUFA concentrations are associated with improved insulin sensitivity, this would provide a mandate for larger and better controlled interventional studies. Thus, we examined the association of omega-3 index with insulin sensitivity and other metabolic indices, in a cohort of overweight middle-aged men enrolled into a randomised clinical trial.

8.3. Methods

Ethics

Ethical approval was granted by the Central Regional Ethics Committee, New Zealand Ministry of Health (CEN/11/07/038). Written and verbal informed consent was obtained from all participants. This study was performed in accordance with all appropriate institutional and international guidelines and regulations for medical research, in line with the principles of the Declaration of Helsinki.

Participants

Volunteers were recruited in 2012 using advertisements in local newspapers that circulate freely in the central Auckland metropolitan area. Overweight (body mass index (BMI) 25–30 kg/m²), middle-aged (35–55 years) men were eligible to participate. The study cohort represents a higher risk group, likely to have early insulin resistance without clinical disease, enabling easier detection of important factors. Note that only males were recruited, so that the effects of the menstrual cycle and/or oral contraceptives on insulin sensitivity (the primary outcome) could be avoided. Exclusion criteria were: diabetes mellitus, hypertension (systolic BP >145 mmHg or diastolic >95 mmHg), known dyslipidaemia, the use of tobacco, or prescription medications likely to affect BP, lipid profile or insulin sensitivity. Participants taking fish oil or other omega-3 supplements were asked to stop supplementation 4 weeks prior to the first assessment.

Study design

All participants were assessed on two occasions, 16 weeks apart, corresponding to the baseline samples of a crossover clinical trial. Importantly, there were no differences in omega-3 index between the two assessments. Clinical assessments were carried out between 07:00 and 09:00 at the Maurice & Agnes Paykel Clinical
The primary outcome was insulin sensitivity measured with a 75 g oral glucose tolerance test (OGTT) using the Matsuda method. Blood samples were collected at 0, 30, 60, 90, and 120 minutes for glucose and insulin measurements. The Matsuda index has a strong correlation with the hyperinsulinaemic euglycaemic clamp ($r=0.77$), and excellent reproducibility during multiple measures. The oral disposition index (a measure of β-cell function corrected for insulin sensitivity) was also calculated. During the OGTT, blood was drawn into collection tubes containing DPP-IV inhibitor at all time points measured. The active GLP-1 concentrations during the OGTT were used to calculate the area under the curve (AUC).

Fasting blood samples were also used to measure n-3 and n-6 PUFA concentrations in red blood cells. These concentrations reflect assimilation of dietary PUFA over a longer period of time than those in plasma, so that they are less prone to short-term fluctuation. Further, as this measure reflects lipid concentrations in the cell membrane, it may be more relevant than plasma concentration to physiological processes such as eicosanoid synthesis.

In addition, fasting samples were used to assess other measures of metabolic disease risk, including uric acid, free fatty acid, and highly-sensitive C-reactive protein (CRP) concentrations, as well as lipid profile (triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations). Auxological assessment included height measurement using a Harpenden stadiometer. Weight and body composition were assessed using whole-body dual-energy X-ray absorptiometry (DXA, Lunar Prodigy 2000, General Electric, Madison, USA).

24-hour ambulatory BP monitoring was carried out prior to each clinical assessment. Participants were fitted with a Spacelabs 90207 or 90217 monitor (Spacelabs Medical Inc., Redmond, USA), with each subject being assigned the same device model for all assessments. Measurements were performed every 20 minutes between 07:00 and 22:00, and every 30 minutes from 22:00 to 07:00. Only profiles with >14 daytime and >7 night time recordings over a 24-hour period were analysed.

Carotid artery intima-media thickness was also measured to assess possible treatment effects, as it is a validated and reproducible measure that is predictive of cardiovascular and cerebrovascular risks. Carotid intima-media thickness was measured using an M-Turbo ultrasound system (Sonosite, Bothel, USA) by a trained
investigator [BBA], with longitudinal images attained using a standard protocol. The right common carotid artery was scanned from both posterolateral and anterolateral views. Digitally stored images were analysed using computer software automated callipers to measure the far wall (SonoCalc v.4.1, Sonosite). The maximal thickness measurement from both views (approximately 10 mm proximal to the carotid bulb) was used for comparative analysis. To assess reproducibility, triplicate measures were taken of seven healthy volunteers over a 7-day interval, and resulted in an intra-observer CV of 3.7% (unpublished data).

Lifestyle factors were recorded with an itemised food diary and physical activity recall. Three-day dietary records were collected prior to clinical assessments. Each dietary report encompassed an itemized nutritional intake recorded during two week days (Monday to Friday) and one weekend day. Nutritional intake was recorded using standard household measures, as well as the information from food labels where appropriate. Participants were instructed by a trained investigator [BBA], who also reviewed all food records with each participant to address unclear descriptions, errors, omissions, or doubtful entries. Records were subsequently entered into Foodworks software (v6.0, Xyris Software, Brisbane, Australia) by the trained investigator [BBA]. Physical activity levels were assessed using the International Physical Activity Questionnaire (IPAQ), covering four domains of physical activity: work-related, transportation, housework/gardening, and leisure time.

Geo-coded deprivation scores were derived from current address using the New Zealand Index of Deprivation 2006 (NZDep2006). This index is based on household census data reflecting nine aspects of material and social deprivation to divide New Zealand into tenths (scored 1-10) by residential address. Scores of 1 represent the least deprived areas and 10 the most deprived. Scores are derived from units covering a small area, each reflecting approximately 90 people. Ethnicity was recorded by self-report using a prioritised system, such that if multiple ethnicities were selected, the patient was assigned to a single category, following a hierarchical system of classification.

Assays

Insulin concentrations were measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, USA) by microparticle enzyme immunoassay with an inter-assay coefficient of variation (CV) of 5.4%. Glucose, triglyceride, total cholesterol, HDL-C, LDL-C, free fatty acid, uric acid, and highly-sensitive CRP concentrations were also measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation) by enzymatic colorimetric assay (Roche) with all CVs lower than 3.2%. Active GLP-1 levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Millipore); this assay had a CV of 7.2%. 

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Erythrocyte fatty acid analysis

Fatty acid profile was analysed via direct transesterification of the washed erythrocyte fraction of blood, followed by gas chromatography. Methanol:toluene 2 ml (4:1 v/v) (containing C19:0 (20 µg/ml) as internal standard) was added to the sample. Acetyl chloride (200 µl) was added while vortexing and then heated (1 hour, 100°C). The tubes were then cooled in water (5 minutes), had K$_2$CO$_3$ 6% (5 ml) added, and were subsequently centrifuged (3000 x g, 5 min, 4°C). The upper toluene phase containing the fatty acid methyl esters was collected and stored in a gas chromatograph vial at -20°C for analysis.

Methylated fatty acid samples were analysed by gas chromatography using a fixed carbon-silica column 30 m x 0.25 mm (DB-225) (J&W Scientific, Folsom, CA, USA). The gas chromatograph was equipped with a flame ionization detector, autosampler, and autodetector. Injector and detector ports were set at 250°C. Oven temperature was programmed: 170°C for two minutes, increased 10°C/minute up to 190°C, where it remained constant for one minute. Temperature then increased 3°C/minute up to 220°C, which was maintained for a total run time of 30 minutes per sample. A split ratio of 10:1 and an injection volume of 3 µl were used. A known fatty acid mixture was compared to analysed samples to identify peaks according to retention time; their concentration was determined using a 6890 Series gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) with Chemstations Version A. 04.02 (Chemstations Inc., Houston, TX, USA).

Statistical analyses

The omega-3 index was calculated by adding erythrocyte EPA and DHA % (weight/weight) values. Stratified analyses were carried out separating participants into tertiles according to omega-3 index. Thus, we compared participants in the two lower omega-3 index tertiles (LOI) with those in the highest tertile (HOI). Potential demographic differences between groups were assessed using one-way ANOVA and non-parametric Kruskal-Wallis, test in Minitab v.16 (Pennsylvania State University, State College, PA, USA). Random-effects mixed models with repeated measures were used to compare outcomes of interest between LOI and HOI men, using SAS v.9.3 (SAS Institute, Cary, NC, USA). All models accounted for important confounding factors, namely age, total body fat percentage, socioeconomic status (NZDep2006), physical activity levels (IPAQ), and the amount of saturated fat consumed. Birth order was also controlled for when assessing outcomes associated with glucose homeostasis and ambulatory BP. Similar multivariate models and simple correlations were also used to test for linear associations with omega-3 index. All statistical tests were two-tailed and significance level maintained at 5%. Note that pairwise correlations between the independent variables were assessed, and none met the lowest threshold criteria for collinearity ($r>0.5$) as described in Dormann et al. Parameters of glucose homeostasis and inflammatory markers were log-transformed to approximate normality.
Data Papers

Demographic data are presented as means ± standard deviations; other data are means and 95% confidence intervals, adjusted for the confounders in multivariate models.

8.4. Results

Forty seven men aged 46.5 ± 5.1 years and of BMI 27.4 ± 1.8 kg/m² were studied. The majority of participants (87%) were of European descent.

Linear associations

Increasing omega-3 index was correlated with higher insulin sensitivity (r=0.23; p=0.025), higher disposition index (r=0.20; p=0.054), lower fasting glucose concentrations (r=-0.23; p=0.029) (Fig. 1), and lower nocturnal systolic BP (r=-0.21; p=0.047). Multivariate analyses showed that increasing omega-3 index tended to be associated with higher insulin sensitivity (p=0.079) and lower nocturnal systolic (p=0.077) and diastolic (p=0.073) BP. Importantly, increasing omega-3 index was correlated with lower CRP concentrations (r=-0.39; p<0.0001) (Figure 7), and this association was corroborated by multivariate analysis (p=0.049).

Stratified analyses

Omega-3 index in HOI (n=16) men were 42% higher than in LOI (n=31) group (p<0.0001; Table 2). HOI men had higher red cell phospholipid concentrations of oleic acid (p=0.040), EPA (p<0.0001), and DHA (p<0.0001) than LOI men (Table 2). In contrast, HOI men had lower concentrations of stearic acid (p=0.001), linoleic acid (p=0.004), dihomo-γ-linolenic acid (p=0.001), and arachidonic acid (p<0.0001) (Table 2). Participants in both groups were of similar age and socioeconomic status, had similar energy intake and consumption of saturated fat, and also engaged in comparable levels of physical activity (Table 3). However, HOI men were slightly leaner, by a difference of 1.1 kg/m² in BMI (p=0.041; Table 3). This difference highlights the importance of controlling for body fat (amongst other confounders) in all subsequent multivariate analyses.
Figure 7: Linear associations between omega-3 index and parameters of glucose homeostasis and CRP.

Parameters of interest have been log transformed.

Insulin sensitivity was 43% higher in HOI than in LOI men (Matsuda index 6.83 vs 4.78; p=0.009) (Table 4), despite adjustment for important confounders that also affected insulin sensitivity, including age (p=0.005), socioeconomic status (p<0.0001), birth order (p=0.017), and total body fat percentage (p=0.010). The HOMA-IR index of insulin resistance corroborated these findings, showing that HOI men tended to be less insulin resistant than LOI men (p=0.055; Table 4). In addition, fasting insulin concentrations were 25% lower in HOI than in LOI men (p=0.038; Table 4). Consistent with improved insulin sensitivity, FFAs were 21% lower (p=0.024) in HOI than in LOI men. In addition, the disposition index was 70% higher in HOI men (p=0.013; Table 4), however there were no differences in fasting active GLP-1 (not shown) or the active GLP-1 response during the oral glucose tolerance test (Table 4).
Table 2: Fatty acid profile in overweight middle-aged men in the upper tertile and lower two tertiles of omega-3 index.

<table>
<thead>
<tr>
<th></th>
<th>LOI</th>
<th>HOI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>C16:0 (palmitic acid)</td>
<td>22.5 ± 0.8</td>
<td>22.4 ± 0.6</td>
<td>0.60</td>
</tr>
<tr>
<td>C16:1n-7 (palmitoleic acid)</td>
<td>0.42 ± 0.14</td>
<td>0.41 ± 0.15</td>
<td>0.80</td>
</tr>
<tr>
<td>C18:0 (stearic acid)</td>
<td>18.7 ± 0.8</td>
<td>18.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>C18:1n-9 (oleic acid)</td>
<td>14.3 ± 0.8</td>
<td>14.7 ± 0.6</td>
<td>0.040</td>
</tr>
<tr>
<td>C18:1n-7 (vaccenic acid)</td>
<td>1.38 ± 0.20</td>
<td>1.37 ± 0.12</td>
<td>0.60</td>
</tr>
<tr>
<td>C18:2n-6 (linoleic acid)</td>
<td>10.3 ± 1.4</td>
<td>9.5 ± 0.7</td>
<td>0.004</td>
</tr>
<tr>
<td>C18:3n-6 (γ-linolenic acid)</td>
<td>0.23 ± 0.10</td>
<td>0.21 ± 0.07</td>
<td>0.56</td>
</tr>
<tr>
<td>C18:3n-3 (α-linolenic acid)</td>
<td>0.17 ± 0.08</td>
<td>0.34 ± 0.86</td>
<td>0.37</td>
</tr>
<tr>
<td>C20:0 (arachidonic acid)</td>
<td>0.43 ± 0.22</td>
<td>0.41 ± 0.19</td>
<td>0.64</td>
</tr>
<tr>
<td>C20:1n-9 (eicosenoic acid)</td>
<td>0.32 ± 0.18</td>
<td>0.34 ± 0.15</td>
<td>0.47</td>
</tr>
<tr>
<td>C20:2n-6 (eicosadienoic acid)</td>
<td>0.38 ± 0.21</td>
<td>0.42 ± 0.20</td>
<td>0.27</td>
</tr>
<tr>
<td>C20:3n-6 (dihomo-γ-linolenic acid)</td>
<td>2.25 ± 0.33</td>
<td>1.97 ± 0.43</td>
<td>0.001</td>
</tr>
<tr>
<td>C20:4n-6 (arachidonic acid)</td>
<td>17.8 ± 1.2</td>
<td>15.7 ± 1.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C22:5n-3 (eicosapentaenoic acid)</td>
<td>1.27 ± 0.36</td>
<td>2.10 ± 0.66</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C22:6n-3 (docosahexaenoic acid)</td>
<td>3.78 ± 0.53</td>
<td>4.00 ± 0.59</td>
<td>0.073</td>
</tr>
<tr>
<td>Omega-3 index</td>
<td>7.1 ± 0.8</td>
<td>10.1 ± 1.5</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

HOI = upper tertile of omega-3 index, LOI = the lower two tertiles. All data are % wt/wt in erythrocyte membrane fatty acids and are presented as means ± standard deviations. Omega-3 index represents the sum of EPA+DHA concentrations.

Table 3: Characteristics of overweight middle-aged men in the upper tertile of omega-3 index compared to the two lower tertiles.

<table>
<thead>
<tr>
<th></th>
<th>LOI</th>
<th>HOI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>46.0 ± 5.0</td>
<td>47.4 ± 5.3</td>
<td>0.38</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.8 ± 1.7</td>
<td>26.7 ± 1.7</td>
<td>0.041</td>
</tr>
<tr>
<td>Socioeconomic status (NZDep2006)</td>
<td>4.0 ± 2.5</td>
<td>3.9 ± 2.0</td>
<td>0.88</td>
</tr>
<tr>
<td>Physical activity levels (IPAQ)</td>
<td>4322 ± 4058</td>
<td>4442 ± 4038</td>
<td>0.75</td>
</tr>
<tr>
<td>Total energy intake (kJ/day)</td>
<td>9150 ± 3043</td>
<td>10063 ± 2202</td>
<td>0.29</td>
</tr>
<tr>
<td>Saturated fat intake (g/day)</td>
<td>30.4 ± 13.7</td>
<td>31.1 ± 8.6</td>
<td>0.86</td>
</tr>
</tbody>
</table>

HOI = upper tertile of omega-3 index, LOI = the lower two tertiles. Data are means ± standard deviations.
Table 4: Study outcomes in overweight middle-aged men in the upper tertile of omega-3 index compared to the two lower tertiles.

<table>
<thead>
<tr>
<th>LOI</th>
<th>HOI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

**Glucose homeostasis**
- Insulin sensitivity (Matsuda index) 4.78 (4.14–5.52) 6.83 (5.53–8.43) 0.009
- Disposition index 4.37 (3.50–5.47) 7.44 (5.34–10.37) 0.013
- HOMA-IR 1.82 (1.58–2.11) 1.41 (1.14–1.74) 0.055
- Fasting glucose (mmol/l) 5.39 (5.19–5.59) 5.35 (5.21–5.49) 0.73
- Fasting insulin (mU/l) 7.46 (6.43–8.66) 5.60 (4.50–6.97) 0.038

**24-hour ambulatory BP**
- Daytime systolic (mmHg) 126.6 (123.2–130.1) 124.8 (119.7–130.0) 0.57
- Daytime diastolic (mmHg) 80.0 (77.4–82.6) 78.1 (74.3–82.0) 0.43
- Night time systolic (mmHg) 112.6 (109.8–115.5) 106.6 (102.4–110.9) 0.025
- Night time diastolic (mmHg) 67.7 (65.6–69.7) 64.2 (61.2–67.3) 0.072
- Systolic dip (%) 10.8 (8.8–12.8) 14.7 (11.7–17.6) 0.039
- Diastolic dip (%) 15.4 (12.9–17.8) 17.7 (14.1–21.4) 0.30

**Carotid-intima media thickness (mm)**
- 0.80 (0.75–0.84) 0.84 (0.77–0.91) 0.30

**Other metabolic markers**
- Free fatty acids (mmol/l) 0.38 (0.34–0.43) 0.30 (0.26–0.36) 0.024
- Uric acid (µmol/l) 360 (337–384) 354 (321–387) 0.77
- CRP (mg/l) 1.16 (0.88–1.55) 0.69 (0.48–1.01) 0.033
- Active GLP-1 (AUC) 1129 (913–1346) 950 (645–1255) 0.35

**Lipid profile**
- Total cholesterol (mmol/l) 5.16 (4.92–5.40) 4.94 (4.59–5.29) 0.31
- LDL-C (mmol/l) 3.51 (3.28–3.73) 3.41 (3.09–3.73) 0.64
- HDL-C (mmol/l) 1.05 (0.97–1.13) 1.05 (0.94–1.18) 0.92
- Total cholesterol : HDL-C 4.86 (4.46–5.30) 4.63 (4.10–5.23) 0.52
- Triglycerides (mmol/l) 1.14 (1.00–1.29) 1.01 (0.85–1.22) 0.31

HOI = upper tertile of omega-3 index, LOI = the lower two tertiles. Data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models, including the repeated measures within each participant. GLP-1 AUC represents the area under the curve measured during a 120-minute glucose tolerance test. Statistically significant values at p<0.05 are shown in bold.

HOI men displayed a more favourable BP profile in the night time (Table 4). Thus, participants with the highest omega-3 index displayed lower nocturnal systolic BP (-6.0 mmHg; p=0.025), with a similar trend for nocturnal diastolic BP (-3.5 mmHg; p=0.072) (Table 4). HOI men also had better (greater) nocturnal systolic dip (p=0.039; Table 3). There were however, no significant differences in daytime BP (Table 4).

Men in the HOI group displayed 41% lower CRP concentration (p=0.033) suggesting reduced systemic inflammation (Table 4). There were no differences in carotid intima-media thickness or lipid profile (Table 4).
Data Papers

**n-6 PUFA and n-6:n-3 ratio**

Exploratory analyses were subsequently carried out to identify possible associations between n-6 PUFA concentrations and the n-6:n-3 ratio with metabolic parameters. Multivariate models yielded no significant associations between either n-6 or n-6:n3 ratio with any outcome response. Stratified analyses also compared the highest tertiles of n-6 PUFA and n-6:n-3 ratio with their respective lower tertiles. There were no observed differences for n-6, but the tertile with lowest n-6:n-3 ratio had lower CRP concentrations than the tertile with the highest ratio (0.85 (95% CI 0.63–1.13) vs 1.49 (95% CI 1.00–2.22) mg/l: p=0.032).

### 8.5. Discussion

This study showed that within a cohort of overweight middle-aged men, increasing omega-3 index was associated with greater insulin sensitivity and a more favourable metabolic profile. The tertile with highest omega-3 index had insulin sensitivity that was 43% greater than the two lower tertiles. In addition, they had improved β-cell function (greater oral disposition index), lower free fatty acid and CRP concentrations, lower night time systolic BP and greater nocturnal systolic dipping.

A favourable metabolic profile in individuals with higher omega-3 index may be associated with lower risk of T2DM, the metabolic syndrome, and cardiovascular disease. Notably, the omega-3 index is a reliable measure of n-3 PUFA intake over several months, but does not necessarily indicate sustained exposure in the longer-term. Thus, it is possible that some participants in the HOI group have not had sustained n-3 PUFA intake over the preceding years, which would be required for long-term metabolic and cardiovascular changes. Therefore, only differences in dynamic outcomes (e.g. insulin sensitivity, CRP and free fatty acid concentrations and BP) would be expected, as observed in our subjects.

To the best of our knowledge, the association between increasing omega-3 index and improved insulin sensitivity has not been previously shown in adulthood. However, in line with our results, a study has reported a relationship between higher omega-3 index and lower HOMA-IR (a proxy of insulin resistance) in obese children.

The reduction in insulin sensitivity seen with obesity appears to be due to adipose tissue expansion and inflammation, leading to abnormal adipose endocrine function and release of excess FFAs into
Raised FFAs have a central role in the development of both hepatic and muscle insulin resistance\textsuperscript{226}, as well as important pro-inflammatory effects\textsuperscript{852}. In our cohort, the effects of n-3 PUFA on insulin sensitivity may be mediated through reduction of FFAs. Long-chain n-3 PUFA are agonists of the nuclear transcription factors PPAR-\(\alpha\) in liver\textsuperscript{490,555}, as well as PPAR-\(\gamma\)\textsuperscript{555} and the recently characterised G-protein linked receptor GPR-120\textsuperscript{121} in adipose tissue. Thiazolidinediones are also PPAR-\(\gamma\) agonists, and may provide insight into the effect of n-3 PUFA via this nuclear receptor. Through PPAR-\(\gamma\), thiazolidinediones increase the concentration of high molecular weight adiponectin\textsuperscript{360}, reduce adipose tissue inflammation, and cause differentiation of preadipocytes\textsuperscript{853}. In turn, this improves adipose tissue storage function and reduces the release of FFAs\textsuperscript{855}.

In rats, n-3 PUFA appears to increase insulin sensitivity primarily through GPR-120, which inhibits adipose tissue inflammation via the NF-\(\kappa\)B pathway\textsuperscript{121}. However, the importance of this mechanism to the anti-inflammatory actions of n-3 PUFA is controversial\textsuperscript{356}. Adipose tissue inflammation could also be reduced by changes in the balance of inflammatory and pro-resolving eicosanoids\textsuperscript{854}. Importantly, these resolvins (enzymatic oxidation products of n-3 PUFA) are significantly more potent agonists of GPR-120 and PPAR-\(\gamma\) than their parent fatty acids\textsuperscript{356}, suggesting they may have an important role in mediating the increased insulin sensitivity associated with higher omega-3 index. Our data are consistent with these mechanisms, as higher concentrations of n-3 PUFA were associated with lower concentrations of FFAs and CRP (an inflammatory marker).

Our results are at odds with systematic reviews and large randomized controls examining the influence of supplemental n-3 PUFA on glycosylated haemoglobin (HbA1c) in adults with T2DM. The ORIGIN study examined the effects of n-3 PUFA and glargine (a basal insulin) in a large cohort made up mostly of adults with T2DM\textsuperscript{654}. There were no effects of n-3 PUFA on cardiovascular outcomes or HbA1c. However, 50\% of subjects were randomized to basal insulin and 59\% were taking at least one oral glucose-lowering medication. Further, the majority were hypertensive and had a history of myocardial infarction or stroke. As such, study participants had significant metabolic dysfunction, morbidity, and cotreatment. Systematic reviews have shown that n-3 PUFA do not affect glycaemic control in diabetes\textsuperscript{529,855}. However, adults with T2DM have more severe metabolic dysfunction than our participants who were simply overweight. We speculate that interrelated pathophysiological processes in T2DM (e.g. accumulation of intracellular lipid in liver and muscle and impaired insulin secretion) interact synergistically, such that n-3 PUFAs may reduce adipose inflammation and FFAs but do not influence the glycaemic control of diabetics.

The importance of the n-6:n-3 ratio to health outcomes is still debatable. Determining the relative importance of this ratio versus raw n-3 PUFA concentrations is difficult because they are not independent; supplementing
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n-3 PUFA leads to both an increase in n-3 levels and a decrease in n-6:n-3 ratio. It is logical to hypothesise that the agonist functions of n-3 PUFA would be proportional to n-3 concentration itself, but where n-3 PUFA is in direct competition with n-6 PUFA (such as for production of eicosanoids), the ratio of these PUFA subtypes could be more important. Our findings are in agreement with this hypothesis, as both lower n-6:n-3 ratio and increasing omega-3 index were associated with lower CRP concentrations, which was likely due to an altered balance of pro-inflammatory n-6-derived and pro-resolving n-3-derived eicosanoids. Importantly, the n-6:n-3 ratio was not associated with outcomes related to insulin sensitivity, which are more likely mediated through n-3 PUFA agonism of PPAR-γ and GPR-120.

The omega-3 index was also positively associated with the disposition index in both continuous and stratified analyses, suggesting an improvement in β-cell function with increasing omega-3 index. The oral disposition index has been shown to be predictive of future development of T2DM\(^{445}\). The mechanism for a relationship between omega-3 index and disposition index is unclear. Activation of GPR-120 by free EPA and DHA in gut enteroendocrine cells leads to an increase in GLP-1, which enhances glucose-stimulated insulin secretion\(^{856}\). However, there was no association between the omega-3 index and GLP-1 in plasma during the oral glucose tolerance test. A direct effect of EPA and DHA on the β-cell is also possible and is consistent with our data. Although activation of GPR-40 on the β-cell surface has a direct and positive effect on insulin secretion\(^{120}\), GPR40 is unlikely to mediate this effect as it is more potently activated by saturated rather than unsaturated fatty acids. It is plausible that GPR-120 is also expressed on the β-cell surface, as GPR-120 mRNA has been found within β-cells. However, evidence for the GPR-120 receptor on the β-cell surface or a direct physiological role of GPR-120 on insulin secretion is lacking\(^{857}\).

We also observed an association between higher omega-3 index and lower night time BP and greater nocturnal systolic dipping. In particular, reduction in the normal systolic BP dipping during sleep is an independent risk factor for cardiovascular disease\(^{858}\). As these effects were observed while the participants were asleep, they would not have been identifiable without 24-hour ambulatory measurement. It is not surprising that we did not detect a relationship between the omega-3 index and daytime BP. The reported effects of n-3 PUFA on daytime BP are subtle (reduction of systolic BP by approximately 2 mmHg)\(^{643}\), and in our relatively small cohort they are likely to have been confounded by the effects of daily stress and activity.

Our ability to show an association between higher omega-3 index and improved insulin sensitivity is likely due to the relatively high plasma concentrations of n-3 PUFA observed in our participants. In our study, mean omega-3 index was 7.1% in the two lower tertiles, which would place the majority of these participants in the highest quartile of the omega-3 index reported in the Physicians’ Health Study\(^{633}\). Thus, we speculate that n-3
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PUFA concentrations in participants from previous studies\(^{526,527,859}\) were not sufficiently high to lead to detectable improvements in insulin sensitivity.

Within our cohort, the HOI group had lower BMI than the LOI group (a difference of 1.1 kg/m\(^2\)). As body composition is an important determinant of insulin sensitivity\(^{185}\), it was paramount to consider body fat as a potential confounder, so that differences in adiposity were controlled for in our statistical analyses. An inverse association between adiposity and plasma n-3 PUFA\(^{860}\) or omega-3 index has been previously observed\(^{525}\), but there is no evidence that increased fat mass causes a reduction in circulating omega-3 levels. On the other hand, there is evidence from animal models that omega-3 supplementation leads to a reduction in adiposity, but the human evidence is limited and conflicting\(^{861}\). Nonetheless, a recent systematic review observed a small effect of omega-3 supplementation on body composition; participants taking fish oil lost 0.59 kg more than controls, with a reduction in BMI of 0.24 kg/m\(^2\)\(^{862}\). As this effect was small, it is unlikely that the differences in BMI between groups in our study were a result of the differences in omega-3 index.

In this study, we have controlled for important confounders that are known to affect insulin sensitivity and other metabolic outcomes, including saturated fat consumption, physical activity levels, socioeconomic status, total body fat, and age. Further, the improved insulin sensitivity in the highest omega-3 index tertile was associated with lower FFAs, in fitting with the proposed mechanisms of action.

However, our study has important limitations. As this was an observational study, our findings are not sufficient to establish a causative relationship between omega-3 index and the metabolic differences identified. It is possible that other unknown factors (not controlled for in our statistical models) accounted for both the higher n-3 PUFA concentrations and the improved metabolic indices in the highest tertile. As we had a relatively low number of participants (n=47) there was insufficient power to better examine a dose/response relationship. Lastly, we studied a relatively narrow range of individuals (overweight middle-aged males living in a large urban centre, mostly of New Zealand European ethnicity), which may limit wider applicability of our findings, particularly to women and to persons not in the overweight category.

In conclusion, higher n-3 PUFA concentrations in red cell phospholipids were associated with improved insulin sensitivity, lower free fatty acid and CRP concentrations, as well as improved nocturnal BP profile in a group of overweight middle-aged men. Randomized controlled trials (that carefully control for potential confounders such as diet, physical activity, and body composition) are required to adequately investigate the effects of supplementation with long-chain n-3 PUFA on insulin sensitivity and metabolism.
Chapter 9. Supplementation with a blend of krill and salmon oil is associated with increased metabolic risk in overweight men

9.1. Preface

This chapter contains a modified version of a manuscript published in the journal *American Journal of Clinical Nutrition*.

- **Authors:** Benjamin B. Albert, José GB. Derraik, Christine M. Brennan, Janene B. Biggs, Manohar L. Garg, David Cameron-Smith, Paul L. Hofman, Wayne S. Cutfield
- **Journal:** American Journal of Clinical Nutrition
- **Year of Publication:** 2015
- **Volume:** 102
- **Page:** 49-57
- **Impact Factor when published:** 6.80
9.2. Introduction

The prevalence of obesity has increased rapidly in both developed and developing nations. Obesity is linked to essential hypertension, dyslipidaemia, and T2DM, through pathologically reduced sensitivity to insulin. This insulin resistance is a common pathological factor behind an oncoming 'tsunami' of non-communicable disease.

Lifestyle factors (such as diet and physical activity) influence insulin sensitivity both directly and through changes in adiposity. While weight-loss and increased physical activity improve insulin sensitivity, for a large proportion of the population these are difficult to achieve. In contrast, small dietary modifications, such as supplementation with nutraceuticals are easier to attain. Thus, if a dietary supplement can improve insulin sensitivity in at-risk groups, it may be possible to lower the incidence of T2DM, cardiovascular disease, and other aspects of the metabolic syndrome.

Fish and krill oils principally contain the long-chain omega-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Epidemiological and animal evidence suggests these n-3 PUFA may improve insulin sensitivity and metabolic risk, but randomized controlled trials have yielded conflicting results. Higher dietary or plasma n-3 PUFA concentrations are associated with lower risk of diabetes. In addition, a higher omega-3 index (weight/weight% of EPA + DHA red cell phospholipid), which reflects dietary intake over a period of months, is associated with greater insulin sensitivity in children and overweight men. Further, it is clear that fish oil supplementation reverses insulin resistance in the rat and that the G-protein linked receptor GPR-120 plays a key role.

A recent systematic review that included 11 randomized controlled trials and 618 participants, concluded that n-3 PUFA supplementation did not influence insulin sensitivity. However, the individual trials were highly heterogeneous, including participants with and without T2DM and utilising a wide range of n-3 PUFA sources, doses and control oils. Fish oil, in association with weight-loss or caloric restriction improved insulin sensitivity, as did switching to a Mediterranean diet, but it is difficult to isolate the specific effects of n-3 PUFA due to the complexity of these interventions. Replacing non-oily fish in the diet with oily fish improved insulin sensitivity in a trial of 12 healthy older adults, but in a larger study of 162 adults, addition of fish oil to a diet high in saturated or monounsaturated fat diet did not influence insulin sensitivity.
Krill is an increasingly popular source of marine n-3 PUFA that differs from fish oil. Unlike fish oil which contains triglycerides, the n-3 PUFA in krill oil are predominantly in phospholipid form\textsuperscript{463,866}. Also, krill oil contains astaxanthin, a carotenoid pigment and powerful antioxidant\textsuperscript{470}. Not surprisingly, differential effects of krill and fish oils have been described in mice\textsuperscript{867}. However, the effect of krill oil supplementation on insulin sensitivity in humans has not been reported. Therefore, we aimed to investigate whether supplementation with a blend of krill and salmon oil (KS oil) would lead to changes in insulin sensitivity in a double-blind randomized controlled crossover human trial.

\textbf{9.3. Methods}

\textit{Ethics}

Ethical approval was granted by the Central Regional Ethics Committee, New Zealand Ministry of Health (CEN/11/07/038). This trial was prospectively registered with the Australian New Zealand Clinical Trials Registry (ACTRN12611000602921). Written and verbal informed consent was obtained from all participants. This study was performed in accordance with all appropriate institutional and international guidelines and regulations for medical research, in line with the principles of the Declaration of Helsinki.

\textit{Participants}

Volunteers were recruited in 2012 using advertisements in local newspapers that circulate freely in the central Auckland metropolitan area (New Zealand). Overweight (body mass index (BMI) 25–30 kg/m\textsuperscript{2}), middle-aged (35–55 years) men were eligible to participate. The study cohort represents a high-risk group, likely to have early insulin resistance without metabolic decompensation to clinical disease (e.g. diabetes mellitus), thus making it easier to detect a potential insulin sensitising effect of KS oil. Note that only males were recruited, so that the confounding effects of the menstrual cycle and/or oral contraceptives on insulin sensitivity (the primary outcome) could be avoided. Exclusion criteria were: diabetes mellitus, hypertension (systolic BP >145 mmHg or diastolic >95 mmHg), known dyslipidaemia, the use of tobacco, or prescription medications likely to affect BP, lipid profile, or insulin sensitivity. Participants taking fish oil or other omega-3 supplements were asked to stop supplementation four weeks prior to the first assessment and to maintain a similar diet and level of physical activity for the duration of the study.
Data Papers

Randomization and masking

Randomized allocation of participants to treatment arm was done using computer random number generation. The code was kept by a third party, and was not released until after statistical analysis. Both researchers and participants were blinded to the contents of capsules being taken. To maintain integrity of the trial evaluation, statistical analysis was carried out on encoded data, so that the data analyst was also blinded to treatment.

Study design

This study was a 24-week randomised double-blind controlled crossover trial. Participants were randomized to receive capsules with active treatment or control for 8 weeks (Figure 8). After an 8-week washout period, participants switched over to the other treatment for a further 8 weeks (Figure 8).

The active treatment contained krill (88%) and salmon (12%) oils. This oil was 42.1% phospholipid by weight, and each 1000 mg gelatine capsule contained 46 mg EPA and 31 mg DHA (Table 5). Participants were instructed to take 5 capsules as a single dose, once a day, with a glass of water, equating to a daily supplementation with 450 mg of n-3 PUFA (including 230 mg EPA + 154 mg DHA). The control intervention consisted of 1000 mg of canola oil, also presented in a gelatine capsule, minimally coated in fish oil (<5 mcg) to match odour and flavour. Canola oil primarily contains oleic acid (Table 5), and there is no reliable evidence that a low dose of canola oil affects insulin sensitivity. Both KS and control oil capsules were manufactured by Nutrizeal Ltd (Nelson, New Zealand), and their contents were verified independently by gas chromatography (Table 5).
Figure 8: Summary of krill trial recruitment process and execution.

I_X indicates timing of assessments. Two participants withdrew during the first treatment phase due to allergic reaction to krill oil, while two subjects withdrew after crossover due to serious injury or engagement in marathon training.

Table 5: Fatty acid concentration of the control (canola oil) and krill/salmon oil.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 (palmitic acid)</td>
<td>18.22 ± 1.45</td>
<td>81.94 ± 4.59</td>
</tr>
<tr>
<td>C16:1n-7 (palmitoleic acid)</td>
<td>0.68 ± 0.03</td>
<td>11.98 ± 2.23</td>
</tr>
<tr>
<td>C18:0 (stearic acid)</td>
<td>16.49 ± 0.76</td>
<td>16.18 ± 0.82</td>
</tr>
<tr>
<td>C18:1n-7 (cis-vaccenic acid)</td>
<td>2.49 ± 0.15</td>
<td>5.60 ± 0.50</td>
</tr>
<tr>
<td>C18:1n-9 (oleic acid)</td>
<td>611.19 ± 51.80</td>
<td>196.89 ± 33.92</td>
</tr>
<tr>
<td>C18:2n-6 (linoleic acid)</td>
<td>31.21 ± 2.33</td>
<td>5.55 ± 0.87</td>
</tr>
<tr>
<td>C18:3n-3 (α-linolenic acid)</td>
<td>41.74 ± 2.74</td>
<td>11.02 ± 0.49</td>
</tr>
<tr>
<td>C18:3n-6 (γ-linolenic acid)</td>
<td>nil</td>
<td>8.31 ± 0.34</td>
</tr>
<tr>
<td>C20:0 (arachidic acid)</td>
<td>nil</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>C20:1n-9 (eicosenoic acid)</td>
<td>nil</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>C20:2n-6 (eicosadienoic acid)</td>
<td>nil</td>
<td>0.23 ± 0.10</td>
</tr>
<tr>
<td>C20:3n-6 (dihomo-γ-linolenic acid)</td>
<td>nil</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>C20:4n-6 (arachidonic acid)</td>
<td>nil</td>
<td>5.49 ± 0.67</td>
</tr>
<tr>
<td>C20:5n-3 (eicosapentaenoic acid)</td>
<td>nil</td>
<td>45.90 ± 4.07</td>
</tr>
<tr>
<td>C22:5n-3 (docosapentaenoic acid)</td>
<td>nil</td>
<td>2.40 ± 0.45</td>
</tr>
<tr>
<td>C22:6n-3 (docosahexaenoic acid)</td>
<td>nil</td>
<td>30.76 ± 2.04</td>
</tr>
</tbody>
</table>

Concentrations are in mg/g of oil, determined by gas chromatography. Data are means ± standard deviations, from analysis in triplicate. The krill/salmon is a blend of krill oil (88%) and salmon oil (12%).
To establish their oxidative state, both trial oils were also analysed for peroxide value (PV) and anisidine value (AV) according to the European Pharmacopoeia methods. Average values were calculated based on triplicate measures of oil pooled from 10 capsules. Assay characteristics were determined using fish oil capsules that were not used in this study. The peroxide value had inter- and intra-assay coefficients of variation (CV) <3% and a lower limit of detection of 0.3 meq/l, while the anisidine value had inter- and intra-assay CV <4%. In addition, the Totox value was calculated as (2 x PV) + AV. The KS oil had a peroxide value below the lower limit of detection of 0.3 meq/l and an anisidine value of 11.0, indicating a Totox value of <11.6. The control oil (canola) had a peroxide value of 3.1 meq/l and an anisidine value of 2.3, indicating a Totox value of 8.5. Thus, both oils were relatively unoxidised and within international recommendations. Nonetheless, to minimise oxidation of the oil prior to consumption, capsules were provided in sealed amber glass jars and participants were instructed to keep them in the refrigerator.

Clinical assessments

All participants were assessed on four occasions: immediately before and after each 8-week intervention. Clinical assessments were carried out between 07:00 and 09:00 at the Maurice & Agnes Paykel Clinical Research Unit (Liggins Institute, University of Auckland), after an overnight fast and no strenuous activity over the previous 24 hours.

The primary outcome was insulin sensitivity measured with a 75 g oral glucose tolerance test using the Matsuda method. Blood samples were collected at 0, 30, 60, 90, and 120 minutes for glucose and insulin measurements. The Matsuda index has a strong correlation with the hyperinsulinaemic euglycaemic clamp (r=0.77), and excellent reproducibility during multiple measures. The oral disposition index (a measure of β-cell function corrected for insulin sensitivity) and the homeostasis model assessment of insulin resistance (HOMA-IR) were also calculated.

Fasting blood samples were used to assess other measures of metabolic disease risk, including uric acid, free fatty acid, and highly-sensitive C-reactive protein (CRP) concentrations, as well as lipid profile (triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), Apo-A and Apo-B concentrations). Auxological assessment included height measurement using a Harpenden stadiometer. Weight and body composition were assessed using whole-body dual-energy X-ray absorptiometry (DXA, Lunar Prodigy 2000, General Electric, Madison, USA).
24-hour ambulatory BP monitoring was carried out prior to each clinical assessment. Participants were fitted with a Spacelabs 90207 or 90217 monitor (Spacelabs Medical Inc., Redmond, USA), with each subject being assigned the same device model for all assessments. Measurements were performed every 20 minutes between 07:00 and 22:00, and every 30 minutes from 22:00 to 07:00. Only profiles with >14 daytime and >7 night time recordings over a 24-hour period were analysed.

Carotid artery intima-media thickness was also measured, as it is a validated and reproducible measure that is predictive of cardiovascular and cerebrovascular risks. Carotid intima-media thickness was measured using an M-Turbo ultrasound system (Sonosite, Bothel, USA) by the same trained investigator, with longitudinal images attained using a standard protocol. The right common carotid artery was scanned from both posterolateral and anterolateral views, with digitally stored images analysed using computer software automated callipers to measure the far wall (SonoCalc v.4.1, Sonosite). The maximal thickness measurements from both views (approximately 10 mm proximal to the carotid bulb) were used for comparative analysis, with an intra-observer CV of 3.7%.

Lifestyle factors were recorded with an itemised food diary and physical activity recall. Three-day dietary records were collected prior to clinical assessments. Each dietary report encompassed an itemized nutritional intake recorded during two week days (Monday to Friday) and one weekend day. Nutritional intake was recorded using standard household measures, as well as the information from food labels where appropriate. Participants were instructed by a trained investigator, who also reviewed all food records with each participant to address unclear descriptions, errors, omissions, or doubtful entries. Records were subsequently entered into Foodworks software (v6.0, Xyris Software, Brisbane, Australia) by the trained investigator. Physical activity levels were assessed using the International Physical Activity Questionnaire (IPAQ), covering four domains of physical activity: work-related, transportation, housework/gardening, and leisure time.

Geo-coded deprivation scores were derived from current address using the New Zealand Index of Deprivation 2006 (NZDep2006). This index is based on household census data reflecting nine aspects of material and social deprivation to divide New Zealand into tenths (scored 1-10) by residential address. Scores of 1 represent the least deprived areas and 10 the most deprived. Scores are derived from units covering a small area, each reflecting approximately 90 people. Ethnicity was recorded by self-report using a prioritised system, such that if multiple ethnicities were selected, the patient was assigned to a single category, following a hierarchical system of classification (order of priority was Maori, Pacific Island, Asian, and New Zealand European).
Assays

Insulin concentrations were measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, USA) by microparticle enzyme immunoassay with an inter-assay CV of 5.4%. Glucose, triglyceride, total cholesterol, HDL-C, LDL-C, Apo-A, Apo-B, free fatty acid, uric acid, and highly-sensitive CRP concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation) by enzymatic colorimetric assay (Roche) with all CVs lower than 3.2%.

Erythrocyte fatty acid analysis

Fatty acid profile was analysed via direct transesterification of the washed erythrocyte (RBC) fraction of blood, followed by gas chromatography. Methanol:toluene 2 ml (4:1 v/v) (containing C19:0 (20 µg/ml) as internal standard) was added to the sample. Acetyl chloride (200 µl) was added while vortexing and then heated (1 hour, 100°C). The tubes were then cooled in water (5 minutes), had K₂CO₃ 6% (5 ml) added, and were subsequently centrifuged (3000 x g, 5 min, 4°C). The upper toluene phase containing the fatty acid methyl esters was collected and stored in a gas chromatograph vial at -20°C for analysis.

Methylated fatty acid samples were analysed by gas chromatography using a fixed carbon-silica column 30 m x 0.25 mm (DB-225) (J&W Scientific, Folsom, CA, USA). The gas chromatograph was equipped with a flame ionization detector, autosampler, and autodetector. Injector and detector ports were set at 250°C. Oven temperature was programmed: 170°C for two minutes, increased 10°C/minute up to 190°C, where it remained constant for one minute. Temperature then increased 3°C/minute up to 220°C, which was maintained for a total run time of 30 minutes per sample. A split ratio of 10:1 and an injection volume of 3 µl were used. A known fatty acid mixture was compared to analysed samples to identify peaks according to retention time; their concentration was determined using a 6890 Series gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) with Chemstations Version A. 04.02 (Chemstations Inc., Houston, TX, USA). The omega-3 index was calculated as the weight/weight% of EPA + DHA in red cell phospholipid.
Data Papers

Power and sample size calculation

The power calculation was based on data from an Auckland population of adult males also overweight and of similar age\textsuperscript{332}. Based on a mean Matsuda index of 6.67 and standard deviation of 3.73\textsuperscript{332}, a total sample of 42 participants would have at least 90% power at 5% level of significance (two-sided) to detect a 20% change in Matsuda index, assuming a correlation of 0.5 between measurements on the same subject. To allow for a 20% drop out rate during the study, the recruitment target was 50 participants.

Safety assessment

All adverse events were recorded throughout the study, and participants were monitored for any adverse events such as gastrointestinal symptoms, allergy, and bleeding. A general medical examination and liver function tests were also carried out. Further, if hypertension or carotid atherosclerosis was identified during pre-screening or clinical assessment, an immediate referral was made to the general practitioner.

Treatment compliance

Participants were over-issued with capsules by an amount unknown to them, and asked to return all unused capsules. Treatment compliance was estimated from returned capsules.

Blinding success

During the final clinical assessment, all participants were asked if they were able to identify the capsules (i.e. KS oil or control) they took last. Their responses were noted, and the effectiveness of treatment blinding was assessed by the Bang’s blinding index (BBI)\textsuperscript{872}. In this study, blinding success was determined as per Moroz et al.’s thresholds: BBI ≥ 0.2 unblinded, −0.2 < BBI < 0.2 random guesses, and BBI ≤ −0.2 opposite guesses\textsuperscript{873}. 

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**Statistical analysis**

Linear mixed regression models with a random patient effect (SAS v.9.3, SAS Institute, Cary, NC, USA) were adopted to assess the main treatment effect. Models accounted for treatment period (first leg or crossover). Importantly, regression models also adjusted for the baseline value of the outcome response to gain statistical efficiency and power (i.e. baseline data were included in the model as covariates). The interaction between treatment and treatment period was also evaluated for each outcome response, in order to assess the success of the washout. Parameters of glucose homeostasis and inflammatory markers were log-transformed to approximate normality. Physical activity levels, dietary intake, and n-3 PUFA levels were compared between treatments with paired t-tests. All statistical tests were two-tailed and significance level maintained at 5%. Descriptive data are presented as means ± standard deviations; other data are means and 95% confidence intervals, adjusted for treatment period and baseline values.

**9.4. Results**

**Participants**

A total of 51 men were recruited into the clinical trial, but 4 were subsequently excluded (Figure 8). Two participants dropped out of the trial soon after baseline due to an allergic reaction to KS oil (unmasked by a third party). As a result, the intention to treat principle was not followed. We analysed data on 47 participants (Figure 8), who were aged 46.5 ± 5.1 years and with BMI of 27.4 ± 1.8 kg/m². The majority of participants (87%) were of European descent.

**Primary outcome**

Insulin sensitivity was 14% lower with KS oil supplementation than with control oil (Matsuda index 4.57 vs 5.33, respectively; \( p=0.049 \)) (Table 6). While there was no significant change in insulin sensitivity from baseline on control oil \( (p=0.23) \), participants experienced an 18% decrease in Matsuda index \( (p=0.002) \) while taking KS oil (Table 7). The deterioration in insulin sensitivity after KS oil was corroborated by HOMA-IR that increased by 0.39 \( (p=0.016) \), and fasting insulin that increased by 1.39 mU/l \( (p=0.048; \text{Table 7}) \). There was no change in disposition index.
Data Papers

As previously reported, there was a positive association between omega-3 index and insulin sensitivity at baseline. To investigate for possible effects of components of KS oil other than the n-3 PUFA, mediated analysis was carried out controlling for the effects of the omega-3 index on the primary outcome. This analysis showed that after controlling for the likely positive effects of blood EPA and DHA (i.e. omega-3 index), the reduction of insulin sensitivity after KS oil supplementation was more marked; 27% lower than control (4.04 vs 5.53; p=0.009) (Table 7). When omega-3 index was accounted for, there was no change in insulin sensitivity (compared to baseline) with control (p=0.88), but participants experienced a 24% reduction in Matsuda index after taking KS oil (p=0.0008) (Figure 9).

Secondary outcomes

There were no significant differences between control and KS oils in the range of secondary outcomes assessed (Table 6). However, participants taking KS oil tended to have higher concentrations of Apo-B than when on control oil (p=0.053; Table 6). In addition, there was a trend towards an increase in carotid intima-media thickness with KS oil (p=0.068; Table 6).

Within-group changes from baseline were assessed in post-hoc analyses (Table 7). When compared to baseline, canola oil did not lead to any significant changes in the parameters assessed. However, KS oil supplementation was associated with increases in carotid artery intima-media thickness (+39 µm; p=0.001) and concentrations of Apo-B (+8.02 mg/dl; p=0.009), total cholesterol (+0.31 mmol/l; p=0.0004), LDL-C (+0.27 mmol/l; p=0.001), and HDL-C (+0.08 mmol/l; p=0.034) (Table 7).
Table 6: Primary and secondary outcomes among overweight middle-aged men, following supplementation with control or krill/salmon oil.

<table>
<thead>
<tr>
<th>Primary outcome</th>
<th>Control</th>
<th>Krill/salmon oil</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary outcome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin sensitivity (Matsuda index)</td>
<td>5.33 (4.73, 6.00)</td>
<td>4.57 (4.06, 5.14)</td>
<td>0.049</td>
</tr>
<tr>
<td><strong>Secondary outcomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>89.0 (88.5, 89.5)</td>
<td>88.7 (88.2, 89.2)</td>
<td>0.45</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>28.0 (27.6, 28.3)</td>
<td>28.0 (27.7, 28.4)</td>
<td>0.82</td>
</tr>
<tr>
<td>Android fat to gynoid fat ratio</td>
<td>1.27 (1.25, 1.29)</td>
<td>1.28 (1.26, 1.30)</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>Glucose homeostasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.85 (1.66, 2.07)</td>
<td>2.02 (1.80, 2.26)</td>
<td>0.26</td>
</tr>
<tr>
<td>Disposition index</td>
<td>5.37 (4.32, 6.67)</td>
<td>5.47 (4.39, 6.78)</td>
<td>0.87</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.30 (5.19, 5.40)</td>
<td>5.40 (5.30, 5.51)</td>
<td>0.092</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>7.53 (6.67, 8.50)</td>
<td>8.22 (7.28, 9.27)</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>24-hour ambulatory blood pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daytime systolic (mmHg)</td>
<td>126.2 (124.3, 128.1)</td>
<td>125.6 (123.7, 127.5)</td>
<td>0.61</td>
</tr>
<tr>
<td>Daytime diastolic (mmHg)</td>
<td>79.2 (77.9, 80.6)</td>
<td>78.9 (77.5, 80.3)</td>
<td>0.72</td>
</tr>
<tr>
<td>Night time systolic (mmHg)</td>
<td>110.7 (108.4, 112.9)</td>
<td>109.6 (107.3, 111.9)</td>
<td>0.42</td>
</tr>
<tr>
<td>Night time diastolic (mmHg)</td>
<td>66.6 (64.9, 68.4)</td>
<td>65.6 (63.8, 67.3)</td>
<td>0.36</td>
</tr>
<tr>
<td>Systolic dip (%)</td>
<td>12.2 (10.6, 13.9)</td>
<td>12.6 (10.9, 14.2)</td>
<td>0.74</td>
</tr>
<tr>
<td>Diastolic dip (%)</td>
<td>15.8 (13.7, 17.9)</td>
<td>16.7 (14.6, 18.8)</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Carotid-intima media thickness</strong> (mm)</td>
<td>0.819 (0.801, 0.836)</td>
<td>0.843 (0.826, 0.861)</td>
<td>0.068</td>
</tr>
<tr>
<td><strong>Other metabolic markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.37 (0.32, 0.41)</td>
<td>0.39 (0.35, 0.44)</td>
<td>0.43</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>366 (355, 377)</td>
<td>364 (352, 375)</td>
<td>0.76</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.01 (0.77, 1.31)</td>
<td>1.10 (0.84, 1.43)</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.17 (5.00, 5.33)</td>
<td>5.38 (5.21, 5.55)</td>
<td>0.11</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.60 (3.44, 3.76)</td>
<td>3.74 (3.58, 3.90)</td>
<td>0.20</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.15 (1.08, 1.23)</td>
<td>1.17 (1.09, 1.24)</td>
<td>0.79</td>
</tr>
<tr>
<td>Total cholesterol : HDL-C</td>
<td>4.82 (4.52, 5.12)</td>
<td>4.79 (4.49, 5.09)</td>
<td>0.89</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.14 (1.03, 1.25)</td>
<td>1.18 (1.07, 1.29)</td>
<td>0.64</td>
</tr>
<tr>
<td>Apolipoprotein A (mg/dl)</td>
<td>162 (156, 168)</td>
<td>162 (157, 168)</td>
<td>0.93</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dl)</td>
<td>104 (98, 110)</td>
<td>110 (104, 116)</td>
<td>0.053</td>
</tr>
</tbody>
</table>

n=47, supplementation was for 8 weeks. Control was canola oil.
Table 7: Changes in primary and secondary outcomes in comparison to baseline among overweight middle-aged men, following supplementation with control or krill/salmon oil.

<table>
<thead>
<tr>
<th>Primary outcome</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin sensitivity (Matsuda index)</td>
<td>-0.38 (-1.00, 0.25)</td>
<td>-0.98 (-0.36, -1.61)*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary outcomes</th>
<th>Anthropometry</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>0.43 (-0.09, 0.94)</td>
<td>0.12 (-0.40, 0.63)</td>
<td></td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>0.08 (-0.29, 0.45)</td>
<td>0.14 (-0.23, 0.52)</td>
<td></td>
</tr>
<tr>
<td>Android fat to gynoid fat ratio</td>
<td>-0.00 (-0.02, 0.02)</td>
<td>0.00 (-0.02, 0.02)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glucose homeostasis</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA-IR</td>
<td>0.16 (-0.16, 0.47)</td>
<td>0.39 (0.07, 0.70)*</td>
</tr>
<tr>
<td>Disposition index</td>
<td>0.98 (-1.34, 3.29)</td>
<td>-0.40 (-2.90, 2.10)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>-0.06 (-0.16, 0.05)</td>
<td>0.05 (-0.06, 0.15)</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>0.70 (-0.67, 2.08)</td>
<td>1.39 (0.01, 2.77)*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>24-hour ambulatory blood pressure</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daytime systolic (mmHg)</td>
<td>0.19 (-1.72, 2.10)</td>
<td>-0.39 (-2.32, 1.55)</td>
</tr>
<tr>
<td>Daytime diastolic (mmHg)</td>
<td>-0.13 (-1.50, 1.24)</td>
<td>-0.44 (-1.83, 0.94)</td>
</tr>
<tr>
<td>Night time systolic (mmHg)</td>
<td>-0.03 (-2.28, 2.21)</td>
<td>-1.11 (-3.36, 1.16)</td>
</tr>
<tr>
<td>Night time diastolic (mmHg)</td>
<td>0.09 (-1.66, 1.84)</td>
<td>-0.98 (-2.74, 0.78)</td>
</tr>
<tr>
<td>Systolic dip (%)</td>
<td>0.24 (-1.39, 1.87)</td>
<td>0.60 (-1.05, 2.25)</td>
</tr>
<tr>
<td>Diastolic dip (%)</td>
<td>-0.41 (-2.50, 1.67)</td>
<td>0.54 (-1.57, 2.65)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carotid-intima media thickness (mm)</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.007 (-0.016, 0.030)</td>
<td>0.039 (0.016, 0.062)**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other metabolic markers</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>-0.02 (-0.06, 0.02)</td>
<td>0.01 (-0.04, 0.05)</td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>8.3 (-2.8, 19.3)</td>
<td>6.2 (-4.9, 17.3)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.45 (-0.50, 1.39)</td>
<td>0.19 (-0.76, 1.13)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipid profile</th>
<th>Control</th>
<th>Krill/salmon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>0.10 (-0.07, 0.26)</td>
<td>0.31 (0.14, 0.47)**</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>0.14 (-0.03, 0.30)</td>
<td>0.27 (0.11, 0.43)**</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.07 (-0.01, 0.14)</td>
<td>0.08 (0.01, 0.15)*</td>
</tr>
<tr>
<td>Total cholesterol : HDL-C</td>
<td>-0.16 (-0.46, 0.14)</td>
<td>-0.18 (-0.48, 0.12)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>-0.05 (-0.16, 0.06)</td>
<td>-0.01 (-0.11, 0.10)</td>
</tr>
<tr>
<td>Apolipoprotein A (mg/dl)</td>
<td>2.00 (-3.57, 7.57)</td>
<td>2.24 (-3.32, 7.81)</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dl)</td>
<td>1.29 (-4.66, 7.23)</td>
<td>8.02 (2.07, 14.0)**</td>
</tr>
</tbody>
</table>

**n=47, supplementation was for 8 weeks. Control was canola oil. Results from linear mixed regression models with a random patient effect. Data are means and 95% confidence intervals adjusted for treatment period and baseline values.**

*p<0.05, **p<0.01, and ***p<0.001 for a change from baseline associated with a particular treatment.
Figure 9: Mediated analysis showing insulin sensitivity and change in insulin sensitivity in overweight middle-aged men, following supplementation with control or krill/salmon oil

n=47, supplementation was for 8 weeks. Control was canola oil. Data are means and 95% confidence intervals adjusted for treatment period, baseline values, and the participants' concentrations of EPA+DHA in erythrocyte membrane fatty acids. *p<0.05 and *p<0.01 for placebo vs krill/salmon oil; †††p<0.001 for the change from baseline associated with a particular treatment.
Other adverse events

Apart from the described reports of allergy that led to the withdrawal of two participants, the main adverse event associated with the consumption of KS oil was a high incidence of eructation described as 'fishy burps'. Over the course of the trial, 22 participants (47%) reported that they experienced 'fishy burps' while on KS oil compared to just one participant (2%) on control (p<0.0001).

Other adverse events were reported by participants while taking KS oil, including mild gastrointestinal symptoms (n=2), increased bowel frequency (n=2), and localized 'pimples' (n=1). Participants taking control also reported mild gastrointestinal symptoms (n=1) and increased bowel frequency (n=3).

Compliance

The compliance of participants with study protocol was high, with 93.2% of capsules being taken overall (SD=5.8%), although it varied among participants from 78.1 to 100%. Compliance was very similar in the first (93.9 ± 5.9%) and crossover (92.4 ± 8.1%) phases of the trial (p=0.21), and it was not affected by treatment (p=0.61), ethnicity (p=0.52), age (p=0.27), or socioeconomic status (p=0.93).

Blinding

Among participants who were last on KS oil (n=23), the majority (12; 52%) correctly guessed its content, while 4 (17%) guessed wrong, and 7 (30%) answered that they did not know. For those on control (n=24), there were 12 correct guesses (50%), 3 incorrect ones (13%), and 9 (38%) did not know. Bang's index was 0.35 for KS oil (95% CI: 0.04, 0.66) and 0.38 for control (95% CI: 0.10, 0.65). Based on Moroz et al.'s thresholds, treatment blinding was not successful, with a considerable proportion of participants correctly guessing beyond chance the last treatment they were on. We know that one participant intentionally cut a capsule to identify its content, but we are unaware of others doing the same. Participants who reported 'fishy burps' appeared to be more likely to correctly guess the treatment than those who did not experience them (odds ratio 3.54 (95% CI: 0.93, 13.51); p=0.065), suggesting that this adverse effect contributed to unblinding. Importantly, there were no differences in diet or physical activity levels during control and KS oil interventions (Table 8), which indicates that unblinding was not associated with modification of important behaviours that influence insulin sensitivity.
Table 8: Physical activity, dietary intake, and n-3 PUFA concentrations among study participants following supplementation with control or krill/salmon oil.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Krill/salmon oil</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical activity levels (IPAQ)</td>
<td>3635 ± 3747</td>
<td>3549 ± 2641</td>
<td>0.67</td>
</tr>
<tr>
<td>Total energy intake (kJ/day)</td>
<td>8932 ± 2312</td>
<td>9102 ± 2985</td>
<td>0.51</td>
</tr>
<tr>
<td>Saturated fat intake (g/day)</td>
<td>28.8 ± 11.5</td>
<td>28.9 ± 15.9</td>
<td>0.81</td>
</tr>
<tr>
<td>Fish meals per participant per week</td>
<td>1.6 ± 1.1</td>
<td>1.5 ± 1.1</td>
<td>0.33</td>
</tr>
<tr>
<td>EPA (% wt/wt)</td>
<td>1.54 ± 0.73</td>
<td>2.47 ± 0.62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHA (% wt/wt)</td>
<td>6.41 ± 1.53</td>
<td>7.06 ± 1.19</td>
<td>0.001</td>
</tr>
<tr>
<td>Omega-3 index</td>
<td>7.87 ± 2.10</td>
<td>9.53 ± 1.67</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

n=47, supplementation was for 8 weeks. Control was canola oil. Results from paired t-tests. Data are means ± standard deviations. EPA and DHA concentrations are % weight/weight in erythrocyte membrane fatty acids. Omega-3 index represent the sum of EPA+DHA concentrations.

Dietary intake & physical activity

There was no evidence of dietary alterations or changes in physical activity levels while on control or KS oil supplementation (Table 8). KS oil supplementation increased blood concentrations of EPA by 60% (p<0.0001) and DHA by 10% (p=0.025), leading to a 21% difference in omega-3 index between the two treatments (Table 8).

Washout period

There were no statistically significant interactions between treatment and treatment period for any of the primary or secondary outcomes (data not shown). Thus, there was no observed carry-over effect between treatment periods, and the washout phase was likely successful.

9.5. Discussion

Contrary to our initial hypothesis, in this double-blind controlled crossover trial there was a reduction in insulin sensitivity after supplementation with the krill/salmon blended oil compared to control (canola oil). Importantly, there were no significant changes in body composition, physical activity or diet, the major lifestyle factors that are known to affect insulin sensitivity. Thus, our findings suggest that krill and/or salmon oil supplementation may increase the risk of developing T2DM and cardiovascular disease.
In addition, a post-hoc analysis showed there was an increase in LDL-cholesterol and Apo-B concentrations, as well as a subtle increase in carotid artery intima-media thickness following KS oil supplementation, suggesting a more atherogenic lipid profile. However, as significant differences were not seen in the primary analysis, these findings must be interpreted with caution, and need to be corroborated in other studies.

The control treatment in this study was canola oil, which confers the advantage of matching the intervention for caloric density and fat content, as well as reducing the risk of unblinding. Canola oil is primarily made up of oleic acid, and there is no reliable evidence that oleic acid improves insulin sensitivity or lipid profile. However, canola oil also contains phytosterols, and this may mediate a beneficial effect of high doses of canola oil (20–25 g/day) on serum triglycerides and cholesterol absorption. The dose of canola oil in this study was low (5 g/day) and we observed no effects on serum triglycerides or any other metabolic outcomes after canola oil supplementation. Thus, it appears that canola oil had no metabolic effects in this study and was an appropriate control treatment.

The KS oil treatment involved a 5 g/day intake, which equated to low dose n-3 supplementation (400 mg/day) providing 80% of the daily intake of EPA and DHA recommended by the National Heart Foundation of Australia. Despite a relatively low dose of n-3 PUFA, KS oil supplementation led to a 60% increase in EPA concentrations compared with control, a magnitude similar to previous studies that have shown metabolic and anti-inflammatory effects of n-3 PUFA supplementation. It seems unlikely that the n-3 PUFA in the KS oil were the cause of the observed reduction in insulin sensitivity, as when the omega-3 index was controlled for, the adverse effect of KS oil on insulin sensitivity became more marked (Figure 9). Further, we have shown that in this cohort of overweight middle-aged men, the omega-3 index at baseline was associated with greater insulin sensitivity, lower nocturnal BP, as well as lower free fatty acid and CRP concentrations, suggesting metabolic benefits to those with higher n-3 PUFA levels. Importantly, previous epidemiological studies and clinical trials of fish oil supplementation in healthy people do not support an adverse metabolic effect of n-3 PUFA in men. Oils derived from marine species are complex in their chemical composition, where n-3 PUFA make up a fraction of their content. Thus, it is important to consider the composition of the trial oil, as the potential benefits to insulin sensitivity of n-3 PUFA appear to have been masked by the adverse effects of other compounds.

As the trial oil was a blend of krill (88%) and salmon oil (12%), it is not possible to ascertain whether the metabolically adverse effect was due to krill oil, salmon oil, or both. However, it is probable that the compound(s) mediating the metabolically adverse effect are found in krill oil rather than salmon oil. Firstly, as above, previous interventional trials of fish oil supplementation have not shown impairment of insulin sensitivity. Secondly, in a small study designed to compare the effects of krill and fish oil on plasma
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n-3 PUFA, large but not statistically significant increases in fasting insulin (44%) and HOMA-IR (55%) were observed in the krill but not the fish oil group. While this study was underpowered to detect a statistically significant difference in fasting insulin or HOMA-IR between groups, these data also suggest a detrimental effect of krill oil on insulin sensitivity, corroborating our findings.

Krill are crustaceans and are evolutionarily very distant from fish (i.e. vertebrates). Thus, the biologically active compounds present in krill and fish oils are likely to be distinct. Krill oil contains the carotenoid pigment astaxanthin that has antioxidant capacity, but which is unlikely to mediate the adverse metabolic effects observed. There are no data on the effects of astaxanthin on human metabolism, but in vitro evidence suggests that it may modulate PPAR-γ. This is a nuclear receptor with important metabolic effects, and PPAR-γ agonists are known to increase insulin sensitivity. Further, astaxanthin supplementation has been shown to improve insulin sensitivity in animal models.

Unlike fish oil where fatty acids are predominantly incorporated into triglycerides, in krill oil the fatty acids are largely present as phospholipids. This is also unlikely to explain the observed reduction in insulin sensitivity. The krill/salmon oil used in this study was 42% phospholipid by weight, the vast majority (76%) in the form of phosphatidyl-choline. The health effects of dietary phospholipids are not well understood, but limited evidence suggests that a higher phosphatidyl-choline fraction of membrane phospholipid is associated with greater insulin sensitivity. Thus, if supplementation with krill oil leads to a greater cell membrane phosphatidyl-choline fraction, a reduction in insulin sensitivity would not be expected.

Nonetheless, the phospholipids present in krill oil are amphipathic, having hydrophilic and lipophilic properties. As a result, after extraction, krill oil may still contain amphipathic or water soluble compounds (such as proteins) that would not be present in fish oil supplements. We speculate that there are compound(s) present in krill oil that impair insulin sensitivity, and that they are either not found in fish, or if present in fish are mostly excluded during oil extraction. Identification of these compound(s) could lead to a better understanding of the metabolic effects of marine oils that are not sourced from fish, and possibly to processes that would exclude them from supplements.

Aside from fishy eructations, adverse events from the KS oil supplementation were uncommon. However, two participants (4%) had an urticarial reaction to KS oil, suggesting a greater than expected incidence of allergy (crustacean allergy has a reported incidence of 1.6%). The presence of traces of protein in krill oil is a likely causative factor, as tropomyosins have been identified as the major allergens in Antarctic krill. Further, krill tropomyosins show significant homology to other crustaceans eaten as food, and immunoglobulin E against
shrimp antigens have been shown to cross-react with krill. Thus, people with known allergy to shellfish, especially crustaceans, should avoid krill oil.

This randomized controlled crossover trial was robust and adequately powered to assess the effects of supplementation with the KS oil on insulin sensitivity. The major strengths of this study were the use of detailed metabolic assessments, recruitment of a cohort that was healthy, yet at increased metabolic risk, and careful assessment of compliance, physical activity, and diet, which did not differ between groups. In addition, participants, investigators, and the data analyst remained blinded to the identity of treatment oils until after data analysis was complete.

Among the limitations, krill oil has a relatively low concentration of fatty acids, so that the dose of n-3 PUFA provided by the KS supplement was relatively low at 400 mg/day. Because the trial oil was a blend of krill and salmon oil, it remains unclear as to whether the metabolically adverse effect was due to krill oil, salmon oil or both. Further, because of the chemical differences between krill oil and other marine oils, these data cannot be generalised to fish, calamari, or algal sources of n-3 PUFA. In addition, we studied a relatively narrow range of individuals (overweight middle-aged males living in a large urban centre, mostly of European ethnicity), which may limit wider applicability of our findings, particularly to women. Recent evidence that the effects of EPA and DHA on platelet aggregation and haemostatic markers are sex-specific raises the possibility that the metabolic effects of n-3 PUFA could also differ according to sex.

In conclusion, this study provides evidence that supplementation with a blend of krill and salmon oil leads to a reduction in insulin sensitivity in overweight men. Further, a post-hoc analysis suggested that this blended oil may also lead to a less favourable lipid profile. The cause of these adverse effects is unclear and probably not associated with the n-3 PUFA, but rather likely effects of other chemical constituents of krill or salmon oil. We speculate that the adverse effects are more likely to be due to the krill oil component, and caution against the use of krill oil in those at increased risk of T2DM or cardiovascular disease, at least until there are more data on the metabolic effects of individual constituents of krill and salmon oil.
Chapter 10. Oxidised fish oil in rat pregnancy causes high newborn mortality and increases maternal insulin resistance

10.1. Preface

This chapter contains a modified version of a manuscript accepted by the American Journal of Physiology. Regulatory, Integrative and Comparative Physiology.

- **Authors:** Benjamin B Albert, Mark H Vickers, Clint Gray, Clare M Reynolds, Stephanie A Segovia, José G B Derraik, Paul A Lewandowski, Manohar L Garg, David Cameron-Smith, Paul L Hofman, Wayne S Cutfield
- **Journal:** American Journal of Physiology. Regulatory, Integrative and Comparative Physiology
- **Impact Factor:** 3.17
- **In Press**
10.2. Introduction

Fish oils are rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs) and are the most common non-vitamin non-mineral supplements in the US, taken by 10% of adults. They are frequently consumed during pregnancy because of claimed benefits to neurodevelopment of the offspring, particularly in childhood. However, the evidence for neurodevelopmental benefits from n-3 PUFAs intake during pregnancy is poor, and a recent report showed no effects on objective assessments of cognition, language, and executive function at 4 years of age. Furthermore, a recent systematic review and meta-analysis showed no benefit of n-3 PUFAs during pregnancy on pre-eclampsia, intra-uterine growth retardation, post-partum depression, or childhood development. In contrast, a number of animal studies have shown adverse effects on neurological development of the offspring following a maternal diet rich in n-3 PUFAs, although in some the dose would be unrealistic for a human diet. Nonetheless, in New Zealand studies in women who were pregnant or undergoing assisted fertility indicated 14-20% of women were taking fish oil. The global consumption of n-3 PUFA supplements continues to grow, despite the increasing number of studies failing to show improvements in hard outcomes.

Pregnancy is a critical time period when considering the safety of medications, and the same should apply to dietary supplements. Fetal life is a highly vulnerable stage, when complex embryological processes can be disrupted by external factors (such as chemicals) that may be harmless to the mother. Further, perinatal factors play an important role in programming body composition and cardio-metabolic risk later in life.

The n-3 PUFAs in fish and other marine oils (e.g. krill, calamari, and algae) are easily oxidised to a variety of lipid peroxides and other secondary oxidation products. In a recent comprehensive survey of fish oil products on the New Zealand market, 83% exceeded the recommended level of lipid peroxides, 33% by more than two fold. In addition, 25% exceeded the recommended level of secondary oxidation products (aldehydes and ketones). Similar results have been demonstrated in studies from North America and Africa. This implies that most women taking fish oil during pregnancy are routinely exposed to oxidation products. Further, antioxidant status is reduced during pregnancy and extremely low in the early embryo, so that pregnancy may be a time of special vulnerability to lipid peroxides. However, there is insufficient evidence to establish a safe level of oxidation in fish oils for consumption.

In animal models, exposure to oxidised lipids has been shown to cause harm, including growth retardation, organ toxicity, and accelerated atherosclerosis. However, the effects of consuming oxidised lipids during pregnancy are unknown. For this reason, in a study primarily designed to investigate the effects of
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fish oil supplementation during pregnancy on the adult offspring of rats fed a high-fat diet, we have also included oxidised fish oil groups. As obesity is associated with increased oxidative stress and greater production of oxidised lipids, the effects of an oxidised lipid supplement may be more marked in obese mothers. This manuscript describes the effects of the oxidised fish oil on neonatal viability and maternal insulin sensitivity, while examining for a possible interaction with a maternal obesogenic diet.

10.3. Methods

Ethics

Ethics approval was granted by the Animal Ethics Committee at the University of Auckland (Approval 001175). This study was performed in accordance with all appropriate institutional and international guidelines and regulations for animal research.

Study design

Female Sprague-Dawley rats (n=75) were housed under standard conditions at 25oC with a 12-h light: 12-h dark cycle. Animals were assigned to one of two isocaloric diets ad libitum for ten days prior to mating (Figure 10): high-fat diet (D12451, Research Diets Inc., New Brunswick, NJ, USA) containing 45% kcal as fat or control diet (D12450H) containing 10% kcal as fat. The two diets were otherwise nutrient-matched. Neither diet contained the long-chain n-3 PUFAs eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Note that the maternal high-fat diet adopted approximated commonly consumed western style diets and has metabolic effects similar to maternal obesity.
Female rats were time-mated using an oestrous cycle monitor (EC-40, Fine Science Tools, San Francisco, CA, USA) at a mean age of 113 days. Day 1 of pregnancy was determined by detection of spermatozoa by vaginal lavage, when pregnant dams were individually housed, continued on the study diet, and were allocated to one of three treatment (gavage) groups: unoxidised fish oil (UnFO), oxidised fish oil (OxFO), or water (Control). On each day of pregnancy, 1 ml of the treatment was administered by oral gavage (Figure 10). Note that an oil was not used as the control treatment in this study, as there are insufficient data to identify an oil that would have no effect on insulin sensitivity and offspring phenotype.

Food intake and body weight were recorded every 3rd day. At the time of birth, the gavage treatment was stopped, but the same maternal diets were fed ad libitum throughout lactation (until postnatal day 21) (Figure 10). On postnatal day 2, offspring were counted, sexed (by measuring anogenital distance), weighed and measured; all dead pups were sexed and counted; litter size was randomly adjusted to 8 pups to ensure standardized metabolic demand on individual dams. Pups not allocated to litters were killed by decapitation, and plasma, packed blood cells, liver, and heart were collected for analyses (Figure 10).

Offspring were weaned on day 21 (Figure 10). The day after weaning, dams were fasted overnight and killed by sodium pentobarbitone anaesthesia (60 mg/kg; intraperitoneal) followed by decapitation. Blood was collected in heparinised tubes, and stored on ice until centrifugation and removal of plasma for analysis. Liver, heart, and the retroperitoneal fat pads were collected and weighed. Fasting glucose and insulin were used to
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calculate HOMA-IR\textsuperscript{438}, which is a validated surrogate measure of insulin sensitivity in rats that is well-correlated (r=0.71) with the hyperinsulinaemic euglycaemic clamp\textsuperscript{438}.

10.3.1 Treatment oil

The treatment fish oil was produced from hoki (\textit{Macruronus novaezelandiae}) liver (Seadragon Marine Oils, Nelson, New Zealand). Half of the oil was frozen and used as the 'unoxidised fish oil' (UnFO). The remainder was treated by bubbling oxygen for 30 days under a fluorescent lamp at room temperature to produce the 'oxidised fish oil' (OxFO). Oxidation was assessed by measuring the peroxide value (PV) and anisidine values (AV)\textsuperscript{573}. The unoxidised oil had a PV of 2.7 meq/l and AV of 0.6, which are well within recommended indices of oxidation\textsuperscript{576,577,820} and lower than those of any fish oil available on the New Zealand market\textsuperscript{818}. In contrast, the oxidised oil had PV 48.8 meq/l and AV 4.5, greatly exceeding recommended levels\textsuperscript{576,577,820}. The fatty acid content of both oils was measured independently by gas chromatography (Table 9). The UnFO and OxFO had similar concentrations of fatty acids, except for a small reduction (10\%) in EPA and DHA concentrations in the OxFO oil. Thus, the treatment oils differed primarily in the concentration of lipid peroxides. The study oils were stored in small aliquots, frozen, sealed, and stored in darkness. Each day a new aliquot was thawed prior to use, so that further oxidation of oils prior to gavage was minimised. This is important as if fish oil is mixed into food, the peroxide value increases significantly over 24 hours in the food bowl\textsuperscript{903}.

10.3.2 Assays

Creatine kinase (CK), aspartate aminotransferase (AST), FFAs (FFA) and lactate dehydrogenase (LDH) were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) with all coefficients of variation (CV) less than 6\%. Insulin was measured by rat-specific ELISA (Crystal Chem, Illinois, USA) with CV of 11\%. Total superoxide dismutase (SOD) activity was measured with a commercial enzyme assay kit as per the manufacturer's instructions (Sapphire Bioscience, Mulgrave, Australia) with a CV less than 6\%. An EIA kit was used to measure the DNA oxidation by-product 8-hydroxy-2-deoxy-guanosine (8-OH-2dG) as a marker of oxidative stress\textsuperscript{904}. DNA was extracted from samples using a DNA isolation kit (Promega, Sydney, Australia). Each sample was then diluted, so that 50 mg of DNA were used in the 8-OH-2dG assay, which was carried out as per the manufacturer’s instructions (Sapphire Bioscience, Mulgrave, Australia) with a CV of 3.5\%.

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Statistical analyses

Rat dam outcomes were compared between groups using general linear regression models. Newborn survival was assessed using generalized linear mixed models, including the dam identification code as a random factor. Offspring outcomes were assessed using random effects mixed models, also including the dam identification code as a random factor. Dietary intake over the course of the study was assessed using linear regression models with repeated measures. Note that all models assessing the effects of gavage also adjusted for diet (high-fat or control). The associations between SOD and 8-OH-2dG were evaluated using Pearson's correlation coefficients and simple linear regressions. Statistical analyses were carried out in SAS v.9.4 (SAS Institute, Cary, NC, USA) and Minitab v.16 (Pennsylvania State University, State College, PA, USA). All tests were two-tailed with significance level maintained at 5%. Unless stated otherwise, data in the text are means.

Table 9: Fatty acid concentrations and oxidative indices in unoxidised and oxidised fish oil.

<table>
<thead>
<tr>
<th>Fatty acid concentration (mg/g of oil)</th>
<th>UnFO</th>
<th>OxFO</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 (palmitic acid)</td>
<td>105.53 ± 2.01</td>
<td>100.66 ± 4.08</td>
<td>0.87</td>
</tr>
<tr>
<td>C16:1n-7 (palmitoleic acid)</td>
<td>15.87 ± 0.34</td>
<td>14.96 ± 0.62</td>
<td>0.20</td>
</tr>
<tr>
<td>C18:0 (stearic acid)</td>
<td>28.26 ± 0.56</td>
<td>26.47 ± 1.39</td>
<td>0.34</td>
</tr>
<tr>
<td>C18:1n-7 (cis-vaccenic acid)</td>
<td>4.35 ± 0.05</td>
<td>4.10 ± 0.16</td>
<td>0.055</td>
</tr>
<tr>
<td>C18:1n-9 (oleic acid)</td>
<td>425.59 ± 7.99</td>
<td>400.97 ± 15.3</td>
<td>0.42</td>
</tr>
<tr>
<td>C18:2n-6 (linoleic acid)</td>
<td>3.12 ± 0.04</td>
<td>3.04 ± 0.08</td>
<td>0.42</td>
</tr>
<tr>
<td>C18:3n-3 (α-linolenic acid)</td>
<td>3.52 ± 0.05</td>
<td>5.32 ± 0.32</td>
<td>0.26</td>
</tr>
<tr>
<td>C18:3n-6 (γ-linolenic acid)</td>
<td>1.68 ± 0.24</td>
<td>1.54 ± 0.19</td>
<td>0.52</td>
</tr>
<tr>
<td>C20:0 (arachidic acid)</td>
<td>0.72 ± 0.01</td>
<td>0.68 ± 0.03</td>
<td>0.26</td>
</tr>
<tr>
<td>C20:1n-9 (eicosenoic acid)</td>
<td>0.47 ± 0.01</td>
<td>0.43 ± 0.03</td>
<td>0.15</td>
</tr>
<tr>
<td>C20:2n-6 (eicosadienoic acid)</td>
<td>1.67 ± 0.03</td>
<td>1.53 ± 0.07</td>
<td>0.11</td>
</tr>
<tr>
<td>C20:3n-6 (dihomo-γ-linolenic acid)</td>
<td>4.10 ± 0.11</td>
<td>3.79 ± 0.23</td>
<td>0.42</td>
</tr>
<tr>
<td>C20:4n-6 (arachidonic acid)</td>
<td>2.90 ± 0.08</td>
<td>2.80 ± 0.18</td>
<td>0.75</td>
</tr>
<tr>
<td>C20:5n-3 (eicosapentaenoic acid; EPA)</td>
<td>42.53 ± 0.76</td>
<td>38.34 ± 1.58</td>
<td>0.004</td>
</tr>
<tr>
<td>C22:5n-3 (docosapentaenoic acid)</td>
<td>1.09 ± 0.03</td>
<td>1.21 ± 0.18</td>
<td>0.87</td>
</tr>
<tr>
<td>C22:6n-3 (docosahexaenoic acid, DHA)</td>
<td>89.00 ± 1.77</td>
<td>79.87 ± 3.35</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Oxidative Indices

<table>
<thead>
<tr>
<th></th>
<th>UnFO</th>
<th>OxFO</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxide value (meq/kg)</td>
<td>1.46 ± 0.03</td>
<td>48.81 ± 1.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Anisidine value</td>
<td>0.62 ± 0.03</td>
<td>4.53 ± 0.43</td>
<td>0.006</td>
</tr>
</tbody>
</table>

UnFO: Unoxidised fish oil, OxFO: Oxidised fish oil. Data are means ± standard errors. Fatty acids concentration is in mg/g, determined by gas chromatography from 6 replicates. Oxidative indices determined from 3 replicates.
10.4. Results

There was no evidence of an interaction between gavage and diet on the two main outcomes: maternal insulin sensitivity and newborn survival. Thus, to better characterise the effects of oxidised fish oil and to increase statistical power, the dams and litters exposed to the same gavage treatment were pooled into the three groups: UnFO, OxFO, and Control. However, as expected, high-fat diet, in the absence of fish oil, tended to lead to poorer insulin sensitivity in the dams compared with control diet (HOMA-IR 1.96 vs 0.97; \( p=0.064 \)), highlighting the need to adjust for maternal diet when evaluating outcomes in the three pooled groups.

Pregnancy & newborn survival

Of the 75 rat dams that were mated, 49 became pregnant and successfully had pups, including 17 dams in Control, 17 in UnFO, and 15 in OxFO. Supplementation (fresh fish oil, oxidised fish oil, or water) had no effect on litter numbers, sex ratio of the offspring, or frequency of non-pregnancy. However, oxidised fish oil during pregnancy had a marked adverse effect on offspring survival at day 2 (Figure 11), leading to much greater odds of mortality than in the Control [odds ratio 8.26 (95% CI 2.04-33.33)] and UnFO [odds ratio 13.7 (95% CI 3.08-62.50)] groups. Notably, the effects of oxidised fish oil on neonatal mortality were nearly identical irrespective of maternal diet (Figure 12).

Offspring outcomes

Amongst pups who were alive at day 2, supplementation had no effect on body weight of live pups, but UnFO pups were longer than controls (+1.3 mm; \( p=0.041 \)) (Table 9). There were no differences in relative organ weights amongst the culled offspring at day 2, or in random blood glucose, FFA, total protein, AST, CK or LDH concentrations (Table 10). However, SOD activity was higher in the UnFO group (0.20 U/ml) compared with Control (0.14 U/ml; \( p=0.001 \)) and OxFO (0.15; \( p=0.005 \)) pups, while 8-OH-2dG levels were lower in the UnFO group (48 pg/ml) compared with Control (63 pg/ml; \( p=0.023 \)) (Table 10). In addition, SOD activity and 8-OH-2dG were inversely correlated among UnFO pups (\( r=-0.65; \ p=0.001 \)), but not among the other two groups (Figure 13).
Maternal outcomes

At weaning (postnatal day 21), dams in the three gavage groups were of similar size and had similar organ weights (data not shown). However, dams supplemented with oxidised fish oil had higher fasting blood glucose concentrations (5.56 mmol/l) than dams from Control (4.80 mmol/l; p=0.012) and UnFO (4.72 mmol/l; p=0.007) groups (Table 10). Dams from the oxidised group were also more insulin resistant than controls, as indicated by differences in HOMA-IR (p=0.044; Table 10).

Figure 11: Overall mortality by postnatal day 2 among rat pups, separated by maternal treatment.

Treatment was with water (control, n=17 dams), unoxidised fish oil (UnFO, n=17), or oxidised fish oil (OxFO, n=15).

Figure 12: Overall mortality at postnatal day 2 among rat pups, separated by both maternal diet and maternal treatment.
### Table 10: Outcomes among rat dams and their respective litters according to treatment during pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>UnFO</th>
<th>OxFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td><strong>Weight at mating (g)</strong></td>
<td>315 ± 8</td>
<td>319 ± 8</td>
<td>329 ± 8</td>
</tr>
<tr>
<td><strong>Pregnancy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight at day 22 (g)</td>
<td>446 ± 11</td>
<td>445 ± 11</td>
<td>455 ± 12</td>
</tr>
<tr>
<td>Pregnancy weight gain (%)</td>
<td>41.3 ± 2.5</td>
<td>39.9 ± 2.5</td>
<td>39.1 ± 2.7</td>
</tr>
<tr>
<td>Daily energy intake (kcal/g of body weight)</td>
<td>0.21 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td><strong>Litter characteristics (day 2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litter size (n)</td>
<td>12.8 ± 0.6</td>
<td>13.0 ± 0.6</td>
<td>12.4 ± 0.7</td>
</tr>
<tr>
<td>Sex ratio (% male per litter)</td>
<td>51 ± 3</td>
<td>46 ± 3</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>Weight of live pups (g)</td>
<td>5.5 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>Length of live pups (mm)</td>
<td>47.1 ± 0.4</td>
<td>48.4 ± 0.4†</td>
<td>47.4 ± 0.5</td>
</tr>
<tr>
<td><strong>Dams post-weaning (day 22)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>352 ± 7</td>
<td>343 ± 8</td>
<td>339 ± 9</td>
</tr>
<tr>
<td>Retroperitoneal fat weight (% body weight)</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>4.7 ± 0.3</td>
<td>5.0 ± 0.3</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.80 (4.46–5.16)*</td>
<td>4.72 (4.38–5.09)**</td>
<td>5.56 (5.10–6.07)</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>239 (167–343)</td>
<td>270 (186–393)</td>
<td>378 (240–595)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.42 (0.98–2.07)*</td>
<td>1.58 (1.07–2.33)</td>
<td>2.64 (1.65–4.25)</td>
</tr>
</tbody>
</table>

Treatment was water (Control), unoxidised fresh fish oil (UnFO), or oxidised fish oil (OxFO). Daily energy intake includes the caloric value of the gavage treatment (i.e. oil or water). Data are means ± standard errors (except for data on glucose homeostasis that are geometric means and 95% confidence intervals), adjusted for diet (high-fat or control). *p<0.05 and **p<0.01 vs OxFO; †p<0.05 vs Control.

### Table 11: Characteristics of culled rat offspring on postnatal day 2 according to maternal treatment during pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>UnFO</th>
<th>OxFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>46</td>
<td>36</td>
<td>20</td>
</tr>
<tr>
<td><strong>Biochemical marker</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random blood glucose (mmol/l)</td>
<td>6.65 ± 0.65</td>
<td>6.22 ± 0.74</td>
<td>6.18 ± 0.93</td>
</tr>
<tr>
<td>Brain (% body weight)</td>
<td>4.33 ± 0.12</td>
<td>4.50 ± 0.13</td>
<td>4.43 ± 0.16</td>
</tr>
<tr>
<td>Heart (% body weight)</td>
<td>0.65 ± 0.03</td>
<td>0.71 ± 0.03</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>4.57 ± 0.15</td>
<td>4.51 ± 0.17</td>
<td>4.61 ± 0.21</td>
</tr>
<tr>
<td><strong>Organ sizes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td><strong>Redox markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD (U/ml)</td>
<td>0.14 ± 0.01</td>
<td>0.20 ± 0.01††**</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>8-OH-2dG (pg/ml)</td>
<td>63 ± 4</td>
<td>48 ± 5†</td>
<td>57 ± 5</td>
</tr>
<tr>
<td><strong>Biochemical markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.37 ± 0.04</td>
<td>0.29 ± 0.04</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>2.26 ± 0.14</td>
<td>2.19 ± 0.14</td>
<td>2.38 ± 0.16</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>397 ± 51</td>
<td>376 ± 51</td>
<td>340 ± 63</td>
</tr>
<tr>
<td>CK (U/l)</td>
<td>114 ± 19</td>
<td>106 ± 18</td>
<td>119 ± 22</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>1767 ± 242</td>
<td>1418 ± 243</td>
<td>1461 ± 298</td>
</tr>
</tbody>
</table>

Treatment was water (Control), fresh fish oil (UnFO), or oxidised fish oil (OxFO). Data are means ± standard errors, adjusted for diet (high-fat or control). Note that aside from glucose, all biochemical markers were performed in a reduced group, where pups were randomly selected from each litter. Also note that 4 pups in the OxFO group had blood taken for biochemistry but did not have organs weighed. **p<0.01 vs OxFO; †p<0.05 and ††p<0.01 vs Control.
High-fat vs control maternal diet

As noted previously, there was no interaction between maternal diet and gavage treatment for the main study outcomes. However, unoxidised fish oil gavage led to increased SOD activity (p<0.001 vs Control; p<0.0001 vs OxFO), but only amongst pups exposed to a maternal control diet (Table 12). Unoxidised fish oil led to a reduction in relative liver weight of pups only when the dam consumed a high-fat diet (p=0.022 vs Control; p=0.020 vs OxFO). This was not observed with the control diet (Table 12). Among rat dams fed a control diet, OxFO dams had higher fasting glucose (p=0.007) and tended to be more insulin resistant (p=0.051) than those who received control gavage (Table 12). There appears to have been a similar effect amongst dams fed a high-fat diet, but there was reduced statistical power due to lower numbers of dams in the OxFO group (Table 12).
Table 12: Study outcomes of rat dams (post-weaning, day 22) and culled rat offspring (postnatal day 2) according to maternal diet and treatment during pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>High-fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control UnFO OxFO</td>
<td>Control UnFO OxFO</td>
</tr>
<tr>
<td><strong>DAMs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>332 ± 11</td>
<td>344 ± 11</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>5.8 ± 0.5</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.43 (4.01–4.89)</td>
<td>4.54 (4.12–5.02)*</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>177 (101–311)</td>
<td>249 (142–438)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.97 (0.55–1.71)</td>
<td>1.40 (0.79–2.48)‡</td>
</tr>
<tr>
<td><strong>OFFSPRING</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Random blood glucose (mmol/l)</td>
<td>5.23 ± 1.36</td>
<td>5.25 ± 1.26</td>
</tr>
<tr>
<td>Brain (% body weight)</td>
<td>4.04 ± 0.19</td>
<td>4.36 ± 0.16</td>
</tr>
<tr>
<td>Heart (% body weight)</td>
<td>0.65 ± 0.04</td>
<td>0.70 ± 0.03</td>
</tr>
<tr>
<td>Liver weight (% body weight)</td>
<td>4.44 ± 0.21</td>
<td>4.92 ± 0.19</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>SOD (U/ml)</td>
<td>0.13 ± 0.01</td>
<td>0.23 ± 0.01***†††††</td>
</tr>
<tr>
<td>8-OH-2dG (pg/ml)</td>
<td>53 ± 6</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.24 ± 0.07</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>2.48 ± 0.22</td>
<td>2.52 ± 0.20</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>307 ± 63</td>
<td>435 ± 62</td>
</tr>
<tr>
<td>CK (U/l)</td>
<td>119 ± 32</td>
<td>114 ± 30</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>1672 ± 287</td>
<td>1850 ± 266</td>
</tr>
</tbody>
</table>

Treatment was with water (Control), unoxidised fish oil (UnFO), or oxidised fish oil (OxFO). Data are means ± standard errors (except for data on glucose homeostasis that are geometric means and 95% confidence intervals), with offspring data adjusted for sex. Note that aside from glucose, all offspring biochemical markers were performed in a reduced group, where pups were randomly selected from each litter. Also note that 2 pups in each OxFO group had blood taken for biochemistry but did not have organs weighed.
Figure 13: The association between SOD activity and 8-OH-2dG concentrations in the red blood cells of new born pups separated by maternal treatment.

Maternal treatments were A: water, B: unoxidised fish oil, C: oxidised fish oil. Blood samples were collected from rat pups that were alive at day 2 and culled on that day. SOD: superoxide dismutase, 8-OH-2dG: 8-hydroxy-2’deoxyguanosine.
Data Papers

10.5. Discussion

In this trial of fish oil supplementation during rat pregnancy, oxidised fish oil led to an 8- to 13-fold increase in the odds of newborn mortality. Importantly, there was no increase in mortality in the groups fed unoxidised fish oil compared to controls, indicating that the adverse effect on newborn survival was due to the presence of lipid oxidation products. Further, dams exposed to the oxidised fish oil in pregnancy were more insulin resistant at weaning, which was the end of the study and 21 days after last exposure. However, the effects of the oxidised fish oil on the newborn pup survival did not differ between dams consuming a high-fat or a control diet. This suggests that the known adverse effects of a high-fat diet on maternal metabolism did not alter the toxicity of oxidised fish oil to newborn pups. These data show that lipid peroxides formed in oil rich with n-3 PUFA are harmful in rat pregnancy, raising the possibility that they may also have an adverse effect in human pregnancy.

The oxidised fish oil was characterised by a high concentration of lipid peroxides, which could have caused oxidative stress (and the consequent toxic effects). Oxidative stress has important effects during pregnancy, as it contributes to the teratogenic effects of drugs and has a role in complications such as spontaneous abortion, pre-eclampsia and intrauterine growth restriction. Oxidised lipids influence placental development, lipid metabolism, and lipid transport, and lipid peroxide levels are markedly higher in serum and placenta of pre-eclamptic pregnancies. Thus, in principle, the increased newborn mortality in the OxFO group could have been mediated by effects on maternal and placental health. Alternatively, lipid peroxides could have crossed the placenta and directly affected the fetus, but there is only weak evidence to suggest that lipid peroxides can cross the placenta.

Our findings indicate that the redox effects of oxidised and unoxidised fish oils on the pups differed. However, it was not possible to determine the mechanisms underlying the increased newborn mortality, or whether oxidative stress played a role. Nonetheless, previous studies showed that fish oil supplementation increases endogenous antioxidant enzymes and reduces oxidative damage. Consistent with these studies, supplementation with unoxidised fish oil was associated with reduced 8-OH-2-dG levels (a marker of oxidative DNA damage in the pups) and increased activity of superoxide dismutase (SOD, a key antioxidant enzyme). Further, 8-OH-2-dG and SOD activity were negatively correlated among the UnFO pups. In contrast, neither these beneficial effects nor the correlation between 8-OH-2-dG and SOD were observed in the OxFO pups. It should be noted that due to limited amount of blood samples that can be extracted from newborn pups, markers of lipid and protein oxidative damage could not be measured.
Surprisingly, and at odds with the high newborn mortality in the OxFO group, no differences were identified between groups for a range of markers of cellular toxicity (e.g. FFA, CK, and LDH) or in body weight or relative organ mass. However, the earliest samples collected were from offspring alive on postnatal day 2 (at the time of a planned cull to standardize litter size), when a large number of pups had already died. Consequently, there was a 'healthy survivor' bias, i.e. the most severely affected pups had died and could not be assessed, so that we sampled those least affected by the toxicity of the oxidised fish oil.

Interestingly, oxidised fish oil supplementation dams led to greater insulin resistance in dams 3 weeks beyond the last dose. Furthermore, as the mothers were culled at this stage, we do not know whether this effect would have been sustained even longer (i.e. a long-term reprogramming of metabolism). Conversely, no insulin-sensitising effect of unoxidised fish oil was apparent 3 weeks after the end of supplementation. Short-term improvements in insulin sensitivity with n-3 PUFAs have been demonstrated in rodents \[121,387,388\], but it is likely that this effect was lost in our unoxidised fish oil group over time. Nonetheless, we report for the first time that oxidised fish oil increases insulin resistance, the opposite effect reported for unoxidised fish oil \[121,387,388\]. The cause of this increased insulin resistance is unknown, but it could be due to induction of systemic inflammation, as oxidative stress activates the proinflammatory NF-κB pathway \[336\], which is central to the inflammation associated with obesity and insulin resistance \[214,237,241\].

It is difficult to extrapolate our findings directly to humans. The dose of fish oil in this study was 1 ml/day, providing approximately 80 mg of DHA and 40 mg of EPA, similar to the dose previously shown to reverse insulin resistance in rats \[121\]. Determining an equivalent dose in humans is difficult as too little is known about the relative affinity between humans and rats of receptors and transcription factors involved in the metabolic effects of n-3 PUFAs, or in factors related to the toxicity of lipid peroxides. In regards to the large differences in body size, Reagan-Shaw et al. recommended conversion of doses between species based on body surface area \[910\], and based on this standard the peroxide dose given to rats in this study was high (890 meq/m²/day). From a survey of the lipid peroxide content of supplements on the New Zealand market \[818\], we estimate that the average woman would need to consume at least 40 ml/day fish oil to have equivalent exposure (more if she consumes one of the less oxidised products). It is unlikely that doses this high would be taken in human pregnancy. Nevertheless, no safe level of peroxide exposure has been established, and pregnancy is a time of special vulnerability, where endogenous antioxidant status is reduced \[898\]. Importantly, the relationship between dose and effect is unknown, and consumption of lower doses of lipid peroxides may still lead to adverse effects. Although there is currently no specific evidence of harm from the consumption of oxidised fish oil in human pregnancy, this issue has not been systematically studied. Thus, a biologically important effect in human pregnancy cannot be disregarded.
Interestingly, many possible causes for the variability of outcomes in trials of n-3 PUFAs in pregnancy have been raised, but the potential for oxidation to change the biological activity of trial oils has not been recognised. In the present study, we showed that oxidised fish oil causes maternal insulin resistance in rats, the opposite of the well-described insulin-sensitising effects of fish oil. Therefore, it is possible that trials and meta-analyses assessing the effects of fish oil on a range of outcomes (including gestational diabetes, birth weight, and offspring metabolism) may have been confounded by oxidation.

Our study had a number of strengths. First, we used an oxidised fish oil that was derived from the fresh fish oil, and thus identical except for the presence of oxidation products. Second, we utilized small frozen aliquots of trial oils with delivery by gavage, which ensured the correct dose was given, and that inadvertent oxidation of the unoxidised oil did not occur. Third, the control dams also underwent daily gavage to prevent confounding by the stress of the procedure itself. However, this study also has limitations. It was not designed to identify the mechanism of harm, and assessment of pups on day 2 (after 25% of pups had died in the OxFO group) introduced a 'healthy survivor' bias, and could not discriminate between stillborn pups and those that died after delivery. Further, the dose used and the severity of oxidation was relatively high, and the possible effects of lower doses in human pregnancy cannot be extrapolated.

10.6. Perspectives and Significance

This is the first study to show negative effects of oxidised fish oil in pregnancy, specifically, increased maternal insulin resistance and increased newborn mortality in rats. It represents an important proof of concept that supplementation with oxidised fish oil in pregnancy may be harmful. This is relevant as fish oil supplements are often oxidised at the time of purchase, and are commonly taken during pregnancy. Therefore, there is a pressing need for studies examining dose-response relationships between oxidised fish oil consumption and pregnancy outcomes. Given our findings, the lack of compelling evidence that fish oil supplementation is beneficial in human pregnancy, and previous studies showing adverse effects, caution is advised when considering taking fish oil supplements in pregnancy. However, it should be pointed out that our findings are unlikely to apply to fresh oily fish. Clinical trials of n-3 PUFAs should ensure that adopted supplements are independently verified to be unoxidised, and their oxidative state should be reported. Lastly, future studies should focus on three particular aspects: 1) whether there is a dose-response relationship between lipid peroxide intake and both maternal and newborn outcomes, and whether doses more comparable to human supplementation are also harmful; 2) whether the addition of antioxidants can mitigate any adverse outcomes; and 3) the identification of the exact mechanisms leading to newborn mortality (including a wider range of markers of oxidative stress and assessments in fetal life).
Chapter 11. Fish oil supplementation to rats fed a high-fat diet during pregnancy prevents the development of impaired insulin sensitivity in male adult offspring

11.1. Preface

This chapter contains a modified version of a manuscript under review by *Diabetes*.

- **Authors:** Benjamin B Albert, Mark H Vickers, Clint Gray, Clare M Reynolds, Stephanie A Segovia, José GB Derraik, Manohar L Garg, David Cameron-Smith, Paul L Hofman, Wayne S Cutfield
- **Journal:** Diabetes
- **Impact Factor:** 8.10
11.2. Introduction

There has been a dramatic increase in the incidence of obesity and overweight worldwide\(^2,3\). As a result, in western countries up to 60% of women of reproductive age are either overweight or obese\(^18,19,686\), and gestational diabetes affects up to 12% of pregnancies\(^687\).

Maternal pre-pregnancy obesity is associated with adverse changes in body composition in the offspring including macrosomia (and occasionally intrauterine growth retardation), greater adiposity in childhood, and obesity in later life, thus perpetuating a vicious cycle of metabolic disorders across generations\(^703,704,916\). Maternal obesity appears to be the strongest predictor of offspring obesity\(^156,705\), and even outside the obese range increasing pre-pregnancy BMI is associated with greater adiposity and an adverse metabolic phenotype in the offspring. This includes lower insulin sensitivity, an unfavourable adipokine profile, as well as increased blood pressure and triglyceride concentrations\(^157,710\). Thus, maternal obesity is associated with a range of metabolic abnormalities in the offspring\(^704,709\), with an increased risk of type 2 diabetes and metabolic syndrome\(^17\). In addition, gestational diabetes (which is associated with maternal obesity) is also linked to metabolic dysfunction and increased risk of diabetes in the offspring\(^729,732,734,738\). While the mechanisms remain unclear, the adverse effects seen in the offspring appear to be mediated (at least in part) by exaggeration of the normal insulin resistance of pregnancy. This leads to excess delivery of glucose and fatty acids to the fetus\(^697\), and ultimately to lifelong alterations in gene expression through epigenetic changes\(^917-920\).

Adverse metabolic programming of offspring as a consequence of a maternal obesogenic environment has important implications for our increasingly overweight society. The origins of the obesity epidemic may lie in a postnatal environment characterized by abundance of high-energy foods and an increasingly sedentary lifestyle. However, now that obesity is common, a second factor has emerged: the transgenerational perpetuation of obesity and metabolic disease through maternal obesity and insulin resistance\(^20,921\). This highlights pregnancy as a critical early window of opportunity where treatments that reduce fetal overnutrition may have lifelong benefit\(^20,921,922\), potentially improving insulin sensitivity and in turn reducing the risk of type 2 diabetes and cardiovascular disease\(^29\).

In rodent models, insulin resistance is readily induced by a high-fat diet\(^121,387\), even during pregnancy\(^772\). Importantly, in the rat, a maternal HF diet leads not only to reduced maternal insulin sensitivity\(^758,768,772\), but also to an offspring phenotype of leptin resistance with hyperphagia\(^760,761\), increased body weight and adiposity\(^758,760,768,923\), reduced insulin sensitivity\(^758,760,772,924\), dyslipidaemia\(^760\), and higher blood pressure\(^760,768\). This phenotype is similar to the offspring of insulin resistant women\(^17,704,709\).
In non-pregnant rodents, insulin resistance can be prevented by supplementation with fish oil\textsuperscript{121,387}. This effect may be mediated by G-protein coupled receptor 120 (GPR-120), a receptor for n-3 PUFAs\textsuperscript{121}, which is expressed by adipocytes and adipose tissue macrophages, and is up-regulated by a high-fat diet\textsuperscript{62,121}. Through this mechanism n-3 PUFAs reduce adipose tissue inflammation and dysfunction, normalise the adipokine profile and reduce the release of free fatty acids, ultimately leading to improved hepatic and peripheral insulin sensitivity\textsuperscript{121}. GPR-120 is also present in humans, and regulates adipose tissue function and insulin sensitivity\textsuperscript{386}. This raises the possibility that fish oil supplementation in pregnancy could prevent metabolic dysfunction in the offspring of insulin resistant women. Thus, we utilised an established rat model of HF feeding in pregnancy to test the primary hypothesis that maternal fish oil supplementation in pregnancy would prevent the development of insulin resistance in the offspring. In addition, we hypothesised that fish oil would also prevent programmed hyperphagia, obesity, dyslipidaemia, and elevated blood pressure.

### 11.3. Methods

#### Animal Ethics

Ethics approval was granted by the Auckland University Animal Ethics Committee (approval #001175). This study was performed in accordance with all appropriate institutional and international guidelines and regulations for animal research.

#### Study design

A model of maternal obesity was utilised as previously reported by our group, in which a maternal high-fat diet leads to obesity in offspring, independent of postnatal diet\textsuperscript{758}. Fifty-two virgin female Sprague-Dawley rats (age 110 days) were assigned to one of two isocaloric diets \textit{ad libitum} for ten days prior to mating: a high-fat diet (HF, D12451, Research Diets Inc., New Brunswick, NJ USA) which contained 45\% kcal as fat, or a matched Control diet (D12450H) containing 10\% kcal as fat. Rats were time-mated using an oestrous cycle monitor (EC-40, Fine Science Tools, San Francisco, CA, USA). Day 1 of pregnancy was determined by detection of spermatozoa by vaginal lavage, and dams were individually housed and continued on their respective study diet. On Day 1 of pregnancy, dams were further allocated to one of two treatment groups: fish oil (FO) or water (control). On each day of pregnancy, 1 ml of the treatment was administered by gavage. Thus, there were four treatment groups: 1) Con-Con, where dams consumed a control diet and had water by gavage; 2) Con-FO, where dams consumed a control diet and had fish oil by gavage; 3) HF-Con, where dams
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consumed a HF diet and had water by gavage; and 4) HF-FO, where dams had HF diet and fish oil by gavage (Figure 14). Importantly, as dams from all groups underwent daily gavage, any potential stressful effects related to the gavage procedure were controlled for.

The treatment oil was derived from hoki (Macruronus novaezelandiae) liver (Seadragon Marine Oils, Nelson, New Zealand). We independently verified n-3 PUFA content (Table 13) and the oxidative state, using methodology previously described. The oil had very low levels of oxidation, with a peroxide value of 2.7 meq/l and anisidine value of 0.6, well within international recommendations and below all products tested in a recent study of fish oil products available for purchase in the NZ market. One ml/day of fish oil equated to a daily dose of 39 mg eicosapentaenoic acid and 82 mg docosahexaenoic acid. To ensure standardised dosage and to prevent inadvertent oxidation, delivery of the treatment (oil or water) was administered by oral gavage. In addition, the fish oil was stored in small aliquots, frozen, sealed, and kept in darkness, so that each day a new aliquot was thawed prior to use, minimizing any oxidation of the oil.

Maternal food intake and body weight were recorded every 3rd day. At birth, the gavage treatment was stopped, but the same diets were maintained ad libitum throughout lactation. Offspring were counted, sexed (by measuring anogenital distance), weighed and measured on postnatal day 2. On this day, litter size was also randomly adjusted to 8 pups to ensure standardized nutrition until weaning. As the primary aim of this study was to assess metabolic phenotype in adult male offspring, 6 males and 2 females were kept. Pups not allocated to litters were killed by decapitation, when plasma, red blood cells, liver, and heart were collected. Pups were weighed every 3rd day until the time of weaning (day 21), when they were housed in pairs for the remainder of the study (until day 110). All offspring were fed a standard chow diet (Harlan-Teklad Diet 2018, Oxon, UK) ad libitum, with food intakes and body weights recorded every 3rd day.
Figure 14: Diagram describing individual steps of the trial and indicating the timing of assessments.

T1: Virgin female rats were randomised to control or high-fat diet. T2: Females were mated and further randomised to one of the two gavage treatments, i.e. Fish or Control (water). T3: on postnatal day 2 pups were counted, sexed and weighed; excess pups were culled. T4: on postnatal day 21 pups were separated, while dams were culled and had samples taken. T5: final metabolic assessments of adult male offspring.

Table 13: Fatty acid concentrations in the fish oil supplement.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 (palmitic acid)</td>
<td>105.53 ± 4.92</td>
</tr>
<tr>
<td>C16:1n-7 (palmitoleic acid)</td>
<td>15.86 ± 0.84</td>
</tr>
<tr>
<td>C18:0 (stearic acid)</td>
<td>28.26 ± 1.37</td>
</tr>
<tr>
<td>C18:1n-7 (cis-vaccenic acid)</td>
<td>4.35 ± 0.12</td>
</tr>
<tr>
<td>C18:1n-9 (oleic acid)</td>
<td>425.59 ± 19.58</td>
</tr>
<tr>
<td>C18:2n-6 (linoleic acid)</td>
<td>3.12 ± 0.09</td>
</tr>
<tr>
<td>C18:3n-3 (α-linolenic acid)</td>
<td>5.52 ± 0.13</td>
</tr>
<tr>
<td>C18:3n-6 (γ-linolenic acid)</td>
<td>1.68 ± 0.59</td>
</tr>
<tr>
<td>C20:0 (arachidic acid)</td>
<td>0.72 ± 0.03</td>
</tr>
<tr>
<td>C20:1n-9 (eicosenoic acid)</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>C20:2n-6 (eicosadienoic acid)</td>
<td>1.67 ± 0.08</td>
</tr>
<tr>
<td>C20:3n-6 (dihomo-γ-linolenic acid)</td>
<td>4.10 ± 0.27</td>
</tr>
<tr>
<td>C20:4n-6 (arachidonic acid)</td>
<td>2.90 ± 0.19</td>
</tr>
<tr>
<td>C20:5n-3 (eicosapentaenoic acid)</td>
<td>42.53 ± 1.85</td>
</tr>
<tr>
<td>C22:5n-3 (docosapentaenoic acid)</td>
<td>1.09 ± 0.08</td>
</tr>
<tr>
<td>C22:6n-3 (docosahexaenoic acid)</td>
<td>89.00 ± 4.34</td>
</tr>
</tbody>
</table>

Fatty acid concentrations in mg/g of oil were determined by gas chromatography. Data are means ± standard deviations, from analysis of 6 replicates.
Data Papers

From the adult offspring within each of the 4 treatment groups, 12 animals were randomly selected for metabolic assessment.

At postnatal day 90, body composition was quantified using dual x-ray emission absorptiometry (DXA, Lunar Prodigy 2000, General Electric, Madison, USA), while animals were under light isoflurane anaesthesia.

Systolic blood pressure was analysed at 95 days of age by tail cuff plethysmography (Model 179 with an automatic cuff inflation pump (NW20), IITC, Life Science, Woodland Hills, CA), according to the manufacturer’s instructions as previously described. Adult offspring were warmed using a heat lamp and acclimatised to the restraint tube for 15 minutes prior to blood pressure recordings. Three clear pressure recordings were taken per animal (CV <5%) and mean blood pressure calculated.

At day 100, insulin sensitivity was assessed by an oral glucose tolerance test following a 6-hour fast. After collection of tail blood (at T0), a glucose load (1 g/kg of body weight) was given by gavage. Blood was collected from the tail at T30, T60, T90 and T120 minutes. Glucose was measured at each time point, with plasma insulin also measured at T0, T30, T60, T90 and T120. Insulin and glucose concentrations were used to derive the Matsuda index of insulin sensitivity. The Matsuda index of insulin sensitivity has been shown to be closely correlated to the hyperinsulinaemic euglycaemic clamp and have excellent repeatability during multiple measures. Its use in rats has been recently described. In addition, HOMA-IR was calculated, as well as glucose and insulin area under the curve (AUC) using the trapezoidal method.

Terminal sample collection

At day 110, adult offspring were fasted overnight and killed by sodium pentobarbitone anaesthesia (60 mg/kg; intraperitoneal) followed by decapitation. Blood was collected in EDTA tubes and stored on ice until centrifugation and removal of plasma for analysis. Liver, kidneys, heart, and the retroperitoneal and gonadal fat pads were harvested and weighed.

Assays

During the oral glucose tolerance test, glucose concentration was measured by glucometer (Optium Xceed; Abbott Laboratories) from tail whole blood, at the time samples were taken. Insulin and leptin concentrations were measured by ELISA (Crystal Chem, Illinois, USA) with CVs of 11% and 7%, respectively. Glucose, creatinine, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), free fatty
acids, uric acid, triglycerides, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), and total cholesterol were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) with all CVs less than 6%.

**Primary outcome and power analysis**

The primary outcome of this study was the Matsuda index of insulin sensitivity in the adult male offspring. Two major comparisons were required: 1) HF-Con vs Con-Con to show the effect of the high-fat diet in pregnancy and lactation, and 2) HF-Fish vs HF-Con to show the additional effect of further supplementation with fish oil to dams consuming a high-fat diet. Due to limited data regarding the standard deviation of the Matsuda index in rats, sample size was calculated to enable differences to be shown in fasting insulin. Given previous data showing maternal high-fat diet was associated with a mean fasting insulin of 4.89 ng/ml and standard deviation of 0.926 in the adult male offspring, a sample of approximately 12 animals per group was required to have 90% power to detect a 25% change in fasting insulin.

**Statistical analyses**

Outcomes in rat dam were compared using general linear regression models, with diet and gavage included as factors. Offspring outcomes were assessed using linear mixed models, including also dam identification code as a random factor. The interaction between diet and gavage was examined in all models. Differences in weight, food and energy consumption in dams and offspring over time were compared using multiple t tests and Holm-Sidak correction for multiple comparisons. All statistical analyses were carried out in SAS v.9.4 (SAS Institute, Cary, NC, USA), except t tests which were carried out in Prism v.6.03 (Graphpad Software Inc., La Jolla, CA, USA). All tests were two-tailed with significance level maintained at 5%.
11.4. Results

Of the 52 rat dams mated, 34 became pregnant and successfully had pups (Table 14). Neither diet, nor gavage treatment affected litter numbers.

Effects of maternal high-fat diet

Dams from the HF-Con group had greater body weight than Con-Con dams at day 10 of pregnancy and at the end of lactation (Figure 15A), as well as having greater body weight and retroperitoneal fat mass (Table 14). In addition, while there was indication of lower food consumption in HF-Con dams during the last days of pregnancy (days 19-22; p<0.06) there were no differences in total energy intake (Figure 15A). At the end of lactation, HF-Con dams tended to have increased insulin resistance (i.e. higher HOMA-IR; p=0.058), and displayed greater fasting glucose (p=0.018) and triglyceride (p=0.047) levels than Con-Con dams (Table 14).

HF-Con litters had a greater proportion of male pups than the Con-Con group, but there were no differences in birth weight or length (Table 14). Male HF-Con offspring had greater food consumption and body weight than those in the Con-Con group (Figure 15B). This was first detectable at postnatal day 57, with further divergence until day 108 (timing of the final weight measurement). In the final assessments, adult males of the HF-Con group had greater body weight (+98 g; p=0.030) and adiposity (+6.4% of body fat; p=0.008) compared with Con-Con males (Table 15).

Importantly, the male adult HF-Con offspring had lower insulin sensitivity than the Con-Con rats, as indicated by a Matsuda index 38% lower (p=0.036), higher HOMA-IR (+51%; p=0.042), and greater insulin AUC (+63%; p=0.023) (Table 16). This was accompanied by other markers of metabolic dysfunction, including higher systolic blood pressure (+12 mmHg, p<0.0001), and increased triglyceride (+0.55 mmol/l; p=0.014) and leptin (+4.1 ng/ml; p=0.002) concentrations (Table 16). Thus, maternal HF diet led to adverse programming of energy balance, body composition, and metabolism in adult male offspring.
Table 14: Maternal weight and metabolic parameters at key milestones of the study and litter characteristics at birth, according to the maternal diet and the gavage treatment during pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>Maternal control diet</th>
<th>Maternal control diet</th>
<th>Maternal high-fat diet</th>
<th>Maternal high-fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fish oil</td>
<td>Control</td>
<td>Fish oil</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td><strong>Weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to diet allocation (day -10)</td>
<td>301 ± 6</td>
<td>298 ± 10</td>
<td>296 ± 5</td>
<td>303 ± 6</td>
</tr>
<tr>
<td>Mating (day 1)</td>
<td>310 ± 7</td>
<td>307 ± 10</td>
<td>322 ± 7</td>
<td>332 ± 12</td>
</tr>
<tr>
<td>End of pregnancy (day 22)</td>
<td>434 ± 6</td>
<td>444 ± 14</td>
<td>457 ± 16</td>
<td>445 ± 17</td>
</tr>
<tr>
<td>End of lactation (day 40)</td>
<td>342 ± 4</td>
<td>359 ± 13</td>
<td>368 ± 9</td>
<td>360 ± 11</td>
</tr>
<tr>
<td>Gestational weight gain</td>
<td>40 ± 2</td>
<td>45 ± 2</td>
<td>42 ± 4</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>Retroperitoneal fat (% of body weight at day 40)</td>
<td>1.2 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.1†</td>
<td>1.2 ± 0.1#</td>
</tr>
<tr>
<td><strong>Metabolic Parameters (day 40)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.44 ± 0.17</td>
<td>4.56 ± 0.15</td>
<td>5.20 ± 0.32†</td>
<td>4.93 ± 0.18</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>180 ± 19</td>
<td>323 ± 118</td>
<td>403 ± 91</td>
<td>332 ± 58</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.98 ± 0.09</td>
<td>1.84 ± 0.66</td>
<td>2.58 ± 0.58‡</td>
<td>2.01 ± 0.37</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.52 ± 0.03</td>
<td>0.47 ± 0.02</td>
<td>0.68 ± 0.05‡</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td><strong>Birth Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Litter size (n)</td>
<td>11.8 ± 1.0</td>
<td>14.1 ± 0.9</td>
<td>13.7 ± 0.8</td>
<td>11.9 ± 1.1</td>
</tr>
<tr>
<td>Sex ratio (% male per litter)</td>
<td>45 ± 5</td>
<td>42 ± 4</td>
<td>57 ± 3†</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>Weight of pups (g)</td>
<td>5.6 ± 0.3</td>
<td>5.6 ± 0.3</td>
<td>5.4 ± 0.2</td>
<td>6.2 ± 0.4*</td>
</tr>
<tr>
<td>Length of pups (mm)</td>
<td>47.7 ± 0.2</td>
<td>47.4 ± 0.2</td>
<td>46.7 ± 0.4</td>
<td>48.9 ± 0.2**</td>
</tr>
</tbody>
</table>

The control gavage treatment was water. Data are means ± standard errors. ‡p<0.06, †p<0.05, and ††p<0.01 for a maternal diet effect among controls; #p<0.06, *p<0.05, and **p<0.01 for an effect of the gavage treatment between groups exposed to the same maternal diet.
Figure 15: Weight, food consumption, and energy intake in rat dams (A) and male offspring (B) according to the maternal diet (control or high-fat) and gavaged treatment during pregnancy (fish oil or control (water)).

A: body weight (A1), food consumption (A2), and total energy consumption including diet and gavaged oil (A3) of dams from mating (day 0) through to weaning (day 40). B: body weight (B1), food consumption (B2), and cumulative food consumption of male offspring from weaning through to termination (day 110) (B3). Error bars represent standard errors; *p<0.05 for a difference in weight between Con-Con and HF-Con groups across the life span; †p<0.05 for a difference in food intake between Con-Con and HF-Con groups from day 60 when intake stabilized; ‡p<0.05 for a difference between Con-Con and HF-Con groups at termination.
Table 15: Pubertal onset, auxology and food consumption among male offspring according to the maternal diet (control or high-fat) and the gavage treatment during pregnancy (fish oil or control (water)).

<table>
<thead>
<tr>
<th></th>
<th>Maternal control diet</th>
<th>Maternal high-fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fish oil</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Pubertal onset (days)</td>
<td>43.3 ± 0.6</td>
<td>41.3 ± 0.6</td>
</tr>
<tr>
<td>Auxology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight at weaning – 24 d (g)</td>
<td>48 ± 3</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>Weight at 108 d (g)</td>
<td>495 ± 19</td>
<td>541 ± 15</td>
</tr>
<tr>
<td>Length at 108 d (mm)</td>
<td>258 ± 2</td>
<td>258 ± 2</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>22.9 ± 1.1</td>
<td>24.2 ± 1.2</td>
</tr>
<tr>
<td>Fat mass to lean mass ratio</td>
<td>0.30 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>Bone mineral density (g/cm²)</td>
<td>0.154 ± 0.002</td>
<td>0.156 ± 0.002</td>
</tr>
<tr>
<td>Diet at 108 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy intake (kcal/day)</td>
<td>85 ± 3</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Energy intake per body weight (kcal/g/day)</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>Cumulative food intake (g)</td>
<td>705 ± 22</td>
<td>751 ± 13</td>
</tr>
</tbody>
</table>

"Cumulative food intake" reflects the weight of all food consumed by an individual rat since weaning. Data are means ± standard errors. †p<0.05 and ††p<0.01 for a maternal diet effect among the offspring of dams that received control gavage. Note that all study groups consisted of 5 to 7 separate litters.

Table 16: Metabolic parameters among male offspring according to the maternal diet (control or high-fat), and gavaged treatment during pregnancy (fish oil or control (water)).

<table>
<thead>
<tr>
<th></th>
<th>Maternal control diet</th>
<th>Maternal high-fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Fish oil</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Glucose homeostasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matsuda index</td>
<td>1.35 ± 0.17</td>
<td>1.27 ± 0.25</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.02 ± 0.41</td>
<td>3.89 ± 0.63</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.96 ± 0.14</td>
<td>5.46 ± 0.21</td>
</tr>
<tr>
<td>Glucose AUC (min.mmol/l)</td>
<td>847 ± 25</td>
<td>841 ± 28</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>497 ± 63</td>
<td>556 ± 74</td>
</tr>
<tr>
<td>Insulin AUC (x10³ min.pmol/l)</td>
<td>76 ± 9</td>
<td>82 ± 11</td>
</tr>
<tr>
<td>Liver function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>79 ± 4</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>144 ± 13</td>
<td>133 ± 5</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>36 ± 2</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.83 ± 0.09</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>1.70 ± 0.13</td>
<td>1.94 ± 0.11</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.21 ± 0.09</td>
<td>1.46 ± 0.08</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>0.29 ± 0.03</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.68 ± 0.07</td>
<td>0.94 ± 0.09</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid (µmol/l)</td>
<td>71 ± 5</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>1.6 ± 0.3</td>
<td>4.2 ± 1.3*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>124 ± 1</td>
<td>125 ± 1</td>
</tr>
</tbody>
</table>

Data are means ± standard errors. †p<0.05, ††p<0.01, and ††††p<0.0001 for a maternal diet effect among the offspring of dams that received control gavage; #p<0.06, *p<0.05, and **p<0.01 for an effect of the gavage treatment between groups exposed to the same maternal diet.
Effects of supplemental fish oil in the context of a maternal high-fat diet

HF-FO dams were not different from HF-Con dams in terms of body weight, food intake, or total energy intake (Figure 15A). However, HF-FO pups were heavier and longer than HF-Con pups (Table 15).

Male offspring from the HF-FO group were no different from HF-Con in terms of food consumption, or body weight (Figure 15B). However, insulin sensitivity in the HF-FO group was greater than in the HF-Con group as indicated by higher Matsuda index (+85%; p=0.014), lower HOMA-IR (-42%; p=0.021), lower fasting insulin concentrations (-298 pmol/l; p=0.026), and a trend towards reduced insulin AUC (-35%; p=0.052) (Table 16). Of note, HF-FO parameters were similar to the indices of insulin sensitivity in the Con-Con group (Table 16). However, there were no differences in blood pressure, triglycerides, or leptin in adult male rats from HF-FO and HF-Con groups (Table 16).

Effects of supplemental fish oil in control diet

There were no differences between the Con-FO and the Con-Con group in terms of food consumption, body weight, adiposity, insulin sensitivity, lipid profile, or systolic blood pressure (Table 15 and Table 16). However, leptin levels were 2.6 times higher in adult Con-FO males (p=0.027; Table 16).

11.5. Discussion

In this study, a maternal HF diet was used, which is known to induce maternal insulin resistance and metabolic abnormalities in the offspring. As expected, despite a standard postnatal diet the adult male offspring developed obesity associated with increased caloric intake, as well as reduced insulin sensitivity, increased plasma leptin and triglyceride levels, and higher systolic blood pressure. Importantly, fish oil supplementation prevented the impairment of insulin sensitivity in the offspring, suggesting reduced risk of type 2 diabetes and cardiovascular disease. This indicates that maternal fish oil supplementation in pregnancy could be a useful approach to reduce cardio-metabolic risk in the offspring of pregnancies complicated by obesity or gestational diabetes.

However, while fish oil supplementation prevented the development of insulin resistance in the offspring of dams fed a HF diet, it did not prevent other adverse cardiometabolic effects. In rats, a maternal HF diet, whether in pregnancy or lactation, leads to greater adiposity and increased leptin, triglycerides, and blood pressure in
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the adult offspring\textsuperscript{768}. Thus, while fish oil ameliorated some of the adverse effects of a maternal HF diet during pregnancy, it would not have countered the effects of HF diet during lactation since fish oil supplementation was not given during this time. In this context, it was not surprising that the HF-FO offspring displayed a phenotype of greater adiposity, increased leptin and triglyceride levels, and elevated systolic blood pressure compared to Con-Con offspring. Nevertheless, the divergence of effects in insulin sensitivity and other metabolic parameters suggests that there are important differences in the programming of insulin sensitivity from other key aspects of the metabolic phenotype, specifically body composition, blood pressure, and leptin resistance. We speculate that there would have been observed benefits of fish oil on energy balance, adiposity, triglycerides, and blood pressure if supplementation could have been continued throughout lactation.

It should be noted that there is a strong association between insulin sensitivity and blood pressure\textsuperscript{29}. Insulin resistance and the associated hyperinsulinaemia lead to increased blood pressure through effects on the sympathetic nervous system\textsuperscript{252}, renal sodium handling\textsuperscript{29}, and endothelial function\textsuperscript{89}. Thus, it is interesting that the adult offspring exposed to maternal high-fat diet and fish oil in pregnancy had increased systolic blood pressure in adulthood, despite insulin sensitivity that was not different to controls. This may be due to leptin resistance (reflected by an elevated fasting leptin) that has been shown to be induced by a maternal HF diet\textsuperscript{761}, and which was not ameliorated by fish oil supplementation in this study. Leptin resistance is associated with increased appetite\textsuperscript{761}, and activation of the sympathetic nervous system\textsuperscript{777}, thus it may underlie the greater caloric intake, adiposity, and blood pressure seen in the groups exposed to a maternal high fat diet.

Apart from hyperleptinaemia in the adult offspring (suggestive of leptin resistance), there were no other adverse metabolic effects of fish oil supplementation in the offspring of mothers fed a control diet. However, it is possible that minor metabolic defects would have developed with age if study had a longer duration. This highlights the importance of the dietary and metabolic context in which treatments such as fish oil supplementation are given, as while supplementation was beneficial to the offspring of the insulin resistant HF diet dams, there was a negative effect in control dams. In humans, studies investigating the effects of supplemental fish oil during pregnancy on offspring body composition or metabolism have yielded conflicting results in childhood\textsuperscript{803-806} and no effect in young adults\textsuperscript{808}. However, none of these studies enrolled women likely to have abnormally poor insulin sensitivity. We speculate that in human pregnancy, maternal fish oil supplementation will have beneficial effects on offspring metabolism in women who are obese or have gestational diabetes, but in pregnancies with appropriate insulin sensitivity the effect could be adverse or absent.

The maternal HF diet model of developmental programming in rodents appears to be relevant to humans. Obesity, and in particular visceral obesity, is associated with adipose tissue inflammation\textsuperscript{203,208}, which leads to
insulin resistance through abnormal adipokine secretion and free fatty acid release. Similarly, HF diets have been shown to increase inflammation of adipose tissue, through saturated fatty acids binding to toll-like receptor-4. Thus, the obese human condition and the obesogenic rat model are both characterized by adipose tissue inflammation and dysfunction. In addition, women who are obese and pregnant do tend to consume excess saturated fat.

The present study has important strengths. The use of four intervention groups ensured both diet and gavage treatments were controlled for. In addition, we independently showed the fish oil supplement to be unoxidised, and its administration by gavage from small frozen aliquots ensured accurate dosing and prevented oxidation from occurring. This is particularly important as fish oil supplements worldwide are frequently oxidised at the time of purchase, and we showed that highly oxidised fish oil caused maternal insulin resistance and markedly increased newborn mortality in rats (unpublished data). However, this study has limitations. Firstly, fish oil supplementation was started immediately after mating, and thus conception. In humans, the timing of conception is mostly unknown, so that starting supplementation at this point would be difficult to achieve, unless women intending to become pregnant took supplemental fish oil. Secondly, while the dose of fish oil used was similar to those of previous rodent studies, it is difficult to convert it to an equivalent human dose. Based on surface area, it equates to approximately 30 ml in humans, which would be likely to be unacceptable to many women. Further, women wishing to take an equivalent dose of n-3 PUFAs would be seriously hindered by their inability to access fresh unoxidised supplements such as the one used in this study. Lastly, as fish oil could not be given during lactation when the HF diet was continued, it is possible that our study has underestimated the potential benefits of maternal fish oil supplementation until weaning.

This study showed that fish oil supplementation solely during pregnancy in mothers who were fed a high-fat diet led to a sustained beneficial effect, preventing insulin resistance in adult offspring. This suggests that supplementation with unoxidised n-3 PUFA rich oils may have a role in mitigating adverse metabolic programming in the offspring of women who are obese during pregnancy. There is a need for randomised controlled trials of unoxidised n-3 PUFA rich supplements in women who are obese or have gestational diabetes, to examine the long-term effects on insulin sensitivity in the offspring. However, we caution against the use of fish oil supplements in human pregnancy, unless they are known to be unoxidised.
Discussion
Chapter 12. Summary of findings

The primary aim of this research program was to investigate the efficacy of n-3 PUFA-rich oil supplements to treat or prevent the development of insulin resistance. I used a randomised double-blind placebo-controlled crossover trial in a group at risk of metabolic disease, to evaluate possible insulin-sensitising effects of a blended marine oil. While I demonstrated a positive association between n-3 PUFA content of red blood cells and insulin sensitivity, treatment with a krill-based oil led to a reduction in insulin sensitivity. Subsequently, I used an established model of insulin resistant pregnancy to show that maternal supplementation with n-3 PUFAs can prevent the development of insulin resistance in the offspring.

I also identified an important problem with n-3 PUFA supplements that had not been widely appreciated: they oxidise easily and this may change their biological effects. In a comprehensive survey of fish oil products available on the New Zealand market, I showed that most were oxidised beyond recommended limits and had lower n-3 PUFA content than labelled. In a series of invited commentaries I have discussed how this may have confounded the trial literature, limiting our knowledge about the biological effects of n-3 PUFA supplements, especially their effects on cardiovascular, metabolic and pregnancy outcomes. Lastly, I demonstrated in an animal model that an oxidised n-3 PUFA rich oil had adverse effects: supplementation of rat dams with oxidised fish oil lead to both increased newborn mortality and persistent maternal insulin resistance (effects that were not observed with the fresh unoxidised fish oil).

12.1. Are n-3 PUFAs insulin-sensitising in humans?

In rodent models GPR-120, a receptor that has anti-inflammatory effects in adipose tissue, was shown to mediate the insulin-sensitising effects of an n-3 PUFA-rich fish oil. As humans also express this receptor in adipose tissue and individuals with GPR-120 mutation develop insulin resistance, a similar insulin sensitising effect would be expected. Some clinical trials have supported an insulin-sensitising effect of n-3 PUFAs, but many studies have shown no effect. A meta-analysis demonstrated a small insulin-sensitising effect in studies reporting HOMA-IR, but no overall effect of n-3 PUFAs. However, the studies included in the meta-analysis have important limitations (Section 4.4.4), including unusual target groups, inclusion of participants with T2DM, inadequate statistical power, and complex interventions, so that they are too heterogeneous for meta-analysis.

To better address this issue, I enrolled overweight middle-aged men into a randomised placebo-controlled crossover trial, examining the effects of a krill/salmon oil blend on metabolism. Unexpectedly, I found that insulin sensitivity was reduced after the 8-week period of supplementation with the krill/salmon oil. Further,
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compared with baseline, the supplement increased total cholesterol, LDL, Chol:HDL ratio and Apo-B, suggesting a more atherogenic lipid profile. Consistent with this, a subtle increase in carotid artery intima-media thickness was observed. This suggests that if the krill/salmon oil was continued long-term, it may have increased the risk of T2DM and cardiovascular disease. Neither the mechanism(s) of this effect nor the chemical compound(s) responsible could be determined. However, it is most likely that the adverse effects of the krill/salmon blend were not due to the n-3 PUFAs, but were rather mediated by chemical component(s) of the krill oil (Section 9.5).

Interestingly, while the adverse metabolic effect of the krill/salmon blended oil reduced insulin sensitivity, there was a positive association between n-3 PUFA levels in the red cell phospholipid membrane and insulin sensitivity at the baseline assessment prior to randomisation. This is consistent with studies in children and adults where n-3 PUFA levels in muscle or serum were associated with greater insulin sensitivity. Although these studies suggest an insulin-sensitising effect of n-3 PUFAs in humans, these associations do not indicate causation. Further, reverse causation is also possible, where metabolic dysfunction leads to lower n-3 PUFA levels.

Nevertheless, as participants in our RCT were not consuming fish oil supplements at baseline, the association between n-3 PUFA levels and insulin sensitivity would be consistent with greater fish consumption leading to a more favourable metabolic profile. Thus, other beneficial effects of eating fish, aside from the n-3 PUFA content, could be at play. Therefore, associational evidence cannot determine whether n-3 PUFAs are insulin-sensitising in humans, and there remains a need for randomised controlled trials in groups at an increased risk of T2DM and cardiovascular disease, using unoxidised fish oil or preferably a pure n-3 PUFA supplement.

An RCT published since the krill trial fits this requirement, and provides strong evidence for clinically relevant insulin sensitising effects of n-3 PUFAs. Nearly 300 overweight or obese patients with impaired glucose tolerance or impaired fasting glucose were enrolled. They received 3g/day of n-3 PUFAs as concentrated ethyl esters or placebo. The ethyl ester form allows greater concentration and reduces the range of other chemicals present – so that the supplement was quite different to consuming fish meat, or fish oil. This high dose treatment reduced fasting glucose, insulin, triglycerides and HOMA-IR, indicating improved insulin sensitivity. These beneficial effects were detected after 9 months of treatment, but there was further improvement by 18 months, indicating long lasting metabolic effects. Most importantly, this treatment reduced progression to type 2 diabetes mellitus, a clinically important outcome. However, this study requires replication, and further studies to determine whether standard fish oil, or lower doses of n-3 PUFAs have the same effect.
Discussion

12.2. Could n-3 PUFAs be used as a treatment to prevent metabolic programming in insulin resistant pregnancy?

There is substantial evidence from studies in humans\textsuperscript{17,157,703,704,709} and animal models\textsuperscript{758,760,768,772,923,924} that the offspring of pregnancies complicated by obesity or diabetes have adverse long-term programming of metabolism and body composition, leading to a phenotype characterised by insulin resistance and obesity. This is likely to be mediated by altered fetal nutrition and consequent epigenetic changes of metabolically important genes\textsuperscript{917,919}. As an increasing proportion of women of childbearing age are obese, this presents a significant problem for the human species, threatening transgenerational perpetuation of obesity\textsuperscript{20,921}. Therefore, a treatment that could be given to obese pregnant women to prevent adverse programming in the offspring would be transformative. Given the important role that maternal insulin sensitivity has on fetal nutrition\textsuperscript{697}, a potentially insulin-sensitising treatment such as fish oil might reduce fetal over-nutrition and thus adverse programming of metabolism.

As a result, I used a rat model of maternal high-fat diet in pregnancy and lactation, which has been shown to induce abnormal appetite\textsuperscript{760,761}, obesity\textsuperscript{758,760,768,923} and metabolic dysfunction\textsuperscript{758,760,772,924} in the offspring independently of postnatal diet\textsuperscript{758}. As expected, the high-fat diet led to increased weight, adiposity, food consumption, and triglyceride levels, as well as reduced insulin sensitivity in the adult male offspring. I observed that supplementation with unoxidised fish oil during pregnancy prevented the adverse effects on insulin sensitivity, but did not affect adiposity, food consumption, BP or triglyceride levels. This indicates that metabolic risk was mitigated, but not averted.

While we did not examine mechanism in this study, it is important to emphasise that differences in epigenetic marks in metabolically important genes represent a highly plausible potential explanation for the different phenotypes we saw with addition of fish oil, to dams fed a high fat diet. Modification of chromatin (without changes in DNA sequence), including methylation of CpG islands, and differences in histone acetylation, have an important impact on gene transcription\textsuperscript{897}. Both fetal under-\textsuperscript{932-935} and over-nutrition\textsuperscript{917,919,936} have been shown to induce changes in histone acetylation or DNA methylation in metabolically important genes. For example in rats a maternal HFD led to reduced deacetylation of H3 in specific genomic sites, which increased expression of important metabolic proteins including PPAR-\textgreek{a}, PPAR-\textgreek{g} and SREB1-c\textsuperscript{919} and changed the metabolomic profile\textsuperscript{936}.

If our finding in rats, translates to a real benefit of fish oil supplementation during the pregnancy of women who are obese, it could have a very important impact on the rates of cardiometabolic disease in the offspring. This possibility should be investigated in humans using a carefully designed randomised controlled trial and detailed assessment of body composition and metabolism in the offspring.
12.3. **Is oxidation of marine oils important?**

I demonstrated in rats that supplementation with highly oxidised fish oil during pregnancy led to adverse outcomes, specifically increased newborn mortality and maternal insulin resistance. While the dose of lipid peroxides administered was high, the overall fish oil dose was the same as that of a previous study demonstrating insulin-sensitising effects\(^\text{121}\). This is the first study to show that oxidised fish oil has different metabolic effects to unoxidised fish oil. Oxidised fish oil led to insulin resistance, while unoxidised fish oil, at least in rats improves insulin sensitivity\(^\text{121,387}\).
Chapter 13. Strengths and limitations of my research programme

13.1. Strengths

The studies that made up my research program had important strengths. Both the human clinical trial and the animal trial made use of high quality techniques to produce a detailed and reliable metabolic assessment. These include use of oral glucose tolerance tests to derive the Matsuda composite index of insulin sensitivity and dual x-ray absorptiometry to assess body composition. Additionally, in the human trial I utilised ambulatory blood pressure monitoring, ultrasound to measure carotid artery intima-media thickness, and included apolipoprotein B measurement to compliment the serum lipid profile.

The design of these trials was also a major strength. In the human clinical trial, a double-blind randomised controlled crossover design was used, which is the design most likely to remove bias and confounding. Importantly an 8 week wash-out period was used to ensure that the second treatment period was not confounded by residual n-3 PUFAs from the first period. Further, exercise and diet was quantified and shown to not have changed during the trial. This is very important given the strong effects of exercise and diet on insulin sensitivity. I also used a system of over-supply of capsules by an amount unknown to the participants to accurately determine compliance, and objectively assessed blinding which is uncommon in such trials.

In the animal trial we used an intricate 6 group trial design to allow us to characterise the separate effects of high fat diet and control diets, as well as the individual effects of the fish oil, oxidised fish oil and water treatments. The use of gavage was a strength as it ensured accurate dosing of a treatment that the animals may not have reliably consumed, and prevented the unoxidised oil, from oxidising in the cage. In addition because the oxidised fish oil was derived from the same aliquot of unoxidised fish oil, the oils were exactly matched except for the level of oxidation.

In the fish oil quality survey study, we captured nearly all fish oil products on the New Zealand market, making this a comprehensive assessment of quality at the time of the study. Further, I used a systematic rule in selecting each product to avoid products very close or very far away from the best before date – this made it more likely that the products chosen would be representative of other packages not purchased. The method used in the quantitative determination of fatty acid concentration was an important strength. This well-established method has a substantial advantage over the methods used in some studies that claim accurate labelling of content. By using a genuine fatty acid mixture of known concentration, the full fatty acid profile is determined, with quantitative concentration derived from calibration curves for each fatty acid. Other studies have used inappropriate methodology to convert qualitative relative peak area data from the chromatogram, or have not reported this aspect of methodology.
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A final strength of my research programme was the recognition of the similarity of the Krill/Salmon oil trial cohort to that included in a previous RCT conducted at our institute. By recording additional data about early life/family factors, and recontacting men from the previous study to gain additional information I was able to produce greater knowledge from the RCT. The studies in the appendix investigate the effects of birth order and parental age at the time of child birth on insulin sensitivity in adulthood, as well as the lack of relationship between insulin sensitivity and the “dipping” blood pressure phenomena. Given the significant sacrifices made by participants in clinical trials there is an ethical obligation to maximise the scientific knowledge that is generated from them.

13.2. Limitations

The greatest weakness of my research programme was that in the human RCT and the animal trial we focussed on phenotypic description and not on mechanism. This was appropriate at the time that the studies were designed as recent studies reporting the effects of n-3 PUFAs on GPR-120 mediated insulin sensitising and anti-inflammatory effects gave a strong mechanistic rationale. However, when our results were not as predicted we were left with very limited ability to explain them. In particular this applies to the reduction of insulin sensitivity after krill/salmon oil supplementation in overweight men, and the increased neonatal mortality and maternal insulin resistance associated with consumption of oxidised fish oil during rat pregnancy.

The other major weakness of the human randomised controlled trial was the use of the blended krill/salmon oil and not having detailed knowledge of its chemical composition. Although the oil was predominantly krill oil, we cannot determine whether the adverse effect was due to a compound in krill oil, salmon oil, or both, or whether such a compound was unusually high in the trial oil. This limits how sure we can be that other krill based oils will have the same effect, and whether for example elimination of a chemical compound from krill oil could prevent that adverse effect.

The animal trial had other important limitations. Firstly the dose of oil given was high when adjusted for body size. This is common in animal studies and the dose of oil effectively matched the dose used in Oh et al’s seminal study in mice. Nevertheless, this makes the effect harder to extrapolate to human pregnancy. Similarly, the level of oxidation in the oxidised fish oil was greater than those sold at retail. This was justifiable given the study was designed as proof-of-concept that oxidation could change the biological effects of fish oil supplementation. If no effect had been seen it would have been immensely reassuring that differential effects of fish oils with levels of oxidation within the range seen in retail products were unlikely in human pregnancy. However, this also makes our finding more difficult to translate to humans. Another limitation is that dosing began at conception. This is very hard to achieve in human women as the time of conception is usually only determined in retrospect. Realistically in order to dose human women at the time
While gavage administration of oil was a strength of our study it was also a limitation. Gavage is a stressful procedure and it is possible that the stress of gavage interacted with the harmful effects of the oxidised oil to produce a greater harmful effect than would occur with a non-stressful method of administration. Further, while the study diet was continued during lactation, the gavage treatment could not be continued during this period (such stress would have had substantial effects on maternal care). Thus the HF-Fish offspring were exposed to a maternal high fat diet during lactation, without the potentially protective fish oil treatment. As maternal HFD solely during lactation has long term effects on offspring metabolism, this might account for the incomplete protective effect of the maternal fish oil during pregnancy on metabolism in offspring e.g. obesity, hypertension and hypertriglyceridaemia developed in this group despite maintenance of insulin sensitivity.

The fish oil survey study also has limitations. Firstly, as krill, calamari and algal based oils were not assessed no conclusions can be drawn about omega-3 rich oils not extracted from fish. In particular it is possible that oxidation of krill oils might differ because the n-3 PUFAs are in the form of phospholipids, and the presence of the natural antioxidant astaxanthin. Secondly as only a single package of each product was analysed, it is possible that there could be substantial variability between packages and batches of oil. Further, while we showed no relationship between best before date and oxidative state, it is possible that within a single brand, best before date and oxidative state might be associated. Lastly, we did not determine the concentration and range of specific lipid oxidation products, which could be useful when considering the potential biological effects of consuming oxidised marine oils.

Lastly, the additional studies produced from the combined cohorts of the krill/salmon RCT and the previous olive leaf extract trial share limitations. They are based on post hoc analyses, thus they incur greater risk of incorrectly identifying associations due to multiple comparisons. These studies require replication. Lastly, the analysis of the relationship between parental age at the time of childbirth could not determine whether the maternal age, the paternal age, or both affected metabolic phenotype in the offspring. No other study has been able to separate these effects, and a recent study did not record or consider that the paternal age might be important. To separate these effects would require studying families where the parents had highly discrepant ages.
Chapter 14. Marine oils and health revisited: a highly complex intervention, with a confusing, and potentially confounded trial literature

14.1. Preface

This chapter contains a modified version of a manuscript published in the Journal of Nutrition and Intermediary Metabolism.

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In my study of fish oil supplementation during rat pregnancy, oxidation of the fish oil, had a very dramatic impact on its biological effects, changing it from a treatment that improved the metabolic phenotype of the offspring, to one that caused substantial neonatal mortality. Further, the krill/salmon clinical trial result suggested a possible harmful compound in krill, that may have counteracted any positive effect of the n-3 PUFAs it contained. These findings illustrate that marine oils are inherently complex treatments, quite unlike medication. This has potential implications for interpretation of the scientific and clinical literature surrounding n-3 PUFA supplementation. The following commentary discusses these complexities, which may have been overlooked by many scientists and clinicians and considers the appropriateness of the current regulation of health supplements around the world.

14.2. Introduction

In 1971, Bang and Dyerberg reported low rates of cardiovascular disease in Greenland Inuits who consumed a fatty diet made up almost exclusively of oily fish and seal meat, a paradox given the contemporary understanding of the association between dietary fat and cardiovascular disease. While this observation has recently been questioned, it sparked considerable scientific interest. Since then, a vast scientific literature has emerged exploring the health effects of marine oils rich in n-3 polyunsaturated fatty acids (PUFAs), in particular the long chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Alongside, a billion
Dollar industry has arisen\textsuperscript{459}, marketing fish oil and other marine oils to consumers, such that marine oils are now one of the most popular supplements worldwide\textsuperscript{456}. In the USA, they are used by 6.5\% of the population (37\% of supplement users)\textsuperscript{456} and 8\% of college students\textsuperscript{939}. In a study in New Zealand, 15\% of women undergoing fertility treatment were taking marine oil supplements\textsuperscript{461}.

In many ways, scientists and physicians have approached marine oils as they would a medication, investigating their health effects using randomised controlled trials, with controlled doses, for specific indications. Marine oils have a long list of apparent indications including prevention of cardiovascular disease\textsuperscript{554} and cognitive decline\textsuperscript{480}, improvement of infant neurodevelopment\textsuperscript{480}, and treatment of inflammatory diseases such as rheumatoid arthritis and asthma\textsuperscript{352}. Recommended doses differ depending on indication\textsuperscript{352,940}, and products are labelled so that consumers can determine a target dose of n-3 PUFAs.

However, there is increasing evidence to suggest that marine oils are actually ineffective for secondary prevention of cardiovascular disease, which is their highest profile indication\textsuperscript{657,658}. In reality, marine oil supplements are quite unlike medications in many respects, including the complexity of their biological effects, their impurity (containing many chemical compounds), the inaccuracy of labelled content, their potential to degrade to toxic compounds, and limited sales and marketing regulation. Consideration of the complexity of marine oils and the ways they differ from typical drugs may help explain why they appear not to have delivered on the promising tale of the Greenland Inuits.

14.3. n-3 PUFA actions

There is a very large range of physiological effects associated with n-3 PUFAs, which is quite unlike most medications.

\textit{Anti-inflammatory}

They inhibit the inflammatory process at 5 distinct levels:

1) Increasing cell membrane fluidity, which interferes with activation of immune cells such as T-lymphocytes\textsuperscript{467}.
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2) Activating the transcription factors PPAR-α\textsuperscript{333} and PPAR-γ\textsuperscript{355}, and the transmembrane receptor GPR-120\textsuperscript{121}, which inhibit the proinflammatory NF-κB pathway\textsuperscript{338,340}. This reduces the production of inflammatory cytokines such as TNF-α and IL-6 and cell adhesion molecules such as ICAM and VCAM\textsuperscript{352}.

3) Competing with n-6 PUFAs as a substrate for the COX-2 enzyme, shifting the balance of eicosanoids from the proinflammatory n-6 series to the anti-inflammatory or less inflammatory n-3 series\textsuperscript{352}.

4) Competing with n-6 PUFAs as a substrate for endocannibinoid synthesis, which leads to the production of anti-inflammatory endocannibinoids\textsuperscript{352}.

5) Forming protectins and resolvins which have a role in ending the inflammatory response\textsuperscript{352}.

Lipid metabolism

N-3 PUFAs also have important effects on lipid metabolism through interaction with key transcription factors. They activate PPAR-α\textsuperscript{333} and inhibit SREB1-c\textsuperscript{379} and HNF-4α\textsuperscript{380} in the liver. The combined effect is to increase fatty acid β-oxidation for energy production and reduce lipid synthesis\textsuperscript{367,379}, reduce hepatic fat storage\textsuperscript{242,333}, and limit the release of triglycerides into the circulation\textsuperscript{242,333}. PPAR-γ is a key regulator of adipose tissue function that is also activated by n-3 PUFAs\textsuperscript{242}. Activation of PPAR-γ increases adipogenesis\textsuperscript{357}, up-regulates enzymatic pathways involved in uptake and storage of lipid\textsuperscript{358} and insulin signalling\textsuperscript{354}, inhibits free fatty acid release, and normalises adipokine production\textsuperscript{361}. It is noteworthy that the activation of PPAR-γ is the primary mechanism for the insulin-sensitising effects of thiazolidinediones, which are used in the management of diabetes mellitus\textsuperscript{354}.

Redox status

While n-3 PUFAs function as antioxidants\textsuperscript{469}, their impacts on biological systems are complex. Their participation in redox reactions leads to the production of a lipid peroxide radical, which itself is highly reactive\textsuperscript{470}. In one study, lower doses of n-3 PUFAs had an antioxidant effect, but higher doses (≥1600 mg/day) were associated with increased markers of oxidative damage\textsuperscript{471}. Aside from dose, many other factors are likely to influence the redox effect of n-3 PUFAs, including the degree to which the oil was oxidised prior to consumption, the concentration of antioxidants in the oil, and the endogenous antioxidant status of the consumer. Further, as obesity, inflammation, infection, and hyperglycaemia all influence oxidative stress\textsuperscript{299,472}, many aspects of health may modulate the redox effects of marine oils.
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Central nervous system

DHA is a major structural component of the central nervous system and the retina, making up 35% of fatty acids within synaptic membranes. There is rapid uptake of DHA in late gestation and infancy, and deficiency is associated with cognitive defects in animal models. Supplementation with n-3 PUFAs has been investigated as a treatment for a wide range of neurological and psychiatric disorders, and they are frequently taken during pregnancy or infancy with the aim of improving neurodevelopment.

n-3 PUFAs also have anti-arrhythmic properties, and like any fatty acid can be stored in adipose tissue or undergo β-oxidation for energy production.

Diversity of effects

As outlined above, n-3 PUFAs have a wide diversity of effects through many different mechanisms. Whilst it could be assumed that these are synergistic, some of these effects may be conflicting.

Ferrannini observed that medications that have a very specific action (affecting a single metabolic pathway) are usually preferable, because unintended/unpredicted adverse effects are less likely. The statins (a class of drugs that reduce synthesis of cholesterol by inhibiting the enzyme HMG-CoA reductase) are a good example. In contrast, the thiazolidinediones act through PPAR-γ, which is expressed in many tissues, and has many transcriptionally regulating actions. While these drugs do have important insulin sensitising effects, reports have shown an increased risk of congestive heart failure and fractures. From this perspective, n-3 PUFAs, which affect many pathways in addition to PPAR-γ, have greater potential for unpredictable and potentially adverse effects.

Importantly, the multiple mechanisms by which n-3 PUFAs modulate inflammation and metabolism may also make it harder to translate the results of animal studies to humans. To standardise between species, doses are often considered adjusted for weight or body surface area. This is reasonable if there is only one mechanism of action, as if there are differences in the affinity of enzymes or receptors for a drug or compound of interest, these are likely to differ by a constant factor, which may simply change the required dose to achieve a biological effect. However, when there are multiple distinct biological effects, such as for n-3 PUFAs, it is possible that the activity through each mechanism could vary by a different factor. In that case, the overall
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Effect in different species would be very difficult to predict. Notably, the insulin-sensitising effects of marine oils have not clearly translated from rodents\textsuperscript{121} to humans\textsuperscript{531}.

14.4. Marine oils contain more than n-3 PUFAs

The n-3 PUFAs EPA and DHA are considered to be the active compounds in marine oils, and the typical labelled content is between 300-600 mg/g of oil. However, marine oils also contain significant quantities of monounsaturated and saturated fatty acids, as well as small amounts of n-6 PUFAs. Further, in addition to fatty acids, there are other chemical species, including fat-soluble vitamins, carotenoids, phospholipids, cholesterol, and glycerol. In fact, two recent clinical trials that presented an independently measured fatty acid profile of the trial oil showed that the fatty acid content was only 42\% of the oil mass in a krill-salmon blended oil\textsuperscript{863} and 75\% in a fish oil\textsuperscript{946}.

Oils made from krill are becoming an increasingly popular source of n-3 PUFAs. These oils may contain an even wider range of chemical components as the fatty acids are in the form of phospholipids\textsuperscript{463,866}, which have water-soluble and lipid-soluble poles. Thus, krill oils may also contain water-soluble molecules. In a randomised controlled trial of krill-salmon blended oil, supplementation lead to reduced insulin sensitivity, which was unlikely to be mediated by the n-3 PUFA components of the oil\textsuperscript{863}.

14.5. Current understanding of the health effects of marine oils

Early trials

Initial evidence from clinical trials suggested cardiovascular, anti-inflammatory, and cognitive benefits of n-3 PUFA supplementation. It was shown to reduce plasma triglyceride levels\textsuperscript{947}, lower BP\textsuperscript{643}, reduce platelet aggregation\textsuperscript{647}, and stabilise atherosclerotic plaque\textsuperscript{650}. Together these effects would be expected to slow atherosclerosis and reduce the tendency of atherosclerotic plaques to rupture, thrombose, and occlude (i.e. the processes leading to myocardial infarction). Further, the reported anti-arrhythmic effects\textsuperscript{588} might prevent sudden death after myocardial infarction. Some trials are consistent with this, including the open-label GISSI-Prevenzione\textsuperscript{948} and JELIS\textsuperscript{655} trials. Similarly, the anti-inflammatory effects of n-3 PUFAs would suggest a useful role in the treatment of inflammatory diseases\textsuperscript{352}, and benefit has been demonstrated in the treatment of rheumatoid arthritis\textsuperscript{949,950}.  

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**Systematic reviews**

With the growing number of trials of n-3 PUFAs, there has been progression to detailed systematic reviews that have cast doubt on the benefits suggested by early trials. Recent systematic reviews of randomized placebo-controlled trials on the use of n-3 PUFAs for secondary prevention of cardiovascular disease showed no effect on the risk of myocardial infarction or sudden death\(^657,658\). Further, there was no overall effect in the treatment of asthma or inflammatory bowel disease, with individual trials showing conflicting results\(^660,951\). Recent systematic reviews have also found no overall effect of n-3 PUFA supplementation on a variety of pregnancy outcomes, including gestational diabetes, preterm birth, pre-eclampsia, intrauterine growth restriction, and measures of infant neurodevelopment\(^891,952,953\), but yet again the individual trial data are mixed\(^891,952,953\).

**Conflicting results**

Together, these data suggest that n-3 PUFAs have little overall effect on cardiovascular risk, pregnancy outcomes, and some inflammatory diseases. However, other potential explanations for a mixed and overall negative literature have been raised. These include heterogeneity of the study populations and their background fish consumption, the trial dose of n-3 PUFAs, the appropriateness of the chosen placebos and study outcomes, as well as concomitant medications\(^629,659,660\). A possible explanation that has received little attention\(^553,929\), is that there is substantial variability in the composition of marine oils sold at retail, which may often contain potentially toxic oxidation products in excess of industry standards\(^557,558,818\), lower than labelled concentrations of n-3 PUFAs, and variation in the ratio of EPA and DHA\(^818,822-824\).

**14.6. Oxidation of n-3 PUFAs**

n-3 PUFAs are highly prone to oxidative degradation, owing to their many double bonds that have a low activation energy for free radical formation\(^470\). Oxidation is a highly complex process, and the degree and rate of oxidation of a marine oil is influenced by many factors, including the fatty acid composition, antioxidant content, temperature, exposure to oxygen and light, and the presence of water and heavy metals\(^470\). Importantly, added antioxidants reduce, but do not prevent oxidation during storage\(^568\).
Discussion

The initial step of oxidation leads to increased levels of hydroperoxides, which decompose into a variety of radicals. These react with unoxidised PUFAs to form additional hydroperoxides, while also breaking down to form a wide range of secondary oxidation products, such as volatile ketones and alcohols that contribute to the smell of rancid oil\textsuperscript{470}.

While measurement of specific chemical species is difficult, simple industry standard tests provide a proxy measure of oxidative products. The peroxide value (PV) provides a quantitative measure of hydroperoxide concentration. The most common method to estimate secondary oxidation is the p-anisidine value (AV), which measures aldehydic compounds. Note that while the AV can be increased by some flavourings (such as citrus oils)\textsuperscript{954}, it remains the recommended test for estimating secondary oxidation\textsuperscript{578,579}. By measuring both PV and AV, primary and secondary oxidation products are characterised, enabling an overall assessment of the degree of oxidation, which is reflected in the Totox value (2PV + AV). A number of authorities have published maximum limits of oxidation in fish oils, most commonly set as PV<5, AV<20, Totox<26\textsuperscript{577-579}. Notably, there are insufficient data to indicate safe levels of oxidation for human consumption, and these limits based on palatability provide the only available reference point.

There are now multiple studies from around the world indicating that retail n-3 PUFA supplements are frequently oxidised above these limits. These include 31\%\textsuperscript{559} and 50\%\textsuperscript{557} of products tested in independent Canadian studies, 44\% in Brazil\textsuperscript{560}, 84\% in South Africa\textsuperscript{558}, and our study showing 92\% in New Zealand\textsuperscript{818}. Of note, one of the Canadian studies\textsuperscript{557} included plant-based oils, which are less prone to oxidation and therefore underestimated the problem. In addition, Labdoor, a for-profit company from the US that tests and sells supplements, reported that 28\% of products tested had greater than twice the recommended level of lipid peroxides\textsuperscript{561}, the same rate as in our study\textsuperscript{818}. Together this indicates that consumers purchasing marine oil products are likely to be exposed to excess lipid oxidation products. Importantly, it appears that there are no reliable indicators that a consumer could use to select an unoxidised product, since cost, best before date, and country of manufacture were not associated with differences in oxidation levels\textsuperscript{818}.

14.7. Safety of oxidised marine oils

The safety of lipid oxidation products present in marine oils is poorly understood. Studies in a variety of animal models have shown toxic effects including growth retardation\textsuperscript{565} and organ toxicity\textsuperscript{565}. In a rabbit model, supplementation with fish oil led to a marked increase of lipid peroxidation products in serum and accelerated atherosclerosis\textsuperscript{506}. A plausible mechanism is through oxidation of LDL particles, as lipid oxidation products are transported within LDL particles\textsuperscript{509}, which must be oxidised before they can be incorporated into the foam...
Discussion

cells of atherosclerotic plaques. Further, lipid oxidation products are mutagenic, and could increase the risk of malignancy. However, the comparatively high doses and unusual methods of administration used in many animal toxicity models make translation to humans difficult. In one human study, short-term supplementation with oxidised fish oil did not affect markers of oxidative stress, but long-term effects on organ function, atherosclerotic cardiovascular disease or the risk of cancer were not assessed.

It is important to consider that many fish oil consumers may be especially vulnerable to toxic effects. For example, marine oils are used in both primary and secondary prevention of cardiovascular disease. Many of these consumers are at increased cardiovascular risk, for example due to obesity, diabetes, increasing age, or a history of ischaemic heart disease. If oxidised marine oils increase the rate of atherosclerosis, they may be of greater harm in such populations. Pregnant women are another particularly vulnerable population who consume marine oils primarily because of purported benefits to neurodevelopment of the fetus. Pregnancy is a time associated with reduced endogenous antioxidants, and the early embryo has very little antioxidant defence. Our own work in a rat model has shown directly that a highly oxidised fish oil, characterised by a high concentration of lipid peroxides led to both maternal insulin resistance and increased newborn mortality (Chapter 9). A recent clinical study has suggested potential adverse effects of fish oil on neurodevelopment of the offspring, although the oxidative state of the trial oil was not reported.

Given the high frequency of oxidised supplements on the retail market, it is likely that in many clinical trials of n-3 PUFAs the oil has been oxidised. Current evidence is insufficient to determine whether oxidised supplements have poorer efficacy or have adverse effects on health. Thus, oxidation remains a potential explanation for the mixed literature investigating the effects of n-3 PUFA supplementation.

14.8. Marine oils and labelled n-3 PUFA content

Unlike medications where consumers and physicians can rely on accurate and consistent dosing, the accuracy of supplement labels is often poor. Studies of marine oil products from Africa, Australasia, North America, South America and Europe have examined the accuracy of labelled n-3 PUFA content of fish oils. Opperman et al. arbitrarily but reasonably defined adequate accuracy as n-3 PUFA content between 90% to 110% of that labelled. Using this threshold, in 6 of the 10 studies at least 30% of products under-delivered, while in 4 studies, including our own, more than half of the products tested under-delivered.
Discussion

Thus, many products sold at retail have less n-3 PUFA content than labelled. This is a potential problem both for clinical trial participants and consumers, as it limits their ability to take a target dose of n-3 PUFAs. This may also hinder interpretation of the trial literature, as an independent analysis of trial oils is usually not reported, thus trials failing to demonstrate effects may have inadvertently under-dosed participants. As a result, the failure of marine oil supplements to alter hard cardiovascular outcomes in the current literature might be explained by excess oxidation products and inaccurate dosing.

14.9. Benefits of fish consumption: beyond n-3 PUFAs

In contrast to the evidence for marine oil supplements, there is substantial epidemiological evidence that fish consumption is associated with reduced cardiovascular and cerebrovascular risk. Further, greater circulating n-3 PUFA levels are also associated with greater insulin sensitivity and a reduction in sudden cardiovascular death. However, it is important to recognise that in cross-sectional and epidemiological studies n-3 PUFA levels are markers of fish consumption (not fish oil). Thus, it may not be the circulating n-3 PUFAs per se that account for the reduced risk.

Fish consumption differs from supplementation with marine oil by three major factors. Firstly, eating a meal of fish displaces other foods from the diet, such as red meat that is rich in saturated fat. Secondly, fish meat has substantial protein content. Consumption of fish protein has important biological effects in humans, including antihypertensive and insulin sensitising effects. Salmon calcitonin, which inhibits bone resorption in humans, has been detected in plasma after salmon feeding in rats, suggesting it might convey some of the biological effects of salmon consumption. Interestingly, fish protein also reduces urinary 8-OH deoxyguanosine (a marker of oxidative DNA damage), so that when fish meat is consumed, the protein component might help to counter potential oxidative stress associated with the n-3 PUFAs. Thus, some of the beneficial effects of fish consumption are likely to be due to its protein content. Lastly, while consumers of fish oil are frequently exposed to excess oxidation products, this is unlikely to occur with fish consumption, as rancid fish is malodourous and unpalatable.

14.10. Alternative approaches to getting the benefit of n-3 PUFAs

Many problems with the use of marine oils as an intervention have been discussed, including the complex chemical composition, many biological effects, presence of oxidation products, and problems with the accuracy of dosing. In this light, two diametrically opposite directions should be considered.
Discussion

Firstly, rather than using fish oil, oily fish as a whole food may be a better intervention to improve cardiovascular risk. Current evidence clearly indicates that it is beneficial, which is not true of marine oils. The Heart Foundation of Australia recommends that 2-3 servings of fish are consumed each week. This is reasonable but may not be achievable at a population level, since fish is costly, many people do not like it, and there are concerns about the sustainability of fisheries. It appears that the greatest incremental benefit is observed between those who eat fish very rarely and those eating fish once per week, suggesting that one fish meal per week may be an appropriate minimum intake.

Secondly, a precise pharmacological approach might be more efficacious than supplementation with marine oils. Many drugs already modulate the pathways affected by n-3 PUFAs, including the thiazolidinediones (PPAR-γ), fibrates (PPAR-α), and salicylates (COX-2 and NF-κB). Recent evidence showed that the cell surface receptor GPR-120 mediates the insulin-sensitising effect of n-3 PUFAs in rodents, through reduced inflammation of adipose tissue. In obesity, adipose tissue inflammation leads to abnormal release of FFAs and adipokine production, which in turn lead to insulin resistance, the major factor that connects obesity to hypertension, dyslipidaemia, T2DM, and cardiovascular disease. Thus, GPR-120 may be an especially promising target for drug development, which may improve cardiometabolic risk. Importantly, a synthetic GPR-120 agonist confers the same insulin-sensitizing effect as n-3 PUFAs in rodents. Further, GPR-120 also has effects on the regulation of appetite and insulin secretion. While there is currently no data on the effects of a GPR-120 agonist in humans, a report indicating that patients with GPR-120 mutations develop obesity and insulin resistance suggests that the effect could translate to humans. Because of the central importance of insulin sensitivity to cardiometabolic risk, if GPR-120 agonists do improve insulin sensitivity, they could potentially ameliorate much of the disease risk associated with obesity. Notably, their action through a single receptor may increase the likelihood that effects can be successfully translated from rodent models to humans without unexpected adverse effects. Lastly, it is likely that a synthetic agonist would be chemically stable, with reliably reported active ingredient, and require a smaller mass of medication than the many marine oil capsules needed to reach a high n-3 PUFA dose.

14.11. Market success in the face of weakening scientific evidence

The great promise for n-3 PUFAs emerging out of the 1970s has not been confirmed by contemporary trials and systematic reviews. Yet, while the evidence base has eroded, the market for marine oil supplements has grown substantially, and is expected to reach US$ 1.7 billion by 2018. This implies that the marketing is disconnected from the current scientific evidence.
In part, this can be ascribed to the way that health supplements are regulated and marketed in many countries. For example, in the US, Australia, and New Zealand, it is not necessary to prove the efficacy or safety of supplements prior to marketing, and there is no formal post-marketing safety surveillance to detect potential uncommon adverse events. In these countries, while specific claims such as treating or curing a disease cannot be made without strong supporting evidence, ‘structure/function claims’ (i.e. that a supplement can promote, maintain or enhance health or well-being, or affect the structure or function of an organ or the body) can be made with minimal support. In Europe, the bar is set higher as “general health claims” for supplements must be submitted to the European Food Safety Authority for review, who consider the scientific evidence and publish lists of allowable and non-allowable claims. Allowable claims for n-3 PUFA rich supplements in Europe include that they contribute to the normal function of the heart, and to the maintenance of normal BP and triglycerides. In contrast, n-3 PUFA rich supplements cannot be claimed to help maintain normal blood glucose or LDL-cholesterol. This type of regulation, while more costly, reduces the range of general claims to those that are probably true to some extent.

Nevertheless, structure/function claims and even the approved general claims in Europe are likely to confuse many consumers. For example, across all of the discussed regions, fish oil products could not be claimed to “prevent myocardial infarction”, but they could be claimed to “support cardiovascular health”. These two claims are technically very different, but it is likely that many (if not most) consumers would interpret them in the same way. This would hamper the ability of consumers to make an informed decision about supplements, and may in part explain the continued success of marine oils in the face of weakening evidence for benefit. Importantly, outside of Europe, because structure/function claims are vague and not based on scientific evidence, they do not need to be changed in light of new evidence to the contrary.

14.12. Regulation and the unknown risk to consumers of oxidised marine oils

Weak regulation of health supplements around the world enables them to be sold without first determining their safety, and this has the potential to allow harmful substances to be sold as supplements. Marine oils that have become oxidised may be such a product. Reports from around the world including Australasia, Africa, North America, and South America have shown a high frequency of oxidation above recommended limits. Further, not only have some of these studies included products imported from other regions, but most fish oils are actually sourced from the West coast of South America, where they undergo the same initial steps in manufacture. Thus, oxidation of marine oil supplements is likely to be a global problem, and the risk to consumers remains unknown.
In this light, it is important to consider whether regulation is sufficient to keep consumers safe. European regulation of supplements is stronger than in other regions, and the European Food Safety Authority (EFSA) has given some consideration to the oxidation of fish oil supplements\textsuperscript{579}. In their report, they indicated that there were insufficient data to set a safe limit of oxidation products, and recommended that the effects of individual oxidation products on human health be investigated\textsuperscript{579}. However, despite this lack of evidence, as has occurred outside of Europe, marketing has been allowed without any requirement to determine safety prior to sale or afterwards. As a result, this issue has been barely investigated and all oxidation limits are arbitrary and based on palatability, whether published by the EFSA (Europe)\textsuperscript{579}, Food and Drug Administration (USA)\textsuperscript{972}, Therapeutic Goods Administration (Australia)\textsuperscript{973}, Health Canada\textsuperscript{578}, the Codex Alimentarius Commission (draft) representing the World Health Organisation and Food and Agriculture Organisation of the United Nations\textsuperscript{581}, or industry groups such as the Global Organisation for EPA and DHA\textsuperscript{576} or Council for Responsible Nutrition\textsuperscript{974}.

While the appropriateness of these limits is unknown, they are frequently exceeded in retail products\textsuperscript{557-561,818}. This suggests that oxidation of marine supplements is rarely audited, testing is unreliable, or the oil tested is not representative of that available for purchase at retail. Nonetheless, despite this fact, we believe that the most pressing issue in the regulation of marine oil supplements is not enforcement of oxidation limits, but establishment of what those limits should be. Regulation should have compelled studies investigating safe limits of oxidation to be performed and funded, but it is clear that this has not been the case. In the absence of such regulation, this has been left to independent members of the scientific community.
Chapter 15. Conclusions and future directions

The metabolic dysfunction of obesity remains one of the world’s most significant health problems. Treatments that improve insulin sensitivity could be transformative, especially since most weight-loss treatments are ineffective and the only treatment with a dramatic effect (bariatric surgery) cannot be performed on all who would benefit. Although I have shown krill oil to worsen insulin resistance, there is still insufficient data to determine whether fish oil supplements, increasing fish in the diet, pure n-3 PUFA supplements, or a synthetic GPR-120 agonist could have an important impact on metabolic health.

During pregnancy, unoxidised fish oil supplementation in the context of maternal obesity had beneficial effects on offspring metabolism in the rat, so that there is a need to consider application of this research to human pregnancy. However, further questions about supplementation during lactation and the safe level of lipid peroxides must be answered first.

Nonetheless, as previously noted supplementation with marine oils constitutes a complex intervention. In addition to having a broad range of chemical compounds, the n-3 PUFAs that are considered to be the active ingredients affect a wide range of biological processes in many different tissues. Further, oxidation products may also have important adverse biological effects. The current scientific literature, which has increasingly shown no overall benefit of marine oils, may have been confounded by other oil components (including unrecognised oxidation products), as well as inadvertent under-dosing of participants. Thus, there is a pressing need for trials to be conducted with supplements where content is independently verified and the oxidative state is determined. Until these data emerge, we cannot be sure that marine oils are as ineffective as current evidence suggests. Further, the trial literature regarding potential insulin sensitising effects of n-3 PUFAs is highly heterogeneous and confounded by differences in study design, and the patient groups enrolled. It is important that a potentially effective treatment such as marine oils is not discarded out of hand. However, there is also a need for studies investigating the potential toxicity of specific marine oil oxidation products. In the meantime, consumption of oily fish can be recommended to reduce cardiovascular risk, and the development of drugs targeting specific pathways that are affected by n-3 PUFAs may provide greater benefit still. Lastly, the poor regulation of supplements and their label claims serves to confuse consumers, such that the market success of marine oils far exceeds the scientific evidence. As it stands, current evidence suggests that consuming marine oil supplements is not money well spent.
15.1. Key questions remaining, and future directions of my research programme

There are two key important questions that arise from my research: 1) Is oxidised fish oil supplementation also harmful in human pregnancy? and 2) Can unoxidized fish oil supplementation in obese human pregnancy protect the offspring from developing metabolic disease as they age? In addition, the mechanisms through which krill oil impaired insulin sensitivity in adult men, oxidised fish oil increased maternal insulin resistance and neonatal mortality in rats, or the unoxidized fish oil preserved insulin sensitivity in the offspring of high fat diet fed rat dams remain unclear.

I have won the Maurice Paykel Research Fellowship which provides funding for 2 years to plan and begin the animal and human trials necessary to address the most important questions and to further analyse samples from the rat pregnancy study to elucidate mechanism for the protective effect of unoxidized fish oil in pregnancy.

There are other important knowledge gaps in this area which also need to be addressed, but are not part of my plans. Recent evidence that an n-3 PUFA concentrate improved insulin sensitivity and reduced incident diabetes in adults suggests that n-3 PUFAs could be metabolically protective. However, this finding must be replicated before it could be recommended to the public.

It is possible that fish consumption could be more beneficial than fish oil supplementation. Larger studies replacing meals with oily fish are required and should be compared with fish oil supplementation head to head.

A GPR-120 agonist could be a useful drug in adults at risk of metabolic disease, given such an agonist has been shown to be effective in rodents. Such a treatment would not be at risk of oxidation, and there could be other advantages such as reduced risk of adverse effects, if the drug does not affect other metabolic pathways. It is likely that early clinical studies are planned or underway.

15.1.1 Determining whether oxidised fish oil consumption is likely to be a risk to human pregnancy

To estimate the risk of consuming oxidised fish oil during human pregnancy will require a series of studies which we have designed in collaboration with a toxicologist from the Ministry of Primary Industries. These aim to first determine whether a human relevant dose of a highly oxidised fish oil causes harm in rat pregnancy, either on pregnancy outcomes such as neonatal mortality or in the adult offspring. If harm during early pregnancy is shown then a dose-response study will be conducted, and a third study utilising a cull in late pregnancy to assess placental and fetal tissues will be planned to address mechanism. These studies will incorporate detailed measures of phenotype including assessment of glucose and lipid metabolism in mothers and adult offspring.
15.1.2 Determining whether supplementation of obese women during pregnancy with an unoxidised fish oil can improve the metabolic phenotype of the offspring

120 women between 8-12 weeks of pregnancy who are obese (BMI >30kg/m²) but do not have diabetes mellitus, use tobacco or medications that influence blood pressure, lipid metabolism or insulin sensitivity will be recruited.

Women will be randomised to fish oil or control oil (canola oil), taking 3g on every day of pregnancy. The control capsules will be lightly coated in fish oil to give them similar odour. Canola oil contains no long-chain n-3 PUFAs. The primary fatty acid is oleic acid which is already abundant in the diet, and there is no reliable evidence to suggest it has important metabolic effects. 3g of fish oil has been shown to have beneficial metabolic effects in non-pregnant subjects indicating it is sufficient to have biological effects. Women will be asked not to take supplements during pregnancy except for folic acid, iodine, and iron.

The impact of the fish oil treatment on the phenotype of the offspring will be assessed in the first year of life, and include studies of body composition and glucose metabolism after birth, 1 month, 3 months and 12 months. These children will then be assessed in early childhood (age 4-7) when it is possible to use more detailed techniques to assess glucose metabolism. This cohort will then be followed to be reassessed in early adulthood.

15.1.3 Determining the mechanism by which fish oil supplementation to rat dams fed a high fat diet during pregnancy, led to greater insulin sensitivity in the offspring

In the animal study, supplementing rat dams consuming a high fat diet with unoxidized fish oil preserved insulin sensitivity in the adult offspring. The most likely explanation for how this occurred is through alteration of epigenetic marks in metabolically important genes, which change the metabolic phenotype. In principle, greater insulin sensitivity in the adult offspring, after supplementation of the mother with fish oil, could be mediated by changes in the function of adipose tissue, liver or muscle.

Given the cornerstone role that adipose tissue has in the development of the metabolic dysfunction of obesity, adipose tissue function was examined first (by another student, Stephanie Segovia, unpublished). However, there was no difference in the expression of a range of key genes in adipose tissue including IRS-1, TLR-4, PPAR-γ and Glut-4, suggesting that the beneficial effect of the fish oil is unlikely to be mediated by differences in adipose tissue function. As we observed improvement of insulin sensitivity as measured by both Matsuda Index (reflects hepatic and peripheral insulin sensitivity) and HOMA-IR (predominantly reflects hepatic insulin sensitivity), it is likely that there was a beneficial effect on the liver. Importantly, it is known that a
Discussion

Maternal high fat diet does alter gene expression in the liver of offspring\(^{975}\). The next step is to comprehensively detail gene expression in liver from samples taken from the adult offspring in the completed rat study. If there are differences in liver gene expression, we will next look for alterations in epigenetic marks (DNA methylation and histone acetylation) associated with these genes. This study will test two predictions:

- The offspring of dams fed a high fat diet during pregnancy will have altered expression of genes that regulate metabolism or inflammation in liver, compared to those of dams fed a control diet.

- The offspring of dams fed a high fat diet and supplemented with fish oil during pregnancy will have a different pattern of gene expression in liver than those of dams fed a high fat diet, but not supplemented with fish oil.

15.2. Final remarks for consumers

Accumulating evidence over the last 20 years has increasingly questioned the benefit of fish oil supplementation for the prevention of heart disease and stroke, and for beneficial effects to the offspring when taken during pregnancy.

The research presented in this thesis has shown that fish oil supplements sold in retail stores are often rancid (excessively oxidised), and it is not currently possible for the consumer to find out how rancid a retail product is at the time of purchase. When pregnant rats were given rancid fish oil, there was a much greater chance of the newborn dying within the first week of life. The effects in human pregnancy are unknown but are likely to be less severe. When considering whether to start to or continue to take n-3 PUFA supplements, a conservative approach should be favoured. The current evidence for beneficial effects of n-3 PUFA supplementation is poor. Thus, until unoxidised fish oils can be easily identified at the time of purchase we recommend that n-3 PUFA supplements are not used during pregnancy.

We also showed that in middle-aged men, consuming krill oil led to poorer blood glucose regulation. However this study did not determine whether consuming krill oil would increase the risk of diabetes or heart disease. While our data relates specifically to overweight men, it is likely to also apply to overweight women. For this reason, krill oil is not recommended for overweight adults.
The Appendix contains three published manuscripts. These represent the results of 3 additional studies conducted using data collected from the Krill/Salmon randomised controlled trial. These metabolic studies are separated in the appendix as they do not fit neatly within the theme of this thesis.
Appendices

Chapter 16. Among overweight middle-aged men, first-borns have lower insulin sensitivity than second-borns

Preface

This chapter contains a modified version of a manuscript published in the journal *Scientific Reports*.569

- Authors: Benjamin B. Albert, Martin de Bock, José GB. Derraik, Christine M. Brennan, Janene B. Biggs, Paul L. Hofman, Wayne S. Cutfield
- Journal: Scientific Reports
- Year of Publication: 2014
- Volume: 4
- Page: 3906
- Impact Factor when published: 5.58
Appendices

16.1. Introduction

There has been a steady reduction in birth rates throughout the world\textsuperscript{976}. Therefore, average family size has decreased, with a consequent increase in the proportion of first-born children in many countries\textsuperscript{977}. Thus, any adverse health outcomes associated with being first-born would affect an increasing proportion of the world’s population\textsuperscript{978}.

There is some evidence that birth order influences growth and metabolism, from infancy to early adulthood\textsuperscript{978}. First-born babies have lower birth weight, but more rapid growth and weight gain in infancy\textsuperscript{979,980}, such that in childhood they are taller than later-borns\textsuperscript{977,981}. Importantly, first-born children have reduced insulin sensitivity and higher daytime BP\textsuperscript{977}. Although the height discrepancy is reduced by early adulthood, first-borns have greater adiposity\textsuperscript{979,982}. Further, first-borns have been shown to have a less favourable lipid profile in young adulthood, with higher LDL, total cholesterol, and triglyceride concentrations than later-borns\textsuperscript{979}. Thus, being first-born may be associated with persistent changes in metabolism and body composition, that may lead to greater risk of developing T2DM and cardiovascular disease.

While there are limited data on the effects of birth order on growth and metabolism in the first two decades of life, there are no data on such outcomes in mid-adulthood. It is important to assess whether the metabolic effects observed in early years are sustained or magnified throughout adulthood, particularly since middle age is a time where identification of risk factors and early intervention may be most appropriate. Therefore, we aimed to assess whether birth order affects metabolism and body composition in overweight middle-aged men.

16.2. Methods

This methods section is abbreviated, additional details regarding ethics and clinical assessments are presented in Chapter 9.3.

Participants were recruited for two clinical trials investigating the metabolic effects of supplementation with olive leaf extract\textsuperscript{332} or krill oil (unpublished data) (Figure 16). From this group, all participants born at term (37–41 weeks) from singleton pregnancies were included. Note that when assessing birth order, miscarriages of less than 20 weeks were not counted. Where subjects participated in more than one clinical trial, the data from the most recent trial was used.
Assessments

Insulin sensitivity was measured using an oral glucose tolerance test (Matsuda method). Additional assessments and outcomes included lipid profile, weight, BMI, fat mass index, body composition (DXA), 24-hour ambulatory BP monitoring, cIMT, physical activity (IPAQ) and socioeconomic status (NZDep2006).

Statistical Analysis

Demographic characteristics between first- and second-born men were compared using one-way ANOVA and Fisher's exact tests in Minitab v.16 (Pennsylvania State University, State College, PA, USA). Multivariate linear regression models were carried out in SAS v.9.3 (SAS Institute, Cary, NC, USA). All models accounted for important confounding factors, namely age, ethnicity, socio-economic status (NZDep2006), physical activity levels (IPAQ), and mean parental age at childbirth. Age and total body fat percentage were also controlled for when assessing potential differences in lipids and outcomes associated with glucose homeostasis. All statistical tests were two-tailed and significance level maintained at 5%. Demographic data are presented as means ± standard deviations, while other data are means and 95% confidence intervals adjusted for the confounders in multivariate models.

16.3. Results

From the 57 first- and second-born men that participated in both trials, 50 subjects met the inclusion criteria. First- and second-born men were of similar height (p=0.40; Table 17). However, first-born men were 6.9kg heavier (p=0.013) and had greater BMI (29.1 vs 27.5kg/m²; p=0.004) than second-borns (Table 17). There were no significant differences between groups in total body fat and android fat to gynoid fat ratio, but first-borns tended to have a greater fat mass index than second-borns (p=0.068; Table 17).
Appendices

Figure 16: Summary of study recruitment.

All participants from the olive leaf extract and krill oil (unpublished data) trials were overweight middle-aged men recruited in Auckland, New Zealand.

Table 17: Age and anthropometry in first-born and second-born men.

<table>
<thead>
<tr>
<th></th>
<th>First-borns</th>
<th>Second-borns</th>
<th>p-value</th>
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<tbody>
<tr>
<td>n</td>
<td>26</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Ethnicity (European descent)</td>
<td>92%</td>
<td>92%</td>
<td>0.99</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.9 ± 5.6</td>
<td>46.4 ± 5.4</td>
<td>0.37</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176.8 (171.0–180.5)</td>
<td>175.1 (169.7–180.5)</td>
<td>0.40</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>91.0 (83.6–98.4)</td>
<td>84.1 (76.9–91.3)</td>
<td>0.013</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.1 (27.7–30.5)</td>
<td>27.5 (26.1–28.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>32.2 (27.3–37.1)</td>
<td>29.9 (25.1–34.7)</td>
<td>0.21</td>
</tr>
<tr>
<td>Android fat to gynoid fat ratio</td>
<td>1.27 (1.13–1.14)</td>
<td>1.23 (1.09–1.36)</td>
<td>0.42</td>
</tr>
<tr>
<td>Fat mass index (kg/m²)</td>
<td>9.36 (7.74–10.97)</td>
<td>8.26 (6.69–9.84)</td>
<td>0.068</td>
</tr>
</tbody>
</table>

Age data are mean ± SD; other data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models.

Insulin sensitivity in first-born men was 33% lower than in second-borns (4.38 vs 6.51; p=0.014) (Table 18), despite adjustment for confounders including fat mass, socio-economic status, and physical activity levels. However, disposition index was similar in both groups (Table 18). Further, there were no significant differences in ambulatory BP, lipid profile, or carotid intima-media thickness between first- and second-borns (Table 18).
Appendices

Table 18: Study outcomes in first-born and second-born men.

<table>
<thead>
<tr>
<th></th>
<th>First-borns</th>
<th>Second-borns</th>
<th>p-value</th>
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<tr>
<td>n</td>
<td>26</td>
<td>24</td>
<td></td>
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<tr>
<td>Glucose homeostasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td>4.38 (2.72–6.73)</td>
<td>6.51 (4.31–9.56)</td>
<td>0.014</td>
</tr>
<tr>
<td>Disposition index</td>
<td>4.72 (4.65–4.79)</td>
<td>4.71 (4.64–4.78)</td>
<td>0.86</td>
</tr>
<tr>
<td>24-hour ambulatory blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic (mmHg)</td>
<td>128.3 (120.4–136.2)</td>
<td>124.7 (116.9–132.4)</td>
<td>0.23</td>
</tr>
<tr>
<td>Diastolic (mmHg)</td>
<td>80.6 (75.3–85.9)</td>
<td>77.9 (72.7–83.1)</td>
<td>0.18</td>
</tr>
<tr>
<td>Systolic dip (%)</td>
<td>13.5 (7.8–19.2)</td>
<td>12.8 (7.2–18.4)</td>
<td>0.76</td>
</tr>
<tr>
<td>Diastolic dip (%)</td>
<td>19.0 (11.5–26.7)</td>
<td>18.4 (11.0–25.8)</td>
<td>0.81</td>
</tr>
<tr>
<td>Carotid-intima media thickness (mm)</td>
<td>0.71 (0.59–0.82)</td>
<td>0.71 (0.61–0.82)</td>
<td>0.92</td>
</tr>
<tr>
<td>Lipid profile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.11 (3.35–4.88)</td>
<td>4.02 (3.31–4.72)</td>
<td>0.74</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>2.80 (2.09–3.51)</td>
<td>2.56 (1.91–3.21)</td>
<td>0.38</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.05 (0.84–1.26)</td>
<td>1.01 (0.82–1.21)</td>
<td>0.65</td>
</tr>
<tr>
<td>Total cholesterol : HDL-C</td>
<td>3.98 (3.05–4.91)</td>
<td>4.13 (3.28–4.99)</td>
<td>0.66</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.78 (0.41–1.44)</td>
<td>0.93 (0.59–1.26)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models.

16.4. Discussion

This study has shown that within a cohort of overweight middle-aged men, first-borns had greater BMI and lower insulin sensitivity than second-borns. Importantly, the difference in insulin sensitivity was independent of fat mass. Reduced insulin sensitivity is an independent predictor of T2DM, hypertension, coronary heart disease, stroke and cancer in non-obese middle aged men. Recently, there has been increasing interest in two atypical groups. Firstly, the metabolically healthy obese (MHO), who although obese do not display the typical adverse metabolic effects of obesity. Secondly, the metabolically obese but normal weight (MONW), who have unhealthy metabolic markers despite being in the normal BMI range. Environmental and genetic correlates of MHO have been identified. We speculate that birth order, through an influence on the early fetal environment, contributes to the phenotype of insulin resistance and metabolic adversity that characterise the unhealthy obese and MONW.

The mechanisms by which birth order influences long-term metabolism are unknown, but differences in placental blood flow may play a role. During pregnancy, structural changes occur to uterine spiral arteries to facilitate placentation. Following parturition, these changes do not reverse, suggesting a more favourable fetal environment for subsequent pregnancies. Not surprisingly, first-borns are lighter at birth than later-
Nonetheless, while placental blood flow is an important regulator of fetal growth, the metabolic effect of birth order is not solely due to differences in birth weight.

It is worth noting that the difference in insulin sensitivity was also independent of the age of participants and of their parents. Recent studies indicate that there are age-related changes in the β-adrenergic system that lead to altered cardiovascular function and reduced insulin secretion. These observations highlight the importance of accounting for both parental and participants' ages in our analyses. Age-related changes could directly influence the metabolic phenotype of participants, while those born of older mothers could theoretically be affected via altered fetal growth and subsequent metabolic programming.

The major strength of this study is the comprehensive metabolic assessment. Although the study of a narrow BMI range enabled characterization of overweight middle-aged men, this may limit wider application of our findings. Given first-borns had greater BMI than second-borns, using BMI as an inclusion criterion for the parent clinical trials might have introduced a subtle selection bias. Other limitations include the post hoc analysis and a relatively small sample size. In addition, we studied a relatively narrow range of individuals (overweight males living in a large urban centre, mostly of New Zealand European ethnicity), which could also limit the applicability to the general population, particularly females. Lastly, as we did not study sibling pairs, our findings could have underestimated the magnitude of birth order effects on insulin sensitivity and other metabolic outcomes.

In conclusion, first-born overweight middle-aged men have greater BMI and lower insulin sensitivity than second-borns. Larger studies are required to better evaluate the long-term health effects of birth order across the BMI range. Ideally, future research should focus on sibling pairs.
Appendices

Chapter 17. Increasing parental age at childbirth is associated with greater insulin sensitivity and more favourable metabolic profile in overweight adult male offspring

Preface

This chapter contains a modified version of a manuscript published in the journal *American Journal of Human Biology*.¹³²

- **Authors:** Benjamin B. Albert, Martin de Bock, José GB. Derraik, Christine M. Brennan, Janene B. Biggs, Paul L. Hofman, Wayne S. Cutfield
- **Journal:** American Journal of Human Biology
- **Year of Publication:** 2015
- **Volume:** 3
- **Page:** 380-386
- **Impact Factor when published:** 1.88
17.1. Introduction

There has been a progressive increase in parental age at childbirth in most western societies, so that more couples are having children in their thirties and forties\textsuperscript{992}. In the US, the proportion of births occurring to women over 35 years has increased from 9\% in 1990 to 14\% in 2008\textsuperscript{993}. Data from OECD countries show a considerable shift in the mean maternal age at first childbirth over the last 40 years, for example rising by 5.2 years in Iceland and 4.8 years in the Czech Republic\textsuperscript{992}.

Although much attention has been given to the increase in maternal age at childbirth, an upwards shift in paternal age is also taking place\textsuperscript{994}. However, paternal age at childbirth is not routinely recorded in birth registries, and such data are consequently scarce. In England and Wales, mean paternal age reportedly increased from 29.2 years in 1980 to 32.1 in 2002\textsuperscript{995}. In Germany, the median age of married fathers increased from 31.3 to 33.1 years between 1991 and 1999\textsuperscript{996}.

Increasing parental age at childbirth has been linked to adverse outcomes in the offspring, including genetic disease\textsuperscript{995}, malignancy and mental health disorders\textsuperscript{997}. Further, there is strong evidence that older paternal age also has important long-term effects on offspring health\textsuperscript{998}, such as increased risk of autism\textsuperscript{999}. A variety of mechanisms could mediate the health effects of greater parental age, including epigenetic changes\textsuperscript{998,1000} and direct effects of the pre- and post-natal environments.

There are limited and conflicting data on the effects of parental age at childbirth on offspring body composition and metabolism. Recently, increasing parental age at childbirth was found to be associated with taller stature and reduced central adiposity in children\textsuperscript{991,994}. Further, girls born to older mothers appeared to have improved insulin sensitivity as assessed by HOMA-IR\textsuperscript{991}. However, increased maternal age at childbirth has been linked to higher BP in childhood\textsuperscript{1001}, and increased paternal age was associated with a less favourable lipid profile in girls\textsuperscript{994}. In young adults, a large cross-sectional study in men found a greater rate of obesity with increasing paternal age\textsuperscript{1002}, while increasing maternal age was associated with higher risk of T2DM\textsuperscript{1003}.

Thus, although there is some evidence suggesting older parenthood may have favourable metabolic effects in the offspring, the evidence is conflicting and the long-term metabolic implications for the offspring of older parents remain unclear. In particular, there are no data on associated metabolic outcomes in the offspring in adulthood. It is important to assess whether the differences observed in childhood studies\textsuperscript{991,994} persist into
adult life. Therefore, we assessed the impact of parental age at childbirth on insulin sensitivity and other metabolic outcomes in a cohort of overweight middle-aged males.

17.2. Methods

This methods section is abbreviated, additional details are reported in Chapter 9.3.

Participant & Recruitment

Participants were recruited for two clinical trials investigating the metabolic effects of nutritional supplementation with olive leaf extract or krill oil in overweight middle-aged men. This study encompasses a post hoc analysis of their pre-trial baseline data. From this group all participants born at term (37–41 weeks) from singleton pregnancies, who knew the age of both parents when they were born, were included.

Assessments

Insulin sensitivity was measured using an oral glucose tolerance test (Matsuda method). Additional assessments and outcomes included lipid profile, weight, BMI, fat mass index, body composition (DXA), 24-hour ambulatory BP monitoring, cIMT, physical activity (IPAQ) and socioeconomic status (NZDep2006).

Statistical analysis

Maternal and paternal ages are highly correlated, and in this relatively small cohort it would not be possible to differentiate their individual effects on study outcomes. Thus, the main parameter of interest was the mean parental age at childbirth (MPAC), calculated as the average of maternal and paternal ages. However, it was important to account for the age gap between parents, and whether the father or the mother was older. Thus, another parameter was calculated, which was the age difference between mother and father, where a negative difference indicated a mother who was older than the father.
Appendices

Descriptive statistics were obtained in Minitab v.16 (Pennsylvania State University, State College, PA, USA) and are presented as means ± standard deviations. Multivariate linear regression models were carried out in SAS v.9.3 (SAS Institute, Cary, NC, USA). All models accounted for important confounding factors, namely age of the offspring at assessment, physical activity levels (IPAQ), socioeconomic status (NZDep2006), birth order, as well as parental age difference. In addition, BMI was controlled for when assessing potential differences in lipid concentrations and outcomes associated with glucose homeostasis. Subgroup analyses were carried out solely on data for participants of European ethnicity. Where necessary, outcome parameters were log-transformed to approximate normality. All statistical tests were two-tailed and significance level maintained at 5%.

17.3. Results

Demographics

There were 97 subjects in both trials, but since 5 men took part in both studies, there were 92 individuals enrolled. We consequently studied a total of 73 men who met the inclusion criteria and knew the age of both biological parents. When study participants were compared to the 19 excluded subjects, there were no significant differences in age, ethnic composition, socioeconomic status, body composition, insulin sensitivity, BP, or lipid profile between groups.

Participants were aged 46.0 ± 5.4 years (range 34.5–55.6 years), and most (88%) were of European ethnicity. The characteristics of the study cohort are provided in Table 19.

Maternal age at childbirth was 27.6 ± 5.3 years (range 18–45 years) and paternal age was 30.7 ± 5.7 years (range 19–45 years) (Figure 17), with maternal and paternal ages being highly correlated (r=0.71; p<0.0001) (Figure 18). MPAC was 29.2 ± 5.1 years (range 18.5–44.5 years) (Figure 17). For the majority of participants, the father was older than the mother, with a mean parental age difference of 3.2 ± 4.2 years, ranging from -5 to 16 years (Figure 17). MPAC was highly correlated with both maternal (r=0.92; p<0.0001) and paternal (r=0.93; p<0.0001) ages at childbirth, but it was not correlated with parental age difference (r=0.12; p=0.32).
### Table 19: Characteristics of the study population

<table>
<thead>
<tr>
<th>Outcome response</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet and lifestyle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical activity levels (IPAQ)</td>
<td>3716</td>
<td>3728</td>
<td>66 – 19490</td>
</tr>
<tr>
<td>Total energy intake (kJ/day)</td>
<td>9875</td>
<td>2798</td>
<td>3313 – 17378</td>
</tr>
<tr>
<td>Saturated fat intake (g/day)</td>
<td>32.3</td>
<td>13.1</td>
<td>6.2 – 64.5</td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>88.6</td>
<td>9.5</td>
<td>66.2 – 111.8</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>27.6</td>
<td>1.8</td>
<td>25.0 – 30.0</td>
</tr>
<tr>
<td><strong>Glucose homeostasis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin sensitivity (Matsuda index)</td>
<td>6.82</td>
<td>3.85</td>
<td>1.31 – 17.0</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.74</td>
<td>1.27</td>
<td>0.37 – 6.42</td>
</tr>
<tr>
<td>HOMA-β (%)</td>
<td>80.2</td>
<td>43.6</td>
<td>13.7 – 198</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>7.20</td>
<td>4.79</td>
<td>1.68 – 24.3</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.33</td>
<td>0.56</td>
<td>4.16 – 7.57</td>
</tr>
<tr>
<td>Glucose 120 minutes (mmol/l)</td>
<td>5.70</td>
<td>1.70</td>
<td>2.47 – 10.8</td>
</tr>
<tr>
<td><strong>Cardiovascular parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daytime systolic blood pressure (mmHg)</td>
<td>126.4</td>
<td>9.1</td>
<td>107.0 – 160.0</td>
</tr>
<tr>
<td>Daytime diastolic blood pressure (mmHg)</td>
<td>80.2</td>
<td>6.6</td>
<td>69.0 – 107.0</td>
</tr>
<tr>
<td>Night time systolic blood pressure (mmHg)</td>
<td>110.5</td>
<td>8.9</td>
<td>89.0 – 138.0</td>
</tr>
<tr>
<td>Night time diastolic blood pressure (mmHg)</td>
<td>67.0</td>
<td>6.7</td>
<td>53.0 – 88.0</td>
</tr>
<tr>
<td>Night time systolic blood pressure dip (%)</td>
<td>12.6</td>
<td>5.7</td>
<td>1.6 – 30.0</td>
</tr>
<tr>
<td>Night time diastolic blood pressure dip (%)</td>
<td>16.5</td>
<td>7.4</td>
<td>0.0 – 41.2</td>
</tr>
<tr>
<td>Carotid intima-media thickness (mm)</td>
<td>0.800</td>
<td>0.165</td>
<td>0.459 – 1.436</td>
</tr>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.06</td>
<td>0.90</td>
<td>3.21 – 7.75</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.42</td>
<td>0.79</td>
<td>1.83 – 5.67</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.09</td>
<td>0.30</td>
<td>0.61 – 2.23</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.20</td>
<td>0.47</td>
<td>0.53 – 3.10</td>
</tr>
</tbody>
</table>
Appendices

Figure 17: Distribution of parental ages at childbirth in the studied cohort

For parental age difference, negative values indicate a mother older than the father.

Figure 18: The association between maternal and paternal ages at childbirth in the studied cohort
Appendices

Effects of parental age at childbirth

Multivariate models showed that increasing MPAC was associated with a continuous increase in insulin sensitivity (Matsuda index; p=0.008) and a reduction in insulin resistance as assessed by HOMA-IR (p=0.011) in our cohort of overweight middle-aged men (Table 20). In addition, increasing MPAC was associated with lower fasting insulin (p=0.018) and glucose (p=0.033) concentrations (Table 20).

Greater MPAC was associated with reductions in night time systolic (p=0.020) and diastolic (p=0.047) BP, as well as with improved (increased) nocturnal diastolic BP dipping (p=0.046) (Table 20). Increasing MPAC also trended to be associated with a subtle reduction in carotid-intima media thickness (p=0.068). MPAC was not associated with any parameter of body composition or lipid profile (Table 20).

Note that MPAC was not associated with birth weight (p=0.20), physical activity levels (p=0.79), energy intake (p=0.23), or saturated fat consumption (p=0.58). Further, in light of their very strong correlations with MPAC, when assessed separately, maternal and paternal ages yielded continuous associations with study outcomes that were very similar to those obtained for MPAC.

Europeans

Subgroup analyses were carried out on the 64 participants of European ethnicity. Despite a 12% reduction in n, these data corroborated the findings obtained for the whole cohort (Table 21). Increasing MPAC was associated with greater insulin sensitivity (Matsuda index; p=0.007), lower insulin resistance (HOMA-IR; p=0.009), lower fasting insulin concentrations (p=0.009), lower nocturnal systolic BP (p=0.032), and greater nocturnal diastolic dip (p=0.039) (Table 21). However, this subgroup analyses also indicated that increase MPAC was associated with a reduction in carotid intima-media thickness (p=0.035) and a more favourable lipid profile, namely lower LDL-C (p=0.028) and a trend towards lower total cholesterol (p=0.054) (Table 21).
### Table 20: Results from multivariate regression models.

<table>
<thead>
<tr>
<th>Outcome response</th>
<th>β</th>
<th>Standard Error</th>
<th>Lower 95% confidence limit</th>
<th>Upper 95% confidence limit</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.074</td>
<td>0.044</td>
<td>-0.015</td>
<td>0.163</td>
<td>0.10</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>0.183</td>
<td>0.146</td>
<td>-0.110</td>
<td>0.475</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Glucose homeostasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin sensitivity (Matsuda index)</td>
<td>0.193</td>
<td>0.078</td>
<td>0.038</td>
<td>0.349</td>
<td>0.008</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-0.064</td>
<td>0.024</td>
<td>-0.112</td>
<td>-0.015</td>
<td>0.011</td>
</tr>
<tr>
<td>HOMA-β (%)</td>
<td>-0.997</td>
<td>0.923</td>
<td>-2.838</td>
<td>0.853</td>
<td>0.29</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>-0.221</td>
<td>0.091</td>
<td>-0.403</td>
<td>-0.039</td>
<td>0.018</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>-0.030</td>
<td>0.014</td>
<td>-0.058</td>
<td>-0.003</td>
<td>0.033</td>
</tr>
<tr>
<td>Glucose 120 minutes (mmol/l)</td>
<td>-0.034</td>
<td>0.042</td>
<td>-0.119</td>
<td>0.051</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Cardiovascular parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daytime systolic blood pressure (mmHg)</td>
<td>-0.208</td>
<td>0.229</td>
<td>-0.667</td>
<td>0.252</td>
<td>0.37</td>
</tr>
<tr>
<td>Daytime diastolic blood pressure (mmHg)</td>
<td>-0.080</td>
<td>0.158</td>
<td>-0.396</td>
<td>0.235</td>
<td>0.61</td>
</tr>
<tr>
<td>Night time systolic blood pressure (mmHg)</td>
<td>-0.500</td>
<td>0.213</td>
<td>-0.928</td>
<td>-0.073</td>
<td>0.020</td>
</tr>
<tr>
<td>Night time diastolic blood pressure (mmHg)</td>
<td>-0.325</td>
<td>0.168</td>
<td>-0.661</td>
<td>0.011</td>
<td>0.047</td>
</tr>
<tr>
<td>Night time systolic blood pressure dip (%)</td>
<td>0.241</td>
<td>0.156</td>
<td>-0.072</td>
<td>0.554</td>
<td>0.13</td>
</tr>
<tr>
<td>Night time diastolic blood pressure dip (%)</td>
<td>0.413</td>
<td>0.202</td>
<td>0.008</td>
<td>0.818</td>
<td>0.046</td>
</tr>
<tr>
<td>Carotid intima-media thickness (mm)</td>
<td>-0.006</td>
<td>0.003</td>
<td>-0.013</td>
<td>0.001</td>
<td>0.068</td>
</tr>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>-0.025</td>
<td>0.021</td>
<td>-0.066</td>
<td>0.017</td>
<td>0.24</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>-0.028</td>
<td>0.019</td>
<td>-0.066</td>
<td>0.009</td>
<td>0.13</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.003</td>
<td>0.007</td>
<td>-0.012</td>
<td>0.018</td>
<td>0.73</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>-0.007</td>
<td>0.011</td>
<td>-0.030</td>
<td>0.016</td>
<td>0.54</td>
</tr>
</tbody>
</table>

This table shows the estimated mean change in the outcome response per year increase in mean parental age at childbirth (β), with associated standard error and 95% confidence interval P-values in bold are statistically significant at p<0.05.
Table 21: Results from multivariate regression models among participants of New Zealand European ethnicity

<table>
<thead>
<tr>
<th>Outcome response</th>
<th>β</th>
<th>Standard Error</th>
<th>Lower 95% confidence limit</th>
<th>Upper 95% confidence limit</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.089</td>
<td>0.046</td>
<td>-0.002</td>
<td>0.180</td>
<td>0.056</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>0.165</td>
<td>0.145</td>
<td>-0.126</td>
<td>0.457</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Glucose homeostasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin sensitivity (Matsuda index)</td>
<td>0.204</td>
<td>0.084</td>
<td>0.036</td>
<td>0.372</td>
<td>0.007</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-0.070</td>
<td>0.026</td>
<td>-0.122</td>
<td>-0.019</td>
<td>0.009</td>
</tr>
<tr>
<td>HOMA-β (%)</td>
<td>-1.397</td>
<td>0.919</td>
<td>-3.238</td>
<td>0.445</td>
<td>0.13</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>-0.256</td>
<td>0.095</td>
<td>-0.447</td>
<td>-0.066</td>
<td>0.009</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>-0.021</td>
<td>0.013</td>
<td>-0.047</td>
<td>0.005</td>
<td>0.11</td>
</tr>
<tr>
<td>Glucose 120 minutes (mmol/l)</td>
<td>-0.042</td>
<td>0.046</td>
<td>-0.135</td>
<td>0.050</td>
<td>0.36</td>
</tr>
<tr>
<td><strong>Cardiovascular parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daytime systolic blood pressure (mmHg)</td>
<td>-0.232</td>
<td>0.202</td>
<td>-0.638</td>
<td>0.175</td>
<td>0.26</td>
</tr>
<tr>
<td>Daytime diastolic blood pressure (mmHg)</td>
<td>-0.106</td>
<td>0.144</td>
<td>-0.394</td>
<td>0.183</td>
<td>0.46</td>
</tr>
<tr>
<td>Night time systolic blood pressure (mmHg)</td>
<td>-0.513</td>
<td>0.232</td>
<td>-0.978</td>
<td>-0.047</td>
<td>0.032</td>
</tr>
<tr>
<td>Night time diastolic blood pressure (mmHg)</td>
<td>-0.338</td>
<td>0.182</td>
<td>-0.704</td>
<td>0.028</td>
<td>0.070</td>
</tr>
<tr>
<td>Night time systolic blood pressure dip (%)</td>
<td>0.240</td>
<td>0.148</td>
<td>-0.058</td>
<td>0.537</td>
<td>0.11</td>
</tr>
<tr>
<td>Night time diastolic blood pressure dip (%)</td>
<td>0.377</td>
<td>0.178</td>
<td>0.020</td>
<td>0.734</td>
<td>0.039</td>
</tr>
<tr>
<td>Carotid intima-media thickness (mm)</td>
<td>-0.008</td>
<td>0.004</td>
<td>-0.015</td>
<td>-0.001</td>
<td>0.035</td>
</tr>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>-0.040</td>
<td>0.020</td>
<td>-0.081</td>
<td>0.001</td>
<td>0.054</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>-0.042</td>
<td>0.019</td>
<td>-0.080</td>
<td>-0.005</td>
<td>0.028</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.004</td>
<td>0.008</td>
<td>-0.013</td>
<td>0.020</td>
<td>0.65</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>-0.012</td>
<td>0.012</td>
<td>-0.036</td>
<td>0.011</td>
<td>0.30</td>
</tr>
</tbody>
</table>

n=64. Data are the estimated mean change in the outcome response per year increase in mean parental age at childbirth (β), with associated standard error and 95% confidence interval. P-values in bold are statistically significant at p<0.05.
17.4. Discussion

We showed in a cohort of overweight middle-aged men that increasing mean parental age at childbirth was associated with improved insulin sensitivity and a more favourable nocturnal BP profile. In addition, there was evidence of an association between increased MPAC and a subtle reduction in carotid artery intima-medial thickness (which may indicate a slower rate of atherosclerosis) and a more favourable lipid profile. Our study cohort is a group at moderately increased risk of insulin resistance, hypertension, dyslipidaemia and early atherosclerosis, and it appears that parental age could theoretically assist in the modulation of such risks. Importantly, the statistical models controlled for the effects of age, BMI, socioeconomic status, and physical activity, so that our findings suggest that parental age at childbirth may have long-term effects on the metabolic health of the offspring.

Our findings are particularly surprising, and somewhat in contrast with the existing literature. The recent EMAS position statement on late parenthood warns that advanced parental age may adversely impact on long-term offspring health. Increasing parental age has been associated with a number of adverse effects on offspring, particularly in the rate of genetic disease. Mintziori et al. listed numerous long-term adverse outcomes, such as increased rates of childhood asthma, leukaemia, autism, neurocognitive disorders, CNS tumours, leukaemia and type 1 diabetes in association with advanced maternal age, and schizophrenia, autism, bipolar disorder, and cancer in association with advanced paternal age. In contrast, our study suggests that the offspring of older parents may have a more favourable metabolic phenotype, indicative of a lower risk of T2DM and cardiovascular disease. Thus, we speculate that, if our findings are corroborated by other studies, increasing parental age at childbirth may help mitigate the metabolic effects of the high rates of overweight and obesity in western countries.

As maternal and paternal ages were highly correlated, it was not possible to determine whether just one or both were responsible for the more favourable metabolic phenotype with increasing parental age at childbirth. Nonetheless, in principle, the effect of both maternal and paternal ages could be mediated through epigenetic changes, or via effects on the fetal or postnatal environment.

Increased paternal age has been shown to be associated with long-term cognitive and psychiatric outcomes, including greater incidence of schizophrenia, autism, early-onset bipolar disorder, and reduced IQ and social functioning in adolescents. Confirmation of similar effects in rodents suggests that these effects are caused by age itself, rather than environmental factors. The mechanisms underpinning such paternal age effects are unknown, but they may be associated with epigenetics, i.e. those processes that alter gene expression without
changing the sequence of DNA base pairs, including DNA methylation, histone acetylation and non-coding RNAs passed from the sperm to oocyte at fertilisation\textsuperscript{998}. Indeed, the sperm of aging rats show hypermethylation of ribosomal DNA, suggesting a possible effect on cellular metabolism\textsuperscript{1008}. Another potential mechanism for paternal age to affect the metabolism of adult offspring is through increased telomere length. The sperm of older men have longer telomeres and these are inherited by the offspring\textsuperscript{1009}. Telomere length is thought to influence the changes associated with aging, and the rate of telomere attrition has been associated with insulin resistance\textsuperscript{1010}. However, whether insulin resistance causes telomere shortening or longer telomeres have a role in maintaining insulin sensitivity is unclear\textsuperscript{1010}.

Increasing maternal age is associated with changes in gonadotrophins\textsuperscript{1011} and sex steroids\textsuperscript{1012,1013}. Such maternal hormonal changes have been associated with alterations in post-natal growth\textsuperscript{1014} and metabolism\textsuperscript{1015} in offspring. Thus, it is possible that age-related changes in maternal hormones lead to programmed changes in offspring phenotype. In addition, increasing maternal age has also been associated with epigenetic changes\textsuperscript{1000} and altered gene expression\textsuperscript{1016} in oocytes, which could mediate long-term effects on offspring metabolism. Further, there is compelling evidence that environmental influences can result in highly specific epigenetic changes in the germline, which alter phenotype and persist through generations\textsuperscript{1017}. This evidence provides additional support for the hypothesis that the effects of parental age at childbirth on offspring metabolism could be mediated through epigenetic changes.

It is important to consider the possibility that the effect we have described may be due to differences in the post-natal environment. One major environmental factor that varies with parental age is socioeconomic status, as parents who delay fertility tend to be wealthier\textsuperscript{1018}. Socioeconomic status may affect factors such as nutrition\textsuperscript{1019}, physical activity\textsuperscript{1020}, and body composition\textsuperscript{1021}. Although we adjusted for current socioeconomic status in our analysis, we could not adjust for possible socioeconomic differences in childhood. Indeed, variation in the post-natal child-rearing environment across the socioeconomic spectrum does affect childhood growth and body composition\textsuperscript{1022}, leading to a taller and slimmer phenotype in those of higher socioeconomic status\textsuperscript{1022}. However, Stringhini et al. compared the effects of socioeconomic status in childhood and adulthood on the risk of chronic inflammation and T2DM, showing that the adult status was the more important determinant of risk\textsuperscript{521}. This suggests that the differences we have described in this study cannot be solely attributed to differences in socioeconomic status in childhood.

However, irrespective of current socioeconomic status, it is possible that older parents may have greater wisdom about the importance of lifestyle factors such as breastfeeding, healthy eating, and/or physical activity; thus, creating a healthier child-rearing environment. In support of this contention, older mothers are more likely to breastfeed\textsuperscript{1023}, and there is evidence that breastfeeding may be protective against obesity in childhood.
 Appendices

and beyond\textsuperscript{1024}. In addition, older parents are more likely to actively control their children’s diet, although this has not been shown to have an effect on BMI\textsuperscript{1025}. Further, while there is evidence that parental support increases physical activity levels in their children\textsuperscript{1026}, it is not clear if this support varies with parental age. Importantly, older parents may impart their knowledge regarding healthier lifestyles to their offspring, which could improve the offspring's adult environment. In our study, physical activity, energy and saturated fat intake did not vary with parental age, findings that are not suggestive of a persistent effect of parental age on lifestyle in adulthood. Nonetheless, it is plausible that the observed effects of parental age on adult metabolism could be mediated through differences in the child-rearing environment.

The major strength of this study is the detailed metabolic assessment. However, our study has limitations, including the \textit{post hoc} analysis. Because maternal and paternal ages were highly correlated, it was not possible to tease out the individual effects of either variable on study outcomes. Future studies in larger cohorts with parents of more discrepant ages would likely enable isolation of these effects. In addition, our study involved a relatively small sample size of 73 individuals, so that it is not possible to ascertain whether non-significant associations actually did not exist or were undetectable due to a lack of sufficient statistical power. Lastly, we studied a relatively narrow range of individuals (overweight males living in a large urban centre, mostly of New Zealand European ethnicity), which may limit wider applicability of our findings, especially to females.

In conclusion, increasing parental age at childbirth was associated with a more favourable metabolic profile in overweight middle-aged men. Future studies on the effects of parental age at childbirth on the metabolism of adult males and females across the BMI range are required.
Appendices

Chapter 18. Non-dipping and cardiometabolic profile: a study on normotensive overweight middle-aged men

Preface

This chapter contains a modified version of a manuscript published in the journal *Heart, Lung and Circulation*.

- **Authors:** Benjamin B. Albert, Martin de Bock, José GB. Derraik, Christine M. Brennan, Janene B. Biggs, Paul L. Hofman, Wayne S. Cutfield
- **Journal:** American Journal of Human Biology
- **Year of Publication:** 2016 epub ahead of print
- **Impact Factor when published:** 1.44
18.1. Introduction

The role of hypertension in the development of atherosclerotic cardiovascular disease has long been recognised. The advent of 24-hour ambulatory BP monitoring has allowed the assessment of the circadian profile and, in particular, the nocturnal BP and the changes that occur during sleep.

Nocturnal hypertension is associated with greater arterial stiffness, carotid artery intima-media thickness, and urinary albumin excretion, as well as excessive inotropic response to exercise. These translate to greater risk of cardiovascular events and mortality in association with increased nocturnal BP in population cohorts, participants with hypertension, and even those with isolated nocturnal hypertension. Importantly, the nocturnal BP is more strongly predictive of cardiovascular events than daytime BP.

A related measure, the magnitude of the nocturnal decline of BP (i.e. dipping) is also an important cardiovascular risk factor, which is independent of the average BP. Non-dippers (whose BP does not decrease by 10% or more at night) who are hypertensive have greater cardiovascular mortality, greater prevalence of left ventricular hypertrophy, and increased carotid intima-media thickening. Further, non-dipping and nocturnal hypertension have an additive effect on cardiovascular risk and end-organ damage. Importantly, even in normotensive individuals, a non-dipping pattern is associated with greater urinary albumin excretion, elevated left ventricular mass index, and increased rate of cardiovascular events.

Essential hypertension and other aspects of the metabolic syndrome share a common cause: pathological reduction of insulin sensitivity (insulin resistance) with hyperinsulinaemia. However, while there is evidence to suggest that the non-dipping pattern is associated with dysfunction of the autonomic nervous system, the relationship between insulin sensitivity and dipping remains unclear. Thus, we aimed to assess insulin sensitivity and other metabolic features of dippers and non-dippers in a cohort of overweight middle-aged men.
Appendices

18.2. Methods

This methods section is abbreviated, additional details are reported in Chapter 9.3.

Participant & Recruitment

Participants were recruited for two clinical trials investigating the metabolic effects of nutritional supplementation with olive leaf extract or krill oil in overweight middle-aged men. This study encompasses a post hoc analysis of their pre-trial baseline data. Exclusion criteria included obstructive sleep apnoea, diabetes mellitus, pre-diagnosed hypertension or elevated clinic BP at the time of recruitment (systolic BP >145 mmHg or diastolic BP >95 mmHg), known dyslipidaemia, tobacco use, or intake of medication likely to affect BP, lipid profile, or insulin sensitivity. Note that a relatively high 'clinic' BP cut-off was used as the clinical trials aimed to enrol participants with mild metabolic dysfunction. Five subjects participated in both trials, and only the data from the most recent trial were used (Figure 19).

Assessments

24-hour ambulatory BP monitoring was performed before the clinical assessment. Participants were fitted with a Spacelabs 90207 or 90217 (Spacelabs Medical Inc., Redmond, USA). Measurements were performed every 20 minutes between 07:00 and 22:00, and every 30 minutes from 22:00 to 07:00. Only profiles with >14 daytime and >7 night time readings over a 24-hour period were analysed. Nocturnal BP dipping was defined as a reduction ≥10% in mean systolic or diastolic BP during sleep (recorded between 22:00 and 06:00) compared with the mean daytime systolic or diastolic BP. Based on this definition, participants were separated into 'dippers' and 'non-dippers'. Note that participants were aware that 22:00 was the predefined sleep period and were encouraged to retire by that time. In addition, they were asked about their sleep, and where it had been unusually disturbed the BP profile was repeated.

Insulin sensitivity was measured using an oral glucose tolerance test (Matsuda method). Additional assessments and outcomes included lipid profile, weight, BMI, fat mass index, body composition (DXA), 24-hour ambulatory BP monitoring, cIMT, physical activity (IPAQ), socioeconomic status (NZDep2006) and an estimation of risk for obstructive sleep apnoea (Berlin questionnaire).
Statistical Analysis

Demographic characteristics and prevalence of hypertension in dippers and non-dippers were compared using one-way ANOVA or Fisher's exact tests in Minitab (v.16, Pennsylvania State University, State College, PA, USA). Multivariable linear regression models were carried out in SAS v.9.3 (SAS Institute, Cary, NC, USA). All models accounted for important confounders (factors likely to affect metabolic outcomes of interest based on published evidence), namely age, socioeconomic status, physical activity levels, birth order, and total body fat percentage. Height was also included as a covariate when comparing BP outcomes. Where necessary, outcomes were log-transformed to approximate a normal distribution. Statistical tests were two-tailed and significance level maintained at 5%. Demographic data are presented as means ± standard deviations, while other data are means and 95% confidence intervals adjusted for confounders in multivariable models.

Power calculations

Post-hoc power calculations were performed for the 73 participants studied, with a ratio of 2.32 subjects between groups. Based on an observed standard deviation of 0.61 for the log-transformed Matsuda index, this study was powered to detected a 25% difference in means between groups, with 80% power and \( \alpha = 0.05 \). With the same power and \( \alpha \), and an observed standard deviation of 0.14, the study was also able to detect a 13% difference in CIMT. It is important to note however, that our models controlling for important confounding factors increased our study's power to detect statistically significant differences between groups.

18.3. Results

Study participants

There were 97 subjects in both trials, but since 5 men took part in both studies, there were 92 individuals enrolled (Figure 19). Nine participants on anti-hypertensive or lipid-lowering medication and one man subsequently diagnosed with obstructive sleep apnoea were excluded from this study; 9 of the remaining 82 participants had insufficient 24-hour BP readings (Figure 19). We consequently studied a total of 73 men (Figure 19), who were aged 45.8 ± 5.3 years and with BMI of 27.4 ± 1.8 kg/m\(^2\). Most participants were of European descent (86%).
Appendices

Figure 19: Summary of study recruitment.

All participants from the olive leaf extract<sup>332</sup> and krill oil<sup>863</sup> trials were overweight middle-aged men recruited in Auckland, New Zealand.

Across the cohort 9 participants (12%) had systolic and/or diastolic hypertension during the daytime (>138.2/86.4 mmHg)<sup>1057</sup>, so that most were normotensive during the day. There were 51 dippers and 22 non-dippers. In the latter group, all participants were systolic non-dippers, while 13 men were both systolic and diastolic non-dippers. Non-dippers were on average 3.3 years older than dippers (p=0.013). However, there were no differences in anthropometry, ethnic composition, socioeconomic status, and physical activity levels (Table 22). In addition, the proportion of participants at high risk of obstructive sleep apnoea was not different in the two groups (Table 22).

**Blood pressure**

Nocturnal systolic and diastolic BP dippings were on average 14.1 and 19.9% (respectively) among dippers, but 5.3 and 9.2% among non-dippers. There were considerable cardiovascular differences between the two groups (Table 23). Non-dippers had lower daytime systolic (-5.0 mmHg; p=0.022) and diastolic (-3.3 mmHg; p=0.035) BP than dippers (Table 23). Conversely, during sleep, non-dippers had higher systolic (+6.5 mmHg; p=0.003) and diastolic (+5.6 mmHg; p=0.001) BP (Table 23). Non-dippers tended to have a lesser reduction in heart rate during sleep compared to dippers (8.0 vs 10.9%; p=0.066) and had a greater rate of nocturnal diastolic hypertension (Table 23).
Table 22: Demographic data among dippers and non-dippers in a cohort of middle-aged overweight men recruited in Auckland, New Zealand

<table>
<thead>
<tr>
<th></th>
<th>Dippers</th>
<th>Non-dippers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>51</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.8 ± 5.4</td>
<td>48.1 ± 4.3</td>
<td>0.013</td>
</tr>
<tr>
<td>Socioeconomic status (NZDep2006)</td>
<td>4.0 ± 2.3</td>
<td>3.9 ± 2.3</td>
<td>0.97</td>
</tr>
<tr>
<td>Physical activity levels (MET-minutes per week)</td>
<td>2880 [3003]</td>
<td>2933 [6458]</td>
<td>0.68</td>
</tr>
<tr>
<td>Ethnicity (New Zealand European)</td>
<td>88%</td>
<td>82%</td>
<td>0.48</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>179.3 ± 6.5</td>
<td>178.4 ± 6.7</td>
<td>0.59</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>88.1 ± 9.4</td>
<td>87.4 ± 10.7</td>
<td>0.73</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3 ± 1.7</td>
<td>27.4 ± 2.0</td>
<td>0.64</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>28.1 ± 5.5</td>
<td>28.9 ± 6.7</td>
<td>0.60</td>
</tr>
<tr>
<td>Android fat to gynoid fat ratio</td>
<td>1.27 ± 0.19</td>
<td>1.31 ± 0.19</td>
<td>0.36</td>
</tr>
<tr>
<td>High risk of obstructive sleep apnoea</td>
<td>12%</td>
<td>21%</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Where appropriate, data are means ± standard deviations, except for physical activity levels where the medians and interquartile ranges are provided. Risk of obstructive sleep apnoea was assessed using the Berlin Questionnaire\textsuperscript{1056}. P-value statistically significant at p<0.05 is shown in bold.

**Metabolic parameters**

There were no observed differences in glucose homeostasis between groups (Table 23). There were also no differences in lipid profile or hsCRP concentrations (Table 23).

Pearson’s correlation coefficients showed no associations between insulin sensitivity and BP dipping (Table 24). However, insulin sensitivity was negatively correlated with daytime diastolic (r=-0.26; p=0.025), nocturnal systolic (r=-0.25; p=0.034), and nocturnal diastolic (r=-0.31; p=0.007) BPs (Table 24). Insulin sensitivity was also correlated with mean arterial pressure in both the daytime and night time (Table 24).

There were no associations between triglyceride and hsCRP levels with the main outcomes of interest, in particular CIMT or the magnitude of nocturnal dipping.
Non-dippers had CIMT that was 9% greater (approximately 72 μm thicker) than dippers (p=0.036; Table 23). Note that age was the strongest predictor of CIMT (β=0.011; p=0.0005), and its exclusion from the multivariable model would increase the difference between groups to 99 μm (p=0.007).

Daytime BPs were not associated with CIMT (Table 24). However, both systolic (r=-0.32; p=0.005; Figure 20) and diastolic (r=-0.26; p=0.025) dippings were negatively correlated with CIMT, while a positive association was observed for nocturnal systolic BP (r=0.24; p=0.038). These patterns were corroborated by multivariable models showing greater CIMT to be associated with lower systolic (β=-0.007; p=0.012) and diastolic (β=-0.004; p=0.042) dippings, but with increased nocturnal systolic BP (p=0.042).
Table 23: Study outcomes among dippers and non-dippers in a cohort of middle-aged overweight men.

<table>
<thead>
<tr>
<th></th>
<th>Dippers</th>
<th>Non-dippers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>51</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>24-hour ambulatory blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daytime systolic (mmHg)</td>
<td>127.6 (124.2–131.1)</td>
<td>122.6 (118.5–126.8)</td>
<td>0.022</td>
</tr>
<tr>
<td>Daytime diastolic (mmHg)</td>
<td>81.0 (78.5–83.4)</td>
<td>77.7 (75.0–80.7)</td>
<td>0.035</td>
</tr>
<tr>
<td>Nocturnal systolic (mmHg)</td>
<td>109.7 (106.4–113.0)</td>
<td>116.2 (112.1–120.4)</td>
<td>0.003</td>
</tr>
<tr>
<td>Nocturnal diastolic (mmHg)</td>
<td>64.7 (62.1–67.2)</td>
<td>70.3 (67.1–73.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>24-hour mean systolic (mmHg)</td>
<td>123.3 (120.0–126.7)</td>
<td>121.1 (117.1–125.1)</td>
<td>0.29</td>
</tr>
<tr>
<td>24-hour mean diastolic (mmHg)</td>
<td>76.7 (74.4–79.1)</td>
<td>75.6 (72.8–78.4)</td>
<td>0.44</td>
</tr>
<tr>
<td>Daytime systolic hypertension</td>
<td>4 (8%)</td>
<td>0</td>
<td>0.31</td>
</tr>
<tr>
<td>Daytime diastolic hypertension</td>
<td>7 (14%)</td>
<td>1 (5%)</td>
<td>0.42</td>
</tr>
<tr>
<td>Nocturnal systolic hypertension</td>
<td>6 (12%)</td>
<td>5 (23%)</td>
<td>0.24</td>
</tr>
<tr>
<td>Nocturnal diastolic hypertension</td>
<td>6 (12%)</td>
<td>10 (45%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Carotid intima-media thickness (µm)</td>
<td>749 (697–800)</td>
<td>820 (757–884)</td>
<td>0.036</td>
</tr>
<tr>
<td>Glucose homeostasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin sensitivity (Matsuda index)</td>
<td>5.85 (4.76–7.19)</td>
<td>5.45 (4.23–7.04)</td>
<td>0.60</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>5.55 (4.59–6.71)</td>
<td>5.66 (4.48–7.15)</td>
<td>0.88</td>
</tr>
<tr>
<td>Lipid profile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.88 (4.53–5.24)</td>
<td>4.91 (4.49–5.34)</td>
<td>0.81</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.29 (2.97–3.61)</td>
<td>3.27 (2.88–3.66)</td>
<td>0.90</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.06 (0.93–1.19)</td>
<td>1.12 (0.96–1.28)</td>
<td>0.47</td>
</tr>
<tr>
<td>Total cholesterol : HDL-C</td>
<td>4.65 (4.18–5.19)</td>
<td>4.39 (3.85–5.02)</td>
<td>0.42</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.09 (0.94–1.26)</td>
<td>0.99 (0.83–1.19)</td>
<td>0.33</td>
</tr>
<tr>
<td>Inflammatory marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highly-sensitive CRP (mg/l)</td>
<td>1.05 (0.71–1.55)</td>
<td>1.12 (0.69–1.80)</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Data are means and 95% confidence intervals adjusted for other confounding factors in the multivariable models, except for hypertension data that are numbers of participants and percentages in parentheses. Cut-offs for the diagnosis of hypertension were systolic >138.2 mmHg and diastolic >86.4 mmHg during the daytime, and systolic >119.5 mmHg and >70.8 mmHg at night [45]. P-values statistically significant at p<0.05 are shown in bold.
Table 24: Pearson's correlation coefficients for the associations between ambulatory blood pressure parameters with insulin sensitivity (Matsuda index) and carotid intima-media thickness.

<table>
<thead>
<tr>
<th></th>
<th>Matsuda index</th>
<th>Carotid intima-media thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p-value</td>
</tr>
<tr>
<td><strong>Daytime</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>-0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>Diastolic</td>
<td>-0.26</td>
<td><strong>0.025</strong></td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>-0.23</td>
<td><strong>0.049</strong></td>
</tr>
<tr>
<td><strong>Night time</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>-0.25</td>
<td><strong>0.034</strong></td>
</tr>
<tr>
<td>Diastolic</td>
<td>-0.31</td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>-0.31</td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td><strong>Dipping</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>Diastolic</td>
<td>0.13</td>
<td>0.28</td>
</tr>
</tbody>
</table>

P-values statistically significant at $p<0.05$ are shown in bold. Note that correlations are reported for the log-transformed Matsuda index.

Figure 20: The association between systolic blood pressure dipping and carotid intima-media thickness
Discussion

18.4. Discussion

This study showed in a cohort of predominantly normotensive overweight males, non-dippers had lower BP in the daytime, but higher BP in the night time compared to dippers. Despite these differences, non-dippers and dippers had similar average BP and rates of hypertension during the day. Importantly, we observed no association between BP dipping and insulin sensitivity triglycerides, or hsCRP. However, non-dippers had CIMT that was 9% greater, which is consistent with the increased cardiovascular risk reported in non-dippers and in association with higher nocturnal BP\textsuperscript{1033}. Notably, these findings were observed despite adjustment for the important confounders known to influence BP, such as age, height, total body fat percentage, ethnicity, and physical activity levels. Further, there were no differences in socioeconomic status between groups.

The differences in BP profile observed between dippers and non-dippers are consistent with previous studies of normotensive individuals\textsuperscript{858,1046} and some studies of hypertensive subjects\textsuperscript{858,1043,1058}. However, one investigation on hypertensive participants has shown increased daytime BP in non-dippers\textsuperscript{1059}. Nonetheless, it appears that the presence or absence of the dynamic dipping pattern is independent of average BP.

Insulin resistance and hyperinsulinaemia (to which obesity and inactivity are major contributors) have a causative role in the pathogenesis of essential hypertension\textsuperscript{29,1050,1051}. This occurs through three major effects: 1) increased sympathetic activation\textsuperscript{251,252}, 2) increased sodium retention\textsuperscript{91}, and 3) impaired insulin signalling in endothelial cells. The latter leads to reduced nitric oxide synthesis and subsequent vasoconstriction\textsuperscript{249}. Consistent with this, across our entire cohort, lower insulin sensitivity correlated with higher systolic and diastolic BPs, but was not associated with dipping. In addition a range of other factors linked to metabolic health did not differ between dippers and non-dippers, including body composition, physical activity, insulin sensitivity, triglycerides, and hsCRP. This suggests that within overweight middle-aged men, insulin sensitivity does not appear to be an important factor affecting nocturnal BP dipping.

However, the reported relationship between insulin sensitivity and dipping is conflicting\textsuperscript{1044,1060-1067}. BP dipping is dependent on cardiac innervation\textsuperscript{1068}, and non-dippers have been shown to have dysfunction of the autonomic nervous system\textsuperscript{1029,1052-1054}. Consistent with autonomic dysfunction, we observed a trend to a lesser fall in nocturnal pulse rate in non-dippers. It is plausible that insulin resistance could contribute to a reduced nocturnal decline in BP and heart rate, by increasing sympathetic activity\textsuperscript{251,252}.
Discussion

The relationship between dipping and insulin sensitivity has revealed conflicting results depending upon the characteristics of the group studied, and is complicated by methodological flaws. In the context of more severe insulin resistance there is evidence for an association. However, in studies of subjects who are only overweight or hypertensive (similar to our participants), there are inconsistent reports of this relationship. In agreement with some studies, we report a lack of association between insulin sensitivity and nocturnal dipping whereas others found non-dippers had poorer insulin sensitivity.

Importantly, we used the Matsuda Index, a more accurate measure of insulin sensitivity than HOMA-IR which was used in these previous studies. Whilst Björklund et al. used the gold standard hyperinsulinaemic euglycaemic clamp technique to directly measure insulin sensitivity in elderly hypertensives, the study was flawed by an incorrect definition of nocturnal dipping.

In more insulin resistant groups such as those with non-alcoholic fatty liver disease (NAFLD) and T2DM, an association is more clearly seen between reduced insulin sensitivity and nocturnal non-dipping. NAFLD subjects have severe insulin resistance and hyperinsulinaemia, and frequently have autonomic dysfunction. Notably, among subjects with NAFLD and in a group with a high prevalence of NAFLD, insulin sensitivity was associated with non-dipping. Further, non-dipping appears to be more common in those with T2DM, which is characterised by severe insulin resistance and hyperglycaemia. However in type 2 diabetics, the relative contributions of insulin resistance and hyperglycaemia are unclear, as hyperglycaemia even without significant insulin resistance (e.g. in type 1 diabetes mellitus) leads to autonomic dysfunction. Thus, we speculate that while the association between non-dipping and insulin sensitivity is weak or absent in patient groups where insulin resistance is mild, there may be a stronger association in more severely insulin resistant groups, probably mediated through hyperinsulinaemia and its effects on sympathetic activity.

In our predominantly normotensive cohort, non-dippers had increased CIMT, which is consistent with previous evidence in hypertensive non-dippers. In contrast, a previous study of normotensive individuals in Japan showed no difference in CIMT between dippers and non-dippers. However, they studied a rural and relatively elderly Japanese population, compared to our urban, overweight, middle-aged, male, and predominantly European participants. Importantly, while greater nocturnal BP was associated with increased CIMT, daytime BPs were not, underscoring the importance of the nocturnal decline in BP. In addition, we observed a continuous association between decreasing nocturnal BP dipping and increasing CIMT. As CIMT is a marker of atherosclerosis and an independent predictor of vascular events across the adult age range, our findings suggest that overweight but otherwise healthy non-dippers may have a greater rate of atherosclerosis and be at increased cardiovascular risk.
Discussion

Perhaps surprisingly, despite increased CIMT in non-dippers, there were no differences in lipid profile between groups. This suggests that within our cohort the difference in CIMT between groups was not mediated through dyslipidaemia. Nonetheless, a larger study in non-dippers who were hypertensive did find features suggestive of a more atherogenic lipid profile\(^{1071}\). Thus, it is unclear whether the lack of an association between CIMT and lipid profile in our study was a result of inadequate statistical power or because this relationship does not exist amongst normotensive non-dippers.

It is important to consider the reproducibility of the dipping BP profile. Ambulatory BP profiles have been shown to be reliable\(^{1073-1076}\) and more reproducible than clinic BP\(^{1074,1075}\). However, although dipping status has been described as reliable\(^{1077}\), there is evidence that 40% of people will change their dipping status during a second 24-hour ambulatory BP measurement\(^{1058,1078}\). Further, the definition of dipping based on a 10% nocturnal BP decline is arbitrary\(^{1053}\). Thus, during re-test, a small change in the nocturnal decline could change an individual’s classification, although there is no compelling reason to believe that for example a 9% dip should carry a substantially greater risk than an 11% dip. Our data support the arbitrary nature of dipping status, as there was a continuous negative association between the nocturnal reduction in BP and CIMT without a threshold (Figure 20). Further, this continuous relationship corroborates our stratified analysis, suggesting that the difference in CIMT we have demonstrated between dippers and non-dippers is not simply an artefact of our group classification.

The major strengths of this study are the detailed metabolic assessment, which used a robust method to measure insulin sensitivity in comparison to other studies that have used only fasting insulin and glucose (e.g. HOMA-IR), and the statistical models that adjusted for important confounders (such as height, body fat, and physical activity levels). In particular, our analysis controlled for the small difference in age between dippers and non-dippers, as CIMT has been shown to increase with age\(^{1079}\). However, our study has limitations, including the post hoc analysis. Our sample size was small with a relatively low number of non-dippers, and relied on a single 24-hour BP profile. In addition, we defined night time using specific time points rather than sleep diaries, so that our nocturnal recordings could have included measurements taken when the participant was still awake and active. We also studied a relatively narrow range of individuals (overweight males living in a large urban centre, mostly of New Zealand European ethnicity), which may limit wider applicability of our findings, especially to females and those who are obese (BMI ≥30 kg/m\(^2\)) or of non-European ethnicity. Lastly, as this was a cross-sectional study, the actual rate of atherosclerosis could not be assessed, and it is not possible to determine causation.

Nonetheless, this study in overweight normotensive men adds to the evidence that amongst subjects expected to have only mild metabolic dysfunction, the non-dipping BP pattern is independent of insulin sensitivity.
Discussion

However, as previous studies in similar groups are conflicting and most have used HOMA-IR (a surrogate index solely derived from fasting values), further studies using more accurate measures of insulin sensitivity are required to better characterise this association. In addition, our study highlights the importance of the nocturnal decline in BP. Even in the context of a normal average BP, non-dipping status was associated with increased CIMT in overweight middle-aged men. Thus, non-dipping, which is known to be associated with increased cardiovascular risk in normotensive men, men may be associated with a greater rate of atherosclerosis.
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