CURCUMIN AND LONG-CHAIN OMEGA-3 POLYUNSATURATED FATTY ACIDS: EFFECTS ON GLYCAEMIC CONTROL AND BLOOD LIPIDS

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Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give my consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provision of the Copyright Act 1968.

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I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of my thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

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Mr Rohith N Thota

13/04/18

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List of research publications included in the thesis

1. Chapter 3

Thota RN, Abbott KA, Ferguson JJA, Veysey M, Lucock M, Niblett S, King K, Garg ML. InsuTAG®: A novel physiologically relevant predictor for insulin resistance and metabolic syndrome. Scientific Reports 2017;7 (1):15204.

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2. Chapter 4

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3. Chapter 5

Thota RN, Acharya SH, Abbott KA, Garg ML. Curcumin and long-chain Omega-3 polyunsaturated fatty acids for Prevention of type 2 diabetes (COP-D): study protocol for a randomised controlled trial. Trials 2016; 17:565.

4. Chapter 6

Thota RN, Acharya SH, Garg ML. Effects of curcumin and/or omega-3 polyunsaturated fatty acids on glycaemic control and blood lipids in individuals with high risk of type 2 diabetes: A randomised controlled trial. Manuscript Submitted MAR 2018.

Other co-authored publications

1. Jameel F**, Thota RN,** Wood LG, Plunkett B, Garg ML. Sex-dependent association between circulating irisin levels and insulin resistance in healthy adults. Journal of Nutrition & Intermediary Metabolism 2015; 2(3–4):86-92.
2. Abbott KA, Burrows TL, **Thota RN**, Acharya S, Garg ML. Do omega-3 PUFAs affect insulin resistance in a sex-specific manner? A systematic review and meta-analysis of randomized controlled trials. American Journal of Clinical Nutrition 2016; 104 (5):1470-84.
3. **Thota RN**, Ferguson JJA, Abbott KA, Dias CB, Garg ML. Science behind the health benefits of omega-3 polyunsaturated fatty acids: biochemical effects vs clinical outcomes. Food and Function 2018; doi:10.1039/c8fo00348c.

Conference presentations

1. Oral presentation on "Sex-dependent relationship between n-3 long-chain polyunsaturated fatty acids and insulin resistance: A Systematic review and Meta-analysis" at Joint Annual Scientific Meeting of the Nutrition Society of NZ and the Nutrition Society of Australia. 1-4 December 2015; Wellington, New Zealand.
2. Oral Presentation on "InsuTAG: A novel and physiologically relevant predictor of insulin resistance and metabolic syndrome" at Asia Pacific Conference on Clinical Nutrition. 26-29 November 2017; Adelaide, Australia.

Scholarships

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Awards and media appearances

1. Best theme paper 2017; Clinical and Experimental Nutrition; by Priority Research Centre for Physical Activity and Nutrition
2. Innovation award 2017 – PhD student category; by Priority Research Centre for Physical Activity and Nutrition
3. Publication of the month OCT 2017 – School of Biomedical Sciences and Pharmacy

Media activities

1. Turmeric + fish oil could prevent type 2 diabetes – ABC NEWS 15 JUL 2015
2. Trial tests the healing properties of turmeric – Newcastle Herald 15 JUL 2015
3. Australian scientists to test Indian spice in type 2 diabetes prevention – China.org.au 17 JUL 2015
4. Australian study investigating Indian spice as type 2 diabetes treatment – Diabetes UK 17 JUL 2015
5. Turmeric may help treat diabetes – Press Club of India – 18 JUL 2015
6. Turmeric and Omega-3 could prevent type 2 diabetes – Saturn Herald 29 JUL 2015
7. Invited to present research during Hunter Science Festival, Science Week – AUG 2015
8. Radio Interview – 'Reducing the risk factors for type 2 diabetes' 2NURFM – 24 NOV 2015 (https://www.youtube.com/watch?v=zVu-uB29Dc8)
9. Radio Interview – Diabetes survey – 2NURFM – 4 JUL 2016 (https://www.youtube.com/watch?v=xzlNLrB0WFo)
10. Radio Interview – Curcumin and Omega-3 fatty acids for management of heart disease risk in type 2 diabetes - 2NURFM – 3 MAR 2017
11. Radio Interview – Curcumin and Omega-3 fatty acids for management of heart disease risk in individuals with type 2 diabetes - ABC Newcastle Drive – 19 OCT 2016

Table of contents

List of figures

List of tables

List of appendices

Abbreviations

ABS	Australian Bureau of Statistics
AE	Adverse events
AIHW	Australian Institute of Health and Welfare
AIP	Atherogenic index of plasma
ALT	Alanine transaminase
AMPK	5' adenosine monophosphate-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ANZCTR	Australian New Zealand Clinical Trials Registry
AST	Aspartate transaminase
ATF2	Activating transcription factor 2
AUSDRISK	The Australian Type 2 Diabetes Risk Assessment Tool
BFM	Body fat mass
BMI	Body mass index
CALFOR-CVD	Curcumin And Long-chain omega-3 polyunsaturated fatty acids for management of CardioVascular health in type 2 Diabetes.
CC	Curcumin alone
CC-FO	Curcumin plus fish oil
CCL-2	CC-chemokine ligand 2
COP-D	Curcumin and/or Omega-3 polyunsaturated fatty acids for Prevention of type 2 Diabetes
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	Docosahexanoic acid
DPP-4	Dipeptidyl peptidase 4
EDTA	ethylenediaminetetraacetic acid

ELK-1	E26 transformation specific containing domain protein 1
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FFA	Free fatty acids
FO	Fish oil alone
FPG	Fasting plasma glucose
GIP	Glucose-dependent insulinotropic polypeptide
GISSI-prevenzione	Gruppo Italiano per lo Studio della Sopravvivenza nell'infarto
GLP-1	Glucagon like peptide-1
GPR	G-protein coupled receptors
HbA1c	Glycosylated haemoglobin
HDL-C	High density lipoprotein cholesterol
HEC	Hyperinsulinemic euglycemic clamp
HMRI	Hunter medical research institute
HNEHREC	Hunter New England Human Research Ethics Committee
HOMA	Homeostatic model assessment
HOMA	Homeostatic model assessment
HOMA-IR	Homeostatic model assessment of IR
ICML	Intramyocellular lipids
IDF	International diabetes federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IKK	Iκb kinase β
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IQR	Interquartile range
IR	Insulin resistance
IRS-1	Insulin receptor substrate-1
IS	Insulin sensitivity

JELIS	The Japan Eicosapentaenoic acid Lipid Intervention Study
JNK	JUN N-terminal kinase
LCn-3PUFA	Long-chain omega-3 polyunsaturated fatty acids
LDL-C	Low density lipoprotein cholesterol
LXRα	Liver X receptor alpha
MCP-1	Macrophage chemoattractant protein-1
MDA	Malondialdehyde
MetS	Metabolic syndrome
MM	Muscle mass
NF-κB	Nuclear factor kappa B
NLRP3	NOD, LRR and pyrin domain–containing 3 inflammasome
OGTT	Oral glucose tolerance test
PBF	Percent body fat
PBG	Postprandial blood glucose
PI	Postprandial insulin
PL	Double placebo
PPAR-α	Peroxisome proliferator-activated receptor alpha sub unit
RHLS	Retirement Health and Lifestyle Study
ROS	Reactive oxygen species
SAE	Serious adverse event
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of mean
SIRT1	Sirtuin1
SOSC3	Suppressor of cytokine signalling 3
STAT3	Signal transducer and activator of transcription 3
SREBP-1c	Sterol regulatory element-binding protein 1
T2D	Type 2 diabetes

TC	Total cholesterol
TG	Triglycerides
TLR4	Toll like receptor-4
TNF-α	Tumor necrosis factor - alpha
UoNHREC	University of Newcastle Human Research Ethics Committee
UPR	Unfolded protein response
VLDL-C	Very low density lipoprotein cholesterol
WC	Waist circumference
WHR	Waist – hip ratio

Synopsis

Type 2 diabetes (T2D) is the most common chronic metabolic disorder resulting from either deficit of insulin secretion and/or action. The transition of normal glucose tolerance to T2D is usually accompanied by a cluster of metabolic risk factors such as low-grade inflammation, oxidative stress, insulin resistance (IR) and dyslipidaemia. IR is one of the marked independent predictors among these cluster of metabolic abnormalities that mediates the transition in high risk states such as obesity, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) to overt T2D. IR also is often associated with decreased clearance of lipids and lipoprotein abnormalities, together representing a greater risk of cardiovascular disease (CVD) in both high risk and individuals with T2D. Several studies have employed lipid ratios, homeostatic models, and anthropometric measures as surrogate markers for predicting IR. However, none of these accounted for both insulin and lipid availability in a single model to predict IR or metabolic syndrome (MetS).

Therefore, the first aim of my PhD project, presented in the chapter 3, was to develop a novel marker for IR and MetS that accounts for both insulin and lipid availability in a single model. We proposed and evaluated a novel physiologically relevant marker, InsuTAG (product of fasting insulin and fasting triglycerides) as a predictor of IR and MetS. Cross-sectional analysis of data from the Retirement Health and Life-style Study (RHLS, n=618) showed that InsuTAG is a strong predictor of IR over existing lipid based surrogate markers and anthropometric measures. Receiver operating curve analysis indicated InsuTAG (93%) as the favourable marker for IR over other lipid based surrogate markers and anthropometry measures. Prevalence of MetS was significantly higher in individuals with InsuTAG values above the optimal cut-off value of 11.2. InsuTAG exhibited a greater area under than curve than HOMA-IR for identifying MetS. Together these observations indicate the potential of InsuTAG for predicting IR and MetS.

Despite effective lifestyle and pharmacological interventions, the prevalence of T2D is growing at an alarming rate in Australia, in line with global prevalence. Failure of long term compliance to these interventions is a major barrier for their effectiveness in halting the transition to T2D in high risk state individuals, indicating a necessity for alternative effective approach.

Given the fact that pathogenesis of T2D is chronic, complex and often involving multiple pathological pathways, use of well tolerated dietary bio-active compounds appears to be a potential strategy for delaying the onset of T2D. Several pre-clinical and *in-vitro* studies have reported the ability of dietary bio-actives to down regulate multiple pathological mechanisms (chronic low-grade inflammation, IR, oxidative stress and β-cell dysfunction) that are involved in the pathogenesis of T2D. We hypothesised that a combination of two lipid-lowering and anti-inflammatory dietary bio-active compounds, curcumin and long-chain omega-3 polyunsaturated fatty acids (LCn-3PUFA), could potentially act in multiple pathways to improve the glycaemic control in individuals at high risk of developing T2D.

My second aim, presented in chapter 4, was to evaluate the acute effects of curcumin and/or LCn-3PUFA on glycaemic responses. Therefore, in a randomised, cross over trial we investigated the postprandial glucose and insulin response to a single dose of curcumin and/or LCn-3PUFA in healthy individuals. The glucose levels were reduced by curcumin at as early as 30 min, and the maximum effect was observed at 60 min post meal consumption. Curcumin was found to be effective for lowering the insulin demand to control postprandial glucose levels. Similar results were observed following dietary supplementation with curcumin plus LCn-3PUFA. It was apparent that the postprandial effects on glycaemic control were primarily due to curcumin even in the combined treatment group. Thus, providing basis for long-term supplementation study with curcumin for glycaemic control.

In chapter 5, a detailed study protocol for 2x2 factorial placebo controlled, double blinded randomised trial with long term (12 weeks) curcumin and LCn-3PUFA supplementation (COP-D trial) was presented. In chapter 6, we examined the effects of curcumin with or without LCn-3PUFA on glycaemic control and blood lipid levels in people at high risk of T2D. 12 weeks of supplementation with curcumin has effectively reduced the fasting insulin levels and IR in individuals with high risk of T2D. Parallel to these results, both curcumin and LCn-3PUFA were able to reduce the fasting triglycerides and atherogenic index of plasma, however the magnitude of reduction was greater with LCn-3PUFA supplementation. InsuTAG levels were also reduced with curcumin and LCn-3PUFA supplementation. However, this study failed to show any complimentary effects with concurrent administration of curcumin and LCn-3PUFA. Though IR and fasting triglycerides, were effectively reduced by these two bio-actives, we did not find any

beneficial effects of curcumin and LCn-3PUFA supplementation on fasting glucose and glycosylated haemoglobin levels.

In chapter 7, we designed a study to target commonly prevalent dyslipidaemia with curcumin and/or LCn-3PUFA in individuals with T2D (CALFOR-CVD trial). Participants were randomised to either placebo or curcumin or LCn-3PUFA, or curcumin plus LCn-3PUFA for six weeks. This pilot study has demonstrated that supplementation of curcumin can effectively reduce the TG. Contrasting to the results from chapter 6, magnitude of reduction in triglycerides in this study was higher with curcumin than LCn-3PUFA. Preliminary observations also presented a non-significant, but a noteworthy reduction of 0.5 mmol/L in total cholesterol and LDL-Cholesterol with curcumin supplementation. In line with observations from the COP-D trial, curcumin and LCn-3PUFA did not have any complimentary and/or added benefits.

In conclusion, the results presented in this thesis demonstrate that InsuTAG has the potential to predict IR and MetS. This provides a basis for further research to validate InsuTAG with gold standard technique for IR and a longitudinal data analysis to determine the ability of InsuTAG to predict T2D in general population. With regards to the intervention trials, our hypothesis of targeting multiple pathways (IR and dyslipidaemia) in high risk and T2D patients with curcumin and LCn-3PUFA supplementation was successful. However, this thesis failed to provide any evidence on beneficial effects of combining curcumin and LCn-3PUFA for better glycaemic control to delay the onset of T2D. This could partly be due to presence of any unknown interactions between the two bio-actives or may be due to uncertainties in co-administration of curcumin and LCn-3PUFA. Thus, paving a way for further research to investigate beneficial effects with single formulation (curcumin and LCn-3PUFA) for achieving glycaemic control. This thesis constitutes a noted contribution to the research area of biomarkers and novel intervention strategies for T2D, and also presents a set of riddles that provides an extensive scope for future research.

Thesis layout

This thesis by publication is presented as 7 chapters: general introduction and literature review, general methodology chapter, 2 papers published in peer reviewed journals, 2 papers submitted for publication (under review), and one is presented as an unpublished chapter.

- **Chapter 1- General introduction and literature review**

  This chapter describes prevalence, economic and health burden of T2D, prediabetes and pathological mechanisms involved in the progression of prediabetes state to overt T2D. This chapter also focuses on the role of bio-active compounds for prevention or delaying the onset of T2D.

- **Chapter 2 – General methodology**

  This chapter describes detailed methodology, trial design and statistical analysis employed in the clinical trials (Chapter 4, 6 and 7).

- **Chapter 3 – InsuTAG: A novel and physiologically relevant marker of insulin resistance and metabolic syndrome.**

  This chapter is presented as published paper "Thota RN, Abbott KA, Ferguson JJA, Veysey M, Lucock M, Niblett S, King K, Garg ML. InsuTAG: A novel physiologically relevant predictor for insulin resistance and metabolic syndrome. Scientific Reports. 2017; 7(1):15204." The paper presents necessity for developing a novel marker, cross sectional study methodology, statistical analysis, results and discussion on whether InsuTAG has the potential to identify insulin resistance and metabolic syndrome.

- **Chapter 4 – Curcumin alleviates postprandial glycaemic response in healthy subjects: A cross over, randomised controlled study.**

  This chapter is presented as a paper (submitted MAR 18). It describes the study aims, design, methods, results and discussion on the effects of curcumin and omega-3 fatty acids for controlling the post-prandial glucose responses to a standardised high carbohydrate-fat meal in healthy adults.

- **Chapter 5 – Curcumin and long-chain Omega-3 fatty acids for Prevention of type 2 diabetes (COP-D): study protocol for a randomised controlled trial.**

  This chapter is presented as a published paper 'Thota RN, Acharya SH, Abbott KA, Garg ML. **C**urcumin and long-chain **O**mega-3 polyunsaturated fatty acids for **P**revention of type 2 **D**iabetes **(COP-D)**: study protocol for a randomised controlled

trial. Trials. 2016; 17:565'. This chapter presents the detailed study protocol of COP-D trial and provides a rationale for conducting a clinical trial with curcumin and LCn-3PUFA for controlling risk factors associated with progression of T2D.

- **Chapter 6– Effects of curcumin and/or omega-3 polyunsaturated fatty acids on glycaemic control and blood lipids in individuals with high risk of T2D: A randomised controlled trial.**

  This chapter is presented as a submitted version ' Thota RN, Acharya SH, Garg ML. Effects of curcumin and/or omega-3 polyunsaturated fatty acids on glycaemic control and blood lipids in individuals with high risk of type 2 diabetes: A randomised controlled trial. (Manuscript submitted MAR 18). This chapter describes the aims, methods, results and discussion from COP-D trial.

- **Chapter 7 – Curcumin and/or omega-3 polyunsaturated fatty acids for amelioration of diabetic dyslipidaemia: a pilot randomised controlled trial**

  This chapter presents the study aims, design, methods and results from pilot randomised controlled trial, '**C**urcumin **A**nd **L**ong-chain omega-3 polyunsaturated fatty acids **FOR** management of **C**ardio**V**ascular health in individuals with type 2 **D**iabetes (**CALFOR-CVD** trial).

- **Chapter 8 – General discussion**

  This chapter focuses on discussion on overall results from the clinical trials, strengths and limitations of the respective trials, significance of this research in nutrition and diabetes research areas and future directions.

CHAPTER 1

General Introduction

1.1 Overview

This chapter begins by presenting the global and regional (Australia) prevalence of T2D, impaired glucose tolerance (IGT), and their associations with other chronic diseases and economic burden. A multi-stage model development of T2D is presented with an emphasis on glucotoxicity, lipotoxicity and low-grade inflammation. The background then moves to the limitations of the existing markers for identification of insulin resistance (IR) and metabolic syndrome (MetS), highlighting the need for an improved marker. A brief description is presented on the current lifestyle and pharmacological interventions for prevention and management of T2D and the barriers associated with their effectiveness for preventing T2D. Meticulous search in the literature for bio-active compounds, presented with four potential compounds, curcumin, long-chain omega-3 polyunsaturated fatty acids (LCn-3PUFA), chlorogenic acid and resveratrol, for evaluating in the clinical trials to target multiple risk factors associated with the development of T2D. Following this, a rationale was presented for evaluating curcumin and/or LCn-3PUFA to target multiple glycaemic and lipid based risk factors in this thesis. Finally, this chapter ends by outlining the research hypothesis and aims of this thesis.

1.2 Background and context

1.2.1 Prevalence and diagnostic criteria of T2D and prediabetes

Diabetes is a chronic, non-communicable metabolic disorder characterised by the presence of hyperglycaemia, resulting from deficiency in secretion of insulin and its action or both. According to the latest edition (2017) of International Diabetes Federation (IDF) Atlas, approximately 425 million people were affected with diabetes in 2017 accounting for 4 million deaths world-wide (1). T2D is the most common form of diabetes, accounting for 90% of the diabetes cases. This number is expected to exceed 629 million within the next 20 years. In addition to this wide spread prevalence of T2D, there are 352.1 million people with IGT who are at a high risk of developing T2D. This number is expected to increase by 234 million in next 20 years (1).

Australian Bureau of Statistics (ABS) National Health Survey (2014-15) reported that nearly 1 million Australians aged 18 years and above had T2D based on measured and self-reported data (Diabetes compendium 2014-15, Australian Institute of Health and Welfare (AIHW, Australian Government).

The prevalence of diabetes has tripled between 1989–90 and 2014–15. The proportion of people with diabetes has increased from 1.5% to 4.7%. These numbers could be underestimated, as this report did not include the number of undiagnosed cases. For every four diagnosed cases, they found one undiagnosed case, as the participants were unaware of their diabetes status. According to World Health Organisation (WHO) criteria, T2D can be diagnosed (Table 1.1) on any of the following conditions along with presence of classic symptoms (frequent urination, increases thirst and hunger)

Fasting plasma glucose (FPG) ≥ 7.0 mmol/L

Or

Oral glucose tolerance test (OGTT) with FPG ≥ 7.0 mmol/L and/or

2-hour plasma glucose ≥ 11.1 mmol/L

Or

Glycosylated haemoglobin (HbA1c) ≥ 6.5% (48 mmol/mol)

Or

Random plasma glucose level ≥ 11.1 mmol/L

Table 1.1: Diagnostic criteria for T2D and prediabetes

Parameter	T2D (WHO, ADA, ADS)	IFG and IGT (WHO)	Prediabetes (ADA)	Prediabetes (ADS)
HbA_{1c} (%)	≥ 6.5%	NA	≥ 5.7 and < 6.5%	NA
FPG (mmol/L)	≥ 7.0 mmol/L	IFG ≥ 6.1 and < 7.0	≥ 5.6 and < 7.0	≥ 6.1 and < 7.0
OGTT (mmol/L)	2h plasma glucose ≥ 11.1 mmol/L	IGT ≥ 7.8 and < 11.1	2h, ≥7.8 and < 11.1	2h, ≥ 7.8 and < 11.1
Random glucose	≥ 11.1 mmol/L With classic symptoms	NA	NA	NA

WHO – World Health Organisation; **ADA** – American Diabetes Association; **ADS –** Australian Diabetes Society; **IFG –** impaired fasting glucose; **IGT –** impaired glucose tolerance; **HbA1c –** Glycosylated haemoglobin; **NA –** not applicable; **OGTT –** oral glucose tolerance test; Adapted from WHO website, ADA (2), ADS (3)

1.2.2 Economic burden and health consequences of T2D

Global epidemic of diabetes is one of the major threats to the human health of 21st century. Over the years the epidemiological studies, global advocates (IDF), and the governments have been producing the information on the economic burden and incidence of diabetes complications. In 2017, the total expenditure worldwide on people with diabetes was 9.4 billion US dollars (1). The total annual cost for Australians with T2D is up to $6 billion including healthcare costs. Compared with other nations, the highest mean expenditure estimate per person with diabetes was in Australia, with 5,560 international dollars (1). Estimated per person costs could be even higher in individuals with diabetes and its micro or macrovascular complications. Recent hospital care statistics by AIHW reported that there were over 1 million hospitalisations in Australia, where diabetes (primarily T2D or T2D with associated co-morbidity) was recorded as the primary diagnosis, representing a silent pandemic and economic burden in Australia, in line with global figures.

Chronic hyperglycaemia in diabetes leads to many long term microvascular complication in eyes (diabetic retinopathy), nerves (diabetic neuropathy), kidneys (diabetic nephropathy) and macrovascular complications in heart (CVD) and blood vessels (atherosclerosis) (4). Approximately 70% of patients with diabetes die due to CVD. As a result, an epidemic of diabetes is followed by an epidemic of CVD (5). According to the AIHW database, Diabetes caused 10 percent of all deaths in Australia, T2D being major contributor the mortality rates. In addition to concerning numbers from diabetes, people with prediabetes (IGT and IFG) also represent a high risk of macrovascular complications (6, 7). Thus, indicating several risk factors such as glucotoxicity, lipotoxicity and inflammation (described in section 1.2.3) present in prediabetes could put them at high risk state of both T2D and CVD. (8)

1.3 Multi-stage model development of T2D

The pathophysiology of T2D is multi-dimensional, varying from deficit in insulin secretion from the pancreatic β-cells to IR in peripheral tissues. Inadequate control on pathogenic metabolic consequences such as abdominal adiposity (particularly in obese individuals), increased hepatic glucose production, hyperglycaemia and loss of β-cell mass over prolonged periods results in progression to T2D from state of normal glucose

tolerance. The multi-stage development model of T2D is presented based on a model proposed by described Weir and Weir (9)

In obese individuals, lipid storage may exceed the adipose tissue capacity resulting in fat accumulation in non-adipose tissues (such as skeletal muscle, liver and pancreas). This lipid overspill blocks insulin binding to its receptors resulting in abnormalities of insulin signalling and glucose uptake, ultimately leading to IR in non-adipose tissues. Under these conditions, pancreatic β-cells increase insulin secretion to compensate (stage 1, β-cell compensation) for the minimised effectiveness of insulin in a tendency to maintain the blood glucose levels within the normal range (Figure 1.1).

Overtime, pancreatic β-cell secretions fail (stage 2, stable β-cell adaptation) to meet insulin demands and IR progress to a stage of IGT along with changes in β-cell mass and function. Progress from stage 1 to stage 2 occurs because of failure in compensatory mechanism adapted by β-cells. Blood glucose levels during this stage lie between 5.0 and 7.3 mmol/L and may not be restored back to normal levels. During this stage significant changes occur in β-cell function due to multiple pathological mechanisms like glucotoxicity, lipotoxicity and chronic low-grade inflammation.

Glucotoxicity: Glucotoxicity refers to modulation of β-cell function in response to even a slight increase in glucose level. In glucotoxicity, the balance between the production of reactive oxygen species (ROS) by mitochondria and anti-oxidant mechanisms worsens, leading to increased oxidative stress in pancreatic β-cells (10). Oxidative stress plays a key role in initiating early pathological mechanisms in β-cell dysfunction through activation of pro-inflammatory kinases and suppression of insulin promoter factor 1 (pdx-1) (11). ROS mediated stress mechanisms are also activated in the endoplasmic reticulum (ER) leading to imbalances between protein accumulation and its protein folding capacity. Disruption of ER homeostasis due to these imbalances results in accumulation of unfolded proteins, causing 'ER stress' (12). Pancreatic β-cells are sensitive to these stress mechanisms because misfolding of pro-insulin in ER hinders the insulin synthesis. During ER stress, innate cellular mechanisms activate a signalling pathway called 'unfolded protein response (UPR)'. In mild and tolerable conditions UPR promotes cell survival, however, if the UPR fails to restore homeostasis in ER apparatus under prolonged stress conditions, it triggers complex pathological mechanism through pro-inflammatory and apoptotic mechanisms leading to cell death (13).

Lipotoxicity: The concept of 'lipotoxicity' refers to multiple pathophysiological mechanisms associated with excessive accumulation of lipids in non-adipose tissues (14). Although T2D is strongly linked with imbalance in glucocentric pathways, lipotoxicity might play a key role in development of T2D by interfering with glucose uptake causing peripheral IR. Randle's hypothesis introduced the interplay between glucose and lipid metabolism through "glucose-fatty acid cycle", which describes the free fatty acid (FFA) mediated impairment in glucose metabolism and insulin sensitivity (IS) (15). Intake of high calorie diets on a regular basis results in breakdown of lipid regulatory system, leading to impairment in lipid homeostasis. This breakdown results in overspill of fatty acids from adipose tissue to non-adipose tissues including skeletal muscle and pancreas (16).

Skeletal muscle: In skeletal muscle, fat is stored as either metabolically inert extramyocellular TG or intramyocellular lipids (ICML) as fat droplets (17). Excessive FFA promotes accumulation of ICML in skeletal muscle. Clinical and preclinical studies indicated a significant negative correlation between the accumulation of ICML and IS (18, 19). Accumulation of surplus FFA and their metabolites activate serine/threonine kinase cascade in myocytes. Serine phosphorylation causes reduction in insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and failure in translocation of GLUT4 transporter to plasma membrane, resulting in insulin signalling deficit and decreased glucose uptake (20). Fatty acid overspill in skeletal muscle also activates toll like receptor-4 (TLR4) that is implicated in developing IR (21). Preclinical studies and in-vitro studies suggest FFA acts through TLR4 to supress insulin stimulated glycolysis and glucose uptake (21). Muscle TG do not directly cause these pathological changes in the insulin signalling pathways, but they more prominently act through their metabolites like intracellular fatty acyl CoA and diacylglycerol (22). To a limited extent another lipid metabolite, ceramide also exacerbate IR in obesity and T2D. Increased ceramide content in muscles impair insulin action on glucose uptake through inhibition of insulin stimulated Akt/ protein kinase B activation (23).

Pancreas: FFA accumulation in pancreas results in abnormalities of insulin secretion as a result of fatty acid mediated reduction in expression of glucokinase, GLUT2 glucose transporter, pro-insulin/insulin ratio and Pdx-1(24). FFA also cause β-cell apoptosis, leading to decrease in β-cell mass and insulin secretion (24). Extent of damage caused by

FFA in β-cell death and caspase activation increase with increase in glucose concentration, suggesting synergism between the toxicity of glucose and fatty acids (25).

Inflammation: Several cross-sectional studies have reported the presence of elevated low-grade pro-inflammatory markers in high-risk and T2D individuals (26-29). Inflammation mediated mechanisms such as macrophage infiltration; pro-inflammatory signalling and inflammatory markers (cytokines and adipokines) have been shown to be associated with the pathogenesis of T2D.

Macrophage infiltration: Despite the potential role of macrophage in providing host defence, they are also implicated in the pathogenesis of obesity-driven IR and T2D (30). Pre-clinical studies reported presence of two types of macrophages from adipose tissue of diet-induced obese rodents. M1 type macrophages are predominant, which is pro-inflammatory in function. On the contrary, M2 type macrophages are anti-inflammatory in action. This classification laid down the basis for phenotypic switch from M2 to M1 during progression from lean to obese state (31). Adipocytes secrete chemokines such as macrophage chemoattractant protein-1 (MCP-1) (also known as CC-chemokine ligand 2, CCL-2) that is primarily involved in recruitment of monocytes (precursors of macrophages). Consistent with the implications of macrophage infiltration in insulin resistance, obese rodents and humans has increased expression of CCL-2 in adipose tissue (32, 33).

Pro-inflammatory kinases signalling: Disruptions in metabolic pathways by above mentioned pathogenic mechanisms is believed to be initiated by pro-inflammatory signalling through activation of stress-induced kinases IκB kinase β (IKK) and JUN N-terminal kinase (JNK) (34, 35). The activation of these kinases suggests that they have a significant impact in development of IR and T2D (36). In obese conditions IKK-β either activates nuclear factor kappa B (NF-κB) or increase expression of NF-κB target genes like TNF-α, IL-6 and IL-1β. These cytokines promote IR in the tissues where they are activated such as in adipose tissue and may be transported to other tissues like liver and skeletal muscle (37). In addition to this kinase family, JNK is another important kinase which is involved in pro-inflammatory signalling by activation of E26 transformation specific containing domain protein 1 (ELK-1), activating transcription factor 2 (ATF2) and JUN through binding to their specific receptors. But the impact of JNK on IR in obesity is relatively less when compared to activation of other kinase (38). Liver and

adipose tissue are primary sites for activation of IKK and JNK kinases and has direct impact on IR (39). In liver, activation of these kinase occurs both in hepatocytes and macrophages which upregulate pro-inflammatory cytokines (34, 35). Activation of these pathways in muscle and hypothalamus has indirect effects on insulin resistance. Muscle related activation of IKK or JNK are involved in serine phosphorylation of insulin substrate receptor which down regulate insulin signalling.

In hypothalamus, activation of these kinases is linked with leptin signalling axis affecting feeding behaviours and promoting obesity (40). NF-κB pathway is also activated in pancreatic islets through actions of glucose and IL-1, which cause glucotoxicity in β-cells leading to decreased insulin secretion and β-cell mass (41).

Interluekin-6 (IL-6) & interleukin 1-β (IL-1β): IL-6 has emerged as a potential pro-inflammatory cytokine that is involved in pathogenesis of obesity-driven IR and T2D (29, 42). High circulating levels of IL-6 in obese individuals predict the future risk of developing T2D (29). IL-6, in the recent studies, demonstrated the capability of inducing IR in hepatocytes and adipocytes (42, 43). Effects of IL-6 on insulin signal impairment is regulated through signal transducer and activator of transcription 3 (STAT3), a suppressor of cytokine signalling 3 (SOSC3) pathway (44). In contrast to these actions in adipocytes and hepatocytes, IL-6 exhibits beneficial role in insulin regulated glucose metabolism in skeletal muscle, particularly during intense exercise (45). Pre-clinical and in-vitro studies on IL-1β has provided substantial evidence of cytokine mediated apoptosis in pancreatic β-cells (46, 47). IL-1β, along with activation of pro-apoptotic CD95 receptor promotes hyperglycaemia induced β-cell apoptosis and impairment of insulin secretion (48). High glucose concentration driven upregulation of IL-1β is regulated primarily through NOD, LRR and pyrin domain–containing 3 (NLRP3) inflammasome. Activation of inflammasome leads to activation of caspase-1 and subsequent processing and release of mature IL-1β (48). FFA infusion in rodents and humans cultured islets also shown to stimulate IL-1β secretion. This might be due to direct activation of TLR4 or TLR2 (49).

Adipokines: Over the last two decades, the view of adipose tissue has been switched from fat storage organ to endocrine organ with discovery of metabolic active proteins secretion (adipokines). Adipokines function as classic hormones that release from adipose tissue and communicate with other organs like brain, liver and muscle.

These are involved in various physiological processes like inflammation, feeding behaviours and energy expenditure. Adipose tissue secretes several adipokines which are pro-inflammatory (leptin, resistin, chemerin) and anti-inflammatory (adiponectin and omentin-1) in function (50, 51). Leptin and adiponectin are two major adipokines which are involved in modulating inflammatory and metabolic effects. Leptin regulate feeding behaviours by interacting with central nervous system (hypothalamus and hippocampus). Mouse models lacking leptin gene (ob/ob) exhibited abnormal feeding behaviours along with other pathological features like obesity and IR (52).

Moreover cross sectional studies in obese individuals indicated strong positive correlation of IR with high circulating leptin levels (53, 54). Adiponectin is an anti-inflammatory adipokine that may exert its beneficial effects in multiple ways such as stimulation of IL-10 and IL-1RA (55) and activation of AMPK (56). Contrary to plasma leptin, plasma adiponectin levels are decreased in the obese and T2D individuals (57-59).

After years of stable stage 2, β-cells fail to compensate insulin secretion for elevated glucose levels (stage 3, unstable early decompensation). Fasting blood glucose levels exceeds 7.3 mmol/L. This stage is very unstable, characterised by presence of inadequate β-cell mass and may not be associated with any classical symptoms of T2D (9). Once the individuals progress to stage 4 (stable decompensation), they have enough β-cell insulin secretion capacity to remain in this stage. Overall insulin secretion decrease and severe β-cell differentiation are persistent in this stage (9). While genetic influence may cause quick destruction of pancreatic β-cells that can lead to stage 5 (severe decompensation). This is the infrequent last stage of diabetes which characterized by glucose levels above 22 mmol/L and ketoacidosis. The individuals at this stage have a marked loss of β-cells and become truly dependent on insulin for their survival (9).

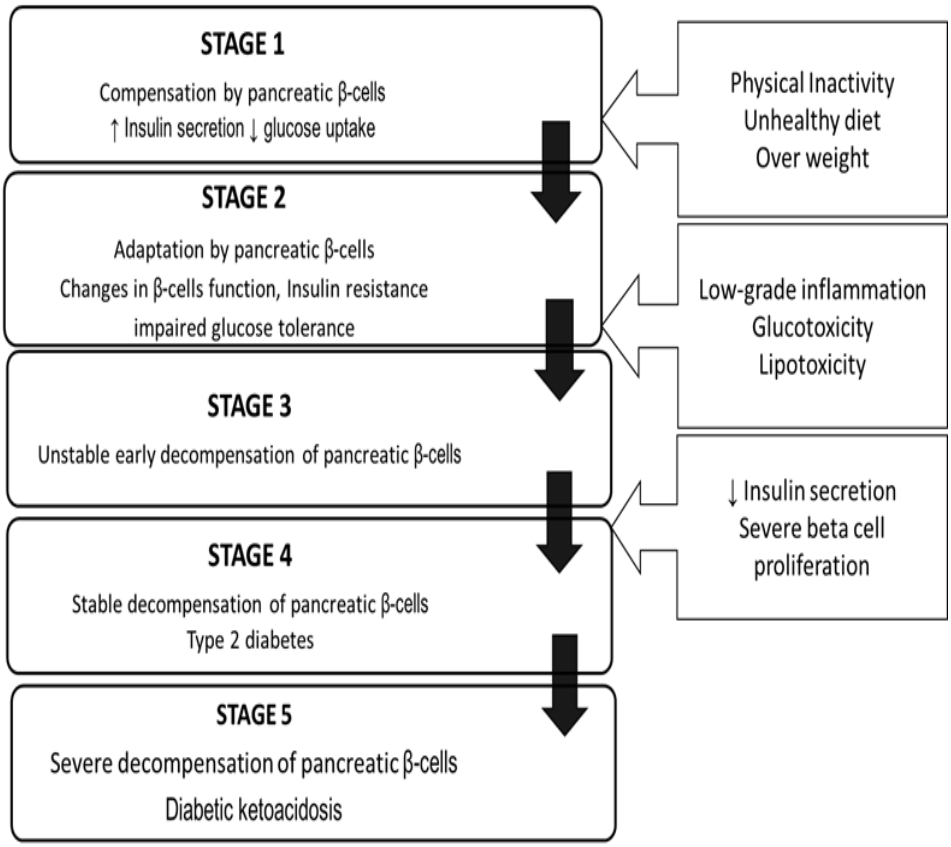

Figure 1.1: Multi-stage model development of T2D

1.4 Necessity for the improved markers

Recognition of the importance of IR in the pathogenesis of T2D have prompted the derivation of a number of indices and surrogate markers to quantify IR (60, 61). Among these, the hyperinsulinemic euglycemic clamp (HEC) technique has been described as the gold standard, providing a direct measure of IR (62). However, cost, expertise, and the requirement for intravenous insulin infusions and frequent blood sampling limits the application of HEC in epidemiological studies and routine clinical investigations. Homeostatic model assessment (HOMA) is a simple minimally invasive model that predicts IR using fasting steady-state glucose and insulin levels and that has been shown to be highly correlated with clamp insulin sensitive index values (63). However, HOMA does not consider the level of blood lipids, such as triglycerides (TG) or HDL-Cholesterol (HDL-C), despite lipid availability in circulation having an important role in IR and its metabolic complications. Chronic overnutrition resulting in disruption of insulin signalling pathways (such as phosphatidylinositol (3,4,5)-triphosphate) leads to over secretion of very low-density lipoprotein (VLDL) and possible decrease in the clearance of TG rich lipoproteins. As a result, levels of circulating TG are increased (64), that is

commonly observed in IR state and MetS (65). Insulin-mediated suppression of hepatic glucose production is still preserved in IR state, however suppression of VLDL-TG secretion is less pronounced (66), indicating rise in TG could be an early manifestation of IR. Cross-sectional studies (67, 68) and mechanistic studies (69) have shown a positive association between circulating TG levels and both IS and action. Kriketos et al. 2003 has shown that both skeletal muscle TG and circulating TG were inversely associated with whole body IS (69). Mingrone *et al* 1997 showed reversibility of IR by lowering plasma TG in obese individuals with diabetes (70); together these observations suggest the prime role of lipid availability as marker of IR and its associated complications. Therefore, there is a necessity for a marker to consider both fasting state insulin levels along with lipid availability that would provide more physiological basis for identifying IR and may even identification of T2D.

1.5 Current available interventions for prevention and management of T2D

Diabetes prevention studies have found that lifestyle interventions aiming at weight reduction through dietary intake changes and increase in physical activity significantly reduce the risk of developing T2D (71, 72). Lifestyle interventions result in weight loss and also improved IS and β-cell function (73-75). However, maintaining weight loss and physical activity is very difficult over longer period of time. Lack of compliance to these interventions is barrier for effectiveness in halting the progression to T2D in high risk individuals; indicating that dietary and life style modifications alone are insufficient. Over the last few years significant number of oral pharmacological agents have been employed for treating T2D. These include sodium glucose transport inhibitors, α-glucosidase inhibitors, glucagon like peptide-1 (GLP-1) agonists, dipeptidyl peptidase 4 (DPP-4) inhibitors, glinides, sulfonylureas and metformin. All these oral anti-hyperglycaemic therapies were proven to be effective only in ameliorating the hyperglycaemic conditions. Moreover current anti-diabetic medications have persistent side effects, such as body weight gain and hypoglycaemia in patients on sulphonylureas and insulin; gastrointestinal problems with metformin and acarbose; weight gain and bone fractures with thiazolidinediones and genital or urinary tract infections predominantly in females with sodium glucose co-transporter inhibitors (76). As prediabetes is not a full-blown disease state, to date, there are no approved drugs for management of prediabetes. Lack of

compliance to lifestyle interventions and pharmacological agents, highlights the necessity of more safe and efficacious interventions for management of prediabetes.

1.6 Role of bio-actives in prevention of T2D

Dietary bio-actives have emerged as an attractive strategy after the increase in evidence of adipose tissue and skeletal muscle inflammation in pathogenesis of IR and T2D; as these agents have shown to influence a number of inflammatory and oxidation pathways at cellular level with relatively higher safety profiles (77, 78). Several bioactive compounds have proven to be effective in *in-vitro* studies and their mechanism of action have been delineated, but translation of these effects in-vivo is still a matter of debate for their efficacy. A meticulous search for dietary bio-actives within the scientific literature resulted in a four potential dietary bio-actives (curcumin, LCn-3PUFA, chlorogenic acid and resveratrol) that might provide beneficial effects by lowering the risk factors to delay the onset of T2D. These agents demonstrated improvement in IR and by modulating the inflammatory pathways and oxidative stress in *in-vitro* and *in-vivo*.

1.6.1 Curcumin

The spice, turmeric contains wide range of chemical constituents like volatile oils (3-5%) and colouring agents, knowns as curcuminoids (5%) like curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcumin that is responsible for colouring properties of turmeric is now being evaluated for therapeutic properties. Curcumin (Figure 1.2) is a hydrophobic molecule which is mostly soluble in oils, ethanol and acetone (79).

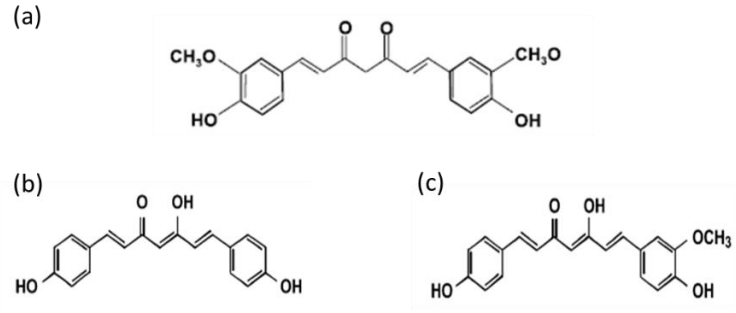

Figure 1.2: Structures of a. curcumin b. demethoxycurcumin c. bisdemethoxycurcumin

Absorption and metabolism of curcumin

Previous studies in rats and humans have reported limited bioavailability of curcumin. Less than 1% of curcumin enter blood stream when administered orally (80). Pharmacodynamic and pharmacokinetic studies on curcumin supplementation at doses of 144 -180 mg/day for 29 days reported absence of curcumin in plasma and urine samples of colorectal cancer patients. Analysis of dried faeces obtained from study volunteers confirmed excretion of curcumin (81). Another study in the healthy human volunteers reported presence of curcumin in plasma (less than 10ng/mL) following 1hour of consuming 2g dose of curcumin (82). To overcome the problem of bioavailability, curcumin has been delivered in different formulations like Nano formulations, liposomal encapsulations, phytosome and solid lipid curcumin particle. Curcumin, on oral administration, is rapidly metabolised to tetrahydrocurcumin and hexahydrocurcumin and excreted via bile. It appears to be easily conjugated to form curcumin glucuronide and sulfonate derivatives in the liver. It is metabolised to tetrahydrocurcumin and hexahydrocurcumin when administered intraperitoneally or systemically (80).

Existing Pre-clinical and clinical evidence

Several preclinical studies demonstrated potential effects of curcumin in ameliorating the risk factors associated with development of T2D. Oral supplementation of curcumin (50-250 mg/kg BW) for seven weeks in streptozotocin-induced diabetic rats resulted in decreased plasma glucose and lipid levels along with increase in glucose uptake, phosphorylation of AMPK, glycogen synthesis (particularly in muscle) (83). Similar effects of curcumin on glycaemic parameters were observed in alloxan-induced diabetic rats. Single oral dose (0.08 g/kg BW) administration of curcumin in diabetic rats for 21 days decreased hyperglycaemia and oxidative stress (84). Curcumin also exhibited potential effects on IR and hyperglycaemia in diet-induced experimental diabetes rodent models. Oral supplementation of curcumin at 80 mg/kg BW for 15 days in high fat diet fed diabetic Sprague Dawley rats exhibited anti-hyperglycaemic effects along with improvement in IS (85). In genetic diabetic rodent models like wild type C57BL/6J, C57 BL/6J, type 2 diabetic KK-Ay mice and db/db, curcumin exhibited glucose lowering effects, anti-inflammatory and insulin sensitising effects at dose ranges of 0.02% to 3% added to diet (86). Despite considerable evidence of blood glucose lowering effects of curcumin in preclinical studies, to date only a few clinical studies have been carried out to evaluate the use of curcumin for prevention or management of T2D.

First scientific report on blood glucose lowering effects of curcumin in a single type 2 diabetic patient was published in 1972 (87). In 2008, a randomised controlled trial was carried out to evaluate the effects of curcumin in 72 middle aged T2D subjects. This study reported that curcumin (300 mg/day) supplementation for 8 weeks resulted in significant improvement in endothelial function with concurrent decrease in malondialdehyde (MDA), IL-6, and TNF-α levels (88). Curcumin (1500 mg/day) supplementation in individuals with pre-diabetes (n=240) for 9 months decreased their homeostatic model assessment of IR (HOMA-IR) scores and FPG levels (89). In addition to these marked effects in the same study, curcumin also increased serum adiponectin levels and β-cell function. In the control pre-diabetes group subjects, 16.4% were detected with diabetes whereas not even a single participant was detected with T2D in curcumin receiving group (89). Another 3-month randomised intervention trial in obese and overweight T2D subjects reported a decrease in the FPG levels, HOMA-IR, FFA, TG and increased lipoprotein lipase in subjects receiving curcumin (300 mg/day) (90).

Tissue specific effects of curcumin

Adipose tissue: Metabolic effects of curcumin in adipocytes are primarily attributed to its anti-inflammatory properties. Curcumin has been shown to decrease inflammation in adipocytes via multiple pathways such as inhibition of macrophage infiltration, scavenging ROS and down regulation of pro-inflammatory cytokine signalling. *In-vitro* studies involving treatment with curcumin (0.1-10μM) macrophage cell lines and 3T3-L1 adipocytes cultured in adipose tissue conditioned medium reported suppression of TNF-α and MCP-1 release in a dose-dependent fashion (91). This anti-inflammatory effect of curcumin in macrophages and adipocytes was supported by another study in LPS treated mouse RAW 264.7 macrophages. Curcumin (100 μM) and its analogues supressed NF-κB DNA binding, NFκB nuclear translocation and down-stream inflammatory mediators like pro-inflammatory cytokines mRNA expressions in these LPS treated macrophages (92). Weisberg et al. demonstrated that curcumin supplementation in high fat diet fed genetically obese (ob/ob) mice resulted in amelioration of diabetes associated symptoms along with a decrease in IR. Multiple mechanisms like decrease macrophage accumulation in WAT, down-regulation of pro-inflammatory cytokines and increase in adiponectin levels are exhibited by curcumin in improving IS (93). Moreover, curcumin treatment increased adiponectin levels and supressed weight gain in high fat diet fed C57BL/6J mice (Model resembling development of human IR) (94). In the same study,

curcumin also improved IS and glucose disposal. These mechanisms highlight curcumin supplementation as an attractive strategy to decrease macrophage infiltration and inflammation in adipose tissue.

Liver

Activation of AMPK: Oral supplementation with curcumin for 8 weeks increased expression of AMPK by 50% in diabetic db/db mice (95). Curcumin mediated activation of AMPK could supress the hepatic glucose production, resulting in decreased blood glucose levels.

Cytoprotection: Hepatocytes incubated with curcumin (1-10μM) provided protection against FFA mediated impairment in mitochondrial function. In addition to this, curcumin treatment also inhibited lipoapoptosis (FFA induced cell death) and ROS production in cultured hepatocytes (96). These cytoprotective mechanism and restoration of mitochondrial function by curcumin might be due to its capacity in increasing levels of key mitochondrial biogenesis regulating transcription factors like PGC-1α, nuclear respiratory factor -1 and mitochondrial transcription factor A (96).

Improvement in insulin signalling: Curcumin supplementation in HFD fed C57BL/ 6J mice significantly improved insulin signalling in hepatocytes (94). Curcumin restored the HFD mediated impairment in insulin stimulated PKB phosphorylation. This effect was seen in hepatocytes pre-treated with glucose-oxidase, which is known to induce oxidative stress (94). Another study reported that curcumin supplementation in drinking water significantly increase high fructose induced decrease in P-IR and P-IRS protein levels. Along with this, curcumin also reduced high serum levels of insulin and leptin in the fructose fed hypertriglyceridemia rats (97).

Skeletal muscle: Presence of pathological conditions like oxidative stress, chronic low-grade inflammation and IR impairs metabolic signalling and mitochondrial biogenesis. Curcumin (50mg/kg and 100 mg/kg per body weight) supplementation in Wistar rats increased expression of mitochondrial function markers like COX-IV and oxidative phosphorylation subunits in isolated rat gastrocnemius and soleus skeletal muscle (98). Additionally, curcumin also increased mtDNA copy number compared to control group, which is probably one of the best biomarker for mitochondrial biogenesis. Curcumin mediated activation of sirtuin1 (SIRT1) expression is observed only in Gastrocnemius muscle, but phosphorylation of Thr^{172} residue of AMPK catalytic unit was observed in both type of skeletal muscle (98). Curcumin prevented reduction in 2-deoxy-[^{3}H]-D-

glucose uptake along with increase in GLUT4 expression in cultured L6 myotubes in a dose-dependent manner (83). Curcumin supressed the total acetyl CoA carboxylase (ACC) activity, which led to decreased malonyl CoA production in these myotubes. This resulted in enhancement of fatty acid oxidation along with reduction in fatty acid accumulation in skeletal muscle (83). Curcumin supplementation in high fat diet fed rats resulted in a reduction of elevated MDA levels, muscular MDA and MDA levels in mitochondrial fraction in compared to the control group (99). Curcumin also activated antioxidant enzymes like nrf2 signalling and induced hemeoxygenase-1 (HO-1) in skeletal muscle of these rodents (99). Together, these results indicate curcumin has potential to decrease accumulation of fatty acids along with improvement in cellular anti-oxidant signalling to protect skeletal muscle against peripheral IR.

Pancreatic β-cells: Dietary supplementation with curcumin in rats and pre-treatment with curcumin in cultured pancreatic cells, demonstrated anti-apoptotic effects against streptozotocin- induced apoptosis in pancreatic cells (100). Curcumin decreased the intracellular effects of calcium and caspase 12 that are known to mediate the apoptotic process. Curcumin attenuated ER-mediated apoptosis mechanism in pancreatic tissue and islet cells by restoring Bax/BCl-2 ratio and normal mitochondrial cytochrome-c levels compared to the control group (100). Stimulation of volume regulated anion channel may be one of the primary mechanisms for anti-hyperglycaemic effects of curcumin. Curcumin directly stimulated volume regulated anion channels in rat pancreatic β-cells (101). This effect was accompanied by generation of electrical activity and depolarisation which resulted in enhanced insulin release. Additionally, β-cell volume was also decreased by curcumin which might be due to a decrease in cellular Cl^- concentration (101). Curcuminoids increased expression of anti-oxidant enzymes HO-1 and NADPH:quinone oxidoreductase 1 at mRNA levels up to 12-fold and at protein levels by 6 fold in human islets (used for human transplantation) (102). Increased expression of these anti-oxidant enzymes in β-cells was observed by immunofluorescence staining of islets (102). These observations suggest curcumin might be beneficial in improving cellular defense mechanisms against oxidative stress. Curcumin has been shown to regulate the insulin secretions under glucose stimulated conditions in cultured human islets (103). Curcumin treatment in β-cell lines also increased intracellular cAMP level that is instrumental in glucose and incretin-stimulated insulin secretion and pancreatic β-cell health. Curcumin in a dose-dependent fashion decreased mRNA expression of

phosphodiesterase enzymes PDE 3B, PDE 8A and PDE 10A which decrease insulin secretion in β-cell lines and human islets (103). Together, cytoprotective and anti-inflammatory effects, reduction in fatty acid accumulation and amelioration of IR in pre-clinical models provide a considerable evidence for exploring the potential of curcumin to lower the risk factors such as IR in a clinical trial to achieve glycaemic control.

1.6.2 Long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA)

LCn-3PUFA are essential, must be obtained through diet, as humans lack the enzymes to synthesise these fatty acids. LCn-3PUFA includes eicosapentaenoic acid (EPA; 20:5n-3), docosahexanoic acid (DHA; 22:6n-3) (Figure 1.3) (104). Rich sources of LCn-3PUFA include cold water fishes such as salmon, mackerel, herring, tuna and trout. Certain plant foods like flaxseeds, nuts, canola oil and other plant based oils also provide n-3PUFA as α-Linoleic acid, the metabolic precursor for EPA and DHA (105). Novel sources have been employed to meet the increasing demand of LCn-3PUFA like food fortification with LCn-3PUFA, oils enriched with DHA from cultured algae like *crypthecodinium cohnii* , *echium plantagineum* seed oil – rich in stearidonic acid (intermediate in metabolism of LCn-3PUFA), transgenic varieties of canola and soybean oils rich in stearidonic acid, EPA and DHA have also been developed (105) .

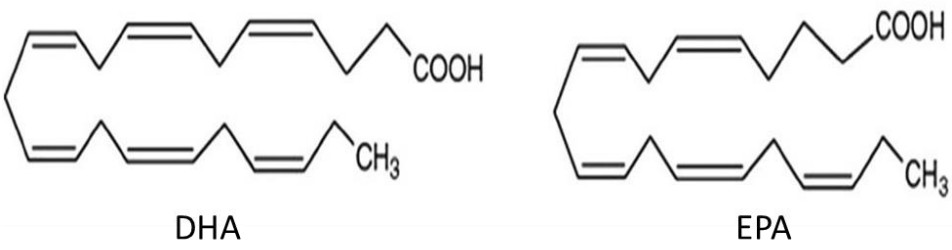

Figure 1.3: Structures of Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA)

Regular consumption of LCn-3PUFA from either fish or fish oil has a significant impact on physio-chemical properties of the membranes, cellular function and intracellular signalling pathways. Cell membranes enriched with LCn-3PUFA suppress the expression of adhesion proteins and pro-inflammatory mediators (106). LCn-3PUFA also cut down the production of pro-inflammatory eicosanoids (thromboxane, prostaglandins and leukotrienes of 2-series) and act as a precursor for the formation of resolvins and protectins that are known to possess anti-inflammatory properties. Epidemiological, experimental and human interventional studies have reported beneficial effects of LCn-

3PUFA in the prevention of inflammatory and metabolic diseases including diabetes (107-109).

Epidemiological studies

The basis for current interest in employing LCn-3PUFA for preventing or delaying onset of diabetes is partly based on observations from epidemiological studies, demonstrating lower prevalence of T2D in Greenland Inuit, Alaskans and Faroe Islands (110, 111) who regularly consume sea food rich diets. Lower prevalence rates of T2D in these populations are attributed to higher circulating levels of LCn-3PUFA. Epidemiological studies reported an inverse correlation between regular consumption of fish and prevalence of diabetes (112-116). Recently Takkunen *et al* carried out a prospective study in a cohort of 407 obese and IGT participants and demonstrated that elevated plasma levels of LCn-3PUFA are associated with lesser prevalence of T2D over the extended 11-year follow up period. Elevated serum EPA, DHA and DPA levels are associated with higher IS, providing a plausible explanation for the lower prevalence rates of diabetes (109). Plasma and serum levels of LCn-3PUFA reflect recent intakes of these fatty acids and do not indicate long-term status. The observed association between plasma/serum levels and T2D needs to be validated using objectives measures of long-term status such as erythrocyte LCn-3PUFA levels (omega-3 Index)

Preclinical and in-vitro studies

Several preclinical and in-vitro studies have demonstrated beneficial effects of LCn-3PUFA in ameliorating risk factors for diabetes such as sub-clinical inflammation, lipotoxicity and IR. High fat diet is the most common experimental diet used in pre-clinical research to induce IR, abnormal body fat composition, glucose intolerance, hyperinsulinemia, and lipid abnormalities. In high fat diet fed rodents, LCn-3PUFA have been shown to exhibit significant effects on restoring normal body composition and protecting the animals from IR (117). Administration of LCn-3PUFA in the diet (5-44%) protected the high fat diet fed animals from developing lipid abnormalities and impaired glucose homeostasis (118-120). The beneficial results obtained from these studies are partly attributed to LCn-3PUFA mediated increase in adiponectin levels, which is believed to be underlying mechanism for improvement in IS (118). LCn-3PUFA also have been shown to delay the development of dietary fructose or sucrose induced dyslipidaemia, IR in peripheral tissues and hyperglycaemia (121). LCn-3PUFA supplementation as fish oil normalised the plasma glucose levels, NEFA, TG, glycogen

synthesis in skeletal muscle and IS in rats fed with high cholesterol diets (122, 123). Few studies in genetic models of T2D exhibited dose-dependent effects of LCn-3PUFA on plasma glucose levels. Single administration of DHA at a dose level of 500 mg/kg BW to KK-Ay mice significantly reduced the plasma glucose levels and FFA at 10 h after oral administration in comparison to the control group (124). In addition, administration of DHA (100 mg/kg) in the same study significantly suppressed the blood glucose and TG levels in 30 days (124). In another study in rats, EPA (1.0g EPA rich oil/ kg per day) treatment for 25 weeks decreased plasma lipids, hepatic TG, and abdominal fat deposits (125). EPA has noteworthy effects on glucose tolerance, but did not reach statistical significance, indicating EPA supplementation might be beneficial in improving peripheral IR and high blood glucose levels in long term (125).

Tissue specific physiological effects of LC-n3PUFA

Liver:

Activation of peroxisome proliferator-activated receptor alpha sub unit (PPAR-α)

LCn-3PUFA are natural agonists of PPAR-α subunits (126). Carboxylic group and hydrophobic chain in structure of LCn-3PUFA allows optimal binding to PPARα. Activation of PPARα by LCn-3PUFA results in increased fatty acids β-oxidation in peroxisomes (lesser extent in mitochondria) and decreased lipogenesis. The increase in fatty acid oxidation leads to decrease accumulation of fatty acids (lipotoxicity) in liver along with improvement in IS in hepatocytes (126).

Down regulation of sterol regulatory element-binding protein 1 (SREBP-1c)

SREBP-1 regulates expression of various genes involved in the synthesis of cholesterol, fatty acids and TG. During hyperinsulinemic conditions, SREBP-1c increases lipogenesis causing dyslipidaemia and associated pathological conditions like MetS. LCn-3PUFA exhibits effects on SREBP-1 though transcriptional and post transcription mechanisms. This down regulation might be mediated through activation of liver X receptor alpha (LXRα) (127) at transcription level and PPARα (128) activation post transcriptionally resulting in a decline in de novo lipogenesis and enhancement in IS (129).

Activation of 5' adenosine monophosphate-activated protein kinase (AMPK)

AMPK is a master regulator protein which maintains hepatic glucose and lipid homeostasis during nutritional stress conditions. Activation of AMPK in liver is associated with fatty acid β-oxidation and inhibition of glucose synthesis (130). The exact mechanism for activation of AMPK by LCn-3PUFA is not yet described, but it is believed

to be regulated through increasing adiponectin levels and controlling phosphatases acting on AMPK dephosphorylation (131). Stimulation of AMPK by LCn-3PUFA along with its effects on PPARα and SREBP-1c result in significant reduction in TG with overall improvement in IS of hepatocytes.

Adipose tissue

Stimulation of PPARγ subunit expression

LCn-3PUFA through PPARγ stimulation, mechanism of action like that of thiazolidinedione class of anti-diabetic drugs (132), balances adipokine secretions, decrease pro-inflammatory signalling and increase IS (133-135).

Regulation of adipokine secretions

Several pre-clinical studies reported the potential of EPA and DHA to modulate leptin and adiponectin secretions (136). In-vitro studies involving treatment of mouse 3T3-L1 adipocytes with EPA demonstrated dose-dependent stimulation on leptin mRNA expression (137). On the contrary, in vivo studies in mice models demonstrated prolonged intake of LCn-3PUFA diets significantly decreased plasma leptin levels (138). Insulin sensitising properties of LCn-3PUFA are partly attributed to its capacity to increase circulating adiponectin levels. Many pre-clinical studies reported increase in adiponectin levels in short span after feeding with EPA/DHA enriched diets (118). Clinical studies provided equivocal results on effects of LCn-3PUFA on adiponectin. Three months dietary supplementation with EPA along with calorie restrictions increased plasma adiponectin levels in human obese subjects (139). Whereas another study reported that dietary supplementation with LCn-3PUFA did not increase plasma adiponectin levels in overweight individuals (140). The mechanisms through which LCn-3PUFA increases adiponectin levels are presently not clear. Activation of AMPK and to some extent PPAR dependent mechanism might be involved in increasing adiponectin levels (141).

Skeletal muscle:

Decrease accumulation of fatty acid metabolites

DHA at a dose of 400µM has been shown to decrease diacylglycerol accumulation in L6 myotubes (142). EPA protected high fat diet fed rodents from developing IR and improved glycogen synthesis in skeletal muscle (143).

Increased glucose uptake in myotubes

EPA at a very high dose of 600 µM increased glucose uptake in human myotubes (144). This observation was further strengthened by another in vivo study which reported

decrease in muscle ceramide and long chain acyl CoA content in mice fed with diet supplemented with fish oil (145). However, the clear mechanism through which it decreases the accumulation of fatty acid metabolites is still to be identified.

Effects of LCn-3PUFA on inflammatory markers and pro-inflammatory gene expressions
Clinical studies with supplementation of EPA + DHA at dose of 3.1 – 8.4g / day have reported 30-55% decrease in production of reactive oxygen species by cultured human neutrophils (146, 147). Cell culture studies reported EPA and DHA have potency to inhibit pro-inflammatory cytokines IL-β, TNFα and NF-κB expression by macrophages (148-150). LCn-3PUFA mediate inhibition of NF-κB expression through multiple pathways which interferes with TLR4 activation by saturated fatty acids and lipopolysaccharides, prevents IKK phosphorylation, PPAR mediated inhibition of NFκB DNA binding activity and DHA derived active anti- inflammatory metabolic compounds like resolvins and protectins (151). These observations are further strengthened by other studies, which reported decreased production of IL-1β, TNFα and IL-6 from rodent macrophages (*ex vivo* stimulated with endotoxins, lipopolysaccharides) after treatment with LCn-3PUFA (152-154). Recent human intervention studies with LCn-3PUFA at a dose levels between 2-4g/ day reported decrease in inflammation and pro-inflammatory markers (155, 156). Recently published research study on G-protein coupled receptors (GPR) reported that anti-inflammatory activity of LCn-3PUFA is mediated through activation of GPR 120 (157). GPRs are important signalling molecules that are involved in several cellular and physiological functions. LCn-3PUFA stimulates GPR 120 in macrophages and adipocytes, through which it exhibits its anti-inflammatory mechanisms. This involves inhibition of transforming growth factor β-activated kinase 1 (TAK 1) through β-arrestin1/TGF-β activated kinase 1/MAP3K7 binding protein 1(TAB1) dependent mechanism. This inhibition leads to down regulation in pro-inflammatory pathways like IKKβ/ NF-κB and JNK/ AP1 (157). Another interesting mechanism was observed through GPR 120 activation. DHA stimulated GPR 120 in 3T3 L1 adipocytes, which mediated increase in uptake of glucose through GLUT4 translocation (157). However this effect is limiting because GPR 120 is not expressed in skeletal muscle, which is primary organ for major glucose uptake. Inhibition of inflammasome became an attractive strategy to down regulated inflammation or inflammation driven diseases in the recent days. Inflammasome is a cystolic protein that comprises nucleotide binding domain, leucine rich proteins (NLRs) and caspase-1. It

promotes release of several pro-inflammatory cytokines. Recent study by Yan et al. demonstrated LCn-3PUFA abolished activation of NLRP3 inflammasome along with inhibition in caspase -1activation and IL-1β secretion (158).

Clinical evidence

Despite potential effects of LCn-3PUFA in animal models on IS and glucose homeostasis, interventional studies with fish oil in humans provided ambiguous results over glucose control. Several randomised controlled trials in obese and diabetic individuals constantly reported triglyceride lowering effects of LCn-3PUFA (159-162). However, with respect to glucose homeostasis, studies either reported no change or deterioration in glucose levels after LCn-3PUFA administration (163, 164). LCn-3PUFA intervention in obesity, which is considered as primary risk factor for development of T2D, exhibited weight reduction when combined with calorie restrictions (165) but not in other intervention studies (166). Frida *et al* reported improvement in glucose and insulin homeostasis in obese adolescents with supplementation with high physiological dose of LCn-3PUFA, indicating reversal of IR at very early stages (167). These findings are supported by other LCn-3PUFA supplementation studies with or without calorie restriction (166, 168). Systemic review and meta-analysis reported lack of LCn-3PUFA effects on IS (169). Some limitations like insufficient data from randomised controlled trials, dose and duration of intervention still demand for more randomised controlled trials to evaluate effects of LC-n3PUFA on IS. According to the recent review on clinical data of LCn-3PUFA in T2D (164), LCn-3PUFA have effectively reduced TG in human studies on T2D patients with unclear effects on glucose metabolism. This study indicated necessity for long-term quality trials to examine the efficacy of LCn-3PUFA on glucose metabolism, which is in line of argument provided by another review. Caterina *et al.* (163) suggested that LCn-3PUFA might reduce the burden of macrovascular complications in T2D patients. But the dose of LCn-3PUFA to exhibit beneficial effects on macrovascular complications in these populations is still a matter of debate. Prospective clinical endpoint trial GISSI-prevenzione (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto) reported 0.85g EPA+DHA/day is beneficial for preventing sudden death, which is comparatively much lower to triglyceride reduction dose (3-6g/ day) (170). JELIS (The Japan Eicosapentaenoic Acid Lipid Intervention Study) study reported intake of 1.5g EPA/day is required for slowing down the vascular disease progression and prevention of thrombosis (171).

The established data on anti-inflammatory and triglyceride reducing effects of LCn-3PUFA might be an important aspect to be considered for reducing low grade systemic inflammation, which is progenitor for several pathological mechanisms like glucotoxicity and lipotoxicity in different tissues.

1.6.3 Chlorogenic acid

Chlorogenic acid is a natural phenolic compound (Figure 1.4), which is the second major component after caffeine in coffee. It is also present in other food sources like apples, plums and berries (172). Regular coffee consumers obtain up to 1g of chlorogenic acid from their daily coffee consumption (173). Chemically chlorogenic acid is an ester form of caffeic acid and quinic acid (172).

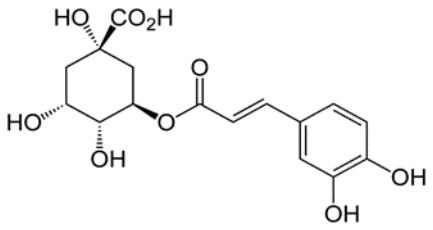

Figure 1.4: Structure of chlorogenic acid

Multiple mechanisms are believed to be modulated by chlorogenic acid for ameliorating the symptoms of T2D. Chlorogenic acid and its synthetic derivatives have shown to inhibit glucose-6-phosphatase which catalyses the final step of glycogenolysis and gluconeogenesis. Similar to that of α-glucosidase inhibitors like acarbose and voglibose, chlorogenic acid significantly inhibited α-glucosidase activity for controlling postprandial glycaemia (174). Another potential mechanism for beneficial effects of chlorogenic acid in IR and T2D is activation of AMPK. Stimulation of glucose uptake and increase in GLUT4 expression in the skeletal muscle by chlorogenic acid is attributed to AMPK activation (175, 176). Chlorogenic acid exhibited activation of PPARα (hepatic) and PPARγ in visceral adipose tissue in animal models (177). Chlorogenic acid significantly upregulated mRNA expression and protein levels of PPARα along with the modulation of adipokine secretion in *db/db* mice, attributing its effects on glucose and lipid homeostasis (178). In addition to these mechanistic pre-clinical studies, the effect of chlorogenic acid on glucose metabolism was also evaluated in humans. In a randomised controlled trial supplementation of 1g of chlorogenic acid showed significant reduction in glucose and insulin levels, 15 min after start of oral glucose tolerance test (OGTT). It

reduced the early glucose responses to OGTT, providing a basis for hypothetical correlation of beneficial effects of coffee consumption in T2D (179). Johnson et al. carried out a randomised trial to examine the glycaemic effects of chlorogenic acid and caffeine. Caffeinated coffee beverage comparatively increased glucose and insulin levels higher than decaffeinated coffee. Moreover decaffeinated coffee increases the postprandial circulating glucagon like peptide (GLP-1) levels and significantly attenuated glucose-dependent insulinotropic polypeptide (GIP) (180). Kempf et al. carried out a single blinded clinical trial to evaluate the efficacy of coffee consumption on sub-clinical inflammation and other risk factors for T2D. They found coffee consumption increased serum levels of chlorogenic acid, caffeine and caffeic acid metabolites. Along with increase in chlorogenic acid levels, coffee consumption demonstrated beneficial effects on subclinical inflammation and HDL levels (181).

1.6.4 Resveratrol

Resveratrol is a naturally occurring polyphenol (figure 1.5), profoundly present in red wine, grapes and peanuts (182).

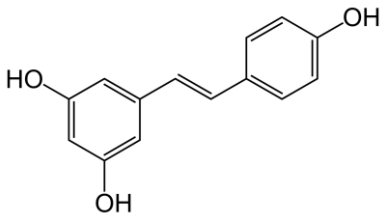

Figure 1.5: Structure of resveratrol

Preclinical studies demonstrated that resveratrol can alleviate T2D by modulating several mechanisms (183, 184). The potential mechanism involves activation of SIRT-1, which in general is activated through exercise and calorie restriction (185, 186). SIRT-1 helps in maintaining glucose homeostasis through regulating insulin secretion, cytoprotection in β-cells, decreasing inflammation, controlling fatty acid oxidation and mitochondrial biogenesis (187). Besides activation of SIRT-1, resveratrol demonstrated glucose lowering (188, 189) and cardio-protective effects (190). Majority of its anti-diabetic activity is attributed to its activation of AMPK in diabetic rodent models (184, 191). *In-vitro* study on treatment of resveratrol in β-cell lines and human islets demonstrated pancreatic β-cell function enhancement by inhibiting phosphodiesterase enzyme in a dose-dependent manner (0.1-10µmol/l) (192). Resveratrol exhibited protective effects on streptozotocin induced diabetic nephropathy in rodents by up-regulating AMPK

expression and decreased pro-inflammatory cytokines (193). Hypoglycaemic effects of resveratrol in type 2 diabetic mice model (db/db mice) is attributed to its enhanced endogenous GLUT 4 translocation. It also increased phosphorylation of AMPK in skeletal muscles along with Akt which resulted in higher glucose uptake in skeletal muscles, providing beneficial effects of resveratrol in ameliorating IR (194). Although there is ample evidence suggesting beneficial effects of resveratrol in pre-clinical and in-vitro mechanistic studies for managing T2D, clinical studies to evaluate the hypoglycaemic effects of resveratrol are lacking. *Movahed et al.* carried out a randomised, double blinded, placebo controlled trial to evaluate hypoglycaemic effects of resveratrol (1g/ day) in conjunction with standard oral anti-hyperglycaemic agents in patients with T2D. After 45 days of intervention, Resveratrol receiving group exhibited pronounced decrease in blood glucose levels along with HbA1c and Insulin levels when compared to control group (189). *Bhatt et al.* designed an open labelled, randomized, controlled trial to evaluate the potential of resveratrol (250 mg/ day) along with oral anti-diabetic agents for improving the glycaemic profiles in individuals with T2D. This study had reported a significant decrease in HbA1c and systolic blood pressure (SBP) (195). However, there is a necessity of independent, long term intervention trials to evaluate the anti-diabetic effects of resveratrol.

1.7 Conclusion of the literature review and project rationale

A detailed literature review on development of T2D indicates IR (196), chronic low grade inflammation (197) and elevated TG (198) appears to be the primary risk factors associated with development of T2D. The evidence on the effects of chlorogenic acid and resveratrol on IR and inflammation are ambiguous and unclear. Limited preclinical evidence could be a barrier to evaluate the chlorogenic acid and resveratrol in clinical trials for lowering the risk of T2D. Existing evidence and tissue specific effects presented in section 1.6.1 and 1.6.2, suggests both curcumin and LCn-3PUFA possess potent anti-inflammatory effects. Moreover, the glucose and IR lowering effects by curcumin and effective reduction in dyslipidaemia by LCn-3PUFA, suggests that combination of these two agents may provide effective overall metabolic control to lower the risk of T2D than either of the supplements alone (Figure 1.6).

Few preclinical studies have already reported synergistic anti-inflammatory effects on curcumin and DHA combination (199-201). Till date no interactions or counter effects

were presented in the literature on combination curcumin and LCn-3PUFA. Clinical evidence presented on curcumin and LCn-3PUFA in section 1.6.1 and 1.6.2, indicates 1g of curcumin (Meriva formulation) alone and 1.3 g of LCn-3PUFA alone exhibits anti-inflammatory effects. Moreover this dose of curcumin (202) and LCn-3PUFA (203) is relatively safe and well tolerated. A low dose combination would be optimal to test for any additive or synergistic effects to target multiple risk factors associated with T2D.

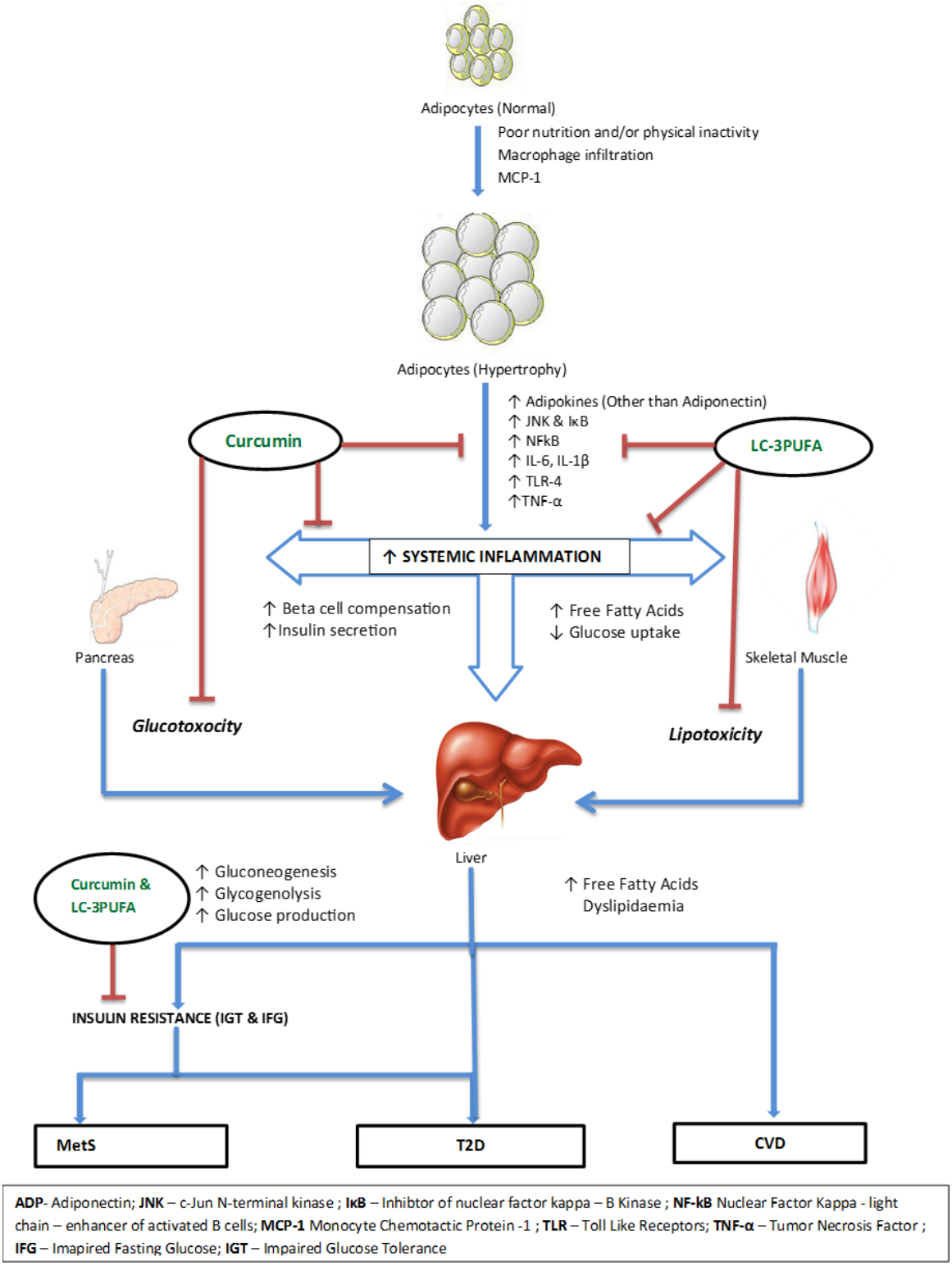

Figure 1.6: Rationale for combination of curcumin and LCn-3PUFA

1.8 Research Aims

1. To investigate whether a novel and physiologically relevant marker, InsuTAG (product of fasting insulin and TG) has the potential to predict IR and MetS.
2. To evaluate the effects of a single dose of curcumin and/or LCn-3PUFA on postprandial glucose and insulin responses in healthy individuals.
3. To evaluate the effects of curcumin and/or LCn-3PUFA supplementation on glycaemic control and blood lipids in individuals at high risk of T2D.
4. To evaluate the effects of curcumin and/or LCn-3PUFA supplementation on diabetic dyslipidaemia.

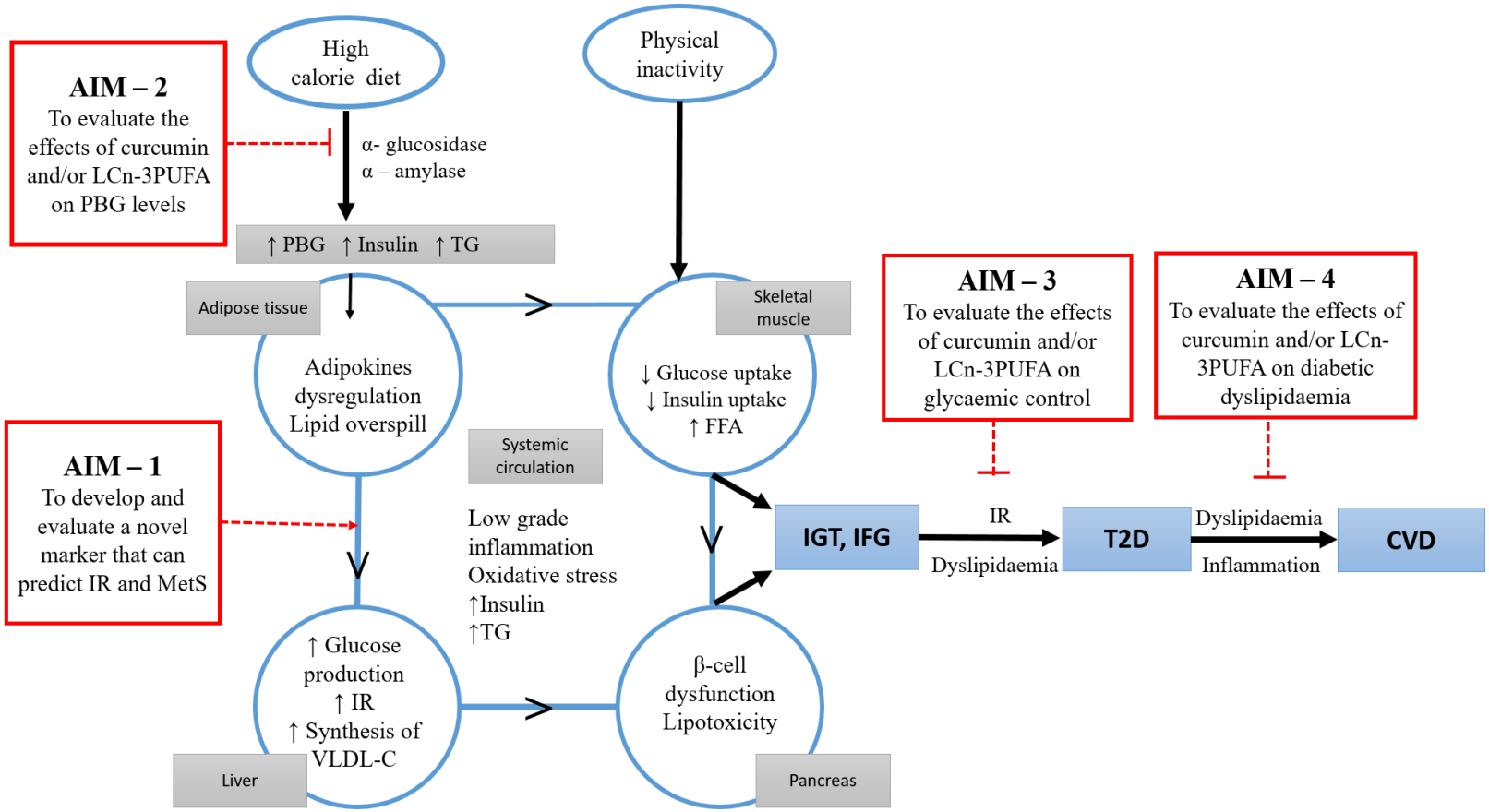

Figure 1.7: Summary of literature review and research aims

CVD – Cardiovascular disease; **FFA** – Free fatty acids; **IFG** – Impaired fasting glucose; **IGT** – Impaired glucose tolerance; **IR** – Insulin resistance; **LCn-3PUFA** – Long-chain polyunsaturated fatty acids; **MetS** – Metabolic syndrome; **PBG** – Postprandial blood glucose level; **T2D** – Type 2 diabetes; **TG** – Triglyceride; **VLDL** – VLDL-cholesterol

CHAPTER 2

General Methods

2.1 Participant Recruitment

Participants were recruited from the Newcastle, Hunter and Central Coast regions using media advertisements and social media (Facebook and Twitter). Recruitment flyers (Appendices 1-3) approved by the University of Newcastle Human Research Ethics Committee (UON HREC) and Hunter New England Health Human Research Ethics Committee (HNEHREC) were placed on notice boards of University of Newcastle buildings, local pathology centres, pharmacies, general practise health centres (with consent from participating GPs) and local shopping centres. Participants were also recruited from the Hunter Medical Research Institute (HMRI) Volunteer Register, as well as the clinics across John Hunter/Newcastle Community Health Centre and Belmont Hospital diabetes clinics and Medicare Local. Potential participants before consenting to participate in one of the studies were screened either through telephone or in person by a study co-ordinator or one of the study investigators and were provided with study information sheets (Appendices 4-6). All the participants provided their written informed consent (Appendices 7-9).

2.2 Anthropometry and Body Composition

Height (cm) of the study participants was measured using a wall mounted roll up stadiometer (SE206, Seca). Waist circumference (WC) (in cm) was measured using a tape measure positioned about halfway between the bottom of the lowest rib and the top of hip bone, roughly in line with the belly button. Body composition measurements (weight, body mass index; BMI, percent body fat, PBF; muscle mass, MM; body fat mass, BFM; waist-hip ratio, WHR) were performed on study participants in fasting state at baseline and post-intervention visit days using direct segmental multi-frequency bioelectrical impedance (InBody 230, Biospace Co., Ltd. Seoul, Korea).

2.3 Questionnaires

2.3.1 Medical History

Medical history, medications and supplements use, and demographic information was obtained from the participants via self-administered medical questionnaire (Appendices 10-12) at baseline visit in trial-1 (section 2.6.1), trial-2 (section 2.6.2) and trial-3 (section 2.6.3).

2.3.2 Nutrition assessment

Participants in trial-1 (section 2.6.1) were asked to provide a 24-hour recall of their previous day food intake (Appendix 13). This dietary information was processed through FoodWorks Version: 8.0.3551 (Xyris Software (Australia) Pty Ltd) to check whether their dietary energy and macronutrients remained unchanged during the study period. Similarly, participants in Trial-2 (section 2.6.2) and Trial-3 (section 2.6.3) reported their food and beverage consumption over 2 weekdays and 1 weekend day (appendix 14). These food diaries were analysed through FoodWorks, Xyris (version 8.0) to estimate participants' habitual dietary intakes. Participants were advised not to change their routine dietary habits during the respective study duration.

2.3.3 Physical Activity

International physical activity questionnaire–short form (appendix 15) designed to capture the frequency, duration and intensity of physical activity undertaken during the previous seven days was used to assess physical activity of the participants in the trial-1 (section 2.6.1) and a long form version (appendix 16) for the participants in trial-2 (section 2.6.2) and trial-3 (section 2.6.3). All the participants were asked to maintain their baseline physical activity levels during the study period to avoid confounding effects on the outcome variables.

2.4 Randomisation

Allocation of intervention was performed by a software-based (Random Allocation Software 1.0.0) randomisation technique, using alpha-numeric codes (eg. 00A, 00B) in blocks of 8.

2.5 Interventions

- **Placebo** (PL, 2 x placebo tablets matching for curcumin plus 2 x 1000 mg corn oil capsules per day)
- **Curcumin** (CC, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg corn oil capsules per day)
- **LCn-3PUFA** (FO, 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2 g DHA + EPA plus 2 x placebo tablets matching for curcumin)
- **Curcumin plus LCn-3PUFA** (CC-FO, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA + EPA)

2.6 Clinical Trials

2.6.1 Trial 1- Acute postprandial cross over study

Ethics approval and trial registration

The study protocol was approved by the University of Newcastle Human Research Ethics Committee (H-2014-0385). The study was registered on Australian New Zealand Clinical Trials Registry (ANZCTR) under study number ACTRN12618000047291.

Study aims and design

- To examine the effects of a single dose of curcumin and/or LCn-3PUFA on postprandial glucose responses in healthy individuals.
- To examine, the effects of a single dose of curcumin and/or LCn-3PUFA on postprandial insulin and TG in the same individuals.

This randomised, placebo controlled, single blinded (participants), cross over study consisted of 4 test days, each separated by a wash out period of one week. Participants received a single dose of one of the following dietary supplements just prior to consuming the standardised breakfast, in a random order according to the computer generated sequence (block size of four): Placebo (PL, 2 x placebo tablets matching for curcumin plus 2 x 1000 mg corn oil capsules per day), curcumin (CC, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg corn oil capsules per day), LCn-3PUFA (FO, 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2 g DHA + EPA plus 2 x placebo tablets matching for curcumin) and curcumin plus LCn-3PUFA (CC-FO, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA + EPA).

Sample size

The primary outcome for this study was change in blood glucose (Δglucose) levels at 60 min. Data from our previous study (204) indicate that the difference in the response of subjects is normally distributed with standard deviation 0.11. With the expected 10% difference in the mean Δglucose response at 60 min with a probability (power) 0.8, a sample size of n=12 subjects were required in a cross-over design. The Type I error probability associated with this test of this null hypothesis is 0.05.

Primary and secondary outcome

The primary outcome for this study was change in blood glucose (Δglucose) levels at 60 min, post meal consumption. Secondary outcome measures include postprandial insulin (PI) and TG.

Statistical analysis

The data of all variables included in the analysis were tested for normality by deriving studentized residuals, kernel density plots and Shapiro-Wilk's tests and are presented as mean ± standard deviation (SD) or median (interquartile range, IQR) as appropriate. Log transformation was used for variables with non-normal distribution. Differences between fasting concentrations of the biochemical variables, diet and macronutrient intake were evaluated by repeated measures analysis of variance. Change Area under the curve (AUC) (0-120) for postprandial blood glucose (PBG), PI and TG responses were constructed using trapezoid rule. Differences between the study interventions were tested for statistical significance using repeated measures analysis of variance (ANOVA). When this analysis was significant, a paired t-test or Wilcoxon signed-rank test was performed to compare the interventions pairwise. Analysis of covariance (ANCOVA) and two-way ANOVA was used to evaluate the effects of confounding variables on primary outcomes. Significance was set at $p<0.05$. All statistical analyses were conducted using Stata version 14.1 (StataCorp, Texas, USA).

2.6.2 Trial 2 - **C**urcumin and/or **O**mega-3 polyunsaturated fatty acids for **P**revention of T2**D** (COP-D trial)

Ethics approval and trial registration

This randomised controlled trial was approved by the UON HREC (H 2014-0385) and the Hunter New England Area Health Service Human Research Ethics Committees (HNEHREC) (16/03/16/3.02). The trial has been registered with Australia New Zealand Clinical Trial Registry (ACTRN12615000559516).

Study aims and design

- To evaluate the effects of curcumin and/or LCn-3PUFA supplementation on glycaemic control.
- To evaluate the effects of curcumin and /or LCn-3PUFA supplementation on blood lipid levels and inflammation.

This was a twelve-week 2x2 factorial, double-blinded, randomised controlled trial. Participants who attended the baseline visit at Nutraceutical Research Program clinical trial facility or John Hunter hospital, were randomised to one of the four interventions: Placebo (PL, 2 x placebo tablets matching for curcumin plus 2 x 1000 mg corn oil capsules per day), curcumin (CC, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg corn oil capsules per day), LCn-3PUFA (FO, 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2 g DHA + EPA plus 2 x placebo tablets matching for curcumin) and curcumin plus LCn-3PUFA (CC-FO, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA + EPA).

Inclusion and exclusion criteria

Participants with high risk of developing T2D (assessed through Australian diabetes risk assessment tool, AUSDRISK) (appendix 19) or with IFG or IGT were screened through telephone interviews based on the inclusion criteria: age between 30-70 years; BMI must lie between 25-45 kg/m^2; diagnosed either with IFG (FPG 6.1-6.9 mmol/L), IGT (2-hour plasma glucose ≥7.8 mmol/L - <11.1 mmol/L) or both; HbA1c levels lie between 5.7-6.4% or they obtain a score of 12 or more in the AUSDRISK tool assessment (a non-invasive questionnaire to determine the risk of developing diabetes). Exclusion criteria included diagnosis with T2D; history of severe neurological diseases or seizures; gall bladder problems; pregnancy or planning to become pregnant or breastfeeding; on pacemakers; consuming >2 serves of oily fish per week or take supplements known to influence blood glucose levels.

Sample size

Sample size was calculated using a computer program (PS Power and Sample Size Calculations Version 3.0), based on the previous data on mean changes in HbA1c (standard deviation of 0.5 units). Seventeen participants in each intervention group were required to give 80% power at 0.05 significance level for detection of 10% reduction in HbA1c. Accounting for drop-out rate of 20% we needed to recruit 20 participants for each treatment group.

Primary and secondary outcome measures

The primary outcome in this trial was to evaluate the effects of curcumin and/or LCn-3PUFA on parameters relating to glucose control i.e. HbA1c, FPG, FI and IR.

Secondary outcomes included lipid profile (total cholesterol, TC; TG; HDL-C, HDL-C; LDL-C; and TC: HDL-C), C-reactive protein (CRP) and whole blood cell count.

Statistical analysis

Data collected at the baseline was analysed for normality using histograms and Shapiro-Wilk's test and presented as mean ± SEM or median (IQR) as appropriate. Significant changes in the baseline data between the groups were assessed through ANOVA or Kruskal-Wallis when the normality assumption was not met. Post-intervention data was presented as mean ± SEM or median (IQR) of absolute change (post-intervention value minus baseline value) for log transformed values and blood cell count and as relative change [(absolute change/baseline value) * 100] for other variables. Changes from the base-line to post-intervention within-treatment group were assessed through paired t-test or Wilcoxon signed-rank test. Significant effects of intervention on mean changes in the variables between the groups were measured using two-way ANOVA with post hoc (tukey's) analysis. Correlations between the independent variables and dependent variables were obtained using Pearson product-moment correlation coefficient analysis. Based on the significant correlation values, ANCOVA was performed on an optimal set of confounding variables.

2.6.3 Trial 3 - **C**urcumin **A**nd/or **L**ong-chain omega-3 polyunsaturated fatty acids **FOR** management of **C**ardio**V**ascular health in individuals with T2**D** (CALFOR-CVD trial)

Ethics approval and trial registration

This study was approved by the HNEHREC (16/07/20/3.02) and this approval was registered with UON HREC. The trial has been registered with Australia New Zealand Clinical Trial Registry (ACTRN12616001483448).

Study aims and design

- In the current study we aimed to evaluate effects of curcumin and LCn-3PUFA on dyslipidaemia in individuals with T2D.
- To evaluate the effects of curcumin and LCn-3PUFA on FPG and FI and SBP.

This was a six-week 2x2 factorial, double-blinded, randomised controlled trial. Participants were advised to attend two visits (baseline and post-intervention) at either Nutraceutical Research Program clinical trial facility or John Hunter hospital, during the study period. At the baseline visit participants were randomised to one of the four

interventions: PL, 2 x placebo tablets matching for curcumin plus 2 x 1000 mg corn oil capsules per day, CC, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg corn oil capsules per day, FO, 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2 g DHA + EPA plus 2 x placebo tablets matching for curcumin and CC-FO, 2 x 500 mg curcumin (Meriva®) tablets, providing 180 mg of curcumin plus 2 x 1000 mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA + EPA).

Inclusion and exclusion criteria

Study participants were screened in person or though telephone based on the inclusion criteria: age between 40 and 75 years; diagnosed with T2D (duration less than 15 years); body mass index (BMI) between 25 and 45 kg/m^2; if willing not to change their usual medication during the study period. Exclusion criteria included; pregnancy or planning to become pregnant or breastfeeding; failure to provide informed consent; diagnosed with type 1 diabetes; currently on insulin treatment; diagnosed with cancer; glomerular filtration rate less than 45; diagnosed with painful peripheral neuropathy; history of severe neurological diseases (Parkinson's, multiple sclerosis, epilepsy); consuming more than 2 serves of oily fish per week; taking regular dietary supplements known to influence blood lipid levels; sensitivity/ intolerance to the products involved in this study; unwilling to fast for 10 hours before giving blood sample.

Sample size

Based on the data (standard deviation - 0.5 unit) of the TG levels of people with T2D from previous study (205), a sample size of seventeen participants in each treatment group were required to give 80% power at 0.05 significance level for detecting a 0.5 mmol/L drop in TG (calculated using PS Power and Sample Size Calculations Version 3.0). To allow for dropouts we needed to recruit 4x20 = 80 participants according to the inclusion criteria.

Primary and secondary outcomes

The primary outcome in this trial was to evaluate the effects of curcumin and/or LCn-3PUFA on serum TG. Secondary outcomes included other blood parameters TC; HDL-C, LDL-C, and TC: HDL-C, atherogenic index of plasma (AIP), CRP, FPG, FI and liver function tests (Aspartate transaminase, AST; and Alanine transaminase, ALT).

Statistical analysis

Data collected at the baseline was tested for normality using histograms and Shapiro-Wilk's test and presented as mean ± SEM or median (IQR) as appropriate. Changes from the base-line to post-intervention within-treatment group were assessed through paired t-test or Wilcoxon signed-rank test. Significant changes in the baseline data between the groups were assessed through ANOVA or Kruskal-Wallis for non-parametric data. Post-intervention data was presented as mean absolute change (post-intervention value minus baseline value). Significant effects of intervention on mean changes in the variables between the groups were measured using two-way ANOVA with post hoc (tukey's) analysis.

2.7 Blood collection

A 25 ml fasting (at-least for 10 hours) blood samples were collected from participants according to the standard operating procedures at the nutraceutical research program during baseline and post-intervention visits in COP-D and CALFOR-CVD studies into ethylenediaminetetraacetic acid (EDTA), fluoride/oxalate and serum clot + Gel + Clot activator vacutainers by a trained phlebotomist. In the study 1, both finger prick and venous blood was collected for estimating the required biochemical measurements. Blood collected into EDTA tubes, was then centrifuged at 1000xg for 15 minutes, aliquoted and stored at -80^{O}C until further analysis of erythrocyte fatty acids. Blood collection was terminated if the participant experienced any adverse reactions or wishes to terminate the procedure. Participants will be advised to avoid lifting anything heavy for 24 hr after blood collection. In case of any adverse event, principal investigator was notified with-in 24 hours of occurrence.

2.8 Biomarker analysis

2.8.1 Markers of glycaemic control

HbA1c (marker of long term glycaemic control), FPG (marker of current BGL status), FI, fructosamine (marker of short term glycaemic control) were used as markers of glycaemic control. HbA1c was measured through Bio-Rad Variant II HbA1c testing system by Hunter Area Pathology Service (HAPS), an accredited pathology laboratory for compliance with National Pathology Accreditation Advisory Council Standards. FPG and FI was measured using standard laboratory techniques by HAPS.

2.8.2 Lipid parameters

TC, TG, LDL-C, HDL-C and TC: HDL-C were analysed using standard laboratory techniques by HAPS.

2.8.3 Inflammation and other markers

Serum high sensitive CRP and whole blood cell count, AST and ALT was measured using VP autoanalyzer by HAPS. BP was measured using digital sphygmomanometer (Microlife®, Switzerland). Average of three SBP and diastolic blood pressure (DBP) readings was considered as the final measurement. Erythrocyte fatty acids were determined using direct transesterification followed by gas chromatography (Hewlett Packard 7890A Series GC with Chemstations Version A.04.02) (290).

2.8.4 Formula based markers

AIP values are derived by using formula log (TG: HDL-C) (207). Homeostasis model assessment (HOMA) (www.dtu.ox.ac.uk/homacalculator) calculator was used to estimate IR and IS (HOMA %S).

2.8.5 Safety monitoring and compliance

The study coordinator will inform the principal investigator of all adverse events (AEs) and serious adverse events (SAEs), who will then follow procedures for unbinding as necessary and notify the relevant bodies. Depending on the nature of AE or SAE, it may be necessary for intervention to cease and/or for the participant to be withdrawn from the study. All SAE that may be related or unrelated to the investigational product will be documented in the SAE form and will be reported immediately to the HREC. Should there be a serious adverse event deemed related to the investigative treatment that increases the risks to participants, the study will be stopped, an investigation conducted, and findings generated before the study is resumed. The compliance to the study interventions were measured during the follow-up and post-intervention visit using a capsule count and capsule intake log. Erythrocyte fatty acid analysis is also used to check the compliance for LCn-3PUFA.

CHAPTER 3

InsuTAG: A novel physiologically relevant predictor for insulin resistance and metabolic syndrome

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(Statement of authorship is attached as appendix. Appendix-19)

3.1 Abstract

The aim of this study was to investigate whether a novel physiologically relevant marker, InsuTAG (fasting insulin X fasting triglycerides) can predict insulin resistance (IR) and metabolic syndrome (MetS). Data of 618 participants from the Retirement Health and Lifestyle Study (RHLS) were evaluated for the current study. IR was defined by homeostatic model assessment (HOMA-IR) scores. Pearson correlations were used to examine the associations of InsuTAG with HOMA-IR and other markers. Predictions of IR from InsuTAG were evaluated using multiple regression models. Receiver operating characteristic curves (ROC) were constructed to measure the sensitivity and specificity of InsuTAG values and to determine the optimum cut-off point for prediction of IR. InsuTAG was positively correlated with HOMA-IR ($r=0.86$; $p<0.0001$). InsuTAG is a strong predictor of IR accounting for 65.0% of the variation in HOMA-IR values after adjusting for potential confounders. Areas under the ROC curve showed that InsuTAG (0.93) was superior to other known lipid markers for predicting IR, with a sensitivity and specificity of 84.15% and 86.88%. Prevalence of MetS was significantly ($p<0.0001$) higher in subjects with InsuTAG values greater than optimal cut-off value of 11.2. Thus, InsuTAG appears to be a feasible marker of IR and MetS.

3.2 Introduction

The rise in sedentary lifestyle and ready access to calorie-dense processed foods in the modern-day world has created a convenient environment for the development of complex metabolic abnormalities in humans. Insulin, secreted by the pancreas, is one of the key anabolic hormones that tightly regulates glucose and lipid homeostasis (208). To maintain this homeostasis, insulin supresses hepatic glucose production, enhances glucose uptake in muscle and fat tissues, increases lipogenesis and regulates hepatic transport of very low-density lipoprotein (VLDL) associated triglycerides (TG) (208). Chronic over-nutrition results in disruption of insulin signalling pathways leading to increased hepatic secretion of VLDL and possible decrease in the clearance of TG rich lipoproteins. As a result, levels of circulating TG are increased (64), and this is commonly observed in insulin resistance (IR) and metabolic syndrome (65). Insulin-mediated suppression of hepatic glucose production is still preserved in IR, however, suppression of VLDL-TG secretion is less pronounced (66), indicating a rise in TG which may represent an early manifestation of IR. Cross-sectional studies (67, 68) and mechanistic studies (69) have shown a positive association between circulating TG levels and both IS and action. Kriketos et al. 2003 has shown that both skeletal muscle TG and circulating TG were inversely associated with whole body IS (69). Mingrone *et al* 1997 showed reversibility of IR by lowering plasma TG in obese individuals with diabetes (70); together these observations suggest that circulating TG may serve as a marker of IR and its associated complications.

Evidence of a relationship between TG and IR has escalated following the identification of "metabolic syndrome" (MetS), a condition representing a cluster of metabolic abnormalities with central obesity and IR postulated as core components (209-211). A considerable body of evidence suggests that obesity-associated IR is an independent risk factor and a central component in the pathogenesis of T2D and cardiovascular disease (CVD), both of which have reached epidemic proportions worldwide (212, 213). Evidence that IR causes T2D comes from cross-sectional studies demonstrating the presence of IR in a majority of T2D patients, and prospective studies demonstrating the development of IR long before the onset of T2D (213, 214). Dyslipidaemia associated with IR increases the risk of the developing CVD in T2D (215, 216). It is therefore important to quantify IR and identify MetS for primary and secondary prevention of these metabolic diseases.

Recognition of the importance of IR have prompted the derivation of a number of indices and surrogate markers to quantify IR (60, 61). Among these, the hyperinsulinemic euglycaemic clamp (HEC) technique has been described as the gold standard, providing a direct measure of IR (62). However, cost, expertise, and the requirement for intravenous insulin infusions and frequent blood sampling limits the application of HEC in epidemiological studies and routine clinical investigations. Homeostatic model assessment (HOMA) is a simple minimally invasive model that predicts IR using fasting steady-state glucose and insulin levels and that has been shown to be highly correlated with clamp insulin sensitive index values (63). However, HOMA does not consider the level of blood lipids, such as TG or HDL-Cholesterol (HDL-C), despite lipid availability in circulation having an important role in IR and its metabolic complications. In the current study we propose and evaluate a novel tool for estimating IR, InsuTAG. This model is based on the product of fasting blood insulin and fasting blood lipid (TG) levels. InsuTAG is unique in its incorporation of a measure of hyperinsulinemia and a measure of circulating TG in the general population, for identification of IR and its metabolic complications.

3.3 Methods

This study is a sub-study of the Retirement Health and Lifestyle (RHLS), a cross-sectional study of Australians aged 65 years and older, living in the Central Coast of New South Wales, Australia. Inclusion and exclusion criteria for participants in RHLS study has been described in detail elsewhere (217, 218). In brief, participants were invited to participate in the RHLS if they were: aged between 65 years or over; living in the Wyong Shire or Gosford City local government areas and living independently in retirement villages or within the community. Participants were included in the present study if: their diabetic status could be determined; their plasma fasting glucose, insulin and TG levels were available; and if they were not taking any TG-lowering medication. Participants provided written informed consent and ethics approval for this study was obtained from the University of Newcastle Human Research Ethics Committee (reference no. H-2008-0431) and the Northern Sydney Central Coast Health Human Research Ethics Committee (reference no. 1001-031M). All testing was performed in accordance with the approved guidelines.

Demographic information, medical history, and information relating to medication and supplement intake were obtained from participants via interviewer-administered and self-administered questionnaire. Height and weight of study participants were measured using a portable stadiometer (design no. 1013522 Surgical and Medical products) and digital scales (Tanita HD 316 or Wedderburn UWPM150). BMI was calculated using the standard formula [weight (kg)/height $(m)^2$]. WC was measured with a non-elastic measuring tape at the midpoint between the iliac crest and coastal margin in the mid-auxiliary line. All anthropometric measurements were conducted by trained research officers.

Information on dietary intake was obtained from study participants using a self-administered semi-quantitative food frequency questionnaire (FFQ) adapted from a validated Commonwealth Scientific and Industrial Research Organisation Human Nutrition FFQ (219). Diet, energy and nutrient intake information was analysed using Food Works Professional (2009 edition, version 6.0.2562, Xyris software, Brisbane, Australia) in association with the following databases; Australia (fatty acids), Abbott products, AusFoods (brand) 2006, AusNut (all foods) 2007, and the New Zealand Vitamin and Mineral Supplement 1999.

Physical activity was assessed during the interviewer-administered questionnaire using questions designed to capture the frequency, duration and intensity of physical activity undertaken during the previous seven days. The questions were adapted from validated questionnaires measuring physical activity, and captured both incidental (e.g. household chores, gardening) and intentional (e.g. recreational sports, strength training) physical activity. Blood pressure measurements were taken with an OMRON 1A2 digital automatic blood pressure monitor in accordance with the "Measuring Blood Pressure" protocol published by the National Heart Foundation in 2008.

Blood samples were collected by trained phlebotomists following an overnight fast of at least 10 hours. Blood glucose control related parameters [fasting glucose (mmol/L), fasting insulin (mIU/L) and HbA1c], lipid profile [TG, total cholesterol, LDL-C and HDL-C; mmol/L] and CRP (mg/mol) were analysed by Hunter Area Pathology Service using standard laboratory procedures. Fasting insulin and glucose were used to measure IR using HOMA-IR scores (fasting insulin X fasting glucose/22.5). Study participants with HOMA-IR values ≥2.5 were categorised as insulin resistant. TyG index (fasting

glucose X fasting TG) and TG/HDL-C ratio were also assessed. InsuTAG was calculated by multiplying fasting insulin (mU/L) and fasting TG (mmol/L). Participants were categorised as having MetS according to IDF criteria for abdominal obesity (waist circumference ≥ 94cm for males, or ≥ 80cm for females) plus any two of the following conditions (or self-report of receiving treatment for any of those conditions): high TG (≥1.7mmol/L); high fasting glucose (≥5.6mmol/L); high BP (≥ 130mmHg systolic or ≥85mmHg diastolic); and/or low HDL-C (<1.03mmol/L for males, <1. 29 mmol/L for females).

The data of all variables included in the analysis were tested for normality using histograms and Shapiro-Wilk's tests and are presented as mean ± SD or median (IQR) as appropriate. Heavily skewed parameters were log-transformed (log base e) prior to correlation and regression analyses. Distributions were reassessed after transformation and in all instances the log transformation successfully achieved a normal distribution. Bivariate relationships between continuous variables were assessed using Pearson's Product-moment correlation. Standard multiple regression was used to assess the relationship of HOMA-IR with InsuTAG and other surrogate markers of IR, with and without adjustment for the potentially confounding variables: age, gender, WC and blood levels of CRP. Logistic regression was used to determine whether an increase in InsuTAG score was associated with increased odds of having IR or MetS. Receiver operating characteristic (ROC) curves were constructed for InsuTAG and other surrogate markers of IR to assess whether they were effective in identifying either IR and/or MetS. For each marker, the area under the curve (AUC) was compared against HOMA-IR using the Stata command *roccomp*. Youden's index was used to determine the optimum cut-off point, the point which has the greatest sensitivity and sensitivity, Participants were categorised according to whether their InsuTAG scores fell below or above the suggested cut-off point. Group differences were assessed using independent sample t-tests, Mann-Whitney U tests, or chi-squared analysis as appropriate. Significance was set at $p<0.05$. All statistical analyses were conducted using Stata version 14.1 (StataCorp, Texas, USA).

3.4 Results

After screening the 618 participants recruited for the RHLS study with exclusion criteria, 486 participants were included in the analysis. One hundred and thirteen participants were excluded because of self-reported diabetes. An additional 19 participants were excluded

because they had blood glucose levels ≥7 mmol/L (n=9); self-reported use of oral anti-hyperglycaemic agents (n=4); had no blood glucose values recorded (n=3); and/or self-reported taking TG lowering medications (n=3). Participant characteristics are outlined in Table 3.1. The study population was predominantly Caucasian (n=469, 96.50%), included more females (n=283, 58.23%) than males (n=203, 41.77%), had a mean ± SD age of 77.78 ± 7.16 years, and a mean ± SD body mass index (BMI) of 28.05 ± 4.64 kg/m^2. A total of 82 (16.87%) participants were categorised as IR according to HOMA-IR ≥ 2.5 and 167 (37.03%) were identified as having MetS according to the International Diabetes Federation (IDF) criteria. Fasting glucose, insulin, TG, weight, waist circumference (WC) and BMI were significantly ($p<0.0001$) higher in individuals with IR compared with insulin-sensitive individuals (Table 3.1). InsuTAG scores ranged from 0.55 to 132.07 across the whole participant group with a median (25th-75th percentile) IsnuTAG score of 7.22 (4.04-11.33)

There is a strong and highly significant positive correlation between InsuTAG and HOMA-IR ($r=0.86$; $p<0.0001$) (Figure 3.1) when compared with the association between HOMA-IR and the other lipid indices, TyG index (TGX glucose) ($r=0.43$; $p<0.001$) and TG/HDL-C ratio ($r=0.38$; $p<0.001$) (Table 3.2). InsuTAG is also positively associated with BMI ($r=0.51$, $p<0.001$) and WC ($r=0.48$, $p<0.001$).

Multiple regression analyses were used to evaluate the relationships between InsuTAG and other surrogate markers of IR with HOMA-IR (Table 3.3). Model 1 presents the unadjusted analyses. Model 2 present the analyses adjusted for age, gender, WC and C-reactive protein (CRP). All surrogate markers of IR were significant predictors of HOMA-IR, both independently and after adjusting for covariates ($p<0.0001$), however, InsuTAG accounted for a greater proportion of variability in HOMA-IR (Model 1, $R^2=0.739$; Model 2, $R^2=0.738$) than did the TyG index (Model 1, $R^2=0.183$; Model 2, $R^2=0.303$) or the TG/HDL-C ratio (Model 1, $R^2=0.145$; Model 2, $R^2=0.284$). Evaluation of the partial correlations indicated that, after adjusting for covariates, InsuTAG accounted for 65.0% of the variability in HOMA-IR scores, whereas TyG and TG/HDL-C were independently associated with only 7.9% and 5.4% of the change in HOMA-IR scores, respectively. Examination of the regression coefficient revealed that, after adjusting for covariates, a 10% increase in InsuTAG resulted in a corresponding increase of 2.57% in HOMA-IR ($p<0.0001$). In addition, logistic regression showed that after adjusting for age, gender, WC and CRP, each one-unit increase in InsuTAG increased the

odds of having IR by 20% (OR (95%CI): 1.20 (1.15-1.26), p<0.0001) and MetS by 16% (OR (95%CI): 1.16 (1.11-1.21), p<0.0001).

ROC curves of InsuTAG and surrogate markers of IR were plotted to compare the predictive values for IR and MetS (Figure 3.2). InsuTAG had significantly greater Area under the curve (AUC) values (AUC=0.93, p<0.001) than TyG index (AUC=0.72) or TG/HDL-C (AUC=0.70) for the identification of IR. For the identification of MetS, the AUC of InsuTAG (0.79) was significantly higher than HOMA-IR (0.73, p=0.001) (Figure 3.3), fasting insulin (0.69, p=0.000) and waist circumference (0.72, p=0.009). The optimal cut off value for InsuTAG in identifying IR in the current study population was determined to be 11.2, with a sensitivity of 84.15% and specificity of 86.88%. At this cut-off, the sensitivity and specificity for MetS was 49.70% and 90.49% respectively (Table 3.4). Positive and negative predictive values along with likelihood ratios are also presented in Table 3.4. After determining the cut-off values for InsuTAG, the study population were categorised into two groups: InsuTAG <11.2 (n=364), and InsuTAG ≥11.2 (n=122). 74.34% of participants with InsuTAG values ≥11.2 were identified with MetS compared to only 24.56% with InsuTAG values <11.2 (Table 5, p<0.0001). All the key components of the MetS, WC (104.65 ± 12.45 vs 94.51 ± 11.79 cm); fasting glucose (5.46 ± 0.51 vs 5.14 ± 0.54 mmol/L); fasting TG [1.87 (1.43-2.31) vs 1.01 (0.79-1.30) mmol/L]; and Total-C/HDL-C [3.60 (3.00-4.70) vs 2.90 (2.40-3.50)] were significantly (p<0.0001) higher in participants with InsuTAG values above the cut-off compared with participants InsuTAG values below the cut-off (Table 5). BMI, diastolic blood pressure, fasting insulin and HOMA-IR values were also significantly higher in the InsuTAG ≥ 11.2 group, whereas systolic blood pressure, glycosylated haemoglobin (HbA1c), total cholesterol and low density lipoprotein-cholesterol (LDL-C) did not differ between the two groups (Table 3.5). The lipid index values, TyG [10.29 (7.69-12.60) vs 5.25 (4.00-6.54)] and TG/HDL-C [1.41 (1.07-1.91) vs 0.66 (0.48-0.89)] were almost doubled in participants with InsuTAG values above the cut-off.

Table 3.1: Participant characteristics of all participants and for participants stratified into subgroups by IR status

	All Participants n = 486	Insulin Sensitive n = 404	Insulin Resistant n = 82	*p*-value
Gender (n, (%))				
Male	203 (41.77)	172 (42.57)	31 (37.80)	0.425 *
Female	283 (58.23)	232 (57.43)	51 (62.20)	0.425 *
Age (years)	77.78 ± 7.16	78.03 ± 7.24	76.54 ± 6.62	0.085
Waist Circumference (cm)	97.03 ± 12.73	95.21 ± 12.06	106.34 ± 12.03	<0.0001
Height (m)	1.63 ± 0.09	1.63 ± 0.09	1.63 ± 0.10	0.837
Weight (kg)	74.37 ± 14.59	72.36 ± 12.81	84.28 ± 14.35	<0.0001
BMI (kg/m^2)	28.05 ± 4.64	27.29 ± 4.36	31.76 ± 4.18	<0.0001
Metabolic Syndrome† (n, (%))	167 (37.03)	112 (29.55)	55 (76.39)	<0.0001
Ethnicity (n, (%))				
Caucasian	469 (96.50)	392 (97.03)	77 (93.90)	0.160 ‡
Aboriginal/Pacific Islander	6 (1.23)	5 (1.24)	1 (1.22)	0.998 ‡
Asian	1 (0.21)	0 (0.00)	1 (1.22)	0.026 ‡
Don't know/didn't respond	10 (2.06)	7 (1.73)	3 (3.66)	0.261 ‡
Dietary Energy Intake (Cal/day)	1971 ± 725	1968 ± 735	1983 ± 679	0.865
Protein (g/day)	90.82 ± 35.20	90.87 ± 35.63	90.58 ± 33.25	0.946
Fat (g/day)	68.95 ± 31.87	69.42 ± 32.89	66.67 ± 26.46	0.478
Saturated fat (%Energy/day)	10.69 ± 3.24	10.73 ± 3.38	10.50 ± 2.43	0.562
Fibre (g/day)	31.81 ± 14.90	31.99 ± 15.25	30.91 ± 13.08	0.549
Physical Activity§ (n, (%))				
High	120 (25.16)	108 (27.20)	12 (15.00)	0.020 ‡
Moderate	263 (55.14)	213 (53.65)	50 (62.50)	0.141 ‡
Low	74 (15.51)	62 (15.62)	12 (15.00)	0.888 ‡
Sedentary	20 (4.19)	14 (3.53)	6 (7.50)	0.102 ‡
Systolic Blood Pressure (mmHg)	145.99 ± 20.90	146.00 ± 21.68	145.94 ± 15.99	0.984
Diastolic Blood Pressure (mmHg)	74.64 ± 9.70	74.30 ± 9.64	76.58 ± 9.91	0.090
Fasting Glucose (mmol/L)	5.22 ± 0.55	5.14 ± 0.51	5.64 ± 0.54	<0.0001
Fasting Insulin (μIU/L)	6.0 (4.0-8.6)	5.4 (3.8-7.2)	13.1 (12.0-16.8)	<0.0001 ‖
HbA1c (%)	5.72 ± 0.34	5.70 ± 0.31	5.85 ± 0.44	0.0002
HbA1c (mmol/mol)	39 ± 3.7	39 ± 3.4	40 ± 4.8	0.0002
Total Cholesterol (mmol/L)	4.69 ± 1.02	4.72 ± 1.01	4.57 ± 1.08	0.241
Triglycerides (mmol/L)	1.15 (0.86-1.55)	1.10 (0.83-1.45)	1.54 (1.01-2.00)	<0.0001 ‖
LDL-C (mmol/L)	2.58 ± 0.91	2.58 ± 0.91	2.53 ± 0.94	0.645
HDL-C (mmol/L)	1.49 (1.23-1.74)	1.53 (1.29-1.77)	1.25 (1.06-1.54)	<0.0001 ‖
Total-C/HDL-C	3.10 (2.50-3.80)	3.00 (2.50-3.70)	3.35 (2.80-4.30)	0.002
HOMA-IR	1.39 (0.90-2.06)	1.20 (0.84-1.69)	3.20 (2.87-4.04)	<0.0001 ‖
TyG	5.88 (4.46-8.12)	5.61 (4.13-7.47)	8.16 (5.88-11.12)	<0.0001 ‖
TG/HDL-C	0.77 (0.54-1.24)	0.72 (0.51-1.13)	1.18 (0.70-1.77)	<0.0001 ‖

Data reported as count, mean ± SD or median (IQR, expressed as the 25^{th}-75^{th} percentile) unless otherwise specified. Insulin resistance categorised as HOMA-IR ≥ 2.5. MetS categorised according to IDF criteria. Difference between groups (Insulin Sensitive versus Insulin Resistant) assessed using two-tailed independent sample t-tests unless otherwise specified. * Categorical data assessed using chi-squared analysis. † Incomplete data for MetS for 35 participants (Insulin sensitive: n=25; Insulin resistant: n=10). ‡ Differences assessed using a two-sample test of proportion. § Incomplete data for physical activity for 9 participants (Insulin sensitive: n=7; Insulin resistant: n=2). ‖ Non-parametric data assessed using Mann-Whitney U test. BMI: body mass index. Total-C/HDL-C: Total cholesterol/HDL-cholesterol. HOMA-IR: Homeostatic model assessment for Insulin Resistance. TyG: Triglycerides x glucose. TG/HDL-C: Triglycerides/HDL-cholesterol

Table 3.2: Correlations between contributors to insulin resistance, InsuTAG, and other surrogate markers of insulin resistance

	Age	BMI	WC	CRP	InsuTAG†	HOMA-IR†	T:HDLratio	TyG†	TG/HDL-C
Age (years)	-	-0.14**	-0.08	-0.01	-0.10*	-0.09*	-0.15***	-0.07	-0.07
BMI (kg/m^2)	-0.14*	-	0.79***	0.24***	0.51***	0.50***	0.21***	0.35***	0.35***
WC (cm)	-0.08	0.79***	-	0.16***	0.48***	0.47***	0.24***	0.35***	0.40***
CRP (mg/L)	-0.01	0.24***	0.16***	-	0.18***	0.15***	0.12**	0.12**	0.14**
InsuTAG†	-0.10	0.51***	0.48***	0.18***	-	0.86***	0.47***	0.79***	0.75***
HOMA-IR†	-0.09	0.50***	0.47***	0.15***	0.86***	-	0.23***	0.43***	0.38***
Total-C/HDL-C	-0.15**	0.21***	0.24***	0.12**	0.47***	0.23***	-	0.58***	0.73***
TyG†	-0.07	0.35***	0.35***	0.12**	0.79***	0.43***	0.58***	-	0.91***
TG/HDL-C†	-0.07	0.35***	0.40***	0.14**	0.75***	0.38***	0.73***	0.91***	-

Pearson product-moment correlation coefficients are presented. †Data $\log_e$ transformed prior to analysis. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. BMI: Body Mass Index. WC: Waist Circumference. CRP: C-reactive protein. InsuTAG: Fasting insulin (μIU/L) x triglycerides (mmol/L). HOMA-IR: Fasting glucose (mmol/L) x insulin (μIU/L). Total-C/HDL-C: Total cholesterol (mmol/L)/HDL cholesterol (mmol/L). TyG: Fasting triglycerides (mmol/L) x glucose (mmol/L). TG/HDL-C: Fasting triglycerides (mmol/L)/HDL-cholesterol (mmol/L).

Table 3.3: Regression models for predicting IR

Surrogate Markers of IR	Model 1				Model 2					
	Model Coefficients		*Model Statistics*		*Model Coefficients*				*Model Statistics*	
	β-coefficient	p	adj. R^2	p	β-coefficient	Partial R^2	Semi-Partial R^2	p	adj. R^2	p
InsuTAG			0.739	<0.0001					0.738	<0.0001
InsuTAG*	0.637	<0.0001			0.613	0.650	0.487	<0.0001		
Age	-	-			0.0002	<0.0001	<0.0001	0.930		
Gender	-	-			-0.022	0.0009	0.0002	0.524		
WC	-	-			0.003	0.0077	0.002	0.058		
CRP	-	-			0.00005	<0.0001	<0.0001	0.988		
TyG			0.183	<0.0001					0.303	<0.0001
TyG*	0.559	<0.0001			0.342	0.079	0.060	<0.0001		
Age	-	-			-0.003	0.002	0.001	0.406		
Gender	-	-			0.176	0.022	0.016	0.001		
WC	-	-			0.020	0.149	0.120	<0.0001		
CRP	-	-			0.007	0.003	0.002	0.214		
TG/HDL-C Ratio			0.145	<0.0001					0.284	<0.0001
TG/HDL-C*	0.380	<0.0001			0.217	0.054	0.041	<0.0001		
Age	-	-			-0.003	0.001	0.001	0.426		
Gender	-	-			0.214	0.032	0.024	<0.0001		
WC	-	-			0.021	0.157	0.132	<0.0001		
CRP	-	-			0.006	0.003	0.002	0.260		

*IR defined by HOMA-IR value, InsuTAG, TyG and TG/HDL-C loge transformed prior to analysis. Model 1: Unadjusted estimates. Model 2: Adjusted for age, gender, waist circumference, CRP. A 1% increase in InsuTAG corresponds with a 0.265% increase in HOMA-IR or a 10% increase in InsuTAG corresponds with an increase to HOMA-IR of 2.57%

Table 3.4: Predictive values of proposed InsuTAG cut-off of 11.2 for the identification of IR and MetS

	Sensitivity	Specificity	PV +	PV -	LR +	LR -
Insulin Resistance	84.15%	86.88%	0.56	0.96	6.41	0.18
Metabolic Syndrome	49.70%	90.49%	0.75	0.75	5.23	0.56

IR classified according to HOMA-IR ≥ 2.5. MetS classified according to IDF criteria. PV+: Positive predictive value. PV-: Negative predictive value. LR+: Positive likelihood ratio. LR-: Negative likelihood ratio.

Table 3.5: Participant characteristics and metabolic parameters of participants stratified into subgroups according to the proposed InsuTAG cut-off of 11.2.

	InsuTAG < 11.2 n = 364	InsuTAG ≥ 11.2 n = 122	*p*-value
Gender (n, (%))			
Male	153 (42.03)	50 (40.98)	0.839 *
Female	211 (57.97)	72 (59.02)	0.839 *
Age (years)	78.18 ± 7.20	76.57 ± 6.94	0.031
Waist Circumference (cm)	94.51 ± 11.79	104.65 ± 12.45	<0.0001
Height (m)	1.63 ± 0.09	1.62 ± 0.10	0.762
Weight (kg)	71.62 ± 13.43	82.57 ± 14.87	<0.0001
BMI (kg/m^2)	26.96 ± 4.21	31.25 ± 4.36	<0.0001
Metabolic Syndrome† (n, (%))	84 (24.56)	83 (74.34)	<0.0001 *
Dietary Energy Intake (Cal/day)	1959 ± 732	2008 ± 705	0.516
Protein (g/day)	90.98 ± 36.30	90.36 ± 31.80	0.868
Fat (g/day)	68.89 ± 32.59	69.10 ± 29.75	0.951
Saturated Fat (%Energy/day)	10.73 ± 3.41	10.59 ± 2.68	0.671
Fibre (g/day)	31.68 ± 14.80	32.18 ± 15.22	0.749
Physical Activity‡ (n, (%))			
High	95 (26.61)	25 (20.83)	0.203 §
Moderate	194 (54.34)	69 (57.50)	0.544 §
Low	55 (15.41)	19 (15.83)	0.912 §
Sedentary	13 (3.64)	7 (5.83)	0.269 §
Systolic Blood Pressure (mmHg)	145.22 ± 21.25	148.49 ± 19.63	0.183
Diastolic Blood Pressure (mmHg)	74.03 ± 9.77	76.64 ± 9.26	0.021
Fasting Glucose (mmol/L)	5.14 ± 0.54	5.46 ± 0.51	<0.0001
Fasting Insulin (μIU/L)	5.0 (3.7-6.9)	11.3 (8.5-14.4)	<0.0001 ‖
HbA1c (%)	5.71 ± 0.32	5.76 ± 0.39	0.213
HbA1c (mmol/mol)	39 ± 3.20	39 ± 4.30	0.213
Total Cholesterol (mmol/L)	4.66 ± 1.00	4.78 ± 1.07	0.269
Triglycerides (mmol/L)	1.01 (0.79-1.30)	1.87 (1.43-2.31)	<0.0001 ‖
LDL-C (mmol/L)	2.56 ± 0.89	2.63 ± 0.97	0.443
HDL-C (mmol/L)	1.57 (1.32-1.82)	1.26 (1.08-1.52)	<0.0001 ‖
Total-C/HDL-C	2.90 (2.40-3.50)	3.60 (3.00-4.70)	<0.0001
HOMA-IR	1.15 (0.82-1.62)	2.67 (2.05-3.55)	<0.0001 ‖
TyG	5.25 (4.00-6.54)	10.29 (7.69-12.60)	<0.0001 ‖
TG/HDL-C	0.66 (0.48-0.89)	1.41 (1.07-1.91)	<0.0001 ‖

Data presented as mean ± SD or median (IQR, expressed as the 25th-75th percentile) unless otherwise specified. Metabolic Syndrome categorised according to IDF criteria. Differences between groups (InsuTAG <11.2 versus InsuTAG ≥ 11.2) assessed using two-tailed independent sample t-test unless otherwise specified. * Differences assessed using chi-squared analysis. † Incomplete data for metabolic syndrome for 35 participants (InsuTAG < 11.2: n=26; InsuTAG ≥ 11.2: n=9). ‡ Incomplete data for physical activity for 9 participants (InsuTAG < 11.2: n=7; InsuTAG ≥ 11.2 n=2). § Differences assessed using a two-sample test of proportion. ‖ Non-parametric data assessed using Mann-Whitney U test. BMI: body mass index. Total-C/HDL-C: Total cholesterol/HDL-cholesterol. HOMA-IR: Homeostatic model assessment for Insulin Resistance. TyG: Triglycerides x glucose. TG/HDL-C: Triglycerides/HDL-cholesterol.

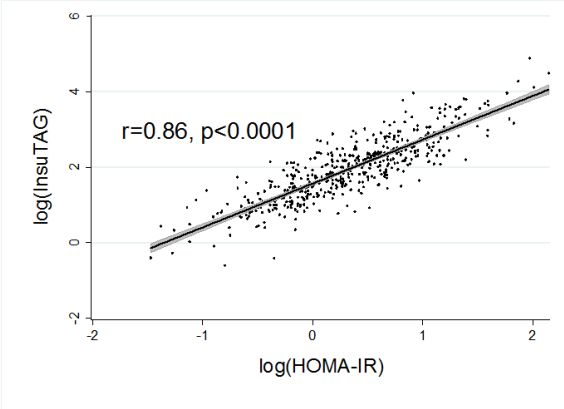

Figure 3.1: Scatterplot of InsuTAG and HOMA-IR.
InsuTAG and HOMA-IR both $\log_e$ transformed. Solid black line: line of best fit. Grey shaded area: 95% confidence interval

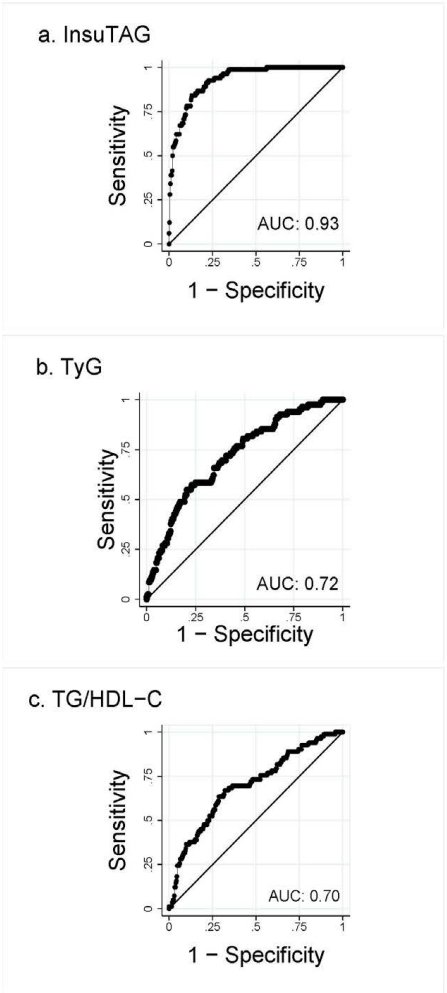

Figure 3.2: Receiver operating characteristic (ROC) curves for identifying Insulin Resistance (IR) using surrogate markers of IR.

IR categorised as HOMA-IR ≥ 2.5. ROC curve for InsuTAG (a); ROC curve for TyG (b); ROC curve for TG/HDL-C (c). AUC: Area under the curve.

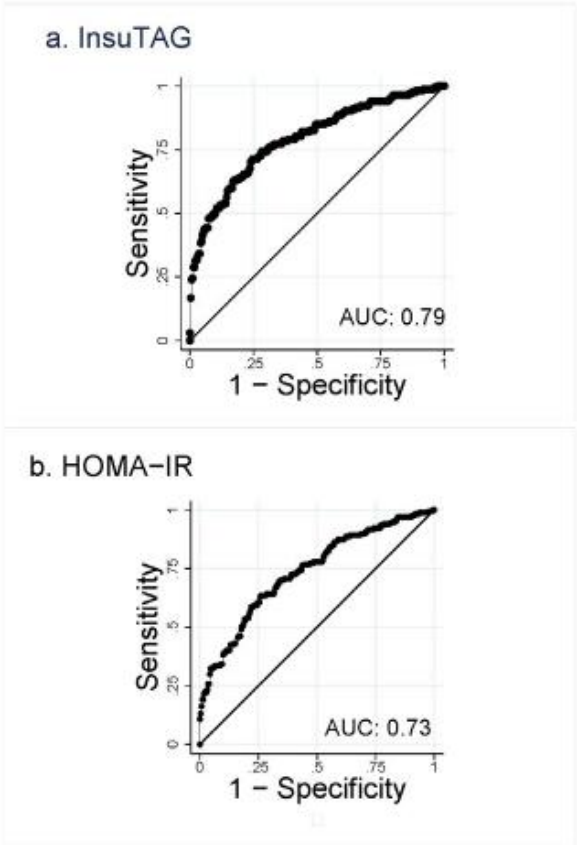

Figure 3.3: Receiver operating characteristic (ROC) curves for identifying Metabolic Syndrome (MetS) using InsuTAG and HOMA-IR.
MetS categorised according to IDF criteria. ROC curve for InsuTAG (a); ROC curve for HOMA-IR (b). AUC: Area under the curve.

3.5 Discussion

Many studies have evaluated lipid ratios, homeostatic models and individual metabolic variables for predicting IR (68, 220, 221), however, none account for fasting insulin and fasting TG in a single model or formula. The current study has evaluated the use of InsuTAG, a novel marker derived from the product of two key continuous variables, fasting insulin and fasting TG, as a predictor for IR and metabolic abnormalities. InsuTAG demonstrated a stronger positive association (0.86) with HOMA-IR than the other individual metabolic markers and lipid surrogate markers analysed in this study. InsuTAG, TyG and TG/HDL-C were all independent predictors of IR in regression models; InsuTAG was the highest (65.0%) contributor to prediction of IR in the study population. ROC analysis also indicated InsuTAG (AUC 0.93) was the favourable marker over TyG index and TG/HDL-C for predicting IR.

HOMA-IR is the most common or frequently used index for assessing IR and closely mirrors HEC values (222). Given that it is not feasible to conduct HEC in large study populations, in this study HOMA-IR scores of ≥2.5 were used to identify IR. Previous studies proposed surrogate lipid-based markers such as TyG index and TG/HDL-C ratio as an alternate approach to predict IR (68, 223). Consistent with previous reports (220, 224, 225), the current study found significant and positive associations between HOMA-IR and BMI, WC and lipid ratios. InsuTAG demonstrated a strong positive association with HOMA-IR, representing a close association with IR, higher than that of other lipid-based surrogate markers and anthropometric measurements analysed in this study population. Multivariate regression analysis indicated that both TyG index and TG/HDL-C were independent predictors for IR, findings that are consistent with previous published studies (69, 220). Amongst these independent predictors, InsuTAG accounted for the greatest variance in IR for this study population, accounting for 73.9% (without adjusting for covariates) and 65% (after adjusting for covariates) of variance in HOMA-IR. Along with the benefit of accounting for two key factors involved in the development of IR, these results indicate that InsuTAG may be a reliable and physiologically relevant marker for IR that can be easily calculated from routine clinical investigations. However, as HOMA-IR is not a gold standard method of determining IR, further validation studies of the predictive capacity of InsuTAG using clamp study IS index values are required.

AUC values from the ROC analysis showed that InsuTAG represented 93% probability of identifying individuals with IR in this study population, comparatively greater than the AUC's of other lipid markers. The high sensitivity and specificity values of InsuTAG and positive likelihood ratio of 6.41 (Table 4) provides reasonable justification to explore InsuTAG as a diagnostic tool for IR. ROC analysis identified 11.2 as the optimal cut-off value for predicting IR in this study population. The corresponding value for identification of MetS was 8.0. The AUC for InsuTAG (0.79) suggests it is higher to HOMA-IR (0.73), fasting insulin (0.69) and waist circumference (0.72) for predicting MetS in this study population. Metabolic characteristics of participants with InsuTAG scores above and below the suggested cut-off were compared. Significant differences in WC, BMI, fasting glucose, fasting insulin, TG, HDL-C and blood pressure, all of which are key components of MetS, were observed. There was no significant difference in participant characteristics between sub-groups stratified by the InsuTAG cut-off values

for predicting IR (11.2) and MetS (8.0), other than minor changes in sensitivity and specificity values for MetS. No further conclusions on these associations can be made at this point warranting further exploration in prospective studies to determine whether InsuTAG alone can predict the development of MetS.

We do acknowledge some limitations to this study. The study population is older and predominantly Caucasian, limiting the generalisability of InsuTAG to younger people and other ethnicities. Since it is less feasible to conduct HEC in studies with larger sample sizes, InsuTAG values were compared with HOMA-IR values rather than IS index values from clamp studies. Additional exploration of InsuTAG using glucose clamp studies is required to further validate this marker. Furthermore, because of the cross-sectional design of this study, no causal relationships can be determined. Longitudinal studies are required to evaluate whether InsuTAG can predict development of IR and MetS.

Surrogate markers for IR are less invasive and closely mirror correlation with HEC for metabolic and CVD risk. A recently published paper [(20)] on correlation of surrogate indices with HEC, concluded that surrogate markers including fasting insulin provided the most information relating to IR, compared with other complex and invasive procedures. The combination of fasting insulin and an indicator of reduced lipid clearance could provide more reliable information on IR and metabolic abnormalities.

In conclusion, we have proposed and evaluated a novel marker for IR that accounts for both fasting insulin and TG. It is simple to calculate and feasible for large cohort studies. This study substantiates and shows InsuTAG as a predictor of IR and a predictor of MetS with higher sensitivity and specify values over other anthropometry and existing lipid surrogate indices. Further research is required to validate InsuTAG against HEC and determine whether it can accurately predict the development of IR and MetS in prospective studies.

CHAPTER 4

Curcumin alleviates postprandial glycaemic response in healthy subjects: A cross-over, randomized controlled study

The contents of this chapter have been submitted for publication:

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(Statement of authorship is attached as appendix-20)

4.1 Abstract

Background and Aims: Reduction in postprandial glycaemic response may lower the risk of developing diabetes and cardiovascular disease. In the current study, we evaluated the effects of a single dose of curcumin and/or long-chain omega-3 polyunsaturated fatty acids on postprandial glucose and insulin responses in healthy individuals.

Methods and Results: This was a randomised, placebo controlled, single-blinded, crossover study. Sixteen (n=16) healthy volunteers were randomised to receive either double placebo (PL), curcumin (CC), fish oil (FO) and curcumin plus fish oil (CC-FO) capsules/caplets prior to a standard meal on 4 test days separated by 1-week wash out period. Blood glucose, serum insulin and TG were measured at regular intervals between 0-120 min. Difference between the treatments was measured using repeated measures analysis of variance and pair-wise comparisons using Wilcoxon signed-rank or paired t-test as appropriate. Blood glucose levels were significantly lower in the CC (60.6%, P=0.0007), CC-FO (51%, P=0.002) and FO (29.7%, P=0.046) treatment groups at 60 min following the meal consumption compared with PL group. Compared with PL, change area under the curve (AUC) for change in blood glucose level was reduced by CC (36%, P=0.003) and CC-FO (30%, 0.004), but not FO alone (p=0.105). Both CC (P=0.01) and CC-FO (P=0.03) treatments significantly lowered the meal stimulated postprandial insulin response (AUC) by 26% in comparison with PL.

Conclusions: Curcumin, but not fish oil, reduces postprandial glycaemic response and insulin demand for glucose control. Dietary supplementation with curcumin has the potential to reduce the risk of developing metabolic abnormalities.

Key words: curcumin, long-chain omega-3 polyunsaturated fatty acids, postprandial glucose, postprandial insulin, postprandial triglycerides, T2D.

4.2 Introduction

Chronic elevation in postprandial blood glucose (PBG) and concomitant increase in insulin levels by calorie-dense foods has detrimental effects on metabolic regulation in many insulin sensitive tissues such as liver, pancreas and muscle in healthy individuals (226). It has been hypothesised that sustained levels of high PBG increase oxidative stress and activation of pro-inflammatory pathways, eventually leading to the development of chronic metabolic diseases such as diabetes and CVD (227). Long-term studies with pharmacological therapies such as α-glucosidase inhibitors (228, 229) that are effective in lowering PBG, have shown to reduce diabetes and cardio-metabolic disease risk. In recent years, interest in the relationship between diet and health has promoted the use of nutrients or dietary components (such as fibre, resistant starch) for lowering the PBG (230, 231). Currently these dietary interventions are being subjected to rigorous scientific evaluation for their beneficial effects in either preventing or delaying the onset of the metabolic diseases.

Curcumin, a dietary bio-active compound derived from turmeric, is currently being evaluated in multiple clinical trials for its beneficial effects in diabetes and CVD (232, 233). *In-vitro* studies demonstrated potent inhibitory effects of curcuminoids and its synthetic analogues on α-glucosidase enzyme and α-amylase, the key regulators of carbohydrate digestion (234, 235). The ability of curcumin to bind to the carbohydrate binding sites (234) and inhibit the carbohydrate digestive enzymes indicates that it might have the potential to lower the PBG. We hypothesised that administration of curcumin prior to meal consumption may lower the postprandial glycaemic response and the amount of insulin required to control the rise in PBG levels.

Short term exposure of pancreatic β-cells to fatty acids has been shown to induce insulin secretion (236). *In-vitro* studies have reported that glucose stimulated insulin secretion is greatly influenced with the degree of saturation of the fatty acids (237). Long term exposure to poly- and mono- unsaturated fatty acids have shown cytoprotective effects on pancreatic β-cells, in contrast to the deleterious effects of long-chain saturated fatty acids (238, 239). Substituting dietary saturated fat with LCn-3PUFA in meals has shown improvements in the IS in individuals with and without T2D (240-242). However, results from multiple studies with long-term supplementation of LCn-3PUFA for improving IR are ambiguous (243). In the current study, we hypothesised to evaluate effects of single

dose of curcumin with or without LCn-3PUFA on PBG, serum insulin and triglyceride levels.

4.3 Methods

4.3.1 Subjects

Sixteen apparently healthy men and women were recruited from the Hunter Region, New South Wales, Australia via displaying recruitment flyers and media publicity. They were screened through telephone interviews based on the inclusion criteria: 18-45 years of age and body mass index less than 30 kg/m^2. Exclusion criteria included diagnosis with any chronic metabolic disease (T2D and CVD), auto-immune disease (rheumatoid arthritis, lupus, type 1 diabetes), liver disease, history of severe neurological diseases or seizures, currently on any medications/supplements known to influence blood glucose level, pregnancy or planning to become pregnant, breastfeeding, unwilling to provide informed consent and sensitivity/ intolerance to curcumin, FO, and/or food products (dairy products, peanuts, wheat, protein and gluten). The study protocol was approved by the University of Newcastle Human Research Ethics Committee (H-2014-0385). All participants gave their written informed consent before entering the study. The study was registered on Australian New Zealand Clinical Trials Registry (ANZCTR) under study number ACTRN12618000047291.

4.3.2 Standard meal

On the test days, participants were provided with standard breakfast: two slices of toasted white bread, peanut spread (22g) and 250 mL chocolate flavoured milk (OAK). The macronutrient composition of this meal is 56g carbohydrate, 22g fat and 21g protein. Participants consume the test meal within 15 min.

4.3.3 Study design

This randomised, placebo controlled, single blinded (participants), cross over study consisted of 4 test days, each separated by a wash out period of one week (Figure 4.1). Following the screening, the eligible participants were asked not to change their dietary habits and level of physical exercise before the test days and during the washout periods. They received necessary instructions for each test day: 12-h overnight fasting, avoiding high caloric meal, alcohol consumption, and any vigorous physical activity 24-h before the test days. They were also asked to collect a 24-hour record of their previous day food intake. This dietary information was processed through FoodWorks Version: 8.0.3551

(Xyris Software (Australia) Pty Ltd) to check whether their dietary energy and macronutrients remained unchanged during the study period. Physical activity of the participants was assessed on the first test day using the International Physical Activity Questionnaire – short form designed to capture the frequency, duration and intensity of physical activity undertaken during the previous seven days. Participants received a single dose of one of the following dietary supplements just prior to consuming the standardised breakfast, in a random order according to the computer generated sequence (block size of four): 2x placebo tablets matching for curcumin plus 2x placebo capsules matching for FO (PL) or 2x 500 mg curcumin tablets (each tablet containing 90 mg curcumin) plus 2x placebo capsules matching for FO (CC), 2x 1000 mg FO capsules (each capsule containing 120mg EPA + 430mg DHA) plus 2x placebo tablets matching for curcumin (FO) and 2x 500 mg curcumin tablets plus 2x 1000 mg FO (CC-FO).

4.3.4 Test day protocol

After the participants arrival at the Nutraceutical Research Program clinical trial facility on the first test day in fasting state, medical history and demographic related information were obtained from participants via self-administered questionnaires. Height of the study participants were measured using a wall mounted roll up stadiometer (SE206, Seca). Body mass index (BMI) and body composition were determined using direct segmental multi-frequency bioelectrical impedance (InBody 230, Biospace Co., Ltd. Seoul, Korea). Blood samples were collected by finger prick for the measurement of fasting glucose levels (BG star glucometer, Sanofi). An 8-ml venous blood sample was also collected and sent to the accredited Hunter Area Pathology Service for the measurements of fasting serum insulin and TG. No other food or drink was allowed during the study period. Capillary and venous blood sampling was repeated at 30, 60 &120 min and 60 & 120 min respectively.

4.3.5 Statistics

The primary outcome for this study was change in blood glucose (Δglucose) levels at 60 min. Data from our previous study (204) indicate that the difference in the response of subjects is normally distributed with standard deviation 0.11. With the expected 10% difference in the mean Δglucose response at 60 min with a probability (power) 0.8, a sample size of n=12 subjects were required in a cross-over design. The Type I error probability associated with this test of this null hypothesis is 0.05. The data of all variables included in the analysis were tested for normality by deriving studentized residuals,

kernel density plots and Shapiro-Wilk's tests and are presented as mean ± SD or median (interquartile range) as appropriate. Log transformation was used for variables with non-normal distribution. Differences between fasting concentrations of the biochemical variables, diet and macronutrient intake were evaluated by repeated measures analysis of variance. Change AUC's (0-120) for postprandial glucose, insulin and triglyceride responses were constructed using trapezoid rule. Differences between the study interventions were tested for statistical significance using repeated measures analysis of variance (ANOVA). When this analysis was significant, a paired t-test or Wilcoxon signed-rank test was performed to compare the interventions pairwise. Analysis of covariance (ANCOVA) and two-way ANOVA was used to evaluate the effects of confounding variables on primary outcomes. Significance was set at $p<0.05$. All statistical analyses were conducted using Stata version 14.1 (StataCorp, Texas, USA).

4.4 Results

4.4.1 Participant characteristics

Fifteen participants (8 males and 7 females) aged 26.33 ± 5.04 years with BMI of 24.91 ± 4.17 kg/m^2 (table 4.1) completed the study. One female failed to follow-up after two visit days because of diagnosis with infection and is excluded from the analysis. Insulin value for one participant on single test day (allocated to FO) was not included because of the unreliable data from the reanalysis of sample by pathology.

4.4.1 Physical activity and dietary intake

Physical activity analysis indicated seven participants with low physical activity, five with moderate and three with high physical activity levels. There were no significant differences in energy, carbohydrate, sugar, starch, protein, total fat, polyunsaturated and monounsaturated fat and fibre intake between 24-hour period preceding each of the four test days (table 4.2)

4.4.2 Postprandial glucose levels

There were no significant differences observed between the baseline glucose levels at the start of each of the 4 test days (table 4.3). Increase in glucose levels post-meal consumption was lower in the CC treatment group at all the time points compared with the PL group. There was no difference between the change in glucose levels between PL and the FO groups. Repeated measures ANOVA model with time and treatment was significant ($P = 0.005$) for ΔGlucose levels (figure 4.2) and change AUC for glucose ($P=0.007$) (table 4.3). Although there was 15.7% reduction in glucose levels with CC at

30 min compared with PL, it did not reach significance (P = 0.208). Pair-wise analysis indicated 61%, 51% and 30% lower Δglucose levels at 60 min in the CC (P=0.0007), CC-FO (P=0.002) and FO (P=0.046) groups respectively, compared with PL group. No significant differences were observed between CC, FO, CC-FO and PL at 120 min. There were no significant differences observed between the CC, FO and CC-FO at all the three-time points, however the magnitude of the effect on Δglucose reduction was different between the three treatments. Change AUC (0-120 min) for glucose was significantly lower with CC (36.2%; P=0.003) and CC-FO (29.9%; P=0.004), but not with FO (15.4%; P = 0.105) (Table 4.3). Change AUC for Glucose were not significantly different between the active treatment groups.

4.4.3 Postprandial insulin

There were no significant differences observed between the baseline insulin levels at the start of each of the 4 test days (table 4.3). Mean changes in insulin levels at all the time points were lower following CC treatment. However, repeated measures ANOVA indicated that the model with time and treatment did not reach significance for the change in the insulin (Δinsulin) levels (P=0.168), but the change AUC for Insulin was significant (P=0.03). Pairwise analysis shown change AUC values for insulin was significantly lower only with CC (26.5%, P=0.01) and CC-FO treatments (25.8%, P=0.03) but not with FO treatment (17.5%, P=0.140) compared with PL group. There were no significant differences observed between the effects of CC, FO and CC-FO treatments on Δinsulin levels (figure 4.3).

4.4.4 Postprandial triglycerides

There were no significant differences observed between the baseline triglyceride levels at the start of each of the 4 test days (table 4.3). As expected, the postprandial triglyceride levels were increased at all the time points following meal consumption compared with baseline. Repeated measures ANOVA change with time and treatment model for change in triglyceride levels (Δtriglycerides) did not reach significance (P=0.074) (figure 4.4). Similar results were observed with for the change AUC triglycerides (P=0.66).

4.4.5 Confounding variables

No significant interaction was observed between gender and treatment effect for Δglucose at 60 mins (P = 0.795), change AUC glucose (p=0.995), change AUC insulin (p=0.305) or Δtriglycerides at 60 min (0.958).

No significant interaction between treatment effect and physical activity or BMI for Δglucose at 60 mins (interaction with physical activity: p=0.604; interaction with BMI: p=0.701); change AUC glucose (interaction with physical activity: p=0.173; interaction with BMI: p=0.365), or change AUC Insulin (interaction with PA: p= 0.969; interaction with BMI: 0.06). Physical activity has a significant interaction with Δtriglycerides (0-120 min; p=0.002) and change AUC (r=-0.33, p=0.003) for triglycerides.

Table 4.1: Baseline characteristics of the participants

Variables	All participants (n=15)
Age (years)	26.33 ± 5.04
Males/Females	8/7
Weight (kg)	71.08 ± 15.03
Muscle mass (kg)	28.12 ± 6.04
Body fat mass (kg)	20.77 ± 9.09
Fat free mass (kg)	49.25 ± 10.98
Body mass index (kg/m^2)	24.91 ± 4.17
Percentage body fat	28.42 ± 8.98
Waist hip ratio	0.86 ± 0.05
Basal metabolic rate	1457 ± 216.10
Physical activity (metabolic equivalents-minutes/ week)	678 (462,1986)
Physical activity (category, n)	Low (n=7) Moderate (n=5) High (n=3)
Fasting glucose (mmol/L)	5.42 ± 0.37
Fasting insulin (mU/L)	8.00 ± 2.42
Fasting triglycerides (mmol/L)	0.88 ± 0.26

Data are reported as means ± SD or median (25^{th} and 75^{th} percentile) as appropriate. SD-standard deviation.

Table 4.2: Composition of the habitual diets as consumed 24 hours before the PL, FO, CC and CC-FO test days

	PL	FO	CC	CC-FO	*P*-value
Energy (kJ/day)	8449 ± 2882.1	8613 ± 1557.3	8625 ± 1755.7	8966 ± 1690.8	0.787
Carbohydrate (g/day)	227.9 ± 84.5	245 ± 58.6	241.21 ± 59.3	247 ± 69.4	0.380
Protein (g/day)	91.3 ± 35.8	86.4 ± 17.9	93 ± 21.6	101.8 ± 29.2	0.411
Total fat (g/day)	71 (50, 93)	72 (55, 106)	83 (67, 91)	72 (55, 100)	0.673
Polyunsaturated fat(g/day)	11 (9, 17)	11 (6, 15)	12 (8, 17)	12 (8, 14)	0.383
Monounsaturated fat (g/day)	29 (19, 35)	28 (22, 39)	30 (25, 34)	29 (22, 41)	0.841
Sugar(g/day)	43 (33, 108)	96 (56, 103)	80 (40, 106)	51 (32, 112)	0.241
Starch (g/day)	159 ± 57.9	151 ± 49.6	133.4 ± 52.4	178.1 ± 57.6	0.141
Fibre (g/day)	24 (16, 32)	20 (15, 25)	20 (17, 23)	26 (20, 34)	0.453

Data are reported as mean **±** SD or median (25^{th} and 75^{th} percentile) as appropriate. Significant changes in dietary intake between the groups (ANOVA), $P < .05$. SD- Standard deviation

Table 4.3: Baseline values, change area under the curve (AUC) for glucose, insulin, triglycerides in response to PL, FO, CC and CC-FO

Parameters	**Time (min)**	**PL**	**FO**	**CC**	**CC-FO**	**Two-way ANOVA Model (time x treatment) P-value**	**Pair-wise analysis P-value**		
							PL VS FO	**PL VS CC**	**PL VS CC-FO**
Fasting glucose (mmol/L)	0	5.26±0.3	5.24±0.37	5.40±0.45	5.35±0.48	0.441	-	-	-
Glucose change AUC (mmol. min/L)	0-120	2.71±1.1	2.28±1.2	1.73±0.7	1.9±1.03	0.007	0.105	0.003	0.004
Fasting insulin (mIU/L)	0	7.6±2.5	8.2±2.5	8.8±3.7	9.0±3.1	0.104	-	-	-
Insulin change AUC (mIU. min/L)	0-120	62.7 (40.4, 103.5)	51.3 (34.8, 69.8)	38.05 (23.9, 61)	51.4 (27, 69.8)	0.02	0.140	0.01	0.03
Fasting triglycerides (mmol/L)	0	1.0 ± 0.5	1.0±0.3	1.1±0.70	1.01±0.38	0.534	-	-	-
Triglycerides change AUC (mmol. min/L)	0-120	0.3 (0.2,0.5)	0.4 (0.2,0.6)	0.2 (0.1, 0.6)	0.52 (0.33, 0.72)	0.100	-	-	-

Data are presented as mean ± SD or median (25th and 75th percentile). AUC – area under the curve. PL-double placebo, FO- fish oil alone, CC- curcumin alone, CC-FO- curcumin plus fish oil.

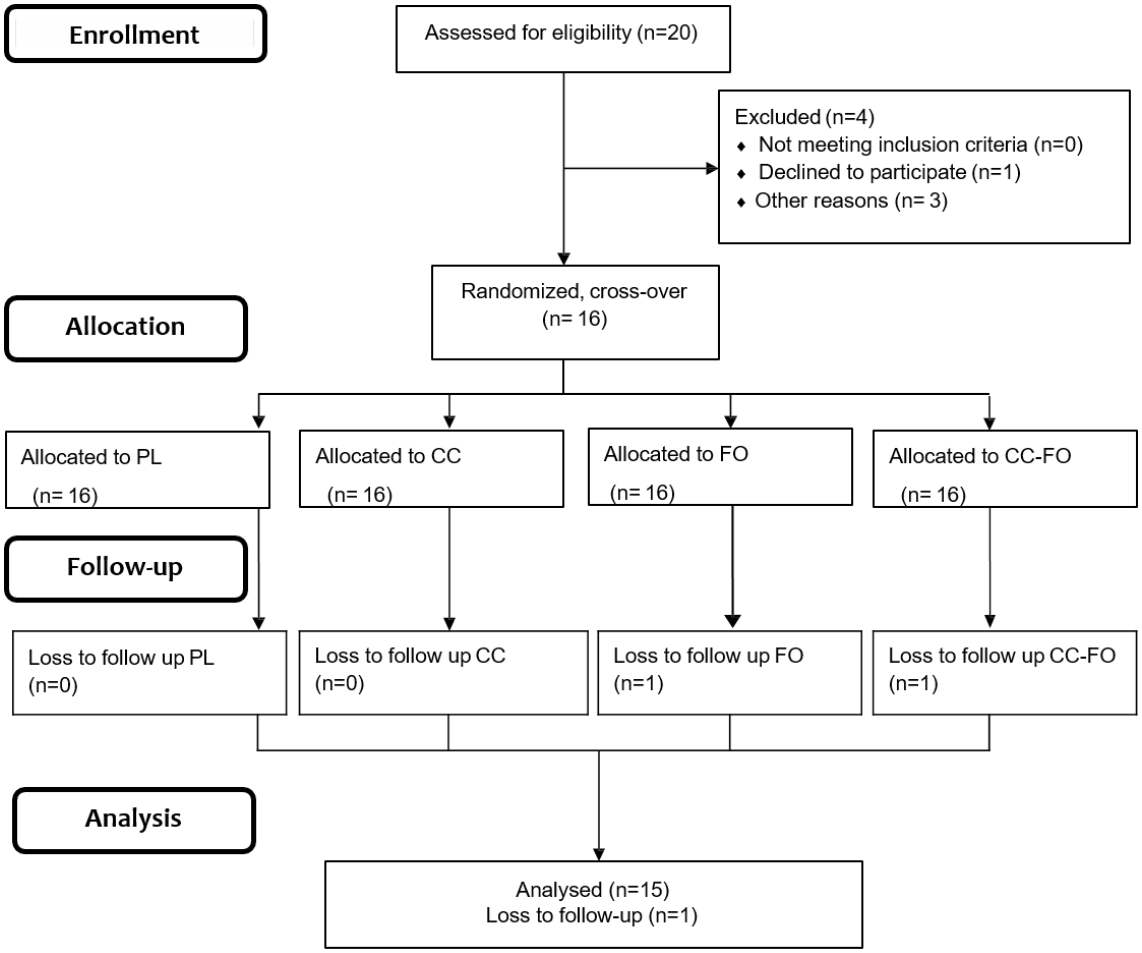

Figure 4.1: CONSORT flow chart

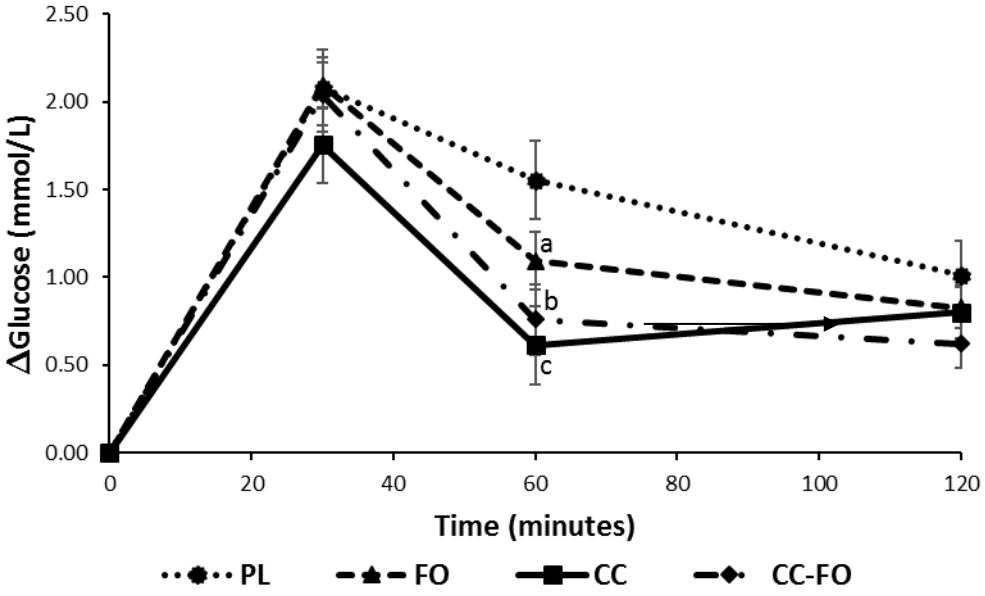

Figure 4.2: Changes in the mean blood glucose levels (Δglucose) over 120 minutes
Data are reported as Mean ± SEM. Lower case letters represent the significance difference between PL and active treatment groups (a P=0.046; b P=0.002; c P=0.0007). PL, double placebo; FO, fish oil; CC, curcumin; CC-FO, curcumin plus fish oil.

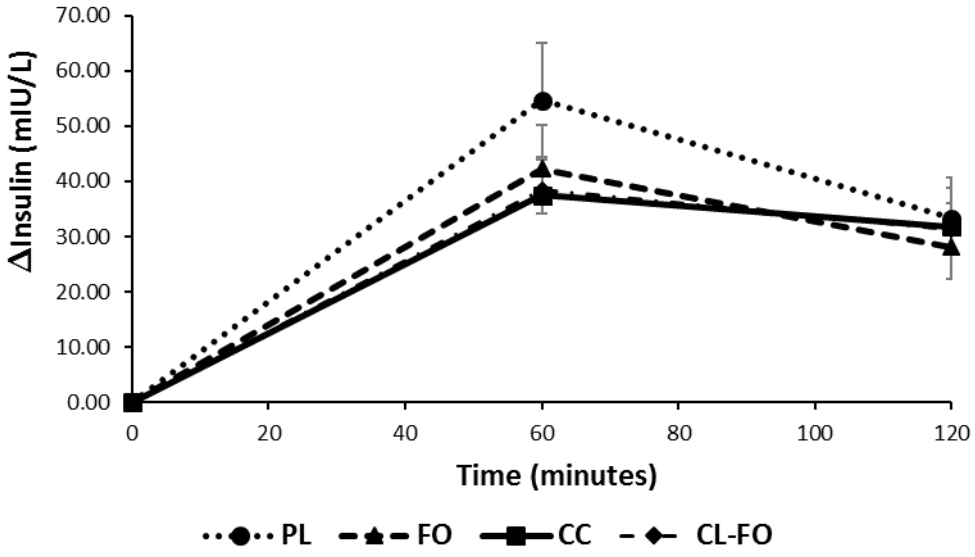

Figure 4.3: Changes in the mean serum insulin levels (Δinsulin) over 120 minutes
Data are reported as Mean ± SEM. PL, double placebo; FO, fish oil; CC, curcumin; CC-FO, curcumin plus fish oil.

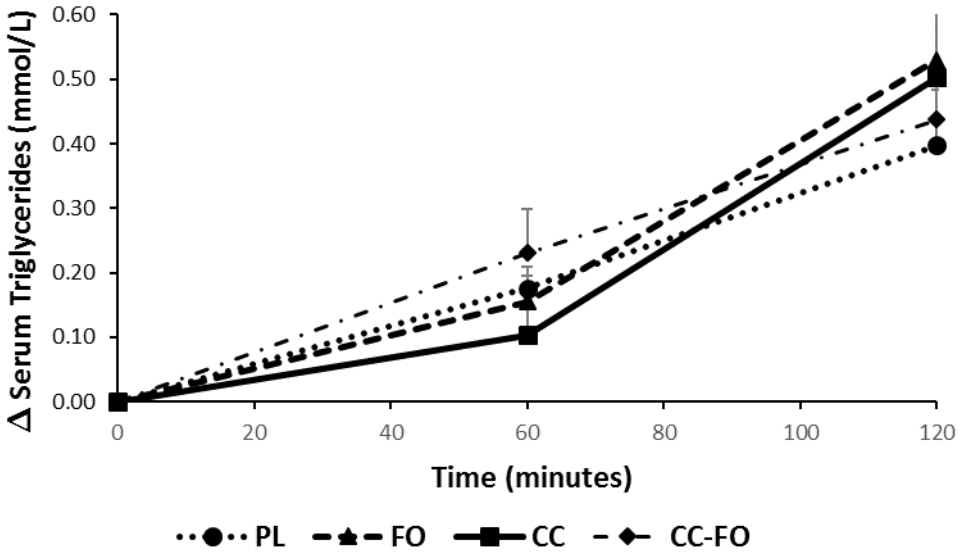

Figure 4.4: Changes in the mean serum triglyceride levels (Δtriglycerides) over 120 minutes.

4.5 Discussion

Results presented in the current study demonstrate that curcumin, but not LCn-3PUFA, has the potential to effectively reduce the rate of rise in PBG and insulin levels. Only CC and CC-FO significantly lowered the change AUC of the glucose and the amount of insulin (AUC) required to control postprandial rise in the blood glucose levels compared to the PL. Significant reduction in Δglucose was observed with CC (61%) and CC-FO (50%) treatments. PBG following CC treatment was lower (16%) than the placebo group at as early as 30 minutes from baseline, however, the difference did not reach statistical significance.

The digestion of carbohydrates is a multistep process involving digestive enzymes in the oral cavity and small intestine. Among these digestive enzymes, in the first step, pancreatic α-amylase in the small intestine regulates the hydrolysis of long-chain polysaccharides (244). The resulting breakdown products are then processed by α-glucosidase resulting in hydrolysis of disaccharides (maltose) into glucose units (245). Current therapeutics including miglitol and acarbose target α-glucosidase to reduce the rate of digestion of carbohydrates into simple sugars (245). Inhibition of pancreatic α-amylase by curcumin in addition to α-glucosidase inhibition might provide as an alternative intervention with enhanced efficacy for the regulation of postprandial glycaemia while minimising the magnitude of the side effects (246). Molecular docking studies have previously reported that curcumin has the potential to bind to the

carbohydrate biding sites with higher inhibition (IC_{50}) activity for both α-glucosidase and α-amylase compared with acarbose (235). In the current study, curcumin significantly reduced the PBG response to standardised carbohydrate rich (composed of 50% starch and 50% sugar meal) meal without increasing the insulin levels, suggesting that the glucose control by curcumin may or possibly be achieved through inhibition of both the digestive enzymes. With relatively high safety profiles, curcumin appears to be a potential candidate for optimising the PBG levels. In contrast with our findings, a previous study involving turmeric extract (*Curcuma longa*) (247), the only source of dietary curcumin, has shown to be ineffective in lowering PBG levels. We used a phytosome formulation of curcumin in the study, which ensures the improved bio-availability of curcumin than the natural parent compound. Pharmacokinetic studies reported 30-60 minutes as t_{max} of the phytosome formulation of curcumin and its metabolites (248, 249), potentially explaining the greater reduction in the PBG is achieved with highest concentration of curcumin in the blood. In animal models of insulin resistance, oral administration of curcumin has been shown to significantly improve the IS and glucose metabolism (93). Curcumin has also been reported to exhibit reductions in fasting insulin and IRin individuals with pre-diabetes (250). Reduced rise in meal-induced insulin by curcumin in the current study compared to the placebo group is suggestive of the beneficial effects of curcumin on IRpossibly by regulation of the post-meal insulin responses and/or preservation of the β-cell function.

In line with previous studies, physical activity was inversely correlated with plasma triglycerides (251). ANCOVA analysis indicated that physical activity has significant interaction with treatment effects on triglycerides. The effects of curcumin on Δ triglycerides in two-hour period did not reach significance. Given the fact that our hypothesis was primarily based on evaluation of curcumin and/or LCn-3PUFA on PBG, the two-hour period was insufficient to demonstrate effects on post-prandial triglycerides. A postprandial study design with time longer than 2-hours is necessary to study the effects of curcumin on postprandial lipids.

Replacement with LCn-3PUFA or meals rich in LCn-3PUFA has been shown to influence the IS without affecting the glucose metabolism in in-vitro (251, 252) and pre-clinical studies (253). On the other hand, clinical trial reported no effect of LCn-3PUFA on IS or PBG (245, 254), leading to ambiguity and necessity for further research. Interestingly FO significantly reduced the Δglucose at 60 min by 30% compared to the placebo.

However, there was no effect of FO either on change AUC of the glucose or insulin or triglycerides. Pharmacokinetic studies indicate the plasma levels of LCn-3PUFA are increased significantly at 2 hours and 4 hours and peak at 6 hours post-supplementation (255). The current study failed to demonstrate any effects of LCn-3PUFA in the limited two-hour study period. A longer study time may be necessary to evaluate the effects of LCn-3PUFA and any of its complementarity with curcumin on PBG and insulin response. In conclusion, we demonstrated that curcumin has the potential to lower postprandial glucose and insulin responses, thus preserving the β-cell function and protection from deleterious consequences of PBG. This study failed to provide any evidence for the benefits of combining LCn-3PUFA with curcumin on postprandial carbohydrate or lipid metabolism. Long-term supplementation studies are warranted to determine whether curcumin and/or its combination with LCn-3PUFA can provide beneficial effects on dysregulated postprandial metabolism.

CHAPTER 5

Curcumin and long-chain Omega-3 polyunsaturated fatty acids for Prevention of type 2 Diabetes (COP-D): Study protocol for a randomised controlled trial

The contents of this chapter have been published:

Thota RN, Acharya SH, Abbott KA, Garg ML. Curcumin and long-chain Omega-3 polyunsaturated fatty acids for Prevention of type 2 Diabetes (COP-D): study protocol for a randomised controlled trial. Trials. 2016; 17:565.

(Statement of authorship is attached as appendix-21)

5.1 Abstract

Background: Life-style interventions including increase in physical activity and dietary counselling shown the ability to prevent T2D in high risk state individuals, but the prevalence is still skyrocketing in Australia, in line with global prevalence. Currently no medicines are approved by the Therapeutic Goods Administration in Australia for management of pre-diabetes. Therefore, there is a need of developing a safer, biologically efficacious and cost-effective alternative for delaying transition of individual health state from pre-diabetes into T2D. In the current trial we propose to evaluate the effects of curcumin and/or long chain omega 3 polyunsaturated fatty acids on improving glycosylated haemoglobin as a primary outcome, along with secondary outcomes being glycaemic indices, lipid profile and inflammatory parameters.

Methods/design: Eighty individuals diagnosed with pre-diabetes, aged between 30-70 years, will be randomly assigned to double placebo, curcumin alone, fish oil alone or double active groups according to computer generated randomisation sequence for 12 weeks. At baseline visit and post-intervention visits participants will be asked to provide blood samples and undergo body composition measurements. Blood sample is used for estimating glycaemic profiles, lipid profiles and inflammatory parameters (C-reactive protein, whole blood cell count, adiponectin, leptin, interleukin-6). Interim visit includes review on compliance to supplements based on capsule log and capsule count, adverse events and anthropometric measurements. In addition to these procedures, participants provide self-reported questionnaires on dietary intake (using 3-day food record), physical activity questionnaire, and medical history.

Discussion: This trial aims to determine whether curcumin and/or long chain omega 3 polyunsaturated fatty acids affect surrogate markers of glycaemic control which is relevant to delaying T2D. Till date 38 participants completed the trial. No changes have been made to the clinical protocol post recruitment. If successful, this trial will provide considerable evidence for performing a larger trial to investigate whether this combination can be administered for prevention or delaying the onset of T2D in high risk individuals.

5.2 Background

Type 2 diabetes (T2D) is a chronic and non-communicable metabolic disorder characterised by hyperglycaemia resulting from defective secretion and/or action of insulin. The prevalence of T2D has been increasing rapidly worldwide at an alarming rate due to over nutrition, physical inactivity, urbanisation (especially in developing countries) and genetic predispositions (256-259). People with prediabetes, are at a high risk of developing T2D, have intermediate glucose levels, elevated but not high enough to be diagnosed as T2D. They are classed as having either impaired glucose tolerance (IGT), impaired fasting glucose (IFG) or both. In 2015, over 318 million people were living with pre-diabetes worldwide, and this number is expected to rise to 471 million by 2035 (260). 5-10% of the individuals diagnosed with prediabetes continue to progress to overt T2D annually (261). Despite its complex and heterogeneous nature, T2D often co-exists with obesity, hypertension and dyslipidaemia which are also common features of ageing (262-265). Recent advances in the molecular biology have provided in-depth understanding of the mechanisms that are involved in the pathobiology of T2D highlighting the role of glucotoxicity, lipotoxicity, oxidative stress and endoplasmic reticulum stress (14, 266, 267). An elevated level of sub-clinical inflammation for prolonged periods is believed to be the primary trigger preceding by all of the above mechanisms (268). These pathological mechanisms mediate a reduction in the insulin stimulated glucose uptake leading to insulin resistance. IR present throughout the pre-diabetic stage acts as a conduit between the progression of subclinical inflammatory state to T2D.

The presence of IR in prediabetes also modulate the risk of developing T2D, and individuals with combined IGT and IFG are at greater risk of developing T2D than IFG or IGT alone (71, 261). IR is prevalent in both the cases, but people with IFG have more hepatic IR and those with IGT have more skeletal muscle resistance with less effect on hepatic IS (269, 270). Along with IR β-cell dysfunction is present in both IFG and IGT individuals. But they differ in time of insulin secretion response during OGTT. Individuals with IGT have both impaired early and late insulin response, in contrast to IFG individuals where only early phase insulin response is impaired (269). These observations indicate IR and moderate β-cell dysfunction is already present in individuals with IFG and IGT (prediabetes).

Diabetes prevention studies have found that lifestyle interventions aiming at weight reduction through dietary intake changes and increase in physical activity significantly reduce the risk of developing T2D (71, 72). Lifestyle interventions resulting in weight loss and also improved IS and β-cell function (73-75). However, maintaining weight loss and physical activity is very difficult over longer period of time. Lack of compliance to these interventions is barrier for effectiveness in halting the progression to T2D in high risk individuals; indicating that dietary and life style modifications alone are insufficient. As prediabetes is not a full blown disease state, to date, there are no approved drugs for management of prediabetes. Lack of compliance to lifestyle interventions and pharmacological agents, highlights the necessity of safe and efficacious interventions for management of prediabetes.

Subclinical inflammation, being the primary trigger for many pathological mechanisms in development of T2D, anti-inflammatory bio-actives such as curcumin and long chain omega 3 polyunsaturated fatty acids (LCn-3PUFA) appear to be an attractive strategy to delay risk of developing T2D in individuals with prediabetes. These bio-active compounds influence multiple numbers of physiological mechanisms with relatively higher safety profiles (77, 271). These bio-actives could provide beneficial effects in IR and T2D by the following pathways: 1. down-regulating inhibitor of kappa B kinase (IKK)/nuclear factor kappa B (NFκB), c-Jun N-terminal kinases (JNK) pathways for lowering chronic low grade inflammation; 2. scavenging the reactive oxygen species and reducing oxidative stress; 4. Cytoprotection for improving β-cell function; 5. reducing the accumulation of fatty acid metabolites for increasing IS; 6. Up-regulation of 5' AMP-activated protein kinase (AMPK); 7. Modulate peroxisome proliferator-activated receptor gamma (PPARγ) agonistic activity.

Some preclinical studies reported synergistic anti-inflammatory effects of curcumin and DHA combination. These observations suggest curcumin might provide synergistic anti-inflammatory effects with LCn-3PUFA in the prevention of T2D (199-201). In this study we aim to perform a two by two factorial double blinded randomised placebo controlled trial to evaluate the complimentary or synergistic effects of curcumin and/or LCn-3PUFA primarily on glycaemic profiles and in individuals with pre-diabetes (IFG, IGT or Both). In addition to these parameters we are also measuring lipid profile and inflammatory parameters to determine effects of curcumin and/or LCn-3PUFA on cardiovascular risk factors and chronic low grade inflammation.

5.3 Method/design

This is a twelve week 2x2 factorial, randomised, double blinded (study investigator and participants) placebo controlled intervention trial for prevention or delaying onset of T2D using curcumin and/ or fish oil as supplements. Eighty individuals diagnosed with prediabetes (IFG – 6.1-6.9; IGT-2h - ≥ 7.8 and ≤ 11.1; HbA1c 5.7-6.5) aged between 30-70 years will be screened for enrolment at two sites; Nutraceuticals Research Group (NRG) and John Hunter Hospital (JHH) in Newcastle, Australia. Potential participants who meet the inclusion and exclusion criteria will be randomised to receive double placebo (corn oil capsules or curcumin placebo tablets), curcumin plus placebo matching for fish oil, fish oil plus placebo matching for curcumin or both curcumin and fish oil. Participants visit the trial sites 3 times at baseline (0 week), interim (6 week) and post-intervention (12 week). Figure 1 shows flow chart of study design, and the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) checklist is presented as additional file 1.

5.3.1 Study Aims

The primary aim is to evaluate the potential of curcumin and/or LCn-3PUFA for improvements in the glycosylated haemoglobin in individuals with pre-diabetes. Secondary outcomes include; FPG, insulin, fructosamine, IS (Homeostatic model assessment), lipid profile (total cholesterol, triglycerides, HDL-C, LDL-C), CRP, IL-1β, IL-6, adiponectin and leptin. Erythrocyte fatty acid composition and plasma curcumin content are measured to determine the compliance to the intervention.

5.3.2 Inclusion criteria

To be eligible to participate in trial, participants should be within the age range of 30-70 years. Their BMI must lie between 25 – 45 kg/m^2. They must be diagnosed either with IFG (fasting glucose 6.1-6.9 mmol/L), IGT (2-hour OGTT plasma glucose ≥7.8 mmol/ L and <11.1 mmol/L) or both. They can also take part in trial if their glycated haemoglobin levels lie between 5.7-6.4% or they obtain a score of 12 or more in the AUSDRISK tool assessment (a non-invasive questionnaire to determine the risk of developing diabetes). The participants should not take part in any clinical trial (particularly with new investigational drugs) before starting this trial. They must be readily available for 3 months and should be willing to maintain similar dietary patterns and physical activity during the trial period.

5.3.3 Exclusion criteria

Participants are excluded if they are unwilling to provide blood sample during initial and final visits. Based on the medical questionnaire they will also be excluded if they have established T2D, gall bladder problems, pace maker implants, severe neurological diseases or seizures. Women who are pregnant or lactating will be excluded. People who consume more than 2 serves of oily fish per week or take any other dietary supplements known to influence blood glucose levels are also ineligible to participate in the trial. Based on their medication intake from medical questionnaire, they will be excluded from trial if they are taking aspirin, warfarin, clopidogrel, ibuprofen, naproxen, dalteparin, enoxaparin and heparin to avoid any possible drug interaction with curcumin.

5.3.4 Sample size calculation

Based on the HbA1c data of the pre-diabetes people from other studies (272, 273), with standard deviation of 0.5 units in HbA1c, a sample size of seventeen participants in each treatment group will give 80% power to detect a 0.5 units drop in HbA1c at type 1 error (alpha)= 0.05. To allow for dropouts we will recruit 4x20 = 80 participants according to the inclusion criteria.

5.3.5 Participant recruitment

Participants will be recruited from the Hunter region in NSW using media advertising and social media (Facebook and Twitter) approved by the University of Newcastle Media and Marketing Department. Recruitment flyers will be placed on noticeboards at the University of Newcastle, local pharmacies, local pathology centres (with permission), and newsletters. Participants will also be recruited from the Hunter Medical Research Institute (HMRI) Volunteer Register, as well as from clinics across John Hunter/Newcastle Community Health Centre and Belmont Hospital diabetes clinics, Medicare local and GP practices database with consent from participating GPs. Interested participants will contact the study coordinator through email or phone, who will screen the participants according to inclusion and exclusion criteria. Potential participants will receive participant information sheet and the health, diet (3-day food record) & physical activity questionnaire (IPAQ long form) after telephone screening. Participants will return the completed questionnaires along with a signed consent form. Study investigators will use demographic and lifestyle details to determine their suitability before visiting the NRG or JHH for a baseline visit.

5.3.6 Baseline assessments

After returning their signed consent forms, participants are provided with baseline visit details (table 5.1). Participants will need to complete 3-day food record, physical activity (IPAQ), and medical questionnaires before visiting trial site. Participants will visit either NRG or JHH after a minimum of 10 hours overnight fasting. Baseline assessments include anthropometry measurements using bioelectric impedance and 20 mL blood sample collection for assessment of plasma glucose levels, insulin, fructosamine, HbA1c, TC, TG, HDL-C, LDL-C, CRP, WBC, leptin, adiponectin and other inflammatory cytokines (IL-1β, IL-6).

5.3.7 Randomisation

Eligible participants who attend the baseline visit are randomised into treatments with either double placebo, curcumin alone, fish oil alone or double active (curcumin and fish oil) groups according to a computer generated randomisation sequence. This randomisation is done in equal block sizes of 8, to ensure the balance between equal numbers of males and females. Randomisation is carried out by an individual who is not involved in trial data collection and analysis. Investigational product will be carefully stored (under the responsibility of the investigator) in a locked, limited access area, safe and separated from drugs. Only authorized persons will have access to the storage place capsules will be stored in sealed HD-PE bottles, and stored at room temperature (15-25°C) in a dry place, protected from light and away from heat sources. The active and placebo capsules are identical, encapsulated and matched for colour. To increase the compliance of participants to the intervention, they are provided with capsule log sheet during the study period to record their capsule intake. At the end of the study they are advised to return capsule containers along with their log sheet.

5.3.8 Intervention

Following randomisation, participants will receive one of the following interventions:

a. Placebo: 2X500mg CN-Placebo capsules plus 2X1000mg corn oil capsules per day
b. Curcumin (Meriva curcumin): 2X500mg CN capsules (providing 180 mg curcumin/day) plus 2X1000mg corn oil capsules per day
c. LCn-3PUFA(EPAX1050): 2X1000mg FO capsules (providing 1.2g EPA/DHA) plus 2X500mg CN-Placebo capsules per day

d. Combination (curcumin and LCn-3PUFA): 2X500mg CN capsules (providing 180 mg curcumin/day) plus 2X1000mg FO capsules per day (providing 1.2g EPA/DHA)

The participants are advised to take 4 capsules daily (2 with morning meals and 2 with evening meals) with no changes in their dietary intake or physical activity during the study period.

5.3.9 Interim visit

Participants are scheduled for interim visit after 6 weeks of the intervention period (table 5.1). They will need to provide completed diet and physical activity questionnaires. Compliance for intervention is measured by capsule count and capsule log sheet. Follow-up visit include only 10 hr overnight fasting and anthropometric assessments using bio-electric impedance. Any adverse events or reports of difficulties in compliance to intervention are recorded during this visit.

5.3.10 Post intervention visit

Post intervention visit is scheduled on the last day of the participant's intervention period (table 5.1). All baseline measures, including, 10 hr overnight fasting, anthropometric measurements and blood sample collection will be repeated on this visit. They will also be asked to fill out 3-day food record and physical activity questionnaires. Participants are advised to return capsule containers along with capsule log sheet on the final visit day.

5.3.11 Safety and compliance monitoring

During follow-up and post intervention visit, any serious adverse events (SAE), adverse events (AE) related with intervention, compliance to intervention, or issues with other procedures (e.g. blood collection, anthropometric measurements etc.) are discussed with participants and recorded. The study coordinator will inform the principal investigator of all AEs and SAEs, who will then follow procedures for unblinding as necessary and notify the relevant bodies. Depending on the nature of AE or SAE, it may be necessary for treatment to cease and/or for the participant to be withdrawn from the study. Should the need for unblinding in the curcumin or LCn-3PUFA group vs. placebo group arise, subjects may inform the study coordinator or anyone from the research team. For emergency situations, subjects will be given an emergency contact card, which will provide general study information and contact information for the unbiased randomizers.

Compliance of participant to the intervention is measured using capsule count and capsule log record. Erythrocyte fatty acid analysis is used to measure compliance of participant to fish oil. Serum levels of curcumin is measured using HPLC procedure; this will be used a measure to determine compliance of participants to curcumin tablets.

5.3.12 Ethics

This trial has been approved by the University of Newcastle and the Hunter New England Area Health Service Human Research Ethics Committees, Approval No H-2014-0385 and HNE HREC 16/03/16/3.02 respectively. The trial is registered with Australia New Zealand Clinical Trial Registry (Trial ID ACTRN12615000559516).

5.3.13 Data collection and outcome measures

The primary aim of this trial is to measure the difference between glycosylated haemoglobin, between double placebo, curcumin, LCn-3PUFA and double active groups. Secondary outcomes include: anthropometric measurements (body weight, body fat mass, waist-hip ratio, waist circumference, BMI); glycaemic profiles and IS, lipid profile (TC, TG, HDL-C and LDL-C) and inflammatory parameters (CRP, whole blood cell count, IL-6, IL-1β, adiponectin and leptin). Other outcomes include dietary intake measures and physical activity from questionnaires (table 5.1).

Body weight, BMI, body fat mass, percent body fat, waist-hip ratio and skeletal muscle mass are measured using bioelectric impedance (InBody 230). These parameters are measured in fasting state at baseline, follow-up and post intervention visits. Because changes in the dietary pattern such as increasing in turmeric or fatty fish intake during the trial period might alter the effects of intervention on clinical outcomes, the participants are advised to maintain consistent dietary pattern during the trial period. They will be asked to complete 3-day food record during each visit, to get an appropriate picture of dietary intake pattern of participants and identify any major changes in dietary habits of participants. These food diaries will be analysed with Food Works Professional 8 2015 (Xyris software (Australia) Pvt.Ltd.). Along with dietary intake, participants will be advised to maintain similar physical activity during the 12-week trial period. International Physical Activity Questionnaire (IPAQ long version) is used to assess the levels of physical activity. This version allows the participants to record the number of hours or minutes they spent on job related physical activity, travel related physical activity, and recreation or sport related physical activity during the last 7 days.

It also has a provision for recording number of days, hours or minutes participant spend on walking and sitting. The physical activity of the participant will be reported in METs-minutes/ week. This will also help us to determine impact of different levels of physical activity on intervention.

Glycaemic profiles of the participants will be measured by Hunter New England Area Health Pathology Services (HNEHAPS) including fasting glucose levels, fasting insulin, glycosylated haemoglobin, and fructosamine. IR will be calculated by HOMA-IR (Glucose X Insulin/22.5), IS by HOMA S (1/[(insulin × glucose)/22.5]) and QUICKI (1/(log [FIRI in mU/l] + log [FPG in mg/dl]). The β-cell function will be measured using HOMA B (20Xinsulin/glucose-3.5 %). Lipid profile triglycerides, total cholesterol, HDL-C, LDL-C, Total/HDL ratio, CRP and whole blood cell count will also be measured by HNEHAPS Plasma pro-inflammatory markers including IL-1β, IL-6, adiponectin and leptin will be measured by human antibody specific Enzyme-Linked Immunosorbent Assay kits at our laboratory in NRG.

5.3.14 Data analysis

Data obtained from the all participants will be analysed according to the intention to treat principle. In case of missing data or dropouts, pairwise deletion will be used to analyse the significant effect of intervention in participants who have completed the trial. Normality of baseline data will be examined using histograms with a normal distribution curve overlayed and Shapiro Wilk's test. Based on the distribution of data, the outcome measures will be analysed using ANOVA (normal distribution) or wilcoxon signed rank test (non-parametric data). Two-way ANOVA with post hoc comparisons (Bonferroni correction) will be used to determine the effect of intervention on different variables and also to determine synergistic and/or complimentary effects between the two interventions (curcumin and LCn-3PUFA). ANCOVA will be used to assess the effects of confounding factors on treatment that include age, gender, BMI physical activity levels and dietary intake. Significance will be set at *P-value* <0.05. Statistical analysis will be performed using GraphPad Prism version 6 and IBM SPSS 22 software.

5.4 Discussion

Parallel to increase in the number of individuals with T2D, the prevalence of people with prediabetes is also increasing exponentially (260). Currently prediabetes is managed by life style modifications to delay onset or prevent progression to T2D.

To date there are no pharmacological agents approved for treatment of prediabetes. Lifestyle modifications alone are insufficient to bring down the prevalence of prediabetes, so there is a need for long standing safe and cost effective agents that can act through multiple mechanisms to improve IS and β-cell function. In this trial we are evaluating effects of diet derived bio-actives curcumin and/or LCn-3PUFA.These are derived from turmeric powder and seafood respectively and have the potential to influence multiple mechanisms. To the best of our knowledge, till date this is the first clinical trial designed to evaluate the combination of curcumin and LCn-3PUFA in prediabetes. If application of curcumin and/or LCn-3PUFA is successful in improving IS and glycaemic profiles in this trial, this combination could be examined in large scale trials that could provide a better alternative for management of pre-diabetes to prevent or delay onset of T2D. In addition to the effects of curcumin and LCn-3PUFA on glycaemic profiles, this combination might also improve underlying chronic low grade inflammation, which is involved in down regulation of insulin signalling and activity pathways. The anti-inflammatory and triglyceride lowering effects of curcumin and LCn-3PUFA might also provide an alternative option to bring down cardiovascular risk factors in those with prediabetes, which is not seen with conventional oral anti-diabetic agents. Therefore, this trial might be an important step in evaluating the effects of bio-actives for both alleviating hyperglycaemic and cardiovascular risk factors in individuals who are at high risk of developing T2D.

Curcumin, an anti-inflammatory bio-active derived from spice turmeric, in preclinical and *in-vitro* studies shown to act on multiple targets for delaying risk of developing T2D; via down regulation of low grade inflammation, cytoprotection of β-cells, improvement in β-cell function and decreasing fat accumulation in non-adipose tissues. Curcumin has been shown to decrease inflammation in adipocytes via multiple pathways such as inhibition of macrophage infiltration, scavenging ROS and down regulation of pro-inflammatory kinase (NF-κB and JKK) signalling. *In-vivo* studies with curcumin supplementation in high fat diet fed genetically obese (ob/ob) mice resulted in amelioration of diabetes associated symptoms along with a decrease in IR(93). Multiple mechanisms like decrease macrophage accumulation in WAT, down-regulation of pro-inflammatory cytokines and increase in adiponectin levels are exhibited by curcumin for improving IS (93).

Curcumin increased expression of AMPK in diabetic db/db mice, which might be a possible mechanism in supressing the hepatic glucose production, resulting in decreased blood glucose levels (95). Curcumin exhibit cytoprotective mechanism increasing levels of key mitochondrial biogenesis regulating transcription factors like PGC-1α, nuclear respiratory factor -1 and mitochondrial transcription factor A (96). Stimulation of volume regulated anion channel may be one of the primary mechanisms for anti-hyperglycaemic effects of curcumin. Curcumin directly stimulated volume regulated anion channels in rat pancreatic β-cells. This effect was accompanied by generation of electrical activity and depolarisation which resulted in enhanced insulin release (101). Curcuminoids increased expression of anti-oxidant enzymes Heme-oxygenase1 and NADPH:quinone oxidoreductase 1 at mRNA levels up to 12-fold and at protein levels by 6 fold in human islets (used for human transplantation) (102). These observations suggest curcumin might be beneficial in improving cellular defense mechanisms against oxidative stress. Direct stimulation of pancreatic β-cells and cellular defence against oxidative stress are two mechanisms which provide a considerable evidence for exploring the potential of curcumin in controlling or preventing T2D.

Meta-analysis and randomised controlled trials have reported LCn-3PUFA have either no effects or worsening effects on glycaemic profiles and IS (163, 169). On the contrary epidemiological studies reported an inverse correlation between regular consumption of fish and prevalence of diabetes (112-116). Recently published prospective study in a cohort of 407 obese and IGT participants and demonstrated that elevated plasma levels of LCn-3PUFA are associated with lesser prevalence of T2D over the extended 11 year follow up period (109). Elevated serum EPA, DHA and DPA levels are associated with higher IS, providing a plausible explanation for the lower prevalence rates of diabetes (109). In line with epidemiological studies, *in-vitro* and pre-clinical studies have reported beneficial effects of LCn-3PUFA on glycaemic profiles and IRthrough amelioration of chronic low grade inflammation and lipotoxicity. Administration of LCn-3PUFA in the diet (5-44%) protected the high fat diet fed animals from developing lipid abnormalities and impaired glucose homeostasis (118-120). LCn-3PUFA are natural agonists of PPAR-α subunits; Carboxylic group and hydrophobic chain in structure of LCn-3PUFA allows optimal binding to PPARα (126). Activation of PPARα by LCn-3PUFA results in increased fatty acids β-oxidation in peroxisomes (lesser extent in mitochondria) and

decreased lipogenesis. The increase in fatty acid oxidation leads to decrease accumulation of fatty acids (lipotoxicity) in liver along with improvement in IS in hepatocytes (126).

Stimulation of AMPK by LCn-3PUFA along with its effects on PPARα and SREBP-1c result in significant reduction in triglycerides with overall improvement in IS of hepatocytes. LCn-3PUFA through PPARγ stimulation, mechanism of action similar to that of thiazolidinedione class of anti-diabetic drugs (132), balances adipokine secretions, decrease pro-inflammatory signalling and increase IS (133-135). LCn-3PUFA has been shown *in-vitro* studies to decrease diacylglycerol, muscle ceramide and long chain acyl CoA accumulation in myotubes that protected high fat diet fed rodents from developing IRand improved glycogen synthesis and glucose uptake in skeletal muscle (19, 142, 144, 145). Clinical studies with supplementation of EPA + DHA at dose of 3.1 – 8.4g / day have reported 30-55% decrease in production of reactive oxygen species by cultured human neutrophils (146, 147). Cell culture studies reported EPA and DHA have potency to inhibit pro-inflammatory cytokines IL-β, TNFα and NF-κB expression by macrophages (148-150). Recent human intervention studies with LCn-3PUFA at a dose levels between 2-4g/ day reported decrease in inflammation and pro-inflammatory markers (155, 156). Recently published research study on G-protein coupled receptors (GPR) reported that anti-inflammatory activity of LCn-3PUFA is mediated through activation of GPR 120 (274). LCn-3PUFA stimulates GPR 120 in macrophages and adipocytes, through which it exhibits its anti-inflammatory mechanisms. This involves inhibition of transforming growth factor β-activated kinase 1 (TAK 1) through β-arrestin1/TGF-β activated kinase 1/MAP3K7 binding protein 1(TAB1) dependent mechanism. This inhibition leads to down regulation in pro-inflammatory pathways like IKKβ/ NF-κB and JNK/ AP1 (274). LCn-3PUFA abolished activation of NLRP3 inflammasome along with inhibition in caspase -1activation and IL-1β secretion (158). The established data on anti-inflammatory (Inhibition of inflammasome and GPR 120 activation) and triglyceride reducing effects of LCn-3PUFA might be an important aspect to be considered for reducing low grade systemic inflammation, which is promoter for several pathological mechanisms like glucotoxicity and lipotoxicity in different tissues. Regardless of availability on large volume ambiguous clinical data, cellular and physiological mechanisms in different tissues still make LCn-3PUFA as a promising agent for delaying or reducing the risk of T2D.

Combination of curcumin and LCn-3PUFA might provide either synergistic or complimentary effects in prediabetes, which is evaluated through this 2x2 factorial study design. To date previous studies did not report curcumin interfering with LCn-3 PUFA binding to GPR120, thereby preventing the competitive binding for same receptor. Substantiating evidence of blood glucose lowering effects of curcumin and triglyceride lowering effects of LCn-3PUFA might provide improved metabolic health in pre-diabetics. Cytoprotective effects of curcumin might provide additional benefits to maintain integrity of pancreatic β-cells. This trial therefore looks at the potential of these two bio-actives in decreasing the risk factors for developing T2D.

5.5 Strengths and limitation of study design

The strengths of this COP-D trial include valid study methodology; factorial design to evaluate the individual effect and complimentary or synergistic effects of both interventions, double blinding of both participants and investigators to reduce bias. The participants are advised to maintain a constant dietary and physical activity pattern, which is evaluated though questionnaires, to reduce effect of confounding factors on intervention. Use of capsule log, capsule count, erythrocyte fatty acids estimation using gas chromatography and measurement of serum curcumin levels using HPLC provides best measure for compliance of interventions. No toxic or adverse events in previous studies are reported at the dose of curcumin and LCn-3PUFAs used in this trial. There might also be potential limitations for this study, because we are measuring only risk factors like fasting glucose and insulin, lipid parameters and pro-inflammatory cytokines, we cannot translate these results completely into prevention or delaying onset T2D. A follow-up study with a larger sample size and prolonged duration would be required to substantiate whether curcumin and/or LCn-3PUFA could actually prevent or delay onset of T2D. If we can obtain considerable evidence of beneficial effects of curcumin and LCn-3PUFA this trial can provide the basis for a safe and cost effective approach to decrease the risk of diabetes

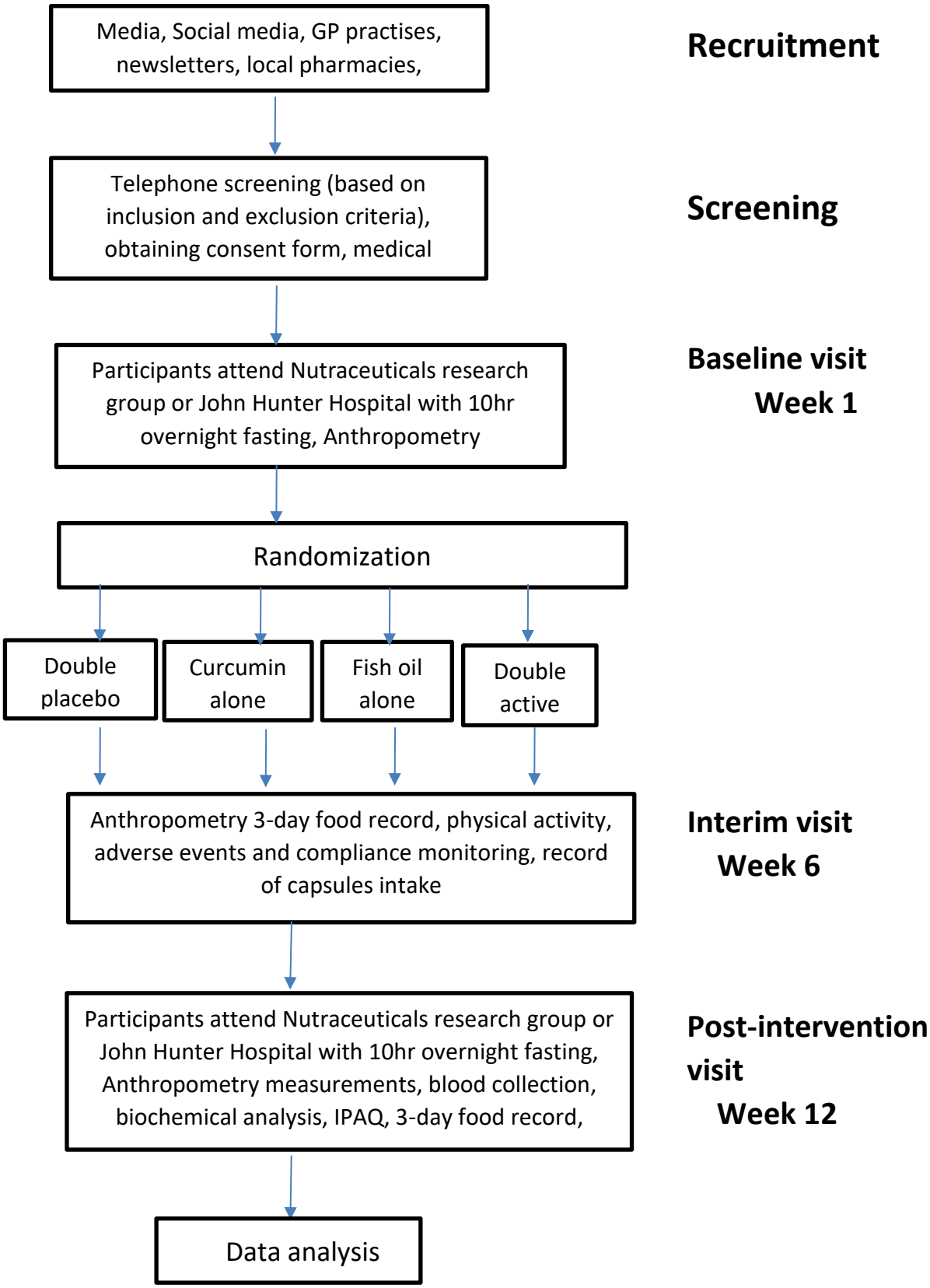

Figure 5.1: Trial protocol flow chart

Table 5.1: Study timeline and Assessments

ASSESSMENTS				
Screening **(**telephone**)**	X			
Informed consent (mail)	X			
IPAQ questionnaire (mail)	X			
3-day food record (mail)	X			
Medical questionnaire (mail)	X			
		WEEK 1	**WEEK 6**	**WEEK 12**
		BASELINE (VISIT1)	**TEST (VISIT 2)**	**TEST (VISIT 3)**
Randomization		X		
Intervention allocation		X		
Anthropometry measurements		X	X	X
Blood sample		X		X
Food diary		X	X	X
Physical activity		X	X	X
Biochemical analysis		X		X
Fasting plasma glucose		X		X
Fasting plasma insulin		X		X
HbA1c		X		X
Fructosamine		X		X
HOMA IR, HOMA S, HOMA β, QUICKI		X		X
Total cholesterol		X		X
Triglycerides		X		X
HDL-C , LDL-C, Total/HDL ratio		X		X
CRP		X		X
Whole blood cell count		X		X
Il-6, Il-1β, Adiponectin, leptin		X		X
Capsule count and log record			X	X
Plasma curcumin concentration				X
Erythrocytes fatty acids content		X		X
Adverse Event reporting			X	X

IPAQ- International physical activity questionnaire; **HbA1c** – glycosylated haemoglobin; **HOMA IR** – homeostatic model assessment for insulin resistance; **HOMA S** – Insulin sensitivity; **HOMA β** – β-cell function; **QUICKI** quantitative insulin sensitivity check index; **HDL-C** – High density lipoprotein cholesterol; **LDL-C** – Low density lipoprotein cholesterol; **CRP-** c-reactive protein; **IL-6 -** interleukin 6; **IL-1β** – Interleukin 1 β

CHAPTER 6

Effects of curcumin and/or omega-3 polyunsaturated fatty acids on glycaemic control and blood lipids in individuals with high risk of Type 2 diabetes: A randomised controlled trial

The contents of this chapter have been submitted for publication:

Thota RN, Acharya SH, Garg ML. Effects of curcumin and/or omega-3 polyunsaturated fatty acids on glycaemic control and blood lipids in individuals with high risk of Type 2 diabetes: A randomised controlled trial. (Manuscript submitted Feb 2018)

(Statement of authorship is presented as appendix-22)

6.1 Abstract

Aims: This study was aimed to evaluate the effects of curcumin and/or long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA) supplementation on glycaemic control and blood lipid levels in individuals at high risk of developing T2D.

Methods: This was a 2x2 factorial, randomised, double-blinded, placebo-controlled study. Participants were allocated to either double placebo (PL) or curcumin plus placebo matching for LCn-3PUFA (CC), or LCn-3PUFA plus placebo matching for curcumin (FO), or curcumin plus LCn-3PUFA (CC-FO) for twelve weeks. Primary outcome of the trial was glycaemic indices (HbA1C, fasting glucose and insulin) with secondary outcome being blood lipids.

Results: A total of sixty-four participants (PL, n=16; CC, n=15; FO, n=17, CC-FO, n=16) were included in the final analysis. Insulin sensitivity (IS) was significantly improved in the CC supplemented group (32.7±10.3 %) compared to PL (P=0.009). FO and CC-FO tended to improve IS by 14.6±8.5% and 8.8±7.7% respectively, but the difference did not reach significance. Fasting glucose and HbA1c remained unchanged across all the groups. Triglyceride levels were further increased in the PL (26.9±7.4%), however, CC and CC-FO supplementation reduced the triglycerides [CC (-0.79%; P=0.019); CC-FO (-5.7% P=0.003)]. FO resulted in the greatest reduction in triglycerides (-16.4±4.5%, P=0.000) and atherogenic index.

Conclusions/interpretation: Improvement in IS with curcumin and reduction in dyslipidaemia by LCn-3PUFA appears to be attractive strategies for lowering the risk of developing T2D in high risk individuals. This study failed to demonstrate any immediate benefits of combined treatment with curcumin and LCn-3PUFA on glycaemic control.

Key words: Curcumin, dyslipidaemia, omega-3 polyunsaturated fatty acids, insulin sensitivity, randomised controlled trial

6.2 Introduction

The period of transition from normal glucose tolerance to overt T2D is primarily mediated by Insulin resistance (IR), a marked independent predictor of progression to T2D in high risk (e.g. obese populations, pre-diabetics) individuals (275, 276). The deficiency of insulin arising due to IR results in progressive deterioration of glucose homeostasis. Results from the British Whitehall II study indicated that IR commences several years prior to diabetes development indicating lowered insulin sensitivity (IS) and reduced β-cell function in the pre-diabetic stage (277). IR is often associated with decreased clearance and increase in the hepatic secretion and of very low-density lipoproteins. As a result, levels of circulating TG are increased, a commonly observed in individuals with IR and MetS (208). IR, together with dyslipidaemia, represents a greater risk of developing both T2D and CVD in high risk individuals (278). Interventions targeting IR and dyslipidaemia might help delay the progression to disease state. Since T2D involves a multifactorial pathogenesis with longer duration, well-tolerated interventions that are cost effective with multiple mechanisms might provide a better solution for lowering the risk of T2D.

Curcumin, a bioactive compound isolated from turmeric, has been shown to provide beneficial effects on IR and glucose intolerance in in-vitro and preclinical studies, (279, 280). These effects were mediated via lowering of low grade inflammation via down-regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and cytoprotection of pancreatic β-cells by increasing the concentrations of anti-oxidant enzymes (279). In mice models of diabetes, curcumin increased the expression of 5' adenosine monophosphate-activated protein kinase (AMPK), a key regulator of glucose and lipid homeostasis (281, 282). Clinical trials with curcumin supplementation (205, 250, 283) have shown promising results, therefore, further substantiation of the beneficial effects of curcumin on glycaemic control is warranted.

LCn-3PUFA (eicosapentaenoic acid, EPA; docosahexaenoic acid DHA) have been shown to be associated with lower incidence of diabetes (284). LCn-3PUFA mediated increase in IS has been proposed to be a possible underlying mechanism for lowering the incidence of T2D (284). In the preclinical studies, LCn-3PUFA increased IS via peroxisome-proliferator activated receptor-gamma (PPARγ) activation (285) that

mediates the adipokines secretions, particularly adiponectin (132); and by GPR 120 activation, that is involved in down regulating the pro-inflammatory pathways (274). However, the evidence from the prospective studies, meta-analysis and randomised controlled trials has been contradictory, leading to ambiguity over beneficial effects of LCn-3PUFA (286, 287). LCn-3PUFA have long been to be known to reduce plasma triglycerides (TG), primarily through reducing hepatic very low-density lipoprotein (VLDL-TG) production, and to some extent from increasing the clearance of VLDL-TG (288). As increased VLDL-TG production is an early manifestation of IR, LCn-3PUFA mediated reduction in VLDL-TG secretion might provide a complimentary beneficial effect on IR along with established triglyceride lowering effects in individuals at high risk and those with T2D. Since IR is a hallmark for progression of T2D, intervening IR might provide both beneficial effects on glycaemic control and preservation of β-cell function. Parallelly lowering the dyslipidaemia is of prime importance to alleviate the risk factors associated with IR. In this study we aimed to evaluate the effects of curcumin and/or LCn-3PUFA on glycaemic indices (HbA1c, fasting glucose and Insulin). In addition, the effects of supplementing curcumin and LCn-3PUFA on lipid and inflammatory markers were also examined.

6.3 Methods

6.3.1 Subjects

Participants with high risk of developing diabetes (assessed through Australian diabetes risk assessment tool, AUSDRISK) or with IFG or IGT, were recruited from the Hunter Region, New South Wales, Australia through recruitment flyers and media advertisements. They were screened through telephone interviews based on the inclusion criteria: age between 30-70 years; body mass index (BMI) must lie between 25-45 kg/m^2; diagnosed either with IFG (fasting glucose 6.1-6.9 mmol/L), IGT (2-hour plasma glucose ≥7.8 mmol/L - <11.1 mmol/L) or both; HbA1c levels lie between 5.7-6.4% or they obtain a score of 12 or more in the AUSDRISK tool assessment (a non-invasive questionnaire to determine the risk of developing diabetes). Exclusion criteria included diagnosis with T2D; history of severe neurological diseases or seizures; gall bladder problems; pregnancy or planning to become pregnant or breastfeeding; on pacemakers; consuming >2 serves of oily fish per week or take supplements known to influence blood glucose levels. All participants gave their written informed consent.

This study was approved by the University of Newcastle and the Hunter New England Area Health Service Human Research Ethics Committees. The trial has been registered with Australia New Zealand Clinical Trial Registry (ACTRN12615000559516).

6.3.2 Study design

The detailed protocol of the current trial was described in our previous publication elsewhere (289). This was a twelve-week 2x2 factorial, double-blinded, randomised controlled trial. Allocation of intervention was performed by a software-based (Random Allocation Software 1.0.0) randomisation technique, using alpha-numeric codes in blocks of 8. Participants who attended the baseline visit at Nutraceutical Research Program clinical trial facility or John Hunter hospital, were randomised to one of the four interventions: Placebo (PL, 2 x placebo tablets matching for curcumin plus 2x1000mg corn oil capsules per day), curcumin (CC, 2x500mg curcumin (Meriva®) tablets, providing 180mg of curcumin plus 2x1000mg corn oil capsules per day), LCn-3PUFA (FO, 2x1000mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA+EPA plus 2xplacebo tablets matching for curcumin) or double active (CC-FO, 2x500mg curcumin (Meriva®) tablets, providing 180mg of curcumin plus 2x1000mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA+EPA). Participants were advised to take two allocated tablets/capsules with morning and evening meals. They were also advised to maintain their routine dietary intake and physical activity during the study period. The compliance to the study interventions were measured during the follow-up and post-intervention visit using a capsule count and capsule intake log. Erythrocyte fatty acid analysis is also used to check the compliance for FO and CC-FO. Changes in the medications or any illness during the study duration were also recorded.

6.3.3 Data collection and outcome measures

Primary and secondary outcomes

The primary outcome in this trial was to evaluate the effects of curcumin and/or LCn-3PUFA on parameters relating to glucose control i.e. glycosylated haemoglobin (HbA1c), fasting glucose, fasting insulin and IR. Secondary outcomes included lipid profile (total cholesterol, TC; TG; HDL-Cholesterol, HDL-C; LDL-Cholesterol, LDL-C; and Total: HDL-cholesterol ratio, TC: HDL-C), C-reactive protein (CRP) and whole blood cell count. Fasting (at-least for 10 hours) blood samples were collected from participants during baseline and post-intervention visits into EDTA, fluoride/oxalate and serum clot

+ Gel + Clot activator vacutainer by a trained phlebotomist. HbA1c was measured through Bio-Rad Variant II HbA1c testing system by Hunter Area Pathology Service (HAPS), an accredited pathology laboratory for compliance with National Pathology Accreditation Advisory Council Standards. Fasting glucose, lipids, CRP and whole blood cell count was measured using VP autoanalyzer by HAPS. Homeostasis model assessment (HOMA) calculator (https://www.dtu.ox.ac.uk/homacalculator/) was used to estimate IR and IS (%S). Atherogenic index of plasma (AIP) values was derived by using formula log (TG: HDL-C). InsuTAG values were derived by using the formula fasting insulin x fasting triglycerides (206). Erythrocyte fatty acids were determined using direct transesterification followed by gas chromatography (Hewlett Packard 7890A Series GC with Chemstations Version A.04.02) (290).

Anthropometry and body composition measurements

Body composition measurements (weight, percent body fat, muscle mass) were performed on baseline and post-intervention visit days using direct segmental multi-frequency bioelectrical impedance (InBody 230, Biospace Co., Ltd. Seoul, Korea) Height (cm) of the study participants was measured using a wall mounted roll up stadiometer (SE206, Seca). Waist circumference (cm) was measured using a tape measure positioned between about halfway between the bottom of the lowest rib and the top of hip bones, roughly in line with the belly button.

Questionnaires (diet, physical activity and medical history)

At baseline, medical history and demographic information was obtained from participants via self-administered questionnaire. Participants were asked to complete a three-day food diary before the baseline and 12-week visit days. These food diaries were analysed through FoodWorks, Xyris (version 8.0) to estimate the measure of participants' habitual dietary intakes. Physical activity of the participants was assessed using the International Physical Activity Questionnaire (IPAQ) – long form version.

6.3.4 Statistical analysis

Sample size was calculated using a computer program (PS Power and Sample Size Calculations Version 3.0), based on the previous data on mean changes in HbA1c (standard deviation of 0.5 units). Seventeen participants in each intervention group were required to give 80% power at 0.05 significance level for detection of 10% reduction in HbA1c. Accounting for drop-out rate of 20% we needed to recruit 20 participants for each treatment group.

Data collected at the baseline was analysed for normality using histograms and Shapiro-Wilk's test and presented as mean±SEM (standard error of the mean) or median (IQR, interquartile range) as appropriate. Significant changes in the baseline data between the groups were assessed through analysis of variance (ANOVA) or Kruskal-Wallis when the normality assumption was not met. Post-intervention data was presented as mean±SEM or median (IQR) of absolute change (post-intervention value minus baseline value) for log transformed values and blood cell count and as relative change [(absolute change/baseline value) * 100] for other variables. Changes from the base-line to post-intervention within-treatment group were assessed through paired t-test or Wilcoxon signed-rank test. Significant effects of intervention on mean changes in the variables between the groups were measured using two-way ANOVA with post hoc (tukey's) analysis. Correlations between the independent variables and dependent variables were obtained using Pearson product-moment correlation coefficient analysis. Based on the significant correlation values, Analysis of covariance (ANCOVA) was performed on an optimal set of confounding variables.

6.4 Results

6.4.1 Baseline characteristics

Eighty-one participants were randomised in this study. Number of participants allocated to the individual intervention groups and drop-outs are presented in the Consolidated Standards of Reporting Trials (CONSORT) flow chart (figure 6.1). There were no statistically significant differences observed in all the baseline characteristics between the four groups (Table 6.1 and 6.2).

6.4.2 Body composition

Comparisons between the groups showed no significant differences observed between the mean changes in body composition measurements (body weight, muscle mass, BMI, percent body fat, waist circumference (data not presented) with all the three interventions.

6.4.3 Glycaemic indices

After 12 weeks of intervention with curcumin and/or LCn-3PUFA, no significant changes were observed in fasting glucose (Figure 6.2 A) and HbA1c (table 6.3). Fasting insulin was significantly (P=0.005) reduced only in CC group [-18.79 (27.6) %] from baseline (Figure 6.2 B). When compared between the groups, CC lowered the fasting insulin

significantly (P=0.002), but not FO (P=0.054) and CC-FO (P=0.101) compared with PL group (figure 6.2 B).

Similar trends were observed in the changes with HOMA2 IR (Figure 6.2 C) and HOMA-S (Figure 6.2 D) within curcumin treatment group and between CC and PL group.

6.4.4 Blood lipids

Post intervention, TG levels were significantly (P=0.007) reduced (-16.54 ± 4.5 %) in FO group and increased (26.8 ± 7.4 %, P=0.007) in PL group (figure 6.3 A). However, when compared between the PL group (0.24, P=0.001), reduction in TG was significant with FO (P=0.000), CC (P=0.019) and CC-FO (P=0.003) (Figure 6.3 A). Similar trends observed with absolute change in AIP with FO (-0.25, P=0.004) and PL groups (figure 6.3 B). AIP was significantly reduced in FO (P=0.000), CC (P=0.025), and CC-FO (P=0.008) groups compared with PL group. There were no statistically significant differences observed in TC (table 6.3), HDL-C (figure 6.3 C), LDL-C and TC: HDL-C between the groups (table 6.3). Both CC and FO exhibited similar magnitude of reductions in absolute change of InsuTAG by -0.29 (0.013) and -0.3 (0.009) respectively from baseline (Figure 6.3D). When compared between the groups, significant reductions in InsuTAG were observed with supplementation of CC (0.001), FO (0.001) and CC-FO (0.030) compared to PL.

6.4.5 Inflammation and blood cell count

Both CC and FO and CC-FO did not exhibit any significant effects on CRP in with-in group or between the group analyses (table 6.3). WBC counts were significantly reduced in the CC and FO group from the baseline (table 6.3). However, this trend on white cells count by CC and FO were not seen in the between-group analysis when compared with PL (table 6.3).

6.4.6 Dietary intake, physical activity, compliance and adverse effects

Comparisons between the groups showed no significant differences, both baseline and post-intervention, on mean diet intake and physical activity levels of the participants (table 6.4). Post intervention, mean changes in body composition measurements (body weight, muscle mass, BMI, percent body fat, waist circumference) were also not changed in the participants (data not presented). The mean compliance of the participants to the study products was 94.86±5.8% (capsule count). Erythrocyte fatty acid content of DHA and EPA were significantly increased by FO (EPA 29.3 (59.5) %; DHA 54.0 (43.6) %) and CC-FO (EPA 27.8 (61.8) %; DHA 39.6 (55.9) %). CC and FO were well tolerated

by the participants and no adverse events were reported during the study period. ANCOVA analysis indicated physical activity as potential confounder for relative change in the fasting insulin levels (P=0.026) and fasting triglycerides (P= 0.013), but the effect of CC (P=0.012) for fasting insulin and FO (0.018) remained after adjusting for physical activity.

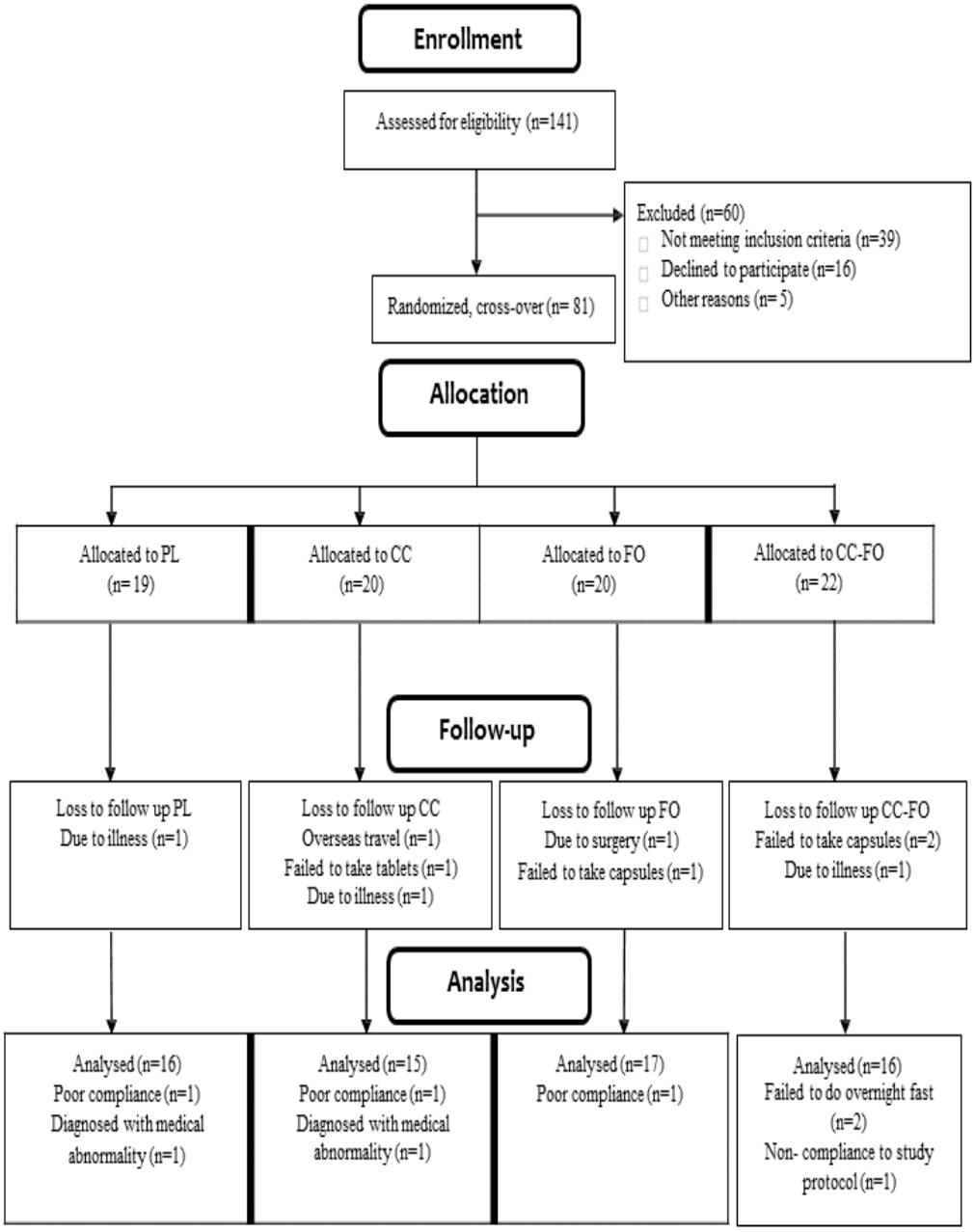

Figure 6.1: Consolidated Standards of Reporting Trials (CONSORT) flow chart

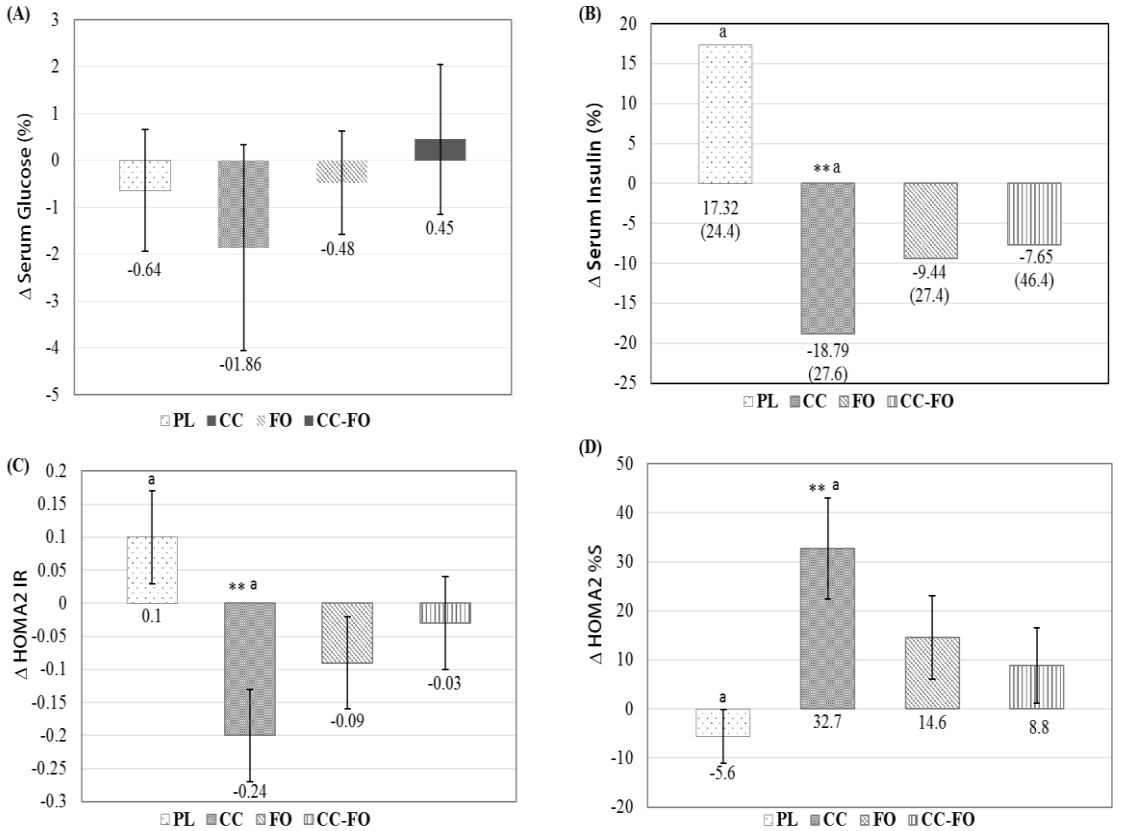

Figure 6.2: Changes in the outcome measures from baseline to post intervention with-in and between double placebo (PL), curcumin (CC), fish oil (FO) and curcumin + fish oil (CC-FO)

A. Change in the serum glucose (%); B. Change in the serum insulin (%). C. Change in homeostatic model assessment for insulin resistance (log transformed) (HOMA2 IR); D. Change in insulin sensitivity. Data presented as mean±SEM or median (IQR) as appropriate. Significant changes from baseline indicated by *p<0.05, **p<0.01. Values with a common letter indicate significant differences between the groups.

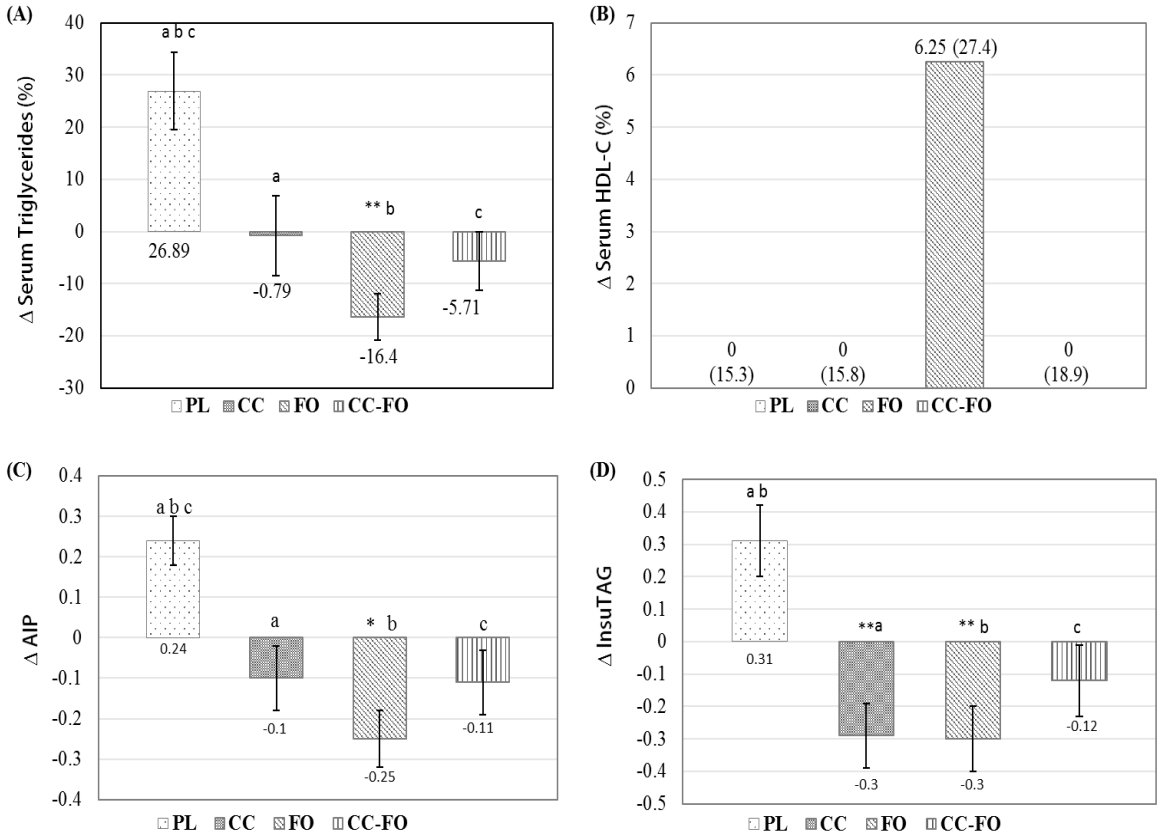

Figure 6.3: Changes in the outcome measures from baseline to post intervention and between double placebo (PL), curcumin (CC), fish oil (FO) and curcumin + fish oil (CC-FO);

A. Change in the serum triglyceride (%). B. Change in the serum HDL-Cholesterol (%); C. Change in atherogenic index of plasma (AIP) D. Change in InsuTAG (log transformed). Data presented as mean ± SEM or median (IQR) as appropriate. Significant changes from baseline indicated by *p<0.05, **p<0.01. Values with a common letter indicate significant differences between the groups.

Table 6.1: Baseline general characteristics of the study population:

Characteristics	Total (n=64)	PL (n=16)	CC (n=15)	FO (n=17)	CC-FO (n=16)	P-value
Age (years)	55 ± 1.3	50 ± 2.5	55 ± 2.8	58 ± 2.5	57 ± 2.2	0.106
Males/females (n/n)	26/38	7/9	6/9	7/10	6/10	-
Ethnicity – no (%)						
Caucasian	50 (78.12)	12 (75)	12 (80)	13 (76.5)	13 (81)	-
Asian	7 (10.93)	2 (12.5)	2 (13.3)	1 (5.8)	2 (12.5)	-
Others	7 (10.93)	2 (12.5)	1 (6.6)	3 (17.6)	1 (6.25)	-
Anthropometry measures						
Body weight (kg)	88.5 ± 2.1	91.0 ± 4.6	88.1 ± 3.5	85.7 ± 4.9	89.9 ± 3.33	0.266
Muscle mass (kg)	32.4 ± 1.2	32.7 ± 1.2	34.2 ± 2.3	32.6 ± 3.6	30.3 ± 1.5	0.432
Body mass index (kg. m^{-2})	31.1 ± 0.7	31.9 ± 1.7	30.9 ± 1.2	30 ± 1.4	31.7 ± 1.2	0.468
Waist circumference(cm)	104.7 ± 1.5	105.7 ± 3.6	104.9 ± 2.7	102.5 ± 3.4	105.8 ± 1.9	0.086
Waist-hip ratio	0.96 ± 0.01	0.97 ± 0.01	0.96 ± 0.01	0.96 ± 0.06	0.99 ± 0.01	0.568
Percent body fat (%)	36.1 ± 1.3	35.3 ± 2.2	34.8 ± 2.5	35.3 ± 2.7	39.2 ± 2.4	0.507
Family history – no (%)						
Family history of diabetes	31 (48.4)	8 (50)	11 (73.3)	4 (23.52)	8 (50)	-
Family history of cholesterol	32 (50)	9 (56.25)	8 (53.33)	8 (47.05)	7 (43.75)	-
Family history of blood pressure	13 (81.25)	13 (81.25)	9(60)	12(70.5)	10(62.5)	-
Alcohol intake	54 (78.1)	10 (62.5)	15(100.0)	14 (82.3)	15(100.0)	-
Smoking	5 (7.8)	3 (18.7)	1 (6.6)	0 (0.0)	1 (6.3)	-
MEDICATION AND SUPPLEMENT USE -no. (%)						
Anti-hypertensives	19(29.68)	3(18.75)	6(40)	7(41.17)	3(18.75)	-
Anti-depressants	10(16.6)	6(37.5)	3(20)	0(0)	1(6.25)	-
Cholesterol lowering medications	10(16.6)	1(6.25)	3(20)	4(23.52)	2(12.5)	-
Antacids	9(14.06)	2(12.5)	4(26.67)	3(17.6)	0(0)	-
Supplements no	32(50)	7(43.75)	11(73.3)	8(47.05)	6(37.5)	-

Data is presented as mean ± SEM or median (IQR), unless otherwise specified. n, number of participants; PL, double placebo; CC, curcumin; FO, Fish oil; CC-FO, curcumin plus fish oil. Kg, kilogram; cm, centimetre; %, percent; Kj, Kilojoule; METs, Metabolic equivalents. SEM, Standard error of the mean; IQR, interquartile range.

Table 6.2: Baseline blood parameters of the trial participants

CHARACTERISTICS	Total (n=64)	PL (n=16)	CC (n=15)	FO (n=17)	CC-FO (n=16)	P-VALUE
Glycaemic control parameters						
Fasting plasma glucose (mmol/l)	5.6 ± 0.1	5.2 ± 0.1	5.7 ± 0.2	5.7 ± 0.1	5.8 ± 0.2	0.168
Fasting serum insulin (mIU/L)	11.5 ± 0.8	11.5 ± 1.4	10.9 ± 1.5	12.6 ± 2.2	11.0 ± 1.5	0.957
HOMA2 IR	1.3 (0.9)	1.4 (1)	1.3 (0.6)	1.3 (0.9)	1.3 (1.3)	0.977
HOMA2 %S	76.1 (53.4)	73.7 (53.7)	79.4 (37.9)	76.6 (53.2)	78.4 (75.6)	0.975
InsuTAG	78.4 (89.5)	111.5 (119.6)	78.5 (85.0)	100.4 (103.7)	72.4 (57.6)	0.455
HbA1c (%)	5.5 ± 0.04	5.3 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	0.207
Fructosamine (umol/L)	229.4 ± 3.4	217.9 ± 8.5	233.3 ± 7.4	229.2 ± 5.7	237.6 ± 5.2	0.285
Lipid parameters						
Total cholesterol (mmol/L)	5.6 ± 0.1	5.7 ± 0.4	5.4 ± 0.3	5.7 ± 0.2	5.4 ± 0.2	0.795
LDL-C (mmol/L)	3.6 ± 0.1	3.8 ± 0.3	3.5 ± 0.2	3.7 ± 0.2	3.4 ± 0.2	0.583
HDL-C (mmol/L)	1.4 ± 0.0	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	0.195
Triglycerides (mmol/L)	1.4 ± 0.1	1.7 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	0.170
Total: HDL-C	4.2 (1.6)	4.5 (2.1)	4.3 (2.0)	4.1 (0.9)	3.8 (1.4)	0.120
Inflammation and blood cell count						
CRP (mg/L)	2 (3)	2.3 (4.7)	1.7 (2)	2.5 (3.4)	1.3 (2.4)	0.250
White blood cells (10^9/L)	6.8 ± 0.2	7.0 ± 0.5	7.0 ± 0.5	7.0 ± 0.3	6.3 ± 0.5	0.680
Neutrophils (10^9/L)	3.9 (1.5)	4.0 (1.7)	4.3 (2.2)	3.9 (0.8)	3.6 (2.0)	0.579
Lymphocytes (10^9/L)	1.9 ± 0.9	2.0 ± 0.7	2.1 ± 0.2	2.3 ± 0.2	1.8 ± 0.9	0.241
Monocytes (10^9/L)	0.5 (0.2)	0.4 (0.3)	0.5 (0.2)	0.5 (0.3)	0.5 (0.2)	0.203
Red blood cells (10^9/L)	4.7 (0.6)	4.8 (0.8)	4.6 (0.7)	4.7 (0.5)	4.9 (0.7)	0.821
Platelets (10^9/L)	229 (73.5)	223 (46)	232 (74)	243 (104)	241 (77)	0.893

Data reported as means ± SEM. or median (IQR). PL, double placebo; C, curcumin; FO, fish oil; CC-FO, curcumin + fish oil; HOMA2 IR, homeostatic model assessment for insulin resistance; HOMA2 %S, insulin sensitivity; HbA1c, glycosylated haemoglobin; CRP, C-reactive protein; LDL-C, LDL-Cholesterol; HDL-C HDL-cholesterol; Total: HDL-C, Total cholesterol to HDL-C ratio.

Table 6.3: Changes in the outcome measures from baseline to post-intervention

CHARACTERISTICS	PL (n=16)	CC (n=15)	FO (n=17)	CC-FO (n=16)	P-VALUE
Glycaemic control parameters					
Fasting plasma glucose (%)	-0.6 ± 1.3	-1.9 ± 2.2	-0.5 ± 1.1	0.5 ± 1.6	0.851
HbA1c (%)	1.7 ± 0.6*	1.0 ± 0.7	2.0 ± 0.8*	2.2 ± 1.2	0.765
Fructosamine (umol/L)	2.2 (14.7)	0.8 (13.3)	2.7 (9.1)	1.7 (10.6)	0.776
Lipid parameters (%)					
Total cholesterol (mmol/L)	0 (3.7)	1.92 (14.4)	0 (10.8)	2.7 (19.2)	0.767
LDL-C (mmol/L)	-2.7 (10.2)	-1.6 (25.1)	3.0 (17.9)	6.5 (29.7)	0.120
HDL-C (mmol/L)	0 (15.3)	0 (15.8)	6.3 (12.5)	0 (19.0)	0.333
Total: HDL-C	3.3 (12.4)	-5.2 (9.7)	-2.6 (16.5)	2.6 (12.2)	0.180
Inflammation and blood cell count					
CRP (mg/L)	-0.3 (1.2)	0.3 (0.6)	0.3 (1.4)	0 (0.9)	0.080
White blood cells (10^9/L)	0.2 (1.1)	-0.7 (1.3)*	-0.4 (0.8)*	-0.4 (1.2)	0.339
Neutrophils (10^9/L)	0.2 (0.8)	-0.1 (0.8)	-0.5 (0.9)	-0.3 (1.1)	0.070
Lymphocytes (10^9/L)	0.04 ± 0.1	-0.3 ± 0.1*	0.1 ± 0.1	0.1 ± 0.1	0.024
Monocytes (10^9/L)	-0.01 ± 0.0	-0.1 ± 0.0**	0.0 ± 0.0	-0.01 ± 0.0	0.290
Platelets (10^9/L)	3 (27)	2 (19)	2 (29)	-10 (24)	0.618

Data presented as mean±SEM or median (IQR) as appropriate.
Significant change from baseline, *p<0.05, **p<0.01 represents the significant differences from the baseline with-in treatment groups.
PL, placebo; CC, curcumin; FO, fish oil; CC-FO, curcumin + fish oil; HbA1c, glycated haemoglobin; LDL-C, LDL-cholesterol; HDL, HDL-cholesterol; Total: HDL, total cholesterol-to-HDL ratio; CRP, C-reactive protein;

Table 6.4: Changes in the dietary intake, physical activity and fatty acid composition of the red blood cell membranes of the participants

CHARACTERISTICS	PL (n=16)	CC (n=15)	FO (n=17)	CC-FO (n=16)	P-value
Dietary intake					
Baseline (Kj)	8790 ± 632.85	9614 ± 534.9	8602 ± 317.3	8209 ± 432.7	0.257
Change	4 ± 467.0	-327 ± 452.6	-591 ±397.6	811 ± 446.6	0.131
Physical activity					
Baseline (METs-minutes/ week)	3859 (5109)	1692 (1597)	2919 (4087.5)	2970.5 (2718.2)	0.257
Change	-349 (3103.3)	61 (1508)	-539 (2012)	113 (1120.2)	0.409
Erythrocyte fatty acid composition (%, w/w)					
AA					
Baseline	15.6 (2.5)	16 (2.2)	15.5 (3.2)	15.7 (1.6)	0.148
Change (%)	-1.9 (9.7) [a]	-2.7 (8.3)	-10.9 (10.3)**	-12.2 (7.0)**[a]	0.003
EPA					
Baseline	0.96 (0.33)	1.11 (0.77)	1.05 (0.43)	1.10 (0.36)	0.067
Change (%)	-3.02 (19.04)[b,c]	-12.78 (4.07)*	29.34 (59.47)**[b]	27.77 (61.88)**[c]	0.0001
DHA					
Baseline	5.7 (1.4)	6.5 (2.3)	6.3 (2.4)	6.4 (2.8)	0.169
Change (%)	1.19 (16.4)[d,e]	-3.69 (22.6)	53.96 (43.6) **[d]	39.63 (56.0)**[e]	0.0001
***Omega*-3 Index**					
Baseline	6.63 (1.8)	7.3 (2.3)	7.6 (2.6)	7.7 (3.3)	0.190
Change	0.82 (16.38)[f,g]	-4.16 (18.8)	46.86 (44.53)**[f]	35.12 (56.69)**[g]	0.0001

Data is presented as mean ± SEM or median (IQR) as appropriate.
Significant change from baseline, *p<0.05, **p<0.01 represents the significant differences from the baseline with-in treatment groups. PL, placebo; CC, curcumin; FO, fish oil; CC-FO, curcumin + fish oil; AA, Arachidonic acid; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; *n*-3 Index Omega-3 index. METs-minutes/week, metabolic equivalent minutes per week.

[a] AA was significantly reduced in CC-FO (P=0.0035) group compared to PL.

[b] EPA was significantly (P=0.0016) increased in the FO group compared to PL.

[c] EPA was significantly increased in CC-FO (P=0.0009) group compared to the PL.

[d] DHA was significantly increased in the FO (P<0.001) group compared to PL.

[e] DHA was significantly increased in the CC-FO (P=0.0018) group compared to PL.

[f] Omega-3 Index was significantly increased in the FO (P<0.001) group PL.

[g] Omega-3 Index was significantly increased in the CC-FO group compared to PL(P=0.0006).

Table 6.5: Mean changes in outcome measures stratified by sex within PL, CC, FO and CC-FO groups

	PL		CC		FO		CC-FO	
Variable	**Males (n=7)**	**Females (n=9)**	**Males (n=6)**	**Females (n=9)**	**Males (n=7)**	**Females (n=10)**	**Males (n=6)**	**Females (n=10)**
Fasting glucose (%)	-2.3 ± 1.8	0.7 ± 2.0	-4.5 ± 4.1	-0.1 ± 2.7	-0.7 ± 3.4	-0.4 ± 2.4	0.1 ± 2.2	0.7 ± 2.4
HbA1c (%)	2.0 ± 1.5	1.5 ± 0.7	0.4 ± 0.9	1.4 ± 1.0	1.3 ± 1.3	2.6 ± 0.9	3.9 ± 2.3	1.2 ± 1.3
Fasting insulin (%)	26.5 ± 29.6	10.1 ± 7.7	-26.6 ±7.1	-13.6 ± 7.6	-10.2 ± 10.3	-1.2 ± 9.5	-9.6 ± 7.5	7.5 ± 12.4
HOMA2 IR	0.1 ± 0.2	0.1 ± 0.1	-0.3 ± 0.1	-0.2 ± 0.1	-0.1 ± -0.1	-0.1 ± 0.1	-0.1 ± 0.1	0.0 ± 0.1
HOMA2 %S	-5.4 ± 10.5	-5.8 ± 6.2	45.9 ± 18.2	23.9 ± 0.3	20.3 ± 13.3	10.6 ± 11.4	15.6 ± 3.6	10.8 ± 10.6
InsuTAG	0.3 ± 0.2	0.3 ± 0.1	-0.5 ± 0.2	-0.2 ± 0.1	-0.4 ± 0.1	-0.2 ± 0.2	-0.3 ± 0.1	-0.0 ± 0.2
TG (%)	21.4 ± 8.6	31.2 ± 11.6	-7.5 ± 15.1	3.8 ± 8.5	-19.5 ± 6.6	-14.3 ± 6.3	-14.2 ± 9.3	-0.6 ± 7.0
HDL-C (%)	-3.0 ± 5.8	-2.2 ± 2.2	6.2 ± 6.3	1.5 ± 3.4	10.7 ± 3.6	2.3 ± 5.2	8.6 ± 1.2	1.2 ± 7.9
AIP	0.2 ± 0.1	0.2 ± 0.1	-0.1 ± 0.2	-0.0 ± 0.1	-0.3 ± 0.1	-0.2 ± 0.1	-0.3 ± 0.1	-0.0 ± 0.1
TC (%)	0.6 ± 3.1	-0.3 ± 1.1	5.5 ± 10.6	0.7 ± 3.7	3.2 ± 3.7	6.8 ± 6.2	7.6 ± 6.6	5.7 ± 4.1
LDL-C (%)	-3.3 ± 2.7	-4.3 ± 2.8	11.0 ± 17.6	0.4 ± 5.4	5.5 ± 3.8	11.6 ± 8.4	11.3 ± 7.3	8.4 ± 5.8

Data are presented as mean ± SEM.

PL, placebo; CC, curcumin; FO, long chain omega-3 polyunsaturated fatty acids; CC-FO curcumin plus LCn-3PUFA. HbA1c, Glycosylated haemoglobin; HOMA2 IR, Homeostatic model assessment for insulin resistance (log transformed); HOMA2 S, Homeostatic model assessment for insulin sensitivity; TG, Triglycerides; HDL-C high density lipoprotein-cholesterol; AIP, Atherogenic index of plasma; TC, total cholesterol, LDL-C, low density lipoprotein- cholesterol.

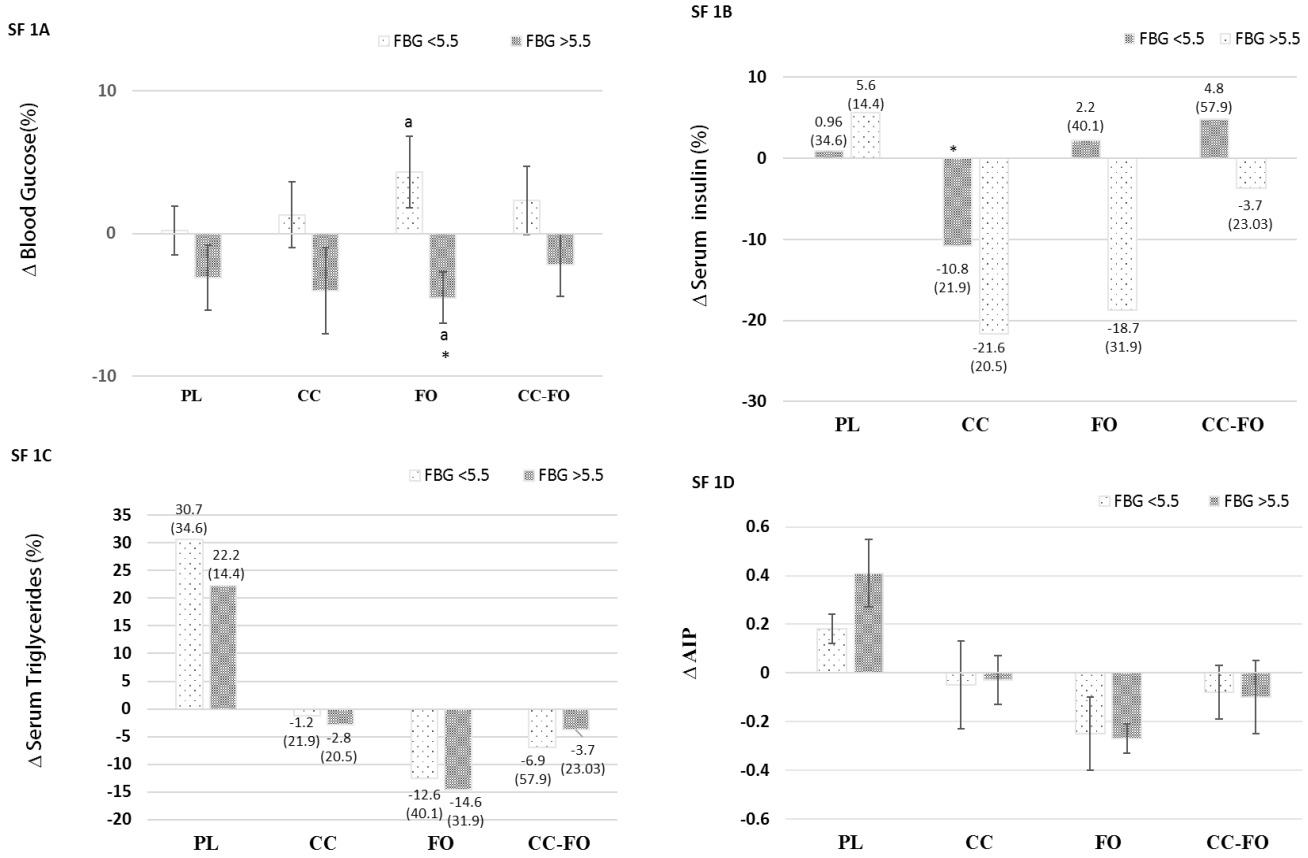

Figure 6.4: Changes in the outcome measures from baseline to post-intervention in people with FPG >5.5 and <5.5 in PL, CC, FO and CC-FO groups.

Data is presented as mean ± SEM or Median (IQR) as appropriate**.** PL, double placebo; CC, curcumin; FO, Fish oil; and CC-FO, curcumin + fish oil; 1A. Change in the fasting blood glucose (%). 1B. Change in the serum insulin (%); 1C. Change in serum triglyceride (%) 1D. Change in atherogenic index of plasma (%). FBG >5.5: PL (n=4); CC (n=9); FO (n=10); CC-FO (n=12); FBG <5.5: PL (n=12); CC (n=6); FO (n=7); CC-FO (n=6). Significant changes from baseline indicated by * $p<0.05$. Means with same lower letter indicate significant differences –a. Fasting blood glucose levels were significantly lower in people with baseline FBG levels >5.5 with FO supplementation (P=0.008) compared to people with baseline FBG < 5.5.

6.5 Discussion

In attempts to find well-tolerated candidates with potential to act through multiple pathways to lower the risk or to the delay the progression of T2D, we evaluated curcumin and/or LCn-3PUFA for achieving the glycaemic control in individuals at high risk of developing T2D. The results presented demonstrate that supplementation with curcumin is effective in lowering the serum insulin levels along with improvement in IS. Curcumin also showed a significant reduction in the serum TG and AIP. However, the magnitude of reduction for the serum triglycerides and AIP was higher in the individuals receiving fish oil. The effects in participants receiving both curcumin and LCn-3PUFA were in line with the individual supplementation groups, however we did not find any additive or complimentary benefits. Despite improving the risk factors (such as insulin resistance) by curcumin and LCn-3PUFA, there were no immediate beneficial effects observed on glycaemic control in 12 weeks.

In preclinical studies with diet-induced IR models, curcumin mediated amelioration of IR was primarily through decreased NFκB activity, increased anti-oxidant enzymes (Foxo1) and activation of AMPK α-subunit by phosphorylating Thr-172 residue, thereby increasing the IS and overall glucose control (6, 23). In the current study, curcumin effectively lowered the fasting insulin levels and IR (HOMA2 IR) and improved the IS (evaluated through HOMA2 %S), in line with reports from the other clinical trials (10, 11). We have previously demonstrated that acute supplementation with same dose of curcumin significantly lowered the meal stimulated postprandial insulin response (AUC) by 26% (Thota et al, under review) compared with placebo. Controlling postprandial insulin responses could be one of the potential underlying mechanisms for reduction in the elevated fasting concentrations of insulin by curcumin. In contrast to the glucose lowering reports from other previously published studies (10, 11) with longer duration of supplementation, in this study there was no significant effects of curcumin on both fasting glucose and HbA1c. This controversy may be explained by the fact that fasting blood glucose levels were only marginally higher in the current study and that twelve weeks may be inadequate to influence changes in HbA1c. Moreover, the dose and formulation of the curcumin employed in these trials were completely different.

The effects of curcumin on blood lipids are still unclear (24, 25). Recent publication from our lab on randomised controlled trial with curcumin supplementation reported enhancement of the cholesterol lowering properties of phytosterols (26). In the current study, curcumin did not

influence cholesterol levels, however it prevented the rise in serum triglycerides that was otherwise evident in the placebo group. The concentration of fasting serum insulin was positively correlated with serum triglycerides (r=0.274, P=0.02). Curcumin supplementation blocked the rise in triglycerides, which could probably be mediated via reduction in the insulin levels. AIP, an independent indicator of coronary artery disease and atherosclerosis (27-29) was significantly lowered by curcumin compared to the placebo group, in line with a randomised controlled trial on beneficial effects of curcumin on atherogenic risks (30).

Several clinical trials and meta-analysis have confirmed triglyceride lowering effects of LCn-3PUFA (31, 32). In the current study, LCn-3PUFA supplementation effectively reduced the circulating triglycerides and AIP. Significant elevation in the erythrocyte levels of both EPA and DHA in the FO receiving groups also confirms its LCn-3PUFA mediated triglyceride lowering effects. On the other hand, a clear trend was observed for improvements in IS (p=0.054) with FO supplementation. These observations on beneficial effects of LCn-3PUFA for improving IS and glucose intolerance is in support with the existing study reports (33-35). A meta-analysis from our research group has previously reported that trials with supplementation of FO ≥6 weeks, showed a significant improvement in IR in women, but not in men (35). This sex dependent effect could partly be one of the key reasons that could be affecting overall outcomes on IS with FO supplementation. Improving omega-3 index (sum of EPA and DHA in erythrocytes) has been associated with increased IS and lower incidence of diabetes (36). FO supplementation improved the omega-3 index of participants to a significant extent, suggesting a favourable metabolic outcome. One of the key mechanism of LCn-3PUFA may be via resolution of inflammation, which plays a key role in resolving persistent tissue low-grade inflammation in chronic metabolic diseases (37, 38). This is believed to be mediated through changes in the membrane phospholipid composition of the cells participating in inflammation process. Studies in humans with doses ranging from (1.3g to 2.5g per day) have shown decreased production of arachidonic acid, a precursor for pro-inflammatory eicosanoids (39). In the current study, with 1.3g EPA + DHA per day, we have shown significant reduction in the erythrocyte levels of arachidonic acid, indicating reduced production of pro-inflammatory molecules. As CRP is more reflective of any acute inflammation or even an infection (40) than resolution of inflammation, we could not find any changes in CRP with FO supplementation in this study.

We have recently introduced a novel marker with high sensitivity and specificity values, InsuTAG, that considers both insulin and triglycerides for identifying individuals with IR and MetS (24). Supplementation of curcumin significantly reduced the InsuTAG levels in the current study, which could primarily be a result from reduction in the fasting insulin. LCn-3PUFA supplementation also shown similar significant reduction in the InsuTAG levels. LCn-3PUFA mediated reduction in InsuTAG could be mainly from lowering the plasma triglycerides. Though the effects on IR are mediated through different mechanism by curcumin and LCn-3PUFA, overall reduction in the InsuTAG implies potential attractive strategy for lowering the multiple risk factors for cardio-metabolic disease.

Recruitment of participants with high risk of developing diabetes, we used blood glucose levels and AUSDRISK questionnaire to determine the risk of diabetes. Subgroup analysis indicated heterogeneity in study population, with more than half the number of the participants having fasting blood glucose levels >5.5 mmol/L. FPG was significantly lowered from baseline with LCn-3PUFA (P=0.008) supplementation in participants with baseline blood glucose level >5.5. Also, there was a clear trend in beneficial effects of LCn-3PUFA on fasting serum insulin (figure 6.4) in participants with high baseline blood glucose levels, indicating LCn-3PUFA supplementation could be of more beneficial in people with IFG than normal glucose levels. On the other hand, there was no significant difference in the effects of curcumin on these parameters between the two groups, except a significant reduction in the fasting insulin from baseline in participants with baseline blood glucose levels >5.5 mmol/L. Moreover, we have not particularly stratified the number of participants in groups based on sex. To examine the treatment effects on relative changes in glucose and lipid variables separately in males and females has led to interesting observations. The mean changes in fasting insulin, fasting triglycerides, HDL-C and HOMA-S with FO and curcumin were distinctively different in males and females, with supplementation greatly favouring males (table 6.5). One of the reasons for these differences could be slightly higher (but not significant) baseline levels of the above parameters in men than women (except for HDL-C). Together these sub-group analysis reports indicate gender stratification and a study population with high baseline glucose status is necessary to further substantiate the beneficial effects of curcumin and LCn-3PUFA in lowering the risk of T2D. This study failed to provide any evidence on benefits of combined supplementation with curcumin and LCn-3PUFA in the current study.

This could partly be due to uncertainties of co-administration of the two bio-actives, heterogeneity of the study population, gender balance in the groups or may be due to unknown interactions between these bio-actives. Further investigations are warranted on a single formulation with curcumin and LCn-3PUFA in a long-term trial to evaluate and elucidate the effects of curcumin and LCn-3PUFA on glycaemic control. In conclusion, curcumin supplementation has a favourable outcome on improving IS. On the other hand, LCn-3PUFA has shown profound effects on dyslipidaemia and AIP. Together, these results are indicative of a potential strategy for lowering the key risk factors in multifaceted progression of T2D.

CHAPTER 7

Curcumin and/or omega-3 polyunsaturated fatty acids for amelioration of diabetic dyslipidaemia: A pilot randomised controlled trial

7.1 Abstract

Background/Aims: Dyslipidaemia, particularly increase in triglycerides (TG) and low levels of HDL-cholesterol, are common features in individuals with T2D, accounting for increased risk of CVD. Our previous study has demonstrated the beneficial effects of curcumin and/or long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA) supplementation on dyslipidaemia and atherogenic index of plasma (AIP) in individuals with high risk of T2D. In the current study we aimed to evaluate the effects of curcumin and LCn-3PUFA on dyslipidaemia in individuals with T2D.

Methods: This study was a 2x2 factorial, randomised, double-blinded, placebo-controlled study. Participants were allocated to either double placebo (PL) or curcumin plus placebo matching for LCn-3PUFA (CC), or LCn-3PUFA plus placebo matching for curcumin (FO), or curcumin plus LCn-3PUFA (CC-FO) for six weeks. Primary outcome of the trial was serum TG with secondary outcome being fasting total cholesterol, LDL-cholesterol, HDL-cholesterol, Total-to-HDL-Cholesterol ratio, AIP and glycaemic control indices.

Results: A total of thirty participants were recruited for this study and twenty-seven participants (PL, n=7; CC, n=7; FO, n=6, CC-FO, n=7) were included in the study final analysis. Serum TG were significantly reduced in the CC (-0.5 ± 0.2 mmol/L; $P<0.05$) and the FO (-0.3 ± 0.1 mmol/L; $P<0.05$) groups post intervention from baseline. Compared with PL, only curcumin exhibited a significant reduction in TG ($P<0.05$). Similar significant reductions were observed in AIP with-in CC (-0.3 ± 0.1; $P<0.05$) and FO (-0.2 ± 0.1, $P<0.05$) groups. However the reduction was not significant when compared with PL. CC supplementation exhibited a non-significant reduction of -0.5 ± 0.2 mmol/L and -0.4 ± 0.2 mmol/L of total cholesterol ($P=0.079$) and LDL-cholesterol respectively ($P=0.129$). CC-FO did not exhibit any significant reduction in both TG and other blood lipids.

Conclusions/interpretation: This pilot study provides preliminary evidence for the potential of curcumin and LCn-3PUFA for aggressive control of diabetic dyslipidaemia. Although a moderate increase in HDL-C was found in the CC-FO group, there were no other benefits observed with the combination. A long-term supplementation study with larger sample size would potentially be required to validate these results.

7.2 Introduction

Cardiovascular disease (CVD) is one of the major causes of morbidity and mortality in individuals with T2D, relative to those without T2D (291). T2D increases the risk of CVD by two to four-fold compared with risk in non-diabetic individuals, accounting for almost seventy percent of the deaths (292) in people with T2D. Although a number of factors such as insulin resistance, MetS, lack of physical activity and high blood pressure could contribute to the CVD risk in T2D, lipid abnormalities, in particular diabetic dyslipidaemia, is a major contributor to accelerate the development of CVD (293). Increased triglycerides (TG), low HDL-cholesterol (HDL-C) and small dense LDL particles are the characteristic features of diabetic dyslipidaemia (294). IR state is the most common basis for hypertriglyceridemia (HTG) in T2D (295). The suppression of fatty-acid release from adipose tissue is impaired in the postprandial state, resulting in an increased transfer of free fatty acids to the liver, inducing overproduction of VLDL (295). HTG potentially accounts for lowering the HDL-C concentrations, either through transfer of redundant phospholipids from VLDL to HDL or by increasing the exchange of esterified cholesterol from HDL to VLDL and of TG to HDL particles (296). As a results HDL is catalysed faster leading to the low levels of circulating HDL-C. LDL-C and TC levels are not profoundly different between the individuals with and without diabetes (297). Due to the hyperglycaemia, LDL particles are glycated and preferentially taken up by macrophages leading to the formation of foam cells, which is hallmark of early stage atherosclerosis (298). Also with presence of HTG, primarily smaller dense LDL particles are formed, further increasing the atherogenic risk (299). Overall these observations indicate that the individuals with T2D need a tight control of dyslipidaemia compared to those without T2D to lower the risk of CVD.

Currently available data supports the potential of long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA) to lower the risk of death resulting from CVD (300). Large volume of evidence has reported that LCn-3PUFA reduces TG by 20-30% in both healthy as well type 2 diabetics (301, 302). TG lowering ability of LCn-3PUFA is primarily attributed to reducing the synthesis and secretion of VLDL-C from liver and to some extent from increased clearance of VLDL-C.

Several clinical studies have also shown that supplementation with LCn-3PUFA induce modest elevations in HDL-C (10-13%) in individuals with hyperlipidaemia (303, 304). The key

mechanism behind the HDL-raising effect of LCn-3PUFA is the reduction in cholesteryl ester transfer protein (CETP) activity (305). In addition, supplementation with LCn-3PUFA has shown favourable shift from small dense to less atherogenic large buoyant LDL particles (306). Together these observations highlight the potential role of LCn-3PUFA for lowering the diabetic dyslipidaemia.

Curcumin, a dietary bio-active derived from turmeric, is now rigorously being evaluated in clinical trials for its anti-inflammatory and glucose lowering properties (233, 307, 308). Preclinical studies with hypercholesteremic rats have shown the ability of curcumin to modulate the key enzymes (cholesterol 7α-hydroxylase and 3-hydroxy-3-methyl-glutaryl-CoA reductase) involved in the cholesterol synthesis and metabolism (309). There are also few preclinical reports on moderate increase in HDL-C with curcumin (310). However, currently available evidence on effects of curcumin supplementation for blood lipids in clinical studies is very limited. Recently published clinical trial from our lab has shown the ability of curcumin to potentiate the cholesterol lowering property of phytosterols (311). Meta-analysis of randomised controlled trials with curcumin supplementation on blood lipids has reported ambiguous conclusions, one reporting clear significant reduction in LDL-C and TG and the other reporting no significant reduction in any blood lipid parameters (312, 313).

Recently we have demonstrated that supplementation with curcumin and LCn-3PUFA has beneficial effects on insulin resistance, dyslipidaemia and atherogenic index of plasma (AIP) in individuals with high risk T2D (Thota et al 2018, under review). Based on these observations, in the current study we hypothesised that in individuals with T2D, curcumin and LCn-3PUFA may exhibit greater reduction in TG and AIP, thus providing beneficial effects on diabetic dyslipidaemia.

7.3 Methods

7.3.1 Subjects

Participants diagnosed with T2D were recruited from the Hunter Region, New South Wales, Australia through the local endocrinologists, general practises, recruitment flyers and media advertisements. They were screened in person or though telephone based on the inclusion criteria: age between 40 and 75 years; diagnosed with T2D (duration less than 15 years); body mass index (BMI) between 25 and 45 kg/m^2; on stable medication during the study period. Exclusion criteria included; pregnancy or planning to become pregnant or breastfeeding; failure

to provide informed consent; diagnosed with type 1 diabetes; currently on insulin treatment; diagnosed with cancer; glomerular filtration rate less than 45; diagnosed with painful peripheral neuropathy; history of severe neurological diseases (Parkinson's, multiple sclerosis, epilepsy); consuming more than 2 serves of oily fish per week; taking regular dietary supplements known to influence blood lipid levels; sensitivity/ intolerance to the products involved in this study; unwilling to fast for 10 hours before giving blood sample. This study was approved by the University of Newcastle and the Hunter New England Area Health Service Human Research Ethics Committees. The trial has been registered with Australia New Zealand Clinical Trial Registry (ACTRN12616001483448).

7.3.2 Study Design

This was a six-week 2x2 factorial, double-blinded, randomised controlled trial. Participants were advised to attend two visits (baseline and post-intervention) at either Nutraceutical Research Program clinical trial facility or John Hunter Hospital, during the study period. At the baseline visit participants were randomised to one of the four interventions: Placebo (PL, 2 x placebo tablets matching for curcumin plus 2x1000 mg corn oil capsules per day), curcumin (CC, 2x500mg curcumin (Meriva®) tablets, providing 180mg of curcumin plus 2x1000mg corn oil capsules per day), LCn-3PUFA (FO, 2x1000mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA+EPA plus 2xplacebo tablets matching for curcumin) or double active (CC-FO, 2x500mg curcumin (Meriva®) tablets, providing 180mg of curcumin plus 2x1000mg fish oil (EPAX 1050 TG) capsules providing 1.2g DHA+EPA). Allocation of study interventions was performed by a software-based randomisation technique, using alpha-numeric codes (e.g. 00A) in the blocks of 8. They were also advised to maintain their routine dietary habits, physical activity and medication intake during the study period. The compliance to the study interventions were measured using a capsule count and capsule intake log on the post-intervention visit. Red blood cell fatty acid (%) analysis is also used to check the compliance for FO and CC-FO.

7.3.3 Data collection and outcome measures

Primary and secondary outcomes

The primary outcome in this trial was to evaluate the effects of curcumin and/or LCn-3PUFA on serum TG. Secondary outcomes included other blood parameters (total cholesterol, TC; HDL-Cholesterol, HDL-C; LDL-Cholesterol, LDL-C; and Total: HDL-cholesterol ratio, TC: HDL-C), AIP, C-reactive protein (CRP), fasting glucose and insulin and liver function tests (Aspartate transaminase, AST; and Alanine transaminase, ALT). Fasting (at-least for 10 hours) blood samples were collected from participants during baseline and post-intervention visits into fluoride/oxalate and serum clot + gel + clot activator vacutainer by a trained phlebotomist. Lipid profile, glucose parameters, CRP and liver function tests were measured by Hunter Area Pathology Service (HAPS), an accredited pathology laboratory for compliance with National Pathology Accreditation Advisory Council. Atherogenic index of plasma (AIP) values were derived by using formula log (TG: HDL-C) (207). Red blood cell fatty acids (%) were determined using direct transesterification followed by gas chromatography (Hewlett Packard 7890A Series GC with Chemstations Version A.04.02) (22).

Anthropometry, body composition and blood pressure

Anthropometry and body composition measurements were performed on baseline and post-intervention visit days using direct segmental multi-frequency bioelectrical impedance (InBody 230, Biospace Co., Ltd. Seoul, Korea). Waist circumference (cm) was measured using a tape measure positioned between about halfway between the top of the hip bone and bottom of the ribs. Blood pressure was measured using digital sphygmomanometer (Microlife®, Switzerland). Average of three systolic and diastolic blood pressure readings was considered as the final measurement.

Questionnaires (diet, physical activity and medical history)

Medical history, medications and demographic information was obtained from participants via questionnaire at baseline visit. Participants were asked to complete a three-day food diary before the baseline and post intervention visits. These food diaries were analysed through FoodWorks, Xyris (version 8.0) to estimate participants' habitual dietary intakes. International Physical Activity Questionnaire (IPAQ) – long form version was used to estimate physical activity of the individuals in four domains: work, leisure, transportation and household.

7.3.4 Statistical analysis

Based on the data (standard deviation - 0.5 unit) of the TG levels of people with T2D from previous study (205), a sample size of seventeen participants in each treatment group were required to give 80% power at 0.05 significance level for detecting a 0.5 mmol/L drop in TG (calculated using PS Power and Sample Size Calculations Version 3.0). To allow for dropouts we needed to recruit 4 x 20 = 80 participants according to the inclusion criteria. Data collected at the baseline was tested for normality using histograms and Shapiro-Wilk's test and presented as mean ± SEM (standard error of the mean) or median (IQR, interquartile range) as appropriate. Changes from the base-line to post-intervention within-treatment group were assessed through paired t-test or Wilcoxon signed-rank test. Significant changes in the baseline data between the groups were assessed through analysis of variance (ANOVA) or Kruskal-Wallis for non-parametric data. Post-intervention data was presented as mean absolute change (post-intervention value minus baseline value). Significant effects of intervention on mean changes in the variables between the groups were measured using two-way ANOVA with post hoc (tukey's) analysis.

7.4 Results

7.4.1 Baseline participant characteristics

Based on the sample size and power analysis, we aimed to recruit n=80 participants for this study. However, extreme difficulties were experienced in recruitment of people with T2D. Despite extensive efforts over 14 months (SEP 16 – NOV 17) to recruit through media coverage, advertisement on the notice boards, stalls at local shopping centres, endocrinology clinics, Medicare Local, several GP practices, National Diabetes Service Scheme database, we were unable to recruit the required number of patients. Thus raising the concerns over feasibility of conducting clinical trials in people with type 2 diabetes in the community set up. After exhaustion of all avenues for recruitment, it was decided to terminate the trial, therefore, this chapter described as pilot study on data collected from n=32 people with type 2 diabetes who completed the intervention trial. As such the results presented in this chapter represents pilot data and the observations merits confirmation in a larger trial with a greater number of participants.

Thirty-two participants were randomised into four groups PL, CC, FO and CC-FO for six weeks in this study (figure 7.1). Five participants were excluded from the final analysis (non-

compliance to the protocol, n=3; started on new medication, n=1; outlier in the data, n=1). A total of twenty-seven participants were included in the study final analysis (figure 7.1). There were no statistical significant differences observed in participant characteristics between the groups (table 7.1 and table 7.2). Though the study was randomised and double blinded, we observed that the baseline levels of duration of T2D, waist circumference, blood lipids (TC, TG, LDL-C), fasting glucose and insulin were relatively higher in individuals randomised to PL and CC groups than FO and CC-FO groups.

7.4.2 Blood lipids

Six weeks of intervention with CC and FO have shown significant reductions in the serum TG. Post-intervention, fasting serum TG was significantly reduced by 0.5 ± 2 mmol/L (P=0.033) in CC group and by 0.3 ± 0.1 mmol/L (P=0.029) in FO group from baseline respectively (figure 7.2 A). However, when compared with PL, only CC supplementation was significant (P=0.033) in reducing the serum TG. Although there was slight reduction in TG with CC-FO (-0.1 ± 0.1 mmol/L) (figure 7.2A), there was no significant difference observed in either baseline to post-intervention or compared to PL. Similar trends were observed with CC (-0.3 ± 0.1, P=0.042) and FO (-0.2 ± 0.1, P=0.025) on AIP (figure 7.2B). However, this reduction was not significant when compared between the groups. With regards to HDL-C, there was no significant difference observed between the groups or change from the baseline in the intervention groups (figure 7.2 C). But, the improvement in the HDL-C (0.1 ± 0.02 mmol/L) is relatively higher compared to the PL (0.01 ± 0.02 mmol/L), CC (0.01 ± 0.04) and FO (-0.01 ± 0.03) (figure 7.2 C). There was a non-significant reduction in both TC (-0.5 ± 0.2 mmol/L, P=0.079) and LDL-C (-0.4 ± 0.2 mmol/L, P=0.129) reduction only with CC supplementation from baseline to post intervention (figure 7.2 B and 7.2 C). Similar trend was observed with changes in the TC:HDL-C following CC supplementation (figure 7.2 E).

7.4.3 Glucose control and other secondary outcome measures

Post-intervention, no significant changes were observed in the fasting glucose and insulin with-in and between the treatment groups (table 7.3). However, there was a noteworthy drop in fasting glucose (1.1 ± 0.8 mmol/L) levels in CC group. Similarly, no significant changes were observed in CRP, liver function test, blood pressure between and with-in the groups (table 7.3).

7.4.4 Dietary intake, physical activity, compliance and fatty acid changes

Dietary intakes (Kj) and physical activity (MET-mins/week) were not significantly different between the groups (table 7.4). However, the dietary intake of the participants in the CC-FO group was comparatively lower and with higher physical activity levels than the other groups. Post-intervention, the body composition measurements of the participants were also not changed (data not presented).

The mean compliance of the participants to the study intervention determined from the capsule count was 97.2 ± 0.6 %. The study products were well tolerated by the participants, and no adverse event was reported during the study period.

Six weeks intervention with FO and CC-FO significantly improved the red blood cell membrane status of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (table 7.4). To our surprise, the increase in DHA was higher in FO (3.0 ± 0.5 units) alone relative to the CC-FO group (2.3 ± 0.3). On the other hand, there was a drop of -0.2 ± 0.3 units in the mean erythrocyte DHA content in CC group.

Table 7.1: Baseline general characteristics of the trial participants

CHARACTERISTICS	Total (n=27)	PL (n=7)	CC (n=7)	FO (n=6)	CC-FO (n=7)	P-VALUE
Age (years)	62.3 ± 2.0	61.1 ± 4.3	62 ± 3.8	62.1 ± 5.4	63.9 ± 3.6	0.930
Males/females	17/10	5/2	4/3	3/3	5/2	-
Duration of T2D (years)	6.4 ± 0.9	5.6±1.2	8.1±2.0	7.5±2.5	4.5±1.5	0.495
Anthropometry measures						
Body weight (kg)	92.9 ± 3.7	101.7 ± 8.2	94.8 ± 9.6	80.4 ± 4.2	92.8 ± 4.6	0.262
Muscle mass (kg)	33.9 ± 1.3	38.2 ± 2.2	31.9 ± 3.2	31.4 ± 1.6	33.6 ± 2.8	0.252
Body mass index (kg. m^{-2})	31.4 ± 1.0	32.8 ± 2.4	33.6 ± 2.1	27.4 ± 1.4	31.2 ± 1.8	0.175
Waist circumference(cm)	111.5 ± 2.9	114.7 ± 7.1	115.1 ± 5.9	99.8 ± 4.3	114.6 ± 4.3	0.214
Waist-hip ratio	0.98 ± 0.01	0.98 ± 0.03	1.01 ± 0.01	0.96 ± 0.02	0.99 ± 0.03	0.528
Percent body fat (%)	34.2 ± 1.9	31.7 ± 4.8	39.2 ± 2.9	29.9 ± 3.1	35.2 ± 4.1	0.369
Family history						
Family history of diabetes – n (%)	17 (62.9)	6 (85.7)	4 (57.1)	2 (33.3)	5 (71.4)	-
Family history of cholesterol - n (%)	13 (48.1)	4 (57.1)	3 (42.9)	2 (33.3)	4 (57.1)	-
Family history of blood pressure – n (%)	16 (59.3)	4 (57.1)	4 (47.1)	3 (50)	5 (71.4)	-
Alcohol intake – n (%)	25 (78.1)	7 (100)	6 (85.7)	5 (83.3)	7 (100.0)	-
Smoking – n (%)	4 (14.8)	2 (28.6)	1 (14.3)	0 (0.0)	1 (14.3)	-
Blood pressure						
Systolic blood pressure (mmHg)	136.7 ± 2.9	136.6 ± 5.2	142.3 ± 4.7	138 ± 9.64	130.1 ± 4.1	0.544
Diastolic blood pressure (mmHg)	78.9 ± 1.3	78.7 ± 2.4	83.1 ± 1.9	79.1 ± 2.6	74.6 ± 3.1	0.143
MEDICATION AND SUPPLEMENT USE						
Anti-hyperglycaemic no. (%)	20 (74.1)	5(71.4)	4(57.1)	5(83.3)	6(85.7)	-
Anti-hypertensive no. (%)	18(66.7)	3(42.9)	5(71.4)	4(66.6)	6(85.7)	-
Cholesterol lowering medications no. (%)	15(55.6)	2(28.6)	4(57.1)	4(23.52)	5(71.4)	-
Supplements no. (%)	11(40.7)	3(42.9)	3(42.9)	1(16.6)	4(57.1)	-

Data is presented as mean ± SEM or median (IQR), unless otherwise specified. n, number of participants; PL, double placebo; CC, curcumin; FO, Fish oil; CC-FO, curcumin plus fish oil. Kg, kilogram; cm, centimetre; %, percent. SEM, Standard error of the mean; IQR, interquartile range.

Table 7.2: Baseline blood parameters of the study participants

Characteristics	Total (n=27)	PL (n=7)	CC (n=7)	FO (n=6)	CC-FO (n=7)	P-VALUE
Lipid parameters						
Total cholesterol (mmol/L)	4.8 ± 0.2	5.1 ± 0.3	5.2 ± 0.5	4.2 ± 0.4	4.6 ± 0.5	0.402
LDL-C (mmol/L)	2.8 ± 0.2	3.0 ± 0.3	3.0 ± 0.4	2.3 ± 0.4	2.7 ± 0.4	0.554
HDL-C (mmol/L)	1.1 ± 0.04	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.195
Triglycerides (mmol/L)	1.8 ± 0.1	2.0 ± 0.1	2.3 ± 0.3	1.5 ± 0.2	1.5 ± 0.2	0.402
Total: HDL-Cholesterol	4.1 (1.8)	4.4 (1.6)	4.7 (1.5)	3.6 (0.5)	3.5 (2)	0.071
AIP						
Glycaemic control parameters						
Fasting plasma glucose (mmol/l)	7.3 (2.6)	8.7 (2)	8 (5.7)	6.7 (0.8)	6.4 (3)	0.404
Fasting serum insulin (mIU/L)	10.6 (7.7)	11.9 (17.5)	11.5 (5.8)	7.9 (9.5)	10.6 (5.5)	0.610
Inflammation and blood cell count						
CRP (mg/L)	1.9 (3.9)	1.5 (1.7)	4.1 (4.9)	1.3 (0.6)	5 (5)	0.049
Liver Function Tests						
Alanine aminotransferase (U/L)	30 (18)	27 (8)	23 (42)	34.5 (15)	30 (9)	0.814
Aspartate transaminase (U/L)	26 (10)	25 (10)	22 (12)	30 (9)	26 (10)	0.700

Data is reported as means ± SEM. or median (IQR). PL - double placebo; CC- curcumin; FO-fish oil; CC-FO curcumin + fish oil; AIP, atherogenic index of plasma; CRP, C-reactive protein; LDL-C, LDL-Cholesterol; HDL-C HDL-cholesterol.

Table 7.3: Changes in the outcome measures from baseline to post-intervention

CHARACTERISTICS	PL (n=7)	CC (n=7)	FO (n=6)	CC-FO (n=7)	P-VALUE
Glycaemic control parameters					
Fasting plasma glucose (mmol/L)	1.51 ± 1.2	-1.1 ± 0.8	0.7 ± 0.5	0.5 ± 0.4	0.206
Insulin (mU/L)	2.3 (17.3)	1.7 (1.5)	0.6 (5.2)	-2.7 (6.3)	0.399
Inflammation					
CRP (mg/L)	0.3 (0.8)	0.4 (3.3)	-.1 (0.6)	-0.6 (3.8)	0.842
Liver function tests					
Alanine aminotransferase (U/L)	-0.7 ± 5.7	0.4 ± 2.7	1.6 ± 3.9	0.1 ± 3.7	0.984
Aspartate transaminase (U/L)	-0.3 ± 3.4	3.1 ± 2.0	1.5 ± 1.6	-0.9 ± 1.9	0.630
Blood pressure					
Systolic blood pressure (mmHg)	1.3 ± 2.9	-2.9 ± 3.2	-4.8 ± 7.0	-2.7 ± 3.2	0.764
Diastolic blood pressure (mmHg)	-0.7 ± 1.3	-6.3 ± 2.9	-4.8 ± 2.6	-4.0 ± 1.9	0.362

Data is presented as mean ± SEM or median (IQR) as appropriate. PL, placebo; CC, curcumin; FO, fish oil; CC-FO, curcumin + fish oil.

Table 7.4: Changes in the dietary intake, physical activity and fatty acid composition of the study participants

CHARACTERISTICS	PL (n=7)	CC (n=7)	FO (n=6)	CC-FO (n=7)	P-VALUE
Dietary intake					
Baseline (kj)	8478 ± 955.9	8529 ± 537.1	8724 ± 636.1	7226 ± 547.7	0.420
Change	249.1 ± 659.0	-165.1 ± 574.7	264.5 ± 227.8	1754.4 ± 687.4	0.131
Physical activity					
Baseline (METs-minutes/ week)	1188 (1615)	1663 (1986)	2036 (1886)	3024 (3553)	0.503
change	73 (2820)	540 (864)	65 (526)	117 (537)	0.374
Erythrocyte fatty acid composition (%, w/w)					
AA					
Baseline	16.5 ± 0.8	16.8 ± 0.6	17.3 ± 1.2	18.1 ± 0.4	0.490
Change	0.5 ± 0.4	0.6 ± 0.2*	-0.4 ± 0.7	-0.6 ± 0.4	0.133
EPA					
Baseline	1.26 (0.6)	1.0 (0.2)	1.0 (0.4)	0.9 (0.8)	0.484
Change	-.23 (0.2)[a,b]	0.07 (0.2)	0.5 (0.3)*[a]	0.5 (0.8)[b]	0.009
DHA					
Baseline	7.0 (5.3)	6.4 (1.8)	5.7 (2.5)	6.5 (2.7)	0.882
Change	-0.8 ± 0.6[c,d]	-0.2 ± 0.3[ef]	3.0 ± 0.5**[ce]	2.3 ± 0.3***[df]	0.0000
Compliance (capsule count)	96.3 ± 1.7	97.7 ± 0.9	96.5 ± 1.8	98.2 ± 1.0	0.701

Data is presented as mean ± SEM or median (IQR) as appropriate. Significant change from baseline, * p<0.05, ** p<0.01, *** p<0.001 represents the significant differences from the baseline with-in treatment groups. PL, placebo; CC, curcumin; FO, fish oil; CC-FO, curcumin + fish oil; AA, Arachidonic acid; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid. METs-minutes/week, metabolic equivalent minutes per week.

[a,b] EPA was significantly increased in the FO (P=0.001) and CC-FO (P=0.002) groups compared to PL. [c,d] DHA was significantly increased in FO (P=0.000) and CC-FO (0.000) group compared to the PL. [e,f] DHA was significantly increased in the FO (P=0.000) group and CC-FO (P=0.002) compared to CC

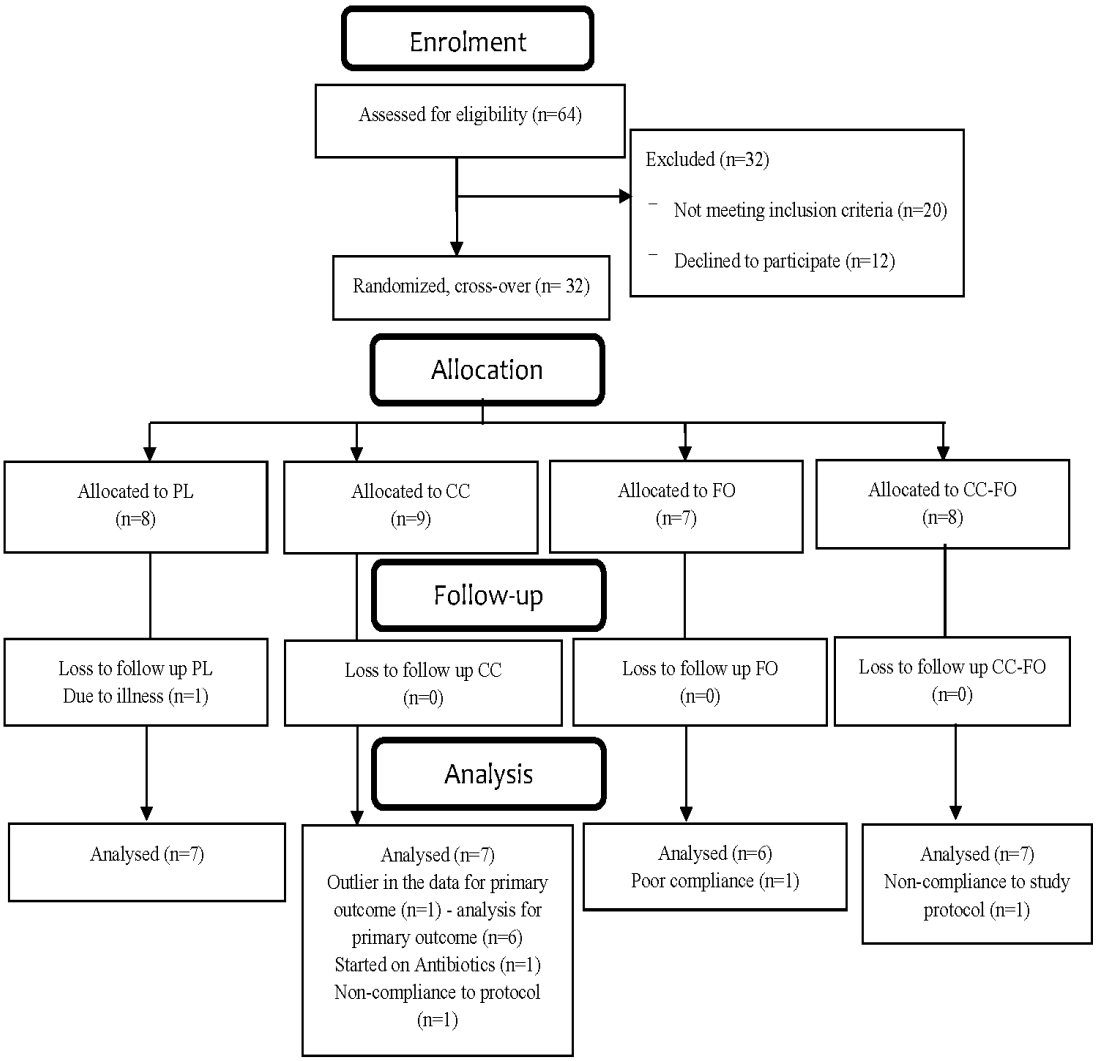

Figure 7.1: Consolidated Standards of Reporting Trials (CONSORT) flow chart

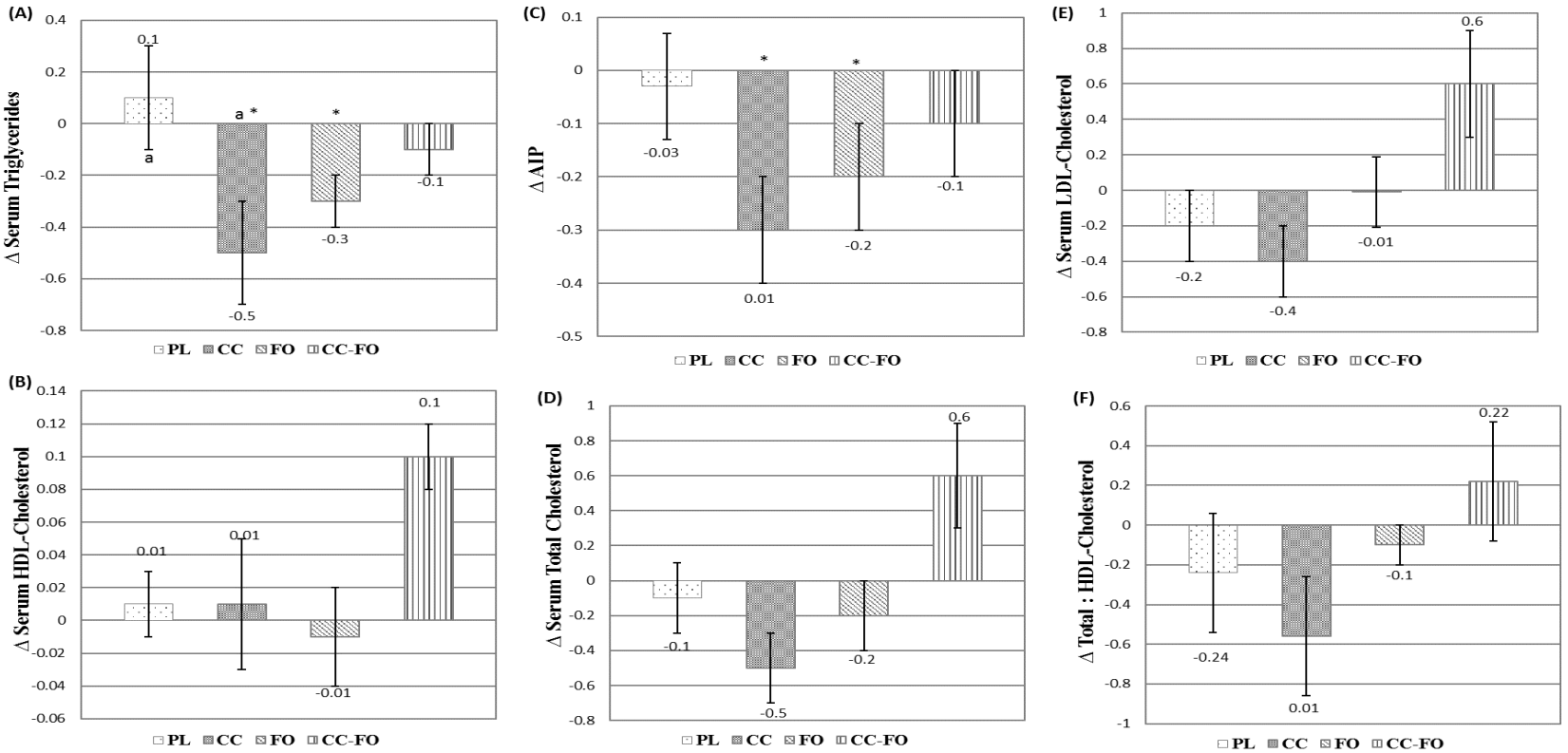

Figure 7.2: Changes in the blood lipids from baseline to post-intervention with-in and between the PL, CC, FO, and CC-FO.

Data presented as mean ± SEM. Significant changes from baseline indicated by * $p<0.05$. Means with same lower letter indicate significant differences between the groups. PL, double placebo (PL), curcumin (CC), fish oil (FO) and curcumin + fish oil (CC-FO); A. Change in the serum triglycerides; B. Change in the serum HDL-Cholesterol C. Change in atherogenic index of plasma; D. Change in total cholesterol; E. Change in LDL-Cholesterol; F. Change in total: HDL-C ratio.

7.5 Discussion

The results presented in this pilot study showed that both curcumin and LCn-3PUFA reduce TG and AIP in individuals with T2D. The magnitude of reduction was relatively higher with curcumin supplementation when compared with other groups. There was a moderate increase in HDL-C only in CC-FO group. No other benefits were observed in CC-FO. There was a trend in reduction of TC (-0.5 mmol/L) and LDL-C (-0.4 mmol/L) with curcumin supplementation. No increase in the LDL-C or TC concentrations with LCn-3PUFA supplementation was observed. Though the sample size of the current study is relatively small, observations from these preliminary analyses indicate the potential of curcumin and LCn-3PUFA for control of diabetic dyslipidaemias.

Though the preclinical evidence is in support of beneficial effects of curcumin supplementation for dyslipidaemia (309, 314, 315), clinical evidence to substantiate the lipids lowering effects of curcumin is not well established. Limited number of clinical trials with curcumin supplementation have reported either a moderate reduction (205, 316) or no effect (317) on serum TG. Recently published two systematic reviews and meta-analyses (313, 318) on effects of curcumin on blood lipids have presented contrasting results. Further dissecting the results from these papers, it is noteworthy to observe that both the meta-analyses conclusions were based on heterogenous population. Secondly, the results included in the meta-analysis were not specifically related to curcumin, but also with turmeric powder and other unspecified curcuminoid mixtures. In our previous study with curcumin supplementation in individuals with prediabetes or high risk of T2D, we found that curcumin augmented the increase in the TG levels and significantly reduced the AIP levels (Thota *et al* 2018, under review). With the higher baseline levels of TG and AIP in the current study, curcumin exhibited a greater magnitude of TG (-0.03 mmol/L vs -0.5 mmol/L) and AIP (-0.1 vs -0.3) lowering effects relative to the previous study with prediabetes or high- risk individuals. The exact mechanism of how curcumin lowers the TG is not known, however, preclinical studies indicate that curcumin supresses the hepatic fatty acid synthase and increases the β-oxidation of fatty acids to lower the TG (319).

Similarly, curcumin exhibited a trend in reducing the TC and LDL-C, in line with previous trials (311, 320). The magnitude of reduction in TC (-0.5 mmol/L) and LDL-C (-0.4 mmol/L) is relatively higher in this study population when compared with the individuals with prediabetes (TC, -0.03 mmol/L; LDL-C, -0.06 mmol/L). Preclinical studies with both diet-induced (279) and genetically modified models (315) have shown potent glucose lowering effects of curcumin. Similarly, small number of clinical studies have also reported glucose lowering effects of curcumin (250, 311, 320). In line with these reports, we have observed a reduction in fasting glucose levels. However, the small sample size could be one of the potential limitation to obtain the significance from other groups.

The current interest in application of LCn-3PUFA for controlling dyslipidaemia can be attributed to the numerous TG lowering reports of LCn-3PUFA from the previous studies in individuals with T2D (302). LCn-3PUFA have long been to be known to lower the TG, primarily through reducing hepatic VLDL-C production, and to some extent from clearance of VLDL-C (288). Given the fact that VLDL-C synthesis is comparatively higher in individuals with T2D than those without T2D, LCn-3PUFA has the potential to exert a greater magnitude of reduction in TG levels. In the current study we observed a significant reduction in TG post-intervention with LCn-3PUFA from baseline. However, we did not find any difference between the magnitude of reduction in TG with LCn-3PUFA supplementation in individuals with prediabetes and T2D. This may be partly due to the fact the baseline TG levels of individuals allocated to FO group are comparatively in the normal range and lower than CC group. Though there are few reports available on moderate increase in HDL-C with LCn-3PUFA, in the current study analysis we did not find any increase in HDL-C. Interestingly, when LCn-3PUFA supplemented concurrently with curcumin in CC-FO group, there was a moderate increase in HDL-C. In contrast to the previous reports on moderate increase in the TC and LDL-C (321), particularly with DHA rich LCn-3PUFA supplements (322, 323), in the current study we did not find any increase in both the parameters.

Previous studies on supplementation with LCn-3PUFA reported an increase in the FPG levels in individuals with T2D (324, 325). LCn-3PUFA supplementation increase PPAR-α, thereby increasing the hepatic oxidation and uptake of fatty acids. Consequent decrease in free fatty acids and TG could increase the hepatic gluconeogenesis, thereby causing an elevation in the plasma glucose levels. In the current study, though the participants were

on concurrent medication with glucose lowering agents, we still observed a slight (but not statistically significant) rise in the glucose levels. However, the glucose levels were relatively lower in the CC-FO group, indicating concurrent administration with curcumin might potentially lower the rise in fasting glucose levels associated with LCn-3PUFA supplementation.

In line with our previous study (Thota et al 2018, under review), this study also failed to show any beneficial effects of concurrent administration of curcumin and LCn-3PUFA. To the best of our knowledge, these two trials are the first studies to provide and evaluate the potential benefits of combining curcumin and LCn-3PUFA. Attempts have been made to couple DHA with curcumin (Patent number: WO2007103435A2) to enhance the bioavailability of curcumin. The inventors in the application have mentioned that esters can be formed when curcumin is coupled with DHA through a synthetic multi-step process, indicating the potential of ester or complex formation. The application also claims with DHA-curcumin approach, curcumin content was increased in the red blood cells and a higher bio-availability. However, no biological evidence was provided regarding this DHA-curcumin complex. To our surprise, we found a decrease in the red blood cell DHA and EPA content when curcumin is co-administered with FO in this study, in line with our previous study (Thota et al 2018, under review) observations. There might be a possible interaction or complex formation in the gastrointestinal tract with co-administration of curcumin and LCn-3PUFA that could eliminate either curcumin and/or DHA when administered together. Based on these observations, there is great necessity to examine the potential interactions between LCn-3PUFA and curcumin.

There are two major limiting factors for the current study. One of the primary limiting factors is the feasibility of recruiting T2D patients. During this study, we have implemented several recruitment strategies such as recruitment through the local general practises, endocrinologists, radio-interviews, media advertisements and social media to meet the target sample number. However, this study failed to recruit the target sample number. Secondly, despite randomisation, we have observed the baseline levels of blood lipids and glucose levels and duration of T2D relatively higher in the CC and PL groups than FO and CC-FO groups. This may have added to the inconclusive observations between the groups. In conclusion, this pilot study results have presented the potential of curcumin and LCn-3PUFA for controlling diabetic dyslipidaemia. Future studies are required to analyse the potential interactions between curcumin and LCn-3PUFA.

Moreover a randomised controlled trial with target sample size and adequate power might validate and substantiate the beneficial effects of curcumin and LCn-3PUFA on diabetic dyslipidaemia.

CHAPTER 8

General Discussion

(Key findings and future directions, limitations, conclusions)

8.1 Key findings

8.1.1 Novel marker, InsuTAG, shows superiority in identifying IR and MetS than the existing markers

The observations presented in chapter 3 were the first reports on the potential of InsuTAG for identifying IR and MetS. Given the prominent role of insulin and TG in all the major tissues (adipose, liver, and skeletal muscle) both in fasting and fed states, that are implicated in the development of T2D, we hypothesised InsuTAG is a physiologically relevant and better indicator of metabolic abnormalities. Correlation analysis indicated strong association with IR over other lipid based surrogate markers and established anthropometry markers such as BMI and WC. InsuTAG represented 93% of the probability of identifying IR in an individual with a sensitivity of over 84%. Positive likely hood ratio of 6.4 provides further evidence for exploring InsuTAG as a diagnostic tool for IR. MetS represents a cluster of high risk metabolic abnormalities for development of both T2D and CVD. The current criteria for MetS (according to IDF) is broad and based on different components: Central obesity (defined as WC with ethnicity specific values) plus any two of the following four factors: TG ≥1.7 mmol/L; HDL-C <40 mg/dL (1.03 mmol/L) for males < 50 mg/dL (1.29 mmol/L) for females; SBP ≥ 130 or DBP ≥ 85 mm Hg or treatment of previously diagnosed hypertension or raised (≥5.6 mmol/L) FPG. InsuTAG exhibited 79% probability for identifying individuals with MetS, which is comparatively higher than waist circumference (72%), which is the key component of MetS definition criteria. All the key high risk factors such as BMI, FPG, HDL-C and SBP were significantly higher in the individuals with InsuTAG values higher than optimal cut-off of 11.2. Moreover, we can rule out the MetS with 90% confidence, indicating InsuTAG can be a relevant screening tool for identifying MetS in large cohorts. Therefore, a single InsuTAG value can be a better representative of MetS than the current available broad diagnostic criteria.

Though the relationships between the InsuTAG and other metabolic risk factors is observational and casual relationship, high sensitivity and positive likelihood ratio values strengthens the potential of InsuTAG as a predictor of IR and MetS. Additional validation of InsuTAG is required using gold standard HEC studies and a longitudinal study to predict whether it has the potential to predict development of metabolic diseases.

8.1.2 Curcumin effectively lowered PBG levels, but did not show any effect on FPG and HbA1c

The results presented in the chapter 3, demonstrate that curcumin has the potential to effectively reduce the blood glucose levels following consumption of standardised high carbohydrate and fat meal. To the best of our knowledge this is the first study presenting the effects of curcumin on postprandial glycaemia. Significant reduction in Δ glucose levels was observed with curcumin (61%) compared with placebo (figure 8.1). PBG following curcumin treatment was lower (16%) than the placebo group at as early as 30 minutes from baseline, however, the difference did not reach statistical significance. On the other hand, long term supplementation with same dose of curcumin (chapter 6 and 7) did not show any effects on FPG or HbA1c. The results presented in the chapter 6 and 7 were in contrast to the previous trials that reported glucose lowering effects of curcumin (89). One possible reason for lack of effects could be that the glucose and HbA1c levels of our COP-D trial participants were only marginally higher than normal level. Half of the study population were presented with FPG and HbA1c in the prediabetes range, whereas the rest of population exhibited normal and HbA1c. This heterogeneity could also be one of the reason limiting the efficacy of curcumin to lower the glucose and HbA1c levels. The type of formulation of curcumin could also be another barrier to compare with other studies to address these discrepancies in the results. In addition, previous published study (86) reporting blood glucose reduction by curcumin was conducted in an Asian population, therefore, Geographical location could have been a factor. Limited evidence on glucose lowering effects of curcumin in Caucasian population still exists, therefore, future studies are required to substantiate the glucose lowering reports of curcumin arising from both pre-clinical and clinical studies. Notably, although there was a substantial (1.1 mmol/L) reduction in fasting blood glucose levels following curcumin supplementation in the CALFOR-CVD trial, it failed to reach the statistical significance because of the small sample size. Magnitude of reduction in glucose levels with curcumin was comparatively higher in individuals with T2D (-1.1 mmol/L) compared to the individuals with high risk of T2D (-0.1 mmol/L). Therefore, the glucose lowering effects of curcumin could possibly dependent on the baseline glucose levels of the individuals.

In the current treatment algorithm for T2D, acarbose (inhibitor of glucosidase) is used as an adjunct along with metformin to control PBG, to provide overall glycaemic control

and lower HbA1c levels. Molecular docking studies have reported that curcumin has higher binding capacity to α-glucosidase than acarbose (235), suggesting that curcumin may have a place in the control of blood glucose levels following meal consumption. Since curcumin is well tolerated and is effective in lowering the PBG, it may be a promising candidate for evaluation in combination with glucose-lowering medications such as metformin to provide overall glycaemic control. Future long term supplementation studies will be required to evaluate and validate the potential of curcumin to lower the FPG and HbA1c levels.

8.1.3 Curcumin effectively lowered both postprandial & fasting insulin levels and improved IS

Curcumin supplementation significantly lowered both the postprandial (presented in chapter 3) in healthy, and fasting (presented chapter 6) insulin levels in individuals with high risk of diabetes. Curcumin did not significantly affect the insulin levels at individual time points, however it reduced the overall change in AUC values for insulin by 26%. Thus, indicating curcumin supplementation could lower the requirement for insulin levels following meal consumption, which may further preserve the β-cell secretion capacity. In preclinical studies with diet-induced IR models (presented in section 1.7.1.2), curcumin mediated amelioration of IR was primarily through decreased NFκB activity, increased anti-oxidant enzymes (Foxo1) and activation of AMPK α-subunit by phosphorylating Thr-172 residue, thereby increasing the IS and improving the glucose control (94, 279). In the current study, curcumin effectively lowered the fasting insulin levels and improved the IS (evaluated through HOMA2 %S) (figure 8.1), in line with reports from the other clinical trials (205, 250). Though multiple mechanisms were proposed for curcumin mediated reduction in IR, the post-prandial control of insulin release appears to be the primary mechanism through which curcumin lowers the FI.

The current medications for T2D (also potential medication for pre-diabetes) are unidirectional aimed at lowering hyperglycaemia. Though thiazolidinedione class of drugs were proven to improve IS, they are associated with high risk of heart failure, weight gain and bone fractures. Evidence provided in this thesis on effects of curcumin on IS provides preliminary evidence to explore curcumin along with these pharmacological agents to improve the IS in the future studies.

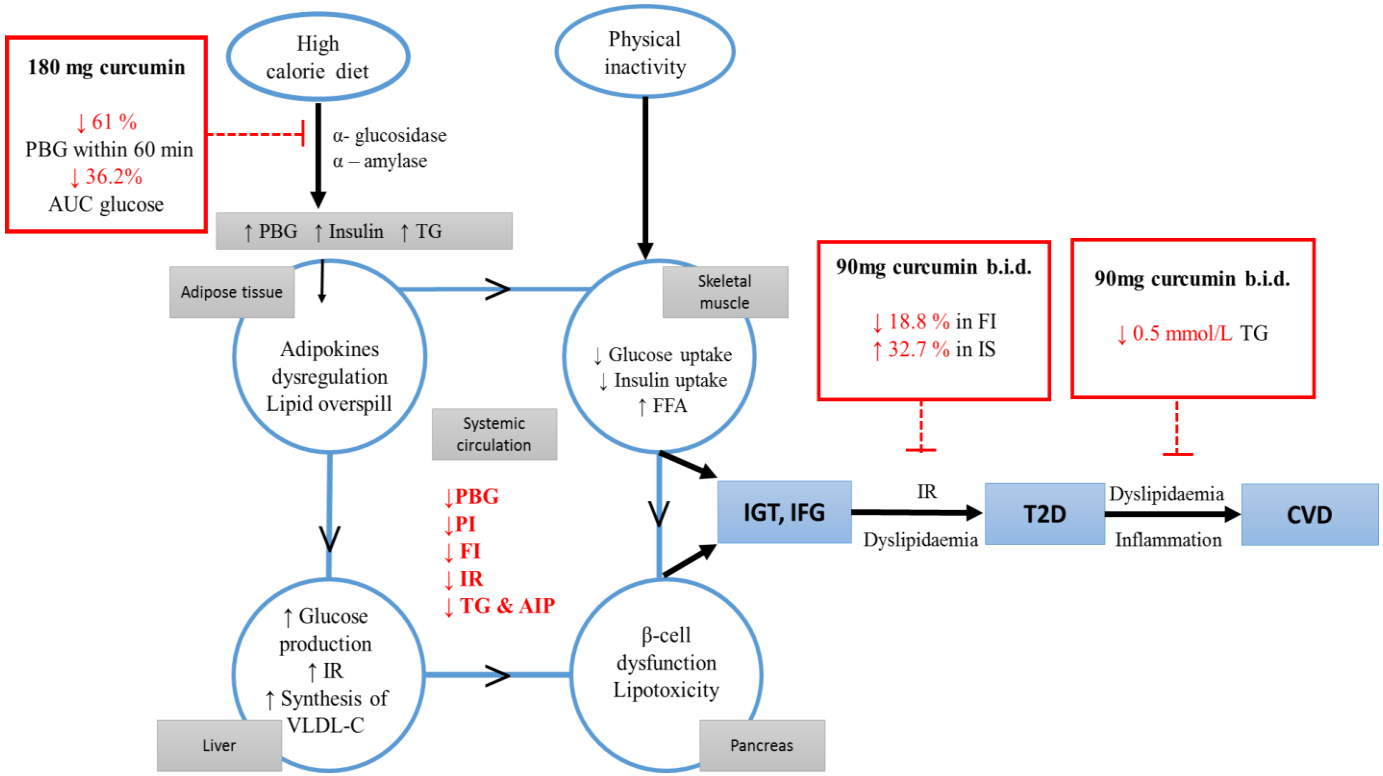

Figure 8.1: Summary of the effects of curcumin on glycaemic control and blood lipids

AIP – Atherogenic index of plasma; **AUC** – Area under the curve; **CVD –** Cardiovascular disease; **FI** – Fasting insulin; **FFA** – Free fatty acids; **IFG** – Impaired fasting glucose; **IGT** – Impaired glucose tolerance; **IR** – Insulin resistance; **IS** – Insulin sensitivity; **PBG** – Postprandial blood glucose; **PI –** Postprandial insulin; **TG** – Triglyceride; **T2D** – Type 2 diabetes

8.1.4 Magnitude of reduction in blood lipids by curcumin was higher in individuals with T2D compared with the reductions in those with high risk of T2D

Results presented in chapter 6 indicate that curcumin supplementation augmented the increase in the TG levels and significantly reduced the AIP levels in individuals with high risk of T2D. With the higher baseline levels of TG and AIP in individuals with T2D, curcumin exhibited a greater magnitude of TG (-0.5 mmol/L vs -0.03 mmol/L) and AIP (-0.3 vs -0.1) lowering effects. The exact mechanism of how curcumin lowers the TG is not yet clear. However, preclinical studies indicate that curcumin supress the hepatic fatty acid synthase and increases the hepatic fatty acid β-oxidation activity to lower the TG (319). Similarly, curcumin exhibited a clear trend in reducing the TC and LDL-C in individuals with T2D. The magnitude of reduction in TC (-0.5 mmol/L) and LDL-C (-0.4 mmol/L) is relatively higher than compared to the reductions in individuals with high risk of T2D (TC – 0.03 mmol/L; LDL-C 0.06 mmol/L).

Though the preclinical evidence is in support of beneficial effects of curcumin supplementation for dyslipidaemia (309, 314, 315), clinical evidence to substantiate the lipid lowering effects of curcumin is not well established. Few clinical trials with curcumin supplementation have reported either lowering (205, 316) or no change effect (317) on serum TG. Recently published two systematic review and meta-analysis (313, 318) on effects of curcumin on blood lipids have presented contrasting results. Further dissecting the results from these papers, it is noteworthy to observe that both the meta-analysis conclusions were based on heterogenous population. Secondly, the results included in the meta-analysis were not specifically related to curcumin, but also with turmeric powder and other unspecified curcuminoid mixtures. Thus, with current available clinical evidence, a clear direction on curcumin supplementation effects on blood lipids is lacking. With a higher magnitude of reduction of blood lipids by curcumin in individuals with T2D, clinical trials with required power and sample size may be a promising direction for further study.

8.1.5 LCn-3PUFA lowers FPG in individuals with baseline FPG level above 5.5 mmol/L

There is considerable evidence in the literature showing an increase in FPG following LCn-3PUFA supplementation, however, this evidence was primarily obtained from the clinical trials with T2D patients. The effects of LCn-3PUFA on FPG have not yet been properly evaluated in the individuals with IFG or IGT. As presented in the chapter 6, LCn-3PUFA did not exhibit any effects on FPG in individuals with high risk of T2D. Subgroup analysis of this population indicated heterogeneity in study population, with more than half the number of the participants having fasting blood glucose levels >5.5 mmol/L. FPG was significantly lowered from baseline with LCn-3PUFA (P=0.008) supplementation in participants with baseline blood glucose level >5.5 mmol/L. Therefore, a low dose LCn-3PUFA supplementation (<1.5g/day) could potentially offer FPG lowering effects in individuals with IFG or IGT. This insightful observation can limit the barriers for clinical trials designed to evaluate LCN-3PUFA effects on glycaemic control.

8.1.6 LCn-3PUFA supplementation reduces erythrocyte arachidonic acid levels, but has no effect on CRP

Failure to resolve inflammation has been shown to invariably result in persistent chronic low grade inflammation, dictating the aetiology of chronic diseases such as T2D (326, 327). One of the key mechanism of LCn-3PUFA is resolution of inflammation, which is mediated through changes in the membrane phospholipid composition of the cells participating in inflammation process. Studies in humans with doses ranging from (1.3g to 2.5g per day) have shown decreased production of arachidonic acid, a precursor for pro-inflammatory eicosanoids (328). In addition, specialised endogenous novel lipid mediators derived from LCn-3PUFA such as E- and D-series resolvins and protectins have shown to promote the resolution of inflammation (151). In the COP-D study, supplementing 1.3g EPA + DHA per day has shown significant reduction in the erythrocyte levels of arachidonic acid, indicating reduced production of pro-inflammatory molecules. Since CRP is more reflective of any acute inflammation or even an infection (329) than resolution of inflammation, we could not find any changes in CRP with LCn-3PUFA supplementation in this study (figure 7.2).

8.1.7 Both LCn-3PUFA and curcumin exhibit similar magnitude of reduction in InsuTAG

Curcumin Supplementation significantly reduced (-0.29 units) the InsuTAG levels in individuals with high risk of T2D, which could primarily be a result from reduction in the fasting insulin. LCn-3PUFA supplementation also shown similar level reduction in the InsuTAG levels (-0.30 units). However, the LCn-3PUFA mediated reduction in InsuTAG could be mainly arising from reduction in the plasma TG. Though the effects on IR are mediated through different mechanism by curcumin and LCn-3PUFA, overall reduction in the InsuTAG implies potential attractive strategy for lowering the multiple risk factors for cardio-metabolic disease.

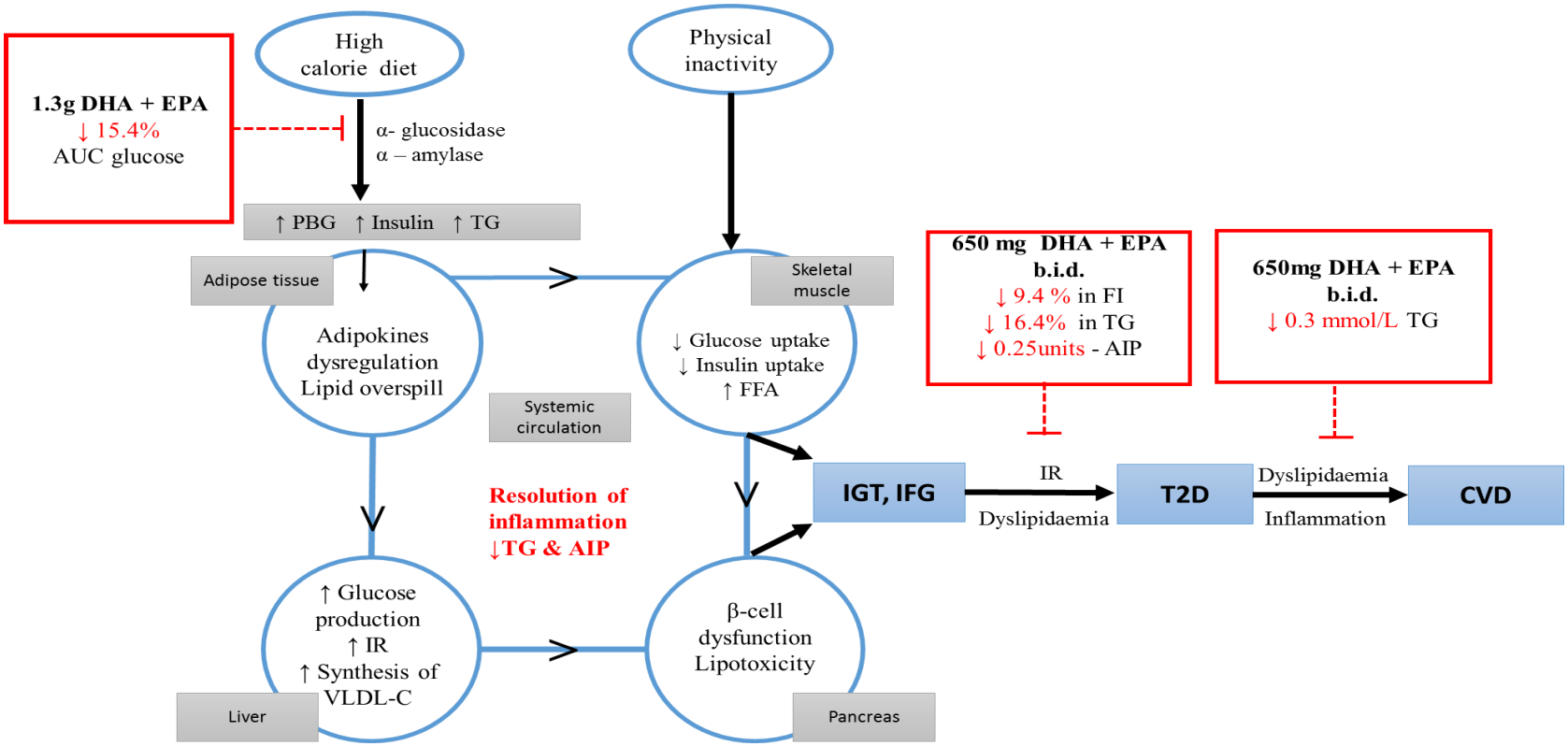

Figure 8.2: Summary of the effects of LCn-3PUFA on glycaemic control and blood lipids

AIP – Atherogenic index of plasma; **AUC** – Area under the curve; **CVD –** Cardiovascular disease; **DHA** – Docosahexaenoic acid; **EPA** – Eicosapentaenoic acid; **FI** – Fasting insulin; **FFA** – Free fatty acids; **IFG** – Impaired fasting glucose; **IGT** – Impaired glucose tolerance; **IR** – Insulin resistance; **IS** – Insulin sensitivity; **PBG** – Postprandial blood glucose; **TG** – Triglyceride; **T2D** – type 2 diabetes; **b.i.d**. - two times a day

8.1.8 Complementary or added benefits with curcumin and LCn-3PUFA were not observed in all the three intervention studies

All the three trials presented in this thesis failed to demonstrate any complimentary and/or synergistic effects of concurrent administration of curcumin and LCn-3PUFA. From the observations, two potential limiting factors were noted as a barrier to effectiveness of combination:

1. *Effects of curcumin on erythrocyte fatty acid composition when co-administered with LCn-3PUFA*

   Unexpectedly, we found a decrease in the red blood cell DHA and EPA content when curcumin is co-administered with FO in our trials (chapter 6 and 7) raising the possibility of interactions or complex formation suggesting reduced efficacy of LCn-3PUFA and/or curcumin for biological functions. There is a remote possibility that curcumin and LCn-3PUFA may make insoluble complexes in the gastrointestinal tract reducing the bioavailability of both the bioactive compounds. Successful attempts have been made to synthesise esters of curcumin and LCn-3PUFA (Patent number: WO2007103435A2). This patent application also claimed that DHA-curcumin complex increased the erythrocyte curcumin content in the animal studies however no clinical evidence has been presented to date. Based on these observations, there is great necessity to examine the potential interactions between LCn-3PUFA and curcumin to avoid potential interactions in the future trials. Pharmacokinetic and isobolographic analysis should be carried out to determine the optimal dose ratio for combining curcumin and LCn-3PUFA. This analysis will further help prevent the interactions between these two dietary bio-actives to minimise subadditive effect of curcumin and LCn-3PUFA.

2. *Co-administration of curcumin and fish oil*

   To the best of our knowledge, the studies described in this thesis are the first to examine the beneficial the combined effects of curcumin and LCn-3PUFA on glycaemic control and blood lipids in individuals with high risk of T2D and those diagnosed with T2D. We designed a 2x2 factorial trial to individually determine the effects of curcumin and LCn-3PUFA alone to compare against the combined group (curcumin + LCn-3PUFA) rather than testing it in a single formulation.

Participants in both the trials were advised to take the curcumin tablets and the fish oil capsules together at the breakfast and the dinner times. Anecdotally, study participants reported difficulties in taking the capsule and tablets at the same time on the follow-up visits. Due to the short half-life of curcumin, complementary and/or synergistic effects with LCn-3PUFA can only be expected if the two supplements are taken together at the same time. Future studies with single pharmaceutical formulation or in a single functional food are warranted to further elucidate any beneficial effects of the concurrent dietary supplementation with curcumin and LCn-3PUFA.

8.2 Limitations

8.2.1 Feasibility of recruiting study participants with pre-diabetes and T2D

Though we had world-wide media attention for these trials with curcumin and LCn-3PUFA, the target number of participant recruitment particularly for the intervention trials in pre-diabetes and T2D was highly challenging. We implemented several strategic plans such as presentations to the GPs, recruitment of participants through the pharmacy practices by involving pharmacy students, Facebook pages, Twitter updates and screening stalls at University open days and local shopping complexes. The major limiting factor for recruitment was the limited awareness about pre-diabetes among the community members. As pre-diabetes is not a fully blown disease state, it was challenging to articulate the high risks of pre-diabetes to the potential participants.

The supplement use was relatively less compared with those with prediabetes or high risk of T2D. Irrespective of continuous screenings of participants at the local diabetes clinics and media advertisements the response from the participants for participation was very weak. Moreover, presence of co-morbidities such as cancer, CVD and diabetic neuropathy were the major limiting factor to meet the study recruitment target.

8.2.2 Heterogeneity in the study populations

Though the overall study population in COP-D trial was insulin resistant, participants (previously diagnosed with prediabetes) did not exhibit the glucose or HbA1c values in the range of IGT or IFG at the baseline visits. Moreover, participants with high risk score in AUSDRISK tool, did not have blood glucose levels in the range of pre-diabetes. As a result, half of the study population have had high FPG and HbA1c levels and rest in the normal ranges. Thus impacting the overall outcomes of the curcumin and LCn-3PUFA in

the trial. Despite blinding in the CALFOR-CVD study design, the participants allocated to the placebo and curcumin alone groups exhibited higher FPG, TG, TC and LDL-C compared to those in fish oil alone and combined group. Together these limitations served as a barrier to the conclusive results on the combination of curcumin and LCn-3PUFA in both the clinical trials.

8.2.3 Different formulations of curcumin and fish oil

Curcumin is available in different formulations such as Longvida, curcumin C3 complex, curcumin+pipperine, curcuminoids mixture etc. Dose and bio-availability of curcumin varied greatly between these formulations. Similarly with fish oil, the composition of EPA and DHA was different between the formulations depending on the dose requirement and type of delivery of these fatty acids. In the current study we have employed Meriva (curcumin phospholipids) and DHA rich fish oil (delivering 1.3g DHA + EPA). This made it challenging to compare the current studies with existing clinical data (as they have used different dose levels and formulations) on curcumin and LCn-3PUFA.

8.3 Conclusions

The results presented in our chapter 3 on InsuTAG are a significant addition to the body of the existing knowledge on disease risk indicators. These are the first reports on development of this novel marker and evidence for its potential to predict IR and MetS in general population. The results presented in the later of part of thesis are the first clinical evidence on combination of curcumin and LCn-3PUFA that were evaluated to influence multiple risk factors to lower the metabolic abnormalities. Curcumin alone has the potential to lower the PBG in healthy individuals and IR in individuals with high risk of T2D. In addition to these results, curcumin has shown effective TG lowering effects along with notable reductions in TC and LDL-C in individuals with T2D. LCn-3PUFA alone exhibited TG reduction in both individuals with and without T2D. Additionally LCn-3PUFA demonstrated a trend in reducing the FI and IR in individuals with high risk of T2D. It is noteworthy to observe that LCn-3PUFA significantly reduced the FPG and FI in individuals with FPG above 5.5 mmol/L, thus indicating supplementation of LCn-3PUFA may benefit individuals with IFG or IGT rather than normal glucose tolerant or overt T2D. These results have expanded our knowledge on how nutritional interventions can elicit their physiological responses depending on the study population and baseline

characteristics of the participants. Though curcumin and LCn-3PUFA alone have shown beneficial effects on glycaemic control and dyslipidaemia (figure 8.1 & 8.2), the data presented in this thesis failed to show any added or complimentary benefits of combining curcumin and LCn-3PUFA.

The above mentioned limitations that acted as a barrier to the effectiveness of combination are manageable. Taking into account of these limitations in the future studies with combination of curcumin and LCn-3PUFA can minimise the barriers to their effectiveness. Overall, the data presented in this thesis represented significant and novel observations together with a set of unresolved questions, further providing aims for future research on prognostic indicators and application of dietary bio-actives for lowering the risk of metabolic diseases.

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Appendix 1: Recruitment flyer – Acute postprandial study

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Project Supervisors:
Professor Manohar Garg
Dr Sham Acharya

VERSION 1.2: 7 SEP 2017

WOULD YOU LIKE TO KNOW ?????

HOW YOUR BODY RESPONDS TO A MEAL CONSUMPTION

RESEARCH STUDY
www.newcastle.edu.au

Researchers from the **University of Newcastle and John Hunter Hospital** are conducting a research study to examine the role of **dietary supplements** on blood sugar and fat levels after meal consumption in healthy volunteers.

The study may be suitable for you:

- If you are aged between 18-45 years
- If you have body mass index (BMI) is below 30

BMI = Body weight (in kilograms) / Height x Height (in meters)^2

THIS RESEARCH HAS BEEN APPROVED BY THE UNIVERSITY OF NEWCASTLE HUMAN RESEARCH ETHICS COMMITTEE– H-2014-0385

If interested, please contact us on:

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Appendix 2: Recruitment flyer – COP-D study

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Project Supervisors
Professor Manohar Garg
Dr Sham Acharya

DO YOU OR SOMEONE YOU KNOW HAVE

VERSION 1.0; 23 MAR 2016

BORDERLINE DIABETES (pre-diabetes)?

NUTRACEUTICALS RESEARCH GROUP IS CONDUCTING A RESEARCH STUDY ON CURCUMIN AND OMEGA 3 FATTY ACIDS

FOR PREVENTION OF TYPE 2 DIABETES.

THE STUDY MAY BE SUITABLE FOR YOU IF YOU ARE:

- Aged between 30-70
- Diagnosed with Borderline diabetes (pre-diabetes)
  or
- Score >12 in AUSDRISK tool

(CONTACT US TO CALCULATE YOUR SCORE)

THIS STUDY IS APPROVED BY HUNTER NEW ENGLAND HUMAN RESEARCH ETHICS COMMITTEE - 16/03/16/3.02

If you would like to know more about this study, please contact a study coordinator on:

02 4921 5636 (Rohith Thota)

E: **prediabetesnewcastle@gmail.com**

4921 5636 prediabetesnewcastle@ gmail.com	4921 5636 prediabetesnewcastle@ gmail.com	4921 5636 prediabetesnewcastle@ gmail.com	4921 5636 prediabetesnewcastle@ gmail.com	4921 5636 prediabetesnewcastle@ gmail.com	4921 5636 prediabetesnewcastle@ gmail.com	4921 5636 prediabetesnewcastle@ gmail.com

Appendix 3: Recruitment flyer – CALFOR-CVD Study

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Project Supervisors
Professor Manohar Garg
Dr Sham Acharya

VERSION 1.1; 29 JUN 2016

RESEARCH STUDY
www.newcastle.edu.au

DO YOU OR SOMEONE YOU KNOW HAVE

TYPE II DIABETES?

Researchers from **University of Newcastle** and **John Hunter Hospital** are conducting a research study on **Curcumin and Omega 3 fatty acids** for management of **blood lipids** in **type 2 diabetes**.

The study may be suitable for you if you are:

- Aged between 40-75
- Diagnosed with type 2 diabetes (<15 years)
  (Currently not on insulin treatment)

THIS RESEARCH HAS BEEN APPROVED BY THE HUNTER NEW ENGLAND HUMAN RESEARCH ETHICS COMMITTEE OF HUNTER NEW ENGLAND LOCAL HEALTH DISTRICT – 16/07/20/3.02

If you would like to know more about this study, please contact

a study coordinator on:

02 4921 5636

(CALFOR-CVD study)

E: calforcvd2016@gmail.com

Appendix 4: Participant information sheet - Acute postprandial study

PARTICIPANT INFORMATION SHEET
Nutritional supplements for prevention of type 2 diabetes – Postprandial Study
INVESTIGATORS: Prof. Manohar Garg, Dr Sham Acharya, Mr Rohith Thota & Ms Melissa Fry
Version 1.2; 07/09/2017

You are invited to participate in the research project identified above which is being conducted by Mr Rohith Thota at the University of Newcastle. This research is a part of Rohith Thota's PhD studies at the University of Newcastle, supervised by Professor Manohar Garg from the School of Biomedical Sciences and Pharmacy, and Dr Sham Acharya from the Hunter New England Local Health District.

Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish.

Why is the research being done?
Postprandial (post-meal consumption) blood sugar level has been implicated in the development of obesity, type 2 diabetes and cardiovascular disease. It has been indicated that a rapid rate of rise followed by slower decline in the blood glucose levels post meal consumption are linked with fat deposition and adversely affect metabolic health. Curcumin, a bio-active ingredient obtained from the spice turmeric, is a potent anti-inflammatory agent, which is being now evaluated for its effects for controlling post meal blood sugar levels. Long chain omega 3 fatty acids (eicosapentanoic acid and docosahexaenoic acid) are established triglyceride lowering agents which are primarily obtained from fish or fish oil. The current purpose of this study is to evaluate the efficacy of a single dose of dietary supplements (curcumin and/or fish oil) on the post meal blood glucose rise in healthy individuals.

Who can participate in this study?
This study is suitable for you, if

- You are aged between 18-45 years
- You have body mass index (BMI) < 30 Kg/m^2

This study is not suitable for you, if

- You are diagnosed with any chronic or metabolic disease (eg: type 2 diabetes, cardiovascular disease)
- You are diagnosed with any auto-immune disease (rheumatoid arthritis, lupus, type 1 diabetes)
- You are diagnosed with liver disease
- You are on any medications for control of blood glucose levels
- You have a history of severe neurological diseases or seizures
- You are taking regular dietary supplements known to influence blood glucose level
- You are pregnant, planning to become pregnant or breastfeeding
- You cannot provide informed consent
- You have Sensitivity/ intolerance to curcumin and/or fish oil, and food (dairy products, wheat, cow's milk, protein and gluten)

What if I don't want to take part in this study, or if I want to withdraw later?
Participation in this study is voluntary. It is completely up to you whether or not you participate. If you decide not to participate, it will not affect how you are treated now or in the future.
New information about the trial supplements being studied may become available during the course of the study. You will be kept informed of any significant new findings that may affect your willingness to continue in the study. If you wish to withdraw from the study once it has started, you can do so at any time without having to give a reason. You can also withdraw your data from being included in the data analysis of study at any point of time before completion of the analysis. Choosing to withdraw from the study will not affect the relationship with staff at the University of Newcastle.

What does this study involve?
If you agree to participate in this study, you will be asked to sign the participant consent form. Throughout the study, you will need to visit the Nutraceuticals Research Group on 4 occasions for two and half hours (one visit per week over 4 weeks):
At the initial (week 1) visit we will be asking you to do the following;

- Complete a brief medical questionnaire (provides information about your medical history, supplement intake and medications)
- Complete a physical activity questionnaire (provides information about your physical activity levels)
- Complete a 24-hour food recall (provides information about your dietary and nutrient intake)
- The day before your appointments you will be asked to refrain from physical activity and alcohol consumption and visit the trial site after an overnight fasting state of at the least for 10 hours.

- Consume the provided standard meal in the night before the visit day.
- Provide us with finger prick blood sample for testing glucose levels at 4 intervals (0, 30 min, 60 min, 120 min)
- Provide 8 mL of blood (collected through venepuncture that involves three separate site punctures) for 3 times in two hours (0, 1 hour, 2 hour)

On each visit, the study participants will be given a standard breakfast along with one of the following dietary supplements in a random order:
1) 2 x placebo tablets (matching for curcumin) + 2 x placebo capsules (matching for fish oil)
2) 2 x 500 mg curcumin tablets + 2 x placebo capsules (matching for fish oil)
3) 2 x 1000 mg fish oil capsules + 2 x placebo tablets (matching for curcumin)
4) 2 x 500 mg curcumin tablets + 2 x 1000 mg fish oil capsules
Placebos are tablets or capsules that look like the tablets or capsules under investigation, but contain no active ingredients.

At the remaining visits (week 2, 3 & 4) we will be asking you to do the following:
- Complete a physical activity questionnaire
- Complete a 24-hour food recall
- Provide Finger prick and venous blood sample (similar procedures to the first visit) after an overnight fast (not eating for 10 hours)

Are there risks to me in taking part in this study?
There are some risks of having blood collected, including bruising or bleeding from the first collection site, as well as rarely fainting and dizziness. However, these risks are minimal and a qualified and experienced phlebotomist will take your blood in order to minimize these risks. In case of any adverse events, participants have an opportunity to report to study coordinator or principal investigator through their contact details provided in this information sheet. In case of any adverse events, participants will be provided with results and will be referred to their GP further review/assessment.

Will I benefit from the study?
This study aims to further medical knowledge and may improve scope for using nutritional supplements in controlling sugar levels after a meal consumption for the prevention of obesity, diabetes and cardiovascular disease.

Will taking part in this study cost me anything, and will I be paid?
Participants who complete the study will each receive $20 for their expenses for each visit (parking and travel). There is no extra payment involved. There will be no reimbursement for participants who do not attend the Nutraceuticals Research Group for the baseline visit or do not qualify for the study.

What will happen to my Blood sample after it has been used?
Blood samples will be collected per standard operating procedures at the NRG. All blood samples will be destroyed after analysis.

How will my confidentiality be protected?'
Your information will be treated with the same respect for privacy and confidentiality as is undertaken for all medical information collected about you during your visits to your local doctor. Access to your personally identifiable information will be limited to the named investigators only.
All collected information on data sheets will be physically stored in a locked filing cabinet in the Medical Science Building accessible to the investigators at the University of Newcastle. All data will only be identifiable by a participant identification number with no other identifying details on file. During statistical data analysis the database will be stored in a password protected computer file on a computer that is kept in a locked room. After completion of computer data analysis, the information will be transferred onto a CD before it will be removed and permanently deleted from the computer file. The CD will also be stored in a locked cabinet in a locked room of the principal investigator.

All data for the study will be kept by the Chief Investigator for the period of 15 years following the completion of the study. Only staff of the University of Newcastle conducting this research will have access to this information.

What happens with the results?
Results of this research will be published in scientific journals and be published in Rohith Thota's PhD thesis. Individual participants will not be identified in any reports arising from the project.
In any publication, information will be provided in such a way that you cannot be identified. Results of the study will be provided to you, if you wish.

What should I do if I want to discuss this study further before I decide?
When you have read this information, Professor Manohar Garg or Mr. Rohith Thota will discuss it with you and any queries you may have. If you would like to know more at any stage, please do not hesitate to contact him on phone (4921 5638) or Email: c3216266@uon.edu.au

Who should I contact if I have concerns about the conduct of this study?
Further information about this or any other research project undertaken in the department at the University of Newcastle can be obtained from Principal Investigators of the study, Professor Manohar Garg and Dr. Shamsunder Acharya or Mr. Rohith Thota (4921 5636), Email: c3216266@uon.edu.au

Thank you for taking the time to consider this study. If you wish to take part in it, please sign the attached consent form. This information sheet is for you to keep.

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Ms. Melissa Fry
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Email: Melissa.fry@newcastle.edu.au

This project has been approved by the University of Newcastle Human Research Ethics Committee H-2014-0385.

Should you have concerns about your rights as a participant in this research, or you have a complaint about the manner in which the research is conducted, it may be given to the researcher, or, if an independent person is preferred, to the Human Research Ethics Officer, Research Office, The Chancellery, The University of Newcastle, University Drive, Callaghan NSW 2308, Australia, Telephone (02) 4921 6333, Email: Human-Ethics@newcastle.edu.au.

Appendix 5 : Participant information sheet – COP-D study

PARTICIPANT INFORMATION SHEET

CLINICAL TRIAL

NUTRITIONAL SUPPLEMENTS FOR PREVENTION OF TYPE 2 DIABETES

Investigators: Professor Manohar Garg, Dr. Sham Acharya, Mr. Rohith Thota & Ms. Melissa Fry

Version 1.4; 06/05/2015

You are invited to participate in a research study on nutritional supplements (Curcumin and/ or omega 3 fatty acids) for preventing type 2 diabetes by improving glycemic profiles and insulin resistance in individuals with pre-diabetes.

The study is being conducted by researchers from School of Biomedical Sciences And Pharmacy at the University of Newcastle as a part of PhD studies of Mr. Rohith Thota under the supervision of Professor Manohar Garg and Dr. Sham Acharya

Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish.

1. **What is the purpose of this study?**

The purpose is to investigate whether the nutritional supplements will improve the glycemic profiles (Glucose levels, insulin levels and HbA1c) in the individuals with pre-diabetes.

2. **Who can participate in this study?**

You are eligible to participate in the study based on inclusion and exclusion criteria

Inclusion criteria:

- Age – 30-70 years ; gender – both males and females
- No participation in any clinical trial for at least 3 months
- An HbA1c of 5.7% - 6.4%
- Impaired Glucose Tolerance (IGT):

ƒ- 2-hour OGTT plasma glucose ≥7.8 mmol/ L and <11.1 mmol/L

- Impaired fasting glucose (IFG):

Fasting plasma venous glucose measurement 6.1–6.9 mmol/L

- 12 or more score or High risk individuals in AUSDRISK assessment tool
- BMI 25 – 45 Kg/m^2

Exclusion criteria Volunteers will be excluded if they have/are

- Pregnancy or lactation
- Established type 2 diabetes
- Allergic to sea foods
- People with gall bladder problems
- People with pace maker implants
- Currently on medication with Aspirin and Warfarin
- History of severe neurological diseases or seizures
- History of using new investigational drug three months prior to this trial
- Consuming more than 2 serve of oily fish per week
- Taking regular dietary supplements known to influence blood glucose level
- Unwilling to fast for 10hr before obtaining blood sample

3. What if I don't want to take part in this study, or if I want to withdraw later?

Participation in this study is voluntary. It is completely up to you whether or not you participate. If you decide not to participate, it will not affect you now or in the future.

New information about the trial supplements being studied may become available during the course of the study. You will be kept informed of any significant new findings that may affect your willingness to continue in the study. If you wish to withdraw from the study once it has started, you can do so at any time without having to give a reason. You can also withdraw your data from being included in the data analysis of study at any point of time before completion of the analysis.

4. What does this study involve?

If you agree to participate in this study, you will be asked to provide your GP details and sign the participant consent form. This study will be conducted over 3 months. Study participants are put into groups and given

1) Placebo: 4 capsules/day (2 each for curcumin and fish oil placebos)

2) Curcumin (2 cap @500 mg each) providing 180 mg curcumin plus 2 placebo capsules/day

3) n-3PUFA (2 cap @1000mg each) providing 1.2g EPA/DHA plus 2 placebo capsules/day

4) Curcumin (2 cap @500 mg each providing a total of 180 mg curcumin) and n-3PUFA (2 cap @1000mg each providing a total of 1.2g EPA/DHA) per day

The results are compared to see whether one supplement is better. To ensure the groups are similar to start with, a computer allocates each study participant into a group randomly, like the flip of a coin. Neither the researchers nor the study participant can decide which supplement the participant receives. The study participants do not know which supplement receiving group they are in. Neither the researcher nor the study participant knows which supplement the participant is receiving. You will be advised to consult your doctor for

management of any medication to be taken with the meal on the days of fasting blood collection during week 1 and 12.

Throughout the study, you will need to visit the NRG at University of Newcastle or John Hunter Hospital on 3 occasions; week 0, week 6 & week 12. At week 0 and 12 we will be asking you to do the following;

- Complete a brief medical questionnaire
- Complete a physical activity questionnaire
- Complete food diary
- Give body weight, height and body composition measurement
- Donate blood (20mL) after an overnight fast

At week 6 we will be asking you to do the following:

- Follow up of supplement intake
- Anthropometrical Measurements (Height, weight, BMI, waist and hip measurements)
- Complete a physical activity questionnaire
- Complete food diary
- Providing the supplements for next 6 weeks

6. Are there risks to me in taking part in this study?

There are some risks of having blood collected, including bruising or bleeding from the first collection site, as well as rarely fainting and dizziness. However these risks are minimal and a qualified and experienced phlebotomist will take your blood in order to minimize these risks. In case of any adverse events, participants have an opportunity to report to study coordinator or principal investigator through their contact details provided in this information sheet. Participant's GP will be informed of their adverse event for further review/assessment if required. You may request information regarding the outcome from any examinations and laboratory tests that are undertaken in the study. Feedback of results from the investigation and other information regarding to the outcome of the research will be made available to you and your GP. The results of the study will be posted as individual letter at the completion of study.

8. Will I benefit from the study?

This study aims to further medical knowledge and may improve scope for use of nutraceuticals in preventing or delaying onset of type 2 diabetes.

9. Will taking part in this study cost me anything, and will I be paid?

Participants who complete the study will each receive $40 as a compensation for their time and expenses. Pro-rata payment will be made to participants who withdraw prior to study completion, $20 per initial and final visit. There will be no reimbursement for participants who did not attend the NRG or John Hunter Hospital for the screening/baseline visit or did not qualify for the study

10. What will happen to my Blood sample after it has been used?

Blood samples will be collected according to standard operating procedures at the NRG. All blood samples will be destroyed after analysis.

11. 'How will my confidentiality be protected?'

Your information will be treated with the same respect for privacy and confidentiality as is undertaken for all medical information collected about you during your visits to your local doctor. Access to your personally identifiable information will be limited to the named investigators only. All collected information on data sheets will be physically stored in a locked filing cabinet in the Medical Science Building accessible to the investigators at the University of Newcastle. All data will only be identifiable by a participant identification number with no other identifying details on file. During statistical data analysis the database will be stored in a password protected computer file on a computer that is kept in a locked room. After completion of computer data analysis, the information will be transferred onto a CD before it will be removed and permanently deleted from the computer file. The CD will also be stored in a locked cabinet in a locked room of the principal investigator.

All data for the study will be kept by the Chief Investigator for the period of 20 years following the completion of the study. Only staff of the University of Newcastle conducting this research will have access to this information.

12. What happens with the results?

Results of this research will be published in scientific journals and be published in Rohith Thota's PhD thesis. Individual participants will not be identified in any reports arising from the project. In any publication, information will be provided in such a way that you cannot be identified. Results of the study will be provided to you, if you wish.

13. What should I do if I want to discuss this study further before I decide?

When you have read this information, Professor Manohar Garg or Mr. Rohith Thota will discuss it with you and any queries you may have. If you would like to know more at any stage, please do not hesitate to contact him on phone (4921 5638) or Email: c3216266@uon.edu.au

14. Who should I contact if I have concerns about the conduct of this study?

Further information about this or any other research project undertaken in the department at the University of Newcastle can be obtained from Principal Investigators of the study, Professor Manohar Garg and Dr. Shamsunder Acharya or Mr. Rohith Thota (4921 5636), Email: c3216266@uon.edu.au

Thank you for taking the time to consider this study.

If you wish to take part in it, please sign the attached consent form.

This information sheet is for you to keep.

Professor Manohar Garg
Principal Investigator 1

Dr. Sham Acharya
Principal Investigator 2

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This project has been approved by the University of Newcastle Human Research Ethics Committee H-2014-0385.

Should you have concerns about your rights as a participant in this research, or you have a complaint about the manner in which the research is conducted, it may be given to the researcher, or, if an independent person is preferred, to the Human Research Ethics Officer, Research Office, The Chancellery, The University of Newcastle, University Drive, Callaghan NSW 2308, Australia, Telephone (02) 4921 6333, Email: Human-Ethics@newcastle.edu.au.

Appendix 6: Participant information sheet – CALFOR-CVD study

PARTICIPANT INFORMATION SHEET
CLINICAL TRIAL

CURCUMIN AND LONG CHAIN OMEGA-3 FATTY ACIDS FOR MANAGEMENT OF CARDIOVASCULAR HEALTH IN INDIVIDUALS WITH TYPE 2 DIABETES (CALFOR-CVD TRIAL)

Investigators: Professor Manohar Garg, Dr. Sham Acharya, Mr. Rohith Thota & Ms. Melissa Fry

Version 1.3; 22/09/2016

You are invited to participate in a research study on dietary supplements (curcumin and/ or omega 3 fatty acids) for managing cardiovascular health by improving blood lipid profiles and reducing chronic inflammation in individuals with type 2 diabetes.

The study is being conducted by researchers from the Nutraceuticals Research group, School of Biomedical Sciences and Pharmacy, University of Newcastle and from the John Hunter Hospital, as a part of PhD studies by Mr. Rohith Thota under the supervision of Professor Manohar Garg and Dr. Sham Acharya.

Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish.

What is the purpose of this study?

Curcumin, a bio-active ingredient obtained from the spice turmeric, is a potent anti-inflammatory agent, which is being now evaluated for its effects on blood lipid levels. Long chain omega 3 fatty acids (eicosapentanoic acid and docosahexaenoic acid) are established triglyceride lowering agents which are primarily obtained from fish or fish oil. The purpose of this study is to investigate whether the nutritional supplements curcumin and/or long chain omega 3 fatty acids will improve the blood lipid profile in individuals with type 2 diabetes.

Who can participate in this study?

This study is suitable for you if

- You are aged between 40 and 75 years;
- You are diagnosed with type 2 diabetes (duration less than 15 years)
- Your body mass index (BMI) lies between 25 and 45 kg/m^2
- You are willing not to change your usual medication during the study period.

This study is not suitable for you if:

- You are pregnant, planning to become pregnant or breastfeeding
- Cannot provide informed consent
- You have type 1 diabetes
- You are currently on insulin treatment
- You are diagnosed with cancer
- You have a glomerular filtration rate less than 45
- You have been diagnosed with painful peripheral neuropathy
- You have a cardiac pacemaker
- You have a history of severe neurological diseases (Parkinson's, multiple sclerosis, epilepsy)
- You are consuming more than 2 serves of oily fish per week
- You are taking regular dietary supplements known to influence blood lipid levels
- You have sensitivity/ intolerance to the products involved in this study
- You are unwilling to fast for 10 hours before giving blood sample

What if I don't want to take part in this study, or if I want to withdraw later?

Participation in this study is voluntary. It is completely up to you whether or not you participate. If you decide not to participate, it will not affect how you are treated now or in the future.

New information about the trial supplements being studied may become available during the course of the study. You will be kept informed of any significant new findings that may affect your willingness to continue in the study. If you wish to withdraw from the study once it has started, you can do so at any time without having to give a reason. You can also withdraw your data from being included in the data analysis of study at any point of time before completion of the analysis. Choosing to withdraw from the study will not affect the care you receive at the John Hunter Hospital or your relationship with staff at the University of Newcastle.

What does this study involve?

If you agree to participate in this study, you will be asked to sign the participant consent form. This study will be conducted over 6 weeks.

Throughout the study, you will need to visit the Nutraceuticals Research Group at the University of Newcastle or the John Hunter Hospital on 2 occasions;

At the initial (week 1) visit we will be asking you to do the following

- Complete a brief medical questionnaire (provides information about your medical history, supplement intake and medications)
- Complete a physical activity questionnaire (provides information about your physical activity levels)
- Complete a 3-day food diary (provides information about your dietary and nutrient intake)

- Provide a 25 mL blood sample after an overnight fast

Study participants are put into groups and given (according to a computer generated sequence) one of the following:

1) 2 X placebo tablets (matching for curcumin) + 2 X placebo capsules (matching for fish oil) per day

2) 2 X 500 mg curcumin tablets + 2 X placebo capsules (matching for fish oil) per day

3) 2 X 1000 mg fish oil capsules + 2 X placebo tablets (matching for curcumin) per day

4) 2 X 500 mg curcumin tablets + 2 X 1000 mg fish oil capsules per day

Placebos are tablets or capsules that look like the tablets or capsules under investigation, but contain no active ingredients.

You will not be asked to make any other changes to your usual diet or lifestyle. The results are compared to see whether one supplement or the combination makes any difference. To ensure the groups are similar to start with, a computer allocates each study participant into a group randomly, like the flip of a coin. Neither you nor the researchers will know which supplement you receive.

At the final visit (week 6) we will be asking you to do the following:

- Complete a physical activity questionnaire
- Complete a 3-day food dairy
- Provide a 25 mL blood sample after an overnight fast (not eating for 12 hours)
- Return the provided tablet/capsule containers and tablet/capsule log sheet provided

Are there risks to me in taking part in this study?

There are some risks of having blood collected, including bruising or bleeding from the collection site, as well as, rarely, fainting and dizziness. However, these risks are minimal and a qualified and experienced phlebotomist will take your blood in order to minimize these risks. In case of any adverse events, participants have an opportunity to report to the study coordinator or principal investigator through the contact details provided in this information sheet. Your GP will be informed of any adverse event (with your permission) for further review/assessment if required.

Some potential side effects including constipation, flatulence, yellow hard stools, nausea and vomiting, dyspepsia, gastritis, distention and epigastric burning, have been observed in previous studies when consuming very high doses (> 8 g) of curcumin. The available amount of curcumin in the product (Meriva) used in this trial is higher than that of other curcurmin supplements; therefore some of the above mentioned side effects may occur. Fish oil supplements up to 3 g/day are generally safe; however, minor gastrointestinal side symptoms such as bloating, nausea and bad breath might be experienced. To avoid these side effects, we recommend taking fish oil and curcumin

capsules with food. It is possible that there may be minor drug interactions between curcumin and/or fish oil supplements and blood-thinning medications; however, these have not been seen in human studies. Participants can contact the study coordinator at any time during the study period to report any adverse event or side effect.

You may request information regarding the outcome from any examinations and laboratory tests that are undertaken in the study. Feedback of results from the investigations and other information regarding the outcome of the research will be made available to you and your GP (with your permission). The results of the study will be posted as individual letters at the completion of study.

Will I benefit from the study?

There may or may not be any direct benefit to you. This study aims to further medical knowledge and may improve scope for use of dietary supplements in managing cardiovascular health in individuals with type 2 diabetes in a natural way.

Will taking part in this study cost me anything, and will I be paid?

Participants who complete the study will each receive compensation for their expenses (parking and travel). There is no extra payment involved. There will be no reimbursement for participants who did not attend the Nutraceuticals Research Group or John Hunter Hospital for the screening/baseline visit or did not qualify for the study.

What will happen to my blood sample after it has been used?

Blood samples will be collected according to standard operating procedures at the Nutraceuticals Research Group. All blood samples will be destroyed after analysis.

'How will my confidentiality be protected?'

Your information will be treated with the same respect for privacy and confidentiality as is undertaken for all medical information collected about you during your visits to your local doctor. Access to your personally identifiable information will be limited to the named investigators only.

All collected information on data sheets will be physically stored in a locked filing cabinet in the Medical Science Building accessible to the investigators at the University of Newcastle. All data will be identifiable only by a participant identification number with no other identifying details on file. During statistical data analysis, the database will be stored in a password-protected computer file on a computer that is kept in a locked room. After completion of computer data analysis, the information will be transferred onto a CD before it will be removed and permanently deleted from the computer. The CD will also be stored in a locked cabinet in a locked room of the principal investigator.

All data for the study will be kept by the Chief Investigator for a period of 15 years following the completion of the study. Only staff of the University of Newcastle conducting this research will have access to this information.

What happens with the results?

Results of this research will be published in scientific journals and be published in Rohith Thota's PhD thesis. Individual participants will not be identified in any reports arising from the project.

In any publication, information will be provided in such a way that you cannot be identified. Results of the study will be provided to you, if you wish.

What should I do if I want to discuss this study further before I decide?

When you have read this information, Professor Manohar Garg or Mr. Rohith Thota will discuss it with you and answer any queries you may have. If you would like to know more at any stage, please do not hesitate to contact them by phone (4921 5636) or email: calforcvd2016@gmail.com .

Who should I contact if I have concerns about the conduct of this study?

Should you have concerns about your rights as a participant in this research, or if you have a complaint about the manner in which the research is conducted, it may be given to the researcher, or, if an independent person is preferred, to Dr Nicole Gerrand, Manager, Research Ethics and Governance Unit, Hunter New England Human Research Ethics Committee, Hunter New England Local Health District, Locked Bag 1, New Lambton NSW 2305, telephone (02) 49214950, email: hnehrec@hnehealth.nsw.gov.au.

Thank you for taking the time to consider this study.

If you wish to take part in it, please sign the attached consent form.

This information sheet is for you to keep.

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Nutraceuticals Research Group

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This research has been approved by the Hunter New England Human Research Ethics Committee of Hunter New England Local Health District – 16/07/20/3.02.

Appendix 7: Participant consent form – Acute post-prandial study

PARTICIPANT CONSENT FORM

V 1.1; 07/09/2017

I, ...[name] of

……..... [address]

have read and understand that the study will be conducted as described in the information statement, a copy of which I have retained.

I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.

I agree to participate in this study and understand that I can withdraw at any time without providing a reason.

I understand that I will need to visit the Nutraceuticals Research Group, University of Newcastle, on 4 occasions (1, 2, 3 & 4 weeks) for two and half hours.

I understand that I will be required to donate blood sample multiple times (as described in the information statement), complete a 24-hour food recall, a physical activity questionnaire, and a medical history questionnaire.

I have had the opportunity to have questions answered to my satisfaction.

I understand that my personal information will remain confidential to the researchers.

I hereby agree to participate in this research study.

NAME: _____

SIGNATURE: _____

DATE: _____

Declaration by person conducting the consent process

I, the undersigned, have fully explained this research to the participant named above.

NAME: _____

SIGNATURE: _____

Appendix 8: Participant consent form – COP-D study

Patient consent form
Version 1.3, 28/01/2016
NUTRITIONAL SUPPLEMENTS FOR PREVENTION OF TYPE 2 DIABETES

I give my consent to participate in the above research project designed to test the efficacy of nutritional supplements on glycaemic profiles and insulin resistance in individuals with pre-diabetes for delaying or preventing onset of type 2 diabetes.

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that I will need to visit the Nutraceuticals Research Group, University of Newcastle, or John Hunter Hospital on 3 occasions (0^{th} week, 6^{th} week & 12^{th} week). ☐
4. I understand I need to take active supplement capsules or placebo during study through random allocation ☐
5. I understand that at my first and last visit, I will be required to donate blood after an overnight fast, complete a medical questionnaire, height and body composition measurements. ☐
6. I understand that I will also be required to complete a food dairy and physical activity questionnaire. ☐
7. I understand my personal information will remain confidential to the researchers. ☐
8. Feedback of results from the investigation and other information regarding to outcome of research will be made available to me and will be posted as individual letter at the completion of study. ☐
9. I agree to my GP being informed of my participation in the study. ☐
10. I agree to provide the details of my GP ☐
    Details of GP:
    Name:
    Address:

    Email: Phone no.:

I agree to take part in the above study.

Signed by participant

Print Name:

Date:

Phone: Email:

Appendix 9: Participant consent form – CALFOR_CVD trial

CURCUMIN AND LONG CHAIN OMEGA-3 FATTY ACIDS FOR MANAGEMENT OF CARDIOVASCULAR HEALTH IN INDIVIDUALS WITH TYPE 2 DIABETES

(CALFOR-CVD TRIAL)

PARTICIPANT CONSENT FORM

V1.0; 19/06/2016

I,..... [name] of

……..... [Address]

have read and understand that the study will be conducted as described in the Information Statement, a copy of which I have retained.

I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.

I understand that my participation in this study will allow the researchers and others, as described in the Information Statement, to have access to my medical record, and I agree to this.

I agree to participate in this study and understand that I can withdraw at any time without providing a reason.

I understand that my personal information will remain confidential to the researchers.

I have had the opportunity to have questions answered to my satisfaction.

I hereby agree to participate in this research study.

NAME:

_____

SIGNATURE:

_____

DATE:

_____

Declaration by person conducting the consent process

I, the undersigned, have fully explained this research to the patient named above.

NAME:_____

SIGNATURE:

_____

Appendix 10: Medical history and screening form – Acute postprandial study

MEDICAL HISTORY AND SCREENING FORM

Version 1.1; 23 AUG 2017

All information provided in this questionnaire will be **strictly confidential**. Your name will **not** be stored with any of your responses. Please answer all relevant questions to the best of your ability. ***Some of these questions will be used to determine your eligibility to participate in the trial.***

If you have any questions regarding the questionnaire or the information it contains please contact **Rohith Thota** on **(02) 4921 5636** or email him on **c3216266@uon.edu.au**

General Information

Participant:

Name _____

Address _____

Contact phone numbers _____

Birth date _____

Please answer all the following questions as they <u>best</u> describe you.

What is your gender?

☐ Male
☐ Female

In what country were you born?

☐ Australia
☐ Overseas (Please specify) _____
If you were born overseas, is English your first language? Yes / No
How long have you lived in Australia for? _____
Do you need an interpreter? Yes / No

How would you describe your ethnicity?

☐ Caucasian
☐ Aboriginal and/or Torres Strait Islander
☐ Asian
☐ Pacific Islander
☐ Middle Eastern
☐ Other (please specify) _____
☐ I'd prefer not to respond

What is the highest level of education you have achieved?

☐ Primary School
☐ Some High School
☐ School Certificate (or equivalent)
☐ Higher School Certificate (or equivalent)
☐ Certificate or Diploma
☐ Bachelor Degree
☐ Postgraduate degree

Present Medical History

Please answer the following questions.

- Age _____
- BMI _____

Comments: _____
_____
_____
_____

Do you now have or have you recently (in the previous 12 months) experienced:

☐ Chronic or recurrent coughs?
☐ Increased anxiety or depression?
☐ Problems with recurrent fatigue, thirst, trouble sleeping or increased irritability?
☐ Migraine or recurrent headaches?
☐ Stomach or intestinal problems, such as recurrent heartburn, ulcers, constipation or diarrhea?

- ☐ Significant unexplained weight loss?
- ☐ Lung and respiratory diseases
- ☐ Severe neurological diseases or seizures
- ☐ Under investigation drug three months prior to this trial?

<u>Women only</u> please answer the following:

Are you currently pregnant? Yes / No
Are you currently breastfeeding? Yes / No
Have you ever been diagnosed with polycystic ovarian syndrome? Yes / No
Which <u>best</u> describes your menopausal status? ☐ Premenopausal ☐ Postmenopausal
Are you currently using any hormonal contraceptives (e.g. the pill, implanon impant)? Yes / No
If yes, please specify _____
Are you on any type of hormone replacement therapy? Yes / No
If yes, please specify _____

<u>Men only</u> please answer the following:

Are you currently taking any medications or supplements to increase testosterone? Yes / No

Do you have any medical conditions which may affect your testosterone levels? Yes / No
If yes, please specify _____

Men and women answer the following:

Are you currently taking any regular **prescription medications**? Yes / No
*If yes please list **all** prescription medications you are taking, including dosage*: _____
_____
_____

Are you currently taking any **over-the counter medications**? Yes / No
*If yes please list **all** over-the counter medications you are taking, including dosage*: _____
_____
_____

Are you currently taking any **vitamins or dietary supplements**? Yes / No
*If yes please list **all** vitamins and/or dietary supplements you take, including dosage*: _____
_____
_____

Have you recently (in the previous 12 months) been hospitalized? Yes / No
If yes please list hospitalizations, including dates of and reasons for hospitalization:_____
_____
_____

Please list any allergies you have to any **food** or **drugs**:_____
_____
_____

Past Medical History

Do you have a history of any of the following? (Please check all that apply).

- ☐ Heart attack
- ☐ Dizziness or fainting spells
- ☐ Pneumonia
- ☐ Bronchitis

- ☐ Epilepsy or seizures
- ☐ Stroke
- ☐ Nervous or emotional problems
- ☐ Anemia
- ☐ Asthma
- ☐ Other lung disease
- ☐ Jaundice or gall bladder problems

Family Medical History

Have you or your blood relatives had any of the following (include grandparents, aunts and uncles, but exclude cousins, relatives by marriage and half-relatives)?

Check those to which the answer is yes (leave others blank).

- ☐ High blood pressure
- ☐ High cholesterol
- ☐ Type 2 diabetes
- ☐ Obesity (BMI > 30kg/m^2)

Smoking

Have you ever smoked cigarettes? Yes / No (*If no, skip to diet section*)

If you did or now smoke cigarettes, how many per day? _____ Age started _____

If you have stopped smoking, when was it? _____

If you now smoke, how long ago did you start? _____

Diet

How many meals do you usually eat each day? _____

Do you ever drink alcoholic beverages?

☐ Yes ☐ No

If yes, what is your approximate intake of these beverages?

Beer:

☐ None ☐ Occasional ☐ Often If often, _____ per week

Wine:

☐ None ☐ Occasional ☐ Often If often, _____ per week

Spirits:

☐ None ☐ Occasional ☐ Often If often, _____ per week

At any time in the past, were you a heavy drinker (consumption of six ounces of spirits per day or more)?

☐ Yes ☐ No

Comments: _____

_____

_____

Do you usually use oil or margarine in place of high cholesterol shortening or butter?

☐ Yes ☐ No

Do you usually abstain from extra sugar usage (Above 10 tea spoons of table sugar)?

☐ Yes ☐ No

Do you usually add salt at the table?

☐ Yes ☐ No

Do you eat differently on weekends as compared to weekdays?

☐ Yes ☐ No

Appendix 11: Medical history and screening form – COP-D study

MEDICAL HISTORY AND SCREENING FORM

General Information

Participant:

Name _____

Address _____

Contact phone numbers _____

Birth date _____

Sex:

☐ Male ☐ Female

Education:

☐ Grade School ☐ Jr. High School ☐ High School

☐ College (2-4 years) ☐ Graduate School ☐ Degree _____

Present Medical History

Check those questions to which you answer yes (leave the others blank).

- ☐ Has a doctor ever said your blood sugar was high?
- ☐ Has a doctor ever told your HbA1c levels was high?
- ☐ Has a doctor ever told you your cholesterol level was high?
- ☐ Duration of pre-diabetes?

Comments: _____

_____

_____

_____

Do you now have or have you recently experienced:

- ☐ Chronic or recurrent cough?
- ☐ Increased anxiety or depression?
- ☐ Problems with recurrent fatigue, thirst, trouble sleeping or increased irritability?
- ☐ Migraine or recurrent headaches?
- ☐ Stomach or intestinal problems, such as recurrent heartburn, ulcers, constipation or diarrhea?
- ☐ Significant unexplained weight loss?
- ☐ lung and respiratory diseases
- ☐ Severe neurological diseases or seizures
- ☐ Under investigation drug three months prior to this trial?

Women only answer the following. Do you have?

- ☐ Menstrual period problems
- ☐ Pregnancy

Are you on any type of hormone replacement therapy? _____

Men and women answer the following:

List any prescription medications you are now taking: _____

_____

_____

List any self-prescribed medications, dietary supplements, or vitamins you are now taking: _____

_____

_____

List hospitalizations, including dates of and reasons for hospitalization:_____
_____
_____

List any drug allergies: _____
_____
_____

Past Medical History

Check those questions to which your answer is yes (leave others blank).

- ☐ Heart attack
- ☐ Diabetes or abnormal blood-sugar tests
- ☐ Dizziness or fainting spells
- ☐ Epilepsy or seizures
- ☐ Stroke
- ☐ Nervous or emotional problems
- ☐ Anemia
- ☐ Pneumonia
- ☐ Bronchitis
- ☐ Asthma
- ☐ Other lung disease
- ☐ Jaundice or gall bladder problems

Familial Diseases

Have you or your blood relatives had any of the following (include grandparents, aunts and uncles, but exclude cousins, relatives by marriage and half-relatives)?

Check those to which the answer is yes (leave other blank).

- ☐ High blood pressure
- ☐ Elevated cholesterol
- ☐ Diabetes
- ☐ Obesity (20 or more pounds overweight)

Smoking

Have you ever smoked cigarettes?

☐ Yes ☐ No

(If no, skip to diet section)

If you did or now smoke cigarettes, how many per day? _____ Age started _____

If you have stopped smoking, when was it? _____

If you now smoke, how long ago did you start? _____

Diet

Number of meals you usually eat per day: _____

Do you ever drink alcoholic beverages?

☐ Yes ☐ No

If yes, what is your approximate intake of these beverages?

Beer:

☐ None ☐ Occasional ☐ Often

If occasional or often, _____ per week

Wine:

☐ None ☐ Occasional ☐ Often

If occasional or often, _____ per week

Spirits:

☐ None ☐ Occasional ☐ Often

If occasional or often, _____ per week

At any time in the past, were you a heavy drinker (consumption of six ounces of spirits per day or more)?

☐ Yes ☐ No

Comments: _____

_____

_____

Do you usually use oil or margarine in place of high cholesterol shortening or butter?

☐ Yes ☐ No

Do you usually abstain from extra sugar usage (Above 10 tea spoons of table sugar)?

☐ Yes ☐ No

Do you usually add salt at the table?

☐ Yes ☐ No

Do you eat differently on weekends as compared to weekdays?

☐ Yes ☐ No

Appendix 12: Medical questionnaire – CALFOR-CVD study

C*URCUMIN* ***A****ND* ***L****ONG CHAIN OMEGA-3 FATTY ACIDS* ***FOR*** *MANAGEMENT OF* ***C****ARDIO****V****ASCULAR HEALTH IN INDIVIDUALS WITH TYPE 2* ***D****IABETES* ***(CALFOR-CVD TRIAL)***

MEDICAL QUESTIONNAIRE ***V1.2; 31 AUG 2016***

General Information

Participant:

Name _____

Address _____

Contact phone numbers _____

Birth date _____

Sex:

☐ Male ☐ Female

Education:

☐ Jr. High School ☐ High School

☐ Diploma ☐ Bachelor Degree or Bachelor Honors degree

☐ Master's degree ☐ Doctoral degree

Present Medical History

Check those questions to which you answer yes (leave the others blank).

- ☐ Diagnosed with type 2 diabetes?
- ☐ Has a doctor ever told your blood lipid levels were high?
- ☐ Duration of Type 2 diabetes _____

Do you now have or were you recently diagnosed with any following conditions?

- ☐ Type 1 diabetes
- ☐ Started on insulin treatment
- ☐ Cancer
- ☐ Painful peripheral neuropathy
- ☐ Pace maker implants
- ☐ Parkinson's (or) multiple sclerosis, epilepsy

Women only answer the following. Do you have?

- ☐ Menstrual period problems
- ☐ Pregnancy

Are you on any type of hormone replacement therapy? _____

Men and women answer the following:

List any prescription medications you are now taking: _____

_____

_____

List any self-prescribed medications, dietary supplements, or vitamins you are now taking:___

_____

_____

List hospitalizations, including dates of and reasons for hospitalization: _____

_____

_____

List any drug allergies:_____

_____

_____

Familial Diseases

Have you or your blood relatives had any of the following (include grandparents, aunts and uncles, but exclude cousins, relatives by marriage and half-relatives)?

Check those to which the answer is yes (leave other blank).

- ☐ High blood pressure
- ☐ Elevated cholesterol
- ☐ Diabetes
- ☐ Obesity (10 or more kilograms overweight)

Smoking

Have you ever smoked cigarettes?

☐ Yes ☐ No

(If no, skip to diet section)

If you did or now smoke cigarettes, how many per day? _____ Age started _____

If you have stopped smoking, when did you stop? _____

If you now smoke, how long ago did you start? _____

Diet

Number of meals you usually eat per day: _____

Do you ever drink alcoholic beverages?

☐ Yes ☐ No

If yes, what is your approximate intake of these beverages?

Beer:

☐ None ☐ Occasional ☐ Often

If occasional or often, _____ per week

Wine:

☐ None ☐ Occasional ☐ Often

If occasional or often, _____ per week

Spirits:

☐ None ☐ Occasional ☐ Often

If occasional or often, _____ per week

At any time in the past, were you a heavy drinker (consumption of six ounces of spirits per day or more)?

☐ Yes ☐ No

Comments: _____

_____

_____

Appendix 13: 24 hour food recall – Acute postprandial study

Instructions for recording food and drink consumption

"Version 1.0"

You are asked to recall everything you have eaten and drunk for a period of 24 hours. The information you record is very important to the success of this study. These instructions will help you to fill in your food and drink consumption in the most accurate manner.

Instructions:

- **Write down everything you ate and drank (including water) for the past 24 hours.**
- **Use as many pages as you need (pages are provided):**
- List the food and give a description, including method of cooking (e.g. grilled rump steak or fried rump steak). An example of how to record the information is given below.
- State the weight of the food or use standard household measure, such as a cup, a tablespoon, a teaspoon.
- Specify brand names of products for processed foods (e.g. Nestle diet yoghurt)
- Include supplements, and any condiments such as sauce or salad dressing

Example:

Time/Meal (Breakfast/Lunch/Dinner)	**Food/Drink + description**	**Quantity**
7 am (Breakfast)	Kellogg's corn flakes	1 cup
	Dairy Farmer's lite milk	100 mL
	Boiled egg	2 medium
	Black coffee – Nescafe instant	1 cup
12 noon (Lunch)	Salad sandwich – wholegrain bread	2 slices
	Baby spinach leaves	1 cup
	Tomato slices	3 slices
	Edgell's tinned beetroot	30 grams
	Grated carrot	1 tablespoon
	Red delicious apple	1 large
	Nestle tropical fruit diet yoghurt	125 mL
	Water	500 mL
3pm (Dinner)	Diet coke	500 mL

Participant ID

Time/Meal (Breakfast/Lunch/Dinn	Food/Drink + description	Quantity

Food diary

Appendix 14: 3-day food record – COP-D and CALFOR-CVD trial

Instructions for recording food and drink consumption

You are being asked to record everything you eat and drink for a period of 3 days. Choose consecutive days made up of 2 week days and 1 weekend day. The information you record is very important to the success of this study. These instructions will help you to fill in your food and drink consumption in the most accurate manner.

If you have any questions, please do not hesitate to contact Rohith Thota on 4921 5638.

Instructions:

- **Write down everything that you eat and drink (including water) for n=3 consecutive days (2 week days and one weekend day).**
- **Complete the record in the time frame you are given by Rohith Thota**
- **Start a new page each day (pages are provided):**
  - List the food and give a description, including method of cooking (e.g. grilled rump steak or fried rump steak). An example of how to record the information is given below.
  - State the weight of the food or use standard household measure, such as a cup, a tablespoon, a teaspoon.
  - Specify brand names of products for processed foods (e.g. Nestle diet yoghurt)
  - Include supplements, and any condiments such as sauce or salad dressing
- **Try to record each item when you eat or drink so that nothing is forgotten.**

Example:

Time/Meal (Breakfast/Lunch/D	Food/Drink + description	Quantity
7 am (Breakfast)	Kellogg's corn flakes	1 cup
	Dairy Farmer's lite milk	100 mL
	Boiled egg	2 medium
	Black coffee – Nescafe instant	1 cup
12 noon (Lunch)	Salad sandwich – wholegrain bread	2 slices
	Baby spinach leaves	1 cup
	Tomato slices	3 slices
	Edgell's tinned beetroot	30 grams
	Grated carrot	1
	Red delicious apple	1 large
	Nestle tropical fruit diet yoghurt	125 mL
	Water	500 mL
3pm (Dinner)	Diet coke	500 mL

Food diary

Participant code___ **Day 1 _____**

Time/Meal (Breakfast/Lunch/Din	Food/Drink + description	Quantity

Researchers: Professor Manohar Garg, Dr. Sham Acharya & Mr. Rohith Thota

Participant code: _____ **Day 2 :** _____

Time/Meal (Breakfast/Lunch/Dinn	Food/Drink + description	Quantity

Participant code: _____ Day 3: _____

Time/Meal (Breakfast/Lunch/Din	Food/Drink + description	Quantity

Appendix 15: International Physical Activity Questionnaire – Short form

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (August 2002)

SHORT LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is supported to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective*. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

Appendix 16: International Physical Activity Questionnaire – Long form

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → ***Skip to question 6***

Appendix 17: The Australian Type 2 Diabetes Risk Assessment Tool

The Australian Type 2 Diabetes Risk Assessment Tool (AUSDRISK)

1. Your age group

Under 35 years	☐	0 points
35 – 44 years	☐	2 points
45 – 54 years	☐	4 points
55 – 64 years	☐	6 points
65 years or over	☐	8 points

2. Your gender

Female	☐	0 points
Male	☐	3 points

3. Your ethnicity/country of birth:

3a. Are you of Aboriginal, Torres Strait Islander, **Pacific Islander or Maori descent?**

No	☐	0 points
Yes	☐	2 points

3b. Where were you born?

Australia	☐	0 points
Asia (including the Indian sub-continent), Middle East, North Africa, Southern Europe	☐	2 points
Other	☐	0 points

4. Have either of your parents, or any of your brothers or sisters been diagnosed with diabetes (type 1 or type 2)?

No	☐	0 points
Yes	☐	3 points

5. Have you ever been found to have high blood glucose (sugar) (for example, in a health examination, during an illness, during pregnancy)?

No	☐	0 points
Yes	☐	6 points

6. Are you currently taking medication for high blood pressure?

No	☐	0 points
Yes	☐	2 points

7. Do you currently smoke cigarettes or any other tobacco products on a daily basis?

No	☐	0 points
Yes	☐	2 points

If you scored 6-11 points in the AUSDRISK you may be at increased risk of type 2 diabetes. Discuss your score and your individual risk with your doctor. Improving your lifestyle may help reduce your risk of developing type 2 diabetes.

8. How often do you eat vegetables or fruit?

Every day	☐	0 points
Not every day	☐	1 point

9. On average, would you say you do at least 2.5 hours of physical activity per week (for example, 30 minutes a day on 5 or more days a week)?

Yes	☐	0 points
No	☐	2 points

10. Your waist measurement taken below the ribs (usually at the level of the navel, and while standing)

Waist measurement (cm) ☐

For those of Asian or Aboriginal or Torres Strait Islander descent:

Men	Women		
Less than 90 cm	Less than 80 cm	☐	0 points
90 – 100 cm	80 – 90 cm	☐	4 points
More than 100 cm	More than 90 cm	☐	7 points

For all others:

Men	Women		
Less than 102 cm	Less than 88 cm	☐	0 points
102 – 110 cm	88 – 100 cm	☐	4 points
More than 110 cm	More than 100 cm	☐	7 points

Add up your points ☐

Your risk of developing type 2 diabetes within 5 years*:

☐ *5 or less: Low risk*
Approximately one person in every 100 will develop diabetes.

☐ *6-11: Intermediate risk*
For scores of 6-8, approximately one person in every 50 will develop diabetes. For scores of 9-11, approximately one person in every 30 will develop diabetes.

☐ *12 or more: High risk*
For scores of 12-15, approximately one person in every 14 will develop diabetes. For scores of 16-19, approximately one person in every 7 will develop diabetes. For scores of 20 and above, approximately one person in every 3 will develop diabetes.

**The overall score may overestimate the risk of diabetes in those aged less than 25 years.*

If you scored 12 points or more in the AUSDRISK you may have undiagnosed type 2 diabetes or be at high risk of developing the disease. See your doctor about having a fasting blood glucose test. Act now to prevent type 2 diabetes.

Appendix 18: SPIRIT checklist for trial protocol

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	____1__ _____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	___1 & 12 _____
	2b	All items from the World Health Organization Trial Registration Data Set	___na to trial___
Protocol version	3	Date and version identifier	__na to paper_
Funding	4	Sources and types of financial, material, and other support	_ na to paper _____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	____1& 20____
	5b	Name and contact information for the trial sponsor	_____na _____
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	_ na to trial _____

	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	___na to trial__
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	__4-7, 14-17_____
	6b	Explanation for choice of comparators	__14-17 _____
Objectives	7	Specific objectives or hypotheses	_____7 _____
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	_____7 _____
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	_____7, 9_____
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	____8__ _
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	_____10 _____

	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	_____11_____
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	_____10, 12___
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	____ 8 __
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	_____9, 12 _____
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure 1)	_____9-12_____
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	___8 ___
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	____9__ __

Methods: Assignment of interventions (for controlled trials)

Allocation:

Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	_____10_____

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	_____10_____
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	_____10_____
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	____7_____
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	___11 _

Methods: Data collection, management, and analysis

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	__12_
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	__na to paper _
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	__na to paper_
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	___13___
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	____13___

	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	__na to paper_

Methods: Monitoring

Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	___na to paper___
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	___na to paper _
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	__na to paper__
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	__na to paper__

Ethics and dissemination

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____12_____
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	___na to paper_
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	___9___ _
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	___na to trial_

Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	__na to paper_
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	__20 _
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	__na to trial___
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	__na to trial__
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	___na to paper_
	31b	Authorship eligibility guidelines and any intended use of professional writers	___na to paper_
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	___na to paper_
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	__na to paper_
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	___na to paper___ __

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

Appendix 19: Statement of contribution and collaboration for chapter 3

Statement of contribution and collaboration for chapter 3

I attest that Research Higher Degree candidate Rohith N Thota contributed to the following publication:

Thota RN, Abbott KA, Ferguson JJA, Veysey M, Lucock M, Niblett S, King K, Garg ML. InsuTAG: A novel physiologically relevant predictor for insulin resistance and metabolic syndromes. Scientific Reports. 2017; 7 (1):15204.

Rohith N Thota contributed to the design of the study, including concept, data analysis and wrote the manuscript. Professor Manohar Garg contributed to the study design and manuscript within his capacity as a primary supervisor. Ms Kylie Abbott was involved with study data analysis and writing of the manuscript. Mrs Jessica Ferguson contributed in editing the manuscript. Professor Martin Veysey, Associate Professor Prof Mark Lucock, Dr Katrina King and Dr Suzanne Niblett provided the required data from Retirement Health and Life Style study and was involved in revising the final manuscript.

Mr Rohith N Thota — Date: 14/03/2018

Ms Kylie Abbott — Date: 15/03/2018

Mrs Jessica Ferguson — Date: 15/03/2018

Professor Martin Veysey — Date: 21/3/18

Associate Professor Mark Lucock — Date: 21st March 2018

Dr Suzanne Niblett — Date: 20th March 2018

Dr Katrina King — Date: 16/03/2018

Professor Robert Callister — Date: 26/3/18
Deputy Head of Faculty of Health and Medicine (Research and Research Training)

Appendix 20: Statement of contribution and collaboration for chapter 4

Statement of contribution and collaboration for chapter 4

I attest that Research Higher Degree candidate Rohith N Thota contributed to the following publication

Thota RN, Dias CB, Abbott KA, Acharya SH, Garg ML. Curcumin alleviates postprandial glycaemic response in healthy subjects: A cross-over, randomized controlled study. 2018; Submitted to Nutrition, Metabolism & Cardiovascular Diseases.

Rohith N Thota contributed to design of the study, including concept, recruitment of participants, conducted study visits, data analysis and wrote the manuscript. Professor Manohar Garg contributed to the study design and manuscript within his capacity as a primary supervisor. Ms Kylie Abbott and Dr Cintia Dias involved with study data analysis and reviewing of the manuscript. Dr Sham Acharya contributed to the study design and manuscript within his capacity as a co-supervisor.

Mr Rohith N Thota — Date: 14/03/2018

Ms Kylie Abbott — Date: 15/03/2018

Dr Cintia Dias — Date: 19/03/2018

Dr Shamasunder Acharya — Date: 23/3/2018

Professor Manohar Garg — Date: March 15, 2018

Professor Robert Callister — Date: 26/3/18

Deputy Head of Faculty of Health and Medicine (Research and Research Training)

Appendix 21: Statement of contribution and collaboration for chapter 5

Statement of contribution and collaboration for chapter 5

I attest that Research Higher Degree candidate Rohith N Thota contributed to the following publication

Thota RN, Acharya SH, Abbott KA, Garg ML. Curcumin and long-chain Omega-3 polyunsaturated fatty acids for Prevention of type 2 Diabetes (COP-D): study protocol for a randomised controlled trial. Trials. 2016; 17:565.

Rohith N Thota contributed to the literature review and study protocol and wrote the manuscript. Professor Manohar Garg contributed to the study design and manuscript within his capacity as a primary supervisor. Ms Kylie Abbott involved with reviewing of the manuscript. Dr Sham Acharya contributed to the study design and manuscript within his capacity as a co-supervisor.

Mr Rohith N Thota Date: 14/03/2018

Ms Kylie Abbott Date: 15/03/2018

Dr Shamasunder Acharya Date: 23/3/2018

Professor Manohar Garg **Date:** March 15, 2018

Professor Robert Callister Date: 26/3/18
Deputy Head of Faculty of Health and Medicine (Research and Research Training)

Appendix 22: Statement of contribution and collaboration for chapter 6

Statement of contribution and collaboration for chapter 6

I attest that Research Higher Degree candidate Rohith N Thota contributed to the following publication

Thota RN, Acharya SH, Garg ML. Effects of curcumin and/or omega-3 polyunsaturated fatty acids on glycaemic control and blood lipids in individuals with high risk of type 2 diabetes: A randomised controlled trial. 2018; submited to Metabolism – Clinical and Experimental.

Rohith N Thota contributed to the design of the study including concept, recruitment of participants, conducted study visits, data collection and analysis, and wrote the manuscript. Professor Manohar Garg contributed to the study design and manuscript within his capacity as a primary supervisor. Dr Sham Acharya contributed to the study design and manuscript within his capacity as a co-supervisor.

Date: 14/03/2018

Mr Rohith N Thota

Dr Shankasunder Acharya

Date: 25/3/2018

Professor Manohar Garg

Date: March 15, 2018

Professor Robert Callister

Date: 26/3/18

Deputy Head of Faculty of Health and Medicine (Research and Research Training)