THE CARDIO-METABOLIC EFFECTS OF LOW DOSE GLUCOCORTICOIDS AND THE TREATMENT OF PREDNISOLONE-INDUCED HYPERGLYCAEMIA

by

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SUMMARY

Glucocorticoids are anti-inflammatory agents that are commonly prescribed in low doses (e.g prednisolone <10 mg/day) long-term to attenuate inflammatory disease progression and in higher doses (e.g prednisolone >20 mg/day) short-term to treat an acute inflammatory exacerbation. Low dose glucocorticoids can cause insulin resistance and postprandial hyperglycaemia. However, whether this translates into an increased cardiovascular risk is unclear. Although it is known that higher prednisolone doses can increase blood glucose, optimal treatment of prednisolone-induced hyperglycaemia has not been defined. This PhD project comprised three studies investigating the acute and chronic cardio-metabolic effects of low dose prednisolone in the same cohort and an open randomized-controlled trial comparing the efficacy and safety of two insulin regimens in hospitalized patients with prednisolone-induced hyperglycaemia.

In Study 1 acute, but not chronic, prednisolone was associated with a reduction in sympathetic nervous system activity and postprandial fall in augmentation index indicating reduced arterial stiffness. There was no change in fasting reactive hyperaemia index (RHI), a marker of endothelial function, with low dose prednisolone. Moreover, there was an attenuated postprandial fall in RHI in patients taking chronic prednisolone that almost reached statistical significance. To further investigate the effect of prednisolone on endothelial function, the effects of rheumatoid arthritis *per se* and low dose prednisolone on arginine metabolomics were assessed (Study 2). Patients with rheumatoid arthritis have higher plasma asymmetric dimethyl arginine (ADMA) than controls, a potent inhibitor of endothelial nitric oxide synthase (e-NOS). Chronic, but not acute, prednisolone reduced ADMA, which may contribute to better endothelial function in patients taking long-term prednisolone.

In study 3, prednisolone did not affect resting energy expenditure or diet-induced thermogenesis. However, acute and chronic prednisolone induced insulin resistance, which was associated with attenuated postprandial suppression of fat oxidation. Patients on long-term prednisolone had higher fasting non-esterified fatty acids (NEFA), but there was no difference in insulin-mediated suppression of NEFA. These results suggest that prednisolone causes greater insulin resistance in skeletal muscle than in adipocytes.

Finally, in Study 4 the efficacy and safety of isophane and glargine-based basal bolus insulin regimens were compared in hospitalized patients with prednisolone-induced hyperglycaemia. Glycaemic control was assessed using a continuous glucose monitoring system. There were no significant differences in the percentage time outside a target glucose range of 4-10 mmol/L or hypoglycaemia with the two insulin regimens.

In summary, acute and chronic low dose prednisolone causes greater insulin resistance in skeletal muscles than in adipocytes. However, this does not translate into increased arterial stiffness or endothelial dysfunction in patients with rheumatoid arthritis. A reduction in the elevated levels of inhibitors of e-NOS associated with rheumatoid arthritis could explain why patients on long-term prednisolone had better endothelial function. There was no difference in the efficacy or safety of isophane- and glargine-based insulin regimens in the treatment of prednisolone-induced hyperglycaemia. The study demonstrates that a starting daily insulin dose of 0.5 units/Kg is safe treatment of prednisolone-induced hyperglycaemia in patients who are not already taking insulin, but that a 30% increase in daily insulin dose is insufficient in insulin-treated patients.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Anjana Radhakutty

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PUBLICATIONS ARISING FROM THIS THESIS

The following are manuscripts from the work conducted during the candidature, which have been published.

- **Radhakutty A,** Mangelsdorf BL, Drake SM, Samocha-Bonet D, Jenkins AB, Heilbronn LK, Smith MD, Thompson CH, Burt MG. Effect of acute and chronic glucocorticoid therapy on insulin sensitivity and postprandial vascular function. Clinical Endocrinology 2016;84(4):501-8.
- **Radhakutty A,** Mangelsdorf BL, Drake SM, Samocha-Bonet D, Jenkins AB, Heilbronn LK, Smith MD, Thompson CH, Burt MG. Effects of prednisolone on energy and fat metabolism in patients with rheumatoid arthritis: tissue-specific insulin resistance with commonly used prednisolone doses. Clinical Endocrinology 2016;85(5):741-47.
- **Radhakutty A,** Mangelsdorf BL, Drake SM, Rowland A, Smith MD, Mangoni AA, Thompson CH, Burt MG. Opposing effects of rheumatoid arthritis and low dose prednisolone on arginine metabolomics. Atherosclerosis 2017;266:190-95.
- **Radhakutty A,** Stranks JL, Mangelsdorf BL, Drake SM, Roberts GW, Zimmermann AT, Stranks SN, Thompson CH, Burt MG. Treatment of prednisolone-induced hyperglycaemia in hospitalized patients: Insights from a randomized, controlled study. Diabetes, Obesity and Metabolism 2017;19(4):571-78.
- **Radhakutty A,** Burt MG. Management of endocrine disease: Critical review of the evidence underlying management of glucocorticoid-induced hyperglycaemia. European Journal of Endocrinology 2018;179(4):R207-R218.

Abstracts

- **Radhakutty A,** Mangelsdorf BL, Drake SM, Samocha-Bonet D, Jenkins AB, Heilbronn LK, Smith MD, Thompson CH, Burt MG. Effect of therapeutic glucocorticoids on insulin sensitivity, cardiovascular risk and energy metabolism in patients with inflammatory arthritis. Proceedings of the 56th Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology, Melbourne, Australia, August 2014. Abs# 114.
- **Radhakutty A**, Mangelsdorf BL, Drake SM, Rowland A, Smith MD, Mangoni AA, Thompson CH, Burt MG. Effect of low dose glucocorticoid therapy on arginine metabolism in patients with rheumatoid arthritis. Proceedings of the 57th Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology, Adelaide, Australia, August 2015. Abs#188.
- **Radhakutty A,** Mangelsdorf BL, Drake SM, Samocha-Bonet D, Jenkins AB, Heilbronn LK, Smith MD, Thompson CH, Burt MG. Mechanistic determinants of arterial stiffness in patients with inflammatory arthritis exposed to mild glucocorticoid excess. The Endocrine Society's 97th Annual Meeting, San diego, USA, March 2015. P- 20792.
- **Radhakutty A,** Stranks JL, Mangelsdorf BL, Drake SM, Roberts GW, Zimmermann AT, Stranks SN, Thompson CH, Burt MG. Randomized-controlled study of isophane and aspart insulin versus glargine and aspart insulin to treat prednisolone-induced hyperglycaemia in hospitalized patients. Proceedings of the 58th Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology, Sydney, Australia, August 2016. Abs#174.
- **Radhakutty A,** Stranks JL, Mangelsdorf BL, Drake SM, Roberts GW, Zimmermann AT, Stranks SN, Thompson CH, Burt MG. Treatment of prednisolone-induced hyperglycaemia in hospitalized patients: Insights from a randomized controlled study. The Endocrine Society's 99th Annual Meeting, Florida, USA, April 2017. P- 30735.

ABBREVIATIONS

ADMA	asymmetric dimethyl arginine
AGAT	arginine:glycine amidinotransferase
AIx	augmentation index
AIx75	augmentation index normalised to heart rate of 75 beats per minute
ASL	arginosuccinate lyase
ASS	arginosuccinate synthase
A-V	arterio-venous
AUC	area under the curve
BGL	blood glucose level
BMI	body mass index
BRS	baroreceptor sensitivity
CGMS	continuous glucose monitoring system
CHOox	carbohydrate oxidation
CIT	cold induced thermogenesis
CKD-EPI	chronic kidney disease- epidemiology collaboration equation
CRP	C - reactive protein
CV	coefficient of variation
DDAH	dimethyl arginine dimethyl amino hydrolase
DIT	diet-induced thermogenesis
DXA	dual energy x-ray absorptiometry
e-NOS	endothelial nitric oxide synthase
eGFR	estimated glomerular filtration rate
FMD	flow mediated dilatation
Fox	fat oxidation
GC	glucocorticoids
GLUT	glucose transporter translocation
HbA1c	glycosylated haemoglobin

HDL	high density lipoprotein
LBM	lean body mass
LCMS	liquid chromatography mass spectrometry
LDL	low density lipoprotein
MAGE	mean amplitude of glycaemic excursions
MAP	mean arterial pressure
MMA	mono methyl arginine
NEFA	non-esterified fatty acid
NO	nitric oxide
PAT	peripheral arterial tonometry
PI-3	phosphatidylionositol-3
PVA	pulse volume amplitude
RA	rheumatoid arthritis
REE	resting energy expenditure
RHI	reactive hyperaemia index
ROS	reactive oxygen species
SDMA	symmetric dimethyl arginine
SEM	standard error of mean
VCO2	carbon dioxide production
VO2	oxygen consumption
VLDL	very low density lipoprotein

CHAPTER 1. INTRODUCTION

1.1. Introduction

The objectives of this thesis were to 1) assess the cardio-metabolic effects of low dose glucocorticoid therapy, and 2) determine whether matching insulin pharmacokinetics to the pattern of prednisolone-induced hyperglycaemia improves glycaemic control. For the first objective, three linked studies using the same patient cohort were performed assessing the acute and chronic effects of low dose prednisolone treatment on vascular function and energy metabolism in subjects with rheumatoid arthritis. For the second objective, a randomized controlled study investigating whether an isophane-based insulin regimen is safer and more effective than a glargine-based regimen was conducted in hospitalized patients with prednisolone-induced hyperglycaemia.

The first endogenous glucocorticoid, cortisone was discovered in 1930's by Kendall and Reichstein. Cortisone was first administered to patients with rheumatoid arthritis in 1948 by Hench, and became the first effective anti-inflammatory therapy (Hench et al., 1949). Kendall, Reichstein and Hench were awarded the Nobel Prize for Physiology or Medicine in 1950 for their significant discoveries relating to the hormones of the adrenal cortex. Subsequently, glucocorticoids have been used widely as anti-inflammatory and immunomodulatory agents.

Despite the availability of newer therapeutic options, the prevalence of glucocorticoid use is increasing (Fardet et al., 2011). While high dose glucocorticoids (prednisolone >10 mg/day) are used to treat acute inflammation, low dose glucocorticoids (prednisolone <10 mg/day) are often used chronically to attenuate disease progression. Long term low dose glucocorticoids are most commonly prescribed to the elderly (Fardet et al., 2011, Walsh et al., 1996), and the most common indication for use is inflammatory rheumatologic disease (Fardet et al., 2011). Patients with rheumatoid arthritis are at increased risk of cardiovascular disease and mortality (Maradit-Kremers et al., 2005a, Maradit-Kremers et al., 2005b, Nicola et al., 2005). Whether glucocorticoids contribute to increased cardiovascular risk in rheumatoid arthritis has been a subject of controversy for decades (Maxwell et al., 1994, Nashel, 1986, Saag, 2001, Girod and Brotman, 2004).

The side effects associated with glucocorticoid use depends on the dose and duration of treatment

(Souverein et al., 2004, Wei et al., 2004). High dose glucocorticoids cause insulin resistance (Rizza et al., 1982, Dirlewanger et al., 2000, Nicod et al., 2003, Tappy et al., 1994) and have adverse effects on lipid profile, body weight and fat metabolism. Moreover, in epidemiologic studies they are associated with a significantly increased risk of cardiovascular disease (Isomaa et al., 2001). However, evidence linking low dose glucocorticoids, which are more commonly used therapeutically, and increased cardiovascular disease is conflicting (Wei et al., 2004, Listing et al., 2015, Svensson et al., 2005, Capell et al., 2004). Moreover, much of the evidence linking cardiovascular disease and glucocorticoid use comes from observational studies rather than randomized controlled studies. These are potentially confounded by indication bias as it is challenging to separate the effects of glucocorticoids from the adverse effects of the disease process for which the glucocorticoids are prescribed. Consequently, it is important to characterise potential mechanisms by which glucocorticoids might increase cardiovascular risk.

Our unit previously reported that older patients with inflammatory rheumatologic disease treated with long term low dose prednisolone have higher post glucose load plasma glucose concentration, but a slightly lower fasting plasma glucose concentration compared to matched controls with an inflammatory rheumatologic disease who are not taking prednisolone (Burt et al., 2012). Using gold standard metabolic techniques, we have also shown that acute and chronic low dose prednisolone treatment in elderly patients with inflammatory arthritis reduced hepatic and peripheral insulin sensitivity (Petersons et al., 2013). This is pertinent as insulin resistance is an independent risk factor for cardiovascular disease (Hanley et al., 2002, Isomaa et al., 2001, Bonora et al., 2007). The interplay between fasting and post-challenge glucose and cardiovascular risk is controversial and is a subject of ongoing studies (Stacey et al., 2019, Tamita et al., 2012, Wei et al., 2000, Decode Study Group, 2001). However, the Decode study group and others have reported that post challenge hyperglycaemia has a stronger association than fasting glucose with cardiovascular and all-cause mortality (Decode Study Group, 2001, Pyorala et al., 1979, Bonora and Muggeo, 2001, Ceriello et al., 2004, Hanefeld et al., 1996, Shaw et al., 1999). Compared to impaired fasting glucose, abnormal glucose tolerance has also been associated with a greater risk for future cardiovascular events in patients after acute myocardial infarction (Tamita et al., 2012).

Varying effects on cardiovascular risk markers have been reported with exogenous glucocorticoids. Studies have shown no increase in carotid-intima media thickness (Hafstrom et al., 2007) or augmentation index (Petersons et al., 2017), and no change in (Hafstrom et al., 2007) or improved endothelial function (Petersons et al., 2017) in patients with rheumatoid arthritis on long term prednisolone. Improvement in arterial stiffness was also demonstrated in patients with polymyalgia rheumatica treated with prednisolone (Schillaci et al., 2012), whereas increased hydrocortisone replacement in ACTH deficient patients was associated with endothelial dysfunction (Petersons et al., 2014). The assessment of vascular function was, however, performed in the fasting state in these studies. As low dose prednisolone predominantly increases postprandial glucose, assessment of vascular function during the postprandial period might provide important insights into the vascular effects of prednisolone.

Hyperglycaemia is a common side effect associated with moderate to high dose glucocorticoids (Hougardy et al., 2000, Braithwaite et al., 1998) . Hospitalized patients prescribed glucocorticoid treatment have a 50% increase in relative risk of new onset hyperglycaemia (Breakey et al., 2016b). In hospitalized patients, hyperglycaemia has been associated with increased duration of hospital stay and mortality, with the association with mortality being stronger in patients with new onset hyperglycaemia than in patients with known diabetes (Umpierrez et al., 2002, Capes et al., 2000, Burt et al., 2013b, Capes et al., 2001, Farrokhi et al., 2011). Despite its high prevalence, the best treatment for glucocorticoid induced hyperglycaemia is still unclear. Current guidelines recommend treatment of inpatient glucocorticoid-induced hyperglycaemia with subcutaneous basal-bolus insulin, without specifying the insulin formulation in detail (Moghissi et al., 2009, Umpierrez et al., 2012). A morning dose of prednisolone, a commonly prescribed glucocorticoid, has little effect on overnight glucose concentration and predominantly causes hyperglycaemia in the afternoon and evening (Yuen et al., 2012, Burt et al., 2011). It is currently unclear as to whether the predominant rise in glucose in the afternoon and evening after a morning dose of prednisolone is related to the time course of prednisolone action or a greater effect of prednisolone on postprandial than fasting glucose. This pattern of hyperglycaemia has been demonstrated in prednisolone treated patients prescribed glargine based basal bolus insulin regimen (Burt et al.,

2015). Matching the pharmacokinetics of the basal insulin prescribed (eg: Isophane insulin) with this circadian pattern of hyperglycaemia may achieve better glycaemic control as well as reduce the risk of overnight hypoglycaemia. However data to support isophane over glargine as basal insulin in the treatment of prednisolone induced hyperglycaemia is limited, with studies showing varying results (Ruiz de Adana et al., 2015, Grommesh et al., 2016, Dhital et al., 2012).

The first section in this chapter will discuss the various non-invasive markers used to assess cardiovascular risk in this thesis, and the relationship between post challenge hyperglycaemia and cardiovascular risk. Possible mechanisms by which glucocorticoid excess increases cardiovascular risk will also be reviewed. The second section will focus on the effects of glucocorticoids on energy and fat metabolism. The physiology behind the techniques used in this thesis for the assessment of energy expenditure and substrate metabolism will be described. Also, the current literature on the effects of glucocorticoids on resting energy expenditure, diet-induced thermogenesis, lipolysis and fat oxidation will be reviewed. The last section of this chapter will review the mechanisms and treatment options for acute glucocorticoid induced hyperglycaemia.

1.2. Non-invasive assessment of cardiovascular risk markers

1.2.1. Introduction

Cardiovascular disease, a leading cause of mortality, has a long asymptomatic phase of development. The progression of cardiovascular disease can be estimated using various non-invasive techniques. In this thesis, the techniques employed to assess cardiovascular risk include assessment of arterial stiffness, endothelial function, measurement of arginine metabolites and baroreceptor sensitivity.

1.2.1.1. Assessment of arterial stiffness

There is currently great emphasis on the role of arterial stiffness in the development of cardiovascular disease (Laurent et al., 2006). Arterial stiffness is determined by vessel wall structure, but is also dependent on autonomic tone and endothelial function. Arterial stiffening can lead to increased left ventricular afterload (Laurent et al., 2006), left ventricular hypertrophy (Toprak et al., 2009) and impaired coronary perfusion (Ikonomidis et al., 2008).The risk associated

with increased arterial stiffness is considered to be similar to that with other established cardiovascular risk markers (Vlachopoulos et al., 2010b). Arterial stiffness is also considered to be an independent predictor of all-cause mortality in addition to predicting cardiovascular outcomes (Vlachopoulos et al., 2010b).

The methodologies used to assess arterial stiffness fall into three categories: 1) measuring local arterial stiffness by relating change in the area of an artery to distending pressure, 2) regional arterial stiffness by measuring pulse wave velocity, and 3) systemic arterial stiffness using pulse wave analysis. In this thesis, pulse wave analysis was assessed at the radial artery by applanation tonometry with a SphygmoCor device (AtCor Medical, New South Wales, Australia) and a high-fidelity micromanometer (SPC-301, Millar Instruments, TX, USA).

Applanation tonometry is considered the gold standard and is the most widely used technique for pulse wave analysis (O'Rourke et al., 2001). It is easy to perform and has good reproducibility (Laurent et al., 2006) . The technique of non-invasive aortic pulse wave analysis depends on accurate recording of the radial pressure wave and its calibration against brachial pressure, which is then used to generate the ascending aortic pressure waveform via use of a computerized generalized transfer function (Figure 1).

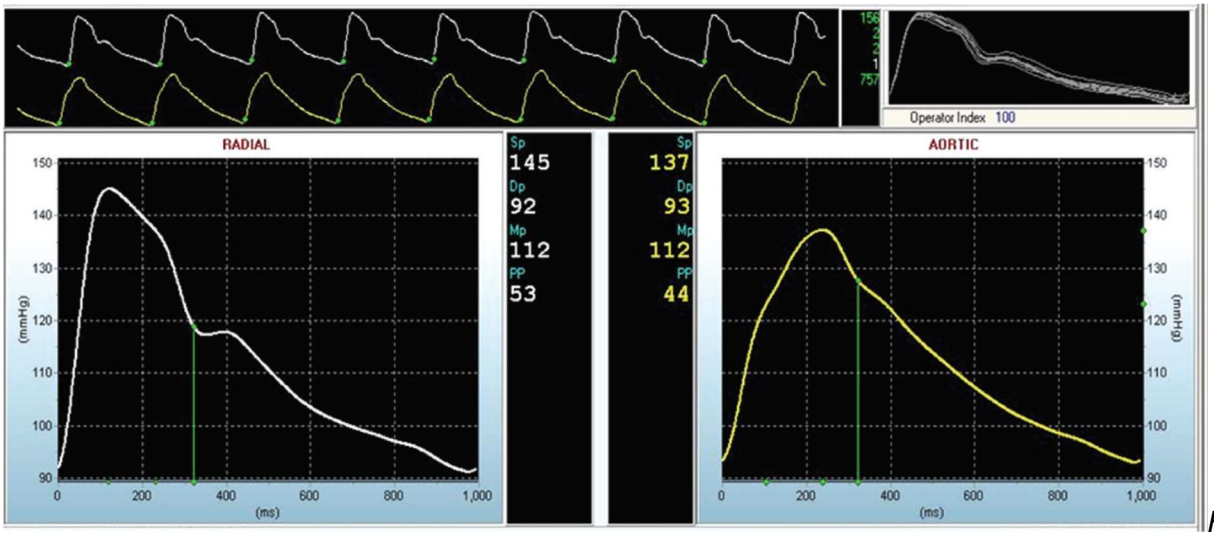

Fig 1: Radial artery applanation tonometry recording. The upper long panel shows the radial pressure waveform above the derived central pressure waveform. The upper right panel shows the overlaid radial waveforms, including the operator index, and the middle panel shows the quality control indices. The bottom left panel demonstrates a magnified radial arterial waveform. The bottom right

panel provides a magnified derived central pressure waveform. Figure reproduced from Stoner L,et al. International Journal of Vascular Medicine. 2012.

Augmentation pressure and augmentation index are then derived from the aortic pulse pressure wave form. Augmentation index is considered to be a composite measure of aortic wave reflection and systemic arterial stiffness (O'Rourke et al., 2001). Augmentation index should be normalized for a heart rate of 75 beats per minute (AIx75), as a 10 bpm increase in heart rate can result in a 4% reduction in augmentation index. Limitations of augmentation index include error introduced by using brachial pressure for calibration, and that it can be influenced by multiple variables including mean arterial pressure, age, height and aortic pulse wave velocity (Laurent et al., 2006).

Augmentation index has shown to be a predictor of cardiovascular events in elderly patients with end stage renal failure (London et al., 2001) and in patients with hypertension (Weber et al., 2005) and coronary artery disease (Williams et al., 2006), independent of traditional cardiovascular risk factors. A systematic review and meta-analysis including 5648 subjects followed up for a mean of 45 months reported that a higher augmentation index was associated with cardiovascular events and all-cause mortality in a range of patient populations (Vlachopoulos et al., 2010a). These findings add to the mounting evidence that augmentation index is a surrogate marker of arterial stiffness and left ventricular systolic loading and a valid method to predict cardiovascular risk (Laurent et al., 2006).

1.2.1.2. Clinical assessment of endothelial function

The importance of endothelial function to the cardiovascular system is well established (Widlansky et al., 2003), with studies showing higher risks of cardiovascular events in people with impaired endothelial function (Yeboah et al., 2007). Damage to endothelial cells by systemic risk factors reduces nitric oxide bioavailability leading to impaired endothelial function. Endothelial dysfunction promotes inflammation, thrombosis and cellular adhesion facilitating atherosclerosis.

Flow mediated vasodilatation (FMD) using brachial ultrasound provides a measure of nitric oxide-mediated endothelium dependent vasodilatation and is considered the gold standard technique for estimation of endothelial function. In FMD the brachial artery diameter proximal to the antecubital

fossa is measured at rest and after arterial occlusion. Flow mediated vasodilatation is expressed as the change in the final diastolic diameter of the brachial artery during reactive hyperaemia relative to the baseline value. Brachial flow mediated dilatation is lower in the presence of traditional cardiovascular risk factors and also predicts risk of cardiovascular events (Yeboah et al., 2007, Widlansky et al., 2003). However, limitations of FMD include that it is highly operator dependent, requires expensive equipment and is technically challenging.

Emerging evidence supports the assessment of reactive hyperaemia index (RHI) using peripheral arterial tonometry (PAT) as a measure of endothelial function. Similar to the principles used in FMD, PAT involves measuring pulse amplitude in the fingertip at rest and following the induction of reactive hyperaemia by arterial occlusion. Studies have demonstrated a direct contribution by nitric oxide to the digital PAT ratio (Nohria et al., 2006). There is a moderate but statistically significant correlation between FMD and PAT hyperaemia ratio in most studies (Dhindsa et al., 2008, Kuvin et al., 2007, Kuvin et al., 2003), while others have shown only a correlation at baseline (Lee et al., 2012). This suggests that brachial and digital vasodilation may in part be mediated by different mechanisms.

However, the evidence is clear that PAT is a predictor of cardiovascular risk. RHI derived from PAT has been associated with cardiovascular events (Rubinshtein et al., 2010). Studies have also demonstrated a progressively lower reactive hyperaemia index with increasing burden of cardiovascular risk factors (Kuvin et al., 2003, Bonetti et al., 2004). Furthermore, a study comparing both FMD and PAT with coronary angiography demonstrated that both markers were significantly lower in patients with coronary artery disease (Kuvin et al., 2007). The advantages of PAT over FMD are that it is operator independent, easy to perform, with studies showing good reproducibility (Bonetti et al., 2003, Tomfohr et al., 2008). PAT has been used in the studies described in this thesis to assess endothelial function.

1.2.1.3 Measurement of arginine metabolites

The endothelium plays a crucial role in vascular homeostasis through the synthesis of endogenous nitric oxide, a potent vasodilator, by the enzyme e-NOS (Ignarro, 2002). In addition to significant anti-inflammatory, anti-thrombotic and anti-atherosclerotic effects, nitric oxide also exerts important

effects on vascular tone (Ignarro and Napoli, 2004). Reduced nitric oxide synthesis by e-NOS has been demonstrated in conditions with endothelial dysfunction (Ignarro and Napoli, 2004) and is strongly and independently associated with increased cardiovascular morbidity and mortality (Lerman and Zeiher, 2005).

L-arginine is the main substrate for endothelial nitric oxide synthesis by e-NOS, which releases citrulline as a by-product (Fig 2). The plasma concentration of L-arginine is affected by dietary intake, arginine synthesis in the kidney and its metabolism by arginases and other arginine catabolic enzymes, and can influence endothelial nitric oxide production (Wang et al., 2006). (Fig 2). Critically, methylated arginine metabolites are important regulators of e-NOS action. ADMA, symmetric dimethyl arginine (SDMA) and mono methyl arginine (MMA) are synthesized intracellularly by methylation of arginine residues in proteins and are released during proteolysis. ADMA and MMA are powerful competitive inhibitors of e-NOS (Boger et al., 1998, Vallance et al., 1992). A minor increase in plasma ADMA adversely affects endothelial nitric oxide production, vascular tone and arterial blood flow (Murray-Rust et al., 2001). MMA has a similar action to ADMA, whereas SDMA decreases nitric oxide production indirectly by reducing L-arginine bioavailability and increased production of reactive oxygen species (Mangoni, 2009).

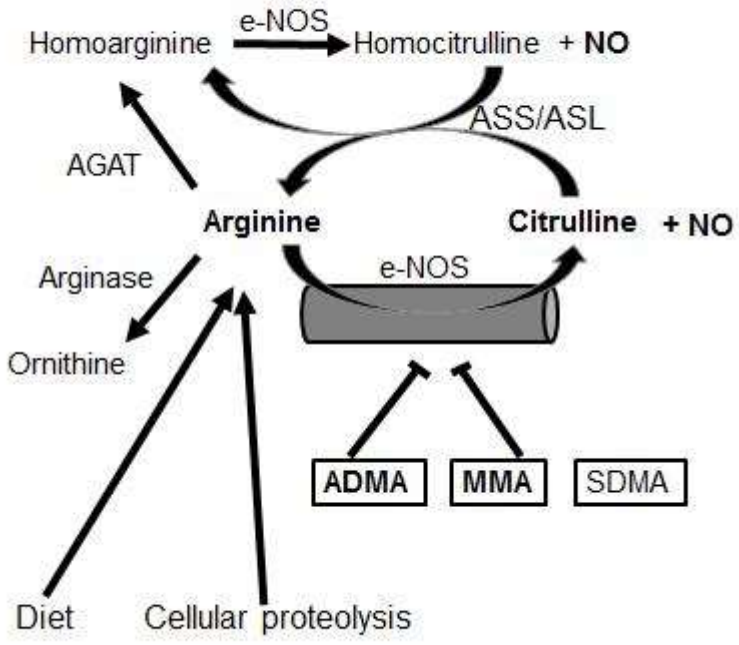

Fig 2: Simplified diagram showing the principal pathways of arginine metabolism. ADMA indicates asymmetrically dimethylated arginine; MMA, monomethylated arginine; SDMA, symmetrically dimethylated arginine AGAT, arginine:glycine amidinotransferase; ASL, arginosuccinate lyase; ASS, arginosuccinate synthase; NO, nitric oxide; e-NOS, endothelial nitric oxide synthase

The measurement of arginine metabolites is an alternate method to assess endothelial function and cardiovascular risk. Vallance, et al first reported an association between increased ADMA and risk for atherosclerosis in patients with end stage renal disease (Vallance et al., 1992). Subsequently, several studies have shown a robust statistical association between ADMA and cardiovascular disease and mortality in a variety of populations comprising a broad range of cardiovascular risk (Zoccali et al., 2001, Krempl et al., 2005, Conroy et al., 2003, Leong et al., 2008, Maas et al., 2007). In the Framingham offspring cohort, a 0.13 µmol/L increase in ADMA was associated with a 21 % increase in risk of cardiovascular events (Boger et al., 2009). Emerging evidence suggests that other arginine metabolites might also influence cardiovascular risk. Positive associations between plasma SDMA and MMA concentrations and cardiovascular events, with similar predictive values to that of ADMA, were noted in population based studies (Kiechl et al., 2009, Chirinos et al., 2008).

There is growing evidence that other pathways of arginine metabolism affect cardiovascular risk. Arginine is also a substrate for arginase which converts arginine to ornithine and urea (Fig 2). An increase in arginase activity reduces availability of arginine for e-NOS and decrease in nitric oxide production (Pernow and Jung, 2016). In addition, increased arginase activity can increase reactive oxygen species (ROS) by uncoupling of e-NOS, causing further endothelial damage (Romero et al., 2008). These effects have been shown to contribute to vascular injury in diabetes, with improved endothelium dependent vasodilatation noted in the forearm of patients with type 2 diabetes mellitus and coronary artery disease following arginase inhibition (Beleznai et al., 2011, Shemyakin et al., 2012). Since the commencement of this thesis, a study by Chandrasekharan, et al, demonstrated increased arginase activity in patients with rheumatoid arthritis, suggesting this pathway may affect cardiovascular risk in this patient group (Chandrasekharan et al., 2018).

Homoarginine is also considered to be a cardiovascular risk marker, although the mechanism of

action is less understood. Homoarginine is a weak substrate for e-NOS. Low plasma homoarginine concentrations were associated with 3.6-fold higher cardiovascular mortality and 2.7-fold higher all-cause mortality after adjustment for potential confounders in patients undergoing coronary angiography, with similar associations reported in patients with diabetes on maintainence haemodialysis (Marz et al., 2010). Plasma homoarginine is also considered an independent marker of all-cause mortality with the potential to improve risk stratification in patients with chronic heart failure (Atzler et al., 2013).

1.2.1.4. Assessment of autonomic nervous system activity

Alteration in autonomic nervous system activity, characterised by reduced vagal activity relative to sympathetic nervous system activity has been demonstrated in patients with cardiovascular disease (Eckberg et al., 1971) and has been implicated in triggering sudden cardiac death (Lown and Verrier, 1976, Schwartz et al., 1992). Baroreceptor sensitivity (BRS) is considered a marker of the reflex increase in vagal activity and decrease in sympathetic activity that occurs in response to a sudden increase in blood pressure (La Rovere et al., 1995). A reduction in baroreceptor sensitivity is associated with a reduction in parasympathetic activity and / or an increase in sympathetic activity. In contrast to previous techniques for assessing BRS which were invasive and had poor reproducibility, BRS is now commonly assessed non-invasively based on the concept that small variations in arterial blood pressure activate baroreceptors. Baroreceptor sensitivity was measured using the sequence method in this thesis, which predominantly reflect parasympathetic nervous system activity. Urinary noradrenaline excretion was used to assess sympathetic activity in the studies included in this thesis.

Alterations in measures of autonomic nervous system activity have been associated with cardiovascular risk. Increases in urinary noradrenaline and metabolite concentrations, efferent muscle sympathetic nerve activity, and plasma noradrenaline levels have been reported in hypertension (Lembo et al., 1992), insulin resistance and diabetes (Chan et al., 1995, Facchini et al., 1996), obesity (Schlaich et al., 2015) and are associated with adverse cardiovascular outcome (Cohn et al., 1984, Hasking et al., 1986, Julius, 1993). In a study involving 78 patients after their first myocardial infarction, a low baroreceptor sensitivity predicted total cardiac mortality after two

years (La Rovere et al., 1988). A subsequent study showed an association between reduced baroreceptor sensitivity and life threatening arrhythmic events, but not all-cause mortality (Farrell et al., 1992). Currently, there is evidence that reduced baroreceptor sensitivity is an independent predictor of cardiovascular mortality in patients post myocardial infarction (La Rovere et al., 1998) and with congestive cardiac failure (Pinna et al., 2005).

1.3. Post-challenge hyperglycaemia, cardiovascular risk and mortality

Prednisolone, the most commonly prescribed semi-synthetic glucocorticoid, predominantly causes hyperglycaemia after a glucose load, with no increase in fasting glucose (Burt et al., 2012). There is strong evidence demonstrating an association between post-challenge glucose concentration and cardiovascular disease, and cardiovascular and all-cause mortality (Pyorala et al., 1979, Decode Study Group, 2001, Bonora and Muggeo, 2001, Ceriello et al., 2004, Hanefeld et al., 1996, Shaw et al., 1999). The DECODE study, which analysed data from more than 20,000 subjects, reported that post-challenge glucose elevation is associated with a greater risk of death from all cause and cardiovascular disease than impaired fasting glucose, with subjects in the upper range of impaired glucose tolerance (10.0-11.1 mmol/L) having a similar risk of death to subjects with diabetes defined by fasting plasma glucose of ≥7 mmol/L (Decode Study Group, 2001). In contrast, subjects with impaired fasting glycaemia do not have increased mortality (Decode Study Group, 2001). Other large epidemiological studies have also reported similar results (Balkau et al., 1998, Meigs et al., 2002, Barrett-Connor and Ferrara, 1998). In a prospective randomized controlled study, in patients with newly diagnosed type 2 diabetes mellitus, postprandial blood glucose, but not fasting blood glucose, was an independent predictor for future myocardial infarction and death (Hanefeld et al., 1996). In a post hoc analysis of the same study, postprandial glucose reduction by insulin therapy in older patients with a recent myocardial infarction was shown to reduce cardiovascular events after a recent myocardial infarction (Raz et al., 2009).

1.3.1. Effects of post-challenge hyperglycaemia on cardiovascular risk markers

Augmentation index decreases postprandially and the reduction is proportional to the carbohydrate content of the meal and the insulin and glucose responses elicited (Greenfield et al., 2007). A hyperinsulinaemic euglycaemic clamp study demonstrated rapid reduction in augmentation index

during sequential supraphysiological insulin infusion suggesting insulin to be the predominant determinant of the postprandial change in augmentation index (Westerbacka et al., 1999b). Insulin resistance has been shown to attenuate insulin's dilatory effects on augmentation index (Westerbacka et al., 1999b). Compared to non-obese insulin sensitive subjects, the reduction in augmentation index by insulin infusion was impaired and delayed in obese subjects with insulin resistance (Westerbacka et al., 1999a). In a meal study, insulin resistant subjects demonstrated an attenuated postprandial reduction in augmentation index suggestive of increased arterial stiffness, with no difference in augmentation index in the fasting state, reinforcing the importance of extending studies of vascular function to the postprandial period (Greenfield et al., 2007). Furthermore, subjects with impaired glucose tolerance, but not those with impaired fasting glucose, have increased arterial stiffness (Li et al., 2012).

Similar to these findings on markers of arterial stiffness, endothelial function estimated by RHI was significantly lower post meal in subjects with post-challenge hyperglycaemia compared to subjects with normal glucose tolerance, with no significant difference in the fasting period (Crandall et al., 2009). Hence assessment of vascular function during the postprandial period in prednisolone treated patients may provide further insights into its cardiovascular effects.

1.3.2. Mechanisms of increased cardiovascular risk with glucocorticoid excess

Endogenous (Cushing's syndrome) and exogenous glucocorticoid excess are associated with hypertension, dyslipidaemia and reduced fibrinolytic potential which can contribute to increased cardiovascular risk (Sholter and Armstrong, 2000). Hypertension affects more than 80% subjects with Cushing's syndrome (Sholter and Armstrong, 2000, Arnaldi et al., 2003) and 20% subjects on exogenous glucocorticoids (Whitworth, 1987). High dose glucocorticoids also increase systolic and diastolic blood pressure (Whitworth et al., 1989, Mangos et al., 2000, van Raalte et al., 2013a). Increased plasma volume, elevated peripheral vascular resistance and increased cardiac output are possible mechanisms of hypertension in glucocorticoid excess. In addition, endothelial dysfunction with reduced nitric oxide availability (Whitworth et al., 2005), increased pressor responsiveness to angiotensin II (Sato et al., 1994), up-regulation of the sympathetic nervous system (Connell et al., 1987), polycythaemia, weight gain and obstructive sleep apnoea could

contribute to hypertension in glucocorticoid treated patients (Pimenta et al., 2012).

Dyslipidaemia in Cushing's syndrome occurs in 40-70 % of patients and is characterised by increased plasma levels of total, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol and triglycerides, and a reduction in high density lipoprotein (HDL) cholesterol (Taskinen et al., 1983). Some, but not all, studies report that exogenous glucocorticoids are associated with an adverse lipid profile (Sholter and Armstrong, 2000, Arnaldi et al., 2003). While few studies reported an increase in total cholesterol, triglycerides and LDL cholesterol with high dose glucocorticoids (Ettinger et al., 1987, Stern et al., 1973, el-Shaboury and Hayes, 1973), others have demonstrated an increase in HDL cholesterol with no changes in LDL cholesterol and triglyceride levels (Zimmerman et al., 1984, Ettinger and Hazzard, 1988).

Cushing's syndrome causes a prothrombotic state predominantly due to an increase in von Willebrand factor and factor VIII, as well as increasing synthesis of fibrinogen and plasminogen activator inhibitor type 1 (Arnaldi et al., 2003). The effects of exogenous glucocorticoids on coagulopathy is less clear (Sholter and Armstrong, 2000). An increased risk of thromboembolism with high dose glucocorticoids has been demonstrated in patients with inflammatory arthritis, bronchial asthma and in healthy subjects (Isidori et al., 2015, Majoor et al., 2016, Stuijver et al., 2013, Johannesdottir et al., 2013), while no increased thromboembolic markers were noted with an increase in hydrocortisone dose in patients with hypopituitarism (Peacey et al., 2012). It is possible that exogenous glucocorticoids may have differential effects on pro-coagulant, anti-coagulant and fibrinolytic factors depending on the condition for which they are administered (van Zaane et al., 2010). Variability in glucocorticoid dose may be a factor, although no clear evidence of a dose-response relationship has been demonstrated (van Zaane et al., 2010).

In contrast to the effects of high dose glucocorticoids, the cardiovascular risk associated with mild glucocorticoid excess is less certain. Low dose prednisolone therapy (<7.5 mg/day) was not associated with hypertension in a cross-sectional (Jackson et al., 1981) and randomized controlled study (van Raalte et al., 2013a). No significant differences in lipid levels in patients on low dose prednisolone versus placebo were demonstrated in a randomised controlled trial, but 63 % in the prednisolone arm had stopped treatment before the end of follow up (Hafstrom et al., 2007). Other

prospective as well as retrospective studies have shown an increase in HDL cholesterol (Dahlqvist et al., 2006, Garcia-Gomez et al., 2008) and decrease in total, LDL cholesterol and triglycerides (Heldenberg et al., 1983) in rheumatoid arthritis patients on low dose prednisolone.

1.3.3. Insulin resistance and cardiovascular risk

Glucocorticoid excess could also indirectly affect cardiovascular function by inducing insulin resistance and increasing visceral adiposity, both of which are independent risk factors for cardiovascular disease (Hanley et al., 2002, Isomaa et al., 2001, Britton et al., 2013, Yip et al., 1998). Much evidence indicates that insulin resistance and its associated comorbidities (metabolic syndrome) increase risk of cardiovascular disease (Miranda et al., 2005, Reaven, 1988). As shown in Figure 3, insulin exerts its biological effects by binding to specific cell surface receptors. This binding activates second messengers which initiate a series of phosphorylation-dephosphorylation cascades stimulating glucose transport, glucose phosphorylation, glycogen synthase (which controls glycogen synthesis), phosphofructokinase and pyruvate dehydrogenase (which regulates glycolysis and glucose oxidation). These metabolic effects of insulin are mediated through the phosphatidylionositol-3 (PI-3) kinase pathway (Kanai et al., 1993). In addition to the effects on glucose metabolism, activation of PI-3 kinase pathway also activates nitric oxide synthase, with consequent nitric oxide production. Nitric oxide is a potent vasodilator and anti-atherogenic agent. Hence, a defect in the PI-3 kinase pathway not only results in hyperglycaemia, but also causes hypertension and accelerated atherosclerosis.

Insulin is also a potent growth factor and the growth promoting effects of insulin are mediated via the mitogen-activated protein (MAP) kinase pathway (Figure 3). Stimulation of this pathway catalyses phosphorylation of transcription factors that promote cell growth, differentiation and proliferation. Blockade of the MAP kinase pathway inhibits the growth promoting effects of insulin without having any effect on the metabolic effects of insulin (Lazar et al., 1995). In insulin-resistant individuals, there is marked resistance in the PI-3 kinase pathway, but the MAP kinase pathway is unaffected (Cusi et al., 2000).

In summary, acutely insulin resistance can cause endothelial dysfunction due to inhibition of PI-3 kinase pathway (Defronzo, 2006), whereas chronically hyperinsulinaemia associated with insulin

resistance can also cause proliferation of vascular smooth muscles and an increase in inflammatory factors through stimulation of MAP kinase pathway promoting atherogenesis.

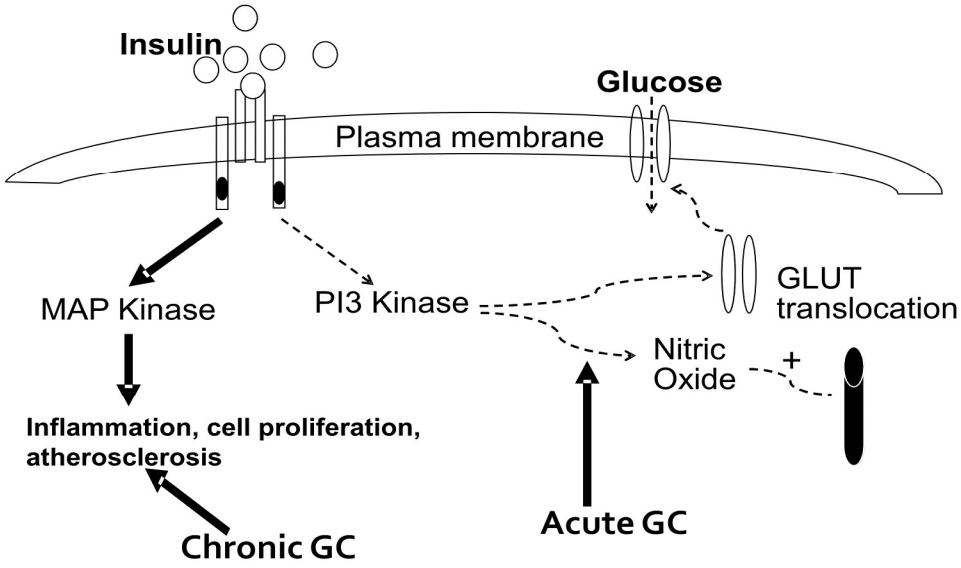

Figure 3: Simplified figure showing the Insulin signal transduction system in individuals with insulin resistance. MAP kinase, Mitogen activated protein kinase; PI3 Kinase, Phosphatidyl ionositol-3 kinase; GLUT translocation, glucose transporter translocation; Acute GC, acute use of glucocorticoids; Chronic GC, chronic use of glucocorticoids; Dotted arrows, refers to inhibition.

1.4. Effects of glucocorticoids on energy and fat metabolism

1.4.1 Introduction

Cushing's syndrome is characterised by a decrease in lean body mass and an increase in fat mass, particularly around the truncal region (Burt et al., 2006). High dose exogenous glucocorticoids (prednisolone > 20 mg/day) also increase central adiposity (Fardet et al., 2011), which is associated with a threefold increased risk of cardiovascular events and stroke (Fardet et al., 2012). The effects of lower therapeutic doses of glucocorticoids (prednisolone <10 mg/day) on adiposity is less clear, with increase in central adiposity reported in some, but not other studies (Burt et al., 2007b, Nordborg et al., 1998). Defining the perturbations in energy and fat metabolism with low dose glucocorticoids will aid in understanding their contribution to adiposity.

1.4.1.1. Assessment of energy and substrate metabolism

Energy metabolism refers to the complex biochemical process whereby the chemical energy of

food is converted to heat and to various energy rich intermediaries like ATP. Carbohydrates, proteins and lipids are the essential substrates for generation of energy. The most common way of extracting the chemical energy of a substrate is by oxidation to carbon dioxide and water. In post absorptive states, the substrates for energy production are mobilized from the endogenous stores such as triglycerides stored in adipose tissue, from hepatic glycogen and by gluconeogenesis. Following the ingestion of a mixed-meal, oxidation of dietary carbohydrates replaces endogenous carbohydrates and lipids as the major source of energy (Kelley and Mandarino, 2000). In patients with insulin resistance, the capacity to switch from predominantly fat oxidation during fasting to carbohydrate oxidation after a meal is attenuated (Galgani et al., 2008). This inability to switch from oxidising fat in the fasting state to carbohydrate in the postprandial state and back again, termed as metabolic inflexibility has been implicated in the accumulation of lipids in the liver and skeletal muscles and the subsequent development of insulin resistance (Galgani et al., 2008). More recent studies have demonstrated that physical inactivity triggers metabolic inflexibility in lean healthy men even preceding the development of glucose intolerance, suggesting that metabolic inflexibility could be a biomarker for glucose intolerance and increased risk of metabolic disease (Rudwill et al., 2018). It is debated whether metabolic inflexibility is a cause or consequence of insulin resistance, and studies are underway to detect if targeting these changes in fat metabolism reduces insulin resistance (Kelley et al., 2002).

The measurement of heat energy plays a central role in the study of energy homeostasis. The generation of heat from substrate metabolism is proportional to energy expenditure and, therefore, metabolic rate (Kenny et al., 2017). Direct and indirect calorimetry are major complementary methods for measuring energy production. In direct calorimetry, energy expenditure is estimated by measuring the amount of heat generated by the body within an insulated environment (Simonson and DeFronzo, 1990). Limitations of direct calorimetry are the requirement for expensive and cumbersome apparatus and lack of information regarding the type of substrate utilized for generating energy.

Indirect calorimetry is the most widely used method for quantifying rates of energy production. Estimation of energy expenditure by indirect calorimetry is based on the principle that energy

(heat) can be determined by measuring oxygen consumption (VO_2) and carbon dioxide production (VCO_2). Direct and indirect calorimetry have been shown to generate near identical results in studies (Simonson and DeFronzo, 1990, Webb et al., 1988). The basic principles of indirect calorimetry are well established as are assumptions underlying the stoichiometry of macronutrients (Ferrannini, 1988, Frayn, 1983). Indirect calorimetry also provides insight into whether energy is derived from fat or carbohydrate. Based on the different stoichiometry of the oxidative metabolism of carbohydrate and fat, indirect calorimetry allows the estimation of fat (Fox) and carbohydrate (CHOox) oxidation. Whereas the oxidation of glucose produces one molecule of carbon dioxide for each molecule of oxygen consumed, oxidation of fat consumes 78 oxygen molecules to generate 55 molecules of carbon dioxide.

$$\text{Glucose } (C_6H_{12}O_6) + 6\ O_2 \longrightarrow 6\ H_2O + 6\ CO_2$$

$$C_{55}H_{104}O_6 + 78\ O_2 \longrightarrow 55\ CO_2 + 52\ H_2O$$

The ratio of carbon dioxide production to oxygen consumption, referred to as the respiratory quotient (RQ), will be equal to 1.0 when purely glucose is metabolised, 0.705 when fat is exclusively metabolised and will be between 0.705 and 1.0 when fat and carbohydrate undergo simultaneous metabolism.

1.4.1.2. Resting energy expenditure and diet-induced thermogenesis

Total daily energy expenditure is comprised of resting energy expenditure (REE), adaptive thermogenesis and physical activity. Fat free mass is the greatest determinant of REE, which comprises of the sleeping metabolic rate and the energy of arousal, and represents about 55-60 % of total daily energy expenditure (Ravussin and Bogardus, 1989, Simonson and DeFronzo, 1990). Despite a marked increase in truncal and total fat, no perturbations in REE were noted in Cushing's syndrome (Burt et al., 2006). While infusions of high dose glucocorticoids paradoxically increase energy expenditure in the first 24 hours (Brillon et al., 1995, Djurhuus et al., 2002), REE is not significantly different after 2-21 days of glucocorticoid administration (Chong et al., 1994, Gravholt et al., 2002, Horber et al., 1991, Short et al., 2004). Similarly no changes were noted in REE with acute and chronic low dose prednisolone (Burt et al., 2007b, van Raalte et al., 2011a).

The available data suggests that stimulation of appetite and not alteration in energy metabolism is the major contributor to glucocorticoid associated adiposity (Tataranni et al., 1996).

Adaptive thermogenesis represents 10-15 % of total energy expenditure and comprises the regulated production of heat in response to cold (cold induced thermogenesis, CIT) and diet (diet induced thermogenesis, DIT). Large inter-individual differences in CIT and DIT have been demonstrated (van Marken Lichtenbelt et al., 2002). An inverse correlation between DIT and percentage body fat has been demonstrated, with a 4% reduction in DIT associated with a 9 % increase in the percentage of body fat (Schutz et al., 1984). It has been postulated that reduced efficiency of adaptive thermogenesis might increase susceptibility to obesity (Eikelis and Esler, 2005), with studies demonstrating reduced DIT (de Jonge and Bray, 1997) and CIT (Wijers et al., 2010) in obese compared to lean individuals.

At the commencement of this thesis the effects of glucocorticoids on DIT had not been studied. DIT comprises obligatory (heat generated by digestion and absorption of food) and facultative (regulated heat production to dissipate food energy) components, with the sympathetic nervous system being an important regulator of the facultative component of DIT (van Baak, 2008, Vosselman et al., 2013). As glucocorticoids reduce sympathetic activity (Lenders et al., 1995), they could potentially reduce DIT, which can contribute to increased adiposity. In a recently published study, prednisolone 15 mg/day for one week was associated with a reduction in the metabolic and thermogenic activity of brown adipose tissue, an important regulator of DIT, and enhanced propensity for lipid synthesis after a mixed-meal (Thuzar et al., 2018). In the studies included in this thesis, the effects of acute and chronic low dose prednisolone on energy expenditure before and after a mixed-meal was assessed using indirect calorimetry, and DIT was calculated as the percentage increase in energy expenditure (Vosselman et al., 2013).

1.4.1.3. Lipolysis

Circulating chylomicron and VLDL triglycerides, from the gut and liver respectively, are hydrolysed by lipoprotein lipase (LPL) located in the luminal side of capillary endothelium to NEFA, which are then re-esterified to triglycerides in the cells. Lipolysis is the breakdown of triglycerides in adipose tissue in a stepwise manner by the lipase enzymes (adipose triglyceride lipase and hormone-

sensitive lipase), resulting in the release of free fatty acids and glycerol. Complete lipolysis of triglyceride releases three fatty acids and one glycerol molecule. As glycerol cannot be transported back to the adipocyte, the appearance of glycerol in the circulation is a direct measure of adipose tissue lipolysis. NEFAs mobilised from stored triglycerides can either undergo beta-oxidation within the mitochondria to generate ATP or are re-esterified to triglycerides.

Lipolysis is closely regulated in a reciprocal, tissue-specific manner such that after a meal, when glucose and chylomicrons are in abundant supply, adipose tissue LPL is upregulated, whereas its activity in muscle is suppressed (Frayn et al., 1997), thus facilitating the storage of triglyceride in adipose tissue. Insulin is a major regulator of lipolysis, and stimulates LPL activity and inhibits the release of NEFA from adipose tissue (Coppack et al., 1994). In addition, insulin also promotes the re-esterification of NEFA to triglyceride within adipose tissue (Campbell et al., 1992). During the post-absorptive state, when insulin levels are low, adipose tissue lipolysis is stimulated by adrenaline and noradrenaline (Coppack et al., 1994).

A number of different techniques can be used to assess lipolysis. Fatty acid turnover can be estimated using isotope dilution techniques involving infusion of stable or radioactive isotopomers of fatty acids (e.g.13C1-palmitate) and whole body lipolysis can be estimated using glycerol tracers to measure rates of appearance (Ra) (Wolfe and Peters, 1987). Regional rates of lipolysis can be assessed by arteriovenous (A-V) sampling or microdialysis techniques measuring glycerol concentrations (Frayn et al., 1997). NEFA levels have also been used in studies as an indirect marker of lipolysis (Johnston et al., 1982, van Raalte et al., 2010).

1.4.1.4. Effects of glucocorticoids on lipolysis and fat oxidation

The current evidence on the effects of glucocorticoids on whole body lipolysis have been inconsistent. An increase in adipose tissue lipolysis during short term infusions of glucocorticoids was reported (Divertie et al., 1991), while other studies showed an increase in lipolysis only after a mixed-meal, with no changes in the fasting state (Dinneen et al., 1993). In contrast, in healthy subjects, administration of prednisolone (30 mg and 7.5 mg prednisolone daily for 2 weeks) was shown to decrease fasting and insulin-mediated suppression of whole body lipolysis and NEFA in a dose dependent manner (van Raalte et al., 2011a). Studies examining the chronic effects of

glucocorticoids on lipolysis have demonstrated no change in systemic or subcutaneous adipose tissue lipolysis (Gravholt et al., 2002, Miyoshi et al., 1988, Johnston et al., 1982) similar to results in patients with Cushing's syndrome (Birkenhager et al., 1976, Saunders et al., 1980).

Several investigators have also explored differences in the effects of glucocorticoids on lipolysis in different areas of body fat, also with inconsistencies in their results. Samra et al (Samra et al., 1998) demonstrated an increase in systemic lipolysis and reduced subcutaneous adipose tissue lipolysis at supraphysiological cortisol concentrations. In contrast, an increase in both systemic and subcutaneous adipose tissue lipolysis was noted during a pancreatic clamp using low dose insulin (Djurhuus et al., 2002). This suggests that insulin levels might have an influence on the effects of glucocorticoids on fat metabolism.

There are fewer studies assessing the effects of glucocorticoids on fat oxidation. Fat oxidation has been shown to be unaltered in Cushing's syndrome (Burt et al., 2006). While infusion of high doses of glucocorticoids increases fat oxidation in the first 24 hours (Brillon et al., 1995, Djurhuus et al., 2002), after 2-21 days of glucocorticoids no significant changes in fat oxidation were reported (Chong et al., 1994, Gravholt et al., 2002, Horber et al., 1991, Short et al., 2004).

The limitations of the studies assessing the effects of glucocorticoids on fat metabolism include that most studies have been performed in healthy subjects or using high dose glucocorticoid infusions and hyperinsulinaemic clamps, conditions which differ from common clinical glucocorticoid use. Also, there are no data on the effects of glucocorticoids on postprandial substrate metabolism. It is possible that, similar to the predominant postprandial hyperglycaemia seen with low dose glucocorticoids, glucocorticoid-induced insulin resistance results in metabolic inflexibility and changes in fat metabolism that predominantly manifest in the postprandial period. In the studies included in this thesis, we have undertaken a systematic analysis of lipolysis, carbohydrate oxidation and fat oxidation during the fasting and postprandial period in patients on acute and chronic prednisolone.

1.4.1.5. Mechanisms of glucocorticoid induced visceral adiposity

Endogenous and exogenous glucocorticoid excess is associated with preferential accumulation of

intra-abdominal adipose tissue. The mechanisms behind glucocorticoid associated obesity are not fully explained, with several proposed mechanisms. Glucocorticoid activity promotes lipoprotein lipase expression in omental fat more than subcutaneous fat (Fried et al., 1998). This in turn would increase the amount of free fatty acid available for uptake in the visceral region predisposing to visceral adiposity. Glucocorticoids and insulin have also been shown to act synergistically in adipocytes to promote lipogenesis and differentiation of preadipocytes to mature adipocytes (Hauner et al., 1987, Gathercole et al., 2007, Gathercole et al., 2011). Increased glucocorticoid receptor expression (Rebuffe-Scrive et al., 1990) and increased expression of pre-receptor modulating enzyme 11-beta hydroxyl steroid dehydrogenase type 1 in adipocytes are other proposed mechanisms for increased visceral adiposity with glucocorticoids (Bujalska et al., 1997).

1.5. Treatment of glucocorticoid induced hyperglycaemia

1.5.1 Introduction

It has been known for decades that high dose glucocorticoid therapy is associated with an increase in blood glucose levels (Ward et al., 1953). Glucocorticoid dose and duration of therapy are important predictors of the development of diabetes (Clore and Thurby-Hay, 2009). Glucocorticoids cause higher increases in blood glucose levels in patients with known diabetes (Yuen et al., 2012, Burt et al., 2011). Furthermore, 50–70% of hospitalized patients without known diabetes prescribed moderate-to-high glucocorticoid doses develop hyperglycaemia (Donihi et al., 2006, Fong and Cheung, 2013). Despite its high prevalence, the optimal treatment of glucocorticoid induced hyperglycaemia is still unclear.

In this section of thesis, the mechanism of hyperglycaemia with glucocorticoids, the evidence behind treatment of acute glucocorticoid induced hyperglycaemia and the various treatment options will be reviewed.

1.5.1.1. Mechanism of glucocorticoid induced hyperglycaemia

Multiple pathways of carbohydrate metabolism are affected by glucocorticoid treatment. Glucocorticoids induce peripheral insulin resistance (Rizza et al., 1982, Tappy et al., 1994). High dose glucocorticoids impair oxidative and non-oxidative glucose disposal, whereas, in patients on

long-term low-dose prednisolone, non-oxidative glucose disposal is predominantly reduced (Tappy et al., 1994, Petersons et al., 2013). Glucocorticoids reduce glycogen synthase activity in skeletal muscle, thus contributing to a reduction in peripheral non-oxidative glucose disposal (Henriksen et al., 1997). High and low dose glucocorticoids also cause hepatic insulin resistance resulting in increased hepatic glucose output (Rizza et al., 1982, Petersons et al., 2013, Dirlewanger et al., 2000). Acutely, glucocorticoids also reduce insulin secretion, which is in part secondary to a reduction in incretin-induced insulin secretion (Eriksen et al., 2015).

1.5.1.2. Impact of acute hyperglycaemia on hospital stay and mortality

There is little direct evidence that treatment of glucocorticoid induced hyperglycaemia is associated with reduced morbidity and mortality in hospitalized patients. In a study done in patients with pneumonia treated with prednisolone, differences in blood glucose levels did not influence the time to clinical stability (Popovic et al., 2016). However, in other clinical scenarios, inpatient hyperglycaemia is associated with increased mortality, morbidity and duration of hospital stay (Umpierrez et al., 2011). The association between hyperglycaemia and mortality is stronger in patients with new onset hyperglycaemia than in those with pre-existing diabetes (Umpierrez et al., 2002). As 50-70% of hospitalized patients can develop new onset hyperglycaemia with glucocorticoids, their morbidity and mortality could be increased (Fong and Cheung, 2013, Donihi et al., 2006). Endocrine Society guidelines state all hospitalized patients prescribed glucocorticoids should be screened for hyperglycaemia with point of care glucose testing and those with hyperglycaemia should be treated (Umpierrez et al., 2012).

1.5.1.3. Treatment of acute glucocorticoid induced hyperglycaemia

Current clinical practice guidelines recommend that glucocorticoid induced hyperglycaemia in hospital should be treated with subcutaneous basal bolus insulin at a starting daily insulin dose of 0.3–0.5 units/kg body weight or a continuous insulin infusion if there is severe or persistent hyperglycaemia (Umpierrez et al., 2012). The rapid onset of action and flexibility in dosing makes subcutaneous basal bolus insulin a preferred option in patients with acute hyperglycaemia on glucocorticoids. There are limited data on the use of other hypoglycaemic agents in the management of glucocorticoid induced hyperglycaemia in the hospital setting.

Prednisolone is the most commonly prescribed semisynthetic glucocorticoid, which is often administered in moderate to high doses acutely as an anti-inflammatory agent (Fong and Cheung, 2013, Overman et al., 2013). Studies have demonstrated a circadian pattern of hyperglycaemia with prednisolone (Burt et al., 2011). When prednisolone is administered as a single oral morning dose, the glucose concentration peaks 8 h later at about 16:00 h (Fig. 4) (Burt et al., 2011). In patients without known diabetes, the blood glucose returns to normal by midnight with little or no overnight hyperglycaemia (Burt et al., 2011). Patients with diabetes have greater degree of hyperglycaemia, but the circadian pattern of hyperglycaemia is similar (Burt et al., 2011, Yuen et al., 2012). These studies suggest that optimal glucose lowering therapy in prednisolone-treated patients should be directed at the time period between midday and midnight.

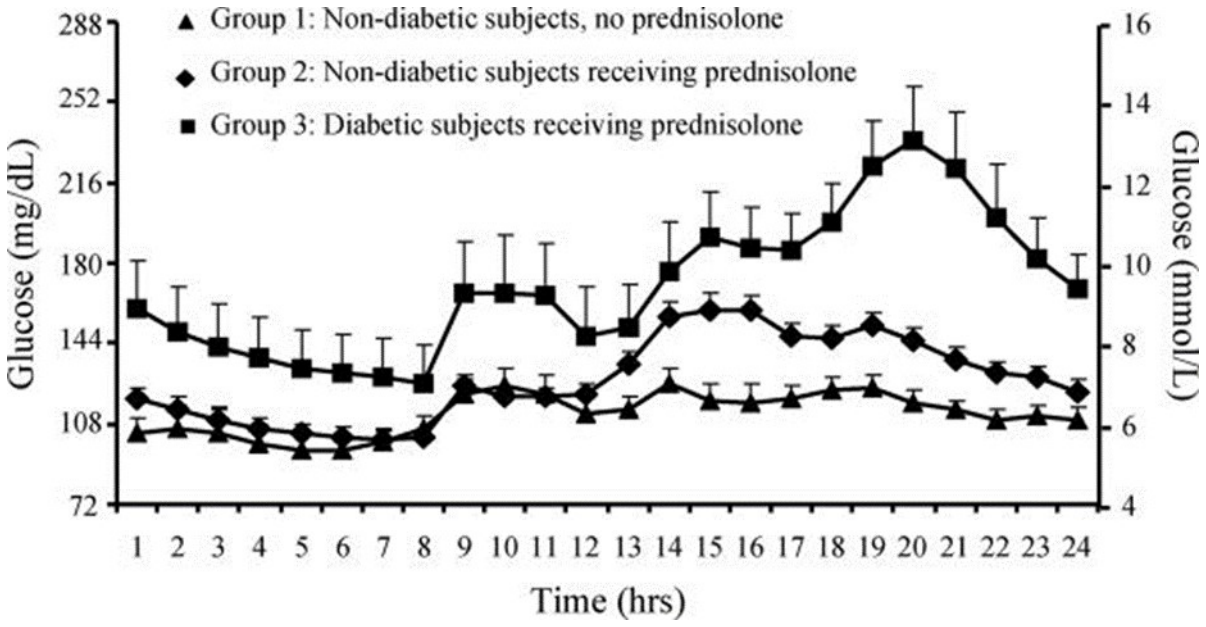

Fig 4: Continuous monitoring of circadian glycaemic patterns in patients receiving prednisolone for Chronic Obstructive Airway Disease. Figure reproduced from Burt MG, et al. JCEM,2011

1.5.1.3.1. Insulin therapy for prednisolone induced hyperglycaemia

The circadian pattern of hyperglycaemia with prednisolone suggests the use of insulin isophane alone or in combination with short-acting insulin to treat prednisolone-induced hyperglycaemia. When administered as a single morning dose, insulin isophane's onset of action, peak effect and duration of action match the pattern of prednisolone-induced hyperglycaemia (Clore and Thurby-

Hay, 2009). Thus, insulin isophane has the potential to better treat daytime hyperglycaemia and avoid overnight hypoglycaemia arising from longer acting basal insulin preparations.

However, the data to support isophane insulin in this context are limited. Prior to the commencement of this thesis, in a retrospective study comparing insulin isophane and insulin glargine as the basal insulin in hospitalized patients with hyperglycaemia on prednisone, isophane-treated patients required 20% less insulin to achieve the same degree of glycaemic control (Dhital et al., 2012). There have been two prospective studies in addition to my own since the commencement of my thesis specifically investigating treatment of hyperglycaemia in patients prescribed glucocorticoids. In patients with type 2 diabetes and respiratory disease treated with methylprednisolone or deflazacort, no significant differences in glucose control were demonstrated between patients randomized to a glargine or isophane based basal bolus regimen (Ruiz de Adana et al., 2015). Another study reported that, when glucocorticoids were administered, additional isophane insulin to the standard hospital insulin regimen resulted in a lower mean glucose concentration on the third day of insulin therapy (Grommesh et al., 2016). However, in that study patients treated with isophane were prescribed a daily insulin dose that was 16% higher than patients treated with standard hospital insulin (Grommesh et al., 2016) a dose difference which will have influenced glycaemic control. Therefore, studies have not demonstrated a conclusive benefit of insulin isophane over insulin glargine in the treatment of glucocorticoid induced hyperglycaemia.

1.6. STATEMENT OF AIMS

In summary, it is unclear whether low dose glucocorticoid therapy is associated with increased cardiovascular risk. Studies have reported increased hepatic and peripheral insulin resistance with low dose prednisolone, and insulin resistance is considered to be an independent risk factor for cardiovascular disease. However, studies assessing cardiovascular risk markers in patients on low dose glucocorticoids have yielded variable results. Most of the current evidence is from studies undertaken in the fasting state in healthy young adults. As glucocorticoids cause predominant postprandial hyperglycaemia, it is possible that the vascular dysfunction associated with low dose glucocorticoids is predominantly manifested in the postprandial period. Furthermore, the findings in healthy cohorts cannot be translated to patients with chronic inflammatory diseases who are most

often prescribed long term low dose glucocorticoids and in whom the underlying disease process may have an impact of the vascular effects of glucocorticoids. Hence, postprandial assessment of vascular function in patients with chronic inflammatory arthritis prescribed acute and chronic glucocorticoid therapy may provide important insights in to the acute and chronic effects of low dose glucocorticoids on cardiovascular risk.

Glucocorticoid use can cause postprandial hyperglycaemia. However, the effects of glucocorticoids on postprandial energy and fat metabolism are unclear. Current evidence suggests that glucocorticoids have no effect on REE, but the effects of glucocorticoids on DIT have not been studied. Reduced DIT may contribute to increased adiposity and metabolic syndrome. The current evidence reporting the effects of glucocorticoids on fat metabolism is inconsistent, with most studies performed using high dose glucocorticoid infusions and hyperinsulinaemic clamps; conditions which differ from common clinical glucocorticoid use. Defining the perturbations of energy and fat metabolism associated with typical therapeutic glucocorticoid doses will help determine the contribution of glucocorticoids to adiposity and increased cardiovascular risk.

Hyperglycaemia in hospitalized patients is associated with increased morbidity, length of hospital stay and mortality. The optimal treatment of glucocorticoid induced hyperglycaemia in hospitalized patients is still unclear. The circadian pattern of hyperglycaemia demonstrated in patients prescribed morning prednisolone suggests that insulin therapy directed at the time period between midday and midnight with a basal-bolus regimen could be safer and provide better glycaemic control. However, data to support isophane insulin in lieu of glargine insulin in this context are limited.

I hypothesized that:

1) Similar to its effects on blood glucose, low dose prednisolone adversely affects postprandial vascular function.

2) Patients with rheumatoid arthritis have alterations in arginine metabolism that influence the effect of prednisolone on vascular function.

3) Prednisolone-induced changes in energy and substrate metabolism predominantly manifest

in the postprandial period.

4) Matching insulin pharmacokinetics to the pattern of prednisolone-induced hyperglycaemia will reduce nocturnal hypoglycaemia and better treat postprandial hyperglycaemia.

The major aims of this thesis were to determine:

1) Whether low dose prednisolone causes postprandial vascular dysfunction.
2) The effect of rheumatoid arthritis on arginine metabolism and the effects of low dose prednisolone on arginine metabolism in these patients.
3) The effect of low dose prednisolone on postprandial energy and substrate metabolism.
4) Whether an insulin regimen comprising insulin isophane and prandial aspart is safer and more effective treatment than insulin glargine and prandial aspart in hospitalised patients with prednisolone-induced hyperglycaemia.

CHAPTER 2. METHODS

2.1. Introduction

This thesis comprised of four main groups of investigations:

1) Assessment of cardiovascular risk

2) Assessment of energy expenditure, carbohydrate and fat metabolism

3) Body composition and other laboratory investigations

4) Continuous glucose monitoring

Cardiovascular risk was assessed using validated non-invasive measures of arterial stiffness, endothelial function, autonomic nervous system activity and measurement of specific arginine metabolites. I performed the clinical assessment of all cardiovascular risk markers. The analysis of arginine metabolites described in Chapter 4 was performed by Dr Andrew Rowland, Department of Clinical Pharmacology, Flinders University. I developed the protocol for the mixed-meal test reported in Chapters 3 and 5, which had not been previously performed in our Unit, after liaising with Dr Dorit Samocha-Bonet, Garvan Institute of Medical Research, Sydney. I undertook indirect calorimetric assessment of energy expenditure and substrate oxidation with assistance from research nurses Ms Brenda Mangelsdorf and Ms Sophie Drake. Glucose was analysed in-house at the Endocrine Research Unit, Repatriation General Hospital. The analysis of insulin, C-peptide and NEFA samples for the studies reported in Chapters 3 and 5 were performed at the Royal Adelaide Hospital, Adelaide by me under the supervision of Associate Professor Leonie Heilbronn. Associate Professor Arthur Jenkins, Garvan Institute of Medical Research, Sydney, provided assistance with the calculation of glucose sensitive insulin secretion. Dual energy X-ray absorptiometry (DXA) scans were performed at the Bone Densitometry Unit, Repatriation General Hospital and analysed by Ms Kirsty Czechowicz. All other laboratory testing was performed by SA Pathology. Research nurses Ms Brenda Mangelsdorf, Mrs Sophie Drake and Dr Jessica Stranks assisted me with the recruitment of inpatients and CGMS insertion for the study in Chapter 6. I performed the randomization of all the consented patients and the daily insulin dose titrations for

most participants, with assistance from Associate Professor Morton Burt, Associate Professor Stephen Stranks and Dr Jessica Stranks. The CGMS data analysis of all the involved participants were analysed by me with assistance from Mrs Sophie Drake.

2.2. Markers of cardiovascular risk

Markers of cardiovascular risk were assessed in studies reported in Chapters 3 and 4. Subjects after overnight fasting were rested supine for a 20 minute acclimatization period in a quiet, temperature controlled room, and tests performed in a standardized order, starting with pulse wave analysis, estimation of autonomic nervous system activity and RHI. Following this, a blood sample for estimation of arginine metabolites was collected. A mixed-meal was administered over 15 minutes and vascular risk assessment repeated in a standardized order.

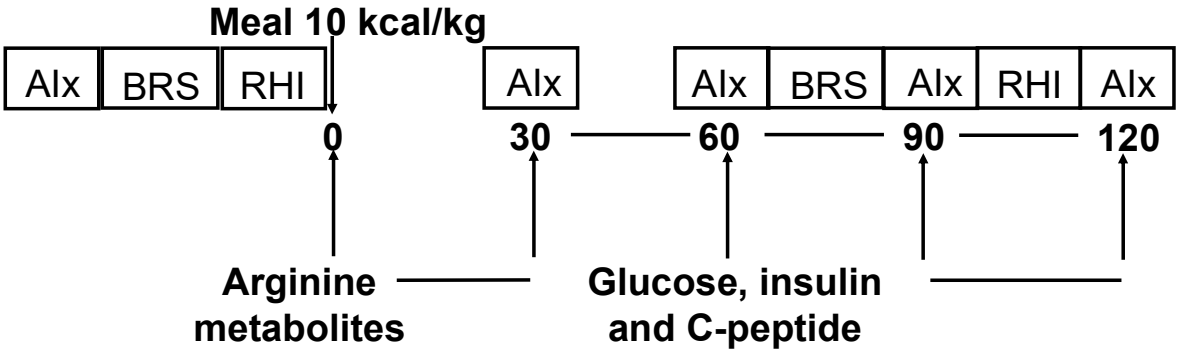

Fig 2.1: Study protocol. AIx: Augmentation index, BRS: Baroreceptor sensitivity, RHI: Reactive hyperaemia index.

2.2.1. Pulse wave analysis

2.2.1.1. Introduction

Arterial stiffness was estimated using pulse wave analysis. The principles underlying this technique are described in Chapter 1.

2.2.1.2. Study protocol

Pulse wave analysis was performed at the radial artery by applanation tonometry with a

SphygmoCor device (AtCor Medical, New South Wales, Australia) and a high-fidelity micromanometer (SPC-301, Millar Instruments, TX, USA). Six measurements in the fasting state and 3 measurements at each specified postprandial time point were averaged.

2.2.1.3. Calculations

The radial artery wave form was recorded by placing a probe on the wrist, and pressure applied to applanate the artery, creating a signal which approximates arterial pressure. The radial artery wave form was calibrated against brachial pressure, and a generalized transfer factor was then used to generate the corresponding aortic waveform (Karamanoglu et al., 1993, Takazawa et al., 1996, Chen et al., 1997). Figure 2.2 shows the typical features of the aortic pressure waveform from which augmentation index is calculated as the increment in pressure from the first shoulder in the aortic pressure wave to the peak of this wave. As augmentation index is also strongly influenced by heart rate (Wilkinson et al., 2000), augmentation index was normalized to a heart rate of 75 beats per minute using an automated equation (AIx75). The quality control indices within the SphygmoCor device software, including average pulse height, pulse height variation, diastolic variation and shape deviation were used to verify recorded waveforms. If the quality index was <80%, the waveform was discarded and the measurement repeated. The area under the curve for AIx75 over two hours was calculated to estimate postprandial arterial stiffness.

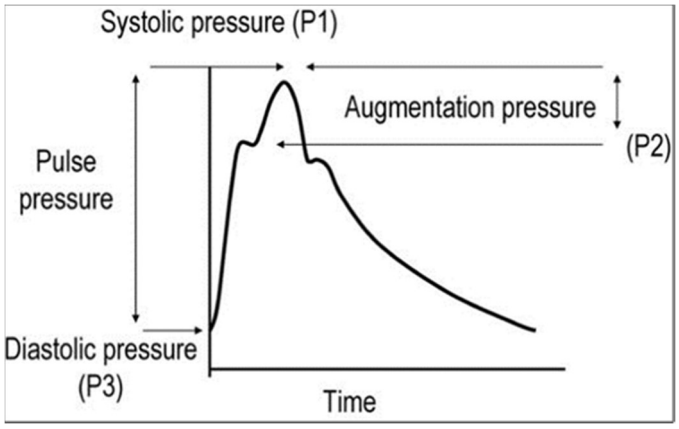

Figure 2.2: Carotid pressure waveform recorded by applanation tonometry. The height of the late systolic peak (P1) above the inflection (P2) defines the augmentation pressure, and the ratio of augmentation pressure to pulse pressure defines the augmentation index, AIx (in percent). Figure reproduced from Laurent et al (Laurent et al.,2006)

2.2.1.4. Reproducibility

Before starting Study 3, I assessed the day-to-day reproducibility of AIx75 in my hands in 6 healthy controls (Table 2.1). The intra-class correlation for AIx75 was 0.97.

Subject	**AIx75 (Visit 1)**	**AIx75 (Visit 2)**	**∆ AIx75**
1	18.3 ± 1.8	25.5 ± 2.1	7.2
2	40.5 ± 1.2	40.8 ± 0.8	0.3
3	9.8 ± 2.8	9.5 ± 1.4	0.3
4	7.0 ± 5.3	7.7 ± 7.4	0.7
5	24.2 ± 4.3	27.8 ± 2.8	3.7
6	7.3 ± 1.4	11.2 ± 2.8	3.8

Table 2.1. AIx75 in 6 healthy controls measured on two different occasions

2.2.2. Reactive hyperaemia index

2.2.2.1. Introduction

RHI estimated using PAT was used as a measure of endothelial function in the studies included in this thesis. The principles underlying this technique are described in Chapter 1.

2.2.2.2. Study protocol

I performed PAT using an Endo-PAT 2000 device (Itamar Medical, Caesarea, Israel) to estimate endothelial function. The identical finger of both hands was placed in a fingertip probe, which was inflated to 10 mm Hg below the diastolic pressure or 70 mm Hg, whichever was lower. The uniform applied pressure field across the finger prevents venous pooling and partially unloads arterial wall tension, and the volume changes in the fingertip were digitally recorded as pulse amplitude. Recordings were taken simultaneously from both fingers, and the response in the control finger not experiencing hyperaemia was used to adjust for systemic effects.

After a baseline recording of 5 minutes, a blood pressure cuff was inflated in one arm to

suprasystolic pressures, or a maximum of 280 mm Hg for 5 minutes, to achieve occlusion of blood flow. After the blood pressure cuff release, recording was continued for another 5 minutes to determine the degree of reactive hyperaemia.

2.2.2.3. Calculations

The pulse amplitude responses were digitalised and analysed, and RHI calculated by an automated computer algorithm. First, the pulsatile arterial volume ratio was calculated for each arm by dividing the pulsatile arterial volume 90 to 150 seconds post-occlusion (red segment in Fig 2.3) by the average of baseline readings in the same arm (green segment in Fig 2.3) (Hamburg et al., 2008). The RHI was then calculated as the ratio of the occluded arm pulsatile arterial volume ratio to that in the control arm. Even though a normal reference range for RHI has not been published, several studies have associated lower RHI with endothelial dysfunction (Bonetti et al., 2004, Kuvin et al., 2003). The manufacturer's advice based on early published data is that an RHI <1.67 denotes endothelial dysfunction.

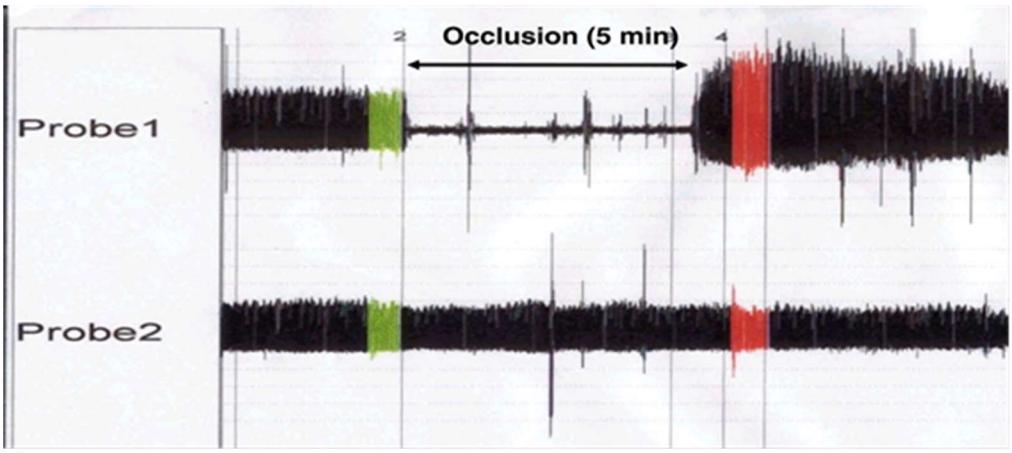

Fig 2.3.An example of peripheral arterial tonometry pulse volume amplitude (PVA) recording in a single individual before and during reactive hyperaemia (RH). Amplitude in probe 1 disappears during inflation of the blood pressure cuff on the upper arm to suprasystolic pressures. After the release of the occlusion, the PVA reaches maximum at 1 min (red-shaded area). PVA during RH divided by the PVA at baseline (green-shaded area) calculates digital pulse arterial volume ratio. Probe 2 is on the index finger of the contralateral, non-study arm and is utilized to define any drift in PVA due to "systemic factors" during the study. Adapted from Nohria et al.,2006

The portion of the peripheral arterial tonometry hyperaemic response shown to be dependent on endothelial nitric oxide release occurs from 60 to 120 seconds following release of the cuff (Nohria

et al., 2006). Notably, the relationship between cardiovascular risk factors and reactive hyperaemic response is maximized in the 90 to 120 second time interval (Hamburg et al., 2008). The combination of these two parameters are well represented in the automated test period.

2.2.2.4. Reproducibility

Several studies have assessed the reproducibility of Endo-PAT 2000 device, with the CV ranging between 13-18 % (Moerland et al., 2012, Brant et al., 2013). The average day-to-day CV for reactive hyperaemia index at our institution was previously reported to be 11.4% (Burt et al., 2013a).

2.2.3. Autonomic nervous system activity

2.2.3.1. Introduction

Urinary noradrenaline excretion was measured during the study period to assess sympathetic nervous system activity and spontaneous baroreceptor sensitivity (BRS), which predominantly reflects parasympathetic nervous system activity, was calculated by the sequence method using a Taskforce Hemodynamic Monitor 3040i (CN Systems, Graz, Austria).

2.2.3.2. Spontaneous baroreceptor sensitivity

2.2.3.2.1. Study protocol

After subjects had rested supine for 10 minutes, BRS was assessed over a 20-minute period in a quiet room with the subjects rested throughout the test. Subjects with premature ventricular complexes and on beta blockers were excluded. A continuous electrocardiogram recording and “flying V” finger cuff for non-invasive measurement of beat-by-beat arterial pressure were placed on the subject, allowing simultaneous computer analysis of heart rate and blood pressure variability. The finger blood pressure cuffs were calibrated automatically against blood pressure measured using a standard brachial cuff.

2.2.3.2.2. Calculations

The sequence method is based on the computer identification of spontaneously occurring sequences of four or more consecutive beats characterized by either a progressive rise in systolic blood pressure and lengthening of R-R interval or by a progressive decrease in systolic blood

pressure and shortening of R-R interval. The slope of the regression line between systolic blood pressure and R-R interval changes is taken as an index of baroreceptor sensitivity (Parati et al., 2000). BRS measurement using sequence method has good intra and inter-observer reproducibility with a CV of 1-6% (Gao et al., 2005).

2.2.3.3. Sympathetic nervous system activity

For the study described in Chapter 3, urine was collected for the study duration (6 hours) and hourly noradrenaline excretion estimated. Noradrenaline was quantified by SA Pathology using liquid chromatography / mass spectrometry (Whiting, 2009). The coefficient of variation (CV) for urinary noradrenaline measurement is 5.1% at 300 nmol/L.

2.2.4. Arginine metabolomics

2.2.4.1. Introduction

For the study in Chapter 4, 7 key components of arginine metabolism that are directly or indirectly involved in the regulation of endothelial function: arginine, homoarginine, citrulline, ornithine, asymmetric dimethyl arginine (ADMA), mono methylated arginine (MMA) and symmetric dimethyl arginine (SDMA) were estimated.

2.2.4.2. Study Protocol

Blood samples were collected in EDTA tubes for the measurement of 7 key components of arginine from all participants after at least 12 hours of overnight fast. Blood samples were centrifuged at 4,000 rpm at 4^0 Centigrade for 10 min and plasma frozen at -80^0 Centigrade until analysis.

2.2.4.3. Laboratory analysis

2.2.4.3.1. Sample preparation

Samples were prepared for analysis by solvent extraction. 100 µL of sample was mixed with 500 µL of assay precipitating solution (0.1% formic acid in methanol) and vortexed for 30 sec. This mixture was centrifuged for 5 min at 16000 g, and a 400 µL aliquot of the resulting supernatant was evaporated to dryness using a MiVac centrifugal vacuum concentrator (GeneVac, Sydney, Australia). Dried eluates were then reconstituted in 200 µL ammonium formate (10 mM with 1%

methanol). A 175 µL aliquot was then transferred to a Waters Acquity auto-sampler vial (Waters Australia, Sydney, Australia) containing an liquid chromatography-mass spectrometry (LC-MS) grade glass insert for analysis (van Dyk et al., 2015).

2.2.4.3.2 Chromatography and Mass spectrometry

Chromatographic separations were performed on a Waters ACQUITY™ T3 HSS C18 analytical column (150 mm × 2.1 mm,1.8 µm; Waters Corp., Milford, USA) using a Waters ACQUITY Ultra Performance LC™ (UPLC) system. Column elutant was monitored by mass spectrometry, performed on a Waters Q-ToF Premier™ quadrupole; orthogonal acceleration time of flight tandem mass spectrometer (Q-ToF-MS) operated in positive ion mode with electrospray ionisation (ESI+) (van Dyk et al., 2015).

2.2.4.3.3 Assay recovery and reproducibility

For all analytes, the percent relative standard deviation (%RSD) was <3% for intra-day variability, and <6% for inter-day variability (van Dyk et al., 2015).

2.3. Assessment of Carbohydrate and Fat metabolism

2.3.1. Introduction

A mixed-meal test was used in the studies described in this thesis for assessment of insulin sensitivity, secretion and to facilitate assessment of postprandial vascular function and quantification of diet-induced thermogenesis. An advantage of a mixed-meal test relative to other methods of insulin sensitivity is that it reflects glucose dynamics and insulin action under physiological conditions including accounting for the incretin effect (Cersosimo et al., 2014).

2.3.2. Mixed-meal test protocol

All subjects attended our research unit after at least 12 hours of overnight fast. An intravenous cannula was then inserted and baseline blood samples collected to measure glucose, insulin, C-peptide and NEFA. A standardised mixed-meal was then administered over 15 minutes (10 kcal/kg body weight, 45% carbohydrate, 40% fat and 15% protein), and further blood samples for glucose, insulin, C-peptide and NEFA were taken at 30-minute intervals for 120 minutes.

2.3.2.1. Estimation of Insulin sensitivity

Insulin sensitivity was estimated by the composite insulin sensitivity index (Matsuda index). The ISI comp is an estimate of insulin sensitivity derived from frequently sampled oral glucose tolerance test. However, calculation of Matsuda index from a mixed meal test has also been reported and classifies insulin sensitivity in healthy adults, and prednisolone-induced changes in insulin sensitivity similarly to a euglycaemic-hyperinsulinaemic clamp (Tan et al., 2015, van Raalte et al., 2011b).

The formula used to calculate ISI comp is:

$$\frac{10000}{\sqrt{I0 \times G0 \times Im \times Gm}}$$

Where:

I0 = Fasting insulin concentration (μU/mL)

G0 = Fasting glucose concentration (mg/dL)

Im = Mean insulin concentration (μU/mL) during the mixed-meal study, calculated by: (I0+I30+I60+I90+I120)/5

Gm = Mean glucose concentration (mg/dL) during the mixed-meal study, calculated by: (G0+G30+G60+G90+G120)/5

2.2.2.2 Estimation of insulin secretion

Insulin secretion at baseline and each 30-minute time point after mixed-meal was estimated by the C-peptide deconvolution method (Sze et al., 2011). Area under the curves (AUC) for glucose and insulin secretion over 120 minutes were calculated using the trapezoidal method (Tai, 1994). Glucose-sensitive insulin secretion was then calculated by: *AUC insulin secretion / AUC blood glucose*

2.3.3. Continuous glucose monitoring

2.3.3.1. Introduction

Continuous glucose monitoring system (CGMS) constantly measure the glucose level of subcutaneous interstitial fluid, and communicate wirelessly to a receiver that displays the most recent sensor glucose level. In the study described in Chapter 6, CGMS monitoring was used for assessment of glycaemic control in addition to capillary blood glucose monitoring performed four times daily.

2.3.3.2. Study protocol

All patients underwent monitoring of interstitial glucose using CGMS (iPro2, Medtronic/Minimed, Northridge, CA, USA). A sensor was placed into the subcutaneous tissue of the abdomen by a member of the research team. Interstitial glucose concentrations were recorded every 5 minutes for up to 4 days using a glucose-oxidase based method. Point of care capillary blood glucose levels were performed at 07.00, 12.00, 17.00 (before each main meal) and at 21.00 hours with a ward glucose meter (Freestyle optium H, Abbott, Victoria, Australia) and used to calibrate the CGMS. The overall mean absolute relative difference, which is the average of the absolute differences between paired capillary blood glucose and interpolated CGM readings, and expressed as a percentage of the corresponding capillary blood glucose readings was 16.2 % for ipro 2 in previous studies (Freckmann et al., 2013) .The test-retest reliability for ipro 2 under standardized conditions in individuals with type 2 diabetes ranged from 0.77 to 0.95 ($P<0.05$) for mean glucose and meal, exercise, and nocturnal glycaemia with CV ranging from 3.9% to 11.7% (Terada et al., 2014).

2.3.3.3. Calculations

Interstitial glucoses were averaged for each hour of CGMS and were then used to calculate the percentage of time glucose was outside a target range of 4-10 mmol/L, mean glucose concentration, percentage of time in hypoglycaemia (defined as glucose < 4 mmol/L) and severe hypoglycaemia (defined as glucose < 2.8 mmol/L). Glycaemic variability was estimated by the mean amplitude of glycaemic excursion (MAGE) (Service et al., 1970).

2.3.4. Lipids and adipocyte insulin sensitivity

Adipocyte insulin resistance index was calculated as the product of fasting insulin and NEFA concentrations (Malin et al., 2014). Insulin-mediated suppression of NEFA was calculated as the percentage decrease in fasting NEFA concentration divided by the mean plasma insulin concentration during the mixed-meal test (Abdul-Ghani et al., 2008).

2.3.5. Laboratory analysis for assessment of carbohydrate and fat metabolism

2.3.5.1. Insulin and C-peptide

For the studies in Chapter 3 and 5, I analysed the insulin and C-peptide samples using a radioimmunoassay technique (EMD Millipore, Toronto, Ontario, Canada). Whole blood samples collected were kept on ice immediately after venesection. Serum for insulin and C-peptide was obtained by centrifuging the blood samples rested for a minimum of 30 minutes, for 16 minutes at 3000 revolutions per minute at 4^0 Centigrade. The serum samples were stored in house at -80^0 Centigrade until analysis. Samples were run undiluted in duplicates using 50 µL of serum. Quality control results were within one standard deviation of the mean at all concentrations. If the difference between duplicate results of a sample was >10% CV, samples were repeated.

2.3.5.2. Non-esterified fatty acids

Serum free fatty acid concentrations were measured by enzyme colorimetry using a Beckman Synchron CX5 analyser (WAKO NEFA C kit, Denver, CO). Whole blood samples collected for NEFA was put on ice immediately after venesection. After resting for a minimum of 30 minutes, samples were centrifuged at 4^0C for 16 minutes at 3000 revolutions per minute. Serum was then stored at -80^0 Centigrade. NEFA samples were analysed undiluted, with 5 µL of serum. Grossly haemolysed samples were excluded from the analysis as haemolysis can cause false positive results. The intra assay CV of this method was <2%.

2.3.6. Indirect calorimetry

2.3.6.1 Introduction

Indirect calorimetry measures gas exchange, ie, whole body O2 uptake and CO2 release, and energy expenditure and substrate oxidation can be calculated using the principles described in

Chapter 1. In the study in Chapter 5, energy expenditure and substrate oxidation were estimated in the fasting state and 2 hours after a mixed-meal.

2.3.6.2. Study protocol

Indirect calorimetry was performed using a ventilated hood technique. With the subject rested supine, a clear plastic hood attached to a metabolic measurement system (Parvo Medics True One 2400 Metabolic Measurement System, Parvo Medics, Sandy, UT) was placed over the subject's head. Air is drawn through the system, thereby maintaining a slightly negative pressure inside the hood. The flow rate through the hood is approximately five times that of the subject's resting minute ventilation (Simonson and DeFronzo, 1990). The volume of air flowing through the hood is measured by a pressure transducer, and the minutely VO2 and VCO2 measurements are automatically reported. After an equilibrium period of 10 minutes, resting energy expenditure and substrate oxidation rates were calculated from the next 20 minutes of indirect calorimetry recordings.

2.3.6.3. Calculations

REE , CHox and Fox were calculated using the equation laid out by Frayn (Frayn, 1983). DIT was calculated as the percentage increase in energy expenditure after the mixed meal (Vosselman et al., 2013).

REE (kcal/min) = 3.91 x VO2 +1.1 X VCO2 – 3.34 X Nu

$$\text{DIT (kcal/min)} = \frac{\text{EE2-EE1}}{\text{EE1}} \times 100$$

Where: EE1 = Baseline energy expenditure

EE2 = Energy expenditure 2 hours after mixed-meal

Carbohydrate oxidation rate (mg/min) = $[(4.55 \times VCO_2 \div 1000) – (3.21 \times VO_2 \div 1000) - (2.87 \times N_u)] \times 1000$

Fat oxidation rate (mg/min) = $[(1.67 \times V0_2 \div 1000) – (1.67 \times VCO_2 \div 1000) – (1.93 \times N_u)] \times 1000$

Where:

VO_2 = Total oxygen production (L/min)

VCO_2 = Carbohydrate consumption (L/min)

N_u = Urinary nitrogen excretion (g/min)

Urinary urea was estimated from the urine collected over the duration of the study (6 hours) and urinary nitrogen excretion calculates as:

N_u (g/min) = [(urinary urea excretion (mmol) ÷ 480) x 60.05526] ÷ 1000

2.4. Body composition

2.4.1. Whole and regional body composition by DXA

2.4.1.1. Introduction

DXA is a useful tool to assess body composition, and is simple and quick to perform with minimal radiation exposure. The fundamental principle of DXA is the transmission of x-rays through the body at high and low energy levels and measurement of the differential attenuation by components of differing densities, like bone and soft tissue or, when no bone is present, fat and fat free soft tissue (Toombs et al., 2012) . This in turn results in a three-compartment model comprising of fat mass, lean body mass and bone. DXA can also be used for assessment of regional body composition. The measurement of central abdominal fat by DXA correlates strongly with that measured by CT which is considered to be the gold standard (Carey et al., 1996). The limitations of DXA arise from the assumption of constant hydration of lean body mass and homogeneity of body compartments. DXA also does not provide information on components within lean body mass and fat mass, like skeletal muscle and visceral fat (Laskey, 1996).

2.4.1.2. Study protocol

For the study in Chapter 5, fat mass and lean body mass were measured by DXA on a GE Medical Systems Lunar Prodigy (GE Healthcare General Electric Company), which also quantified central abdominal fat. All scans were performed on the same scanner, with subjects weighed using the same scale immediately prior to assessment.

2.4.1.3. Calculations

Total tissue mass, total fat mass, lean body mass and bone mineral content were estimated, and fat free mass was calculated as the sum of lean body mass and bone mineral content. Central abdominal fat was estimated by manually tracing the area bordered by the upper margin of the second and the lower margin of the fourth lumbar vertebral bodies and the outer margin of the ribs in all subjects (Carey et al., 1996). CV for fat mass and lean body mass by DXA are 2.9 and 1.4% respectively (O'Sullivan et al., 1994).

2.5. Other laboratory investigations

2.5.1. Blood glucose

For the study in Chapter 3 and 5, whole blood glucose was measured at the bedside immediately after venesection on an in-house glucose analyser (YSI 2300 STAT Plus, Yellow Springs Instrumentation, Ohio, USA) by an immobilised glucose oxidase method, which was self-calibrated against a standard solution.

2.5.2. Glycosylated haemoglobin

For the study in Chapter 6, glycosylated hemoglobin (HbA1c) was measured at SA Pathology using boronate affinity chromatography on a Primus PDQ (Immuno, Sydney, Australia). The between-run CV was 2.6 % at an HbA1c of 5.4% and 2.9% at an HbA1c of 9.6%.

2.5.3. Lipid profile

Fasting lipid profiles were measured by SA pathology using enzymatic colorimetry (Roche Modular P Unit; Roche Diagnostics GmbH). The between-run CV for cholesterol was 1.9 % at 2.7 mmol/L and 1.7 % at 6.5 mmol/L, for triglycerides was 3.0 % at 1.2 mmol/L and 2.1 % at 2.1 mmol/L, and for HDL cholesterol was 3.9 % at 0.7 mmol/L and 3.8 % at 1.4 mmol/L. LDL cholesterol was calculated using the Friedewald equation.

2.5.4. C-reactive protein

C-reactive protein (CRP) was measured by SA Pathology using a Tinaquant immunoturbidimetric assay (Roche Diagnostics GMBH, Mannheim, Germany) on a Roche Modular Analyser (Hitachi

High-Technologies Corporation, Tokyo, Japan). The limit of detection was 0.3 mg/L. The between-run CV was 3.6 % at a CRP of 3.9 mg/L and 2.3 % at a CRP of 49.5 mg/L.

2.5.5. Urinary urea

Urinary urea was analysed by kinetic UV spectrophotometric assay (Roche Diagnostics GMBH, Mannheim, Germany) on a Roche/Hitachi Modular Analyser (Hitachi High Technologies Corporation, Tokyo, Japan) at SA Pathology. The between run CV for urine urea was 2.9 % at 1456 mmol/L and 3.1 % at 279 mmol/L

2.5.6. Renal function

Serum creatinine was measured by SA Pathology staff using a Roche automated clinical chemistry analyser (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany) and estimated glomerular filtration rate (e-GFR) was assessed using the chronic kidney disease-epidemiology collaboration equation (CKD-EPI).

2.6. Physical activity questionnaire

Physical activity was assessed using the Modified Baecke Questionnaire, which is a composite score based on household activity, sports and exercise and other leisure activities. A higher score reflects higher levels of physical activity. A score between zero and 9.4 represents low physical activity, between 9.4 and 16.5 represents moderate physical activity and greater than 16.5 represents high physical activity (Voorrips et al., 1991).

2.7. Statistics

Statistical analysis was performed using IBM SPSS version 20 for Windows (IBM, New York, USA). A p-value of <0.05 was considered statistically significant. Subject characteristics are presented as mean ± standard deviation if the data were Normally distributed and median (interquartile range) if the distribution was not Normal. All other data are presented as mean ± standard error of mean. Unpaired variables were analysed using unpaired t-tests if Normally distributed or Mann-Whitney U tests if the distribution was not Normal. Paired variables were analysed using paired t-tests. One way analysis of variance was used for the 3-group analysis in Chapter 4. In Chapter 6, univariate regression analysis was performed to assess the relationship

between relevant variables and the percentage of time spent with glucose outside the target range of 4-10 mmol/L on Day 1. Variables that were statistically significant in univariate analyses were then included in a multivariate analysis. Changes in variables over three days of insulin treatment were assessed using two way repeated measures analysis of variance.

CHAPTER 3. EFFECT OF ACUTE AND CHRONIC GLUCOCORTICOID THERAPY ON INSULIN SENSITIVITY AND POSTPRANDIAL VASCULAR FUNCTION

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Author roles

AR was responsible for study design, subject recruitment, data acquisition, laboratory analysis, data analysis and manuscript preparation. BLM was responsible for data acquisition. SMD was responsible for data acquisition. DSB was responsible for the mixed-meal protocol and manuscript revision. ABJ was responsible for insulin data analysis and manuscript revision. LKH was responsible for laboratory analysis and manuscript revision. MDS was responsible for subject recruitment and manuscript revision. CHT was responsible for study design, supervision and manuscript revision. MGB was responsible for obtaining funding, study design, data analysis, supervision and manuscript revision.

SUMMARY

Objective: Postprandial hyperglycaemia is associated with increased arterial stiffness and cardiovascular events. Low-dose prednisolone causes insulin resistance that typically manifests as postprandial hyperglycaemia. We investigated whether prednisolone causes postprandial vascular dysfunction in a cohort of patients with rheumatoid arthritis.

Design: An open interventional and cross-sectional study were undertaken.

Patients and measurements: Eighteen subjects with rheumatoid arthritis who had not taken oral glucocorticoids for ≥6 months were studied before and after prednisolone 6 mg/day for 7 days to determine the acute effects of prednisolone. Pre-prednisolone data were compared to 18 subjects with rheumatoid arthritis taking long-term (>6 months) prednisolone (6.5±1.8 mg/day) to assess the chronic effects of prednisolone. Augmentation index (by applanation tonometry) and reactive hyperaemia index (by peripheral artery tonometry) were measured before and after a mixed meal (10 kcal/kg, 45% carbohydrate, 15% protein, 40% fat). Insulin sensitivity was estimated by the Matsuda index and sympathetic nervous system activity from urinary noradrenaline excretion.

Results: Matsuda index was lower after acute (2.0±1.0 vs 3.6±1.1, p=0.01) and chronic (1.9±1.0 vs 3.6±1.1, p=0.04) prednisolone. Postprandial augmentation index was lower after acute prednisolone (2551±197 vs 2690±272 %*min, p≤0.001), but not chronic prednisolone. There were no significant differences in reactive hyperaemia index with acute or chronic prednisolone. Noradrenaline excretion was lower after acute (54±8 vs 93±23 nmol/6h, p=0.02), but not chronic, prednisolone.

Conclusions: Prednisolone-induced insulin resistance is not associated with postprandial vascular dysfunction in patients with rheumatoid arthritis. Reduced sympathetic activity may contribute to the reduction in postprandial arterial stiffness with acute prednisolone.

3.1. Introduction

Glucocorticoids are anti-inflammatory agents that are widely used to treat a range of inflammatory and autoimmune diseases. Despite an expanding range of therapeutic options, the prevalence of glucocorticoid use is increasing (Fardet et al., 2011). Although high dose glucocorticoids are often needed acutely, daily prednisolone-equivalent doses of less than 10 mg are usually prescribed as long-term therapy (Fardet et al., 2011).

While high dose glucocorticoid therapy is associated with an increased risk of cardiovascular disease, the effect of low dose prednisolone on cardiovascular risk is uncertain (Gaujoux-Viala and Gossec, 2014). Epidemiologic studies, which can be confounded by indication bias, have reported conflicting results (Wei et al., 2004, Listing et al., 2015). The sample size and duration of randomized-controlled studies are insufficient to assess cardiovascular events (Svensson et al., 2005, Capell et al., 2004). Lacking definitive data, assessments of vascular function may provide important insight into the cardiovascular effects of low dose prednisolone and clarify the risks of using glucocorticoids to treat chronic inflammatory disease.

Augmentation index is a measure of left ventricular afterload that is predominantly determined by arterial stiffness (Laurent et al., 2006). A higher augmentation index indicative of increased arterial stiffness is associated with an increased risk of cardiovascular disease, independent of traditional cardiovascular risk factors. Arterial stiffness is affected by vessel wall structure, but is also dependent on autonomic tone and endothelial function. Studies in healthy adults have shown impairment in microvascular function with low dose prednisolone (van Raalte et al., 2013a). However, long-term prednisolone did not increase carotid intima media thickness or cause endothelial dysfunction in patients with rheumatoid arthritis (RA) (Hafstrom et al., 2007), and in patients with polymyalgia rheumatica there was a fall in fasting augmentation index after 4 weeks of prednisolone (Schillaci et al., 2012).

It is important to consider that these vascular effects of prednisolone were observed in fasting patients (Schillaci et al., 2012, Hafstrom et al., 2007). We previously reported that low dose prednisolone predominantly causes postprandial hyperglycaemia (Burt et al., 2012). In patients

with type 2 diabetes mellitus or impaired glucose tolerance, when insulin resistance results in postprandial hyperglycaemia it is more strongly associated with cardiovascular events (Decode Study Group, 2003), and arterial stiffness (Li et al., 2012), than when insulin resistance causes fasting hyperglycaemia. Furthermore, vascular dysfunction in these patients may only manifest during the postprandial period (Crandall et al., 2009, Greenfield et al., 2007).

We hypothesized that, similar to its adverse effects on blood glucose concentration, prednisolone-induced insulin resistance would predominantly adversely affect postprandial vascular function. As such, the aim of this study was to investigate the effect of low dose prednisolone on postprandial glycaemia, insulin sensitivity and postprandial arterial stiffness in patients with RA. We also investigated whether changes in autonomic nervous system activity or endothelial function underlie any changes in arterial stiffness.

3.2. Materials and Methods

3.2.1. Subjects and study design

Subjects aged 50 years or older with RA were recruited from the rheumatology outpatient clinic at Repatriation General Hospital, Adelaide, Australia. We studied 18 subjects who had not been administered any oral glucocorticoids for at least 6 months (non-GC users) and 18 subjects taking a stable continuous oral prednisolone dose of 4-10 mg/day for at least 6 months (GC users). The two groups were matched for age and sex. Non-GC users were studied before and after a 7 day course of oral prednisolone 6 mg daily to determine the acute effects of prednisolone. Baseline data from non-GC users were compared with data from chronic GC users to determine the chronic effects of prednisolone. Subjects with atrial fibrillation, Raynaud's phenomenon, on oral hypoglycaemic medications, insulin or beta blockers were excluded from the study. The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee, Flinders Medical Centre, and all subjects provided written informed consent in accordance with the Declaration of Helsinki.

3.2.2. Study protocol

Subjects attended the Endocrine Research Unit at Repatriation General Hospital at 0830 h after a

12 hour overnight fast. All subjects took their regular medications in the morning prior to arrival, including prednisolone. Basic anthropometric measures were recorded. At each visit, after resting supine for a 20 minute acclimatization period, subjects underwent fasting assessment of vascular function. An intravenous cannula was then inserted and baseline blood samples collected to measure glucose, insulin, C-peptide and non-esterified fatty acid levels (NEFA). A mixed-meal was then administered over 15 minutes (10 kcal/kg body weight, 45% carbohydrate, 40% fat and 15% protein), and further blood samples for glucose, insulin, C-peptide and NEFA were collected at 30-minute intervals for 120 minutes. Vascular function was reassessed after the mixed-meal in a standardized order.

3.2.2.1. Pulse wave analysis

The augmentation index was calculated by Pulse wave analysis. Applanation tonometry was performed by one operator (AR) with a SphygmoCor device (AtCor Medical, New South Wales, Australia) and a high-fidelity micromanometer (SPC-301, Millar Instruments, TX, USA). Six measurements in the fasting state and 3 measurements at each specified postprandial time point were performed and averaged. The quality control indices within the SphygmoCor device software were used to verify recorded waveforms. If the quality index was <80%, the waveform was discarded and the measurement repeated. To correct for the effect of pulse rate, augmentation index results were normalized for a heart rate of 75 beats per minute (AIx75). The day-to-day intra-class correlation for AIx75 for this operator is 0.97. The area under the curve (AUC) for AIx75 over two hours was calculated to estimate postprandial arterial stiffness.

3.2.2.2. Autonomic nervous system activity

Urinary noradrenaline excretion was measured during the 6 hour study period by liquid chromatography / mass spectrometry to estimate sympathetic nervous system activity. The coefficient of variation (CV) for urinary noradrenaline measurement is 5.1% at 300 nmol/L. Spontaneous baroreceptor sensitivity, which predominantly reflects parasympathetic nervous system activity, was calculated by the sequence method using a Taskforce Hemodynamic Monitor 3040i (CNSystems, Graz, Austria) (Parati et al., 2000). A continuous electrocardiogram recording and “flying V” finger cuff for non-invasive measurement of beat-by-beat arterial pressure were

placed on the subject, allowing simultaneous computer analysis of heart rate and blood pressure variability. After subjects had rested supine for 10 minutes, baroreceptor sensitivity was assessed over a 20-minute period.

3.2.2.3. Peripheral arterial tonometry

Peripheral arterial tonometry was performed using an Endo-PAT 2000 device (Itamar Medical, Caesarea, Israel) to estimate endothelial function. After a baseline pulse amplitude measurement was obtained from both hands, local ischaemia to the arm was induced by inflating a blood pressure cuff to suprasystolic pressures for five minutes. The pulse amplitude response to hyperaemia is recorded electronically in both fingers (the non-occluded arm serves as control). The time period between 90 and 150 seconds after deflation was used for automated calculation of the reactive hyperaemia index (Matsuzawa et al., 2010). The average day-to-day CV for reactive hyperaemia index at our institution is 11.4% (Burt et al., 2013a).

3.2.2.4. Insulin sensitivity and secretion

Whole blood glucose was measured at the bedside immediately after venesection on an in-house glucose analyser (YSI 2300 STAT Plus, Yellow Springs Instrumentation, Ohio, USA) by an immobilised glucose oxidase method. Insulin and C-peptide were measured by radioimmunoassay (EMD Millipore, Toronto, Ontario, Canada). Quality control results were within one standard deviation of the mean at all concentrations. Insulin sensitivity was estimated by the composite insulin sensitivity index (Matsuda index) (Tan et al., 2015), and insulin secretion at baseline and each 30-minute time point by the C-peptide deconvolution method (Sze et al., 2011). Glucose-sensitive insulin secretion was then calculated by dividing the AUC for insulin secretion over two hours by the AUC for blood glucose over two hours.

3.2.2.5. Physical activity

Physical activity was assessed using the Modified Baecke Questionnaire (Voorrips et al., 1991). The questionnaire is designed to estimate habitual physical activity in the elderly, and is a composite score based on household activity, sports and exercise and other leisure activities. Higher levels of physical activity are reflected by a higher score.

3.2.2.6. Other laboratory analysis

C-reactive protein was measured using a Tinaquant immunoturbidimetric assay (Roche Diagnostics GMBH, Mannheim, Germany) on a Roche Modular Analyser (Hitachi High-Technologies Corporation, Tokyo, Japan). The limit of detection was 0.3 mg/L. The between-run CV was 3.6 % at a CRP of 3.9 mg/L and 2.3 % at a CRP of 49.5 mg/L.

3.2.3. Statistical analysis

Statistical analysis was performed using IBM SPSS version 20 for Windows (IBM, New York, USA). A p-value of <0.05 was considered statistically significant. Subject characteristics are presented as mean ± standard deviation except characteristics that were not Normally distributed, which are expressed as median (interquartile range). All other data are presented as mean ± standard error of mean. Changes in variables in non-GC users after 7 days prednisolone were analysed using paired t-tests. Hereafter in the manuscript these results are reported as the acute effects of prednisolone. GC users were compared with baseline data from non-GC users using unpaired t-tests if Normally distributed or Mann-Whitney U tests if the distribution was not Normal. Differences between these two groups are reported in the manuscript as the chronic effects of prednisolone.

The primary endpoint is change or difference in the AUC for AIx75. In the acute study, a sample size of 18 subjects has >80% power to detect a 500 %*minute change in the AUC AIx75 (assuming a SD of 700 %*minute). A 500 %*minute change in the AUC AIx75 corresponds to an average 4% change in augmentation index which has been associated with a 10-15 % increase in cardiovascular mortality (Vlachopoulos et al., 2010a). In the chronic study, a sample size of 18 subjects per group has >80% power to detect a 680 %*minute difference in the AUC AIx75 (assuming a SD of 700 %*minute).

3.3. Results

3.3.1. Subject characteristics

GC users were taking a mean prednisolone dose of 6.5 ± 1.8 mg/day, with a mean duration of continuous prednisolone therapy of 62 ± 62 months. There were no significant differences in sex

distribution, age, body mass index, height, C-reactive protein, physical activity score, use of disease-modifying antirheumatic drugs or history of hypertension or ischaemic heart disease between GC and non-GC users (Table 1).

3.3.2. Carbohydrate metabolism

Fasting glucose (5.3 ± 0.3 vs 4.6 ± 0.1 mmol/L, p=0.02) and glucose AUC over two hours (844 ± 58 vs 733 ± 27 mmol/L*minute, p=0.03) were higher after acute prednisolone. There were no significant differences in fasting (4.9 ± 0.2 vs 4.6 ± 0.1 mmol/L, p=0.20) or glucose AUC over two hours (822 ± 40 vs 733 ± 27 mmol/ L*minute, p=0.07) with chronic prednisolone, although the higher glucose AUC with chronic prednisolone approached statistical significance. The Matsuda index was significantly lower after both acute (p=0.01) and chronic (p=0.04) prednisolone (Figure 1A). There were no significant differences in insulin secretion with either acute or chronic prednisolone (Figure 1B).

3.3.3. Non-esterified fatty acids

There was no significant difference in fasting (0.7 ± 0.1 vs 0.6 ± 0.1 meq/L, p = 0.24) or NEFA AUC over 2 hours (39.5 ± 3.5 vs 34.5 ± 3.1 meq/L*min, p = 0.12) after acute prednisolone. Fasting (1.0 ± 0.1 vs 0.6 ± 0.1 meq/L, p = 0.002) and NEFA AUC over 2 hours (47.1 ± 3.9 vs 34.5 ± 3.1 meq/L*min, p = 0.02) were significantly higher in patients taking chronic prednisone.

3.3.4. Pulse wave analysis

There were no significant differences in fasting AIx75 with either acute (28.0 ± 1.7 vs 29.2 ± 2.0 %, p=0.46) or chronic (29.1 ± 2.1 vs 29.2 ± 2.0 %, p=0.98) prednisolone. Following the meal there was a fall in AIx75 in all groups indicating a reduction in arterial stiffness (Figure 2A). There was a greater reduction in the AUC for AIx75 after acute prednisolone (Figure 2B, p<0.001). The AUC for AIx75 was not significantly different with chronic prednisolone (Figure 2B).

3.3.5. Autonomic nervous system activity

There was a reduction in urinary noradrenaline excretion after acute prednisolone (Figure 3A, p=0.02). Urinary noradrenaline excretion was not significantly different with chronic prednisolone (Figure 3A). There were no significant differences in fasting (data not shown) or post meal (Figure

3B) baroreceptor sensitivity with acute or chronic prednisolone.

3.3.6. Peripheral arterial tonometry

There were no significant differences in fasting reactive hyperaemia index with either acute (2.2 ± 0.2 vs 2.4 ± 0.1, p=0.33) or chronic (2.3 ± 0.2 vs 2.4 ± 0.1, p=0.90) prednisolone. Following the meal there was a fall in reactive hyperaemia index in all groups indicating a reduction in endothelial function (Figure 4). There was no significant difference in the post-meal reduction in reactive hyperaemia index after acute prednisolone (Figure 4). In chronic prednisolone users, the post-meal fall in reactive hyperaemia index was approximately 60% of that in non-GC users, but this difference was not statistically significant (Figure 4, p=0.09). There was not a significant correlation between the post meal change in reactive hyperaemia index and NEFA AUC (r = 0.26, p = 0.13).

3.4. Discussion

This study assessed the effects of acute and chronic low dose prednisolone on carbohydrate metabolism and fasting and postprandial vascular function in patients with RA. We found that low dose prednisolone increased postprandial glucose concentration secondary to a reduction in insulin sensitivity. However, this was not associated with adverse changes in vascular function.

High dose glucocorticoids can cause hyperglycaemia because they reduce insulin sensitivity and secretion (Rizza et al., 1982).However, fewer studies have investigated the effects of lower glucocorticoid doses on carbohydrate metabolism. One cross-sectional study reported no significant differences in insulin sensitivity and secretion between patients with RA on long-term low dose prednisolone versus prednisolone naive patients (Hoes et al., 2011). In contrast, we found that low dose prednisolone in patients with inflammatory arthritis reduced hepatic and peripheral insulin sensitivity, assessed using gold standard metabolic techniques (Petersons et al., 2013). In this study we employed a physiologic (mixed meal) test and also found that low dose prednisolone reduced insulin sensitivity, with no significant change in insulin secretion. These data suggest that, even at low doses, prednisolone has an adverse effect on carbohydrate metabolism.

As glucocorticoid receptors are present in vascular endothelial cells, prednisolone could affect vascular function directly. Alternatively, prednisolone could alter vascular function indirectly

secondary to reducing insulin sensitivity. Vascular tissues are an important physiological target for insulin (Serne et al., 2007), and insulin resistance is an established risk factor for cardiovascular disease (Kim et al., 2006). Acutely, insulin resistance can increase arterial stiffness because of reduced signalling through the phosphatidyl ionositol-3 kinase pathway leading to reduced nitric oxide-mediated vasodilatation. Chronically, enhanced stimulation of the mitogen activated protein kinase pathway by hyperinsulinaemia can cause vascular smooth muscle proliferation and excessive production of inflammatory cytokines, contributing to accelerated atherosclerosis (DeFronzo, 2010). We hypothesized that adverse changes in arterial stiffness would be greater in patients taking chronic than acute prednisolone, because of the combination of attenuated and enhanced signalling via the phosphatidyl ionositol-3 kinase and mitogen activated protein kinase pathways, respectively.

Patients with insulin resistance have an attenuated postprandial reduction in augmentation index (Greenfield et al., 2007). We hypothesized that prednisolone-induced insulin resistance would result in similar adverse changes in vascular function. However, contrary to our hypothesis, acute prednisolone use was associated with a greater postprandial fall in augmentation index. In patients on long-term prednisolone there was no suggestion of a higher postprandial augmentation index indicative of increased arterial stiffness. We previously reported that fasting augmentation index was lower in hypopituitary patients after a 7 day increase in glucocorticoid dose (Petersons et al., 2014). These data suggest that low dose glucocorticoids do not induce deleterious changes in arterial stiffness that will increase cardiovascular risk.

The autonomic nervous system is an important regulator of cardiovascular function. A prednisolone dose of 20 mg/day for 7 days was reported to reduce sympathetic nervous system activity (Lenders et al., 1995), with *in vitro* studies suggesting that this effect is mediated by non-genomic pathways (Park et al., 2008). We found that lower prednisolone doses are also acutely associated with reduced sympathetic nervous system activity. Urinary noradrenaline excretion was also 27% lower in patients on long term prednisone but this difference was not statistically significant. Reduced sympathetic nervous system activity is a likely mechanism to explain a reduction in augmentation index with low dose prednisolone.

There were no significant changes in spontaneous baroreceptor sensitivity with either acute or chronic low dose prednisolone. These findings in patients with RA are similar to those in young healthy men (van Raalte et al., 2013b), and hypopituitary patients (Petersons et al., 2014), exposed to mild glucocorticoid excess. Measurements of baroreceptor sensitivity using the sequence method predominantly reflect parasympathetic, and not sympathetic, nervous system activity (Parati et al., 2000). Our findings suggest that the fall in augmentation index with low dose prednisolone is not mediated by an increase in parasympathetic nervous system activity.

In this study, neither acute nor chronic low dose prednisolone significantly affected fasting reactive hyperaemia index, a marker of endothelial function. There are conflicting data on the effects of glucocorticoids on fasting endothelial function. There were no significant changes in flow-mediated dilatation with short-term prednisolone in healthy adults (Brotman et al., 2005), or long-term prednisolone in patients with RA (Hafstrom et al., 2007). In contrast, there was a dose dependent reduction in microcapillary recruitment with acute prednisolone in healthy young adults (van Raalte et al., 2013a), and a reduction in fasting reactive hyperaemia index after 7 days of an increased glucocorticoid replacement dose in hypopituitary patients (Petersons et al., 2014). The contrasting findings in different studies may reflect different methods of assessment of endothelial function, glucocorticoid dose and formulation or susceptibility to the effects of glucocorticoids in different patient groups.

Reactive hyperaemia index falls after a glucose load in patients with insulin resistance and post-glucose load hyperglycaemia, but not in patients with normal glucose tolerance (Crandall et al., 2009). However, despite reduced insulin sensitivity and consequent postprandial hyperglycaemia, there was no change in the postprandial reduction in reactive hyperaemia index after acute prednisolone. Furthermore, there was a trend to an attenuated postprandial fall in reactive hyperaemia index in patients on long-term prednisolone suggestive of better endothelial function. This occurred despite higher NEFA concentrations in patients on chronic prednisolone, which have been associated with postprandial endothelial dysfunction and atherosclerosis (Steinberg and Baron, 2002). One possible explanation for these findings is that low dose prednisolone acts directly on the endothelium to improve endothelial function. Alternatively the anti-inflammatory

effect of prednisolone in the long-term could improve endothelial function, similar to other disease modifying drugs (Ablin et al., 2006). Although there were no significant differences in C-reactive protein or in the use of disease-modifying antirheumatic drugs between the two groups, it is possible that prednisolone affected a component of the inflammatory cascade that modulates endothelial function. Whatever the mechanism, our study suggests that in contrast to the general population, prednisolone-induced postprandial hyperglycaemia in subjects with rheumatoid arthritis is not associated with postprandial endothelial dysfunction.

This study does not provide direct evidence of the effect of low dose prednisolone on cardiovascular events. However, augmentation index (van Raalte et al., 2013a), reactive hyperaemia index (Matsuzawa et al., 2010), and baroreceptor sensitivity (Parati et al., 2000), are all well-validated markers of cardiovascular risk independent of traditional cardiovascular risk factors. The effect of low dose prednisolone on all 3 markers of cardiovascular risk was either neutral or in a direction associated with reduced cardiovascular events. Given the lack of direct evidence of the cardiovascular effects of low dose prednisolone, this study provides some confidence that low dose prednisolone can be used to attenuate disease progression in this patient group without increasing cardiovascular risk.

The strengths of this study include that we simultaneously assessed carbohydrate metabolism and fasting and postprandial vascular function in our patient groups. However, we acknowledge this study has limitations. Firstly, in this study with a relatively small sample size, the acute study analysed using paired t-tests has greater statistical power than the chronic study, which was analysed using appropriate statistical tests for independent groups. A lack of statistical significance in the chronic study may reflect its lesser statistical power rather than attenuation of the effect of prednisolone. As such, our findings should not be interpreted as indicating that the acute and chronic effects of prednisolone on augmentation index and noradrenaline secretion differ. However, it is relevant that the direction of change of all measures of vascular function in the cross-sectional study was similar to the acute study. This suggests that chronic prednisolone does not exert opposite (adverse) effects on vascular function. Secondly, inherent in any cross-sectional study is the possibility that an unmeasured variable affected results. However, the groups were

well matched for a number of key variables (Table 1). Thirdly, calculation of a Matsuda index from a mixed meal test is not as well validated as from a glucose load. However, calculation of a Matsuda index from a mixed meal test classifies differences in insulin sensitivity in groups of healthy adults (Tan et al., 2015) and prednisolone-induced changes in insulin sensitivity (van Raalte et al., 2011b) similarly to a euglycaemic-hyperinsulinaemic clamp. Finally, our study does not investigate the effect of prednisolone doses above 10 mg/day, which have been associated with a greater risk of cardiovascular events (Listing et al., 2015).

In summary, we have demonstrated that low dose prednisolone treatment in patients with RA was associated with a higher postprandial glucose secondary to a reduction in insulin sensitivity. However, these changes in carbohydrate metabolism were not associated with adverse effects on markers of arterial stiffness, endothelial function or autonomic nervous system activity. In fact, arterial stiffness was acutely reduced by low dose prednisolone, which is likely to be secondary to a prednisolone-induced reduction in sympathetic nervous system activity. Our findings suggests that in contrast to the relationship in the general population, postprandial hyperglycaemia may not be a marker of cardiovascular risk in patients with RA on low dose prednisolone.

Acknowledgements: The authors are grateful to the subjects who generously volunteered for the study.

3.5. Table 1: Subject characteristics

	Non-GC users (n=18)	GC users (n=18)	P-value
Female (n, (%))	12 (67)	12 (67)	1.00
Age (years)	64 ± 7	66 ± 7	0.33
BMI (kg/m^2)	28.1 ± 5.2	27.9 ± 6.1	0.95
Height (m)	1.65 ± 0.08	1.68 ± 0.07	0.24
C-reactive protein (mg/L)*	2.4 (1.1-4.5)	1.6 (0.5-7.6)	0.44
Physical activity score	12.8 ± 5.7	10.5 ± 5.5	0.22
DMARDs (n)	11	9	0.50
Ischaemic heart disease (n)	1	1	1.00
Hypertension (n)	5	4	0.70
Statins (n)	5	3	0.69
Anti-hypertensives (n)	5	3	0.69

*Data are mean ± SD unless otherwise stated, * = median (interquartile range), n = number of patients with specified variable. GC = glucocorticoid, BMI = body mass index, DMARDs = disease modifying anti-rheumatic drugs.*

3.6. Figures and figure legends

Figure 1: Insulin sensitivity (Matsuda index, 1A) and glucose-sensitive insulin secretion (1B) in 18 subjects with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white bars) and after (grey bars) 7 days prednisolone and 18 subjects with rheumatoid arthritis on long-term prednisolone (GC users, black bars). Data are mean ± SEM.

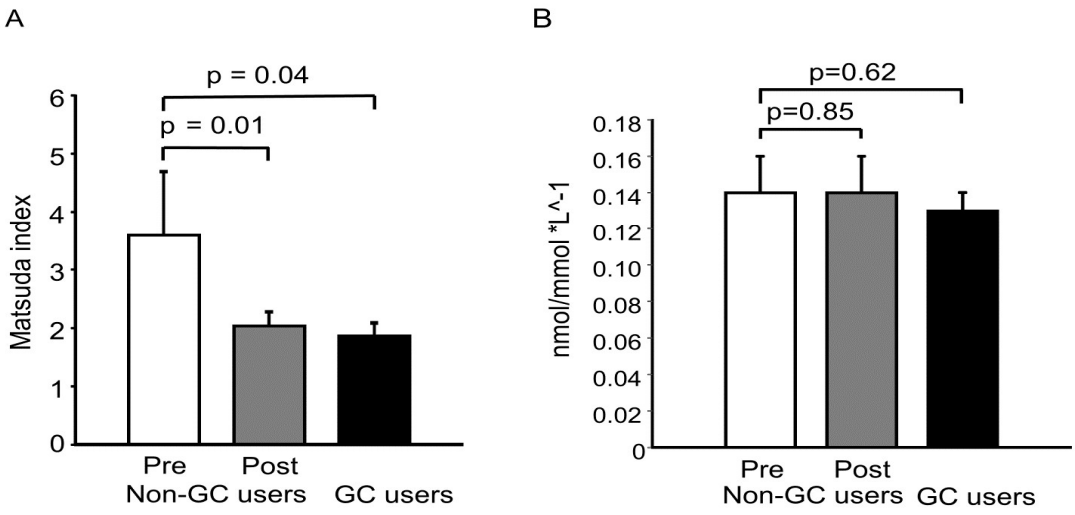


Figure 2: Time course for augmentation index (AIx75) over 2 hours (2A) and area under the curve for augmentation index (AIx75 AUC) over two hours (2B) in 18 subjects with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white line) and after (grey line) 7 days prednisolone and in 18 subjects with rheumatoid arthritis on long-term prednisolone (GC users, black line). Data are mean ± SEM.

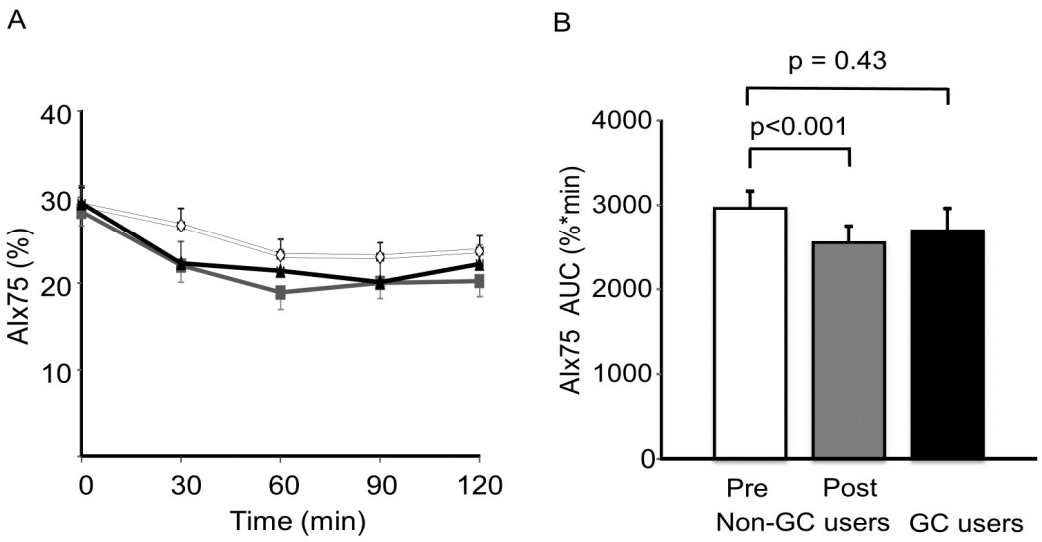


Figure 3: Urinary noradrenaline excretion (3A) and postprandial baroreceptor sensitivity (3B) in 18 subjects with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white bars) and after (grey bars) 7 days prednisolone and 18 subjects with rheumatoid arthritis on long-term prednisolone (GC users, black bars). Data are mean ± SEM.

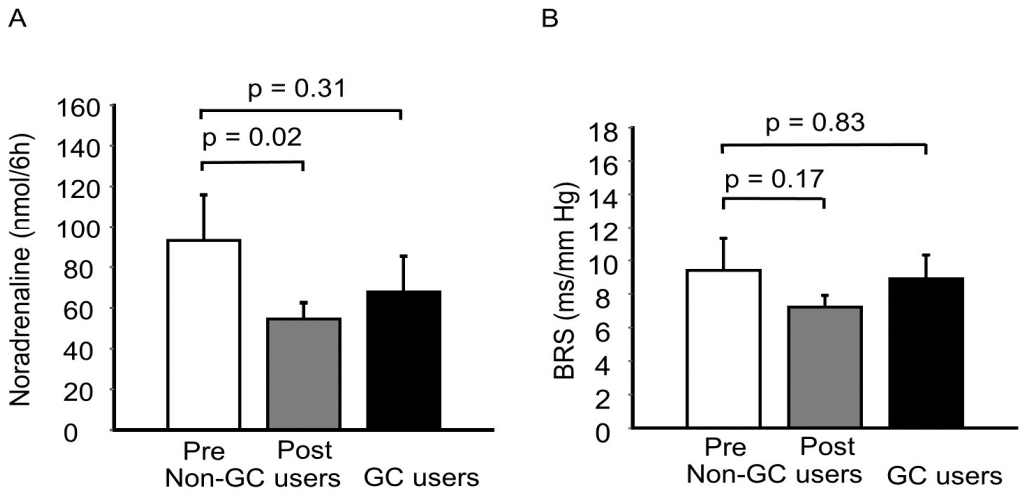
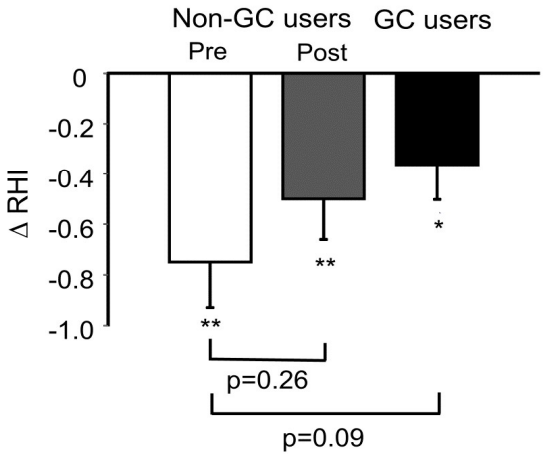


Figure 4: Post-meal change in reactive hyperaemia index (RHI) in 18 subjects with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white bars) and after (grey bars) 7 days prednisolone and 18 subjects with rheumatoid arthritis on long-term prednisolone (GC users, black bars). Data are mean ± SEM. * p<0.05 for within group change in RHI after the meal, **p<0.005 for within group change in RHI after the meal.



CHAPTER 4. OPPOSING EFFECTS OF RHEUMATOID ARTHRITIS AND LOW DOSE PREDNISOLONE ON ARGININE METABOLOMICS

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Author roles

AR was responsible for study design, subject recruitment, data acquisition, data analysis and manuscript preparation. BLM was responsible for data acquisition. SMD was responsible for data acquisition. AR2 was responsible for laboratory analysis and manuscript revision. MDS was responsible for subject recruitment and manuscript revision. AAM was responsible for study design, data analysis and manuscript revision. CHT was responsible for study design, supervision and manuscript revision. MGB was responsible for obtaining funding, study design, data analysis, supervision and manuscript revision.

Abstract

Background and Aims: The effects of low dose prednisolone on circulating markers of endothelial function, the arginine metabolites asymmetric dimethyl arginine (ADMA), mono methyl arginine (MMA), and homoarginine, are uncertain. We assessed whether patients with rheumatoid arthritis have perturbations in arginine metabolite concentrations that are reversed by low dose prednisolone.

Methods: Eighteen rheumatoid arthritis patients who had not taken prednisolone for >6 months (non-glucocorticoid (GC) users), 18 patients taking continuous oral prednisolone (6.5±1.8 mg/day) for >6 months (GC users) and 20 healthy controls were studied. Fasting plasma concentrations of ADMA, MMA, and homoarginine were measured by ultra-performance liquid-chromatography. Baseline data from non-GC users were compared with healthy controls to assess the effect of rheumatoid arthritis. The change in arginine metabolites in non-GC users after 7 days of prednisolone (6 mg/day) was used to assess the acute effects of prednisolone. Baseline data from non-GC users were compared with GC users to assess the chronic effects of prednisolone.

Results: Non-GC users had higher ADMA (0.59±0.03 vs 0.47±0.01 μM, p=0.004) and MMA concentrations (0.10±0.01 vs 0.05±0.00 μM, p <0.001) than controls. The only change with acute prednisolone was a reduction in homoarginine (1.23±0.06 vs 1.08±0.06 μM, p=0.04) versus baseline. GC users had lower concentrations of ADMA (0.51±0.02 vs 0.59±0.03 μM, p=0.03) than non-GC users.

Conclusion: Rheumatoid arthritis patients have higher concentrations of ADMA and MMA, inhibitors of endothelial function. Chronic, but not acute, prednisolone therapy is associated with a lower ADMA concentration, suggesting a salutary effect of long-term glucocorticoid treatment on endothelial function.

4.1. Introduction

Rheumatoid arthritis is associated with a 30-60% increased risk of cardiovascular events (Watson et al., 2003, Mutru et al., 1989, Wallberg-Jonsson et al., 1997, Turesson et al., 2004, Solomon et al., 2003, Avina-Zubieta et al., 2013) and a 50% increased risk of death from cardiovascular disease (del Rincon et al., 2001). Glucocorticoids are often prescribed to patients with rheumatoid arthritis, but there are concerns regarding potential adverse cardiovascular events in these patients already at high cardiovascular risk (Conn, 2001, Saag, 2001). While high dose glucocorticoids are associated with increased cardiovascular events, it is unclear whether lower doses (e.g prednisolone <10 mg/day) that are commonly prescribed long-term alter cardiovascular risk (Gaujoux-Viala and Gossec, 2014). Some epidemiological studies have reported an increase in cardiovascular events with low dose prednisolone, while others have reported no effect (Wei et al., 2004, Listing et al., 2015). Furthermore, the sample size and duration of randomized-controlled studies of glucocorticoid therapy in patients with rheumatoid arthritis are insufficient to assess cardiovascular events (Capell et al., 2004, Svensson et al., 2005).

Endothelial dysfunction is a key event in the pathogenesis of atherosclerosis and develops early in the course of rheumatoid arthritis (Kerekes et al., 2008, Bergholm et al., 2002). A patient's vasodilatory response to hypoxia is often used to assess endothelial function. However, the effect of glucocorticoids on endothelial function assessed by this approach is uncertain. Endothelial function was reduced after an increase of glucocorticoid dose in hypopituitary patients (Petersons et al., 2014) and in patients with IgA nephropathy prescribed glucocorticoids (Uchida et al., 2006). In contrast, glucocorticoids did not change endothelial function in healthy adults (Brotman et al., 2005) or patients with rheumatoid arthritis (Hafstrom et al., 2007). Moreover, we recently reported that endothelial function is not affected by acute prednisolone, but is better in patients on long-term prednisolone (Radhakutty et al., 2016b, Petersons et al., 2017). These contrasting findings suggest that the effects of glucocorticoids on endothelial function might differ depending on the patient group, the methods used to assess vasodilation, and the dose and duration of glucocorticoid treatment.

The measurement of circulating arginine metabolites is an alternative method to assess

endothelial function and cardiovascular risk. Asymmetric dimethyl arginine (ADMA) is a competitive inhibitor of endothelial nitric oxide synthase (e-NOS), the enzyme that converts L-arginine to citrulline and releases nitric oxide. ADMA is positively associated with endothelial dysfunction (Schulze et al., 2006) and cardiovascular mortality (Miyazaki et al., 1999, Zoccali et al., 2001). Emerging evidence suggests that other arginine metabolites also influence cardiovascular risk. Mono methyl arginine (MMA), another inhibitor of e-NOS, and symmetric dimethyl arginine (SDMA), which reduces L-arginine bioavailability, are also associated with atherosclerosis and cardiovascular events (Chirinos et al., 2008, Wang et al., 2009, Bode-Boger et al., 2006). L-arginine is also metabolized by arginase to ornithine and by arginine: glycine amidino transferase (AGAT) to homoarginine. Perturbations in these pathways have also been associated with vascular dysfunction and increased cardiovascular mortality (Marz et al., 2010, Pernow and Jung, 2016).

Increased ADMA concentrations in patients with rheumatoid arthritis have been linked to endothelial dysfunction and impaired endothelial repair (Surdacki et al., 2007, Spasovski et al., 2013). However, little is known about the effect of rheumatoid arthritis on other arginine metabolites. High dose glucocorticoids increased ADMA in patients with IgA nephropathy (Uchida et al., 2006) and arginase activity in an animal model (Erisir et al., 2003). However, it is not clear whether the typical therapeutic glucocorticoid doses prescribed to patients with rheumatoid arthritis affect arginine metabolite concentrations.

We hypothesized that 1.) Patients with rheumatoid arthritis have alterations in arginine metabolism that will influence the effect of prednisolone on endothelial function and 2.) The acute and chronic effects of prednisolone on arginine metabolism differ. Consequentially, the aims of this study were firstly to assess whether patients with rheumatoid arthritis have perturbations in arginine metabolism and then to assess the acute and chronic effects of low dose prednisolone on arginine metabolism in patients with rheumatoid arthritis.

4.2. Patients and Methods

4.2.1. Subjects and study design

Subjects with rheumatoid arthritis aged 50 years or older were recruited from the rheumatology

outpatient clinic at Repatriation General Hospital, Adelaide, Australia and healthy controls from the general community. We studied 18 subjects who had not been administered any oral glucocorticoids for at least 6 months (non-GC users), 18 subjects taking a stable continuous oral prednisolone dose of 4-10 mg/day for at least 6 months (GC users) and 20 healthy controls with no history of inflammatory disease. The groups were matched for age, sex and renal function and subjects on oral hypoglycaemic agents and /or insulin were excluded from the study. First, we compared arginine metabolite concentrations in non-GC users and controls to assess the effect of rheumatoid arthritis on arginine metabolism. Secondly, non-GC users were studied before and after a 7 day course of oral prednisolone 6 mg daily to determine the acute effects of prednisolone. Finally, baseline data from non-GC users were compared with data from GC users to determine the chronic effects of prednisolone.

The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee, Flinders Medical Centre, and all subjects provided written informed consent in accordance with the 1975 Declaration of Helsinki. The primary analyses of this study investigated the effect of prednisolone on clinical measures of vascular function and energy and substrate metabolism in the rheumatoid arthritis patients; these have previously been reported (Radhakutty et al., 2016b, Radhakutty et al., 2016a).

4.2.2. Study protocol

Subjects attended the Endocrine Research Unit at Repatriation General Hospital at 0830 h after a 12 h overnight fast. All subjects took their regular medications in the morning prior to arrival, including prednisolone. Basic anthropometric measures were recorded. In each study participant, fasting blood samples were collected in EDTA tubes for measurement of 7 key components of arginine metabolism that are directly or indirectly involved in the regulation of endothelial function: arginine, homoarginine, citrulline, ornithine, ADMA, MMA and SDMA. Blood samples were centrifuged at 4,000 rpm at 4^0 Centigrade for 10 min and plasma frozen at -80^0 Centigrade until analysis.

4.2.3. Arginine metabolomics

Samples were prepared for analysis by solvent precipitation. 100 µL of sample was mixed with 400

µL of assay precipitating solution (0.1% formic acid in methanol), centrifuged for 5 min at 16,000 g, and a 400 µL aliquot of the resulting supernatant evaporated to dryness. Dried eluates were then reconstituted in 200 µL ammonium formate for liquid chromatography-mass spectrometry (LC-MS). Chromatographic separations were performed on a Waters ACQUITY^{TM} T3 HSS C18 analytical column (150 mm × 2.1 mm,1.8 µm; Waters Corp., Milford, USA) using a Waters ACQUITY Ultra Performance LC^{TM} system. Column elutant was monitored by mass spectrometry, performed on a Waters Quad-Time of Flight Premier^{TM} quadrupole (van Dyk et al., 2015).

4.2.4. Other laboratory analysis

Serum creatinine was measured using Roche automated clinical chemistry analyser (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany) and estimated glomerular filtration rate (eGFR) was measured using the Chronic Kidney Disease- Epidemiology collaboration equation (CKD-EPI equation). C-reactive protein (CRP) was measured using a Tinaquant immunoturbidimetric assay (Roche Diagnostics GMBH, Mannheim, Germany) on a Roche Modular Analyser (Hitachi High-Technologies Corporation, Tokyo, Japan). The between-run coefficient of variation was 3.6 % at a CRP of 3.9 mg/L and 2.3 % at a CRP of 49.5 mg/L.

4.2.5. Statistical analysis

Statistical analysis was performed using IBM SPSS version 20 for Windows (IBM, New York, USA). A p-value of <0.05 was considered statistically significant. Subject characteristics are presented as mean ± standard deviation if the distribution was Normal and median (interquartile range) if the distribution was not Normal. All other data are presented as mean ± standard error of mean. Subject characteristics in the three groups were compared using one-way analysis of variance. Non-GC users were compared to controls using unpaired t-tests if Normally distributed or Mann-Whitney U tests if the distribution was not Normal. Changes in variables in non-GC users after 7 days prednisolone were analysed using paired t-tests. Hereafter in the manuscript these results are reported as the acute effects of prednisolone. GC users were compared with baseline data from non-GC users using unpaired t-tests if Normally distributed or Mann-Whitney U tests if the distribution was not Normal. Differences between these two groups are reported in the manuscript as the chronic effects of prednisolone. Variables that were significant in univariate

analysis were corrected for potential confounders using analysis of covariance.

The primary end point of this analysis was the difference in concentration of ADMA. A sample size of 18 per group in the cross-sectional study had 80 % power to detect a 0.07 μM difference in ADMA assuming a standard deviation of 0.07. In the longitudinal study, a sample size of 18 per group had 80 % power to detect a 0.05 μM difference in ADMA assuming a standard deviation of 0.07.

4.3. Results

4.3.1. Subject characteristics

There were no significant differences in sex, age, body mass index, eGFR, smoking, history of hypertension, ischemic heart disease or diabetes between the three groups (Table 1). GC users were taking a mean prednisolone dose of 6.5 ± 1.8 mg/day, with a median duration of continuous prednisolone therapy of 48 (6-240) months. There was no significant difference in C-reactive protein (1.6 (0.5-7.6) vs 2.4 (1.1-4.5) mg/L, p=0.44), or in the number of patients taking disease modifying anti-rheumatic drug use (11 vs 9, p= 0.50) between GC and non-GC users.

4.3.2. Arginine metabolomics

4.3.2.1. Effect of rheumatoid arthritis

In univariate analyses, ADMA (0.59 ± 0.03 vs 0.48 ± 0.01 μM, p = 0.004), MMA (0.10 ± 0.01 vs 0.05 ± 0.00, p <0.001), arginine (93.9 ± 4.8 vs 75.0 ± 2.3 μM, p = 0.001) and citrulline (37.1 ± 2.2 vs 29.3 ± 1.1 μM, p = 0.002) concentrations were higher in non-GC users than in controls. The higher concentrations of ADMA (p=0.008, Fig 2A), MMA (p<0.001, Fig 2B), arginine (94.3 ± 4.2 vs 75.0 ± 4.2 μM, p = 0.003) and citrulline (37.1 ± 1.4 vs 28.7 ± 1.4 μM, p<0.001) in non-GC users remained significant after adjustment for age, sex, eGFR, smoking and cholesterol. There were no significant differences in SDMA (0.69 ± 0.06 vs 0.56 ± 0.04, p = 0.08), ornithine (52.3 ± 3.7 vs 56.8 ± 3.3, p = 0.37) and homoarginine (1.23 ± 0.06 vs 1.08 ± 0.06 μM, p = 0.08) concentrations between non-GC users and controls.

4.3.2.2. Acute effects of prednisolone

Homoarginine concentration was significantly lower (Δ -0.15 ± 0.07 μM, p = 0.04) after 7 days prednisolone. There were no significant changes in ADMA (Δ -0.02 ± 0.02 μM, p = 0.47), MMA (Δ -0.002 ± 0.003 μM, p = 0.70), SDMA (Δ -0.08 ± 0.05 μM, p = 0.14), arginine (Δ -5.2 ± 5.0 μM, p = 0.31), citrulline (Δ +0.2 ± 1.6 μM, p = 0.90) or ornithine (Δ +7.8 ± 4.0 μM, p = 0.07) concentrations after acute prednisolone.

4.3.2.3. Chronic effect of prednisolone

In univariate analyses, GC users had lower concentrations of ADMA (0.51 ± 0.02 vs 0.59 ± 0.03 μM, p = 0.03) and SDMA (0.53 ± 0.03 vs 0.69 ± 0.06, p=0.03) than non-GC users. The lower concentrations of ADMA (p = 0.03, Fig 3A), and SDMA (p = 0.02, Fig 3B) in GC users remained significant after adjustment for age, sex, eGFR, smoking cholesterol, CRP and disease modifying anti-rheumatic drug use. There were no significant differences in the concentrations of MMA (0.09 ± 0.00 vs 0.10 ± 0.01 μM, p = 0.12), arginine (86.3 ± 4.7 vs 93.9 ± 4.8 μM, p = 0.27), citrulline (33.6 ± 2.6 vs 37.1 ± 2.2 μM, p = 0.26), ornithine (59.9 ± 5.5 vs 52.3 ± 3.7 μM, p = 0.26) or homoarginine (1.16 ± 0.06 vs 1.23 ± 0.06 μM, p = 0.42) between GC and non-GC users.

4.4. Discussion

This study assessed the effects of rheumatoid arthritis on arginine metabolism and then the acute and chronic effects of low dose prednisolone on arginine metabolism in patients with rheumatoid arthritis. We demonstrated that patients with rheumatoid arthritis had higher concentrations of ADMA and MMA, endogenous inhibitors of e-NOS, than healthy controls. Acute prednisolone treatment resulted in a small reduction in homoarginine, but there were no significant changes in other arginine metabolites. In contrast, rheumatoid arthritis patients on chronic prednisolone treatment had significantly lower concentrations of ADMA and SDMA than patients not on prednisolone. These findings suggest that rheumatoid arthritis *per se* is associated with an increase in plasma concentrations of endogenous inhibitors of nitric oxide synthase, which are likely to contribute to endothelial dysfunction. The reduction in ADMA and SDMA with chronic, but not acute, prednisolone could provide a mechanism that explains why clinical measures of endothelial function improves with chronic, but not acute, prednisolone in this patient group

(Radhakutty et al., 2016b, Petersons et al., 2017).

In this study, patients with rheumatoid arthritis had higher concentrations of ADMA and MMA than controls. The finding of increased ADMA in patients with rheumatoid arthritis is consistent with other studies (Spasovski et al., 2013, Surdacki et al., 2007), in whom ADMA is associated with increased carotid intima media thickness and depleted endothelial progenitor cells (Dimitroulas et al., 2012, Surdacki et al., 2007, Dimitroulas et al., 2017). This study extends these observations by demonstrating that MMA, another inhibitor of e-NOS, is also increased in rheumatoid arthritis. ADMA and MMA are both degraded by dimethyl arginine dimethyl amino hydrolase (DDAH). DDAH activity is reduced in inflammatory states (Ito et al., 1999, Spinelli et al., 2014). Elevations of ADMA and MMA are a potential mechanism underlying endothelial dysfunction in patients with rheumatoid arthritis. SDMA was also increased by 19%, although this difference was not statistically significant. This finding may represent a type 2 error, given the relatively small sample size. Alternatively, SDMA is metabolized by different pathways to ADMA and MMA, and this could explain the discordant results (Mangoni, 2009).

Patients with rheumatoid arthritis also had higher plasma concentrations of arginine and citrulline. However, most of the plasma arginine arises from diet with only a small fraction synthesized from other amino acids (Michel, 2013), while citrulline is predominantly synthesized from glutamate in the small intestine (Morris, 2005). Hence the increased arginine and citrulline concentrations are likely to reflect increased protein catabolism in rheumatoid arthritis (Rall and Roubenoff, 2004) and not increased e-NOS activity. The concentrations of homoarginine and ornithine were similar in patients with rheumatoid arthritis and controls. These metabolic pathways have not been extensively studied in patients with rheumatoid arthritis, although one study also reported homoarginine is not different in patients with rheumatoid arthritis (Kayacelebi et al., 2014). Our study suggests that changes in arginase and AGAT activity do not contribute to endothelial dysfunction in patients with rheumatoid arthritis.

The only significant change in arginine metabolites after acute low dose prednisolone consisted of a reduction in homoarginine concentration. Homoarginine is a weak substrate for nitric oxide synthase that has been negatively associated with cardiovascular morbidity and mortality in

epidemiologic studies (Marz et al., 2010, Atzler et al., 2013). However, the mechanism underlying this association is not well understood and the role of this metabolic pathway in rheumatoid arthritis is unclear (Kayacelebi et al., 2014). There were no significant changes in inhibitors of e-NOS or ornithine, a marker for arginase activity after acute prednisolone. This is consistent with studies reporting that acute low dose prednisolone does not affect endothelial function in patients with rheumatoid arthritis (Hafstrom et al., 2007, Radhakutty et al., 2016b).

In contrast to acute prednisolone and despite greater insulin resistance [21], patients with rheumatoid arthritis on chronic prednisolone treatment had lower ADMA and SDMA concentrations than patients with rheumatoid arthritis who were not taking prednisolone. Previous studies reporting the effects of glucocorticoids on ADMA have been discordant with lower serum ADMA concentrations in patients with Duchenne's muscular dystrophy treated with glucocorticoids (Horster et al., 2015), but an increase in ADMA, coupled with a reduction in flow-mediated vasodilatation, in patients with IgA nephropathy treated with high dose glucocorticoids (Uchida et al., 2006). Moreover, TNF-alpha inhibitors were also shown to reduce ADMA- arginine ratio and improve vascular function in patients with rheumatoid arthritis in some (Angel et al., 2012), but not all [48], studies. We postulate that the effects of glucocorticoids on arginine metabolism are influenced by the glucocorticoid dose and underlying disease state. In patients with an active inflammatory disease, anti-inflammatory treatment is associated with a reduction in ADMA, possibly via increasing DDAH activity (Ito et al., 1999, Spinelli et al., 2014). The reduction in ADMA is consistent with better endothelial function in patients with rheumatoid arthritis prescribed chronic prednisolone.

This study does not provide direct insights on the cardiovascular effects of prednisolone. However, available epidemiologic data suggesting ADMA has an important physiologic role is strong; an increase in serum ADMA concentration of 0.1 μmol/L is associated with a 27 fold increase in relative risk of an acute coronary event (Valkonen et al., 2001). A reduction in ADMA and SDMA, together with a higher fasting and postprandial reactive hyperaemia index (Petersons et al., 2017, Radhakutty et al., 2016b), suggests that chronic low dose prednisolone treatment in patients with rheumatoid arthritis may not worsen endothelial function. Given the lack of direct evidence of the

cardiovascular effects of low dose prednisolone in literature, our study give some reassurance that long-term low dose prednisolone can be used to attenuate disease progression in this patient group without increasing cardiovascular risk.

We acknowledge the following limitations of this study. We have only assessed extracellular concentrations of arginine metabolites and must extrapolate these results to assess intracellular e-NOS activity and vascular function. However, studies of enzyme kinetics have shown enhanced cellular uptake of methylarginines and increased NOS inhibition with elevated plasma concentrations (Cardounel et al., 2007). Secondly, there was wide variability in the duration of prednisolone treatment in GC users and this could have affected results. However, the small sample size precludes subgrouping GC users further based on duration of prednisolone use. Thirdly, other markers of endothelial dysfunction such as monocyte chemoattractant protein 1 (MCP1), vascular cell adhesion molecule 1 (VCAM 1), Selectins or interleukin 6 (IL6) were not measured. Fourthly, inherent in any cross-sectional study is the possibility that an unmeasured variable affected results. However, the groups were well matched for a number of key variables (Table 1). We did not have a control group on prednisolone to confirm our hypothesis that patients with rheumatoid arthritis have alterations in arginine metabolism that will influence the effect of prednisolone on endothelial function. Finally, our findings cannot be translated to prednisolone doses of >10 mg/day.

In summary, patients with rheumatoid arthritis have higher concentrations of ADMA and MMA, inhibitors of e-NOS that could contribute to the endothelial dysfunction associated with this disease. Acute and chronic prednisolone treatment have differing effects on arginine metabolomics. While acute prednisolone has little effect, chronic prednisolone reduces ADMA and SDMA concentrations. Reducing these elevated inhibitors of nitric oxide synthesis could explain why endothelial function is better in patients with rheumatoid arthritis prescribed prednisolone long-term.

4.5. Table 1: Subject characteristics.

	Controls (n=20)	Non-GC users (n=18)	GC users (n=18)	p-value
Female (n (%))	16 (80)	12 (67)	12 (67)	0.57
Age (years)	63 ± 6	64 ± 7	66 ± 7	0.24
BMI (Kg/m^2)	28.6 ± 4.2	28.1 ± 5.2	27.9 ± 6.1	0.95
e-GFR (ml/min)	82 ± 17	87 ± 19	82 ± 13	0.61
Smoking (n, %))	0 (0)	2 (11)	1 (6)	0.32
Hypertension (n, (%))	3 (15)	5 (25)	4 (20)	0.63
Ischemic heart disease (n, (%))	0 (0)	1 (6)	1 (6)	0.56
Diabetes (n, (%))	0 (0)	1 (6)	1 (6)	0.56
Anti hypertensives (n)	2	5	3	0.58
Statins (n)	1	5	3	0.17

Data are mean ± standard deviation. GC: glucocorticoid, n: number of subjects with a specified variable, BMI: Body mass index, e-GFR: estimated glomerular filtration rate.

4.6. Figures and figure legends

Figure 1: Arginine metabolism: Simplified diagram showing the principal pathways of arginine metabolism and nitric oxide production: ADMA indicates asymmetric dimethyl arginine; MMA, mono methyl arginine; SDMA, symmetric dimethyl arginine; AGAT, arginine:glycine amidino transferase; ASS/ASL, arginosuccinate synthase/arginosuccinate lyase.

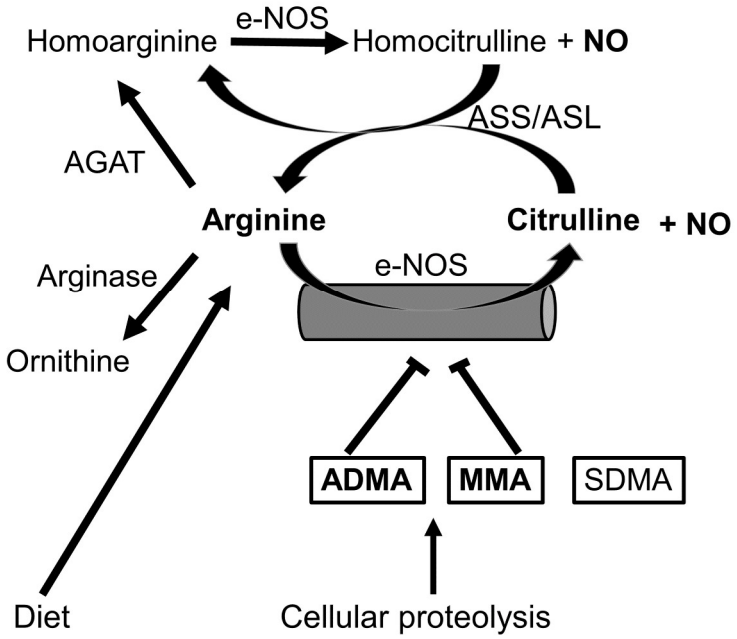


Figure 2: Effect of rheumatoid arthritis on ADMA and MMA: Plasma concentrations of asymmetric dimethyl arginine (ADMA) (A) and mono methyl arginine (MMA) (B) in 20 healthy controls (white bar) and in 18 patients with rheumatoid arthritis who were not taking prednisolone (grey bar). Results are mean ± standard error and are corrected for age, sex, eGFR, smoking and cholesterol.

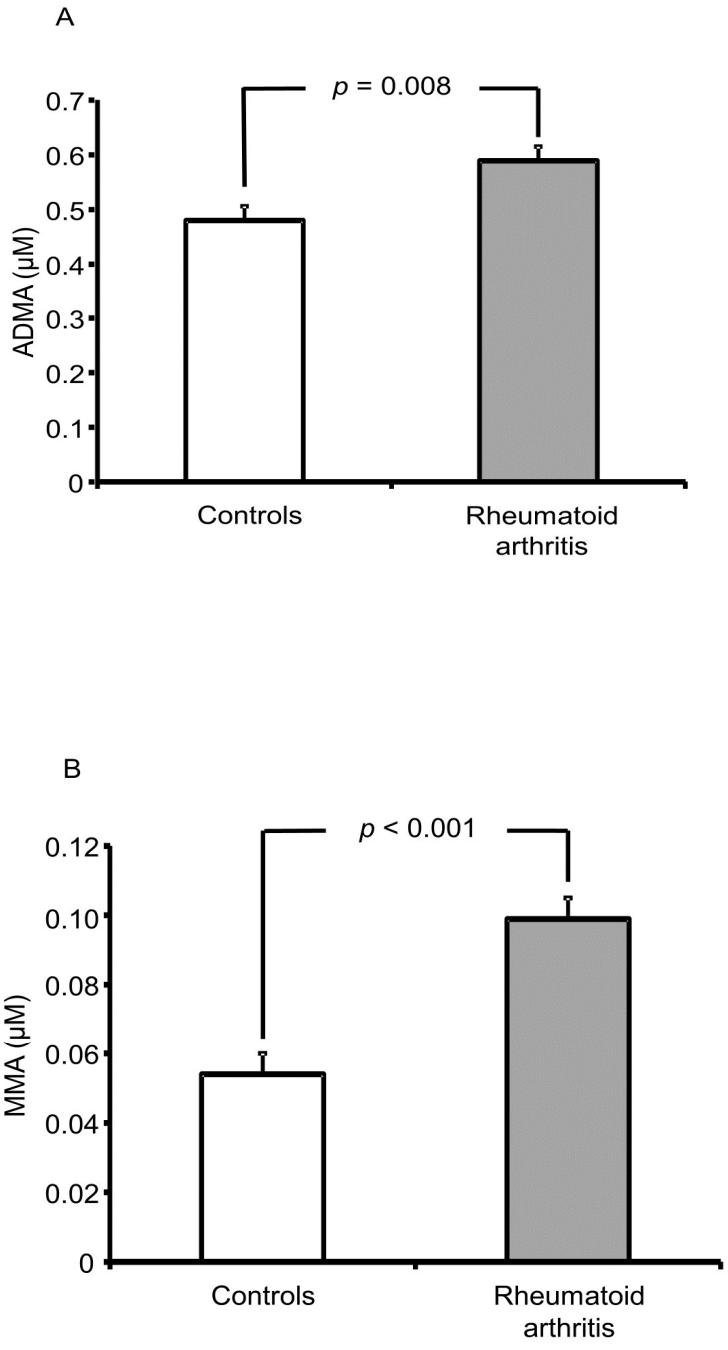
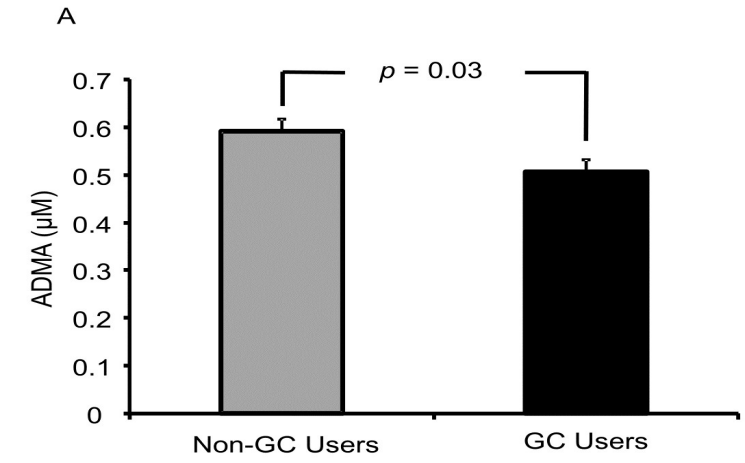
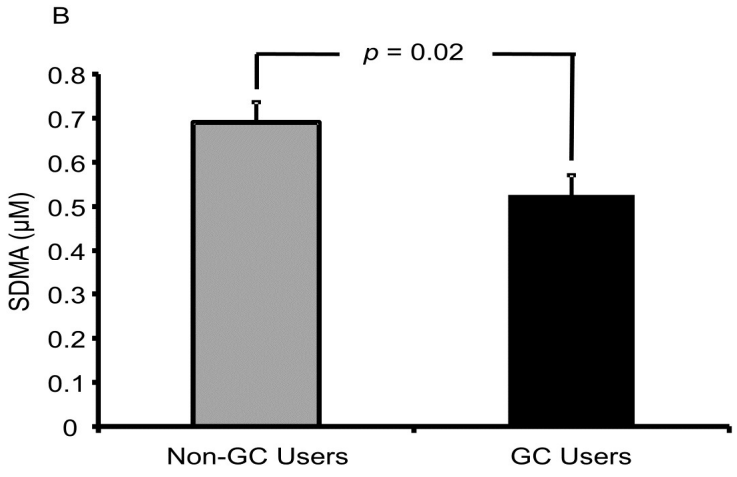


Figure 3: Effect of long-term prednisolone on ADMA and SDMA: Plasma concentrations of asymmetric dimethyl arginine (ADMA) (A) and symmetric dimethyl arginine (SDMA) (B) in 18 patients with rheumatoid arthritis who were not taking prednisolone (non-GC users, grey bar) and 18 patients with rheumatoid arthritis on chronic (>6 months) prednisolone (GC users, black bar). Results are mean ± standard error and are corrected for age, sex, eGFR, smoking cholesterol, CRP and disease modifying anti-rheumatic drug use.





CHAPTER 5. EFFECTS OF PREDNISOLONE ON ENERGY AND FAT METABOLISM IN PATIENTS WITH RHEUMATOID ARTHRITIS: TISSUE-SPECIFIC INSULIN RESISTANCE WITH COMMONLY USED PREDNISOLONE DOSES

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Author roles

AR was responsible for study design, subject recruitment, data acquisition, laboratory analysis, data analysis and manuscript preparation. BLM was responsible for data acquisition. SMD was responsible for data acquisition. DSB was responsible for the mixed-meal protocol and manuscript revision. LKH was responsible for laboratory analysis and manuscript revision. MDS was responsible for subject recruitment and manuscript revision. CHT was responsible for study design, supervision and manuscript revision. MGB was responsible for obtaining funding, study design, data analysis, supervision and manuscript revision.

SUMMARY

Objective: Glucocorticoids can cause postprandial hyperglycaemia, but the effects on postprandial energy and fat metabolism are uncertain. We investigated the effects of acute and chronic low dose prednisolone on fasting and postprandial energy expenditure and substrate metabolism.

Design: An open interventional and cross-sectional study were undertaken

Patients and measurements: Eighteen patients who had not taken oral glucocorticoids for ≥6 months were studied before and after 7 days prednisolone (6 mg/day) to assess the acute effects of prednisolone. Baseline data from patients not on glucocorticoids were compared with 18 patients on long-term prednisolone (6.5±1.8 mg/day for >6 months) to assess the chronic effects. Energy expenditure and substrate oxidation were measured using indirect calorimetry before and after a mixed meal. Adipocyte insulin resistance index and insulin-mediated suppression of NEFA was calculated from fasting and postprandial insulin and NEFA concentrations.

Results: There were no significant differences in resting energy expenditure or diet-induced thermogenesis with prednisolone. Acute (-2.1±6.2 vs -16.3±4.8 mg/min, p=0.01) and chronic (-1.4±2.8 vs -16.3±4.8 mg/min, p=0.01) prednisolone attenuated postprandial suppression of fat oxidation. Chronic (31.6±3.8 vs 17.0±3.3, p=0.007), but not acute, prednisolone increased adipocyte insulin resistance index. However, insulin-mediated suppression of NEFA was not significantly different after acute or chronic prednisolone.

Conclusions: Prednisolone does not alter energy expenditure. However, even at low doses, prednisolone exerts adverse effects on fat metabolism, which could exacerbate insulin resistance and increase cardiovascular risk. Attenuated postprandial suppression of fat oxidation, but not lipolysis, suggests that prednisolone causes greater insulin resistance in skeletal muscle than in adipocytes.

5.1. Introduction

Endogenous glucocorticoid excess (Cushing's syndrome) causes a distinctive change in body composition with reduced lean body mass and increased fat mass, predominantly in the truncal region (Burt et al., 2006). Consequently visceral adiposity is increased, which is associated with increased rates of cardiovascular disease and diabetes. While Cushing's syndrome is rare, exogenous glucocorticoid therapy is prescribed long-term to nearly 1% of the adult population to treat inflammatory disease or attenuate disease progression (Fardet et al., 2011). High dose glucocorticoid therapy (prednisolone >20 mg/day) also causes central adiposity (Fardet et al., 2007) and is associated with a threefold increased risk of cardiovascular event and stroke (Fardet et al., 2012). However, most patients on chronic glucocorticoid therapy are prescribed prednisolone equivalent doses of <10 mg/day (Fardet et al., 2011). The effect of typical therapeutic (lower) glucocorticoid doses on fat mass is less clear, with some studies reporting an increase in central fat mass and others no significant difference (Burt et al., 2007b, Nordborg et al., 1998).

Studies of body composition are valuable but interpretation can be limited by between-subject heterogeneity and relatively small sample size. Defining the perturbations of energy and fat metabolism associated with typical therapeutic glucocorticoid doses will aid an understanding of their contribution to adiposity. While infusion of high doses of glucocorticoids paradoxically increases resting energy expenditure (Djurhuus et al., 2002), during longer-term administration of exogenous glucocorticoids (Chong et al., 1994) and in Cushing's syndrome (Burt et al., 2006), resting energy expenditure is not significantly different to controls. Stimulation of appetite, and not reduced energy utilization, is considered to be the major contributor to glucocorticoid-induced adiposity (Tataranni et al., 1996).

Glucocorticoids predominantly increase glucose concentration in the postprandial period (Burt et al., 2012). However, the effect of glucocorticoids on diet-induced thermogenesis, the increase in energy expenditure after a meal, has not been studied. A reduction in diet-induced thermogenesis, which comprises obligatory (heat generated by digestion and absorption of food) and facultative (regulated heat production to dissipate food energy) components, may contribute to adiposity and the metabolic syndrome (de Jonge and Bray, 1997). As glucocorticoids reduce sympathetic

nervous system activity and insulin sensitivity, even at typical therapeutic doses, this could reduce diet-induced thermogenesis and contribute to adiposity in patients taking glucocorticoids.

Variable effects of glucocorticoids on fat oxidation have been reported (Macfarlane et al., 2008, Peckett et al., 2011). Fasting fat oxidation was increased after a 4-5 hour infusion of hydrocortisone (Divertie et al., 1991). However, there were no significant changes in fasting fat oxidation in patients with Cushing's syndrome (Burt et al., 2006) and taking chronic glucocorticoid therapy (Burt et al., 2007b). The effects of glucocorticoids on postprandial lipid metabolism have not been well characterized.

The aim of this study was to assess the acute and chronic effects of commonly used therapeutic prednisolone doses on fasting and postprandial energy and fat metabolism. We hypothesized that, similar to its effects on postprandial glycaemia, glucocorticoid-induced changes in energy and substrate metabolism would predominantly manifest in the postprandial period.

5.2. Material and Methods

The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee, Flinders Medical Centre, and all patients provided written informed consent in accordance with the Declaration of Helsinki. The effects of prednisolone on whole body insulin sensitivity and vascular function in this cohort have previously been reported (Radhakutty et al., 2016b).

5.2.1. Patients

Patients aged 50 years or older with rheumatoid arthritis were recruited from the rheumatology outpatient clinic at Repatriation General Hospital, Adelaide, Australia. We studied 18 patients who had not been administered any oral glucocorticoids for at least 6 months (non-GC users) and 18 patients taking a stable continuous oral prednisolone dose of 4-10 mg/day for at least 6 months (GC users). The two groups were matched for age and sex. Patients with active synovitis and those prescribed oral hypoglycaemic agents and /or insulin were excluded from the study.

5.2.2. Study design

Non-GC users were studied before and after a 7 day course of oral prednisolone 6 mg daily to determine the acute effects of prednisolone. Baseline data from non-GC users were compared with

data from chronic GC users to determine the chronic effects of prednisolone.

5.2.3. Study protocol

Patients attended the Endocrine Research Unit at Repatriation General Hospital at 0830 h after a 12 hour overnight fast. All patients took their regular medications on the morning prior to arrival, including prednisolone. Basic anthropometric measures were recorded. At each visit, after resting supine for a 20 minute acclimatization period, energy and substrate metabolism were assessed using indirect calorimetry. Blood samples were collected for estimation of glucose, insulin, lipid profile, non-esterified fatty acids (NEFA) and C-reactive protein (CRP). A mixed-meal was then administered over 15 minutes (10 kcal/kg body weight, 45% carbohydrate, 40% fat and 15% protein), and indirect calorimetry repeated two hours after the mixed meal. Further blood samples for glucose, insulin, and NEFA were taken at 30-minute intervals for 120 minutes. Patients then underwent a dual energy X-ray absorptiometry scan to measure whole body composition.

5.2.3.1. Indirect calorimetry

Indirect calorimetry was performed using a ventilated hood technique (Parvo Medics True One 2400 Metabolic Measurement System, Parvo Medics, Sandy, UT). After an equilibrium period of 10 minutes, resting energy expenditure and substrate oxidation rates were calculated from the next 20 minutes of indirect calorimetry recordings using the equations of Ferrannini (Ferrannini, 1988). Diet-induced thermogenesis was calculated as the percentage increase in energy expenditure after the mixed meal (Vosselman et al., 2013).

5.2.3.2. Body composition

Fat mass and lean body mass (LBM) were measured by dual energy X-ray absorptiometry on a GE Medical Systems Lunar Prodigy (GE Healthcare General Electric Company), which also quantified central abdominal fat. Central abdominal fat is the fat within a manually traced region bordered by the upper margin of second and the lower margin of the fourth lumbar vertebral bodies and the outer margin of the ribs (Burt et al., 2007b).

5.2.3.3. Whole body insulin sensitivity

As previously described (Radhakutty et al., 2016b), whole blood glucose was measured at the

bedside immediately after venesection on an in-house glucose analyser (YSI 2300 STAT Plus, Yellow Springs Instrumentation, Ohio, USA) by an immobilised glucose oxidase method. Insulin was measured by radioimmunoassay (EMD Millipore, Toronto, Ontario, Canada). Whole body insulin sensitivity was estimated by the composite insulin sensitivity index (Matsuda index) (Matsuda and DeFronzo, 1999).

5.2.3.4. Lipids and adipocyte insulin sensitivity

Fasting lipid profiles were measured by enzymatic colorimetry (Roche Modular P Unit; Roche Diagnostics GmbH). Serum free fatty acid concentrations were measured by enzyme colorimetry using a Beckman Synchron CX5 analyser (WAKO NEFA C kit, Denver, CO). Adipocyte insulin resistance index was calculated as the product of fasting plasma insulin and fasting NEFA concentration (Malin et al., 2014). Insulin-mediated suppression of NEFA was calculated as the percentage decrease in fasting NEFA concentration divided by the mean plasma insulin concentration during the mixed-meal test (Abdul-Ghani et al., 2008).

5.2.3.5. Physical activity

Physical activity was assessed using the Modified Baecke Questionnaire, which is a composite score based on household activity, sports and exercise and other leisure activities. A higher score reflects higher levels of physical activity (Voorrips et al., 1991).

5.2.3.6. Other laboratory analysis

CRP was measured using a Tinaquant immunoturbidimetric assay (Roche Diagnostics GMBH, Mannheim, Germany) on a Roche Modular Analyser (Hitachi High-Technologies Corporation, Tokyo, Japan). The limit of detection was 0.3 mg/L. The between-run CV was 3.6 % at a CRP of 3.9 mg/L and 2.3 % at a CRP of 49.5 mg/L. Urinary noradrenaline excretion was measured during the 6 hour study period by liquid chromatography / mass spectrometry to estimate sympathetic nervous system activity. The coefficient of variation (CV) for urinary noradrenaline measurement is 5.1% at 300 nmol/L.

5.2.4. Statistical analysis

Statistical analysis was performed using IBM SPSS version 20 for Windows (IBM, New York,

USA). A p-value of <0.05 was considered statistically significant. Patient characteristics are presented as mean ± standard deviation if the distribution was Normal and median (interquartile range) if the distribution was not Normal. All other data are presented as mean ± standard error of mean. Changes in variables in non-GC users after 7 days prednisolone were analysed using paired t-tests, which are hereafter described in the manuscript as the acute effects of prednisolone. GC users were compared with baseline data from non-GC users using unpaired t-tests if Normally distributed or Mann-Whitney U tests if the distribution was not Normal. These results are reported in the manuscript as the chronic effects of prednisolone. As resting energy expenditure is dependent on LBM, it was adjusted for this variable using analysis of covariance.

The primary end point is the change / difference in diet-induced thermogenesis. In the acute study a sample size of 17 had 80% power to detect a 3% change in diet-induced thermogenesis at the 0.05 significance level, assuming a standard deviation of 4%. In the chronic study, a sample size of 16 per group had 80% power to detect a 4% difference in diet-induced thermogenesis at the 0.05 significance level, assuming a standard deviation of 4%.

5.3. Results

5.3.1. Patient characteristics, body composition and noradrenaline excretion

GC users were taking a mean prednisolone dose of 6.5 ± 1.8 mg/day, with a mean duration of continuous prednisolone therapy of 62 ± 62 months. None of the non-GC users reported receiving long-term prednisolone therapy in the past. There were no significant differences in sex distribution, age, body mass index, C-reactive protein, physical activity score or use of disease-modifying anti rheumatic drugs between GC users and non-GC users (Table 1). There were no significant differences in total fat mass, truncal fat mass, central abdominal fat or lean body mass between GC and non-GC users (Table 1). There were no significant differences in serum lipid profile between GC users and non-GC users (Table 1). Urinary noradrenaline excretion was reduced by acute prednisolone (54 ± 8 vs 93 ± 23 nmol/6 hours, p=0.02) but was not significantly different in patients on chronic prednisolone (68 ± 17 vs 93 ± 23 nmol/6 hours, p=0.31). No patient in either group had a diagnosis of diabetes.

5.3.2. Indirect calorimetry

There were no significant differences in resting energy expenditure (1391 ± 52 vs 1383 ± 52 Kcal/day, p= 0.67) or diet-induced thermogenesis (Fig 1) after acute prednisolone. There was no significant difference in resting energy expenditure (1374 ± 27 vs 1408 ± 27 Kcal/day, p = 0.38) or diet-induced thermogenesis (Fig 1) between GC users and non-GC users.

There were no significant differences in fasting fat (29.1 ± 6.0 vs 37.4 ± 3.9 mg/min, p=0.14) or carbohydrate oxidation (88.6 ± 12.4 vs 64.0 ± 8.6 mg/min, p=0.09) after acute prednisolone. There were no significant differences in fasting fat (34.0 ± 4.0 vs 37.4 ± 3.9, p=0.56) or carbohydrate (73.4 ± 9.4 vs 64.0 ± 8.6 mg/min, p=0.47) oxidation between GC users and non GC users. However, acute and chronic prednisolone were both associated with an impaired ability to suppress fat oxidation (Fig 2A) and increase carbohydrate oxidation (Fig 2B) in response to the meal.

5.3.3. Whole body insulin sensitivity

The time course of changes in glucose and insulin concentrations are shown in Fig 3A and 3B respectively. As previously reported, fasting glucose (5.3 ± 0.3 vs 4.6 ± 0.1 mmol/L, p=0.02) and glucose AUC over two hours (844 ± 58 vs 733 ± 27 mmol/L*minute, p=0.03) were higher after acute prednisolone. No significant differences in fasting (4.9 ± 0.2 vs 4.6 ± 0.1 mmol/L, p=0.20) or glucose AUC over two hours (822 ± 40 vs 733 ± 27 mmol/ L*minute, p=0.07) were observed with chronic prednisolone, although the higher glucose AUC with chronic prednisolone approached statistical significance. There were no significant differences in fasting insulin (28.3 ± 2.0 vs 24.2 ± 3.7 µU/ml, p=0.28) or insulin AUC (14.3 ± 1.8 vs 12.5± 1.6 mU/ml*min, p=0.28) with acute prednisolone. Fasting insulin (32.2 ± 2.9 vs 24.2 ± 3.7 µU/ml, p= 0.10) and insulin AUC (17.3 ± 2.2 vs12.5 ± 1.6 mU/ml*min, p=0.09) were increased by more than 30% in patients on chronic prednisolone, but these differences did not reach statistical significance. The Matsuda index was significantly lower after both acute (2.0 ± 1.0 vs 3.6 ±1.1, p=0.01) and chronic (1.9 ± 1.0 vs 3.6 ±1.1, p=0.04) prednisolone.

5.3.4. Adipocyte insulin sensitivity

There was no significant difference in fasting NEFA after acute prednisolone (0.7 ± 0.1 vs 0.6 ± 0.1 mmol/L, p = 0.24). GC users had higher fasting NEFA (1.0 ± 0.1 mmol/L vs 0.6 ± 0.1 mmol/L, p <0.001) than non-GC users. Adipocyte insulin resistance index was higher after chronic, but not acute prednisolone (Fig 4A). However, percentage suppression of NEFA post-meal was not significantly different after acute (85.4 ± 1.9 vs 88.5 ± 1.8 %, p=0.09) or chronic prednisolone. (89.5 ± 1.2 vs 88.5 ± 1.8 %, p=0.63) (Fig 4B). Consequently, insulin-mediated suppression of NEFA was not significantly different after acute or chronic prednisolone (Fig 4C).

5.4. Discussion

This study investigated the effects of low dose prednisolone on fasting and postprandial energy and substrate metabolism in elderly patients with rheumatoid arthritis. Neither acute nor chronic prednisolone were associated with significant changes in resting energy expenditure or diet-induced thermogenesis. In contrast, low dose prednisolone reduced whole body insulin sensitivity, which was associated with attenuated postprandial suppression of fat oxidation. Chronic, but not acute, prednisolone was associated with higher fasting NEFA and adipocyte insulin resistance index. However, acute and chronic prednisolone did not affect postprandial insulin-mediated suppression of NEFA. These data provide insight into tissue-specific differences in glucocorticoid-induced insulin resistance and suggest potential therapeutic targets to reduce the metabolic effects of glucocorticoids.

The effects of glucocorticoids on diet-induced thermogenesis have not been studied. Diet-induced thermogenesis contributes 10-15 % of average daily energy expenditure. Diet-induced thermogenesis is regulated by the sympathetic nervous system (Valensi et al., 1998) and is lower in patients with insulin resistance (Camastra et al., 1999). Previous studies have reported that resting energy expenditure is not reduced in patients with Cushing's syndrome (Burt et al., 2006) and taking exogenous glucocorticoids (Chong et al., 1994, Tataranni et al., 1996). Our study was concordant with these findings, but extended them to demonstrate that there is no significant change in postprandial energy expenditure with acute or chronic low dose prednisolone. In our cohort of patients urinary noradrenaline excretion was reduced by 42% with acute prednisolone

and there was a reduction in Matsuda index with acute and chronic prednisolone. (Radhakutty et al., 2016b). However, these changes did not translate into a reduction in diet-induced thermogenesis. It is possible that the effects of glucocorticoids on postprandial energy metabolism manifest only with higher glucocorticoid doses. However, our study is clinically relevant as we studied commonly used therapeutic doses of prednisolone in typical (older) patients at ambient temperature.

In this study we have systematically assessed the effects of prednisolone on substrate oxidation in the fasting and postprandial state. Previous studies have demonstrated no significant changes in fasting fat oxidation in patients with Cushing's syndrome (Burt et al., 2006) and taking exogenous glucocorticoids (Burt et al., 2007a). Concordant with these studies, there were no significant changes in fasting fat oxidation in this study. However, we have demonstrated for the first time that low dose prednisolone alters postprandial substrate oxidation, with attenuation of suppression of fat oxidation and the concomitant increase in carbohydrate oxidation (Fig 2A and B). There is a parallel between the timing of these postprandial changes in substrate metabolism, termed metabolic inflexibility, and prednisolone-induced changes in blood glucose concentration, which also mainly occurs after a meal (Burt et al., 2012). Postprandial glucose uptake and substrate oxidation predominantly occur in skeletal muscle. Consequently postprandial hyperglycaemia and metabolic inflexibility are consistent with acute and chronic prednisolone causing insulin resistance in skeletal muscle.

In contrast to changes in fat oxidation, elevations of NEFA manifested in the fasting state and only during chronic glucocorticoid excess. Elevated serum NEFA concentrations are indirectly indicative of resistance to insulin-induced suppression of lipolysis in adipocytes. Consistent with previous studies (Petersons et al., 2013), circulating NEFA and adipocyte insulin resistance index were not significantly different after 7 days of low dose prednisolone, suggesting insulin sensitivity in adipocytes was not affected by acute prednisolone. However, adipocyte insulin resistance index was increased in patients on long-term prednisolone, with mean fasting NEFA higher than typical NEFA concentrations after an overnight fast (Karpe et al., 2011). These findings are similar to those observed in patients with Cushing's syndrome, in whom fasting NEFA and subcutaneous

adipose tissue lipolysis are increased (Leal-Cerro et al., 1997), but extend them by demonstrating that mild degrees of chronic glucocorticoid excess attenuate insulin-induced suppression of lipolysis. They do, however, need to be verified using stable isotopic assessment of lipid metabolism.

Previous *in vitro, ex vivo and in vivo* studies have reported that glucocorticoids do not cause insulin resistance in adipocytes (Gathercole et al., 2011, Gathercole et al., 2007, Hazlehurst et al., 2013). In contrast, the elevated fasting NEFA in our study suggests that prednisolone does induce insulin resistance in adipocytes as the relatively low insulin concentrations during fasting do not suppress lipolysis. However, in the postprandial period, during which mean insulin concentrations were more than 100 μU/ml in all groups, insulin-mediated suppression of lipolysis was not significantly affected by prednisolone. As these insulin concentrations did not suppress fat oxidation, this suggests that the degree of glucocorticoid-induced insulin resistance is tissue specific, with lipolysis in adipose tissue less resistant than fat oxidation in skeletal muscle.

These changes in lipid metabolism induced by glucocorticoids may have clinical consequences. It is debated as to whether metabolic inflexibility is a cause or consequence of insulin resistance, and its clinical significance in humans remains to be clarified. However, in an animal model, inhibition of fat oxidation prevented glucocorticoid-induced insulin resistance in muscle (Guillaume-Gentil et al., 1993). Elevated fasting NEFA are associated with fat accumulation in liver and skeletal muscle, a major contributor to insulin resistance (Belfort et al., 2005). Furthermore, patients with higher fasting NEFA have increased rates of hypertension, type 2 diabetes and increased mortality (Miedema et al., 2014). Inhibition of lipolysis with acipimox was reported to reduce dexamethasone-induced insulin resistance (Santomauro et al., 1999). Consequently, reversing prednisolone-induced changes in lipolysis and fat oxidation could potentially ameliorate the adverse metabolic effects of glucocorticoids and this should be the subject of future studies.

Assessing the metabolic effects of glucocorticoids *in vivo* is challenging. In addition to potential direct effects of glucocorticoids, changes in insulin concentration and sensitivity and catecholamine secretion influence their effects on energy and substrate metabolism. These variables can differ in the fasting and postprandial state. Strengths of this study include that we have systematically

assessed the effects of prednisolone on fasting and postprandial energy metabolism and also simultaneously quantified insulin sensitivity and catecholamine secretion in a well matched cohort of patients with rheumatoid arthritis.

However, we acknowledge our study has limitations. Firstly, as our study has a relatively small sample size, this could have resulted in a type 2 error when analyzing some measurements e.g. fasting substrate metabolism. Secondly, we have not used an euglycaemic-hyperinsulinaemic clamp to assess the effect of glucocorticoids on insulin sensitivity, a technique considered to be the gold standard. However calculation of Matsuda index from a mixed-meal produces insulin sensitivity results similar to that from euglycaemic-hyperinsulinaemic clamp studies (van Raalte et al., 2011b). Thirdly, our findings may not be applicable to patients on higher glucocorticoid doses (>10 mg/day). Finally, inherent in any cross-sectional study is the possibility that an unmeasured variable affected results. However, the groups were well matched for a number of key variables.

In summary, we have assessed the effects of commonly used therapeutic prednisolone doses on energy and fat metabolism in a typical (older) patient group with rheumatoid arthritis. Neither acute nor chronic prednisolone was associated with changes in fasting or postprandial energy expenditure. However, prednisolone attenuated postprandial suppression of fat oxidation. Chronic prednisolone also increased fasting NEFA and adipocyte insulin resistance, but did not affect postprandial insulin-mediated suppression of lipolysis. Our study highlights the adverse changes in carbohydrate and fat metabolism associated with mild glucocorticoid excess. The fasting and postprandial changes in fat metabolism demonstrated in our study suggest that glucocorticoids cause greater insulin resistance in skeletal muscle than in adipocytes. Future studies should confirm our findings using more sophisticated metabolic techniques and explore whether therapies targeting these changes in fat metabolism could reduce insulin resistance and the associated metabolic consequences of glucocorticoid therapy.

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5.5. Table 1: Subject Characteristics, body composition and lipid profile

	Non-GC users (n=18)	GC users (n=18)	P-value
Female (n, (%))	12 (67)	12 (67)	1.00
Age (years)	64 ± 7	66 ± 7	0.33
BMI (Kg/m^2)	28.1 ± 5.2	27.9 ± 6.1	0.95
C-reactive protein (mg/L)[a]	2.4 (1.1-4.5)	1.6 (0.5-7.6)	0.44
Physical activity score	12.8 ± 5.7	10.5 ± 5.5	0.22
DMARDs (n)	11	9	0.50
Total fat mass (Kg)	29.6 ± 12.7	29.5 ± 12.9	0.98
Central abdominal fat (Kg)	2.3 ± 1.3	2.2 ± 1.2	0.65
Truncal fat (Kg)	16.2 ± 6.8	15.2 ± 7.0	0.68
Lean body mass (Kg)	43.3 ± 8.4	45.6 ± 8.1	0.41
Total Cholesterol (mmol/L)	5.3 ± 0.8	5.3 ± 1.1	0.87
Triglyceride (mmol/L)	1.3 ± 0.6	1.2 ± 0.8	0.74
LDL cholesterol (mmol/L)	3.0 ± 0.9	3.0 ± 0.9	0.77
HDL cholesterol (mmol/L)	1.7 ± 0.6	1.7 ± 0.5	0.82

Data are mean ± SD unless otherwise stated, [a] = median (interquartile range). n = number of patients with specified variable. GC = glucocorticoid, BMI = body mass index, DMARDs = disease modifying anti-rheumatic drugs

5.6. Figures and Figure legends

Figure 1: Diet-induced thermogenesis (DIT) in 18 patients with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white bars) and after (grey bars) 7 days prednisolone and 18 patients with rheumatoid arthritis on long-term prednisolone (GC users, black bars). Data are mean ± SEM.

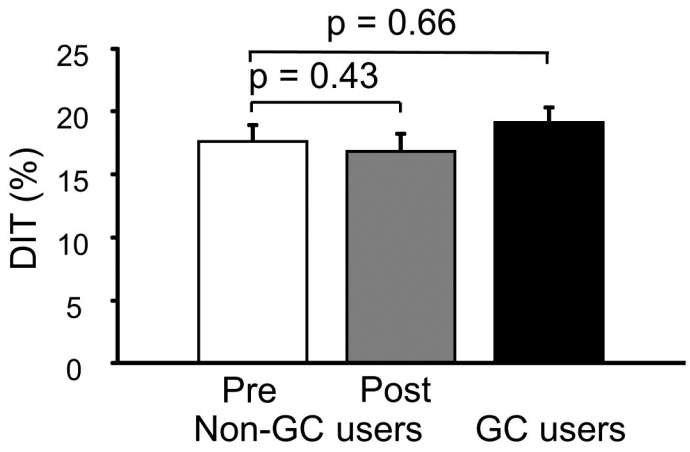


Figure 2: Post-meal change in fat oxidation (A) and carbohydrate oxidation (CHO oxidation) (B) in 18 patients with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white bars) and after (grey bars) 7 days prednisolone and 18 patients with rheumatoid arthritis on long-term prednisolone (GC users, black bars). Data are mean ± SEM.

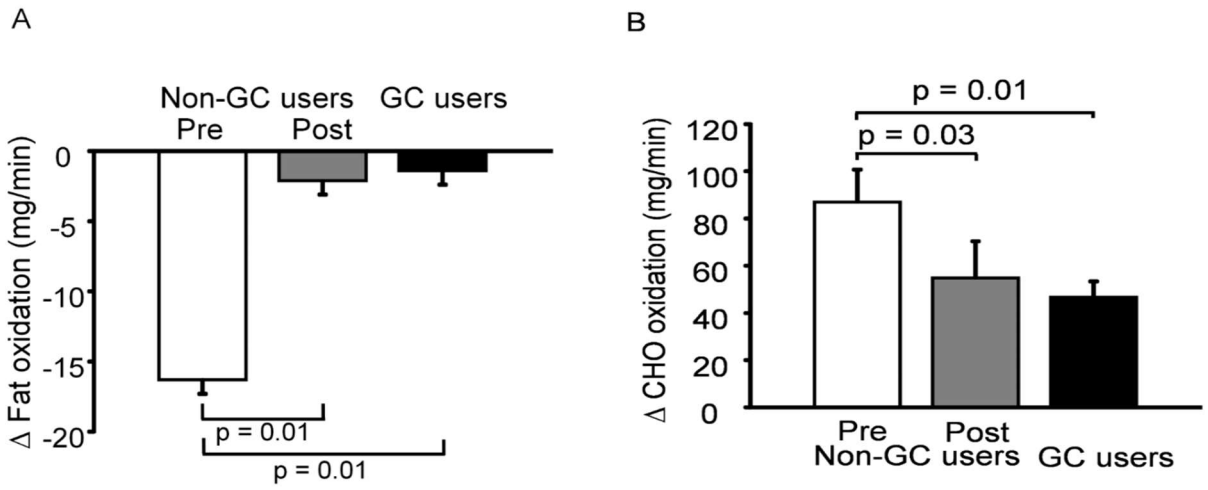


Figure 3: Time course for glucose (A) and insulin (B) after a mixed meal in 18 patients with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white bars) and after (grey bars) 7 days prednisolone and 18 patients with rheumatoid arthritis on long-term prednisolone (GC users, black bars). Data are mean ± SEM.

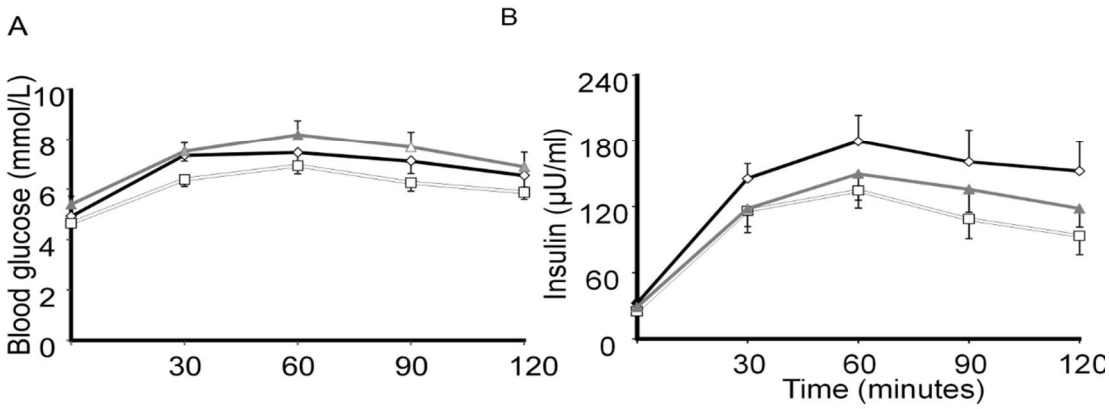
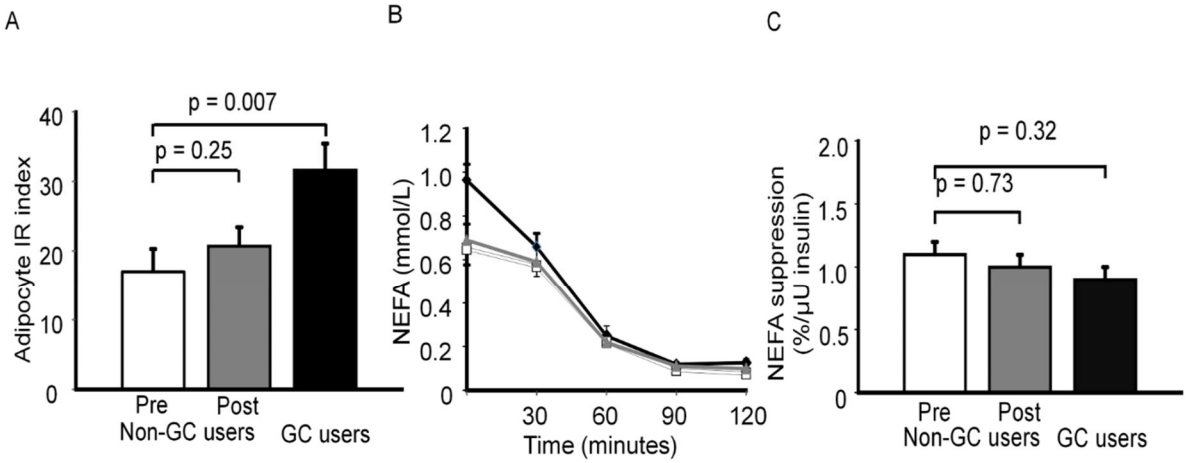


Figure 4: Adipocyte insulin resistance index (Adipocyte IR index) (4A), time course of plasma non-esterified fatty acids (NEFA) (4B) and insulin-mediated suppression of NEFA (4C) in 18 patients with rheumatoid arthritis who do not take oral glucocorticoids (non-GC users) before (white bars) and after (grey bars) 7 days prednisolone and 18 patients with rheumatoid arthritis on long-term prednisolone (GC users, black bars). Data are mean ± SEM.



CHAPTER 6. TREATMENT OF PREDNISOLONE-INDUCED HYPERGLYCAEMIA IN HOSPITALIZED PATIENTS: INSIGHTS FROM A RANDOMIZED-CONTROLLED STUDY

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Author roles

AR was responsible for study design, patient recruitment, randomization, insulin dose titration, data analysis and manuscript preparation. JLS was responsible for patient recruitment, CGMS insertion and insulin dose titration. BLM was responsible for patient recruitment and CGMS insertion. SMD was responsible patient recruitment, CGMS insertion and data acquisition. ATZ was responsible patient recruitment and manuscript revision. GWR was responsible for study design, patient recruitment and manuscript revision. SNS was responsible for study design, insulin dose titration and manuscript revision. CHT was responsible for study design and manuscript revision. MGB was responsible for obtaining funding, study design, data analysis, insulin dose titration, supervision and manuscript revision.

Abstract

Aims: Prednisolone predominantly causes hyperglycaemia between midday and midnight. Consequently glargine-based basal-bolus insulin regimens may under-treat day-time hyperglycaemia and cause nocturnal hypoglycaemia. We investigated whether an isophane-based insulin regimen is safer and more effective than a glargine-based regimen in hospitalized patients.

Materials and Methods: 50 inpatients prescribed ≥20 mg/day prednisolone acutely with one finger prick blood glucose level (BGL) ≥15 mmol/L or two BGLs ≥10 mmol/L within 24 hours were randomised to either insulin isophane or glargine before breakfast and insulin aspart before meals. The initial daily insulin dose was 0.5 U/kg body weight or 130% of the current daily insulin dose. Glycaemic control was assessed using a continuous glucose monitoring system.

Results: On Day 1, there were no significant differences in percentage time outside a target glucose range of 4-10 mmol/L (41.3±5.5 vs 50.0±5.7 %, p=0.28), mean daily glucose (10.2±0.7 vs 10.8±0.8 mmol/L, p=0.57) or glucose <4 mmol/L (2.2±1.1 vs 2.0±1.3 %, p=0.92) in patients randomized to isophane and glargine. In patients treated for 3 days, prednisolone dose reduced (p=0.02) and insulin dose increased over time (p=0.02), but the percentage time outside the 4-10 mmol/L glucose range did not differ over time (p=0.45) or between the groups (p=0.24).

Conclusions: There were no differences in the efficacy or safety of the isophane and glargine-based insulin regimens. We recommend an initial daily insulin dose of 0.5 units/Kg if not on insulin, a greater than 30% increase in pre-prednisolone insulin dose and larger insulin dose adjustments in patients with prednisolone-induced hyperglycaemia.

6.1. Introduction

Hyperglycaemia in hospitalized patients is associated with increased morbidity, length of hospital stay and mortality.(Umpierrez et al., 2002, Capes et al., 2000, Burt et al., 2013b, Krinsley, 2003, Baker et al., 2006, Capes et al., 2001, Barsheshet et al., 2006, Vriesendorp et al., 2004) However, the association with mortality is stronger in patients with new hyperglycaemia than patients with known diabetes.(Umpierrez et al., 2002, Farrokhi et al., 2011) Treatment of hyperglycaemia has been reported to reduce morbidity in hospitalized patients.(Umpierrez et al., 2007, Umpierrez et al., 2011) The Endocrine Society clinical practice guidelines and the American Diabetes Association standards of medical care in diabetes recommend prescribing insulin to maintain glucose below 10 mmol/L in most patients on the general ward.(Umpierrez et al., 2012, Chamberlain et al., 2016)

Prednisolone is commonly prescribed in moderate to high doses as an anti-inflammatory agent to hospitalized patients. However, these prednisolone doses cause hyperglycaemia in 40-70 % of patients.(Hougardy et al., 2000, Braithwaite et al., 1998) Hospitalized patients prescribed glucocorticoid treatment have a 50% increase in relative risk of new onset hyperglycaemia.(Breakey et al., 2016a) The Endocrine Society Guidelines recommend that hospitalized patients prescribed glucocorticoids, including those without known diabetes, undergo point of care glucose testing to screen for hyperglycaemia.(Umpierrez et al., 2012)

Although the effect of glucocorticoid-induced hyperglycaemia on morbidity and mortality is not fully determined,(del Rincon et al., 2004, Cheung, 2016) current guidelines recommend treatment of inpatient glucocorticoid-induced hyperglycaemia with subcutaneous basal-bolus insulin.(Umpierrez et al., 2012, Moghissi et al., 2009) However, they do not specify the insulin formulation in detail. Recent studies provide insight into appropriate insulin formulations in this context by characterizing the circadian pattern of hyperglycaemia induced by prednisolone. They demonstrate that a morning dose of prednisolone, as commonly prescribed, has little effect on overnight glucose concentration and predominantly causes hyperglycaemia in the afternoon and evening.(Burt et al., 2011, Yuen et al., 2012) This pattern of hyperglycaemia is present in patients on prednisolone prescribed glargine-based basal bolus insulin.(Burt et al., 2015) These studies suggest that glucose lowering therapy in prednisolone-treated patients should be directed at the time period

between midday and midnight. Moreover, treatment with a basal-bolus regimen that includes long-acting basal insulin such as glargine might cause overnight hypoglycaemia in prednisolone-treated patients.

We hypothesized that delivering more insulin between midday and midnight and less insulin between midnight and breakfast would reduce nocturnal hypoglycaemia and better treat postprandial hyperglycaemia in prednisolone-treated patients. As such, the aim of this study was to determine whether an insulin regimen comprising isophane and prandial aspart designed to provide more insulin in the afternoon and evening provided safer and more effective treatment of prednisolone-induced hyperglycaemia in hospitalized patients than a standard regimen of insulin glargine and prandial aspart. We also assessed which clinical factors influenced the glycaemic response to insulin therapy.

6.2. Materials and Methods

The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee and Human Research Ethics Committee (The Queen Elizabeth Hospital/ Lyell Mc Ewin Hospital/ Modbury Hospital), and all subjects provided written informed consent in accordance with the Declaration of Helsinki.

6.2.1. Patients

Fifty consecutive consenting hospitalized patients aged 18 years or older who were treated with oral prednisolone ≥ 20 mg/day as a single morning dose for an acute medical condition were recruited from the general medical wards of three tertiary care hospitals in Adelaide, Australia (Flinders Medical Centre, Repatriation General Hospital and Lyell McEwin Hospital) between 2012 and 2016. All patients had hyperglycaemia, defined as two finger prick blood glucose levels > 10 mmol/L or one finger prick blood glucose of > 15 mmol/L in the prior 24 hours. Patients with type 1 diabetes, prescribed chronic glucocorticoid treatment (prednisolone equivalent of >10 mg/day) and pregnant women were excluded from the study.

6.2.2. Study design

An open labelled stratified randomized controlled study was conducted. Subjects were randomized

to insulin isophane and aspart or insulin glargine and aspart. Randomization was stratified for prior insulin treatment in blocks of 8 using sealed envelopes to ensure this variable did not differ in the two groups, as it could result in a different daily insulin dose.

6.2.2.1. Insulin regimens

Oral hypoglycaemic agents were discontinued in both treatment groups. Patients were initially prescribed a total daily insulin of 0.5 units / Kg body weight or 130% of the current daily dose of insulin (whichever was greater).(Umpierrez et al., 2012) In the isophane plus aspart group 50% of the total daily insulin dose was isophane administered at 07.00 hours and 50% of the total daily dose was insulin aspart with 20% of the aspart dose administered before breakfast, 40% before lunch and 40% before dinner. In the glargine plus aspart group 50% of the total daily insulin dose was glargine administered at 07.00 hours and 50% of the total daily dose was aspart administered as three equal doses before breakfast, lunch and dinner. Aspart doses were differently distributed in the isophane group with the aim to deliver more insulin between afternoon and evening coinciding with the circadian pattern of hyperglycaemia associated with prednisolone. In both groups, correctional insulin aspart was administered in addition to prandial insulin if finger prick glucose levels were elevated (glucose 10-15 mmol/L - additional 3 Units insulin aspart; glucose >15 mmol/L - additional 6 Units insulin aspart).

6.2.2.2. Glucose monitoring

All patients underwent monitoring of interstitial glucose using a continuous glucose monitoring system (CGMS) (iPro2, Medtonic/Minimed, Northridge, CA, USA). A sensor was placed into the subcutaneous tissue of the abdomen by a member of the research team. Interstitial glucose concentrations were recorded every 5 minutes for up to 4 days using a glucose-oxidase based method. These were then averaged for each hour of CGMS and were then used to calculate the percentage of time glucose was outside a target range of 4-10 mmol/L, mean glucose concentration, rates of hypoglycaemia defined as glucose < 4 mmol/L and severe hypoglycaemia defined as glucose < 2.8 mmol/L and glycaemic variability estimated by the mean amplitude of glycaemic excursion (MAGE) (Service et al., 1970). Point of care capillary blood glucose levels were performed at 07.00, 12.00, 17.00 (before each main meal) and at 21.00 hours with a ward

glucose meter (Freestyle optium H, Abbott, Victoria, Australia) and used to calibrate the CGMS.

6.2.2.3. Insulin dose adjustments

Insulin doses were reviewed daily by the research team. Isophane and glargine doses were adjusted if the fasting finger prick glucose level was outside the target range of 4-10 mmol/L and aspart doses were adjusted if the finger prick glucose level before the subsequent meal was outside the target glucose range. Each insulin dose was increased by 2 units if the associated finger prick glucose level was 10-15 mmol/L, 4 units if the finger prick glucose level was 15-20 mmol/L and 6 units if the finger prick glucose level was >20 mmol/L. Insulin doses were reduced by 2 units if the associated finger prick glucose level was 2.8-4 mmol/L and 4 units if finger prick glucose level was < 2.8 mmol/L or if there was symptomatic hypoglycaemia. In case of severe hypoglycaemia the study team could either reduce the insulin dose by > 50% or cease insulin.

6.2.3. Laboratory analysis

Glycosylated hemoglobin (HbA1c) was measured using boronate affinity chromatography on a Primus PDQ (Immuno, Sydney, Australia). The between-run coefficient of variation (CV) is < 3% across the measured range. C-reactive protein (CRP) was measured using a Tinaquant immunoturbidimetric assay (Roche Diagnostics GMBH, Mannheim, Germany) on a Roche Modular Analyser (Hitachi High-Technologies Corporation, Tokyo, Japan) with a CV < 4%.

6.2.4. Statistical analysis

Statistical analysis was performed using IBM SPSS version 20 for Windows (IBM, New York, USA). A p-value of <0.05 was considered statistically significant. Subject characteristics are presented as mean ± standard deviation, while all other data are presented as mean ± standard error of mean. Differences between the groups on Day 1 of insulin treatment were assessed by unpaired t-tests. Univariate regression analysis was performed to assess the relationship between relevant variables and the percentage of time spent with glucose outside the target range of 4-10 mmol/L on Day 1. Variables that were statistically significant in univariate analyses were then included in a multivariate analysis. Changes in variables over three days insulin treatment were assessed using two way repeated measures analysis of variance.

The primary end point was the percentage of time interstitial glucose was outside the target range of 4-10 mmol/L during the first 24 hours of treatment as data was available for all patients for this time period. A sample size of 25 subjects per group had 94% power to detect a 2 hour difference in the time outside the target glucose range at the 0.05 significance level, assuming a SD of two hours that was derived from a previous study.(Burt et al., 2011)

6.3. Results

6.3.1. Patient Characteristics

25 patients were randomized to each insulin regimen. However, data from two patients randomized to glargine and aspart could not be included in the analysis; one patient was discharged from hospital earlier than expected and the other had an incomplete CGMS trace.

Approximately 70% of patients had been prescribed prednisolone to treat an acute exacerbation of chronic obstructive airway disease. The remaining patients had received prednisolone for pneumonia, interstitial lung disease or gout. There were no significant differences in age, sex, body mass index (BMI), waist circumference or CRP between the two treatment groups (Table 1). Approximately 70% of subjects had been diagnosed with diabetes prior to hospital admission, but less than 25% of subjects were treated with insulin before admission (Table 1). There were no significant differences in HbA1c or mean blood glucose on Day 0 (measured by point of care capillary blood glucose) between the two groups.

6.3.2. Glycaemic control on Day 1

On Day 1 patients were prescribed prednisolone 20-50 mg / day, with no significant difference between patients randomized to isophane and aspart versus glargine and aspart (33 ± 11 vs 33 ± 8 mg, $p = 0.88$). The total insulin dose on Day 1 was also not significantly different in subjects randomized to isophane and aspart versus glargine and aspart (0.61 ± 0.04 vs 0.67 ± 0.08 units/Kg, $p = 0.57$). The percentage of time spent outside the target glucose range of 4-10 mmol/L (Figure 1A) and mean glucose on Day 1 (Figure 1B) were not significantly different in the isophane- and glargine-based treatment groups. The hourly interstitial glucose profiles in the two groups on Day 1 are shown in Figure 2.

We also examined whether glucose concentrations differed at different times of the day in the two groups. There were no significant differences in the percentage of time spent outside target glucose range of 4-10 mmol/L between 07.00-12.00 hours (39.2 ± 8.2 vs 35.7 ± 7.4 %, p = 0.75), 12.00-17.00 hours (61.6 ± 9.0 vs 60.9 ± 8.2 %, p = 0.95), 17.00-22.00 hours (60 ± 7.2 vs 76.5 ± 7.2 %, p = 0.11) and 22.00-07.00 hours (25.7 ± 7.1 vs 36.7 ± 7.7 %, p = 0.30) between patients treated with isophane and aspart versus glargine and aspart. There were also no significant differences in mean glucose between 07.00-12.00 hours (9.9 ± 0.8 vs 9.1 ± 0.6 mmol/L, p = 0.41) , 12.00-17.00 hours (12.0 ± 0.9 vs 12.0 ± 0.8 mmol/L, p = 1.00), 17.00-22.00 hours (12.2 ± 0.8 vs 13.9 ± 0.9 mmol/L, p = 0.16) and 22.00-07.00 hours (8.2 ± 0.8 vs 9.2 ± 0.6 mmol/L, p = 0.32) between patients treated with isophane and aspart versus glargine and aspart. Glycaemic variability assessed by MAGE was not significantly different in isophane- and glargine-based treatment groups (7.6 ± 0.7 vs 8.8 ± 0.8, p = 0.20).

Rates of hypoglycaemia were also assessed. There was no difference in the time glucose was < 4 mmol/L in the two treatment groups (Figure 1C). Moreover, there was no difference in the percentage of time interstitial glucose was below 4 mmol/L overnight, defined as between 22.00-07.00 hours, in subjects randomized to isophane and aspart versus glargine and aspart (4.4 ± 2.6 vs 3.9 ± 3.0 %, p = 0.89). No patient in either group had severe hypoglycaemia.

6.3.3. Factors affecting glycaemic control on Day 1

As there was marked variability in the time within the target glucose range of 4-10 mmol/L in both groups on Day 1 (Supplemental figure S1), factors that affected glycaemic control were assessed. Despite greater insulin doses on Day 1 (0.97 ± 0.18 vs 0.55 ± 0.01 units/Kg, p <0.001), subjects who had been on insulin prior to study entry spent a greater percentage of time outside the glucose target range (68.3 ± 7.2 vs 39.5 ± 4.1 %, p = 0.002) and had a higher mean glucose concentration (13.0 ± 1.1 vs 9.8 ± 0.5 mmol/L, p = 0.004) than patients not taking insulin before the study. In univariate analyses, the percentage of time outside the target glucose range positively correlated with BMI, HbA1c, mean glucose on Day 0 and prior insulin treatment, but was not significantly correlated with prednisolone dose or CRP (Table 2). These results were not significantly different when insulin regimen was included as a covariate in analyses (data not shown). In a multiple

regression analysis, BMI, mean glucose on day 0 and prior insulin treatment, but not HbA1c, were independently correlated with the percentage of time outside the target glucose range (Table 2). These variables explained 48 % of the variability in time outside the target glucose range.

6.3.4. Changes over three days

This analysis was undertaken in the subgroup of 13 subjects whose hospital admission was of sufficient duration to provide 3 days of CGMS recordings. There was a reduction in prednisolone dose (Figure 3A) and increase in insulin dose (Figure 3B) over time with no significant difference between the groups. However, there were no significant changes over time or between the groups in the percentage of time spent outside the target glucose range (Figure 3C), mean glucose concentration (p=0.30 for change over time, p=0.24 for difference between the groups) or hypoglycaemia (p = 0.21 for change over time, p = 0.96 for difference between the groups).

6.4. Discussion

This study assessed whether an insulin regimen that comprised isophane as the basal insulin prescribed with a greater proportion of aspart between midday and midnight was safer and more effective treatment than a standard glargine-based insulin regimen in hospitalized patients with hyperglycaemia on prednisolone. Contrary to our hypothesis, we did not find an isophane-based regimen had greater efficacy or safety, as the percentage of time glucose was outside the target glucose range of 4-10 mmol/L and glucose < 4 mmol/L were not significantly different with the two insulin regimens. However, the study does provide insight into appropriate insulin doses and dose adjustments in patients on prednisolone with hyperglycaemia and factors that influence the response to insulin therapy.

There have been few prospective studies specifically investigating treatment of hyperglycaemia in patients prescribed glucocorticoids. In patients with type 2 diabetes and respiratory disease treated with methylprednisolone or deflazacort there were no significant differences in glucose control between patients randomized to a glargine or isophane based basal bolus regimen.(Ruiz de Adana et al., 2015) Another study reported additional isophane insulin when glucocorticoids were administered as well as the standard hospital insulin regimen resulted in a lower mean glucose

concentration on the third day of insulin therapy.(Grommesh et al., 2016) In that study patients treated with isophane were prescribed a daily insulin dose that was 16% higher than patients treated with standard hospital insulin.(Grommesh et al., 2016) While this difference in daily insulin dose was not statistically significant, it is likely to have influenced glycaemic control. Consequently, it is not clear whether differences in insulin formulation influence glycaemic control in patients on glucocorticoids with hyperglycaemia.

We tested an insulin regimen for prednisolone-induced hyperglycaemia based on CGMS analysis of glucose concentrations in hospitalized patients taking prednisolone.(Burt et al., 2011) We hypothesized that delivering more insulin during the time period when hyperglycaemia predominates would improve glycaemic control, despite the same overall daily insulin dose. A morning dose of isophane insulin has a pharmacokinetic pattern that reasonably closely approximates the circadian pattern of hyperglycaemia induced by prednisolone. In support of this hypothesis, a retrospective study reported that less insulin was required to provide similar glycaemic control using an isophane-based insulin regimen to treat prednisone-induced hyperglycaemia.(Dhital et al., 2012) The isophane-insulin regimen used in our study also prescribed a greater proportion of short-acting insulin in the afternoon and evening.

In contrast to our hypothesis, there were no significant differences in time outside the target glucose range or mean glucose concentration between the two insulin regimens. There are several possible explanations for this negative result. Firstly, as most patients in our hospital are discharged rapidly, the primary endpoint was glucose control on Day 1 to ensure maximum number of patients were included in the analysis. It is likely that insulin pharmacokinetics become more important during longer-term treatment when insulin doses are closer to being optimized. Secondly, we underestimated the variability in glycaemic response to insulin (Supplemental figure S1), which was more marked than in the smaller cohort in our previous study.(Burt et al., 2011)

Factors that influenced inter-individual variability in glycaemic response on Day 1 were explored. Patients on prior insulin treatment were outside the glucose target range for almost 70% of the time, reflecting substantially poorer glycaemic control than patients who were not taking prior insulin. This demonstrates that a 30% increase in daily insulin dose is insufficient in most insulin-

treated patients with hyperglycaemia on prednisolone. Despite a weight-based dosing regimen, a greater BMI was independently associated with poorer glycaemic control. This may reflect an effect of visceral adiposity, which has been reported to influence the glycaemic response to glucocorticoids.(Darmon et al., 2006) A higher glucose concentration before starting insulin was also associated with poorer glycaemic control. While it may be possible to refine insulin doses based on these factors, they accounted for less than 50 % of the variability in glycaemic response. Other unmeasured variables affecting glucocorticoid metabolism such as 11-beta hydroxy steroid dehydrogenase 1 activity are likely to influence the degree of insulin resistance induced by prednisolone and consequently insulin requirements.(Morgan et al., 2014) A biomarker of glucocorticoid activity could also aid assessment of insulin requirements.(Barclay et al., 2016)

Background glycaemic control and glucocorticoid dose have been considered to be important variables when choosing insulin doses in patients taking glucocorticoids.(Grommesh et al., 2016, Clore and Thurby-Hay, 2009) In our study HbA1c was associated with glycaemic response in a univariate analysis, but was not a significant predictor of response in a multiple regression analysis. There was a weak correlation between HbA1c and day 0 glucose. However, using the collinearity diagnostics in SPSS, there was no collinearity between the variables in the multiple regression model. Consistent with our previous report (Burt et al., 2011) but not others (Gurwitz et al., 1994), there was no association between prednisolone dose and glycaemic control. However, the prednisolone dose range was relatively narrow in this study and this variable is likely to be an important factor if the daily prednisolone dose is reduced below 20 mg.

Minimization of hypoglycaemia is a critical safety factor for an inpatient insulin regimen.(Turchin et al., 2009) We hypothesized that, given its prolonged duration of action, a glargine-based insulin regimen would be associated with overnight hypoglycaemia as the hyperglycaemic effect of prednisolone abated.(Burt et al., 2011) However, there was no difference in daily, overnight or severe hypoglycaemia with the two insulin regimens, with glucose below 4 mmol/L for only 2% of the day. This finding was despite prescribing an initial daily insulin dose of 0.5 units/Kg body weight, which is at the upper end of the recommended dosing range.(Umpierrez et al., 2012) Our study provides reassurance that this starting insulin dose is safe in the vast majority of hospitalized

patients with prednisolone-induced hyperglycaemia.

Finally our study provides insight into appropriate insulin dose adjustments in patients with prednisolone-induced hyperglycaemia. Despite a reduction in prednisolone dose and increase in daily insulin dose, there was no change in percentage of time spent outside the target glucose range or mean glucose over three days. Although this result must be interpreted with caution given the smaller sample size in this analysis, it suggests that greater insulin dose adjustments than employed in this study are required in patients with prednisolone-induced hyperglycaemia. In this study, the supplemental insulin doses were administered as recommended in our local hospital protocol. A higher supplemental insulin dose for hyperglycaemia, in keeping with the latest Endocrine Society guidelines, is probably required in prednisolone-treated patients.(Umpierrez et al., 2012)

There are alternative potential therapeutic approaches to insulin treatment to ameliorate prednisolone-induced hyperglycaemia. Administering prednisolone twice as opposed to once daily reduces mean and peak glucose and glycaemic variability.(Yates et al., 2014) Glucagon-like peptide-1 agonists have been reported to attenuate prednisolone-induced hyperglycaemia in short-term studies, but longer duration studies are lacking.(van Raalte et al., 2011b) These approaches should be explored further in hospitalized patients prescribed prednisolone.

The strengths of our study include the prospective randomized-controlled study design, the inclusion of participants with and without prior history of diabetes and that all participants included had interstitial glucose monitored using CGMS throughout the study period. However, we acknowledge our study has limitations. The main limitations are the short duration of insulin treatment and relatively small sample size given the high degree of interindividual variability, as already outlined in this discussion. Another limitation is that carbohydrate intake was not controlled. However, these limitations reflect routine clinical practice in the hospital setting. Finally, the numbers of patients screened and found ineligible or who declined to participate were not systematically recorded.

In conclusion, prednisolone induced hyperglycaemia predominantly occurs between midday and

midnight and intuitively insulin therapy should be targeted at this time period. However, in the doses used, there were no differences in the efficacy or safety of isophane and glargine-based insulin treatment of prednisolone-induced hyperglycaemia in hospitalized patients. The short duration of hospitalization and marked heterogeneity in the response to insulin therapy are likely to have contributed to the lack of difference between the groups. We recommend a starting daily insulin dose of 0.5 units/Kg in most patients not already taking insulin, greater than a 30% increase in daily insulin dose in patients already taking insulin and larger insulin dose adjustments than utilized in this study, when treating this difficult patient group.

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6.5. Table 1: Patient characteristics

	Isophane + Aspart	Glargine + Aspart	P-value
Number of subjects	25	23	
COAD/Pneumonia/Other* (n)	17 / 2 / 6	17 / 5 / 1	0.18
Age (years)	74 ± 11	70 ± 12	0.29
Female (n, (%))	14 (56)	17 (74)	0.24
BMI (Kg/m^2)	31 ± 7	32 ± 8	0.63
Waist circumference (cm)	113 ± 19	110 ± 19	0.66
C-reactive protein (mg/L)	55 ± 71	35 ± 48	0.28
Known diabetes (n, (%))	19 (76)	15 (65)	0.53
Insulin (n, (%))	5 (20)	5 (22)	1.00
HbA1c (%) (mmol/mol)	7.2 ± 1.2 (55 ± 6)	7.9 ± 2.0 (63 ± 10)	0.12
Mean glucose on day 0 (mmol/L)	12 ± 4	13 ± 4	0.20

*Data are mean ± SD unless otherwise stated; COAD: Chronic obstructive airway disease; *Other: Interstitial lung disease or gout; BMI: Body mass index, HbA1c: Glycosylated haemoglobin*

6.6. Table 2: Univariate and multiple regression analysis showing the relationship between variables and time outside a target glucose concentration range of 4-10 mmol/L

	Univariate		Multivariate	
	R-value	P-value	β coefficient	P-value
BMI	0.35	0.02	0.33	0.004
HbA1c	0.34	0.02	0.04	0.73
Mean glucose (day 0)	0.59	<0.001	0.47	<0.001
Prior insulin treatment	0.43	0.002	0.33	0.005
CRP	-0.07	0.63	NA	NA
Prednisolone dose	0.21	0.15	NA	NA

BMI: Body mass index; HbA1c: Glycosylated haemoglobin; CRP: C-reactive protein; NA = not assessed.

6.7. Figures and Figure legends

Figure 1: The percentage of time spent outside the target glucose range of 4-10 mmol/L (Figure 1A), mean glucose concentration (Figure 1B) and the percentage of time spent with glucose levels < 4 mmol/L (Figure 1C) on Day 1 of treatment, in patients with prednisolone-induced hyperglycaaemia randomised to receive isophane and aspart (white bars) or glargine and aspart (black bars) insulin regimens.

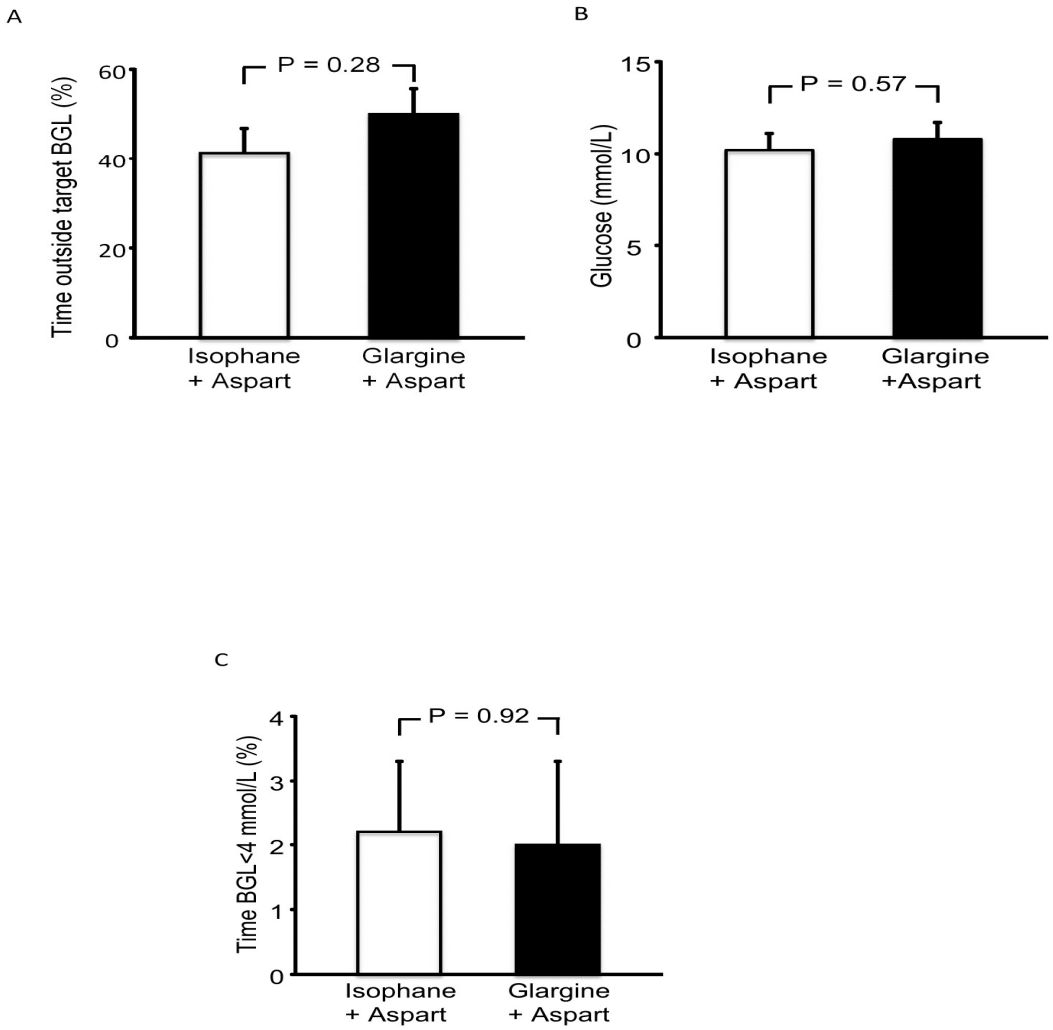


Figure 2*:* Hourly interstitial glucose profile on Day 1 in patients with prednisolone-induced hyperglycaemia randomized to receive isophane and aspart (white lines) or glargine and aspart (black lines) insulin regimens.

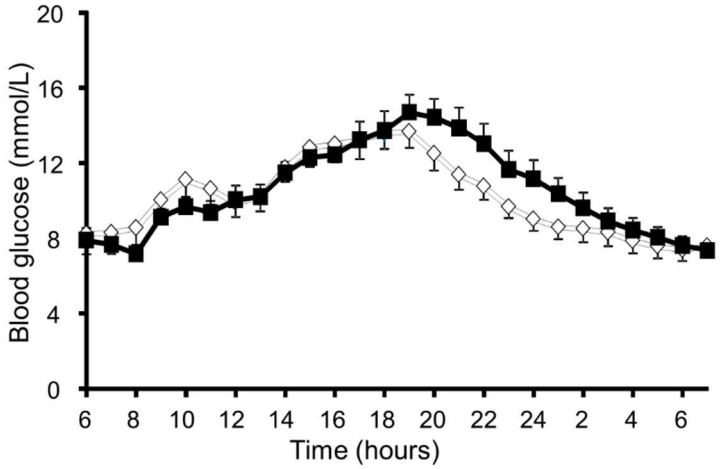
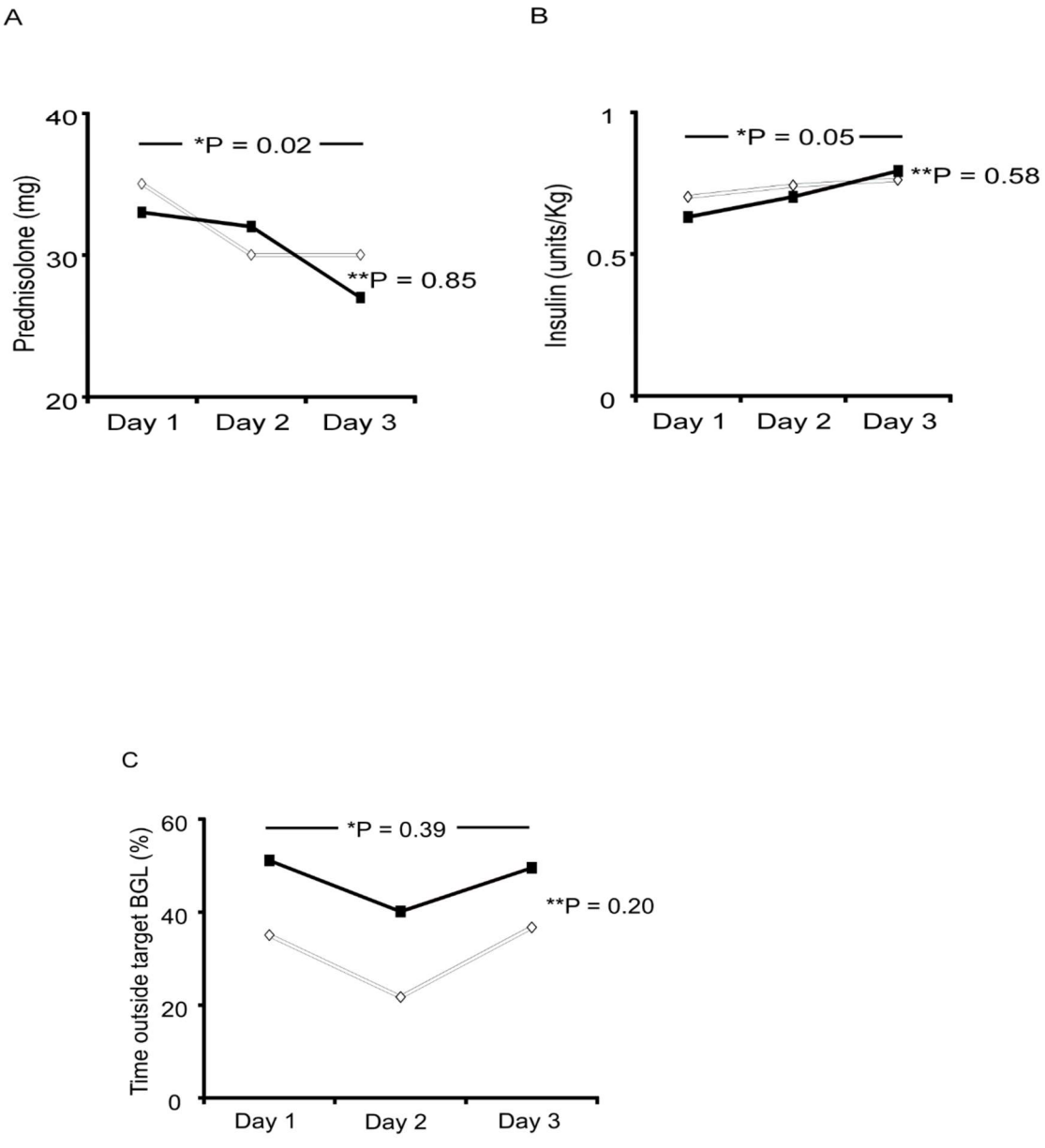
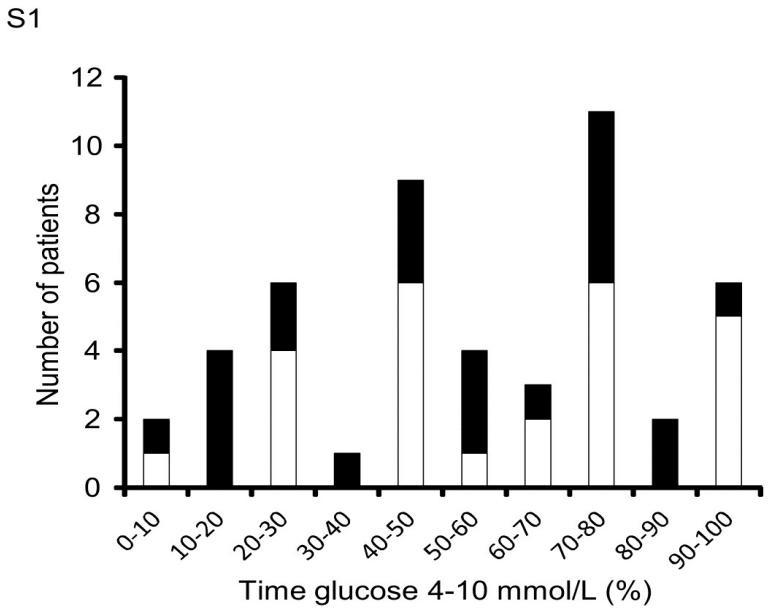


Figure 3: The changes over 3 days of treatment in prednisolone dose (Figure 3A), daily insulin dose (Figure 3B), and percentage of time spent outside the target glucose range of 4-10 mmol/L (Figure 3C) in patients randomised to isophane and aspart (white lines) and glargine and aspart (black lines) insulin regimens. * p value for change over time; ** p value for difference between the groups.



Supplementary figure S1: The percentage of time patients with prednisolone-induced hyperglycemia randomized to isophane and aspart (white bars) or glargine and aspart (black bars) insulin regimens achieved target glycemia (4-10 mmol/L) on Day 1 of treatment.



CHAPTER 7. DISCUSSION

7.1. Introduction

While high dose glucocorticoids are often used to treat an acute exacerbation of an inflammatory illness, when glucocorticoids are prescribed long-term to attenuate inflammatory disease progression the dose is usually lower (e.g prednisolone <10 mg/day) (Fardet et al., 2011). There are limited data on the cardio-metabolic effects of low dose glucocorticoid treatment. A number of conditions for which chronic low dose glucocorticoids are prescribed, such as rheumatoid arthritis, are associated with increased cardiovascular events and mortality. Hence, it is important to understand if low dose glucocorticoid treatment further increases cardiovascular risk in these patients.

Our unit previously demonstrated that older patients with inflammatory rheumatologic disease treated with long term low dose prednisolone have a higher post glucose load glucose, but lower fasting glucose concentration than matched controls who were not taking prednisolone (Burt et al., 2011). We then demonstrated that the glucose elevation was secondary to both hepatic and peripheral insulin resistance (Petersons et al., 2013). In patients with type 2 diabetes mellitus or impaired glucose tolerance, when insulin resistance results in postprandial hyperglycaemia it is more strongly associated with cardiovascular events and vascular dysfunction than when insulin resistance causes fasting hyperglycaemia (Decode Study Group, 2003, Li et al., 2012). Furthermore, vascular dysfunction in these patients may only manifest during the postprandial period (Crandall et al., 2009, Greenfield et al., 2007). At the commencement of this thesis, there were no studies assessing the effects of glucocorticoids on cardiovascular markers in the postprandial period. We hypothesized that assessing the effects of glucocorticoids on postprandial vascular function might provide important insight into their cardiovascular effects and safety.

Endothelial dysfunction, a key event in the pathogenesis of atherosclerosis, occurs early in patients with rheumatoid arthritis. The effect of glucocorticoids on endothelial function is uncertain. Endothelial function was reduced with higher glucocorticoid doses in hypopituitary patients (Petersons et al., 2014) and in glucocorticoid treated patients with IgA nephropathy (Uchida et al.,

2006). However, no significant changes in endothelial function were noted in healthy adults (Brotman et al., 2005) or rheumatoid arthritis patients prescribed glucocorticoids (Hafstrom et al., 2007). These contrasting findings suggest that the effects of glucocorticoids on endothelial function might differ depending on the patient group, the methods used to assess vasodilation, dose and duration of glucocorticoid treatment.

The estimation of arginine metabolites is an alternative method to assess endothelial function. Studies have associated increased levels of methylated arginines (ADMA, MMA and SDMA) (Schulze et al., 2006, Chirinos et al., 2008, Bode-Boger et al., 2006, Wang et al., 2006) and alterations in other arginine metabolites (Marz et al., 2010, Chandrasekharan et al., 2018, Atzler et al., 2013) with endothelial dysfunction and cardiovascular mortality (Zoccali et al., 2001, Miyazaki et al., 1999). Increased ADMA concentrations in rheumatoid arthritis patients have been linked to endothelial dysfunction and impaired endothelial repair (Surdacki et al., 2007, Spasovski et al., 2013). However, little is known about the effects of rheumatoid arthritis on other arginine metabolites or on the effects of therapeutic glucocorticoid treatment on arginine metabolite concentrations. We hypothesized that patients with rheumatoid arthritis have alterations in arginine metabolism that will influence the effect of prednisolone on endothelial function, and that the acute and chronic effects on prednisolone on endothelial function will differ.

The effects of glucocorticoids on postprandial energy and fat metabolism are also unclear. Recent evidence suggests that reduced diet-induced thermogenesis may contribute to increased adiposity and metabolic syndrome (de Jonge and Bray, 1997). While it has been reported that glucocorticoids have minimal effect on resting energy expenditure, at the commencement of this thesis there were no studies assessing the effects of glucocorticoids on diet-induced thermogenesis. Moreover, most studies assessing the effects of glucocorticoids on fat metabolism have been performed using high dose glucocorticoid infusions and hyperinsulinaemic clamps; conditions which differ from common clinical glucocorticoid use. Defining the perturbations of postprandial energy and fat metabolism associated with typical therapeutic glucocorticoid doses will help determine the contribution of glucocorticoids to adiposity and consequently cardiovascular risk.

Hyperglycaemia is a common side effect in patients treated with medium to high dose glucocorticoids (Hougardy et al., 2000). Our unit previously demonstrated that a morning dose of prednisolone, as commonly prescribed, has little effect on overnight glucose concentration and predominantly causes hyperglycaemia in the afternoon and evening (Burt et al., 2011). A similar pattern of hyperglycaemia was also demonstrated in patients on prednisolone prescribed glargine based basal-bolus insulin (Burt et al., 2015). We hypothesized that prescribing isophane insulin as the basal insulin might better treat day-time hyperglycaemia and reduce the risk of overnight hypoglycaemia in prednisolone-treated patients as its pharmacokinetics approximate the circadian pattern of prednisolone-induced hyperglycaemia.

For these reasons, this thesis aimed to determine:

1) Whether low dose prednisolone causes postprandial vascular dysfunction.
2) The effect of rheumatoid arthritis on arginine metabolism and the effects of low dose prednisolone on arginine metabolism in these patients.
3) The effect of low dose prednisolone on postprandial energy and substrate metabolism.
4) Whether an insulin regimen comprising insulin isophane and prandial aspart is safer and more effective treatment than insulin glargine and prandial aspart in hospitalised patients with prednisolone-induced hyperglycaemia.

7.2. Summary and recommendations

I will now address each aim of this thesis in turn. The first aim of this thesis was to determine the effects of low dose prednisolone on postprandial vascular function. Consistent with previous studies (Petersons et al., 2013, Burt et al., 2012), I demonstrated that low dose prednisolone increased postprandial glucose concentration secondary to a reduction in insulin sensitivity. However, the reduction in insulin sensitivity was not associated with adverse changes in fasting or postprandial vascular function (Chapter 3). In fact, acute prednisolone caused a greater postprandial fall in augmentation index, suggestive of reduced arterial stiffness, secondary to reduced sympathetic activity. There was no significant change in augmentation index in patients

taking chronic prednisolone users. No significant change in reactive hyperaemia index, a marker of endothelial function, was noted after acute prednisolone. In patients on chronic prednisolone, there was an attenuated postprandial fall in reactive hyperaemia index suggestive of better endothelial function that almost reached statistical significance. These findings suggest that in contrast to the relationship in the general population, postprandial hyperglycaemia is not associated with vascular dysfunction in patients with rheumatoid arthritis prescribed low dose prednisolone.

The next aim of this thesis was to assess whether patients with rheumatoid arthritis have perturbations in arginine metabolism and then to assess the acute and chronic effects of low dose prednisolone on arginine metabolism in this patient group. I demonstrated that patients with rheumatoid arthritis have elevated concentrations of ADMA and MMA, endogenous inhibitors of e-NOS. Acute prednisolone treatment resulted in a small reduction in homoarginine, but there were no significant changes in other arginine metabolites. In contrast, rheumatoid arthritis patients on chronic prednisolone treatment had significantly lower concentrations of ADMA and SDMA than patients not on prednisolone. These findings suggest that rheumatoid arthritis *per se* is associated with an increase in plasma concentrations of endogenous inhibitors of nitric oxide synthase, which are likely to contribute to endothelial dysfunction. The reduction in ADMA and SDMA with chronic, but not acute, prednisolone could provide a mechanism that explains why clinical measures of endothelial function improves with chronic, but not acute, prednisolone in this patient group and explain the results reported in Chapter 3.

The third study included in this thesis assessed the acute and chronic effects of low dose prednisolone on postprandial energy and substrate metabolism in patients with rheumatoid arthritis. Energy and substrate metabolism were assessed in the fasting state and after a mixed meal using indirect calorimetry as detailed in Chapter 5. This study demonstrated that neither acute nor chronic prednisolone significantly affected resting energy expenditure or diet-induced thermogenesis. In keeping with previous studies (Burt et al., 2007a), no changes in fasting fat or carbohydrate oxidation was noted with prednisolone. However, prednisolone attenuated postprandial suppression of fat oxidation and increased carbohydrate oxidation. These changes in postprandial substrate metabolism have been called metabolic inflexibility and occur in patients

with skeletal muscle insulin resistance. Chronic, but not acute, prednisolone increased fasting NEFA and adipocyte insulin resistance index, indicative of resistance to insulin mediated suppression of lipolysis. However, during postprandial hyperinsulinaemia, insulin mediated suppression of NEFA was unaltered by low dose prednisolone. My findings suggest that the degree of glucocorticoid induced insulin resistance is tissue-specific, with glucocorticoids inducing less insulin resistance in adipose tissue than in skeletal muscle.

The final aim of this thesis was to determine whether an insulin regimen comprising insulin isophane and prandial aspart is a safer and more effective treatment than insulin glargine and prandial aspart in hospitalised patients with prednisolone-induced hyperglycaemia. An open labelled stratified randomized controlled study was conducted including 50 consecutive hospitalized patients with hyperglycaemia on oral prednisolone ≥ 20 mg/day as a single morning dose for an acute medical condition as detailed in Chapter 6. Subjects were randomized to insulin isophane and aspart or insulin glargine and aspart and insulin doses were titrated on a daily basis. Glycaemic control was assessed using CGMS in all participants. No significant differences were noted in the percentage of time glucose was outside the target range of 4-10 mmol/L and glucose < 4 mmol/L between the two treatment groups. We did note that there was the marked inter-individual variability in glycaemic response to a standard insulin dose in both treatment groups. Patients on prior insulin treatment were outside the glucose target range for almost 70% of the time, demonstrating that a 30% increase in daily insulin dose is insufficient in most insulin-treated patients with hyperglycaemia on prednisolone. Greater BMI and higher blood glucose levels prior to insulin initiation also correlated with poor glycaemic control. However, these factors together only contributed to less than 50 % of the variability in glycaemic response.

In conclusion, even at low doses, glucocorticoids reduce insulin sensitivity and have adverse effects on fat metabolism. However, in patients with rheumatoid arthritis, prednisolone-induced insulin resistance is not associated with increased arterial stiffness or endothelial dysfunction. This may be because prednisolone also reduces endogenous inhibitors of NO synthesis, which are increased in patients with rheumatoid arthritis. My studies provide some confidence that low dose prednisolone can be used to attenuate disease progression in rheumatoid arthritis without

significantly increasing cardiovascular risk.

Attenuated postprandial suppression of fat oxidation, but not lipolysis, suggests that prednisolone causes greater insulin resistance in skeletal muscle than in adipocytes. Therapies targeting these changes in fat metabolism could potentially reduce insulin resistance and the associated metabolic consequences of glucocorticoid therapy.

When using a body weight-based dosing regimen there were no differences in the efficacy or safety of isophane- and glargine-based insulin treatment of prednisolone-induced hyperglycaemia in hospitalized patients. The short duration of hospitalization and marked heterogeneity in the response to insulin therapy are likely to have contributed to the lack of difference between the groups. My study did demonstrate that a starting daily insulin dose of 0.5 units/Kg is safe in nearly all patients who are not already taking insulin, but that a 30% increase in daily insulin dose is insufficient in patients already prescribed insulin.

7.3. Future directions

Cushing's syndrome and exogenous glucocorticoids have been associated with increased cardiovascular risk. The studies included in this thesis and earlier studies published by our unit (Petersons et al., 2013, Petersons et al., 2017) have shown that mild glucocorticoid excess and small differences in glucocorticoid dose, of a similar order of magnitude to variability in endogenous glucocorticoid production, cause insulin resistance and adverse effects on fat metabolism. Whether these metabolic side effects of mild glucocorticoid excess occur in subjects with higher endogenous cortisol production requires further investigation. Characterisation of the hypothalamic pituitary adrenal (HPA) axis activity has shown inter-individual variability in cortisol secretion, providing evidence that glucocorticoid tone varies between individuals, even in the absence of endocrinopathy (Girod and Brotman, 2004). Babies with low birth weight have changes in foetal programming that cause long-term hyperactivity of the HPA axis, which is likely to contribute to increased rates of cardio-metabolic disease (Reynolds et al., 2001). Several studies have also shown a relationship between higher endogenous glucocorticoid secretion and glucose intolerance, hypertension, dyslipidaemia and increased cardiovascular mortality (Reynolds, 2013).

However, how increased HPA activity causes increased cardio-metabolic risk is not fully defined. Glucocorticoid-induced insulin resistance, and consequent vascular dysfunction, could be a potential mechanism that links HPA hyperactivity and cardio-metabolic risk, and should be investigated.

I reported no adverse changes in cardiovascular markers in rheumatoid arthritis patients prescribed low dose prednisolone. It is possible that the cardiovascular adverse effects with low dose glucocorticoids are better demonstrated by alternative techniques like cardiac magnetic resonance imaging. Additional research using gold standard cardiovascular research tools like cardiac MRI are required before concluding that low dose glucocorticoid treatment does not further increase cardiovascular risk in this high risk patient cohort.

I demonstrated no changes in DIT with acute or chronic prednisolone treatment. However, I studied the effects of glucocorticoids on energy expenditure in elderly patients with rheumatoid arthritis, as they are the most common cohort of patients in whom low dose prednisolone is prescribed. Recent evidence suggests that brown adipose tissue may contribute to post prandial energy metabolism (Orava et al., 2011). The effects of brown adipose tissue are more evident in younger individuals and when studied at lower than ambient temperatures. Further studies are required involving younger adults to clarify if alteration in DIT plays a role in glucocorticoid induced adiposity. Furthermore, my results may not apply to patients on higher glucocorticoid doses.

Hyperglycaemia in hospitalized patients has been linked to increased morbidity and mortality (Umpierrez et al., 2002, Capes et al., 2000, Burt et al., 2013b). Our unit had earlier shown that prednisolone induces a distinct circadian pattern of hyperglycaemia with glucose elevations mainly in the afternoon and evening (Burt et al., 2011). However, my study demonstrated that a therapeutic approach based on matching insulin pharmacokinetics to this circadian pattern of hyperglycaemia was not superior to conventional basal bolus insulin. The reasons for this negative result could be the wide variability in the therapeutic response to a body weight-based insulin dose and also because the patients were discharged from hospital quickly and insulin pharmacokinetics is likely to be more important when the insulin dose is near optimal. Further studies are required to identify the contribution of variability in glucocorticoid metabolism by 11-beta hydroxyl steroid

dehydrogenase 1 to glucocorticoid-induced insulin resistance and subsequently insulin requirements. Development of biomarkers of glucocorticoid activity would also aid in defining insulin requirements in glucocorticoid treated patients. It will also be important to assess the efficacy of newer therapeutic agents like GLP-1 agonists in the treatment of glucocorticoid-induced hyperglycaemia.

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