Investigation of the effects of VGF overexpression and VGF-derived peptides on lipolysis in 3T3-L1 cells

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MRes Dissertation Declaration

The work presented in this dissertation is my own work except where stated in the text. Technical assistance, where relevant, has been acknowledged.

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Abstract

Obesity is a common problem in the UK affecting 1 in 4 adults and 1 in 5 children, and the prevalence of obesity is steadily increasing. Obesity is becoming more widely recognised as hormonal dysregulation of fat accumulation rather than being solely attributed to caloric energy imbalance. The neuroendocrine pro-hormone VGF is processed into a variety of peptide hormones and has been linked to energy homeostasis and insulin secretion, with expression of VGF in human adipose tissue and plasma levels of VGF-derived peptides correlating negatively with obesity. It has been hypothesised that some VGF-derived peptides may exert opposing effects. The VGF-derived peptide TLQP-21 induces lipolysis in adipocytes, however, the effects of VGF and its derived peptides TLQP-62 and AQEE-30 on adipocyte lipid content *in vitro* have not yet been investigated. Here we show that treatment of 3T3-L1 adipocytes with TLQP-62 significantly increased cellular lipid content, whereas treatment with AQEE-30 and VGF overexpression had no significant effect on lipid content. We found that lipid-based transfection of 3T3-L1 pre-adipocytes was toxic and resulted in cell death, whereas transfection of partially differentiated 3T3-L1 adipocytes was inefficient and induced an insignificant reduction in lipid accumulation compared to adipocytes transfected with a control construct lacking *vgf*. Furthermore, we found that TLQP-62 and AQEE-30 had no significant effect on cellular lipid content when administered in the absence of isoproterenol. However, when administered with isoproterenol, TLQP-62 significantly increased 3T3-L1 lipid content and AQEE-30 significantly inhibited isoproterenol-induced lipolysis in 3T3-L1 adipocytes. Our results demonstrate that TLQP-62 may induce lipogenesis in 3T3-L1 cells via β-adrenergic signalling pathways and supports the hypothesis that some VGF-derived peptides may have opposing effects in energy homeostasis *in vivo*. We anticipate that VGF could provide targets for the treatment of obesity and T2DM, and a greater understanding of the relationships between VGF-derived peptides could be relevant for the development of new intervention strategies.

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Abbreviations

ADP – Adenosine Diphosphate

AMP – Adenosine Monophosphate

ATP – Adenosine Triphosphate

AP-1,-2 – Activating Protein 1, 2

AT – Adipose Tissue

ATF2 – Activating Transcription Factor 2

ATP – Adenosine Triphosphate

BAT – Brown Adipose Tissue

BeAT – Beige Adipose Tissue

BMI – Body Mass Index

BMR – Basal Metabolic Rate

C3a – Complement 3a

C3aR1 – Complement 3a Receptor 1

cAMP – cyclic Adenosine Monophosphate

CNS – Central Nervous System

CRE – cAMP Response Element

CREB – CRE Binding protein

CRP – C Reactive Protein

CT – Computed Tomography

CVD – Cardiovascular Disease

DALY – Disability-Adjusted Life Year

DEE – Daily Energy Expenditure

DNP – 2, 4-dinitrophenol

EGF – Epidermal Growth Factor

ELISA – Enzyme-Linked Immunosorbent Assay

EMA – European Medicine Agency

ERK – Extracellular Receptor Kinase

FA – Fatty Acid

FDA – Food and Drug Administration

FFAs – Free Fatty Acids

GCN5 – Histone acetyltransferase GCN5

GDP – Guanosine Diphosphate

GHSR-1a – Growth Hormone Secretagogue Receptor 1a

GLP-1 – Glucagon-Like Peptide 1

GLP-1RA – Glucagon-Like Peptide 1 Receptor Agonist

IL-6 – Interleukin-6

kcal – Kilocalorie(s)

mRNA – messenger RNA

mTOR – mammalian/mechanistic Target Of Rapamycin

NA – Noradrenaline

NF-κB – Nuclear Factor κ-light-chain-enhancer of activated B cells

NGF – Nerve Growth Factor

NGFI-A – Nerve Growth Factor Inducible protein A

NHS – National Health Service

NRE – Negative Response Element

P38 – P38 Mitogen-activated protein kinase

PC – Prohormone Convertase

PC1/3 – Prohormone Convertase 1/3

PC2 – Prohormone Convertase 2

PCOS – Polycystic Ovary Syndrome

PET – Positron Emission Tomography

PGC-1α – PPARG Coactivator 1α

PKA – Protein Kinase A

PNS – Peripheral Nervous System

PPARα,γ – Peroxisome Proliferator-Activated Receptor α, γ

PRDM-16 – PR Domain Containing 16

RA – Retinoic Acid

ROS – Reactive Oxygen Species

RT-qPCR – Quantitative Reverse Transcription Polymerase Chain Reaction

SIRT1 – Sirtuin 1

SIRT6 – Sirtuin 6

SNP – Single Nucleotide Polymorphism

SP-1 – Specificity Protein 1

T2DM – Type 2 Diabetes Mellitus

T_3 – Triiodothyronine (highly active thyroid hormone)

TAG - Triacylglycerol

TCA cycle – Tricarboxylic Acid cycle

TFX – Transcription Factor X

TLR – Toll-Like Receptor

TNF-α – Tumour Necrosis Factor α

UCP1,2,3 – Uncoupling Protein 1, 2, 3

VAT – Visceral Adipose Tissue

VGF (Non-acronymic) – VGF Nerve Growth Factor Inducible

WAT – White Adipose Tissue

WHO – World Health Organisation

β3-AR – β3 Adrenergic Receptor

β-AR – β-Adrenergic Receptors

1 Introduction

1.1 Overview of Obesity

The World Health Organisation (WHO) defines overweight (BMI ≥ 25) and obesity (BMI ≥ 30) as abnormal or excessive fat accumulation that may impair health. BMI is a commonly used weight-height index for classification of adult weight categories. Calculation of BMI is independent of sex or age in adults and should be considered a rough guide as it cannot distinguish between body mass due to muscle or fat (Meeuwsen et al., 2010) – this is easily circumvented by also measuring the individual's waist circumference (Neovius et al., 2005). In 2016, the WHO estimated that over 1.9 billion adults (approximately 39% of the worldwide adult population) were overweight and of these, 650 million adults (13% of the worldwide adult population) were obese. In children and adolescents (ages 2-18), a percentile scale based on sex and age is used instead of BMI (Apovian, 2016). In this population, overweight and obese are defined as being in the 85th-94th and ≥95th percentiles respectively (Apovian, 2016). Alarmingly, worldwide prevalence of obesity nearly tripled between 1975 and 2016, and 38 million children under the age of 5 were overweight or obese in 2019 (WHO). The most recent UK data shows that prevalence of obesity in the UK is greater than the worldwide population, with 25% of adults classed as obese (Hancock and Timpson, 2022).

1.1.1 Costs of Obesity

It is estimated that overweight and obesity-related conditions cost the NHS £6.1 billion between 2014-2015 (Public Health England, 2017). It is difficult to calculate indirect costs of obesity, such as absence from work, early pension payments, and increased demand on community services, but this was estimated to cost £27 billion in the UK for 2014-2015 (Public Health England, 2017). The UK government predicts that over half of the UK population will be obese by 2050, costing the NHS a projected £10 billion per year and costs to wider society reaching £49.9 billion (not accounting for inflation) (Butland et al., 2007). The most recent UK health survey reported that the majority of adults in England were overweight (67% of men and 60% of women), of which 26% of men and 29% of women were obese, and 2% of men and 4% of women were morbidly obese (BMI ≥ 40) (Health Survey for England, 2019).

1.1.2 Health Consequences of Obesity

Obesity and its complications constitute an important source of morbidity, impaired quality of life, and reduced life expectancy. Obesity is associated with higher rates of death driven by comorbidities including type II diabetes mellitus (T2DM), hypertension, dyslipidaemia, coronary artery disease, joint diseases, polycystic ovary syndrome (PCOS), and cancers including colon, rectum, and prostate in men, and uterus, biliary tree, and breast in women (Abdelaal et al., 2017; Siddiqui, 2008). Every additional 5kg/m^2 in BMI increases a man's risk of oesophageal cancer by 52% and colon cancer by 24%, and increases a woman's risk of endometrial cancer by 59%, gall bladder cancer by 59%, and postmenopausal breast cancer by 12% (Renehan et al., 2008). Excess bodyweight is also linked to many non-fatal, but costly and/or disabling disorders including osteoarthritis (Guh et al., 2009), infertility (Withrow and Alter, 2011), asthma (Thorpe et al., 2004), and sleep apnoea (Vgontzas et al., 1994).

In 2015, high BMI accounted for 4 million deaths (or 7.1% of total deaths) globally, nearly 40% of which occurred in persons who were not obese (GBD 2015 Obesity Collaborators, 2017). In comparison, 6.4 million (or 11.5%) global deaths were attributable to tobacco smoking that same year (Reitsma et al., 2017). According to the disability-adjusted life year (DALY), a measurement of overall disease represented as the number of years lost due to ill-health, poor diet now contributes more to premature death than tobacco smoking. Dietary risks were the leading DALY risk factor in both men (12.2%) and women (9%) in 2015 (Forouzanfar et al., 2016). Dietary risks, high BMI, high systolic blood pressure, high fasting plasma glucose, and high total cholesterol all ranked within the top 10 DALY risk factors for men and women, and together contribute more to cardiovascular and metabolic diseases than any other factors (Forouzanfar et al., 2016).

Wang et al., (2011) used a simulation model to project probable consequences of rising obesity in an aging population over the next two decades. They estimated that, by 2030, there would be 65 million more obese adults in the USA and 11 million more in the UK. This could contribute an additional 6-8.5 million cases of diabetes, 5.7-7.3 million cases of heart disease and stroke, and 492,000-669,000 cases of cancer for the USA and UK combined (Wang et al., 2011).

1.2 Occurrence of Obesity and Failure of Current Treatment Strategies

Weight gain and obesity is caused by a positive net intake of energy due to an imbalance between calories consumed and calories expended. According to the WHO, there has been a global increase in intake of energy dense foods and a decrease in physical activity, which could explain the increasing average body mass trends which are occurring globally, and most drastically in developed countries.

In the UK, between 1986 and 2000, increased energy intake accounted entirely for the increase in body mass of women, but not in men (Scarborough et al., 2010). The discrepancy between the increased average male calorie intake and weight gain could be attributed to reduced energy expenditure due to a more sedentary lifestyle – a factor that may have impacted men more significantly than women over this period due to changes to their traditional manual labour style working roles (Frenkel et al., 1995). A more recent study from the US revealed that increased energy intake was sufficient to explain their obesity epidemic (Swinburn et al., 2009). According to Swinburn, the estimated food energy intake for children increased by 353kcal/day between the 1970s (1971-1976) and 2000s (1999-2002), and estimated food energy for adults increased by 497kcal/day. This study did not reveal differences between sexes, but increased calorie intake is certainly a large factor in weight gain and obesity in the UK and US adult and child populations.

The NHS currently recommends that adults do 150 minutes of moderate intensity activity per week (NHS, 2019). However, this guideline is likely insufficient for individuals aiming to prevent weight gain or regain. Prevention of weight regain in formerly obese individuals requires 60-90 minutes of moderate activity per day, and 45-60 minutes of moderate activity per day are required to prevent the transition from overweight to obese (Saris et al., 2003). Current strategies for weight loss focus on reduced calorie intake (reduced food intake or reduced energy (fat) intake) and increased calorie expenditure (via physical activity), whilst severe cases may require surgery e.g., gastric surgery and/or pharmaceutical treatments to help induce weight loss and manage the comorbidities of obesity e.g., Glucagon-like peptide 1 (GLP-1)

receptor agonists (liraglutide, semaglutide), lipase inhibitors (orlistat), statins (atorvastatin), and insulin.

1.2.1 Failure of Current Treatment Strategies

Reduction of daily calorie intake is a simple and effective method for weight management and tends to require behavioural changes such as counting calories and reducing fat intake (McGuire et al., 1998). The concept is a simple and achievable one, yet failure to lose weight or maintain weight loss is a common issue. Whilst many adults successfully lose weight, the majority experience weight regain. Only an estimated 20% of overweight/obese adults maintain a deliberate 10% reduction of body weight for at least 1 year (Wing and Hill, 2001). Increased hunger and subsequently increased food intake are critical problems during weight loss maintenance in management of obesity. As shown in Figure 1, diet-induced weight loss in obese patients results in elevated fasting serum levels of the appetite-stimulating hormone ghrelin and decreased fasting serum levels of the satiating hormone leptin (Kotidis et al., 2006).

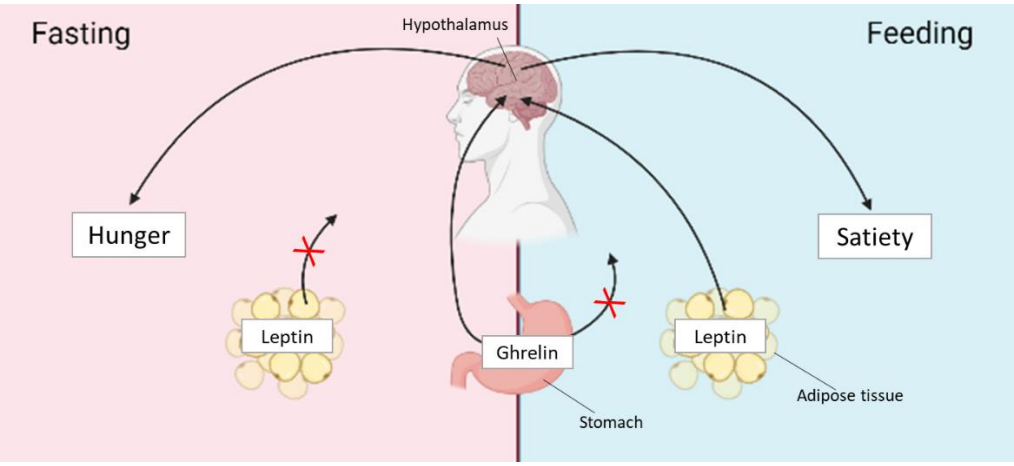


Figure 1. **The roles of leptin and ghrelin in hunger regulation.** Long term diet-induced weight loss mimics the effects of fasting. Created with Biorender.com.

1.3 Targeting Hunger-Regulating Hormones

1.3.1 Leptin

Obese people have elevated leptin and leptin insensitivity (Considine et al., 1996), so administration of leptin and leptin sensitisers e.g., metformin via injection are possible methods of increasing satiety (Quarta et al., 2016). However, elevated leptin levels result in upregulation of ghrelin expression in the stomach (Toshinai et al., 2001) so administration of leptin to overweight and obese individuals may increase hunger signalling and energy intake. Elevated serum leptin is also associated with increased incidences of breast and colon cancer, and leptin may be involved in the development of prostate, ovarian, endometrial, pancreatic, lung, and adrenal cancers (Garofalo and Surmacz, 2006).

1.3.2 Ghrelin

Alternatively, administration of a ghrelin receptor (GHSR-1a) antagonist such as a quinazolinone derivative may alleviate hunger (Rudolph et al., 2007). Daily oral

administration of a GHSR-1a antagonist to diet-induced obese mice resulted in decreased energy (food) intake and fat-specific weight loss (Esler et al., 2007). GHSR-1a has high constitutive signalling which is linked to growth hormone secretion (Pantel et al., 2006) and maintenance of lean mass in a catabolic state (Nørrelund et al., 2001), and mediation of the hyperphagia response elicited by fasting (Fernandez et al., 2018), so administration of an antagonist should not affect these functions. However, ligand binding is required for some signalling pathways such as ERK phosphorylation (Ramirez et al., 2018), inhibition of which can induce apoptosis (Feng et al., 2013). Ghrelin has vasodilatory (Nagaya et al., 2001), cardiovascular protective (Baldanzi et al., 2002), and possibly anti-inflammatory (Dixit et al., 2004) effects in humans which it exhibits (at least partly) by inhibiting cardiomyocyte and endothelial cell apoptosis (Baldanzi et al., 2002). Inhibition of ghrelin signalling in humans would be expected to have opposite effects. Consistent with these expectations, peripheral injection of the ghrelin receptor antagonist [D-Lys-3]-GHRP-6 into rats resulted in increased mean arterial pressure and heart rate (Vlasova et al., 2009).

1.3.3 GLP-1

GLP-1 is an incretin hormone produced and secreted by intestinal enteroendocrine L-cells following food consumption (Holst, 2007). GLP-1 stimulates insulin secretion, inhibits glucagon secretion, regulates the ileal brake mechanism by inhibiting gastrointestinal motility, and is a regulator of appetite and food intake (Holst, 2007). Daily subcutaneous injection of the GLP-1 receptor agonist (GLP-1RA) liraglutide has been approved by the FDA and EMA (Food and Drug Administration and European Medicine Agency) to treat obesity-related comorbidities and achieve weight reduction (Chirstou et al., 2016), and significantly improves several cardiometabolic parameters (Katsiki et al., 2016).

Similarly, weekly administration of the longer-lasting GLP-1RA semaglutide (an antidiabetic medication) significantly lowers blood glucose and blood pressure (Avgerinos et al., 2019), and reduces incidences of cardiovascular morbidity and mortality in T2DM patients (Hedrington et al., 2017). Daily administration of semaglutide also results in greater weight loss than liraglutide at lower doses (O'Neil et al., 2018).

GLP-1RAs are generally well tolerated, with common side effects including gastrointestinal tract disorders e.g., nausea, vomiting, diarrhoea, and dyspepsia (Tan et al., 2017). Rarer side effects include headaches, nasopharyngitis, influenza virus infection, and increased pancreatic lipase and amylase levels (Ahrén et al., 2017; Aroda et al., 2017; Sorli et al., 2017). Similarly to other GLP-1RAs, semaglutide increases heart rate by 1.6-4.0 beats per minute (Christou et al., 2019) though this is deemed clinically insignificant (O'Neil et al., 2018). Elevated heart rate is associated with increased all-cause and cardiovascular mortality (Zhang et al., 2016) so long-term effects of GLP-1RAs should be investigated but may be offset by their weight loss-induced cardiovascular protective effects.

1.3.4 Limitations of Targeting Hunger-Regulating Hormones

Due to their wide range of physiological roles, leptin and ghrelin may not be suitable targets for reducing energy intake and inducing weight loss in overweight and obese individuals. GLP-1 analogues are effective dose-dependent anti-obesity treatments but may be limited by uncomfortable short-term side-effects and potentially severe long-term side-effects. Increased energy expenditure is an alternative mechanism for

preventing weight gain and/or inducing weight loss. A method which increases basal energy expenditure could be a useful treatment for overweight and obesity; adipose tissue (AT) may provide a solution.

1.4 Introduction to Brown and White Adipose Tissue

Human AT was only relatively recently recognised as a major endocrine and secretory organ (Trayhurn, 2005). It has a major role in appetite and energy homeostasis, secreting factors which regulate insulin sensitivity and glucose homeostasis, lipid metabolism, reproduction (sex hormone production), angiogenesis, blood pressure regulation, inflammation, immunity, and the acute phase response (Trayhurn and Wood, 2004). AT is comprised of two cell types – white and brown adipocytes – which typically form distinct white (WAT) or brown (BAT) depots. WAT constitutes the majority of adipose in humans and its main function is the storage of lipids, mostly in the form of triacylglycerol (TAG), which can be subsequently mobilised from the cells to maintain energy homeostasis (Vázquez-Vela et al., 2008). BAT is a metabolically active tissue found specifically in the supraclavicular, paraspinal, cervical, abdominal, axillary, and mediastinal regions of the torso (Figure 2) (Leitner et al., 2017). BAT owes its darker colour to its relatively high mitochondrial cytochrome content compared to WAT (Joel and Ball, 1961). Active brown adipocytes contain multiple fat vacuoles (multilocular) within a granular cytoplasm; in contrast, inactive brown adipocytes are unilocular and indistinguishable from white adipocytes under a microscope (Heaton, 1972). Importantly, activated BAT has greater glucose uptake, lipolysis, and energy expenditure than WAT (Blondin et al., 2014; Festuccia et al., 2011). This has made it an attractive potential target for inducing weight loss, increasing plasma TAG clearance, and treating obesity and its comorbidities.

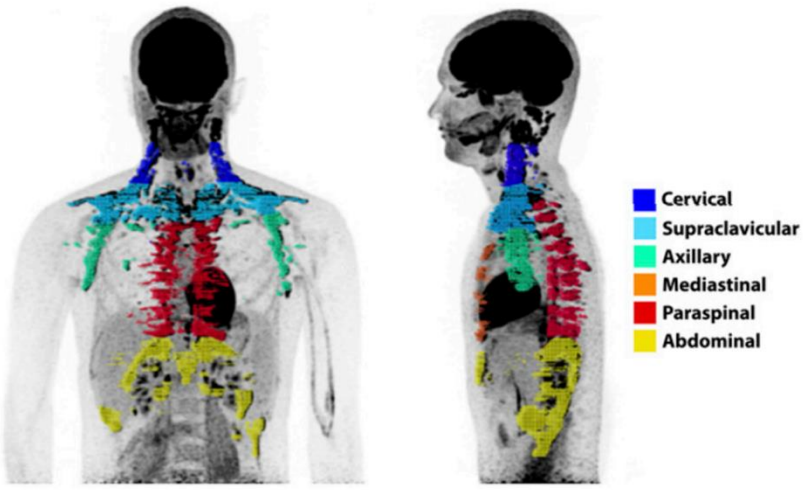


Figure 2. **Regional distribution of BAT depots in a lean man.** From Leitner et al. (2017).

BAT provides a mechanism of heat generation (non-shivering thermogenesis) via 'inefficient' oxidative phosphorylation which uses metabolites from food/energy stores to generate thermal energy rather than chemical energy in the form of ATP (Nedergaard et al., 2001). It is this 'inefficient' energy-production system that has recently thrust BAT into the spotlight as a potential therapeutic target for weight management and metabolic syndrome treatment. Sustainable non-shivering thermogenesis in adult humans amounts to up to 15% of the average daily energy expenditure (DEE), and BAT

thermogenesis alone could account for up to 5% of one's basal metabolic rate (BMR) (van Marken Lichtenbelt and Schrauwen, 2011).

Under aerobic conditions, mitochondria generate energy in the form of ATP using hydrogen sourced from reduced substrates (NADH and $FADH_2$) obtained from the TCA cycle (Bodner, 1986). ATP is a high energy molecule used by cells to drive most chemical reaction pathways necessary for life. Molecular hydrogen is split, and free energy is generated as high-energy electrons are transferred along a series of donor/acceptor proteins collectively called the electron transport chain. As shown in Figure 3, free energy yielded from these redox reactions is used to simultaneously pump protons across the inner membrane from the matrix into the intermembrane space (Hatefi, 1985). Following expulsion from Complex IV, a pair of low-energy electrons combine with $½O_2$ and 2 protons in the matrix cytosol to form water. ATP synthase (often referred to as Complex V) catalyses the phosphorylation of ADP to ATP using free energy yielded from the diffusion of protons back into the matrix (Boyer et al., 1977).

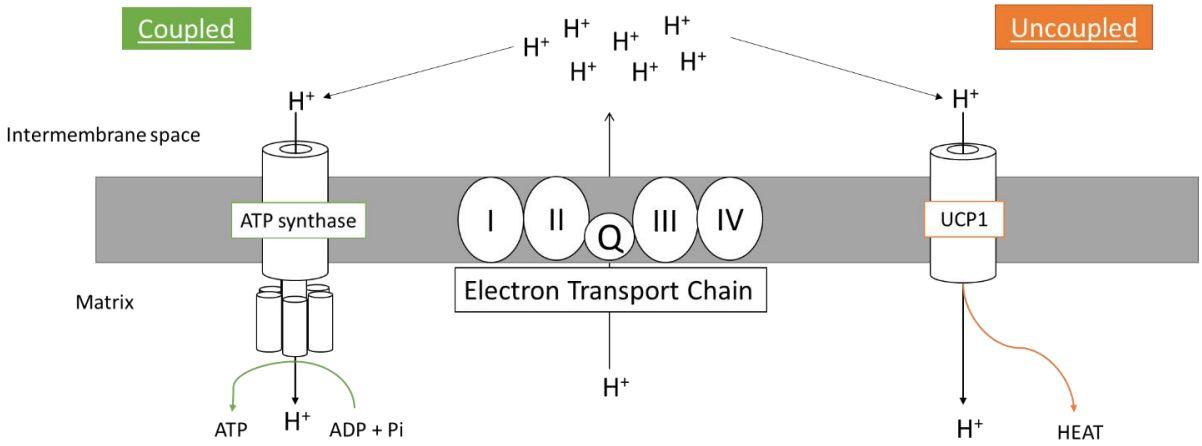


Figure 3. **Schematic diagram comparing coupled and uncoupled mitochondrial oxidative phosphorylation.** Adapted from Brondani et al. (2012).

1.4.1 Mitochondrial Uncoupling as a Weight Loss Tool

Unlike WAT, BAT cells express high levels of uncoupling protein 1 (UCP1) which spans the mitochondrial inner membrane (Frühbeck et al., 2009). As shown in Figure 3, UCP1 allows for passive transport of protons from the intermembrane space into the matrix, thus dissipating the protonmotive force across the membrane without generating ATP (Terada, 1990) and disconnecting ATP synthesis from substrate oxidation.

Energy homeostasis in cells is partly maintained by monitoring ATP:ADP and ATP:AMP ratios (Hardie et al., 2012). As ATP concentrations decrease, the cell responds by increasing energy turnover largely by increasing uptake of glucose and fatty acids which can enter the TCA cycle and provide a source of hydrogen for oxidative phosphorylation (Hardie, 2014). Proton leakage from the intermembrane space decreases the efficiency of ATP generation relative to the amount of substrate metabolised, so it can be presumed that a person with a 'leaky' ATP synthesis system will have increased energy expenditure and a higher BMR, hence increasing a person's uncoupling activity could prevent weight gain and induce weight loss.

1.4.2 Increased UCP1 Expression is Associated with Thermogenic Activity of BAT and Leanness

Increased expression of UCP1 in existing brown adipocytes results in increased uncoupled energy expenditure (Ruan et al., 2011). Alternatively, ablation of UCP1 in mice results in complete loss of thermogenic capacity of brown adipocytes (Matthias et al., 2000). Understandably, no human studies involving ablation of UCP1 have been conducted, but data regarding a single-nucleotide polymorphism (SNP) in the distal enhancer of the human *UCP1* gene has been reported. At position -3826 upstream of the transcription initiation site, an A/G SNP has been detected, with the A allele being associated with a greater level of expression of *UCP1* (Esterbauer et al., 1998). Individuals homozygous for the A allele (A/A) have higher thermogenic responses following a high-fat meal (Nagai et al., 2003) and the A/G and G/G genotypes are associated with higher BMI, larger waist circumference, and greater subcutaneous fat mass (Sramkova et al., 2007).

1.4.3 DNP – Uncontrolled uncoupling

2, 4-dinitrophenol (DNP), a by-product generated in World War 1 munition factories, is the most prominent uncoupler of oxidative phosphorylation and is an effective weight loss agent in humans (Germain et al., 2020; Sousa et al., 2020). DNP is an uncoupler which is clinically proven to induce significant and dose-dependent sustained weight loss and was commercialized as an anti-obesity agent in the 1930's before being withdrawn due to toxicity in 1938 (Goldgof et al., 2014). Dunlop (1934) reported average metabolic rate increases of 12%, 25%, and 35% 24 hours after administration of 1mg, 2mg, and 3mg of DNP per kg of bodyweight respectively. Similarly, Tainter et al. (1935) reported that metabolic rate increased by 11% per 100mg of DNP up to 300mg when administered daily for at least 1 week, and a dose of 400mg elicited a much greater response (up to 95% increase in metabolic rate). Increases in metabolic rate following DNP consumption were associated with greatly increased lipid metabolism, slightly decreased carbohydrate metabolism, and a negligible change in protein metabolism (Tainter et al., 1935). Whilst 3-5mg/kg of DNP is sufficient to induce pyrexia in humans, the margin between febrile and fatal doses is narrow (Dunlop, 1934). In humans, the lethal dose of DNP is between 20-50mg/kg but fatalities have been recorded for doses below 5mg/kg (Grundlingh et al., 2011). Weight loss could be more safely induced by low doses of DNP taken with frequent medical supervision but may result in several non-fatal cumulative effects of DNP consumption which include skin reactions, liver damage, cataract formation, and potentially kidney damage, agranulocytosis, and gastroenteritis (Horner, 1941; Tainter et al., 1934).

Classic symptoms of DNP poisoning include a combination of hyperthermia, tachycardia, diaphoresis, and tachypnoea, eventually leading to death (Grundlingh et al., 2011). Uncoupled oxidative phosphorylation leads to the release of calcium ions from mitochondrial stores, causing muscle contraction which further contributes to hyperthermia (Tewari et al., 2009). DNP is still used commercially as a pesticide and fungicide, and is currently being investigated due to its neuroprotective qualities at low doses (0.5mg per day) (Geisler et al., 2017). DNP is also presently sold and distributed illegally online, usually marketed as 'fat burner' pills which aid weight loss or 'slimming' and tends to be sought after by strength-based athletes during weight loss programmes such as bodybuilders (Grundlingh et al., 2011). By 2011, there had been 62 recorded deaths attributed to DNP since its discovery (Grundlingh et al., 2011), and according to the American Association of Poison Control Centers, at least 3 American people died from DNP use in 2018 (Gummin et al., 2019). It was not until 1978, over 40 years after the

emergence of DNP, that the naturally occurring uncoupler UCP1 was discovered (Foster and Frydman, 1978). Exploitation of the uncoupling potential of UCP1 could more safely induce adipose-specific weight loss.

1.4.4 Noradrenaline Activates BAT – A case study

In humans, the sympathetic neurotransmitter noradrenaline (NA) regulates BAT thermogenesis by activating β-adrenergic receptors (β-ARs) and subsequently stimulating respiration in BAT (Isler et al., 1987). NA stimulates respiration in BAT via upregulation of several thermogenic genes in response to exercise (Dinas et al., 2015) and cold exposure detected by skin and central thermal receptors (Isler, et al., 1987). The effects of cold-induced BAT activation can be replicated by chronic NA administration (Bouillaud et al., 1984).

To gain insight into the capacity for developing thermogenic adipocytes under chronic adrenergic stimulation, Leitner et al., (2017) quantified BAT in a patient with a NA-secreting paraganglioma of the bladder. Total AT in all depots was 486ml, of which 300ml (62%) was active BAT. Extraordinarily, 87% of the patient's abdominal AT was brown (161ml out of 185ml), amounting to more than half of their total active BAT. In contrast, BAT was most abundantly found in the supraclavicular region of healthy lean subjects (118ml on average, or 33% of total BAT), and only 46% of that region's AT was active BAT.

Whether NA-induced thermogenesis is totally UCP1-dependent or acts via other additional pathways is still debated. Hofmann et al., (2001) reported that free fatty acids (FFAs), which can be mobilised by NA, can also activate UCP1. It is likely that FFAs do not directly activate UCP1 but play a role in the transport of H^+ (Winkler and Klingenberg, 1993). UCP2 and 3 have amino acid sequence similarities of 55% and 58% to UCP1 respectively (Nagy et al., 2004). UCP2 is widely expressed (Lentes et al., 1999) whereas UCP3 is most highly expressed in skeletal and cardiac muscle, as well as at lower levels in BAT (Macher et al., 2018; Zaninovich, 2005). Figure 4 illustrates potential mechanisms of proton transport by UCPs. UCP1, 2 and 3 are all inhibited by allosteric binding of purine nucleotides (Berardi and Chou, 2014; Macher et al., 2018; Shabalina et al., 2004). UCP2 proton transport is dependent on FA flippase activity (Berardi and Chou, 2014) whereas UCP1 and 3 proton transport is increased by, but not dependent on, FA flippase activity (Esteves and Brand, 2005; Jiménez-Jiménez et al., 2006). The functions of UCP2 and 3 are debated. Unlike UCP1, ablation of UCP2 and 3 does not result in obesity (Arsenijevic et al., 2000; Gong et al., 2000). Evidence suggests that UCP2 and 3 may not be uncouplers, and are instead involved in mitochondrial FFA transport, lipolysis regulation, and protect mitochondria against reactive oxygen species (ROS) (Nedergaard and Cannon, 2003; Samec et al., 2002). In BAT, FAs likely act as regulators of UCP1 and increase activity by binding to UCP1 and relieving inhibition by purine nucleotides rather than participating in proton transport directly (Huang, 2003; Shabalina et al., 2004). UCP1 likely transports protons via mechanisms (A) and possibly (B) shown in figure 4, with the presence of a bound FFA competitively inhibiting binding of GDP as shown in (E). NA-induced FFA mobilisation is a likely mechanism of UCP1 activation in BAT.

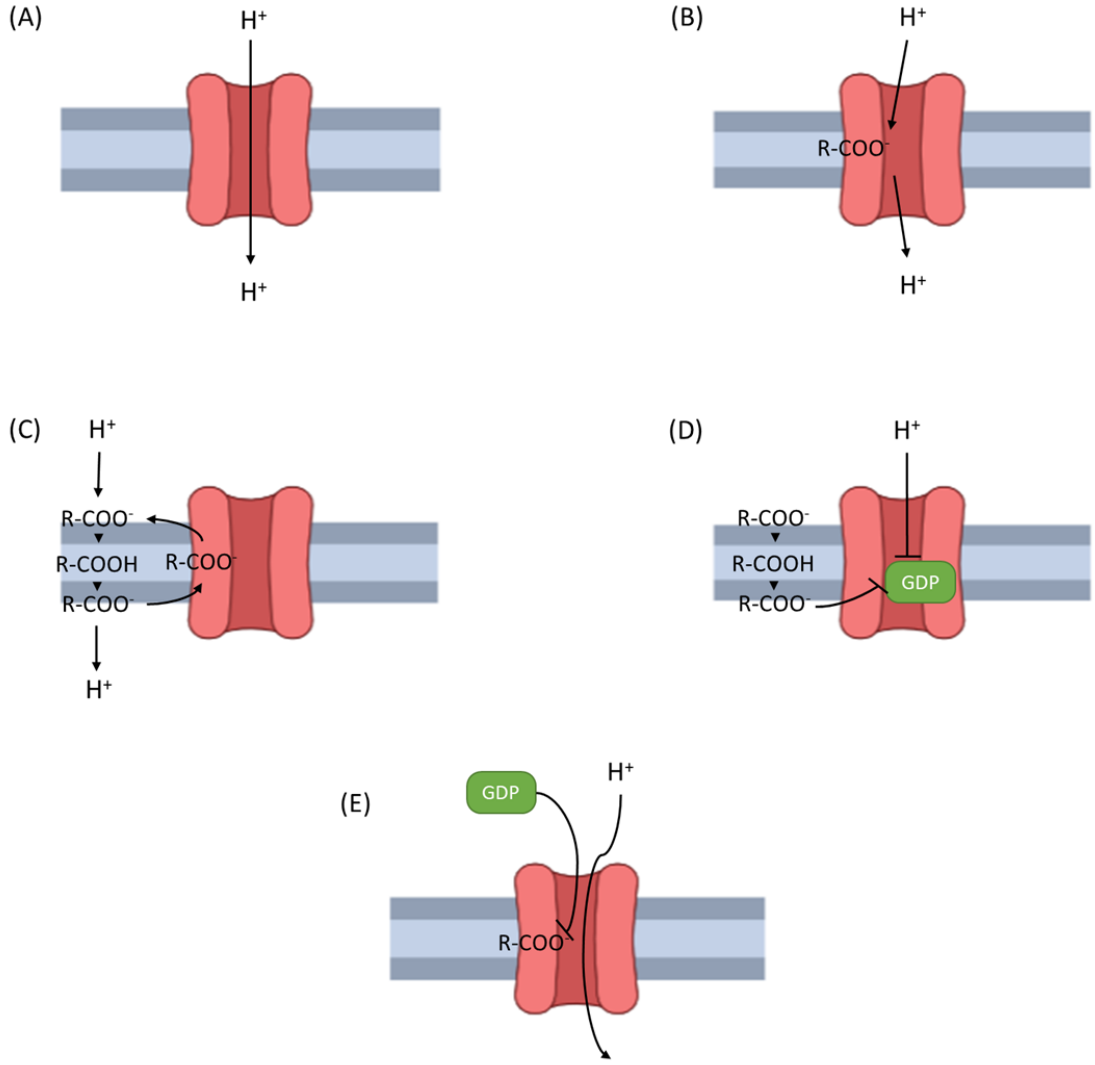


Figure 4. **Schematic diagrams of potential mechanisms of proton transport across the mitochondrial inner membrane (blue) into the matrix by the uncoupling proteins 1, 2, and 3 (red).** Protons may pass through in the absence **(A)** or presence **(B)** of the carboxy group of a fatty acid. **(C)** Fatty acids may be cycled through the membrane via UCP flippase activity. **(D)** Binding of a purine nucleotide may induce a conformational change which inhibits proton transport independent of which mechanism. **(E)** Presence of a FFA bound to UCP may facilitate proton transport by inhibiting GDP binding. Adapted from Ricquier and Bouillaud, (2000) and created with BioRender.com.

1.4.5 β-Blockers Cause Weight Gain by Blocking NE

β3-AR antagonists (β-blockers) block the effects of NE and are used to treat a variety of conditions including heart failure and hypertension (Bangalore et al., 2008). They also tend to induce weight gain in patients – in a year-long human study, the median weight of the group using β-blockers differed by +1.2kg compared to the control group and the majority of this was associated with an initial weight gain in the first few months of treatment followed by a long maintenance period (Sharma et al., 2001). β-blockers reduce energy expenditure through non-direct metabolic effects, causing increased tiredness and reduced anxiety. These effects decrease purposeless movement (fidgeting) which has been shown to significantly contribute to non-exercise-associated-thermogenesis (NEAT) (Levine et al., 1999). β-blockers can also reduce metabolic rate by 10%, or 200kcal/ day for an average person (Pischon and Sharma, 2008), which is

sufficient to account for the average 1.2kg of weight gain reported by Sharma et al. (2001). In contrast, the highly specific β3-AR agonist CL 316,243 increases UCP1 expression in BAT and protects against diet-induced obesity in both rats (Himms-Hagen et al., 1994) and mice (Yoshida et al., 1994), without affecting food intake, whilst chronic administration ameliorates diabetes and obesity (Gavrilova et al., 2000).

1.4.6 Beigeing of WAT

UCP1 expression can also be induced in white adipocytes by cold exposure (Rossato et al., 2014). Cold-induced β3-AR stimulation in WAT upregulates expression of the nuclear receptor protein and transcription factor PPARα, which induces PGC-1α and PRDM16 gene expression (Figure 5) (Hondares et al., 2011). PGC-1α is a transcriptional coactivator that regulates energy metabolism and PRDM16 is a transcriptional coregulator that controls the development of myoblasts to brown adipocytes (Kajimura et al., 2009), represses WAT gene expression, and is required to maintain brown fat identity and function during ageing (Harms, et al., 2014). White adipocytes which are induced to express markers characteristic of BAT phenotypically present as brown/white hybrids with increased numbers of mitochondria, hence they are referred to as beige (or brite) adipocytes. Like BAT, beige AT (BeAT) expends chemical energy in the form of heat, and increased levels of BAT and BeAT correlate with energy expenditure and reduced adiposity (Saito, et al., 2009). Conversely, ablation of PRDM16 in mice results in loss of BAT and BeAT, increased adiposity, and metabolic disease when fed a high-fat diet (Cohen, et al., 2014). Activating the thermogenic capacity of WAT could induce fat-specific weight loss – see section 1.5.4.

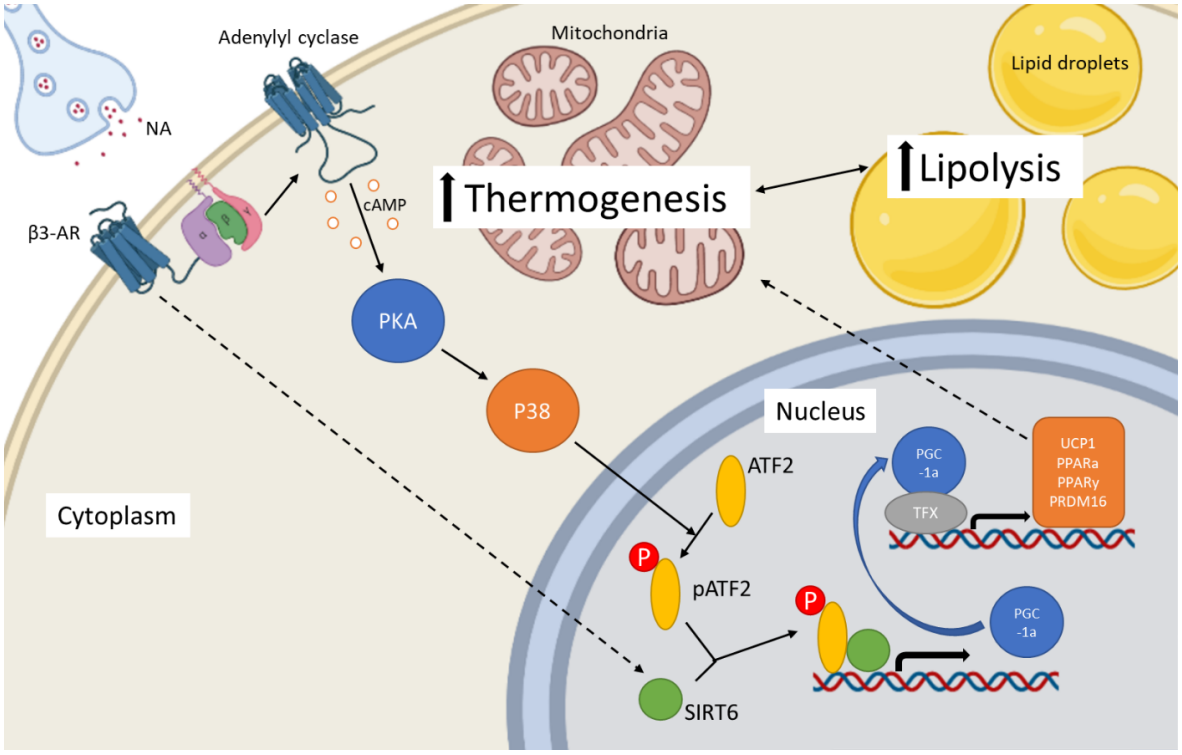


Figure 5. **Noradrenaline induces expression of thermogenic genes in adipocytes.** Norepinephrine activates β3-ARs which activate adenylyl cyclase. cAMP activates kinases which phosphorylate and activate the nuclear protein ATF2 which interacts with SIRT6 via a currently unclear mechanism to induce expression of PGC-1α. PGC-1α induces expression of several thermogenic genes including *UCP1* which results in increased mitochondrial thermogenesis and lipolysis. Adapted from Yao et al., (2017) and created with BioRender.com.

1.5 Factors Affecting the Potential of BAT to Induce Fat-Specific Weight Loss

1.5.1 Age

Quantification of whole-body BAT volume and activity is difficult because brown adipocytes are integrated among WAT and often reside in narrow fascial layers adjacent to other tissues including skeletal muscle, bone, and other organs (Leitner et al., 2017). BAT constitutes a small proportion of total AT in humans and has an inverse relationship with age. An average weight new-born (6.8lb) is estimated to contain approximately 35g (39ml) of BAT (Merklin, 1974), whereas an average young adult (11-43 years old with a normal BMI) contains approximately 50g (56ml) of BAT. Saito et al. (2009) reported that approximately half of their younger adult subjects (23-35 years old) had metabolically active BAT, whereas only 2 out of 24 elderly subjects had active BAT. These 2 male subjects were both relatively lean (BMI = 20.6 and 22.2) in comparison to the other 10 male subjects who reportedly had no active BAT (average BMI = 24.4). When this study was conducted, the consensus was that adults contained no (or very little) BAT, and methods for quantifying BAT volume and activity were relatively insensitive. However, another study reported that active BAT was present in almost all adults (van Marken Lichtenbelt et al., 2009), and a more recent study reported that average volume and activity of BAT is substantially higher than previously estimated (Leitner et al., 2017). Whilst BAT volume and activity does decrease with age, it is substantially higher than was previously thought.

1.5.2 Sex

BAT activity and mass are both reduced in males compared to females and both decrease more drastically in males from around the age of 50 onwards (Pfannenberg et al., 2010). On average, men and women can stimulate up to 24g and 42g of BAT respectively in the absence of cold exposure (Ouellet et al., 2011). An in vitro study on rodent brown adipocytes revealed that testosterone dose-dependently inhibits UCP1 mRNA expression, whilst progesterone stimulated NE-induced UCP1 mRNA expression (Rodriguez et al., 2002). However, adipocytes which were treated with progesterone had more and larger lipid droplets, suggesting an adipogenic role. Progesterone increased UCP1 mRNA expression at concentrations similar to those found in the serum of healthy females (10^{-9}M) but not at higher concentrations. Opposing actions of male and female sex hormones could explain the increased volume, activity, and longevity of BAT in females.

1.5.3 BMI

There is an inverse relationship between BAT volume and adiposity; average BAT volume in lean (BMI <25) male subjects (130 ± 98ml) was higher than in overweight/obese (BMI >25) male subjects (77 ± 69ml), though this difference was not statistically significant (van Marken Lichtenbelt et al., 2009). However, BAT samples taken from the supraclavicular region of all subjects revealed that BAT activity was significantly lower in the overweight/obese subjects. Interestingly, all but one subject in this study had evidence of BAT activity. This subject had the highest BMI (38.7) and percentage of body fat (41.8%) suggesting that BAT could protect against diet-induced obesity. Similarly, transgenic mice deficient in BAT develop obesity in the absence of hyperphagia, indicating increased metabolic efficiency (Lowell et al., 1997). As obesity

progressed, the transgenic mice developed hyperphagia and continued to gain weight, evidencing a role for BAT in energy homeostasis in mice.

A smaller capacity of active BAT may predispose an individual to weight gain and obesity, but it is not causative. It is likely that increased adiposity (possibly contributed to by intrinsically low BAT activity) contributes to reduced BAT activity and thus further weight gain. BMI-related active BAT volume loss is reversible; following gastric bypass-induced weight loss in obese subjects, average BAT volume increased from 14ml to 85ml (Vijgen et al., 2012).

Based on the (modest) estimate that 63g of fully activated BAT could induce a loss of approximately 4.1kg of AT per year (Virtanen et al., 2009), and that 1kg of adipose tissue contains 7,700kcal, the following assumptions can be made: 15.4g of fully activated BAT could induce 1kg of adipose-specific weight loss per year; 1g of fully activated BAT could expend an additional 501kcal per year. Based on the estimates that men and women can stimulate averages of 24g and 42g of BAT respectively in the absence of a stimulant (Oullet et al., 2011), men and women attribute 12,024kcal (1.56kg) and 21,042kcal (2.73kg) of their annual energy expenditure to active BAT respectively.

van Marken Lichtenbelt et al. (2009) estimated average BAT volumes of 130ml (117g) in lean men and 77ml (69g) in obese men. If these BAT deposits could be fully activated, then 58,617kcal (lean men) and 38,577kcal (obese men) could be expended annually, equivalent to 7.6kg and 5.0kg of AT respectively. According to the WHO, in 2015, the global average energy per day was 2940kcal, and 3380kcal per day for industrialised countries, amounting to annual intakes of 1,073,835kcal and 1,234,545kcal respectively. For an average lean and obese man consuming the global average annual calorie intake, 5.46% and 3.59% of calorie intake could be dissipated by fully stimulated BAT respectively; for an industrialised country, this would decrease to 4.75% and 3.12% respectively. This percentage will decrease as calorie intake increases, and it can be presumed that obese individuals will be consuming above average calorie intake. An average obese man consuming the industrialised country average calorie intake +10% (1,357,000 calories) could only dissipate 2.84% of this via fully stimulated BAT. Since BAT potential is reduced with increased adiposity and age, BAT alone may not be a highly effective target for inducing weight loss in obese individuals.

1.5.4 Beigeing of WAT to induce weight loss

Fortunately, WAT can be recruited to perform a similar function to BAT in a process called browning (Schulz et al., 2013). In humans, BAT depots are surrounded by WAT (Figure 6) which has the potential to become thermogenically active, and obese people have significantly more total AT in their BAT depots (Leitner et al., 2017). Leitner et al., (2017) estimated that lean individuals contained an approximate average total of 250ml of activated BAT and 900ml of combined adipose tissue, whereas obese individuals had an approximate average total of 125ml of activated BAT and over 1500ml of combined adipose tissue. This could mean that obese individuals have a greater potential capacity for recruitment of WAT, and recruitment of even a small proportion of this could result in the induction of significant weight loss. 250ml (225g) of activated adipose tissue could increase energy expenditure by 308kcal/day or 112,725kcal/year, resulting in 14.6kg of adipose-specific weight loss – although Leitner et al., (2017) estimated that this could be as high as >520kcal/day based on rat studies.

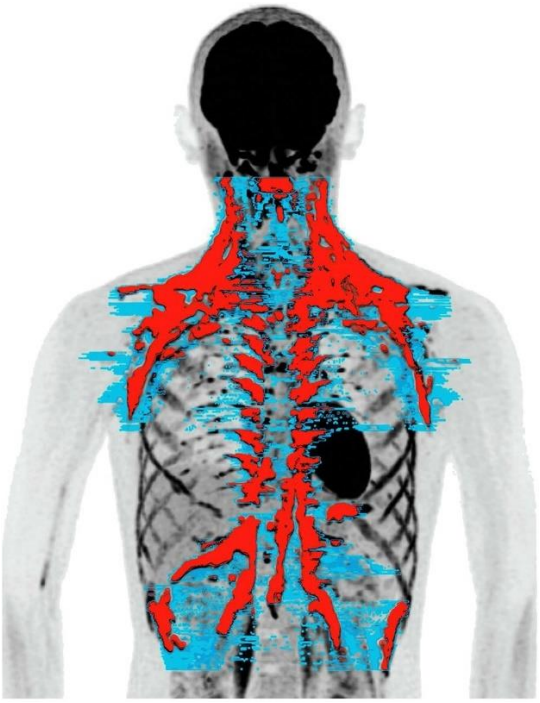


Figure 6. **Activated BAT and activatable WAT in adipose depots.** Red areas represent active BAT. Blue areas represent activatable WAT within BAT depots. Image from Leitner et al. (2017).

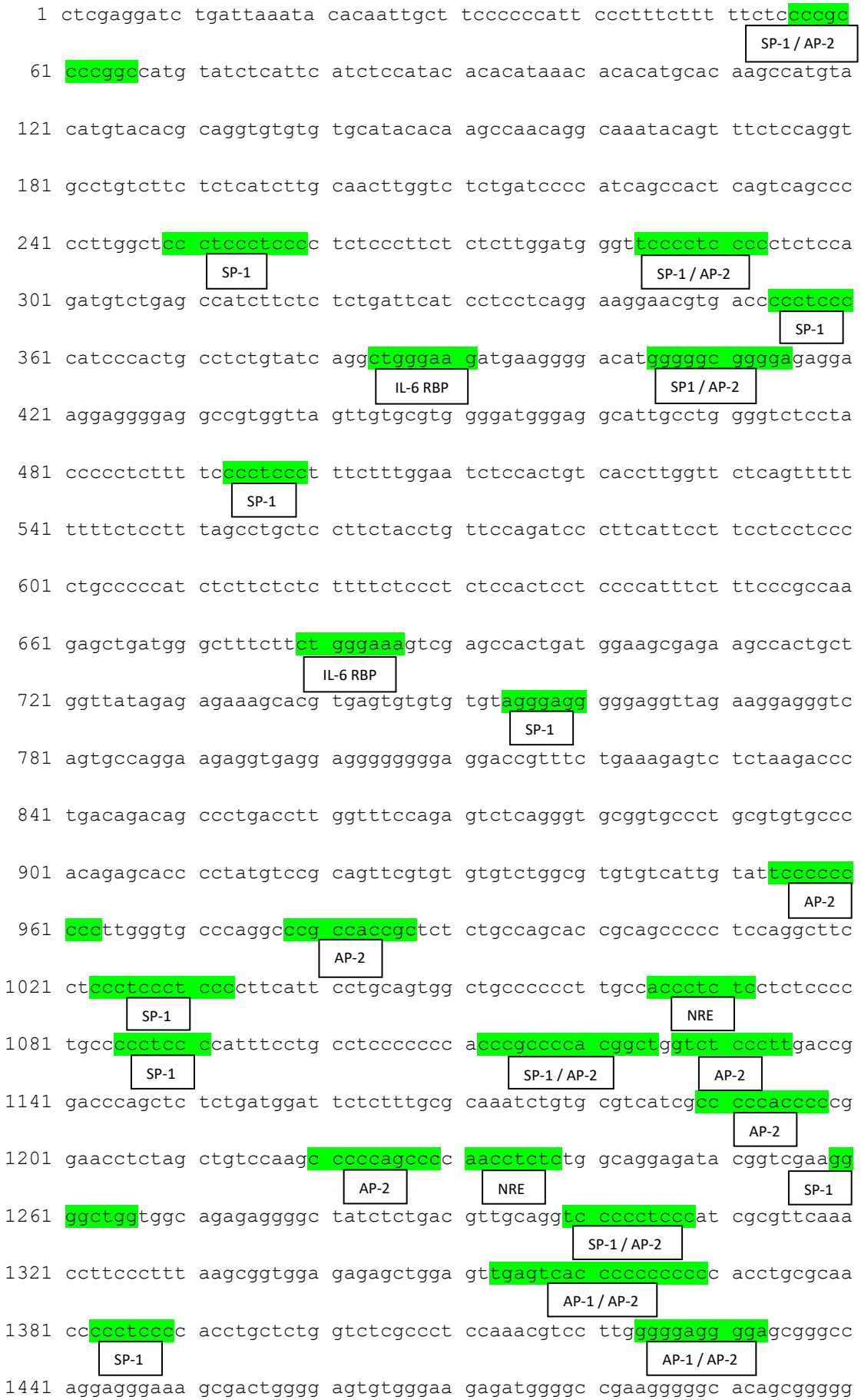
1.6 VGF – A Novel Regulator of Energy Homeostasis

The *vgf* gene encodes a 68kDa (615 amino acid) neurosecretory precursor protein called VGF which is expressed in a subset of neurons in the central and peripheral nervous systems, and specific endocrine cell populations in the gastrointestinal tract, pancreas, adrenal medulla, and adenohypophysis (Levi et al., 2004). It is induced by neurotrophins – a family of proteins identified as survival factors for sympathetic and sensory neurons, and subsequently implicated in the development, survival, and function of CNS and PNS neurons (Skaper, 2007). The precursor protein (pro-VGF) is stored in core vesicles and processed via cleavage by the prohormone convertases PC1/3 and PC2 which are typical of endocrine and neuronal tissue. The products of pro-VGF are released from the vesicles following depolarisation (Trani et al., 2002). VGF is similarly and selectively upregulated by several neurotrophins in neuronal targets, whilst other growth factors elicit only a marginal increase in expression (Hawley et al., 1992; Possenti et al., 1992; Salton, 1991; Salton et al., 1991).

1.6.1 Transcriptional Regulation of VGF

In humans, *vgf* is a single copy gene with its locus at chromosome 7q22 (Canu et al., 1997). The *vgf* gene and its promoter are highly conserved, with greater than 85% similarity between human and rat sequences (Trani et al., 2002). The promoter region, located upstream of the transcription initiation site, contains many consensus motifs for binding of transcriptional regulators (Figure 7A). These include several activating protein 1 and 2 (AP-1 and AP-2) binding sites, specificity protein 1 (SP-1) binding sites, an NF-κB binding site, a cAMP response element (CRE) motif, and a nerve growth factor inducible protein A (NGFI-A) binding site (Canu et al., 1997; Levi et al., 2004; Li et al., 1993).

The *vgf* promoter region contains two elements called V1 and V2 (Figure 7B), which are required for basal expression and induced gene expression by nerve growth factor (NGF), epidermal growth factor (EGF), and cAMP (Luc and Wagner, 1997). The V1 element is essential for promoter function but is not independently sufficient to confer NGF responsiveness, whereas the V2 element can independently stimulate expression of a linked gene in the presence of NGF (Luc and Wagner, 1997). The V2 element contains part of the CRE motif and interacts with CRE binding protein (CREB) and its associated proteins, as well the transcription factors JunB and JunD which are components of AP-1 (Karin et al., 1997). NGF increases JunB-related binding activities, suggesting that NGF may activate *vgf* transcription partly via increased synthesis of a V2 binding protein (Luc and Wagner, 1997).


1 ctcgaggatc tgattaaata cacaattgct tccccccatt ccctttcttt ttctcccgc
SP-1 / AP-2
61 cccggccatg tatctcattc atctccatac acacataaac acacatgcac aagccatgta
121 catgtacacg caggtgtgtg tgcatacaca agccaacagg caaatacagt ttctccaggt
181 gcctgtcttc tctcatcttg caacttggtc tctgatcccc atcagccact cagtcagccc
241 ccttggctcc ctccctcccc tctcccttct ctcttggatg ggttcccctc ccctctcca
SP-1
SP-1 / AP-2
301 gatgtctgag ccatcttctc tctgattcat cctcctcagg aaggaacgtg acccctccc
SP-1
361 catcccactg cctctgtatc aggctgggaa gatgaagggg acatgggggc ggggagagga
IL-6 RBP
SP1 / AP-2
421 aggaggggag gccgtggtta gttgtgcgtg gggatgggag gcattgcctg gggtctccta
481 ccccctcttt tcccctcccc ttctttggaa tctccactgt caccttggtt ctcagttttt
SP-1
541 ttttctcctt tagcctgctc cttctacctg ttccagatcc cttcattcct tcctcctccc
601 ctgccccat ctcttctctc ttttctccct ctccactcct ccccatttct ttcccgccaa
661 gagctgatgg gctttcttct gggaaagtcg agccactgat ggaagcgaga agccactgct
IL-6 RBP
721 ggttatagag agaaagcacg tgagtgtgtg tgtagggagg gggaggttag aaggagggtc
SP-1
781 agtgccagga agaggtgagg agggggggga ggaccgtttc tgaaagagtc tctaagaccc
841 tgacagacag ccctgacctt ggtttccaga gtctcagggt gcggtgccct gcgtgtgccc
901 acagagcacc cctatgtccg cagttcgtgt gtgtctggcg tgtgtcattg tattccccccc
AP-2
961 cccttgggtg cccaggccgg ccaccgctct ctgccagcac cgcagccccc tccaggcttc
AP-2
1021 ctccctccct cccttcattt cctgcagtgg ctgcccccct tgccaccctc tcctctcccc
SP-1
NRE
1081 tgccccctcc ccatttcctg cctcccccccc acccgcccca cggctggtct ccctttgaccg
SP-1
SP-1 / AP-2
AP-2
1141 gacccagctc tctgatggat tctctttgcg caaatctgtg cgtcatcgcc cccacccccg
AP-2
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AP-2
NRE
SP-1
1261 ggctggtggc agagaggggc tatctctgac gttgcaggtc cccctcccat cgcgttcaaa
SP-1 / AP-2
1321 ccttcccttt aagcggtgga gagagctgga gttgagtcac ccccccccc acctgcgcaa
AP-1 / AP-2
1381 cccctcccc acctgctctg gtctcgccct ccaaacgtcc ttgggggagg ggagcgggcc
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AP-1 / AP-2
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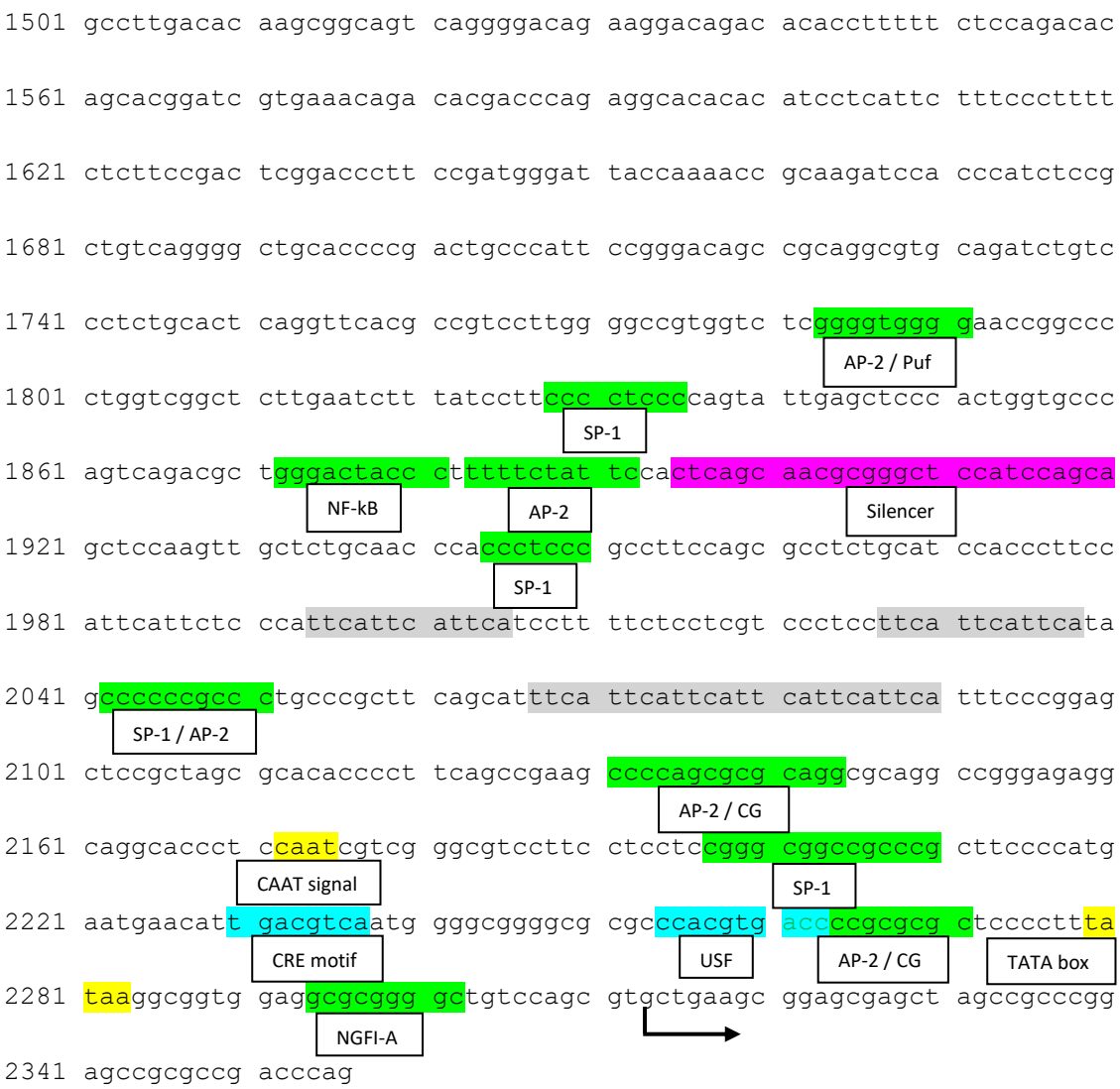

Figure 7A. **Nucleotide sequence of the 5' promoter region of the human *vgf* gene.** Motifs are highlighted and named underneath. Green: Regulatory element binding sites. Pink: Silencer region. Grey: TTCA repeats. Yellow: Transcription factor binding sites. Blue: Motifs encompassed in V2 element. Arrow: Transcription initiation site.

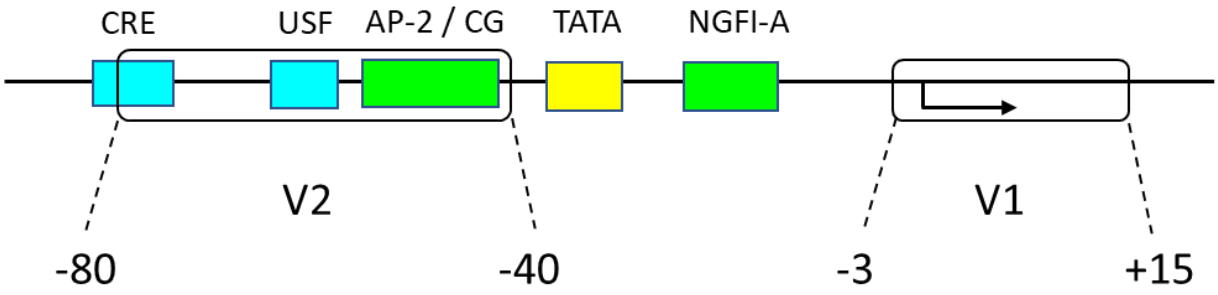

Figure 7B. **Schematic diagram of *vgf* V1 and V2 promoter elements.** V2 is located -80/-40 upstream of the +1 transcription initiation site (black arrow). V2 contains the upstream stimulatory factor, an activating protein 2 motif, and intersects the CRE locus. V1 is located -3/+15 and encompasses the transcription initiation site.

1.6.2 VGF Polypeptide Structure

The VGF polypeptide is a precursor for several biologically active peptides involved in intercellular communication (Bartolomucci et al., 2006; Jethwa et al., 2007; Thakker-Varia et al., 2014). Cleavage of the VGF peptide by PC1/3 and PC2 generates a wide array of neuroendocrine-specific biologically active peptides, including but not limited to those shown in Figure 8. NERP1 and NERP2 are neuroendocrine regulatory factors involved in water homeostasis (D'Amato et al., 2012); TPGH may have neuroprotective qualities (Cocco et al., 2010); TLQP-62 and AQEE-30 induce neurogenesis (Alder at al., 2003) and have anti-depressive effects (Thakker-Varia and Alder, 2009); HHPD41, AQEE-30, AQEE-11, and LQEQ-19 stimulate sympathetic outflow (Hahm et al., 1999; Hahm et al., 2002); NERP2 and TLQP-21 are involved in energy homeostasis (Bartolomucci et al., 2006; Jethwa et al., 2007; Toshinai et al., 2010).

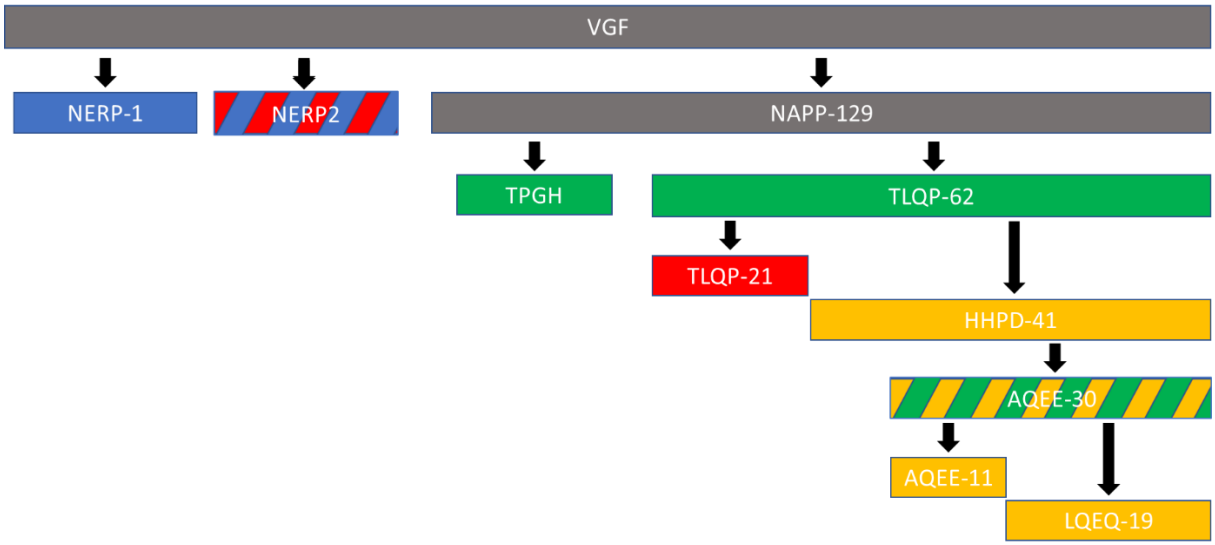

Figure 8. **The VGF peptide and its derivatives.** VGF is cleaved by prohormone convertases 1/3 and 2 generating a range of shorter biologically active peptides. Grey: Not biologically active. Blue: Water homeostasis. Red: Energy homeostasis. Green: Neural health, plasticity, and genesis. Orange: Stimulate sympathetic outflow. Adapted from Lewis et al. (2015).

The true number of VGF-derived peptides may be greater than currently recognised. The human VGF sequence contains at least 10 conserved basic amino acid regions which could be potential PC cleavage sites (Levi et al., 2003), and may also be cleaved by currently unidentified proteases. For example, HFHH-51 (not shown in Figure 8) is generated from TLQP-62 via endoproteolytic cleavage following two arginine residues – characteristic of PC enzymes (Levi et al., 2003). However, other N-terminal VGF fragments do not appear to be cleaved at known PC consensus motifs, so some products of VGF may be produced by currently unidentified endoproteases (Levi et al., 2003).

1.6.3 Function of VGF

VGF null mice (*vgf* -/-) are lean, hyperactive, and hypermetabolic, have reduced leptin levels and adipose stores compared to wild type littermates, and are protected against high fat diet-induced obesity (Hahm et al, 2002), suggesting that VGF is an anabolic regulator of energy homeostasis which reduces metabolic activity. VGF null mice also have improved glucose tolerance and are more sensitive to insulin (Watson et al., 2005). The hypermetabolic effects seen in VGF null mice can be attributed to altered gene

expression in BAT, correlating with increased fatty acid uptake and oxidation, and increased lipolysis, as well as browning of white adipocytes (Watson et al., 2009). Activation of BAT in VGF null mice could be due to increased sympathetic nervous system activity which causes increased mitochondrial number, cristae density, and UCP1 protein levels (Watson et al., 2009).

In contrast to this, Barrett et al., (2005), Jethwa et al., (2006), and Lewis et al, (2017) reported that VGF exerts a catabolic effect in rodents. VGF itself has no known receptor, and receptors for many of the VGF-derived peptides have yet to be discovered despite their biological activity being established. Opposing effects on energy homeostasis by VGF-derived peptides e.g. the anabolic orexin NERP2 (Tashinai et al., 2010) and the catabolic peptide TLQP-21 (Bartolomucci et al., 2006) could explain the apparently opposite effects of the same protein and suggests a 'confused' phenotype for the lean VGF null mouse.

1.6.4 Correlations between VGF, BAT Activity, and Obesity in Males and Females

VGF is predominantly expressed in neural and specific endocrine tissues but was recently reported to be expressed in human AT (Koc et al., 2020). Koc et al. (2020) quantified mRNA encoding VGF and the TLQP-21 receptor C3aR1 in visceral AT and revealed that *vgf* expression was reduced in the obese group (BMI >35) whilst *C3aR1* expression was elevated compared to the non-obese group (BMI <24.9). Gene expression was also evaluated in men and women separately. *C3aR1* expression was significantly higher in obese men and women than their non-obese counterparts and *C3aR1* expression was higher in obese men than obese women (Koc et al., 2020). This difference between obese men and women was mirrored in the non-obese group but at significantly lower levels. *vgf* expression was not significantly different between any of the groups due to the large variability in the data for the non-obese women group, which may have prevented accurate statistical analysis. On average, *vgf* expression was similarly low in obese men and women, elevated in non-obese men, and much higher in non-obese women (Koc et al., 2020).

vgf expression levels were only measured in visceral AT (VAT) and may not be representative of total AT, but this provides evidence of a negative correlation between *vgf* expression in AT and obesity and a positive correlation between *vgf* expression and BAT volume and activity. Lower *vgf* expression in obese individuals correlates with the reduced BAT activity and volumes described in section 1.5.3, and higher *vgf* expression in non-obese women correlates with the higher volume of active BAT in average women (almost double that of average men) – see section 1.5.2.

1.6.5 Correlations between VGF, BAT Activity, and Exercise

As discussed in section 1.4.4, exercise increases activity of BAT and BeAT via NA (Dinas et al., 2015). Exercise also increases VGF expression in mice (Alvarez-Saavedra et al., 2016; Hunsberger et al., 2007). In these studies, the roles of exercise and VGF were being investigated in relation to neurogenic and anti-depressive effects so VGF gene and protein expression was only measured in the brain. Currently, there are no published studies regarding the relationship between exercise and VGF expression levels in AT. However, exercise has been reported to increase expression/activity of several of the general *vgf* regulatory factors (see Figure 7A) in other tissues, including AP-1 in human skeletal muscle (Popov et al., 2019; Yang and Stacey, 1999), AP-2 in the hippocampus of rats (Tong et al., 2001), SP-1 in human skeletal muscle (Christiansen et al., 2017),

and CREB in rat cardiac muscle (Watson et al, 2007), the hippocampus of mice (Chen and Russo-Neustadt, 2009), and in human skeletal muscle (Widegren et al., 1998). Exercise may also increase expression of NGFI-A, which amplifies transcription of *vgf* (D'Arcangelo et al., 1996) via increased vasopressin expression and plasma levels (Brinton et al., 1998; Convertino et al., 1980; Wade, 1984). Thus, exercise could potentially increase thermogenic activity of BAT and BeAT via increased VGF expression in adipocytes.

1.6.6 Correlations between VGF, BAT activity, and Adiposity

Adiponectin is a fat-derived hormone synthesised and secreted by adipocytes which protects against insulin resistance and atherosclerosis (Schöndorf et al., 2005). Low levels of adiponectin are attributed to development of T2DM, CVD, and obesity (Achari and Jain, 2017). Adiponectin, which negatively correlates with adiposity, is an inhibitor of vasopressin (Hoyda et al., 2007; Iwama et al., 2009) and inhibits BAT activity (Qiao et al., 2014). A relationship between low body fat and low VGF expression could explain the reduced BAT activity seen in chronic exercisers and anorexic patients discussed in section 1.4.6. A relationship between high body fat and low vasopressin also exists (Gavalda-Manso et al., 2019). Gavalda-Manso et al., (2019) described an 'obesity paradox' in patients with chronic heart failure, where the obese patients had better survival rates – a phenomenon which was attributed to their lower levels of vasopressin. Figure 9 depicts a schematic representation of the correlations between adiposity, adiponectin, vasopressin, VGF, and thermogenically active AT. As adiposity increases, vasopressin levels decrease via a mechanism independent of adiponectin. Copeptin (the C-terminal fragment of arginine vasopressin pro-hormone) is elevated in obesity (Enhörning et al., 2012), suggesting that the vasopressin pro-hormone is highly expressed and has an inverse relationship with adiponectin but is processed differently in obese individuals.

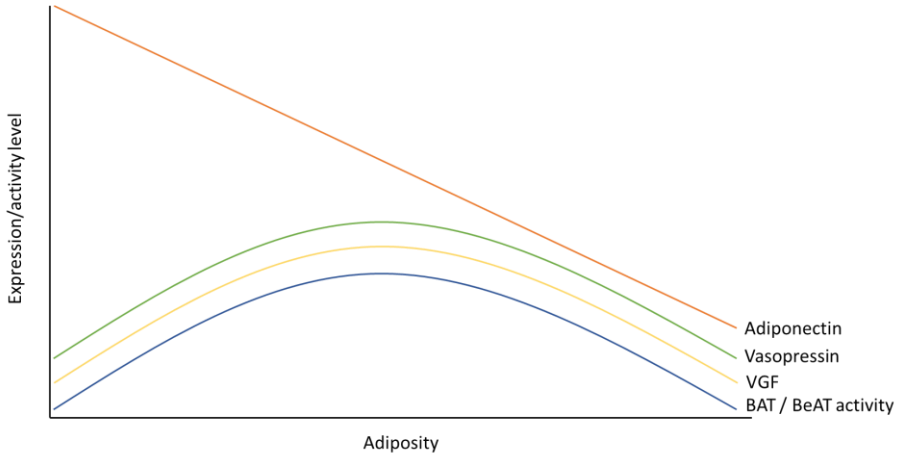

Figure 9. **Schematic diagram depicting correlations between BAT/BeAT activity and expression of adiposity-related factors.** Adiponectin (orange) expression has a negative correlation with adiposity. At low adiposity, vasopressin (green) has a negative correlation with both adiponectin and adiposity. VGF expression (yellow) and BAT/BeAT activity (blue) both correlate with vasopressin levels. Expression/activity levels depicted are not relative.

1.6.7 VGF Expression is Regulated by Vitamins and Thyroid Hormone

In neuroblastoma cells (SH-SY5Y cell line), VGF expression is regulated by vitamin D (1,25-dihydroxyvitamin D_3) and thyroid hormone T_3 (Lewis et al., 2016). Vitamin D upregulates VGF expression and promoter activity (Lewis et al., 2016) and is present at relatively low levels in obese individuals (Liel et al., 1998; Wortsman et al., 2000). In contrast, T_3 reduces VGF expression and promoter activity (Lewis et al., 2016) and is elevated in obese individuals (Reinehr, 2010). T_3 reduces VGF expression in neuroblastoma cells but activates BAT by sympathetic innervation (Yau et al., 2018). In conjunction with SIRT1, T_3 can also activate BAT by decreasing mTOR activity and increasing mitochondrial turnover which is essential for thermogenesis (Yau et al., 2018). However, SIRT1 expression is downregulated by TNF-α and is expressed at low levels in the AT of obese individuals (Pedersen et al., 2008; Serrano-Marco et al., 2012) so elevated T_3 in obesity is likely insufficient to activate BAT. Retinoic acid (RA) is the biologically active metabolite of vitamin A. RA suppresses obesity by inhibiting adipogenesis and increasing energy expenditure (Berry et al., 2012; Berry and Noy, 2009; Noy, 2013), and is another activator of VGF gene expression (Lewis et al., 2016). Vitamin A homeostasis is tightly regulated in humans and obesity leads to tissue, but not serum, deficiency (Trasino et al., 2015). Obese mice generated via high-fat diets or genetic mutations have reduced levels of retinol and impaired vitamin A signalling in multiple organs despite adequate vitamin A intake from diet and elevated serum vitamin A (Trasino et al., 2015). Low levels of SIRT1 expression and deficiencies of vitamins A and D correlate with low levels of BAT activity in obesity, whilst vitamin A and D deficiencies also reduce VGF expression.

1.6.8 Correlations between C3aR1, Adiposity, and Inflammation

In contrast to VGF, increased C3aR1 expression positively correlates with obesity (Koc et al., 2020). TLQP-21 opposes obesity via C3aR1-mediated enhancement of adrenergic-induced lipolysis (Cero et al., 2017) so elevated C3aR1 expression could be expected to negatively correlate with obesity. However, VGF expression was reduced in obese patients and it can be assumed that TLQP-21 levels were also low, so increased C3aR1 expression could be compensatory. However, it is more likely that C3aR1 expression is increased as part of obesity-associated low-grade inflammation (Figure 10). In correlation with obesity, several proinflammatory factors are upregulated including C reactive protein (CRP), Complement 3a (C3a), estradiol, tumour necrosis factor-α (TNF-α), IL-1β, IL-6, toll-like receptors (TLRs),and NFκB (Austin et al., 1991; Bashir et al., 2020; Calabro et al., 2005; Cohen, 1999; Dasu et al., 2010; Fried et al., 1998; Rippee-Brooks et al., 2020; Sundstrom et al., 1989; Vitseva at al., 2012; Winkler et al., 2003). Despite the presence of a NFκB binding site upstream of *vgf* (figure 7A), upregulation of NFκB correlates negatively with VGF expression.

Whether inflammation is causative of increased C3aR1 expression or vice versa is unclear, but increased expression of C3aR1 has been implicated in several inflammatory diseases including optic nerve degeneration (Harder et al., 2020), vascular inflammation and Alzheimer disease (Propson et al., 2021), Lafora disease (López-González et al., 2017), and diabetic nephropathy (Li et al., 2014). Moreno-Viedma et al., (2016) identified 22 dysregulated pathways common to obese adipose tissue and atherosclerotic plaques in insulin resistant and atherosclerotic mice and identified *C3aR1* as the one of highest ranked genes for the inflammatory response pathway. Estradiol is also elevated in obese subjects (Austin et al., 1991; Cohen, 1999), and induces expression of the C3aR1 ligand C3a (Sundstrom et al., 1989). C3a is expressed in the CNS and on neutrophils and monocytes (Boos et al., 2005; Martin et al., 1997). C3a is one of many

peptides involved in the complement immune system and is a mediator of inflammation, fibrosis, and phagocyte recruitment (Frank and Fries, 1991), and activates NFκB via ROS production (Elsner et al., 1994). Administration of a C3aR antagonist ameliorated inflammatory and fibrotic signals in obese diabetic rats (Li et al., 2014) and protected against injury in diabetic nephropathy by inhibiting ROS production (Morigi et al., 2020). Elevated expression of inflammatory factors, including CRP, C3a, and C3aR1, may be maintained in obesity-related chronic low-grade inflammation by several positive feedback loops (Figure 10).

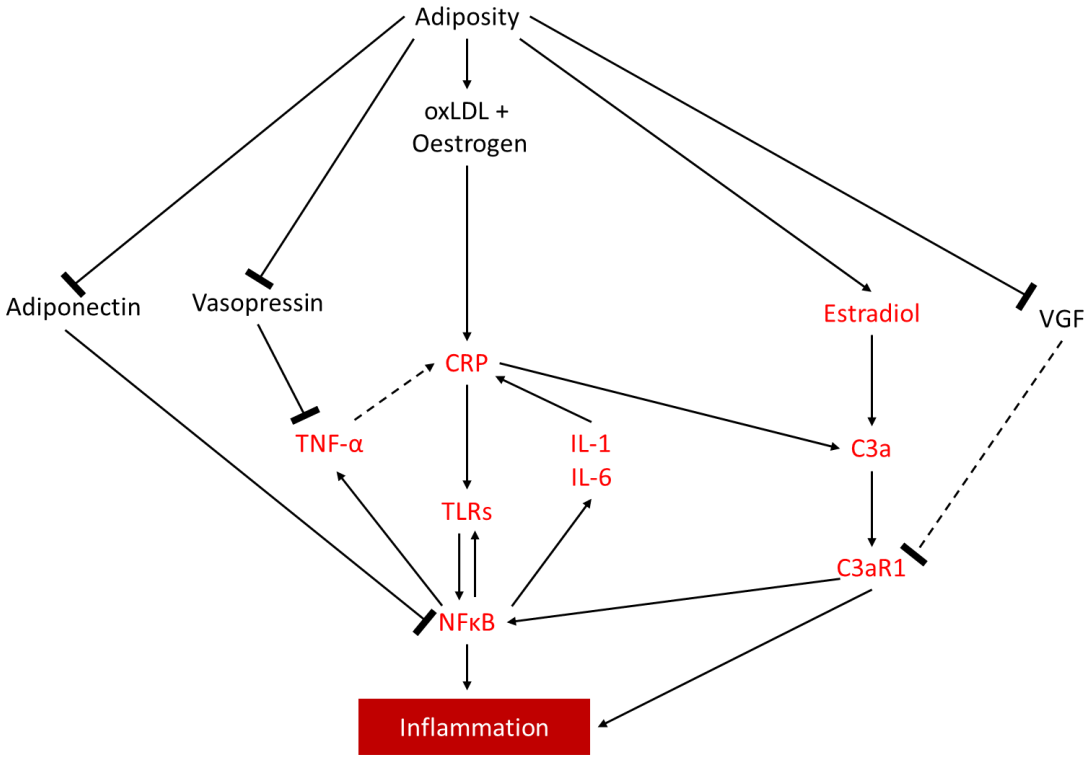

Figure 10. **Chronic low-level inflammation is sustained in obese individuals via several positive feedback loops.** Proinflammatory factors are indicated in red and dashed lines represent tentative relationships. Increased adiposity results in elevated plasma oxLDL and oestrogen which increases production of CRP. CRP activates TLRs and upregulates expression of several inflammatory cytokines and TLRs via NFκB. Simultaneously, elevated estradiol increases expression of C3a which activates its receptor C3aR1 and subsequently activates NFκB via increased ROS production. Adiposity negatively correlates with adiponectin and vasopressin so inhibition of TNF-α and NFκB is alleviated in obesity. VGF expression correlates negatively with C3aR1 expression but is an unlikely direct inhibitor of C3aR1. Rather, VGF may reduce C3aR1 expression via anti-obesogenic effects.

1.6.9 Correlations between C3aR1, Adiposity, and BAT Activity

TLQP-21 exerts its lipolytic effects by enhancing adrenergic-induced lipolysis (Cero et al., 2017). Obesogenic effects of reduced NA transporter availability and reduced NA signalling in obese individuals (Chiang-shan et al., 2014) may be enhanced by low levels of TLQP-21. If reduced expression of VGF is (at least somewhat) causative of reduced BAT volume and activity in obese patients, and BAT volume and activity can be reversibly increased following weight loss, perhaps weight loss causes increased VGF expression in AT. Since variants resulting in increased C3aR1 expression have been recently linked to increased risk of coronary artery disease (Xu et al., 2014), reduction via weight loss could be beneficial. Exercise may decrease expression of C3aR1 (Xia et al., 2020) independently of weight loss, which could explain the anti-diabetic, anti-

obesity, and cardiovascular-protective effects of exercise in obese patients even in the absence of weight loss (Church, 2011; Lee et al., 2005; O'Gorman and Krook, 2011; Voulgari et al., 2018).

1.7 Summary and Aims

We aim to determine the effects of VGF overexpression, and TLQP-62 and AQEE-30 on lipid content in 3T3-L1 adipocytes. The 3T3-L1 cell line is derived from mouse 3T3-L1 cells and can differentiate into adipocyte-like cells under specific conditions (Zebisch et al., 2012). This makes 3T3-L1 cells an appropriate cell model for researching the effects of VGF and its derived peptides in adipocytes and adipose tissue.

1.7.1 VGF Overexpression in 3T3-L1 Adipocytes

VGF exerts different and often opposing effects depending on the target tissue. Despite VGF null mice having a lean phenotype (Hahm et al., 1999), knockdown of VGF in the hypothalamus of mice disrupted metabolism, reduced UCP1 levels in BAT, and resulted in weight gain despite food intake being unaffected (Foglesong et al., 2016). In contrast, overexpression of VGF in the hypothalamus of hamsters resulted in increased energy expenditure and reduced body weight despite hyperphagia (Lewis et al., 2017). VGF null mice are resistant to diet-induced obesity (Hahm et al., 1999) and have high levels of BAT activity and beigeing of WAT (Watson et al., 2009). Despite this, VGF expression is significantly higher in the BAT of lean mice than obese (D'Amato et al., 2015) and VGF expression in WAT correlates negatively with obesity in humans (Koc et al., 2020).

We hypothesise that overexpression of VGF will reduce lipid content in adipocytes. Here, the effects of overexpression of VGF in white adipocytes (3T3-L1 cells) via transfection with a *vgf* construct will be investigated with regards to cellular lipid content.

1.7.2 Treatment of 3T3-L1 Adipocytes with TLQP-62 and AQEE-30

In lean patients, plasma levels of TLQP peptides significantly increase following glucose consumption of glucose, but not in overweight, obese, or T2DM patients (D'Amato et al., 2015). TLQP-62 and TLQP-21 modulate basal and glucose-stimulated insulin secretion by pancreatic cells (Petrocchi-Passeri et al., 2015; Stephens et al., 2012) so reduced expression in overweight and obese patients may contribute to the development of T2DM. In lean mice, TLQP peptide levels significantly increase in both plasma and WAT following glucose consumption, but not in obese mice (D'Amato et al., 2015). TLQP peptide levels in BAT of mice were higher than those in plasma and WAT, but lean mice had significantly higher levels of TLQP peptides in their BAT than obese mice (D'Amato et al., 2015). TLQP peptide levels in BAT did not change following glucose consumption (D'Amato et al., 2015). This suggests that expression levels of TLQP peptides correlate positively with BAT activity and negatively with obesity. TLQP-21 protects against diet induced obesity in mice (Bartolomucci et al., 2009) and is generated alongside AQEE-30 following cleavage of TLQP-62.

We hypothesise that treating 3T3-L1 adipocytes with TLQP-62 and AQEE-30 will induce lipolysis. TLQP-21 enhances adrenergic-induced lipolysis, so 3T3-L1 adipocytes will be treated with the VGF-derived peptides TLQP-62 and AQEE-30 in the absence and presence of the β-agonist isoproterenol, and cellular lipid content will be quantified.

2 Materials and Methods

2.1 Equipment and Reagents

DMSO from Fisher Scientific; Fluorescent Microscope: EVOS Invitrogen by Thermo Fisher Science; Fluorescent plate reader: FLUOstar Omega by BMG Labtech; LB powders (tryptone, yeast extract, NaCl, and agar) from Oxoid; Lipofectamine 2000 from Invitrogen by Life Technologies; NanoDrop from Thermo Fisher Science; PureYield MiniPrep Kit from Promega; VGF and GFP constructs from VectorBuilder; VGF peptides from Pepceuticals Limited; 96 well plates: sterile, black-sided, clear bottom assay plates from Corning Incorporated.

All other solutions and reagents were obtained from Sigma-Aldrich unless otherwise stated.

All cell culture work was carried out in a Class II Microbiology Safety Cabinet.

2.2 Cell Culture Techniques

2.2.1 Buffers and Solutions

Dimethyl sulfoxide (DMSO) from Fisher Scientific.

Hanks' Balanced Salt Solution (HBSS) (1X) from Gibco by Life Technologies.

3-Isobutyl-1-methylxanthine (IBMX).

Phosphate Buffer Saline (PBS) - PBS tablets were diluted in 400ml sterile water and autoclaved in a glass bottle before use.

Trypsin-ethylenediaminetetraacetic acid (EDTA) – diluted in PBS.

2.2.2 Growth Medias

Pre-differentiation Growth Media

Pre-differentiation growth media was prepared by supplementing Dulbecco's Modified Eagle Medium – High Glucose (DMEM-HG) containing 4500mg/L glucose with 10% sterile filtered foetal bovine serum (FBS), 1% penicillin streptomycin, and 1% sodium pyruvate.

Differentiation Growth Media

Differentiation growth media was prepared by supplementing pre-differentiation growth media with dexamethasone [1μM], insulin [1.7nM], IBMX [0.5mM], and rosiglitazone [1μM]. Stocks of differentiation factors were diluted in the following solvents: dexamethasone in ethanol, insulin in PBS, and both IBMX and rosiglitazone in DMSO.

Post-differentiation Growth Media

Post-differentiation growth media was prepared by supplementing pre-differentiation growth media with insulin [1.7nM] and rosiglitazone [1μM].

2.2.3 3T3-L1 Culturing and Differentiation

3T3-L1 Cell Growth and Treatment

3T3-L1 cells were grown in pre-differentiation growth media at 5% CO_2 and 37°C in T75 cell culture flasks. Cells were passaged every 2-3 days as described below.

Harvesting and Passaging of Cells

When cells reached approximately 70% confluency (typically 2-4 days) they were harvested and passaged as follows. All solutions were pre-warmed to 37°C. Media was removed from the flask and the cells were washed with 10ml PBS. 2ml of 0.25% trypsin-EDTA was added and the flask was incubated at 37°C for 3-4 minutes until all cells appeared separate and detached from the flask under a light microscope. Trypsin-EDTA was neutralised by adding 8ml of pre-differentiation growth media and triturating with a pipette. The cells were then transferred to a 30ml tube and centrifuged at 1000rpm at 21°C for 5 minutes. Supernatant was removed and discarded, and the pellet resuspended in 10ml of fresh pre-differentiation growth media. Cells were split appropriately to prevent >70% confluence being achieved. Generally, a 1:10 split (1ml of the cell media was added to a new T75 flask and total volume of media was made up to 13ml) was used.

3T3-L1 Differentiation

3T3-L1 cells were differentiated according to the protocol established by Zebisch et al., 2012, but with the concentrations stated in section 2.2.2. A 96-well plate was seeded with 10,000 cells per well in 200µl pre-differentiation media and incubated at 5% CO_2 and 37°C. 100% confluency was typically reached after 2 days and the media was replaced with fresh pre-differentiation growth media. 2 days post confluency (day 0), pre-differentiation media was replaced with differentiation growth media. 2-3 days following day 0, differentiation media was replaced with post-differentiation growth media and media was refreshed every 2-3 days. 3T3-L1 cells were fully differentiated by days 7-9 (Figure 11).

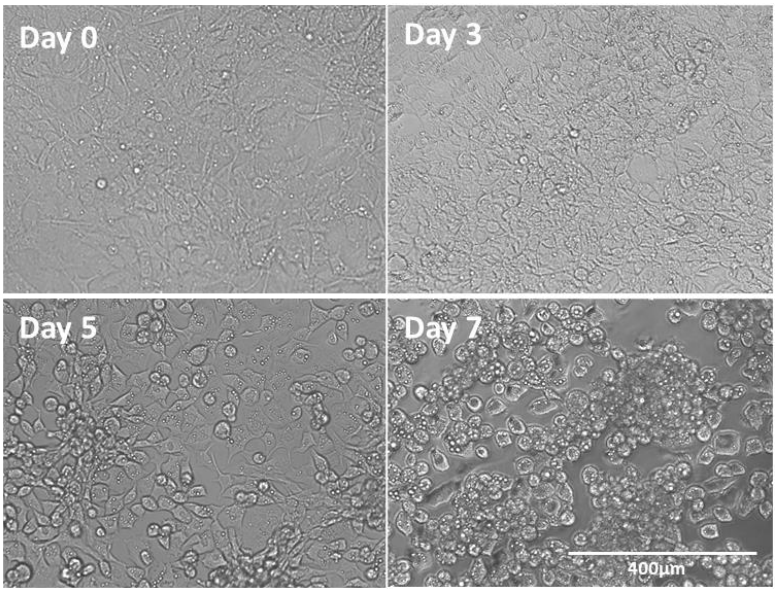

Figure 11. **Differentiating 3T3-L1 cells photographed under phase-contrast light microscope (EVOS Invitrogen by Thermo Fisher Science) at 10x magnification**. Differentiation media was added to cells 2 days post-confluence (day 0), and then switched for post-differentiation media on day 3. Media was refreshed every 2 days until day 7 when cells were fully differentiated. Lipid droplets within cells appear bright on day 7.

2.2.4 Nile Red and DNA Assays

Nile Red Staining and Assay

Nile Red is a lipophilic stain which can be used for imaging and quantification of lipid droplets in 3T3-L1 adipocytes (Song et al., 2015). 1mM Nile Red stock solution was prepared by dissolving 15.8mg Nile Red and 500mg Pluronic F-127 in 50ml DMSO. Aliquots were stored at -20°C. Nile Red 1mM stock solution was diluted in HBSS to 30µM immediately before use and protected from light using foil. Media was removed from the wells and cells were carefully washed twice with HBSS. 100µl of 30µM Nile Red was added per well, then the plate was incubated at 5% CO_2 and 37°C in the dark (wrapped in foil) for 15 minutes. Nile Red solution was then removed from the wells and cells were washed once with HBSS then covered with HBSS. Cells were viewed and photographed using a fluorescent microscope (Figure 12), and fluorescence was measured using a fluorescence plate reader (excitation 485nm, emission 590nm, orbital scanning diameter of 3mm).

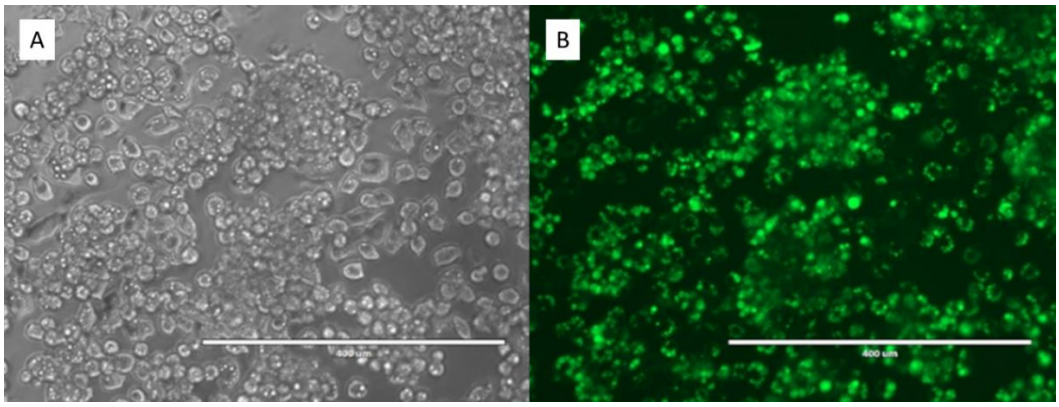

Figure 12. **Nile Red staining of 3T3-L1 cells photographed under EVOS Invitrogen fluorescent microscope**. Cells were photographed under light (A) and GFP fluorescent settings (B). Scale bar (400µm) is shown in both images.

DNA Assay

DNA was assayed using bisbenzimide according to the protocol established by Papaioannou and Ebert, 1988, with modified concentrations. Following the Nile Red assay, HBSS was carefully removed from the wells. Cells were then washed with PBS, covered in 100µl sterile water, and frozen and thawed twice. A standard curve was prepared with serial dilutions of calf thymus DNA in sterile water (40, 20, 10, 5, 2.5, 1.25, 0.6, and 0.3µg/ml). Working dye solution was prepared by diluting stock DNA dye solution (1mg/ml bisbenzimide) in 2x TNE (20mM Tris [pH 7.4], 2mM EDTA, 200mM NaCl) to a final concentration of 2µg/ml. Triplicates of each standard (100µl) were loaded into empty wells with 100µl of working dye solution per well and tapped gently to mix. Prior to conducting a DNA assay on the samples, a standard curve was generated using the fluorescent plate reader (excitation 355nm, emission 460nm) to check that a linear standard curve was generated. 100µl/well of working dye was loaded into samples, gently mixed, and run alongside standards.

2.2.5 VGF Overexpression in 3T3-L1 Cells

VGF-overexpression plasmid harvesting

Two DNA constructs were purchased from VectorBuilder and arrived in the *E. coli* K-12 strain VB Ultrastable. Both constructs contained eGFP (GFP) and were identical except for the absence/presence of the VGF gene in one construct (Figure 13). LB broth (10% tryptone, 5% yeast extract, 5% NaCl, 50µl/ml ampicillin) and LB agar (10% tryptone, 5% yeast extract, 5% NaCl, 10% agar, 50µg/ml ampicillin) were prepared and autoclaved, and 20ml agar plates were poured in a Class II Microbiology Safety Cabinet. The following steps of colony harvesting and DNA isolation were carried out by Kirsty Jewell. VB Ultrastable containing GFP and VGF-GFP were streaked out on LB agar plates and incubated overnight. 3 colonies were harvested from each plate and incubated at 37^{o}C on a rocker in 1ml LB agar broth overnight. Plasmid DNA was isolated according to the Promega PureYield Miniprep kit protocol and eluted in 30µl of water per colony. The DNA concentration of each sample was determined using a Nanodrop spectrophotometer and the DNA of one colony containing VGF was sequenced by Source BioScience International Plc using M13F and M13R primers (Appendix A). BLAST of the DNA sequence (Appendix B) on UniProt revealed 100% similarity to the C-terminal region of *Mus musculus* VGF (Appendix C). Isolated DNA samples were stored at -18^{o}C until later use.

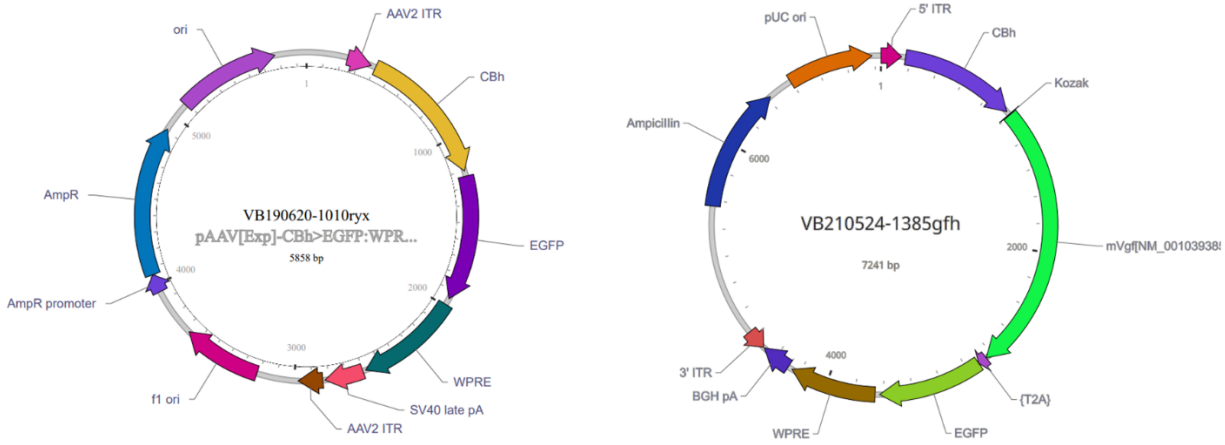

Figure 13. **Vector maps of the GFP (left) and VGF (right) constructs.** The VGF construct was identical to the GFP construct except for the insertion of the mouse Vgf (mVgf) gene and a T2A cleavage site between mVgf and EGFP.

Transfection of undifferentiated 3T3-L1 cells with a VGF-overexpression construct

A 96 well plate was seeded with 10,000 3T3-L1 cells per well. Once confluent (day -2), cells were transfected with either GFP or VGF-GFP constructs according to the Invitrogen Lipofectamine 2000 protocol. Briefly, the transfection media was made by diluting DNA and Lipofectamine 2000 in DMEM-HG containing 1% sodium pyruvate, the DNA and Lipofectamine 2000 medias were mixed in a 1:1 ratio, and incubated at room temperature for 5 minutes. The cells were washed with PBS before 100µl of transfection media was added to each well, containing final quantities of 100ng DNA and 0.4µl Lipofectamine 2000 per well. After 4 hours, the transfection media was removed and 200µl of antibiotic-free pre-differentiation growth media was added to each well. After 2 days (day 0), cells were differentiated according to section 2.2.3. On day 9, a Nile Red assay was performed as described in section 2.2.4.

Transfection of partially differentiated 3T3-L1 cells with a VGF-overexpression plasmid

3T3-L1 cells were seeded into a 96 well plate at a density of 10,000 cells per well and differentiated according to the protocol in section 2.2.3. On day 5 of differentiation, cells were transfected with constructs containing either GFP or VGF-GFP as described above. However, 200µl of transfection media was added per well, and 20µl of FBS was added to each well after 4 hours instead of removing the transfection media. One day after transfection, transfection media was removed, and post-differentiation media was added. A Nile Red assay was performed as described in section 2.2.4 on day 7 (2 days after transfection) and again on day 10 (5 days after transfection).

2.2.6 Treatment of 3T3-L1 Adipocytes with VGF-Derived Peptides

The effects of two VGF-derived peptides (TLQP-62 and AQEE-30) on 3T3-L1 adipocytes were investigated in the absence and presence of the beta-agonist isoproterenol. 3T3-L1 cells were seeded into a 96 well plate at 10,000 cells per well and differentiated as described in section 2.2.3. On day 8, cells were serum starved for 3 hours by replacing

the current media with 200µl of FBS-free DMEM-HG (containing 1% penicillin streptomycin and 1% sodium pyruvate) per well. Serum starving prevents the effects of treatments applied from being inhibited by interacting with FBS and eliminates variability in cell growth by synchronising cell cycles. Serum-free media was removed and replaced with 100µl of serum-free DMEM-HG containing either TLQP-62 or AQEE-30 at concentrations of 5µM and 10µM. Isoproterenol hydrochloride was dissolved in serum-free DMEM-HG to a concentration of 440nM and 10µl was added per well resulting in a final concentration of 40nM, whilst 10µl of serum-free DMEM-HG was added to wells not being treated with isoproterenol. Two positive controls of known lipolysis-inducers were set up, containing final concentrations of 40nM isoproterenol hydrochloride and 10mM dibutyryl cAMP (dbcAMP), to confirm that the cells were functioning as expected and a suitable model for this experiment. After 3 hours of treatment, cells were analysed by Nile Red staining and a DNA assay was performed as described below.

2.2.7 Analysis and Presentation of Results

Results data were analysed with one- and two-way ANOVA as appropriate using GraphPad Prism 9, and significant results were analysed post hoc with Bonferroni correction with a significance level of 0.05 using Genstat 19th edition. All results are presented with standard error.

3 Results

For all data presented here, 3 biological replicates were performed and analysed. Experiments were carried out once only.

The mouse-derived 3T3-L1 cell line was selected as a suitable cell model for investigating the effects of rodent VGF and VGF-derived peptides. It is currently unknown whether 3T3-L1 cells express and process VGF. 3T3-L1 cells do express the TLQP-21 receptor C3aR1 (Hannedouche et al., 2013), and express and secrete NGF (Peeraully et al., 2004) which induces an increase in VGF mRNA in the neuroendocrine cell line PC12 (Levi et al., 2004). It is possible that 3T3-L1 cells express and process VGF given that the VGF gene is conserved, and VGF and VGF-derived peptides are expressed in human and mouse AT (Benchoula et al., 2021; D'Amato et al., 2015).

3.1 VGF Overexpression

Undifferentiated (day -2) and partially differentiated (day 5) 3T3-L1 cells were transfected with GFP and VGF-GFP (VGF) constructs using Lipofectamine 2000.

3.1.1 Transfection of Pre-Adipocytes

Transfection of undifferentiated cells resulted in significantly lower Nile Red staining than non-transfected cells (Figure 14) and lipid droplets were not visible (Figure 15).

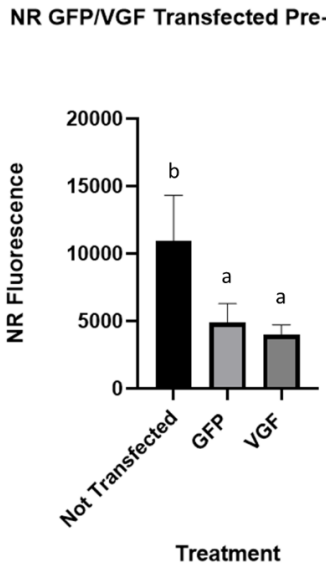

Figure 14. **Nile Red assay results of 3T3-L1 adipocytes transfected before differentiation.** 3T3-L1 pre-adipocytes were transfected with constructs containing GFP and VGF-GFP (VGF) on day -2 of differentiation. Nile Red assay was performed on day 7 of differentiation. Both transfected groups had significantly lower Nile Red staining than the non-transfected group according to one-way ANOVA ($p < 0.0001$).

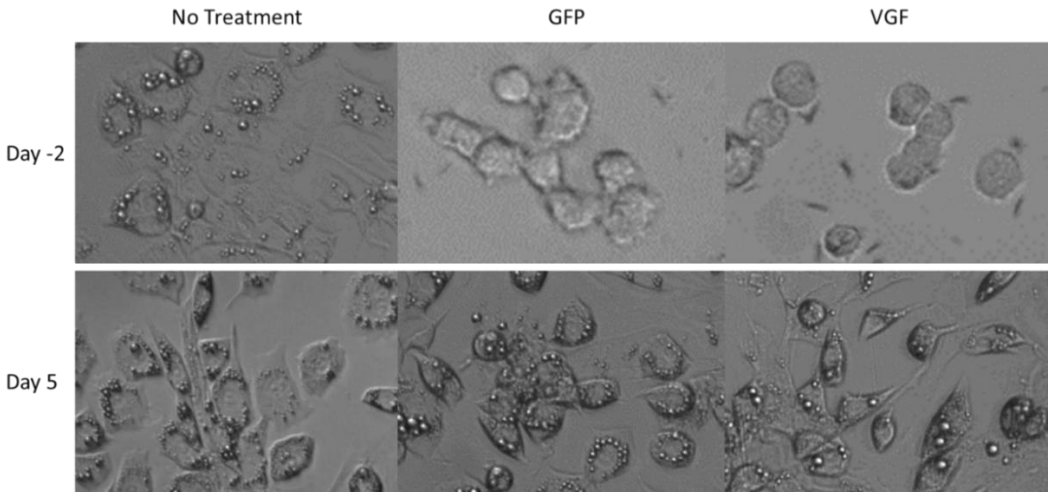

Figure 15. **Transfection of 3T3-L1 pre-adipocytes inhibited the differentiation process.** Cells were photographed on day 7 of differentiation. The day of transfection is indicated on the left. Transfection of pre-adipocytes on day -2 with GFP and VGF constructs resulted in cell death and adipocytes lacking lipid droplets. Transfection of partially differentiated adipocytes (day 5) did not appear to inhibit differentiation and lipid droplets were visible in both transfected groups.

Based on the visibility of GFP 2 days post-transfection, transfection of 3T3-L1 pre-adipocytes with the GFP construct appeared to be successful whereas transfection with the VGF construct appeared unsuccessful (Figure 16).

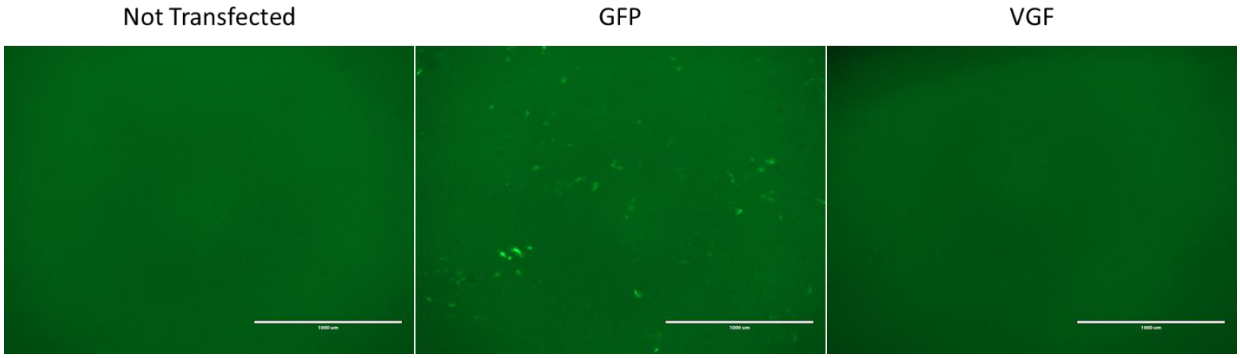

Figure 16. **GFP visualised 2-days post-transfection of 3T3-L1 pre-adipocytes.** GFP was only visible in the GFP transfected cells. Transfection with the VGF-GFP construct appeared unsuccessful.

3.1.2 Transfection of Partially Differentiated Adipocytes

Transfection of 3T3-L1 cells on day 5 of differentiation did not appear to affect development and lipid droplets were clearly visible (Figure 15). GFP expression was visible at a low level in 3T3-L1 adipocytes 2 days post-transfection (Figure 17).

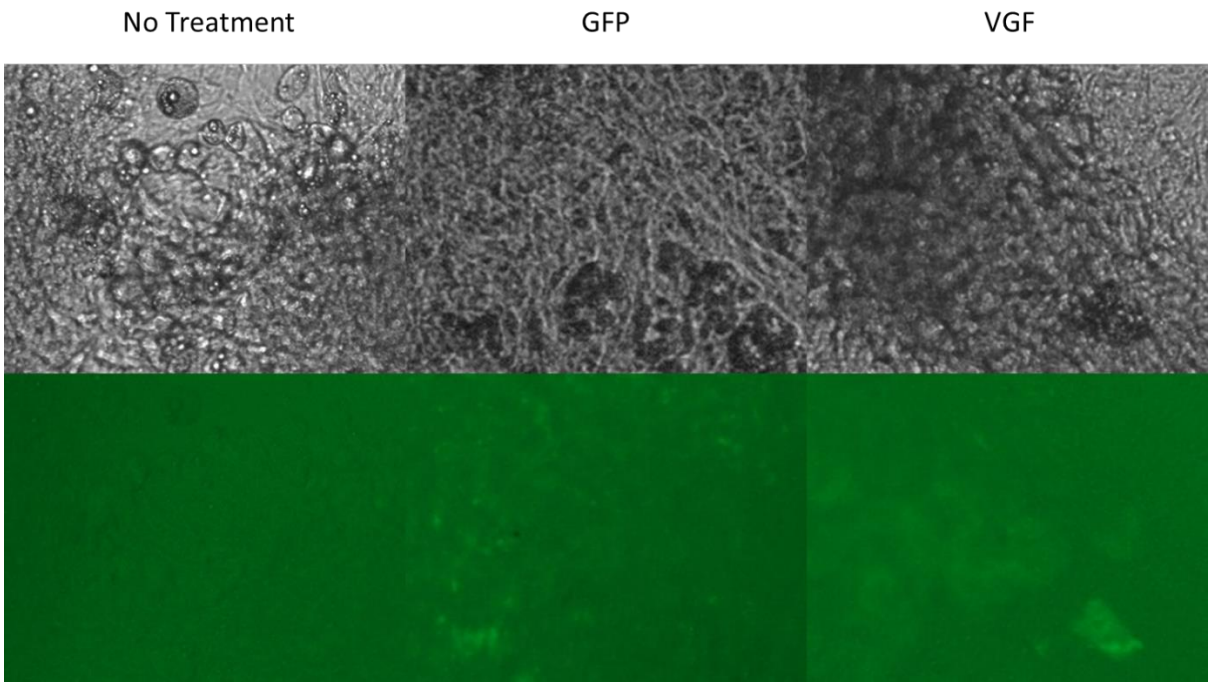

Figure 17. **GFP expression in 3T3-L1 adipocytes transfected on day 5 of differentiation.** GFP expression was visible 2 days post-transfection when 3T3-L1 cells were fully differentiated (day 7).

3T3-L1 adipocytes were stained with Nile Red 2 and 5 days after transfection (Figure 18). 2-way ANOVA suggested that there was a significant increase in Nile Red staining for all groups between days 2 and 5, but no significant difference between groups on each day. However, Bonferroni correction revealed that there were no significant differences between treatment groups or day, and no interaction.

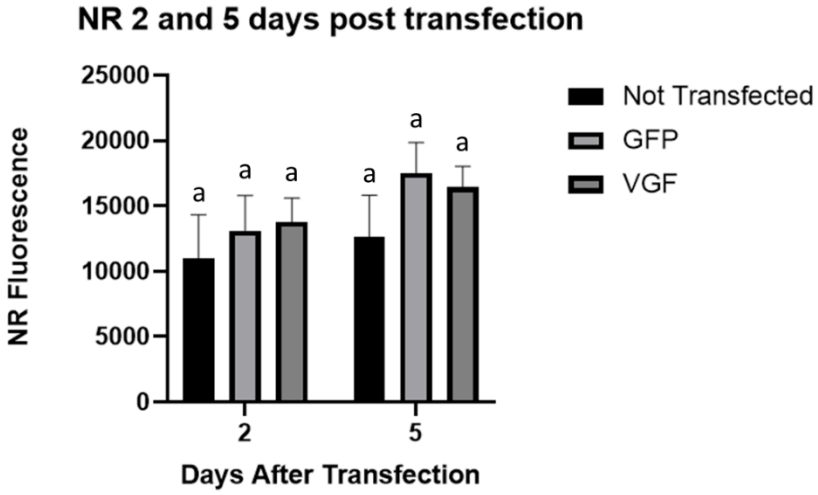

Figure 18. **Nile Red assay of 3T3-L1 adipocytes 2- and 5-days post-transfection.** Nile Red staining of all groups increased significantly between 2- and 5-days post transfection (days 7 and 10 of differentiation respectively).

The change in Nile Red staining between days 2 and 5 post-transfection was calculated by subtracting the Nile Red value for day 2 from the Nile Red value for day 5 (Figure 19). 1-way ANOVA revealed a significant difference in Nile Red staining between GFP and not transfected cells. Bonferroni correction was applied and confirmed that the GFP group had significantly greater Nile Red staining whereas VGF was not significantly different to either group.

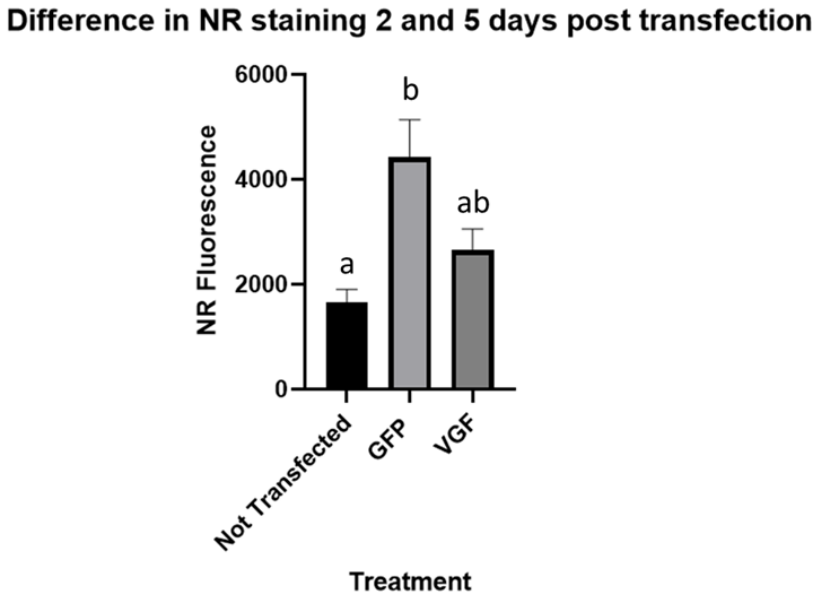

Figure 19. **Increase in Nile Red staining between day 7 and day 10 of differentiation.** GFP transfected cells had a significantly greater increase in Nile Red staining over the 3-day period compared to not transfected cells. The differences between the VGF and GFP, and VGF and not transfected groups were insignificant.

3.2 Treatment of 3T3-L1 Adipocytes with TLQP-62 and AQEE-30

DNA and Nile Red assay results of the controls are shown in Figure 20. According to the DNA assay, both isoproterenol- and dbcAMP-treated groups had a significantly greater number of cells per well than the untreated cells. According to Nile Red staining, untreated cells had significantly greater lipid content than both isoproterenol- and dbcAMP-treated cells, with isoproterenol-treated cells having significantly lower Nile Red staining than dbcAMP-treated cells. Nile Red staining was corrected for the number of cells per well inferred from DNA content, revealing that both isoproterenol- and dbcAMP-treated cells had significantly lower cellular lipid content than untreated cells.

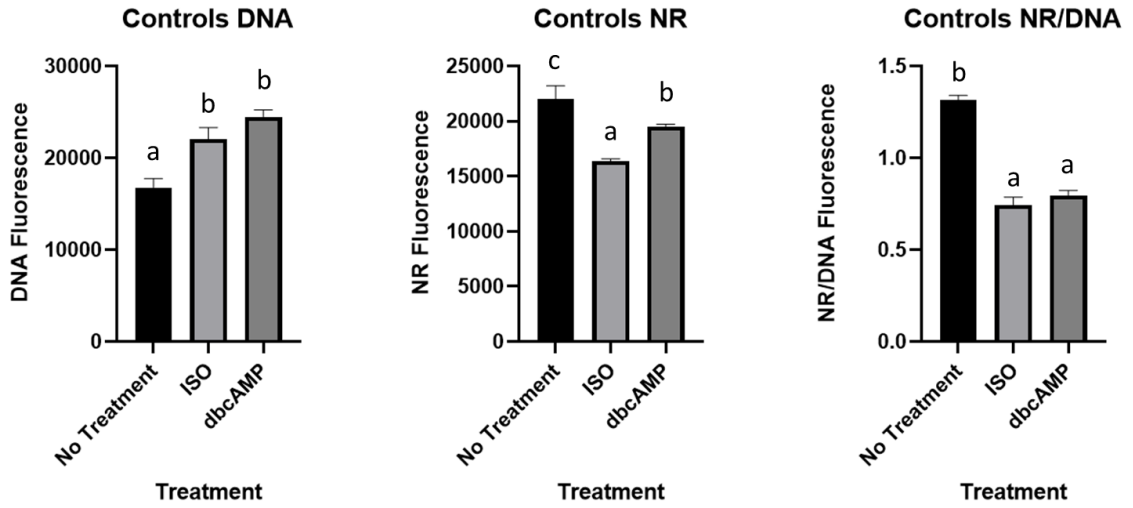

Figure 20. **DNA and Nile Red assay results for the control groups.** Both isoproterenol (ISO)- and dbcAMP-treated cells had significantly greater DNA fluorescence compared to the untreated cells. Untreated cells had significantly greater Nile Red (NR) staining than isoproterenol- and dbcAMP-treated cells, and isoproterenol-treated cells had significantly lower NR staining than dbcAMP-treated cells. Following correction for DNA present in each well, both isoproterenol- and dbcAMP-treated cells had significantly lower NR/DNA fluorescence compared to untreated cells.

Following treatment of fully differentiated 3T3-L1 adipocytes with TLQP-21 and AQEE-30 for 3 hours, a Nile Red assay was performed, followed by a DNA assay. Nile Red staining was corrected for the number of cells per well for each treatment group (Figure 21). Bonferroni correction revealed a significant interaction between treatment with peptides and treatment with isoproterenol ($p < 0.001$). Cells treated with the peptides in the absence of isoproterenol showed no significant difference in cellular lipid content compared to untreated cells and had significantly greater lipid content than isoproterenol-treated cells. Treatment of 3T3-L1 adipocytes with isoproterenol significantly reduced cellular lipid content, but the addition of TLQP-62 and AQEE-30 ablated this effect. Whilst treatment with TLQP-62 and AQEE-30 in the presence of isoproterenol resulted in significantly greater cellular lipid content compared to cells treated with just isoproterenol, only treatment with TLQP-62 in the presence of isoproterenol resulted in significantly greater lipid content compared to untreated cells. Treatment with 10μM TLQP-62, but not 5μM TLQP-62, and isoproterenol resulted in significantly greater cellular lipid content than cells treated with TLQP-62 only.

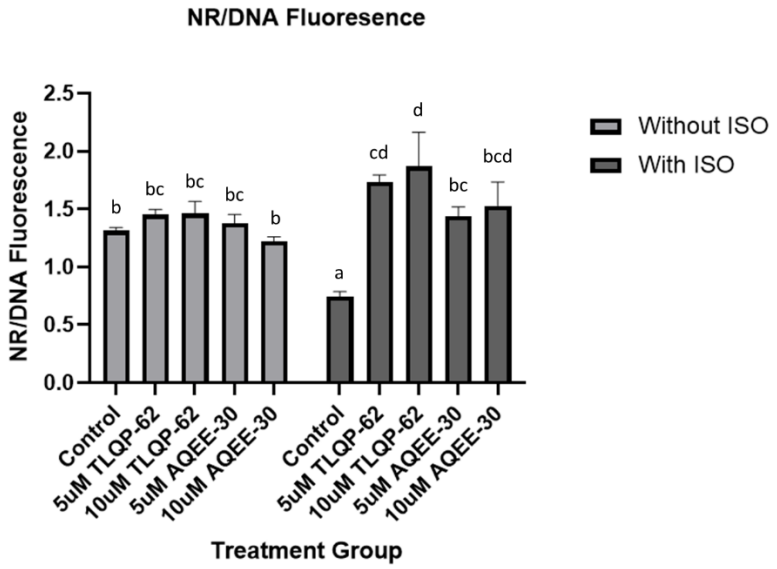

Figure 21. **NR/DNA staining of 3T3-L1 adipocytes treated with TLQP-62 and AQEE-30 in the absence and presence of isoproterenol.** Treatment with TLQP-62 and AQEE-30 in the absence of isoproterenol resulted in no significant difference compared to the control (untreated) group. Treatment with TLQP-62 and AQEE-30 in the presence of isoproterenol resulted in significantly greater Nile Red/DNA fluorescence compared to the control (isoproterenol) group. Treatment with TLQP-62, but not AQEE-30, in the presence of isoproterenol resulted in significantly greater lipid content compared to untreated cells. Treatment with 10μM TLQP-62 + ISO also resulted in significantly greater cellular lipid content than cells treated with TLQP-62 only.

Cells were also photographed following Nile Red staining as shown in Figures 22 and 23 below. In agreement with the Nile Red assay results, 3T3-L1 adipocytes treated with TLQP-62 in the absence of isoproterenol were not noticeably brighter than untreated cells, whereas cells treated with TLQP-62 in the presence of isoproterenol had more intensive Nile Red staining compared to both untreated and isoproterenol-treated cells (Figure 22).

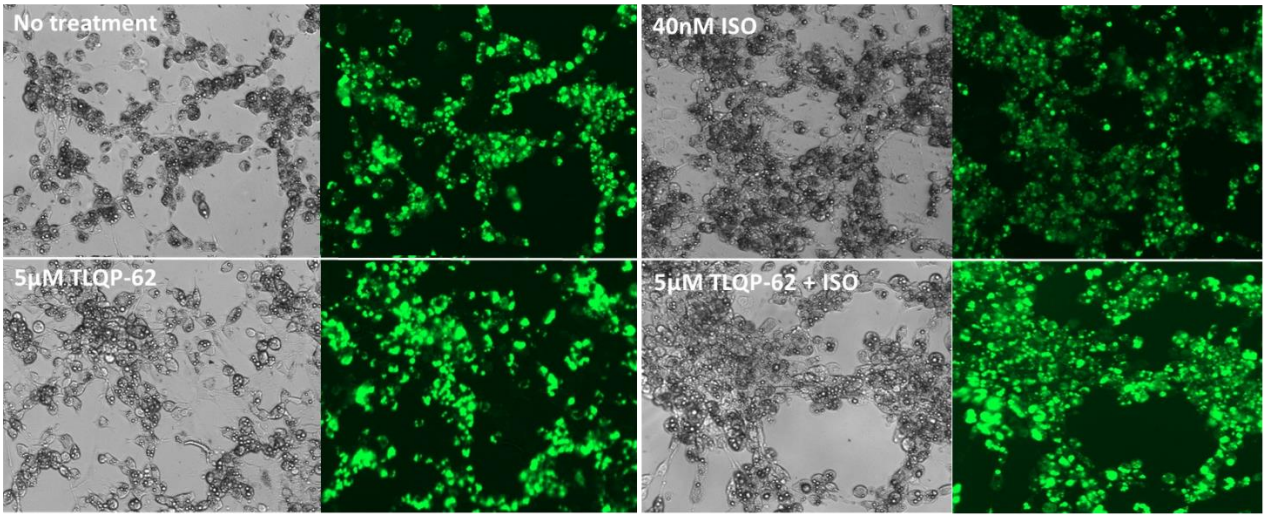

Figure 22. **3T3-L1 adipocytes stained with Nile Red and photographed with EVOS Invitrogen fluorescent microscope under light and GFP settings.** Cells were treated with TLQP-62 in the absence (bottom left) and presence (bottom right) of isoproterenol (ISO). Relevant controls containing either no or 40nM ISO (top left and top right) are shown above.

Similarly, 3T3-L1 adipocytes treated with AQEE-30 in the absence of isoproterenol do not appear brighter (Figure 23). Cells treated with AQEE-30 in the presence of isoproterenol do appear brighter than cells treated with just isoproterenol, though not as bright as cells treated with TLQP-62 in the presence of isoproterenol and of similar brightness to untreated cells.

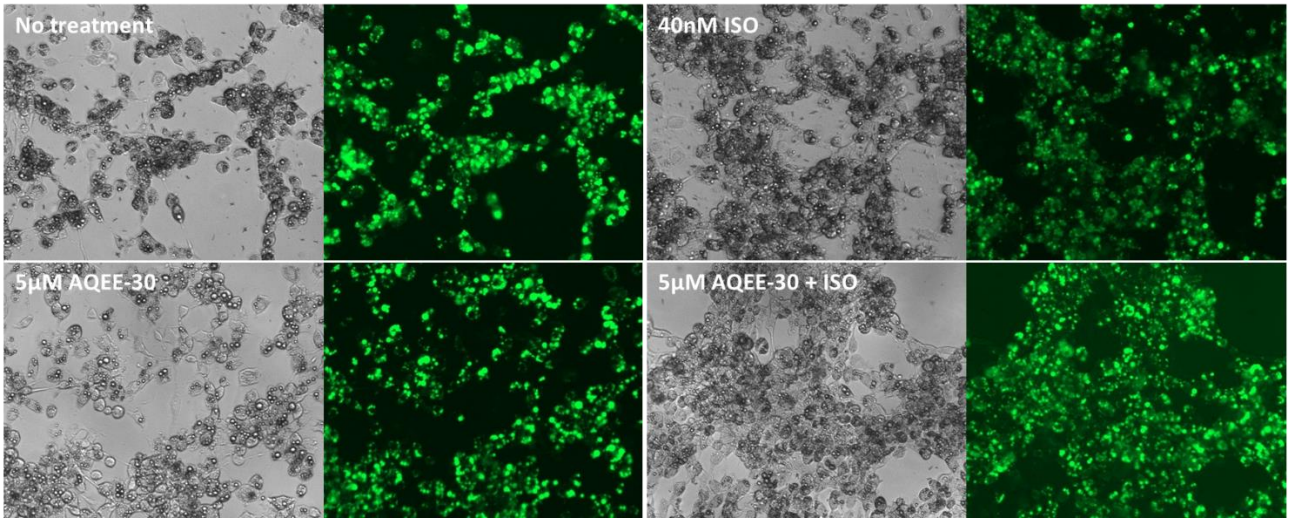

Figure 23. **3T3-L1 adipocytes stained with Nile Red and photographed with EVOS Invitrogen fluorescent microscope under light and GFP settings.** Cells were treated with AQEE-30 in the absence (bottom left) and presence (bottom right) of isoproterenol (ISO). Relevant controls containing either no or 40nM ISO (top left and top right) are shown above.

4 Discussion

4.1 VGF Overexpression in 3T3-L1 cells

3T3-L1 cells were transfected with a construct containing GFP (control) or VGF-GFP to test the hypothesis that VGF overexpression would decrease lipid content of adipocytes. Transfection of 3T3-L1 pre-adipocytes with GFP and VGF constructs using Lipofectamine 2000 resulted in cell death and inhibited the differentiation process. The Nile Red assay showed that there was significantly less cellular lipid in the transfected cells compared to cells which had not been transfected, and transfected cells had no visible lipid droplets. Transfection with the larger construct containing VGF appeared unsuccessful, suggesting that the transfection reagent was responsible for inhibited differentiation, not the constructs.

Transfection of 3T3-L1 cells on day 5 of differentiation did not appear to negatively affect the cells. However, the transfection was inefficient as GFP was visible at a low level. This was most likely due to the cells being partially differentiated and no longer proliferating. The cells continued to accumulate lipid in the days following transfection according to Nile Red staining and lipid droplets were visible. Between 2- and 5-days post-transfection, Nile Red staining increased insignificantly in all groups. When comparing the change in Nile Red staining between days 2 and 5, GFP had a significantly greater increase than not-transfected cells, whilst VGF was not significantly different to either group.

4.1.1 Critical Analysis of VGF Overexpression Results

GFP (eGFP) and Nile Red have similar emission peaks (515nm and 488nm respectively) so the presence of GFP will have likely affected the results of Nile Red assays. Since GFP is present in both the GFP and VGF groups, any differences in Nile Red staining could be attributed to the expression of VGF. 2 days post-transfection, GFP and VGF had very similar Nile Red staining with VGF being slightly higher on average. 5 days post-transfection, GFP had slightly higher Nile Red staining than VGF. Bonferroni correction revealed that there was no significant difference in Nile Red staining between GFP and VGF transfected cells 2 and 5 days after transfection. The change in Nile Red staining between 2- and 5-days post-transfection was calculated by subtracting the Nile Red value for day 2 from the Nile Red value for day 5. When comparing the change in Nile Red assay results for both days, VGF increased noticeably but insignificantly less than GFP. The greater increase in Nile Red staining for both transfected groups compared to not-transfected cells could be attributed to GFP expression. A DNA assay was not carried out due to time restraints so it cannot be ruled out that there were more cells present in the GFP samples than the VGF samples. However, GFP and VGF groups had very similar Nile Red staining on day 2 and differentiated 3T3-L1 adipocytes do not proliferate (Masella et al., 2006), so changes in Nile Red staining could be attributed to cellular lipid accumulation over the 3-day period.

The construct was not sequenced following cloning, and no measurements of VGF or VGF-derived peptides were made, so VGF was not definitively expressed, processed, nor secreted by the transfected cells. Since transfection success was validated visually based on GFP expression, it can only be presumed that VGF was also being expressed. A control plasmid expressing a fluorescent marker e.g. dsRED could have been co-transfected as a measure of transfection efficiency. White adipocytes express, process, and secrete VGF *in vivo* (D'Amato et al., 2015) suggesting that 3T3-L1 cells are likely capable of this as well. In support of this, the VGF processing enzymes PC1/3 and PC2

are ubiquitous and are both expressed in 3T3-L1 cells (Croissandeau et al., 2002; Gurriarán Rodríguez et al., 2011), and 3T3-L1 cells secrete a wide variety of large peptide hormones including adiponectin, resistin, and leptin (Blümer et al., 2008; Fasshauer et al., 2001; Yoshida et al., 1997). Transfection appeared successful but inefficient, and an insignificantly reduced increase in Nile Red staining between days 2 and 5 was seen in the VGF-transfected adipocytes compared to GFP. Overexpression of VGF may reduce lipid content in 3T3-L1 adipocytes but this will need to be investigated further.

4.1.2 Future Research of VGF Overexpression in 3T3-L1 Cells

Future research into the effects of VGF expression on adipocyte lipid content could optimise transfection of 3T3-L1 cells. Transfection of 3T3 cells with Lipofectamine 2000 has a relatively low efficacy compared to other common cell lines. Since the Lipofectamine 2000 used for these transfections had expired in 2017, this could explain the very low transfection efficiency seen. A lower concentration of newer Lipofectamine 2000 could possibly yield increased transfection efficiency of pre-adipocytes with reduced toxicity and less disruption to the differentiation process. Alternatively, transfection of pre-adipocytes could be carried out using different reagents, such as Lipofectamine 3000 (which is more efficient than Lipofectamine 2000) or the low toxicity reagents FuGENE and ViaFect. Since the size of the construct or overexpression of a large protein could affect transfection efficiency and results, the control vector could contain a scrambled peptide sequence in place of the VGF gene. Lipid-based transfection is inefficient for 3T3-L1 adipocytes so a viral vector, such as a lentivirus, would be more suitable (Carlotti et al., 2004) and would likely yield more significant results if VGF expression does have an effect of adipocyte lipid content. Electroporation has a 50-80% transfection efficiency in 3T3-L1 cells and could be more suitable than lipid transfection (Okada et al., 2003). If 3T3-L1 cells express VGF endogenously, cells could be treated with upregulators e.g., vitamin D and retinoic acid and downregulators e.g., T_3 hormone. Over/underexpression of VGF could be validated by quantifying VGF mRNA in the cells with quantitative reverse transcription PCR (RT-qPCR). VGF expression, processing, and secretion could be confirmed by western blots of the cells and media with several primary antibodies specific to products of VGF e.g., NERP, N-terminus of TLQP peptides, and N- terminus of AQEE peptides. Enzyme-linked immunosorbent assays (ELISA) could be used to quantify VGF-derived peptides but would not be able to distinguish their molecular weights.

Ideally, an expression marker which does not interfere with Nile Red and DNA (bisbenzimide) assays should be included in the transfection constructs. Blue fluorescent proteins such as BFP, mTurquoise, and mCerulean may be more suitable. BFP has excitation and emission peaks at 380nm and 448nm respectively and would be visible using the DAPI setting of the EVOS Invitrogen microscope making it an ideal marker for future experiments. Additionally, a glycerol assay on the media could be used to infer the rate of lipolysis and could be used alongside Nile Red staining as evidence for increased lipolysis or reduced lipid accumulation. As is discussed below in section 4.2, VGF-derived peptides may exert their effects via β-adrenergic signalling, so VGF over/underexpression could be investigated in the absence and presence of a β-agonist.

If VGF expression does significantly affect 3T3-L1 lipid content, this could be followed up by investigating beigeing in adipocytes. Gene and protein expression levels of common BAT markers (UCP1, PGC-1α, and PRDM16) could be quantified with RT-qPCR and ELISA. BAT has greater mitochondrial density than WAT so mitochondria could also be quantified via staining with a fluorescent dye e.g., MitoTracker.

4.2 Treatment of 3T3-L1 Adipocytes with VGF-Derived Peptides

4.2.1 TLQP-62 and AQEE-30 inhibit isoproterenol-induced lipolysis

Following the insignificant results from VGF overexpression, 3T3-L1 adipocytes were treated with two VGF-derived peptides: TLQP-62 and AQEE-30. The results showed that neither peptide significantly affected lipid content in the absence of isoproterenol. When administered in the presence of isoproterenol, both peptides significantly increased cellular lipid content compared to the control group which was treated with just isoproterenol. Administration of TLQP-62, but not AQEE-30, in the presence of isoproterenol resulted in significantly greater cellular lipid content than the untreated cells. This suggests that TLQP-62 and AQEE-30 both inhibit isoproterenol-induced lipolysis, with TLQP-62 being a more potent inhibitor than AQEE-30. Administration of TLQP-62 in the presence of isoproterenol also resulted in significantly greater cellular lipid content than cells treated with TLQP-62 only. TLQP-62 may induce lipogenesis via β-adrenergic signalling.

4.2.2 VGF-Derived Peptides may Protect Against T2DM

These results show that TLQP-62 may function as a β-adrenergic blocker. β-adrenergic stimulation simultaneously induces insulin release (Porte, 1967), inhibits insulin-stimulated glucose uptake (Abramson and Arky, 1968), and increases insulin-independent glucose uptake (Shimizu et al., 1996). Similarly, β-adrenergic blockage prevents adrenaline and isoproterenol from inhibiting insulin-stimulated glucose uptake and dampen the rebound of plasma glucose after insulin-induced hypoglycaemia (Abramson and Arky, 1968). In lean and non-T2DM patients, plasma NA and TLQP peptide levels increase significantly following glucose consumption (Astrup et al., 1991; D'Amato et al., 2015). TLQP-62, and possibly NA and TLQP-21, increase basal and glucose-induced insulin secretion by pancreatic cells (Corda et al., 2021; Christiansen et al., 2015; Moin et al., 2012; Petrocchi-Passeri et al., 2015; Porte, 1967; Stephens et al., 2012). Following glucose consumption, plasma TLQP peptide levels do not increase significantly in overweight, obese, and T2DM patients (D'Amato et al., 2015), and plasma NA does not increase in obese T2DM patients (Astrup et al., 1991). D'Amato et al. (2015) did not differentiate between levels of TLQP-62 and TLQP-21, so it is possible that these peptides are present in different ratios during fasted and fed states.

TLQP-62 may increase glucose uptake and lipogenesis, and inhibit adrenergic-induced lipolysis in 3T3-L1 cells, as was speculated by Sadahiro et al., 2015. In contrast, TLQP-21 enhances β-adrenergic-induced lipolysis (Cero et al., 2017). Lean patients have higher fasting plasma NA and TLQP peptide levels compared to obese patients (Astrup et al., 1991; D'Amato et al., 2015) but express lower levels of the TLQP-21 receptor C3aR1 in their AT (Koc et al., 2020). Lean, non-T2DM patients may have higher adrenergic-induced glucose uptake and lipolysis during fasting compared to overweight, obese, and T2DM patients. In lean, non-T2DM patients, glucose consumption is followed by enhanced insulin secretion mediated by increased NA and TLQP peptide levels. Lean, non-T2DM patients express lower levels of the TLQP-21 receptor C3aR1 (Koc et al., 2020) so may have reduced inhibition of insulin-induced uptake whilst benefiting from TLQP-21-induced enhanced islet β-cell survival and function, and possibly enhanced insulin secretion. Exogenous NA and TLQP-21 have short half-lives of 1.5 and 0.97 minutes respectively (Benedict et al., 1978; Guo et al., 2018), whilst the half-life of TLQP-62 has not yet been reported. This suggests that glucose consumption induces short-term increases in plasma NA and TLQP peptide levels. TLQP-62 may temporarily alleviate NA-induced inhibition of insulin-stimulated glucose uptake, allowing efficient

blood glucose clearance and preventing hyperglycaemia. Post-feeding, plasma NA, insulin, and TLQP peptide levels all decrease to their baseline levels over time, allowing TLQP-21-enhanced NA-induced insulin-independent glucose uptake and lipolysis to maintain blood glucose levels and lipid homeostasis during fasting.

Overweight and obese patients may have reduced fasting NA-induced glucose uptake and lipolysis due to reduced NA plasma levels (Astrup et al., 1991). Whilst NA and TLQP peptide plasma levels decrease with obesity, expression of the TLQP-21 receptor C3aR1 increases (Koc et al., 2020). Increased C3aR1 expression may be due to obesity-induced chronic low-grade inflammation (Koc et al., 2020). The C3aR1 ligand C3a is elevated in obesity (Pomeroy et al., 1997) and has insulin-like lipogenic and antilipolytic effects (Lim et al., 2013; Saleh et al., 2011). C3aR1 antagonists attenuate diet-induced obesity and insulin resistance in rats (Lim et al., 2013), and C3aR1 null mice are resistant to diet-induced obesity (Mamane et al., 2009). Following glucose consumption, NA plasma levels increase in overweight and obese patients, possibly resulting in increased adrenergic-induced inhibition of glucose uptake alongside reduced TLQP-62-induced alleviation of glucose uptake inhibition, resulting in peripheral insulin resistance and hyperglycaemia-induced hyperinsulinemia (DeFronzo, 1982). Reduced plasma NA and TLQP peptide levels relative to lean patients may also reduce β-islet cell survival and function. Alongside C3a-induced lipogenesis and insulin resistance, obesity-related reduced expression of VGF and its derived peptides could contribute to increased adiposity, and the initiation of insulin resistance and metabolic syndrome via dysregulation of adrenergic signalling pathways influencing plasma glucose uptake during feeding and fasting (Figure 24).

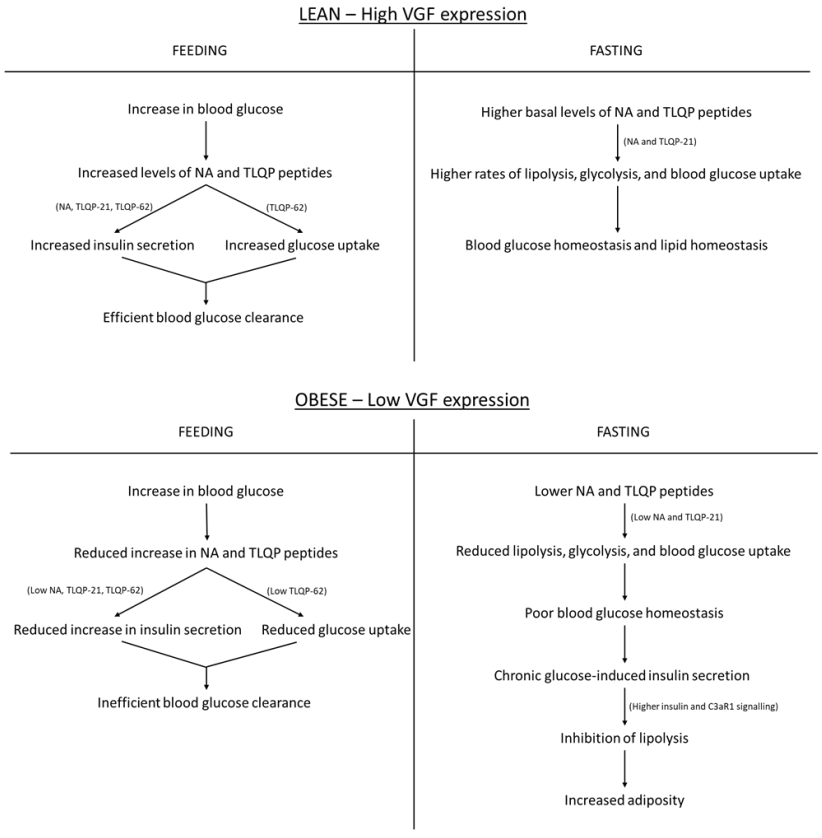

Figure 24. **Proposed mechanisms of NA and VGF-derived peptide mediated blood glucose and lipid homeostasis in lean (top) and dysregulation in obese (bottom) patients during feeding and fasting states.** Reduced expression of VGF and its derived peptides in obesity may contribute to inefficient blood glucose clearance, reduced glycolysis and lipolysis, and facilitate fat-specific weight gain. It is possible that TLQP-62 is present at a higher ratio to TLQP-21 during feeding, and at a lower ratio to TLQP-21 during the fasted state.

4.2.3 Future Research of the Effects of VGF-Derived Peptides on 3T3-L1 Adipocytes

To confirm that these specific peptides inhibited isoproterenol, this experiment could be repeated using scrambled peptides as a second negative control (as well as untreated cells). TLQP-21 enhances isoproterenol-induced lipolysis in 3T3-L1 adipocytes (Cero et al., 2016). These experiments could be repeated alongside TLQP-21 to confirm its adrenergic-induced lipolysis enhancing effects in our cell line, and simultaneous administration of both TLQP-21 and TLQP-62 could reveal if TLQP-62 inhibits the effect of TLQP-21. To determine whether TLQP-62 and TLQP-21 are present in vivo in different ratios in response to feeding and fasting, plasma levels of TLQP-62 and TLQP-21 before, immediately after, and at regular intervals e.g., every 30 minutes for 2-4 hours after a meal could be measured and compared between lean, obese, and T2DM patients. Plasma glucose, insulin, and NA could also be measured, and the response to meals of different macronutrient profiles could also be compared.

Non-alcoholic fatty liver disease is commonly associated with obesity, insulin resistance, and T2DM (Adams and Lindor, 2007). Lipolysis can be induced in hepatocytes via β-adrenergic stimulation (Schott et al., 2017), so the effects of VGF-derived peptides in the absence and presence of a β-agonist could also be investigated in liver cell lines e.g., HepG2.

Future experiments could investigate the effects of the AQEE-30 precursor HHPD-41, and the AQEE-30-derived peptides AQEE-11 and LQEQ-19. The potential isoproterenol inhibitor TLQP-62 is processed into the adrenergic enhancer TLQP-21, and the products of AQEE-30 could also have different effects on isoproterenol-induced lipolysis to their precursor. Mutations in the VGF processing hormone PC1/3, which is responsible for the production of TLQP-62 and its derived peptides, have been linked to obesity (Ramos-Molina et al., 2016. High PC1/3 activity may result in a higher ratio of the VGF end products TLQP-21, AQEE-11, and LQEQ-19 compared to intermediate products such as TLQP-62 and AQEE-30. It is possible that these 'end product' peptides are cleaved further but this has yet to be reported. Under the assumption that the overall effect of VGF is reduced lipid content, it could be expected that AQEE-11 and LQEQ-19 either enhance or have no effect on adrenergic-induced lipolysis. It is unlikely that these peptides would be present on their own in vivo, so cells could be treated with combinations of VGF-derived peptides and in different ratios. As with future VGF expression experiments, peptide treatments could be followed up by investigating beigeing in adipocytes. Mitochondrial density and gene and protein expression levels of common BAT markers could be measured.

4.3 Conclusion

In summary, overexpression of VGF in 3T3-L1 adipocytes may be linked to reduced lipid content but needs to be investigated further. We disproved our hypotheses that TLQP-62 and AQEE-30 would decrease lipid content of 3T3-L1 adipocytes. Both TLQP-62 and AQEE-30 inhibited isoproterenol-induced lipolysis in 3T3-L1 adipocytes. Administration of TLQP-62 with isoproterenol to 3T3-L1 adipocytes resulted in significantly greater cellular lipid content than TLQP-62-treated and untreated cells.

Future research could investigate the effects of VGF expression in adipocytes by upregulating and downregulating endogenous VGF, optimising transfection efficiency of adipocytes and pre-adipocytes with VGF, and investigating the effects of VGF in the absence and presence of a β-agonist. The presence and identification of VGF and VGF-

derived peptides in cells and growth media could be detected and quantified via RT-qPCR and western blotting/ELISA. Lipid content of 3T3-L1 cells treated with the AQEE-30-derived peptides AQEE-11 and LQEQ-19, and with differing combinations of VGF-derived peptides could also be investigated. Beigeing effects of VGF and its derived peptides could be investigated by measuring gene and protein expression of BAT markers e.g., UCP1, PGC-1α, and PRDM16 via RT-qPCR and ELISA, and with mitochondrial staining.

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Appendices

M13F primer seqeunce: TGT AAA ACG ACG GCC AGT
M13R primer seqeunce: CAG GAA ACA GCT ATG ACC

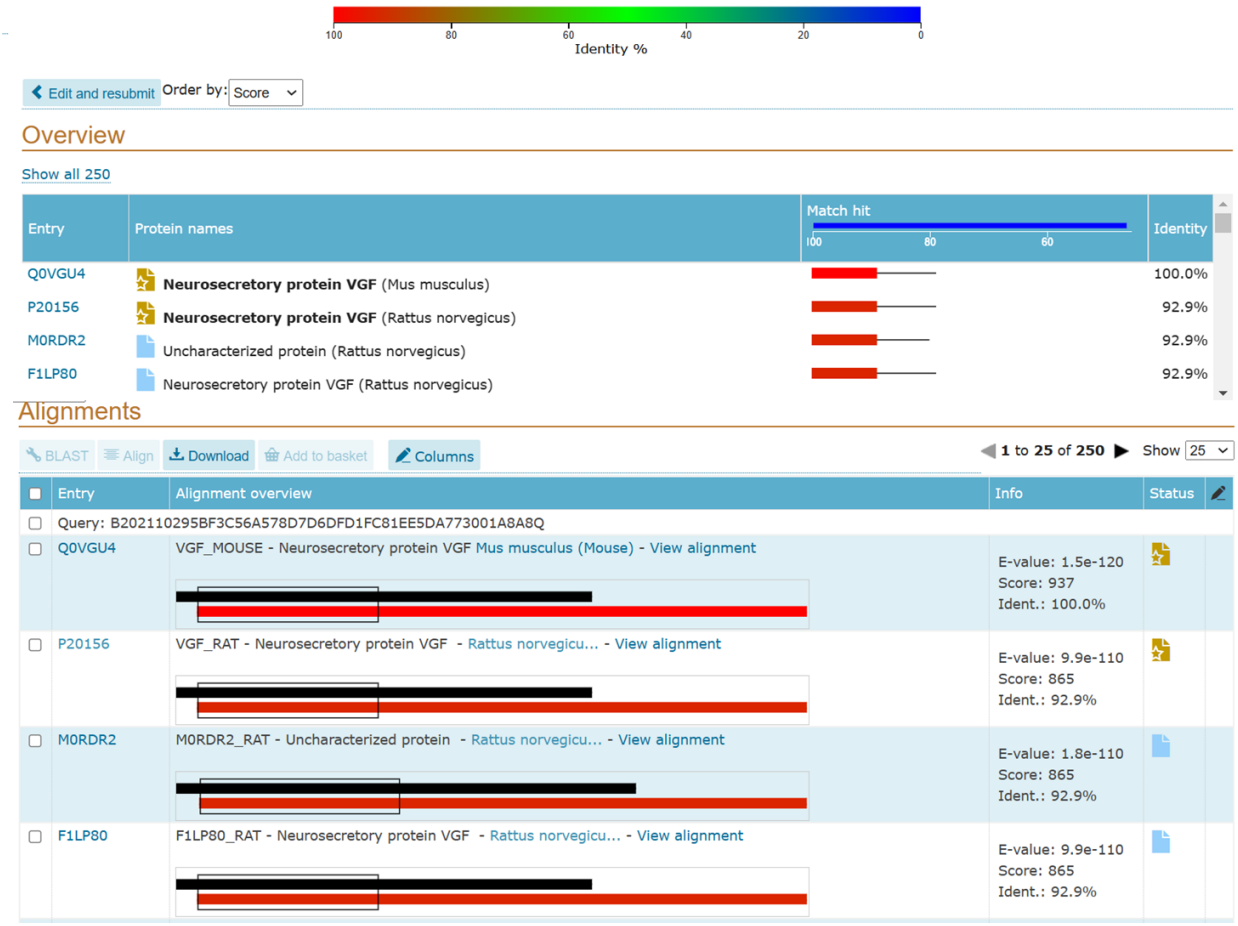
Appendix A. **Sequences of the primers used by Source BioScience to sequence the DNA isolated from VB Ultrastable containing mVgf.** M13 primers bind to the pUC origin of replication which is located upstream of mVgf in the VGF construct.

NNNNNNNNNNNNNNNGCTGCGCGTCTCTTGTTGGCGCTTAGCATTGCTCGGACTGAAATCTCGAAGTTCTTGG
AGCAAGGATGCTAGCGCCTCCAGCTCTTCTGAGCGGTCGTCCTCCTCGGGATCGCGGTCCTGAGTCTGAGGGCG
AGGGGGGGCCACAGCGGGCGCTTGGATTTCTGGTGCCGGGAGGCTATGGGTCTGACTGCGCACGGACTCGGTCA
GCAGAGCTTCTGCTGCTTCTTCGGGTGTTCCCTGCTGGGAACCTCCCGGGACCGACGGGGGCGAGGCCGGACGG
TCCAGTGCCTGCAACAGTACCGAGGCCAGCGCCCGGGGATCCACGCCCTGGAAGAGCTCTCCCTGGTCCTGAGG
CTCGGGATTCCGAGCAGCTCGGACCTCTGGTACGCCGTCATCCTTTGGCCGGGACACTGCGTCCTCAGCTACCT
GCCCATTATGCTCAGAGCTGAGGGGAGGAGGAAAAACATCGGGGCGCCCGGGGGGCGCTGCTCCCAACCCCTGG
ATCAGTAGAAGGAAGCAGAAGAGGACGGATGCCGGCAACGTGAAGGTTTTCATGGTGGCAGCCTGCTTTTTTGT
ACAAACTTGCCAACCTGAAAAAAAGTGATTTCAGGCAGGTGCTCCAGGTAATTAAACATTAATACCCCACCAAC
CAACCATCCCTTAAACCCTTACCTCTTGCTCAGCTAATTACAGCCCGGAGGAGAAGGGCCGTCCCGCCCGCTCA
CCTGTGGGAGTAACGCGGTCAGTCAGAGCCGGGGCGGGCGGCGCGAG

Rev. comp:

CTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCACAGGTGAGCGGGCGGGACGGCCCTTCTCCTC
CGGGCTGTAATTAGCTGAGCAAGAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTAC
CTGGAGCACCTGCCTGAAATCACTTTTTTTCAGGTTGGCAAGTTTGTACAAAAAAGCAGGCTGCCACCATGAAA
ACCTTCACGTTGCCGGCATCCGTCCTCTTCTGCTTCCTTCTACTGATCCAGGGGTTGGGAGCAGCGCCCCCCGG
GCGCCCCGATGTTTTTCCTCCTCCCCTCAGCTCTGAGCATAATGGGCAGGTAGCTGAGGACGCAGTGTCCCGGC
CAAAGGATGACGGCGTACCAGAGGTCCGAGCTGCTCGGAATCCCGAGCCTCAGGACCAGGGAGAGCTCTTCCAG
GGCGTGGATCCCCGGGCGCTGGCCTCGGTACTGTTGCAGGCACTGGACCGTCCGGCCTCGCCCCCGTCGGTCCC
GGGAGGTTCCCAGCAGGGAACACCCGAAGAAGCAGCAGAAGCTCTGCTGACCGAGTCCGTGCGCAGTCAGACCC
ATAGCCTCCCGGCACCAGAAATCCAAGCGCCCGCTGTGGCCCCCCCTCGCCCTCAGACTCAGGACCGCGATCCC
GAGGAGGACGACCGCTCAGAAGAGCTGGAGGCGCTAGCATCCTTGCTCCAAGAACTTCGAGATTTCAGTCCGAG
CAATGCTAAGCGCCAACAAGAGACGCGCAGCNNNNNNNNNNNNNNN

Appendix B. **Sequence of DNA isolated from VB Ultrastable containing mVGF.** Sequencing results obtained using the M13F primer (top) and the reverse compliment sequence (bottom). DNA was isolated from VB Ultrastable containing VGF using Promega Miniprep kit and was sequenced by Source BioScience.



Appendix C. **DNA isolated from VB Ultrastable shared 100% sequence similarity to *Mus musculus* VGF.** M13F binds the antisense strand, so the reverse complement DNA sequence was used. Presence of mVGF was confirmed by a BLAST of the DNA sequence provided by Source BioScience using UniProt.