METABOLIC PROFILING AND IDENTIFICATION OF
BIOMARKERS FOR WEIGHT LOSS

by

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A thesis submission in partial fulfilment of the degree of

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DECLARATION

This thesis is submitted to the University of Warwick in support of my application for the degree of Master of Philosophy/Doctor of Philosophy (M.Phil/PhD). It has been composed by me and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by myself except in the cases outlined below:

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Parts of this thesis have been published by the author:


Part of this thesis have been presented at conferences, either as poster or as oral presentations. The references for the conference proceeding abstracts are listed below:


ABSTRACT

Obesity is a worldwide rising pandemic disease and a great challenge for healthcare systems due to its associated disorders. Exercise, drugs and all sorts of dietary plans are typical weight loss options that overweight or obese individuals are offered besides more radical actions such as bariatric surgery. However, weight loss is sometimes incremental, especially at the very start of a regimen and this often leads to the patient dropping-out of a dietary program. Hence, there is a need to identify biomarkers that are affected over a 24-hour interval and use them to provide quantitative biofeedback on the efficiency of a diet. It would also allow personalised optimisation of dieting parameters with relevance to short-term. There is also a need to understand the effects of various food on these biomarkers and the mechanisms by which the food affects adipose tissue metabolism in particular.

This thesis describes for the first time the identification of insulin, lactate and angiotensin converting enzyme (ACE) as potential biomarkers of weight loss. Decreased levels of these biomarkers were observed in the urine of individuals following a low-calorie diet. Importantly, these changes preceded weight loss and were consistently associated with weight loss on the long term. Therefore, insulin, lactate and ACE are biomarkers that may be used to assess the metabolic benefits of a diet in its early stages where weight loss is not always a reliable parameter.

Further, nutritional means are identified by which ACE could be regulated and it is found that lemon extract (LE) down regulates ACE activity in adipocytes and is associated with both improved adipose insulin sensitivity and increased lipid mobilization.

In a proof of concept study, biomarkers have been identified that could provide molecular feedback on food intake behaviour.
LIST OF ABBREVIATIONS

WHO- World Health Organization

BMI- Body mass index

POMN- Pro-opiomelanocortin

GWASs- Genome wide association studies

FTO- Fat mass and obesity-associated

SEP- Socioeconomic population

EU- European Union

WC- Waist circumference

VAT- Visceral adipose tissue

NICE- National Institute for Health and Care Excellence

PYY- Peptide tyrosine tyrosine

LDL- Low density lipoprotein

CR- Caloric restriction

IF- Intermittent fasting

ADF- Alternate day fasting

HDL- High density lipoprotein

ACE- Angiotensin converting enzyme

Ang- Angiotensin

AGT- Angiotensinogen

DIT- Diet induced thermogenesis

AgRP- Agouti related protein

IL- Interleukin

TH- T helper

Tregs- Regulatory T cells

CRP- C-reactive protein
TNFα- Tumour necrosis factor α
IBMX- 3-isobutyl methyl xanthine
NCS- New calf serum
LE- Lemon extract
LJ- Lemon juice
ANOVA- One-way analysis of variance
JRES- J-resolved
MPS- media preparation service
HSL- Human Serum Lipase
pHSL- Phospho Human Serum Lipase
AKT- Protein Kinase B
pAKT- Phospho Protein Kinase B
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
HRP- Horse Radish Peroxidase
CST- Cell Signaling Technology
B- Breakfast
L- Lunch
D- Dinner
S- Snack
NAD- Nicotinamide adenine dinucleotide
ELISA- Enzyme-linked immune sorbent assay
OD- Optical Density
DMEM- Dulbecco's Modified Eagle's Medium
PBS- Phosphate Buffered Saline
BSA- Bovine Serum Albumin
SDS- Sodium Dodecyl Sulphate
PAGE- Poly Acrylamide Gel Electrophoresis
APS- Ammonium per Sulphate
TBST- Tris Buffer Saline with Tween-20
TEMED- Tetramethylethylenediamine
RIPA- Radioimmunoprecipitation Assay Buffer
FC- Folin-Ciocalteu
EDTA- Ethylaminediaminetetracetic acid
DMSO- Dimethyl Sulphoxide
RT- Reverse transcriptase
HPLC- High performance liquid chromatography
ACN- Acetonitrile
NMR- Nuclear magnetic resonance
HMDB- Human metabolome database
HMISC- Harrell Miscellaneous
DHP- Digital health platform
TSP- 3-trimethylsilyl propionic-2, 2, 3, 3-d4 acid sodium salt
PPARg- Peroxisome proliferator-activated receptor gamma
FAPB4- Fatty Acid-Binding Protein 4
D- Differentiated cells
LE50- LE dose of 50µg/mL
LE 100- LE dose of 100µg/mL
LE 500- LE dose of 500µg/mL
m/z- Mass/charge
TGs- Triglycerides
HsCRP- High-sensitive C-reactive protein
VLCD- Very low calorie diet
C - Control (C)
A - Abstinence
T - Transition
CVD - Cardiovascular disease
This figure illustrates lifestyle factors associated with healthy, overweight or obese individuals. The figure was designed using Paint and PowerPoint 2013.
1.0 SUMMARY

This chapter introduces the topic of this thesis, “Metabolic profiling and identification of biomarkers for weight loss” and provides the necessary background information. It also discusses the outstanding questions, aims and significance of the thesis and briefly outlines the experimental approaches used to address the questions and aims.

1.1 OBESITY BURDEN

A. TRENDS AND PREVALENCE

Obesity is a global epidemic with increasing incidence rates in developed and developing nations. According to the World Health Organization (WHO), more than 2 billion adults (39% of men and 40% of women) are overweight and >600 million of these are obese (1). In 2016, there were 41 million children (≤5 years) who were overweight or obese (2). The worldwide prevalence has nearly tripled between 1975 and 2016 (3). Figure 1 shows the body mass index (BMI) trend among females and males across the globe. Red, purple and pink colour on the map corresponds to BMI ≥25 kg/m². BMI is defined as weight divided by the square of height (in kg/m²) and is used to classify normal weight (18.5-24.9 kg/m²), overweight (25-29.9 kg/m²), obesity (≥30 kg/m²) and severe obesity (≥40 kg/m²) (4).

![Figure 1: Worldwide distribution of body mass index in 2014. Global BMI standardized based on age for (a) women and (b) men (1).](image-url)
Obesity is associated with several comorbidities such as type 2 diabetes, cardiovascular diseases (CVDs), sleep apnoea, metabolic syndrome and certain types of cancer (5, 6). The progressive excess mortality is mainly due to vascular diseases and is probably largely causal. A study on 900,000 adults from Europe and the United States found that at 30-35 kg/m² the survival rate is decreases by 4 years and at 40-45 kg/m² the survival decreases by 10 years (7).

**B. CAUSES AND RISK FACTORS**

Obesity is an incredibly complex disease that is associated with metabolic, genetic and behavioural deregulations. They should be studied in combination with environmental factors, socio-economic status, behaviour, education and genotype to understand obesity. All these factors affect caloric intake, thermogenesis, lipid utilization, nutrient turnover and differential fat storage in tissues (1, 8).

**I. GENETIC FACTORS**

Inter-individual variations in the body may be due to genetic factors. Body weight is effected by genes coding for pro-opiomelanocortin (POMC), melanocortin 4 receptor, leptin and leptin receptor (1). Studies on twins and adopted children concluded that 25-50% of the risk for obesity is heritable (9). Genome wide association studies (GWASs) have found >300 loci associated with obesity. One such locus is fat mass and obesity-associated (FTO), which regulates adipocyte browning, thermogenesis and appetite (1, 10, 11). Epigenetic modifications are a result of internal (genetics and hormones) and external (diet and physical activity) factors. These are reversible and can be passed on to next generations. For example, increased methylation of CpG and repressive histones on the POMC locus lead to lower leptin response in humans (9, 12, 13). Maternal obesity is associated with decreased methylation of the Znf483 gene, which is linked to adipocyte differentiation and increased risk of obesity in children (9, 14, 15).

**II. SOCIAL AND ECONOMIC FACTORS**

The prevalence of obesity is rising in low socioeconomic population (SEP) groups showing a relationship between SEP and obesity. In the European Union (EU) alone, 50% of obesity in women and 26% of obesity in men is associated with the educational status (16). Low SEP are two times more likely to become obese than people of average socioeconomic
status (17). For various reasons, mothers from SEP are more often overweight and less likely to breastfeed than women from average socioeconomic status. In general, babies that are not breastfed and are born to obese mothers are more likely to have poor eating habits and become overweight in the later years of their lives (16).

III. OTHER FACTORS

Obesity is often believed to be caused by an imbalance between energy intake [EI] (calories consumed) and expenditure (calories burnt) (18). This can be caused by a number of factors, for example, an increase in intake of high energy yielding food which are high in fat (19). There is also a shift towards overeating due to inexpensive, easily accessible processed and large portions (20). Another cause is sedentary life style or low physical activity due to the nature of work and increased urbanization (or also culture). The other contribution factors to the obesity epidemic are sleep debt, shift work, ambient temperature, drugs, endocrine disrupters, ethnicity, age, intrauterine conditions, the microbiome, cigarette smoking and infections (21).

C. COST OF OBESITY

Obesity imposes a large economic burden on countries, individuals and families. The cost of obesity can be broken down into direct and indirect costs. The direct cost is the one incurred by the health care systems for treating the condition and associated co-morbidities. The global impact of obesity was estimated to be US $2.0 trillion in 2014 (8). In 2015, UK spent ~£4.2 billion as a direct cost of obesity (22). The US alone spends 20.6% of its healthcare budget on treating obesity and obesity related diseases (23).

Indirect costs involve morbidity (presenteeism, absenteeism and disability) and mortality costs (premature death). Presenteeism is the cost accrued by the employee who is unable to work at full capacity. Absenteeism is the time during which an individual is absent from work because of illness. Disability is the absence from work due to physical or mental incapability. Premature mortality occurs from obesity related mortality and leads to the lost productivity costs. All these factors lead to cost that are incurred by the workplace, government and/or families. The UK incurred a total of £27 billion as indirect costs in 2015 (22).

Many low and middle income countries are facing a "double burden" of disease. They are not only dealing with infectious diseases and under-nutrition but also experiencing a rapid increase in non-communicable disease like obesity (24). Children in such countries suffer
from an increased risk of under-nutrition while being exposed to high fat, sugar, salt, micronutrient deficient food, which are lower in cost and nutrient value (2).

1.2 OBESITY: CLINICAL DEFINITION AND MEASUREMENT

Obesity is defined as an abnormal deposition of fat that poses a health risk. BMI is the most commonly used tool to measure adiposity, however it might not be the most efficient method as it is unable to provide information about body fat distribution (25). For example, elderly people lose muscle with age making BMI a less accurate indicator of body fat (26, 27).

BMI can be complemented with waist circumference (WC) or biometric impedance measurements, which gives a better representation of adiposity distribution to differential fat under the skin (subcutaneous) and intra-abdominal (visceral) (28). Visceral adipose tissue (VAT) is associated with metabolic risk and cardiovascular diseases (29). According to the National Institute for Health and Care Excellence (NICE) guidelines, WC>94cm (male) and WC ≥85cm (females) indicates a higher deposition of visceral fat (25). Biometric impedance involves estimation of body composition, particularly body fat. The health risk associated with obesity is identified by combining these methods, as shown below:

<table>
<thead>
<tr>
<th>BMI (kg/m²)</th>
<th>Waist Circumference</th>
<th>Risk of co-morbidities</th>
</tr>
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<tbody>
<tr>
<td>&lt;18.5</td>
<td>&lt;94 cm (men)</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>&lt;85 cm (women)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥94 cm (men)</td>
<td>Average</td>
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<tr>
<td></td>
<td>≥85 cm (women)</td>
<td></td>
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<tr>
<td>18.5-22.9</td>
<td>&lt;94 cm (men)</td>
<td>Average</td>
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<td>≥94 cm (men)</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>≥85 cm (women)</td>
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<tr>
<td>23-24.9</td>
<td>&lt;94 cm (men)</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>&lt;85 cm (women)</td>
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</tr>
<tr>
<td></td>
<td>≥94 cm (men)</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>≥85 cm (women)</td>
<td></td>
</tr>
<tr>
<td>25-29.9</td>
<td>&lt;94 cm (men)</td>
<td>Moderate</td>
</tr>
<tr>
<td>&lt;85 cm (women)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>≥94 cm (men)</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>≥85 cm (women)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;94 cm (men)</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>&lt;85 cm (women)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥94 cm (men)</td>
<td>Very Severe</td>
<td></td>
</tr>
<tr>
<td>≥85 cm (women)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1: Identification of risk level for obesity related co-morbidities by combining BMI and Waist Circumference.** Each BMI category in association with waist circumference is used to identify the risk for developing obesity related co-morbidities (25).

1.3 OBESITY TREATMENT

A. BARIATRIC SURGERY

Use of bariatric surgery is recommended to individuals with BMI >40 kg/m² or BMI >35 kg/m² with associated comorbidities or BMI 30-35 kg/m² with type 2 diabetes (30). The different options available are shown in Table 2.

<table>
<thead>
<tr>
<th>Type</th>
<th>Roux-en-Y Gastric Bypass</th>
<th>Sleeve Gastrectomy</th>
<th>Gastric Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>A transection is made in the stomach to create a gastric pouch of ~1 ounce capacity</td>
<td>A majority of the stomach (80%) is removed</td>
<td>Involves placement of an adjustable gastric band just near the proximal stomach to constrict the size of the gastric pouch and outlet.</td>
</tr>
<tr>
<td>Mechanism</td>
<td>The pouch limits the amount of food eaten and is digested further down in intestine</td>
<td>Ghrelin, the appetite stimulation hormone reduces feeling of hunger and gastric</td>
<td>Limits the food eaten</td>
</tr>
</tbody>
</table>
leading to decreased absorption of calories | emptying is increased |  
---|---|---
Reversible | No | No | Yes
Average Weight Loss | 60-80% of excess body weight is lost over 12-18 months following the surgery | 50-60% of excess body weight is lost over 2 years following the surgery | 40-60% of excess body weight is lost over 3-4 years following the surgery
Risks | Bowel obstruction, dumping syndrome, gallstones, hernias, low blood sugar, malnutrition, perforation and ulcers | Gastrointestinal obstruction, hernias, gastroesophageal reflux, low blood sugar, malnutrition and vomiting | Infection, deep vein thrombosis or pulmonary embolism and internal bleeding

Table 2: Bariatric surgery. Different types of bariatric surgeries available (30-32).

Although surgeries lead to up to 60% average weight loss, the patients need to be on a lifelong controlled diet and regular exercise (32). Thus, there is a need for identification of other alternatives to treat obesity.

B. DRUGS AND FUNCTIONAL FOOD

The use of drugs is limited to people who have a BMI >30 kg/m² or >27 kg/m² with associated morbidities (33). They do not cure obesity but help with weight loss. These drugs are approved in conjunction with diet and exercise [discussed later] (34, 35). Before prescribing the medication, the health professional will consider:

i. likely benefits of weight loss
ii. possible side effects
iii. current health issues
iv. medications
v. family's medical history
vi. cost

The medication needs to be reconsidered if no weight is lost after 12 weeks (36). Orlistat is the only drug approved for use in the UK. The different types of FDA-approved drugs available are shown in Table 3.
<table>
<thead>
<tr>
<th>Drug: Orlistat</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanism of Action</td>
<td>Fat absorption/ reduction</td>
</tr>
<tr>
<td>Average Weight Loss (%)</td>
<td>4</td>
</tr>
<tr>
<td>Approved use</td>
<td>Long term oral use</td>
</tr>
<tr>
<td>Adverse Effects</td>
<td>Malabsorption, Vitamin deficiencies, Oily stools and GI discomfort</td>
</tr>
<tr>
<td>Receptor Molecular Pathway</td>
<td>Lipase inhibitor</td>
</tr>
<tr>
<td>Target Tissue</td>
<td>Bowel/gut</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug: Lorcaserin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanism of Action</td>
<td>Appetite suppressant</td>
</tr>
<tr>
<td>Average Weight Loss (%)</td>
<td>3</td>
</tr>
<tr>
<td>Approved use</td>
<td>Long term oral use</td>
</tr>
<tr>
<td>Adverse Effects</td>
<td>Dizziness, Headaches, GI disturbances, Insomnia and Fatigue</td>
</tr>
<tr>
<td>Receptor Molecular Pathway</td>
<td>5HT2c receptor agonist</td>
</tr>
<tr>
<td>Target Tissue</td>
<td>Hypothalamus and brainstem</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug: Liraglutide</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanism of Action</td>
<td>Appetite suppressant</td>
</tr>
<tr>
<td>Average Weight Loss (%)</td>
<td>6</td>
</tr>
<tr>
<td>Approved use</td>
<td>Long term use by subcutaneous injection</td>
</tr>
<tr>
<td>Adverse Effects</td>
<td>GI discomfort, Hypoglycaemia Nausea and Diarrhoea</td>
</tr>
<tr>
<td>Receptor Molecular Pathway</td>
<td>GLP-1 receptor agonist</td>
</tr>
<tr>
<td>Target Tissue</td>
<td>Hypothalamus</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug: Phentermine/ topiramate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanism of Action</td>
<td>Appetite suppressant</td>
</tr>
<tr>
<td>Average Weight Loss (%)</td>
<td>9</td>
</tr>
<tr>
<td>Approved use</td>
<td>Long term oral use</td>
</tr>
<tr>
<td>Adverse Effects</td>
<td>Neurological dysfunction, Constipation, Dizziness, Headaches and Insomnia</td>
</tr>
<tr>
<td>Receptor Molecular Pathway</td>
<td>Release of serotonin, norepinephrine, and dopamine; inhibition of ionic conductance</td>
</tr>
</tbody>
</table>
and carbonic anhydrase; inhibition of voltage-gated sodium channels

<table>
<thead>
<tr>
<th>Target Tissue</th>
<th>Hypothalamus and brainstem</th>
</tr>
</thead>
</table>

**Drug: Bupropion/ naltrexone**

<table>
<thead>
<tr>
<th>Mechanism of Action</th>
<th>Appetite suppressant, Increased satiety perception</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Weight Loss (%)</td>
<td>6</td>
</tr>
<tr>
<td>Approved use</td>
<td>Long term oral use</td>
</tr>
<tr>
<td>Adverse Effects</td>
<td>Depression, Nausea/vomiting, Headaches, Dizziness and Cardiovascular impairment</td>
</tr>
<tr>
<td>Receptor Molecular Pathway</td>
<td>Dopamine and norepinephrine reuptake inhibitor; opioid receptor blocker and POMC activation</td>
</tr>
<tr>
<td>Target Tissue</td>
<td>Hypothalamus</td>
</tr>
</tbody>
</table>

**Table 3: FDA approved drugs for treatment of obesity.** The table discusses the different drugs available for obesity treatment along with their mechanism of action, effects, target tissues and average weight loss. Adapted from (35).

These drugs have several side effects ranging from diarrhoea, oily stools, headache, insomnia, nausea, vomiting, increased blood pressure, tachycardia, myocardial infraction to stroke. Therefore, efforts have been made to find other active compounds to treat obesity.

Several bioactive compounds found in plants can also be used for the treatment of obesity. Functional foods are defined as dietary components that have a health benefit beyond the provision of basic nutrition (37). Food components can modulate hunger, satiety and energy expenditure (EE). In particular, benefits of plant bioactive compounds on metabolism have become a focus of multidisciplinary studies; and several plant extracts are studied in the prevention of obesity (38). Based on their nutritional value, such extracts have physiological benefits and reduce the risk of chronic diseases (39, 40). Phenols, anthocyanins and tannins, found in tea, berries and peas, have been demonstrated to decrease lipids, through the inhibition of lipase activity (41). Starch with high glycaemic index is associated with weight gain (42, 43) by causing increased insulin secretion (44). Diets rich in protein from vegetables (e.g. legumes) induce satiety and suppress intake and appetite (45, 46) by increasing peptide tyrosine tyrosine (PYY) levels and decreasing ghrelin hormone (47, 48). Raspberries decrease serum glucose, insulin levels, leptin and body weight in mice fed high fat diet (49). Mango peel and bilberry extract inhibit adipocyte differentiation in the 3T3-L1 adipocyte cellular
model (50, 51). Citrus fruit species are associated with decreased inflammation and reduced oxidative stress markers in humans (52). Other studies have found bergamot, grapefruit and orange juice to be associated with decreased total cholesterol, low density lipoprotein (LDL) and glucose concentration in humans (53-55). Similar results have also been found in rats after lemon juice administration (56).

To summarize, the different plant-based compounds that are prescribed or used to reduce weight gain are shown in Table 4.

| Appetite                          |  |
|-----------------------------------|--|---|
| **Molecule**                      | **Mechanism** |
| Saponins (gingenoside)            | Satiety        |
| Fibre and polysaccharides (diverse)|               |
| Terpenes (geniposide)             |               |
| Steroidal glycosides (P57A53)    |               |
| Polyphenols (proantho-cyanidins)  |               |
| Proteins (diverse)                |               |

| Energy Expenditure                |  |
|-----------------------------------|--|---|
| **Molecule**                      | **Mechanism**             |
| Alkaloids (capsaicin)             | Thermogenesis             |
| Flavonoids (diverse)             |                           |
| Polyphenols (catechin)            |                           |
| Fatty acids (MUFA, PUFA, ...)     | Beta-Oxidation Lipolysis/anti-lipogenesis |
| Curcumin                          | Induction of brown fat-like phenotype |

| Metabolism                        |  |
|-----------------------------------|--|---|
| **Molecule**                      | **Mechanism** |
| Saponins (astragaloside IV, gingenoside) | Lipase inhibition, fat depletion |
| Fibre                             | Lipid uptake reduction; Reduction of energy dietary value; Secretion of anorectic peptides |
| Pseudo-tetrasaccharide, acarbose  | Amylase inhibitor |
| Polysaccharides (diverse)         | Fat depletion |
| Alkaloids (berberine betaine, piperine, capsaicin) | Browning Fat depletion |
Table 4: Bioactive compounds currently prescribed or investigated to treat obesity (35). The table describes the different compounds, their source and mechanism of action.

| Polyphenols (resveratrol, proanthocyanidins, epicatechin, diverse from tea) | Adipogenesis inhibition; Fat depletion and absorption Lipase/amylase inhibition |

C. EXERCISE

Physical activity is recommended for weight loss and management of obesity. Physical activity for around 1 hour per day is recommended to maintain the lost weight (1, 57). Physical activity is defined as a bodily movement that require energy expenditure (58). Apart from weight loss, it also helps lowering the risk of diabetes and CVDs. However, studies with people doing exercise intervention have also seen that it yields smaller changes than what could be predicted (59). This is due, at least partly, to the fact that energy expenditure has a complex relationship with energy intake, exercise, food type, composition and hormones (60). Sex, age, BMI, ethnic background and genetics also play a role in a person’s response to exercise (60, 61).

D. DIETS

Different dietary plans are widely available. They include caloric restriction (CR), the Atkins Diet, low fat diet, crash diet, Mediterranean diet, intermittent fasting and the ketogenic diet to name some of the most popular. Several of these diets have overlapping principles. For example, the Atkins diet is a low carbohydrate, low calorie diet focusing on maintaining of low insulin levels in the body (62). In the low fat diet, caloric consumption is achieved based on the principle that 1 gram of fat contains more than twice the calories than that of 1 gram of carbohydrates (63). The most commonly followed diets revolve around CR or even complete fasting. CR diets typically reduce daily energy intake by 30-50% (64). It can be difficult to follow on the long term, as it requires continuous tracking of energy intake (65). Another available approach is intermittent fasting (IF). It involves restriction of energy intake for 1-3 days in a week (66) by introducing short to medium term fasting (8 to 16 hours) to 1-3 days a week. For the remaining days, one is allowed ad-libitum food consumption. In mice, IF has shown a positive effect on prolonging life span, glucose tolerance and insulin sensitivity (67, 68). Alternate day fasting (ADF) is a type of IF involving one day of fasting (energy restriction...
day) followed by a day where ad libitum food consumption is allowed, alternating fasting and non-fasting days (66). Decreases in glucose and insulin concentrations have been observed in all types of diets and are modulated by the number of fasting days (56). All these diets induce weight loss to various extents. However, weight maintenance is very variable amongst the diets, and largely depends on the individual’s adherence to the diet (65).

Besides food composition, another factor amenable to behaviour change is meal timing. Different studies have found evidence supporting that consuming a regular breakfast is associated with a lower body weight (69-72). There is also a plethora of metabolic studies that support that eating breakfast is preferable over eating dinner due to a phenomenon referred to as “afternoon diabetes”, in which insulin sensitivity is higher early during the day than it is on the evening (73-75). A crossover study comparing days with “breakfast and lunch” versus days with “lunch only” showed decreased clock gene expression in type 2 diabetic subjects in comparison to healthy individuals, while skipping of breakfast showed an altered response for clock gene profiles in both groups (76). On the other hand several studies indicate that body weight and food intake in individuals eating breakfast were relatively similar than those who omitted this meal (77-80). Gill and Panda (81) have demonstrated the extent to which adults display an irregular daily and weekly rhythm of eating and fasting, could be manoeuvred to obtain desirable health benefits. It might be possible to cater to such personal preferences, at least to some extent, without compromising weight loss success.

E. SUPPORT NETWORKS

Many studies have shown that dieting is more successful when supported by social interactions, such exercising with a friend or dieting as a group (82, 83). A support network is a platform (in the real world and/or electronic) where people trying to lose weight come together sharing their stories, challenges, victories, feelings and provide support to others to help achieve their goals. People also share their food recipes, diet plan and physical activity routine on such networks. There are different types of networks available like Weight watchers and Slimming World. They allow people to remain enthusiastic, motivated, set goals, track progress and engaged in the weight loss plan being followed by them (62).
F. TECHNOLOGY AND APPS

With the explosion of social network and social media, technological support has dramatically increased in the last decade. Technological support available to help with weight loss includes diet trackers and activity monitors. Recording of eating patterns has been recognized as an effective step in managing weight gain / weight loss (84, 85). While the traditional paper version of the commonly used dietary questionnaire is considered tiring (86), there are numerous computer-assisted versions (87, 88) as well as apps and websites available for personal tracking of food intake (89, 90). However, regardless of interface, mis-reporting of food intake is a well-documented problem (86, 91), as it depends on self-awareness, honesty and motivation of the user (85). Often, unconscious bias of self-observation leads to under-realization of food eaten (92).

Energy expenditure on the other hand can potentially be tracked without bias using activity monitors, but they do not provide a direct link to weight loss. They may show the number of steps walked or run, calories consumed and/or heartbeat per minute but no direct information on weight loss (65). Even if a device that accurately measures caloric intake and expenditure was widely available, the information may not be sufficient to motivate a user to make changes in their behaviour that would result in weight loss. Also, these devices are not 100% tailored to an individual. They only give an estimate of the calories burnt based on weight and height, sometimes including gender and age, but they do not include any information on fat distribution or hormonal patterns. This information is therefore generic and there is a need for identification of parameters (such as biomarkers) to provide tailored feedback during dieting. This may palliate the fact that even when meticulously keeping records of food intake, individuals still find it challenging to lose weight (93). This is because the relationship between caloric intake and weight loss is not always linear (94). As a result, current approaches to lose weight loss generally do not work well (95), and the weight loss market is missing a device that is more directly coupled to the desired outcome, weight loss.

1.4 MOLECULAR INFORMATION AND WEIGHT LOSS

A. MARKERS OF ADIPOSY

Quantitative biomarkers of weight loss do not exist yet, but biomarkers have been considered for a number of related areas. For example, urinary metabolic markers for
cardiovascular disease, blood pressure and adiposity have been identified (96, 97). Several metabolomics studies involving untargeted proton (\(^1\)H) nuclear magnetic resonance spectroscopy (NMR) and ion exchange chromatography (IEC) on obese human and mice urine samples have identified metabolites associated with BMI and adiposity (96, 98), summarized in Figure 2.

**Figure 2:** Urinary metabolic signatures of BMI and Adiposity in urine. *The image was designed using Paint and PowerPoint 2013. The information for the metabolic signatures was obtained from (99).*

One of these biomarkers is lactate, which has been studied as a potential marker for biofeedback in the thesis. Lactate is the by-product of glucose utilization by the organs and tissues during hypoxia or glycolysis (100). It helps to modulate oxygen release in hypoxic tissues and acts as a substrate for gluconeogenesis (101, 102). It also acts as a substrate for lipogenesis in liver and other tissues (103-105). Release of lactate in adipose tissue is often attributed to be a result of hypoxia, acidosis and stress (106, 107). Lactate production is known to increase *in vitro* in large adipocytes from obese animals and can reach up to 50-70% of glucose metabolized (108). Indeed, baseline lactate concentrations are higher in obese subjects as compared to lean subjects (109). This is due to the increased production of lactate as a result of increased adipose cell size. Since adipose cells in overweight and obese people are larger, there is constriction of blood vessels in adipose tissues. This restriction creates a hypoxic environment in the tissue leading to increased production of lactate (110-112).
B. MARKERS OF LONG-TERM WEIGHT LOSS

Several blood and physical markers are used to measure weight loss. It can involve measurement of hip/waist ratio, BMI, body fat and/or weight for physical markers and high-density lipoprotein (HDL), low-density lipoprotein (LDL), fasting glucose in serum. In most diets the weight lost is rapidly regained after the end of the diet (the “yo-yo” effect), therefore investigating possibilities to use molecular information to predict whether the weight is kept off on a long-term is an interesting question. To find a marker for long-term weight loss, a panel of biomarkers was tested before and after an eight-week diet, and weight loss maintenance was determined after six months (96). In this study, angiotensin converting enzyme (ACE) is shown (amongst the extensive blood profiling for diverse protein and steroid hormones) to be the only potential predictor for sustained weight loss (113). At the end of the eight-week intervention, individuals with weight loss displayed decreased ACE concentration (~12%) (113), which supports the previously reported decreased ACE activity in overweight/obese adults after dieting (114, 115). Building on this finding, ACE was investigated as a potential marker for biofeedback on a 24-hour interval.

ACE is a zinc metallopeptidase enzyme involved in the conversion of Angiotensin (Ang) I to Angiotensin II (113, 116). Ang I is obtained by cleavage of Angiotensinogen (AGT) with the help of renin. Ang II is well known for its role in increased blood pressure and retention of salt and water (116). Ang II is further degraded into the amino acid peptide fragment Ang (1-7) by ACE 1, a homologue of ACE (117). Like ACE, ACE 1 is also a membrane protein and is known to shed at its carboxy-terminus in vitro to yield soluble ACE 1 (118). This process is catalysed by the metalloprotease ADAM 17 (119). Detection of ACE usually refers to detection of circulating soluble ACE 1, which has been observed in human urine using an activity assay based on fluorescence quenching (119). The circulating ACE lacks the transmembrane and cytosolic domains and has a molecular weight of 65 kDa. Like most enzymes, ACE does not act alone but in complex inter-relationships with numerous other regulatory agents. Animal models have shown increased adipose specific angiotensin (AGT) expression, secretion is involved in adipose tissue development (120).

The renin angiotensin system (RAS) is one of the most important systems that regulate cardiovascular and fluid homeostasis (121). Along with conversion of Ang I to II, ACE is also involved in breakdown of bradykinin into inactive products (122). Inhibition of ACE lowers Ang II levels leading to decreased sympathetic stimulation, vasoconstriction and platelet aggregation (123). Lower Ang II levels also help in maintaining cell β function by decreasing production of aldosterone (124). Increased bradykinin levels increase vasodilation, improve glucose uptake leading to improved insulin sensitivity (125-128). These benefits are
observed in people taking ACE inhibitors with a risk of developing cardiovascular diseases. A study on 3577 individuals taking Ramipril (an ACE inhibitor) showed a reduced risk of myocardial infarction, stroke, or cardiovascular death by 25% after 4.5 years (129).

**Figure 3: Conversion of Angiotensin I to Angiotensin II by ACE and functions of Angiotensin II.**

**C. MARKERS OF NUTRITIONAL INTAKE**

Several clinical studies have demonstrated an effect of nutrients and lifestyle on the prevention of metabolic disease and CVDs. Thus, identification of markers for different foods can help improve health but also improve compliance to the diet plan (130). Double labelled water (water with isotopes of deuterium and oxygen 18) is used to measure energy expenditure (EE) in an individual (131). Urine samples are collected to determine the rate of disappearance of each isotope form the body via mass spectrometry. This disappearance rate is further used to indirectly calculate carbon dioxide production to estimate total EE. EE is lower in obese and overweight population in comparison to healthy individuals. To be in an energy balance, energy intake (EI) should be equal to EE. Increased EE is associated with underreporting of food intake. Urine nitrogen is used as a biomarker for protein intake through dietary means (132). Constant dietary intake over longer periods are also associated with daily nitrogen intake and excretion (132). Hydrocarbons in breath are used as a means to measure lipid peroxidation. Peroxidation of n-6 and n-3 fatty acid release pentane and ethane respectively in to breath (133). Supplementation with β carotene reduces breath pentane levels significantly (134).

Markers for α and β carotene, lycopene and β-cryptoxanthin are correlated with serum cholesterol levels (135). Biomarkers for different foods include NMNA, a niacin-related
(vitamin B3) metabolite marker for coffee drinking (136), proline betaine for citrus fruit consumption (137) and O-acetyl carnitine for red meat intake (138). Dietary iron and zinc intakes are positively associated with CRP, an inflammatory marker (132, 139).

Although not a direct biomarker for nutritional intake, it is well known that in non-diabetic individuals insulin concentrations vary dramatically with food intake. Previous work from our team has investigated insulin as a potential marker for biofeedback as a proof of concept (140) and we have compared lactate and ACE biomarker studies with the insulin results in this thesis (see chapter 3 and 5) (65). Insulin is a small peptide hormone, whose secretion is stimulated by increased levels of glucose in blood (141). Insulin regulates blood glucose homeostasis by increasing glucose uptake in muscles and fat cells and inhibits its production in liver (141). It varies with food intake in terms of meal composition, timing and quantity (74, 75, 142). Moreover, its concentration and action are under circadian regulation, giving rise to “afternoon diabetes” (73-75). This finding has been used to suggest that diet plans should incorporate meal timing (143) with the breakfast meal being the largest meal as opposed to the most common behaviour of highest food consumption in the evening (81, 143). Indeed, it is shown that a high-carbohydrate breakfast promotes greater weight loss than a diet low in carbohydrates by reducing food-cravings and increased satiety (144-146).

D. MARKERS OF HUNGER AND SATIETY

Appetite can be expressed in terms of physical behaviour, peripheral physiology and central nervous system functions (147). People stop eating due to absence of hunger or because they feel full (148). Diet induced thermogenesis (DIT) is the amount of energy used above the basal rate during the breakdown of food in the body. DIT and satiety are positively correlated and DIT decreases hunger (149-151). Ghrelin is found to be associated with hunger. Infusion of ghrelin in healthy subjects lead to enhanced appetite and energy intake levels (152). Plasma concentration for ghrelin decreases after administration of glucose (153) and carbohydrates (154) but there is no suppression after high fat administrations (154, 155). Insulin and glucose are therefore indirectly related to satiety. There are also several biomarkers that are associated with the neurons involved in maintaining energy balance and homeostasis. Neuropeptide Y and agouti related protein (AgRP) are located in the hypothalamus and their expression is activated in conditions of fasting, negative energy balance and they increase the hunger level (9). Pro-opiomelanocortin (POMC) neurons release anorexic neuropeptide that reduces food intake (156). It is a target for certain anorexic drugs while leptin activates POMC neurons (157).
E. MARKERS OF INFLAMMATION

Normal adipose tissue expresses anti and pro inflammatory regulators to allow functioning of adipose tissue (158). Interleukin (IL) 4 is a major anti-inflammatory regulator that controls the expression of T helper (TH) type 2 cells, regulatory T cells (Tregs) and macrophages. Macrophage phenotype is divided into groups namely, classically activated macrophages (M1) and alternatively activated macrophages (M2). Expression of M2 macrophages by IL4 promotes systemic insulin sensitivity in lean mice (159, 160). Many pro-inflammatory marker levels increase in obese subjects. A cross-sectional meta-study has found association between C-reactive protein (CRP), a marker for systemic inflammation, and free fat mass (161-163). Increased levels of CRP and triglyceride (TG) are found in overweight women in comparison to normal weight women (164). With nutrition overload, lipid activation and increased energy storage, there is a switch from M2 to M1 (158). Adipocytes secrete adipokines such as leptin and adiponectin, which have opposing effects on immune cell functions. Leptin has pro-inflammatory effects and increases with increased nutrition uptake and stimulates production of IL 1, 6 and 12, and tumour necrosis factor α (TNFα), while adiponectin has anti-inflammatory effects and decreases with increase in adiposity (158). The overexpressed pro-inflammatory cytokines is found in obese individuals and one third of total circulating concentrations of IL-6 originate from adipose tissue (165). Levels of IL 6 decreases after 12 months of bariatric surgery in morbidly obese patients (166).

The increased energy substrates in obesity leads to increased reactive oxygen species (ROS) signalling and mitochondrial dysfunction (167). This is also associated with decreased insulin sensitivity (167). c-Jun N-terminal kinases (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are regulated by ROS and are associated with obesity induced insulin resistance (168). JNK is involved in insulin secretion in beta cells and insulin sensitivity in liver and muscle cells (169). NF-κB is part of pro-inflammatory signalling pathway. In the presence of high ROS levels, JNK and NF-κB are activated and their activation has shown to alter insulin sensitivity (170).

1.5 MODELS OF OBESITY

Several animal and cellular models are used to better understand the disease and find treatments of obesity in humans. Different animal models of obesity are listed in Table 4, and different cellular models of obesity are listed in Table 5.
<table>
<thead>
<tr>
<th>Model name</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ob/ob mouse</td>
<td>leptin deficiency</td>
</tr>
<tr>
<td>db/db mouse</td>
<td>leptin receptor</td>
</tr>
<tr>
<td>s/s mouse</td>
<td>disrupted STAT3 signal of leptin receptor</td>
</tr>
<tr>
<td>Zucker mouse</td>
<td>mutated leptin receptor</td>
</tr>
<tr>
<td>Koletsky rat</td>
<td>mutated leptin receptor (null mutation)</td>
</tr>
<tr>
<td>ZDF rat</td>
<td>mutated leptin receptor (fa/fa)</td>
</tr>
<tr>
<td>Wistar Kyoto fatty rat</td>
<td>Zucker /fa/fa x Wistar-Kyoto</td>
</tr>
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</table>

*Table 5: Examples of animal models of obesity, adapted from (171)*.

<table>
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<th>Model name</th>
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<td>Pre-adipose cells</td>
</tr>
<tr>
<td>C3H10T1/2</td>
<td>Stem cells</td>
</tr>
<tr>
<td>ES cells</td>
<td>Stem cells</td>
</tr>
<tr>
<td>DFAT cells</td>
<td>Pluripotent cells</td>
</tr>
</tbody>
</table>

*Table 6: Cellular models of obesity, adapted from (172).*

**A. 3T3-L1**

3T3-L1 adipocytes are the most commonly used *in vitro* model to study fat cell biology, adipogenesis, metabolism and action of hormones on adipose tissue function (172). 3T3L1 cells were obtained from mouse embryos. L1 is a continuous sub-strain of 3T3 and was originally deposited by Massachusetts Institute of Technology (MIT) in 1974. It was deposited without the passage number information and a seed stock with “unknown+4” passage number was developed (172). When undifferentiated, these cells have a fibroblast like morphology and are adherent in nature. The pre-adipocytes stage of the cells is maintained by keeping the confluency up to 70%. The induction of lipid formation occurs by exposure to insulin, dexamethasone and the phosphodiesterase inhibitor 3-isobutyl methyl xanthine (IBMX). Finally, after 4 days of exposure, the cells differentiate and there is accumulation of
lipids in the cells. Adipocyte differentiation can also be increased in the presence of rosiglitazone, an agonist of the peroxisome proliferative activator receptor γ (173). However, no significant difference in lipid accumulation was observed between cells treated or not with rosiglitazone and therefore rosiglitazone has not been used in any of the experiments in this thesis.

![Image of undifferentiated and differentiated adipocytes]

*Figure 4: Morphology of undifferentiated 3T3-L1 pre-adipocytes (left) and fully differentiated adipocytes (right). Typical images were taken using EVOS XL Core.*

### 1.6 OPEN QUESTIONS, THESIS GOALS AND RESEARCH PROGRAM

#### A. OUTSTANDING QUESTIONS

There is a range of approaches available to lose weight from surgical or drug-based interventions to different diet programs. Weight loss plans involve following a certain diet regime and/or monitoring net caloric intake. To date, no dietary intervention combines molecular measurements with digital technology tracking life-style parameters such as food intake or exercise, despite the availability of many food tracker apps and websites.

All weight loss programs give a broad description of the diet plan, which is often arbitrary. In addition, every individual has a different metabolism and responds differently to a diet plan (65). Thus, personalised optimisation of the diet plan needs to be understood to determine how diet parameters can be adapted to an individual’s metabolism. Therefore, there is an unmet need to identify markers that could be correlated with life style and dietary habits.
Every molecule is part of a complex network and with many other metabolic agents, substrates and regulatory mechanisms so multiple markers might be identified.

While most diets are conducted on a long-term (e.g. several weeks), very little work has focused on a short-term modulation of biomarkers in response to a diet. Therefore, this thesis is centred on 24-hour intervals to quantitatively investigate different dietary conditions, to allow the optimization of dieting behaviours in the short-term.

Previous work in my primary supervisor’s lab showed the potential for quantifying insulin values in urine and potentially using that information to provide feedback to participants on how well they are doing with respect to their weight loss goal (140). However, insulin values alone may not replace or even help interpret a person’s metabolic profile or state, as it interacts with a large number of other metabolic agents, substrates and regulatory mechanisms. Thus, two additional molecules, lactate and ACE, have been chosen along with insulin, to investigate in detail how meal composition and timing may be optimized to assist dieting efforts. Individuals may use lactate and ACE urine levels as a quantitative, molecular guide to provide feedback even before weight loss is observed. Insulin levels in urine samples were quantified as part of the previous work but their relation with lactate and ACE has been studied as a part of this thesis. Thus, the major questions addressed in the thesis are whether or not lactate and/or ACE could be relevant biomarkers for weight loss to give feedback even before weight loss is observed and in the case of ACE, what the mechanisms underlying the connection to weight loss are.

**B. AIMS OF THE THESIS**

This thesis aims at identifying biomarkers associated with weight loss that may prove useful for personalised feedback. It also involves exploration of lemon (a functional food) effects on adipocyte differentiation, and the role that ACE plays in weight loss associated with a lemon juice diet.

The thesis is divided into clinical and cellular studies, as outlined below. Lastly, a pilot clinical study on the lemon juice diet is described.

**I. CLINICAL STUDY**

A clinical study was designed to test the proof of concept of using several candidate biomarkers for potential biofeedback to people undergoing a weight loss intervention by demonstrating their correlation with weight loss.
1) Identification of molecular markers (lactate, insulin and ACE) for short-term biofeedback (24 hour periods) [Chapter 3 and 5] (65, 174)

2) What are the effects of different meal plan options on weight loss and molecular biomarkers? [Chapter 3 and 4] (65)

3) What are the correlations of lactate, insulin and ACE levels with weight loss? [Chapter 3 and 5] (65, 174)

4) Identification of new biomarkers by NMR [Chapter 6]

II. CELLULAR STUDY

Lemon helps in losing weight (175) and might act as an ACE inhibitor (56). In the clinical study (see section I), ACE levels correlated with weight loss in overweight and obese population. Thus, cellular studies were conducted to establish the relationship between lemon and ACE expression, insulin sensitivity and fat accumulation in 3T3-L1 adipocytes.

5) What are the effects of lemon extract on 3T3-L1 adipocytes differentiation? [Chapter 7]

6) What are the different components in lemon extract (LE) that may be responsible for the effects observed? [Chapter 7]

III. LEMON JUICE STUDY

In the cellular study, it was established that LE is associated with lipid breakdown in 3T3 L1 adipocytes. To investigate the relevance of this observation in vivo, a pilot study was conducted to address the following question:

7) What are the effects of lemon juice (LJ) on weight loss in humans on a short term? [Chapter 8]
C. OUTLINE OF EXPERIMENTAL APPROACHES

I. CLINICAL STUDY

Ethics approval for the clinical studies was obtained from the Warwick Medical School Ethics committee BSREC (Appendix I and II).

1) To quantify lactate, fluorescence based measurements were conducted involving conversion of lactate into pyruvate. ACE quantification was carried out using an ELISA kit from R&D Systems, Biotechne, UK.

2) Statistical analyses were performed using IBM SPSS Statistics 24 and R. Associations between different variables were calculated using bivariate Pearson Coefficient analyses. Nonparametric Mann-Whitney U test was performed in some cases as indicated. One-way analysis of variance (ANOVA) was used to compare control groups with other groups.

3) Different meal plan groups were studied using Nonparametric Mann-Whitney U and ANOVA tests.

4) 1D NOESY and J-resolved (JRES) nuclear magnetic resonance (NMR) spectra were collected with the goal of identifying new biomarkers. The data was analysed using metabohunter software.

II. CELLULAR STUDY

5) To understand the effects of lemon extracts (LE) on ACE mRNA expression, Taqman RTPCR was conducted, and effects on ACE protein expression were studied via Western blot analysis. The relationship between insulin sensitivity and LE was established by studying protein expression of known signalling molecules in the insulin pathway via Western blot. Furthermore, the effects of LE on lipolysis was established through Western blots, free glycerol assays and imaging techniques.

6) HPLC-MS was performed to separate and identify different components in LE.
III. LEMON JUICE STUDY

7) In a pilot study, the effects of lemon juice supplementation on ACE levels were investigated. Association between ACE levels and weight loss was studied.

D. CONTRIBUTIONS OF THIS THESIS

I. CLINICAL STUDY

1) Conclusive evidence was presented for the novel concept of biomarkers for weight loss (as opposed to obesity and other long-term consequences of lifestyle).

2) It was found that skipping a meal in a day regardless of which one, while also recording all food and exercise events and collecting urine samples for subsequent molecular profiling, resulted in consistent weight loss, in comparison to control days in which any number of meals was allowed.

3) With a meal-skipping diet, it was shown that insulin, ACE and lactate concentrations in urine correlate with weight loss, making these molecules potential candidates for quantitative feedback on whether dieting is working or not to the people trying to control their weight.

4) More than 200 different compounds were identified in urine through NMR. The number of compounds identified varied with levels of biomarkers at the end of a dieting and a control day.

II. CELLULAR STUDY

5) Cellular studies demonstrated a connection between LE and ACE regulation in adipocytes. LE down regulated ACE activity and RNA expression in adipocytes. This was also associated with improved adipose insulin sensitivity and increased lipid mobilization.

6) HPLC-MS allowed identification of the different compounds in LE, which was used in cellular studies. Citric acid and a mix of flavonoids and limonoids were identified in the extracts.
III. LEMON JUICE STUDY

7) The pilot study with LJ in humans supported the previous findings that LJ has an effect on weight loss. This loss of weight was sustained for several days even when going back to a normal feeding routine.

E. SIGNIFICANCE

A contribution to understanding the molecular mechanism(s) of the metabolic marker(s) identified during this research may have implications for obesity management and weight regain. The current technologies only allow keeping a record of activities such as exercise and food intake. The latter usually rely on self-reporting, which is prone to under-realization of the amount of food consumed (176). Weight change during dieting is usually not sufficiently immediate to keep participants motivated. Thus, identification of changes in metabolic markers over 24-hour periods may provide a better understanding on how the body responds to certain interventions. The personalized molecular information may potentially help in understanding daily nutritional requirements and help dieters plan their activities (e.g.: food intake, exercise) accordingly. This may help reduce the obesity’s adverse effects on general health.
CHAPTER TWO

EXPERIMENTAL PROCEDURES

2.0 LIST OF SOLUTIONS

The solutions listed below were used during the cellular experiments to conduct cell culture, western blot and cell staining.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routine Medium</td>
<td>DMEM/F12+ 10% heat inactivated NCS</td>
<td>From University of Warwick media preparation service (MPS)</td>
</tr>
<tr>
<td>PenStrep Solution</td>
<td>10000 I.U. /mL / 10000 μg/mL Penicillin/Streptomycin</td>
<td>MPS</td>
</tr>
<tr>
<td>Differentiation Medium</td>
<td>DMEM/F12+ 10% heat inactivated FBS, 1 μg/mL Insulin, 250 nM Dexamethasone, 0.5 mM Dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine</td>
<td>MPS</td>
</tr>
<tr>
<td>Maintenance Medium</td>
<td>DMEM/F12+ 10% FBS, 1 μg/mL Insulin</td>
<td>MPS</td>
</tr>
<tr>
<td>PBS</td>
<td>8 g/L NaCl, 0.2 g/L KCl, 1.44g/L Na2HPO4, 0.24 g/L KH2PO4, Adjusted to pH 7.4 with HCl</td>
<td>MPS</td>
</tr>
<tr>
<td>Trypsin/EDTA in HBSS</td>
<td>Ca, Mg: 8 g/L NaCl, 0.4 g/L KCl, 48 mg/L Na2HPO4, 1 g/L glucose, 60 mg/L KH2PO4, Phenol red to 1 part in 100,000, 2.5 g/L Trypsin (1:250), 0.38 g/L EDTA, 0.35 g/L NaHCO3</td>
<td>MPS</td>
</tr>
</tbody>
</table>
Freezing medium | DMEM+10% DMSO | MPS
---|---|---
1.5M Tris-HCl (pH 8.8) | Tris base 0.18 g/mL, adjust pH with 6N HCl | MPS
0.5M Tris-HCl (pH 6.8) | Tris base 0.06 g/mL, adjust pH with 6N HCl | MPS
Sample Buffer | 0.5M Tris-HCl (pH6.8), 10% SDS, 25% glycerol, 1% bromophenol blue, 0.4mL β-mercaptoethanol | Sigma-Aldrich
Running Buffer | 25mM Tris, 190 mM glycine, 0.1% SDS, pH 8.8 | MPS
Transfer Buffer | 25mM Tris, 190 mM glycine, 20% methanol | MPS
TBST | 50mM Tris, 150mM NaCl, 0.1% Tween-20, Adjust pH to 7.4 with HCl | MPS
Oil Red O Stock Solution | 3 mg/mL Oil Red O in 100% isopropanol | Sigma Aldrich
RIPA | 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4 | Sigma Aldrich
Glycine-hydrazine buffer | 0.6 M 398 glycine and 0.5 M hydrazine, pH 9.2 | Sigma Aldrich
Washing Buffer | 0.05% Tween® 20 in PBS, pH 7.2-7.4 | MPS
Reagent Diluent | 1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered | MPS

Table 7: List of solutions used in the experiments below.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Serum Lipase (HSL)</td>
<td>25</td>
</tr>
<tr>
<td>Phospho Human Serum Lipase (pHSL)</td>
<td>153</td>
</tr>
<tr>
<td>Protein Kinase B (AKT)</td>
<td>34</td>
</tr>
</tbody>
</table>
Table 8: List of antibodies used in western blot. All antibodies were bought from Cell Signaling Technology (CST).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho Protein Kinase B (AKT)</td>
<td>10</td>
</tr>
<tr>
<td>Perilipin</td>
<td>47</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>42</td>
</tr>
<tr>
<td>Horse Radish Peroxidase (HRP)</td>
<td>100</td>
</tr>
<tr>
<td>ACE 1</td>
<td>1</td>
</tr>
</tbody>
</table>

2.1 CLINICAL PROCEDURES

2.1.1 STUDY DESIGN

Participants were recruited through flyer and/or newsletter advertisements at the University of Warwick. They needed to be 18 years or older and not on any medication to be eligible for the study. Exclusion criteria included pregnancy and diagnosed diabetes. The ethics approval was given by Warwick Medical School Ethics committee BSREC (protocol identification REGO-2014-1318). All participants were informed about the study requirements and written consent from each participant were obtained. Participants provided information about their age, BMI, sex, weight (recorded every morning) and ethnicity.

2.1.2 HEALTH PLATFORM

Intel IDX is a framework that allows building of cross-platform mobile applications, (Windows, Android and iOS) along with a web browser interface. It was used to develop the mobile and web application, available at https://agper.lnx.warwick.ac.uk/mobileHealth-web/. The programming was done using technologies such as HTML, Javascript and CSS. To enable device specific optimal user experiences, a responsive web design approach was adopted in implementing the application. Mobile app functionality and front-end browser was built using AngularJS (a JavaScript framework). All back-end support for the web interface and mobile app were built using Java and MariaDB database server. Programming language R was used
to generate back end automation for providing users with graphical feedback. Data communication between the mobile and web applications and server was through HTTPS.

2.1.3 MEAL PLANS

A diet day was defined as a day where the participant omitted one of the 3 main meals i.e. breakfast (B), lunch (L) or dinner (D). The total calories for the day were to be less than ~1200KCal (± 50KCal). An optional snack (S) (<250± 50KCal) could be eaten only once in a day at a time chosen by the participant. All the meals (B, L, D, S) were defined according to i) calorie intake (<250KCal for S and >250KCal for B, L, D) summed over nearby entries (within 30 min) and ii) timing, being B before noon, L between noon and 3 pm and D after 4 pm (Figure 1A). A control day was defined a day where the participant had three or more meals on that day.

According to these terms, the different meal plans were defined to describe an individual’s preference: i) B, L, [S]; ii) B, D, [S]; iii) L, D, [S]; iv) B or L or D, [S] for dieting days; and v), B,L,D, [S] or >3 meals for control days, where [S] denoted optional snack intake. Entries in the health platform were used to identify the meal plan an individual chose every day. The different meal plans were compared to weight changes observed over the respective 24-hour period. Only those records were analysed that had a minimum of two meal records for the day (from 00:00h to 12:00h day +1). Any food item was allowed, and no specifications were given to participants for food composition.

The caloric intake was estimated by the meal information provided by the user. The participants either used the pre-entered meal options from the health platform or entered descriptions of their meals. The meal breakdown in protein, fat and carbohydrate was obtained from the fatsecret database (available at https://platform.fatsecret.com). For analysis purposes, it was converted into KCal by multiplying gram of protein, fat and carbohydrate by 9, 4 and 4, respectively. The fasting period (during the day) was obtained by subtracting the time of the second meal from the first meal of the day. Similarly, the overnight fasting time was obtained for days where weight information was provided on the following day.

2.1.4 URINE SAMPLE COLLECTION

Urine samples were collected in the home/work/leisure environment each time participants needed to empty their bladder. The total urine output was entered in the health
platform. Approximately 2-3 mL of urine was transferred using plastic pipettes to a pre-labelled 2 x 1.5 mL Eppendorf tubes. Participants placed samples in a secure container in a freezer until transferred (on dry ice) to the university. For short term, samples were stored at 4°C and later transferred to -80°C freezer (at University of Warwick) after 24 hours.

2.1.5 SURVEY DESIGN

A short-self-administered questionnaire, designed using Google forms, was emailed to all participants who expressed an interest in the study, including those who decided to not continue with enrolment. The individuals participating in the survey were not linked to their identification in the platform, as the survey document (Google forms) and the digital health platform were independent of each other. This optional survey was completed by 48 people; and all 48 individuals had consented to participate in the survey.

The survey was divided into three parts:

Section I: Motivation

Survey consisted of questions evaluating the understanding of an individual’s motivation in participating in the study. Options for participation included interest in losing weight, dieting, metabolic profile, health platform, being involved in medical research or any other reasons not specified in the list. These parameters were analysed together and when separated into 10-year bin-sized age groups.

Section II: Dropout

The survey was used to identify the reasons for dropout such as difficulty of following a restrictive diet, complicated sample collection, time consuming, complicated health platform, no more interest in losing weight and diet was not as expected. The participants were also provided a free text field (to catch all possible reasons) to enter other factors that contributed to their dropout.

Section III: Feedback

The health platform was developed in-house and the concept of identifying life-style markers was being tested. To develop this further, it was necessary to take participants feedback on the health platform and sample collection. Their personal input was requested on suggestions for the platform’s improvement.
2.1.6 LACTATE QUANTIFICATION IN URINE SAMPLES

Lactate dehydrogenase is the enzyme responsible for the interconversion of lactate into pyruvate following reduction of nicotinamide adenine dinucleotide (NAD) to its reduced form (NADH).

\[
\text{NADH} + \text{H}^+ \xrightarrow{} \text{NAD} + \text{H}_2\text{O} \xrightarrow{} \text{NADH} + \text{H}^+
\]

*Figure 5:* Conversion of lactate to pyruvate and vice-versa

As it is a reversible reaction, to measure lactate there was a need of excess NAD (Figure 5). It is necessary to trap the formed pyruvate with hydrazine present in glycine-hydrazine buffer (Sigma Aldrich, UK) as it would prevent the formed pyruvate to go back in the reversible reaction forming lactate.

The samples were centrifuged before analysis at 12,000 rpm for 15 minutes at room temperature. The experiments were done in 96-well solid black fluorescence plates. 10 mM lactate stock solution was prepared in glycine-hydrazine buffer. The standard reactions (25-100 μM lactate) were prepared. A reaction mixture contacting 10 mg NAD, 2.0 mL glycine-hydrazine solution, 4.0 mL water and 0.1 mL L-lactate dehydrogenase (mammalian cells only have L-form of the enzyme). 20, 20 and 130 μL of sample, standard and reaction mixture were added to each well respectively. The plate was then incubated for 15 minutes at 37°C. The fluorescence was read with excitation at 345-355nm and emission at 450-460nm using Perkin Elmer Wallac 1420 Victor 2 Microplate Reader.

2.1.7 ACE QUANTIFICATION IN URINE SAMPLES

ACE was measured using an immune-sandwich based ELISA method based on the manufacturer’s instructions (R&D Systems, UK). First, a 96-well plate was coated with 100 μL per well of the diluted Capture Antibody (R&D Systems). The goat anti-human ACE Capture Antibody was diluted to the working concentration (8000 ng/mL) in PBS. The plate was sealed and incubated overnight at room temperature. Each well was washed 3 times with 400 μL of the wash buffer. Each well was then blocked with 300 μL of reagent diluent and the plate was incubated at room temperature for a minimum of 1 hour, after which the plate was washed as above.
100 μL of urine samples or standards in reagent diluent was added to each well. The plate was sealed and incubated for 2 hours at room temperature. The plates were then washed as previously. 100 μL of the biotinylated goat anti-human ACE detection antibody (2000 ng/mL) diluted in reagent diluent was added to each well. The plates were incubated for 2 hours at room temperature. The plates were then washed as previously. 100 μL of the working dilution (1:10) of Streptavidin-HRP was added to each well. The plate was incubated for 20 minutes at room temperature in the dark. The plate was then washed as previously. 100 μL of substrate solution (1:1 mixture of H₂O₂ and Tetramethylbenzidine) was added to each well and incubated for 20 minutes at room temperature. Reaction was stopped by adding 50 μL of stop solution (2N H₂SO₄) per well. The optical density (OD) of each well was measured at 450 nm corrected at 540 nm (to allow correction for optical imperfections) using Synergy HTX multi-mode plate reader.

2.1.8 INSULIN QUANTIFICATION IN URINE SAMPLES

Insulin in samples was measured using a Mesoscale Discovery Human Insulin Kit containing (catalogue number: K151 BZC-2) 96 well plates coated with insulin antibodies obtained from Mesoscale Inc. (www.mesoscale.com). The assay was performed according to the manufacturer’s instructions. The plates were analysed on a SECTOR Imager 6000 system. All samples (urine and plasma) were centrifuged prior to analysis at 12000 rpm for 5 min at room temperature. Insulin calibrators (supplied by the kit) were run in duplicate to generate an 8-point standard curve covering the 0–50,000 pg/mL range. The standard curve was modelled using least squares fitting algorithms so that signals from standards with known concentrations of insulin can be used to calculate insulin concentrations in samples. The MSD Discovery Workbench® analysis software was used to calculate the concentration of insulin in samples. The software uses a 4-parameter logistic model and includes a 1/Y² weighting function. This allows for a better fit of data over a wide dynamic range (3–4 logs), particularly at lower insulin concentrations. The wide dynamic range of the assay allowed for the quantification of insulin in urine without the need for dilution nor concentration.
2.2 CELLULAR PROCEDURES

2.2.1 CELL CULTURE AND ADIPOCYTE DIFFERENTIATION

3T3-L1 cells (obtained from Dr. Mark Christian at University of Warwick) were cultured in DMEM/F12 + 10% NCS with Pen-Strep antibiotic solution. The cells were incubated at 37 °C and 5% CO2. They were kept below 50% confluency during routine culture. For differentiation, cells were plated and cultured to confluency (Day 0). They were then left for two days at confluence prior to addition of the induction medium (Day 2). These confluent cells were treated for 48 hours (until Day 4) with induction medium. The cells were then treated with maintenance medium for 48 hours (until Day 8).

2.2.2 TRYPsinIZATION

Cells were first washed with PBS prior to trypsinization. 1 mL of trypsin was added to 10 cm dish and T-25 flask and 2mL to T-75 flask. Cells were incubated at 37°C for 2 minutes to allow complete detachment of the cells from the surface (10 cm, T-25 and T-75). 9 mL (10 cm dish), 4 mL (T-25 flask) and 13 mL (T-75 flask) of DMEM/F12 + 10% NCS was added to inactivate the trypsin. Cells were removed from the plate by pipetting up and down and transferred to new dishes in 1:10 dilution.

2.2.3 FREEZING

For long-term storage of 3T3-L1 cells, up to 50% confluent plates (10 cm or T75-flask) were trypsinized as shown above. The cells were centrifuged at 1,000 rpm for 10 minutes. The supernatant was discarded, while the pellet was suspended in 3 mL of freezing medium. 1 mL aliquots were prepared in cryogenic vials and kept in a Styrofoam box at -80°C freezer overnight. The following day, vials were moved to a liquid nitrogen container.

2.2.4 THAWING

The cryogenic vial was moved from liquid nitrogen to 37°C water-bath to allow thawing. The cells were transferred to a 15 mL falcon tube and slowly 5 mL of DMEM/F12
+ 10% NCS was added to the tube. Cells were pelleted at 1,000 rpm for 10 minutes; the supernatant was discarded and 9 mL of fresh DMEM/F12 + 10% NCS (supplemented with PenStrep) was added. The cells were re-suspended and transferred to a 10 cm dish at 37°C, 5%CO₂ incubator. The medium was changed 4-6 hours later to minimise DMSO exposure.

2.2.5 LEMON EXTRACT TREATMENT

One lemon was squeezed into a 50 mL falcon tube. This solution was then frozen by placing the 50 mL falcon tube at -20°C for 24 hours. The frozen solution was kept on dry ice for 2 hours before freeze-drying. The solution was lyophilised overnight in an instrument named Alpha 2-4 LD plus made by Martin Christ Gefriertrocknungsanlagen GmbH (Germany). The lyophilised powder was re-suspended in 10 mL of DMEM/F12. The pH was adjusted to 7 by adding sodium hydroxide.

2.3 ANALYTICAL PROCEDURES

2.3.1 RNA EXTRACTION

RNA was extracted using QIAzol Lysis Reagent. After removing the maintenance medium, 1 mL of QIAzol reagent was added directly to the cells in the culture dish per 10 cm² of dish surface area. The cells were lysed directly by pipetting the cells up and down several times. The homogenised sample was then incubated at room temperature for 5 minutes followed by addition of 0.2 mL of chloroform per 1 mL of lysis reagent. The tube was shaken vigorously for 15 seconds and centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous layer was removed and transferred to a new tube. 0.5 mL of 100% isopropanol (per 1 mL of lysis reagent) was added and incubated for 10 minutes at room temperature. The solution was then centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was discarded, leaving only the RNA pellet. This was then washed with 1 mL of 75% ethanol (per 1 mL of lysis reagent) and vortexed briefly before centrifuging at 7,500 g for 5 minutes at 4°C. The wash was discarded and the pellet was air dried for 10 minutes. The pellet was re-suspended in 50 µL of nuclease free water. The RNA solution was incubated at 55°C on a heat block for 10 minutes to complete solubility of RNA. The extracted RNA was quantified using a NanoPhotometer N60. RNA was used for further work if OD₂₆₀/OD₂₈₀ was ≥1.90. It was either used for downstream applications or stored at -70°C.
2.3.2 CDNA SYNTHESIS

The cdNA synthesis was performed using a High-Capacity RNA to c-DNA kit (Thermo Scientific, UK). The Reverse transcriptase (RT) enzyme allows conversion of RNA to cdNA. 100 ng of total RNA is used in a 20 µL reaction. All the kit components were thawed on ice. The volume of the components needed were as follow:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+RT reaction (µL)</td>
</tr>
<tr>
<td>2X RT Buffer Mix</td>
<td>10</td>
</tr>
<tr>
<td>20X RT Enzyme Mix</td>
<td>1</td>
</tr>
<tr>
<td>RNA sample</td>
<td>Up to 9µL</td>
</tr>
<tr>
<td>Nuclease- free water</td>
<td>Q.S. * to 20µL</td>
</tr>
<tr>
<td>Total per reaction</td>
<td>20</td>
</tr>
</tbody>
</table>

*Table 9: cdNA Synthesis Reaction Mix. *Quantity Sufficient

Aliquots of RT reaction mix were added to the reaction tubes and briefly centrifuged to spin down the contents and to eliminate any air bubbles. The tubes were then loaded to the thermal cycler with following cycling conditions:

<table>
<thead>
<tr>
<th>Setting</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37</td>
<td>95</td>
<td>4</td>
</tr>
<tr>
<td>Time</td>
<td>60</td>
<td>5</td>
<td>∞</td>
</tr>
</tbody>
</table>

*Table 10: cdNA Synthesis Reaction Setting

The reverse transcription run was started and the cdNA generated was stored at 2-8°C for short-term storage and at -25°C for long-term storage.

2.3.3 REAL TIME POLYMERASE CHAIN REACTION (RTPCR)

RTPCR was performed on a 96 well reaction plate using Taqman primers (Taqman Gene expression Assay, ThermoScientific, UK) for the genes of interest. The cdNA samples and Taqman Gene expression Assay were thawed on ice. The 20 µL reaction was prepared as follow:
### Table 1: RT PCR Reaction Mix

The plate was sealed and centrifuged briefly before loading in to the Applied Biosystems 7500 Fast Real-Time PCR System instrument. Real time PCR was run as follows:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp (°C)</th>
<th>Time (mm:ss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>50</td>
<td>2:00</td>
</tr>
<tr>
<td>Hold</td>
<td>95</td>
<td>0:20</td>
</tr>
<tr>
<td>Cycle (40 Cycles)</td>
<td>95</td>
<td>0:03</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0:30</td>
</tr>
</tbody>
</table>

### Table 2: RTPCR Reaction Setting

The data was exported into an Excel file and analysed for fold change using the equations below:

\[
\Delta C_t = C_t \text{ gene test} - C_t \text{ endogenous control}
\]

\[
\Delta \Delta C_t = \Delta C_t \text{ sample1} - \Delta C_t \text{ calibrator}
\]

Fold Change= RQ = Relative quantification = \(2^{-\Delta \Delta C_t}\)

### 2.3.4 PROTEIN EXTRACTION

LE-treated cells along with untreated cells (control) were used to study protein expression levels. Each well was washed with 1 and 5 mL of ice-cold PBS solution in 12 and 6 well plate, respectively. 200 µL of lysis buffer solution was added to each well. Protease inhibitor cocktail Set V (Calbiochem, Germany) and phosphatase inhibitor cocktail 2 P5726

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X TaqMan® Gene Expression Assay</td>
<td>1</td>
</tr>
<tr>
<td>2X TaqMan® Gene Expression Master Mix‡</td>
<td>10</td>
</tr>
<tr>
<td>cDNA template (100ng)</td>
<td>4</td>
</tr>
<tr>
<td>RNase free water</td>
<td>5</td>
</tr>
<tr>
<td>Total per reaction</td>
<td>20</td>
</tr>
</tbody>
</table>
(Sigma-Aldrich, UK) were added at a concentration of 10 µL/mL of RIPA lysis buffer. The cells were scraped quickly using a cell scraper, collected in a 1.5 mL Eppendorf tube and placed on ice. The tubes were vortexed strongly every 2 minutes and kept on ice for 10 minutes. The samples were then centrifuged at 12,000 rpm form 10 minutes at 4°C. The supernatant was collected in a fresh Eppendorf. An aliquot was taken for quantification and/or further analysis and the extracted protein was stored at -20°C.

2.3.5 PROTEIN QUANTIFICATION

Protein concentrations were determined using the Modified Lowry’s Protein Assay. A calibration curve was obtained by making 0, 1, 5, 25, 125, 250, 500, 750, 1000 and 1500 µg/mL BSA concentrations using a 2 mg/mL BSA stock solution. 1X Folin-Ciocalteu (FC) reagent was prepared by diluting the supplied reagent (1:1) with ultrapure water. 40 µL of each standard and sample was added into a microplate followed by 200 µL of Modified Lowry Reagent. The plate was mixed for 30 seconds and incubated at room temperature for 10 minutes. 20 µL of prepared 1X FC Reagent was added to each well and mixed 30 second followed by incubation at room temperature for 30 minutes. Protein quantification was determined at 750 nm absorbance, using a Synergy HTX multi-mode plate reader. The concentration was determined using a BSA standard curve.

2.3.6 SDS-PAGE

The acrylamide separating gel was made of 12% resolving and 4% stacking gels. 10 mL resolving gel and 5 mL stacking gel was prepared. 30 µg protein sample were mixed with 4X sample buffer solution and gel was run in running buffer solution at 50 Volts for 5 minutes and at 100V for 90 minutes.

<table>
<thead>
<tr>
<th>Gel Type</th>
<th>Acrylamide (mL)</th>
<th>Tris-HCl (mL)</th>
<th>Water (mL)</th>
<th>10% SDS (µL)</th>
<th>10% APS (µL)</th>
<th>TEMED (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolving</td>
<td>4</td>
<td>2.5 (1.5M, pH 8.8)</td>
<td>3.3</td>
<td>100</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>(12%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 13: SDS-PAGE Gel Composition

<table>
<thead>
<tr>
<th></th>
<th>0.7</th>
<th>1.25</th>
<th>2.94</th>
<th>50</th>
<th>10</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(0.5M, pH6.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3.7 WESTERN BLOTTING

The electrophoresis gel was equilibrated in ice-cold transfer buffer solution for 10 minutes. Two sponges and six Whatman paper (for 1 gel) were also equilibrated in the same buffer. A polyvinylidene fluoride membrane (Thermo Scientific, UK) was activated in methanol for 10 minutes before being incubated in the transfer buffer for 10 minutes. These were assembled in a transfer sandwich cassette with the gel on anode and membrane on the cathode (Biorad system, UK). The cassette was then placed in the transfer tank and transfer was performed at 100 Volts for 45 minutes. The membrane was then blocked in 3% BSA in 1X TBST solution for 1 hour at room temperature before being incubated overnight at 4°C under rotation in the primary antibody solution (1:1000) against the protein of interest. The blot was washed (3 times) with 25 mL TBST for 5 minutes and it was incubated with the appropriate secondary antibody (1:10,000) in 1X TBST for 1 hour at room temperature. The membrane was washed as before and dried for up to 5 second before being transferred to saran wrap. 600 µL of SuperSignal West Pico PLUS (Thermo Scientific, UK) developing solution was distributed evenly on the blot and the wrap was closed. Using secondary antibody as the enzyme label, luminescent substance in SuperSignal West Pico PLUS was oxidized by hydrogen peroxide and luminesced. The signal was detected when the blot was sensitized on photographic film. The image was taken using an Image Quant LAS4000 apparatus.

2.3.8 FREE GLYCEROL RELEASE ASSAY

1x 12-well plate with differentiated 3T3L1 adipocytes was treated with LE, PBS (negative control) for 0, 2, 4, 6, 24 and 72 hours and 1 µM isoproterenol (positive control). The cells were pre-incubated with 1400 µL DMEM/F12 medium solution (without phenol red) for 2 hours. The cells were then either treated with LE or PBS for 72 hours. Prior to treatment (t=0) and at t=2, 4, 6, 24 and 72 hours after treatment, a media aliquot (200 µL) was taken and immediately frozen. After t=72 aliquot, the cells were lysed for protein extraction followed by protein quantification. Glycerol amount in each media was quantified using a free
glycerol kit from Sigma-Aldrich, UK. A standard curve was performed using glycerol standard solution. 40 µL of media was added into 200 µL of glycerol free reagent solution in a 96-well plate. The samples were incubated at room temperature for 15 minutes and absorbance was measured at 540 nm using a Synergy HTX multi-mode plate reader. The amount of free glycerol was calculated using the standard curve and the amount was normalized by the amount of protein.

2.3.9 INSULIN SENSITIVITY ASSAY

Cells were differentiated on a 12-well plate (Sec 2.2.1) until day 7. The cells were deprived of serum overnight. On Day 8, 3 out of 12 wells were incubated with 100 µg/mL of LE for 10 hours and then treated with 100 nM of insulin for 15 minutes. Proteins were extracted as described above and insulin receptor downstream target AKT was analysed by western blot.

2.3.10 ACE INHIBITORY ACTIVITY ASSAY

ACE inhibitory activity was measured by a fluorometric assay following the method of (177). A potential ACE inhibitor would prevent conversion of Abz-GLY-PHe(NO2)-Pro to Abz-GLY by inhibiting ACE enzyme. 50 µL of LE (0, 50, 100 and 500 µg/mL) followed by 50 µL ACE (3 mU/mL) solution was added in each well of a black 96-well plate. The mixture was incubated at 37°C for 10 minutes. 200 µL of Abz-GLY-PHe(NO2)-Pro in 150mM Tris base (pH 8.3) with 1.125 M NaCl was added to each well and fluorescence was measured at 360 nm (excitation) and 430 nm (emission) for t=0 using a Synergy HTX multi-mode plate reader. The plate was then incubated for 30 minutes and reading was taken as mentioned above. The % inhibitory activity was calculated following the method in (178). Briefly, it was calculated as shown below:

\[
\%\text{inhibitory activity} = 100 \times \frac{(A-C)}{(A-B)}
\]

where A was the absorbance of ACE solution in buffer; B was the absorbance of buffer and C is the absorbance of ACE in LE.
2.3.11 CELL VIABILITY ASSAY

The cells were trypsinized as above and 50 µL of cell suspension was added in an eppendorf. Equal parts of 0.4% trypan blue dye were added to the cell suspension and mix by pipetting up and down. Place the cover slip on the haemocytometer and 10-20 µL of cell suspension on one side of the haemocytometer. Place the haemocytometer on the stage of a light microscope and the cells were counted by the in each large corner. The percentage of viable cells were calculated by dividing the number of viable cells by the number of total cells and multiplying by 100.

2.4 IMAGING TECHNIQUES

2.4.1 OIL- RED O STAINING

Oil red O staining was used to stain and detect lipids in cultured adipocytes. The medium was removed and cells were gently washed twice with a PBS solution. Cells were then fixed in 10% formalin (Sigma-Aldrich, UK) for 1 hour. Cells were washed with water and incubated with 60% isopropanol for 5 minutes. Oil Red O stock solution was prepared by reconstituting 3 mg/mL of Oil Red O powder in 100% isopropanol. The solution was mixed and left undisturbed for 20 minutes. The working solution was prepared by mixing 3 parts of stock solution with 2 parts of water. The solution was mixed and left undisturbed for 10 minutes before being filtered through Whatman filter paper. The cells were then covered with the Oil Red O working solution for 20 minutes. The cells were then washed with water 5 times and covered with water to view under the microscope. The images were taken using EVOS XL Core. For quantification, the water was removed, and the wells were allowed to dry. Oil Red O dye was eluted in 1 mL of 100% isopropanol and incubated for 10 minutes with gentle shaking. The isopropanol was pipetted up and down several times ensuring that the dye is in solution. Absorbance was taken at 500 nm with 100% isopropanol as blank using a Synergy HTX multi-mode plate reader.
2.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

A Merck instrument equipped with L-7100 pump, L-7455 UV diode array detector, D-7000 chromate-integrator and a column separator was used for analysis. LE was separated on a C-18 column (250 mm x 5 mm x 4.6 mm). The mobile phase consisted of 0.04% formic acid in water (A) and acetonitrile (ACN) in 0.04% formic acid (B). A gradient program was used as shown below. The flow rate for the mobile phase was 1mL/min. The column temperature was 25°C.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A: Water (0.04% Acetic Acid)</th>
<th>B: Acetonitrile (0.04% Acetic Acid)</th>
<th>Flow Rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>60</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>5</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>95</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>95</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table 14: Low resolution HPLC gradient program*

2.6 MASS SPECTROSCOPY

Following the optimisation of the HPLC gradient, different compounds in LE were identified using mass spectrometry. Data was acquired on the MaXis II Q-TOF instrument coupled with Dionex 3000RS UHPLC and the column used was Agilent Zorbax C18, 100 x 2.1 mm. Mobile phase used was water/ACN with 0.1% ammonium, formic acid. The same gradient as in Table 14 with a reduced flow rate of 0.2 mL/min was used.
2.7 NUCLEAR MAGNETIC RESONANCE (NMR)

Urine samples were thawed and centrifuged at 12,000 rpm for 10 minutes. 60 µL of urine buffer was added to NMR tube followed by 540 µL of urine sample. Urine buffer (100 mL) was prepared as mentioned in (179).

1.5 M KH$_2$PO$_4$ buffer was prepared in D$_2$O. 100 mg TSP and 13 mg NaN$_3$ was dissolved in 6 to 10 mL of D$_2$O. Both solutions were mixed by sonication. The solution might appear cloudy, but it disappeared when the pH was adjusted to 7.4 by adding KOH pellets. The solution was transferred to a 100 mL volumetric flask and the volume was adjusted with D$_2$O.

To aid in identification of metabolites found on diet and control day (see 2.1.3), 1D NOESY and 2D J-resolved (JRES) was performed on a Bruker spectrometer at 300K. The experiment was done as described in (1). A standard 2 mM sucrose sample (containing 0.5 mM TSP, 2 mM NaN3 in 90% H2O:10% D2O) was loaded to check the performance of the water suppression functionality. The temperature was equilibrated after 5 minutes. The experimental parameters (Table 15 and 16) for urine were loaded.

<table>
<thead>
<tr>
<th>Pulse program</th>
<th>noseygppr1d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time domain</td>
<td>65536</td>
</tr>
<tr>
<td>Dummy scans</td>
<td>4</td>
</tr>
<tr>
<td>Scans</td>
<td>32</td>
</tr>
<tr>
<td>Sweep width</td>
<td>20 ppm</td>
</tr>
<tr>
<td>Acquisition time</td>
<td>2.726 s</td>
</tr>
<tr>
<td>Relaxation delay</td>
<td>4 s</td>
</tr>
<tr>
<td>Receiver gain</td>
<td>90.5s</td>
</tr>
<tr>
<td>Dwell time</td>
<td>41.6 µs</td>
</tr>
<tr>
<td>Mixing time</td>
<td>0.01 s</td>
</tr>
<tr>
<td>Line broadening</td>
<td>0.3 Hz</td>
</tr>
</tbody>
</table>

*Table 15: Experimental Parameters for Urine for 1D NOESY (179).*

<table>
<thead>
<tr>
<th>Pulse program</th>
<th>Jresprqrf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time domain</td>
<td>8192 in F2</td>
</tr>
<tr>
<td></td>
<td>40 in F1</td>
</tr>
<tr>
<td>Dummy scans</td>
<td>16</td>
</tr>
<tr>
<td>Scans</td>
<td>2</td>
</tr>
<tr>
<td>Parameter</td>
<td>Value</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Sweep width</td>
<td>16.7 ppm in F2</td>
</tr>
<tr>
<td></td>
<td>0.13 in F1</td>
</tr>
<tr>
<td>Acquisition time</td>
<td>0.41s in F2</td>
</tr>
<tr>
<td></td>
<td>0.26 in F1</td>
</tr>
<tr>
<td>Relaxation delay</td>
<td>2s</td>
</tr>
<tr>
<td>Receiver gain</td>
<td>90.5</td>
</tr>
<tr>
<td>Dwell time</td>
<td>50µs</td>
</tr>
<tr>
<td>Line broadening</td>
<td>0.3Hz in F2</td>
</tr>
<tr>
<td></td>
<td>0.3 in F1</td>
</tr>
</tbody>
</table>

*Table 16: Experimental Parameters for Urine for 2D JRES (179).*

Peak assignments relied on established literature, such as the human metabolome database (HMDB) (2) and human urine metabolome (3). The peak assignment was decided using metabohunter (4). The Bruker Topspin 4.0.4 software package was used to quantify metabolites.

### 2.8 STATISTICAL PROCEDURES

#### 2.8.1 CLINICAL STUDY

Statistical analyses were performed using IBM SPSS Statistics 24 and R. ANOVA was used to understand the effect of meal plan choice on weight loss. The control group was compared with BL, BD, LD and B or L or D using SPSS software. Tukey posthoc test was conducted on ANOVA data for further analysis of difference in each group. Similarly, control group was also compared to BL, BD, LD and B or L or D with or without consumption of a snack.

To understand the effect of caloric intake, total insulin, total lactate and overnight fasting time, Pearson Coefficient analysis was conducted using SPSS. This informed about the measure of the strength and direction of association that exists between two variables. To address the missing values, pairwise cases were excluded. Nonparametric Mann-Whitney U test was performed to compare healthy, overweight and obese groups.

The parameters extracted from digital health platform and biomarker profile, were analysed using bivariate Pearson Coefficient analysis using R. Harrell Miscellaneous (hmisc)
and corrplot R packages were used for the data analysis and graphic representation. The following script was used for the analysis:

Install.packages ("Hmisc")

Install.packages ("corrplot")

mydata=read.csv("filename.csv")

mcor=cor(mydata, use="pairwise.complete.obs", method="pearson")

cor.mtest <- function(mat, ...) {
  mat <- as.matrix(mat)

  n <- ncol(mat)

  p.mat<- matrix(NA, n, n)

  diag(p.mat) <- 0

  for (i in 1:(n - 1)) {
    for (j in (i + 1):n) {
      rcorr
      tmp <- cor.test(mat[, i], mat[, j], ...)
    }
  }

  colnames(p.mat) <- rownames(p.mat) <- colnames(mat)
}

p.mat=cor.mtest(mydata)

rcorr(as.matrix(mydata[,1:21]))

flattenCorrMatrix <- function(cormat, pmat) {
  ut <- upper.tri(cormat)

  data.frame(row = rownames(cormat)[row(cormat)[ut]], column = rownames(cormat)[col(cormat)[ut]], cor =(cormat)[ut], p = pmat[ut])
}
res<-rcorr(as.matrix(mydata[,1:21]))

flattenCorrMatrix(res$r, resp.mat$P)

m= flattenCorrMatrix(res$r, res$P)

corrplot(mcor, type="lower", order="alphabet", p.mat=p.mat, sig.level=0.01, tl.col="red", tl.srt=45, tl.cex=0.75)

2.8.2 CELLULAR STUDY

Results were expressed as mean ± standard error mean (SEM). Differences between cells or treatments were tested for statistical significance using the unpaired Student’s t test.
LACTATE AND INSULIN ARE PREDICTORS OF WEIGHT LOSS OVER SHORT TERM (24 HOUR) PERIODS

This figure illustrates personalised the feedback strategy with focus on lactate and insulin. The figure was designed using Paint and PowerPoint 2013.
3.0 SUMMARY

Numerous diets, apps and websites help guide and monitor dietary behaviour with the goal of losing weight, yet dieting success is highly dependent on personal preferences and circumstances. To enable a more quantitative approach to dieting, an integrated platform was developed that allowed tracking of life-style information alongside molecular biofeedback measurements using lactate and insulin levels in urine samples.

To facilitate weight loss, participants (≥18 years) omitted one main meal from the usual three-meal routine. Daily caloric intake was restricted to ~1200 KCal with one optional snack ≤250 KCal. A mobile health platform (personalhealth.warwick.ac.uk) was developed and used to maintain diaries of food intake, weight, urine collection and volume. A survey was conducted to understand participants’ willingness to collect samples, motivation for taking part in the study and reasons for dropout.

Meal skipping resulted in weight loss after a 24-hour period in contrast to 3-meal control days regardless of the meal that was skipped, breakfast, lunch or dinner (p<0.001). Common reasons for engagement were interest in losing weight and personal metabolic profile. Total insulin and lactate values varied significantly between healthy and obese individuals at p=0.01 and 0.05, respectively.

In a proof of concept study with a meal-skipping diet, it has been shown that insulin and lactate values in urine correlate with weight loss, making these molecules potential candidates for quantitative feedback on diet efficiency to people dieting.

3.1 INTRODUCTION

Male obesity rates rose from 13.2% to 24.4% and from 16.4% to 25.1% in women over the period 1993 to 2012 (180). Obesity’s health related concerns (discussed in chapter 1) may be excellent motivating factors to lose weight for some individuals. However, achieving weight loss is challenging and failure to obtain results is demoralising (65). Most typical weight loss programs include a diet regime (with or without drugs), a fitness regime or a combination of these approaches. There is a need of a device that involves use of biomolecules levels of a dieter to provide a biological feedback to understand the effects of lifestyle habits. The prerequisite for such a device is the identification of quantitative biomarkers of weight loss that have the potential to provide a feedback on the efficiency of the diet on a short term to the dieting individual, especially when weight loss is not always observed yet.
Insulin and lactate were chosen as these are both known to vary with consumption of glucose, as well as with caloric intake overall (96, 181-183). Skipping meals is often suggested for weight loss but there are controversies, in particular regarding omitting breakfast. Currently, there is an accumulation of facts with no consensus of the issue. Eating breakfast lowers body weight and leads to lower caloric intake in comparison to skipping this meal (69-72). Many studies indicate that breakfast eaters and skippers didn’t vary significantly in terms of body weight and nutrient intake (77-80). The concept of eating or skipping breakfast is controversial since some studies say it has negative impacts and some say this has no influence at all on body weight. Besides food composition, another factor amenable to behaviour change is meal timing.

From a metabolic perspective it might be most efficient for weight loss to skip meals other than breakfast. This might be true especially for omitting dinner (65). However, from a behavioural and/or cultural perspective, breakfast and lunch may be the easiest meals to skip, and dinner the most difficult (65). Weighing between these alternate strategies requires quantitative analysis. Finally, in order to develop a tool for personalised feedback, understand the willingness of individuals to collect samples to obtain metabolic biomarker information has to be investigated.

Here, the goal was to lay the foundation for a molecular feedback approach to assist dieting efforts. The aim was to obtain a better understanding of the complex interplay between possible molecular biomarkers of dieting behaviour, individuals’ personal preferences, their willingness to collect information and weight loss success. Specifically, 52 dieters were asked to record meal timing and composition using an electronic diary interface, along with collection of urine samples, and weight. The urine samples were analysed for lactate and insulin levels and the information was integrated with the digital health platform. The results provide a strong proof of concept that each molecule studied may be used for biofeedback on dieting effects, with a stronger correlation for insulin.

3.2 RESULTS

3.2.1 STUDY DESIGN AND DATA COLLECTION

Initially, 146 individuals (recruited using flyer and newsletter advertisements) expressed interest in the study. Of these, 52 individuals became study participants (77% females and 23% males) and provided recorded data and urine samples (Figure 6a).
Participants received access to a web application at personalhealth.warwick.ac.uk, as well as an app “Digital Health Platform” for android and apple devices available in Google play and iTunes stores, respectively. Through this platform, participants entered weight, food and liquid intake (caloric intake), exercise, and urine sample collection and volume details. It was used to provide information on calories consumed during each meal and over a 24-hour period. The mobile health platform created a timeline of the logs or events that were entered by the user. This electronic information was sent to a web server that allowed users to store their information securely and access it anywhere using either a web browser based interface or a native mobile application from their smart phones or tablets. In addition to being a tool for logging time and other parameters, the application also served to seamlessly share information between the user and the analyst. It allowed researchers and cohort group participants to register, and manage the logistics of data collection. Researchers obtained analysis files in an anonymized fashion only through the website administrator. Ease of use and cross-platform support were the most important among the factors considered in the design of the health platform.

Urine samples were used to measure insulin and lactate levels, which were uploaded onto the platform. Participants collected samples and life-style data for control and diet days (see Methods).
**Figure 6: Study Design.** (a) Meal plan layout for the participants in a 24-hour period. (b) Flow diagram of the study design. (c) Comparison between demographic features of the study participants and the UK population. Overall numbers for the UK population was the arithmetic middle between the male and female values based on the assumption that the distribution of male and females in the statistics was approximately 50%. In this study, there was more females than males, so the overall number was obtained directly from the raw data.
3.2.2 DEMOGRAPHICS OF STUDY PARTICIPANTS

According to the UK Health and Social Care Information Centre, the prevalence of overweight individuals in the UK population is age and gender dependent, with 9% (male) and 13% (female) in the 16–24 age group and 13% (male) and 35% (female) in the 50-69 age group (180). A similar pattern characterized the participants in this study (Figure 6). Grouping participants by age showed that the number of overweight study participants was lowest among younger adults (20-29 year old group, Figure 7a), increasing through middle age (ages 30-59, Figure 7b-d), and only reducing among the oldest participants (ages 60-69, Figure 7e). The majority of study participants were in the normal and overweight groups (Figure 6f). The mean BMI of 27.2 kg/m² observed in the UK population (180) parallels that of 27.0±5 kg/m² (mean ± standard deviation) in this study. Similarly, the weight and height values split by gender also mirrored those of the UK population (Figure 6c). This indicated that the sample of 52 participants was a good representation of the UK population. The mean BMI of males and females in the study were 26.0 and 28.0 kg/m², respectively, which indicated that they were significantly different (p<0.001) within the study group (Figure 8).

Figure 7: Demographic of study participants. Comparison of BMI of participants across different age groups (20-29, a; 30-39, b; 40-49, c; 50-59, d; 60-9, e). Comparison of BMI of participants across all age groups. BMI values are available for 31 participants out of 52. Significance levels are marked as follows: *p<0.05; **p<0.01.
3.2.3 DIET BEHAVIOUR: CALORIC INTAKE PATTERN

The timings of health platform entries on the 149 days of data entered by participants showed a wide spread from 7 am - midnight on a 24-hour scale (Figure 9a), with only night time (midnight to 7 am) receiving very few entries, in line with previous observations (81). There were a higher percentage of total entries on the health platform in the mornings and evenings, namely 33% and 32% of the total entries, respectively (Figure 9b). Many of the morning entries were weight and urine sample collections. When only food entries were plotted, entries clustered in the morning (around 7 am), at lunchtime (around 1 pm), and in the evening, peaking at 6 pm (Figure 9c). When entries were quantified by calories consumed, it could be seen that the largest calorie intake was in the evening, with 22% and 51% of the total calories recorded from 7-11 am and 4-9 pm, respectively (Figure 9d). A more detailed breakdown of entries as % food events per hour is shown in Figure 9e. Purple indicates meals, green snacks and blue/brown low-calorie drinks (including water and coffee). Most entries for caloric intake of >250KCal (i.e. a meal) were observed in just one hour from 6-7 pm (Figure 9e). Many breakfast (B) “meals” were low in calories and were therefore classified here as snacks (S), see below. Caloric intake was significantly different for males and females (p=0.05, Figure 8).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male</th>
<th>Female</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting Time</td>
<td>10.7±3.2</td>
<td>9.3±2.8</td>
<td>0.7</td>
</tr>
<tr>
<td>BMI</td>
<td>26.2±5.3</td>
<td>28.2±5.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total Calories</td>
<td>1329.7±736.1</td>
<td>1142.6±561</td>
<td>0.35</td>
</tr>
<tr>
<td>Weight Change</td>
<td>0.5±0.6</td>
<td>0.5±0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Figure 8: Gender Differences. Comparison of different parameters across male and female groups with significance at p=0.05.
Figure 9: More calories are consumed at dinner and breakfast and dinner combination were more popular than breakfast and lunch. (a) Polar plot of all entries of each individual plotted against the time of day (angular axis). Data from 50 individuals are shown. 24 hr rose plots showing (b) percentage of total entries from individuals, (c) percentage of ingestion events and (d) % of calories consumed. (e) Percentage of food events in 1 hr bins. The radial axis for each rose plot shows % of events.
3.2.4 DIET BEHAVIOUR: MEAL PLAN PREFERENCES

Out of the 52 participants, at least one entry with two meals (see Methods) was available for 43 people. Participants could freely choose the number of days they participated in the study, thus the number of days for which data was available varied for each participant and between participants. The majority followed the study plan for 1-2 days, while one participant collected data for up to 24 days. Thus, the 43 individuals collectively provided data for 147 days consisting of both, control (28) and diet (119) days. Participants were given a relatively free choice in meal plans, with the only restrictions being the omission of one of the 3 main meals and the total number of calorie, as described in Methods.

The meal plan choices made by participants on the 147 days is shown graphically in Figure 10a. BL, the meal plan that would be metabolically optimal from a theoretical perspective (see Introduction), or the slightly modified BLS meal plan, were followed only on 10 days. 19 days corresponded to the LD plan, while the largest number of 42 days was in the BD or BDS category. BDS was followed on 39% of the dieting days, and was thus the most popular meal choice, while the B[S] plan accounted for only 8% of the dieting days.

A graph of the spread of meal timing of individuals showed that participants followed similar eating patterns for all days if they provided samples and data for more than one day (Figure 10b). Another frequently followed meal plan was that of the single meal: 46 days had only one meal B, L or D (sometimes plus optional snack, B[S] or L[S] or D[S]). This large number likely arose from classification of what participants might have thought of as “meals” as snacks based on the 250KCal cut-off. In total, there were 26 control days (18% of the 147 days), where people have had at least three meals (BLD, BLD[S] or more). On control days, whilst caloric intake was significantly higher (p <0.01) than on diet days, there were many days of low calorie intake as well. The control group is a group of days where the participants did not receive the intervention. Notably, individuals did not lose weight on calorie- restricted control days, suggesting that meal timing played an important role, perhaps more than caloric intake for losing weight (Figure 10c).
Figure 10: Skipping a meal resulted in weight loss. (a) Distribution of meal plans. The meal plan preference is calculated by assigning each day to a meal plan category (BL (“Plan 1”), BD (“Plan 2”), LD, Control and B or L or D) based on the definitions described in Methods. (b) Eating duration of individuals, error bars are standard deviations where individuals have provided more than one day data. (c) Total caloric consumption by 52 participants across
meal groups (BL, BD, Control, LD and B or L or D). **Caloric intake is significantly different between group at p=0.01.

### 3.2.5 DIETING SUCCESS BY STUDY PARTICIPANTS’ MEAL PLAN CHOICE

Weight change data were available for 43 out of the 52 participants for at least one 24-hour period, therefore reducing the total of 147 days to 126 days. For ease of analysis, the weight change values were grouped into 3 groups: weight loss when the weight difference between the beginning and end of the 24-hour period was >0kg, weight gain for <0kg, and no change =0kg. Figure 11a shows the % of participants with weight change in each of these groups. One could clearly see that all diet meal plans resulted more often in weight loss as compared to the control days. Figure 11b shows the more detailed split into sub-groups taking whether or not a snack was eaten into account. Overall there did not appear to be a negative consequence of having the additional snack, although the size of the data was too small to ascertain the statistical significance of this statement. Because there was meal plan information for 21 days without weight change information, a fourth group “NA” (purple) was included in Figure 11a, b.

ANOVA was conducted to compare the control group with each of the other groups in Figure 11a (i.e. BL, BD, LD and B or L or D). Each group was significantly different from the control group (p<0.01). This indicated that skipping a meal resulted in weight loss irrespective of which meal of the day was skipped. Comparing weight loss with total caloric intake showed an inverse relation with Pearson Correlation significant at p=0.05 (Figure 11c). Finally, on days when participants achieved weight loss, the length of overnight fasting periods was inversely correlated to weight loss expressed as negative kg values (Pearson Correlation R=-0.21, p=0.016) (Figure 11d), i.e. the longer the fasting the greater the weight loss.
Figure 11: Weight loss is associated with fasting time and consumption of calories. (a) Effect of dieting on weight with respect to different meal groups. N/A refers to the days for which weight loss data is not available. Weight change is defined as weight loss (any change > 0kg), weight gain (any change <0kg), and no change (=0kg). One-way ANOVA analysis comparing control with other meal groups show significant difference at p=0.01** (p<.001) (b) Effect of dieting on weight with respect to different meal plan subgroups. (c) Plot of total caloric intake against weight difference. Pearson’s R= -0.21 correlation is significant at the 0.05 level (p <0.05). (d) Plot of overnight fasting time against weight difference. Pearson’s R=-0.21 correlation is significant at the 0.05 level (p <0.05).

3.2.6 MOTIVATION: REASONS TO PARTICIPATE IN THE STUDY

A survey was conducted to understand the reasons people were interested in the study. This included both, weight loss and urine sample collection (Figure 12). Interest in losing weight, involvement in research and knowledge of their metabolic profile were the main drivers behind the participant enrolment. There might be a difference in motivation for different age groups, as the 20-29 and 40-49 year age-group more often reported interest in their metabolic profile (33%), while the 30-39, 50-59 and 60-69 year age groups were more
motivated by losing weight (32, 32 and 20 % respectively). However, because of the small number of participants, it cannot be said if these differences were statistically significant.

Figure 12: Survey Questionnaire. Questionnaire designed with input from the research team and generated on google forms. For i), ii), ix) and xv) participants were allowed to choose more than one option.
3.2.7 DROPOUT ANALYSIS

Dropout rates in weight loss studies have been a prominent concern when promoting lifestyle and dietary changes in overweight and obese populations, as well as affecting the validity and generalisation of conclusions in weight loss studies (184). In this study, a similar trend was observed. At the first meeting, people were informed of the study requirements, which resulted in 70 out of the 146 initial participants to drop out of the study. The 76 remaining individuals who provided written consent for participation in the study received sample collection kits. 52 out of these 76 participants provided urine samples and life-style information through the online/mobile platforms. Thus, the dropout rate after the first meeting of 48% reduced to 16% when comparing to the initial number of people interested in the study, and 34% when comparing to the previous step (Figure 6b).

To identify the reasons for dropout, a survey was designed (Figure 12). Busy schedule, complicated sample collection and loss of motivation corresponded to 25%, 21% and 18% of the reasons chosen by people who participated in the survey, respectively. Apart from pre-defined reasons, individuals also entered their personal hurdles through a free text option. Participants found it difficult to follow caloric restriction guidelines due to their active work life or the psychological stress given by the word “diet”. The fear of eating more after a day of dieting also made people drop out from the study. In addition, since the individuals in the study were UK based, they found it difficult to maintain the food diaries, as the fatsecret database used was an American food database.

3.2.8 MOLECULAR INSULIN AND LACTATE BIOMARKER CORRELATE WITH LIFE-STYLE DATA

The urine samples collected by the participants were used to measure insulin and lactate levels and a 24-hour profile was obtained based on the values. Based on the profile and data entered in the health platform, all possible variables (23 in total) were studied (Figure 13). The intra-individual variation of the biomarkers and caloric intake over a 24-hour period has been shown in Appendix V. The levels of biomarkers changed with different food eaten by the individual. Blue, green and pink corresponds to ACE, insulin and lactate respectively. Yellow triangles are the urine stamps, blue square corresponds to the weight of an individual and red circles corresponds to the multiple food entries added in the health platform in a day.
**Variables**

**Extracted from biomarker profiles**

- Volume of urine in 24 hour (mL)
- Biomarker mass in 24 hour for individuals who provided 3 or more urine samples
- Fasting biomarker value of 24 hours
- Last biomarker value of 24 hour
- Biomarker value following day
- Minimum biomarker value in 24 hour
- Maximum biomarker value in 24 hour
- Ratio of maximum/minimum
- Time of minimum biomarker value
- Time of maximum biomarker value
- Biomarker before breakfast
- Biomarker before lunch or dinner
- Total amount of biomarker/calories consumed over 24 hr
- Ratio of last biomarker value/following day value

**Variables from DHP entries**

- Fasting time between breakfast and or dinner
- Total calories consumed over 24 hr
- Macronutrient content (g and kcal): Carbohydrate, Protein, Fat

**Anthropometrics**

- Weight difference (kg) (weight before breakfast minus weight following morning)
- BMI

**Other**

- Age
- Gender

**Figure 13: Variables found.** Variables extracted from biomarker profiles and entries on the digital health platform (https://personalhealth.warwick.ac.uk/).

The cross-correlation matrix of all the 23 extracted parameters from biomarker profiles and the digital health platform are shown for the complete cohort in Figure 14 for an overall summary. The weight difference showed a positive correlation with BMI while a negative correlation with carbohydrates, fat and lactate levels before the second meal of the day and total calories was observed. This relationship between body weight loss and different variables over 24-hour might be associated with fluid loss and rather than loss of adipose tissue and or fat mass. Care needs to be taken with the interpretation as there has been no adjustment for the use of multiple comparison, thus leading to potential Type II error.
Figure 14: Correlation plot of measured variables. The correlation (or lack thereof) between the parameters is shown for 147 days. Correlations between the parameters were scaled from 1.0 to -1.0. Blue indicates positive correlation while red indicates negative correlation. X indicates no correlation between the two parameters.

Furthermore, as expected, the total lactate and insulin parameters were strongly correlated with other parameters such as first, last, maximum, minimum and following day lactate and insulin concentrations. In particular, the weight difference (expressed as negative kg) showed a correlation with total calorie intake, which was significant at R=0.04 (p<0.05). Total insulin and total lactate were positively correlated to the total calorie intake (p<0.001, R=0.35 and R=0.03, respectively). Fasting, total, last, following day and maximum amounts for insulin and lactate had significant correlation with carbohydrate, fat and protein content in the meals (Figure 15d, e).
After taking out the outliers from the Figure 15 a, b and c, the correlation between weight difference and total caloric intake was maintained significant at p<0.05. Similarly, positive association between total insulin and total caloric intake was also retained significant at p<0.01. However, correlation is lost between total lactate and total caloric intake which was found otherwise significant at p<0.001.

**Figure 15:** Individual correlation plots of selected parameters. (a) Weight difference versus total calories. (b) Total insulin versus total calories. (c) Total lactate versus total calories. (d) Insulin parameters correlation with nutritional parameters: Panel I. Carbohydrate. Panel II. Fat. Panel III. Protein. (e) Lactate parameters correlation with nutritional parameters, as in (d). Significant correlations with macronutrient content were marked by ** or *, when significant at p=0.01 or p=0.05, respectively.

### 3.2.9 BIOMARKER AND BMI

BMI was correlated with several variables (Figure 14), therefore pre-defined BMI groups were investigated to analyse if they differed in correlation of variables discussed above.
Segregation of the data into different BMI groups showed loss of correlation between weight loss and other variables in the obese and overweight groups while being sustained in the healthy group. Particularly, the insulin biomarker profiles in the overweight and obese group were dampened in comparison to the healthy group (Figure 17a). Total, fasting, last, following day and maximum insulin values were significantly higher in the obese group in comparison to healthy individuals (Figure 17b). Also, total and last lactate amounts increased in obese people in comparison to the healthy group (Figure 18a). Furthermore, total, maximum and minimum lactate values were higher in obese than in overweight individuals (Figure 18b), in accordance with previous findings of increased lactate levels in obese individuals.

I. Healthy Weight BMI <25.00
II. Overweight BMI 25.00-29.99
Figure 16: Correlation plot of measured variables. Correlation plot of measured variables for healthy individuals with BMI up to 25 (panel I), in the overweight category with BMI in the range 25-30 (panel II) and the obese category with BMI >30 (panel III).
Figure 17: Insulin response is dependent on BMI. (a) Spread of total, fasting, last, following day insulin and total calories of all the participants in comparison to BMI. (b) Comparison of insulin parameters among healthy, overweight and obese participants. Significance levels are marked as follows: *p=0.05, **p=0.01, p=0.001.

Figure 18: Lactate response is dependent on BMI. (a) Spread of total, fasting, last, following day lactate and total calories of all the participants in comparison to BMI. (b) Comparison of
lactate parameters among healthy, overweight and obese participants. Significance levels are marked as follows: *p=0.05, **p=0.01, p<0.001.

3.2.10 BIOMARKER AND WEIGHT LOSS

Because weight loss was the identified desired outcome in the study, weight change versus biomarker levels were studied, as shown in Figure 19. Because of inaccuracies inherent to measuring weight, the weight change values were grouped into 4 groups: weight loss >0.5kg, weight loss 0.1-0.5kg, weight gain and no change. It was apparent that total insulin values varied most dramatically in the weight gain group, and was overall higher in the no weight and weight gain categories. Similar patterns were also observed for fasting, last, following day and maximum insulin values. This graph thus emphasized that insulin values, even individual ones, as opposed to all values collected over a 24-hour period, were potentially useful biomarkers for immediate feedback on dieting efficiency, with low values being likely predictive of weight loss, information which could only be obtained the day following a diet, too slow to be sufficiently motivating.

Figure 19: Insulin response is associated with weight loss. Weight loss is associated with low insulin values. Weight change was grouped into four groups, no weight difference, weight gain or weight loss between 0.1-0.5 kg and >0.5 kg. Significance levels are marked as follows: *p<0.05; **p<0.01.
3.3 DISCUSSION

The escalating obesity epidemic that might in part even be related to the recent decline in life expectancy in the USA (185, 186) requires novel approaches suitable to help people lose weight. In this study, the first attempt at developing quantitative, molecular feedback mechanisms for people dieting has been described. While biofeedback has been well established to be successful in diabetes (187), it has not been studied in people with no obvious signs of a disease. The approach used in this study, also differed from previous efforts at identifying biomarkers of sustained weight loss which had for example identified ACE levels, amongst others (113). While extremely useful, this information was long-term, and could not be used for immediate feedback to dieters. The present study has filled this gap. For the first time, it was demonstrated that metabolic markers could be used in conjunction with food intake behaviour and have the potential to predict weight loss. Thus, a person on a diet, in the future, could measure their insulin (or to a lesser extent, lactate) values and make a decision if it is acceptable to eat another meal that day, or what type of meal it should be. The current study has provided the proof of concept that biomarker measurements could be used in this context. Limitations of the study were the short-term nature of the diet (24-hour periods, as opposed to more realistic weeks/months of dieting) and the length and cost of the assay of insulin, and the need for urine samples. Thus, both assays for urine require a laboratory setting, making it not yet feasible to conduct a long-term study or investigate the effect on behaviour.

We are currently in the process of developing a rapid, cheap and home-based sensor for insulin and lactate (140), which would enable us to address these limitations in the future. As the majority of participants only provided data for 2-3 times 24-hour periods, a long term trial would be needed to demonstrate if similar conclusions could be reached over longer periods of dieting.

The study was intended as a proof of concept to demonstrate if molecular measurements might provide reliable information during dieting efforts. The most useful information for a dieter is weight loss. Thus, the main purpose of the study was to identify if there might be any correlation between molecular data and weight loss. Because this was an observational study with a relatively small number of participants (52), the treatments (which meal to skip and on what day) were not assigned randomly. Thus, the protocol of measurement, as well as sampling might cause the study not to be representative of the general population. Sources of sampling bias could be due to this being a volunteer sample, as well as a convenience sample imposed by the requirement to transfer urine samples to the laboratory for measurements. The bias associated with this was made evident by the large disparity between male and female participants (77% female, 23% male). Consequently, there might be
a sampling bias because participants were not chosen at random and they might exhibit different lifestyles. Since most participants worked or studied at the University of Warwick, participants could not be considered representatives of the UK population (although some of the demographics were similar), nor could the conclusions necessarily be extrapolated to people from other countries.

Another source of sampling bias introduced by the observational nature of the study was that participants were given the liberty to choose what days to diet, as well as what meals to skip. This has resulted in different meal plans to be followed for different number of days. Therefore, there was another instance of non-probability sampling, thus creating a possibility for statistical bias. There were also sources of response bias because participants were asked to record their data in an app, this meant that participants might forget or neglect to record data. Also, participants could have entered incorrect values for meal calories, thus indicating voluntary response bias. Another form of response bias, more unique to this study, was improper measurements bias by the participants.

Users were asked to record their weight, as well as collect samples of their urine. Incorrect sample storage, errors in measuring urine volume, could all result in inaccurate entries in the digital health platform. The body weight was self-reported by the participants. Improper weight and height measurement and difference in weighing scales might have generated variations in the data obtained. This limitation could be addressed by performing the height and weight measurements (performed by researches involved in the study) in the standardised conditions at the start and the end of the study. Providing guidelines like weighing before or after going to the toilet in the morning, measurement with or without clothes could also increase consistency in the measurements by the participants. Individuals entered information on health platform for 146 days but weight information was only provided for 126 days. The missing weight information for those 20 days could have affected the dieting success and biomarker levels and meal plan choice. Incorrect use of weighing machine, height measurement by the participants could have resulted in misclassification of individuals in BMI groups.

Lastly, no conclusions on causation were intended or could be inferred due to the high likelihood of confounding variables. One such variable was the fact that some individuals recorded data on consecutive days, while others on single days separated by days without data entries. There could have been an effect on some of the measurements after consecutive days of skipping meals. This, in turn, might have affected the conclusions of the study. Another possibly confounded variable was which meal was omitted. For example, participants might have skipped a meal that they regularly take, as opposed to skipping a meal where they
regularly skip. Also, other daily activities might also have had an effect on an individual’s weight loss and thus been confounded with other variables in the study. While the platform contained an entry form for physical activity, few entries were made.

In summary, the present study contained a number of sources for potential bias that could be addressed in future efforts. Most importantly, the data collected provided the necessary information to design a larger study in which participants would be randomly assigned to meal plans over longer periods of time. Given that the last insulin and lactate measurements of the day were the most informative, a future study could restrict sample collection to these samples, allowing for data collection over an entire diet period, which normally would take place over several weeks. Once participants would carry out these molecular measurements at home, recruiting participants outside of the university campus would allow broadening of the participant profiles.

Extensions to the study could also include improvements to the digital health platform. The current app provided the setting that allowed recording of life-style related data, including weight, food and drink intake, exercise, and urine sample collection details. It also provided automation for the analysis of the data. To broaden the use of the app additional access to local based food information databases need to be included (such as TESCO/Sainsbury’s basket for UK users). Increase in user-friendliness of the app could also help to target a wider audience. With the wide-spread use of smartphones and tablets, apps that run on these devices have become a structural part of our lives (188). 74% of European and 73% of American adolescents use a smartphone on a regular basis (188). With the increase in abundance of such technologies came the development of fitness and health apps that could provide behavioural interventions (188, 189). However, Alley et al. (2017), have shown that there are only 25 apps that directly target sedentary behaviour, physical activity and/or diet. No app so far has provided personalised feedback using molecular measurement information. This was the gap this study was aiming to fill, which we hope could help target behaviour change in individuals, or in obesity clinics, weightwatcher programs and other organizations that aim to assist individuals or patients making life-style changes.
CHAPTER FOUR

EFFECT OF LACTATE AND INSULIN ON DIFFERENT MEAL PLANS

This figure illustrates personalised feedback strategy with focus on the effects of meal plan choice. The figure was designed using Paint and PowerPoint 2013.
4.0 SUMMARY

Any diet might result in weight loss but all are prone to inter-individual variations in terms of behaviour, preferences and biological response. In this study, the effects of different meal plan on lactate and insulin levels were examined allowing integration of molecular information with meal scheduling choices.

Participants followed the similar instructions described in Chapter 3. Participants omitted one out of the 3 main meals. They also used the health platform to maintain diaries of food intake, weight, urine collection time and volume.

Analysis of insulin and lactate levels taken at different time of day revealed that insulin levels on the morning (before eating) of the diet day, before the second meal of the diet day and on the morning of the day following the diet day were all higher in the control group in comparison to diet groups (where one of the 3 main meal was skipped; p<0.01). Insulin levels on breakfast, dinner and a snack (BDS) meal option showed a correlation with weight loss and fasting time significant at p=0.05.

In this study the effects of different meal options on potential biomarker of weight loss (lactate and insulin) have been demonstrated. This information might be used as a therapeutic approach to improve health, in spite of the variation in the daily food consumed.

4.1 INTRODUCTION

Inter-individual behavioural and biological differences are amongst the causes for the lack of consensus on optimal diet recommendations (63). Setting realistic goals is perhaps the most important factor in dieting to avoid failure (63, 190). Even if a specific diet recommendation is scientifically sound, it may not be the most successful, due to human behaviour and inter-individual variations and preferences. In chapter 3, we have established that reduced food intake to 2 instead of 3 daily meals (either breakfast, lunch or dinner) resulted in weight loss regardless of which meal was omitted (65).

In this study, the effects of different meal options chosen by the participants (when given flexibility in which meal they could skip) on lactate and insulin have been examined in more details.
4.2 RESULTS

4.2.1 CALORIC PATTERN & MEAL PLANS CHOICE

52 participants followed the study as described in the chapter 2 section 2.1. They provided food intake information and urine samples for 147 days. Of the 147 days, there were 11, 42, 15, 46 and 24 days for B/L, B/D, L/D, one meal per day and control days, respectively (Figure 20). The spread of BMI across different groups was similar showing that variations in biomarker level (discussed later; Figure 21) were not affected by participants’ BMI but due to their meal plan choice.

Figure 20: Study Design. Flow diagram of the study design.

Figure 21: Meal choice distribution in different BMI groups.

Analysis of the health platform entries (of food entries) revealed that the participants followed the guideline of consumption of 1200 Kcal on diet days and >1200 Kcal on control days
(Figure 22A) with a few outliers. The highest caloric consumption was 3673 Kcal. Carbohydrate, protein and fat proportions in meals taken by the control group were higher compared to any of the diet groups (p=0.05) (Figure 22B, C & D) at the exception of the BL group that had higher carbohydrate intake (p=0.02; Figure 22B). The average carbohydrate consumption (KCal) was 640.17, 508.30, 943.81, 474.04 and 370.17 in B/L, B/D, control, L/D and “one meal in a day” group respectively. 168.49, 203.05, 354.76, 200.57 and 159.31 of protein (KCal) was consumed in B/L, B/D, control, L/D and “one meal in a day” group respectively. 234.28, 307.82, 634.77, 392.27 and 245.11 of fat (KCal) was consumed in B/L, B/D, control, L/D and “one meal in a day” group respectively.

Figure 2: Caloric intake of study participants. Participants’ caloric pattern in the different meal groups. Significance levels are marked as follows: *p<0.05, **p<0.01.
4.2.2 OVERALL COMPARISON OF ALL DIET GROUPS COMBINED WITH THE CONTROL GROUP

ANOVA was used to compare the control group against all the diet groups combined. Confirming the findings described in Chapter 3, there was a significant (at p=0.001) difference in the calories consumed (Figure 23A) between control and diet groups. It also showed that the level for lactate and insulin were different in control and diet groups (combined).

Insulin levels in all the diet groups were consistently lower in comparison to the control group, regardless of the time of day they were measured. For instance, the mean value for the insulin levels in the morning of the diet was 34.19 µg in diet group vs. 67.87 µg in control group (p=0.06) (Figure 23B). Total insulin levels (which include all insulin concentrations recorded throughout the diet day) was 115.31 µg in diet groups vs. 297.11 µg in the control group (p<0.00010 (Figure 23C). Similarly, following day, maximum insulin level in a 24-hour period, insulin before first and second meal of the day were all higher in control groups than in the diet groups (Figure 23 B&C).

Similar analysis of lactate levels revealed similar trends as insulin (Figure 23D). The mean total lactate was 339.77 and 259.68 µg in control and diet groups, respectively, significant at p=0.15 (Figure 23D). The mean following day lactate was 89.08 and 52.13 µg in control and diet groups, respectively, significant at p=0.34 (Figure 23D). Similar decrease in maximum lactate levels was observed in diet groups in comparison to the control group (Figure 23D).
Figure 23: Comparison of control vs. all diet groups together. (a) Caloric comparison in control vs. diet groups together. Data from 52 individuals are shown. (n= 24 days and n= 123 days in control groups and diet group, respectively)(b) & (c) Insulin amount from 52 individuals. (n= 24 days and n= 123 days in control groups and diet group, respectively) (d) Lactate amount comparison from 52 participants. (n= 24 days and n= 123 days in control groups and diet group, respectively). Significance levels are marked as follows: *p<0.05; **p<0.01.

4.2.3 INSULIN AND LACTATE PROFILE VARIES WITH DIFFERENT MEAL PLANS

Next, a correlation between meal plan choice and the variation in insulin and lactate values was investigated. Consistent with the results described above, it was observed that the insulin parameters (first, maximum, total and insulin before first meal of the day) were higher
in the control group in comparison to each of the different meal plans, significant at p=0.05 (Figure 24). The minimum insulin was only higher in the control group when compared to the “single meal in a day” group at p=0.01. However, it was also found that the differences amongst different meals plans contributed to the overall effect. In particular, for the BDS meal plan, the total, maximum, minimum was significantly lower in comparison to the “single meal in a day” group (B or L or D) at p=0.01, 0.032, 0.038 respectively. Similarly, insulin before first meal of the day was significantly higher at p=0.268 in BDS in comparison to the “single meal in a day” group.

Albeit with less significance, the lactate values also followed this trend, with minimum and maximum lactate being significantly higher and lower when comparing the BDS and the “one meal in a day” groups at p= 0.15 and 0.004, respectively.

Figure 24: Insulin profile in different meal groups. Insulin variation in each group based on participants entries. Data from 52 individuals are shown. *Insulin amounts significantly different at p=0.05.

4.2.4 BDS CORRELATION WITH WEIGHT LOSS

The group who chose to have “one single meal a day” logged significantly more data on food with caloric content of 250 KCal or less in comparison to other groups. This had generated technical issues as the criteria for a snack was defined as any food bringing a maximum of 250 KCal and thus defining which of breakfast, lunch or dinner was omitted was unclear. Participants might be having what they considered a meal but since the total caloric
intake was equivalent to that a snack, this pseudo meal was considered as a snack. B/L, B/D, and control group had a small size of 11, 15 and 24 of days respectively. Weight change was observed in all the groups (see Chapter 3) but the small sample size hindered statistical analysis of lactate and insulin trends in these groups. Thus, the BDS group with a total of 42 days was studied hereafter.

Following a Pearson correlation, individuals following BDS group showed a significant correlation with weight loss. Also, in BDS group, insulin before first meal of the day, maximum (at any point over a 24-interval), last (at the end of a 24-hour period) and total insulin amounts had a negative correlation with weight loss (Figure 25). This suggested that BDS plan was associated with weight loss after a 24 h interval, at least partly, due to lower insulin levels. By following BDS meal, one could attain lower insulin values, which favoured weight loss at the end of a 24-hour interval. Total urine volume and carbohydrate consumption also had a negative correlation with weight loss, significant at p=0.05 (Figure 24).

Figure 25: BDS is correlated with weight difference. Effects of BDS on weight difference showing Pearson’s correlation is significant at the 0.05 level (p <0.05).
4.2.5 INSULIN LEVELS IN BDS CORRELATION WITH FASTING TIME

Insulin levels in BDS group also showed a significant correlation with fasting time. Higher fasting length associated with decreased amounts of insulin before first meal of the day, maximum, following day, last and total insulin at the end of a 24-hour period (p=0.05; Figure 26).

Figure 26: BDS is correlated with fasting time. Effects of BDS fasting time showing Pearson’s correlation is significant at the 0.05 level (p <0.05).
4.3 DISCUSSION

An important determinant for an ideal dietary intervention is to be culturally acceptable, safe and nutritionally adequate (191). It should also enhance the possibility of long-term compliance and weight maintenance. Different dietary plans have been tested and found to be beneficial for weight loss over short and long terms. Although recommendation and guidelines are usually provided for every diet type, yet there is no diet that provided personalized optimization. The current chapter filled this gap. For the first time, biomarker changes have been identified over a 24-hour interval on the basis of the meal plan chosen. The insulin levels in BDS where individual omitted lunch had a correlation with weight loss and fasting time.

In the study, lunch was the most popular meal to be omitted and dinner was the least popular. Most of the participants either studied or worked at the University of Warwick, therefore meal preferences could not be extrapolated to the general population. Skipping breakfast and eating heavy dinners have been found to be associated with abnormal metabolisms (192) and higher BMI (193, 194). Individuals participating in this study belonged to all ranges of BMI and it was found that any variation in lactate and insulin levels was independent of BMI.

The adherence of the participants was observed for the total calories consumption in a day on both control and diet days. Long-term adherence is not only important for initial weight loss but also weight maintenance (195). Adhering to a dietary intervention is difficult for many individuals (83). It has been found to be associated to weight loss success (196-198), which could be non-existent over a 24-hour period (65). In the study, the guidelines were followed even at instances where more than a single 24-hour data and urine sample collection was done. The participants could choose which days to diet, as well as which meals to omit. The different meal options chosen in the study showed different effects on insulin and lactate levels. Therefore, providing the molecular information over a short time might become a source of motivation leading to personalization of diet planning and ensuring better adherence.

Insulin levels in BDS were associated with weight difference and fasting time. Weight loss was observed in every diet plan but due to small sample size, only BDS was studied for statistical significance for insulin levels. Only 7.97, 10.86 and 17.39% of the total participants followed B/L, L/D and control meal plan, respectively. 30.42% of the participants chose the BDS plan next to “one meal in a day” plan, which was followed for 33.33% participants. As it was also the most popular plan in the study showing that popular choice also had a significant consequence. Such information might help initiate a behaviour change in
individuals, or in weightwatcher programs, obesity clinics, and other organizations that aim to assist individuals making life-style changes (65).

All analyses were based on a short-term interval study, therefore a longer intervention study would be necessary to investigate the effects of meal plan choice on insulin and lactate levels. Extension of the study involving the effects of different food items on insulin and lactate could help create a database which could replace the need of maintenance of food diaries in the future. The level of insulin and lactate in the urine could be used to determine the type of food eaten by an individual. Food diaries, questionnaires are often considered tiring (86), although there are numerous technology-based platforms such as websites (87, 88), apps and computer-assisted versions available for personal tracking of food intake (89, 90). Misreporting of food intake is a well-documented problem (86, 91). Making food diaries depend on motivation, honesty and self-awareness of the user (85) and suffer from the underestimation of food consumed (92). Creating a database with trend and levels of lactate and insulin of food items could help overcome such an issue.
CHAPTER FIVE

ANGIOTENSIN CONVERTING ENZYME AS A PREDICTOR OF WEIGHT LOSS

This figure illustrates personalised feedback strategy with focus on Angiotensin Converting Enzyme (ACE). The figure was designed using Paint and PowerPoint 2013.
5.0 SUMMARY

Angiotensin Converting Enzyme (ACE) expression and activity is associated with obesity. It has been identified as a circulating factor that might predict sustained weight loss over longer times. Here, the aim was to evaluate whether ACE might be an early marker (over a 24 hour period) for weight loss. ACE quantification was performed in urine, using ELISA kit from R&D Systems, UK.

32 participants (78% females and 21% males; BMI 28.47 ± 4.87 kg/m²) followed a 1200 KCal diet with an optional daily (<250 KCal) snack. Participants used an in-house generated health platform to provide urine and daily recordings of food intake and physical activity.

Following a day of dieting, ACE levels positively correlated with weight loss (p<0.05) and that this reduction was significantly more robust in individuals with a BMI >25 (p<0.005).

In summary, the study demonstrated that ACE levels correlate with BMI and weight loss, as early as after 1 day of dieting, and thus ACE could be a potential early “biofeedback” marker for weight loss and diet efficiency.

5.1 INTRODUCTION

Quantitative biomarkers have not been considered for providing early (especially over a 24-hour period) feedback to an individual undergoing a weight loss intervention. The goal is to identify a molecular marker that reports on dieting efficiency so that it can be used for feedback on this efficiency while weight loss may still not be observed. It is established previously that insulin and lactate (Chapter 3) could be potential early markers for dieting and this chapter introduces an additional marker, ACE, that may also be used as a feedback on diet efficiency.
5.2 RESULTS

5.2.1 ACE CORRELATE WITH LIFE-STYLE DATA

The participants followed as described in Chapter 2 section 2.1. The multiple urine samples collected by the participants were used to measure ACE levels using the method described in Chapter 2. These values were then used to define a total of 14 parameters relating to biomarker profile or lifestyle data entered (Table 17).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracted from Biomarker Profile</td>
<td></td>
</tr>
<tr>
<td>Volume of urine in 24-hour (mL)</td>
<td>Total volume of urine produced over a 24-hour period</td>
</tr>
<tr>
<td>First ACE value of 24-hour</td>
<td>Obtained from the urine sample provided just before the first meal of the day</td>
</tr>
<tr>
<td>Last ACE value of 24-hour</td>
<td>Obtained from the last urine sample provided over a 24-hour period</td>
</tr>
<tr>
<td>Total ACE value of 24-hour</td>
<td>Summation of ACE values obtained from all the urine samples provided over a 24-hour period</td>
</tr>
<tr>
<td>Following Day ACE</td>
<td>Obtained from the first urine sample of the 24-hour period (valid where participant provided more than 1-day worth of samples)</td>
</tr>
<tr>
<td>Minimum ACE value in 24-hour</td>
<td>The smallest amount of ACE amongst all the urine samples provided over a 24-hour period</td>
</tr>
<tr>
<td>Maximum ACE value in 24-hour</td>
<td>The largest amount of ACE amongst all the urine samples provided over a 24-hour period</td>
</tr>
<tr>
<td>Time of maximum ACE in 24-hour</td>
<td>Time stamp of the urine sample entry in the health platform that corresponds to the maximum ACE value</td>
</tr>
<tr>
<td>Time of minimum ACE in 24-hour</td>
<td>Time stamp of the urine sample entry in the health platform that corresponds to the minimum ACE value</td>
</tr>
</tbody>
</table>
**Table 17: Variables found.** Variables extracted from ACE profile obtained from the urine samples and entries on the digital health platform (https://personalhealth.warwick.ac.uk/).

The cross-correlation matrix of all the 14 extracted variables from biomarker profiles and the digital health platform has been shown (for the complete cohort) in Figure 27 for an overall summary. As discussed in chapter 3, most of the participants provided data for 2-3 days. First ACE was correlated with following day, last, maximum, minimum and total ACE (Figure 27).
Figure 27: Correlation Matrix for ACE. Correlations between the variables (shown in table 1). They were scaled from 1.0 to -1.0. Blue indicates positive correlation while red indicates negative correlation. X indicates no correlation between the two parameters which is significant at p=0.05. The size of the circles correspond to the strength of the correlation. Bigger the circle, stronger is the correlation between 2 variables.

5.2.2 EFFECTS OF BMI ON ACE LEVELS

BMI correlated with ACE levels on the following day (Figure 27). Therefore, it was further investigated whether this association was also found in pre-defined BMI groups and whether correlations between ACE levels at different times (e.g.: first collection, last collection) could be found in these BMI groups.
There was an association between BMI and ACE level on the following day (Figure 28). Moreover, a linear trend was observed in BMI > 25 group for the following day ACE levels (Figure 29). No correlation was observed between ACE levels in the first or last collection and any BMI group (Figure 28). The decrease in ACE levels after a day of dieting was higher in individuals with BMI ≥ 25 (p < 0.005; Figure 30).

Figure 28: Spread of ACE values in comparison to BMI. ACE levels were quantified from the urine samples collected over the period of the study. First ACE was obtained from the urine sample provided just before the first meal of the day. Last ACE was obtained from the last sample collected in a 24 hour period. Total ACE is the summation of ACE values obtained from all the urine samples provided over a 24-hour period. Following day ACE was obtained from the first urine sample of the 24-hour period (valid where participant provided more than 1 day worth of samples).
**Figure 29: Trend in following day ACE and BMI.** BMI≥25 group and following day ACE was positively correlated ($R^2=0.292$) significant at $p=0.015$. Following day ACE obtained from the first urine sample of the 24-hour period (valid where participant provided more than 1 day worth of samples).

**Figure 30: ACE response is dependent upon BMI.** Following day ACE correlation was studied in different BMI groups. Following day ACE obtained from the first urine sample of the 24-hour period (valid where participant provided more than 1 day worth of samples). Significance level marked as **$p<0.01$.**
5.2.3 DEPENDENCE OF WEIGHT LOSS ON ACE LEVEL

As correlation between BMI and following day ACE has been identified, further examination was done to find a correlation between ACE levels and weight loss. Although correlation between weight loss and ACE level was not found when examining the whole cohort without any parameter refinement (Figure 27), a strong association between the following day ACE levels and weight loss in individuals with a BMI ≥25 group was found, significant at $R^2=0.274$ and $p=0.015$ (Figure 31).

![Figure 31: ACE as an early biomarker for weight loss in individuals with a BMI≥25. Plot of following day ACE against weight difference. $R^2=0.274$ and $p=0.015$.](image)

5.2.4 CORRELATION AMONG ACE, INSULIN AND LACTATE

Pearson Correlation was conducted to find association between ACE and insulin variables. First ACE correlated positively to first insulin, whilst last ACE correlated negatively with maximum and total insulin (Figure 32). Following day ACE also had a positive correlation with following day, maximum, minimum and total insulin (Figure 32). To a lesser extent, ACE levels were also correlated to lactate values. Last ACE correlated positively to maximum and total lactate (Figure 33). Maximum ACE correlated to maximum lactate and minimum ACE correlated to minimum lactate (Figure 33). In chapter 3, it was shown that insulin and lactate levels (such as following day level) correlated with weight loss. Correlation with following day ACE variable showed that it might be usable in an exchangeable manner while analysing weight loss in an individual.
Figure 32: Correlation Matrix for ACE and Insulin. Correlations between the parameters were scaled from 1.0 to -1.0. Blue indicates positive correlation while red indicates negative correlation. X indicates no correlation between the two parameters which is significant at p=0.05.
**Figure 33: Correlation Matrix for ACE and Lactate.** Correlations between the parameters were scaled from 1.0 to -1.0. Blue indicates positive correlation while red indicates negative correlation. X indicates no correlation between the two parameters which is significant at \( p=0.05 \).

### 5.3 DISCUSSION

ACE has been identified as an important predictor for sustained weight loss through profiling for blood protein and steroid hormones after a low caloric diet for 8 weeks (113). The extent of reduction in ACE separated the individuals who continued to lose weight from individuals who regained weight during a six-month maintenance period (113). Therefore, ACE appeared to be a good marker correlated with weight loss on a long term. However, the
Goal here was to identify markers on a very short term, as early as 24 hours after the start of a diet regimen, to provide feedback to the individuals but also reassure the individuals about the efficiency of their diet. Thus, it was investigated whether correlation could be found between urine ACE levels and parameters such as BMI and weight loss.

Following day ACE levels correlated with BMI however, a stronger association was observed in BMI ≥25 group (overweight and obese individuals). The spread of ACE values among different BMI groups suggested that ACE might only be used as a predictor in overweight/obese population. This makes ACE a good “indicator” on the diet efficiency in individuals who are overweight/obese as it could give a feedback to the population who might require weight management the most. However, the size of the study limited the effect of this inference. Conducting a similar study over a larger cohort, and identifying ACE level patterns could palliate this. In addition, out the 147 days for the total study (Figure 5), ACE information was only available for 92 days and as such, the missing information might have affected the correlation between ACE levels and BMI and weight loss.

Another confounding variable was that some individuals recorded data on consecutive days, while others recorded data on single days separated by days without data entries. There could have been an effect on some of the measurements after consecutive days of omitting meals. This, in turn, might have affected the conclusions of the study. Lastly, individuals provided data collected for at least one 24-hour period, with a few exceptions where participants provided data for more than 2 weeks. Thus, there is a need for data collection over longer periods to determine whether ACE could be used as a potential biomarker prudentially for overweight or obese individuals.

In conclusion, the study demonstrated that ACE levels vary within a 24-hour interval after following a calorie-restricted diet. The inter-individual variation of ACE has shown that it could potentially be used as an early biofeedback marker on dieting and weight loss.
This figure illustrates the personalised feedback strategy with focus on identification of new biomarkers with NMR. The figure was designed using Paint and PowerPoint 2013.
6.0 SUMMARY

\(^1\)H NMR spectra of urine contains thousands of sharp lines from predominantly low molecular weight metabolites. The peaks were assigned based on the chemical shifts using metabohunter software. Testing of the 3 urine samples from the clinical study showed identification of different metabolites on days with varied evening insulin levels.

6.1 INTRODUCTION

Metabolic phenotyping of biological fluids involve the profiling of metabolites to understand their variations in response to genetic variation, lifestyle, drugs or any other stimuli (181). The profiles can help understanding the effects of these variations to evaluate the mechanisms involved in those interactions mentioned above (182, 183). Phenotyping has also been used to understand interactions between an individual and his/her nutrition and gut microbiome (184). Serum, urine and plasma are the most commonly studied fluids as the preparation for them is easier in comparison to tissue samples (185). NMR and MS are the most used techniques for metabolic profiling (186-188). Here, an exploratory study for identification of other metabolites apart from lactate, insulin and ACE using NMR spectroscopy has been discussed.

6.2 RESULTS

6.2.1 SAMPLE CHARACTERISTIC

To identify other makers for weight loss, a total of 3 samples from the clinical study were tested (described chapter 2, 3 and 4). As last insulin of a day has been identified as a potential biofeedback marker for weight loss (chapter 3), so samples corresponding to the last urine sample collected over three different 24 hour periods were tested. The insulin values of the samples have been listed in Table 18.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Insulin (ng/mL)</th>
<th>Insulin (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>240</td>
<td>40</td>
</tr>
</tbody>
</table>
Table 18: Insulin levels in the urine samples tested via NMR.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>210</td>
<td>48</td>
</tr>
</tbody>
</table>

6.2.2 WATER SUPPRESSION

Water is present in biological samples at very high concentrations (10,000 times) than metabolites of interest. Thus, an unprepared magnetic resonance spectrum would be dominated by a major dominating water peak, making small molecules barely undetectable. To visualize these small molecules, the water peak must therefore be suppressed. Water suppression was done as mentioned in (179). To check the performance of water suppression, 2mM sucrose sample prepared in in 2 mM sodium azide and 0.5mM 3-trimethylsilyl propionic-2, 2, 3, 3-d4 acid sodium salt (TSP) in 10% D2O: 90% H2O was tested. The suppression was accomplished using pre-saturation and relaxation delay of 10 seconds in 8 scans. The spectrum before pre-saturation had a major water peak at 4.7 ppm (Figure 34). The spectrum after pre-saturation was suppressed and the line width was less than half height of TSP (internal standard) peak (Figure 35).

Figure 34: 2mM sucrose 1D NMR spectrum before pre-saturation. The internal standard TSP is at 0ppm.
Figure 35: 2mM sucrose 1D NMR spectrum after pre-saturation. The internal standard TSP is at 0ppm.

6.2.3 1D NOESY

6.2.3.1 URINE SPECTRA COMPARISON

1D NOESY spectra were obtained for the samples based on the parameters mentioned in Chapter 2. Overlay of spectra of sample 2 (low insulin level) against high insulin samples (#1 and 3) showed differences in the intensities of several peaks. Also, greater number of peaks were observed in sample 2 in comparison to sample 1 and 3 (Figure 36 and 37). Similarly, comparison of both high insulin samples (sample 1 and 3) showed similar NMR spectra (Figure 38).
Figure 36: Comparison of sample 1 vs. 2. 1D NOESY spectrum is obtained with TSP as internal standard.

Figure 37: Comparison of sample 3 vs. 2. 1D NOESY spectrum is obtained with TSP as internal standard.
Figure 38: Comparison of sample 1 vs. 3. 1D NOESY spectrum is obtained with TSP as internal standard.

6.2.3.2 METABOLITE IDENTIFICATION

The Bruker Topspin 4.0.4 software package was used for peak picking and peak assignments relied on established literature, specifically, the human metabolome database (HMDB), human urine metabolome (99) and metabohunter (199) as shown in Figure 40. Some of the peaks identified have been listed in Table 19. On analysing the peaks and their intensities using metabohunter, a total of 663 and 329 compounds were observed in samples with low and high insulin respectively.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Creatinine</td>
</tr>
<tr>
<td>2</td>
<td>Citric acid</td>
</tr>
<tr>
<td>3</td>
<td>Cis-aconitic acid</td>
</tr>
<tr>
<td>4</td>
<td>Formic acid</td>
</tr>
<tr>
<td>5</td>
<td>Methanol</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---------------------</td>
</tr>
<tr>
<td>6</td>
<td>Guanidoacetic acid</td>
</tr>
<tr>
<td>7</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>8</td>
<td>L-cysteine</td>
</tr>
<tr>
<td>9</td>
<td>Glycolic acid</td>
</tr>
<tr>
<td>10</td>
<td>Creatine</td>
</tr>
<tr>
<td>11</td>
<td>Isocitric acid</td>
</tr>
<tr>
<td>12</td>
<td>Hippuric acid</td>
</tr>
<tr>
<td>13</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>14</td>
<td>L-alanine</td>
</tr>
<tr>
<td>15</td>
<td>L-lysine</td>
</tr>
<tr>
<td>16</td>
<td>2-hydroxyglutaric acid</td>
</tr>
<tr>
<td>17</td>
<td>D-glucose</td>
</tr>
<tr>
<td>18</td>
<td>Indoxyl sulfate</td>
</tr>
<tr>
<td>19</td>
<td>Trimethyl-N-oxide</td>
</tr>
<tr>
<td>21</td>
<td>L-lactic acid</td>
</tr>
<tr>
<td>22</td>
<td>Taurine</td>
</tr>
<tr>
<td>23</td>
<td>L-threonine</td>
</tr>
<tr>
<td>24</td>
<td>Dimethylamine</td>
</tr>
<tr>
<td>25</td>
<td>Pyroglutamic acid</td>
</tr>
<tr>
<td>26</td>
<td>Trigonelline</td>
</tr>
<tr>
<td>27</td>
<td>Sucrose</td>
</tr>
<tr>
<td>28</td>
<td>Trimethylamine</td>
</tr>
<tr>
<td>29</td>
<td>L-cystine</td>
</tr>
<tr>
<td>30</td>
<td>Mannitol</td>
</tr>
<tr>
<td>31</td>
<td>L-histidine</td>
</tr>
<tr>
<td>32</td>
<td>Imidazole</td>
</tr>
<tr>
<td>33</td>
<td>Mandelic acid</td>
</tr>
<tr>
<td>34</td>
<td>Dimethylglycine</td>
</tr>
<tr>
<td>35</td>
<td>Cis-aconitic acid</td>
</tr>
<tr>
<td>36</td>
<td>Urea</td>
</tr>
<tr>
<td>37</td>
<td>Phenol</td>
</tr>
<tr>
<td>38</td>
<td>Isobutyric acid</td>
</tr>
<tr>
<td>39</td>
<td>Methylsuccinic acid</td>
</tr>
<tr>
<td>40</td>
<td>3-aminoisobutyric acid</td>
</tr>
<tr>
<td>41</td>
<td>L-fucose</td>
</tr>
</tbody>
</table>
Table 19: List of metabolites identified using human metabolome database (HMDB).

<table>
<thead>
<tr>
<th>No.</th>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>N-acetylneuraminic acid</td>
</tr>
<tr>
<td>43</td>
<td>Acetoacetic acid</td>
</tr>
<tr>
<td>44</td>
<td>Alphaaminoadipic acid</td>
</tr>
<tr>
<td>45</td>
<td>Phenylacetylglutamine</td>
</tr>
</tbody>
</table>

Figure 39: Peak Assignment. Peak assignment was done using the human metabolome database (HMDB), the human urine metabolome and the metabohunter (99, 199).

6.2.4 2D J-RESOLVED (JRES) SPECTRUM

2D JRES spectra were obtained for the urine samples selected from the clinical study. A representative spectrum obtained is shown in Figure 40. The analysis was done using a spin couple software, available at http://emar.riken.jp/spincpl/ (200). This database has analysed standard chemical compounds in metabolic pathways for 2D-Jres spectrum. From the JRES spectrum obtained, peak picking was done using the Bruker Topspin 4.0.4 software package. Chemical shift (ppm) and J-value (interaction between two nuclear spins) were obtained. This data was incorporated to the software, which generated a list of compounds identified in the samples. No new additional compounds were identified in comparison to compounds
identified from 1D NOESY. Thus, the analysis of the remaining evening samples would be done using 1D NOESY technology only.

**Figure 40: 2D JRES spectrum of sample 2.** \( F1 \) represents the J-coupling values and \( F2 \) represents the chemical shift.

### 6.3 DISCUSSION

It was found that 2 samples with similar insulin levels had similar spectra (Figure 38). These samples being from 2 different individuals suggested that there was little inter-individual variability. However, analysis of additional samples would further be performed to confirm or infirm this trend.

The metabolite profile in urinary samples that had low or high insulin levels was also identified. There was a clear variation in type of metabolites found in these 2 types of samples (Figure 37). In particular, more metabolites were identified in samples associated with low insulin than in samples with high insulin levels. This suggested that these metabolite patterns variations might model weight loss. These initial finding would be completed by further investigating whether a diet could be characterized by a specific metabolite profile.
LEMON EXTRACT REDUCES ANGIOTENSIN CONVERTING ENZYME (ACE) FUNCTION AND INCREASES INSULIN SENSITIVITY AND LIPOLYSIS IN MOUSE ADIPOCYTES

This figure illustrates the effects of lemon extracts on Angiotensin Converting Enzyme (ACE) expression/activity in mouse adipocytes. The figure was designed using Paint and PowerPoint 2013.
7.0 SUMMARY

ACE was identified as a potential biomarker for diet efficiency feedback (Chapter 5) and ACE expression varied with weight loss in overweight and obese people. In recent years, efforts have been made to identify means other than drugs, which might support or even increase weight loss. Interest has been focused particularly on the effects of specific nutrients, called functional foods, on health improvement. Lemon extracts (LE) appeared to improve weight loss and reduce ACE activity. Interestingly, ACE inhibitors (captopril, cilazapril and ramipril), which alter ACE activity, improve insulin sensitivity. Here, the aim was to establish the connection between LE and ACE and investigate the effects of LE on adipose tissue metabolism, using the 3T3-L1 adipocyte cell line.

LE treatment dramatically decreased lipid accumulation in vitro and lipolysis was increased in response to LE, as shown by a 5.5 ± 0.09 and 16.6 ± 1.2-fold-change in perilipin and pHSL protein expression levels respectively. ACE gene expression increased 12 ± 0.05-fold during adipose differentiation. Interestingly, LE was reported to inhibit ACE and it was found that a short treatment with LE decreased ACE expression both at gene (80 ± 0.49%) and protein (55 ± 0.37%) levels. Consistently with reports showing that ACE reduction was associated with increased insulin sensitivity, it was found that LE (which decreases ACE expression) improved insulin sensitivity evidenced by a (3.74 ± 0.54) fold increase in the levels of insulin receptor downstream targets pAKT and GLUT4.

This established for the first time a molecular mechanism by which ACE might relay the effects of LE on lipid accumulation and insulin sensitivity. This strongly supported that LE-induced ACE inhibition promoted increased insulin sensitivity and breakdown of lipids.

7.1 INTRODUCTION

ACE activity is a critical component of RAS and is responsible for the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) (201). Ang II is involved in decreased insulin sensitivity, increased reactive oxygen species generation, decreased glucose uptake, regulation blood pressure and electrolyte balance (202). Increased production of Ang II is associated with increased lipogenesis in human adipose cells (203). It could induce differentiation leading to formation of mature adipocyte (204).

An individual’s metabolism is not only affected by genetics but also physical activity, diet, nutrition or lifestyle. Dietary interventions could be an effectual option for obesity and
its related disorders management (205, 206). Other studies have found bergamot, grapefruit and orange juice to be associated with decreased total cholesterol, low density lipoprotein (LDL) and glucose concentration (53-55) in humans. Administration of lemon juice decreased total cholesterol, LDL and glucose concentration in rats (56). Lemon extracts (LE) decreased ACE activity in rats (56).

The current study focuses on the effects of lemon extract on ACE expression, insulin sensitivity and fat accumulation in 3T3-L1 adipocytes. This would allow exploring the molecular mechanism by which ACE might relay the effects of LE.

7.2 RESULTS

7.2.1 LEMON EXTRACT DECREASES ACE GENE AND PROTEIN EXPRESSION AS WELL AS ACE ENZYMATIC ACTIVITY

A significant (p<0.002) and robust (12 ± 0.05-fold) increase in ACE gene expression was observed during 3T3L1 adipogenesis (Figure 41A). Incubation of mature differentiated 3T3L1 adipocytes with total LE (100 µg/mL; LE was prepared by freeze drying one squeezed lemon) resulted in a significant reduction of ACE gene expression, which was decreased by 80% ± 0.49% after 10 hours (Figure 41B). Consistently with this, ACE 1 protein expression was significantly decreased by ~40-55 ± 0.37% (p=0.01) (Figure 41C&D) in differentiated 3T3L1 adipocytes treated with LE. LE effects on ACE expression were dose dependent (Figure 41D) and importantly, ACE inhibitory activity was gradually inhibited (15-87%) with increasing concentrations of LE (Figure 41E). ACE activity was studied using by measuring the conversion of ACE substrate Abz-GLY-PHe(NO2)-Pro into Abz-GLY product. Consistently with previous report (56), it was observed that LE prevented this conversion and therefore inhibited ACE activity.
Figure 41: Total lemon extracts decrease ACE expression and activity. A) ACE mRNA expression levels were quantified at the indicated days during 3T3L1 adipocyte differentiation. n=3 independent experiments, P<0.001. B) Fully differentiated 3T3L1 adipocytes were treated or not with lemon extracts (100µg/mL) for the indicated times and ACE mRNA expression levels were quantified. n=3 experiments, P<0.05. C) 3T3L1 differentiated adipocytes were incubated with 50,100 or 500µ/mL of lemon extract for 10 hours. ACE I and GLUT4 protein expression were assessed by immunoblotting. GAPDH was used as internal control. n=3. D) Signal quantification of ACE I and GLUT4 immunoblot. E) LE inhibitory effects on ACE activity. Cells were treated with LE (50, 100 and 500 µg/mL) and conversion of Abz-GLY-PHe(NO2)-Pro to Abz-GLY was quantified as described in methods. n=3. **significant at p=0.01 and *significant at p=0.05.
7.2.2 LEMON EXTRACT DECREASES LIPID DROPLETS SIZE AND NUMBER IN 3T3-L1 ADIPOCYTES

Peroxisome proliferator-activated receptor gamma (PPARγ) and Fatty Acid-Binding Protein 4 (FABP4) are known markers of late adipose differentiation (22). Differentiated mature adipocytes treated with LE showed similar PPARγ and FABP4 gene expression compared to control untreated adipocytes, suggesting that LE treatment was not affecting the adipogenesis process (Figure 42A).

Unexpectedly nonetheless, visualization of adipocytes by Oil red O staining showed that LE-treated adipocytes displayed a drastic reduction in lipid droplet numbers compared to control untreated cells (Figure 42B). The number of Oil red O stained cells after a 24-hour treatment with LE was decreased by up to 90% (p=0.034) (Figure 42C). Staining quantification showed a significant decrease (p<0.001) in absorbance for LE-treated cells, compared to control cells (Figure 42D).

Figure 42: Lemon Extract decreases lipid droplets in 3T3-L1 adipocytes. A) Pparg and fabp4 mRNA expression levels were quantified in adipocytes treated with and without LE (100µg/mL) for 8 days. n=3. B) Lipids were visualized using Oil Red staining. Images are
representative of 3 experiments. C) Staining was quantified using ImageJ by calculating number of oil red stained cells in the section shown in B). D) Quantified oil red stain in treated and untreated adipocytes. **significant at p=0.01 and *significant at p=0.05.

7.2.3 STIMULATION OF LIPOLYSIS BY LEMON EXTRACT

Perilipin and Hormone Sensitive Lipase (HSL) are known markers for mobilization of lipids through lipolysis (207). To understand the apparent decrease in lipid droplets, protein expression for perilipin and pHSL were analysed. Increased perilipin protein levels were observed with increasing dose of LE (Figure 43A&B). There was a 1.8, 3.8 and 5.5-fold increase of perilipin protein expression in 10 hours LE-treated cells, compared to untreated cells. Isoproterenol, a potent stimulator of lipolysis, was used as a positive control during the experiment. Consistently, an increase in phosphorylation levels of HSL (pHSL) in response to increasing doses of LE was also found. A 2.6, 6.5 and 16.6 fold change in pHSL levels in LE-treated differentiated cells was observed in comparison to control untreated differentiated cells (Figure 43C&D) after 10 hours of treatment. Supporting this, a free glycerol release assay (see chapter 2) was performed on mature adipocytes at t= 2, 4, 6, 10 and 24 hours (Figure 43E) with different doses of LE. Hydrolysis of triglycerides through lipolysis lead to the release of free glycerol and free fatty acids (FFA). A release of 23.08, 29.19 and 39.22 µg/mg of total protein of free glycerol was observed after 24-hour of treatment of 3T3-L1 cells with LE does of 50, 100 and 500 µg/mL respectively. At LE dose of 500µg/mL, a 4.02-fold higher release of free glycerol at t=2h in comparison to t=0h (Figure 43E). A similar increase (~3.5-4.2 folds) was seen at t=10 and t=24 h for 50 and 100 µg/mL LE dose. To confirm that LE did not lead to cell death, cell viability assay was performed. The cells were treated with LE for 72 hours and on an average 89.95±0.54, 92.12±0.24 and 91.87±0.12 % were viable even after 72 hours (Figure 43F).
Figure 43: Stimulation of Lipolysis by Lemon Extract. A) Perilipin protein expression was assessed by immunoblot in cells treated with the indicated LE concentrations and stimulated or not with 1µM isoproterenol. Blot for protein expression for perilipin and GADPH (loading control) in untreated differentiated cells (D), differentiated cells treated with LE dose of 50µg/mL (LE50), differentiated cells treated with LE dose of 100µg/mL (LE100), differentiated cells treated with LE dose of 500µg/mL (LE500) and differentiated cells treated with isoproterenol (positive control). B) Signal quantification for protein expression for perilipin and GADPH (loading control) protein expression. C) Blot for protein expression for pHSL and HSL in D (untreated differentiated cells), differentiated cells treated with LE dose of 50µg/mL (LE50), differentiated cells treated with LE dose of 100µg/mL (LE100) and differentiated cells treated with LE dose of 500µg/mL (LE500). D) Quantified protein expression for pHSL and HSL in untreated differentiated cells (D), differentiated cells treated with LE dose of 50µg/mL (LE50), differentiated cells treated with LE dose of 100µg/mL (LE100) and differentiated cells treated with LE dose of 500µg/mL (LE500). E) Free glycerol release from treated and untreated cells after t=0, 2, 4, 6, 10 and 24 hours. F) Cell viability
at LE dose of 50, 100 and 500 µg/mL. **significant at p=0.01 and *significant at p=0.05. For D) *significant at p=0.01 and #significant at p=0.05.

7.2.4 LEMON EXTRACT INCREASES INSULIN SENSITIVITY

Protein Kinase B (AKT) is an important signalling molecule in the insulin pathway (208). Levels of Akt phosphorylation at the insulin site (S473) were increased in control cells after insulin stimulation, with no effects on total AKT expression. Interestingly, LE incubation accentuated the insulin response, evidenced by a 3.74-fold increase in AKT Ser473 phosphorylation levels (compared to control; Figure 44A&B). A similar response was observed for GLUT4 expression, which was also increased in LE-treated cells (compared to untreated cells; Figure 44C&D).

Figure 44: Lemon Extract increases Insulin Sensitivity in differentiated adipocytes. A) 3T3L1 differentiated adipocytes were incubated or not with lemon extract for 10 hours before being treated with 100nM insulin for 10 min. Total AKT and phosphorylated AKT on S473 protein levels were assessed by immunoblotting in untreated differentiated cells (D), differentiated cells treated with LE (D+LE), differentiated cells treated with 100nM insulin (D+I) and differentiated cells treated with LE and 100nm insulin (D+LE+I). n=3, p<0.05. B) Signal quantification of protein expression for AKT and pAKT in D (untreated differentiated cells), differentiated cells treated with LE (D+LE), differentiated cells treated with 100nM insulin (D+I) and differentiated cells treated with LE and 100nm insulin (D+I) and differentiated cells treated with LE and 100nm insulin.
(D+LE+I). C) 3T3L1 differentiated adipocytes were incubated or not with lemon extract for 10 hours before being treated with 100nM insulin for 10 min. GLUT4 and GAPDH (loading control) (B) were assessed by immunoblotting in untreated differentiated cells (D), differentiated cells treated with LE (D+LE), differentiated cells treated with 100nM insulin (D+I) and differentiated cells treated with LE and 100nm insulin (D+LE+I). n=3, p<0.05. D) Quantified protein expression for GLUT4 and GADPH in untreated differentiated cells (D), differentiated cells treated with LE (D+LE), differentiated cells treated with 100nM insulin (D+I) and differentiated cells treated with LE and 100nm insulin (D+LE+I). **significant at p=0.01 and *significant at p=0.05.

7.2.5 IDENTIFICATION OF LEMON CONSTITUENTS BY HPLC AND MS/MS

Having determined the effects of LE on adipocyte lipid utilization, further investigation was conducted to identify the compounds in the extract that could have been involved in the LE-induced increased lipolysis. HPLC has been routinely used to identify active components in plant extracts (38, 209, 210). For optimisation of HPLC conditions for LE, a low resolution HPLC was conducted. The low resolution HPLC created a chromatogram as shown below. The optimised conditions were run thrice to ensure reproducibility of the spectrum.
Figure 45: HPLC chromatogram of lemon extract. The extract was run C18 column with mobile phase of water and acetonitrile; repeated thrice.

Further, high resolution HPLC followed by mass spectroscopy was performed to obtain details on the nature of the different compounds of the extract. A total of 16 compounds were identified (Table 20) through Mass/charge (m/z) (mass/charge/) fragmentation. This was compared to compounds in the PubChem database for identification. An example m/z fragmentation for citric acid has been shown in Figure 46 (remaining could be found in appendix III).

<table>
<thead>
<tr>
<th>RT(min)</th>
<th>[M-H]-</th>
<th>Mol. Formula</th>
<th>MS Fragments</th>
<th>Name</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>191.02</td>
<td>C6H7O7</td>
<td>133, 111</td>
<td>(iso)citric acid</td>
<td>Organic acid</td>
</tr>
<tr>
<td>5.9</td>
<td>771.201</td>
<td>C33H39O21</td>
<td>695, 547, 415, 375, 353, 285, 191</td>
<td>Kaempferol-Osophoroside O-glucoside</td>
<td>Flavonol acylated glycoside</td>
</tr>
<tr>
<td>13.1</td>
<td>711.28</td>
<td>C34 H47 O16</td>
<td>693, 549, 341</td>
<td>Nomilinic acid -17- O-glucoside</td>
<td>Limonoid</td>
</tr>
<tr>
<td>14.7</td>
<td>693.2775</td>
<td>C34 H45 O15</td>
<td>531, 443, 341</td>
<td>Nomilinic acid -4- O-glucoside</td>
<td>Limonoid</td>
</tr>
<tr>
<td>No.</td>
<td>Mol. Wt.</td>
<td>C, H, O</td>
<td>M.p. (°C)</td>
<td>Compound Name</td>
<td>Class</td>
</tr>
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<td>-----</td>
<td>----------</td>
<td>--------</td>
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<td>---------------</td>
<td>-------</td>
</tr>
<tr>
<td>2.6</td>
<td>651.261</td>
<td>C29H31O17</td>
<td>507, 417, 341</td>
<td>Kaempferol acetyl dihexoside</td>
<td>Flavonoid-3-o-glycosides</td>
</tr>
<tr>
<td>13.1</td>
<td>649.251</td>
<td>C32H41O14</td>
<td>413, 341</td>
<td>Limonin glucoside</td>
<td>Limonoid</td>
</tr>
<tr>
<td>8.9</td>
<td>625.17</td>
<td>C28H33O14</td>
<td>383, 312</td>
<td>Diosmetin-6, 8-di-C-hexoside (Lucenin-2,4'-methyl ether)</td>
<td>Flavonoid-7-o-glycosides</td>
</tr>
<tr>
<td>2.3</td>
<td>623.162</td>
<td>C28H31O16</td>
<td>605, 533, 503, 579, 443</td>
<td>6,8-C,C-Diglucosyldiosmetin isomer</td>
<td>C-Flavone glycoside</td>
</tr>
<tr>
<td>2.7</td>
<td>623.156</td>
<td>C28H31O16</td>
<td>605, 533, 503, 413, 329</td>
<td>C,C-Diglucosyldiosmetin</td>
<td>C-Flavone glycoside</td>
</tr>
<tr>
<td>14.9</td>
<td>609.182</td>
<td>C28H33O15</td>
<td>301.07</td>
<td>Diosmetin-7-O-rutinoside (diosmin)</td>
<td>Flavonoid-7-o-glycosides</td>
</tr>
<tr>
<td>15.7</td>
<td>609.182</td>
<td>C28H33O15</td>
<td></td>
<td>Chrysoeriol 7-rutinoside</td>
<td>Flavonoid-7-o-glycosides</td>
</tr>
<tr>
<td>13.6</td>
<td>607.166</td>
<td>C25H31O15</td>
<td>299,284</td>
<td>Diosmetin 7-O-neohesperidoside</td>
<td>Flavone</td>
</tr>
<tr>
<td>14.7</td>
<td>607.166</td>
<td>C25H31O15</td>
<td>299,283.8</td>
<td>Chrysoeriol 7-O-neohesperidoside</td>
<td>Flavonoid-7-o-glycosides</td>
</tr>
<tr>
<td>13.3</td>
<td>595.166</td>
<td>C27H31O15</td>
<td>505, 457, 427, 421, 409, 391, 379, 355, 337, 325, 307, 295</td>
<td>Apigenin-6,8-di-C-glucoside</td>
<td>Flavonoid</td>
</tr>
</tbody>
</table>
Table 20: List of compounds found in lemon extract.

<table>
<thead>
<tr>
<th>Retention</th>
<th>Mass (m/z)</th>
<th>Formula</th>
<th>Description</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.0</td>
<td>287.055</td>
<td>C_{15}H_{11}O_{6}</td>
<td>(2S)-Eriodictyol flavone</td>
<td><img src="image" alt="Flavone Structure" /></td>
</tr>
<tr>
<td>36.7</td>
<td>269.24</td>
<td>C_{17}H_{37}O_{2}</td>
<td>Apigenin flavone</td>
<td><img src="image" alt="Apigenin Structure" /></td>
</tr>
</tbody>
</table>

Figure 46: Iso-citric acid compound chromatogram found in lemon extract.

7.3 DISCUSSION

This study demonstrated that ACE expression in 3T3-L1 adipocytes treated cells with LE decreased by ~80% after 10 hours of exposure. A study in overweight women drinking lemon juice (lemon juice with a mixture of maple and palm syrup), following abstinence from solid food for 7 days reduced body fat and increased insulin sensitivity (175). It was shown that differentiation to mature adipocytes induced an increase in ACE gene expression. This supported previous studies showing that higher ACE expression was associated with increased adiposity and fat deposition in murine and human (211, 212). Increased production of Ang II in adipose tissue has also been observed in diet-induced obese models (213, 214). The inhibitory effect of LE showed that it has a potential to act as a potential ACE inhibitor. Lemon
and lime juice has been found to inhibit ACE activity in a dose dependent manner in mouse models (56).

3T3-L1 cells showed improved insulin sensitivity after treatment with LE, evidenced by increased p-AKT and GLUT4 levels. Several studies testing citrus fruits have found increased insulin stimulated glucose uptake and improved glycemia and HbA1c (209, 210, 215) in humans. Several flavonoids (a naturally occurring plant based nutrients) have been associated with biological activities. Some of the flavonoids found in citrus fruits are naringin, hesperidin, eriodictyol and nobiletin (210). These inhibit accumulation of triglycerides (TGs), increased HDL and decreased VLDL-TG section (216, 217). Healthy volunteers on hesperidin for 4 weeks showed a reduced cytokines and inflammatory markers in circulation (218-221). All the 16 compounds (a mix of flavonoids and limonoids) identified in LE (used in this study) haven’t been tested for their health benefits. These should be tested to identify the active compound leading to lipid mobilization and increased insulin sensitivity shown in this chapter.

To investigate the induction of lipolysis, protein expression of perilipin, pHSL were measured in combination with free glycerol release from treated and untreated adipocytes. In the LE-treated cells, an increase in free glycerol release along with perilipin and pHSL protein expression was observed, which supported a probable increased in lipid breakdown (222, 223). The reduction of lipid content has often been associated with increased insulin sensitivity and glucose utilization (224). Consistently with this, expression of GLUT4 (the insulin-dependent glucose transporter in adipocytes) (225) and pAKT were elevated after LE incubation.

The pathway for the potential effectiveness of lemon extract for weight loss can be described in 2 steps. LE decreased the ACE levels in the in-vitro model leading to increased insulin sensitivity by potentially increasing glucose uptake demonstrated by increased pAKT and GLUT4 signalling molecules which are involved in insulin signalling pathway. ACE was also involved in lipogenesis and/or adipose tissue development, so LE potentially acting as an ACE inhibitor induced lipolysis in the adipocyte demonstrated by increased pHSL levels.

A study involving drinking lemon juice decreased body fat, waist-hip ratio and decreased high-sensitive CRP in serum (175). Similar studies on citrus fruits such as orange juice, bergamot extract and grapefruit have found beneficial effects on lipid variables (53, 54, 221). Drinking orange juice every day lowered concentrations of LDL, LDL/HDL ratio (55). Another study involving supplementation of every meal with grapefruit decreased body weight (54). It would be interesting to investigate if drinking lemon juice has an effect on the circulating ACE levels in blood/urine.
LEMON JUICE SUPPLEMENTATION STUDY IN HUMANS (PILOT)

This figure illustrates lemon juice effects on Angiotensin Converting Enzyme (ACE) in humans. The figure was designed using Paint and PowerPoint 2013.
8.0 SUMMARY

ACE has been identified as a marker that correlated with weight maintenance, which is defined by a sustained weight without regain after a period of weight loss. In addition, chapter 5 described that ACE correlated with weight loss in individuals who fall in the overweight or obese category (BMI≥25) and provided an early feedback on diet potency. Supporting a role of ACE in diet feedback, chapter 6 demonstrated the effects of LE on mouse adipocyte leading to improved insulin sensitivity and lipid breakdown.

In this chapter, the effects lemon juice (LJ) dietary supplementation on ACE levels and weight loss have been discussed. In this pilot study, it was found that drinking LJ was positively correlated with weight loss and that weight loss was improved when LJ was combined with a calorie-restricted diet. Lastly, this weight loss was sustained even when normal (non-restricted) dietary habits were resumed.

8.1 INTRODUCTION

As established in the previous chapter, LE has been found to be associated with increased lipid mobilization in adipocytes. LE also decreased ACE mRNA expression, activity and protein expression in 3T3-L1 cells. Drinking LJ has been found to decrease body fat, waist-hip ratio in serum in humans (175). Drinking orange juice daily lowers concentrations of LDL (55) and supplementation of every meal with grapefruit also decreases body weight (54).

In this pilot study, the effects of LJ supplementation on weight loss in the context a very low-calorie diet (VLCD), were investigated. The programme itself is not only restricted to ingesting lemon juice, but is also complemented with a mixture of Neera syrup (provides necessary minerals like iron, manganese, zinc, magnesium, calcium, sodium, copper and potassium.) which provides 600 to 800 KCal a day (175, 226). Urine samples were provided over the course of several days and ACE was quantified as described in the Methods chapter.

<table>
<thead>
<tr>
<th>Batch Amount</th>
<th>Syrup</th>
<th>Lemon</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 mL</td>
<td>10 mL</td>
<td>1</td>
</tr>
<tr>
<td>1800 mL</td>
<td>60 mL</td>
<td>6</td>
</tr>
</tbody>
</table>

*Table 21: Composition of LJ consumed during the study.*
8.2 RESULTS

8.2.1. STUDY DESIGN AND CHARACTERISTICS

The study was followed for 7 days with 3 control (C), 2 abstinence (A) and 2 transition (T) days. “Abstinence days” were defined as 24-hour solid-food fasts. No solid food was allowed but liquids were allowed. The individual drank 1.5-2 L of LJ in the form of a Neera syrup that associates a blend of maple and palm tree syrups with LJ. The syrup has been described to have high amounts of minerals and trace elements to sustain a metabolism (175). The “Transition days” were defined as a combination of solid food (with no restriction on caloric intake) and 1.5- 2 L of LJ intake (also in the form of a Neera syrup). The “Control days” were defined as a 24-hour period without any food restriction. There was no intake of LJ but other liquids were allowed in similar volume (1.5-2 L) as on Abstinence or Transition days. On Control and Transition days, the solid food intake was similar to the routine of the participant. Abstinence, Transition and Control days were consecutive days, without any break (Table 22).

<table>
<thead>
<tr>
<th>Day #</th>
<th>Day Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (C)</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Lemon Juice only (Neera syrup) (A-Abstinence day)</td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Lemon juice (Neera syrup)+ Solid food (T-Transition day)</td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Control (C)</td>
</tr>
</tbody>
</table>

Table 22: Meal Plan for ACE study.

8.2.2 CALORIC INTAKE PATTERN

The average total calories were 562Kcal on Abstinence days, 1744 Kcal on Transition days and 1508 KCal on Control days. On Abstinence days, lemon juice was taken along with a blend of Neera syrup, making the total caloric intake to ~550 KCal.
Figure 47: Caloric spread during the lemon juice study. C-Control day; A-Abstinence day; T-Transition day.

8.2.3 WEIGHT LOSS PATTERN

Figure 48 shows the weight loss during the LJ programme on Control, Abstinence and Transition days. Any change in weight >0Kg was defined as weight loss, any change in weight <0Kg was defined as weight gain and any change in weight =0Kg was defined as no change. The weight was measured on every morning during the study. Following the day 1 of the study, weight gain was observed on the morning of the first abstinence day (day 2). As apparent, 4.5 kg weight (based on scale measurement) was lost on the second abstinence day showing that drinking only lemon juice (during the day) affected weight loss. A small weight loss remained during transition days. During the last two days (control), the weight lost appeared to be sustained that was lost during the abstinence (day 2 and 3) and transition days, i.e. lemon juice + solid food days (day 4 and 5).
Figure 48: Weight Change Pattern: Relation between weight and weight lost and the type of the day. C-Control day; A-Abstinence day; T-Transition day. The primary y-axis show the weight of the participant on the day (bars) and the secondary y-axis show the weight change (line).

8.2.4 ACE LEVELS NEGATIVELY CORRELATED WITH CALORIE INTAKE

ACE levels were measured using the method described in Chapter 2. Based on the entries in the health platform, the average caloric intake was similar on transition and control days but drastically reduced on Abstinence days, as shown in Figure 1. Interestingly, the average ACE levels on transition and control days were very similar (877 and 790 ng, respectively) but total ACE was significantly increased (1517 pg) during Abstinence days where caloric intake was low (Figure 49). This suggested a correlation between calorie intake and ACE levels, however statistical significance was not reached due to the low number of participants.
8.3 DISCUSSION

A calorie-restricted diet supplemented with LJ appeared to alter ACE levels. The evidence suggested that the effects of LJ extended over subsequent days, as the ACE patterns were similar for Abstinence days (only LJ- day 3 and 4) and the subsequent Transition days (day 5 and 6). However, the sample size was too limited to reach statistical significance and the study would have to be confirmed in a larger cohort. The days with only LJ were recorded to have a caloric input of ~500kcal. In addition, to confirm the additional effects of LJ, controls would have to be taken with similar calorie on control days and abstinence days. According to Bayer et al (2006), in the LJ programme, the participants needed to follow 2-3 days of abstinence (LJ only) and 3-4 days of transition period (LJ + solid food). However, there were no indication of the effects of LJ on metabolism or general health, and no information is yet available on how many days of abstinence, transition (as well as control) are needed to obtain weight loss. The pattern of A, A, T, T and C was chosen arbitrarily, however additional studies would have to be conducted to evaluate the influence of the different combinations on weight loss (discussed in Chapter 9).
This figure summarizes the personalised feedback strategy for a short term feedback. The figure was designed using Paint and PowerPoint 2013.
9.0 SUMMARY

This chapter discusses the highlights of the thesis and the future work that could be performed for taking the future steps toward personalised molecular feedback for weight loss.

9.1 CONCLUSIONS

A molecular feedback approach to assist dieting efforts and behavioural responses of people using a web- and mobile-based application was investigated. Skipping a meal in a day regardless of which one resulted in consistent weight loss for that day, in comparison to control days in which any number of meals was allowed. Insulin, ACE and lactate levels in urine showed correlations to BMI, caloric patterns and weight difference. Breakfast/Dinner (BDS) was the most commonly chosen meal plan where insulin showed a negative and positive correlation with weight differences and fasting time, respectively. ACE is the least studied molecule in terms of its association with dieting. Thus, cellular studies were conducted to identify nutritional means by which ACE could be regulated, assisting in weight loss. It was found that lemon extracts down regulated ACE activity in adipocytes and was associated with both improved adipose insulin sensitivity and increased lipid mobilization. Further, to support the in-vitro studies, a pilot scale study in humans was done suggesting the hypothesis that a low ACE level at the end of the day might signal weight loss for that day (as evidenced by the weight decrease the following morning). Furthermore, as expected the LJ diet appeared to alter ACE levels. The evidence so far suggested that the effects extended over subsequent days, as the ACE patterns were similar for LJ only days and the subsequent transition days.

9.2 FUTURE WORK

9.2.1 SHORT TERM GOAL

I. NEW MARKER IDENTIFICATION THROUGH NMR

The feasibility of detecting variations in molecular markers over short periods of time (minimum 24-hour interval) has been established and correlated with weight change.
Encouraged by the results obtained, it is proposed to test the urine samples from the meal skipping study via NMR. This would allow identification of patterns in a 24-hour urinary profile of participants and potential other metabolites that might have potential to assist weight loss efforts. So far only 3 samples have been studied but testing all the samples obtained over 147 days, could help in identification of different molecules involved, thereby shedding light on possible mechanisms involved in the process of weight loss. Testing multiple days from a single participant would also enable understanding of intra and inter variability in the urinary profile.

II. IDENTIFICATION OF EFFECTS OF LE COMPONENTS

Different compounds in LE have been identified using HPLC-MS techniques. LE down regulated ACE activity in 3T3-L1 adipocytes and was associated with both improved adipose insulin sensitivity and increased lipid mobilization. The next step would be to identify the bioactive compounds in LE that are associated with these effects. HPLC with mass spectrometry techniques could be used to extract different fractions and later test these fractions for their potential metabolic effects. Incidentally, some of those compounds are commercially available and could also be studied with respect to their contribution to the observed metabolic effects.

9.2.2 LONG TERM GOALS

I. EFFECT OF LE ON ACE IN ANIMAL MODEL

Studying the change in expression of ACE in adipose tissue of control mice (C57Blk6) before and after an overnight fast would allow exploration of the results observed in 3T3-L1 cell line. Overnight fasted animals would be sacrificed on the morning to collect tissue samples for ACE mRNA/protein quantification. The selection of a mouse model is motivated by their routine use as a mammalian model of obesity and diabetes. Mice provide an excellent fit for obesity and insulin-related studies since the mechanisms leading to obesity and underlying insulin function are remarkably similar to those in humans and technical approaches for modelling aspects of diet-induced metabolic disorders are established and standardized worldwide (171).
II. EFFECT OF LJ ON ACE IN HUMANS

The pilot scale study suggested that drinking LJ led to a low ACE level at the end of the day that might signal weight loss for that day (as evidenced by the weight decrease the following morning). More participants should be recruited to determine if similar patterns could be achieved and identify the reproducibility of the data obtained. The days with just LJ were recorded to have a caloric input of 200-500 KCal. Samples need to be collected for at least 2 days to verify how ACE levels would effected on days with similar caloric intake but without LJ. According to Bayer (2006), the lemon dieting program needs to be followed with 2-3 days of abstinence (lime juice only) and 3-4 days of transition period (lime juice + solid food). Identification of total days of abstinence and transition period needs to be conducted to understand the required days to attain weight loss and for the maintenance of the lost weight.

To achieve this, different groups need to follow the study for different combinations of abstinence (lemon juice only) and transition period (lemon juice + solid food) and observe the weight change at the end of the intervention.

III. EFFECT OF LJ ON APPETITE

ACE interacts with uncoupling protein-2 (UCP2) (found in the hypothalamus) leading to regulation of neurons involved in food intake during fasting (226-228). It would be interesting to study the effect of lemon on an individual’s appetite. A survey should be designed, in which participants would rate their hunger on a scale of 0 to 5 while following the LJ plan. This option would be available as a part of the platform (designed for the meal skipping study) where they maintain their daily food and urine entries. This scale would also be used to rate their hunger on a control day (no caloric restriction) before they eat a meal. They would also rate their hunger before and after drinking lemon juice on abstinence and transition days. Analysis of these data points would help to identify if drinking LJ influenced an individual’s appetite. Similarly, effects on appetite could also be studied in animal models by analysing signs of withdrawal, tolerance and consumption behaviour.
LIST OF TRAINING COMPLETED DURING PhD

Metabolomics Phenotyping in Disease Diagnostics and Personalised Health Care - Date:
27-30th October 2015 at Imperial College London.
BIBLIOGRAPHY

limon) and lime (Citrus aurantifolia) juices. Comparative Clinical Pathology. 2015;24(6):1395-406.


90. Obesity – Android Apps on Google play [Available from: https://play.google.com/store/search?q=obesity&c=apps&docType=1&sp=CAFiCQoHb2Jlc2JoXoGFADAAQQKQAQ3D%3D%3AS%3AANO1ljJqCJM&hl=en_GB.


128. Henriksen EJ, Jacob S, Kinnick TR, Youngblood EB, Schmit MB, Dietze GJ. ACE inhibition and glucose transport in insulinresistant muscle: roles of bradykinin and nitric


224. Sears B, Perry M. The role of fatty acids in insulin resistance. Lipids in health and disease. 2015;14:121-.
17th April 2015

PRIVATE
Prof Judith Klein
Translational & Systems Medicine
WMS
University of Warwick
Coventry
CV4 7AL

Dear Prof Klein,

Study Title and BSREC Reference: Measuring insulin in urine to assist weight-loss efforts,
REGO-2014-1318

Thank you for submitting your revisions to the above-named project to the University of Warwick's Biomedical and Scientific Research Ethics Sub-Committee for approval.

I am pleased to confirm that approval is granted and your study may commence.

Please keep a copy of the signed version of this letter with your study documentation.

Yours sincerely

[Signature]

Professor Scott Weich
Chair
Biomedical and Scientific
Research Ethics Sub-Committee

Biomedical and Scientific
Research Ethics Sub-Committee
A010 Medical School Building
Warwick Medical School,
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Tel: 02476-528207
Email: BSREC@Warwick.ac.uk
PRIVATE
Prof Judith Klein
Warwick Medical School
University of Warwick
Coventry
CV4 7AL

21 July 2016

Dear Professor Klein,

Study Title and BSREC Reference: Measuring insulin in urine to assist weight-loss efforts
REGO-2014-1318 AM01

Thank you for submitting a substantial amendment application for the above-named project to the University of Warwick's Biomedical and Scientific Research Ethics Sub-Committee.

I am pleased to confirm that the changes that you wish to make to this study have been approved.

Please keep a copy of the signed version of this letter with your study documentation.

Yours sincerely

Professor Scott Welch
Chair
Biomedical and Scientific Research Ethics Sub-Committee

Biomedical and Scientific Research Ethics Sub-Committee
A010 Medical School Building
Warwick Medical School,
Coventry, CV4 7AL.
T: 02476 528207
E: BSREC@Warwick.ac.uk
http://www2.warwick.ac.uk/services/ris/research_integrity/researchethicscommittees/bsrec
Dear Professor Klein,

Study Title and BSREC Reference: *NMR analysis to identify markers for weight loss*
REGO-2018-2218

Thank you for submitting the above-named project to the University of Warwick Biomedical and Scientific Research Ethics Committee for research ethical review.

I am pleased to advise that research ethical approval is granted.

In undertaking your study, you are required to comply with the University of Warwick’s Research Data Management Policy, details of which may be found on the Research and Impact Services’ webpages, under “Codes of Practice & Policies” » “Research Code of Practice” » “Data & Records” » “Research Data Management Policy”, at:
http://www2.warwick.ac.uk/services/ris/research_integrity/code_of_practice_and_policies/research_code_of_practice/datacollection_retention/research_data_mgt_policy

You are also required to comply with the University of Warwick’s Information Classification and Handling Procedure, details of which may be found on the University’s Governance webpages, under “Governance” » “Information Security” » “Information Classification and Handling Procedure”, at:
http://www2.warwick.ac.uk/services/gov/informationsecurity/handling.

Investigators should familiarise themselves with the classifications of information defined therein, and the requirements for the storage and transportation of information within the different classifications:

- Information Classifications:
  http://www2.warwick.ac.uk/services/gov/informationsecurity/handling/classifications
- Handling Electronic Information:
  http://www2.warwick.ac.uk/services/gov/informationsecurity/handling/electronic/
- Handling Paper or other media
  http://www2.warwick.ac.uk/services/gov/informationsecurity/handling/paper/

Please also be aware that BSREC grants ethical approval for studies. The seeking and obtaining of all other necessary approvals is the responsibility of the investigator.
Figure 50: Apigenin compound chromatogram found in lemon extract.
Figure 51: (2S)-Eriodictyol compound chromatogram found in lemon extract.
Figure 5: Apigenin-6,8-di-C-glucoside compound chromatogram found in lemon extract.
Figure 53: Chrysoeriol-7-O-neohesperidoside compound chromatogram found in lemon extract.
Figure 54: Neodiosmin compound chromatogram found in lemon extract.
Figure 55: Chrysoeriol 7-rutinoside compound chromatogram found in lemon extract.
Figure 56: Diosmetin-7-O-rutinoside (diosmin) compound chromatogram found in lemon extract.
Figure 57: Isorhamnetin-3-O-rutinoside compound chromatogram found in lemon extract.
Figure 58: Diglucosylidiosmetin isomer C-flavone glyc. compound chromatogram found in lemon extract.
Figure 59: Diosmetin-6, 8-di-C-hexoside compound chromatogram found in lemon extract.
Figure 60: Limonin Glucoside compound chromatogram found in lemon extract.
Figure 61: Kaempferol acetyl dihexoside compound chromatogram found in lemon extract.
Figure 62: Nomilinic acid-4-Oglucoside Limonoid compound chromatogram found in lemon extract.
Figure 63: Nomilinic acid -17- O-glucoside Limonoid compound chromatogram found in lemon extract.
Figure 64: Kaempferol-Osophoroside-Oglucoside compound chromatogram found in lemon extract.
Figure 65: The intra-individual variation of the biomarkers and caloric intake over a 24-hour period. Blue, green and pink corresponds to ACE, insulin and lactate respectively. Yellow triangles are the urine stamps, blue square corresponds to the weight of an individual and red circles corresponds to the multiple food entries added in the health platform in a day. The image was created by Dr. Joan Planas using R.
APPENDIX VI