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Liver enzymes but not free fatty acid levels predict markers of insulin sensitivity in overweight and obese, non-diabetic subjects.

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Abbreviations

ALP: alkaline phosphatase

ALT: alanine transaminase

AST: aspartate transaminase

BMI: body mass index

CV: coefficients of variation

DHA: docosaexaenoic acid

eGFR: estimated glomerular filtration rate

EPA: eicosapentaenoic acid

FFA: free fatty acids

FISK: Fatigue Impact Scale

GGT: gamma-glutamyl transpeptidase

HOMA: Homeostasis Model of Assessment

ICAM-1: intercellular adhesion molecule 1

IPAQ: International Physical Activity Questionnaire

IR: insulin resistance

NQSP: National Glycohemoglobin Standardization Program

LCPUFA: polyunsaturated fatty acids

r: spearman's correlation coefficients

T2DM: type 2 diabetes mellitus

WC: waist circumference

WHpR: waist to hip ratio

WHtR: waist to height ratio

Abstract

Whilst obesity is a key predisposing risk factor in the development of insulin resistance and type 2 diabetes mellitus, not all obese subjects develop insulin resistance. This study aimed to identify key anthropometric and biochemical parameters that predict insulin sensitivity in overweight and obese adults. Based on previous literature, we hypothesised that markers of insulin sensitivity would be negatively correlated with plasma concentrations of free fatty acids (FFA) and liver enzymes. Forty non-diabetic adult subjects (body mass index (BMI) ≥ 25.0kg/m^2) were recruited. Data collection included anthropometric measurements and fasting plasma samples for the quantification of liver enzymes (ALT, AST, GGT), blood lipid profile and markers of insulin sensitivity. Questionnaires relating to dietary intake, physical activity and fatigue were also completed. Insulin and Homeostasis Model of Assessment (HOMA) scores were significantly correlated with indirect measures of central obesity ($P<0.05$). Glycosylated haemoglobin, insulin and HOMA scores for Insulin Resistance (IR) were all positively correlated with selected liver function markers ($P<0.05$). HOMA-IR scores were significantly positively correlated with and plasma phospholipid levels of n-3 fatty acids ($P=0.04$) and n-3:n-6 ratio ($P<0.05$) and negatively correlated with n-6 fatty acids ($P=0.03$). No significant correlations were found between markers of insulin sensitivity and cholesterol levels, physical activity or self-reported fatigue. These results have reinforced the integral role of liver function in development of insulin resistance. Despite previous data linking elevations in FFA to the development of insulin resistance, we found no relationship between these variables in this study.

Keywords: human, obesity, insulin, liver, n-3 fatty acids, n-6 fatty acids.

1. Introduction

Obesity is thought to have a causal effect on the development of skeletal muscle insulin resistance and the subsequent development of type 2 diabetes mellitus (T2DM)[1]. Central adiposity is one of the key risk factors for the development of insulin resistance and progression towards T2DM and forms the basis of the portal/visceral hypothesis for the development of insulin resistance[2]. According to this hypothesis, increased adiposity (specifically visceral adiposity), leads to greater movement of free fatty acids (FFA) and inhibition of insulin action via Randle's effect in insulin-sensitive tissues[3]. Thus, the presence of an increased supply of FFA reduces muscle glucose utilisation, leading to reduced glucose clearance in the periphery and enhancing insulin secretion. In addition, the prolonged lipotoxic effect of these fatty acids on the pancreatic β-cells is posited to link obesity, insulin resistance and development of T2DM[4]. With recent literature questioning the validity of this hypothesis, alternative theories including the ectopic fat storage syndrome[5] and the endocrine paradigm[6] have been proposed. Prior to the onset of diabetes, insulin resistant individuals are able to compensate for increases in blood glucose by increased insulin secretion by pancreatic β cells. T2DM develops when this process is no longer sufficient to maintain normal fasting levels of blood glucose[7].

Healthy diet and regular physical activity can prevent or delay the onset of T2DM[8]. Whilst lipids have a greater energy density per gram than carbohydrates or proteins, selective intake of specific fatty acids, in particular the n-3 and n-6 polyunsaturated fatty acids (PUFA), can differentially impact upon satiety and adiposity through their influence on adipokine production[9, 10]. Both the n-3 and n-6 PUFA are essential fatty acids, that is, they must be obtained from dietary or supplementary sources[11]. While intakes of sufficient quantities of both n-3 and n-6 PUFA is necessary for the maintenance of optimal health[11], it also

appears that n-3 and n-6 PUFA have distinct biological actions in relation to metabolic health.

Previous studies have reported that increased n-3 PUFA status is associated with a lower body mass index (BMI) and waist circumference (WC), suggesting that the n-3 fatty acids may be protective against weight gain, abdominal obesity[12] and the effects of non-alcoholic fatty liver disease[13]. Similarly, n-3 fatty acids stimulate production of the insulin-sensitising hormone, adiponectin, which would be expected to have a positive effect on insulin sensitivity[14]. In contrast, there have been no reports linking increased n-6 PUFA intake with markers of metabolic health. Enhanced n-6:n-3 ratios are believed to result in a pro-inflammatory state[15], favouring the development metabolic syndrome and associated conditions such as non-alcoholic fatty liver disease[16].

Increased fatigue levels have also been correlated positively with percentage body fat[17] and obesity[18-20]. Further, self-reported fatigue is negatively associated with the likelihood of getting recommended levels of physical activity[21]. Thus, obesity-associated increases in fatigue are hypothesised to play an integral role in poor weight management, and restricted physical activity levels[20]. This has further ramifications for self-esteem and eventually the health system, work force and the economy[22] making primary prevention of obesity and subsequent fatigue a necessity.

Whilst obesity remains a key predisposing risk factor in the development of insulin resistance, the reasons why not all obese subjects progress to develop insulin resistance remain unclear. Based on previous literature, we hypothesised that markers of insulin sensitivity would be positively correlated with plasma FFA levels and liver enzymes and negatively correlated to plasma levels of n-3 PUFA and the n-3:n-6 PUFA ratio. This study aims to identify key anthropometric and biochemical parameters that predict insulin

sensitivity in overweight and obese adults with potential implications for the future identification of at risk individuals and future weight management strategies. With a focus on identifying parameters that may indicate increased risk of insulin sensitivity within the general population, consideration must be given to both male and female participants from broad age range.

2. Methods

2.1 Subjects

Forty subjects were recruited from within the greater Brisbane area for a study assessing the effects of n-3 fatty acid supplementation on adipokine levels. Equal proportions of males and females were recruited, with a total of 10 overweight (5 males, 5 females) and 30 obese subjects (15 males, 15 females) , between 18 and 80 years of age. Study criteria required that subjects have a BMI $\geq$25.0 kg/m^2. Exclusion criteria included previous diagnosis with type 1 or type 2 diabetes mellitus, weight loss of more than 10% body weight within the last six months, active substance abuse (alcohol or drug dependency), smoking, breast feeding or pregnancy, concomitant use cholesterol medications or nutritional supplements. Written informed consent was obtained from all subjects.

2.2 Study Design

Upon recruitment, subjects' anthropometric measures were taken and fasting blood samples collected. Anthropometric measures included height, weight, WC and hip circumference. WC was measured to the nearest 0.1 cm at the umbilicus level. Hip circumference was measured to the nearest 0.1cm at the widest point between the iliac crest and buttock. BMI was calculated as weight in kilograms divided by the square of the height in meters, squared (kg/m^2). BMI groups were based on World Health Organisation classifications for adults[23]. An overweight BMI range was categorised as 25.0 kg/m^2 $\leq$ BMI $>$ 30.0 kg/m^2 and a

BMI≥30.0 kg/m^2 was classified as obese. Fasting blood samples were utilised for the quantification of adipose hormones (total adiponectin and leptin), markers of insulin sensitivity, electrolyte and liver function, cholesterol, FFA and plasma phospholipid fatty acids. Homeostasis Model of Assessment (HOMA) scores for Insulin Resistance (IR) and pancreatic beta cell function (β) were calculated based on fasting glucose and insulin levels (HOMA-IR = (fasting plasma insulin x fasting plasma glucose)/22.5; HOMA-β = (20 x fasting plasma insulin)/(fasting plasma glucose- 3.5))[24]. It was not possible to carry out all analyses in every individual due to the inability to collect a sufficient volume of blood from some individual participants to conduct every assay. In this case a subset of the analyses were performed for this participant.

Each subject also completed a validated 72hour dietary recall[25] and International Physical Activity Questionnaires (IPAQ)[26-28] at this time. Dietary records were analysed in Foodworks to determine the mean reported energy intake for each subject (2007; Xyris Software, Brisbane, Australia). Self-reported fatigue was measured using the Fatigue Impact Scale (FISK) which examines patient perceptions of the functional limitations imposed by fatigue on cognitive, physical, and psychosocial functioning[29].

The study design was approved by Metro South Human Research Ethics Committee (HREC/10/QPAH/141) and The University of Queensland Medical Research Ethics Committee (2010001200).

2.4 Assays

Adiponectin and and intercellular adhesion molecule 1 (ICAM-1) were measured by Cardinal Bioresearch, Brisbane, utilising Multiplex ELISA techniques on a Luminex platform. These assays were performed with Human Adiponectin/Acrp30[30] and Human ICAM-1/CD54 Biotinylated Affinity Purified PAb antibodies[31] supplied by R&D Systems, following the

standard procedures outlined in the corresponding Human Obesity MultiAnalyte Profiling Base Kit[32]. Acylated ghrelin was measured utilising Millipore Human Ghrelin (active) ELISA kits supplied by Abacus ALS, Australia[33]. FFA were measured using a NEFA assay from Wako Diagnostics in Osaka, Japan[34]. Plasma phospholipid fatty acid concentrations were measured by gas chromatography in conjunction with FOODplus Research Centre at The University of Adelaide[14]. All other pathological markers were analysed by Pathology Queensland (Princess Alexandra Hospital, Brisbane).

2.5 Statistical Analyses

All data are expressed as means ± standard deviation. Differences between overweight and obese groups for each parameter were assessed using standard t-tests. Relationships between variables were assessed by nonparametric correlation analysis (Spearman's rank correlation coefficient). P values <0.05 were considered of statistical significance. All statistical analyses were performed in SPSS version 20.0.

3. Results

Mean and standard deviation data for anthropometric measures, markers of insulin sensitivity and selected metabolic parameters are summarised for overweight and obese participants in Table 1. Subjects were aged between 23 and 79 years with a mean BMI of 32.8kg/m^2. Indirect measures of adipose tissue distribution including WC, waist:height ratio (WHtR) and waist:hip ratio (WHpR) were higher in obese than overweight subjects. There were no significant differences between overweight and obese groups in relation to plasma levels of glucose (P=0.22), insulin (P=0.49), HbA1C (National Glycohemoglobin Standardization Program (NGSP); P=0.20) or HOMA-IR (P=0.97).

Mean reported energy intake for the subjects was 4246 ± 1930 kJ. No significant difference was observed between the mean energy intake of overweight and obese subjects (P=0.57).

Mean self-reported physical activity level across all subjects was 4585.2 ±6517.3 MET-minutes/week. Mean total self-reported fatigue scores as determined by FISK Questionnaires was 32.0 ± 29.2, on a scale of 0 (No Problem) to 160 (Extreme Problem). No significant difference in physical activity (as measured by IPAQ) or fatigue scores were observed between overweight and obese subjects (P=0.91 and P=0.44 respectively).

Spearman's correlation coefficients (r) and coefficients of variation (CV) for plasma glucose concentrations are shown in Table 2. There were no statistically significant correlations observed between plasma glucose levels and anthropometric parameters including BMI or waist circumference. Plasma glucose concentrations were positively correlated with plasma phospholipid proportions of total n-3 PUFA (r=0.40, P=0.01), eicosapentaenoic acid (EPA; r=0.48, P<0.01), docosaexaenoic acid (DHA; r=0.33, P=0.04) and the n-3:n-6 ratio (r=0.40, P=0.01). Conversely, glucose levels were inversely correlated with n-6 fatty acid content of plasma phospholipids (r=-0.33, P=0.04). Other biochemical markers associated with glucose included calcium (inverse relationship; r=-0.37, P=0.02) and acylated ghrelin (positive relationship; r=0.34, P=0.04).

Spearman's correlation coefficients (r) and coefficients of variation (CV) for plasma insulin concentrations are also summarised in Table 2. Positive correlations were observed between insulin and anthropometric measures included waist circumference (r=0.61, P<0.01) and waist:height ratio (WHtR; r=0.48, P=0.01). Of further interest, insulin was positively correlated with a number of electrolyte and liver function parameters including alanine transaminase (ALT; r=0.44, P=0.02), gamma-glutamyl transpeptidase (GGT; r=0.49, P<0.01) and estimated glomerular filtration rate (eGFR; r=0.56, P=0.02). Correlations between insulin and other metabolic parameters included an inverse relationship to adiponectin (r=-0.49,

P<0.01) and positive relationships with ICAM-1 (r=0.49, P<0.01) and plasma phospholipid percentages of total saturated fatty acids (r=0.56, P<0.01).

Spearman's correlation coefficients (r) and coefficients of variation (CV) for glycosylated haemoglobin levels (HbA1c (NGSP)) are also summarised in Table 2. HbA1C (NGSP) was correlated with a number of liver enzymes including alkaline phosphatase (ALP; r=0.47, P<0.01), ALT (r=0.44, P<0.01), aspartate transaminase (AST; r=0.42, P<0.01), GGT (r=0.38, P=0.02) and inversely correlated with eGFR (r=-0.50, P=0.498).

HOMA-IR and HOMA-β scores, derived from calculations pertaining to fasting glucose and insulin levels, were also assessed for correlations with anthropometric and metabolic parameters. A summary of the results from Spearman's correlations of HOMA-IR scores are shown in Table 3. Significant correlations were observed between HOMA-IR and anthropometric parameters including BMI (r=0.38, P=0.04), WC (r=0.60, P<0.001) and WHtR (r=0.50, P<0.01), whilst HOMA-β was only significantly correlated with WC (r=0.41, P=0.03).Of the electrolyte and liver function parameters, HOMA-IR was significantly positively correlated with GGT (r=0.39, P=0.04) and eGFR (r=0.57, P=0.02), with inverse correlations to phosphate (r=-0.39, P=0.04). Conversely, HOMA-β was positively correlated with ALT (r=0.54, P<0.01), AST(r=0.44, P=0.02) and GGT (r=0.55, P<0.01). No direct correlations were observed between HOMA-IR and cholesterol, however, HOMA-β was significantly inversely correlated with LDL cholesterol (r=-0.43, P=0.02).

As with glucose, significant positive correlations were observed between HOMA-IR scores and selected plasma phospholipid percentages including total saturated fatty acids (r=0.55, P<0.01), total n-3 fatty acids (r=0.38, P=0.04), EPA (r=0.42, P=0.02) and the n-3:n-6 ratio (r=0.37, P=0.046), with inverse correlations to total n-6 percentages (r=-0.40, P=0.03). Conversely, the only significant correlations observed between plasma phospholipids and

HOMA-β were with the total saturated fatty acid content (r=0.41, P=0.03). Additional negative correlations were observed between HOMA-IR and adiponectin (r=-0.46, P=0.01), as well as a positive correlation with ICAM-1 (r=0.43, P=0.02). HOMA-β was negatively correlated with adiponectin (r=-0.42, P=0.02) and acylated ghrelin (r=-0.38, P=0.0499) yet positively correlated with ICAM-1 (r=0.43, P=0.02).

No significant correlations were observed between cholesterol measures (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides) and markers of insulin sensitivity including glucose, insulin, HbA1C and HOMA-IR ($P \geq 0.05$). No significant correlations were observed between plasma free fatty acid levels and markers of insulin sensitivity including glucose (r=-0.18, P=0.27), insulin (r=0.01, P=0.95), HbA1C (NGSP; r=-0.06, P=0.71) and HOMA-IR (r=-0.08, P=0.69).

Of further interest, self-reported fatigue levels were inversely correlated with plasma phosphate concentrations (r=-0.33, P=0.04) and positively correlated with C-reactive protein (r=0.57, P<0.01). Within this cohort, no significant correlations were observed between self-reported fatigue and anthropometric markers, such as BMI (r=0.17, P=0.28), glucose (r=-0.24, P=0.14), HbA1C (NGSP; r=-0.02, P=0.31) or HOMA-IR (r=0.18, P=0.35).

4. Discussion

Obesity is a key pre-disposing risk factor in the development of insulin resistance and T2DM, however, not all obese subjects develop insulin resistance. This study aimed to identify key anthropometric and biochemical parameters that predict insulin sensitivity in overweight and obese adults.

Previous studies suggest that central adiposity is associated with an increased risk of insulin resistance and T2DM[3]. WC serves as simple, more convenient means of approximating central obesity[35]. Thus, correlations between markers of insulin sensitivity (insulin and

HOMA-IR scores) and both WC and WHtR support the association between central adiposity and insulin resistance within overweight and obese subjects.

The liver plays an integral role in maintaining normal glucose concentrations[36]. Following the development of hepatic insulin resistance, insulin is no longer able to suppress hepatic glucose production, resulting in elevated blood glucose concentrations[36]. In cases of cirrhosis, liver transplantation has been shown to improve whole-body insulin sensitivity, highlighting the important role of the liver in the regulation of glucose homeostasis[37]. In the present study, we also found correlations between selected electrolyte and liver function parameters and markers of insulin sensitivity in overweight and obese subjects. Specifically, insulin levels were positively correlated with ALT, and HOMA-IR was positively correlated with GGT. Both HbA1C and HOMA-β were correlated positively with ALT and GGT. These results are consistent with previous studies suggesting that these two enzymes are important biomarkers for metabolic syndrome[38, 39], and elevated levels of ALT[40] and GGT[41] have previously been associated with later development of diabetes. ALT and GGT have been also been inversely correlated with adiponectin levels. As adiponectin levels are also inversely correlated with obesity[42], it is plausible that adiponectin may provide a link between elevations in liver enzymes and hyperinsulinemia in obesity, as well as the subsequent development of metabolic syndrome.

The importance of adipose tissue in the regulation of metabolic processes including insulin metabolism was clearly demonstrated through significant inverse correlations between adiponectin levels and both insulin and HOMA-IR. This is not surprising given that adiponectin levels decrease as the degree of adiposity increases and this hormone is also known to promote glucose uptake in the liver and skeletal muscle[43], with low adiponectin concentrations linked to T2DM[44]. Concurrent correlations were also observed between

markers of insulin sensitivity (insulin and HOMA-IR scores) and inflammatory molecule ICAM-1, further supporting previous data pertaining to correlations between inflammation, adiponectin levels and the subsequent development of insulin resistance[45].

Ghrelin is a gut-derived hormone which, in addition to its role as a hunger signal, also has an established role in the regulation of fat mass and glucose homeostasis[5]. This is supported by the results of the present study, which identified a significant positive correlation between acylated ghrelin and both glucose and HOMA-β.

It is clear that the relative proportions of fatty acids incorporated within the phospholipids are related to these markers of insulin sensitivity, specifically noting inverse correlations between markers of insulin sensitivity (glucose and HOMA-IR) with n-6 fatty acids and positive correlations with total n-3 fatty acids, EPA, DHA and the ratio of n-3:n-6 fatty acids. Given these correlations, it would therefore be plausible that supplementing with n-3 fatty acids may result in increased glucose and HOMA-IR scores in obese participants whilst n-6 fatty acids would have the opposite effect. Results of previous studies have demonstrated insufficient evidence to suggest impaired glycaemic control associated with use of n-3 in patients with T2DM, showing no significant changes in fasting glucose, fasting insulin or body weight[46]. This is of particular interest, given that n-3 has been shown to increase adiponectin levels in obese subjects[47] and increased adiponectin levels are associated with improvements in insulin sensitivity[43, 48]. Further research is required in order to fully understand these apparently conflicting results.

Previous literature suggests that mobilisation of FFA in the circulation also promote insulin resistance[49]. By contrast, this study found no direct correlations between FFA and markers of insulin sensitivity. Further, there were no clear correlations between markers of insulin sensitivity (glucose, insulin, HbA1C or HOMA scores) and physical activity or self-reported

fatigue. Fatigue scores were correlated with C-Reactive Protein suggesting that inflammation may play a role, though there was no significant correlation to ICAM-1. Further investigation is still needed to better understand and treat fatigue in obesity, given the significant limitations this has on quality of life[50], weight management[20] as well as the ability to maintain the recommended levels of physical activity in obese subjects[21].

These results support previous literature regarding the integral role of the liver in the development of insulin resistance. Significant correlations are observed between markers of insulin sensitivity and liver enzymes as well as adipose derived hormone adiponectin and inflammatory marker ICAM-1 suggesting a plausible link between liver function, adiposity and the development of insulin resistance. Despite previous data linking elevations in FFA to the development of insulin resistance, there were no significant correlations between these factors observed in overweight and obese subjects. Thus, authors partially accept the original hypothesis, noting that markers of insulin sensitivity were positively correlated with liver enzymes but not plasma FFA levels.

Positive correlations to n-3 fatty acids and markers of insulin sensitivity suggests that theoretically, caution may be warranted when supplementing obese participants with these fatty acids though previous literature does not indicate significant adverse clinical implications related to n-3 intake. Additional correlations observed between markers of insulin sensitivity and n-6 fatty acids as well as n-3:n-6 ratios provide alternate directions for future research.

There are limitations inherent within the design and outcomes of any study. Whilst the sample size of this study was sufficient in meeting the desired aims, future trials incorporating a larger cohort would allow for additional sub-analyses for variables such as age and gender. In addition, all subjects were drawn from the same region, it is therefore

plausible that this population may not be indicative of other geographical locations. In relation to age, ethnicity and socio-economic status, the characteristics of the subjects indicate that they were relatively representative of an urban Australian population. Finally, the correlations between anthropometric/biochemical parameters and adipose hormones or markers of insulin sensitivity do not necessarily indicate the existence of a causal relationship. Thus, further intervention studies are necessary to investigate the extent of the clinical implications associated with these findings.

Insulin resistance and the subsequent development of T2DM remain primarily lifestyle disorders. With correlations to liver function and plasma phospholipid fatty acid concentrations it would follow that these parameters may serve as markers for identifying overweight and obese individuals at a greater risk of developing insulin resistance as well as potential therapeutic targets though further randomised clinical trials are needed.

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