DIETARY MANAGEMENT OF POLYCYSTIC OVARY SYNDROME

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DECLARATION

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 consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

I acknowledge that copyright of published works contained within this thesis (as listed below) resides with the copyright holders/s of these works.

Moran LJ, Noakes M, Clifton PM, Wittert GA, Williams G, Norman RJ. 2006, Short term meal replacements followed by dietary macronutrient restriction enhance weight loss in Polycystic Ovary Syndrome. The American Journal of Clinical Nutrition; 84(1):77–87.

Moran LJ, Noakes M, Clifton PM, Wittert G, Tomlinson L, Galletly C, Luscombe N, Tomlinson L, Norman RJ. 2004, Ghrelin and measures of satiety are altered in polycystic ovary syndrome but not differentially affected by diet composition. Journal of Clinical Endocrinology and Metabolism, 89(7):3337–44.

Moran LJ, Noakes M, Clifton P, Wittert GA, Norman RJ. 2007, Weight loss, CRP and adiponectin in overweight women with and without Polycystic Ovary Syndrome. The Journal of Clinical Endocrinology and Metabolism, in press.

SIGNED.. DATED…………………

DESCRIPTION OF THESIS

Chapters 2–6 were submitted for publication prior to the completion of this thesis. Chapters 2 and 4 have been accepted and published, Chapters 3 has been accepted and is in press and Chapter 5 is currently under review. For this reason, this thesis was prepared in a similar style to a Thesis by Publication. The bulk of the study methodology is included within the relevant chapters conforming to the style of the relevant journal to which the chapters were submitted. Additional methodological information is provided in Appendix 2. Where new information pertinent to the topic of the chapter has been published after the relevant paper, it is discussed in the final conclusion as opposed to the Chapter/Paper discussion being amended. Paper co-authors are acknowledged in the Acknowledgement Section and Appendix 3 contains the published papers.

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ABSTRACT

Background

Polycystic ovary syndrome (PCOS) is a common endocrine condition in women associated with obesity, reproductive and metabolic abnormalities. It improves with weight loss, however currently no specific dietary recommendations exist and there may be abnormalities in appetite regulation in PCOS that contribute to difficulty in weight management.

Aims

To assess the effect of 1) short and long-term weight loss and weight maintenance strategies on weight loss, reproductive and metabolic parameters in overweight women with PCOS and to 2) assess the relative effect of weight loss on cardiovascular risk factors and 3) post-prandial appetite, appetite hormones (ghrelin, CCK, PYY) and food intake in overweight women with and without PCOS.

Results

Overweight women with PCOS followed an 8-week weight loss (2 meal replacements/day, 4904.4±127 kJ, n=32) followed by a 6 month carbohydrate (<120 g/day) or fat restricted (<50 g/day) weight maintenance regime (n=23). Reductions in weight (5.6±2.4 kg) and improvements in body composition, insulin, reproductive hormones and menstrual cyclicity occurred and were sustained equivalently for both diet groups. We then assessed the effect of weight loss (4.2±0.7 kg over 8 weeks as described above) in overweight women with (n=15) and without (n=17) PCOS on cardiovascular risk factors. All subjects had similar improvements in body composition, triglycerides, reproductive hormones and fasting and post-prandial insulin. C-reactive protein decreased with weight loss for non-PCOS women (-1.2±0.5 mg/L, P=0.025) but not for PCOS women.

We finally assessed appetite regulation in PCOS. Women with (n=20) and without (n=12) PCOS followed a standard protein (55% carbohydrate, 15% protein) or high protein diet (40% carbohydrate, 30% protein) for 16 weeks (~6000 kJ/day). Non-PCOS subjects were more satiated (P=0.001) and less hungry (P=0.007) after the test meals and had a 70% higher fasting baseline ghrelin (P=0.011), a greater increase in fasting ghrelin (57.5 versus 34.0%, P=0.033), a greater post-prandial ghrelin decrease at week 16 (113.5±46.3 versus 49.3±12.2 pg/mL, P=0.05) and a greater maximal decrease in post-prandial ghrelin (-144.1±58.4 versus -28.9±14.2 pg/mL, P=0.02) following weight loss than subjects with PCOS. Lastly, women with (n=14) and without (n=14) PCOS undertook an 8-week weight loss regime (4.2±0.7 kg as described above). At week 0 and 8, women with PCOS again displayed lower ghrelin levels (P=0.01 and P=0.097 respectively) and a lesser post-prandial ghrelin decrease (P=0.048 and P=0.069 respectively) but similar post-prandial appetite, buffet consumption and fasting or post-prandial peptide YY and cholecystokinin compared to women without PCOS.

Conclusion

Meal replacements and moderate macronutrient restriction are effective strategies for the dietary management of PCOS. Equivalent weight losses improved cardiovascular risk factors similarly for overweight women with and without PCOS with the exception of CRP which did not decrease with weight loss for overweight women with PCOS. PCOS status is associated with altered fasting and post-prandial ghrelin levels but is not consistently associated with other impairments in post-prandial gut peptides or food intake. Further investigation is required to assess if appetite regulation is impaired in PCOS and the optimal strategies and amount of weight loss for improvement of reproductive and metabolic parameters in PCOS.

PUBLICATIONS ARISING FROM THIS THESIS

Moran LJ, Noakes M, Brinkworth G, Norman RJ. 2006, Diet, Nutrition and Exercise in Reproduction. Reproductive BioMedicine Online, 12(5):569–578

Moran LJ, Noakes M, Clifton P, Wittert GA, Le Roux C, Ghatei M, Bloom S, Norman RJ. 2006, Post-prandial ghrelin, cholecystokinin, PYY, appetite and food consumption before and after weight loss in overweight women with and without Polycystic Ovary Syndrome. The Journal of Clinical Endocrinology and Metabolism, (Submitted 23rd January 2007).

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PRESENTATIONS ARISING FROM THIS THESIS

Oral presentations

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2005:

Dietetics Association of Australia State Conference, Adelaide, Australia

'Diet and Polycystic Ovary Syndrome', **Moran LJ**.

Poster presentations

2006:

International Congress of Obesity, Sydney, Australia

'Weight loss does not lower CRP in overweight women with Polycystic Ovary Syndrome', **Moran LJ**, Noakes M, Clifton P, Wittert GA, Norman RJ.

2005:

North American Society for the Study of Obesity International Conference,

Vancouver, Canada

Australasian Society for the Study of Obesity National Conference, Adelaide, South Australia

Nutrition Society of Australia National Conference, Melbourne, Victoria

‘Effective weight loss and maintenance strategies in polycystic ovary syndrome’, **Moran LJ,** Noakes M, Clifton PM, Wittert GA, Williams G, Norman RJ.

2004:

Nutrition Society of Australia National Conference, Brisbane, Queensland

Australasian Society for the Study of Obesity, Brisbane, Queensland

‘Short term meal replacements followed by dietary macronutrient restriction enhance weight loss in Polycystic Ovary Syndrome’, **Moran LJ,** Noakes M, Clifton PM, Wittert GA, Williams G, Norman RJ.

2003:

Endocrine Society of Australia National Conference, Melbourne, Victoria

Australian Medical Research Council National Conference, Adelaide, South Australia

Nutrition Society of Australia National Conference, Hobart, Tasmania

Dietetics Association of Australia State Conference, Adelaide, South Australia

‘Ghrelin and measures of satiety are altered in polycystic ovary syndrome but not differentially affected by diet composition’, **Moran LJ**, Noakes M, Clifton PM, Wittert G, Tomlinson L, Galletly C, Luscombe N, Tomlinson L, Norman RJ.

ABBREVIATIONS

α-MSH: α-melanocyte stimulating hormone

ADP: Adenosine diphosphate

AgRP: Agouti-related peptide

AI: Adequate intake

AMH: Anti-mullerian hormone

Apo A-IV: Apolipoprotein A-IV

ATP: Adenosine triphosphate

AUC: Area under the curve

BIA: Bioelectrical impedance analysis

BMI: Body mass index

BNRP: Bombesin/bombesin related peptides

CART: Cocaine and amphetamine regulated transcript

CC: Carbohydrate counting

CCK: Cholecystokinin

CHO: Carbohydrate

CIGMA: Continuous infusion of glucose with model assessment

CRF: Corticotropin-releasing factor

CRP: C-reactive protein

CV: Coefficient of variation

CVD: Cardiovascular disease

DBP: Diastolic blood pressure

DEXA: Dual X-ray absorptiometry

DHEA: Dehydroepiandrosterone

DHEAS: Dehydroepiandrosteronesulfate

5α-DHT: 5α- Dihydrotestosterone

DHT: Dihydrotestosterone

ER: Energy restriction

FAI: Free androgen index

FC: Fat counting

FFA: Free fatty acid

FSH: Follicle-stimulating hormone

FSIVGTT: Frequently sampled intravenous glucose tolerance test

hCG: Human chorionic gonadotrophin

GH: Growth hormone

GHRH: Growth hormone releasing hormone

GHS-R: Growth hormone secretagogue receptor

GI: Glycaemic index

GL: Glycaemic load

GLP-1: Glucagon-like peptide 1

Glucose-6-P: Glucose-6 phosphate

GLUT4: Glucose transporter 4

GnRH: Gonadotrophin releasing hormone

HA: Hyperandrogenism

HDL-C: High density lipoprotein cholesterol

HOMA: Homeostasis model assessment

HP: High protein

3βHSD: 3β-hydroxysteroid dehydrogenase

17βHSD: 17β-hydroxysteroid dehydrogenase

20αHSD: 20α-hydroxysteroid dehydrogenase

HSD: Hydroxysteroid dehydrogenase

IGF: Insulin-like growth factor

IGFBP: Insulin-like growth factor binding proteins

IGT: Impaired glucose tolerance

IL: Interleukin

IR: Insulin resistance

IRS: Insulin receptor substrate

IST: Insulin sensitivity test

ITT: Insulin tolerance test

IVF: In vitro fertilization

LDL-C: Low-density lipoprotein cholesterol

LH: Luteinising hormone

LP: Low protein

MAPK: Mitogen activated protein kinase

MCH: Melanin-concentrating hormone

MTT: Meal tolerance test

MUFA: Monounsaturated fatty acid

NIH: National Institute of Health

NPY: Neuropeptide Y

OGTT: Oral glucose tolerance test

OXM: Oxyntomodulin

P450AR: Cytochrome P450 aromatase

P450cscc: Cytochrome P450 side chain cleavage

P450c11AS: Cytochrome P45011 aldosterone synthetase

P450c11B: Cytochrome P450 11-hydroxylase

P450c17α: Cytochrome P450 17 α hydroxylase

P450c17,20: Cytochrome P450 17,20 lyase

P450c21: Cytochrome P450 21-hydroxylase

PAI-1: Plasminogen-activator inhibitor activity

PCO: Polycystic Ovary Syndrome

PCOS: Polycystic Ovary Syndrome

PI3-K: Phosphatidlyinositol 3-kinase

POMC: Pro-opiomelanocortin

PP: Pancreatic polypeptide

PPAR: Peroxisome proliferator activator receptor

PUFA: Polyunsaturated fatty acid

PVN: Paraventricular nucleus

PYY: Peptide YY

QUICKI: Quantitative insulin sensitivity check index

RDI: Recommended dietary intake

REE: Resting energy expenditure

RR: Relative risk

RQ: Respiratory quotient

SFA: Saturated fatty acid

SHBG: Sex-hormone binding globulin

SP: Standard protein

StAR: Steroidogenic acute regulatory protein.

T2DM: Type II diabetes mellitus

TFM: Total fat mass

TFFM: Total fat free mass

TNF-α: Tumour necrosis factor α

TSH: Thyroid stimulating hormone

VAS: Visual analogue scores

VLCD: Very low calorie diets

VLDL: Very low density lipoprotein

VO_{2max} : Maximal oxygen consumption

WHR: Waist-hip ratio

WM: Weight maintenance

CHAPTER 1: LITERATURE REVIEW

1.1: INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common clinical condition in women of reproductive age associated with reproductive and metabolic dysfunction. Treatment has traditionally focused on fertility and hormonal therapy. However, obesity, central obesity and insulin resistance (IR) are strongly implicated in its aetiology and improving these factors has proved successful, sparing the need for costly assisted reproduction. Lifestyle modification programs with an emphasis on behavioural management and dietary and exercise interventions have been successful in the general population in reducing cardiovascular risk factors and the risk of Type 2 Diabetes Mellitus (T2DM) and the metabolic syndrome and have had some initial success in improving fertility outcomes in PCOS. However, there are no dietary recommendations for PCOS and it is not known if standard dietary management of obesity and metabolic dysfunction is appropriate. Women with PCOS may represent a population with unique barriers to weight loss and maintenance including abnormalities in appetite regulation. However, the impact of these on weight loss and weight maintenance is currently unknown. Furthermore, effective means of maintaining weight loss and reproductive and metabolic improvements are unknown in both PCOS and the general population. This literature review will summarise current knowledge on appetite regulation in PCOS and the rationale for different weight loss and weight maintenance strategies in PCOS. Sections 1.2 and 1.3 will provide an overview of obesity and IR. Section 1.4 will discuss the presentation and aetiology of PCOS and section 1.5 and 1.6 will discuss the pathophysiology of PCOS, particularly with regards to the influence of hyperinsulinaemia and IR. Section 1.7 will discuss dietary management of overweight and obese women with PCOS and section 1.8 will discuss potential barriers to the implementation of dietary management of obesity in PCOS. Section 1.9 will summarise my thesis rationale, hypothesis and aims.

1.2: OVERWEIGHT AND OBESITY

1.2.1: Overview of overweight and obesity

Obesity is a serious and prevalent condition constituting a significant economic burden in Western countries (1) and it is considered a global epidemic by the World Health Organisation (2). It is defined as a condition where the amount of adipose tissue is increased to a point where it can adversely affect health (3). Body mass index (BMI, weight in kg per height in m^2) is a useful clinical tool that correlates reasonably well with adiposity although it does not distinguish between lean and fat mass unlike more precise measures such as skinfold thickness, underwater weighing, dual energy X-ray absorptiometry, magnetic resonance imaging and infrared spectroscopy). In Australia 56% of the adult population are overweight or obese (BMI $\geq$ 25 kgm^{-2}), 18% are obese (BMI $\geq$ 30 kgm^{-2}) and 30% of females are overweight or obese and 22% of females are obese (4). Additionally, the proportion of adults who are obese is increasing and has doubled in the past 20 years in Australia and the majority of other Western countries (4). As more developing countries adopt Western lifestyles and diets, this trend is likely to spread. This rising prevalence is an important health issue due to the clear association of obesity and abdominal obesity with an increased risk of IR, impaired psychosocial health, T2DM, cardiovascular disease (CVD), osteoarthritis, sleep apnoea and breast and uterine cancer (2).

The location of adipose tissue (peripheral or abdominal) also adversely affects health. The abdominal adipose tissue consists of visceral (the mesenteric and the greater and lesser omental depots contained within the body cavity surrounding the internal organs) and subcutaneous tissue (under the skin). Abdominal visceral fat correlates more strongly with IR and metabolic and reproductive fitness than subcutaneous fat (5-8) although the subcutaneous depot is likely to also contribute to metabolic abnormalities (9). Waist-hip ratios (WHR) or waist circumferences (measured midway between the lowest rib and the iliac crest) provide a reasonable estimate of abdominal fat. More sensitive imaging tools such as computed

tomography or magnetic resonance imaging also distinguish between visceral and subcutaneous fat, however these are not practical in a clinical setting. In women, a WHR >0.8 or a waist circumference ≥80 cm indicates increased risk of obesity-associated metabolic complications and ≥88 cm indicates substantially increased risk (1).

1.3: INSULIN RESISTANCE AND HYPERINSULINAEMIA

1.3.1: Insulin resistance and hyperinsulinaemia

1.3.1.1: Insulin resistance

IR is defined as the reduced ability of insulin to exert its physiological effect at normal concentrations and is manifested peripherally at the tissues or centrally at the liver. This is observed through impaired insulin-stimulated glucose uptake at the muscle, adipose tissue and liver; hepatic glucose overproduction and release; increased lipolysis at the adipose tissue and consequent increased circulating free fatty acids (FFA); reduced lipogenesis from circulating triglycerides and impairment of glycogen synthesis (10-13). Additional actions of insulin may also be impaired including protein synthesis, vasodilation and stimulation of nitric oxide in the vascular endothelium), vascular cell growth, activation of the sympathetic nervous system, promoting cell differentiation (preadipocytes to adipocytes) and metabolic and mitogenic gene expression (10-12). As a consequence of tissue IR, pancreatic β-cell secretion and circulating levels of insulin are increased to provide sufficient insulin to elicit action on glucose homeostasis. This produces the compensatory hyperinsulinaemia commonly observed in people with IR. The presence of IR and hyperinsulinaemia increases the likelihood of a variety of abnormalities including glucose intolerance, dyslipidaemia, endothelial dysfunction, dysregulated coagulation and inflammation, hemodynamic dysfunction, abnormal uric acid metabolism and disordered breathing during sleep (Table 1.1). These increase the risk of developing a range of clinical syndromes including T2DM, CVD, the

metabolic syndrome, essential hypertension, non-alcoholic fatty liver disease, some types of cancer, sleep apnoea and PCOS (discussed in section 1.5.3) (10-14).

Table 1.1: Abnormalities associated with insulin resistance and hyperinsulinaemia

NOTE: This table is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.

Adapted from (14)

1.3.1.2: Mechanisms of insulin resistance

IR occurs due to abnormalities in any of the downstream facets of the insulin signalling pathway (13) (Figure 1.1) including downregulation of adipose tissue GLUT4 or impaired translocation of skeletal muscle GLUT4 (15), impaired muscle and adipocyte insulin receptor number binding and activity, reductions in expression and action of downstream proteins including IRS-1 and PI3-K and upregulation of enzyme activity involved in suppressing insulin signalling or inhibitory molecules in the insulin signalling pathway including phosphotyrosine phosphatase, protein kinase C substrates and Rad (13, 15-21). In general, the activity of the MAPK pathway appears to be preserved (22), although some members of the MAPK family (including p38 and JNK) that could regulate both the metabolic and mitogenic actions of insulin are dysregulated in T2DM or hyperinsulinaemia (23, 24).

Figure 1.1: Insulin receptor signalling

NOTE: This figure is included on page 6 of the print copy of the thesis held in the University of Adelaide Library.

Insulin binding initiates signal transduction by induced tyrosine autophosphorylation of the insulin receptor. The activated receptor tyrosine phosphorylates intracellular substrates such as insulin receptor substrates (IRS). The metabolic actions of insulin are instigated through activation of phosphatidylinositol 3-kinase (PI3-kinase), translocation of the glucose transporter GLUT4 and glucose uptake at the cell membrane and phosphorylation of other downstream molecules. The mitogenic responses of insulin are initiated following downstream activation of IRS's, the Ras complex and the mitogen activated protein kinase (MAPK) pathway.

Adapted from **herkules.oulu.fi/ isbn9514264266/html/x482.html**

1.3.1.3: Obesity and insulin resistance

Obesity, specifically abdominal obesity and visceral obesity are important contributors to IR (25, 26). A cohort study of pre-menopausal women showed that baseline visceral fat and waist circumference were significant correlates of the development of IR at 7 years (27). Clearance of insulin is additionally reduced with fat localised in the upper body, further contributing to hyperinsulinaemia (28). An increase in adipose tissue mass leads to increased FFA release derived from lipolysis of stored adipocyte triacylglycerol. FFA impair insulin-mediated glucose uptake in skeletal muscle, adipocytes and the liver, decrease hepatic insulin sensitivity and increase hepatic glucose output through preferential oxidation of FFA over glucose (29-31). In visceral fat compared to subcutaneous fat, catecholamine induced lipolysis is increased (32) and insulin-mediated suppression of lipolysis reduced, increasing FFA turnover and release (33, 34) which can increase very low density lipoprotein (VLDL) triglyceride and glucose synthesis (33, 35, 36). Differential expression of adrenergic receptors can also contribute to these differences, with visceral adipocytes expressing elevated lipolytic type 1 and 2 β-adrenergic receptors (37, 38) and reduced levels of anti-lipolytic α2-receptors (39) and reduced inhibition by α2 receptor agonists (40).

Circulating factors elevated in obesity that can influence IR include adipocytes-secreted proteins (adipokines such as tumour necrosis factor-α or TNF-α, interleukin-6 or IL-6, IL-8, resistin, leptin and adiponectin) and inflammatory cytokines (13, 41) (Table 1.2). TNF-α is proposed to play a role in the induction of IR. TNF-α administration to cultured cells decreases GLUT4 expression, increases FFA secretion and mediates IRS-1 serine phosphorylation which inhibits insulin receptor and insulin-like growth factor (IGF) receptor tyrosine kinase activity and stimulates IGFBP production (42, 43). Some investigators report TNF-α is elevated in obesity and abdominal obesity (44). IL-6 and IL-8 secretion is elevated in visceral compared to subcutaneous adipocytes (45, 46) and related to IR in some reports (46, 47). It is controversial whether resistin is associated with obesity and IR (48) and it may

play a greater role in immune and inflammatory processes in humans (49). Leptin is a hormone involved in long-term regulation of energy homeostasis that reduces food intake through hypothalamic action (discussed in section 1.8.3) (50). Leptin is produced in the adipose tissue in proportion to body fat content and in human obesity circulating leptin and adipocyte leptin mRNA levels are commonly increased (51). Conversely, leptin is potentially elevated in subcutaneous compared to visceral adipose tissue (52) and is proposed to have insulin sensitising effects (53) and effects on reducing insulin secretion (54), increasing lipid oxidation and decreasing lipid synthesis (55) in skeletal muscle, the pancreatic β cells and other tissues. An additional proposed insulin sensitiser is adiponectin. Adiponectin administration enhances hepatic insulin action and reduces circulating glucose (56), of which levels negatively correlate with insulin sensitivity and are reduced in obesity (57). The mechanism for this is unclear but it may either interact with TNF-α or the other interleukins or decrease muscle and liver triglyceride content and enhance intracellular fatty acid oxidation (58). Peroxisome proliferator activator receptor (PPAR) γ regulates the expression of genes involved in the differentiation of pre-adipocytes into adipocytes (59). Activation of this pathway by synthetic ligands such as thiazolidinediones stimulates preferential subcutaneous adipocyte differentiation (60). It also enhances insulin sensitivity through increasing insulin-stimulated GLUT4 activity and muscle glycogen synthesis (61) and potentially through redistributing fat into adipocytes and away from hepatocytes and monocytes (62, 63). There is conflicting data on the relative expression and activity of PPARγ in visceral compared to subcutaneous tissue (64, 65). Inflammatory cytokines can induce the suppressors or cytokine signalling proteins (SOCS) which bind to the insulin receptor and block its signalling (66). There are thus a variety of circulating factors that play a potential mechanistic role in inducing IR in a state of obesity and abdominal or visceral obesity.

Table 1.2: Factors secreted from the adipocyte

NOTE: This table is included on page 9 of the print copy of the thesis held in the University of Adelaide Library.

Adapted from (67)

1.3.1.4: Measurement of insulin resistance

The gold standard for measurement of insulin sensitivity is the euglycaemic hyperinsulinaemia clamp which measures the insulin action on glucose concentration under steady state conditions (68). IR has been defined as the lowest decile (69) of insulin sensitivity as assessed by the euglycaemic hyperinsulinaemic clamp in a non-obese, nondiabetic and normotensive population. However, this is intensive and difficult to perform clinically and a range of surrogate measures have been developed including insulin or insulin/glucose responses in oral-glucose tolerance tests (OGTT) (70-72), continuous infusion of glucose with model assessment (CIGMA) (73), insulin sensitivity tests (IST) (74), short insulin tolerance tests (ITT), minimal model assessment during a frequently sampled intravenous glucose tolerance test (FSIVGTT) (75, 76) and hyperglycaemic clamps (68). For clinical use, measures have been developed which are derived from fasting insulin and glucose and reflect basal IR, or more specifically basal hepatic insulin sensitivity and hepatic glucose production (77). These include fasting insulin (78), glucose/insulin ratios (79), the homeostasis model assessment estimate of insulin sensitivity (HOMA) (80) and the

quantitative insulin sensitivity check index (QUICKI) (81). Surrogate measures of basal β-cell function include fasting insulin, HOMAβ and the insulinogenic index (fasting insulin/glucose) (80, 82). For clinical use, the euglycaemic clamp correlates reasonably well with estimates of insulin sensitivity from the OGTT (r=0.7) (71), HOMA (r=0.83), (80), QUICKI (r=0.78) and fasting insulin (r=0.6) (83). However, their use is unclear as considerable variation exists between insulin assays (84) and there is a lack of exact population reference values to allow specific diagnosis or comparison between studies (77).

1.4: PREVALENCE AND DEFINITION OF PCOS

1.4.1: Definition and overview of PCOS

PCOS is associated with a variety of clinical and metabolic presentations (Figure 1.2). These include the primary reproductive and endocrine manifestations of infertility, menstrual dysfunction, hirsutism, acne and hyperandrogenism (HA) and an elevated risk of pregnancy complications including gestational diabetes, pregnancy-induced hypertension, pre-eclampsia and preterm birth (85). There is an increase in the prevalence of obesity and abdominal obesity (discussed in section 1.2) and IR and hyperinsulinaemia (discussed in section 1.3) in PCOS. Furthermore, PCOS is associated with an adverse cardiovascular risk profile. There is an increase in traditional and novel risk factors for CVD, including dyslipidaemia (decreased plasma high-density lipoprotein cholesterol (HDL-C), increased plasma triglycerides and increased small dense low-density lipoprotein cholesterol (LDL-C)), impaired fibrinolysis, increased inflammation, endothelial dysfunction and hypertension and an elevated prevalence of subclinical and clinical atherosclerosis (86-89). While the prevalence of the metabolic syndrome, impaired glucose tolerance (IGT) and T2DM are elevated in PCOS (90, 91), it is as yet unclear if long-term cardiovascular morbidity and mortality are increased (92, 93). PCOS may also be associated with an elevated prevalence of obstructive sleep apnoea (94-96) and endometrial cancer (97, 98).

Figure 1.2: Clinical features associated with Polycystic ovary syndrome

NOTE: This figure is included on page 11 of the print copy of the thesis held in the University of Adelaide Library.

Adapted from (99)

PCOS constitutes a significant health burden across the lifespan with metabolic consequences that may persist into the menopause (100), although HA and menstrual irregularity may improve with increasing age (101, 102). The signs of PCOS can manifest themselves in adolescence and can include premature adrenarche (early maturation of the adrenal gland cortex and activation of adrenal androgen production) which may contribute to precocious puberty or premature pubarche (the appearance of pubic hair before the age of 8 years) (103, 104). Delayed menarche (onset of menstruation) has also been documented by some (105) but not all investigators (106). Disturbingly, the metabolic complications associated with PCOS are also being documented at this early stage with T2DM, IGT and the metabolic syndrome all reported in lean and overweight adolescents with PCOS (107, 108). PCOS is thus associated with a diverse short and long-term presentation that may adversely affect women at varying stages of their life. This constitutes a significant financial burden of which it is estimated that 30.1% is associated with hormonal treatment of menstrual dysfunction, 12.2% infertility care, 14.2% treatment of hirsutism and 40.5% of T2DM related costs (109).

1.4.2: Diagnosis of PCOS

PCOS is extremely heterogeneous and not all women present with all symptoms (110). Furthermore, the criteria for diagnosis are controversial and inconsistent. National Institute of Health (NIH) diagnostic criteria have been used for the past 15 years (Table 1.2) (111) comprising biochemical or clinical HA and anovulatory irregular cycles (in the absence of any other contributing features such as congenital adrenal hyperplasia, Cushing Syndrome, hyperprolactinaemia and thyroid disease). According to these criteria, 4–8% of women exhibit PCOS (112-115). Menstrual disturbances consist of anovulation, amenorrhoea (lack of menstruation for > 3 months) (present in 29–52% of women with PCOS) and oligomenorrhoea (irregular menstruation) (present in 19–51% of women with PCOS) (116-118). Assessment of clinical HA involves subjective clinical scoring of degrees of hirsutism (excessive growth of terminal hair in women in a male like pattern) such as the Ferriman-

Gallwey score or later modifications (119, 120) and severity and presence of acne (121, 122), seborrhoea (accumulation of sebaceous secretion of the skin) and androgenic alopecia (progressive pattern of terminal scalp hair loss in a male pattern). The assessment of biochemical HA is through measurement of the ovarian androgen testosterone as total testosterone (free and bound to sex-hormone binding globulin (SHBG)), free testosterone (measured by equilibrium dialysis) or calculated as a function of total testosterone and SHBG levels (free androgen index (FAI)) or estimated from equilibrium binding equations (123, 124). Elevated total testosterone levels are present in 40–51% of women with PCOS (116, 117). However, there is considerable variation between hormone assays and a lack of accurate ranges for well-defined non-PCOS populations (125).

In 2003 the Rotterdam criteria were developed (124, 126) which included presence of polycystic ovaries (PCO) as a diagnostic criterion, defined as the presence of 12 or more follicles measuring 2–9 mm in each ovary and/or increased ovarian volume (>10 mL) (127). These criteria diagnosed PCOS as including two of the three criteria of HA, irregular anovulatory periods and PCO (Table 1.3), thus introducing additional phenotypic subsets (Table 1.4). PCO are present in up to 22% of women in the general population (128) and are not consistently associated with menstrual dysfunction and HA (110). The endocrine and metabolic sequalae of these subsets is thus unclear (129-131) and there is continued disagreement over the most accurate means of diagnosing PCOS. The prevalence of PCOS using these additional criteria is currently unknown, although logically it would be expected to be much higher than assessed using the NIH criteria. There is a lack of data on the prevalence of PCOS in the Australian population. One study (n=100) documented a 23% prevalence of PCO and 12% prevalence of PCOS, using the criteria of PCO in conjunction with hirsutism or menstrual irregularity (132) although hormonal androgen levels were not measured to determine the presence of hyperandrogenism.

Table 1.3: 1990 National Institute of Health and 2003 Rotterdam criteria for clinical diagnosis of Polycystic ovary syndrome

NOTE: This table is included on page 14 in the print copy of the thesis held in the University of Adelaide Library.

Adapted from (111)

Table 1.4: Phenotypes and estimated prevalence of phenotypes of Polycystic ovary syndrome according to the National Institute of Health 1990 and Rotterdam 2003 Criteria

NOTE: This table is included on page 14 in the print copy of the thesis held in the University of Adelaide Library.

(133, 134)

1.4.3: The relationship of obesity to PCOS

1.4.3.1: Obesity and reproductive parameters

Reproductive dysfunction occurs with both positive and negative extremes of body weight (135, 136). Menstrual disturbances including oligomenorrhoea, amenorrhoea and anovulation are consistently related to obesity in women. In early reports, obesity, assessed as 20% over ideal body weight, was present in 43–48% of women of reproductive age with menstrual disorders (n=160), compared to 13% of age-matched controls (n=201) (137). A direct relationship between menstrual irregularity (138) or infertility (135) and degree of obesity in women of reproductive age has also been reported. In a subset of The National Health and Nurses' Health Study (NHANES), women with ovulatory disorders (n = 2 527) were compared to controls with no history of infertility (n = 46 718). Increased BMI at age 18 was significantly associated with ovulatory infertility for a BMI of 28–29.9 kgm^{-2} (relative risk or RR=2.4) and a BMI ≥ 30 kgm^{-2} (RR=2.7) (139). Once conception is achieved, an increased risk of pregnancy complications including gestational diabetes and miscarriage may result with increased weight (140). Reduced fecundity (the probability of achieving at least one pregnancy during treatment) is additionally observed with increased weight. In n = 3 586 women receiving assisted reproductive treatment, there was a significant linear reduction in fecundity from non-obese to obese women (141).

The link with abdominal obesity and menstrual abnormalities and infertility is also apparent although it not known whether it is the visceral or subcutaneous depot that is related to reproductive dysfunction. In n=11 791 women, Hartz et al 1984 found for women with upper body fat predominance (WHR > 0.8), the RR of irregular menstruation and oligomenorrhoea were 1.56 and 2.29 respectively compared to women with a lower body fat predominance (142). In n=40 980 post menopausal women, WHR was significantly positively associated with a history of infertility (143). Zaadstra et al 1993 documented a 30% decrease in probability of conception for each 0.1 increase in WHR in women of reproductive age

(n=500) presenting for assisted reproductive treatment (8). Android obesity is additionally associated with a low pregnancy rate after in vitro fertilization (IVF) (144).

1.4.3.2: Obesity and PCOS

There is thus a clear association between obesity and abdominal obesity, both in adulthood and childhood, on menstrual abnormalities and consequent infertility independent of PCOS. Obesity and abdominal obesity in adolescence and adulthood and weight gain after adolescence are predictors of the development of hirsutism and menstrual disturbances in PCOS (145). Furthermore, women with PCOS constitute a significant proportion of the infertile population. Women with PCOS tend to have a BMI outside the acceptable range (19–25 kg/m^2) and 40–60% of women with PCOS are overweight or obese (116, 146, 147). These rates may appear similar to the population rates discussed previously (for example, 22.3% and 13.1% of women aged 25–34 and 23.8% and 14.9% of women aged 35–44 were overweight or obese respectively in the 2004–2005 Australian National Health Survey). However, given the ongoing increase in the prevalence of overweight and obesity in Western countries it is likely that at the time of measurement these rates were elevated compared to population means and are in all probability even higher now. In a recent study comparing women with PCOS (n=401) and age-matched controls from the NHANES I study (n=2586), the women with PCOS demonstrated a lower proportion of BMI <25 kg/m^2 and higher proportion of BMI > 30 kg/m^2 and 40 kg/m^2 (148). Women with PCOS also display an increased central distribution of adiposity (149) and WHR measurements > 0.8 have been reported in 63% and 53% of women with PCOS (110, 150) compared to 35% of Australian women (4). This increase in abdominal fat can be observed even in lean women with PCOS compared to weight-matched controls (151, 152) and lean women with PCOS also display an increased amount of visceral fat compared to lean controls (152). A number of investigators have shown that obesity and abdominal obesity worsen the clinical features of menstrual irregularity and infertility in PCOS (116, 146).

1.5: PATHOPHYSIOLOGY AND AETIOLOGY OF PCOS

The physiological process of reproduction occurs through the monthly cycle of follicular maturation, ovulation, fertilisation or resorption of the corpus luteum and is tightly regulated through a complex interaction of hormones from the hypothalamus and pituitary (the hypothalamic-pituitary-ovarian axis). Gonadotrophin-releasing hormone (GnRH) is synthesised by the preoptic and arcuate nucleus areas of the hypothalamus and released in a pulsatile fashion into the hypophyseal portal system and acts on the pituitary to stimulate the synthesis and pulsatile secretion of the gonadotrophins luteinising hormone (LH) and follicle-stimulating hormone (FSH) (153). The primary role of LH is to regulate androgen production in the theca interna and the primary role of FSH is to regulate growth and maturation of ovarian follicles and to stimulate aromatisation of androgens to oestrogens. GnRH and gonadotrophins are differentially regulated by a variety of factors including noradrenalin, serotonin, endogenous opioids, angiotensin II, neuropeptide Y, oxytocin, steroids and ovarian factors (154).

Folliculogenesis is the process of maturation of the primordial follicle to a primary, pre-antral or secondary, antral or tertiary and pre-ovulatory or Graafian follicle (reviewed by (155)). In PCOS, anovulation and menstrual irregularity are characterised by excessive early follicular growth with significantly greater amounts of primary and pre-antral follicles (156), arrested antral follicle development at the 4–7 mm stage and disturbed dominant follicle selection (reviewed by (157, 158)). The follicles commonly display hypertrophied and luteinised theca interna layers and a thickened ovarian tunica (159). The pre-antral follicles express the androgen receptor and androgens augment thecal and granulosa cell growth, inhibit follicular atresia and promote granulosa cell gonadotrophin responsiveness and steroidogenesis both directly and indirectly through increasing FSH receptor, IGF-1 and IGF-1 receptor expression (160-163). The increased pre-antral follicle number is likely due to an increased ratio of intra ovarian androgens to oestrogens. The cause of the arrested antral follicle development is

unclear. Increased FSH levels may be required for cyclic follicle recruitment to occur due to the presence of excessive local negative inhibitors of FSH activity. These may include anti-mullerian hormone (AMH) which is potentially increased in PCOS as a direct consequence of the increased number of pre-antral follicles (164) or increased follicle secretion of inhibin and oestradiol due to increased follicle number or premature development (158). Excessive LH production or earlier LH receptor gain could lead to premature action on granulosa cell LH receptors in the mid-follicular phase and premature arrest of cell proliferation and follicle growth and consequent anovulation (165).

Despite a number of candidate genes being identified, a primary pathological cause of PCOS has not been identified. A polygenic mode of inheritance with a number of predisposing genetic and environmental factors contributing to its presentation is therefore more likely. Candidate genes studied include those involved in steroidogenesis, carbohydrate metabolism and fuel homeostasis, gonadotrophin regulation and action and cardiovascular disease (reviewed by (166, 167)). The exact cause of PCOS is unclear and numerous theories exist. Three main pathophysiological hypothesis have been proposed to explain the endocrine alternations that occur in PCOS: (a) Hypothalamic-pituitary dysfunction involving a primary neuroendocrine defect leading to disruption of the hypothalamic-pituitary axis and excessive LH synthesis and secretion; (b) Primary hypersecretion of androgens involving primarily a defect in ovarian sex steroid synthesis or metabolism and consequent anovulation and (c) Defects in tissue specific IR and consequent hyperinsulinaemia with resultant effects on androgen secretion and anovulation (168). There are thus a wide range of potential central and peripheral abnormalities in PCOS that may interact to contribute to abnormal regulation of gonadotrophin biosynthesis and secretion.

1.5.1: Hypothalamic-pituitary dysfunction in PCOS

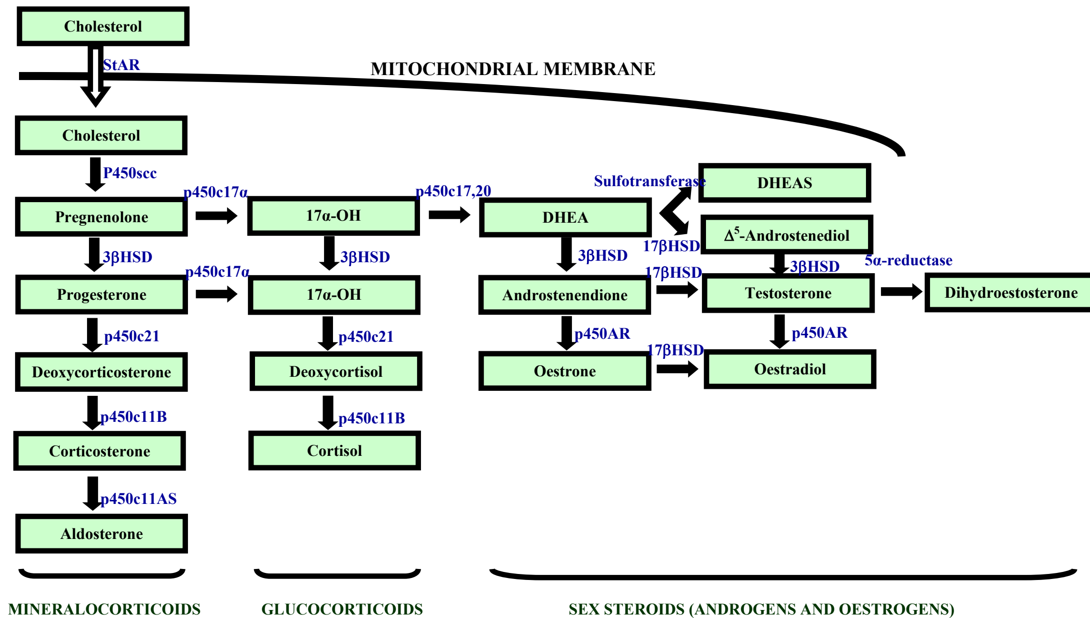
Gonadotrophin abnormalities in PCOS include LH hypersecretion due to increased LH pulse amplitude in 30–90% of women with PCOS (169-173). FSH levels tend to be normal or reduced compared to regularly ovulating women, resulting in an increased LH/FSH ratio (169, 170). Women with PCOS may display increased GnRH pulse frequency which would ultimately favour LH over FSH synthesis (174). Consistent with this, some (172) but not all (175, 176) investigators have demonstrated continual rapid LH pulse frequency and an absence of the normal menstrual variation in LH frequency in women with PCOS (172). This may be either due to a primary hypothalamic defect or an inherent abnormal pituitary response to GnRH stimulation or failure of inhibitory signals to suppress GnRH pulsatility in PCOS. However, a primary hypothalamic defect is unlikely in the majority of cases of PCOS and abnormal gonadotrophin secretion is more likely to be induced by peripheral hormonal contributors including insulin, inhibin, progesterone or oestrogen (reviewed by (177)). The effects of disordered gonadotrophin secretion are also likely to ultimately provide abnormal negative feedback to the hypothalamic pituitary axis and further impede its normal functioning (99).

1.5.2: Excessive androgen production and secretion in PCOS

The process of ovarian and adrenal steroidogenesis is represented in Figure 1.3. A large proportion of women with PCOS demonstrate elevated circulating androgens. These are predominantly of ovarian origin (114, 116), although an adrenal contribution to androgen excess has also been suggested with 20–60% of women with PCOS displaying excessive production and secretion of dehydroepiandrosteronesulfate (DHEAS) and 11-hydroxyandrostenendione (178-180). Ovarian theca cells from women with PCOS display excessive basal and LH-stimulated synthesis and secretion of androgens (dehydroepiandrosterone (DHEA), Δ4-Androstenendione and testosterone) (181, 182).

Increased P450c17 expression, increased P450c17 promoter function and reduced degradation of P450c17 mRNA are demonstrated in cultured ovarian thecal cells from women with PCOS (183-185). Alterations in other steroidogenic enzymes that could contribute to upregulation of ovarian steroidogenesis include increased P450c side chain cleavage (P450cscc), 3β-hydroxysteroid dehydrogenase (3βHSD) and 20α-HSD expression and P450cscc promoter function (182, 186-188) and increased transcription factor expression and mRNA stability (specifically GATA6 which regulates steroidogenic acute regulatory protein or StAR, P450c17 and sulfotransferase) (189). Peripheral androgen production occurs through conversion of androstenedione to testosterone by 17β-HSD in the liver and adipocytes (190) and conversion of testosterone to dihydrotestosterone (DHT) (the active form of testosterone for most receptors) by type 1 or 2 5α-reductase in the skin, brain, liver, prostate, pituitary, epididymis and adipocytes and in the ovary (thecal and granulosa cells) and adrenal gland (190). Increased peripheral steroidogenesis can also contribute to HA and women with PCOS display elevated conversion of circulating DHEA to 5α-DHT and associated metabolites, indicating enhanced 5α-reductase activity (191).

Figure 1.3: Steroid biosynthetic pathways in the adrenal, ovary and peripheral tissue



StAR: Steroidogenic acute regulatory protein.
P450cscc: Cytochrome P450 side chain cleavage
P450c11AS: Cytochrome P45011 aldosterone synthetase
P450c11B: Cytochrome P450 11-hydroxylase
P450c17α: Cytochrome P450 17 α hydroxylase
P450c17,20: Cytochrome P450 17,20 lyase
P450c21: Cytochrome P450 21-hydroxylase
P450AR: Cytochrome P450 aromatase
3βHSD: 3β-hydroxysteroid dehydrogenase
17βHSD: 17β-hydroxysteroid dehydrogenase

The production of the mineralocorticoids (aldosterone) and glucocorticoids (cortisol) occurs in the adrenal cortex (zona reticularis). Production of androgens occurs in the ovary (predominantly androstenedione and testosterone) and adrenal cortex (predominantly DHEA, DHEAS, androstenedione and testosterone). The peripheral tissues are involved in androgen production through conversion of androstenedione to testosterone and testosterone to dihydrotestosterone through 5α-reductase. Production of oestrone and oestradiol occurs in the granulosa cells of the ovary and in the peripheral tissue.

1.5.3: PCOS, insulin resistance and hyperinsulinaemia

Insulin can contribute to the presentation of PCOS through effects on steroidogenesis and increasing intra-ovarian or circulating androgens and through affecting normal gonadotrophin feedback and release (Table 1.5). It can also directly affect folliculogenesis through actions as a mitogenic factor or through stimulating or augmenting local tissue production of growth factors (192). Insulin resistant women with PCOS are more severely clinically affected than insulin sensitive women with PCOS (193) and HA positively correlates with IR in obese and lean women with PCOS (194). As previously discussed in section 1.4.3.2, obesity and abdominal obesity are frequent features of PCOS. Where overweight, women with PCOS will thus exhibit obesity-associated IR and hyperinsulinaemia (discussed in section 1.3.1.3). Additionally, even IR has been documented in lean women with PCOS compared to weight-matched controls and IR and compensatory hyperinsulinaemia are documented in approximately 40–70% of lean and obese women with PCOS compared to weight-matched controls (193, 195-197). This strongly suggests that in a subset of women with PCOS, the IR and consequent hyperinsulinaemia significantly contribute to the aetiology and presentation of PCOS and that these defects in insulin metabolism are separate and additive to that of obesity (198). Not all women with PCOS exhibit hyperinsulinaemia and IR (199, 200) and ethnic background and the heterogeneity and complex aetiology of the syndrome contribute to this discrepancy.

In PCOS, selective tissue sensitivity to the actions of insulin exists and reduced insulin sensitivity has been identified in fibroblasts, skeletal muscle, adipocytes and hepatocytes. The key insulin homeostasis abnormalities identified in PCOS thus far involve defects in receptor and/or post-receptor signal transduction. In cultured fibroblasts from women with PCOS, abnormal constitutive insulin receptor serine phosphorylation and consequent reduced insulin receptor tyrosine kinase activity potentially due to abnormal regulation of a specific serine kinase were observed in approximately 50% of subjects (201, 202). The remaining subjects

displayed IR but with normal insulin receptor phosphorylation, indicating additional downstream anomalies. In cultured adipocytes, intrinsic decreases in maximal insulin-stimulated rates of glucose utilization or transport and GLUT4 (203-208), insulin-stimulated autophosphorylation (205), insulin-induced lipolysis (206, 207) and insulin receptor binding (207) were observed. In vivo and in vitro studies of human PCOS skeletal muscle show reductions in insulin-mediated glucose uptake (209), IRS-1 (209, 210) and IRS-2 associated PI3-K activity (210) and intrinsic increased IRS-1 Ser^{312} phosphorylation which inhibits its insulin-induced tyrosine phosphorylation (210). Although impaired hepatic insulin clearance is observed in lean and obese women with PCOS (211-214), hepatic insulin sensitivity appears to be reduced predominantly in obese women with PCOS as a result of the synergistic effect of obesity and PCOS (195, 203, 215). In vivo factors including adipokines likely further modulate insulin signalling (210). While the above defects appear to affect only the metabolic pathway in skin fibroblasts (216), both the metabolic and mitogenic pathways are affected in skeletal muscle (217). Corbould et al also demonstrated that the mitogenic pathway can directly influence the metabolic pathway through ERK 1/2 or ERK activated kinases phosphorylating IRS-1 on Ser^{312} (217). A variety of additional defects in insulin metabolism have been reported in PCOS. A higher level of β-cell function is present as indicated by insulin hypersecretion (211, 213, 218-220) and increased insulin response to metabolic stimuli (221) which are present in the absence of IR (218, 221). Impaired peripheral tissue degradation of insulin (212) has also been observed, indicating a further mechanism for augmentation of hyperinsulinaemia.

In PCOS the ovarian tissue does not appear to display IR, unlike skeletal muscle, fibroblasts and adipose tissue (222). Insulin stimulates thecal androgen overproduction (223). The exact downstream mediators for this are unknown, although there is evidence that cytochrome P450c17α activity is increased by insulin (224). This may be mediated through PI3-K and AKT activation with the MAPK pathway remaining unaffected (225), although other work

reports that PI3-K is not required for other ovarian specific effects of insulin such as stimulation of progesterone production (226). Of interest, P450c17 can be selectively induced to increase 17,20-lyase activity, leading to excessive androgen production by serine phosphorylation (227). This suggests an intriguing but as yet unconfirmed theory whereby abnormal serine phosphorylation both of the insulin receptor and P450c17 could account for the development and presentation of PCOS (228). Insulin also synergistically increases the action of LH in women with and without PCOS (229, 230), potentially through LH cyclic AMP independent effects that stimulate StAR and p450c17 expression (231). Nestler et al additionally suggested that ovarian steroidogenesis may be mediated through inositolglycan second messengers produced by the insulin-sensitive hydrolysis of a glycolipid in the plasma membrane (222, 232). These may be depleted in PCOS and respond to regeneration with D-chiro-inositol (233). Insulin has also been reported to potentially stimulate adrenal steroidogenesis through increasing P450c17 responsivity to adrenocorticotrophic hormone (234), stimulate oestradiol and progesterone production in human granulosa cells (230) and increase granulosa cell responsiveness to LH (230) or FSH (235).

SHBG is a liver derived plasma steroid-binding protein that additionally acts in androgen and oestrogen steroid signalling mediated through the cell membrane SHBG receptor (236). Its production and secretion is primarily inhibited by androgens, insulin, IGF-1 and potentially triglycerides and promoted by oestrogen, cortisol, iodothyronines and growth hormone (GH) (237-239). SHBG levels are thus lower in obesity and abdominal obesity (240). A decrease in the hepatic production of SHBG by insulin increases total and free or bioavailable androgens (241, 242). Dysregulation of other circulating factors can also modulate the relationship between insulin and steroidogenesis including the IGFs and their binding proteins (IGFBP) (243). Hyperinsulinaemia can activate the ovarian-IGF system through reducing hepatic and ovarian IGFBP production (244) and upregulating IGF-1 receptor expression (245). This sensitises the ovary to the androgenic effects of IGF-I and IGF-II which include augmenting

LH and FSH actions on thecal steroidogenesis (246), granulosa and thecal growth and maturation, oocyte maturation and stimulation of the insulin receptor (243). A complex interrelationship thus exists between obesity, abdominal obesity and IR in the aetiology and pathogenesis of PCOS.

Table 1.5: Insulin effects related to ovarian function

NOTE: This table is included on page 25 of the print copy of the thesis held in the University of Adelaide Library.

Adapted from (243)

In PCOS, HOMA (247), fasting glucose/insulin ratio (79), fasting insulin (248) and QUICKI (249) are reasonably accurate measures of insulin sensitivity and cut-off ranges to define IR have been calculated by a number of authors (250). Conversely, no correlation of the above parameters with euglycaemic clamps was reported in PCOS (251). Where unique varieties of IR or variations in insulin clearance (ie in obese patients with an excess of abdominal fat (252)) or glycemic tolerance (ie when assessing patients with normal or IGT (250)) exist, the relationship between circulating insulin and insulin secretion or IR may be altered and surrogate measures may not be optimal. This may be improved through incorporation of additional metabolic markers such as age, weight, FFA, androgens, SHBG or triglycerides

into a diagnostic model (249, 253-256). PCOS is an extremely heterogeneous condition and the aetiology and treatment may differ depending on the presentation including the degree of IR. As yet there is no optimal simple reliable clinical tool for diagnosing IR in PCOS and development of this for routine use in clinical practice or research would greatly improve the understanding of the pathophysiology of PCOS and the appropriate treatments for differing subsets of PCOS.

1.6: OVERVIEW OF THE PATHOPHYSIOLOGY OF PCOS

There are multiple potential abnormalities present in PCOS that can contribute to the reproductive presentation. Current evidence suggests intrinsic abnormalities in gonadotrophin secretion and ovarian steroidogenesis are likely to play a role. Contributory stimulatory influences such as hyperinsulinaemia are crucial in the presentation of a significant proportion of women with PCOS (Figure 1.4). However, the heterogeneity of PCOS is such that no one factor is likely to explain the bulk of cases and women with PCOS present with variable severities of HA, menstrual dysfunction, IR and weight. Despite this, there is a clear environmental contribution of obesity and consequently additional obesity-induced IR to the pathophysiology of PCOS. Indeed, regardless of the main underlying contributing factor to PCOS (gonadotrophin defect, androgen hypersecretion or PCOS-specific IR), obesity will in the majority of cases further worsen the clinical presentation of PCOS (257). Although obesity is not a universal feature of PCOS, the increasing prevalence of obesity in the general population thus signifies a key contributing factor to reproductive and metabolic morbidity.

Figure 1.4: Polycystic ovary syndrome, the hypothalamic pituitary axis and insulin

NOTE: This figure is included on page 27 of the print copy of the thesis held in the University of Adelaide Library.

Increased ovarian androgen biosynthesis in the polycystic ovary syndrome (PCOS) results from abnormalities at all levels of the hypothalamic–pituitary–ovarian axis. The increased frequency of LH pulses in the PCOS appears to result from an increased frequency of hypothalamic GnRH pulses favouring the production of LH over FSH. This leads to an increase in androgen production by ovarian theca cells. Increased efficiency in the conversion of androgenic precursors in theca cells leads to enhanced production of androstenedione, which is then converted by 17β to form testosterone or aromatized by the aromatase enzyme to form oestrone. Within the granulosa cell, oestrone is then converted into oestradiol by 17β. Insulin acts synergistically with LH to enhance androgen production and inhibits hepatic synthesis of SHBG, the key circulating protein that binds to testosterone and thus increases biologically available testosterone. Testosterone inhibits and oestrogen stimulates hepatic synthesis of SHBG.

(258)

1.7: TREATMENT OF PCOS

1.7.1: Overview of the treatment of PCOS

Treatment of PCOS includes the normalisation of biochemical and clinical HA, the optimisation of reproductive function and outcomes and the management of metabolic morbidity and mortality. Pharmaceutical and surgical treatment of PCOS includes oral contraceptives (oestrogen-progesterone combination therapy) to suppress LH production and enhance SHBG production, anti-androgens (spirinolactone and flutamide) to inhibit androgen (testosterone and 5α-DHT) binding to peripheral androgen receptors, 5α-reductase inhibitors (finasteride) for treatment of hirsutism, glucocorticoids to reduce adrenal androgen levels, ovulation induction agents (such as the oestrogen receptor antagonist clomiphene citrate) and gonadotrophins for the treatment of anovulatory infertility, laparoscopic ovarian surgery (reviewed by (258-260)). Where the clinical features of PCOS are worsened by the presence of IR and obesity, these are important targets for preventative and therapeutic interventions and use of insulin sensitising agents, including the glitazones and metformin, have increasingly been adopted as preferable pharmacological strategies both in isolation or in combination with other pharmacological options to improve treatment response (reviewed by (261)). However, where possible use of lifestyle (dietary and/or exercise) interventions to reduce the features of obesity, IR and hyperinsulinaemia are preferable and cost-effective options for initial treatment strategies in PCOS (262) compared to surgical and pharmacological options.

1.7.2: Dietary management of PCOS

1.7.2.1: Overview of dietary management of PCOS

The dietary management of obesity consists of achieving and maintaining a reduced body weight and preventing further weight gain (263). In obese individuals, energy restriction and weight loss improve glycaemic control (264, 265), dyslipidaemia (265-267),

hyperinsulinaemia and IR (265) and reduce blood pressure (268). Acute energy restriction decreases human endogenous cholesterol synthesis (267), improves central and peripheral insulin sensitivity and glycaemic control due to an enhancement of non-oxidative glucose disposal, a reduction in insulin secretion or reduced hepatic glucose production (265, 269, 270). In the general population, guidelines for obesity management recommend an initial weight loss of ~5–10% for reduction of obesity-related risk factors (271, 272) with long-term goals of achieving and maintaining a reduction in weight of 10–20% and waist circumference of < 88 cm for women (273). There are a range of principles to consider with regards to the optimal dietary treatment of PCOS. The success of a weight loss or weight maintenance strategy depends on a variety of factors. The efficacy of a dietary strategy can be assessed by its ability to induce and maintain weight loss and its effect on the composition of weight loss (fat versus lean body mass), metabolic parameters (glucose and insulin homeostasis, lipid profile, risk factors for cardiovascular disease and diabetes, blood pressure) and reproductive endocrine or clinical outcomes. Furthermore, the nutritional adequacy of a particular dietary strategy needs to be ensured, particularly where followed long-term or pre-pregnancy, and the effect of a diet on micronutrient (vitamin and mineral) status must be assessed (274). A particular dietary strategy or composition may therefore induce either a greater weight loss than an alternative strategy or induce a greater metabolic or endocrine improvement than an alternative strategy with equivalent reductions in weight.

However, although weight loss can be achieved in the short-term, dietary strategies for continued weight loss or prevention of weight regain must be maintained long-term for successful weight maintenance. Many patients who lose weight from dietary weight loss programmes will eventually regain the weight (275, 276). A recent meta-analysis reported approximately 15% of subjects undergoing weight loss interventions maintain either their reduced weight or an overall reduction of 9–11 kg at a follow-up time of up to 14 years (277). When weight loss maintenance is defined as maintaining a reduction of 10% of initial body

weight for 1 year (278), 20.6% of n=228 overweight adults in a random telephone survey were successful weight maintainers (279). Thus, for successful maintenance of a reduced weight the dietary strategy must be sustainable in the long-term (278, 280). The success of a weight loss strategy will be further increased with incorporation of additional principles including regular physical activity, behavioural management strategies, social support and attention to psychological adjustment including behaviour modification and stress management strategies (263, 273).

1.7.2.2: Effects of weight loss on the presentation of PCOS

The majority of studies support the positive effect of modest weight loss (5–10%) on improving risk factors for CVD and T2DM, reproductive endocrine parameters and reproductive outcomes in PCOS (194, 262, 281-285) (Appendix 1). Dietary strategies used in PCOS include structured very low calorie diets (VLCD) (1386–2730 kJ/day over 4–6 weeks) (284-288)), structured low calorie high carbohydrate diets (4200–6300 kcal/day over 1–12 months with diet compositions of 15–20% protein, 20–30% fat and 50–60% carbohydrate), (194, 219, 286, 289-298) and structured lifestyle modification strategies incorporating individualised high carbohydrate low fat diets, exercise prescription and behavioural modification strategies (262, 283, 299-301). These studies consistently demonstrate that weight loss improves insulin sensitivity measured through euglycaemic hyperinsulinaemia clamps (219, 286), fasting glucose/insulin ratios (289), HOMA (302), OGTT-stimulated insulin (194, 285, 291) and fasting insulin (284, 287, 292, 293).

Improvements in HA, measured as decreases in FAI, free or total testosterone and increases in SHBG, are also consistently displayed (219, 283, 284, 287-290, 295, 296, 302). Alterations in LH are less consistently documented with reductions (194, 288, 301), increases (293) and no changes reported (219, 283, 284, 289-292, 296, 297). Hirsutism is both improved (194, 290, 296, 298) or not altered (219, 289, 292, 297, 300, 302-304) following modest weight loss.

Clinically, improvements in menstrual function and ovulation (262, 292, 294, 296, 301) and reductions in ovarian volume and follicle number are documented following modest weight loss (294). In overweight women with PCOS, lifestyle modification techniques led to a weight loss of 6.3 kg over 6 months (283) and 6.8% over 48 weeks (300). There is as yet limited additional data on the effect of weight loss on reducing negative reproductive outcomes or pregnancy complications in PCOS although modest weight loss reduces the risk of developing gestational diabetes (305) and Clark et al reported a reduction in miscarriage rates from 75% pre-treatment to 18% post-treatment in women with PCOS (262). As both T2DM (306) and the metabolic syndrome (307) are more common in PCOS than the overweight population, lifestyle modification strategies therefore also seem appropriate in regards to their reduction of metabolic risks.

1.7.2.3: Degree of weight loss for improving the presentation of PCOS

An important point is that a minimal amount of weight loss (5–10%) over as little as 4 weeks is sufficient to improve the clinical reproductive presentation despite subjects remaining clinically overweight or obese (285, 287). Large reductions in weight may not be needed to restore reproductive function and, therefore, realistic and achievable weight loss goals can be set for overweight infertile women. Indeed, reductions in circulating insulin and testosterone and increases in SHBG are observed as early as 4 weeks post-energy restriction (284, 285), supporting the proposed role of IR and hyperinsulinaemia in HA and clinical reproductive dysfunction in PCOS. This also has important implications for which women with PCOS are likely to experience reproductive benefits from weight loss. In 11/25 women who responded to weight loss with improvements in menstrual cyclicity (ovulation and menstrual cycle length), a significant reduction in fasting insulin and HOMA was observed compared to subjects who showed no improvement in menstrual cyclicity with weight loss (302). This is consistent with the proposed aetiological role of IR in reproductive dysfunction in PCOS and

highlights the importance of reducing IR through obesity treatment as a key initial treatment strategy in PCOS.

As previously discussed (section 1.3.1.3), adiposity, abdominal or visceral adiposity, IR and hyperinsulinaemia are key risk factors for metabolic disease. The reduction in these factors following modest weight loss in PCOS highlights the benefits of weight loss in PCOS on metabolic risk factors. Only a small number of studies have assessed the effect of weight loss on risk factors for CVD and T2DM (219, 286, 298, 302, 304, 308). Modest weight loss (5–14%) improved dyslipidaemia (increased HDL-C, reduced LDL-C, total cholesterol and triglycerides) (286, 302), diastolic blood pressure (DBP) (298), plasminogen-activator inhibitor activity (PAI-1) (a marker of impaired fibrinolysis) (286) and circulating FFA (219). Conversely, other studies showed no improvement in circulating lipids despite weight losses of 5–15% (286, 304, 308). One additional issue of interest is thus whether women with and without PCOS would experience similar metabolic improvements with the same degree of weight loss. There is some evidence that subjects with features of the metabolic syndrome (IR, high liver fat, dyslipidaemia) experience greater improvements in fasting insulin and lipid profiles following equivalent weight losses compared to relatively more metabolically healthy individuals (309-311). This is demonstrated by reductions in fasting insulin and HOMA with weight loss for relatively more IR subjects compared to more insulin sensitive subjects with or without PCOS (302, 312). If this is the case, then potentially where women with PCOS demonstrate a significantly worsened metabolic profile a lesser degree of weight loss may be sufficient to improve risk factors for CVD and T2DM than would be necessary for a less metabolically disturbed population. In isocaloric weight loss studies comparing women with and without PCOS, equivalent weight losses (7.5% reduction in BMI or 5kg reduction in weight) induced similar reductions in fasting insulin for women with or without PCOS (291, 292). However, the differential effect of weight loss on additional risk factors for cardiovascular disease in overweight women with and without PCOS has not been examined.

This could have important implications for the determination of appropriate weight loss targets or identification of particular presentations of PCOS that would benefit from weight loss. Chapter 3 will discuss the effect of identical weight loss interventions with equivalent weight loss on novel and traditional risk factors for CVD and T2DM in overweight women with and without PCOS.

1.7.3: Altering dietary composition in the dietary management of PCOS

A low fat (~30% of energy, saturated fat ~10% of energy, <300 mg cholesterol daily), moderate protein (~15%) and high carbohydrate intake (~55%), in conjunction with moderate regular exercise is recommended by a variety of institutions for the management of obesity and related co-morbidities (263, 273, 313, 314). Some evidence indicates weight is maintained more effectively and compliance is increased when this dietary pattern is followed over longer periods of time compared to fixed energy diets. Toubro et al (315) assessed weight maintenance at 1 year in n=43 obese adults; 65% of the *ad libitum* low fat high carbohydrate group and 40% of the fixed energy group maintained a weight loss of >5 kg after 2 years. In a cross-sectional study assessing the dietary patterns of 438 subjects from the National Weight Control Registry who maintained a weight loss of 30 kg for 5.1 years, successful weight maintenance behaviours included continued consumption of a low energy and low fat diet (316). However, a recent Cochrane review of free-living subjects following low fat versus other diets reported similar drop-out rates for most of the studied research and similar weight losses at 6 months (-5.08 versus -6.5 kg), 12 months (-2.3 versus -3.4 kg) and 18 months (+0.1 versus -2.3 kg) post-intervention, indicating a low fat diet suffers similar compliance issues as other approaches (317).

Furthermore, a number of studies have demonstrated a worsening of the metabolic profile as increases in triglycerides (due to increases in hepatic synthesis of VLDL) (318, 319), decreases in HDL-C (319, 320) and increases in post-prandial insulin and glucose (321) are

observed following a low fat high carbohydrate diet, particularly if weight loss is not achieved. These are factors implicated in the pathogenesis of CVD (322-324) and as such this dietary regime may be potentially detrimental. This may also be more pronounced in individuals with IR where the need to secrete more insulin could lead to additional demands on the β-cell, potential β-cell exhaustion and compromised glucose tolerance or increased hyperinsulinaemia and resultant metabolic consequences (Table 1.5) (14). Alternative approaches are thus being studied which may have more favourable effects on the metabolic profile or may be more effective in achieving and sustaining long-term weight loss. There has been increased community interest in dietary strategies modifying macronutrient contribution (Table 1.6). However, the effect of altering dietary composition in a structured weight loss environment has been poorly studied in PCOS. In this literature review a discussion of the effect of modifying diet composition will be limited to individuals with IR, T2DM or elevated cardiovascular risk factors as these are important features in the aetiology and presentation of PCOS.

Table 1.6: Changes in the macronutrient composition of various diets

Diet description	**Energy (kJ)**	**Fat g/day (% kJ)**	**CHO g /day (% kJ)**	**Protein g/day (% kJ)**	**Alcohol g/day (% kJ)**
Typical American Diet	9240	85 (35)	275 (50)	82.5 (15)	3
Low CHO					
Diet Induction Phase	4838	75 (59)	13 (5)	102 (35)	-
Diet Ongoing Phase	6833	105 (58)	35 (9)	134 (33)	-
Diet Maintenance Phase	8358	114 (52)	95 (19)	125 (25)	-
Moderate-low fat	6140	42 (26)	207 (57)	73 (20)	-
Very low fat	5347	13 (9)	258 (81)	48 (15)	-
Moderate protein moderate CHO low GI	6199	44 (26)	176 (46)	89 (23)	-
High protein moderate CHO	6199	52 (30)	155 (40)	109 (30)	-

CHO: carbohydrate
GI: glycemic index
Adapted from (274, 325, 326)

1.7.3.1: Altering dietary protein amount

The recommended daily intake for protein from the Australian Dietary Guidelines for females aged 19–70 is 0.75 g/kg/day and the estimated average requirements are 0.6 g/kg/day (327). A high protein (HP) diet contains ≥1.6 g/kg ideal body weight/day protein for negative energy balance (≥25% daily energy) and a very HP diet contains ≥2.4 g/kg ideal body weight/day protein (≥35% daily energy) (328). A range of popular dietary protocols advocate moderate increases in protein (to approximately 30% of total energy intake) with concomitant reductions in dietary carbohydrate. In short-term isocaloric studies (8–12 weeks), greater decreases in weight, total fat or abdominal fat (329-331) or no differences (321) were observed for overweight subjects with hyperinsulinaemia, T2DM or elevated baseline triglycerides following a moderate protein (MP) (28–31% protein, 42–44% carbohydrate, 22–28% fat) compared to a low protein (LP) diet (16–18% protein, 55–61% carbohydrate, 20–26% fat). With regards to long-term weight loss and maintenance, increasing dietary protein may be advantageous through effects on increasing satiety (332-336), reducing *ad libitum* food intake (337), increasing post-prandial thermogenesis and resting energy expenditure (REE) (338-340) and preserving lean body mass in weight loss (341). However, this approach has not been more successful compared to a moderate protein intake over 24 months in the general population (342) or over 12 months in women with IR (343).

Despite a lower post-prandial insulin response for HP compared to LP test meals in subjects with hyperinsulinaemia or T2DM (321, 344), following weight loss similar decreases in fasting insulin levels (321, 329, 331, 345) or insulin sensitivity as assessed by HOMA (321) have been reported between HP and LP diets. The effect of a HP diet on lipid profiles, blood pressure and glucose homeostasis is controversial. In short-term (4–6 weeks) weight maintenance trials in hyperlipidaemic (346, 347) or T2DM (348) subjects, significant improvements in dyslipidaemia, post-prandial glucose or blood pressure have been reported by some (346-348) but not all researchers (347, 348) for HP compared to LP diets with

identical fat intake. Similarly, an energy reduced HP compared to a LP diet produces either greater improvements in the lipid profile and post-prandial glucose (321, 330, 331, 345) or similar changes in lipids, blood pressure or fasting and OGTT glucose (321, 329-331, 345).

In overweight women with PCOS, two small studies compared structured low calorie high protein moderate carbohydrate and low protein high carbohydrate diets (~4200–6000 kJ/day over 1–3 months with diet compositions of 30% protein, 30% fat, 40% carbohydrate and 15% protein, 30% fat, 55% carbohydrate). Equivalent retention rates, reductions in weight (3.6–7.5%), fasting insulin and reproductive hormones and improvements in reproductive parameters were observed (302, 303). Modest benefits were observed for the high protein diet in a greater reduction in post-prandial glucose, a sustained reduction in the free androgen index in weight maintenance and a lack of a decrease in HDL-C during weight loss (302).

1.7.3.2: Altering dietary carbohydrate amount

An additional approach is that of a carbohydrate restricted diet (initially 20–30 g/day). A number of short to medium term studies in individuals with dyslipidaemia, T2DM or the metabolic syndrome have demonstrated that when this approach is followed *ad libitum*, greater weight loss occurs compared to an energy restricted low fat approach (moderate calorie restriction and 25–30% calories from fat) (349, 350), with a recent meta-analysis reporting a 3.3 kg difference in weight loss at 6 months (351). The success of this strategy in the short-term may firstly be due the simplicity and therefore the ease of implementation of the dietary message and restriction of food choices. Furthermore, one dietary factor associated with overconsumption is dietary variety, particularly of highly palatable energy dense foods. Flexible dietary strategies restricting intake of certain food classes or macronutrients can be successful due to reduction of dietary variation (352, 353) which reduces food choices and also increases sensory specific satiety, defined as a reduction in palatability of a food being consistently consumed (354). The low carbohydrate approach may also result in a

concomitant increase in dietary protein (355) with its aforementioned effects on satiety. However, weight loss is typically not maintained after 12 months (356) or 36 months (357) indicating that a low-carbohydrate diet is likely to suffer similar problems to other weight maintenance regimes with regards to compliance and sustainability.

The response of measures of insulin sensitivity to a low carbohydrate diet are mixed. Where greater weight losses occurred for a low carbohydrate diet after 6 months, greater (349) improvements in insulin sensitivity (assessed by fasting insulin or the QUICKI index) were reported. At 12 months where equivalent weight losses occurred between the low carbohydrate and standard diets, reductions in insulin sensitivity were the same. Furthermore, fasting insulin decreased after 12 months on energy restricted higher protein high carbohydrate low fat and very low fat diets but not on the Atkins diet (358). A meta-analysis of moderate-term *ad libitum* low carbohydrate diets compared to energy restricted low fat diets reported greater decreases in triglycerides (-0.25 mmol/L) and greater increases in HDL-C (0.12 mmol/L) for the low carbohydrate diets either at 6 months in conjunction with greater weight losses or at 12 months in conjunction with similar weight losses. However, blood pressure was not different between the approaches and greater decreases in total cholesterol (0.23 mmol/L) and LDL-C (-0.14 mmol/L) occurred for the low fat diet (351). In longer-term studies, fasting and OGTT glucose have been reported to be lower (349) or similar (359) for individuals with T2DM following the low carbohydrate diet after 6 months (349) and similar after 12 months (356) and 36 months (357). There thus appear to be both some metabolic benefits of a low carbohydrate diet independent of weight loss and some deleterious effects likely due to the high saturated fat content. It is not known what the combined effect would be on long-term cardiovascular risk.

A number of small pilot studies have assessed flexible carbohydrate restricted weight loss strategies. In overweight women with PCOS (n=15), a high saturated fat low starch diet

(energy intake not reported) over 24 weeks resulted in a 14.3% reduction in weight and a 12 mU/L reduction in fasting insulin with no changes in the lipid profile or fasting glucose (308). In n=5 overweight women with PCOS following a ketogenic diet (<20 g carbohydrate/day) for 6 months, decreases in weight (5 kg), fasting insulin (53.7) and free testosterone (30%) occurred (304). However, these strategies were not compared to conventional structured weight loss management and their comparative success cannot be determined.

1.7.3.3: Altering dietary glycemic index or glycemic load

An alternative dietary modification is altering the type of carbohydrate to produce a lower glycemic response (low glycemic index or low GI). The GI is a classification index of carbohydrate foods based on their effects on post-prandial blood glucose response (360) and the glycemic load (GL) is the product of the GI and the amount of dietary carbohydrate (361). The GL can be reduced by either decreasing the GI of the carbohydrate or by decreasing the amount of carbohydrate with a concomitant increase in protein or fat. In acute intervention studies, high GI meals decrease satiety and increase hunger and subsequent food intake compared to low GI test meals (reviewed by (362)). In the general population, *ad libitum* low GI diets result in either greater (363, 364) or similar (365-367) weight losses compared to a conventional energy restricted low fat diet. In a recent 12 week study in overweight or obese adults (n=129) following one of 4 isocaloric *ad libitum* diets, either high or low in GI and protein, the high carbohydrate low GI and HP high GI diets (with similar GL) had the greatest reduction in fat mass (−4.5±0.5 kg, −4.6±0.5 kg) (368). Similar retention rates (82%), reductions in weight and waist circumference and changes in body composition were observed for overweight hyperinsulinaemic women following a 12 month weight loss maintenance program on a HP (40% low GI carbohydrate, 30% fat, 30% protein) (6.6 kg), high carbohydrate (55% carbohydrate, 30% fat, 15% protein, 25–30 g fibre/day) (4.4 kg) or high fat diet (<20 grams of carbohydrate/day initially with increase up to weight maintenance levels) (5.4 kg) (343). Overweight individuals with at least one metabolic cardiac risk factor

demonstrated equivalent weight losses at 1 year for the Atkins diet (2.1 kg), Ornish diet (10% fat) (3.3 kg), Weight Watchers (3.0 kg) and Zone diet (40% carbohydrate, 30% fat, 30% protein) (3.2 kg) with a similar intervention intensity. At 12 months the drop–out rate was 42%, with a trend for the more extreme approaches (48% and 50% for the Atkins and Ornish diets) to have a higher drop-out rate than the more moderate approaches (35% for the Zone and Weight Watchers diets) (358).

In subjects with T2DM, coronary artery disease or hyperlipidaemia, acute weight loss or weight maintenance low GI diets reduce daylong glucose levels, post-prandial insulin, glycated haemoglobin and reduce triglycerides, total cholesterol or LDL-C, plasminogen activator inhibitor 1, apolipoprotein B, FFA and increase HDL-C (369-374). However, results are not consistently observed (375) and a recent meta-analysis of 15 weight loss and weight maintenance studies reported that low compared to high GI diets reduce total cholesterol with no additional effects on triglycerides, HDL-C, LDL-C, body weight, fasting insulin or glucose (376). In subjects with IGT, the metabolic syndrome, CVD or a family history of CVD, reduced OGTT-stimulated insulin (377, 378), increased early insulin secretion (379), increased insulin sensitivity (measured by the short ITT (380) or euglycaemic hyperinsulinaemic clamp (374)) and increased adipocyte insulin-stimulated glucose uptake (378, 380) were observed for a low compared to a high GI diet in weight maintenance or weight loss. Conversely, no differences have been observed in fasting insulin or HOMA (378, 381) or measures of insulin sensitivity (372) between high GI and low GI diets and one study reported a 20% increase in fasting insulin after 4 months of a low GI diet (382). Although there appears to be some metabolic benefit to a low GI diet, the effect in clinical trials is inconsistent.

The effect of altering dietary GI on reproductive hormonal parameters and fertility has not been studied. In the two studies assessing dietary protein in PCOS (302, 303), the HP diet

represents an overall reduction in GL although the GI of the carbohydrate was not specified. The Diet and Androgens randomised trial studied the effect of an *ad libitum* diet low in animal fat and high in low GI foods, monounsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA) and phytoestrogens compared to usual eating patterns in n = 104 women with high testosterone. The intervention diet reduced weight (4.06 versus 0.54 kg), testosterone (-19.5 versus -7.1%), fasting glucose (-5.7 versus -1.2%) and OGTT-stimulated insulin (-7.7 versus + 9.4%) and increased SHBG (+25.2 versus +3.6%), although it is not possible to determine whether weight loss or one or more of the dietary components produced the beneficial endocrine effect (383).

1.7.3.4: Safety of different dietary compositions

Some concern has been raised as to the potentially detrimental effects of an increased protein intake on bone turnover and renal function. The evidence surrounding these claims has been recently reviewed (384, 385). No difference in markers of bone turnover and calcium excretion were observed in short-term dietary intervention studies (321, 330, 386). Over a 6 month intervention, increased glomerular filtration rate observed with increasing dietary protein (from 70g/day to 108 g/day) was associated with an increased renal mass, suggesting an adaptation to the increased protein intake can minimise detrimental renal effects (387). This suggests that a moderate increase in dietary protein is unlikely to have adverse consequences on bone metabolism or kidney function. Populations at an increased risk of developing kidney disease (T2DM) may respond differently to changes in dietary composition and further longer term studies are required, although a range of short and medium term studies (5–12 weeks) in T2DM examining the effect of increasing dietary protein show glycemic and lipid improvements and no adverse effects (331, 348, 388, 389). With regards to a very low carbohydrate approach, additional safety concerns relate to the effect of the low fibre intake on constipation and long-term cancer and diverticular disease,

the effect of low magnesium, potassium and vitamin C intakes on calciuria and osteoporosis and elevated uric acid levels and effects on gout and potential nutritional inadequacy (274).

1.7.3.5: Summary of dietary management of obesity and overweight in PCOS

There are thus a range of strategies for the dietary management of overweight and obesity in PCOS, although their comparative effectiveness on maintaining weight loss and inducing optimal metabolic improvements is unknown. Chapter 2 will discuss the efficacy and effectiveness of a number of dietary strategies for weight loss and weight maintenance in overweight women with PCOS. Furthermore, there is a lack of long-term studies (>1 year) assessing the efficacy and sustainability of different dietary and lifestyle strategies in overweight women with PCOS. The longest follow-up time thus far is 12-15 months, with reported drop-out rates at this time of 23–39% (262, 300). This indicates relatively poor retention rates and sustainability, although in contrast Gambineri only observed a 5% drop-out rate over 12 months with a structured low calorie diet, flutamide/metformin/placebo trial, monthly follow up visits and overall weight loss of 5 kg (298). In comparison, long-term lifestyle modification trials in overweight people with IGT demonstrate modest sustainable weight losses (5.6 kg with dropout rates of 7.5% over 3 years) through lifestyle intervention (a low fat diet, 150 minutes exercise per week and behaviour management strategies) (390) which reduced the risk of developing T2DM or the metabolic syndrome by 58% and 41% respectively (390, 391). Significant improvements in the cardiovascular risk profile, hypertension, hyperlipidaemia (392), inflammation, fibrinogen (393), were also demonstrated. With regards to metabolic features, these programs are also more successful and cost-effective (394) than the insulin-sensitising agent metformin, commonly used to treat IR and IR-associated features of PCOS. There is thus good evidence that long-term maintenance of a reduced weight will help reduce the risk of IGT and T2DM and aid in long-term management of reproductive fitness in women with PCOS.

1.8: PATHOPHYSIOLOGY RELEVANT TO IMPLEMENTATION OF LIFESTYLE MANAGEMENT IN PCOS

1.8.1: Overview of pathophysiology relevant to lifestyle management implementation in PCOS

Although a high proportion of overweight and obesity is reported in PCOS, it is unclear if women with PCOS are predisposed to obesity or if obesity solely acts to worsen the presentation of PCOS. Furthermore, although it is anecdotally reported that women with PCOS have difficulty in achieving and maintaining weight loss, there is a dearth of research assessing this issue. Indirectly supporting this are a number of studies reporting worsened retention rates for weight loss studies in PCOS (discussed in section 1.7.3.5). Similarly, in short-term weight loss studies, dropout of rates of 26–38% over 1–4 months are reported for women with PCOS (302, 303) compared to rates of 8–9% over 4 months in non-PCOS subjects for comparable short-term weight loss interventions (321, 395, 396). However, both females and subjects of a younger age exhibit higher dropout rates in weight loss interventions (397-399) and it is unclear if these differences in retention rates are due to the population group studied (pre-menopausal women). If women with PCOS exhibit poorer retention rates to weight loss protocols, they may experience greater difficulties with weight loss and maintenance than women without PCOS. The potential reasons for this are unclear.

Body weight is regulated through a balance between energy input and energy output, with weight gain occurring where there is an imbalance between energy input and energy output. The balance between these factors depends on the interaction between a range of biological (appetite, energy expenditure), behavioural (dietary and physical activity patterns) and environmental and social factors (societal change of a more sedentary lifestyle, decrease in physical activity and increases in dietary energy intake possibly mediated by increases in the energy density of food) (400) (Figure 1.5). The biological regulation of energy homeostasis is a complex process that involves the individual and synergistic action of physiological and

psychological factors including energy expenditure, appetite regulation and macronutrient balance. Thus, potential physiological and behavioural mechanisms that could predispose women with PCOS to weight gain or confer resistance to weight loss include those that favour an increase in energy intake (eg physiological or psychological dysregulation of feeding behaviour) or modification of energy intake through nutrition partitioning or substrate oxidation or those that favour a decrease in energy output (eg dysregulation of energy expenditure, reduction in exercise tolerance or change in physical activity behaviour). A specific population may therefore be at a higher risk of developing obesity due to abnormalities in the physiological or psychological components contributing to energy homeostasis, ie they may have a genetically determined increased susceptibility to developing obesity that would particularly manifest in an adverse environment.

It is additionally suggested that the hyperinsulinaemia and IR commonly present in many cases of PCOS is the characteristic that contributes to any difficulty in weight management. However, a range of studies in different ethnic groups have shown that compared to relatively more insulin sensitive subjects, subjects with elevated insulin secretion, hyperinsulinaemia or IR (using surrogate fasting or post-prandial or clamp measures of IR) gain either more weight (401-403), similar amounts of weight (404-408) or lesser amounts of weight (409-412) in free-living observational studies (with follow up times of up to 26 years) or clinical weight loss interventions. The reasons for these discrepant results likely include population differences including subject age, menopausal status, current obesity status, ethnicity or predisposition to metabolic disease. The application of this principle to overweight women with PCOS and IR is also unclear. Hyperinsulinaemia could represent either an adaptation to confer resistance to weight gain through centrally suppressing food intake (discussed in section 1.8.3.3) or partitioning lipids away from adipose tissue storage (413-415) or a mechanism to increase obesity through the inhibition of lipolysis or reduction of the thermic effect of food (416, 417).

Figure 1.5: The major causal linkages among genetics, environmental effects, physiology, behaviour and energy balance

NOTE: This figure is included on page 44 of the print copy of the thesis held in the University of Adelaide Library.

The multiple causal roles exist by which both environmental and genetic effects may exert their influence
(418)

1.8.2: Modification of energy expenditure and energy intake in PCOS

There is little data regarding potential abnormalities in energy output or energy expenditure in overweight women with PCOS. While REE does not differ between women with PCOS and weight-matched controls (419), the thermic effect of food was both similar (419) or decreased (420) in women with PCOS. This reduction in the thermic effect of food significantly correlated with the reduced insulin sensitivity in this group and would account for a weight gain of 1.9 kg per year if maintained in the long-term (420). With regards to voluntary energy expenditure or physical activity, there is some evidence that individuals with IR, the metabolic syndrome, T2DM and first degree relatives of patients with T2DM have reduced cardiorespiratory fitness and physical work capacity (421-424) potentially related to reduced insulin sensitivity (425). Orio et al recently demonstrated reduced maximal workload at peak exercise and VO_{2max} (maximal oxygen consumption) that correlated with increased HOMA in overweight women with PCOS compared to age and BMI-matched controls. VO_{2max} indicates the capacity of an individual to perform aerobic work and a reduced VO_{2max} in women with PCOS could indicate a potential barrier to lifestyle modification through decreasing the capacity for exercise (426). As yet, this hypothesis has not been supported in the literature. However, age and weight matched women with and without PCOS demonstrated similar VO_{2max} and peak workload (419) and lean or overweight women with and without PCOS displayed similar levels of self-reported physical activity (427) although the IR status of the PCOS and non-PCOS populations was not reported in either of these studies.

There is some preliminary evidence indicating that food choices may also be altered in PCOS and it has been suggested by a number of investigators that women with PCOS selectively choose foods that could worsen their presentation of PCOS, ie either foods that would predispose to obesity through their high energy density or foods that could have effects on metabolic parameters independent of weight gain. Holte et al proposed that recurrent hypoglycaemia due to hyperinsulinaemia (428) could lead to decreased post-prandial satiety

or increasing craving for or consumption of high carbohydrate foods (219). Supporting this, in healthy lean men reductions in blood glucose following insulin infusion suggestive of reactive hypoglycaemia were associated with increased energy intake and increased consumption of high fat foods (429). However, the limited number of studies assessing free-living food intake in women with and without PCOS show no major differences in nutrient intake. Lean women with PCOS consume less total energy than weight-matched women without PCOS, suggesting women with PCOS may need to follow more stringent dietary restrictions to maintain weight (427). Conversely, no differences in macronutrient intake or intake of high fat or high carbohydrate foods was observed between lean or overweight subjects with or without PCOS (427). Douglas et al reported similar total energy, macronutrient, micronutrient and high GI food intake in overweight age and BMI-matched women with and without PCOS although the women with PCOS consumed higher amounts of a number of specific high GI foods (white bread, fried potatoes) than the subjects without PCOS (430). Although the women with PCOS were significantly more insulin resistant, measures of IR did not correlate with any parameter of dietary intake (430). Wild et al reported that women with PCOS were more sedentary and consumed a diet higher in saturated fat and lower in fibre, however the subjects with PCOS were significantly heavier (431). Finally, age and BMI-matched women with PCOS displayed a trend towards post-prandial sweet craving but no differences in post-prandial fat craving compared to women without PCOS (432). Both the presence of abnormalities in energy homeostasis and the contribution of IR-related abnormalities in energy homeostasis to the development of obesity in PCOS is unclear.

1.8.3: Appetite regulation overview

The physiological control of eating involves peripheral and central hormonal and neural factors predominantly integrated in the arcuate nucleus in the hypothalamus which contains appetite inhibiting and stimulating neuronal populations. The appetite inhibiting circuit consists of cocaine and amphetamine regulated transcript (CART) and proopiomelanocortin

(POMC) co-expressing neurons which produce α-melanocyte stimulating hormone (α-MSH) which act through the melanocortin type 3 and 4 receptor to inhibit appetite. The appetite stimulating circuit consists of neuropeptide Y (NPY) and agouti related peptide (AgRP) co-expressing neurons which promote feeding behaviour through NPY signalling to the paraventricular nucleus (PVN) and AgRP blocking the melanocortin type 4 receptor (reviewed by (433)). Other regions of the brain involved in regulation of appetite include the hypothalamic PVN, dorsomedial hypothalamus, lateral hypothalamic nucleus and nucleus tractus solitarus and area posterna in the brainstem, parts of the limbic system, the amygdala and the cerebral cortex. Short-term feeding behaviour (day-to-day food intake) is controlled through integrating neuro-endocrine, behavioural and psychological factors. The hypothalamus integrates peripheral signals involved in the regulation of energy homeostasis (circulating appetite hormones), mechanical signals (stretch) and visual, olfactory and social cues (smell, sight, social context of food). The other areas of the brain either integrate signals from neuronal projections from the arcuate nucleus or relay neural (vagal) and hormonal signals from the gastrointestinal tract to the hypothalamus (434) (Table 1.7, Figure 1.6).

Circulating adiposity signals are produced in proportion to body fat stores (such as insulin or leptin) or inverse proportion (such as adiponectin) to body fat stores (discussed in section 1.3.1.3). These adiposity signals reflect long-term energy balance and can additionally affect the central regulation of signals affecting short-term energy balance (day-to-day energy intake and expenditure). Leptin, insulin and adiponectin receptors are present in the brain in regions including the arcuate nucleus (435-438). Central effectors integrate adiposity, satiety and other central nervous system signals and allow for the contribution of additional psychological and environmental factors that can influence meal size such as dietary restraint, sight, smell, texture, memory of food, learning, habits, social situation, stress or emotions to energy homeostasis regulation (439). Adiposity signals and central effectors can alter food intake through changing the sensitivity of the central nervous system to satiety signals (434).

It is proposed that this modulates feeding behaviour in situations of long-term positive or negative energy balance, simplistically through a reduced concentration of adiposity signals (eg leptin, insulin) decreasing the effectiveness of satiety-inducing input signals, reducing catabolic and increased anabolic pathways and overall stimulating feeding and reducing energy expenditure (440). The effect of adiponectin on these pathways is as yet unknown. Therefore although short-term meal regulation can be affected by social, psychological and environmental over physiological cues, longer-term

Table 1.7: Hypothalamic and gut peptides involved in appetite control

NOTE: This figure is included on page 48 of the print copy of the thesis held in the University of Adelaide Library.

(442)

Figure 1.6: Energy homeostasis and peripheral signals

NOTE: This figure is included on page 49 of the print copy of the thesis held in the University of Adelaide Library.

Energy homeostasis is controlled by peripheral signals from adipose tissue, pancreas and the gastrointestinal tract. Peripheral signals from the gut include peptide YY (PYY), oxyntomodulin (OXM), ghrelin, pancreatic polypeptide (PP), glucagon-like peptide 1 (GLP-
1) and cholecystokinin (CCK). These gut-derived peptides and adiposity signals influence central circuits in the hypothalamus and brain stem to produce a negative (-) or positive (+) effect on energy balance. The drive to eat and energy expenditure are thus adjusted so that over time, body weight remains stable.

(443)

Short-term control of food intake occurs through altering the initiation, termination or size of individual meals and key factors are those involved in meal initiation, factors involved in maintaining feeding once a meal has begun and factors involved in mediating meal termination (444). Signals that influence food intake include satiety signals (signals that inhibit further eating and the duration of time until the initiation of the subsequent meal or between-meal hunger) or satiation signals (signals that bring the process of eating to a close when fullness or absence of hunger occurs or meal termination signals representing within-meal hunger) (434, 445). Prior to and during a meal, hormonal and sensory (visual, olfactory, taste) stimuli occur to promote and maintain food intake through neural and hormonal feedback to the central nervous system. Food consumption thus continues until negative physiological feedback stimuli (mechanical and physical stimuli including distension, change in nutrient concentration, pH and osmolarity of gut contents, interaction of nutrients with hormonal receptors on the gastrointestinal tract lining, gut peptides and metabolic effects of absorbed nutrients) override these positive stimuli (444).

The individual and interactive influences of the large number of gastrointestinal hormones (Table 1.7) released post-prandially result in overall satiety and satiation. Potential candidate molecules for satiety effects include ghrelin and PYY and for satiation effects include CCK, GLP-1, bombesin and somatostatin. Short-term satiation is additionally related to physical measures including stomach fullness or distension, the functions of acute satiating signals such as CCK, GLP-1 and bombesin include altering pyloric pressure, stomach motility, stomach muscle relaxation, delaying gastric emptying and increasing gastric distension (446, 447). Food intake is thus determined by meal size (related to satiation), meal frequency (related to satiety) and meal termination (related to the hunger before the meal and the perception of satiety during the meal). Peripheral signals of key importance involved in the regulation of energy homeostasis to be discussed in this literature review in more detail are the anorexigenic gut hormones PYY and CCK and the orexigenic gut hormone ghrelin.

1.8.3.1: Cholecystokinin, peptide YY and ghrelin

CCK is released from the small intestine post-prandially and acts through CCK1 receptors located on the sensory fibres of the vagus nerve innervating areas including the pyloric sphincter and proximal duodenum (448). There is emerging evidence that the CCK2 receptor is involved in mediating the effect of CCK on food intake, demonstrated by the development of obesity and hyperphagia in CCK2 knockout mice (449). Longer post-prandial elevations are observed for protein and fat compared to carbohydrate preloads (450-452). In humans, CCK inhibits gastric emptying, reduces meal size and calorie intake and influences pancreatic, hepatic and gallbladder enzyme secretion to aid digestion (447, 453, 454). CCK is predominantly related to satiation rather than satiety. While CCK administration pre-prandially reduces meal size this only occurs if administered > 15 minutes pre-prandially (455) and intermeal administration does not affect intermeal interval (456). While administration of CCK pre-prandially reduces meal size, when continued over a period of days total food intake and body weight are unaffected due to compensatory increases in meal size (456, 457). However, post-prandial satiety has been found to be both related (458, 459) and unrelated (458, 460) to post-prandial CCK profiles.

Peptide YY (PYY) 3-36 is a peptide secreted from the intestinal L-cells in the gastrointestinal tract predominantly in response to protein (461). It is present throughout the gut with the highest tissue concentrations in the distal regions of the GI tract (462). It is secreted following food intake with peak levels occurring 1–2 hours post-prandially and remaining elevated for 4–6 hours (462) and post-prandial PYY elevations are proportional to meal size (463). This inhibition of food intake occurs both at the next meal and in the cumulative 24-hour intake (463). In addition to its proposed role in appetite regulation, PYY reduces gastric emptying and delays gastrointestinal transit (464). The post-prandial effects of PYY are likely to be mediated by both a neural mechanism through activation of the NPY Y_2 receptor expressed on the NPY/AgRP neurons (465) (as levels rise prior to arrival of nutrients in the intestine) and

an effect of intra-luminal contents (due to the sustained increased levels). PYY appears to be an important factor in the mediation of satiety.

Ghrelin is a 28 amino acid acylated peptide produced primarily by the oxyntic cells in the stomach and the duodenum, ileum, caecum and colon (466). It stimulates GH secretion through its action as an endogenous ligand for the hypothamalamic-pituitary growth-hormone secretagogue receptor (GHS-R). Additionally, it is implicated as an important regulatory peptide in food intake, body weight regulation, adipocyte regulation, endocrine pancreatic function, glucose metabolism and ovarian function (467, 468). Ghrelin levels are elevated during fasting or prior to feeding onset (469-472) and stimulate hunger and food intake (473-475) through action on the AgRP and NPY hypothalamic arcuate nucleus neurons (476) with a potential vagal contribution (477). Once feeding commences, ghrelin levels fall rapidly. This is likely due to the presences of nutrients in the gut or metabolic response to feeding as gastric distension demonstrated through ingestion of water does not alter ghrelin (475). Ghrelin is thus implicated as an acute meal initiator. This effect of ghrelin administration is not attenuated or compensated for after multiple injections, indicating it is may also play a role in longer-term energy homeostasis (475, 478). This may be through alterations in energy expenditure, although this effect appears to be more pronounced in rats than humans (473).

When ghrelin is measured over a 24-hour period with meals administered at set times, post-meal ghrelin suppression is related to the energy content of the immediately preceding meal (479) (480, 481) and correlates with post-meal decreases in hunger and increases in satiety (338), suggesting potential roles in meal initiation and satiety (482). Leidy et al observed the combined energy content of a number of preceding meals is related to the pre-prandial ghrelin increase of subsequent meals (479), thus suggesting a cumulative contribution of energy intake on reducing pre-prandial ghrelin peaks. This could potentially provide a lesser stimulus

for meal initiation and contribute to extending the intermeal interval (479) although this was not found to be the case in a recent study (481).

1.8.3.2: Appetite hormones, obesity and diet composition

The effect of obesity on PYY and CCK is unclear. Both reductions in fasting PYY and impaired post-prandial PYY (463, 483-485) have been observed in overweight subjects. Fasting CCK levels have been variably reported as lower (486), higher (487) or not different (451) between overweight and lean subjects. French et al observed a significantly higher CCK post-prandial response after a high fat preload for obese subjects compared to lean subjects (487). This was additionally related to a lower subjective post-prandial hunger score for the obese subjects (487) that could potentially represent decreased receptor sensitivity to CCK with consequent increased CCK production through negative feedback. Conversely, post-prandial CCK levels were similar between obese versus lean subjects (451, 488, 489) and in studies where CCK was intravenously infused, post-infusion subjective appetite was similar for lean and obese humans suggesting no impairment of CCK sensitivity in obesity (490).

Fasting ghrelin levels have been reported as 27–62% decreased in obese compared to lean subjects (471, 472, 491) and ghrelin negatively correlates with weight (492), fat mass (491, 492), BMI (471) and abdominal fat cell size (492). These data indicate ghrelin is down regulated in obesity. If ghrelin is a physiological meal initiator, this could be interpreted as an appropriate physiological adaptation to the positive energy balance associated with obesity. Fasting ghrelin is also decreased further in both lean and obese Pima Indians (a population predisposed to obesity) compared to age, gender and weight matched Caucasians (491). Conflicting data exists as to the response of ghrelin to meals in obese subjects. English et al observed a 39.5% decrease in plasma ghrelin after a meal in lean subjects, with no decrease in obese subjects and suggest this lack of suppression of ghrelin could lead to increased food consumption (472). However, Cummings et al 2002 reported a similar post-prandial ghrelin

decrease after feeding in obese subjects before and after weight loss and normal-weight controls (470). In n=57 overweight hyperinsulinaemia men and women, we observed that a 9.2 kg weight loss increased fasting ghrelin and improved the post-prandial ghrelin response after weight loss such that the post-prandial nadir occurred at 60 minutes post-prandially post-weight loss compared to 120 minutes post-prandial pre-weight loss (493). This timing of the post-prandial nadir post-weight loss is similar to that observed in lean individuals (469, 472) suggesting a potential improvement of appetite regulation by ghrelin following weight loss.

The effect of macronutrient intake on post-prandial ghrelin is unclear. Maximal suppression of post-prandial ghrelin was reported for high carbohydrate compared with high fat isocaloric weight-maintaining diets (494) and meals (495). Increases in post-prandial ghrelin with high protein loads have been observed in a number of studies (496, 497) while dietary protein was reported as suppressing the post-prandial ghrelin nadir compared to carbohydrates (451). A separate study reported a similar post-prandial ghrelin response for a high fat or high protein equivalent carbohydrate test meal despite a greater satiating effect of the high protein meal (493).

1.8.3.3: Appetite regulation and insulin

As discussed above, it is possible that hyperinsulinaemia and IR could confer resistance to weight loss. However, the influence of insulin on feeding behaviour is still not completely understood. Circulating insulin can reach the brain through receptor mediated transport across the blood brain barrier or to regions of the brain with a less effective blood brain barrier (498). Insulin receptors are present in a number of areas of the brain including the arcuate nucleus, dorsomedial hypothalamus, paraventricular nucleus, suprachiasmatic and periventricular region. Central insulin infusions reduce food intake (499), likely through mechanisms including activation of arcuate nucleus POMC/CART and inhibition of NPY/AgRP neurons (reviewed by (500)). As mentioned above, adiposity signals can also alter food intake through

changing the sensitivity of the CNS to satiety signals (434). One example of this is the effect of increase in leptin or insulin following weight gain on increasing the sensitivity of the central nervous system to CCK, thus increasing the ability of CCK to reduce meal size (501-503). First degree relatives of people with T2DM had significantly lower fasting serum PYY in conjunction with lower insulin sensitivity (504). It is not known if this represents altered PYY regulation in insulin resistant states, as PYY inhibits pancreatic insulin secretion in animal models (505) but not in humans (506).

The relationship of insulin with feeding behaviour may also be through a role in mediating the effects of the peripheral appetite hormones. In human studies, fasting ghrelin negatively correlates with fasting insulin (491), insulin infusions decrease plasma ghrelin (507, 508) and OGTT-induced reductions in ghrelin correlate with insulin sensitivity (509). This suggests that decreases in ghrelin may be related to elevated insulin levels observed acutely following feeding or chronically in obesity (507). This may also aid in explaining the lower fasting ghrelin levels observed in Pima Indians, a population with very high reported rates of both obesity and IR (510). Ghrelin is additionally proposed as an inhibitor of insulin secretion and recent data indicates that the active (acylated) and inactive (des-acylated) forms of circulating ghrelin have differing effects on post-prandial ghrelin regulation with acylated ghrelin increasing glucose and insulin concentrations and des-acylated ghrelin preventing this increase (511).

1.8.3.4: Appetite regulation and reproductive steroids

Alterations in reproductive steroids may also affect feeding behaviour or peripheral appetite hormones. Alterations in eating behaviour have been reported across the human menstrual cycle with general patterns of elevated appetite (512), elevated food cravings and elevated food intake in the post-ovulatory phase compared to the pre-ovulatory or follicular phase reported (reviewed by (513, 514)). This increase in energy intake (515) is potentially

associated with the reduction in insulin sensitivity (assessed as reduction in adipocyte insulin receptor binding) (516) that occurs during the luteal phase of the menstrual cycle. It may also be due to fluctations in reproductive steroids such as oestrogen and progesterone. Receptors for oestrogen and progesterone are present in the hypothalamic arcuate nucleus (517) and circulating steroids can cross the blood brain barrier. Although poorly studied, proposed effects of these steroids or their metabolites on feeding behaviour may occur through the serotonergic system (518) or the gamma-aminobutyric acid-A receptor (519). Furthermore, the effects of oestradiol include inhibition of food intake through increasing the satiating effect of CCK (potentially in the hypothalamus or brainstem), demonstrated in rat models (520) and an increase in CCK in the luteal phase of the menstrual cycle (521). There is additionally a hypothesised excitatory effect of moderate doses of testosterone on food intake through increasing meal number in rats, although the mechanism for this effect is as yet unknown (reviewed by (522)). The effects of testosterone in humans is unclear although androgen or androgen-oestrogen administration in oophorectomized women increased appetite (523). It is unclear if the above mechanisms represent a potential system by which feeding behaviour is altered in PCOS. Although not consistently demonstrated (204) some investigators report that obese women with PCOS have higher circulating oestrogen and oestrone levels compared to lean women with PCOS (257) and elevated balance of active (higher production of oestrone-sulfate) to inactive (decreased production of oestradiol metabolites such as 2-hydroxyoestrogens) oestrogens (257) due to increased peripheral aromatase activity in adipose tissue (524) and obesity-associated reduced SHBG levels. Furthermore, the effect of testosterone on appetite in humans is poorly studied and no significant alterations in food intake were observed over the menstrual cycle for anovulatory women (525).

1.8.3.5: Appetite hormones and PCOS

There is some preliminary evidence that appetite hormones are dysregulated in PCOS as overweight women with PCOS have a reduced post-prandial CCK response compared to weight-matched controls (432). Fasting ghrelin levels are lower in overweight women with PCOS compared to weight-matched controls in some (526, 527) but not all studies (528). Furthermore, women with PCOS who were relatively more insulin sensitive had similar fasting ghrelin levels to controls and significantly higher fasting ghrelin levels than relatively more insulin resistant women with PCOS (526). However, the circulating levels of orexigenic neuropeptide galanin were similar between lean, overweight and obese women with PCOS and controls. Circulating NPY was higher in lean women with PCOS compared to lean controls, similar in overweight women with PCOS compared to overweight controls and lower in obese women with PCOS compared to obese controls (529).

A factor receiving considerable interest in recent years was the potential contribution of leptin to reproductive abnormalities in PCOS. In overweight women with PCOS compared to weight-matched controls both elevations of (530-532) or no differences in circulating leptin (533-535) or decreases in soluble leptin receptor and increases in free leptin (536) or no differences (535) have been reported. Furthermore, treatment with anti-androgens, oestrogens and insulin do not generally affect serum leptin levels in humans (537, 538). Of interest, women with and without PCOS undergoing IVF or gamete intrafallopian transfer who succeeded in becoming pregnant within three cycles had significantly lower follicular fluid leptin levels (539). It is therefore unclear whether differences in leptin independent of obesity exist in PCOS and the potential impact of leptin on reproductive function in PCOS or obesity is also unclear.

As previously discussed, IR and hyperinsulinaemia are observed in lean and obese women with PCOS. The IR and hyperinsulinaemia commonly observed in PCOS could potentially

result in lower fasting ghrelin levels. The reduction in fasting ghrelin may be observed in association with impaired post-prandial ghrelin (as discussed above), which further implies potential abnormalities in appetite regulation in PCOS. However the data is conflicting. Other data reports no changes in circulating ghrelin following insulin infusions modifications (540) and one study additionally reported the post-prandial ghrelin decrease occurred in the absence of a post-prandial insulin increase (541). As yet the relationship between positive and negative energy balance, ghrelin and insulin is unclear in individuals with and without PCOS.

The post-prandial changes in ghrelin, CCK and PYY following a meal may be altered partially or fully in obesity. These changes could be interpreted as compensatory changes to induce negative energy to aid weight loss, alterations caused by the weight gain that are conducive to the maintenance of an increased weight or physiological alterations present in the individual prior to weight gain that predisposed the individual to gain weight. It is therefore not clear if the post-prandial appetite hormone alterations potentially present in obesity alter appetite and feeding behaviour. If women with PCOS are a population predisposed to weight gain, difficulties with weight loss or difficulties with weight maintenance, additional abnormalities in the regulation of peripheral appetite hormones are thus one potential explanation. It is also not clear if modest weight loss would improve any potential abnormality in appetite regulation. The effect of PCOS status on post-prandial CCK has been poorly studied and the effect of PCOS status on post-prandial appetite, ghrelin, PYY and food intake either before or after weight loss has not previously been investigated. This will be examined in Chapter 4 and 5. Furthermore, if the differential satiating effects of altering macronutrient composition is mediated by gastrointestinal hormones where potential differences in appetite hormone regulation exist in obesity or PCOS, these could be partially overcome by selecting macronutrients that maximise the post-prandial appetite hormone response and thus maximise satiety and/or satiation. This has not been investigated in PCOS and will be examined in Chapter 4.

1.9: THESIS AIMS AND HYPOTHESES

1.9.1: Thesis aims

1.9.1.1: Summary of thesis aims

The overall aim of my thesis was to investigate the dietary management of overweight women with PCOS and to examine how modifying dietary composition affects a range of outcomes in PCOS including appetite regulation, risk factors for CVD and T2DM and reproductive outcomes. The principles of dietary management of PCOS are currently those followed by the general population for weight management. However, if women with PCOS are a population with difficulties in appetite regulation, it is crucial to examine the effectiveness of different short and long-term structured and free-living dietary strategies. Furthermore, if women with PCOS are to follow similar dietary advice to the rest of the overweight population, it is crucial to determine if they experience the same benefits of weight loss on risk factors for CVD and T2DM. I therefore examined the effect of equivalent modest weight loss in overweight women with and without PCOS on a range of novel and traditional risk factors for cardiovascular disease. A key factor in implementing dietary management of obesity is the ability of the population to achieve weight loss. I therefore examined potential barriers to achieving weight loss in overweight women with PCOS and without PCOS. Specifically, I examined if differences in appetite hormones, appetite regulation and feeding behaviour occurred in PCOS and the relationship of appetite and appetite hormones to anthropometric and hormonal features of PCOS. I also assessed if post-prandial appetite and appetite hormones were altered by weight loss or by modifying dietary composition.

1.9.1.2: Specific thesis aims

1: To assess the effect of minimal intervention short-term strategies on weight loss, body composition, reproductive and metabolic parameters and menstrual cyclicity in overweight women with PCOS.

2: To assess the effect of two long-term (6 month) minimal intervention dietary strategies (moderate carbohydrate or fat restriction) on maintenance of weight and reproductive and metabolic parameters in overweight women with PCOS.

3: To assess the effect of equivalent modest weight loss on risk factors for Cardiovascular Disease and Type 2 Diabetes Mellitus (adiponectin, C-reactive protein, lipid profile, insulin and glucose homeostasis) in overweight weight-matched women with and without PCOS.

4: To assess post-prandial appetite, appetite hormones (ghrelin, CCK, PYY) and food intake in overweight women with and without PCOS before and after weight loss with a number of dietary compositions.

1.9.2: <u>Specific thesis hypotheses</u>

1: That a short-term minimal intervention dietary strategy (meal replacements) will cause reductions in weight and waist circumference and improve glucose and insulin homeostasis, lipid profile, reproductive hormone profile and menstrual cyclicity.

2: That a longer-term *ad libitum* dietary strategy moderately restricted in carbohydrate with low GI food choices will result in a greater proportional intake of protein, a lower GI and GL and a greater weight loss and improvement in reproductive and metabolic parameters compared to an *ad libitum* dietary strategy moderately restricted in fat with low GI food choices.

3: That overweight women with PCOS will demonstrate a significantly greater improvement in novel and traditional cardiovascular risk factors following an equivalent weight loss compared to overweight women without PCOS.

4: That overweight women with PCOS will have a lesser post-prandial reduction in ghrelin and an impaired post-prandial increase in PYY and CCK compared to weight-matched women without PCOS and that that this will be related to a greater degree of post-prandial subjective hunger and desire to eat, a lesser degree of post-prandial subjective satiety and a greater consumption of food at an *ad libitum* buffet meal.

5: That modest weight loss will improve the post-prandial ghrelin, PYY and CCK profiles and that this will be related to reduced food consumption.

CHAPTER 2: WEIGHT LOSS AND WEIGHT MAINTENANCE STRATEGIES IN POLYCYSTIC OVARY SYNDROME

2.1: ABSTRACT

Polycystic ovary syndrome (PCOS), a common endocrine condition in women, improves with weight loss. Meal replacements for short-term weight loss and strategies for weight maintenance have not been investigated in PCOS. The objective of this study was to assess in overweight women with PCOS the use of meal replacements as a short-term weight loss strategy, followed by a carbohydrate or fat restriction strategy on weight maintenance and consequent improvements in reproductive and metabolic parameters. Overweight women with PCOS (n=43; age=32.1±5.2 years; weight=96.1±18.4 kg; mean±SD) followed an 8 week weight loss (2 meal replacements/day, 4904.4±127 kJ) followed by a 6 month carbohydrate (<120 g/day) or fat restricted (<50 g/day) low glycaemic index and saturated fat weight maintenance regime. Thirty two women completed the weight loss phase and 23 women completed the weight maintenance phase, with similar dropouts in each diet group. During the short-term phase, reductions in weight (5.6±2.4 kg), waist circumference (6.1±2.5 cm), body fat (4.1±2.2 kg), insulin (2.8±1.1 mU/L), total testosterone (0.3±0.7 nmol/L) and free androgen index (3.1±4.6) ($P<0.05$) occurred. These changes were sustained during weight maintenance with no differences between diet groups for any variables. At 6 months, both approaches resulted in a net weight loss of 4.7±4.6 kg. Improvements in menstrual cyclicity occurred for 16/28 (57.1%) of subjects. In conclusion, meal replacements are an effective strategy for the short-term management of PCOS. Advice on moderate fat or carbohydrate restriction was equally effective in maintaining weight reduction and reproductive and metabolic improvements.

2.2: INTRODUCTION

It is unclear which dietary strategies are optimal for weight management in a free-living situation with no more than 15 % of subjects undergoing weight loss interventions maintaining their reduced weight with follow-up times of up to 14 years (277).. Some

evidence indicates weight is maintained more effectively and compliance is increased when an *ad libitum* low fat high carbohydrate dietary pattern is followed (316). However, these regimes may result in poor long-term compliance (317) and a worsening of the metabolic profile (318). Due to potential abnormalities in energy expenditure (420) and appetite hormone homeostasis (432, 526), weight loss and maintenance of a reduced weight may also be more difficult to achieve for women with PCOS. Dietary regimes addressing these abnormalities and offering more intensive or structured weight loss protocols may be preferable for overweight women with PCOS. However, effective dietary approaches to achieve and maintain weight loss and metabolic and reproductive benefits in women with PCOS in a free-living environment have been poorly studied.

Alternative dietary compositions including increasing dietary protein and reducing glycemic index (GI) and glycemic load (GL) may be more successful for optimally improving the metabolic profile (331, 365, 373), improving insulin sensitivity (380, 542) and aiding weight loss and maintenance, through effects on increasing satiety (332, 362) and preserving lean body mass (542, 543). When adopted *ad libitum*, both of these approaches reduced spontaneous energy intake, weight and total fat mass (337, 364) compared to conventional low fat high carbohydrate diets over 6 months. A simple strategy to reduce energy intake is also to focus on restriction of one macronutrient. Severe carbohydrate restriction (20–30 g/day) resulted in greater weight loss than a structured reduced fat intake (moderate calorie restriction, 25–30% calories from fat) over 6 months (349, 350, 355, 359) in the general population. Long-term weight maintenance studies in PCOS tend to be in a highly structured environment with a variety of health professionals (262, 300) and the efficacy of a simple dietary strategy has not been assessed in this population. The objective of this study was to examine the effects of acute energy restriction on reproductive and metabolic characteristics of PCOS. In addition, we wished to compare two different long-term dietary strategies (moderate carbohydrate restriction low GI or moderate fat restriction low GI) on weight loss

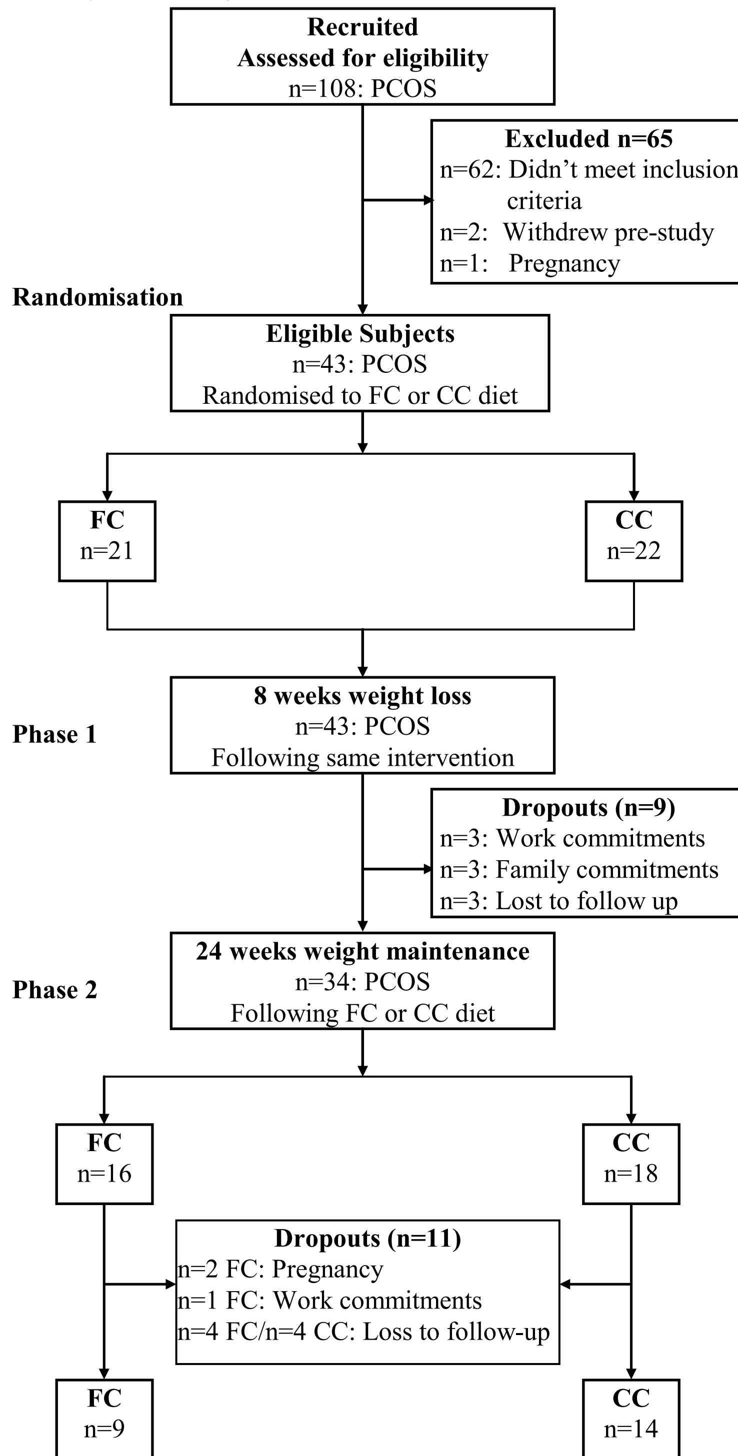
and maintenance, body composition, energy expenditure, hormonal, metabolic and reproductive characteristics in overweight women with PCOS.

2.3: METHODS

2.3.1: Subjects and recruitment

Overweight pre-menopausal women (European Caucasian) with PCOS (n=43) were recruited through public advertisement. Consort criteria are documented in Figure 2.1. The study was approved by the Human Ethics committees of the CSIRO Division of Health Sciences and Nutrition, The Royal Adelaide Hospital and the Womens and Childrens Hospital of South Australia and all subjects gave informed written consent. Inclusion criteria were diagnosis of PCOS according to the Rotterdam consensus group by two of the following three criteria: 1) Menstrual irregularity (cycle length < 26 days or > 31 days or variation between consecutive cycles of > 3 days); 2) Clinical (hirsutism assessed by Ferriman-Gallwey score > 8) or biochemical hyperandrogenism (free androgen index > 5.4 or testosterone > 1.4 nmol/L, defined from a representative population of non-PCOS women (n=80) (544)) or 3) Positive ultrasound presentation of polycystic ovaries by trans-vaginal scan (124). Exclusion criteria were pregnancy, breastfeeding, body mass index (BMI) < 25 kg/m^2, Type II Diabetes Mellitus (T2DM) and related endocrine disorders (excluded by assessment of thyroid stimulating hormone (TSH), prolactin and 17α-hydroxyprogesterone). Use of endocrine hormonal treatment or insulin-sensitising agents was not permitted during both phases of the study and use of oral contraceptives was not permitted during Phase 1 of the study. Subjects were required to cease oral contraceptives 4 weeks and hormonal treatment/insulin-sensitising agents 2 weeks prior to commencement of the short-term study phase. From weeks 8–32 (phase 2), subjects were allowed to take oral contraceptives with ethinyl oestrogen < 35 mcg.

Figure 2.1: Study flow diagram



Flow diagram of subjects enrolment, random assignment and completion of the 32 week protocol, incorporating Phase 1 (8 weeks where all subjects followed the same intervention of twice daily meal replacements) and Phase 2 (24 weeks where subjects were followed either a fat counting (FC) or carbohydrate counting (CC).

2.3.2: Study design

The study was conducted on an outpatient basis over 32 weeks and consisted of 8 weeks of energy restriction with all subjects following the same dietary protocol (phase 1) and 24 weeks of weight maintenance with subjects following either a carbohydrate-counting (CC) or fat-counting (FC) protocol (phase 2). Subjects were stratified to ensure equal distribution for age, BMI, smoking status and use of oral contraceptives and then the two groups were randomized by an independent observer using the computer program Clinstat to the CC or FC protocol before study commencement.

In phases 1 and 2, subjects attended the clinic fortnightly and monthly respectively. At the initial visit, height was measured to the nearest 0.1 cm using a stadiometer (SECA, Hamburg, Germany) with subjects in the free-standing position. At all visits, body weight was measured to the nearest 0.05 kg using calibrated electronic digital scales (Mettler scales, model AMZ14, A&D Mercury, Kinomoto, Japan) while subjects were wearing light clothing with no shoes. BMI was calculated by weight (kg) divided by squared height (m^2) and waist circumference was measured in triplicate directly on the skin with a soft tape to the nearest 0.5 cm at the level of midway between the lateral lower rib margin and the iliac crest. At weeks 0, 2, 4, 6, 8, 20 and 32, resting blood pressure was measured by automated oscillometry (Dinamapt, 845XT/XT-IEC, Tampa, FL, USA), with subjects in a seated position and overnight fasting venous blood samples were taken for assessment of plasma glucose, insulin and ghrelin and serum lipids, C-reactive protein (CRP), testosterone and SHBG. At weeks 0, 8 and 32, in a subset of subjects (n=13) fasting resting energy expenditure (REE) and respiratory quotient (RQ) were measured over 30 minutes by indirect calorimetry using a ventilated canopy and Deltratrac™ metabolic monitor (Datex Division Instrumentarian Corp., Helsinki, Finland). The instrument was calibrated before each set of measurements. After an 8 hour fast, subjects lay supine on a bed in a thermoneutral environment with a clear plastic hood over their head and shoulders and the REE and RQ were recorded for 30 minutes. The first 10 min of data

were discarded to ensure all subjects had reached equilibrium and the remaining 20 min of data were averaged and represented the values for fasting REE and RQ. The intraindividual coefficient of variation (CV) of the Deltatrac system was established to be 1.77±0.41% for fasting REE, 3.10±0.8% for fasting RQ (545). Body composition was measured by tetrapolar single-frequency (50 Hz) bioelectrical impedance analysis (BIA) (ImpDF50; Impedimed Pty Ltd., Qld, Australia) using the offline general algorithm. Total fat mass (TFM) (CV 2.3±8.7%) and total fat-free mass (TFFM) (CV 2.1±0.4%) were assessed. Duplicate measurement were made while subjects were lying supine and with an empty bladder. A 2-hour oral glucose tolerance test (75 g load) (OGTT) was conducted with assessment of venous glucose and insulin at 0 and 120 minutes. Exercise was assessed by a 7-day 24-hour physical activity record at weeks 0, 8, 20 and 32 (546, 547). Daily physical activity was computed in MET-min·day^{-1}, calculated as the MET intensity multiplied by the corresponding minutes of each activity to yield MET minutes, summed across the day and then averaged over the 7 day recording period. The total energy expenditure was expressed as a mean daily energy expenditure expressed in kJ, computed as: EE (kJ) = MET-min x [body weight (in kg) x (4.186/60)] (548). At week 0 dietary restraint, disinhibition and hunger were measured at week 0 by the Three-Factor Eating Questionnaire (549). Health related quality of life was assessed by the validated PCOS quality of life questionnaire at week 0, 8 and 32 (550, 551).

Subjects documented their menstrual cycles for the study duration and for 6 months before study commencement. During phase 1, first morning urine samples were collected twice weekly and assessed for total urinary pregnanediol-3-glucuronide to determine ovulation status. Results were compared with the menses calendars to qualitatively determine ovulation. Improvements in menstrual cyclicity were defined as a change from nonovulatory to ovulatory cycles or from irregular to regular cycles or an improvement in consecutive intercycle variation.

2.3.3: Dietary treatment

Subjects were provided with a pedometer and encouraged to aim for 8000 steps/day throughout the study. For phase 1 (weeks 0–8) subjects followed an energy restricted diet whereby two meals daily were replaced with commercially available meal replacements (Slimfast™, Unilever Australasia, Epping, New South Wales, Australia) (1800 kJ/day) in addition to a low-fat evening meal per day and at least 5 servings of fruit and vegetables per day (3500 kJ) and sample meal plans and recipes. This involved a restriction of dietary intake to approximately 6000±50 kJ/d or approximately two-thirds of normal intake. Slimfast™ products were provided fortnightly. Alcohol consumption was not permitted. For phase 2 (weeks 9–32), subjects followed either a CC (< 120 g carbohydrate/day) or a FC (< 50 g fat/day) regime. Subjects were provided with a carbohydrate/fat counting resource and asked to daily document their carbohydrate or fat intake of selected foods (Table 2.1). All subjects also received advice on reducing the GI and saturated fat content of their diet. A semi ad-libitum approach was followed and subjects were allowed to consume their specified fat or carbohydrate intake (50 g fat or 120 g carbohydrate) and advised to eat until full from unlimited quantities of additional foods from which they were not required to count fat or carbohydrate from. Subjects were allowed to consume moderate amounts of alcohol (maximum of 2 standard drinks/day and at least 2 alcohol free days/week). Subjects met with a qualified dietitian for initial education on quantification and recording of their daily food intake and to assess and modify the dietary regime based on compliance and weight loss. Subjects then met with the dietitian fortnightly for phase 1 and monthly for phase 2. Nutrient intakes were calculated with Diet 4/Nutrient Calculation Software (Xyris Software, Highgate Hill, Australia), based on data from Australian food composition tables. The database had been extensively modified by our group to add new foods and recipes. For phase 1, nutritional intake was assessed from fortnightly 3–day consecutive dietary food records (one weekday and two weekend days) and daily dietary checklists. For pre-study and phase 2 dietary intake, GI and GL were assessed from 3 monthly Food Frequency questionnaires (Anti Cancer

Foundation) and a monthly 24-hour dietary recall. Dietary compliance was determined by subject adherence to the meal replacement regimes for phase 1 and daily fat and carbohydrate counting in phase 2.

2.3.4: Biochemical measurements

Fasting blood samples were taken by venipuncture. Blood for serum was collected in tubes with no additives and allowed to clot at room temperature for 30 min. Blood for plasma was collected in tubes containing sodium fluoride/EDTA for glucose or K/EDTA and 500 KIU/mL blood aprotinin (Roche; IN, USA) for ghrelin measurement and stored on ice. Serum and plasma were isolated by centrifugation for 10 min at 3000g (5°C) (Beckman GS-6R; Beckman, Fullerton, CA) and aliquots were stored at –80°C. Details of all assay methodologies (SHBG, testosterone, total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), insulin, glucose, CRP, total ghrelin, TSH, prolactin, 17-α progesterone and urinary pregnanediol) are described in Appendix 2. The homeostatic model assessment (HOMA) was used as a surrogate measure of insulin sensitivity and was calculated as [fasting serum insulin (mU/L) x fasting plasma glucose (mmol/L)/22.5] (80). The free androgen index (FAI) (testosterone/SHBG x 100) and equilibrium binding equations for determination of free testosterone (123) were used as surrogate estimates of free testosterone. Study subjects were categorized as having T2DM if fasting glucose ≥ 7.0 mmol/L and/or 2 hour OGTT glucose ≥ 11.0 mmol/L, impaired glucose tolerance (IGT) if 2 hour OGGT 7.8–11.1 mmol/L and impaired fasting glucose if fasting glucose 5.5–6.9 mmol/L (552). Study subjects were classified as having the metabolic syndrome if they had three or more of the following abnormalities: waist circumference > 88 cm, fasting triglycerides ≥ 1.7 mmol/L, fasting high density lipoprotein (HDL-C) ≤ 1.3 mmol/L, blood pressure ≥130/≥85 mmHg and fasting glucose ≥ 6.1 mmol/L (553). Biochemical assays were performed in a single assay at the completion of the study and all samples for individuals were analysed in the same assay.

Table 2.1: The food sources that subjects in the fat counting and carbohydrate counting diet groups were required to count grams of fat or carbohydrate from daily

Fat counting	Carbohydrate counting
Meat (red meat, chicken, fish and eggs) Dairy products (milk, cheese, yoghurt, cream) Nuts and seeds Some fruits (avocados) and vegetables (olives) Margarines, butter, fats, oils, creams and salad dressings. Condiments, sauces and salad dressing (if high fat eg creamy or satay sauces, oily pastes or using more than 40 g) Commercial foods	Breads, crackers and crispbreads and cereals Rice, noodles and pasta Some dairy products (milk, yoghurt) Fruit and fruit juice Legumes Some vegetables (potatoes, sweet potatoes, sweet corn, frozen or fresh mixed vegetables) Condiments, sauces and salad dressings (if using more than 40 g) Commercial foods

2.3.5: Statistics

Parametric data were presented as means±SEM except where indicated. Non-parametric data (Three factor eating questionnaire and PCOS quality of life questionnaire) were presented as median±range. Normality was assessed using the Kolmogorov-Smirnoff test. Data were log transformed for analysis where non-normally distributed. For phase 1, results are presented for 34 subjects except ovulation (n=33), menses (n=28), TFM, TFFM (n=33), 2 hour glucose (n=33), REE, RQ (n=13), CRP (n=29) and ghrelin (n=26) due to incomplete data. For phase 2, results are presented for 23 subjects except REE and RQ (n=11), CRP (n=18) and ghrelin (n=19). Five subjects recommenced oral contraceptive/hormonal treatment in phase 2 and their data was excluded from reproductive hormone analysis. Chi-squared tests were used to assess changes in proportions between diet groups. Two-tailed statistical analysis was performed using SPSS for Windows 10.0 software (SPSS Inc, Chicago, USA) with statistical significance set at α level of $p < 0.05$. Baseline data were assessed using a one-way ANOVA for parametric data and a Kruskal-Wallis test for non-parametric data. Comparisons between time points were assessed using two factor ANOVA time as the within subject factor and diet as the between subject factor. In specific analyses, baseline % total fat, % monounsaturated fat and % carbohydrate intake were included as covariates. In the event of an interaction, post-hoc pairwise comparisons (Bonferroni) were performed. Relationships between variables were examined using bivariate and partial correlations and ANCOVA. Weight, insulin, HOMA, testosterone and free testosterone data was assessed as a completers analysis (data from subjects who completed each phase included) and a baseline intention to treat analysis (baseline values from subjects who dropped out carried forward) or carried forward intention to treat analysis (last clinical value from subjects who dropped out carried forward). As there was a time-by-insulin interaction but no time-by-treatment-by-insulin or treatment-by-insulin interaction, subjects with higher insulin levels at baseline (above the median of 10.45 mU/L) were assessed separately from those with lower fasting insulin levels at baseline (below the median of 10.45 mU/L), with insulin status as between-subject factor. This study had 40%

power to detect a difference of 1.6 kg between diet groups for net weight loss to statistical significance of P < 0.05. To confirm the observed differences between the diet groups of 1.6 kg in net weight loss to statistical significance of P < 0.05 and 80% power a total of 37 subjects in each diet group would be needed. This study was able to detect a difference of 2.6 kg between the diet groups at 80% power and statistical significance of P < 0.05.

2.4: RESULTS

2.4.1: Subjects

Thirty-four subjects completed phase 1 (20.9% drop-out rate) and 23 subjects completed phase 2 (27% drop-out rate) and there were no differences in baseline characteristics of study completers in each of the dietary groups (Table 2.2). Study drop-outs are documented in Figure 2.1. Activity levels were comparable between diet groups at week 0 and did not change throughout the study. There were no differences in the baseline dietary restraint (11.5±16), disinhibition (11±13) and hunger scores (6±12) or study drop-outs between diet groups. Two subjects conceived during phase 2 (both from the FC group) and discontinued the intervention. In phase 1, significant differences existed between subjects who dropped out and those who completed the study for total fat intake (40.0±1.7 versus 35.6±0.9%, P=0.02), saturated fat intake (17.7±1.1 versus 13.4±0.5%, P<0.001) and weight loss at week 2 (1.5±0.4 versus 2.4±0.2 kg, P=0.042). In phase 2, significant differences existed between subjects who dropped out and those who completed for fasting insulin and HOMA at the beginning of phase 2 (13.8±2.0 versus 8.7±0.3 mU/L and 3.2±0.5 versus 2.0±0.2 units respectively, P=0.008). There was a trend for subjects who dropped out of all phases of the study to have higher baseline hunger scores than completers (7.4±0.7 versus 5.7±0.6, P=0.077).

Table 2.2: Baseline subject characteristics

	Phase 1 completer (week 0–8) N = 34	
	FC (n = 16)	**CC (n = 18)**
Age (years)	33.2±4.8	32.1±5.5
Weight (kg)	95.8±21.9	96.1±17.7
BMI (kg/m^2)	34.9±7.0	34.9±6.6
Glucose (mmol/L)	5.2±0.4	5.2±0.4
Insulin (mU/L)	10.8±5.2	14.7±8.5
HOMA	2.5±1.3	3.5±2.2
Testosterone (nmol/L)	2.9±1.2	2.5±0.7
Free testosterone (pmol/L)	66.3±39.3	54.8±24.0
SHBG (nmol/L)	28.1±18.5	28.1±13.6
Free androgen index	14.6±12.2	11.6±7.1

Data are expressed as mean ± SD.
Measurements were made at the week 0 visit and were assessed using one-way ANOVA with diet as the fixed factor. There were no baseline differences between the subjects in the FC or CC group.

FC: Fat counting
CC: Carbohydrate counting
BMI: Body mass index
HOMA: Homeostasis model assessment
SHBG: Sex hormone binding globulin.

2.4.2: Physical activity, diet and compliance

All diets were well tolerated with no adverse events. The energy, macronutrient content and micronutrient content of the diet in phase 1 are presented in Table 2.3 and the diet achieved the recommended micronutrient dietary allowance (327). The energy and micronutrient and macronutrient contents of diets prior to study commencement and for phase 2 are presented in Table 2.4. The micronutrient content of the diets prior to study commencement and for phase 2 are presented in Table 2.5. The subjects in the FC and CC diets had no significant difference in micronutrient intake at any time point. From baseline to week 20 and to week 32, total energy, carbohydrate, protein, fat, GI, GL decreased similarly for the FC and CC groups. There were no differences in energy intake, GI or GL between the FC and CC groups at week 20 or 32 and energy intake remained constant from week 20 to 32. From baseline to week 20, % PUFA and cholesterol decreased similarly for the FC and CC groups.

There was a significant time-by-diet interaction from week 0–20–32 for % carbohydrate (P=<0.001), % fat (P=0.002), % SFA (P=0.002), % MUFA (P=0.001) and fibre (P=0.022) and a trend for a time-by-diet interaction for % protein (P=0.056). From week 0–20 and week 0–32, fibre (P<0.001) and % carbohydrate (P=0.014) decreased for the CC group but stayed the same for the FC group and % total fat (P=0.005) decreased for the FC group and stayed the same for the CC group. From week 0–20 there was a time-by-diet effect such that % SFA (P=0.017) and % MUFA (P=0.011) decreased for the FC group and stayed the same for the CC group and % protein increased for the CC group (P<0.001) and did not change for the FC group. At week 20 the FC group had a lower % fat, SFA and MUFA and higher % carbohydrate intake and a trend for a higher GI (P=0.082) and at week 32 there was a trend for the FC group to have a lower % SFA intake (P=0.054). At week 20, on average subjects complied with the prescribed dietary intervention (FC 37±4 grams fat/day, CC 123±14 grams carbohydrate/day). By week 32, subjects were not complying with the prescribed dietary intervention (FC 54±9 grams fat/day, CC 135±11 grams carbohydrate/day).

Table 2.3: Dietary intake during 8 weeks of energy restriction on one dietary pattern (meal replacements)

Phase 1: Week 0–8	Dietary intake	Recommended Dietary Intake (women 18–50 years)	Recommended Dietary Intake (Pregnancy)
Energy (kJ)	4904.4±127.8	-	
Fat (% energy)	21.0±0.8	-	
Carbohydrate (% energy)	52.9±1.0	-	
Protein (% energy)	24.2±0.4	-	
Vitamin C (mg)	83.3±4.2	45	60
Thiamin (mg)	1.36±0.04	1.1	1.4
Riboflavin (mg)	2.05±0.07	1.1	1.4
Niacin (mg)	18.8±0.57	14	18
Calcium (mg)	996.1±29.1	1000	1000
Iron (mg)	14.0±0.3	18	27
Zinc (mg)	13.9±0.3	8	11

Data are expressed as mean ± SEM.

Table 2.4: Dietary intake at baseline and during 24 weeks on either a fat counting (FC) or carbohydrate counting (CC) dietary protocol (week 8 to 32)

		Week 0	Week 20	Week 32	Time (Week 0/20/32)	Time-by-diet (Week 0/20/32)
Energy (kJ/d)	FC	8143±868	5035±412 *	6191±636 *	P<0.001	P=0.764
	CC	8082±414	5287±637 *	5865±511 *		
CHO (g/d)	FC	188±22.2	135±13.4 *	156±15.7 *	P<0.001	P=0.093
	CC	209±10.6	123±13.6 *	135±10.7 *		
CHO (% energy)	FC	39±1.3 [a]	46±2.5 [ab*]	43±2.4 b *	P=0.927	P<0.001
	CC	44±1.6	40±1.4	40±1.4		
Protein (g/d)	FC	100±10.8	68±5.5 *	81±6.9 *	P=0.003	P=0.803
	CC	94±7.2	78±12.0 *	79±8.4 *		
Protein (% energy)	FC	20±1.0	22±0.9	21±0.7	P<0.001	P=0.056
	CC	19±0.9	23±1.1 [b*]	21±1.0		
Fat (g/d)	FC	84±10.4	37±3.6 *	54±8.9 *	P<0.001	P=0.340
	CC	75±5.8	48±6.8 *	56±6.4 *		
Fat (% energy)	FC	38±1.0 [a]	27±2.1 [ab*]	31±2.2 [b*]	P=0.001	P=0.002
	CC	34±1.2	33±1.0	35±1.2		
SFA (% energy)	FC	14.2±0.9	9.5±0.6 [ab*]	11.6±0.9 [b]	P=0.008	P=0.002
	CC	12.9±0.5	12.7±0.5	13.6±0.5		
MUFA (% energy)	FC	13.6±0.4 [a]	9.5±0.7 [ab*]	11.2±1.0 [b]	P=0.002	P=0.001
	CC	12.1±0.4	12.1±0.3	12.5±0.5		
PUFA (% energy)	FC	6.8±0.4	5.3±1.0 *	5.3±0.7	P=0.014	P=0.234
	CC	5.9±0.6	4.8±0.3 *	5.2±0.5		
Chol (mg/d)	FC	273.7±31.6	157.2±12.7	202.9±24.6	P=0.001	P=0.117
	CC	249.0±28.6	213.6±37.2	231.6±20.9		
Fibre (g/d)	FC	20.9±2.4	16.9±2.1	19.1±2.5	P<0.001	P=0.022
	CC	23.2±1.5	14.0±1.4 [b*]	16.2±1.2 [b*]		
Glycemic Index	FC	53.3±1.3	49.5±0.9 *	49.6±1.1 *	P<0.001	P=0.215
	CC	52.4±0.8	46.5±1.1 *	49.2±1.0 *		
Glycemic Load	FC	100.7±13.6	66.6±6.7 *	77.5±8.9 *	P<0.001	P=0.180
	CC	109.5±6.5	57.7±7.1 *	66.4±6.4 *		

Data are expressed as mean ± SEM.

Data were assessed using one-way ANOVA with diet as the fixed factor and repeated measured ANOVA with time as within-subject factor and diet as between subject factors. For all data FC n=9 and CC n=14 except for week 20 (FC n=8, CC n=13).

CHO: Carbohydrate
SFA: Saturated fatty acid
MUFA: Monounsaturated fatty acid
PUFA: Polyunsaturated fatty acid

[*] Effect of time relative to week 0 (week 0 to week 20 or week 0 to week 32), P<0.05.
[a] Significant difference between FC and CC group, P< 0.05.
[b] Effect of time-by-diet for week 0–20 or week 0–32, P<0.05

Table 2.5: Dietary intake (micronutrient) before study commencement (week 0) and during 24 weeks on either a fat counting (FC) or carbohydrate counting (CC) dietary protocol (week 8 to 32)

Micronutrient	RDI/AI	Week 0		Week 20		Week 32	
		FC N=16	CC N=18	FC N=8	CC N=13	FC N=9	CC N=13
Vitamin C (mg)	RDI: 45 (Pregnancy: 60)	113.3±13.6	140.0±20.6	77.6±6.3	78.1±10.5	101.9±15.8	90.6±7.9
Vitamin E (mg)	AI: 7 (Pregnancy: 7)	5.9±0.6	6.3±0.4	3.4±0.3	3.5±0.4	4.7±0.8	4.2±0.4
Thiamin (mg)	RDI: 1.1 (Pregnancy: 1.4)	1.7±0.2	1.7±0.1	1.0±0.1	1.1±0.1	1.2±0.1	1.1±0.1
Riboflavin (mg)	RDI: 1.1 (Pregnancy: 1.4)	2.5±0.2	2.5±0.2	1.8±0.2	1.9±0.1	2.1±0.2	1.8±0.2
Niacin eq (mg)	RDI: 14 (Pregnancy: 18)	23.6±2.4	23.8±1.7	28.3±2.6	31.6±4.5	17.7±1.9	16.2±1.7
Retinol eq (µg)	RDI: 700 (Pregnancy: 800)	840.5±83.4	880.7±76.8	495.4±40.6	517.6±82.1	590.5±91.8	622.7±59.1
Calcium (mg)	RDI: 1000 (Pregnancy: 1000)	980.1±65.7	951.6±56.2	854.0±83.4	831.6±64.5	938.2±65.4	769.7±73.3
Iron (mg)	RDI: 18 (Pregnancy: 27)	13.3±1.3	14.0±1.0	9.1±1.5	9.0±1.2	11.1±1.4	9.6±1.0
Zinc (mg)	RDI: 8 (Pregnancy: 11)	13.2±0.1	13.2±0.9	8.5±0.7	10.4±1.6	10.4±0.9	10.3±1.1
Potassium (mg)	AI: 2800 (Pregnancy: 2800)	2885.7±244.0	3010.4±129.4	2249.1±151.2	2210.0±207.6	2547.7±206.0	2240.2±166.6
Magnesium (mg)	RDI: 320 (Pregnancy: 360)	294.1±20.8	312.9±14.4	224.6±19.6	213.7±20.0	265.1±25.4	223.3±16.6
Phosphorus (mg)	RDI: 1000 (Pregnancy: 1000)	1637.0±128.9	1658.9±85.6	1218.3±100.6	1285.9±126.4	1418.4±95.5	1286.5±102.8
Folate (µg)	RDI: 400 (Pregnancy: 600)	267.0±24.2	296.4±16.9	188.0±21.1	185.6±15.6	225.2±23.9	195.1±14.2

Data are expressed as mean ± SEM.
Measurements were assessed using one-way ANOVA with diet as the fixed factor.

RDI: Recommended dietary intake for women aged 18–50 years
AI: Adequate intake for women aged 18–50 years
FC: Fat counting
CC: Carbohydrate counting

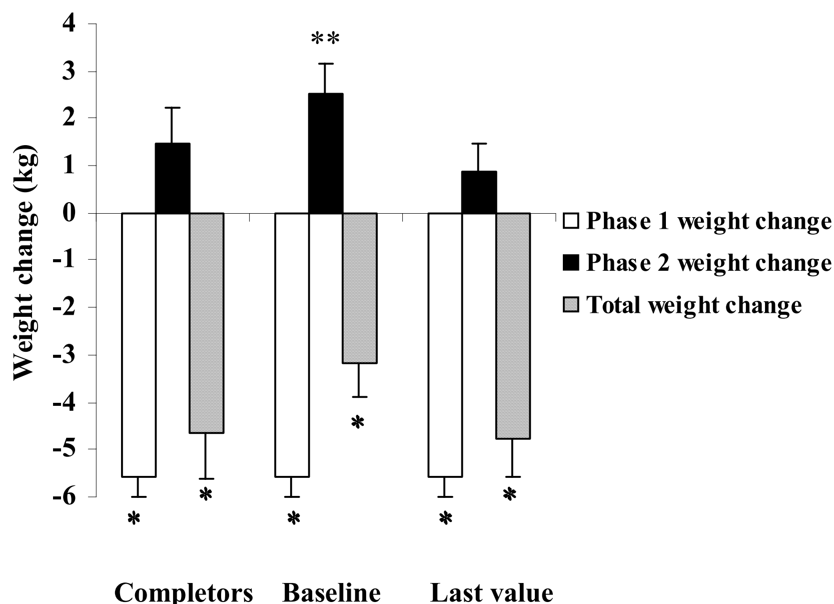
There were no significant differences between the subjects in the FC or CC group for any variables.

2.4.3: Weight loss, body composition, energy expenditure and quality of life

There were no differential effects of diet composition on weight loss, body composition or energy expenditure. Weight decreased during phase 1 (5.6±2.4 kg or 6.0±0.4%, P<0.001) and did not change further during phase 2 (P=0.133) such that at week 32 there was a net weight loss of 4.7±1.0 kg (5.9±2.1 kg for FC, 4.4±0.7 kg for CC) (time-by-diet effect P=0.659) (Table 2.5). At the end of phase 1, 74% of subjects had lost >5% of their body weight. In phase 2 at week 20, 68% of subjects had lost >5% of their body weight. At week 32, 44% of subjects had lost >5% of their body weight. With an intention to treat analysis with the last data point used for subjects that dropped out, the weight loss was still maintained in phase 2. When the baseline data point was used for subjects that dropped out, significant regain of weight occurred from week 8 to 32 (2.5±0.7 kg, P=0.028) such that net weight loss from week 0 to 32 was 3.2 ± 0.7 kg (Figure 2.2).

During phase 1, decreases in waist circumference (6.1±0.4%), TFFM (2.5±0.5%) and TFM (12.3±1.3%) (P<0.001) occurred which were maintained during phase 2, such that decreases in waist circumference (5.9±1.1%), TFFM (2.5±0.9%) and TFM (9.4±2.0%) occurred over the entire study (P≤0.001) (Table 2.5). During phase 1, decreases in REE (expressed as an absolute value) (8.0±1.9%, P=0.003) and RQ (3.3±1.4%, P=0.04) occurred. These decreases were not maintained and there was no overall difference between week 0 and week 32 (Table 2.5). There were no differences between the diet groups for the quality of life domain scores emotional state (4.5±4), hirsutism (3.9±5.2), weight status (2.6±4.4), infertility (6±5.5) and menstrual irregularity (3.75±4.25) at baseline or over the study duration. In phase 1, there was an improvement in the weight status (2.6±4.4 to 3.4±5.6, P=0.013) and the menstrual irregularity domain (3.75±4.25 to 4.5±4.0, P=0.039). There were no changes in the quality of life domains in phase 2 and there were no differences between quality of life measures at study beginning and end.

Figure 2.2: Weight loss for data analysed as completers analysis, baseline value carried forward for study drop-outs and last clinic visit carried forward for study drop-outs



Data are expressed as mean±SEM.

Phase 1: n=34
Phase 2: Completers= Data from subjects who competed each phase included (n=23).
Baseline= Baseline values from subjects who dropped out carried forward (n=34).
Last value=Last value from subjects who dropped out carried forward (n=34).

* Significant effect of time relative to week 0 for all treatments combined, P<0.001.
** Significant effect of time for week 8 to week 32 for all treatments combined, P=0.028
There was no significant time by diet effect.

Table 2.6 Weight, body composition, blood pressure, energy expenditure, lipids, glucose, ghrelin and CRP before and after 8 weeks of energy restriction on one dietary pattern (meal replacements) and 24 weeks of follow-up on either a fat counting (FC) or carbohydrate counting (CC) dietary protocol

	Week 0 Completers Phase 1 N=34		Week 8 Completers Phase 1 N=34		Week 20 Completers Phase 2 N=23		Week 32 Completers Phase 2 N=23	
	CC N=16	FC N=18	CC N=16	FC N=18	CC N=9	FC N=14	CC N=9	FC N=14
Weight (kg) [ae]	95.8±5.5	96.1±4.2	90.3±5.4	90.3±4.1	91.1.±8.9	91.2±5.1	92.4±8.7	92.6±5.0
Waist circumference (cm) [ae]	101.8±3.3	100.2±3.0	95.8±3.5	94.1±2.9	95.5±5.1	94.7±3.3	96.1±4.9	95.8±3.4
Total fat mass (kg) [ae]	36.0±2.4	34.0±1.8	31.7±2.6	30.2±1.9	-	-	33.4±3.9	31.8±2.5
Total fat free mass (kg) [ae]	60.7±3.5	62.2±2.5	59.5±3.2	60.1±2.4	-	-	59.0±5.0	60.8±2.6
SBP (mmHg) [ae]	122.6±3.5	118.0±2.7	111.3±2.8	112.3±2.8	119.5±3.7	114.8±2.9	114.7±4.0	110.6±3.2
DBP (mmHg)	67.5±2.9	66.4±2.1	66.8±2.5	66.9±2.9	66.5±3.0	64.9±3.0	67.8±5.1	66.6±3.3
REE (MJ/day) [a]	7.3±0.3	8.1±0.4	6.7±0.2	7.4±0.4	-	-	6.8±0.3	7.8±0.4
RQ [a]	0.85±0.02	0.83±0.01	0.82±0.01	0.81±0.01	-	-	0.86±0.03	0.84±0.01
Fasting glucose (mmol/L) [ae]	5.2±0.1	5.2±0.1	5.0±0.1	5.1±0.1	5.0±0.1	5.3±0.2	4.9±0.1	4.9±0.2
2 hour glucose (mmol/L)	5.8±0.3	5.4±0.3	5.7±0.3	5.5±0.2	-	-	5.7±0.3	5.8±0.3
Total cholesterol (mmol/L) [abcd]	4.6±0.2	4.7±0.3	3.9±0.2	4.1±0.2	4.5±0.2	4.6±0.2	4.6±0.3	4.7±0.2
LDL-C (mmol/L) [ad]	2.7±0.2	2.9±0.2	2.4±0.2	2.5±0.2	2.5±0.2	2.7±0.2	2.6±0.2	2.8±0.2
HDL-C (mmol/L) [abcd]	1.3±0.1	1.3±0.1	1.1±0.1	1.2±0.1	1.3±0.1	1.3±0.1	1.4±0.1	1.4±0.1
Triglycerides (mmol/L) [ab]	1.2±0.2	1.2±0.1	0.9±0.1	1.0±0.1	1.4±0.2	1.3±0.2	1.2±0.2	1.1±0.1
CRP (mg/L) [a]	2.5±0.4	3.9±0.6	2.3±0.5	3.1±0.3	3.1±0.6	2.7±0.4	2.6±0.5	3.0±0.7
Ghrelin (pg/mL) [a]	562.2±84.6	497.1±43.7	524.5±63.6	574.1±55.1	635.6±13.9	573.5±60.1	597.6±12.4	543.6±45.1

Data are expressed as mean ± SEM.
Data were assessed using repeated-measures ANOVA with time as within-subject factor and diet as between subject factor.

Completers phase 1 or phase 2 refers to subjects who finished phase 1 (week 0–8) or 2 (week 0–32).
For phase 1, n=34 except FFM/FM/2 hour glucose (n=33), REE/RQ (n=13), CRP (n=29), ghrelin (n=26). For phase 2, n=23 except FFM/FM (n=23), REE/RQ (n=11), CRP/ghrelin (n=19).

SBP: Systolic blood pressure
DBP: Diastolic blood pressure
REE: Resting energy expenditure
RQ: Respiratory quotient
LDL-C: Low-density lipoprotein cholesterol
HDL-C: High-density lipoprotein cholesterol
CRP: C-reactive protein
FC: Fat counting
CC: Carbohydrate counting

[a] Significant effect of time from week 0 to week 8 for all treatments combined, P<0.05.
[b] Significant effect of time from week 8 to 20 for all treatments combined, P<0.05.
[c] Significant effect of time from week 20 to 32 for all treatments combined, P<0.05.
[d] Significant effect of time from week 8 to 32 for all treatments combined, P<0.05.
[e] Significant effect of time from week 0 to 32 for all treatments combined, P<0.05.
There was no significant time by diet effect for any variables.

2.4.4: Fasting blood pressure, lipids, CRP, ghrelin, insulin and glucose homeostasis

No time-by-diet interaction was observed for any metabolic variables. In phase 1 of the study, decreases in total cholesterol (2.6±3.3%, P<0.001), triglycerides (12.9±5.2%, P=0.005), LDL-C (12.1±3.3%, P<0.001) and HDL-C (9.5±3.5%, P<0.001) occurred. In phase 2, an increase in these variables was observed such that there was no difference between these values at study commencement and completion. In phase 1, fasting ghrelin increased (7.5±3.6%, P=0.013) and CRP decreased (9.6±8.2%, P=0.018) but at study completion they were not significantly different than at study commencement (Table 2.6).

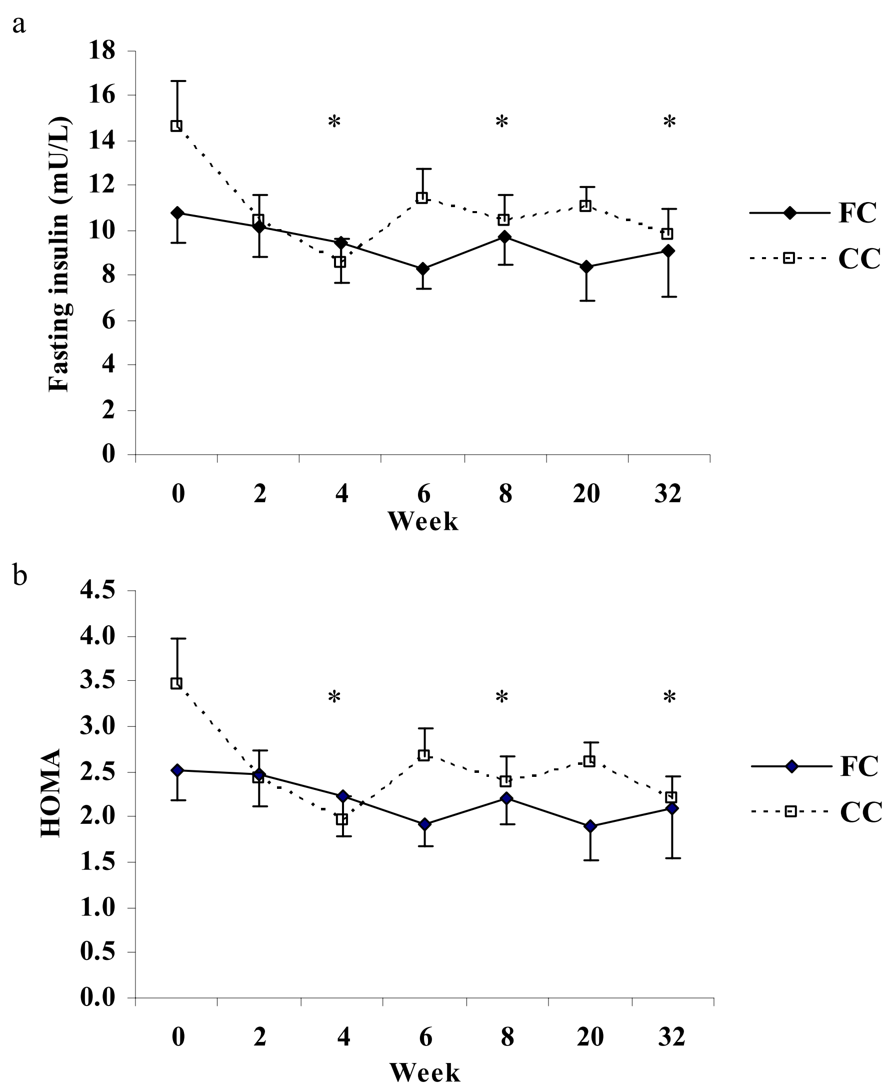
In phase 1, decreases in fasting glucose (1.8±1.0%, P=0.02) and SBP (6.6±1.4%, P<0.001) occurred which were maintained in phase 2 such that a net decrease in fasting glucose (2.9±1.2%, P=0.046) and SBP (6.6±2.2%, P=0.002) occurred. No change in DBP or 2 hour OGTT glucose occurred in phase 1 or phase 2 (Table 2.5). At week 0, 33/43 (76.7%) of subjects had NGT, at week 8 91.1% of subjects had NGT and at week 32 87.0% of subjects had NGT (P=0.003 for change over time). At week 0, 20.9% of subjects met the criteria for the metabolic syndrome compared to 5.9% at week 8 and 4.3 % at week 34 (P=0.085 for change over time).

2.4.5: Insulin homeostasis, reproductive hormones and menstrual cyclicity

There were no differences observed between the diet groups for changes in insulin. In phase 1 of the study, there was a decrease in fasting insulin (2.8±1.1 mU/L, P=0.003) (Figure 2.3a) and HOMA (0.7±0.3, P=0.005) (Figure 2.3b). This occurred by week 4 for insulin (3.9±1.2 mU/L, P=0.013) and HOMA (0.9±0.3, P=0.02), corresponding to a weight loss of 3.8±0.3 kg. These decreases were maintained for phase 2 of the study such that a net decrease in insulin (3.5±1.6 mU/L or 11.9±10.7%, P=0.044) and HOMA (0.9±0.4, P=0.033) occurred. During phase 1, reductions in testosterone (0.3±0.7 nmol/L, P=0.019), FAI (3.1±6, P=0.001) and free

testosterone (10.7±3.3 pmol/L, P=0.001) and increases in SHBG (3.2±1.8 nmol/L, P=0.020) occurred (Figure 2.4a-d). These decreases occurred during weeks 0–2 for FAI (2.4±4.2, p=0.022) and free testosterone (8.8±2.6 pmol/L, P=0.001) and corresponded with a weight loss of 2.4±1.0 kg. In phase 2, the decrease in testosterone was sustained (0.4±0.1 nmol/L, P=0.013) and there was a trend for the decrease in free testosterone to be sustained (8.8±5.6 pmol/L, P=0.087) such that a net decrease occurred from week 0–32. For SHBG and FAI, there was no difference between values at study commencement and completion. When an intention to treat analysis was used for insulin, HOMA, testosterone and free testosterone values, there was no difference in the results using either the baseline value carried forwards or the last clinical value carried forwards such that a significant decrease in insulin, HOMA, testosterone and free testosterone occurred during Phase 1 (week 0–8) and was maintained during Phase 2 (week 8–32) such that an overall decrease occurred from week 0–32 for insulin, HOMA and testosterone and there was a trend for an overall decrease to occur from week 0–32 for free testosterone. There was no change in 2 hour insulin over the study duration (64.2±11.5 mU/L at week 0).

Figure 2.3: Fasting insulin (a) and homeostasis model assessment of insulin sensitivity (b) before and after 8 weeks of energy restriction on one dietary pattern (meal replacements) and 24 weeks of follow-up on either a fat counting (FC) or carbohydrate counting (CC) dietary protocol

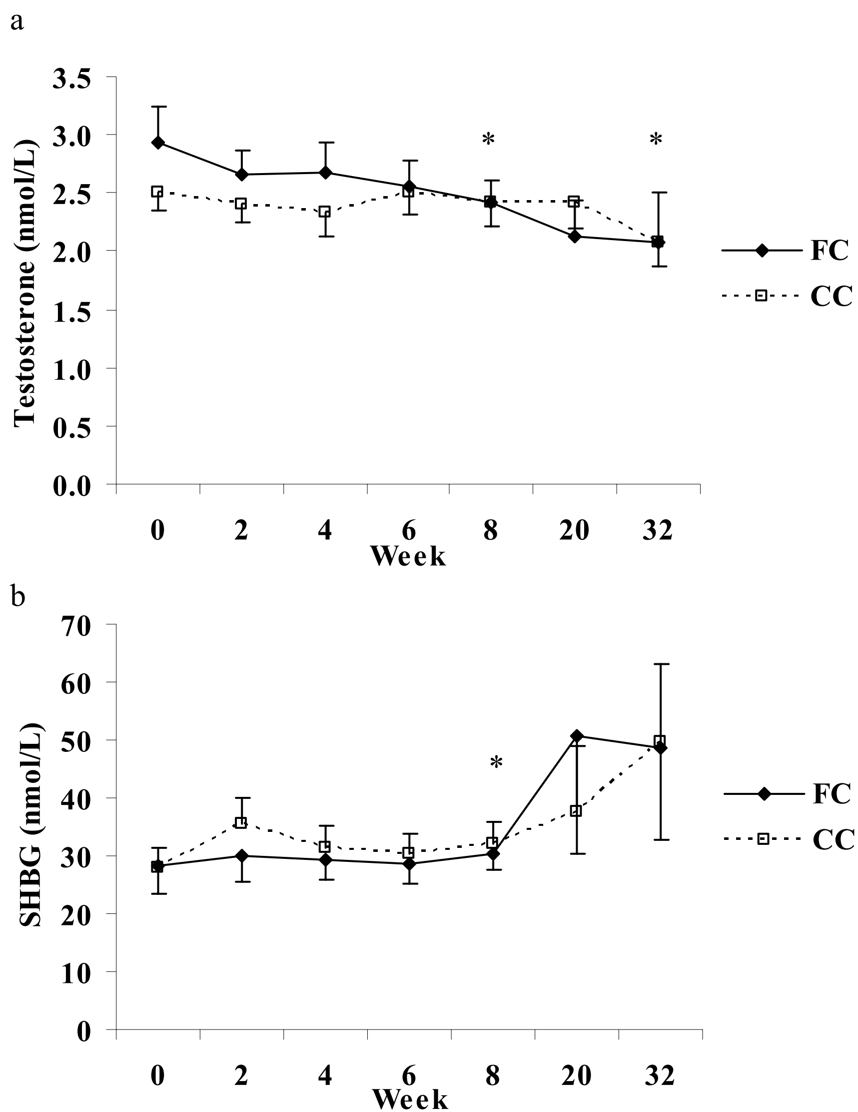


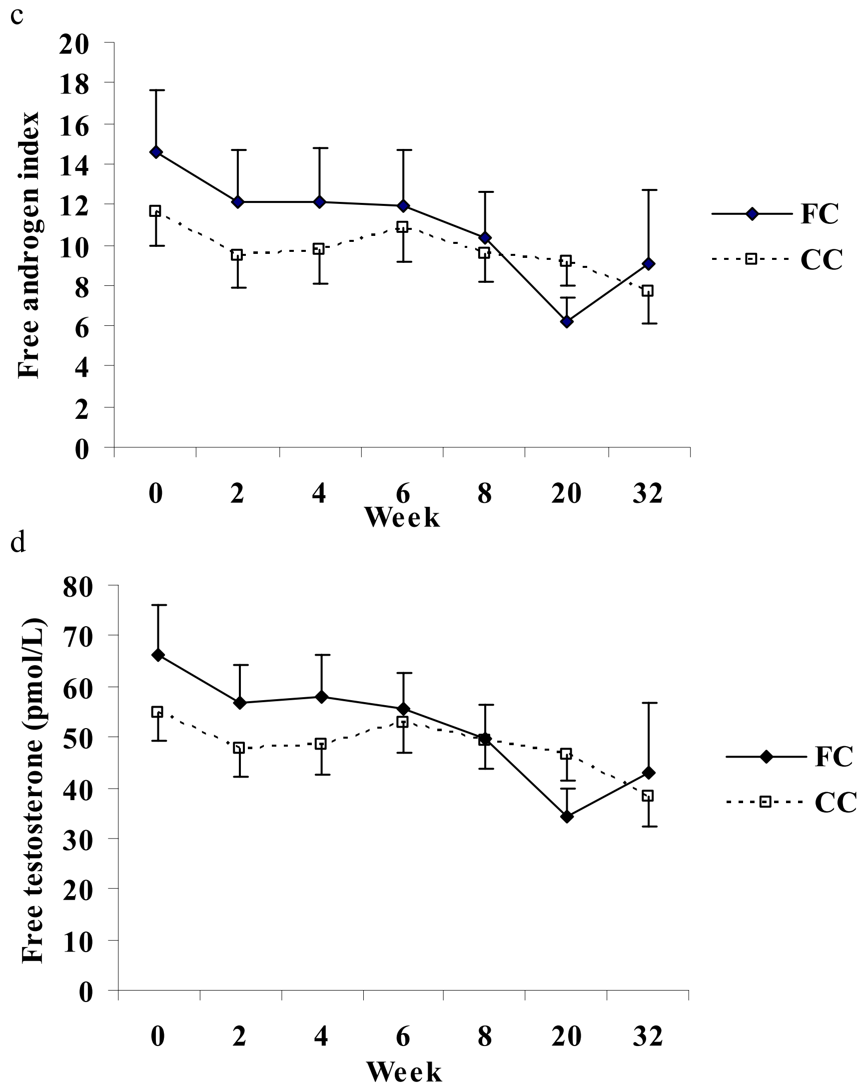
Data are expressed as mean±SEM.

Phase 1 wk 0–8 FC=16, CC=18, phase 2 wk 8–32 FC=9, CC=14.

* Significant effect of time relative to week 0 for all treatments combined, P<0.05.
There was no significant time by diet effect for any variables.

Figure 2.4: Fasting testosterone (a), SHBG (b), free androgen index (c) and free testosterone (d) before and after 8 weeks of energy restriction on one dietary pattern (meal replacements) and 24 weeks of follow-up on either a fat counting (FC) or carbohydrate counting (CC) dietary protocol





Data are expressed as mean±SEM.

Phase 1 wk 0–8 FC=16, CC=18, phase 2 wk 8–32 FC=7, CC=11.

* Significant effect of time relative to week 0 for all treatments combined, $P<0.05$.
† Effect of time relative to week 0 for all treatments combined, P=0.087.
There was no significant time by diet effect for any variables.

The data was subdivided by those with baseline fasting insulin levels above and below the median (10.45 mU/L, baseline fasting insulin 7.9±0.4 versus 17.8±.8 mU/L, P<0.001). At baseline, compared to the subjects with high insulin, the subjects with lower insulin levels had lower 2 hour insulin (36.7±7.2 versus 91.8±20.0 mU/L P=0.015), testosterone (2.3±0.2 versus 3.1±0.3 nmol/L, P=0.023), FAI (9.7±1.5 versus 16.3±2.9, P=0.05) and free testosterone (48.2±5.3 versus 72.2±8.9 pmol/L, P=0.027). From week 0–2, the subjects with high insulin showed a decrease in testosterone (0.4±0.1 nmol/L P=0.007, time*insulin effect P=0.011), FAI (4.0±0.1 P=0.001, time*insulin effect P=0.049) and free testosterone (14.4±3.6 pmol/L P=0.001, time*insulin effect P=0.026) and the subjects with low insulin had no change. From week 0–4, the subjects with high insulin showed a decrease in testosterone (0.3±0.1 nmol/L P=0.004, time*insulin effect P=0.023) and free testosterone (9.1±2.8 pmol/L P<0.001, time*insulin effect P=0.037) and a trend for a decrease in FAI (2.3±0.8 P<0.001, time*insulin effect P=0.056) and the subjects with low insulin had no change.

There were no differences between diet groups for change in reproductive hormones, ovulation or menstrual cyclicity. There were no differences in menstrual cyclicity improvements for subjects with baseline fasting insulin above or below the median. During phase 1, 15/33 subjects ovulated twice, 13/33 subjects ovulated once and 5/33 of the subjects did not ovulate. Over the entire study, there was an improvement in menstrual cyclicity for 16/28 subjects (57.1%). This occurred through resumption of menses (n=1) and ovulation (n=3) for previously amennorheic subjects and improvement in cycle length (n=12) for other subjects. Two pregnancies occurred during the long-term phase (2 fat counting) with weight losses at the estimated time of conception of 6.5 kg and 11.8 kg.

2.5: DISCUSSION

There is considerable research on the effects of weight loss on improving abnormal reproductive and metabolic parameters in women with PCOS (301, 302). We have also shown for the first time that meal replacements are an effective nutritionally adequate short-term strategy for reducing weight and improving body composition, quality of life and metabolic and reproductive parameters. Meal replacements (with or without dietetic input), as part of a reduced energy diet (4–6 MJ/day), are nutritionally adequate and produce comparable or greater weight loss than conventional reduced energy diets (554). Women with PCOS also exhibit high drop-outs and poor compliance to dietary protocols, potentially due to increased difficulty with energy restriction consequent to abnormalities in appetite regulation (432). We observed 20% drop-outs for the 8 week weight loss phase, similar to reported rates of 26–38% over 1–4 months for women with PCOS (302, 303) and higher than 8–9% drop-outs over 4 months for subjects without PCOS (321, 395, 396). Meal replacements may be a preferential weight loss strategy for some individuals through aiding compliance and adherence to a reduced energy diet (554).

Only a subset of subjects (57.1%) had improved menstrual cyclicity following weight loss. We found no differences between responders and non-responders, although we did not perform a complete gonadotrophic or insulin sensitivity assessment. Subjects with PCOS who show menstrual improvements after 4–6 months of weight loss have greater decreases in fasting insulin, HOMA, insulin sensitivity (301, 302), lutenising hormone (LH) or central fat (301) or greater increases in LH and reductions in oestradiol (295). In 182 normogonadotropic oligomenorrheic infertile women undergoing ovulation induction with clomiphene citrate (555), elevated baseline FAI was the most important negative determinant of treatment response. It is currently unclear why reproductive function is restored with energy restriction or weight loss, how long it would take for the short term hormonal changes to translate into improvements in reproductive function or what the key triggering hormonal factor is.

Subjects with elevated baseline insulin had maximal improvements in reproductive hormones after acute energy restriction (week 2–4). However, no differences in menstrual cyclicity were observed for subjects with higher or lower baseline insulin, similarly observed for women with PCOS with or without insulin resistance (IR) undergoing 12 months treatment of metformin or an energy reduced diet (556). Potentially only short-term energy restriction is needed to improve reproductive function, likely attributable to reductions in insulin-stimulated androgen production (229). This is supported by the use of insulin sensitising agents such as metformin and the glitazones restoring menstrual cycles and ovulation (557, 558). However, reproductive improvements have been reported after 4–6 weeks (284, 302) or 3–5 months of energy restriction (262, 302). Weight loss may be more important in sustaining improvements or a specific decrease in weight or insulin (depending on initial weight or IR) may be needed to improve reproductive function. However not all women with PCOS exhibit IR (199), although those that do are more severely clinically affected (193). Moreover, responders to a 6 month weight loss program displayed significant reductions in oestradiol and increases in LH secretion after 7 days of acute energy restriction (295), suggesting other hormonal responses to energy restriction may precede improvements in follicular function.

The aim of the long-term intervention was to compare two education strategies moderately restricting fat or carbohydrate on maintenance of weight and reproductive and metabolic improvements. We achieved a moderately fat or carbohydrate restricted intake for 3 months of weight maintenance with no differential effects of diet group on changes in weight or body composition observed. Severe carbohydrate restriction (20–30g/day) results in greater weight loss than a structured reduced fat intake over 6 months (355, 359), potentially due to simplicity of the education strategy, restriction of food choices or proportionally increased dietary protein intake (355) with effects on satiety (333) and lean mass maintenance (543). We may not have restricted carbohydrate sufficiently to achieve significant increases in protein intake or decreases in food choices to aid reduction in energy intake. Weight loss is

also typically not maintained after 12 months with a low carbohydrate diet (356, 359), signifying similar compliance and sustainability issues to other weight maintenance regimes. With regards to the effect of modulating GI or GL on weight loss, greater reductions in BMI (363, 364) and fat mass (364) were reported for 4 (363) or 12 months (364) of *ad libitum* low GI diet compared to an energy restricted low fat diet in paediatric and adolescent obesity. We observed no differences in GI, GL or weight loss between the CC and FC groups. A larger reduction in GL, through greater reduction in GI or carbohydrate amount, may be needed to translate acute satiating effects of low GI foods into significant clinical practice. The role of GI or GL in weight maintenance is also unclear. When used in conjunction with a 12 month low fat reduced energy increased physical activity program, advice on reducing dietary GI did not have any additional effect on weight loss (7.6 kg) or regain (4.6 kg) in 56 overweight individuals (559).

At study completion, both dietary strategies maintained a weight loss of 4.7 kg. Equivalent sustained reductions in waist circumference, insulin, HOMA and testosterone occurred with both diets, likely due to the similar weight maintenance and potentially the similar GI, GL and protein intake. This is a clinically relevant weight loss that is associated with a reduced prevalence of T2DM (390) and the metabolic syndrome (391) in the general population and improved fertility outcomes in PCOS (262). This sustained weight loss could potentially be due to the reduced GL load compared to baseline, although we observed no relationship between GI, GL and weight loss. Alternatively, the decrease in REE observed during weight loss (560) may predispose subjects to weight regain. We observed a partial return of REE to baseline levels at study completion which may have aided weight maintenance. Indeed, an increase in REE was reported in conjunction with sustained weight maintenance following a physical activity program post-weight loss (561). Given the lack of concomitant change in TFFM in weight maintenance, the mechanism for this effect is unclear and requires further exploration.

The monthly follow-up or physical activity advice may also have aided weight maintenance and dietary compliance (562, 563). However, in the last 3 months of weight maintenance, subjects did not adhere to their daily allocated fat or carbohydrate intake, energy intake increased insignificantly and some metabolic parameters returned to baseline levels, indicating a worsening of dietary compliance. Energy intake, regain of weight and study dropouts may have increased further with a longer period of weight maintenance. In previous weight loss studies in women with PCOS, structured individualised dietary and exercise protocols with input from dietitians, exercise professionals and psychiatrists were successful in achieving weight loss (6.3 kg at 24 weeks (262) or 2.6–6.3 kg at 48 weeks (300)) and sustained improvements in ovulation and fertility. Incorporation of a more structured physical activity protocol, behavioural treatment, group support and increasing intensity or frequency of follow-up and dietary counselling would likely increase long-term compliance (277, 562) and may outweigh the effects of modifying dietary composition. This could also be a more relevant approach in PCOS if appetite regulation is impaired. We also observed no significant differences between the FC and CC approaches for any micronutrients, indicating the nutritional adequacy of both strategies was equivalent. While in the short-term phase the micronutrient intake was sufficient (with the exception of iron intake), we observed an inadequate intake of a variety of micronutrients (including calcium, vitamin E, iron, zinc and folate) in the long-term dietary interventions for both approaches and use of a multivitamin in conjunction energy restricted strategies is a prudent approach.

In conclusion, we have shown for the first time that the use of twice daily meal replacements was an effective and nutritionally adequate strategy for achieving weight loss and associated hormonal and clinical benefits in overweight women with PCOS. Acute energy restriction improved reproductive hormonal parameters in a study subset, implying long-term weight loss may not be needed for fertility improvements in all women with PCOS. We showed for the first time that advice on fat or carbohydrate restriction with moderate targets had no

differential effect on weight maintenance and reproductive and metabolic parameters in PCOS. However, it is possible that more stringent fat or carbohydrate targets may be more effective in achieving the dietary macronutrient restriction such that differential effects on weight and energy intake can be observed. Monthly support may have been important in facilitating weight maintenance and sustaining metabolic and hormonal improvements in conjunction with an improvement in menstrual function in 57% of the subjects. Both these dietary approaches may be useful additional strategies for women with PCOS, affording a degree of choice depending on preference or short term metabolic imperative.

CHAPTER 3: DIFFERENTIAL EFFECT OF WEIGHT LOSS ON CARDIOVASCULAR RISK FACTORS IN OVERWEIGHT WOMEN WITH AND WITHOUT POLYCYSTIC OVARY SYNDROME

3.1: ABSTRACT

Polycystic ovary syndrome (PCOS) is associated with reproductive and metabolic abnormalities. It is unknown whether overweight women with and without PCOS achieve a similar magnitude of benefit from weight loss on cardiovascular risk factors. Overweight BMI-matched women with (n=15) and without (n=17) PCOS (weight 95.3±17.6 kg, BMI 35.6±5.3 kg/m^2, mean±SD) followed an 8 week weight loss regime (2 meal replacements/day, 5217.0±130.0 kJ/day). All subjects had similar reductions in weight (3.9±0.9 kg or 3.8% versus 4.5±1.0 kg or 4.7%), waist circumference, fat mass, triglycerides, free testosterone and fasting and post-prandial insulin. At baseline, C-reactive protein (CRP) between groups was not significantly different (5.5±0.9 mg/L PCOS versus 4.9±0.9 mg/L non-PCOS). There was a significant interaction between PCOS status and CRP (P=0.016), such that CRP decreased with weight loss for non-PCOS women (-1.2±0.5 mg/L, P=0.025) but not for PCOS women. For all women, the change in CRP correlated with the change in weight (r=0.560, P=0.003), fat mass (r=0.477, P=0.016) and post-prandial insulin (r=0.402, P=0.046). Fasting adiponectin was not significantly different between groups before or after weight loss. Subjects with baseline CRP levels below the median (4.52 mg/L) showed reductions in adiponectin (P=0.015) and greater reductions in triglycerides (P=0.001) with weight loss. A 4–5% weight loss improved lipid, glucose and insulin profiles in women with and without PCOS. However, this degree of weight loss was not effective in lowering CRP concentrations in PCOS women, suggesting that greater weight loss is required in this group to achieve equivalent cardiovascular benefit as non-PCOS women.

3.2: INTRODUCTION

Inflammation is proposed as a pathogenic factor in the development of atherosclerosis in the development of endothelial dysfunction, plaque development and the initiation of plaque instability and rupture (reviewed by (564)). High sensitivity C-reactive protein (CRP) is an

acute-phase reactant synthesized in the liver in response to interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) (565). CRP levels independently predict cardiovascular events and coronary heart disease (566) and elevated CRP levels are linked to a number of abnormal physiology and disease states including visceral fat accumulation, insulin resistance (IR), the metabolic syndrome and T2DM (567). CRP has been identified in atherosclerotic plaques (568) and there is evidence that it is both a marker for low grade chronic inflammation and plays an active role in atherosclerosis. In vitro, CRP upregulates angiotensin-1 receptor and promotes migration and proliferation of vascular smooth muscle cells, enhances reactive oxygen species formation (569), increases the expression of endothelial cell adhesion molecules (vascular cell adhesion molecule, intracellular adhesion molecule, E-selectin and monocyte chemoattractant protein-1) (570, 571), has a pro-thrombotic role through the stimulation of plasminogen-activator inhibitor 1 (PAI-1) antigen and activity in aortic endothelial cells (572) and increases macrophage low-density lipoprotein (LDL) uptake (570, 573). The adipose tissue secretes a variety of bioactive substances or adipocytokines that additionally contribute to inflammation and metabolic disease including leptin, TNF-α, IL-6, IL-18, resistin, PAI-1 and adiponectin. Adiponectin levels negatively correlate with insulin sensitivity and are reduced in obesity and visceral obesity (57, 574) and there is some indication that low adiponectin levels may predict cardiovascular disease (575), although the evidence is thus far controversial (576). Adiponectin has important anti-atherogenic and anti-inflammatory actions including inhibition of transcription factor nuclear factors kappa beta signalling, TNF-α secretion from macrophages, the expression of TNF-α induced endothelial adhesion molecules and the macrophage to foam cell transformation (577-580). Adiponectin has additional proposed insulin sensitising roles (58) and an inverse association exists between adiponectin and hsCRP (581).

Peripheral IR is common and present in up to 50–80% of women with PCOS and is further worsened by the presence of obesity (198). Metabolic complications associated with IR are

also increased in PCOS independent of obesity. PCOS is associated with an adverse cardiovascular risk profile (impaired fibrinolysis, endothelial dysfunction, hypertension and hyperlipidaemia) and increased prevalence of the metabolic syndrome, impaired glucose tolerance (IGT) and Type 2 Diabetes Mellitus (T2DM) (90, 91). In PCOS, the contribution of adipocytokines to inflammation is a field of research undergoing extensive investigation. There is emerging evidence that novel cardiovascular risk factors are also dysregulated in PCOS and elevated hsCRP (582, 583) and reduced adiponectin levels (534, 584, 585) have been documented in obese and non-obese women with PCOS compared to age and weight matched controls by some but not all investigators (586-588). Elevations in other pro-inflammatory mediators such as IL-6, TNF-α, procalcitonin and white blood cell or neutrophil count were also observed in women with PCOS compared to age and BMI-matched controls (589-591). It is currently unclear whether the presence of increased inflammation in PCOS is associated with IR, adiposity or adiposity distribution or an inherent feature of the condition.

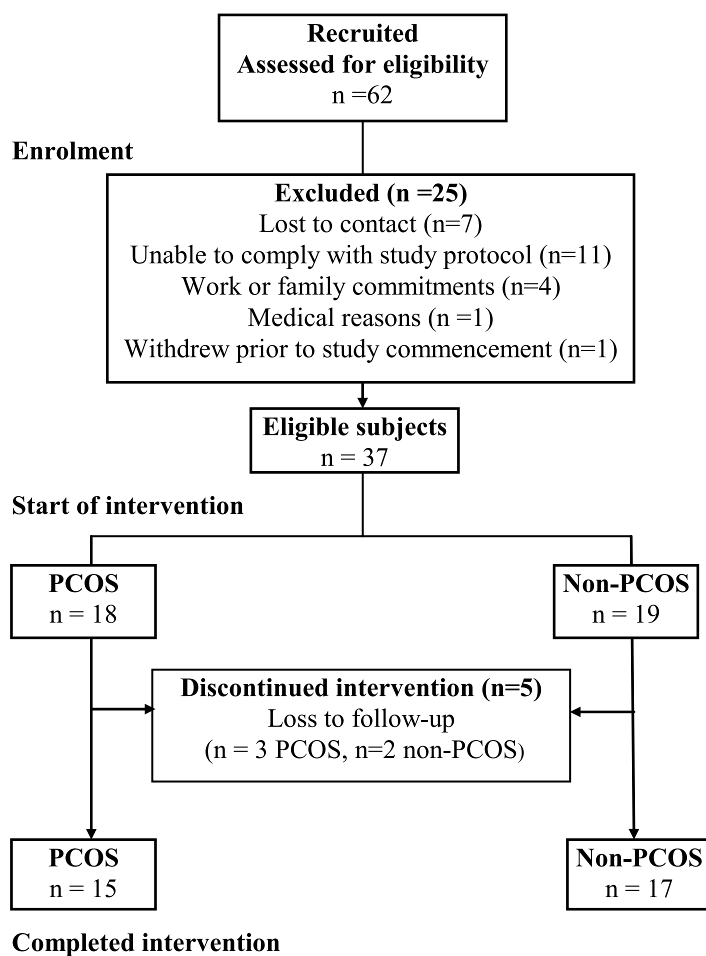
Despite evidence for a greater risk of subclinical atherosclerosis in women with PCOS (87), there is no conclusive evidence that long-term cardiovascular morbidity and mortality are elevated (92). However, a prudent approach should nonetheless target metabolic risk factors at the earliest stage as weight loss normalises cardiovascular and diabetic risk factors and reduces hsCRP in women with PCOS (discussed in Chapter 2) and without PCOS (592-594). In the general population, adiponectin increases following weight loss (592), although the effect of weight loss on adiponectin in PCOS or the relationship between hsCRP and adiponectin with weight loss in PCOS has not yet been studied. The objective of this study was therefore to examine the effect of acute energy restriction on cardiovascular risk factors in overweight women with and without PCOS, and specifically to examine the relationship between hsCRP and adiponectin before and after weight loss in overweight women with and without PCOS.

3.3: METHODS

3.3.1: Subjects and recruitment

Overweight pre-menopausal women (European Caucasian) with (n=19) and without PCOS (n=18) were recruited through public advertisement. The study was approved by the Human Ethics committees of the CSIRO Division of Health Sciences and Nutrition, The Royal Adelaide Hospital and the Womens and Childrens Hospital of South Australia and all subjects gave informed written consent. Inclusion criteria were diagnosis of PCOS according to the Rotterdam consensus group by two of the following three criteria: 1) Menstrual irregularity (cycle length < 26 days or > 31 days or variation between consecutive cycles of > 3 days); 2) Clinical (hirsutism assessed by Ferriman-Gallwey score > 8) or biochemical hyperandrogenism (free androgen index > 5.4 or testosterone > 1.4 nmol/L, defined from a representative population of non-PCOS women (n=80) (544)) or 3) Positive ultrasound presentation of polycystic ovaries by trans-vaginal scan (124). Exclusion criteria were pregnancy, breastfeeding, body mass index (BMI) < 25 kg/m^2, T2DM, use of oral contraceptives, endocrine hormonal treatment or insulin-sensitising agents (subjects were required to cease oral contraceptives 4 weeks and hormonal treatment/insulin-sensitising agents 2 weeks prior to commencement of the study) and related endocrine disorders (excluded by assessment of thyroid stimulating hormone (TSH), prolactin and 17α-hydroxyprogesterone).

Figure 3.1: Study flow diagram



Flow diagram of enrolment, commencement and completion of the 8 week weight loss protocol incorporating for subjects with (n=17) and without (n=15) Polycystic ovary syndrome (PCOS).

3.3.2: Study design and dietary treatment

The study was conducted on an outpatient basis over 8 weeks. Subjects followed an energy restricted diet whereby two meals daily were replaced with commercially available meal replacements (Slimfast™, Unilever Australasia, Epping, New South Wales, Australia) (1800 kJ/day) in addition to a low-fat evening meal per day and at least 5 servings of fruit and vegetables per day (3500 kJ) with sample meal plans and recipes provided. This involved a restriction of dietary intake to approximately 6000±50 kJ/d or approximately two-thirds of normal intake. Slimfast™ products were provided fortnightly. Subjects were allowed to consume moderate amounts of alcohol (maximum of 2 standard drinks/day with at least 2 alcohol free days/week). Subjects met with a qualified dietitian for initial education on the dietary regime and quantification and recording of their daily food intake. Subjects did not meet with the dietitian for the remainder of the study and minimal oral advice was provided. Nutrient intakes were calculated with Diet 4/Nutrient Calculation Software (Xyris Software, Highgate Hill, Australia) based on data from Australian food composition tables. The database had been extensively modified by our group to add new foods and recipes. Nutritional intake was assessed from fortnightly 3–day consecutive dietary food records (one weekday and two weekend days) and daily dietary checklists. Dietary compliance was determined by subject adherence to the Slimfast™ regime.

Subjects attended the clinic fortnightly. At the initial visit, height was measured to the nearest 0.1 cm using a stadiometer (SECA, Hamburg, Germany) with subjects in the free-standing position. At all visits, body weight was measured to the nearest 0.05 kg using calibrated electronic digital scales (Mettler scales, model AMZ14, A&D Mercury, Kinomoto, Japan) while subjects were wearing light clothing with no shoes. BMI was calculated by weight (kg) divided by squared height (m^2). At week 0 and 8, waist circumference was measured in triplicate directly on the skin with a soft tape to the nearest 0.5 cm at the level of midway between the lateral lower rib margin and the iliac crest and body composition was measured

by tetrapolar single-frequency (50 Hz) bioelectrical impedance analysis (ImpDF50; Impedimed Pty Ltd., Qld, Australia) using the offline general algorithm. Total fat mass (TFM) (coefficient of variation (CV) 2.3±8.7%) and total fat-free mass (TFFM) (CV 2.1±0.4%) were assessed. Duplicate measurements were made while subjects were lying supine with an empty bladder. At weeks 0 and 8 overnight fasting venous blood samples were taken for assessment of glucose, insulin, lipids, CRP, adiponectin, testosterone and SHBG. Exercise was assessed by a 7-day 24-hour physical activity record at weeks 0 and 8 (546, 547). Daily physical activity was computed in MET-min·day^{-1}, calculated as the MET intensity multiplied by the corresponding minutes of each activity to yield MET minutes, summed across the day and then averaged over the 7 day recording period. The total energy expenditure was expressed as a mean daily energy cost expressed in kJ, computed as: EE (kJ) = MET-min x [body weight (in kg) x (4.186/60)] (548).

All subjects documented their menstrual cycles for the study duration and for 6 months before study commencement. During the 8 week intervention, first morning urine samples were collected twice weekly and assessed for total urinary pregnanediol-3-glucuronide to determine ovulation status. Improvements in menstrual cyclicity were defined as a change from non-ovulatory to ovulatory cycles or from irregular to regular cycles or an improvement in consecutive intercycle variation. For the fortnight prior to study commencement, subjects weighed themselves daily to ensure weight stability, defined as a weight change of ≤2% of initial body weight. At week 0 and week 8, subjects performed a meal tolerance test (MTT). On the evening prior to the meal tolerance test, all subjects consumed the same meal (3820 kJ, 20% of energy from protein, 17% from fat, 62% from carbohydrate) and refrained from alcohol for 24 hours. A cannula was inserted into a lower arm vein and a fasting blood sample was taken for assessment of plasma glucose and insulin. Subjects consumed a liquid preload of Slimfast™ (936 kJ, 12 g protein, 2 g fat, 35 g carbohydrate) within 5 minutes and subsequent blood samples were taken at 15, 30, 45, 60, 90, 120 and 180 minutes.

3.3.3: Biochemical measurements

Fasting blood samples were taken by venipuncture. Blood for serum was collected in tubes with no additives and allowed to clot at room temperature for 30 min. Blood for plasma was collected in tubes containing sodium fluoride/EDTA for glucose. Serum and plasma were isolated by centrifugation for 10 min at 3000g (5°C) (Beckman GS-6R; Beckman, Fullerton, CA) and aliquots were stored at –80°C. Details of all assay methodologies (SHBG, testosterone, total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), insulin, glucose, CRP, adiponectin, TSH, prolactin, 17-α progesterone and urinary pregnanediol) are described in Appendix 2. The homeostatic model assessment (HOMA) was used as a surrogate measure of insulin sensitivity and was calculated as [fasting serum insulin (mU/L) x fasting plasma glucose (mmol/L)/22.5] (80). A HOMA score >2.61 or a fasting insulin level > 12.1 mU/L was defined as insulin resistant (248). The free androgen index (FAI) (testosterone/SHBG x 100) and equilibrium binding equations for determination of free testosterone (123) were used as surrogate estimates of free testosterone. Biochemical assays were performed in a single assay at the completion of the study and all samples for individuals were analysed in the same assay.

3.3.4: Statistics

Data was presented as means±SEM except where indicated and log transformed for analysis where non-normally distributed. Results are presented for 32 subjects (n=15 PCOS, n=17 non-PCOS), except for post-prandial insulin and glucose (n=15 PCOS, n=15 non-PCOS), CRP (n=12 PCOS, n=15 non-PCOS) and dietary analysis, BIA, waist circumference and menstrual cyclicity (n=14 PCOS, n=17 non-PCOS) due to incomplete data. Subjects with an isolated CRP level > 10mg/L were excluded from the CRP analysis. Two-tailed statistical analysis was performed using SPSS for Windows 10.0 software (SPSS Inc, Chicago, USA)

with statistical significance set at α level of $p \leq 0.05$. Baseline data were assessed using a one-way ANOVA. Comparisons between time points were assessed using repeated measures ANOVA with PCOS diagnosis as between subject factor. In specific analyses, age and weight loss were included as covariates. In the event of an interaction, post-hoc pairwise comparisons were performed. Relationships between variables were examined using bivariate and partial correlations and ANCOVA. Subjects with baseline CRP levels above and below the median of 4.53 mg/L (7.4±0.6 versus 2.5±0.3 mg/L), were assessed separately with baseline CRP status as the between subject factor. This study had 65% power to detect a difference of 1.6 mg/L of CRP between subjects with and without PCOS to statistical significance of $P<0.05$. To confirm the observed differences between subjects with and without PCOS of changes in CRP with weight loss of 1.6 mg/L, to statistical significance of $P<0.05$ and 80% power, a total of 19 subjects for each group would be needed. For changes in adiponectin with weight loss, 124 subjects would be needed in each group to detect a difference of 633.4 ng/mL between the subjects with and without PCOS to statistical significance of $P<0.05$ and 80% power.

3.4: RESULTS

3.4.1: Subjects, physical activity and diet

Thirty two subjects completed the intervention (15 PCOS and 17 non-PCOS) with an overall drop-out rate of 13.5%. Study drop-outs are documented in Figure 3.1. The number of drop-outs was not statistically different between the subjects with and without PCOS. Subject baseline characteristics are shown in Table 3.1. Activity levels were comparable between diet groups at week 0 and did not change throughout the study. The dietary intervention was well tolerated by all subjects with no adverse events documented. The energy intake was 5217.0±130.0 kJ, the fat intake was 34.4±1.9 g/day (24.3±1.1%), the carbohydrate intake was 158.5±5.0 g/day (51.6±1.0%) and the protein intake was 72.6±1.7 g/day (22.4±0.4%) with no differences between the subjects with and without PCOS.

Table 3.1: Subject baseline characteristics

	PCOS (n=15)	**Non-PCOS (n=17)**
Age (years)[a]	31.7±6.2	37.1±4.7
Weight (kg)	95.1±19.3	95.5±16.5
BMI (kg/m^2)	35.7±5.8	35.5±5.1
Waist circumference (cm)	114.4±3.7	112.6±3.4
Fat mass (kg)	35.0±2.2	35.0±1.9
Fat free mass (kg)	60.1±3.0	61.1±2.3
Glucose (mmol/L)	5.3±0.8	5.2±0.5
Insulin (mU/L) [b]	21.5±14.2	12.2±6.4
HOMA [c]	5.3±3.7	2.8±1.6
Testosterone (nmol/L) [d]	3.3±1.0	2.0±0.5
Free testosterone (pmol/L) [d]	82.2±35.9	43.6±13.7
SHBG (nmol/L)	20.8±10.7	24.7±7.7
Free androgen index [d]	21.6±18.2	9.0±4.3

Data are expressed as means ± SD.
Measurements were made at the week 0 visit and were assessed using one-way ANOVA with PCOS status as the fixed factor.

PCOS: Polycystic ovary syndrome
BMI: Body mass index
HOMA: Homeostasis model assessment
SHBG: Sex hormone binding globulin

a P=0.009 for PCOS versus non-PCOS.
b P=0.039 for PCOS versus non-PCOS.
c P=0.053 for PCOS versus non-PCOS.
d P<0.001 for PCOS versus non-PCOS.

3.4.2: Weight loss and body composition

Over the 8 weeks, there was no significant difference in weight loss (P=0.642) between the subjects with and without PCOS (3.9±0.9 or 4% versus 4.5±1.0 kg or 4.7%) with a mean weight loss of 4.2±0.7 kg (4.3%) occurring for combined subjects. There were no differences in the proportion of subjects with and without PCOS who lost greater than 2% (73.3 versus 64.7%) or 5% of their initial body weight (40 versus 47.1%). Equivalent reductions in waist circumference, TFM and TFFM occurred for all subjects (Table 3.2).

3.4.3: Fasting lipids, CRP and adiponectin

At week 0 CRP levels were not different between subjects with and without PCOS (5.5±0.9 versus 4.9±0.8 mg/L, P=0.603). At week 8 there was a trend for subjects with PCOS to have higher CRP levels (5.9±1.0 versus 3.7±0.7 mg/L, P=0.066) (Figure 3.2). There was a significant time-by-PCOS status interaction (P=0.016) such that CRP decreased with weight loss for non-PCOS women (-1.2±0.5 mg/L, P=0.025) but did not change for PCOS women (P=0.418). CRP correlated with SHBG at week 0 (r=-0.598, P=0.001) and week 8 (r=-0.443, P=0.021). There was a trend for CRP to correlate with insulin (r=0.361, P=0.064) and HOMA (r=0.378, P=0.052) at week 0. At week 8 CRP correlated with insulin (r=0.532, P=0.005) and HOMA (r=0.530, P=0.004). These relationships remained after adjusting for weight. The change in CRP with weight loss correlated with the change in weight (r=0.546, P=0.003), FFM (r=0.548, P=0.005), FM (r=0.477, P=0.016) and change in AUC insulin (r=0.456, P=0.025) but not fasting insulin, HOMA or waist circumference. The correlation between the change in CRP and AUC insulin was removed after adjusting for the effect of weight loss. When subjects with baseline CRP levels above and below the median were assessed separately, there was a time-by-baseline CRP effect for changes in adiponectin (P=0.029) and triglycerides (P=0.015). Only subjects with baseline CRP levels below the median showed reductions in adiponectin (P=0.015) and triglycerides (P=0.001) with weight loss.

There was no effect of PCOS status on changes in fasting lipids. Following weight loss, total cholesterol, LDL-C and HDL-C did not change and equivalent decreases in fasting triglycerides (P=0.024) occurred for all subjects (Table 3.2). There were no differences in adiponectin between subjects with and without PCOS at week 0 (P=0.641) or week 8 (P=0.259) and no change in adiponectin with weight loss (P=0.457) or effect of PCOS status (P=0.166) on change in adiponectin with weight loss. Adiponectin correlated with insulin (week 0 r=-0.525, P=0.002; week 8 r=-0.527, P=0.002), HOMA (week 0 r=-0.540, P=0.001; week 8 r=-0.544, P=0.001), AUC insulin (week 0 r=-0.373, P=0.042; week 8 r=-0.449, P=0.013), HDL-C (week 0 r=0.530, P=0.002; week 8 r=0.422, P=0.016), triglycerides (week 0 r=-0.650, P<0.001), CRP (week 8 r=-0.416, P=0.031), fasting glucose (week 8 R=-0.363, P=0.041) and AUC glucose (week 8 r=-0.495, P=0.005), but not weight or body composition and the change in adiponectin did not correlate with the change in any measured variable.

3.4.4: Fasting and post-prandial glucose and insulin

There was no effect of PCOS status on changes in fasting glucose and no changes in fasting glucose with weight loss. There was an effect of PCOS status on MTT glucose (P=0.026) such that the post-prandial glucose response decreased with weight loss only for the subjects with PCOS (P=0.029) (Figure 3.3a). At weeks 0 and 8, subjects with PCOS had significantly higher fasting insulin and HOMA than subjects without PCOS (Table 3.2, Figure 3.3b). At week 0 there was no difference in the proportion of subjects who were insulin resistant with or without PCOS (10/15 versus 8/17, P=0.265) while at week 8, significantly more subjects with PCOS were insulin resistant than subjects without PCOS (9/15 versus 4/17, P=0.026). Following weight loss, similar decreases in fasting HOMA (0.85±0.37 mu/L, P=0.005) (Table 3.2) and insulin (3.4±1.4 mu/L, P=0.002) (Figure 3.3b) occurred in subjects with and without PCOS. Following weight loss, the test meal insulin response similarly significantly decreased for subjects with and without PCOS (P<0.001), although subjects without PCOS had a lower insulin response at both time points (P=0.050 for between subject effect) (Figure 3.3b).

Table 3.2: Weight, body composition, lipids, HOMA and reproductive hormones before and after 8 weeks of energy restriction on one dietary pattern (meal replacements)

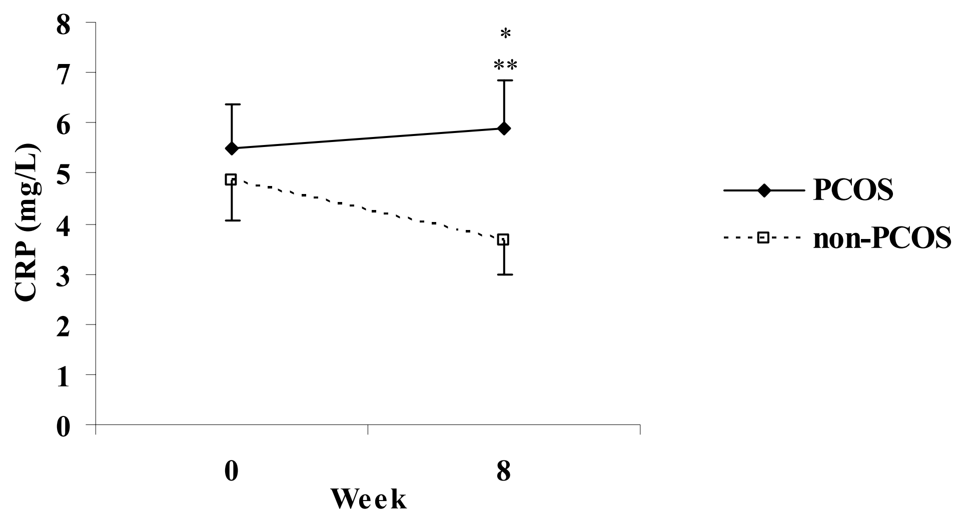
	PCOS		**Non-PCOS**	
	Week 0	**Week 8**	**Week 0**	**Week 8**
Weight (kg) [c]	95.1±5.0	91.2±4.5	95.5±4.0	90.9±4.0
Waist circumference (cm) [c]	114.3±4.0	108.2±4.3	112.5±3.4	105.4±3.5
Total fat free mass (kg) [c]	59.7±3.2	58.5±3.0	61.1±2.3	59.2±2.2
Total fat mass (kg) [c]	35.0±2.4	32.3±2.0	35.0±1.9	31.8±1.9
Total cholesterol (mmol/L)	5.1±0.3	4.9±0.2	5.0±0.2	4.9±0.2
LDL-C (mmol/L)	2.8±0.3	2.8±0.2	2.9±0.2	2.8±0.1
HDL-C (mmol/L)	1.2±0.1	1.2±0.1	1.3±0.1	1.3±0.1
Triglycerides (mmol/L) [a]	2.3±0.4	2.0±0.3	1.7±0.2	1.6±0.2
Adiponectin (mg/L)	7.0±1.1	7.1±0.8	6.8±0.9	7.5±0.6
HOMA [b]	5.3±1.0	4.2±0.9	2.8±0.4	2.2±0.4
Testosterone (nmol/L) [c]	3.3±0.3	2.8±0.2	2.0±0.1	1.7±0.1
SHBG (nmol/L) [b]	20.8±2.8	21.8±2.8	24.7±1.9	28.5±2.6
Free androgen index [c]	21.6±4.7	17.5±4.6	9.0±1.0	7.0±0.9
Free testosterone (pmol/L) [c]	82.2±9.3	66.6±8.6	43.6±3.3	35.0±2.9

Data are expressed as mean ± SEM.
Data were assessed using repeated measured ANOVA with time as within-subject factor and PCOS status as between subject factors.

PCOS: Polycystic ovary syndrome
LDL-C: Low-density lipoprotein cholesterol
HDL-C: High-density lipoprotein cholesterol
SHBG: Sex hormone binding globulin

PCOS n=15 and non-PCOS n=17 except for weight, waist circumference, total fat free mass and total fat mass (PCOS n=14, non-PCOS n=17).
[a] P<0.05 for effect of time (week 0–8).
[b] P≤0.01 for effect of time (week 0–8).
[c] P≤0.001 for effect of time (week 0–8).

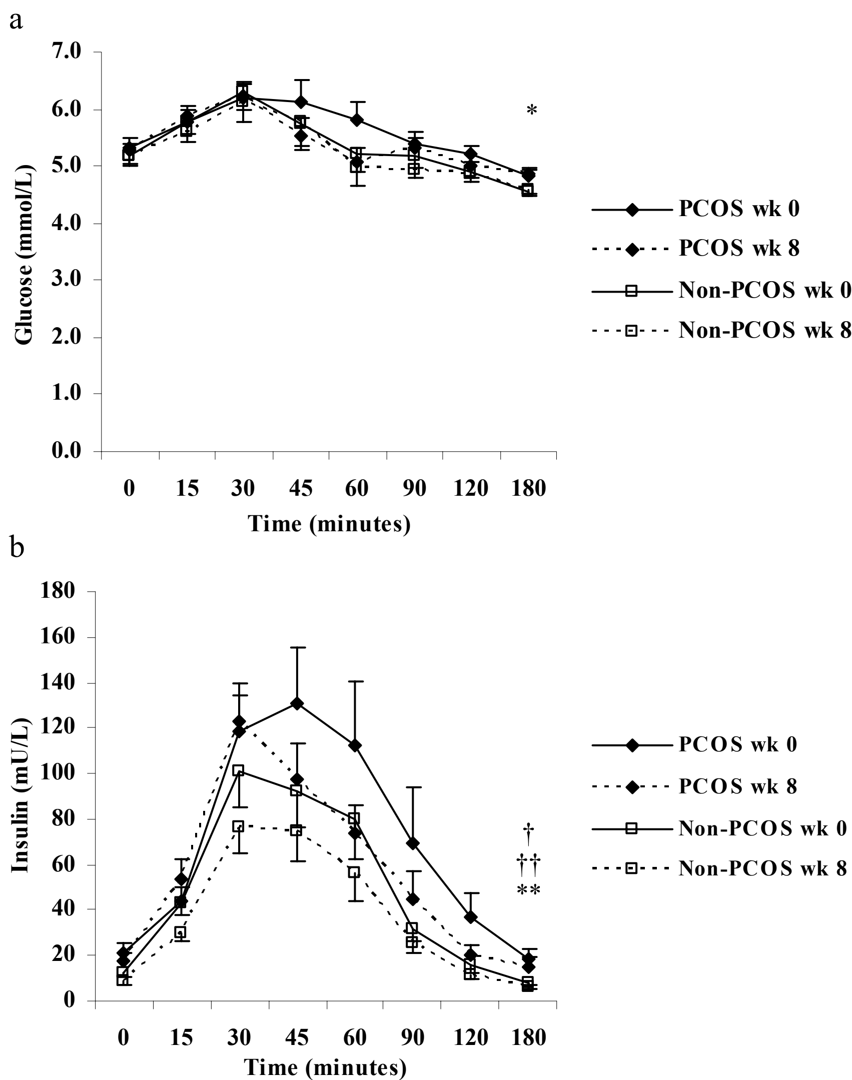
Figure 3.2: C-reactive protein before and after 8 weeks of energy restriction on one dietary pattern (meal replacements)



Data are expressed as mean±SEM.
Data were assessed using repeated measured ANOVA with time as within-subject factor and Polycystic ovary syndrome (PCOS) status as between subject factors.

* Time-by-PCOS status interaction (P=0.016).
** Trend for a difference at week 8 (P=0.066).

Figure 3.3: Glucose (a) and insulin (b) concentrations at baseline and 15, 30, 45, 60, 90, 120 and 180 min after the ingestion of a test meal at week 0 and week 8



Data are expressed as mean±SEM.

Week 0 and 8 data were compared by repeated measures ANOVA with week and blood sampling time as within-subject factors and Polycystic Ovary Syndrome (PCOS) status as between-subject factors.

* Significant week-by-minute-by-PCOS status effect (P=0.026).

** Significant effect of time from week 0 to 8 for fasting (P=0.002) and post-prandial insulin (P<0.001).

† Significant between subject effect of PCOS status for post-prandial insulin at week 0 and week 8 (P=0.05).

†† Significant difference at 0 minutes between subjects with and without PCOS at week 0 (P=0.039) and week 8 (P=0.011).

3.4.5: Reproductive hormones and menstrual cyclicity

There was no effect of PCOS status on changes in SHBG, FAI and free or total testosterone. Following weight loss, testosterone (0.4 nmol/L or 13.5%, P=0.001), FAI (3.0±0.6 or 19.4%, P<0.001) and free testosterone (11.9±2.7 or 17.2%, P<0.001) decreased and SHBG (2.5±0.9 or 10.4% nmol/L, P=0.01) increased, with no differences between the women with and without PCOS (Table 3.2). The subjects without PCOS had double the number of ovulations over the 8 week study duration (1.0 ovulations for subjects with PCOS and 1.9 ovulations for subjects without PCOS, P<0.001). For the subjects with PCOS, 11/15 ovulated at least once during the study and 4/15 were previously amennorheic and spontaneously ovulated or recommenced menstruation during the study.

3.5: DISCUSSION

We compared risk factors for T2DM and cardiovascular disease in overweight women with and without PCOS before and after moderate weight loss. We report that equivalent changes in weight, body composition, insulin homeostasis and reproductive hormones occurred for all subjects when similar dietary protocols and levels of energy restriction are followed. We observed no differences in baseline CRP and adiponectin between women with and without PCOS and no change in adiponectin for all subjects following weight loss. However, women with PCOS displayed no reduction in CRP with weight loss, unlike women without PCOS.

We report for the first time that moderate weight loss (3.9 kg or 4%) does not reduce CRP levels in overweight women with PCOS compared to weight-matched controls undergoing a similar amount of weight loss (4.5 kg or 4.7%). Although the effect of modest weight loss on reducing CRP is well-documented in subjects without PCOS (592-594), there is limited data on the effects of weight loss on reducing CRP in women with PCOS. As discussed in Chapter 2, in overweight women with PCOS a weight loss of 5.6 kg, in association with a reduction in

fasting insulin of 2.8 mU/L, reduced CRP levels from 3.3–2.8 mg/L. Changes in CRP with weight loss have been previously related to changes in weight (594), waist circumference (595) and insulin sensitivity (596). In this study, although we observed equivalent reductions in weight and fasting insulin, significantly more women with PCOS than without PCOS were classified as insulin resistant after weight loss. The metabolic benefits conferred by weight loss, specifically reductions in IR, may therefore be contingent on reduction of a key level of abdominal or visceral abdominal fat (597) and for participants who are relatively more insulin sensitive at baseline, a lesser degree of weight loss may be sufficient to observe metabolic improvements. In support of this, following obesity surgery maximal decreases in CRP occurred for participants who were relatively more insulin sensitive at baseline (596). Conversely, equivalent weight losses in insulin resistant and insulin sensitive subjects (8.7 versus 8.4 kg) induced decreases in CRP only for insulin resistant subjects (3.9–1.2 mg/dL) (598), although this may be accounted for by the relatively low CRP levels prior to weight loss (1.2 mg/L) for the insulin sensitive subjects unlike our controls. Alternatively, the insignificantly greater degree of weight loss observed in this study for the women without PCOS (0.7%) may have been crucial in achieving a key reduction in IR with resultant positive effects on circulating CRP, as no reductions in CRP were observed for subjects who achieved a 3% compared to a 15% weight loss in a 2 year dietary and exercise intervention study (593).

We report for the first time that modest weight loss does not increase fasting adiponectin in overweight women with PCOS. In contrast to previous findings (592, 593), we observed no increase in adiponectin for women without PCOS. Although the similar response for subjects with and without PCOS is to be expected given the comparable reductions in weight and waist circumference, the lack of a reduction for all subjects following weight loss is surprising. In this study the modest weight reduction (<5%) was potentially too small to confer some metabolic benefits to all subjects, as evidenced by the lack of a reduction in adiponectin, total cholesterol, HDL-C and LDL-C. This is supported by observed increases in

adiponectin and improvements in lipid profiles with a 15% weight loss over a 2 year dietary and exercise intervention, with no changes in adiponectin for controls who lost 3% of their initial body weight (593) and no changes in adiponectin following significant reductions in weight (6.81 kg over 9 weeks) (595). However, in a sub-analysis we observed increases in adiponectin levels and reductions in triglycerides levels following weight loss for both women with and without PCOS with significantly lower baseline CRP levels. This suggests that subjects with an adverse inflammatory profile may demonstrate less favourable metabolic improvements following weight loss, although the evidence to support this is currently unclear (599). Despite the significantly higher fasting and post-prandial insulin levels for women with PCOS in this study, all subjects pre-weight loss displayed an equivalently moderately increased metabolic risk profile, including moderately elevated CRP levels (> 3 mg/L) BMI, waist circumference, triglycerides and LDL-C and moderately reduced HDL-C. It is possible that the participants in this study are not representative of the general population where differences in cardiovascular risk profiles are commonly observed between women with and without PCOS (600). Where women with PCOS usually display an elevated cardiovascular risk profile in association with elevated inflammatory markers, it is therefore possible that a greater degree of weight loss may be required to achieve similar metabolic benefits to subjects without PCOS and with a reduced cardiovascular risk profile.

We and other investigators have reported similar CRP and adiponectin levels in overweight women with and without PCOS (586-588), in contrast to alternative reports of elevated CRP (582, 583) and reduced adiponectin levels (534, 584, 585) in lean and overweight women with PCOS compared to weight matched controls. It is unclear why we did not observe differences in CRP or adiponectin levels between women with and without PCOS. Where differences in CRP and adiponectin between women with and without PCOS were observed, this was often in conjunction with significantly elevated surrogate measures of IR for the women with PCOS, supporting the proposed association between cytokines, adipokines,

insulin sensitivity and inflammation (601-603). Although less likely, it is possible that alterations in adiposity, as opposed to insulin sensitivity, are primarily responsible for mediating changes in cytokines and adipokines with weight loss. In a number of studies, weight status was more strongly associated with CRP than surrogate measures of insulin sensitivity (587, 604) and no association was reported between adiponectin and insulin sensitivity as assessed by the euglycaemic hyperinsulinaemic clamp after adjustment for BMI (605). The similar CRP and adiponectin levels may therefore be a function of the similar weight and waist circumference between the women with and without PCOS.

There is some suggestion that the ratio of high to low molecular weight forms of adiponectin influences hepatic insulin sensitivity and that this may be differently modulated by weight loss than total adiponectin (606). We only measured total adiponectin and therefore cannot comment on any differential effect of weight loss on adiponectin molecular forms in women with and without PCOS. It is proposed that obesity-associated increases in TNF-α and IL-6 reduce adiponectin expression and thus insulin sensitivity through inhibiting adiponectin promoter activity and reducing adiponectin mRNA expression and secretion (607, 608) while in PCOS IR is predominantly associated with post-receptor defects in insulin signalling (609). Obesity-associated IR is thus metabolically distinct from PCOS-associated IR (609) and it is possible that adiponectin is not involved in the mediation of IR in PCOS. However, there is a proposed role for androgens in inhibiting adiponectin expression and secretion (610) and the implications of this with regards to circulating adiponectin in PCOS is unclear.

Although the subjects with and without PCOS displayed similar metabolic profiles at baseline, with no differences in weight, fat mass, waist circumference, CRP or lipid profile present, we did not utilize precise measures of body composition, glucose tolerance or insulin sensitivity in this study and are therefore not be able to perform a detailed assessment of metabolic risk to aid in interpretation of results. Although waist circumference is a strong

independent predictor of visceral adipose tissue (611), more definitive measures of visceral adiposity through computed tomography, ultrasonography or magnetic resonance imaging may have elucidated subtle differences between the women with and without PCOS. It is additionally unclear what the optimal measurement for IR is in PCOS. Despite good associations by a variety of investigators of fasting measures of insulin sensitivity with the euglycaemic hyperinsulinaemic clamp (247, 248), no correlation of the above parameters with euglycaemic clamps was observed (251) and HOMA failed to diagnose hyperinsulinaemia (612) in women with PCOS. Furthermore, in this study we were not able to age match our subjects with PCOS and controls. Aging is associated with an increase in insulin resistance and central and visceral adiposity and associated metabolic conditions (613, 614). Conversely, adiponectin has been found to both be unrelated to age in lean women with and without PCOS (585) and to modestly increase with age in the lean and obese general population (615, 616). It is unclear if the potential modest change in adiponectin with age modulates the risk of metabolic disease. Any impairment in metabolic factors in subjects with PCOS (eg adiposity distribution, insulin or glucose homeostasis) may also have been masked by the significantly younger age of this group. However, the lack of a decrease in CRP following weight loss for the women with PCOS is consequently more important as this occurred despite the age difference. Furthermore, inflammatory markers are also affected by menstruation (617) and CRP levels have been observed to be elevated during the early follicular phase and reduced during the midfollicular phase (618). Variations in insulin sensitivity and glucose homeostasis have also been reported over the course of the menstrual cycle (619). Thus the lack of controlling for menstrual cycle phase in the collection of our blood samples is a flaw in this study that may affect the relevance of our results.

Although modest weight loss improves reproductive features in PCOS, effects on the metabolic profile were more varied. Despite improvements in triglycerides and insulin and glucose homeostasis for all subjects, modest weight loss had no effect on CRP or adiponectin

in overweight women with PCOS. Clinically, this suggests that overweight women with PCOS may require a greater weight loss target (>5%) to achieve reductions in inflammatory markers such as CRP. This may be related to the elevated insulin resistance commonly observed in PCOS which either may either not be improved by weight loss in all women with PCOS or may require a greater reduction in weight, abdominal or visceral adiposity, insulin and androgens to be ameliorated.

CHAPTER 4: DIET COMPOSITION, GHRELIN AND SATIETY IN OVERWEIGHT WOMEN WITH AND WITHOUT POLYCYSTIC OVARY SYNDROME

4.1: ABSTRACT

Polycystic ovary syndrome (PCOS) is a common endocrine condition in women of reproductive age. Ghrelin is a hormone implicated in appetite regulation which may be dysregulated in PCOS. The effect of varying dietary composition on ghrelin is additionally unknown. This study examined the effects of a standard protein (SP) (55% carbohydrate, 15% protein) or high protein (HP) diet (40% carbohydrate, 30% protein) on weight loss, body composition, glucose and insulin homeostasis, fasting and post-prandial ghrelin and satiety by visual analogue scores (VAS) in overweight women with (n=20, n=10 SP, n=10 HP) or without PCOS (n=12, n=6 SP, n=6 HP) matched for BMI. The intervention consisted of 12 weeks energy restriction (~ 6000 kJ/day) followed by 4 weeks weight maintenance. Post-prandial insulin, ghrelin and satiety were assessed after a representative meal tolerance test (MTT). Diet composition had no effect on fasting or post-prandial ghrelin or measures of satiety. Non-PCOS subjects had a 70% higher fasting baseline ghrelin (P=0.011), a greater increase in fasting ghrelin (57.5 versus 34.0%, P=0.033), a greater post-prandial ghrelin decrease at week 16 (113.5±46.3 versus 49.3±12.2 pg/mL, P=0.05) and a greater maximal decrease in post-prandial ghrelin following weight loss (-144.1±58.4 versus -28.9 ± 14.2 pg/mL, P=0.02) than subjects with PCOS. Subjects with PCOS were less satiated (P=0.001) and more hungry (P=0.007) after a test meal at week 0 and 16 than subjects without PCOS, although satiety was not related to ghrelin or diet composition. However, subjects with PCOS were significantly less satiated at both week 0 and week 16 than subjects without PCOS (P=0.003). Appetite regulation, as measured by subjective short-term hunger and satiety and ghrelin homeostasis, may be impaired in PCOS.

4.2: INTRODUCTION

Ghrelin is a 28 amino acid acylated peptide produced primarily by the endocrine cells in the stomach (466). It stimulates growth hormone (GH) secretion through its action as an

endogenous ligand for the hypothamalamic-pituitary growth-hormone secretagogue receptor (GHS-R). In addition, it is implicated as an important regulatory peptide in food intake, body weight regulation, endocrine pancreatic function, glucose metabolism and ovarian function (467, 468). Ghrelin levels increase sharply prior to feeding onset (469-472) and stimulates hunger and food intake (473-475) via action on the hypothalamic arcuate nucleus (476). Once feeding commences, either the presence of nutrients in the gut or the metabolic response to feeding leads to the suppression of ghrelin and a resultant change in appetite (469-472). Fasting ghrelin levels are lower in obese individuals (491), an appropriate response to chronic positive energy balance and increase with weight loss (470). There is some suggestion that the decrease in ghrelin following a meal may be impaired partially or fully in obesity (472). This may play a role in the pathophysiology of obesity through compromised meal termination and increased food consumption. Additional factors such as leptin, CCK and peptide YY also play a crucial role in the regulation of energy homeostasis, appetite and ghrelin (620).

In PCOS, fasting ghrelin is decreased compared to controls in some (526, 527) but not all studies (528). Furthermore, plasma ghrelin is lower in Pima Indians, a population predisposed to obesity, compared to age and weight matched Caucasians (491). Post-prandial ghrelin has not previously been examined in either of these populations. It is unclear whether ghrelin homeostasis is impaired in PCOS. The effect of varying dietary composition on ghrelin secretion has not been examined extensively in humans. High protein diets have been proposed to aid in weight loss (337) through an increased satiating effect of dietary protein compared with carbohydrate or fat (332). It is unknown whether this satiating effect is mediated through ghrelin. A greater suppression of post-prandial ghrelin was observed for a high carbohydrate diet compared to an isocaloric high fat meal (495) and diet (494).

The objectives of this study were: (1) To determine the effects of standard or high protein diets on weight loss, body composition, leptin, glucose and insulin homeostasis and fasting

and post-prandial ghrelin in women with or without PCOS and (2) To determine the relationship between satiety, PCOS status, diet composition, leptin and changes in ghrelin homeostasis.

4.3: METHODS

4.3.1: Subjects

Previous study cohorts were used to select a subset of pre-menopausal overweight (BMI > 25 kg/m^2) women with PCOS (302) and without PCOS (321) (European Caucasian). From these studies, 20 overweight women with PCOS and 12 overweight women without PCOS matched for weight and body mass index (BMI) were chosen for further comparison of selected hormonal variables. As a result of this selection, we were unable to completely control for age. Diagnosis of PCOS was by menstrual irregularity (cycle length < 21 days or > 35 days or variation between consecutive cycles of > 3 days) and clinical (hirsutism/acne) and/or biochemical hyperandrogenism (111). Hyperandrogenism was defined from a range obtained from a representative population of non-PCOS women (n=80) (544). Clinical hyperandrogenism was assessed initially by self-reported hirsutism and confirmed by the Ferriman-Gallwey score, with a score > 8 indicating hirsutism (119). PCOS and non-PCOS status was confirmed for all subjects based on these criteria and all non-PCOS subjects displayed menstrual regularity. Inclusion criteria were fasting serum insulin greater than 12 mU/L (non-PCOS subjects) and body mass index greater than 25 kg/m^2. Exclusion criteria were weight greater than 140 kg, smoking, Type 2 Diabetes Mellitus (T2DM), proteinuria, a history of malignancy, liver, unstable cardiovascular, respiratory, or gastrointestinal disease and use of insulin-sensitising agents, hormonal treatment or oral contraceptives and related endocrine disorders (excluded by assessment of thyroid stimulating hormone (TSH), prolactin and 17α-hydroxyprogesterone).

Subjects were eligible for the study if they had not been taking oral contraceptives for more than 4 weeks or hormone treatment or insulin sensitising agents for more than 2 weeks. All subjects gave informed consent for the studies, which were approved by the Human Ethics Committees of The Commonwealth Scientific and Industrial Research Organisation (302, 321), The Royal Adelaide Hospital (321) and The North West Adelaide Health Service (302).

4.3.2: Dietary intervention

Subjects were stratified to ensure equal distribution for known confounding factors of weight, age and desire to conceive (for the PCOS subjects) and fasting serum insulin concentrations at screening, BMI and age (for the non-PCOS subjects). The two groups were then randomized by an independent observer to one of two diets: (1) standard protein (SP) (15% of daily energy as protein, 55% carbohydrate and 30% fat) or (2) high protein (HP) (30% of daily energy as protein, 40% carbohydrate and 30% fat). Energy intake was restricted (~6000 kJ/day) for 12 weeks. Thereafter, subjects were placed on a weight maintenance diet of the same macronutrient composition for 4 weeks. Both diets were nutritionally complete and had a similar fatty acid profile. Alcohol was not permitted. Ten PCOS and 6 non-PCOS women were on the HP diet and 10 PCOS and 6 non-PCOS women were on the SP diet. Subjects met with a registered dietitian fortnightly for initial education on quantification and recording of their daily food intake and to assess and modify the dietary regime based on compliance and weight loss. Nutrient intakes were calculated with Diet 4/Nutrient Calculation Software (Xyris Software, Highgate Hill, Australia) based on data from Australian food composition tables. Nutritional intake was assessed from monthly 3–day consecutive dietary food records (one weekday and two weekend days) and daily dietary checklists. At weeks 0 and 16 dietary compliance was determined by subject adherence to the macronutrient profiles (protein, carbohydrate, fat) and from assessment of random urine samples (302) and 24-hour urine samples (321) for urea excretion relative to urine creatinine.

4.3.3: Study design

Subjects attended the outpatient clinic on two consecutive days monthly. At the initial visit, height was measured to the nearest 0.1 cm using a stadiometer (SECA, Hamburg, Germany) with subjects in the free-standing position. At all visits, body weight was measured to the nearest 0.05 kg using calibrated electronic digital scales (Mettler scales, model AMZ14, A&D Mercury, Kinomoto, Japan) while subjects were wearing light clothing with no shoes. BMI was calculated by weight (kg) divided by squared height (m^2). At weeks 0, 12 and 16, venous blood samples assessed for insulin, glucose and ghrelin were taken after an overnight fast. The homeostasis model assessment (HOMA) was used as a surrogate measure of insulin sensitivity [Fasting insulin (mU/L)*Fasting glucose (mmol/L)/22.5] (80).

At weeks 0 and 16, a 3-hour meal tolerance test (MTT) was performed with a 2700 kJ test meal using the allocated diet with equivalent energy densities (11% protein, 15% fat and 76% carbohydrate for the SP and 31% protein, 14% fat and 55% carbohydrate for the HP diet). Fasting venous blood was taken for measurement of insulin, glucose and ghrelin (time 0). Subjects were then required to consume the meal within 20 minutes and further blood samples were taken for assessment of insulin and glucose at 60, 120 and 180 minutes and ghrelin at 60 and 120 minutes. Subjective hunger, fullness, satiety and desire to eat were assessed using a validated 10 cm linear scale visual analogue scores (VAS) immediately before eating and at 60, 120 and 180 minutes (621). The change in ratings from baseline was quantified (622, 623). A more positive value for satiety and fullness indicates greater satiety and fullness and a more negative value for hunger and desire to eat indicates lesser hunger and desire to eat. Post-prandial ghrelin was measured as the change in ghrelin from 0 minutes to 120 minutes.

At week 0 and 16, total fat mass and total lean mass were assessed by whole-body dual x-ray absorptiometry (DEXA) (Norland Medical Systems Inc., Fort Atkinson, WI) (coefficient of variation of 2.3±0.9% for total fat mass and 2.1±0.4% for lean tissue mass). Abdominal fat

mass was measured from the area demarcated by the ribs at the upper portion and the iliac crests at the lower portion (302, 321). The PCOS subjects attended a weekly exercise/education class (262, 283, 301) and were advised to increase exercise to a minimum of 3 times per week. Exercise levels were documented at baseline, monthly and study completion and categorised according to National Health and Medical Research Council Standards (327). The non-PCOS subjects were asked to continue their usual physical activity levels throughout the study and physical activity levels were not recorded.

4.3.4: Biochemical measurements

Fasting blood samples were taken by venipuncture. Blood for serum was collected in tubes with no additives and allowed to clot at room temperature for 30 min. Blood for plasma was collected in tubes containing sodium fluoride/EDTA. Serum and plasma were isolated by centrifugation for 10 min at 3000g (5°C) (Beckman GS-6R; Beckman, Fullerton, CA) and serum, plasma and urine aliquots were stored at –80°C. Details of all assay methodologies (SHBG, testosterone, total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), insulin, glucose, total ghrelin, leptin, urinary urea, urinary creatinine, TSH, prolactin, 17-α progesterone) are described in Appendix 2.

4.3.5: Statistics

All data are presented as means±SEM. Two-tailed statistical analysis was performed using SPSS for Windows 10.0 software (SPSS Inc, Chicago, USA) with statistical significance set at an α level of $P \leq 0.05$. Baseline measurements were assessed using one way ANOVA. Comparisons between time points were assessed using repeated measures ANOVA with diet and PCOS status as between subject factors. However, there was no difference in weight loss or metabolic, leptin and ghrelin responses to weight loss between subjects with and without

PCOS depending on diet. The analysis was therefore confined to the effect of diet or PCOS status on the observed variables.

In the event of an interaction, post-hoc pairwise comparisons were performed. Relationships between variables were examined using bivariate and partial correlations, ANCOVA and multiple linear regression. VAS were analysed by a 4 way repeated measures ANOVA with week and blood sampling time as the within subject factors and PCOS status and diet as the between subject factor. Differences at each blood sampling time were compared by one-way ANOVA. Total areas under the insulin, glucose and VAS curves (AUC) (satiety and fullness) above baseline during the MTT and for total AUC below the curve (hunger, desire to eat) were calculated geometrically (trapezoidal rule) (624). Dietary variables that differed in energy restriction or weight maintenance (polyunsaturated fatty acid, cholesterol, fibre), weight at week 0 and weight loss over the 16 weeks were additionally used as covariates. Where no significant interaction existed between the diet groups, data was combined to compare the PCOS and non-PCOS subjects. One subject did not comply with the intervention (non-PCOS status, HP diet) and her data was used only for baseline comparisons. Weight maintenance dietary composition data is presented for 31 subjects, DEXA data is presented for 26 subjects and VAS data is presented for 29 subjects due to incomplete data.

4.4: RESULTS

4.4.1: Subjects

The study comprised 32 subjects (mean weight 94.5±2.3 kg, mean BMI 35.4±0.9 kg/m^2, mean age 35.7±1.1 years). Baseline characteristics by diet composition and PCOS status are shown in Table 4.1 and apart from age there was no difference between groups. HP PCOS had greater abdominal fat mass than HP non-PCOS subjects (P=0.024) (Table 4.1).

4.4.2: Diet and compliance

Both diets were well tolerated with no adverse events reported. All subjects complied well with the intervention based on urinary urea/creatinine and individual macronutrient profiles (previously reported). There was a diet effect such that the urinary urea to creatinine ratios were significantly different at study completion ($P<0.001$) (302, 321). For the PCOS subjects, exercise levels were similar between the two dietary groups. As designed, protein intake was higher and carbohydrate intake was lower on the HP than on the SP diet during both energy restriction (26.8 versus 15.9%) and weight maintenance (27.0 versus 15.6%) ($P<0.001$). Minor differences existed between the PCOS and non-PCOS subjects in diet composition for PUFA intake in energy restriction and fibre intake in weight maintenance ($P\leq0.05$). When comparing the SP and HP diets, energy intake was not significantly different in either energy restriction (6.3±0.09 MJ) or weight maintenance (7.75±0.21 MJ) between the two diets. Total fat and saturated fat intake were not different between the two diets in either energy restriction or weight maintenance, however significant differences existed between the two diets for PUFA and cholesterol in energy restriction and weight maintenance and fibre in energy restriction ($P\leq0.05$) (Table 4.2).

Table 4.1: Subject baseline characteristics

Variable (week 0)	PCOS		Non-PCOS		PCOS vs non-PCOS	SP vs HP
	SP	**HP**	**SP**	**HP**		
Age (year)	34.8±1.2*	33.1±1.7	41.1±3.1	36.2±3.2	P=0.04	P=0.19
BMI (kg/m^2)	36.6±2.2	36.0±1.6	34.5±1.6	33.1±1.5	P=0.19	P=0.65
Weight (kg)	94.9±4.8	98.4±4.9	93.2±4.3	88.9±2.5	P=0.25	P=0.90
Fasting glucose (mmol/L)	5.7 ± 0.3	5.5 ± 0.2	5.3 ± 0.2	5.2 ± 0.2	P=0.17	P=0.70
Fasting insulin (mU/L)	21.0±5.9	24.9±3.2	23.7±9.8	18.5±3.9	P=0.75	P=0.90
HOMA	6.0±2.0	6.3±0.8	5.9±2.6	4.3±1.0	P=0.54	P=0.79
Leptin (ng/mL)	66.7±12.8	86.0±14.5	66.8±9.9	60.5±14.9	P=0.41	P=0.51
DEXA: Abdominal fat (kg)	10.5±1.1	10.8±0.6 †	9.1±0.7	8.7±0.4	P=0.06	P=0.96
DEXA: Total fat mass (kg)	47.0±3.5	46.9±3.9	43.1±5.2	43.4±1.3	P=0.35	P=0.94

Data are expressed as mean ± SEM.
Date was assessed by one-way ANOVA with diet or PCOS status as the between subject factors.

SP: Standard protein
HP: High protein
PCOS: Polycystic ovary syndrome
BMI: Body mass index
HOMA: Homeostasis model assessment
DEXA: Dual x-ray absorptiometry

PCOS n=20: SP n=10, HP n=10 (DEXA SP n=9, HP n=8).
non-PCOS n=12: SP n=6, HP n=6 (DEXA SP n=4, HP n=5).
* P=0.040 for SP PCOS compared to SP non-PCOS.
† P=0.024 for HP PCOS compared to HP non-PCOS.

Table 4.2: Dietary intake for 12 weeks of energy restriction and 4 weeks of weight maintenance on a standard or high protein diet

Macronutrients	SP ER	SP WM	HP ER	HP WM
Energy (MJ/day)	6.38±0.14	7.66±0.27	6.17±0.12	7.86±0.35
CHO (% E)	57.0±0.47 [a]	56.5±0.55 [b]	43.9±0.48	44.1±0.77
Protein (% E)	15.9±0.16 [a]	15.6±0.40 [b]	26.8±0.44	27.0±0.56
Total fat (% E)	26.8±0.49	27.7±0.43	27.9±0.60	27.8±0.77
Alcohol (% E)	0.1±0.11	0.0±0.00	0.0±0.00	0.0±0.00
SFA (% E)	8.1±0.23	8.7±0.35	8.5±0.24	8.9±0.31
PUFA (% E)	2.2±0.12 [a]	2.9±0.14 [b]	2.9±0.11	3.1±0.14
MUFA (% E)	12.9±0.35	12.7±0.37	13.9±0.41	13.4±0.54
Cholesterol (mg)	92±4.42 [a]	139±13.62 [b]	169±5.38	207±23.45
Fibre (g)	26±0.76 [a]	29±2.05	20±1.20	26±1.90

Data are expressed as mean±SEM.
Date was assessed by one-way ANOVA with diet as the between subject factors.

ER SP: n=16, HP: n=15; WM SP: n=16: HP n=14.

SFA: Saturated fatty acid
MUFA: Monounsaturated fatty acid
PUFA: Polyunsaturated fatty acid
% E: % of total daily energy intake
CHO: Carbohydrate
SP: Standard protein diet
HP: High protein diet
ER: Energy restriction (weeks 0 – 12)
WM: Weight maintenance (weeks 12 – 16)

[a] significant difference from HP diet in energy restriction (weeks 0 – 12) ($P \leq 0.05$).
[b] significant difference from HP diet in weight maintenance (weeks 12 – 16) ($P \leq 0.05$).

4.4.3: Weight and body composition

Over the 16 weeks there was no significant difference in weight loss between the SP and HP diets (7.0±0.7 versus 7.2±0.9 kg) or the PCOS and non-PCOS subjects (7.0±0.8 versus 7.2±0.6 kg) with a mean weight loss of 7.1±0.6 kg (7.5%) for combined subjects. Weight was maintained during energy balance with a total mean gain of 0.15±0.19 kg and no differences between the SP and HP diets or the PCOS and non-PCOS subjects, indicating good compliance with the weight maintenance regime. There was no differential effect of diet composition or PCOS status on changes in body composition over the 16 weeks, with an overall reduction in BMI (7.5%), total fat mass (13.4%), total lean mass (2.5%) and abdominal fat mass (13.3%) occurring for all subjects (Table 4.3).

4.4.4: Fasting and post-prandial glucose, insulin and HOMA

There was no effect of diet composition or PCOS status on changes in fasting glucose, insulin, HOMA or leptin over the study duration (Table 4.3). There was no effect of diet composition or PCOS status on the area under the insulin curve after the test meal (MTT insulin AUC) at week 0 or week 16 or on the change in MTT insulin AUC with weight loss. The insulin response to the test meal was reduced by 25% after the 16 week intervention. There was no effect of PCOS status on the area under the glucose curve after the test meal (MTT glucose AUC) at week 0 or week 16. However, the SP meal resulted in a 2.9 times greater AUC at week 0 (P=0.019) (3.9±0.9 versus 1.4±0.5 mmol/L/180 minutes) and a 3.4 times greater AUC at week 16 (P=0.024) (3.2±0.7 versus 1.0±0.6 mmol/L/180 minutes) compared to the HP test meal. There was no change in MTT glucose AUC with weight loss (Table 4.3).

Table 4.3: Combined data for weight, body composition, fasting and post-prandial glucose and insulin and homeostasis model assessment before and after 12 weeks of energy restriction and 4 weeks of weight maintenance on a standard protein or high protein diet

Variable	Week 0	Week 12	Week 16
Weight (kg)	94.8±2.4	87.6±2.2 †	87.7±2.2 †
BMI (kg/m^2)	35.6±0.9	32.9±0.9 †	32.9±0.9 †
DEXA: Abdominal fat (kg)	10.0±0.5	-	8.7±0.5 †
DEXA: Total fat mass (kg)	45.7±1.8	-	39.6±1.7 †
DEXA: Total lean mass (kg)	45.4±1.2	-	44.1±1.1 *
Fasting glucose (mmol/L)	5.5 ± 0.1	5.4 ± 0.1	5.5 ± 0.1
Fasting insulin (mU/L)	22.2±2.9	14.9±1.5 *	15.0±1.4 *
HOMA	5.8±0.8	3.6±0.4 *	3.7±0.4 *
Leptin (ng/mL)	73.9±7.1	41.0±4.0 †	52.0±5.8 † ‡
MTT Insulin AUC (mU/L/180 min)	329.6±51.1	-	216.7±38.5 *
MTT Glucose AUC (mg/dL/180 min)	48.3±10.2	-	38.7±8.9

Data are expressed as mean ± SEM.
Data was assessed using a repeated measures ANOVA with diet or Polycystic Ovary Syndrome (PCOS) status as the between subject factors.
N = 31; N = 26 (DEXA).

BMI: Body mass index
MTT: Meal tolerance test
AUC: Area under the curve
HOMA: Homeostasis model assessment
DEXA: Dual x-ray absorptiometry

* effect of time (weeks 0 – 12 or weeks 0 – 16) (P<0.01).
† effect of time (weeks 0 – 12 or weeks 0 – 16) (P<0.001).
‡ effect of time (weeks 12 – 16) (P<0.01).

4.4.5: Fasting and post-prandial ghrelin

There was no difference in baseline fasting ghrelin between the HP and SP diets. Following the dietary intervention, there was no significant differential effect of diet composition on changes in fasting ghrelin between the SP and HP diets with respective increases of 67.9±26.7 pg/mL and 120.6±40.7 pg/mL observed from week 0 to week 16 (P=0.415 for diet x time effect). However, fasting ghrelin was significantly higher by 70.4% in the non-PCOS subjects compared to the PCOS subjects (P=0.011) (355.9±60.6 versus 205.3±23.1 pg/mL) and non-PCOS subjects had a significantly greater increase in fasting ghrelin compared to PCOS subjects from weeks 0 – 16 (57.5 versus 34.0%) (161.7±56.4 versus 55.8±17.2 pg/mL, P=0.033 for diet x PCOS status effect) (Figure 4.1a). Improvement in fasting ghrelin following weight loss occurred in energy restriction. No further changes in fasting ghrelin occurred during the weight maintenance period.

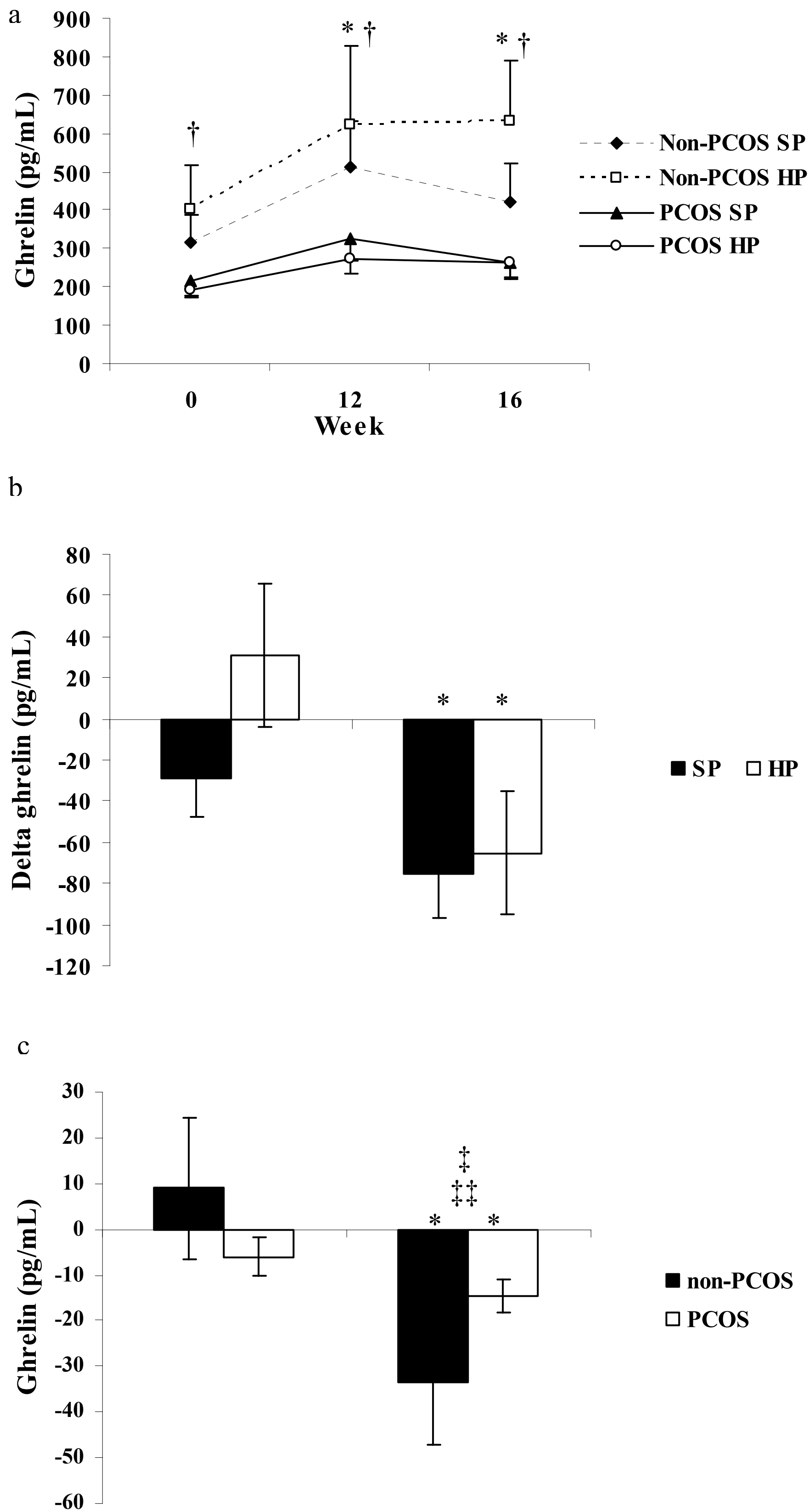
There was no differential effect of diet composition on the ghrelin response to the test meal (MTT ghrelin) at week 0 or 16. For combined subjects at week 0, there was no significant decrease in ghrelin after the test meal. For combined subjects at week 16, there was a significant decrease in MTT ghrelin (-69.8±24.2 pg/mL, P=0.002). There was no effect of diet on the change in MTT ghrelin from week 0 – 16. However, there was a time x PCOS status interaction; the non-PCOS subjects showed a significantly greater decrease in post-prandial ghrelin from week 0 to 16 than the PCOS subjects (-144.1±58.4 versus -28.9±14.2 pg/mL, P=0.02 for PCOS status x time effect) (Figure 4.1b) such that at week 16, the PCOS subjects had a significantly lesser post-prandial decrease (49.3±12.2 versus 113.5±46.3 pg/mL, P=0.05).

4.4.6: Visual analogue scores

There was no differential effect of diet composition or PCOS status on fasting VAS measures. There was no differential effect of diet composition on the MTT VAS measures. Data was therefore combined to assess subjects with and without PCOS. Figure 4.2 shows the average hunger, fullness, desire to eat and satiety response curves for PCOS and non-PCOS groups at week 0 and week 16. There was no significant difference between the PCOS and non-PCOS subjects with regards to changes in MTT VAS AUC scores over time. However, at both week 0 and week 16 the PCOS subjects were significantly more hungry (Figure 4.2a, $P=0.007$) and less satiated (Figure 4.2c, $P=0.001$) than the non-PCOS subjects during the MTT. This effect was apparent by 120 minutes (for satiety at week 0 and 16) and by 180 minutes (for hunger at week 0 and 16) ($P<0.05$). The non-PCOS subjects had a 2.9 times higher AUC for satiety at week 0 and a 1.66 times higher AUC for satiety at week 16 compared to the PCOS subjects ($P=0.003$).

For all subjects, there was a reduction in the desire to eat during the test meal from week 0 to 16 (Figure 4.2d), with a 1.5 times higher AUC at week 16 ($P<0.001$). There was no differential effect of diet composition or PCOS status on any of the fasting VAS values. However, the baseline desire to eat increased from week 0 to week 16 (54.8±5.0 to 72.8±4.7 mm, $P<0.001$).

Figure 4.1: Fasting and post-prandial ghrelin after 12 weeks of energy restriction and 4 weeks of weight maintenance on a standard protein or high protein diet



Data are expressed as mean±SEM.

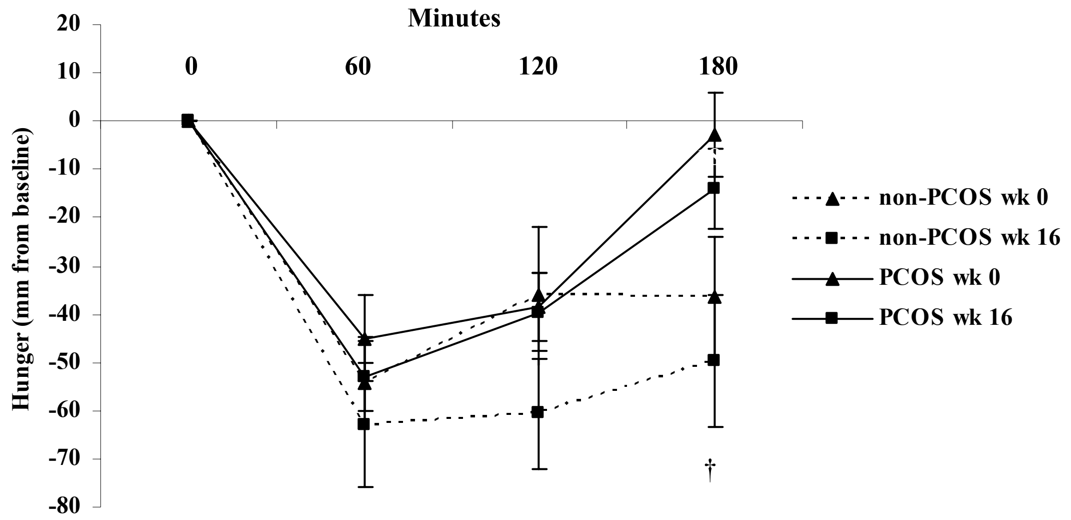
Data was assessed using a repeated measures ANOVA with diet or Polycystic ovary syndrome (PCOS) status as the between subject factors. Differences at each week were compared by one-way ANOVA.

(a) Fasting ghrelin at weeks 0, 12 and 16 for subjects with polycystic ovary syndrome (PCOS) (n=20) and without PCOS (n=11) following a standard protein or a high protein diet for 12 weeks of energy restriction and 4 weeks of weight maintenance.
(b) Ghrelin response to a high protein (HP) or standard protein (SP) meal tolerance test (MTT) (delta ghrelin from 0 – 120 minutes) before (week 0) and after weight loss (week 16) for combined data for subjects with polycystic ovary syndrome (PCOS) (n=20) and without PCOS (n=11).
(c) Ghrelin response to a meal tolerance test (MTT) (combined data for high protein and standardprotein) (delta ghrelin from 0 – 120 minutes) before (week 0) and after weight loss (week 16) for subjects with polycystic ovary syndrome (PCOS) (n=20) and without PCOS (n=11).

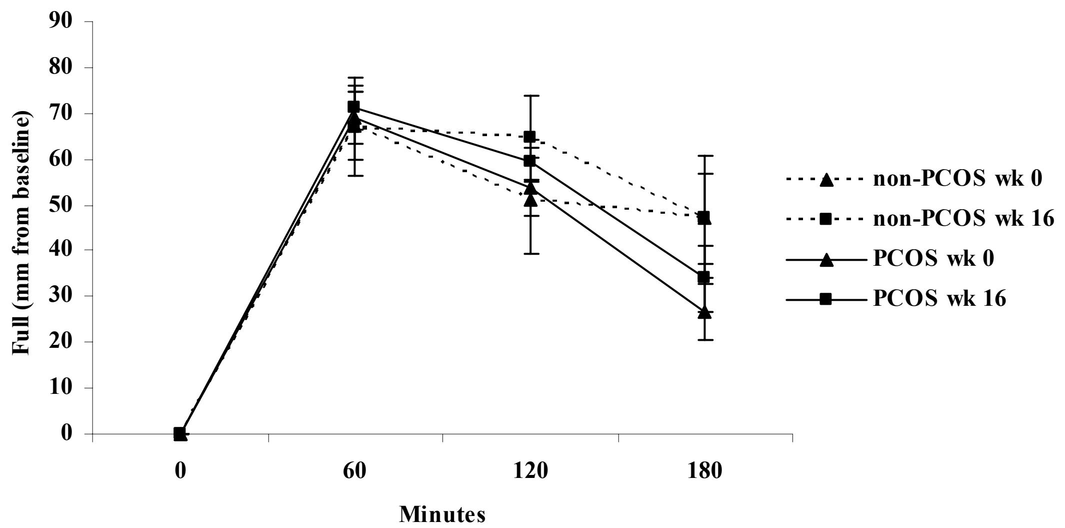
[*] effect of time compared to week 0 ($P \leq 0.05$).
† significantly greater than PCOS for combined SP and HP diets ($P \leq 0.05$) (time x PCOS status, P=0.033)
‡ significantly greater change in delta ghrelin from week 0 to week 16 for the non-PCOS subjects (P=0.033) compared to the PCOS subjects for combined SP and HP diets (P=0.05) (time x PCOS status, P=0.02).
‡‡ significantly greater change in MTT ghrelin at week 16 for PCOS subjects for combined SP and HP diets (P=0.05).
There was no effect of diet composition on any ghrelin parameter.

Figure 4.2: Subjective measures of hunger, fullness, satiety and desire to eat after a test meal before and after 12 weeks of energy restriction and 4 weeks of weight maintenance on a standard protein or high protein diet

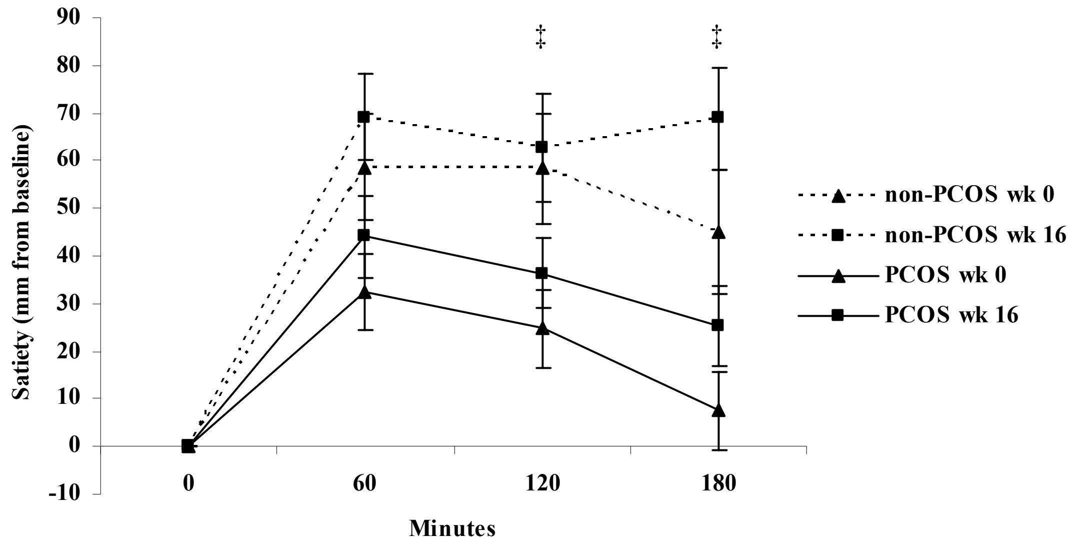
a



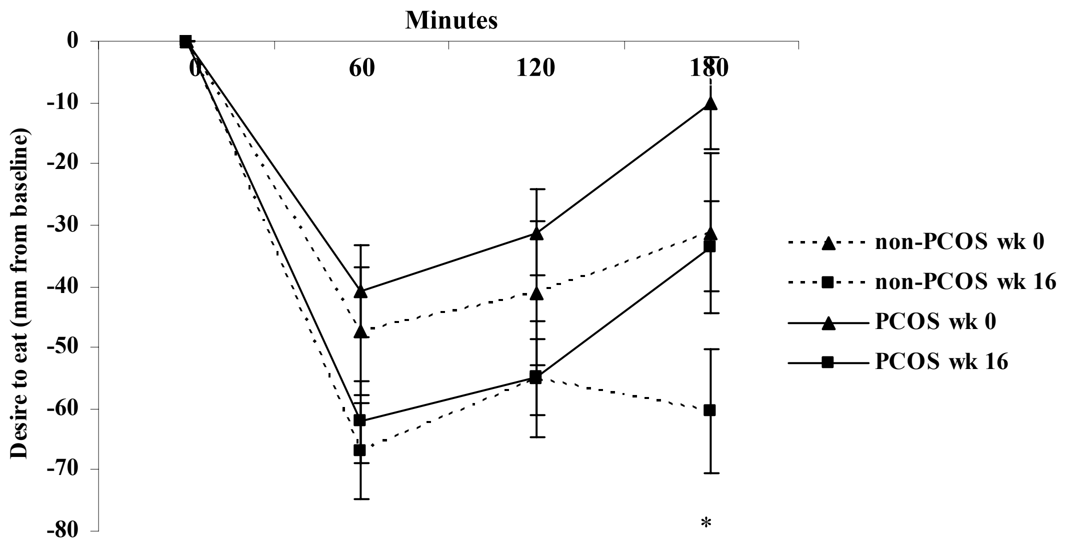
b



c



d



Data are expressed as mean±SEM.
Data were analysed by a 4 way repeated measures ANOVA with week and blood sampling time (minute) as within subject factor and Polycystic ovary syndrome (PCOS) status or diet as between subject factor. Differences at each sampling time were compared by one-way ANOVA.

Changes from baseline in visual analogue scores subjective measures of hunger (a), fullness (b), satiety (c) and desire to eat (d) after a test meal before (week 0; ▲) and after weight loss (week 16; ■) in subjects with PCOS)(n = 10; —) and without PCOS (n = 20; – –).

* effect of time from week 0 to week 16 for combined data (P<0.001)
† P<0.05 for difference between PCOS and non-PCOS subjects at week 0 and 16 (minute x PCOS status, P=0.007)
‡ P<0.05 for difference between PCOS and non-PCOS subjects at week 0 and 16 (minute x PCOS status, P=0.001).

4.4.7: Correlations and multiple regressions

Neither age, fasting insulin nor HOMA correlated with any measure of ghrelin or changes in ghrelin. At week 0, fasting ghrelin correlated with testosterone (r=-0.455, P=0.009), FAI (r=-0.492, P=0.004), BMI (r=-0.5, P=0.004) and abdominal fat (r=-0.604, P=0.001). After correction for BMI at week 0, fasting ghrelin did not correlate with any of the above variables. Multiple regression analysis showed the best predictor of fasting ghrelin at week 0 was abdominal fat at week 0 (r^2=0.365, P=0.001). The best predictor of changes in fasting ghrelin from week 0 – 16 was PCOS status (r^2=0.118, P=0.033). Fasting ghrelin or changes in fasting ghrelin with weight loss did not correlate with any of the VAS markers. Week 0 MTT ghrelin (0 – 120 minutes) correlated with week 0 VAS fullness (0 – 120 minutes) (r=-0.520, P=0.002). AUC desire to eat week 0 correlated with HOMA at week 0 (r=0.490, P=0.004) and insulin at week 0 (r=0.482, P=0.005). Baseline desire to eat at week 0 correlated with testosterone week 0 (r=-0.577, P=0.001) and hunger at week 16 correlated with SHBG week 16 (r=0.631, P=0.005) and weight at week 16 (r=0.497, P=0.006). Fasting leptin correlated with BMI at week 0 (r=0.585, P<0.001) and week 16 (r=0.610, P<0.001), abdominal fat at week 16 (r=0.630, P<0.001) and total fat at week 0 (r=0.573, P=0.002) and week 16 (r=0.694, P<0.001).

4.5: DISCUSSION

As we have previously shown, caloric restriction reduced weight, total and abdominal fat and improved insulin homeostasis with no differential effect of diet composition (302, 321) and the SP test meal resulted in a greater glucose AUC than the HP test meal (302, 321). Although it is anecdotally reported that women with PCOS have difficulty in achieving and maintaining weight loss (625), this has not been confirmed by us or other investigators (291, 292). However, neither the current study nor previous studies (291, 292) were *ad libitum* interventions and therefore do not represent a free-living situation where abnormalities in

energy homeostasis may lead to difficulties in achieving and maintaining a reduced weight. Additionally, exercise recommendations differed between the non-PCOS and PCOS subjects which is a potentially confounding factor in our analysis. However, this would have had a minimal effect on weight loss and body composition. Although physical activity is important in improving insulin resistance (IR) and maintaining weight loss, there is substantial data showing that physical activity adds relatively little to the magnitude of weight loss that occurs in response to short-term caloric restriction (626).

We have confirmed that a 7.5% weight loss in overweight individuals increases fasting ghrelin (470, 627). Similarly Hansen et al (627) reported a 12% increase in ghrelin following a 5 kg weight loss, although contrary data exists showing that there were no increases in either fasting or 24-hour AUC ghrelin after a 3.4 kg weight loss in subjects following an *ad libitum* low fat, high carbohydrate diet (494). The variations in weight loss may account for the discrepant results. Reduced ghrelin in obesity (471, 472, 491) and increased ghrelin in weight loss (470, 627) represent appropriate downregulation and upregulation of ghrelin in positive or negative energy balance. Ghrelin increases sharply prior to feeding onset (469-472) and decreases to reach a trough 1–2 hours post-meal (469), consistent with its proposed role in meal initiation and satiety. We and other investigators (12) observed impaired ghrelin responses to a test meal in overweight subjects which we found was normalised by weight loss. It is proposed that the chronic positive energy balance of obesity maximally suppresses ghrelin and thus limits further short-term regulation by feeding (472). This is potentially an important consequence of obesity and could influence body weight through compromising satiety and influencing inter-meal interval. However, in some studies in overweight individuals, 24-hour ghrelin profiles appear not to be impaired (470, 494).

After weight loss subjects displayed a greater desire to eat before the test meal but a reduced desire to eat during the meal, mirroring the observed changes in fasting and post-prandial

ghrelin. Although we did not observe a direct relationship between subjective measures of hunger and satiety and post-prandial ghrelin, a significant relationship has previously been reported (495). It has been proposed that the metabolic changes associated with weight loss (including decreased insulin and leptin and increased ghrelin) increase hunger and decrease satiety, contributing to the reported poor success of long-term weight maintenance (628, 629). Previous investigators have reported increases (628, 630) or no changes (629, 630) in subjective measures of fasting, 24-hour or post-prandial desire to eat, hunger or satiety following weight loss. It can be speculated that although the desire to eat increases in the fasting state following weight loss, appetite regulation following consumption of a meal improves. The effect of this on food intake in post-obese subjects is unclear but could include improved satiety and satiation which could contribute to reduced food intake and improved weight maintenance. Alternatively, Doucet et al hypothesised that the quality and quantity of foods prepared are likely to be influenced by fasting hunger levels, potentially contributing to passive overconsumption at meal-times (630).

Isocaloric substitution of protein for carbohydrate had no effect on any measures of ghrelin either before or after weight loss. Literature on the effect of varying dietary composition on ghrelin secretion is scarce. Maximal suppression of post-prandial ghrelin was observed following substitution of carbohydrate for fat either acutely (495) or following weight maintenance diets (494). Additionally, Erdmann et al observed an increase in post-prandial ghrelin following a high protein meal compared to a high fat or high carbohydrate meal (631). It is difficult to determine which macronutrient modulates ghrelin. The effect of weight loss on ghrelin may also be stronger than any effect of diet composition and therefore mask any subtle changes that occur. Since the publication of this study, there have been a number of additional papers examining the effect of modifying dietary composition on ghrelin homeostasis. The contribution of these additional papers to the concepts addressed in this thesis will be discussed in the final conclusion (Chapter 6).

Fasting ghrelin has been reported to be decreased in subjects with PCOS compared to controls (526, 527) but not in all studies (528). The reduced fasting ghrelin levels and the relatively smaller increase in ghrelin following weight loss that we have observed in the subjects with PCOS suggest a greater suppression of appetite in obesity and a reduced increase in appetite in weight loss. Moreover we have now demonstrated that subjects with PCOS are significantly hungrier and less acutely satiated after a test meal. These observations suggest that subjects with PCOS have impaired defences against overeating and may not have as strong a drive for meal termination as non-PCOS subjects. The reason for these observed differences in ghrelin between subjects with and without PCOS is unclear. We have confirmed findings that ghrelin negatively correlates with abdominal fat mass (632) and additionally report that abdominal fat was the best predictor of baseline fasting ghrelin. This could indicate a relationship between insulin sensitivity and ghrelin, indeed insulin has been postulated to play an important role in the regulation of ghrelin secretion and feedback (467). However, fasting insulin and HOMA were similar between PCOS and non-PCOS subjects and were not related to any measures of ghrelin. It is thus unclear if insulin resistance plays a role in ghrelin regulation. Although PCOS status was the strongest predictor for changes in fasting ghrelin with weight loss, no other metabolic variables associated with PCOS (testosterone, SHBG, FAI) were related to measures of ghrelin after adjustment for body weight. This has been previously reported (526, 528), although Pagotto et al reported a negative correlation between androstenedione and ghrelin (527). Leptin is proposed to be involved in ghrelin regulation (494) and reproductive physiology (633) and some (531) but not all (634) investigators have reported differing fasting levels between women with PCOS and weight matched controls. Fasting leptin decreased with weight loss as previously reported (628), but we observed no differential effect of diet composition or PCOS status. Baseline fasting leptin levels were similar between PCOS and non-PCOS weight and BMI-matched subjects.

In contrast to our findings, Pagotto et al reported that fasting ghrelin was not altered in subjects with or without PCOS following a weight loss of 3.9–10.2 kg after moderate caloric restriction (1200–1400 kcal/day) for 7 months (527). The reason for these discrepant results is unclear. We did not perform our measurements at a defined stage of the menstrual cycle and in addition the subjects in the two intervention groups were not perfectly matched. The retrospective nature of this study resulted in imperfect matching of subjects between diet groups and between PCOS and non-PCOS subjects. HOMA correlates well with the euglycaemic hyperinsulinaemic clamp (635). However, although it did not correlate with ghrelin in this study, it is only a surrogate marker of insulin sensitivity and may not allow the detection of small changes. We additionally only measured post-prandial ghrelin at a limited number of time points (0, 60 and 120 minutes) which may mask any differences occurring at other time points between either the diet groups or individuals with and without PCOS. Furthermore, the majority of work on ghrelin involves measurement of total ghrelin as opposed to bioactive ghrelin. It is unclear whether these are similarly regulated in human physiology and this needs to be further examined (636).

Nevertheless, this study confirmed that the post-prandial ghrelin response is impaired in obesity and that weight loss increases fasting ghrelin levels. Moreover, weight loss restores the impaired post-prandial ghrelin response and subjective measures of desire to eat appear to reflect the changes in fasting and post-prandial ghrelin. This study additionally supports previous reports of differences in fasting ghrelin between subjects with and without PCOS, and we have found that ghrelin homeostasis and acute measures of satiety and hunger are significantly impaired in women with PCOS both before and after weight loss. The potential impact of these findings on appetite regulation and the pathogenesis of achieving and maintaining a reduced weight remain to be elucidated.

CHAPTER 5: APPETITE HORMONES AND *AD LIBITUM* FOOD CONSUMPTION IN OVERWEIGHT WOMEN WITH AND WITHOUT POLYCYSTIC OVARY SYNDROME

5.1: ABSTRACT

Polycystic ovary syndrome (PCOS) is associated with obesity, reproductive and metabolic abnormalities. It is speculated that there may be abnormalities in appetite regulation in PCOS that favour weight gain or hinder weight loss. Overweight age and weight matched women with (n=14) and without (n=14) PCOS undertook an 8 week diet (5185.3±141.6 kJ/day). At baseline and study end, subjects consumed a test meal (936 kJ, 25% protein, 9% fat, 67% carbohydrate). Sensations of hunger and satiety were assessed by visual analogue scale and blood was sampled for glucose, insulin, ghrelin, cholecystokinin and peptide YY at 0, 15, 30, 45, 60, 90, 120 and 180 minutes. Thereafter a mixed buffet lunch was offered to assess *ad libitum* food intake. The average weight loss of approximately 4 kg was similar in both groups. Before weight loss, women with PCOS displayed significantly lower ghrelin levels (P=0.01) and an impaired post-prandial ghrelin decrease (P=0.048) compared to women without PCOS. After weight loss, there was a trend for women with PCOS to have lower ghrelin levels (P=0.097) and an impaired post-prandial ghrelin decrease (P=0.069) compared to women without PCOS. There were no differences in appetite responses, buffet consumption or fasting or post-prandial peptide YY and cholecystokinin between women with and without PCOS before or after weight loss. PCOS status is associated with lower fasting ghrelin and a smaller post-prandial ghrelin reduction both before and after weight loss but was not associated with other post-prandial gut peptides, subjective satiety or food intake. It is unclear if appetite regulation is impaired in PCOS.

5.2: INTRODUCTION

It is unclear if there are underlying metabolic abnormalities in some presentations of PCOS that either favour weight gain or contribute to difficulties in weight loss. Compared to body mass index (BMI)-matched controls, overweight women with PCOS display lower satiety and higher hunger post-prandially both before and after weight loss (Chapter 4). There is some

evidence that disturbances in appetite hormone regulation in PCOS could account for these reported discrepancies in hunger and satiety. Ghrelin is proposed as an acute meal initiator; ghrelin levels increase pre-prandially and decrease post-prandially and stimulate hunger and food intake (469). Post-prandial ghrelin suppression may be partially or fully impaired in obese compared to lean subjects (472, 480, 493) and overweight PCOS compared to weight-matched non-PCOS individuals (Chapter 4). Cholecystokinin (CCK) is released from the small intestine post-prandially, primarily in response to duodenal protein and fat and inhibits gastric emptying and reduces meal size and calorie intake in humans (637). It has been shown that post-prandial CCK is both elevated in obese compared to lean subjects or unaffected by body weight status (451, 487). In overweight women with PCOS compared to weight-matched controls, a reduced post-prandial CCK response was observed further suggesting a dysregulation of appetite control in PCOS (432). Peptide YY (PYY) is a peptide synthesised in the gastrointestinal tract that increases post-prandially, increases satiety and reduces food intake. It has additional functions including inhibition or reduction of gallbladder secretion, gut motility and pancreatic secretion (462, 463). Both reductions in fasting PYY or impaired post-prandial PYY (463, 483) have been observed in overweight subjects. It is unknown if it is differentially regulated in PCOS.

Although fasting ghrelin increases and the post-prandial ghrelin response improves with weight loss, this may be impaired in women with PCOS (Chapter 4). Fasting PYY increases following diet induced weight loss (638) and fasting or post-prandial CCK are unchanged by weight loss (639). The effect of weight loss on CCK or PYY has not yet been examined in PCOS. The objective of this study was therefore to examine fasting and post-prandial subjective appetite, appetite hormones (ghrelin, PYY, CCK) and *ad libitum* buffet meal consumption before and after weight loss in women with and without PCOS.

5.3: METHODS

5.3.1: Subjects and recruitment

Overweight pre-menopausal (European Caucasian) women with (n=14) and without PCOS (n=14) were recruited through public advertisement. This study population consists of a subset of subjects matched for age, weight and smoking status from the weight loss study described in Chapter 3. The study was approved by the Human Ethics committees of the CSIRO Division of Human Nutrition, The Royal Adelaide Hospital and the Womens and Childrens Hospital of South Australia and all subjects gave informed written consent. Inclusion criteria were diagnosis of PCOS according to the Rotterdam consensus group by two of the following three criteria: 1) Menstrual irregularity (cycle length < 26 days or > 31 days or variation between consecutive cycles of > 3 days); 2) Clinical (hirsutism assessed by Ferriman-Gallwey score > 8) or biochemical hyperandrogenism (free androgen index > 5.4 or testosterone > 1.4 nmol/L, defined from a representative population of non-PCOS women (n=80) (544)) or 3) Positive ultrasound presentation of polycystic ovaries by trans-vaginal scan (124). Exclusion criteria were pregnancy, breastfeeding, BMI < 25 kg/m^2, Type 2 Diabetes Mellitus (T2DM), use of oral contraceptives, endocrine hormonal treatment or insulin-sensitising agents (subjects were required to cease oral contraceptives 4 weeks and hormonal treatment/insulin-sensitising agents 2 weeks prior to commencement of the study) and related endocrine disorders (excluded by assessment of thyroid stimulating hormone (TSH), prolactin and 17α-hydroxyprogesterone).

5.3.2: Study design and dietary treatment

The study was conducted on an outpatient basis over 8 weeks. Subjects followed an energy restricted diet whereby two meals daily were replaced with commercially available meal replacements (Slimfast™, Unilever Australasia, Epping, New South Wales, Australia) (1800 kJ/day) in addition to a low-fat evening meal per day and at least 5 servings of fruit and

vegetables per day (3500 kJ) with sample meal plans and recipes provided. This involved a restriction of dietary intake to approximately 6000 ± 50 kJ/d or approximately two-thirds of normal intake. Slimfast™ products were provided fortnightly. Subjects were allowed to consume moderate amounts of alcohol (maximum of 2 standard drinks/day with at least 2 alcohol free days/week). Subjects met with a qualified dietitian for initial education on the dietary regimes and quantification and recording of their daily food intake. Subjects did not meet with the dietitian for the remainder of the study and minimal oral advice was provided to the PCOS and non-PCOS subjects. Nutrient intakes were calculated with Diet 4/Nutrient Calculation Software (Xyris Software, Highgate Hill, Australia) based on data from Australian food composition tables. Nutritional intake was assessed from fortnightly 3–day consecutive dietary food records (one weekday and two weekend days) and daily dietary checklists. Dietary compliance was determined by subject adherence to the Slimfast™ regime.

Subjects attended the clinic fortnightly. At the initial visit, height was measured to the nearest 0.1 cm using a stadiometer (SECA, Hamburg, Germany) with subjects in the free-standing position. At all visits, body weight was measured to the nearest 0.05 kg using calibrated electronic digital scales (Mettler scales, model AMZ14, A&D Mercury, Kinomoto, Japan) while subjects were wearing light clothing with no shoes. BMI was calculated by weight (kg) divided by squared height (m^2). At weeks 0 and 8, waist circumference was measured in triplicate directly on the skin with a soft tape to the nearest 0.5 cm at the level of midway between the lateral lower rib margin and the iliac crest and body composition was measured by tetrapolar single-frequency (50 Hz) bioelectrical impedance analysis (ImpDF50; Impedimed Pty Ltd., Qld, Australia) using the offline general algorithm. Total fat mass (TFM) (coefficient of variation (CV) 2.3±8.7%) and total fat-free mass (TFFM) (CV 2.1±0.4%) were assessed. Duplicate measurement were made while subjects were lying supine and with an empty bladder. At weeks 0 and 8 overnight fasting venous blood samples

were taken for assessment of glucose, insulin, lipids, CRP, adiponectin, testosterone and SHBG. Exercise was assessed by a 7-day 24-hour physical activity record at weeks 0 and 8 (546, 547). Daily physical activity was computed in MET-min·day^{-1}, calculated as the MET intensity multiplied by the corresponding minutes of each activity to yield MET minutes, summed across the day and then averaged over the 7 day recording period. The total energy expenditure was expressed as a mean daily energy cost expressed in kJ, computed as: EE (kJ) = MET-min x [body weight (in kg) x (4.186/60)] (548).

For the fortnight prior to study commencement, subjects weighed themselves daily to ensure weight stability, defined as a weight change of ≤2% of initial body weight. At week 0 and 8, subjects underwent a meal tolerance test (MTT). They consumed the same meal the evening before (3820 kJ, 20% protein, 17% fat, 62% carbohydrate) and refrained from alcohol for 24 hours. A cannula was inserted into a lower arm vein and an overnight fasting venous blood sample was taken for assessment of plasma glucose and insulin and serum ghrelin, CCK and PYY. Subjects then completed a validated visual analogue scale (VAS) questionnaire to assess subjective hunger (621). The change in ratings from baseline was quantified (622, 623). Subjects consumed a liquid preload of Slimfast™ (936 kJ, 12 g protein 25% protein, 2 g fat 9% fat, 35 g carbohydrate 67% carbohydrate) within 5 minutes and subsequent blood samples and VAS were taken at 15, 30, 45, 60, 90, 120 and 180 minutes after meal consumption. At 180 minutes, subjects were given a mixed buffet-style lunch (12.1 MJ, 15% protein, 44% fat, 41% carbohydrate) whereby each subject served their own meals from designated portions of the foods and ate until satisfied over a 30 minute period. Each food was weighed to the nearest gram before and after eating using digital scales. Total (glucose, insulin, CCK and PYY), incremental (ghrelin) and net (VAS) areas under the curve (AUC) during the 3-hour MTT were calculated geometrically using the trapezoidal rule (624).

5.3.3: Biochemical measurements

Blood for serum was collected in tubes with no additives and allowed to clot at room temperature for 30 minutes. Blood for plasma was collected in tubes containing sodium fluoride/EDTA for glucose or K/EDTA and aprotinin (Roche; IN, USA) 500 KIU/mL blood for ghrelin, CCK and PYY measurement and stored on ice. Serum and plasma were stored at –80°C. Details of all assay methodologies (SHBG, testosterone, insulin, glucose, total ghrelin, total PYY (1-36 and 3-36), CCK, TSH, prolactin, 17-α progesterone) are described in Appendix 2. The homeostatic model assessment (HOMA) was used as a surrogate measure of insulin sensitivity [fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5] (80). The free androgen index (FAI) (testosterone/SHBG x 100) and equilibrium binding equations were used for estimation of free testosterone (123). Biochemical assays were performed in a single assay at the completion of the study and all samples for individuals were analysed in the same assay.

5.3.4: Statistics

Data was presented as means±SEM where parametric. Non-parametric data (three factor eating questionnaire, median weight loss) were presented as median±range. Where data was non-normally distributed, data was log transformed for analysis. Results are presented for 28 subjects (n=14 PCOS, n=14 non-PCOS) except for MTT glucose, insulin, ghrelin, PYY and CCK (n=14 PCOS, n=13 non-PCOS) due to incomplete data. Two-tailed statistical analysis was performed using SPSS for Windows 14.0 software (SPSS Inc, Chicago, USA) with statistical significance set at α level of $p<0.05$. Baseline data were assessed using a one-way ANOVA for parametric data and a Kruskal-Wallis test for non-parametric data. Comparisons between time points were assessed using repeated measures ANOVA with PCOS diagnosis as between subject factor. In the event of an interaction, post-hoc pairwise comparisons were performed. Relationships between variables were examined using bivariate and partial

correlations and ANCOVA. Subjects who lost more weight (above the median of 4.32 kg) were assessed separately from those who didn't lose weight (defined as below the median of 4.32 kg) with weight loss status as the between subject factor.

5.4: RESULTS

5.4.1: Subjects, physical activity, diet, weight loss, body composition and reproductive hormones

Baseline characteristic of the subjects are shown in Table 5.1. There were no differences in the baseline dietary restraint (9.5±13), disinhibition (10±13) and hunger scores (7±14) between the women with or without PCOS. Activity levels were comparable between women with and without PCOS at week 0 and did not change during the study. There were no differences in energy or macronutrient intake between subjects with and without PCOS (5185.3±141.6 kJ, 34.9±2.2% fat, 51.0±1.1% carbohydrate, 22.5±0.5% protein). Equivalent decreases in weight, waist circumference, TFM, TFFM, free testosterone, FAI, testosterone and increases in SHBG occurred for all subjects (Table 5.2).

5.4.2: Fasting and post-prandial insulin and glucose homeostasis

There was no effect of PCOS status on changes in fasting glucose and there was no change in fasting glucose over the study duration. Following weight loss, there was an interaction between MTT glucose and PCOS status ($P=0.017$) such that MTT glucose decreased ($P=0.043$) only for subjects with PCOS and did not change for subjects without PCOS (Figure 5.1a). Decreases in HOMA (13.6%, $P=0.010$), fasting insulin (15.9%, $P=0.004$), AUC insulin (20.0±3.9%, $P<0.001$) and MTT insulin ($P<0.001$) were similar for subjects with and without PCOS. There was no difference in the magnitude of the change for subjects with and without PCOS although subjects without PCOS had a lower fasting insulin, AUC insulin and MTT insulin at all time points (Figure 5.1b, Table 5.3).

Table 5.1: Subject baseline characteristics

	PCOS (n=14)	**Non-PCOS (n=14)**
Age (years)	32.3±5.9	36.2±4.5
Weight (kg)	94.5±19.8	94.9±15.4
BMI (kg/m^2)	35.3±5.7	35.3±4.8
Glucose (mmol/L)	5.3±0.8	5.1±0.6
Insulin (mU/L) [a]	21.5±14.2	12.2±6.4
HOMA [a]	5.5±3.8	2.8±1.8
Testosterone (nmol/L) [a]	3.2±1.0	2.0±0.5
Free testosterone (pmol/L) [a]	81.4±37.1	45.0±14.5
SHBG (nmol/L)	20.5±10.9	24.9±7.9
Free androgen index [a]	22.0±18.9	9.3±4.5

Data are expressed as means±SD.
Measurements were made at the week 0 visit and were assessed using one-way ANOVA with PCOS status as the fixed factor.

PCOS: Polycystic ovary syndrome
BMI: Body mass index
HOMA: Homeostasis model assessment
SHBG: Sex hormone binding globulin

For conversion from mmol/L to mg/dL for glucose, multiply by 18.
For conversion from mU/litre to pmol/litre for insulin, multiply by 6.95.
[a] $P<0.05$ for PCOS versus non-PCOS.

Table 5.2: Weight, body composition, lipids, glucose and reproductive hormones before and after 8 weeks of energy restriction on one dietary pattern (meal replacements)

	PCOS		Non-PCOS	
	Week 0	**Week 8**	**Week 0**	**Week 8**
Weight (kg) [a]	94.5±5.3	90.6±4.8	94.9±4.1	90.2±3.9
Waist circumference (cm) [a]	113.9±4.3	106.9±4.4	111.0±3.1	103.9±3.1
Total fat free mass (kg) [a]	59.5±3.4	58.3±3.2	61.1±2.5	58.9±2.3
Total fat mass (kg) [a]	34.5±2.5	31.8±2.1	34.5±2.0	31.3±1.8
HOMA [ab]	5.5±1.0	4.4±0.9	2.8±0.5	2.0±0.4
Testosterone (nmol/L) [a]	3.2±0.3	2.7±0.2	2.1±0.1	1.8±0.1
SHBG (nmol/L) [a]	20.5±2.9	21.8±3.0	24.9±2.1	28.7±3.0
Free androgen index [a]	22.0±5.0	17.3±4.9	9.3±1.2	7.3±1.0
Free testosterone (pmol/L) [a]	81.4±9.9	64.6±9.0	45.0±3.9	36.5±3.3
Glucose AUC (mmol/L/180min)	989±36	954±26	935±31	912±35
Insulin AUC (mU/L/180min) [ab]	11842±2543	8818±1466	6702±890	5039±690
Ghrelin AUC (pmol/L/180min)	5935±1964	5031±2616	8658±4261	8263±1479
CCK AUC (pmol/L/180min)	369±112	412±94	290±69	304±91
PYY AUC (pmol/L/180min)	3748±279	4036±390	3295±194	3139±256

Data are expressed as mean±SEM.
Data were assessed using repeated measured ANOVA with time as within-subject factor and PCOS status as between subject factors.

PCOS: Polycystic ovary syndrome
SHBG: Sex hormone binding globulin
HOMA: Homeostasis model assessment
AUC: Area under the curve
CCK: Cholecystokinin
PYY: Peptide YY.

PCOS n=14 and non-PCOS n=14 except for weight, waist circumference, total fat free mass, total fat mass, AUC glucose, insulin, ghrelin, CCK and PYY (PCOS n=14, non-PCOS n=13).
[a] P<0.05 for effect of time (week 0–8).
[b] P≤0.05 for difference between subjects with and without PCOS at week 0 and 8.

5.4.3: Fasting and post-prandial ghrelin, PYY, CCK and visual analogue scores

Fasting ghrelin was significantly higher for subjects without PCOS compared to subjects with PCOS at week 0 (P=0.01) and there was a trend for it to be higher at week 8 (P=0.099). There was no change in fasting ghrelin following weight loss for subjects with or without PCOS (Figure 5.1c). At weeks 0 and 8, subjects with and without PCOS showed a post-prandial reduction in ghrelin (P<0.001). Subjects with PCOS displayed significantly lower ghrelin levels at all time points before and after weight loss compared to subjects without PCOS (P=0.023 for between subject effect of PCOS). There was a trend for an effect of weight loss on changes in test meal ghrelin (P=0.097). There was a significant effect of PCOS status on test meal ghrelin (minute-by-PCOS status effect, P=0.023). For subjects with PCOS, the post-prandial ghrelin response was more impaired (indicated by a lesser post-prandial decrease) at week 0 and tended to be more impaired at week 8 (P=0.048 and P=0.069 respectively for time-by-PCOS status effect). The differences in ghrelin between subjects with and without PCOS were removed on adjustment for fasting or post-prandial insulin at weeks 0 or 8. At weeks 0 and 8 fasting ghrelin correlated with fasting insulin (r=-0.518 P=0.006; r=-0.515 P=0.006), HOMA (r=-0.488 P=0.008; r=-0.508 P=0.007), FAI (r=-0.553 P=0.003; r=-0.489 P=0.010) and free testosterone (r=-0.538 P=0.004; r=-0.471 P=0.013). After controlling for weight, all of the above relationships remained with the exception of fasting ghrelin and HOMA at week 0 (r=-0.369 P=0.063).

There was no effect of PCOS status on fasting or post-prandial CCK or PYY. There was no effect of weight loss on fasting CCK (P=0.919) or PYY (P=0.404) or post-prandial CCK (P=0.440) or PYY (P=0.210) (Figure 5.1d, e). CCK and PYY increased post test meal consumption at both weeks 0 and 8 (P< 0.001). All subjects had an increase in their sensation of fasting fullness with weight loss (29.4±3.5 versus 38.4±4.8 mm, P=0.033) and a decrease in their sensation of post-prandial fullness with weight loss (P=0.005), as reflected in the AUC (3326.5±630.0 versus 1130.9±686.4 mm/180 min, P=0.009). The reduction in fullness

was removed when controlled for the change in the total amount of energy consumed at the buffet meal. There were no other differences in fasting or post-prandial VAS measures either before or after weight loss or between subjects with or without PCOS. No post-prandial appetite hormones correlated with each other or with VAS.

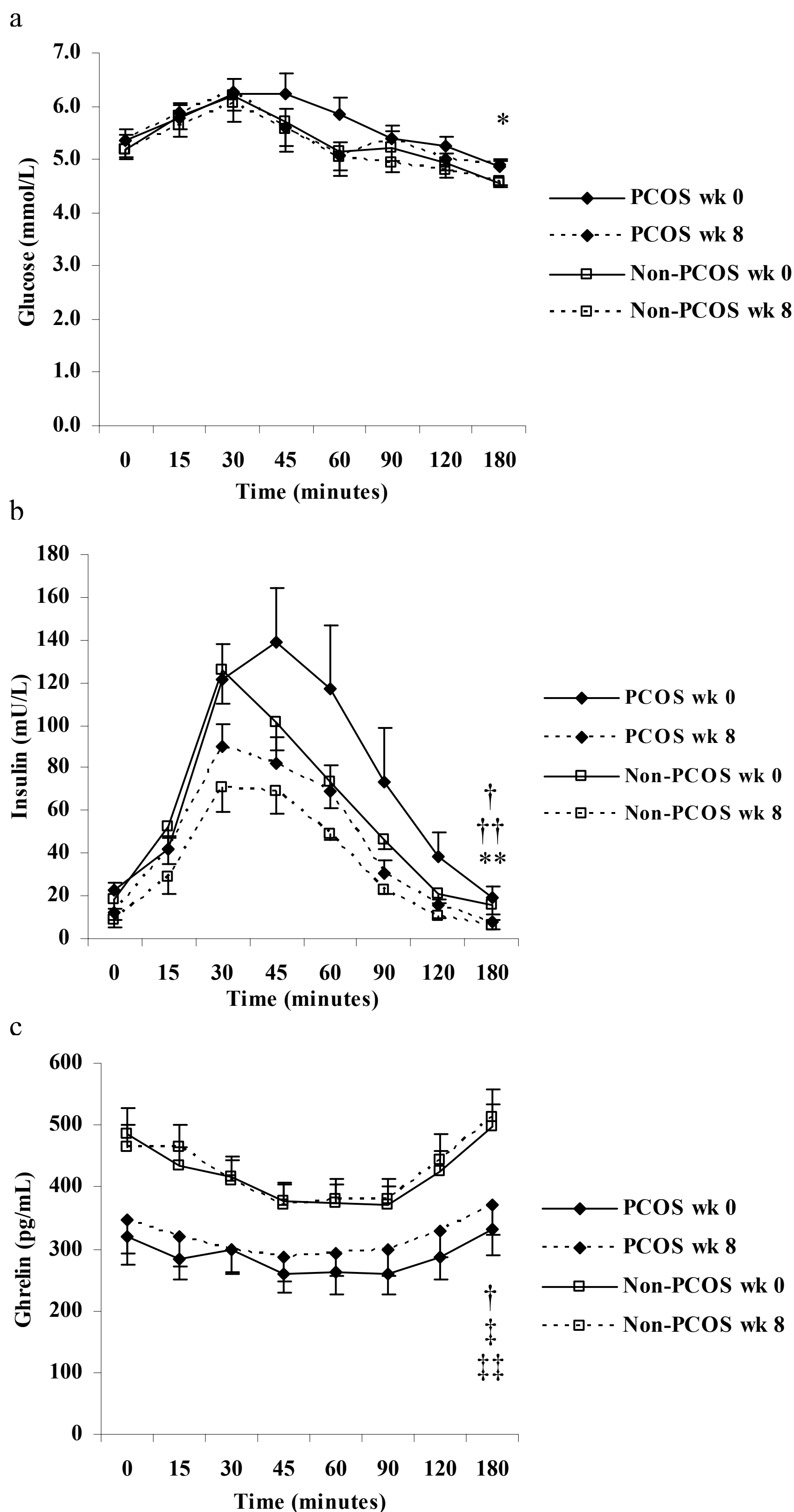
5.4.4: Buffet dietary intake

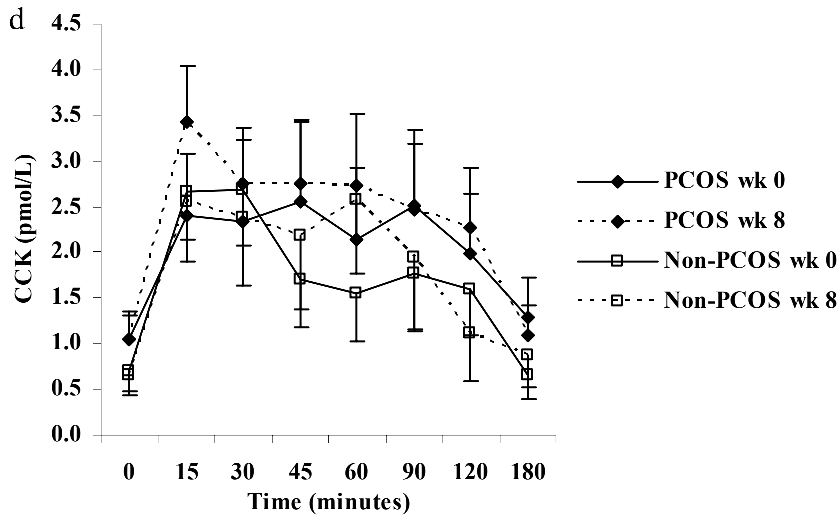
Women with PCOS had a similar energy and macronutrient intake at the buffet meal compared to women without PCOS before and after weight loss (Table 5.3). After weight loss, the amount of food eaten at the buffet meal equivalently decreased by 919.0±260.0 kJ for all subjects (P=0.002), with consequent reductions in total macronutrient intake but no changes in proportional macronutrient intake. The amount of food eaten at the buffet correlated with weight (r=0.489 P=0.008), BMI (r=0.447 P=0.017), total FFM (r=0.498 P=0.007) and total FM (r=0.513 P=0.005) at week 0 but not week 8.

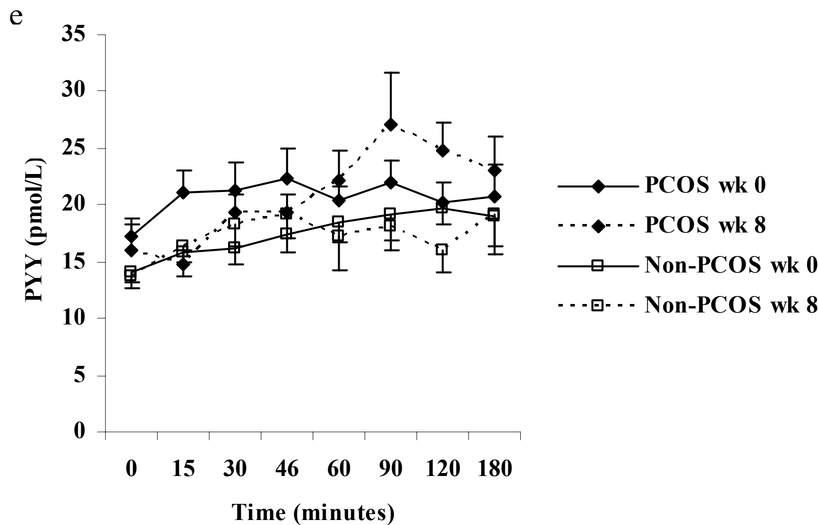
5.4.5: Weight loss status

The subjects were divided into those who lost more weight (7.33 kg; -4.4–13.3 kg) or less weight (1.1 kg, -4.25-+1.9 kg) with equal numbers of subjects with and without PCOS in the weight losers and non-weight losers groups. There was a significant effect of weight loss status on the change in post-prandial PYY (time-by-weight-loss status effect P=0.019) such that subjects that didn't lose weight had an increased post-prandial response over time (P=0.006), while there was no change in post-prandial response for those who did lose weight (P=0.2). There was a significant effect of weight loss status on the change in post-prandial CCK (time-by-weight-loss status effect P=0.022) such that there was a trend for subjects that didn't lose weight to have an increased post-prandial response over time (P=0.065), while there was no change in post-prandial response for those who did lose weight (P=0.155).

Figure 5.1: Glucose (a), insulin (b), ghrelin (c), cholecystokinin (d) and peptide YY (e) concentrations at baseline and 15, 30, 45, 60, 90, 120 and 180 min after the ingestion of a test meal at week 0 and 8 before and after 8 weeks of energy restriction on one dietary pattern (meal replacements)







Data are expressed as mean±SEM.
Week 0 and 8 data were compared by repeated measures ANOVA with week and blood sampling time as within-subject factors and Polycystic ovary syndrome (PCOS) status as between-subject factors.
PCOS n=14 and non-PCOS n=13.

[*] Significant week-by-minute-by-PCOS status effect (P=0.017).
[**] Significant effect of time from week 0 to 8 for fasting (P=0.004) and post-prandial insulin (P<0.001).
† Significant between subject effect of PCOS status (P<0.05).
†† Significant difference at 0 minutes between subjects with and without PCOS at week 0 (P=0.042) and week 8 (P=0.009).
‡ Significant minute-by-PCOS status effect (P=0.023).
‡‡ Significant difference at 0 minutes between subjects with and without PCOS at week 0 (P=0.01) and week 8 (P=0.099).

Table 5.3: Ad libitum energy and macronutrient intake 3 hours after test meal consumption before and after 8 weeks of energy restriction on one dietary pattern (meal replacements)

	PCOS (N=14)		**Non-PCOS (n=14)**	
	Week 0	**Week 8**	**Week 0**	**Week 8**
Energy (kJ) *	4625.8±458.6	3921.4±355.3	5476.3±354.6	4314.6±330.1
Protein (g) *	42.3±4.1	37.9±3.0	48.1±3.0	39.5±2.7
Protein (% energy)	14.9±0.5	15.9±0.6	14.2±0.6	15.0±0.6
Fat (g) *	53.3±6.4	46.2±4.6	67.1±5.2	51.8±5.2
Fat (% energy)	41.1±2.1	43.4±1.5	45.0±1.4	43.2±1.5
Carbohydrate (g) *	110.5±11.0	89.1±9.3	124.5±8.6	100.2±6.8
Carbohydrate (% energy)	41.8±1.9	38.4±1.3	38.7±1.2	39.6±1.0
SFA (% energy)	17.3±1.2	17.3±1.0	19.1±0.9	17.8±0.8
MUFA (% energy)	13.9±1.0	15.1±0.9	15.5±0.9	14.8±0.9
PUFA (% energy)	7.6±0.7	8.7±0.9	7.9±0.6	8.2±0.5
Fibre (g) *	7.7±0.6	6.7±0.5	8.2±0.5	7.1±0.7
Cholesterol (mg) *	80.6±10.2	69.9±8.8	94.2±10.0	72.7±5.6

Data are expressed as means±SEM.
Data were assessed using one-way ANOVA with PCOS status as the fixed factor.

PCOS: Polycystic ovary syndrome
SFA: Saturated fatty acid
MUFA: Monounsaturated fatty acid
PUFA: Polyunsaturated fatty acid

* P≤0.01 for effect of time (week 0–8)

5.5: DISCUSSION

We have confirmed reports on lower fasting ghrelin levels (533) and additionally report a smaller post-prandial reduction in ghrelin in overweight women with PCOS compared to weight and age-matched controls both before and after weight loss. Independent of PCOS status, weight loss reduced *ad libitum* energy intake and did not affect fasting or post-prandial CCK or PYY. In contrast to previous findings (Chapter 4), we observed no change in fasting ghrelin and only a trend for a change in post-prandial ghrelin with weight loss. This discrepancy is likely due to the relatively modest weight loss of 4.2 kg as no changes in circulating ghrelin were observed following weight losses of 3–5% (494).

When ghrelin is measured over a 24-hour period with meals administered at set times, post-meal ghrelin suppression is related to the energy content of the immediately preceding meal (479-481) and correlates with post-meal decreases in hunger and increases in satiety (338), suggesting potential roles in meal initiation and satiety (482). Leidy et al observed the combined energy content of a number of preceding meals is related to the pre-prandial ghrelin increase of subsequent meals (479), thus suggesting a cumulative contribution of energy intake on reducing pre-prandial ghrelin peaks. This could potentially provide a lesser stimulus for meal initiation and contribute to extending the intermeal interval (479) although this was not found to be the case in a recent study (481). The impairment in post-prandial ghrelin secretion observed in obesity (472, 480, 493) may be related to the impairment of appetite regulation in overweight humans, consistent with reports of delayed satiation in overweight subjects (640). Furthermore, positive energy balance may decrease the sensitivity of the central nervous system to ghrelin (641), indicating that it is possible that post-prandial ghrelin and its regulatory role on appetite may be blunted in obesity. The improvement in post-prandial ghrelin with weight loss suggests an improvement in appetite regulation, further supported by the reduction in ad libitum food consumption after weight loss observed here and by other investigators (642).

We and other investigators observed decreased fasting ghrelin levels for overweight women with PCOS compared to age and weight-matched controls ((526, 527, 533) and Chapter 4) which weight loss partially normalised. We also observed a trend for the overweight women with PCOS to show a lesser improvement in post-prandial ghrelin with weight loss. We observed an impaired post-prandial ghrelin response in women with PCOS which weight loss only partially normalised and a trend for the overweight women with PCOS to show a lesser improvement in post-prandial ghrelin with weight loss. This further adds to the findings discussed in Chapter 4 of increased post-prandial hunger, decreased post-prandial satiety and a reduced improvement in post-prandial ghrelin following weight loss for overweight women with PCOS compared to overweight women without PCOS. If additional dysregulation of ghrelin occurs in PCOS to that associated with obesity, this could further potentially contribute to development of obesity or difficulty in weight loss and maintenance. One proposed regulator of ghrelin is insulin which could act to suppress ghrelin secretion both acutely post-prandially (508, 643-645) and chronically in conditions of hyperinsulinaemia such as obesity or PCOS (491). There are a variety of other potential contributory factors to ghrelin regulation in PCOS that warrant further investigation including adiponectin (605). A recent study noted that the change in 24-hour ghrelin profile with weight loss was related to the change in fat-free mass as opposed to fat mass or insulin sensitivity (646). Androgens may also contribute to ghrelin regulation as shown by inverse correlations by us and others of fasting ghrelin with free or total testosterone and FAI (533), lower ghrelin levels in overweight women with PCOS compared to insulin and weight matched controls and increases in circulating ghrelin by anti-androgen therapy (647). The lower ghrelin levels observed in all subjects before weight loss and in the subjects with PCOS are potentially explained by the significantly higher insulin and/or androgen levels for the subjects with PCOS at all time points or the higher insulin levels for all subjects before weight loss. This is also consistent with the differences in fasting and post-prandial ghrelin between women with and without PCOS being ameliorated on adjustment for insulin levels. However, it is unclear

why this did not result in any difference in subjective appetite or buffet meal consumption for women with PCOS compared to controls either before or after weight loss.

The exact effects of ghrelin on meal initiation, meal termination (satiation) or meal frequency (satiety) remain unknown. It has not been established if post-prandial ghrelin suppression would have an effect on spontaneous meal request in a non-structured environment or if pre-prandial increases in ghrelin occur as a physiological meal initiator (648) or as a an anticipatory response to feeding to potentially facilitate digestion (481, 649, 650). Furthermore, in obese compared to lean males a delayed post-prandial ghrelin profile occurred with no resultant differences in subsequent energy intake (451). Thus although differences in post-prandial ghrelin are mediated by potential factors including weight loss and PCOS status, this does not consistently result in differences in appetite or food intake. Alternatively, ghrelin may be more important in regulation of long-term energy homeostasis as indicated by its significant correlation with obesity (491). Ghrelin has a variety of functions in addition to its proposed role in appetite regulation and is associated with the presence of abdominal obesity (651), hyperlipidaemia (low HDL-C) (652-654), insulin resistance (655), hypertension (656) and T2DM (656, 657) and is implicated as an important regulatory peptide in endocrine pancreatic function, glucose metabolism, inflammation, vasodilation and ovarian function (658-662). Ghrelin and the growth hormone secretagogue receptor are present in the pancreas (663) and although the literature is conflicting, ghrelin inhibits both insulin secretion in vivo (664) (665) and inhibits the stimulation by insulin of glycogen synthesis and gluconeogenesis in vitro (658). Ghrelin additionally has effects on the cardiovascular system including reducing mean arterial pressure and increasing artery vasodilatation (659, 666), reducing inflammation through reducing cytokine production and cytokine-stimulated monocyte adhesion to endothelial cells (660). Alternatively, a number of hormones involved in energy homeostasis also affect reproductive functioning including leptin, ghrelin, peptide YY and CCK (reviewed by (667)). Ghrelin and ghrelin receptors have been localised in the

human or rodent ovary, embryo, endometrium, placenta and testis (reviewed by (667)) and ghrelin negatively affects rodent pregnancy outcomes and early embryo development (661) (668). In rodent models, there is a complex effect of ghrelin on the hypothalamic-pituitary axis and ghrelin decreases LH and GnRH secretion and LH responsiveness to GnRH in vivo (669). Additionally, ghrelin mRNA expression is reduced in polycystic ovaries despite similar blood cell levels, intriguingly suggesting a potential contribution of ghrelin regulation to the reproductive function and the presentation of PCOS (662). It is therefore possible that the low ghrelin levels commonly observed in PCOS represent the increased metabolic, diabetic (307, 670, 671) and reproductive dysfunction (258) associated with the condition.

We report for the first time that fasting and post-prandial PYY are similar in age and weight-matched women with and without PCOS before and after weight loss. Post-prandial reductions in PYY occur in conjunction with sustained reductions in subjective fullness (from 30 – 180 minutes post-prandially) (483) and PYY 3-36 infusion reduced food intake for up to 24 hours, indicating a sustained anorexigenic effect (465). Post-prandial PYY profiles are therefore proposed to play a role in both regulating acute satiation or meal size and longer-term satiety or inhibition of further eating (672). Our observed lack of a difference in post-prandial PYY may account for the similar food intake between women with and without PCOS. We also report for the first time that post-prandial PYY is not altered following diet-induced weight loss. Obesity may be associated with reduced post-prandial satiety due to decreased PYY synthesis or release demonstrated by lower fasting and post-prandial PYY levels for overweight compared to lean subjects (463, 483). The lack of a change in PYY with weight loss is therefore unexpected although previously observed increases in fasting PYY following diet-induced weight loss were related to the degree of weight loss (638) and it is possible a greater degree of weight loss is required to alter post-prandial PYY. Alternatively, the lack of improvement in post-prandial PYY following weight loss may reflect increased sensitivity to the effects of PYY or an adaptive response to attempt to restore energy balance.

We observed an increase in the post-prandial PYY response over the study duration for subjects who lost less weight, with no changes occurring for the more successful weight losers. The significance of this novel finding is unclear but could reflect a worsened sensitivity to PYY for subjects resistant to weight loss. However, obese subjects do not appear to suffer PYY resistance, as demonstrated by similar reductions of food intake following PYY 3-36 infusions for obese and lean subjects (463). Alternatively, these results could represent a relative compensatory decrease in post-prandial PYY for subjects who lost weight. Furthermore, the regulation of PYY with regards to long-term changes in energy balance is unclear. The regulation of PYY, ghrelin and CCK appears to be interrelated (442), while PYY infusions do not alter circulating insulin (463) and changes in fasting insulin and PYY with weight loss were unrelated (638).

In contrast to previous reports (432), we observed no differences in fasting or post-prandial CCK in women with and without PCOS either before or after weight loss. The lack of a change in post-prandial CCK before and after weight loss is consistent with previous reports of similar post-prandial CCK profiles between lean and obese age-matched subjects (451), no changes in post-prandial CCK with diet-induced weight loss (639) and similar satiating effects of CCK infusions between lean and obese subjects (490). Conversely, post-prandial CCK was elevated in overweight compared to lean males and females (487). It is unclear whether the similar CCK profiles between women with and without PCOS are related to the consequent buffet meal energy intake as the primary effect of CCK is on inhibiting gastric emptying, increasing satiation and reducing meal size (454, 490). However, a recent study suggested a potential role of CCK in satiety through a prolonged post-prandial elevation of CCK and CCK being a significant predictor of late post-prandial appetite (452). In animal models, CCK administration ameliorates the central orexigenic effect of ghrelin leading to reduced food intake (673). Sustained post-prandial elevations in CCK may therefore augment satiety through suppressing ghrelin and thus diminishing its orexigenic effect. CCK is

released from the small intestine post-prandially predominantly in response to duodenal protein and fat (637). The relatively low amount of protein or fat in our preload may not have provided a sufficient stimulus (25% protein, 67% carbohydrate, 9% fat versus 17% protein, 54% carbohydrate, 28% fat) (432) and is one potential explanation for the different results observed by us and Hirschberg et al.

Our lack of observed differences in PYY may also be affected by the preload macronutrient composition as post-prandial PYY is more strongly stimulated by protein as opposed to fat or carbohydrate (461). Furthermore, PYY is released in proportion to calories ingested (462) and the energy content of the preload (936 kJ) may not have been adequate to detect subtle differences between the women with and without PCOS or before and after weight loss. The length of our post-prandial observation may have been too short to allow meaningful observations, particularly considering the use of a liquid preload (674) and the extended post-prandial elevations in PYY (4–6 hours) (463). The use of the mixed buffet post-test meal allows an assessment of food intake from a varied source. Use of a test meal from a single source would result in decreased food consumption compared to a varied test meal (675) due to sensory specific satiety, defined as a reduction in palatability of a food being consistently consumed (354). Although this is a model that more closely depicts a free-living environment than a non-varied test meal, this may have introduced variability into our results that have clouded any potential relationship existing between appetite, food intake and PCOS or pre and post-weight loss status.

We report for the first time that measures of dietary restraint, fullness and disinhibition to eat are not different between overweight women with PCOS and age and weight matched non-PCOS subjects. However, in attempting to compare the eating behaviour status of our PCOS and non-PCOS population, we did not select a totally restrained population and thus introduced further variability into our results (439). Furthermore, performing the MTT at

defined stages of the menstrual cycle would further strengthen our observations as variations in appetite, appetite hormones (specifically CCK), insulin sensitivity and glucose homeostasis have also been reported over the course of the menstrual cycle (513, 521, 619). We measured total ghrelin which comprises biologically active (Ser3 octanolyated) and inactive ghrelin. The relative ratio of these fractions differs in some physiological states and thus may not be optimal for assessment of ghrelin regulation in diverse conditions such as obesity, weight loss and PCOS (636). Despite attempting to more thoroughly examine the assessment of appetite regulation in PCOS, there are a range of other hormones that contribute to energy homeostasis which we did not measure including glucagon-like peptide 1, glucose-dependent inhibitory polypeptide, oxyntomodulin, peptide PP and pancreatic polypeptide (434). Examination of these factors and any interrelationship between them would further contribute to the understanding of the regulation of energy homeostasis in PCOS and following weight loss.

In conclusion, we observed no differences in post-prandial subjective appetite, PYY and CCK and *ad libitum* food consumption but have confirmed alterations in fasting and post-prandial ghrelin levels in women with PCOS. *Ad libitum* buffet consumption was decreased after modest weight loss in conjunction with an improved post-prandial ghrelin profile but no changes in subjective appetite or circulating CCK or PYY. It is unclear if women with PCOS display abnormal regulation of energy homeostasis or appetite hormones and despite improving some aspects of appetite regulation, the effect of weight loss on post-prandial anorexigenic hormones is unclear in overweight women with or without PCOS.

CHAPTER 6: FINAL DISCUSSION

6.1: THESIS OVERVIEW

The overall aim of this thesis was to investigate the dietary management of overweight women with polycystic ovary syndrome (PCOS) and to examine how modifying dietary composition affects a range of outcomes in PCOS including appetite regulation, risk factors for CVD and T2DM and reproductive outcomes. The ultimate goal of this thesis was to contribute to the clinical dietetic treatment of overweight women with PCOS. The focus of this thesis was to examine a range of dietary strategies on weight loss and maintenance and reproductive and metabolic parameters in overweight women with PCOS (Chapter 2), to identify the relative improvement in cardiovascular risk factors following modest weight loss in overweight women with and without PCOS (Chapter 3) and to examine post-prandial appetite, appetite hormones and food intake in overweight women with and without PCOS before and after weight loss (Chapters 4 and 5).

The aims of the studies described in this thesis were:

1: To assess the effect of minimal intervention short-term strategies on weight loss, body composition, reproductive and metabolic parameters and menstrual cyclicity in overweight women with PCOS.

2: To assess the effect of two long-term (6 month) minimal intervention dietary strategies (moderate carbohydrate or fat restriction) on maintenance of weight and reproductive and metabolic parameters in overweight women with PCOS.

3: To assess the effect of equivalent modest weight loss on risk factors for Cardiovascular Disease (CVD) and Type 2 Diabetes Mellitus (T2DM) (adiponectin, C-reactive protein (CRP), lipid profile, insulin and glucose homeostasis) in overweight weight-matched women with and without PCOS.

4: To assess post-prandial appetite, appetite hormones (ghrelin, cholecystokinin (CCK), peptide-YY (PYY)) and food intake in overweight women with and without PCOS before and after weight loss using with a number of dietary compositions.

The specific hypotheses addressed in this thesis were:

1: That a short-term minimal intervention dietary strategy (meal replacements) will cause reductions in weight and waist circumference and improve glucose and insulin homeostasis, lipid profile, reproductive hormone profile and menstrual cyclicity.

2: That a longer-term *ad libitum* dietary strategy moderately restricted in carbohydrate with low glycemic index (GI) food choices will result in a greater proportional intake of protein, a lower GI and glycemic load (GL) and a greater weight loss and improvement in reproductive and metabolic parameters compared to an *ad libitum* dietary strategy moderately restricted in fat with low GI food choices.

3: That overweight women with PCOS will demonstrate a significantly greater improvement in novel and traditional cardiovascular risk factors following an equivalent weight loss compared to overweight women without PCOS.

4: That overweight women with PCOS will have a lesser post-prandial reduction in ghrelin and an impaired post-prandial increase in PYY and CCK compared to weight-matched women without PCOS and that that this will be related to a greater degree of post-prandial subjective hunger and desire to eat, a lesser degree of post-prandial subjective satiety and a greater consumption of food at an *ad libitum* buffet meal.

5: That modest weight loss will improve the post-prandial ghrelin, PYY and CCK profiles and that this will be related to reduced food consumption.

6.2: WEIGHT MANAGEMENT AND DIETARY COMPOSITION IN PCOS

The structured minimal intervention dietary strategy of meal replacements resulted in modest weight loss (5.6±2.4 kg or 6.0%) and significant improvements in body composition, reproductive hormones and menstrual cyclicity, blood pressure, glucose homeostasis, dyslipidaemia, CRP and surrogate measures of insulin sensitivity, thus fulfilling Hypothesis 1. The literature on weight loss options in PCOS has thus been broadened to include meal replacements which are a commonly used (676) and nutritionally adequate strategy (554) for

achieving weight loss in non-PCOS patients. The carbohydrate and fat restricted weight maintenance approaches had equivalent effects on weight maintenance and reproductive or metabolic parameters, thus leading to a rejection of Hypothesis 2. Despite the potential proposed benefits of modifying diet composition (increasing protein, reducing GI or GL or reducing carbohydrate) on satiety, metabolic parameters and aiding weight loss, the majority of studies in PCOS ((302, 303), Chapter 2, Chapter 4) thus demonstrate equivalent improvements in weight loss, weight maintenance or metabolic or reproductive benefits occurring with weight loss in PCOS. This highlights the importance on focusing on reducing overall energy intake in the treatment of PCOS. However, this research provides preliminary evidence that different flexible dietary strategies can be used to sustain weight loss in addition to standard low fat *ad libitum* advice. Any of the dietary approaches described above would be a reasonable option for overweight women with PCOS seeking to lose weight if they are equivalently nutritionally adequate and have no adverse effects on safety parameters (discussed in section 1.7.3.4).

The primary aim of Chapter 2 was to explore different strategies on long-term maintenance of weight loss in PCOS. A secondary aim was to assess if different dietary strategies could achieve potentially beneficial macronutrient compositions to aid with weight loss maintenance or metabolic or reproductive improvements. We may not have achieved sufficient alterations in macronutrient intake or GI or GL to achieve potential benefits as we did not achieve the desired changes in dietary patterns (proportional protein intake, GI and GL), thus leading to a rejection of Hypothesis 2. However, the aim of this chapter was to observe primarily the effect of flexible dietary advice on energy intake and macronutrient composition and secondarily the effect on weight loss and reproductive and metabolic characteristics. Future work examining the specific effects of modifying macronutrient composition on weight loss, maintenance of lean mass in weight loss and on reproductive and metabolic parameters independent of weight loss (thus removing any confounding effect of

weight loss on masking the beneficial effects of altering diet composition) in PCOS is warranted. There have been a number of additional studies examining this concept in PCOS since these studies were carried out. Douglas et al compared 16 days of a weight maintenance monounsaturated fatty acid (MUFA)-enriched diet (55% carbohydrate, 15% protein, 33% fat; 7% saturated fatty acid (SFA), 6% polyunsaturated fatty acid PUFA, 17% MUFA); low carbohydrate diet (43% carbohydrate, 15% protein, 45% fat; 8% SFA, 17% PUFA, 18% MUFA) and standard diet (56% carbohydrate, 16% protein, 31% fat; 7% SFA, 10% PUFA, 13% MUFA) in 15 overweight women with PCOS (677). A more favourable metabolic response was observed for the low carbohydrate diet, with fasting insulin and total cholesterol decreased relative to the standard diet and post-oral glucose tolerance test (OGTT) insulin decreased relative to the MUFA enriched diet. Similar low density lipoprotein cholesterol (LDL-C) and triglycerides were observed for all approaches. This further supports the concept that alternative dietary options may offer some metabolic benefits in PCOS and a moderately carbohydrate reduced diet, particularly one with a reduced saturated fat content and low GI, may be an alternative option for women with PCOS in weight loss or weight maintenance. The effect of modifying fatty acid composition in insulin resistance (IR) and PCOS is unclear. Overweight women with PCOS (n=17) followed a 3 month weight maintenance polyunsaturated fat (PUFA)-enriched diet (49% carbohydrate, 16% protein, 39% fat, 11% saturated fat (SFA), 12% monounsaturated fat (MUFA), 15% polyunsaturated fat (PUFA). Compared to habitual intake (53% carbohydrate, 15% protein, 33% fat, 12% SFA, 12% MUFA, 7% PUFA), the PUFA-enriched diet increased fasting and OGTT glucose, decreased fasting FFA and did not alter fasting insulin and reproductive hormones (678). There is also a need to expand dietary advice beyond solely weight loss and these findings contribute to the development of dietary or lifestyle recommendations for optimizing reproductive and metabolic parameters in weight maintenance in lean or reduced-obese women with PCOS. However, as yet there is no evidence that modifying macronutrient composition independent of weight loss differentially affects reproductive hormones or menstrual cyclicity.

Although current research has primarily focused on reducing IR through weight loss, the influence of the degree of existing metabolic profile to individual responses to macronutrient composition has been poorly studied. A small study (n=21) examined isoenergetic diets (1680 kJ/day deficit) (60% carbohydrate, 20% fat, 20% protein or 40% carbohydrate, 40% fat, 20% protein) over 16 weeks in obese insulin sensitive (fasting insulin <10 mU/L) or insulin resistant (fasting insulin >15 mU/L) individuals. Insulin resistant individuals lost significantly more weight on the low carbohydrate diet (11.1 kg versus 7.4 kg), while insulin sensitive subjects lost more weight on the high carbohydrate diet (11.3 kg versus 6.2 kg) (312). Similarly, in insulin resistant versus insulin sensitive individuals (n=32, post-hoc defined by high or low 30 minute insulin secretion post-OGTT), randomized to an energy restricted high (60% carbohydrate, 20% fat, 20% protein) or low GL diet (40% carbohydrate, 30% fat, 30% protein) for 6 months, the insulin resistant subjects demonstrated a greater weight loss on the low GL diet (10 versus 6 kg) and the insulin sensitive subjects demonstrated a greater weight loss on the high GL diet (8 vs 6 kg) (679). The reasons for these observed differences is unknown but may involve differential alterations in nutrient oxidation, energy expenditure, post-prandial hyperinsulinaemia or hyperglycaemia or reactive hypoglycaemia induced by different diet compositions in more or less insulin resistant states (680). The only study assessing this concept in PCOS examined carriers (n=4) and non-carriers (n=4) of an insulin receptor substrate (IRS)-1 variant gene (Gly972Arg) which confers impairment in insulin signalling and IR. Carriers exhibited an elevated glucose response to a high GI load (50 g glucose) but a similar response to a low GI load (50 g fructose) compared to non-carriers. Insulin responses to a high or low GI load (50 g fructose) were similar for all subjects (681). There is thus preliminary evidence that different macronutrient profiles are optimal for achieving weight loss in insulin resistant or insulin sensitive individuals without PCOS and potentially with PCOS.

Where a dietary weight loss strategy will be followed, particularly pre-pregnancy, adequate micronutrient intake must be ensured (274). For the meal replacement strategy (Chapter 2), iron intake did not reach the recommended dietary intake (RDI) levels (14 mg/day compared to the RDI of 18 mg/day or 27 mg/day for pregnancy). However, a comparison of micronutrient intake during the intervention to pre-study micronutrient intake demonstrated improvements in zinc and iron intake (13.2 mg/day–14mg/day) although this data is estimated from a direct comparison of FFQ (baseline) and food records (short-term intervention). A similar acute weight loss intervention reported a folate intake of 320.8–330.7 μg/day (554) which is less than the RDI of 400 μg/day (for women aged 18–50 years and recommended for 1 month pre-pregnancy) or 600 μg/day for pregnancy (327). This was again higher than the estimated baseline folate intake (267-296.4 μg/day) (Chapter 2). Both weight maintenance approaches (carbohydrate or fat restriction) also had similar micronutrient profiles although neither was completely nutritionally adequate (Chapter 2). However, the RDI is defined as the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all (97-98%) healthy individuals in a particular life stage and gender group (327) and as such is a generous estimate of nutrient intake that does not necessarily imply inadequacy. Furthermore, the aim of the intervention in Chapter 2 was to examine the effect of different dietary strategies on weight loss and reproductive and metabolic parameters and this study and the dietary interventions in this study was not designed to specifically assess optimal nutritional intake.

Concerns have been raised about the nutritional adequacy of low-carbohydrate diets with some analyses indicating low A, B_6, C, E, thiamine, folate, calcium, magnesium, iron, potassium and fibre (274). In overweight women, an energy restricted high protein (HP) diet resulted in a higher iron, calcium, niacin, riboflavin and thiamine intake compared to an isocaloric high carbohydrate moderate protein (MP) diet (330), indicating that in an energy restricted setting a standard high carbohydrate MP diet may be nutritionally inadequate.

However, the RDI was still not met for calcium or iron on either approach. A conservative approach for overweight women with PCOS undertaking a weight loss intervention would be to advise micronutrient (folate and additional micronutrients) supplementation (eg Elevit which contains 800 μg of folic acid, 60 mg iron, 125 mg calcium, 7.5 mg zinc or Blackmores Pregnancy and Breastfeeding formula which contains 400 μg of folic acid, 10 mg iron, 118 mg calcium, 16 mg zinc) to minimise the risk of micronutrient deficiency or marginal intakes and associated obstetric complications if they conceive during the intervention.

Effective ways to achieve and maintain weight loss in a free-living environment remain unclear and to increase the success of a weight loss regime, a range of elements including modifying dietary composition and incorporation of behavioural and lifestyle factors must be studied. Increased psychological support (682) and adoption of behaviours such as reduced intake of high–energy density foods, reduction in 'non-hungry' eating, eating breakfast almost every day and regular weighing are key features of a successful free-living weight loss regime (273, 563, 683). Adoption of dietary patterns that are nutritionally adequate with a reduced intake of SFA and proportionally increased intake of MUFA, increased intake of fibre from whole-grain cereals and intact fruit and vegetables have also been recommended to optimally improve metabolic parameters in insulin resistant states (684). Use of physical activity will also increase the success of a weight loss or maintenance intervention (563, 683). The relative effects of aerobic and resistance training on IR and weight management in PCOS is a poorly studied area that warrants further attention (685). Furthermore, this thesis did not focus on the use of pharmacological agents or surgery to aid weight loss and the presentation of PCOS in conjunction with dietary management. This includes drugs such as sibutramine (a centrally acting β-phenethylamine derivative that reduces energy intake and increases energy expenditure through inhibiting the re-uptake of noradrenaline and serotonin), orlistat (a lipase inhibitor that reduces gastrointestinal fat absorption) and cannabinoid receptor inhibitors (inhibitors of the endocannabinoids which are endogenous lipids derived from arachidonic

acid that hypothalamically regulate energy homeostasis through cannabinoid 1 and 2 receptors) which are treatments being increasingly studied in overweight subjects with and without PCOS (686-689). An important focus of future research is to compare their relative efficacy and sustainability to standard dietary management (688, 690).

6.3: EFFECT OF WEIGHT LOSS ON REPRODUCTIVE AND METABOLIC PARAMETERS IN PCOS

In overweight women with and without PCOS, an equivalent modest weight loss (4.2±0.7 kg) decreased triglycerides and improved insulin homeostasis for all subjects (Chapter 3). However, only women without PCOS demonstrated a reduction in CRP with weight loss, thus leading to a rejection of Hypothesis 3. In contrast, overweight women with PCOS displayed a reduction in CRP from 3.3–2.8 mg/L following a weight loss of 5.6±2.4 kg (Chapter 2). These two studies are the first to examine the effect of weight loss on CRP in overweight women with PCOS. The reason for these contradictory results is unclear. It is possible that the length of the dietary intervention and degree of weight loss in Chapter 3 was not sufficient to observe differences in all metabolic variables, as shown by unchanged adiponectin occurring with minimal weight loss (3 versus 15%) (593) or short study durations (9 weeks or greater) (595). Additionally, although the subjects in Chapters 2 and 3 had equivalent ages, weight, BMI, fat mass and lipid profiles, in Chapter 3 the subjects with PCOS had significantly higher waist circumferences (114.4 versus 101.0 cm), fasting insulin levels (21.5 versus 12.8 mU/L), triglycerides (2.3 versus 1.2 mmol/L) and CRP (5.5 versus 3.3 mg/L) compared to the subjects with PCOS in Chapter 2. One possible interpretation is that subjects with a significantly elevated metabolic risk profile would require substantially more weight loss or weight loss in combination with pharmacologic therapy to reduce their metabolic risk factors to a similar level to moderately metabolically impaired subjects. This suggests not only that women with PCOS who display an adverse metabolic profile may require a greater degree of weight loss to achieve improvements in metabolic parameters compared to women without

PCOS, but also that the degree of weight loss required may further vary depending on their initial metabolic characteristics including IR. This is supported by the greater reductions in triglycerides and adiponectin occurring for all subjects (PCOS and non-PCOS) with lower baseline CRP levels (Chapter 3). It is also crucial to identify subjects who will respond to weight loss interventions with improvements in menstrual cyclicity and ovulation. Previous work has reported that overweight insulin resistant women with PCOS display reproductive improvements with weight loss compared to relatively less insulin resistant subjects (301, 302). In Chapter 2, although improvements in insulin and reproductive hormones occurred maximally after acute caloric restriction for subjects with elevated fasting insulin at baseline (above the median of 10.45 mU/L), this was not related to reproductive outcomes following weight loss and we were unable to identify the 57% of subjects who responded to weight loss with improvements in menstrual cyclicity and ovulation. These chapters have contributed to the limited research examining the effect of weight loss on reproductive function and novel and traditional risk factors for CVD in PCOS. The implication is that it may be possible to identify pre-intervention which overweight women with PCOS will demonstrate reproductive and/or metabolic benefits with weight loss and which women will require more stringent weight loss goals or additional pharmacological interventions. Although it is not yet possible to identify predictive factors for reproductive or metabolic improvers, these pilot studies provide preliminary observations to support this hypothesis.

In addition to the metabolic improvements observed with a 5–10% weight loss (Chapter 2 and Chapter 3, (219, 286, 298, 302, 304, 308)), in overweight women with PCOS improvements in ovulation, menstrual cyclicity and fertility are observed with modest weight losses (5–10kg) while subjects remain clinically overweight (Chapter 2, (283)). Where pregnancy occurs when women with PCOS are overweight or have T2DM, pregnancy complications such as maternal mortality, congenital abnormalities, miscarriage, gestational diabetes, hypertension and delivery complications are increased (691, 692). Furthermore, maternal

obesity and IR are proposed to contribute to both increased birth weight and an increased risk for childhood obesity (693-696) or decreased birth weight (697), both of which are associated with an increased risk of metabolic disease such as T2DM, CVD and hypertension in adult life. The development of PCOS may additionally be influenced by pre-natal factors. This is demonstrated in animal models by the interaction of intrauterine hyperandrogenaemia with genetic and environmental factors to contribute to the programming of components of the hypothalamic-pituitary-ovarian axis including abnormalities in ovulation, hyperandrogenism, folliculogenesis, disordered ovarian function, impaired fertility, luteinising hormone (LH) hypersecretion, abdominal obesity and impaired insulin secretion or insulin action in offspring (reviewed by (698)). The contribution of maternal obesity to the hypothesized pre-natal development of PCOS is currently unclear and both impaired fetal growth and high birth weight (699, 700) have been reported in women with later development of PCOS. Future research should therefore focus on both the optimal amount of weight loss to restore fertility and to minimize pregnancy complications and post-natal adverse birth outcomes. Additionally, the optimal diet composition during pregnancy for women with PCOS to minimize adverse post-natal outcomes associated with maternal insulin resistance needs to be determined. Recent research on reducing GI in pregnancy has demonstrated promising outcomes of lowering the prevalence of large-for-gestational age birth (701) although the literature is at present contradictory with some data indicating increased risk of small-for-gestational age birth (702).

6.4: APPETITE, APPETITE HORMONES AND FOOD INTAKE

Overweight women with PCOS exhibit abnormalities in subjective post-prandial appetite (Chapter 4) and hormones implicated in the regulation of appetite, specifically lower fasting ghrelin (Chapters 4 and 5), an impaired post-prandial reduction in ghrelin (Chapter 5) and a lesser improvement in fasting ghrelin (Chapter 4) and post-prandial ghrelin (Chapter 5) following weight loss compared to weight-matched controls. This provides preliminary

evidence that women with PCOS have abnormalities in appetite regulation that could predispose them to weight gain or confer resistance to weight loss and maintenance. However, this did not translate to differences in feeding behaviour (*ad libitum* buffet meal consumption) between women with and without PCOS (Chapter 5). Furthermore, we failed to confirm the differences in post-prandial hunger between overweight women with and without PCOS. Fasting and post-prandial appetite hormones more closely associated with satiation and satiety (CCK and PYY) were also similar between women with and without PCOS before or after weight loss (Chapter 5). The implications of these contradictory results are unclear. Future work examining differences in appetite should carefully control for a range of factors. Appetite and food intake vary considerably over the menstrual cycle (with differences of up to 2.5 MJ/day reported for luteal compared to follicular phase intakes (514)), potentially due to fluctuations in reproductive steroids such as oestrogen and progesterone (discussed in section 1.8.3.4). We did not control for menstrual cycle phase in these studies and it is possible that the differences in appetite we observed in Chapter 4 but not Chapter 5 are due to alternative hormonal factors as opposed to ghrelin. The issue of seasonal variation in food intake must also be considered. Modest differences in estimated daily food intake (24 hour recalls) have been reported with a 361 kJ/day (703)–932 kJ/day (704) difference in intake estimated between autumn and spring. Other investigators report that energy intake does not vary significantly by season (705) or report variations in macronutrient intake as opposed to energy intake (704-706). It is unclear if the different times of the year in which the studies in Chapters 4 (April–July) and 5 (February–April) were conducted could impact on the results.

It is proposed that the differences in ghrelin homeostasis between the subjects with and without PCOS are due to the IR commonly observed in PCOS. A recent study demonstrated that post-prandial total ghrelin decreases were strongly positively correlated with insulin sensitivity (measured by IVGTT) in obese children and negatively correlated with post-prandial insulin (707). A dose-dependent suppression of total ghrelin by hyperinsulinaemia

was also observed in subjects without T2DM, while supraphysiological insulin levels were required to suppress ghrelin levels in subjects with T2DM (645). It is possible that the ability of insulin to suppress ghrelin is altered by IR and hyperinsulinaemia and Maffeis et al suggest a potential for the gastric cells to exhibit altered insulin signalling with sustained IR and hyperinsulinaemia and consequent reduced post-prandial ghrelin suppression (707). There is also some evidence that women with PCOS who are relatively more insulin sensitive have similar fasting ghrelin levels to controls and significantly higher fasting ghrelin levels than relatively more insulin resistant women with PCOS (526). Our data supports this in that differences in fasting and post-prandial ghrelin between women with PCOS and controls were removed on adjustment for fasting or post-prandial insulin (Chapter 5) although ghrelin did not correlate with any measure of insulin homeostasis in Chapter 4. It is therefore possible that abnormalities in ghrelin regulation and thus potentially appetite regulation will be more prevalent in insulin resistant women with PCOS. However, although the controls and subjects with PCOS had similar baseline weight and BMI in Chapter 4 and 5, in Chapter 4 the controls and PCOS subjects were matched for fasting baseline insulin levels (20.8±5.6 versus 23.0±3.3 mU/L), while in Chapter 5 the controls were significantly more insulin sensitive that the subjects with PCOS (11.8±1.8 versus 21.1±3.8 mU/L). It therefore appears unlikely that the lack of a difference in subjective appetite observed in Chapter 5 can be accounted for by any variations in IR between subjects. However, the baseline fasting ghrelin levels for the subjects with PCOS in Chapter 5 (348.9 pg/mL) were similar to the baseline fasting ghrelin levels for the controls in Chapter 4 (355.9 pg/mL). If ghrelin is related to appetite regulation and if fasting ghrelin is a marker of post-prandial ghrelin (469), this could indicate the PCOS population in Chapter 4 had significantly more impaired appetite regulation, albeit for reasons separate to their degree of IR. The potential site of action of insulin for regulating ghrelin is unknown (ie at the stomach or hypothalamic level) and post-prandial ghrelin suppression occurs in the absence of insulin (541). Other factors potentially identified in the regulation of

ghrelin include androgens, glucose, somatostatin, growth hormone (GH), leptin, melatonin, thyroid hormones and the parasympathetic nervous system (reviewed by (708)).

If the differences in fasting and post-prandial ghrelin noted in this thesis do translate to alterations in appetite regulation in some or all women with PCOS, the differential effect of modifying macronutrient composition on ghrelin homeostasis offers one target for dietary management to improve satiety and thus aid weight loss and maintenance. Modifying dietary composition before and after weight loss on fasting and post-prandial ghrelin was examined in Chapter 4. Since this chapter was published, there have been a number of additional papers exploring the relationship between modifying dietary composition, appetite hormones, appetite and food intake. In healthy subjects, maximal suppression of post-prandial total (495) and active (709) ghrelin was reported for high carbohydrate compared with high fat isocaloric meals. A number of recent studies have reported that increasing dietary protein compared to carbohydrate or fat results in both reduced energy intake at subsequent meals (451, 452) and prolonged post-prandial total (451, 452, 710) and active (711, 712) ghrelin suppression. Conversely, similar post-prandial reductions were observed in other studies between HP and high fat equivalent carbohydrate meals, despite lower subjective desire to eat for the HP test meal (493). Although we did not observe a difference in post-prandial appetite or ghrelin between the HP low carbohydrate or MP high carbohydrate diets (Chapter 4), this may in part be due to the methodological limitations discussed in section 4.6. If the observed differences in ghrelin in PCOS are related to appetite regulation, there is some evidence that modifying diet composition, specifically increasing dietary protein, may sustain post-prandial ghrelin reductions with potential beneficial effects on satiety. Furthermore, while overweight women (n=17) exhibited similar post-prandial ghrelin responses for a high fat (80% fat, 20% protein) or high carbohydrate (80% carbohydrate, 20% protein) test meal pre-weight loss, the post-prandial ghrelin decrease was maximised on the high carbohydrate test meal following weight loss (11.2 kg) (713). This provides preliminary evidence that a particular dietary approach

post-weight loss may be more favourable for improving appetite hormones and potentially appetite regulation. The effect of GI on ghrelin homeostasis has not been examined. Given the suggestions of impairments in appetite regulation in PCOS demonstrated in this thesis, meal replacements may also be a particularly useful dietary strategy that may aid in weight loss through reducing dietary variety and increasing sensory specific satiety.

6.5: STUDY LIMITATIONS AND FUTURE RESEARCH

The small sample size of these studies resulted in relatively low power for a number of the study outcomes. For example, in Chapter 2 the study was only powered to 40% to achieve significance ($P<0.05$) for the observed difference in net weight loss between the diet groups of 1.6 kg and in Chapter 3 the study was only powered to 65% to achieve significance ($P<0.05$) for the observed difference in change in CRP with weight loss of 1.6 mg/L between subjects with and without PCOS. In future studies, recruitment of larger study populations is crucial to provide sufficient power to detect significant outcomes and the pilot studies within this thesis can aid in the calculation of appropriate sample sizes. For example, in Chapter 2 to confirm the observed differences between the diet groups of 1.6 kg in net weight loss to statistical significance of $P<0.05$ and 80% power, a total of 37 subjects in each diet group would be needed. In Chapter 3 to confirm the observed differences between subjects with and without PCOS of changes in CRP with weight loss of 1.6 mg/L to statistical significance of $P<0.05$ and 80% power, a total of 19 subjects for each group would be needed and n=124 subjects PCOS and non-PCOS subjects would be required to achieve significance ($P<0.05$) for the observed difference in change in adiponectin of 633.4 ng/mL to 80% power.

Furthermore, given the heterogeneity of PCOS these studies lacked sufficient numbers to allow assessment of the baseline characteristics and responses to weight loss of different phenotypic subsets of PCOS. Increased study populations would also ensure adequate numbers of the relevant diagnostic subsets of PCOS to allow sufficient power for sub-

analyses. The studies presented in this thesis generally exhibited high drop-out rates which further contributed to the small samples size. The age (pre-menopausal) and gender (female) of the subjects likely contributed to these attrition rates as these are factors associated with poor weight loss intervention compliance and high drop-outs (397-399). Additional factors associated with high attrition include unrealistic weight loss goals, poor self-esteem, previous dieting history and eating self-efficacy (397-399). Future study sizes can be increased by successful identification of subjects who are more likely to drop-out, thus enabling recruitment of appropriate populations or appropriate monitoring or modification of treatment type, support or intensity in individuals with characteristics of attrition.

Classic PCOS (hyperandrogenism (HA) and ovulatory dysfunction with or without polycystic ovaries (PCO)) is associated with increased metabolic risk and T2DM (306, 671, 714). Within these presentations, the presence of clinical or biochemical HA may modify the metabolic risk with subjects with both biochemical and clinical HA compared to subjects with biochemical HA or subjects with clinical HA having the greatest, intermediate and mildest degree of hyperinsulinaemia and β-cell function respectively (715). The introduction of the Rotterdam diagnostic criteria introduced further phenotypic heterogeneity (Chapter 1, Table 1.4) (133). However, the relative metabolic risk conferred by the presentations of normal menstrual cyclicity with hyperandrogenism and PCO morphology or normoandrogenic with menstrual irregularity and PCO morphology is less well studied and these presentations have both been reported to display similar (134, 716) or milder endocrine and metabolic characteristics compared to classic PCOS (129, 131, 717, 718). The initial metabolic presentation of a subject with PCOS may therefore differ depending on which diagnostic criteria they meet. Epigenetic modification of candidate genes by environmental factors is also a potential contributor to the PCOS phenotype (719). Furthermore, both impaired fetal growth and high birth weight (699, 700) have been reported in women with later development of PCOS and it

has been proposed that adult phenotypes of PCOS can be categorised based on birth weight, with different gonadotrophin, androgen or insulin abnormalities present in each (699).

As discussed above, the initial metabolic characteristics may determine the degree of metabolic (Chapter 2) and reproductive (Chapter 3) improvement with weight loss and the ability to achieve and maintain weight loss (Chapter 4 and 5). Given the extreme heterogeneity present in PCOS, this degree of responsiveness may be based on specific characteristics related to the diagnostic criteria or to other metabolic or endocrine features within the diagnostic subsets. If this is the case, responsiveness to dietary treatment may vary depending either on the phenotypic subset of PCOS or the relative degree of metabolic dysfunction within that subset. In the work presented in this thesis we are unable to speculate on more precise means of defining particular phenotypes of PCOS that will benefit from weight loss. Furthermore, we only used PCO to confirm diagnosis of PCOS in a minority of cases and are therefore unable to examine our data for responsiveness based on these additional phenotypes introduced by the Rotterdam criteria.

Future research should focus on the metabolic and reproductive effects and sustainability, safety and nutritional adequacy of different short and long-term dietary patterns (both with or without supplements) in the general population and in women with PCOS. The majority of studies assessing dietary management of PCOS measure surrogate markers of fertility such as reproductive hormones, menstrual cyclicity and ovulation and future research should focus on incorporation of clinical outcomes such as pregnancy, miscarriage rate and live birth (720). Furthermore, exploring dietary treatment options should therefore be expanded to include not just treatment of PCOS as a broad entity, but treatment of subjects with PCOS and a range of diagnostic, endocrine and metabolic characteristics. This is required before practical implications on the appropriate degree of weight loss for either all subjects with PCOS or the different metabolic and endocrine subsets of PCOS can be explored. The regulation of fasting

and post-prandial ghrelin and its potential relationship with appetite, regulation by insulin and modulation by dietary composition also requires further elucidation in subjects with and without PCOS and in different insulin resistant subsets of women with PCOS. This will ultimately aid further identification of populations with impairments in ghrelin homeostasis and thus potentially appetite regulation and allow the targeting of appropriate dietary interventions for these populations that may experience resistance to weight loss. The ability to predict reproductive and metabolic responsiveness to weight loss interventions and relative success of achieving and maintaining a reduced weight is an ultimate goal for the dietary treatment of PCOS and would allow precise and targeted dietary treatment.

6.6: CONCLUSIONS

Weight loss through energy restriction remains the key treatment goal in overweight women with PCOS. In overweight women with PCOS, a range of short-term weight loss and longer-term weight maintenance strategies have been shown to equivalently reduce weight and improve a range of reproductive and metabolic parameters. Furthermore, with regards to the clinical dietetic management of PCOS, overweight women with PCOS have a range of dietary strategies to follow depending on their preference, past dietary experiences or short-term and long-term treatment aims. Overweight women with PCOS do not routinely display the same degree of metabolic improvements (decreases in CRP) with weight loss compared to overweight women without PCOS. This suggests that the degree of weight loss to aim for in PCOS for optimal metabolic improvements may differ from the general population and may vary depending on initial metabolic characteristics. There is preliminary evidence that women with PCOS have abnormalities in appetite regulation (ghrelin homeostasis), although this is not consistently associated with altered subjective appetite or feeding behaviour. The studies within this thesis have thus significantly contributed to the understanding of and the literature on the dietary management of PCOS. They have explored additional dietary treatment options and expanded on the metabolic and reproductive responses to weight loss that occur in PCOS.

Further investigation is required to assess if appetite regulation is impaired in PCOS, the optimal dietary strategies for weight loss and maintenance and the optimal strategies and amount of weight loss for improvement of reproductive and metabolic parameters in PCOS.

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APPENDIX 1: EFFECT OF DIETARY INTERVENTION IN PCOS ON CLINICAL, ENDOCRINE AND METABOLIC PARAMETERS

Study	Subjects completed	Length	Intervention	Weight loss	Clinical parameters	Endocrine parameters	Metabolic parameters
Pasquali et al 1986 (289)	PCOS n=7 (Diet + placebo) BMI > 28 kg/m^2	3 months	4200–5040 kJ/day 20% P, 50% C, 30% F	8.5% ↓ BMI	3/7 ↑ menstrual cyclicity ↔ hirsutism	↔LH ↔total T	↑glucose/insulin ratio ↑glucose/C-peptide ratio ↔glucose
Kiddy et al 1989 (284)	PCOS n=5 BMI: 36.1 kg/m^2	4 weeks	VLCD, 1386 kJ/day 41% P, 51% C, 8% F	5.2 kg ↓ weight 6.8% ↓ weight	N/A	↓free T ↑SHBG ↔LH, FSH	↓insulin
Pasquali et al 1989 (194)	PCOS n=20 BMI: 32.1 kg/m^2	6–12 months	4200–6300 kJ/day 20% P, 50% C, 30% F,	9.7 kg ↓ weight 9% ↓ % fat 5.8% ↓ WHR	8/20 ↑ menstrual cyclicity 55 % ↓ hirsutism 4/20 pregnant	↓ total T ↓ LH	↓OGGT glucose, insulin ↓insulin ↔glucose
Kiddy et al 1992 (290)	24 PCOS BMI: 34.1 kg/m^2	6–7 months	4200 kJ/day Low fat (20 g /day)	6.9 kg ↓ weight	9/11 ↑ reproductive function 4/10 ↓ hirsutism	↓free T ↑SHBG ↔LH	↓insulin ↓OGGT insulin ↔glucose
Hamilton-Fairley et al 1993 (285)	PCOS n = 6 BMI: 34.2 kg/m^2	4 weeks	VLCD, 1470 kJ/day 41% P, 51% C, 8% F	1.7–9.5 kg ↓ weight 6.6% ↓ weight	N/A	↑SHBG ↔total T	↓OGGT insulin ↔glucose
Clark et al 1995 (283)	N = 13 (8/13 PCOS) BMI: 38.7 kg/m^2	6 months	Lifestyle modification and weekly diet/exercise group High carbohydrate, low fat	6.3 kg ↓ weight ↔ WHR	12/13 resumed ovulation 11/13 pregnant	↓ total T ↑SHBG ↔LH	↓insulin ↓insulin/glucose ratio
Andersen et al 1995 (286)	N = 6 PCOS BMI: 33.6 kg/m^2	24 weeks	4 weeks: VLCD 1768 kJ/day 60% P, 28% C, 12 % F 20 weeks: 4200–6300 kJ/day 22% P, 59% C, 20% F	5% ↓ weight 4% ↓ WHR	2/7 pregnant	↔total T ↔SHBG	↓insulin ↑insulin sensitivity ↓PAI-1 activity ↑fibrinolytic capacity ↔glucose,TG,TC,HDL-C
Holte et al 1995 (219)	N = 13 PCOS BMI: 32.3 kg/m^2	14.9 months	5040 kJ/day	12.5 kg ↓ weight 14% ↓ weight ↓ WC	7/13 ↑ menstrual cyclicity ↔ hirsutism	↓ total T ↑SHBG ↓ FAI ↔LH	↓insulin ↓fasting free fatty acids ↓IVGTT insulin ↑insulin sensitivity ↔glucose
Jakubowicz et al 1997 (291)	PCOS n = 12 BMI: 32.0 kg/m^2	2 months	4200- 5040 kJ/day 25%P, 42% C, 35% F	7.5% ↓ BMI ↔WHR	N/A	↓free T ↓17-α OHP ↑SHBG ↔LH	↓insulin ↓OGTT insulin ↔glucose

Clark et al 1998 (262)	N = 67 (53/67 PCOS) BMI: 37.4 kg/m^2	6 months	Lifestyle modification and weekly diet/exercise group High carbohydrate, low fat	10.2 kg/m^2↓ BMI	60/67 resumed ovulation ↓ miscarriage rate (75–18%) 52/67 pregnant	N/A	N/A
Huber-Buchholz et al 1999 (301)	PCOS n = 18 BMI: 27 – 45 kg/m^2	6 months	Lifestyle modification and weekly diet/exercise group High carbohydrate, low fat	↓ 2–5% weight ↓ abdominal fat, WC	60 % (9/15) ovulated	↓ LH ↔total T ↔SHBG	↓insulin ↑insulin sensitivity ↓OGGT insulin
Wahrenberg et al 1999 (287)	PCOS n = 9 (Diet + placebo) BMI: 37.9 kg/m^2	4 weeks	VLCD Diet composition not specified	8 kg ↓ weight ↔ WHR	N/A	↓free T ↓total T ↑SHBG	↓insulin, glucose 5 – 7 fold improved abdominal adipocyte catecholamine sensitivity
Pasquali et al 2000 (292)	PCOS n = 9 (Diet + placebo) BMI: 39.6 kg/m^2	7 months	5040 – 5880 kJ/day 20% P, 50% C, 30% F	5 kg ↓ weight 4.9% ↓ weight 4.6% ↓ WC 9.6% ↓ VAT/SAT	↓ menstrual cyclicity ↔ hirsutism	↔total T ↔SHBG ↔LH	↓insulin ↔OGGT insulin, glucose
Butzow et al 2000 (288)	PCOS n = 10 BMI: 37.1 kg/m^2	10 weeks	6 weeks: VLCD 2730 kJ/day 4 weeks: Weight maintenance	11% ↓ weight 7.5% ↓ BMI 5% ↓ WHR	N/A	↓total T ↑SHBG ↓LH	↑insulin sensitivity ↓insulin, glucose
Van Dam et al 2002 (293)	PCOS n = 15 BMI: 39 kg/m^2	1 week	4200 kJ/day 43%P, 42% C, 15% F	3 kg ↓ weight 1.6% ↓ body fat	N/A	↓total T ↑24-hour LH	↓insulin, glucose
Crosignani et al 2003 (294)	PCOS n = 25 BMI: 32.1 kg/m^2	6 months	5040 kJ/day 20% P, 55% C, 25% F	6 kg ↓ weight 6% ↓ weight ↓ WC ↔ WHR	↓ ovarian volume, follicle number 72% ↑ menstrual cyclicity 10/20 pregnancy	N/A	N/A
Moran et al 2003 (302)	PCOS n = 28 BMI: 37.4 kg/m^2	16 weeks	12 weeks: 6000 kJ/day 4 weeks: Weight maintenance HP:30% P, 40% C, 30% F HC:15% P, 55% C, 30% F	7.7 kg ↓ weight 7.5% ↓ weight 12.5% ↓ abdominal fat	3/31 pregnancy 11/25 ↓ menstrual cyclicity ↔ hirsutism	↓ total T ↑ SHBG	↓ insulin, HOMA-IR ↓TC, LDL-C, TG ↑HDL-C ↔ glucose
Hays et al 2003 (308)	PCOS n = 15 BMI: 36.1 kg/m^2	24 weeks	High saturated fat low starch 7500 kJ/day	14.3% ↓ weight	N/A	N/A	↓ insulin ↔TC, HDL-C, LDL-C, TG, glucose
Stamets et al 2004 (303)	PCOS n = 26 BMI: 37–38 kg/m^2	4 weeks	Energy deficit 4200 kJ/day HP: 30% P, 40% C, 30% F HC: 15% P, 55% C, 30% F	3.6–4.2% ↓ weight ↔ WHR, WC	14/26 menstrual bleeding ↔ hirsutism	↓ total T ↓ free T ↑ SHBG	↓insulin ↓OGGT insulin ↓glucose/insulin ratio

Van Dam et al 2004 (295)	PCOS n = 14 BMI: 39 kg/m^2	6.25 months	4200 kJ/day 43%P, 42% C, 15% F	10.9–11.5 % ↓ BMI ↓ VAT, SAT)	9/15 ↓ menstrual cyclicity	↓ free T ↑SHBG	↓insulin, glucose ↓HOMA-IR
Hoeger et al 2004 (300)	PCOS n =8 (Diet + placebo) BMI: 40 kg/m^2	48 weeks	Lifestyle modification 2100–4200 kJ deficit/day 150 minutes exercise/week 25% P, 50% C, 25% F, low GI	6.8% ↓ weight	↓ menstrual cyclicity ↔ hirsutism	↔total T ↔SHBG	↔insulin, glucose ↔OGGT insulin
Tolino et al 2005 (296)	PCOS n = 121 BMI: 35 kg/m^2	7 months	4200 kJ/day Diet composition not specified	6 kg ↓ weight	24/114 ↓ hirsutism 30/54 conception 54/66 ↑ menstrual cyclicity	↓ free T ↑SHBG ↔ LH	↓insulin ↓OGGT insulin
Gambineri et al 2005 (297)	PCOS n = 8 (Diet + placebo) BMI: 35.7 kg/m^2	7 months	5040–5964 kJ/day 15%P, 55% C, 30% F	5.3 kg ↓ weight 5.6% ↓ weight ↓ WC, SAT ↔WHR, VAT	↑ menstrual cyclicity ↔ hirsutism	↔SHBG, total T, LH	↓insulin, HOMA-IR ↔glucose
Mavropoulos et al 2005 (304)	PCOS n = 5 BMI: 38.5 kg/m^2	6 months	Ketogenic (< 20 g C/day)	5 kg ↓ weight 12% ↓ weight	2/5 pregnancy ↔ hirsutism	↓ % free T ↔ total T, free T, SHBG	↓insulin ↔glucose, TG, TC, LDL-C, HDL-C, SBP, DBP
Gambineri et al 2006 (298)	PCOS n = 19 (Diet + placebo) BMI: 37 kg/m^2	12 months	5040–5880 kJ/day 20%P, 50% C, 30% F	5 kg ↓ weight 5.1% ↓ weight ↓ WC ↓ VAT, SAT	↑ menstrual cyclicity ↓ hirsutism	↓ total T ↔SHBG	↓ insulin ↓ OGGT insulin ↑HDL-C ↔LDL-C, TG, glucose
Tang et al 2006 (299)	PCOS n = 66 (Diet + placebo) BMI: 37.6 kg/m^2	6 months	Lifestyle modification High carbohydrate/low fat Energy deficit 2100 kJ/day	1.5 kg ↓ weight 1.4% ↓ weight ↔ WC, WHR	58.1% ↑ menstrual cyclicity 2/68 pregnancy	↔ total T, SHBG	↔ insulin, glucose, TC, TG, SBP ↓ DBP

BMI = Body mass index, T = Testosterone, LH = Lutenising hormone, PCOS = Polycystic ovary syndrome, SHBG: Sex hormone-binding globulin, VLCD: Very-low calorie diet, F: Fat, P: Protein, C: Carbohydrate, 17α-OHP:17-α-hydroxyprogesterone, HOMA-IR: Homeostasis model assessment of insulin resistance; OGTT: Oral glucose tolerance test; IVGTT:Intravenous glucose tolerance test; HP: High protein; HC: High carbohydrate: TC: Total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; TG: Triglycerides, WHR: Waist hip ratio, WC: Waist circumference: VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue; SBP: Systolic blood pressure; DBP: Diastolic blood pressure, ↑: increased, ↓: decreased, ↔: unchanged

APPENDIX 2: ASSAY METHODOLOGY

Reproductive hormones

Sex-hormone binding globulin (SHBG) was measured by a non-competitive liquid phase immunoradiometric assay (Orion Diagnostica, Espoo, Finland) (intra-assay CV 1.8–4.9%, lowest detectable limit 0.5 nmol/L) (721). Total testosterone (bound and unbound) (722) (intra-assay CV 2.3–6.22%, lowest detectable limit 0.35 nmol/L), thyroid stimulating hormone (TSH) (intra-assay CV 2.66–7.32%, lowest detectable limit 0.004 mU/L), prolactin (intra-assay CV 2.3–3.39%, lowest detectable limit 0.3 ng/mL) and urinary pregnanediol (723) were measured by chemiluminescent immunoassay (ADVIA Centaur Assay, Bayer Corporation, Inc) (intra-assay CV 3.3–6.8%, lowest detectable limit 1 nmol/L). 17α-hydroxyprogesterone was measured using a commercial radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, Texas) (intra-assay CV 3.7–5.9%, lowest detectable limit 2 ng/mL)(724). The free androgen index (FAI) (testosterone/SHBG x 100) and equilibrium binding equations for determination of free testosterone (123) were used as surrogate estimates of free testosterone.

Lipids, insulin, glucose, C-reactive protein, adiponectin, urea and creatinine

For Chapter 4, total cholesterol (intra-assay CV 1.0%, lowest detectable limit 0.08 mmol/L) (725), triacylglycerol (intra-assay CV 1.5%, lowest detectable limit 0.05 mmol/L) (726) and plasma glucose (intra-assay CV 0.8%, lowest detectable limit 0.11 mmol/L) (727) concentrations were measured on a Cobas-Bio centrifugal analyser (Roche Diagostic, Basel, Switzerland) using enzymatic colorimetric kits (Hoffman-La Roche Diagnostica, Basel, Switzerland) and control sera. Plasma high density lipoprotein cholesterol (HDL-C) concentrations were measured as above for total cholesterol by using the Cobas-Bio centrifugal analyser after precipitation of low density lipoprotein cholesterol (LDL-C) and very-low density lipoprotein with polyethylene glycol 6000 solution (728). Fasting plasma insulin concentrations were measured in duplicate using a commercial radioimmunoassay kit

(Pharmacia AB, Uppsala, Sweden) (intra-assay CV 4.6–9.4%, lowest detectable limit 2.5 mU/L, <0.1% cross-reactivity with C-peptide) (729). For Chapters 2, 3 and 5, serum total (intra-assay CV 0.8%, lowest detectable limit 0.08 mmol/L) and HDL-C (intra-assay CV 0.9%, lowest detectable limit 0.08 mmol/L), triacylglycerol (intra-assay CV 1.5%, lowest detectable limit 0.05 mmol/L), and CRP (intra-assay CV 1.5%, lowest detectable limit 0.03 mg/L) and plasma glucose (intra-assay CV 1.0%, lowest detectable limit 0.11 mmol/L) were measured on a Hitachi Cobas-Bio centrifugal analyzer (Roche Diagostic, Basel, Switzerland) using standard enzymatic kits (Roche Diagostic, Basel, Switzerland) and insulin was measured in duplicate by using a commercial enzyme immunoassay kit (Mercodia, Uppsala, Sweden) (intra-assay CV 2.8–4.0%, lowest detectable limit 1 mU/L, <0.01% cross-reactivity with C-peptide). For Chapters 2 -6, the following modification of the Friedewald equation (730) for molar concentrations was used to calculate LDL-C; LDL-C (mmol/L) = total cholesterol – triacylglycerol/2.18 – HDL-C. HOMA was used as a surrogate measure of insulin sensitivity [fasting insulin (mU/litre)/fasting glucose (mmol/L)/22.5] (80). Adiponectin was measured in duplicate using a commercial enzyme immunoassay kit (R&D systems, Minneapolis, USA) (intra-assay CV 1.78–6.21%, lowest detectable limit 1 ng/mL). Urinary urea and creatinine were measured with the use of the urease enzymatic assay (731) (intra-assay CV 2.03%) and the jaffe reaction technique (732) (intra-assay CV 2.8%) respectively.

Leptin, ghrelin, cholecystokinin and peptide YY

Leptin was measured with a commercially available ELISA kit (Diagnostic System Laboratories, Inc, Webster, TX) (intra-assay CV 2.1%, lowest detectable limit 0.05 ng/mL). Ghrelin (total including Ser3-octanoyl and Ser3-des-octanoyl) was measured with a commercially available RIA kit (Phoenix Pharmaceuticals, Inc, Belmont, CA) (intra-assay CV 5.5%, lowest detectable limit 6.4 pg/mL). CCK was analysed by competitive RIA (Euro-diagnostica, Malmo, Sweden) using an antiserum raised against CCK-8 with cross-reactivity

to CCK-33 (intraassay variation 2.0–5.5%, lowest detectable limit 0.3 pmol/L). CCK was extracted using ethanol; plasma was mixed with an equal part of 96% ethanol, vortexed for 10 seconds, left to stand at room temperature for 10 minutes and then centrifuged at 1700 x g for 15 minutes. The decanted supernatant was evaporated to dryness using a speed-vac concentrator (Savant, Farmingdale, NY) at 37°C then reconstituted to original plasma volume with assay buffer. PYY-like immunoreactivity (YY1-36 and YY3-36) was measured with a specific and sensitive radioimmunoassay using antiserum (Y21) raised against synthetic porcine PYY (Bachem) coupled to bovine serum albumin by glutaraldehyde and used at a final dilution of 1:50,000 (462). This antibody cross-reacts fully with the biologically active circulating forms of human PYY, but not with pancreatic polypeptide, neuropeptide Y, or other known gastrointestinal hormones. 125I-labeled PYY was prepared by the iodogen method and purified by high-pressure liquid chromatography. The specific activity of the 125I-labeled PYY was 54 Bq/fmol. The assay was performed in a total volume of 700 mL of 0.06M phosphate buffer, pH 7.3, containing 0.3% bovine serum albumin. The sample was incubated for 3 days at 41ºC before the separation of free and antibody-bound label by sheep anti-rabbit antibody. Two hundred μL of un-extracted plasma was assayed. Two hundred μL of PYY-free, charcoal-stripped plasma was added to standards and other reference tubes to negate any effects of non-specific assay interference. The assay detected changes of 2 pmol/L, with an intra-assay coefficient of variation of 5.8%.

APPENDIX 3: PUBLISHED PAPERS

CHAPTER 2: Published paper

Moran LJ, Noakes M, Clifton PM, Wittert GA, Williams G, Norman RJ. 2006, Short term meal replacements followed by dietary macronutrient restriction enhance weight loss in Polycystic Ovary Syndrome. The American Journal of Clinical Nutrition; 84(1): 77–87.

Moran, L.J., Noakes, M., Clifton, P.M., Wittert, G.A., Williams, G. and Norman, R.J. (2006) Short term meal replacements followed by dietary macronutrient restriction enhance weight loss in Polycystic Ovary Syndrome.
The American Journal of Clinical Nutrition, v. 84 (1) pp. 77–87 July 2006

NOTE: This publication is included on pages 221 - 231 in the print copy of the thesis held in the University of Adelaide Library.

CHAPTER 3: Published paper

Moran LJ, Noakes M, Clifton PM, Wittert G, Tomlinson L, Galletly C, Luscombe N, Tomlinson L, Norman RJ. 2004, Ghrelin and measures of satiety are altered in polycystic ovary syndrome but not differentially affected by diet composition. Journal of Clinical Endocrinology and Metabolism 89(7):3337–44.

Moran, L.J., Noakes, M., Clifton, P.M., Wittert, G., Tomlinson, L., Galletly, C., Luscombe, N., Tomlinson, L. and Norman R.J. (2004) Ghrelin and measures of satiety are altered in polycystic ovary syndrome but not differentially affected by diet composition.
Journal of Clinical Endocrinology and Metabolism, v. 89 (7) pp. 3337–44 July 2004

NOTE: This publication is included on pages 233 - 240 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1210/jc.2003-031583