Investigating the role of free fatty acid receptor 4 in 3T3-L1 Adipocytes

Woroud Sayel Alshammari

BSc , MSc

Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy

School of Molecular Biosciences

College of Medical, Veterinary & Life Sciences

University of Glasgow

November 2023

Abstract

FFA4 is a G protein coupled receptor activated by long chain fatty acids that has been found to inhibition of inflammation, improve insulin sensitivity and adipogenesis, and regulate hormone secretion from the gastro-intestinal system and pancreatic islets. All these reports indicate the strong relationship between maintaining metabolic homeostasis and FFA4, which puts this receptor as a great potential target to treat obesity and type 2 diabetes. In adipocytes, FFA4 expression increasing during adipocyte differentiation and there is some evidence that inhibiting FFA4 reduces adipogenesis. However, there are still gaps in understanding FFA4 and its function in adipocytes. In particular, most studies have relied on either natural fatty acid to activate the receptor, or genetic approaches to reduce receptor expression. Currently there is a lack of research using pharmacological ligands to understand FFA4 function in adipocytes. The current study is aimed to address this by assessing the effects of FFA4 agonism and antagonism on a 3T3-L1 in vitro model of adipocytes. Cells were treated with either an FFA4 agonist, TUG-891, or an FFA4 antagonist, AH7614.

Initially, pharmacological assays were performed in cell lines that heterologously express mouse ortholog of FFA4 to confirm the pharmacological properties of TUG-891 and AH7614. TUG-891 was found to induce phosphorylation of extracellular signal regulated kinase, elevate Ca^{+2} mobilization, increase levels of ß-arrestin recruitment and induce receptor internalisation. On the other hand, AH7614, effectively inhibited both TUG-891 and fatty acid agonists of FFA4 in calcium mobilisation, ß-arrestin2 recruitment, and internalisation assays. This inhibition appeared to be non-competitive antagonism.

3T3-L1 cells were used as platform for functional experiments to uncover the effects of FFA4 on glucose uptake and lipolysis in mature adipocytes. While acute TUG-891 treatment of 3T3-L1 adipocytes had no significant effect on glucose uptake, AH7614 treatment significantly decreased glucose uptake. In lipolysis assays, a range of concentration of TUG-891 significantly inhibited ß-adrenoceptor stimulated lipolysis, while different concentrations of AH7614 increased this lipolysis.

Observation of the acute effects of FFA4 agonism and antagonism on 3T3-L1 adipocytes inspired me to next explore the effects of TUG-891 and AH-7614 treatment during the adipogenesis process in 3T3-L1 cells. 3T3-L1 adipocytes that were differentiated in the present of TUG-891 or AH7614 went through assays to estimate the effect of agonist and antagonist treatment in adipogeneses. Oil Red O staining revealed that AH7614 significantly reduced lipid content, while TUG-891 had limited effect. This was supported by RT-qPCR showing that AH7614 treatment significantly decreased expression of various adipogenic genes including: FFA4 itself, PPARγ, adiponectin, leptin and GLUT4. In contrasts, while TUG-891 did significantly increase FFA4 expression, it had no effect on the other adipogenic genes. 3T3-L1 adipocyte function in cells differentiated in the presence of TUG-891 and AH7614 was assessed in two ways: by glucose uptake upon insulin stimulation and by measurement of isoprenaline stimulated lipolysis. Consistent with the observed effects on lipid uptake and gene expression, AH7614 treatment during differentiation reduced both insulins stimulated glucose uptake and isoprenaline stimulated lipolysis in the mature adipocytes, while TUG-891 had no significant impact on either readout.

These finding highlighted the role of FFA4 in adipocyte since its activation is curial for adipogenesis. Moreover, the ability of AH7614 to reduce in 3T3-L1 adipocyte raises the possibility of using FFA4 antagonists in the fight against obesity. Yet this suggestion could be complicated, since this FFA4 antagonists also inhibited glucose transport. Together, this thesis uses pharmacological tools to provide new insight into the function of FFA4 in adipocytes and the potential for targeting this receptor in the treatment of metabolic disorders.

Table of Contents

Investigating the role of free fatty acid receptor 4 in 3T3-L1 Adipocytes.....i
Abstract ii
Table of Contents..... iv
List of Tables ix
List of Figures x
List of Publications xii
Acknowledgements xiii
Author's Declaration xiv
Definitions/Abbreviations xv
Chapter 1 Introduction 1
1.1 Obesity 1
1.1.1 Causes of obesity 2
1.1.2 Therapeutics of obesity..... 3
1.1.3 Obesity limitations 8
1.2 Type 2 diabetes mellitus 9
1.2.1 The role of adipose tissue in T2DM 11
1.2.2 Insulin secretion 12
1.2.3 Insulin sensitivity 13
1.2.4 Therapeutics of T2DM..... 14
1.2.4.1 Lifestyle and health behaviour therapy..... 14
1.2.4.2 Pharmacotherapy 15
1.3 Adipocytes 16
1.3.1 White adipose tissue 17
1.3.2 Brown adipose tissue..... 18
1.3.3 Beige adipose tissue..... 19
1.4 Adipocyte functions 20
1.4.1 Adipogenesis..... 22
1.4.2 Glucose transports..... 23
1.4.2.1 GLUT4 pathway in adipose tissue..... 26
1.4.3 Lipolysis 29
1.5 Adipogenesis study models..... 30
1.5.1 *In vivo* models..... 31
1.5.2 *In vitro* models to study adipogenesis and the 3T3-L1 cell line 31
1.6 G Protein-Coupled Receptors 35
1.6.1 GPCRs in drug discovery 40
1.7 Free fatty acid receptors..... 41
1.7.1 FFAs and their types 41

1.7.2 Free fatty acid receptor 1 42
1.7.3 Free fatty acid receptors 2 and 3 43
1.8 Free fatty acid receptor 4 43
1.8.1 FFA4 expression 45
1.8.2 FFA4 signalling 46
1.8.3 FFA4 isoforms 47
1.8.4 FFA4 agonists and antagonists 48
1.8.5 FFA4 receptor in adipose 51
1.8.6 FFA4 receptor's role in glucose uptake 53
1.8.7 FFA4 and adipogenesis 56
1.9 Aim of the study 59
Chapter 2 Materials and Methods 62
2.1 Materials. 63
2.1.1 Tissue Culture 63
2.1.2 Kits 63
2.1.3 Solution 64
2.1.4 pharmacological Compounds 64
2.1.5 cell lines 65
2.1.6 Antibodies 65
2.2 Cell culture methods 66
2.2.1 3T3-L1 cells maintenance. 66
2.2.1.1 Subculturing 3T3-L1 cells. 66
2.2.1.2 3T3-L1 differentiation procedure. 66
2.2.2 FFA4- CHO cell line maintenance 66
2.2.3 Subculturing FFA4- CHO cells 67
2.2.4 HEK293T cells maintenance 67
2.2.5 Subculturing HEK293T cells. 67
2.2.6 FLAG-mFFA4-eYFP Flip-In T-REx cell line maintenance 67
2.2.7 Subculturing FLAG-mFFA4-eYFP Flip-In T-REx cells. 68
2.2.8 Cryopreservation cells. 68
2.2.9 Recovery of Cryopreserved Cell Stock 68
2.2.10 Short term transfection 68
2.2.11 Poly D-lysine cote 69
2.3 Molecular Biology Methods 69
2.3.1 Making L-Broth 69
2.3.2 Making Agar Plates 69
2.3.3 Preparation of Competent Bacteria 69
2.3.4 Transformation of Competent Cells with Plasmid DNA 70
2.3.5 Maxiprep 70

2.3.6 DNA concentration..... 71
2.4 Pharmacological Assays..... 72
2.4.1 ERK Phosphorylation Assay..... 72
2.4.2 Ca^{2+} Mobilization Assay..... 72
2.4.3 ß-Arrestin-2 BRET Assay..... 73
2.4.4 TRUEPATH BRET Assay..... 74
2.4.5 Receptor internalisation by confocal microscopy..... 75
2.5 Gene Expression Analysis..... 75
2.5.1 RNA isolation. 75
2.5.2 RNA concentration..... 76
2.5.3 DNAse digest..... 76
2.5.4 Reverse transcript (making cDNA)..... 76
2.5.5 Real-Time Polymerase Chain Reaction..... 77
2.6 Immunoblot..... 78
2.6.1 Preparation of 3T3-L1 lysates..... 78
2.6.2 Protein concentration determination. 79
2.6.3 SDS-PAGE..... 79
2.6.4 Electrophoretic Transfer into Nitrocellulose Membrane..... 79
2.6.5 Blocking of Membranes and Probing with Primary Antibodies. 80
2.6.6 Secondary Antibodies and Immunodetection..... 80
2.7 Glucose uptake assay. 80
2.8 Oil Red O stain. 81
2.9 Lipolysis. 81
2.10 Nuclear Stain and Cell Count..... 82
2.11 Data analysis..... 82
Chapter 3 Pharmacological evaluation of FFA4 agonist TUG-891 and FFA4 antagonist AH7614..... 85
3.1 Introduction..... 86
3.2 Aims..... 88
3.3 Results..... 89
3.3.1 FFA4 agonist induces the phosphorylation of extracellular signal regulated kinase..... 89
3.3.2 Activation of the FFA4 receptor leads to increases in intracellular Ca^{2+} 90
3.3.3 Activation of mFFA4 coupled to Gi and Gq/11 G proteins..... 92
3.3.4 Activation of the FFA4 receptor induces ß-arrestin recruitment..... 94
3.3.5 AH7614 inhibits the agonist-promoted internalisation of mFFA4, visualised using confocal microscopy..... 98
3.3.6 AH7614 inhibits TUG-891 and aLa action on intracellular Ca^{2+}..... 100
3.3.7 Treatment with AH7614 decreases ß-arrestin recruitment..... 101

3.4 Discussion 104

Chapter 4 Acute treatment with FFA4 agonists and antagonists affects function of 3T3-L1 adipocytes 112

4.1 Introduction 113

4.2 Aims 114

4.3 Results 115

4.1.1 FFA4 expression is upregulated during differentiation of 3T3-L1 adipocytes 115

4.1.2 Adipogenesis marker upregulated during 3T3-L1 differentiation 117

4.1.3 Glucose transporter 4 (GLUT4) upregulated during differentiation of 3T3-L1 adipocytes 119

4.1.4 Acute treatment with an FFA4 agonist, TUG-891, in various conditions does not affect basal or insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes 120

4.1.5 Acute treatment of a range of concentrations of AH7614 decrease basal glucose uptake in 3T3-L1 adipose 123

4.1.6 AH7614 decrease insulin response in insulin-stimulated glucose uptake in 3T3-L1 adipocytes 125

4.1.7 A range of AH7614 concentration decrease the maximal insulin response in Differentiated 3T3-L1 Adipocyte 126

4.1.8 AH7614 does not affect insulin-stimulated AKT phosphorylation in 3T3-L1 adipocytes 128

4.1.9 Treatment with TUG-891 inhibits lipolysis, while AH7614 treatment enhances lipolysis in 3T3-L1 adipocytes 129

4.4 Discussion 131

Chapter 5 Effects of agonism and antagonism of the FFA4 receptor on adipogenesis in 3T3-L1 adipocyte. 142

5.1 Introduction 143

5.2 Aims 144

5.3 Results 145

5.3.1 FFA4 agonist treatment does not affect lipid accumulation during 3T3-L1 differentiation, while antagonist treatment decreases lipid accumulation 145

5.3.2 AH7614 affects 3T3-L1 adipogenesis as early as six days post differentiation 148

5.3.3 FFA4 antagonist treatment decreased 3T3-L1 lipid accumulation in a concentration-dependent manner 151

5.3.4 AH7614 has its strongest effect on 3T3-L1 adipogenesis at early stages of the differentiation 153

5.3.5 AH7614 treatment downregulated adipogenesis markers and glucose transport machinery in gene expression in differentiated 3T3-L1 155

5.3.6 Treatment with AH7614 during 3T3-L1 differentiation decreases glucose uptake 160

5.3.7 Glycerol release upon Isoprenaline stimulation decreased in 3T3-L1 differentiated in the presence of AH7614 163
5.4 Discussion 165
Chapter 6 Final discussion 175
6.1 Key findings 177
6.2 Limitations to this work 181
6.3 Future directions 184
List of References 190

List of Tables

Table 1-1 long term obesity treatment and its targets 6
Table 1-2 The list of drugs used in long-term obesity treatments and its complications: 7
Table 1-3 Glucose Transporter Subclassification, Characteristics, and Tissue Expression 26
Table 1-4 Clinical Trial Agents targeting GPCRs for diabetes and obesity : 38
Table 1-5 Endogenous Activators of FFA4 with Biological Functions 45
Table 1-6 Potency and Selectivity of Agonists and Antagonists for FFA4 Activation 51
Table 2-1 cell culture Materials 63
Table 2-2 List of selected compounds targeting FFA4 64
Table 2-3 list of the cell lines that were used in this study 65
Table 2-4 list of primary antibodies used in this study 65
Table 2-5 A list of secoundry antibodies that were used in this study 65
Table 2-6 Plasmids that were used in this study..... 71
Table 2-7 DNA mixtures for G protein signiling pathways..... 74
Table 2-8 Sequences of the primers used in the Real-Time PCR analysis..... 77
Table 2-9 amplification cycles process..... 78
Table 3-1 pEC50 potency values of agonists at mFFA4 in various Gα assays using BRET 93
Table 4-1. CT values for FFAs in undifferentiated vs differentiated 3T3-L1. ... 116
Table 4-2. CT values for adipogenesis markers that was assayed via RT-qPCR. 118
Table 4-3. CT values for GLUT1 and GLUT4 in RT-qPCR for 3T3-L1 cell line (undifferentiated vs differentiated). 120
Table 5-1: P value for the comparisons between treatments vs DMSO control at different time points for 3T3-L1 cells. 151
Table 5-2: Summary of statistical comparisons when adding AH7614 at different timepoints during 3T3-L1 differentiation. 155

List of Figures

Figure 1-1 Glucose transport 4 pathway in adipocyte: 28
Figure 1-2 Lipolysis pathway in adipocyte: 30
Figure 1-3 Free fatty acid receptor 4 (FFA4) signalling pathway: 47
Figure 3-1 ERK phosphorylation assay using CHO FFA4 cells 89
Figure 3-2 TUG-891 and α-linolenic acid (aLa) elevate intracellular calcium through mFFA4: 91
Figure 3-3 mFFA4 couples to Gq/11 and Gi/o G proteins 93
Figure 3-4 FFA4 receptor induced ß-arrestin recruitment after stimulation by TUG-891 95
Figure 3-5 FFA4 receptor induced ß-arrestin recruitment after stimulation with aLa 96
Figure 3-6 TUG-891 promotes the internalisation of mFFA4, as visualised by confocal microscopy 99
Figure 3-7 Mobilisation inhibition after the use of AH7614 at high concentrations. 101
Figure 3-8 Inhibition of the TUG-891 promotion of ß-arrestin recruitment to mFFA4 103
Figure 3-9 snake plot diagram of HFFA4 107
Figure 4-1 Expression of FFA4 and FFA2 mRNA is unregulated during 3T3-L1 differentiation 116
Figure 4-2 Adipogeneses conformed in 3T3-L1 by Oil Red O stain and adipogeneses marker expression via RT-qPCR. 118
Figure 4-3 Expression of GLUT4 mRNA is unregulated during 3T3-L1 differentiation. 119
Figure 4-4 TUG-891 does not enhance insulin's responses glucose uptake in 3T3-L1 adipocytes..... 121
Figure 4-5 Adding TUG-891 within insulin incubation dose not enhance insulin response in glucose uptake assay in 3T3-L1 adipose. 122
Figure 4-6 Acute treatment with a range of TUG-891 does not enhance glucose uptake in 3T3-L1. 123
Figure 4-7 Acute treatment with a range of AH7614 decrease glucose uptake in 3T3-L1. 124
Figure 4-8 Acute treatment with AH7614 decrease insulin responded glucose uptake in 3T3-L1 125
Figure 4-9 A pre-treatment with a range of AH7614 concentrations decrease the maximal insulin response in differentiated 3T3-L1 adipocyte. 127
Figure 4-10 GLUT4 and P-AKT protein levels were not affected by short-term incubation with AH7614 in 3T3 adipocytes. 129
Figure 4-11 TUG-891 decreases lipolysis, while AH7614 increases lipolysis in 3T3-L1 adipocyte. 130
Figure 5-1 FFA4 antagonist treatment decreases adipogenesis in 3T3-L1 adipocytes. 146
Figure 5-2 Treatment with AH7614 had no significant toxicity on cell growth in 3T3-L1 differentiated cells: 147
Figure 5-3 AH7614 affects adipogenesis byt Day 6 of 3T3-L1 differentiation. .. 150
Figure 5-4 AH7614 has a conceration depond effact on 3T3-L1 adipocyte. 152
Figure 5-5 AH7614 maximum effect on 3T3-L1 adipogenesis reached when adding the antagonist at all differentiation timepoints. 154

Figure 5-6 AH7614 treatment downregulate gene expression for both adipogenesis markers along with glucose transport machinery in 3T3-L1 adipocytes. 159
Figure 5-7 Glucose uptake and GLUT4 protein level both decreased in 3T3-L1 adipocytes that were differentiative in the presence of AH7614. 162
Figure 5-8 Glycerol release decreases in 3T3-L1 adipocytes that were differentiated in the presence of AH7614. 164

List of Publications

Bremner SK, **Al Shammari WS**, Milligan RS, Hudson BD, Sutherland C, Bryant NJ, Gould GW. Pleiotropic effects of Syntaxin16 identified by gene editing in cultured adipocytes. Front Cell Dev Biol. 2022 Nov 18;10:1033501. doi: 10.3389/fcell.2022.1033501. PMID: 36467416; PMCID: PMC9716095.

Acknowledgements

Completing this PhD was a challenging journey, which needed support and lots of love that I luckily both received from my family, supervisors and friends who stayed by my side until the end.

First, I would like to thank my supervisors Brian Hudson and Gwyn Gould. Throughout my PhD, I faced many obstacles and both of you showed patience and supported me. Brian, thank you for believing in me and for the close leading supervision in such a challenging time for me. It was a pleasure to work with someone as smart as you. And a special thanks to you, Gwyn, for continuing to support me even after our paths took different turns. It was such a pleasure to be your student.

Thanks to the lab members of the Tobin group, who helped me, taught me techniques and cheered for me all the way until the end. Thanks to the Hudson Group, my lab colleagues, for their help and support when I was finishing up my last experiments. And my thanks also go to the Gould group members who continued to guide me even after they had moved to another university.

A big thanks to my Glasgow friends Sarah, Nini, Becca , Natasja , Abeer , Marwa and Eloise for their love, support, kind words and laughter, making Glasgow feel like home.

Thanks to my sister Asma who held my hand in this journey. To my niece Dana and my nephews Ali, Sair and Sulaiman for believing in their auntie and their love and cheering for me all the way. Thank you also to my sister Kholoud and my brothers Nader, Maher and Tariq, for being there for me.

Thanks to my lifetime friend, Deema this thesis is for us. Thanks to all my friends back home in Saudi Arabia who motivated me all the way to the end.

Sayel and Huda (my dad and my mom), thank you for waiting for me always, loving me daily and supporting me through life. Without you, none of this would have been possible.

Author's Declaration

November 2023

"I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution."

Woroud Alshammari

Definitions/Abbreviations

2-DG	2-deoxyglucose
7TMRs	seven transmembrane spanning receptors
Acadm	acyl-Coenzyme A dehydrogenase medium chain
ACC	acetyl-coenzyme A carboxylase
Acsl1	acyl-CoA synthetase long-chain family member 1
AKT	protein kinase B
AMPK	AMP-activated protein kinase
Angpt14	angiopoietin-like 4
AT	adipose tissue
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate ATP
BAT	Brown adipose tissue
BMMSCs	bone marrow mesenchymal stem cells
cAMP	Cyclic adenosine monophosphate
CCK	cholecystokinin
CD36	cluster of differentiation 36
CDC	The U.S. Centres for Disease Control and Prevention
CHO	Chinese Hamster Ovary
DEX	dexamethasone
DGLA	dihomo-gamma-linolenic acid DGLA
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Media
DMSO	dimethyl sulfoxide
DPP-IV	Dipeptidyl peptidase-IV
DRPs	Drug-Related Problems
ECL2	extracellular loop 2
EPA	eicosapentaenoic acid
FABP4	FA binding protein 4
Fabp5	FA binding protein 5
FBS	foetal bovine serum
FCS	fetal calf serum FCS
FDA	The Food and Drug Administration
FFA1	Free fatty acid receptor 1
FFA2	Free fatty acid receptors 2
FFA3	Free fatty acid receptors 3
FFA4	Free fatty acid receptor 4
FFA4-L	Free fatty acid receptor 4 long
FFA4-S	Free fatty acid receptor 4 short
FFARs	free fatty acid receptors
FFAs	free fatty acids
FRET	fluorescence resonance energy transfer
GDP	Guanosine diphosphate
GIP	gastric inhibitory peptide

GLP-1	glucagon-like peptide-1
GLUT4	glucose transporter type 4
GLUTs	glucose transporters
GPCRs	G Protein-Coupled Receptors
GRKs	GPCR kinases
GSV	storage vesicle
GTP	Guanosine triphosphate
GTT	glucose tolerance tests
HBSS	Hank's Balanced Salt Solution
HFD	high-fat diet
HPRT	housekeeping gene HPRT
IBMX	isobutymethylxanthin
IR	insulin receptor
IRS1	insulin receptor substrate
ISO	isoprenaline
ITT	insulin tolerance tests
KRP	Krebs–Ringer phosphate buffer
LCFAs	long-chain fatty acids
Lipe	lipase hormone sensitive
MAP	mitogen-activated protein
MCFAs	medium-chain fatty acids
MMLV	Moloney Murine Leukemia Virus
MUFAs	monounsaturated fatty acids
NCS	new-born calf serum
ND	normal diet
NEFAs	non-esterified FAs
OA	oleic acid
OADs	oral anti-diabetic drugs
OP	osteoporosis
ORO	Oil Red O
p-Akt	phosphorylated protein kinase B
PBS	Dulbecco's Phosphate Buffered Saline
PDK	PIP3-dependent kinase
PEI	polyethyleneimine
PI3K	phosphatidylinositol 3-kinases
PIP2	bisphosphate
PKA	protein kinase A
PM	plasma membrane
PPAR-γ	Peroxisome proliferator-activated receptors
PUFAs	polyunsaturated fatty acids
PYY	peptide hormone YY
qRT–PCR	Quantitative real-time PCR
S-V	stromal-vascular
SCFAs	short-chain fatty acids
SFAs	saturated fatty acids
SGLT2	Sodium-Glucose Transport Protein 2

SGLTs	sodium–glucose linked transporters
siRNA	small interfering RNA
SREBP	called Sterol Regulatory Element Binding Proteins
T1DM	Type 1 diabetes mellitus (T1DM)
T2D	type 2 diabetes
TAGs	triacylglycerols
TBS	Tris Buffered Saline
TGN	trans-Golgi network
TNFα	tumour necrosis factor α
TZDs	thiazolidinediones
UCP1	uncoupling protein 1
VLCD	very-low-calorie diet
VLCFAs	very-long-chain fatty acids
WAT	White adipose tissue
αMG	α-methyl-d-glucose
ß1AR	ß1-adrenoceptor

Chapter 1 Introduction

1.1 Obesity

Obesity is a complex multi-factorial condition in which excess body fat negatively impacts health. Body mass index (BMI) (calculated by dividing a person's weight by the square of their height [kg/m2]) is used to define and diagnose obesity (Hampl et al. 2023). This classified number range was created using classification systems (Bray et al. 2018) and obesity guidelines (MD et al. 2014; Pi-Sunyer et al. 1998) and defines an adult's body weight as healthy with a BMI between 18.5 and 24.9 kg/m2, overweight as between 25.0 and 29.9 kg/m2, and obesity as over 30 kg/m2. The BMI-for-age growth charts of the US Centres for Disease Control and Prevention (CDC) define being overweight as having a BMI at or above the 90th percentile of normal weight and obesity as having a BMI above the 95th percentile of normal weight in children and adolescents (Bray et al. 2018). Obesity is classified further into three severity levels: class I (BMI 30.0–34.9), class II (BMI 35.0–39.9) and class III (BMI greater than 40.0)(Lin and Li 2021). It is common to be classified as overweight or obese, and these classifications are more frequently associated with global deaths than the underweight classification (Lin and Li 2021).

Annually, a minimum of 2.8 million individuals loses their lives globally as a result of being overweight or obese. While obesity was initially linked to high-income nations, it has now become widespread in low- and middle-income countries as well (Ahmed & Konje, 2023) . According to the World Health Organization's 2016 estimation, there were approximately 1.9 billion adults who were classified as overweight, with 650 million among them being obese (having a BMI of 30 kg/m^2 or higher). This figure also included 340 million adolescents and 39 million children. These statistics indicate that 39% of adults aged 18 years or older were overweight (39% of men and 40% of women), while 13% were classified as obese. Over the period from 1975 to 2016, the global prevalence of obesity nearly tripled (Ahmed & Konje, 2023). Also, obesity effected children population as according to the world health organisation reports the number of

overweight or obese children under the age of 5 reached 41 million globally in 2019

A raised BMI is a risk factor for noncommunicable diseases, such as diabetes, cardiovascular disease and musculoskeletal cancers, and results in a significant decrease in life quality and expectancy (Lin and Li 2021). Obesity is caused by a long-term energy imbalance between consumed and expended calories (Lin and Li 2021). Globally, obesity continues to rise, resulting in an unprecedented epidemic that shows no signs of abating (Lin and Li 2021).
Many factors, including genetic, epigenetic, and hormonal and behavioural factors contribute to obesity. However, the primary cause is typically having a greater amount of stored energy than is required by the body. Excess calories are deposited as fat, which leads to the pathology of obesity(Lin and Li 2021).

1.1.1 Causes of obesity

Several causes can lead to obesity, including diet, lifestyle, genetic or hormonal factors, or a combination of these factors.

Dietary intake is the most common reason for obesity. Overconsuming foods that are highly calorific, fat-laden, and frequently available in significant portions can lead to an increased daily calorific intake resulting in obesity (Wright and Aronne 2012).

Genetics is another factor for obesity (Jebb 1993). Obesity tends to run in families, but the effects of genotype on the aetiology of obesity may be mitigated or exacerbated by nongenetic factors (Kopelman 2000). Genetic influences appear to operate via susceptibility factorsexcept for obesity-related rare syndromes (Kopelman 2000). These genes increase the risk of developing a trait, but they are neither necessary for its expression nor sufficient for its onset explanation (Kopelman 2000). Researchers have found that many genes are linked to syndromic, non-syndromic, oligogenic and polygenic obesity (Pigeyre et al. 2016). Additionally, researchers theorise that the genetics of leanness are important in their similarity to some of the causes of obesity (Pigeyre et al. 2016). For example, some rare genetic forms of obesity are linked to an issue with how the hypothalamus handles energy(Dubern et al. 2022). These rare

types of obesity are caused by defects in more than 60 genes that code for proteins in the hypothalamic leptin–melanocortin pathway(Dubern et al. 2022).

Several studies have reviewed and linked hormones to obesity. These studies recorded evidence linking different hormonal change patterns to different obesity levels and locations of fat tissue (Kopelman 1994). For example, testosterone is an important hormone that influences how metabolic diseases, such as fat, develop(Kelly and Jones 2015). Males with low testosterone levels tend to have more fat mass and less lean mass(Kelly and Jones 2015). These physical characteristics are linked to metabolic dysfunction, with the lack of testosterone developing an energy imbalance, poor glucose control and decreased insulin sensitivity(Kelly and Jones 2015). Gut hormones are another example. The stomach hormone ghrelin appears to operate as both a meal-initiator and a long-term body weight regulator; therefore, it is identified as having a potential role in hunger signalling (Kopelman 1994). The cause of obesity is usually the result of a combination of factors, which makes the fight against obesity more complicated.

1.1.2 Therapeutics of obesity

Lifestyle modification remains the cornerstone of obesity management. Obese people are advised to lose at least 10% of their body weight through a combination of diet, physical activity, behaviour therapy or lifestyle modification. Consumption of portion-controlled diets can result in significant short-term weight loss. Moreover, high levels of physical activity and ongoing patient–practitioner contact can result in long-term weight control. In many cases, lifestyle changes result in significant weight loss and a noteworthy reduction in cardiovascular risk (Lin and Li 2021).

In the United Kingdom, individuals diagnosed with obesity are enrolled in a weight management plan. The plan commences with lifestyle changes, emphasizing the promotion of healthy eating habits and regular physical activity as essential components of obesity management(Boyers et al., 2021). This encompasses personalized dietary plans, portion control, increased consumption of fruits and vegetables, and a reduction in the intake of sugary drinks and processed foods. Physical activity recommendations often include a combination

of aerobic exercise, strength training, and regular physical activity. Following this, patients may be offered behavioural therapy aimed at modifying attitudes and behaviours related to eating and physical activity. This may involve counselling, self-monitoring, goal setting, and strategies to address emotional eating or triggers for overeating. Structured weight management programs are available to provide support and guidance for individuals seeking weight loss (*Healthy Lives, Healthy People: A Call to Action on Obesity in England - GOV.UK*, n.d.). These programs may include group sessions, educational materials, dietary advice, exercise plans, and ongoing monitoring by healthcare professionals. Finally, healthcare professionals may consider prescribing medications or offering bariatric surgery as additional options for obesity management (Boyers et al., 2021).

Pharmacotherapy is recommended for people with a BMI above 30 (or above 27 for people with comorbidities) who are unable to lose weight through lifestyle changes alone. The Food and Drug Administration (FDA) has approved orlistat, phentermine/topiramate, naltrexone/bupropion and liraglutide for long-term weight management (Tak and Lee 2021).

Orlistat is an anti-obesity drug that has been approved by the US FDA. Orlistat directly targets pancreatic lipase, functioning as a potent and reversible inhibitor of this enzyme (S. Li et al., 2020). Pancreatic lipase plays a crucial role in breaking down dietary fats into smaller molecules that can be absorbed by the body. By inhibiting pancreatic lipase activity, Orlistat effectively curtails the digestion and absorption of dietary fats, resulting in a reduced intake of calories from fat(L. Zhang et al., 2021). This mechanism of action contributes to weight management by facilitating weight loss or preventing weight gain. It has been associated with mild-to-moderate gastrointestinal adverse effects, serious hepatic adverse effects and beneficial effects on non-alcoholic fatty liver disease(NAFLD) (Heck et al. 2000). Additionally, it has been associated with rare cases of acute kidney injury (Filippatos et al. 2008). Orlistat interferes with the absorption of many drugs, which affects their bioavailability and effectiveness (Filippatos et al. 2008).

Another mode of drug is the combining drugs with various modes of action may increase their efficacy through additive or synergistic effects, as well as by potentially overriding the body's natural homeostatic control over body weight (Gadde and Atkins 2020). This was the idea behind introducing phentermine/topiramate and naltrexone/bupropion as a treatment for obesity.

The phentermine/topiramate extended-release capsule is a fixed-dose combination of phentermine and topiramate developed by Vivus company for the treatment of obesity, sleep apnoea syndrome, type 2 diabetes mellitus (T2DM) and NASH(Dhillon 2022; Mauer et al. 2021). The main molecular target of phentermine is the stimulation of norepinephrine release in the central nervous system (G. W. Kim et al., 2014). As a sympathomimetic amine, phentermine binds to and activates specific adrenergic receptors. These receptors are predominantly located in the hypothalamus, a region responsible for regulating appetite and satiety. By activating these receptors, phentermine promotes the release of norepinephrine, which contributes to appetite suppression and helps control food intake. This mechanism of action centers around the hypothalamus' role in appetite regulation and underscores how phentermine affects the release of norepinephrine in the central nervous system(G. W. Kim et al., 2014). On the other hand, Topiramate has multiple molecular targets within the central nervous system. It modulates various neurotransmitter systems, including GABA and glutamate (Shank & Maryanoff, 2008). One of its primary targets is voltage-gated sodium channels, which play a role in generating and propagating neuronal electrical signals. By blocking these channels, topiramate reduces neuronal excitability and abnormal brain activity. Topiramate also inhibits the AMPA/kainate subtype of glutamate receptors(G. W. Kim et al., 2014).This drug has been approved in the US and South Korea for the treatment of obesity in adults (Dhillon 2022).

Additionally, Naltrexone/bupropion is a fixed-dose combination product for treating obese or overweight adults with at least one weight-related comorbidity. The molecular target for Naltrexone is an opioid receptor antagonist. Its primary molecular target is the opioid receptors in the brain(de Laat et al., 2019) . By binding to these receptors, naltrexone blocks the effects of opioids and helps reduce cravings for opioids or alcohol(de Laat et al., 2019). On the other hand Bupropion is a norepinephrine-dopamine reuptake inhibitor

(NDRI) (Deang et al., 2017). Its molecular targets involve the inhibition of the reuptake of norepinephrine and dopamine, two important neurotransmitters in the brain(Deang et al., 2017). By increasing the levels of these neurotransmitters, bupropion helps regulate mood and can contribute to reducing cravings and withdrawal symptoms(Deang et al., 2017).

Finally, liraglutide GLP-1 receptor agonists are also FDA-approved for the long-term treatment of obesity(Mauer et al. 2021). By targeting the GLP-1 receptor, liraglutide helps regulate blood sugar levels, promotes weight loss, and can be used in the treatment of type 2 diabetes and obesity(García-Cantú et al., 2014). Gastrointestinal effects of these drugs include nausea, abdominal cramping and diarrhoea. Liraglutide requires daily injections as oral formulation has not been approved(Mauer et al. 2021).

Table 1-1 below outlines different anti-obesity drugs along with their respective targets and pharmacology. Each drug targets specific molecular pathways or receptors and exerts its pharmacological effects accordingly.

Table 1-1 long term obesity treatment and its targets

Drug	Target	Pharmacology
Orlistat	pancreatic lipase	A potent, specific and irreversible inhibitor
Phentermine/topiramate	stimulation of the release of norepinephrine /Multiple ions channels	Sympathomimetic agent/anti-epileptic drug
Naltrexone/bupropion	opioid receptors in the brain/ neurotransmitters	Opioid antagonist with a high affinity for the μ-opioid receptor/antagonist of the nAch receptor
Liraglutide	GLP-1 receptor	GLP-1 receptor agonists

Table 1-2 The table below summarizes the complications associated with different drugs used in the treatment of obesity in different groups. Each drug has its unique set of complications that should be considered when prescribing

for specific patient types. The references provide further information on the reported complications.

Table 1-2 The list of drugs used in long-term obesity treatments and its complications:

Drug	Type of patient	Complication	Reference
Orlistat	Patients who do not want to take a systemic drug or patients who eat a moderate- or high-fat diet	Headaches, flatulence, cramping, gastric disturbances and rare cases of acute kidney injury	(Filippatos et al. 2008; Mauer et al. 2021)
Phentermine/topiramate	Patients with chronic migraines	Increased heart rate, dizziness, neuropathy, insomnia, anxiety, depression, and cognitive impairment.	(Dhillon 2022; Mauer et al. 2021)
Naltrexone/bupropion	Patients with depression, interested in smoking cessation or with food addiction and strong cravings	Headaches, irritability, anxiety and insomnia.	(Mauer et al. 2021; "Naltrexone/Bupropion for Obesity," 2017; Tak and Lee 2021)
Liraglutide	Patients with coronary artery disease, prediabetes and diabetes	Nausea, abdominal cramping and diarrhoea	(Armstrong et al. 2016; Mauer et al. 2021)

Individuals with a BMI greater than 40 who are unable to lose weight through lifestyle changes or pharmacotherapy may consider bariatric surgery. Individual

metabolic profiles benefit to varying degrees from standard bariatric operations, such as biliopancreatic diversion, sleeve gastrectomy, Roux-en-Y gastric bypass and adjustable gastric banding (Arterburn and Courcoulas 2014). Several studies show the benefits of bariatric surgery extend beyond simply losing weight; the surgery also reduces chronic inflammation associated with obesity, alters biomarkers and gut microbiota and can result in long-term T2DM remission(Lin and Li 2021).

1.1.3 Obesity limitations

Although there is an increased effort in the fight against obesity, there are noticeable therapy limitations.

The side effects and selectivity criteria for patients are the first limitations to occur. In the drugs stated in (table 1-2) some of the reported side effects limited the individuals who could receive treatment. Anti-obesity drugs are recommended for patients with a BMI of more than 30 kg/m2 (or a BMI of at least 27 kg/m2 with obesity-related disorders or risk factors) in addition to lifestyle modifications (Gadde and Atkins 2020). This fact is also true in weight loss surgery since weight loss surgery is recommended for patients exceeding a BMI of 40'.(Arterburn and Courcoulas 2014). Furthermore, several long-term problems were reported after weight loss surgeries, including gastroesophageal reflux disease, dietary deficiencies, incisional hernias and a failure to lose weight (Martínez-Núñez et al. 2017).

The cost of available obesity treatments is also an issue. Anti-obesity medications are rarely reimbursed by insurance, and their high costs can limit patients' options (Gadde and Atkins 2020; Mauer et al. 2021). Moreover, the cost of weight loss surgeries can often range between $15,000 and $25,000 in United states. In United Kingdom the cost of wight lost surgery vary. Gastric Band Surgery range from £5,000 to £8,000 (Hopkins et al., 2016), while Sleeve Gastrectomy costs range from £8,000 to £12,000in the U.K. (Lefere et al., 2023).

However , the long-term weight loss has also been reported as an issue for obesity. A study conducted on anti-obesity drug outcomes for weight loss recorded that after one year, the average weight loss was between 3% and 9%

from randomised controlled studies of currently available anti-obesity medications (Gadde and Atkins 2020). This was considered a negative side effect of bariatric surgeries for weight loss.

Although obesity rates are increasing globally, there are far fewer therapeutic alternatives for weight control medicine than for other chronic conditions, such as hypertension and T2DM (Tak and Lee 2021). give a whole area of studies such as my project.

1.2 Type 2 diabetes mellitus

In a healthy person, blood glucose levels stay balanced due to parallel actions between insulin and glucagon. Diabetes mellitus is defined as prolonged elevated blood glucose levels(Ashcroft and Rorsman 2012). There are two types of diabetes. Type 1 diabetes mellitus (T1DM) accounts for 5%–10% of diabetes diagnoses and typically manifests in childhood. It is an autoimmune illness characterised by the destruction of insulin-producing beta cells and the development of islet cells and results in patients requiring insulin therapy for the rest of their lives. The most common form of diabetes is T2DM, which accounts for approximately 90%–95% of cases(Chatterjee et al. 2017). This form of diabetes is caused by a combination of two main factors: defective insulin secretion by pancreatic ß-cells and the inability of insulin-sensitive tissues to respond to insulin (Roden and Shulman 2019).

Numerous genetic risk loci, or regions of the genome associated with an increased risk of developing type 2 diabetes, have been identified through genome-wide association studies (GWAS) and other genetic studies(Frayling, 2007). These risk loci often contain or are in proximity to genes involved in various biological processes related to glucose metabolism, insulin signaling, and pancreatic beta cell function(Barroso et al., 2003).

Some of the well-established genetic risk loci for type 2 diabetes include TCF7L2, KCNJ11, PPARG, FTO, CDKAL1, and HNF1A (Khatami et al., 2019; Sirdah & Reading, 2020; Stolerman & Florez, 2009). However, it is important to note that these genetic variants individually confer only a modest increase in the risk of developing type 2 diabetes, and the interplay between multiple genetic

variants and environmental factors is crucial in determining an individual's overall risk (Lyssenko et al., 2008). Ongoing research continues to expand our understanding of the genetic architecture of type 2 diabetes and may lead to the identification of additional genetic risk loci in the future.

Lifestyle variables, such as age and obesity, impact T2DM; however, there is also a substantial hereditary component, as several genes are thought to influence the risk of T2DM (Ashcroft and Rorsman 2012). In contrast to T1DM, which necessitates daily insulin injections due to pancreatic insufficiency, T2DM can be managed with various drugs that boost insulin secretion or raise insulin sensitivity(Hauser et al. 2017).

The number of people diagnosed with T2DM has skyrocketed in recent years due to the rise in obesity, inactivity and high-calorie diets that have swept the globe. In 2015, more than 90% of people with diabetes were living with type 2, and the number of people affected is expected to rise to 642 million worldwide by the year 2040(Chatterjee et al. 2017).

The risk of death from any cause is 15% higher for persons with T2DM compared to those without the disease, and it is twice as high for those under the age of 40 (Chatterjee et al. 2017). There are many ways in which diabetes both affects and is affected by other chronic diseases. Co-occurring disorders can make the disease more severe and raise the risk of complications (Teck 2022).

Over time, diabetes can have devastating effects on a wide variety of bodily systems. T2D has two main categories of complications that can arise: macrovascular and microvascular (Gedebjerg et al., 2018).
Macrovascular complications refer to conditions that affect the larger blood vessels in the body (Fowler, 2011). These complications are primarily associated with atherosclerosis, a condition characterized by the buildup of plaque in the arterial walls. Over time, this plaque can narrow and harden the arteries, leading to reduced blood flow to vital organs (Fowler, 2011).

Macrovascular complications in type 2 diabetes commonly include cardiovascular diseases such as coronary artery disease, heart attacks, strokes, and peripheral

arterial disease (K. A. A. Ahmed et al., 2010). These conditions are major contributors to the increased risk of morbidity and mortality in individuals with type 2 diabetes (K. A. A. Ahmed et al., 2010; Fowler, 2011).

On the other hand, microvascular complications involve damage to the small blood vessels throughout the body(Schalkwijk & Stehouwer, 2005). These complications are mainly associated with chronic hyperglycemia (high blood sugar levels) and typically develop over an extended period (Fowler, 2011). Microvascular complications primarily affect the eyes, kidneys, and nerves (Faselis et al., 2019). Diabetic retinopathy can lead to vision impairment or blindness (Kropp et al., 2023).Diabetic neuropathy refers to nerve damage and can result in symptoms such as numbness, tingling, pain, and impaired limb function (Vinik et al., 2006). Diabetic nephropathy affects the kidneys and can progress to chronic kidney disease and end-stage renal disease(Shahbazian & Rezaii, 2013).

Managing type 2 diabetes involves not only controlling blood sugar levels but also addressing the risk factors associated with both macrovascular and microvascular complications(Krentz et al., 2007). This includes maintaining a healthy lifestyle, managing blood pressure and cholesterol levels, regular medical check-ups, and adherence to prescribed medications (Bielecka-Dabrowa et al., 2022). Early detection and timely intervention are crucial for preventing or minimizing the impact of macrovascular and microvascular complications in individuals with type 2 diabetes.

1.2.1 The role of adipose tissue in T2DM

White adipose tissue (WAT) plays an important role in energy storage and the endocrine system, both of which are important in maintaining glucose homeostasis (Rosen and Spiegelman 2006). The WAT contains TGs, which are broken down into energy via lipolysis. While excess circulating free fatty acids (FFAs) contribute to ectopic lipid build-up and the subsequent development of insulin resistance in T2DM, effective regulation of lipolysis and its counterpart, lipogenesis, is crucial in maintaining normal glucose homeostasis (Javeed and Matveyenko 2018). Insulin resistance is defined as insulin's failure to stimulate

adipose glucose transport, enhance lipid uptake and inhibit lipolysis in adipocytes (Samuel and Shulman 2016).

More precisely, the resistance of peripheral tissues, such as adipose, to the action of insulin is a fundamental feature of T2DM. The glucose transporter (GLUT4) is unable to be activated by insulin and is brought to the cell surface from intracellular vesicles. This, in turn, limits the clearance of insulin-induced glucose and results in hyperglycaemia. One of the most important requirements for insulin to mobilise GLUT4 from intracellular deposits is for GLUT4 to be sorted into an insulin sensitive GLUT4 storage location (Lee et al. 2023).

1.2.2 Insulin secretion

Insulin secretion is a vital process that plays a crucial role in maintaining balanced blood sugar levels in the body. When blood glucose levels rise, specialized cells called beta cells in the pancreas detect this increase and respond by secreting insulin into the bloodstream(Rahman et al., 2021). This release of insulin is carefully regulated and orchestrated by a series of events. As glucose enters the beta cells, it undergoes metabolism, generating energy in the form of ATP. Increased ATP levels cause the closure of ATP-sensitive potassium channels, leading to depolarization of the cell membrane (MacDonald et al., 2005).
This depolarization triggers the opening of voltage-gated calcium channels, allowing calcium ions to enter the beta cells. The influx of calcium then triggers the fusion of insulin-containing vesicles with the cell membrane, releasing insulin into the bloodstream (Jing & Sjöholm, n.d.). Once in the bloodstream, insulin binds to receptors on target cells throughout the body, enabling them to take up glucose and utilize it for energy or storage. Through this intricate process of insulin secretion, the body can effectively regulate blood sugar levels, ensuring proper cellular function and energy metabolism.

Medications that target insulin secretion aim to enhance the production and release of insulin from pancreatic beta cells (Doyle & Egan, 2003). Examples of such medications include sulfonylureas and meglitinides. These drugs work by stimulating the beta cells to produce and release more insulin, thereby increasing insulin levels in the bloodstream (Doyle & Egan, 2003).

1.2.3 Insulin sensitivity

Insulin sensitivity holds significant importance in metabolic research and greatly contributes to comprehending the pathophysiology of conditions like obesity, T2D, and metabolic syndrome (Guo, 2014). It characterizes the degree of responsiveness exhibited by specific tissues, notably skeletal muscle, liver, and adipose tissue, towards insulin's actions (da Silva Rosa et al., 2020).

In individuals with heightened insulin sensitivity, these tissues effectively take up and utilize glucose in response to insulin stimulation, facilitating its storage as glycogen or hindering its release from the liver(Norton et al., 2022). Conversely, diminished insulin sensitivity, referred to as insulin resistance, leads to impaired glucose uptake and metabolism, resulting in elevated blood glucose levels (Muoio & Newgard, 2008).

Numerous factors, such as genetic predisposition, lifestyle choices including physical activity and diet, adiposity, and specific hormonal and inflammatory factors, influence insulin sensitivity (Gasmi et al., 2020). The assessment and comprehension of insulin sensitivity play a vital role in evaluating metabolic health and developing therapeutic interventions aimed at enhancing insulin action and mitigating the risk of metabolic disorders(Jyotsna et al., 2023). Medications that target insulin sensitivity aim to improve the response of these tissues to insulin, thereby enhancing glucose uptake and utilization (Cersosimo & DeFronzo, 2006). One commonly used class of drugs for improving insulin sensitivity is the thiazolidinediones (TZDs), which enhance insulin action in peripheral tissues(Quinn et al., 2008).

Maintaining metabolic health therefore relies on both insulin secretion and insulin sensitivity. Disruptions in either process can contribute to the onset of metabolic disorders, including type 2 diabetes. It is vital to comprehend and evaluate both insulin secretion and insulin sensitivity for assessing metabolic health, devising therapeutic interventions, and devising strategies to enhance glucose regulation and reduce the risk of metabolic disorders.

1.2.4 Therapeutics of T2DM

The available therapies for diabetes can be divided into lifestyle changes and pharmacotherapy or a combination of the two.

1.2.4.1 Lifestyle and health behaviour therapy

The importance of dietary therapy in preventing and managing T2DM is often overlooked. Most people with T2DM are overweight, making weight loss the primary goal of dietary therapy. Reducing total and saturated fat levels and increasing complex carbohydrate intake to 50%–55% of calorific intake are common recommendations (Mahler and Adler 1999).

'Reverse T2DM' is a term used in several studies to demonstrate that changing lifestyle and food consumption can lead to the reversal of T2DM. For example, (Perry et al. 2018) conducted a study that found that three days of a very low-calorie diet (VLCD) lowered plasma glucose and insulin concentrations in a T2DM rat model without altering body weight. The lower plasma glucose was associated with a 30% reduction in hepatic glucose production and reduced hepatic triglyceride and diacylglycerol content, which suggests that VLCD reverses hyperglycaemia in a rat model of T2DM by reducing DAG-PKC 3- induced hepatic insulin resistance, hepatic glycogenolysis, acetyl-CoA content, PC flux and gluconeogenesis.

Exercise has been demonstrated to be useful for preventing the onset of T2DM and improving glucose control due to increased insulin sensitivity(Mahler and Adler 1999). Exercising seems to restore insulin sensitivity through several different processes, including decreasing belly fat and FFA levels and increasing GLUT4 in muscle and blood flow to insulin-sensitive tissues(Eriksson et al. 1997).

Controlling the consumption of alcohol and tobacco is an additional lifestyle change that can impact T2DM. Some observational studies have found that moderate alcohol consumption is associated with a decreased incidence of T2DM. A systematic review and dose-response meta-analysis of observational data demonstrated that a decrease in risk was seen at all levels of alcohol intake below 63 g/d (Kautzky-Willer et al. 2016). Moreover, smoking is another contributing factor to the onset of T2DM (Gujral et al. 2013). According to the

CDC, the risk of developing T2DM is increased by 30%–40% among cigarette smokers compared to non-smokers, and diabetics who smoke are more likely to struggle with insulin dosage and overall management of their condition than their non-smoking counterparts.

1.2.4.2 Pharmacotherapy

As T2DM advances, blood glucose levels rise, and cell function gradually diminishes. Patients typically begin with oral anti-diabetic medications but progress to insulin injections.

Exenatide, a GLP-1 receptor agonist (or incretin mimic), was the first G protein-coupled receptor (GPCR)-targeted medication licensed for T2DM in 2005. GLP-1 supplies beta-cell insulin reserves through enhanced insulin mRNA stability, gene transcription and biosynthesis. It increases and stabilises the expression of preproinsulin by stabilising the mRNA that encodes it (Portha et al. 2011).

Metformin remains the medication of choice for individuals with T2DM unless contraindicated, such as in patients with renal impairment. Metformin decreases hepatic glucose production, increases the sensitivity of peripheral tissues and boosts GLP-1 secretion. In addition, metformin efficiently reduces HbA1c levels by 1%–2%, has no effect on body weight, does not cause hypoglycaemia and can have modestly favourable effects on blood pressure and lipid profile (Chatterjee et al. 2017).

A prominent hypothesis proposes that the primary mode of action for metformin involves the inhibition of complex I within the mitochondrial respiratory chain, leading to a decline in ATP synthesis (OWEN et al., 2000). This decrease in cellular energy levels is believed to activate the AMP-activated protein kinase (AMPK) pathway, a crucial sensor of cellular energy status. AMPK activation triggers a range of effects, including the suppression of hepatic glucose production and improved uptake of glucose in peripheral tissues such as skeletal muscle (W. L. Hou et al., 2018). Metformin's metabolic effects may also involve interactions with other molecular targets, such as mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014). However, further research is required to fully understand and delineate the specific molecular

targets and mechanisms through which metformin exerts its therapeutic actions. Unravelling these details could contribute invaluable insights toward the development of novel treatments and the enhancement of metabolic disorder management.

Furthermore, sulfonylureas, such as gliclazide and glimepiride, are generally the treatment of choice for dual therapy due to their demonstrated efficacy, low cost and action on cells to promote insulin secretion. However, these medications have been linked to hypoglycaemia and weight gain, and questions remain about their possible link to poor CVD outcomes(Chatterjee et al. 2017).

Sodium-glucose transport protein 2 (SGLT2) inhibitors are also available. The SGLT2 inhibitors are an FDA-approved class of prescription medications used in conjunction with diet and exercise to reduce blood sugar levels in adults with T2DM. The SGLT2 inhibitors include canagliflozin, dapagliflozin and empagliflozin(Padda et al. 2022). Dipeptidyl peptidase-IV is an enzyme inhibitor found in oral anti-diabetic drugs for the treatment of T2DM and acts by inhibiting the degradation of incretin peptides(Shao et al. 2020; Singh et al. 2021).

Finally, insulin therapy is the most effective treatment for overall glycaemic management, with a reduced HbA1c blood test concentration of 0.5%–2%. However, it also increases the risk of hypoglycaemia, particularly in the elderly, and may provide an average weight gain of four kilograms (Chatterjee et al. 2017).

Many patients with T2DM experience sequenced health problems because their blood sugar levels are not adequately controlled by existing therapies, even when administered in combination. Hence, novel agents that target not only the disease but also its onset, development and progression are needed.

1.3 Adipocytes

For decades, adipose tissue (AT) was considered to be an inert energy storage deposit, but new findings of AT's broader involvement in cell and whole-body communication have sparked a scholarly revolution in this field. As of early

2019, approximately 139,000 citations involving adipocytes or AT can be found (Richard et al. 2020).

Composing of multiple cell types, AT releases numerous cytokines, chemokines and hormones in coordination. About one-third of the cells in AT are adipocytes, while the remainder is a combination of preadipocytes, fibroblasts, endothelial cells, stromal cells, macrophages and other immune cells (Chait and den Hartigh 2020). The AT is a critical component for maintaining lipid and glucose homeostasis. To date, three types of AT have been identified (white, brown and beige), and they are found in various anatomical locations. WAT is found throughout the body, with the highest concentrations located in subcutaneous (under the skin) areas, such as the abdomen, thighs, and buttocks. It also surrounds internal organs, known as visceral fat (Gesta & Kahn, 2017). While brown adipose commonly found in specific locations, including the neck (around the collarbone and upper back), interscapular region (between the shoulder blades), and along the spine (Cypess et al., 2013).Since Beige adipose tissue is a transitional type of adipose tissue that possesses characteristics of both white and brown adipose tissue (Kaisanlahti & Glumoff, 2018). It is formed within white adipose tissue in response to certain stimuli, such as cold exposure or exercise (Bargut et al., 2017). These adipose depots' cellular composition, secretome and location determine their function in health and metabolic disease (Chait and den Hartigh 2020).

1.3.1 White adipose tissue

Essential for energy storage, endocrine communication and insulin sensitivity, WAT makes up the largest portion of AT in the majority of mammals, including humans(Richard et al. 2020). White adipocytes are normally spherical, with a single large lipid droplet pushing all other organelles, including the nucleus, to the cell's perimeter(Richard et al. 2020).

Serving as a key energy reserve for the other organs, WAT plays a crucial role in regulating whole-body energy balance. The ability to store food efficiently during times of food excess is essential for survival amid food shortages(Marcelin et al. 2019). Orans such as liver, muscles, heart and adrenal glands. Within the Liver, WAT releases stored triglycerides in the form of fatty acids, which are

taken up by the liver. The liver then processes these fatty acids through beta-oxidation to generate energy, as well as for the synthesis of lipids and other important molecules (Calderon-Dominguez et al., 2016). On muscles During periods of increased physical activity, white adipose tissue supplies fatty acids to the muscles as an energy source. Fatty acids are transported to the muscle cells, where they undergo beta-oxidation to produce ATP, the primary energy currency of cells (Chouchani & Kajimura, 2019). Additionally, in the heart WAT provides fatty acids to the heart muscle, allowing it to meet its high energy demands(Collins, 2013). Lastly, WAT contributes to the availability of fatty acids, which are precursors for the synthesis of cortisol and other steroid hormones produced by the adrenal glands (Kargi & Iacobellis, 2014).

1.3.2 Brown adipose tissue

Brown adipose tissue (BAT) is abundant in mammals after birth and throughout hibernation. Brown adipocytes have many lipid droplets spread out over an ellipsoid-shaped cell that is full of iron-rich mitochondria, which gives the cell a brownish colour(Richard et al. 2020). Moreover, brown adipocytes are distinguished by several large mitochondria brimming with cristae. These mitochondria show the expression of uncoupling protein 1 (UCP1), a unique protein that decouples oxidative phosphorylation from adenosine triphosphate (ATP) synthesis, which results in heat production (Cinti 2012).

The BAT uses energy to produce heat without shivering, which is essential for maintaining body temperature. It was previously believed that BAT was only present in infants (Cypess et al. 2009; Nedergaard et al. 2007); however, imaging investigations have found metabolically active BAT in the supraclavicular and thoracic areas of adults(Sampath et al. 2016). Additionally, seasonal relationships have been reported, with BAT activity being higher in winter and lower in summer, either due to temperature or the photoperiod (Cypess et al. 2009). Gender is also a factor, as women have more BAT mass and activity than men, and the likelihood of detecting BAT activity in either sex has been demonstrated to be inversely related to age and BMI (Cypess et al. 2009). This difference can be attributed to several factors, including physiological and hormonal factors. One of the main reasons is the influence of sex hormones. Estrogen, a predominant female sex hormone, has been shown to stimulate the

development and activation of brown adipocytes (Kaikaew et al., 2021). Estrogen receptors are present in brown adipose tissue, and the hormone can enhance the thermogenic activity of BAT, leading to increased BAT mass in women (González-García et al., 2017). Additionally, there are differences in overall body composition between men and women. Women generally have a higher percentage of body fat compared to men, and brown adipose tissue contributes to this difference (Wells, 2007).

Most WAT depots are thought to be susceptible to browning under certain conditions, but more research is needed in this area. Cold exposure, exercise, bariatric surgery and severe burns, as well as pharmacological and dietary components, such as conjugated linoleic acid, short-chain fatty acids (SCFAs), capsaicin, non-caffeinated green tea extract, thiazolidinediones (TZDs) and adrenergic receptors, are all examples of physiological stressors that can promote WAT browning (Chait and den Hartigh 2020).

1.3.3 Beige adipose tissue

In addition to WAT and BAT, a third fat type known as browned, beige or ‘brite’ (brown-in-white) fat has been identified. Beige fat, as the name implies, is defined as the presence of brown adipocytes within classic WAT depots (Chait and den Hartigh 2020). There are different ideas about where beige adipocytes originate and how they affect energy homeostasis. One prevalent hypothesis suggests that beige adipocytes originate from a distinct lineage of precursor cells present within WAT (Sanchez-Gurmaches & Guertin, 2014). In response to various stimuli such as cold exposure or certain hormones, these precursor cells undergo a process called "browning" or "beiging," where they differentiate into beige adipocytes (Sanchez-Gurmaches & Guertin, 2014). This cellular transformation involves changes in gene expression, leading to the acquisition of brown adipocyte characteristics and increased thermogenic capacity. Another viewpoint suggests that beige adipocytes may arise from a trans-differentiation process in which existing white adipocytes undergo a shift in phenotype to resemble brown adipocytes(Park et al., 2014). This process, known as "brown-like" or "beige conversion," can be triggered by various factors, including cold exposure, exercise, and certain signaling molecules (Harms & Seale, 2013).

While beige fat shares some characteristics with traditional BAT, such as lowering systemic TGs, it is thought to be physiologically distinct from BAT, with differential expression of genes involved in metabolism, inflammation and transcription (Chait and den Hartigh 2020).

1.4 Adipocyte functions

The significance of AT becomes evident through its diverse functions, which include the distribution and storage of energy, thermogenesis, and the secretion of hormones and adipokines.

First, AT is a key organ for keeping the body's energy levels stable. Adipocytes' main function is to store the extra energy as TGs. The storage of energy in the form of triacylglycerols (TAGs) was the first known function of adipocytes (Knebel et al. 2019). The secretory functions of AT and the synthesis of adipocyte-specific proteins were not discovered until the mid-1980s. At that time, adipsin, a serine protease, was discovered to be secreted by cultured adipocytes and was found to be reduced in obese mouse models compared to lean littermates. Another function of AT is to produce acylation-stimulating protein, a member of the alternative complement family that is involved in lipid storage(Cianflone et al. 1999; Cook et al. 1987; Saleh et al. 2011). In this role, AT goes through hyperplasia to make more adipocytes and hypertrophy to enlarge them. This allows AT to grow when there is more food than necessary. When needed, such as during fasting or exercise, TGs stored in AT are broken down into fatty acids so the rest of the body can use them for energy. As a result, the quantity of stored triglycerides TGs constantly fluctuates, with hormonal fluctuations predominantly regulating the balance between energy storage and expenditure. As a central hub for energy balance, AT connects and caters to the energy requirements of various organ systems, including the liver, skeletal muscles, heart, pancreas, and brain (Pozo & Claret, 2018).

Second, although white adipose tissue (WAT) primarily functions to store energy, brown adipocytes possess the unique ability to efficiently burn fatty acids generated during adaptive thermogenesis(Castro et al., 2017). In mammals, BAT plays an important role in thermoregulation, as it is high in mitochondria and

expresses UCP1, allowing heat production by uncoupling ATP synthesis (Chait and den Hartigh 2020).

Finally, AT is now recognised as a key aspect of the endocrine system, capable of secreting adipokines, growth factors, cytokines and chemokines(Chait and den Hartigh 2020). Adipokines play vital roles in metabolic processes within metabolically active tissues, such as the liver, skeletal muscles, and brain. These bioactive molecules are involved in various activities, including oxidation of fatty acids, synthesis of new lipids, production or uptake of glucose (gluconeogenesis), insulin signaling, and expenditure of energy. Adipokines exert their effects on these tissues, influencing their metabolic functions and contributing to overall energy balance and regulation(Ren et al., 2022). Some examples of notable adipokines Leptin , Adiponectin, Resistin and Visfatin. Leptin is one of the most well-known adipokines. It is involved in regulating energy balance by suppressing appetite and increasing energy expenditure. Leptin acts on the hypothalamus in the brain to regulate food intake and body weight (Meier & Gressner, 2004). Adiponectin enhances insulin sensitivity and has anti-inflammatory properties(Febriza et al., 2019) and regulates glucose and lipid metabolism. Reduced levels of adiponectin are associated with insulin resistance and metabolic disorders such as obesity and type 2 diabetes (Jung & Choi, 2014). Resistin has been implicated in insulin resistance and inflammation(Kusminski et al., 2005). It is thought to play a role in the development of obesity-related insulin resistance and may contribute to chronic inflammation(Kusminski et al., 2005). Visfatin, also known as nicotinamide phosphoribosyltransferase (NAMPT), is an adipokine that is involved in the regulation of glucose metabolism and insulin sensitivity (El-Mesallamy et al., 2011; Sommer et al., 2008). It has been suggested to have a role in the development of insulin resistance and type 2 diabetes (El-Mesallamy et al., 2011; Sommer et al., 2008).

The adipose organ is a complex structure with highly plastic capabilities, including its parenchymal cells' ability to reprogramme their genes and transdifferentiate into cells with different appearances and physiology. It is envisaged that the adipose organ's adaptability will be used in the next

generation of therapeutic strategies to tackle the rising prevalence of metabolic illnesses, such as obesity and T2DM (Cinti 2012).

1.4.1 Adipogenesis

The transformation of preadipocytes into adipocytes is known as adipogenesis (de Sá et al. 2017). Adipogenesis follows a multi-phase process(Guru et al. 2020). The expression pattern of transcripts and proteins involved in adipogenesis is arranged according to the level of adipogenesis. Different transcription factors, such as PPAP-γ and C/EBP, are activated in adipocytes (Guru et al. 2020), and precursor cells cannot be separated from mature adipocytes without these elements (Guru et al. 2020). The expression pattern of the proteins and RNAs involved are coordinated based on the stage of adipogenesis (Guru et al. 2020; Huang et al. 2011). A transcription factor and additional adipogenesis regulator called sterol regulatory element binding protein (SREBP) controls FFAS and is involved in lipid metabolism (Huang et al. 2011).

The key early adipogenesis regulators are PPAP-γ and SREBP proteins, which control adipocyte differentiation(Guru et al. 2020; Hsu et al. 2012). In contrast, fatty acid binding protein 4 (FABP4), adiponectin, IL-6, leptin, GLUT4, cluster of differentiation 36 (CD36) and insulin receptor substrate (IRS1) are in charge of adipocyte formation(Hsu et al. 2012). Later adipocyte differentiation stages are determined by adipocyte-specific genes, such as FAS, FABP4 and acetyl-CoA carboxylase (ACC), and SREBP, PPAP-γ and C/EBP regulate the manufacture of the associated fatty acids and TGs (Hsu et al. 2012).

While adipogenesis is a fundamental process in developing AT, the excess energy and nutrition can contribute to adipocytes expanding in size and number, which is a leading cause of obesity. Increasing the size of extant adipocytes (hypertrophy) or differentiating new adipocytes to increase their number (hyperplasia) contributes to the abnormal expansion of WAT associated with obesity (Jakab et al. 2021; Lai et al. 2016). As a crucial factor in determining the number of adipocytes, it is a potential obesity treatment (Jakab et al. 2021). Therefore, it is essential to identify the molecular mechanisms underlying adipogenesis that could function as therapeutic targets. Furthermore, weight

loss is regarded as a significant health benefit (Jakab et al. 2021). Existing anti-obesity treatments have limited efficacy, potential adverse effects and drug interactions, which highlight an ongoing need for novel, effective and safe ingredients (Jakab et al. 2021). As a result, the project focused on the agonist and antagonist effects of the FFA4 receptor on adipogenesis.

1.4.2 Glucose transports

Glucose transporters are expressed in all cells (Navale and Paranjape 2016). For most living cells, glucose serves as a major source of energy. However, glucose molecules cannot pass through the cell's lipid membrane by simple diffusion because of their polar nature and size.

To demonstrate, the lipid bilayer that forms the cell membrane is made up of nonpolar fatty acid chains, creating a hydrophobic interior. This hydrophobic environment prevents polar molecules like glucose from easily crossing the membrane(*Biomembranes: Molecular Structure and Function - Robert B. Gennis - Google Books*, n.d.). The polar nature of glucose arises from the presence of hydroxyl (-OH) groups, which make it soluble in water. Since the interior of the lipid membrane is nonpolar, glucose molecules cannot pass through it without the help of specialized transporters(Pozo & Claret, 2018). Additionally, the size of glucose molecules is relatively large approximately 9 angstroms (Si et al., 2011) compared to the small molecules that can passively diffuse through the membrane. The cell membrane allows the passive diffusion of small nonpolar molecules, such as oxygen and carbon dioxide(N. J. Yang & Hinner, 2015). However, larger polar molecules like glucose require specific transport mechanisms to facilitate their entry into or exit from the cell(N. J. Yang & Hinner, 2015).

Therefore, a family of structurally-related transport proteins known as glucose transporters regulates the entry of glucose molecules into the cells. The group of structurally-related transport proteins can be categorized into two types: sodium-glucose linked transporters (SGLTs) and facilitated diffusion glucose transporters (GLUTs)(Pessin & Bell, 2003). The SGLTs consist of 14 transmembrane helices, with each helix having terminals oriented towards either the extracellular space or the COOH end(Tyagi et al., 2011). While the

SGLT family consists of proteins ranging in size from 60 to 80 kDa and having 580 to 718 amino acids(Navale and Paranjape 2016), GLUTs are membrane-spanning proteins with intracellular amino and carboxyl terminals. The amino acid sequence of GLUT proteins exhibits a range of 28% to 65% identity with GLUT1. Through analyses of multiple sequence alignments, this similarity has led to the identification of three subclasses of facilitative transporters (classes I, II, and III)(Mueckler & Thorens, 2013).

SGLT symports (transporters that move substances in the same direction) facilitate the co-transport of glucose and sodium ions (E. M. Wright et al., 2011). In order to transport glucose against its concentration gradient, SGLTs utilize the sodium concentration gradient established by the sodium-potassium ATPase as a source of chemical potential. These SGLTs are located on the luminal surfaces of cells lining the small intestine, where they facilitate the absorption of glucose from ingested food. Additionally, they are present in the renal tubules, where they assist in the reabsorption of glucose from the glomerular filtrate (E. M. Wright et al., 2011).

The GLUTs use a facilitated diffusion method to carry glucose across the plasma membrane (Navale and Paranjape 2016). For example, GLUT1 to GLUT4 are class I facilitative glucose transporters; each is expressed in different tissue and simulates according to different hormones or molecules. In contrast, GLUT2 is found mostly in beta cells of the pancreas, liver and kidney. In marine creatures, GLUT2 operates as a glucose sensor, while human beta cells primarily express GLUT1(Navale and Paranjape 2016). Additionally, GLUT3 is mostly found in the brain, whereas GLUT4 is a glucose transporter that is insulin-responsive and present in the heart, skeletal muscle, AT and brain. It is found in the cytoplasm of cells as vesicles, from which it is translocated to the plasma membrane by insulin (Navale and Paranjape 2016). The four members of the class II facilitative glucose transporters are GLUT5, GLUT7, GLUT9 and GLUT11 (Navale and Paranjape 2016). GLUT5 is predominantly a fructose transporter found in cells of the small intestine, testes and kidney, where it performs crucial physiological and pathological roles. GLUT7 has a high affinity for both glucose and fructose and is found in cells of the small intestine, colon, testes and prostate (Li et al. 2004).GLUT9, a member of the facilitative glucose transporter family, belongs to Class II of glucose transporters. It serves as a specialized transporter for urate, a

waste product of purine metabolism. GLUT9 facilitates the transport of urate across cell membranes, particularly in the kidney and liver (Doblado & Moley, 2009). While GLUT11 facilitates both glucose and fructose transport and has been found in the human heart, skeletal muscle, kidney, placental, AT and pancreatic cells (Navale and Paranjape 2016; Sasaki et al. 2001). The five known glucose facilitative transporters of class III are GLUT6, GLUT8, GLUT10, GLUT12 and GLUT13. GLUT6 is predominantly expressed in cells of the brain, spleen and peripheral leukocytes, while GLUT8 is primarily expressed in testicular germ cells (Godoy et al. 2006); GLUT8 is a glucose transporter with high affinity, whereas fructose and galactose inhibit this transport. Additionally, GLUT10 is found in the skeletal muscle, heart, lungs, brain, placenta, kidney, liver and pancreas. The cells of AT, the small intestine, skeletal muscle and the placenta express GLUT12 (Navale and Paranjape 2016).

Glucose transporters vary not only in locations and cell types but also in the hormones and initiators used for them to translocate to cell membranes (Navale and Paranjape 2016). For example, GLUT1 is associated with the bidirectional transport of glucose in hepatocytes, which is regulated by hormones, such as the thyroid hormone (Navale and Paranjape 2016). Another example is the insulin hormone, which simulates GLUT4 translocation but does not mediate GLUT8 and GLUT6 (Navale and Paranjape 2016).

Table 1-3 presents a summary of glucose transporters, their subclassification, characteristics, and tissue expression.

Table 1-3 Glucose Transporter Subclassification, Characteristics, and Tissue Expression

Glucose Transporter	Subclassification	Characteristics	Tissue Expression
GLUT1	Class I	glucose transport	human beta cells
GLUT2	Class I	transport of glucose and galactose	Liver, pancreatic ß-cells, small intestine
GLUT3	Class I	glucose transport	Neurons, placenta
GLUT4	Class I	Insulin-regulated transporter	Adipose tissue, skeletal muscle
GLUT5	Class II	Fructose transporter	Small intestine, testes
GLUT7	Class II	Fructose and glucose transporter	small intestine, colon, testes and prostate
GLUT9	Class II	Urate transporter	kidney proximal tubule
GLUT11	Class II	glucose and fructose transport	human heart, skeletal muscle, kidney, placental
GLUT6	class III	glucose transport	brain, spleen and peripheral leukocytes
GLUT8	class III	glucose transport	testicular germ cells
GLUT10	class III	glucose transport	skeletal muscle, heart, lungs, brain, placenta, kidney, liver and pancreas
GLUT12	class III	glucose transport	AT, the small intestine, skeletal muscle and the placenta express

1.4.2.1 GLUT4 pathway in adipose tissue

GLUT4 is distributed between the plasma membrane, the trans-Golgi network (TGN), endosomes and small heterogeneous vesicles consisting of endosomal system sorting intermediates and GLUT4 storage vesicles (GSV) (Leto and Saltiel 2012). Insulin treatment of muscle or adipose cells stimulates exocytosis of

GLUT4 from multiple intracellular compartments, resulting in an increase of GLUT4 at the plasma membrane for glucose transport into the cell (Leto and Saltiel 2012; Sheena et al. 2011).

Furthermore, GLUT4 primarily promotes insulin-stimulated glucose transport through translocation and activation mediated by the phosphatidylinositol 3-kinase (PI3K)/phosphorylated protein kinase B (p-Akt) pathway (Gandhi et al. 2014). The amount of GLUT4 in adipose cell plasma membrane (PM) is determined by a dynamic equilibrium between exocytosis and internalisation (Hou and Pessin 2007). Upon insulin activation, 50% of intracellular GLUT4 is translocated to the PM, resulting in a more than tenfold increase in the quantity of transporter on the cell surface (Lizunov et al. 2005).

This carefully regulated sequence of events is launched by a cascade of signalling molecules activated by insulin, and these signals must influence these compartments to trigger GLUT4 translocation (Thong et al. 2005).

The translocation of GLUT4 starts with the insulin receptor (IR). Insulin interaction induces conformational changes within the receptor and promotes the subunit's intrinsic tyrosine kinase activity. Then, the phosphorylated receptor attracts and phosphorylates on tyrosine residues of the insulin receptor substrate 1 (IRS-1), which subsequently recruits dimeric PI3 kinase via SH2 domains on the p85 subunit. After that, PI3 kinase catalyses the phosphorylation of phosphatidylinositol bisphosphate (PIP2) at the PM to PIP3, which is reversed by PTEN. The PIP3 then binds PIP3-dependent kinase (PDK) and Akt, allowing PDK to phosphorylate and activate Akt. Activated Akt further phosphorylates and deactivates the Rab10 GAP, AS160, allowing persistent Rab10 activation, which is essential for GSV trafficking to the PM and surface expression (Carmichael et al. 2019). This process is demonstrated in (Figure 1-1).

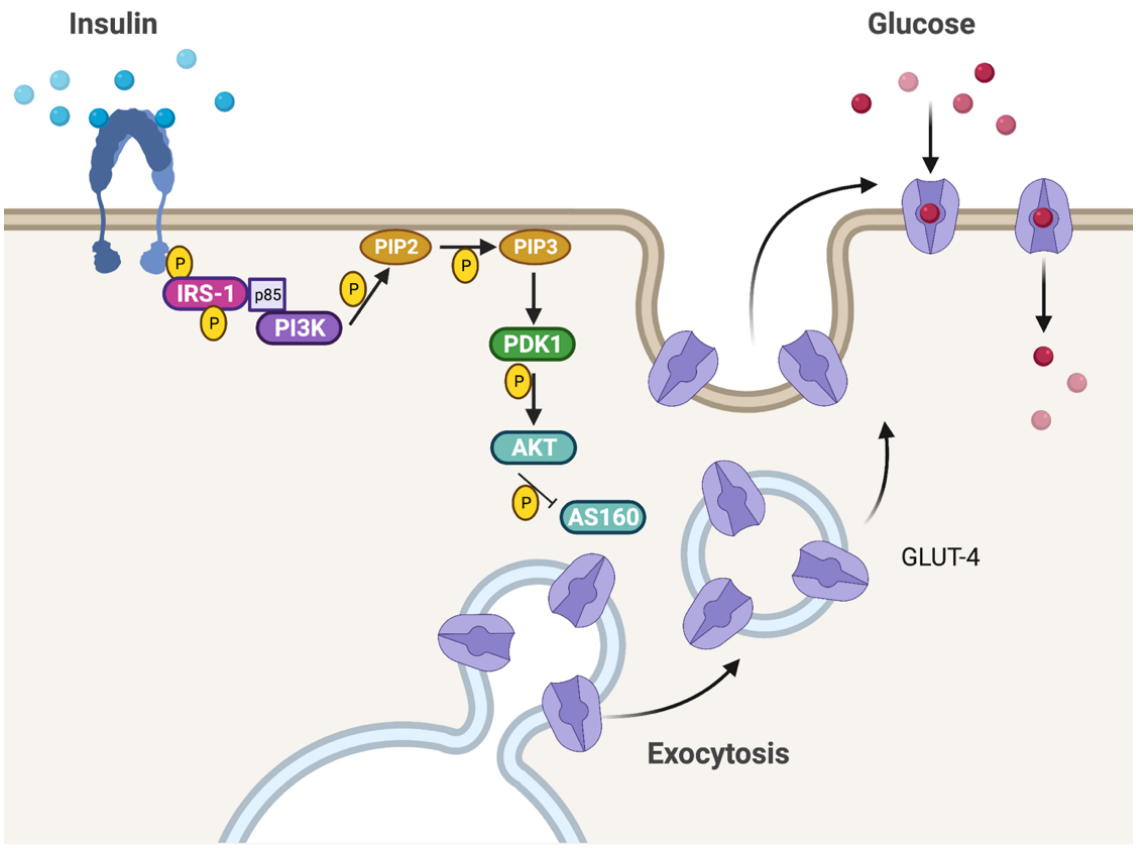


Figure 1-1 Glucose transport 4 pathway in adipocyte:
Upon binding with insulin, the insulin receptor, a type of receptor tyrosine kinase, undergoes dimerization and autophosphorylation. This phosphorylated receptor then triggers a cascade of events. It recruits and phosphorylates insulin receptor substrate 1 (IRS-1) on tyrosine residues. Subsequently, IRS-1 recruits dimeric PI3 kinase through SH2 domains on the p85 subunit. PI3 kinase catalyzes the phosphorylation of phosphatidylinositol bisphosphate (PIP2) at the plasma membrane, resulting in the formation of PIP3 (reversed by PTEN). PIP3 then acts as a docking site for PIP3-dependent kinase (PDK) and Akt. PDK phosphorylates and activates Akt. Once activated, Akt phosphorylates and deactivates AS160, a Rab10 GAP protein. This deactivation allows sustained activation of Rab10, which is crucial for the trafficking of GLUT4 storage vesicles (GSVs) to the plasma membrane, leading to the surface expression of GLUT4. Created with BioRender.com

1.4.3 Lipolysis

As an endocrine organ, WAT plays an important role in the regulation of metabolic homeostasis by generating a variety of factors, including leptin, adiponectin and tumour necrosis factor α (TNFα) (Gotoh et al. 2007). In periods of excess energy, the WAT stores TAG as the most efficient form of energy storage. Frequently, abnormal secretion of these factors causes metabolic disorders (Gotoh et al. 2007).

Lipolysis can be defined as the metabolic mechanism that catabolises TAG in cellular lipid droplets. The hydrolysis of TAG produces non-esterified FFAs, which are then employed as energy substrates, necessary precursors for lipid and membrane production or mediators in cell signalling processes (Lass et al. 2011). Lipolysis occurs in almost all tissues and cell types, but it is most abundant in WAT (Lass et al. 2011).

The process starts with catecholamines and other hormones that stimulate lipolysis in adipocytes during times of increased energy demand. The interaction of hormones with GPCRs raises the level of adenosine 3′,5′-monophosphate (cAMP) and activates cAMP-dependent protein kinase (protein kinase A) (Gotoh et al. 2007). Thus, GPCRs are crucial for numerous aspects of cellular function. They belong to a large protein family that shares structural motifs, such as seven transmembrane helices, and can activate heterotrimeric G proteins, such as Gs, Gi and Gq. Several second messenger pathways, including modulation of cAMP production, the phospholipase C pathway, ion channels and mitogen-activated protein kinases, are activated and induced by the binding of ligands to GPCRs (Gotoh et al. 2007).

Studies show that selective activation of FFA4 inhibited lipolysis in primary white adipocytes (Satapati et al. 2017). Compound B, an FFA4 selective agonist, was administered orally to lean rodents, and plasma FFA and glycerol levels were measured (Satapati et al. 2017). At 0.5, 2, and 4 hours after administration of the FFA4 agonist, plasma FFA levels were significantly lower than in control rats (Satapati et al. 2017). The response of primary adipocytes isolated from FFA4 KO mice was compared to wild mice of the same age and gender. Dose-dependent suppression of glycerol release by compound B was observed in adipocytes from

WT mice, whereas the effect was significantly diminished in adipocytes from FFA4 KO mice. This confirmed that compound B acts by activating FFA4, and the effect is mediated by the FFA4 receptor (Satapati et al. 2017). In agreement with this finding, Husted et al. (2020) stated that compound A, a selective FFAR4 agonist, suppressed isoproterenol-induced intracellular Cyclic adenosine monophosphate cAMP build-up in primary cultures of murine white adipocytes similar to the antilipolytic control drug nicotinic acid. In the same study, findings showed that in fasting lean mice, ingesting compound B reduced the level of circulating non-esterified FAs (NEFAs) to a similar extent as nicotinic acid. In line with the known anti-lipolytic activity of FFAR4, plasma NEFAs and glycerol levels were higher in FFAR4-deficient mice compared to littermate controls. This process is summarised in (Figure 1-2).

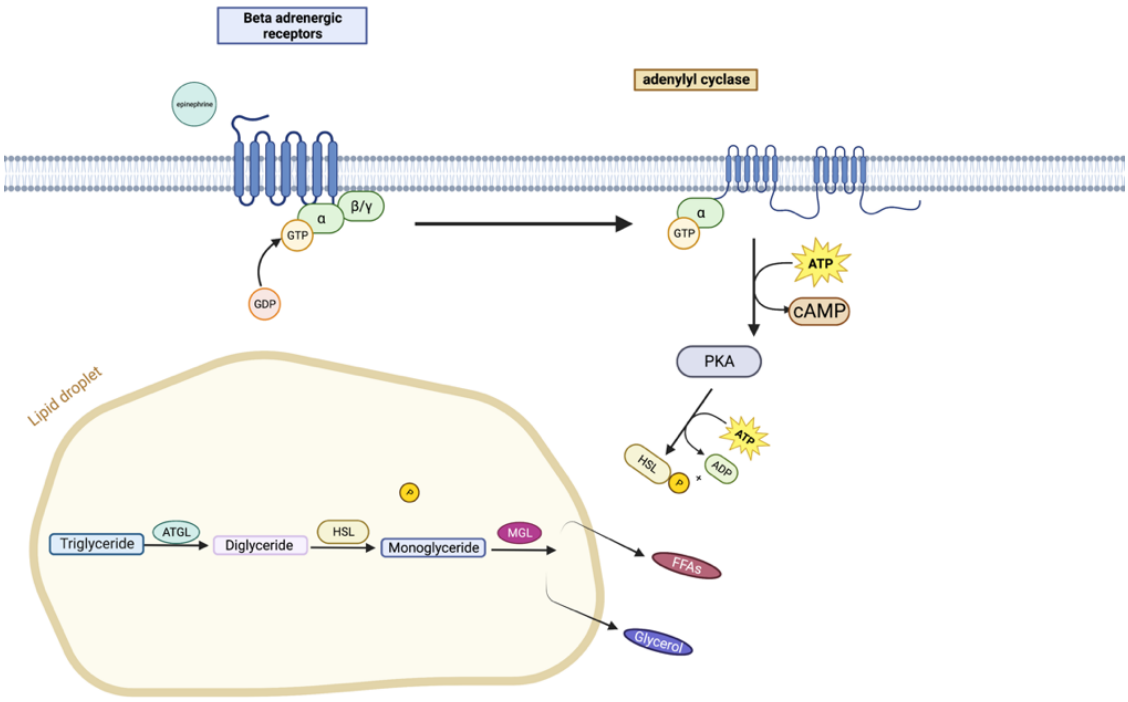


Figure 1-2 Lipolysis pathway in adipocyte:
The lipolysis pathway in adipocytes begins with the stimulation beta adrenergic receptors on the cell surface by adrenaline) or norepinephrine. This stimulation activates hormone-sensitive lipase (HSL), the key enzyme involved in lipolysis. HSL then catalyses the hydrolysis of stored triglycerides within lipid droplets, breaking them down into free fatty acids and glycerol.

1.5 Adipogenesis study models

The models to study and understand adipogenesis are under constant development. These models can be divided into two categories: *in vivo* and *in vitro* models.

1.5.1 *In vivo* models

The implantation of precursor cells in athymic rodents is one of the earliest *in vivo* methods used to examine adipocyte development (de Sá et al. 2017). Athymic rodents are a strain with a genetic mutation that results in thymic dysfunction and immunosuppression (de Sá et al. 2017). These mice are valuable for scientific research because they do not reject cells from mice, humans or other species (Ruiz-Ojeda et al. 2016). For this reason, they have been used to investigate the development of adipocytes in whole animals. Over three decades ago, the implantation of 3T3-F442A preadipocytes into athymic rodents produced ectopic fat at the injection site(de Sá et al. 2017; Green and Kehinde 1979). Despite the ectopic fat pads generated in athymic mice being vascularised, an apparent limitation of these experiments is that the effects observed may not be representative of what occurs in mice with a functional immune system (de Sá et al. 2017).

Another method is the generation of whole-body and tissue-specific knockouts, which has significantly increased our understanding of *in vivo* adipocyte development. The majority of these animal models express transgenes throughout their entire lives, including during gestation. Therefore, the mice's phenotype can be linked to events that occurred during development. In more complex transgenic models, inducible expression or knockout of a specific transcription factor in the adult animal has been utilised (de Sá et al. 2017).

Another effective *in vivo* technique is lineage tracing. In lineage tracing, a single cell is marked so that the mark is passed on to the cell's offspring. This yields numerous labelled clones that provide information regarding the number of progeny of the progenitor cell, their location and their differentiation status (de Sá et al. 2017; Kretzschmar and Watt 2012). For example, this method studies the origin and cellular transformation of white, brown and beige adipocytes during adipogenesis (Sanchez-Gurmaches and Guertin 2014).

1.5.2 *In vitro* models to study adipogenesis and the 3T3-L1 cell line

Various animal and human cell culture models are available for investigating the *in vitro* adipogenic differentiation process in relation to obesity and its

comorbidities (de Sá et al. 2017; Ruiz-Ojeda et al. 2016). Historically, rodent cells have been the most used, although feline and porcine cells have also been utilised to a lesser degree (Ruiz-Ojeda et al. 2016).

Multiple established cell lines consist of pluripotent fibroblasts that can differentiate into myocytes, chondrocytes and adipocytes, among others (de Sá et al. 2017). The cells most frequently used to investigate adipogenesis are fibroblast-like preadipocytes that are committed to differentiating into adipocytes; these include 3T3-L1, 3T3-F442A, OP9, 1246, Ob1771 TA1 and 30A5 preadipocytes (de Sá et al. 2017; Ruiz-Ojeda et al. 2016).

Many insights into the biology of adipocytes have been gleaned from cell culture models, most notably 3T3-L1 cells (Wolins et al. 2006). Both 3T3-L1 cells and 3T3-F442A cells are derived from disaggregated 17- to 19-day-old Swiss 3T3 mouse embryos and have a fibroblast-like morphology that can acquire an adipocyte-like phenotype under the appropriate conditions (Armani et al. 2010; Green and Kehinde 1976; Green and Meuth 1974; Ruiz-Ojeda et al. 2016).

To convert 3T3-L1 cells from their fibroblast phenotype into adipocytes, they must be treated with adipogenic agents such as insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX), which increase intracellular cAMP levels in the presence of foetal bovine serum (Caprio et al. 2007). This differentiation process was demonstrated in a report by Zebisch et al. that using rosiglitazone as an extra adipogenic agent, 3T3-L1 cells can develop in 10 to 12 days and remain that way for at least 10 cell culture passages (Zebisch et al. 2012). The cells should begin to accumulate lipids in the form of lipid droplets about four days following the addition of the agents; these droplets should increase in size and number over the course of cultivation(Zebisch et al. 2012). Triglyceride synthesis and lipid build-up cause the 3T3-L1 adipocyte's shape to change, giving it the appearance of an adipose cell signet ring (Guru et al. 2020). The build-up of lipid droplets in 3T3-L1 adipocytes fully differentiated within 10 to 12 days (Guru et al. 2020). At this stage, the 3T3-L1 cell line's high level of morphological and functional differentiation *in vitro* makes it a good model for examining intracellular transport, anti-adipogenesis and therapeutic targeting (Guru et al. 2020).

Those features are the reason why 3T3-L1 cells have been used extensively to assess the effects of substances or nutrients on adipogenesis, define the underlying molecular mechanisms of adipogenesis and assess the potential use of various substances and nutrients in the treatment of obesity (Kang et al. 2016; Lai et al. 2016; Tutino et al. 2016).

The key benefits of 3T3-L1 cells are that they are less expensive to utilise and easier to culture than newly separated cells, such as mature adipocytes (Ruiz-Ojeda et al. 2016). They are homogeneous in terms of cell population and can withstand a greater number of passes. As a result, these cells respond uniformly to treatments and modifications of the experimental environment (Poulos et al. 2010).

The 3T3-F442A cells are also derived from Swiss 3T3 mouse embryos and can accumulate lipid, as early exposure to glucocorticoids, such as DEX (Poulos et al. 2010), is not required to initiate their adipogenic differentiation (Ruiz-Ojeda et al. 2016). Using 3T3-F442A cells, researchers have examined the effects of compounds on the differentiation process. In addition, gene silencing via small interfering RNA (siRNA) has been used to investigate the role of alkaline phosphatase in lipid metabolism, gene expression and adipokine secretion (Hernández-Mosqueira et al. 2015). Regarding *in vitro* adipogenic differentiation research, the 3T3-F442A cell line has been utilised considerably less frequently than 3T3-L1 cells (Ruiz-Ojeda et al. 2016). The 3T3-L1 cell line model can accomplish adipogenesis. In the 3T3-L1 fibroblast cell line, adipocyte characteristics, such as expression of adipocyte markers and lipid aggregation, are chemically induced. In experimental investigations, 3T3-L1 differentiation is an efficient and convenient method for obtaining adipocyte-like cells (Guru et al. 2020).

Another cell model is the OP9 mouse stromal cell line, which is a novel adipocyte cell culture model (Ruiz-Ojeda et al. 2016). This cell line was derived from the calvaria of rodents born without functional macrophage colony-stimulating factor in their genes (Wolins et al. 2006). These OP9 cells are stromal cells derived from mouse bone marrow that accumulate large

triacylglycerol-filled vesicles after 72 hours of adipogenic stimulation, making them an appropriate model for high-throughput screening (Wolins et al. 2006).

The explored adipocyte cell is the budling unit for AT (de Sá et al. 2017). This was explored in both *in vitro* and *in vivo* models. Using 3T3-L1 cells allows tracking and recording of the compound being studied during 3T3-L1 differentiation since it takes place over a few days, as mentioned in. Several compounds or nutrients that have been shown *in vivo* to inhibit adiposity have been studied for their mechanisms of action during the differentiation process using 3T3-L1 cells (Mammi et al. 2016). Moreover, during the differentiation of 3T3-L1 cells, several endocrine disruptors and obesogenic compounds have also been evaluated (Regnier et al. 2015), which is the main focus of this project.

3T3-L1 cells and OP9 cells offer distinct advantages and disadvantages in biological research. 3T3-L1 cells are widely available and relatively easy to culture and maintain in the laboratory. They are commonly used in adipogenesis research, making them suitable for studying adipocyte differentiation, lipid metabolism, and obesity-related mechanisms (Ruiz-Ojeda et al., 2016). However, one disadvantage of 3T3 cells is their lower sensitivity to differentiation stimuli, requiring extended culture periods to induce adipocyte differentiation (Ruiz-Ojeda et al., 2016). Additionally, 3T3 cells may not fully recapitulate the complexity of *in vivo* adipose tissue (Dufau et al., 2021). On the other hand, OP9 cells exhibit high sensitivity to differentiation signals, particularly valuable for studying hematopoietic cell development and differentiation (Figueiredo et al., 2015). They efficiently support the differentiation of pluripotent stem cells into various hematopoietic lineages and can maintain hematopoietic stem cells in culture(Vodyanik et al., 2005). However, a drawback of OP9 cells is their specialization for hematopoietic differentiation, limiting their applicability to studies outside the hematopoietic lineage (Cichocki et al., 2023). Moreover, OP9 cells needs careful handling and specific culture conditions are required to maintain their differentiation-supporting properties (Yamashita et al., 2009).

Most of the research used the 3T3-L1 cell line with the FFA4 receptor or FAs (Huang et al. 2006; Hudson et al. 2013; Koh et al. 2021; Morrison and McGee

2015; Pei et al. 2022; Perera et al. 2003; Reusch et al. 2000; Trigatti et al. 1991),which can be a direct comparison of the finding as a first step in the project. Using another cell line may involve a future study.

1.6 G Protein-Coupled Receptors

The most successful family of drug targets are GPCRs due to their relevance in health and disease, as well as their potential for therapeutic intervention by small compounds acting as regulators (Hauser et al. 2017). A high number of GPCR-targeted drugs have been studied in diabetes and obesity clinical trials, with 27 and seven agents targeting GPCRs, respectively (representing 10% of the total number of agents in clinical trials) (Hauser et al. 2017). The increasing market share of medications for metabolic diseases is evident in the substantial number of GPCR-targeted agents undergoing clinical trials for diabetes and obesity. In these trials, there are 27 agents targeting GPCRs for diabetes and seven for obesity, collectively accounting for approximately 9% of the total number of agents being investigated.

Diabetes mellitus affects an estimated 415 million individuals globally, with 90% of cases being type 2 diabetes (Brennan et al., 2019). Unlike type 1 diabetes, which requires regular insulin injections due to insufficient insulin production by the pancreas, type 2 diabetes can be managed with medications that stimulate insulin secretion or enhance insulin sensitivity (Tan et al., 2019).

The first GPCR-targeted drug for type 2 diabetes, exenatide, was approved in 2005(Davenport et al., 2020). Several other peptidic GLP1 receptor agonists, such as liraglutide, lixisenatide, dulaglutide, and albiglutide, have since been approved, primarily administered via injections(Latif et al., 2021). However, semaglutide, a once-weekly GLP1 analogue, demonstrated improved glycemic control in a phase II trial and is now being tested for oral dosing in phase III trials(Rasmussen, 2020). Advances in small-molecule screening techniques have identified non-peptide agonists, such as TTP273, that target the GLP1 receptor and are currently in phase II trials (Deng et al., 2020).

The challenges associated with managing type 2 diabetes and related complications like diabetic neuropathy and foot ulcers have prompted further

investment in GPCR-targeted agents (Deng et al., 2020). One such agent is MBX-2982, a small-molecule GPR119 agonist currently in phase II trials, which increases both insulin secretion and GLP1 release(Ritter et al., 2016). The FFA1 receptor is another novel target for stimulating insulin secretion, although the development of the small-molecule agonist fasiglifam was discontinued due to hepatotoxicity(Kaku et al., 2015). Additionally, the dopamine D2 receptor is being explored as a novel target for diabetes treatment. Bromocriptine, the first dopaminergic agent, was recently approved for improving glycemic control and glucose tolerance in type 2 diabetes (Lopez Vicchi et al., 2016).

In the realm of obesity, an encouraging candidate is setmelanotide, which acts as a selective melanocortin receptor agonist specifically targeting the MC4 receptor. Phase II trials investigating its effectiveness in treating rare genetic disorders associated with obesity have yielded promising results. As a result, setmelanotide has progressed to the phase III development stage (Hauser et al., 2017).

Additionally, RM-493 is an investigational drug that is being studied for its potential use in the treatment of obesity(Chen et al., 2015). It is a melanocortin 4 receptor (MC4R) agonist, similar to setmelanotide. MC4R is a receptor involved in regulating appetite and energy balance(Chen et al., 2015). phase II for this has been initiated (*Rhythm Initiates Phase 2 Clinical Trial of RM-493 for Obesity | Fierce Biotech*, n.d.).

Also, Cannabinoid receptor type 1 (CB1) receptor activity is being studied to regulate appetite and metabolism for weight management by antagonist TM38837 at phase 1 clinical trial (Klumpers et al., 2013). Additionally, GLP-1 receptor have been targeted to developed an anti-obesity drugs by using agonists of GLP-1R (C. Liu et al., 2020). Promising results have emerged from both animal experiments and clinical trials, indicating that GLP-1R agonists exhibit enhanced effectiveness in treating or preventing obesity (J. Y. Wang et al., 2023). For example, Tirzepatide, a synthetic peptide consisting of 39 amino acids, has emerged as a novel dual GIP/GLP-1 receptor agonist based on the native GIP sequence (Min & Bain, 2021). Preclinical trials, as well as phase 1 and 2 clinical trials, have demonstrated that tirzepatide exhibits robust glucose-

lowering effects and promotes weight loss, with side effects comparable to established GLP-1 receptor agonists (Min & Bain, 2021).

Targeting the Ghrelin receptor is considered a viable approach for treating obesity. Ghrelin is responsible for stimulating appetite and can be regulated by focusing on the Ghrelin receptor (Colldén et al., 2017). Modulating the Ghrelin receptor holds the potential to effectively manage hunger, leading to weight loss (Colldén et al., 2017). appetite and block nutrient absorption, potentially reducing weight gain (Altabas & Zjačić-Rotkvić, 2015). As a Therapeutic vaccines and drugs targeting appetite-stimulating hormones, including ghrelin, are being studied for obesity treatment. The ghrelin receptor plays a crucial role in regulating appetite and metabolism, and anamorelin is a new oral ghrelin receptor agonist and it reached phase 1 of clinical trial (Blum et al., 2019).

Neuropeptide Y receptor receptors are involved in appetite stimulation, and their modulation is being explored for appetite control and weight management by simultaneously targeting several systems—of which the NPY system is likely to be a prime component (Beck, 2006). Despite efforts, the development of drugs has proven challenging, and no successful clinical applications have been achieved thus far(Tang et al., 2021).

Orexin receptors play a role in regulating sleep-wake cycles and appetite and targeting these receptors may have implications for obesity treatment (Couvineau et al., 2019). By pharmacologically inhibiting OX-A receptor type 1, the negative effects on α-MSH signaling, as well as the resulting hyperphagia, obesity, and steatosis, were effectively countered (Morello et al., 2016). By pharmacologically inhibiting OX-A receptor type 1, the negative effects on α-MSH signaling, as well as the resulting hyperphagia, obesity, and steatosis, were effectively countered (Morello et al., 2016). Additionally, Serotonin receptors are involved in appetite regulation, and specific subtypes, such as the 5-HT2C receptor, are being investigated for their potential in weight management (Yuen et al., 2020).

Table 1-4 provides a summary of clinical trial agents being tested as medications for diabetes and their corresponding GPCR targets

Table 1-4 Clinical Trial Agents targeting GPCRs for diabetes and obesity :

Agents	Target	Clinical trials	Metabolic disorder
Exenatide	GLP1 receptor agonists	approved	Diabetes
Liraglutide		approved	
Lixisenatide		approved	
dulaglutide,		approved	
Albiglutide		approved	
Semaglutide	GLP1 analogue	tested for oral dosing in phase III trials.	
TTP273	an orally bioavailable non-peptide agonist of the GLP1 receptor	currently in phase II trials	
MBX-2982	a small-molecule GPR119 agonist	Phase II	
TAK-875	agonist fasiglifam on FFA1	Stopped due to due to hepatotoxicity	
bromocriptine	dopamine D2 receptor	approved	
Setmelanotide	melanocortin-4 receptor (MC4R)	Phase III	Obesity
RM-493		Phase II	
TM38837	Cannabinoid receptor type 1 (CB1) receptor	Phase I	
Tirzepatide	GLP-1 receptor agonist	Phase I and Phase II	
Anamorelin	ghrelin receptor agonist	Phase I	

Approximately 30% of the existing therapeutic treatments available in the market specifically focus on targeting these receptors(Piper et al., 2022). Furthermore, these receptors are also the target of over a quarter of the top 100 best-selling medications, generating annual revenues in the billions of dollars (J. Liu et al., 2021; Midlam, 2021).

Regarded as one of the most effective therapeutic targets for a wide range of disorders, GPCRs mediate a variety of key physiological activities. With over 800 genes in the human genome, GPCRs, also known as seven transmembrane-spanning receptors, make up about 4% of the human genome that codes for proteins and are the largest family of cell surface proteins. Their primary function is to convert a wide range of extracellular stimuli, such as biogenic amines, peptides, hormones, neurotransmitters, ions, odorants and photons, into intracellular signals that regulate a variety of physiological processes, such as cell metabolism, differentiation, growth, neurotransmission and sensory perception(Nieto Gutierrez and McDonald 2018).

All GPCRs have seven helices that cross the membrane and are connected by three intracellular loop regions and three extracellular loop regions. They also have an amino-terminal domain on the outside and a carboxyl tail on the inside. When a ligand binds to the extracellular domains of a receptor, the receptor changes shape in a way that is specific to the ligand. This allows the receptor to bind to its cognate G-protein (heterotrimeric guanine nucleotide-binding protein) at the plasma membrane. This causes the G-protein subunit to release guanosine diphosphate in exchange for guanosine triphosphate (GTP) and the GTP-bound α-subunit to separate from the - βγ-dimer (Nieto Gutierrez and McDonald 2018).

Typically, the orthosteric binding site of endogenous ligands is in the upper extracellular region of the receptor, beneath extracellular loop 2 (de Felice et al. 2021).At the intracellular level, GPCRs interact with the G-protein heterotrimer complex (G) via a process that is allosterically controlled by ligand-induced conformational changes that activate a specific signal cascade based on the kind of interacting G-protein (Gs, Gi, Go, Gq/11, G12/13)(de Felice et al. 2021; Kamato et al. 2015). Following this, GPCRs are phosphorylated by specific GPCR kinases in the presence of continuous agonist stimulation. The binding of β-arrestins to the phosphorylated GPCRs stops G-protein signalling and leads to a coordinated process of desensitisation, inactivation and internalisation. The β-arrestins also help form multi-molecular complexes and provide a way for G-protein-independent signalling of GPCRs, such as those involving mitogen-

activated protein kinases, receptor and non-receptor tyrosine kinases, PI3K and others (Zhang and Xie 2012).

The GPCR superfamily is a prime target for therapeutic intervention, as GPCR signal transduction is essential for most physiological systems, including vision, smell and taste, as well as neurological, cardiovascular, endocrine and reproductive functions (Katritch et al. 2013). Continuing efforts in drug development are directed towards improving therapeutics for numerous recognized GPCR targets and expanding the range of GPCRs amenable to targeting (Saikia et al., 2018). Trends in modern pharmacology include the discovery of allosteric and/or functionally selective modulators, in addition to modulating GPCR signal activation with agonists and inhibition with antagonists and inverse agonists (Mantas et al., 2021)

1.6.1 GPCRs in drug discovery

As pharmacological targets, GPCRs have long been of particular interest because they control many different physiological processes and have druggable sites on the cell surface(Hauser et al. 2017). Medicines that target GPCRs account for approximately 27% of the global therapeutic medication market (Hauser et al. 2017). The FDA recently reported 70%, 33%, and 25–30% success rates for all target GPCR families in stages I, II and III, respectively (Hauser et al. 2017).

With crystal structures of 43 distinct receptors and 196 ligand complexes, there is a wealth of templates for structure-based drug discovery and design(Hauser et al. 2017). Modification of GPCRs via allosteric regions distinct from the native ligand binding site might modify the structure, dynamics and function of the receptor to gain a potential therapeutic advantage, such as enhanced spatial and temporal selectivity (Hauser et al. 2017).

Examples of GPCRs as drug targets include ß1-adrenoceptor (ß1AR) antagonists, which are used to treat cardiovascular illness (beta blockers), and ß2AR agonists, which are used to treat asthma (Congreve et al. 2020).

1.7 Free fatty acid receptors

It is now recognised that four GPCRs are activated by FFAs (Im 2013). Due to their physiological importance in numerous biological processes, the four free fatty acid receptors (FFARs) have generated significant attention to date (Kimura et al. 2020). Namely, they are FFA1 (previously known as GPR40) and FFA4 (previously GPR120), which are activated by medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs), whereas FFA2 (previously GPR43) and FFA3 (previously GPR41) are activated by short-chain fatty acids (SCFAs) (Kimura et al. 2020).

Each FFAR can function as an FFA sensor with selectivity for a specific FFA carbon chain length. These FFARs have been linked to physiological processes such as insulin and incretin hormone release, adipocyte differentiation, anti-inflammatory properties, neural responses and taste preferences. It is thought these FFAR physiological functions could govern energy and immunological homeostasis (Kimura et al. 2020). It is for this reason that FFARs have gained interest as therapeutic targets for illnesses of the immune system and energy metabolism (Weyand and Goronzy 2016).

1.7.1 FFAs and their types

Free fatty acids are carboxylic acids with long aliphatic chains that can be saturated or unsaturated (Kimura et al. 2020). Most natural FAs are composed of an unbranched chain with an even number of carbon atoms ranging from 4 to 28(Kimura et al. 2020). According to their length, FAs are categorised as SCFAs, MCFAs, LCFAs or very-long-chain fatty acids (VLCFAs), which have more than 22 carbon atoms (Ulven and Christiansen 2015). They can also be divided into saturated fatty acids (SFAs), monounsaturated fatty acids (containing a single cis-alkene) and polyunsaturated fatty acids (PUFAs) (containing several methylene-interrupted cis-alkenes) (Ulven and Christiansen 2015). Saturated FAs lack carbon–carbon double bonds, while unsaturated FAs have one or more such bonds (Kimura et al. 2020). The geometric disparities between various types of unsaturated FAs, as well as between SFAs and unsaturated FAs, play a crucial role in numerous biological processes and the building of biological structures, such as cell membranes (Kimura et al. 2020).

Free fatty acids are found in organisms as three types of esters: TGs, phospholipids and cholesterol esters(Kimura et al. 2020). To be produced, FFAs are generated by lipolysis from AT and other cell types. In addition to their traditional responsibilities as sources of energy and structural components, FFAs are emerging as active participants in a variety of biological processes. They are important, not only as sources of energy but also as signalling molecules that control different cellular processes and physiological functions, depending on the length of their carbon chains (Kimura et al. 2020).

1.7.2 Free fatty acid receptor 1

Free fatty acid receptor 1 was identified as the first GPCR to be activated by FFAs and first described as an uncharacterised 7-TMD sequence on chromosome 19q13 in humans. In 2003, three distinct investigations indicated that MCFAs and LCFAs activate the receptor (Sawzdargo et al. 1997;Briscoe et al. 2003).

Early research on FFA1 immediately identified pancreatic islets and, in particular, the ß-cells as having high levels of the receptor, but subsequent investigations have also revealed FFA1 expression in the cells of the islets (Briscoe et al. 2003;Tomita et al. 2006). Its primary role is to regulate glucose-dependent insulin secretion by mediating GLP-1 secretion (Xiong et al. 2013). In addition, FFA1 is expressed by numerous enteroendocrine cell types, including L cells that produce GLP-1, peptide hormone YY cells that release cholecystokinin and K cells that secrete gastric inhibitory peptide (Edfalk et al. 2008;Liou et al. 2011;Sykaras et al. 2012). Skeletal muscle, heart, liver, bone, brain and monocytes are among the other tissues where FFA1 has been reported to be expressed(Briscoe et al. 2003;Cornish et al. 2008; Kotarsky et al. 2003).

Signals from FFA1 activation are mostly transmitted via Gq/11-family G-proteins, and the pharmacological Gq/11 inhibitor YM-25489 has been utilised in multiple experiments to validate this finding (Fujiwara et al. 2005). Furthermore, FFA1 signals through Gs, Gi/o and ß-arrestin2 pathways but largely signals via Gq/11 G-proteins to increase insulin secretion(Itoh et al. 2003;Kotarsky et al. 2003;Shimpukade et al. 2012).

Free fatty acid receptor 1 is widely expressed by pancreatic ß cells, and because FAs are known, at least in acute conditions, to stimulate insulin secretion from pancreatic islets in a glucose-dependent manner, the effectiveness of synthetic small-molecule activators of FFA1 to imitate this action has been extensively explored(Milligan et al. 2017). Free fatty acid receptor 1 has been a promising pharmacological target for T2D, and several FFA1 molecules have entered clinical trials(Watterson et al. 2014). Of these, TAK875 (Fasiglifam) progressed to phase III clinical trials but was ultimately terminated due to liver toxicity (Defossa and Wagner 2014; Kaku et al. 2013;Shavadia et al. 2019).

1.7.3 Free fatty acid receptors 2 and 3

Human FFA2 and FFA3 SCFA receptors are activated by micromolar amounts of acetate, propionate and butyrate (Offermanns 2014). At a similar time frame to the diaphanization of FFA1, two additional 7-TMD sequences, GPR43 and GPR41, found on chromosome 19q13.1 in humans, were coupled with SCFAs as endogenous ligands (Sawzdargo et al. 1997). Investigation of the signalling pathways of FFA2 and FFA3 has revealed that FFA3 links largely, if not exclusively, to Gi/o-family G-proteins and is thus best appreciated for its ability to inhibit adenylyl cyclase to reduce intracellular cAMP levels(Brown et al. 2003;Stoddart et al. 2008). FFA2 receptors couple to Gαq/11 and Gαi/o G-proteins and ß-arrestin2(Hudson et al. 2012; Nilsson et al. 2003)

Earlier research revealed that several immune cells contain both SCFA receptors, with FFA2 being abundantly expressed in monocytes and polymorphonuclear cells (Brown et al. 2003). In the gut, both FFA2 and FFA3 are expressed largely by enteroendocrine cells (Nøhr et al. 2013). Expression of FFA2 and FFA3 in AT has also been observed, while the expression of FFA3 has been controversial, with much research identifying only FFA2 (Hong et al. 2005; Hudson et al. 2013).

1.8 Free fatty acid receptor 4

Hirasawa et al. discovered in 2005 that GPR120, an orphan GPCR, was activated by LCFAs(Hirasawa et al. 2004). This receptor was activated by a variety of LCFAs, particularly PUFAs. While FFA4 is activated by many of the same ligands as FFA1, in terms of sequence identity, it is only distantly linked to the other

FFA family members. However, numerous studies have conclusively proven that FFA4 is an LCFA receptor, and as such, it is now officially recognised as FFA4 and a member of the FFA family of receptors(Davenport et al. 2013).

These LCFAs act as endogenous ligands for FFA4 and play a significant role in regulating metabolic processes(Hara et al., 2013). Studies have indicated that activation of FFA4 by specific LCFAs can lead to beneficial effects on glucose homeostasis, insulin sensitivity, and anti-inflammatory responses(Im, 2018; Moraes-Vieira et al., 2016; Ulven & Christiansen, 2015). Additionally, FFA4 activation has been associated with improvements in lipid metabolism, adipogenesis, and the release of gut hormones involved in satiety regulation(Gribble et al., 2017; Im, 2018). Understanding the interaction between LCFAs and FFA4 can provide insights into potential therapeutic strategies for metabolic disorders, including obesity and type 2 diabetes. Further investigation into the molecular mechanisms and therapeutic potential of FFA4 agonists holds promise for the development of novel treatments targeting this receptor.

Endogenous activators of Free Fatty Acid Receptor 4 (FFA4) have garnered significant interest in recent research. FFA4 is a G protein-coupled receptor found in various tissues, including the gastrointestinal tract, adipose tissue, and immune cells(Im, 2018; Moniri, 2016; Vieira et al., 2016).

Several endogenous molecules have been identified as activators of FFA4, including long-chain fatty acids (LCFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are omega-3 polyunsaturated fatty acids (Calder & Yaqoob, 2009). It is an essential fatty acid, meaning that it cannot be synthesized by the human body and must be obtained through the diet or supplementation. EPA is abundantly found in fatty fish such as salmon(Calder & Yaqoob, 2009).Some of the biological functions associated with EPA's interaction with FFA4: anti-inflammatory effects, regulation of insulin sensitivity and glucose homeostasis, modulation of lipid metabolism, and regulation of gut hormone release(Flachs, 2014)

Additionally, Linoleic acid is an essential omega-6 polyunsaturated fatty acid that can activate FFA4. It is found in various vegetable oils and has been

associated with various metabolic and inflammatory processes (Saini & Keum, 2018; Takic et al., 2022). Also, Palmitic acid is a monounsaturated fatty acid that is naturally produced in the body. It has been shown to activate FFA4 and is involved in regulating insulin sensitivity and lipid metabolism(Im, 2018).Other endogenous ligands include certain monoacylglycerols, such as 2-arachidonoyl glycerol (2-AG), and the bile acid derivative, 3-oxo-α,ß,ß-trifluoro-4'-pyridinecarboxylic acid (TZP-101)(Fisk, 2021; Juárez-Hernández et al., 2016; Kimura et al., 2020). Table 1-5 presents summary of the most known endogenous activators of Free Fatty Acid Receptor 4 (FFA4), their associated biological functions.

Table 1-5 Endogenous Activators of FFA4 with Biological Functions

Endogenous Activator	Biological funcation	Reference
Eicosapentaenoic acid (EPA)	anti-inflammatory effects, regulation of insulin sensitivity and glucose homeostasis, modulation of lipid metabolism	(Flachs, 2014)
Linoleic acid	involvement in metabolic and inflammatory processes	(Kapoor & Huang, 2006)
Palmitic acid	regulation of insulin sensitivity and lipid metabolism	(Im, 2018)

Activation of FFA4 by these endogenous activators has been associated with a range of physiological effects, including anti-inflammatory responses, regulation of glucose homeostasis, insulin sensitivity, lipid metabolism, and satiety regulation. Understanding the role of endogenous activators of FFA4 contributes to the broader understanding of the receptor's function and its potential as a therapeutic target for metabolic diseases (Freitas & Campos, 2021; Milligan, Alvarez-Curto, et al., 2017). Further research is needed to explore the full spectrum of endogenous activators and their specific roles in FFA4-mediated signaling pathways.

1.8.1 FFA4 expression

FFA4 was initially identified to be expressed at the highest levels in the lower intestine, lung, spleen and AT in human, mouse and rat studies (Milligan et al.

2017). Detailed research on FFA4 expression in the intestine revealed that enteroendocrine cells on each of the L, K and I cells express FFA4(Milligan et al. 2017). It is expressed more extensively in the intestine, including in intestinal epithelial cells as well as enteroendocrine cells (Milligan et al. 2017). Furthermore, FFA4 is expressed in colon, pancreas, AT, brain, thymus, skeletal muscle, lung and spleen enteroendocrine cells (Miyauchi et al. 2009), and it is also expressed in multiple immune cells, such as macrophages and monocytes, that are linked to FFA4 anti-inflammation action (Oh et al. 2010, 2014).

1.8.2 FFA4 signalling

FFA4 has been reported to signal through three primary pathways (Figure 1-1). Several studies have shown that FFAR4 is coupled with Gq proteins and that activation of FFAR4 results in increased intracellular Ca^{2+} levels(Kimura et al. 2020;Milligan et al. 2017). There is additional evidence that certain signals related to FFA4's physiological role reflect the activation of Gi/o-family G-proteins(Barkan, 2017) .For example, it blocks the FFA4-mediated regulation of ghrelin secretion from ghrelin-containing mice gastric cells as well as inhibiting somatostatin release from pancreatic islet delta cells, implying the existence of such a route (Milligan et al. 2017). Moreover, the internalisation of the receptor from the surface of transfected cells was a crucial experiment used to identify LCFAs as agonists of FFA4. Indeed, when an agonist is bound, FFA4 internalises quickly and significantly (Hudson et al. 2013). This is connected to the receptor's strong, agonist-induced association with Arrestins 2 and 3, which is largely determined by the phosphorylation status of several serine and threonine residues in the receptor's intracellular C-terminal tail (Milligan et al. 2017).

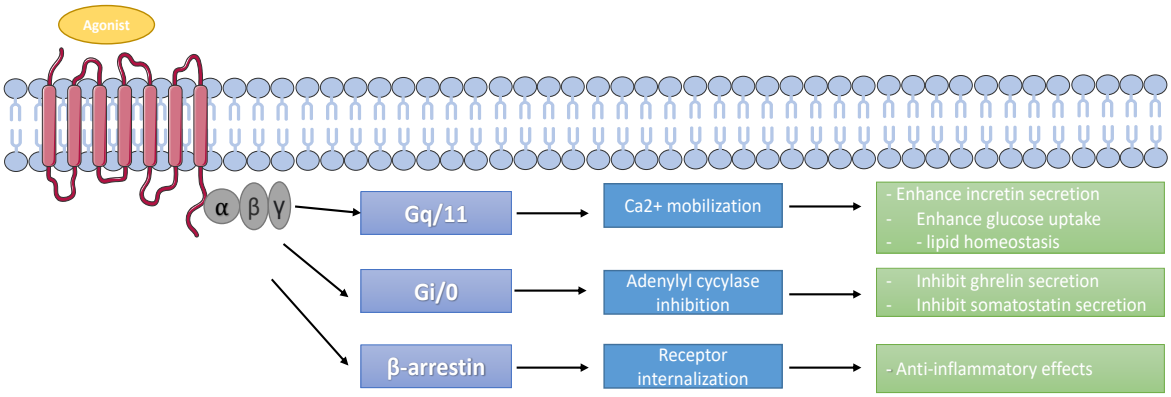


Figure 1-3 Free fatty acid receptor 4 (FFA4) signalling pathway:

Free fatty acid receptor 4 (FFA4) signals via Gαq/11 and Gαi G-protein signalling, in addition to coupling to β-arrestin2. Ligands (yellow) bind to FFA4, and this stimulates G-protein binding to the GPCR. After FFA4 expression in heterologous cell systems, agonist-induced interactions with members of the Gq/G11 G-protein family are found, resulting in increased intracellular [Ca2+]. This route is also important for many of FFA4's physiological effects. However, ghrelin release is sensitive to pertussis toxin therapy, indicating a function for Gi-family G-proteins in this outcome. Several efforts to develop synthetic FFA4 agonists have used receptor β-arrestin interaction assays, and essential physiological roles for such arrestin-mediated interactions include modulation of macrophage anti-inflammatory mediator production.

1.8.3 FFA4 isoforms

Free fatty acid receptor 4 can exist in two forms, which appear to be species dependent. The short isoform contains 361 amino acids, whereas there is also a splice variant containing an additional 16 amino acids inside ICL3, known as the long isoform(Moore et al. 2009). The human FFA4 gene can be alternatively spliced to yield either a 361-amino acid protein (FFA4-S) or a longer isoform (FFA4-L) that retains exon 3 to encode for an additional 16 amino acids within the third intracellular loop, a receptor domain that affects intracellular signalling and desensitisation(Burns and Moniri 2010; Moore et al. 2009). To date, expression of FFA4-L has been discovered solely in the human colon or colon epithelial cell lines, and the colon expresses the greatest FFA4 transcript in the gastrointestinal tract(Galindo et al. 2012;Hirasawa et al. 2005; Kim et al. 2015). It is not detected in rodents, felines, canines, bovines or nonhuman primates, indicating that this isoform is a separate human gene product (Moore et al. 2009;Song et al. 2015; Takemitsu et al. 2015).

Prior research has established that the two receptor isoforms respond similarly to FFA agonists (Burns and Moniri 2010; Galindo et al. 2012; Moore et al. 2009).

Similar to other Gq/11-coupled GPCRs, FFA4-S agonism with FFAs induces robust Ca^{2+}-mobilisation and downstream activation of PKC or ERK1/2, as well as G-protein independent effects via recruitment of -arrestin-2 partner proteins, which are capable of scaffolding other signalling molecules (Burns et al. 2014; Hudson et al. 2013; Oh et al. 2010; Watson et al. 2012). In contrast, agonism of native FFA4-L does not promote Ca^{2+}-mobilisation but preserves ß-arrestin-2 recruitment, indicating that the additional 16 amino acids inside the third intracellular loop of FFA4-L oppose intrinsic G-protein coupling to this isoform (Watson et al. 2012).

1.8.4 FFA4 agonists and antagonists

Several investigations have now conclusively proven that FFA4 is activated by LCFAs (Milligan et al. 2017). A variety of synthetic agonists have been created, though, with the hypothesis that therapeutic benefits may result from regulating the activity of FFA4. Many of these agonists were created with the intention of treating metabolic disorders, including T2DM. Given the closeness between FFA1 and FFA4's activating ligands, it is not surprising that many early FFA4 agonists were also agonists for FFA1(Milligan et al. 2017). Initially, a synthetic FFA1 agonist, GW9508, was discovered to also have activity on FFA4, but with a much lower potency than it has for FFA1(Briscoe et al. 2006). In the absence of FFA4-selective synthetic agonist ligands, GW9508 was used as an FFA4 agonist in multiple experiments employing cells and tissues that lacked measurable quantities of co-expressed FFA1 (Milligan et al. 2017).

To characterise agonists selective for FFA4 exclusively, peroxisome proliferator-activated receptors PPAR-γ active compounds were changed to produce a ligand, 4-4-[2-(phenyl-pyridin-2-yl)-amino)-phenyl-butyric acid, which was later designated NCG21 (Shimpukade et al. 2012). However, this compound has only modest potency and moderate selectivity for FFA4 over FFA1 (Milligan et al. 2017). Since it has been shown to stimulate FFA4, it is not surprising that chemically altering these agonists results in robust FFA4 activation (Grygiel-Górniak 2014). Nonetheless, given that this chemical was still an FFA1 activator, it was evident that novel FFA4 compounds that were more selective and powerful were required (Suzuki et al. 2008).

The first reported potent and selective FFA4 agonists were identified by screening representatives from a collection of known FFA1 agonists (Shimpukade et al. 2012). This ligand 4-[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy-benzenepropanoic acid (TUG-891) was the first significant advance in generating FFA4-selective agonist ligands (Milligan et al. 2017). In experiments based on the induced contacts between the receptor and ß-arrestin 2, this compound demonstrated high efficacy in both human and mouse FFA4 and 1,000-fold selectivity over human FFA1(Milligan et al. 2017). TUG-891 exhibited signalling features similar to the LCFA α-linolenic acid at human FFA4, including stimulation of Ca^{2+} mobilisation, ß-arrestin-1 and ß-arrestin-2 recruitment and extracellular signal-regulated kinase phosphorylation (Hudson et al. 2013). It has also been reported to be a potent molecule in human and mouse FFA4 but with reduced selectivity over mouse FFA1(Hudson et al. 2013).

Efforts to produce a selective agonist for FFA4 did not stop there. Oh et al. (2014) demonstrated that a small molecule FFA4 agonist developed by Merck, Compound A, is selective for FFA4 vs FFA1, and this compound was used to demonstrate its effects in obese mice (Oh et al. 2014). The study described Compound A as a powerful anti-inflammatory and a selective high-affinity, orally absorbable small molecule FFA4 agonist, has on macrophages *in vitro* and in obese mice *in vivo*. Treatment with an FFA4 agonist improves glucose tolerance, reduces hyperinsulinemia, boosts insulin sensitivity and reduces hepatic steatosis in obese mice fed a high-fat diet (HFD). As a result, it is possible that FFA4 agonists will one day be used to treat T2D and other conditions where the body is resistant to insulin (Oh et al. 2014).

A mutational study of FFA4's orthosteric binding site revealed that Arg99 was critical to the activity of carboxylic residues present in both LCFAs and many synthesised FFA4 ligands (Shimpukade et al. 2012). That is why all the synthetic agonists described above contain carboxylic groups that mimic those of endogenous agonists. However, a few classes of non-carboxylic acid FFA4 agonists have now been developed, including GSK137647A and TUG-1197.

The study of diarylsulfonamide FFA4 agonists by GlaxoSmithKline led to the discovery of a selective FFA4 agonist GSK137647A [4-methoxy-N-(2,4,6-trimethylphenyl) benzenesul fonamide (Sparks et al. 2014). It has been found in

recent research that GSK137647A showed greater than 50-fold selectivity for FFA4 over FFA1 and that this is preserved across species (Milligan et al. 2017). However, problems with agonist solubility may limit its application *in vivo* (Sparks et al. 2014). TUG-1197 2-[3-(pyridin-2-yloxy) phenyl] -2,3 dihydrobenzo[d]isothiazole 1,1-dioxide, another sulphonamide ligand developed based on a patent published by Banyu Pharmaceuticals has no detectable action at FFA1(Milligan et al. 2017).

There are only two antagonists known to block FFA4 activity, both derived from the same chemical series. AH-7614 (4-methyl-N-9H-xanthen-9-yl-benzenesulfonamide) was reported based on GlaxoSmithKline's study of sulfonamide-containing FFA4 ligands (Sparks et al. 2014), while TUG-1506 (4-methyl-*N*-[9*H*-thioxanthen-9-yl] benzenesulfonamide) was developed as a later chemical derivative of AH-7614 (Watterson et al. 2017). These are both non-competitive negative allosteric modulators of FFA4 signalling and do not bind to the same site as the LCFAs (Milligan et al. 2017).

FFA4 agonist and antagonist chemical structures are displayed in Table 1-6 presents the potency and selectivity of various compounds acting as agonists and antagonists for FFA4 activation.

Table 1-6 Potency and Selectivity of Agonists and Antagonists for FFA4 Activation

Compound	Type	Potency	Selectivity	Reference
α-linolenic acid (ALA)	Endogenous	5.16 ± 0.08	FFA1/FFA4	(Hudson et al., 2014)
Palmitic acid		6.12 ± 0.08	FFA1/FFA4	(Briscoe et al., 2006)
Eicosapentaenoic acid (EPA)		5.68 ± 0.06	FFA1/FFA4	(Briscoe et al., 2006)
GW9508	Agonist	5.89±0.04	FFA1/FFA4	(Briscoe et al., 2006)
NCG21		5.62 ± 0.10	FFA4/FFA1	(Shimpukade et al., 2012)
TUG-891		7.36 ± 0.15	FFA4/FFA1	(Shimpukade et al., 2012)
Compound A		7.62 ± 0.11	FFA4	(Briscoe et al., 2006)
GSK137647A		6.3 ± 0.2	FFA4	(Sparks et al., 2014)
TUG-1197		6.8	FFA4	(Azevedo et al., 2016)
Metabolex-36		5.9	FFA4	(Stone et al., 2014)
AH7614	Antagonist	8.1	FFA4	(Hansen & Ulven, 2017)

1.8.5 FFA4 receptor in adipose

As mentioned in Section 1.7.1, FFA4 is highly expressed in AT. Adipose tissues most known classes are types of white adipose tissues and brown adipocytes have both been shown to significantly contribute to obesity-related disease (Hajer et al. 2008). In particular, WATs serve as the primary site for the storage of FAs in the form of lipid droplets, which can be used as a source of energy (Hajer et al. 2008). Frequently, abnormal WAT secretion results in metabolic disorders; this leads to an interest in FFA4 receptors as a potential target for obesity treatment.

A number of studies have highlighted the link between adipose function and the FFA4 receptor, either by studying the effect of PUFAs (the ligands for FFA4) or

by examining the receptor expression in adipose and assaying its activity influence on adipose functions.

For example, in a 12-month study established to examine the effect of omega-3 fatty acids (ω-3 FAs) on non-alcoholic fatty liver disease, the effects of 1 g of eicosapentaenoic acid (EPA) per day on 42 patients with NAFLD were evaluated. Compared to the control group, the treatment group demonstrated an increase in ultrasound-measured liver fat quantity, as well as decreases in TGs and liver enzyme, following treatment(Capanni et al. 2006). Moreover, Zhu et al. investigated the effects of 6 g of EPA plus DPA and docosahexaenoic acid (DHA) per day on subjects with NAFLD and hypertriglyceridemia; patients were randomly assigned to treatment or control groups. The study lasted six months, and the authors noted an improvement in ultrasound-measured liver lipid content and serum levels of TG and liver enzyme in the treatment group versus the control group (Zhu et al. 2008).

Moreover, the level of FFA4 receptor mRNA increased as 3T3-L1 cells differentiated into adipocytes (Gotoh et al. 2007). In human AT, human preadipocytes and cultured adipocytes, identical outcomes were observed(Gotoh et al. 2007). In addition, the use of siRNA to inhibit FFA4 expression inhibited adipocyte differentiation (Gotoh et al. 2007). Moreover, in humans, FFA4 expression in AT is substantially higher in obese individuals than in lean controls (Ichimura et al. 2012). In addition, FFA4 has clinical implications in obesity, as a variant of FFA4 containing a p.R270H mutation that inhibits FFA4 activity has been associated with an increased risk of obesity in European populations. This indicates that FFA4 participates in the regulation of body weight (Ichimura et al. 2012).

Furthermore, results showed (Ichimura et al. 2012)that FFA4-deficient rodents fed an HFD developed obesity, glucose intolerance and a fatty liver characterised by decreased adipocyte differentiation and lipogenesis and increased hepatic lipogenesis. More importantly, FFA4-deficient mice developed glucose intolerance, obesity and fatty liver, and a malfunctioning version of this receptor (R270H) has been linked to obesity and other metabolic disorders in humans (Villegas-Comonfort et al. 2017). Additional evidence for this suggested

that FFA4 has a role in satiety by promoting the production of peptide YY, a hormone that has been demonstrated to decrease food intake in both mice and humans (Moodaley et al. 2017).

Therefore, the research and characterisation of FFA4 agonists are crucial in applying the therapeutic potential of FFA4 agonists to metabolic disorders associated with AT.

1.8.6 FFA4 receptor's role in glucose uptake

As mentioned in Section 1.4, T2D is a global health issue due to its devastating long-term consequences, including cardiovascular disease, retinopathy, neuropathy and nephropathy (Sparks et al. 2014). More than 300 million people worldwide suffer from it, and deaths from the disease are expected to rise by 50% over the next decade, making it the seventh-largest cause of death by 2030 globally (Sparks et al. 2014). This, along with the serious complications of diabetes, has led to studies investigating the role of FFA4 in glucose uptake, which is one of the main mechanisms that exhibit dysfunction in patients with T2D.

An example of such a study is Oh et al. 2010), which used primary AT and 3T3-L1 adipocytes that were treated with GW9508 or DHA, an omega-3 FA (ω-3 FA), for 30 minutes before measuring baseline and insulin-stimulated GLUT4 translocation and 2-deoxyglucose translocation. Results showed that DHA stimulated FFA4 in 3T3-L1 adipocytes, increasing GLUT4 translocation to the cell surface and enhancing glucose uptake (Oh et al. 2010). This effect was approximately 30%–50% that of the maximum insulin response, and the actions of DHA on glucose uptake were in addition to those of a sub-maximally-stimulating concentration of insulin. This suggests that these insulinometic effects contribute to the overall insulin-sensitising actions of FFAs receptors that are activated by omega-3 (Oh et al. 2010). Agreeing with these results, in WT and GPR120 KO mouse primary AT, it was found that an increase in glucose transport and the translocation of GLUT4 to the plasma membrane were caused by ligand-stimulated FFA4 (Oh et al. 2010). Nonetheless, it should be highlighted that when TUG-891, a selective agonist for FFA4, was used on the same cell line, the

responses were modest. In glucose uptake assays, for instance, the response of TUG-891 was only 8% of the maximal insulin response(Hudson et al. 2013).

The effect of FFA4 simulation was noticed not only in adipose but also in other cell types. In skeletal muscles, DHA increased glucose absorption in an AMP-activated protein kinase (AMPK)-dependent way. For DHA-mediated AMPK activation and glucose absorption, an FFA4-mediated rise in intracellular Ca^{2+} was essential. DHA also promoted GLUT4 translocation in an AMPK-dependent manner (Kim et al. 2015). Furthermore, using CACO-2 cells with the presence of tumour necrosis factor-α (TNF-α) has shown that pre-incubation with EPA, an omega-3 PUFA, prevented the inhibitory effect of the cytokine on α-methyl-d-glucose (αMG) uptake (Castilla-Madrigal et al. 2018).

Although the study used DHA, EPA and GW9508 to evoke the role of FFA4 as a potential target for drug development against T2D, both ligands are not a selective agonist for FFA4; therefore, there is a need to explore mechanisms such as glucose uptake and GLUT4 translocation using a potent selective agonist for FFA4, such as TUG-891, in this project.

In addition, in another study, mice were fed either an HFD or a normal diet for 10 weeks. In fasting states, HFD-treated WT mice showed a significant rise in plasma insulin as well as an increase in plasma glucose, indicating the establishment of insulin resistance. However, DHA supplementation alleviated HFD-induced increases in plasma glucose and insulin but not supplementation of dihomo-gamma-linolenic acid DGLA or oleic acid OA, which are ω-6 FAs, indicating that DHA has a positive effect on HFD-induced insulin resistance. As a follow up, in HFD-treated mice with or without DHA supplementation, glucose tolerance tests (GTTs) and insulin tolerance tests (ITT) were performed; GTTs revealed that mice given DHA were more glucose tolerant than WT mice, and DHA significantly increased insulin sensitivity in HFD-treated mice, as determined by the reduction in plasma glucose after insulin delivery, according to ITTs (Yan et al. 2013).

In light of the suggestion that chronic inflammation is an important pathogenetic factor in the development of insulin resistance and T2D (Donath and Shoelson

2011), FFA4 plays a crucial function in mediating the anti-inflammatory effects of ω-3 FAs on macrophages, thereby contributing to the maintenance of insulin sensitivity via inhibition of inflammation (Yan et al. 2013). Although the mechanisms are still unclear, ω-3 FAs may have an anti-inflammatory effect in a number of inflammatory human disorders. Yan et al. 2013) demonstrated that stimulation of macrophages with ω-3 FAs, such as EPA, DHA and other family members, prevented the activation of the NLRP3 inflammasome and the subsequent activation of caspase-1 and secretion of IL-1b. Inflammasome suppression brought on by ω-3 FAs was also demonstrated to be mediated through FFA4 and downstream scaffold protein B-arrestin-2 (Yan et al. 2013). Importantly, in an HFD-induced T2D mouse, ω-3 FAs also reduced NLRP3 inflammasome-dependent inflammation and metabolic dysfunction. Yan et al. 2013)'s findings indicate a mechanism by which ω-3 FAs suppress inflammation and guard against diseases brought on by inflammation, and they point to the potential clinical application of ω-3 FAs for the treatment of gout, auto-inflammatory syndromes and other NLRP3 inflammasome-driven inflammatory diseases (Yan et al. 2013).

It is also important to note that FFA4 has insulin-sensitising and anti-inflammatory effects on not only macrophages but also adipocytes (Villegas-Comonfort et al. 2017). In general, FFA4's modulatory effects on insulin sensitivity and glucose uptake raise the possibility that it could be targeted as an anti-diabetic medication(Zhang and Leung 2014).

Diabetes-linked mutations in Free Fatty Acid Receptor 4 (FFA4) have been identified and studied to understand their impact on receptor signaling and their association with diabetes pathogenesis. One specific mutation that has been investigated is located in the intracellular loop 3 (ICL3) of FFA4(T. Liu et al., 2022). The position of this mutation within ICL3 and its relative impact on signaling provide valuable insights into the functional significance of intracellular loops and the C-terminal tail in GPCR (G-protein coupled receptor) function(T. Liu et al., 2022). Intracellular loops, including ICL3, play crucial roles in GPCR signaling by interacting with intracellular signaling proteins and modulating downstream signaling cascades(X. E. Zhou et al., 2017). They contribute to receptor activation, desensitization, and internalization processes.

The C-terminal tail of GPCRs is also involved in signaling regulation, receptor trafficking, and interaction with intracellular effectors(X. E. Zhou et al., 2017).

The mutation within ICL3 may affect the conformational changes of FFA4 upon ligand binding or its coupling to downstream signaling proteins(X. E. Zhou et al., 2017). For example, if the mutation disrupts the interaction between ICL3 and G-proteins or other intracellular effectors, it could impair the receptor's ability to activate downstream signaling pathways(T. Liu et al., 2022). Consequently, this may lead to dysregulation of insulin sensitivity, glucose homeostasis, and other metabolic processes associated with diabetes.

1.8.7 FFA4 and adipogenesis

FFA4 is implicated in crucial homeostatic processes, including adipogenesis, anti-inflammatory processes, glucose uptake and insulin sensitivity, as well as the secretion of hormones (Gotoh et al. 2007; Ichimura et al. 2012; Song et al. 2017). Ichimura et al. 2012) found that FFA4 expression in AT is considerably higher in obese people than in lean controls in humans. Exon sequencing of FFA4 in obese people revealed a harmful non-synonymous mutation (p.R270H) that decreases FFA4 signalling function. Furthermore, among European populations, the p.R270H variation increases the risk of obesity (Bonnefond et al. 2015; Ichimura et al. 2012). The R270H FFAR4 mutation has also been demonstrated to influence the risk of T2D when combined with dietary fat intake(Lamri et al. 2016). This important role and relevant studies have highlighted the FFA4 receptor as a target in the field of drug discovery (Gotoh et al. 2007; Ichimura et al. 2012; Song et al. 2017).

Further studies provide more data that show that FFA4 is a key player in the development of both white and brown adipocytes (Otto and Lane 2008; Song et al. 2017). As was discussed in chapter 4 of this project, FFA4 cannot be found in preadipocytes or undifferentiated 3T3-L1 cells, but it is highly expressed in differentiated 3T3-L1 cells, which are a well-known cell line for studying white adipocytes (Otto and Lane 2008), and in human white adipocytes(Otto and Lane 2008; Song et al. 2017). The expression of FFA4 goes up when isobutylmethylxanthine, DEX and insulin are added to the differentiation medium, which is a typical adipogenic induction cocktail for white adipocytes *in*

vitro (Otto and Lane 2008). This suggests that this receptor is probably involved in adipocyte differentiation (Otto and Lane 2008; Song et al. 2017). Moreover, experiments on humans showed a similar trend, that FFA4 is expressed much more in obese people than in lean people (Ichimura et al. 2012).

Not only has the influence of the FFA4 receptor on WAT been identified, but evidence also implies that the receptor has an impact on BAT. In a study using RNA-sequencing to analyse the mouse BAT transcriptome, Quesada-López et al. (2016) found that thermogenic activation induces the FFA4 gene. FFA4 activation induces BAT activity and promotes the browning of white fat in mice, whereas cold-induced browning is impaired in FFA4-null mice (Quesada-López et al. 2016). Moreover, GW9508 is a selective agonist for GPR120 that has been shown to stimulate GPR120, which in turn may boost BAT activity as well as n-3 PUFAs (Kim et al. 2016; Quesada-López et al. 2016). Upregulation of critical thermogenic gene expression and increased rates of brown and beige adipocyte adipogenesis were seen after treatment with EPA and GW9508(Kim et al. 2016; Quesada-López et al. 2016; Song et al. 2017). In addition, the browning of subcutaneous WAT in response to cold exposure is impaired in FFAR4-deficient rodents (Quesada-López et al. 2019).

These findings about FFA4 expression level in AT encourage more research to examine its role in adipogenesis in several models. Using 3T3-L1 cells, Song et al. (2016) showed that as 3T3-L1 adipocytes underwent adipogenic differentiation, FFA4 expression increased markedly, whereas shFFA4-transfected cells significantly inhibited adipogenic differentiation (Song et al. 2016).

Before testing TUG-891, the well-established FFA4 agonist, researchers used omega-3 fatty acids to link the function that occurs in *in vitro* or *in vivo* systems with the FFA4 receptor. In Hilgendorf et al. (2019)'s study, FFA4 agonists and ω-3 fatty acids were used on adult mesenchymal stem cells, including preadipocytes, which have a sensory organelle called the main cilium. The study found that the omega-3 fatty acid causes FFA4 to promote adipogenesis by localising to cilia in a way that depends on TULP3 (Hilgendorf et al. 2019). This happens by quickly triggering the production of cAMP inside cilia, FFAR4 agonists and ω-3 fatty acids, causing mitosis and adipogenesis to occur(Hilgendorf et al.

2019). The study demonstrated that ω-3 fatty acids in the diet selectively increase the number of adipocytes to make new fat cells and store SFAs, which keeps healthy fat tissue in balance(Hilgendorf et al. 2019).

Studies then used TUG-891 as the selective agonist of FFA4 and found that it induced dose-dependent accumulation of intracellular triglycerides in shFFA4-transfected cells but had no effect on adipogenesis (Song et al. 2016). Using a luciferase reporter assay, TUG-891 significantly increased PPARγ activation via a GPR120-dependent pathway. Moreover, during the adipogenic differentiation of 3T3-L1 adipocytes, TUG-891 increased intracellular calcium mobilisation and ERK1/2 phosphorylation. Investigations into 3T3-L1 went further to examine the cellular effect of FFA4 receptors in adipogenesis. Gotoh et al. 2007) found that the major adipogenic regulator PPARγ and the GPR120 gene both express their mRNA during the differentiation of 3T3-L1 cells. PPARγ and the adipogenic marker gene, FA binding protein 4 (FABP4), were shown to have lower levels of mRNA when FFA4 was knocked down (Gotoh et al. 2007). This finding was also true in another cell system. Ichimura et al. (2012) also demonstrated that adipogenesis-related genes, such as PPARγ and FABP4, are downregulated in adipocytes derived from FFA4 −/− MEF cells.

Research on FFA4 receptors was expanded to another cell system – the bone marrow mesenchymal stem cells (BMMSCs) – a model that was used to understand osteoblasts. In summary, the abnormal osteoblast/adipocyte ratio is determined by the imbalance between the decreased osteogenic and increased adipogenic differentiation tendency of BMMSCs (Gao et al. 2015; Idris et al. 2009), and this imbalance will cause more fat to reside in the bone marrow cavity, modulating the bone formation process and causing osteoporosis4. Numerous fundamental factors, including -catenin, Runx2, and Ppar-, play a significant role in the bi-potential differentiation of BMMSCs (Gao et al. 2015; Hoffman and Benoit 2015; Shin et al. 2009; Sun et al. 2012). Gao et al. (2015) reported that TUG-891 acts as a positive enhancer in the adipogenic process at low concentrations, while higher concentrations induce the opposite developmental direction, osteogenesis.

In summary, high levels of FFA4 expression in AT have been linked to its role in controlling several metabolic processes associated with fat storage. In recent years, FFA4 has garnered a lot of attention, and various research groups have produced compelling evidence suggesting that FFA4 could be used as a potential therapeutic target to repair metabolic imbalances associated with obesity and T2D. In animal models, several synthetic FFA4 agonists have been found to have anti-diabetic benefits. Many FFA4 agonists and antagonists are poised to enter human clinical studies in the next few years. But the specific role of FFA4 in fat tissue has not been well shown yet. Before medication trials can move forward, however, several important concerns about FFA4's function in adipogenesis and adipose metabolism must be answered. Important steps in treating obesity and diabetes include studying the ligands of FFA4 in detail and identifying the antagonist input as a therapy.

This process can be studied using *in vitro*-differentiated adipocyte models, such as 3T3-L1, that, once differentiated, develop characteristics of adipose cells *in vivo* (Gregoire et al. 1998). This change from fibroblast into started by treat the cells with hormones that promote cells differentiation in adipocyte direction. Namely, in 3T3l1 differentiation, culture media containing foetal calf serum, DEX, IBMX, troglitazone and insulin has been used in combination with inducing agents (Gregoire et al.,1998). In several weeks, the cells reach an adipose-cell-like phenotype, which presents the opportunity to perform several assays to determine key features in AT functions.

This transformation promotes AT features that relate to lipid metabolism, such as the ability to store FAs and glycerol release and secretion, in addition to insulin-dependent glucose transport that controls glucose circulation inside and outside of the cell (Ghaben and Scherer 2019).

1.9 Aim of the study

The main aim of this study is to uncover how pharmacological manipulation of FFA4 affects adipogenesis and adipocyte function using the 3T3-L1 model. Two pharmacological tool compounds will be used, TUG-891 a potent agonist of FFA4 or AH7614, an FFA4 antagonist. Prior to the using TUG-891 and AH7614 in the 3T3-L1 adipocyte model, their pharmacological properties were tested in a

heterologous cell system. This work focuses on addressing three central questions about FFA4 function, with key objectives for each listed below:

1. To define the pharmacological properties of TUG-891 and AH7614 at the mouse orthologue of FFA4:
    - Use common heterologous expression systems, including CHO, HEK293T and Flp-IN T-Rex 293 cells to express the mouse (m) orthologue of FFA4.
    - Employ cells expressing mFFA4 to assess how FFA4 agonists, including TUG-891, affect Extracellular signal-regulated kinases ERK1/2 phosphorylation, intracellular Ca^{2+} mobilisation, ß-arrestin recruitment, and receptor internalisation.
    - Use the TRUPATH bioluminescence resonance energy transfer (BRET) assay to establish which G proteins couple with FFA4.
    - Confirm the properties pharmacological properties of AH7614 to inhibit FFA4 signalling using Ca^{2+} mobilisation, ß-arrestin recruitment, and receptor internalisation assays.
2. Establish the effects of acute agonism or antagonism of FFA4 on glucose uptake and lipolysis in 3T3-L1 adipocytes.
    - Use RT-qPCR to characterise changes in expression of the FFA family of GPCRs during adipogenic differentiation of 3T3-L1 cells.
    - Assess the effects of TUG-891 and AH7614 treatment on insulin dependent and insulin independent glucose uptake in 3T3-L1 adipocytes
    - Evaluate the effects of TUG-891 and AH7614 on ß-adrenoceptor stimulated lipolysis in 3T3-L1 adipocytes
3. Determine how long-term treatment of 3T3-L1 adipocytes with FFA4 ligands throughout differentiation affects adipogenesis
    - Track changes in the adipogenesis process of 3T3-L1 cells treated with TUG-891 or AH7614 by measuring total lipid accumulation
    - Screen for changes in adipogenic gene expression on 3T3-L1 differentiated in the presence of TUG-891 or AH7614.
    - Establish how 3T3-L1 cells differentiated in the presence of TUG-891 or AH7614 have altered insulin dependent glucose uptake, or ß-adrenoceptor stimulated lipolysis.

Together, these studies aiming to evaluate the role FFA4 in adipocyte differentiation and function, providing insighted into the potential for this receptor in the treatment of metabolic disorders like T2DM or obesity.

Chapter 2 Materials and Methods

2.1 Materials.

2.1.1 Tissue Culture

A summary of the material that were used in cell culture is listed in table 2.1

Table 2-1 cell culture Materials

Marital	Supplier
Dulbecco's Modified Eagle Media (DMEM) Foetal bovine serum (FBS) Hygromycin B sodium bicarbonate solution	Invitrogen Life Technologies, Paisley, UK
Penicillin/Sterptomycin Glutamine Poly-D-Lysine Trypsin-EDTA solution 0.25% Tween-20 Ampicillin dimethyl sulfoxide (DMSO) Triton X-100	Sigma-Aldrich Company Ltd., Poole, Dorset, UK
Dulbecco's Phosphate Buffered Saline (PBS) HBSS - Hank's Balanced Salt Solution	Thermo Fisher Scientific

2.1.2 Kits

CisBio phospho-ERK (Thr202/Tyr204) kit (P/N: 64AERPEH)

Qiagen RNeasy plus mini kit (P/N: 74134)

High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM, P/N: 4368813).

QIAfilter® maxiprep kit (Qiagen)

The Wizard® Plus SV Minipreps DNA purification system (Promega)

2.1.3 Solution

Fixing solution – 4% (w/v) Paraformaldehyde (PFA) in PBS

TBS-Tween – TBS and 0.1% (v/v) Tween-20.

Transfer buffer – 25 mM Tris-base, 192 mM glycine, and 20% ethanol.

Tris Buffered Saline (TBS) - 20 mM Tris-HCl, pH 7.6, 137 mM NaCl.

Tris-Glycine SDS Running buffer – 5 mM Tris-Cl, 250 mM glycine, 0.1% SDS.

SDS-PAGE gel resolving buffer 75mM Tris-HCl, pH 8.8, 0.2% (w/v) SDS

SDS-PAGE gel stacking buffer 25mM Tris-HCl, pH 6.8, 0.2% (w/v) SDS

Krebs-Ringer phosphate buffer (KRP) 10 mM $NaHCO_3$, 120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$.

PBS 140mM NaCl 3mM KCl, 1.5mM KH_2HPO_4, 8mM Na_2HPO_4

RIPA Buffer 50mM Tris, HCl pH8, 150mM NaCl, 2mM $MgCl_2$, 1% Triton, 0.5% sodium deoxycholate (w/v), 0.1% (w/v) SDS, 1mM DTT, 50 units/ml Benzonase

L-Broth Medium (LB) 10g Tryptone, 5g Yeast extract, 10g NaCl

2.1.4 pharmacological Compounds

Summary of compounds targeting the FFA4 that were used in this project can be found in Table 2.2.

Table 2-2 List of selected compounds targeting FFA4

Compound	Supplier
TUG-891	Tocris P/N: 4601
TUG-1197	From Trond Ulven, University of Copenhagen
AH7614	Tocris biotech P/N: 5256

And one compound was used to target M1 receptor Acetylcholine chloride ≥99% from Sigma, P/N: A6625.

2.1.5 cell lines

A summary of the cell lines that were used in this study are listed in Table 2.3.

Table 2-3 list of the cell lines that were used in this study

Cell Line	Discerption	Source
3T3-L1	Mammalian cell, mouse, fibroblasts can be differentiated to mature adipocytes.	American Type Culture Collection (ATCC)
HEK293T	Human embryonic kidney	American Type Culture Collection (ATCC)
FLAG-FFA4-eYFP Flp-In	mFFA4-eYFP constructs using the Flp-In T-RExTM system	(Hudson et al., 2013)
FFA4- CHO	Chinese Hamster Ovary (CHO) cells previously generated using the Flp-InTM system to stably express mouse FFA4-HA	(Butcher et al., 2014)

2.1.6 Antibodies

A summary of the primary and secondary antibodies that were used in this study can be found in table

Table 2-4 list of primary antibodies used in this study

Antigen	host species	working dilution	Supplier
GLUT4	Rabbit	1:500	A combination of rabbit polyclonal antibodies raised against the C terminus of GLUT4 and the N-terminus of GLUT4. Made as described in (Brant et al., 1992)
Phospho-Akt	Rabbit	1:1000	Cell Signaling (9220)

Table 2-5 A list of secoundry antibodies that were used in this study

Antigen	working dilution	Supplier
IRDye 800CW Donkey anti- Rabbit IgG (H+L)	1:10000	LI-COR Biotechnology (926- 32213)
IRDye® 800CW Donkey anti- Mouse IgG (H + L)	1:10000	LI-COR Biotechnology (926- 32212)

2.2 Cell culture methods

2.2.1 3T3-L1 cells maintenance.

3T3-L1 cells were cultured in Falcon T75 flasks or desired plates using Falcon plastic from VWR. 3T3-L1 fibroblasts (passage 7 to 11) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% new-born calf serum (NCS) and Penicillin-Streptomycin. Cells were cultured at 37°C in an incubator with 10% CO_2 and every two days the medium replaced on the cells.

2.2.1.1 Subculturing 3T3-L1 cells.

When cells in reached 70-80% confluency, growth medium was aspirated, and the cells gently washed with sterile PBS before sterile trypsin (0.05% in EDTA) was added. Cells were then incubated at 37°C for 4 minutes or until the cells began to detach from the surface. Detached cells were diluted in 1:8 – 1:10 dilution with appropriate DMEM growth medium was added to new T25 flasks as required in the upcoming experiments.

2.2.1.2 3T3-L1 differentiation procedure.

3T3-L1 cells were grown to 100% confluency in 10% NCS/DMEM in desired wells plates for each experiment and maintained for 48 hours post-confluence before starting differentiation. To initiate differentiation, culture medium was aspirated and replaced with DMEM containing with 10% foetal calf serum (FCS), 0.5 mM IBMX, 5 µM troglitazone 0.25 µM dexamethasone and insulin (1 µg/ml). After 3 days, the medium was replaced with DMEM supplemented with 10% FCS, insulin (1 µg/ml) and 5 µM troglitazone. The cells were cultured for 3 days then the medium was replaced with DMEM containing 10% FCS and Penicillin-Streptomycin . At day 8-12 post differentiation the cells were well-differentiated into adipocytes and ready to be used for experimentation.

2.2.2 FFA4- CHO cell line maintenance

Cells were cultured in T75 flasks or dishes using Ham's Nutrient Mixture F-12 from Sigma, P/N: N6658 medium supplemented with 10% FBS, 400 µg/mL hygromycin B (v/v). Cells were kept in the incubator at 37 °C and 5% CO2.

Medium replaced on the cells every 3 days until cells reached the desired confluency.

2.2.3 Subculturing FFA4- CHO cells

When the cells reached 80% confluency culture medium was removed and cells were washed with sterile 1X PBS supplemented with 1 mM EDTA and then incubate with PBS/1mM EDTA on cells for 5 minutes incubator at 37 °C and 5% CO_2 to detach the cells from the flask. Detached cells were diluted with appropriate medium and transferred to a sterile flask containing fresh cell culture medium.

2.2.4 HEK293T cells maintenance

Cells were cultured in T75 flasks or dishes using Dulbecco's modified Eagle's media (DMEM) supplemented with 10% (v/v) FBS, 0.292 g/L L-glutamine, 100 units/mL penicillin and streptomycin. Cells were maintained at 37 °C and 5% CO2 in the incubator.

2.2.5 Subculturing HEK293T cells.

Upon reaching 80% confluency, media was aspirated. Cells were first washed with sterile 1X PBS before addition of pre-warmed 0.25% trypsin-EDTA and incubation for 5 minutes to allow detachment of cells from the flask. DMEM was added to make a cell suspension. Cells (1:10 or 1:20 ratio) were transferred to new flasks or dishes with fresh culture media.

2.2.6 FLAG-mFFA4-eYFP Flip-In T-REx cell line maintenance

Cells were cultured in Falcon T75 flasks or dishes using DMEM media (4500 mg/L glucose + L-glutamine, − pyruvate) supplemented with 10% FBS (v/v), 5 µg/mL blasticidin , 100 units/mL penicillin streptomycin and 200 µg/mL hygromycin B. Cells were grown in an incubator at 37 °C and 5% CO_2. Medium replaced on the cells every 4 days as feed or until ready to be split.

2.2.7 Subculturing FLAG-mFFA4-eYFP Flip-In T-REx cells.

After reaching 80% confluency, media was aspirated. Then 0.25% trypsin-EDTA were added to the flask and incubated for 5 minutes to allow detachment of cells from the flask. DMEM was added to make a cell suspension. Cells (1:10 or 1:20 ratio) were transferred to new flasks or dishes with fresh culture media.

2.2.8 Cryopreservation cells.

Cells lines were cultured as previously described in T75 flasks until it reached 70-90% confluency. DMEM growth medium was aspirated washing with sterilised PBS then incubation with trypsin for 5 minutes at 37oC in an incubator followed by tapping flask to dislodge cells n. Then DMEM growth medium was added to neutralise the trypsin. The trypsin/cell mix was then transferred to a falcon tube and centrifuged at at 1000 x gfor 5 minutes. The culture medium was gently aspirated, and the cell pellet was resuspended in 1 ml of freezing media containing foetal calf serum supplemented with 10% DMSO. The cells in freezing medium were transferred to polypropylene cryogenic tubes and placed in a Mr FrostyTM freezing container. Cells were stored overnight at -80°C before. Next day, the cells were moved into liquid nitrogen container for storage the vials were transferred to liquid nitrogen or -80 freezer.

2.2.9 Recovery of Cryopreserved Cell Stock

Cell cryogenic vials were removed from liquid nitrogen and rapidly thawed in a 37°C water bath. Vials were then transferred to a cell culture sterile flow hood where they were transferred to a T75 flask containing 10 ml of appropriate culture media for the desire cell line and incubated at 37°C in 10% CO_2 incubator. The next day the medium was aspirated to remove dead cell and was replaced with fresh growth medium.

2.2.10 Short term transfection

For short term transfection was done using 1 mg/ml polyethyleneimine (PEI) that was used to generate a transfection that can be used in an assay one time only. HEK293 cells were seeded on to 10 cm2 dish to a volume of 10 ml media. Transfection of cells was performed when the cells had reached 60-70%

confluency. Cells were transfected A mixture of 2.5 μg DNA was prepared in (150 mM) NaCl. PEI (1 mg/mL) was diluted as 1:6 ratio in 250 µl NaCl (150 mM). Two mixtures were added together, vortexed and incubated for 15 minutes at room temperature. Cell media was replaced by fresh media and the DNA/PEI mixture was added drop-wise into the media. Transfected plates were incubated in incubator with 5% CO_2 and maintained at 37°C. Assays with these cells 24 hours post transfection.

2.2.11 Poly D-lysine cote

A 10 ml of serum-free culture media has a 1 mg of Poly D lysin was divided in 96 well plat either white 96 well plates or black clear bottom 96 well plates as 20ul per well. After 5 minutes plates were saved in 4 °C until used.

2.3 Molecular Biology Methods

2.3.1 Making L-Broth

In 500 ml of H_2O, we add 10g of Tryptone, 5g Yeast extract and 10g NaCl then pH was adjusted to 7 then the volume completed to 1000ml with H_2O. The mixture was stirred adequately and autoclaved at 126 °C. The solution was allowed to cool and stored at room temperature.

2.3.2 Making Agar Plates

In this step the same L- Broth that was made in section 2.3.1 was used, but with the addition of Bacto-agar (1.5% w/v) prior to the autoclave step. After removing the bottles from the autoclave, it was left to cool to approximately 50 ºC before ampicillin was added to a final concentration of 100 mg/ml. After that the liquid agar was poured into 10-cm-petri dish and allowed to solidify before storing at 4°C.

2.3.3 Preparation of Competent Bacteria.

In transformation (which is the next step) a Escherichia coli laboratory strain XL-1 blue was used. XL1 Blue cells were first streaked out on an agar plate with no antibiotics and grown overnight at 37°C. Then a single colony is picked and grown in 5ml of L-broth overnight at 37°C. This culture is then transferred into

100 ml of LB-broth and grown until the optical density at 550 nm is 0.48. After chilling on ice for 5 min the cells are spun at 2000 x g for 10min at 4°C in 50ml sterile falcon tubes. Each pellet is re-suspended in 20ml of solution 1 that contained: 1 M Potassium acetate, 1 M RbCl2 , 1 M CaCl2 , 1 M MnCl2 and 80% (w/v) glycerol and pH adjusted to 5.8 with 100mM Acetic acid. The cells were incubated on ice for 5 min then centrifuged at 2000 x g again for 10 min. Pellet were then re-suspended in 2ml of Solution 2 that contained: 100 mM MOPS pH 6.5, 1M $CaCl_2$, 1M $RbCl_2$ and 80% (w/v) glycerol and pH adjusted to 6.5 with concentrated HCl. The solution was filter-sterilised through a 0.2 μm syringe filter and stored at 4 °C. Solution 2 added by gentle pipetting and chilled on ice for a further 15min.Cells are then aliquoted in 220μl volumes and stored at -80 °C.

2.3.4 Transformation of Competent Cells with Plasmid DNA

10-100ng of the desired DNA was incubated with 50 μl of competent cells on ice for 30 min. Then the cells were heated for 90 seconds at 42°C and returned to ice for 2 min. then 450 μl of LB-broth was added immediately after, and cells were allowed to recover and express antibiotic resistance genes by incubation at 37 °C for 45-60 min in a shaking incubator. After that 50-200ul is added to an agar plate containing 100 mg/ml ampicillin. The plates are incubated overnight at 37°C and transformed colonies selected and grown up overnight in 5ml of LB-broth containing 100 mg/ml ampicillin.

2.3.5 Maxiprep

Purification of DNA was carried out as per manufacturer's instructions for QIAfilter® maxiprep kit (Qiagen) that was used to produce larger scale DNA samples. First, a 100 ml culture of transformed bacteria was pelleted by centrifugation 4000x g at 4 °C for 30 minutes. All traces of media were removed, and the pellet re- suspended in 10 ml of cold buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/μl Rnase A). Cell lysis was achieved by the addition of 10 ml buffer P2 (200 mM NaOH, 1% (w/v) SDS) and incubated for 10 minutes at room temperature. Then 10 ml of buffer P3 (3.0 M potassium acetate pH 5.5) was added to neutralise the reaction and the solution was immediately applied to a QIAfilter® cartridge and left for 10 minutes at room temperature to settle.

Aside, a Qiagen tip 500 was equilibrated by the addition of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol).

After 10 minutes, the lysed cells were added onto the equilibrated tip and allowed to filter through. The column was washed with 60 ml of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol).

The DNA was eluted by the addition of 15 ml of buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol) to the tip. The DNA was precipitated by the addition of 10.5 ml isopropanol and pelleted by centrifugation at 12,000x g for 30 minutes at 4°C. The DNA pellet was washed with 5 ml of 70% (v/v) ethanol and then centrifuged for 15 minutes at 12,000x g at 4°C. The supernatant was removed, and the pellet allowed to air dry prior to resuspension in 500 ml of sterile water.

2.3.6 DNA concentration

Concentration of DNA samples was examined by the absorbance of a 1:100 dilution of the sample at 260 nM. An A_{260} value of 1 unit was taken to correspond to 50 μg/ml of double stranded DNA. The A_{280} value of sample was also measured to assess the purity of the DNA solution. A DNA solution with A_{260}/A_{280} ratio of between 1.7 and 2.0 was considered pure enough for use.

A summary of the plasmids that were used in this study can be found in Table 2-6

Table 2-6 Plasmids that were used in this study

Plasmid	Vector	Source
NLuc-h-arrestin 3	pcDNA3	(Prihandoko et al., 2016)
mNG CAXX	pcDNA5 FRT/TO	(Prihandoko et al., 2016)
PCDNA3		Life Technologies
mFFA4-HA	pCDNA5	(Prihandoko et al., 2016)
Gαq	pcDNA3 / Rluc8	(Olsen et al., 2020)
Gαi2	pcDNA3 / Rluc8	(Olsen et al., 2020)
Gα15	pcDNA3 / Rluc8	(Olsen et al., 2020)
Gβ3	pcDNA5	(Olsen et al., 2020)
Gγ-GFP2 (Gγ9 - γ8-γ13)	pcDNA5	(Olsen et al., 2020)

2.4 Pharmacological Assays

2.4.1 ERK Phosphorylation Assay

Chinese hamster ovary (CHO) cells expressing mouse FFA4 receptor was cultured as described then seeded as 35,000 cells per well in a 96 well plate. Plates were then cultured overnight before being used in the assay. Cells were washed 2x with 100 µL F12 ham serum free medium and serum starved for 5 h with 90 µL of serum free medium. Following serum starvation, 10 µL of serially diluted FFA4 agonists (TUG-891 or TUG1197) in serum free medium were added to cells so that the final concentration of agonist ranged from 0.1 nM to 3.16 µM agonist treatment for 5 minutes.

Medium was removed and cells were lysed in 50 µL of lysis buffer (prepared according to the manufacturer's instructions) for 30 min by shaking plates at 600 xg at room temperature.

16 µL of cell lysate and 4 µL of a 1:1 mixture of cryptate and d2 antibodies were added to each well of a 384 well optiplate (PerkinElmer).

The cryptate antibody is a specific antibody which is labelled with Eu3+-cryptate and acts as a donor with emission at 620 nm. The second specific antibody is labelled with d2 and acts as the acceptor antibody with emission at 665 nm. One antibody binds to the phosphorylated motif on the protein and the second binds the protein independently of its phosphorylation state. When both antibodies meet, phosphorylated ERK1/2 occur and the Eu3+ cryptate and d2 are in close proximity and initiate a fluorescence resonance energy transfer (FRET) signal which is proportional to the phosphorylation of ERK1/2. This signal can be measured using a ClarioStar plate reader fluorescence was measured. For data analysis, the ratio of fluorescence at 665 over 620 nm was calculated for sample wells and plotted against the logarithm of agonist compound concentrations.

2.4.2 Ca^{2+} Mobilization Assay

FLAG-mFFA4-eYFP Flp-In T-REx cells were cultured until reach 80% confluence then subculture and seeded at 80,000 cells per well in pre coted poly D-lysine 96 black clear bottom plates and placed in the incubator for 24 hours. The flowing day the medium was changed with medium that either contained

doxycycline to induce receptor expression (100 ng/ml), or with regular culture medium, then the plates were placed in the incubator for another 24 hours. On the day of the assay, cells were loaded with treated with doxycycline for 18-24 hours to induce receptor expression. Cells were loaded with the Ca2+-sensitive dye Fura-2 AM (1.5 μM) in media and incubated at 37 °C for 45 minutes.

The cells were then washed twice with HBSS (for 500 ml NaHco3 solution was added to a cold 50 mL HBSS solution then a 19 gram HEPES was added to the solution then the pH was adjust to 7.4 using NAOH then the volume was completed to 500ml with H2O) and incubated at 37°C with 100 μL HBSS per well for 15 minutes. For antagonist assay cells were pre-incubate with 2X antagonist concentrations that were made in HBSS and then with 2X ligand solution that also was made with HBSS (130 μl/well) in a full 96-well plastic plate. The plate was then read in the FlexStation™ II at 37 ^{0}C. Baseline fluorescence was measured for 16 seconds; test compounds were then added, and fluorescence was measured for an additional 74 seconds

2.4.3 β-Arrestin-2 BRET Assay

HEK 293T cells were cultured until 80% confluency, then split flask into 10 cm dishes and placed in the incubator at 37 °C and 5% CO2 for 24 hours until they reached 70-60% confluency. Then dishes were transfected with mFFA4-HA receptor or hFFA4-HA, fluorescent energy acceptor (mNG-CAXX) and bioluminescence donor ß-arrestin of the bright Nanoluc BRET donor (NLuc-h-arrestin 3) in a 3:1 ratio using PEI.
Dishes were placed in the incubator at 37 °C and 5% CO2 for 24 hours. Cells were detached by incubating with trypsin and then centrifuged then the media were removed and the cells were vortexed with the required amount of media to plate 50,000 cell per well plated in white 96 well plates that were pre-coated with poly D-lysine. Plates were maintained for a further 24 hours in the incubator at 37 °C and 5% CO_2 overnight prior to the experiment.

In the day of the experiment, cells were washed twice with HBSS and then incubated in HBSS for 30 min at 37°C. cells then were loaded with nano-glow substrate before a further incubation in dark for 10 min at 37 ^{0}C. cells were moved to PHERAstar plat reader then the light emissions was measured at 475

nm and 535 for 5 minutes before adding the Agonist TUG-891 and α-linolenic acid which is the natural activator for FFA4 were added at the relevant concentrations and the measurement was completed for 30 mints in total . BRET ratios were obtained by dividing the emission at 535 nm by the emission at 475 nm and subtracting the 535 nm/475 nm ratio for cells expressing only the Nluc-ß-arrestin donor.

2.4.4 TRUEPATH BRET Assay

HEK cells were cultured to 70% confluency, then split 10 cm dishes and placed in the incubator at 37 °C and 5% CO_2 for 24 hours. The dishes were then transfected with the desire bio censers. For Gq pathway M1 receptor as a reference in this pathway. the transfection was made either with 1.25 μg M1 or mFFA4 receptor then 1.25 μg Gαq and 1.25 μg G ß3 and finally 1.25 μg Gγ9. For Gi pathway cells were transfected with 1.25 μg mFFA4 receptor then 1.25 μg Gαi2 and 1.25 μg G ß3 and finally 1.25 μg Gγ8. For a subunit from Gq/11 which is G15 cells were transfected with 1.25 μg mFFA4 receptor then 1.25 μg Gαi2 and 1.25 μg G ß3 and finally 1.25 μg Gγ13

A summary of the DNA mixture for each pathway can be found on table 2.7.

Table 2-7 DNA mixtures for G protein signiling pathways

Receptor	Gα	G ß	Gγ
M1	Gq	ß3	γ9
MFFA4-HA	Gi2	ß3	γ8
MFFA4-HA	G15	ß3	γ13

Then 50 mM NaCl was added to each DNA mixture of 150 mM NaCl
And 1 mg/ml polyethyleneimine was prepared then Add to each tube. The tubes then Incubated at room temperature for 15 minutes. Medium was changed on the dishes before adding the DNA mix to each assigned Dish. The dished placed back in the incubator at 37 °C and 5% CO_2 for another 24 hours.
The medium was removed from the dishes and trypsin was added to detach cells. Once cells detached, growth medium was added to each dish to inactivate the trypsin. The medium was transferred to a 15 ml tube then centrifuged at

2000 xg for 5 minuets. The supernatant was removed, and cells were resuspended in growth medium and plated at 50,000 cells per well in white 96 well plates pre-coated with poly D-lysine. Plates were then placed back in the incubator at 37 °C and 5% CO_2 for another 24 hours.

In the day of the assay cells were washed twice with HBSS then incubated in HBSS at 37 °C for 30 minutes. Then cells were loaded with Prolume purple substance to sense BRET signal. Then a rang of Agonist TUG-891 and α-linolenic acid which is the natural activator for FFA4 were prepared in 1% DMSO HBSS with a range of concentrations then added Independently to the plates then the plates. Plates then placed in CLARIOstar to read the signal for 5 minutes.

2.4.5 Receptor internalisation by confocal microscopy

FLAG-mFFA4- eYFP Flp-In T-REx cells were cultured until reach 80% confluence then subculture and seeded at 20,000 cells per well in pre coted poly D-lysine chamber slide and placed in the incubator at 37 °C and 5% CO_2 for 48 hours. Then doxycycline (100 ng/ml) were used to induce mFFA4 receptor and cells were incubated for another 24 hours in the incubator at 37 °C and 5% CO_2. In the experiment day cells were washed with HBSS then added to each well-known volume of HBSS. First, 10μM TUG-891 were added and Images were taken after addition of ligand every 1 minutes for 45 minutes. Then cells were pre-treated with 10μM AH7614 for 5 minutes then 10μM TUG-891 after the addition Images were taken every 1 minutes for 45 minutes. The images were manipulated using FIJI.

2.5 Gene Expression Analysis

2.5.1 RNA isolation.

RNA was isolated from both 3T3-L1 fibroblast and Adipocytes by using the Qiagen RNeasy Plus Mini kit following the manufacturers protocol. Cells were homogenisation in RLT Buffer containing 10% ß-mercaptoethanol, the homogenate was centrifuged at 10,000 x *g* for 30 sec in a gDNA eliminator column then an equal amount of 70% ethanol was added to the mixture then

applied to a RNeasy Mini spin column and centrifuged at 8000 x *g* for 15 sec at room temperature The flow-through was discarded and the column was washed twice with wash buffer, then centrifuging for 2 min. RNA was eluted in 30-40 µL nuclease-free water.

2.5.2 RNA concentration.

RNA concentration was determined by measuring absorbance at 260 nm using a Nanodrop spectrophotometer. RNA purity was assessed according to the absorbance ratios at 230/260 nm and 260/280 nm. RNA was stored at –80°C.

2.5.3 DNAse digest

DNAse digest was performed to ensure RNA samples extracted from the cells were free of DNA contaminants. The reaction was set up with the following this steps:
1 µg of RNA in 8 µL nuclease free water and 2 µL of a 1:1 mixture of DNase amplification grade and 10X DNase I reaction buffer. This mixture was incubated at room temperature for 15 min before 1 µL of DNase I stop solution was added to stop the reaction.

2.5.4 Reverse transcript (making cDNA)

For cDNA synthesis, extracted RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied BiosystemsTM, P/N: 4368813). Reactions were set up in as follows; 1 µg RNA template was mixed with random hexamers (50 ng/µl final concentration) and dNTPs (final concentration 2.5mM for each dATP, dTTP, dCTP, dGTP),
Mix – 100 mM DTT – RNAse-free water Reaction and Moloney Murine Leukemia Virus MMLV Reverse Transcriptase enzyme. Then the mixtures were incubated in a thermal cycler using the following conditions: 1. Annealing 10 min 25°C 2. Extension 50 min 37°C 3. Inactivation 15 min 70°C 4m, before being held at 4 °C. Then cDNA samples were stored at -20°C until quantitative real-time (qRT)-PCR was performed.

2.5.5 Real-Time Polymerase Chain Reaction

Quantitative PCR analysis was performed via QuantStudioTM 5 Real-Time PCR System (Thermo Fisher Scientific) using the fluorescence channel for SYBR Green. A q-PCR master mix composed of 10 μL fast sybr green, 5 μM forward primer and 5 μM reverse primer, was prepared and made up to 16.8 μL with nuclease free H_2O. Each reaction was set up in a total volume of 20 μL in duplicate in 384-well PCR plates.

Following is the list of primer as shown in table used in this study

Table 2-8 Sequences of the primers used in the Real-Time PCR analysis

Gene Name	Gene Symbol	Oligonucleotide sequence (59-39)	
		Forward 5' - 3'	Reverse 5' - 3'
Free fatty acid receptor 1	FFA1	TTTCTGCCCTTGGTCATCAC	CTAGCCACATTGGAGGCATT
Free fatty acid receptor 2	FFA2	AGAGGAGAACCAGGTAGAAG	TCTTTCCGGAGAGTTCTGAC
Free fatty acid receptor 3	FFA3	CACCATCTATCTCACCGCCC	TATGACGTAGACCACGCTGC)
Free fatty acid receptor 4	FFA4	GGCACTGCTGGCTTTCATA	GATTTCTCCTATGCGGTTGG
Glucose transporter 1	Glut1	TCAACACGGCCTTCACTG	CACGATGCTCAGATAGGACATC
Glucose transporter 4	Glut4	GTAACTTCATTGTCGGCATGG	AGCTGAGATCTGGTCAAACG
leptin		TCTCCGAGACCTCCTCCATCT	TTCCAGGACGCCATCCAG
Protein kinase B	AKT	CTCATTCCAGACCCACGAC	ACAGCCCCGAAGTCCGTTA
Adiponectin	Adipo	GGAACTTGTGCAGGTTGGAT	CCTTCAGCTCCTGTCATTCC
Insulin receptor	IRS	GACTATATGCCCATGAGCC	GACATGTTCATGTAGTCACC
Syntaxin 4		CGACAGGACCCATGAGTTGAGGC	GGTGATGCCAATGATGACAGCCAA
hypoxanthine-guanine phosphoribosyl transferase	mHPRT	AGGCCAGACTTTGTTGGATTTGAA	CAACTTGCGCTCATCTTAGGCTTT
Syntaxin 6	-	CGACTGGACAACGTGATGAA	CTGGGCGAGGAATGTAAGTG
Peroxisome proliferator-activated receptor gamma	PPARγ	ATTGAGTGCCGAGTCTGTGG	GGCATTGTGAGACATCCCCA

Plates were read on a QuantStudioTM 5 Real-Time PCR System.

The amplification cycles were set up using the following conditions: as showed in the table

Table 2-9 amplification cycles process

Stage	Temperature	Time
Preheating	50°C	2 min
Denaturing	95°C	5 sec
Annealing	58°C	60 sec
Repeat steps 2–3 (x44)		
melt curve Heating	95°C	1 sec
Annealing	60°C	5 sec
Heating	95°C	5 sec

Quantification of the fold change in gene expression was determined with the $2^{\Delta\Delta Ct}$ method. In the $2^{\Delta\Delta Ct}$ method the fold of gene expression is calculated relative to the sample control after first correcting for the expression of a housekeeping gene. First, for all the samples, the difference in Ct values between all the gene was determined by Delta Ct = Ct (housekeeping gene HPRT) – Ct (each sample). Then a Delta delta Ct was calculated using the reference sample in this study which was undifferentiated 3T3-L1 cells. ΔΔCt values were calculated by subtracting the ΔCt value of the target gene from the ΔCt value of a reference condition. Then $2^{-\Delta\Delta CT}$ were calculated and used to determine the fold of expression.

2.6 Immunoblot

2.6.1 Preparation of 3T3-L1 lysates

On day 9 of differentiation 3T3-L1 adipocytes in 6 well plates were incubated at 37°C in a 10% (v/v) CO_2 in serum-free DMEM for 2 hours. Cells were subsequently incubated with test substances (DMSO or AH7614) for 10 minutes at 37°C in 10% (v/v) CO_2 then cells were 1 μm insulin stimulated or 20 minutes. The medium was removed, prior to addition of ice-cold lysis buffer to each well. The cells were then scraped off using a cell lifter and transferred into pre-cooled 1.5 ml microcentrifuge tubes and incubated on ice for 30 min. The extracts were centrifuged at 21,910 x g for 3 min at 4°C. The supernatants were stored at -80°C.

2.6.2 Protein concentration determination.

Protein concentrations in lysates were assayed following the Bradford assay. First, 990 μL of H_2O, 10 μL of lysate buffer control and 1 mL of bradford reagent were added to 3 mL cuvettes. Absorbance at a wavelength of 595 nm was measured in a spectrophotometer (Eppendorf BioPhotometer) and concentrations interpolated from a standard curve created by measuring the absorbance of standards with known concentration. Then for each sample 10 μl of protein samples were mixed with 1 ml Bradford's Reagent.

2.6.3 SDS-PAGE.

Cell lysates were mixed 1:1 2X Laemmli sample buffer (312.5 mM Tris-base, pH 6.8, 10% w/v SDS, 50% v/v glycerol, 250 mM DTT and 0.01% bromophenol blue) to give a 1X final. Samples then were heated to 60°C in a heating block for 10 min. sampleas were then resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A 10% SDS- PAGE gel were made(375 mM Tris (pH 8.8), 0.1% SDS (w/v), 0.1% APS (w/v), and 200 nM TEMED, diluted in distilled water , 20 μg of protein per lane was loaded and gels were run at 80 V for 90 min and until the dye front had migrated to the bottom of the gel and good separation of the molecular weight markers had been obtained.

2.6.4 Electrophoretic Transfer into Nitrocellulose Membrane

After separating proteins using SDS-PAGE, gels were removed and placed on top of 8 x 9 cm nitrocellulose membrane that was then placed between two pieces of filter paper (9 x 10cm and 3mm thick). Filter paper was pre-soaked in transfer buffer (25 mM Tris, 200 mM glycine, 20% (v/v) ethanol) and were placed between transfer sponges and inserted into a holder cassette which was inserted into the Bio-Rad electrophoresis tank and electrophoresed for 120 min at 60V. After protein transfer was completed, nitrocellulose membranes were removed from the transfer cassette and the efficiency of the transfer were determined by the presence and intensity of the pre-stained molecular weight standards. Also, by briefly staining the nitrocellulose membrane with Ponceau red to confirm sample protein transfer.

2.6.5 Blocking of Membranes and Probing with Primary Antibodies.

After staining the membrane with Ponceau Red, the membrane was washed extensively with TBS (20 mM Tris-HCl, pH 7.5 and137 mM NaCl). Non-specific sites on the nitrocellulose membranes were blocked by incubating the membrane in TBS with 5% (w/v) milk powder for 60 min at room temperature. After that the primary antibodies were applied and incubated with shaking, overnight at 4°C in the same blocking buffer.

2.6.6 Secondary Antibodies and Immunodetection

Following overnight incubation in primary antibody, nitrocellulose membranes were washed three times for 5 min with TBST. Secondary antibodies were prepared the same way as primary antibodies and nitrocellulose membranes were incubated in secondary antibodies in the dark for 1 h at room temperature with shaking. Membranes were then washed three times for 5 min with TBST followed by one wash with TBS for 5 min and finally one wash with H_2O for 5 min. proteins were visualised using LICOR Odyssey SA scanner using the appropriate lasers. Using protein ladder the molecular wight for the appearing band was determined.

2.7 Glucose uptake assay.

3T3-L1 fibroblast were split in 12 well plates and differentiated to adipocytes as described in section 2.2.1.2. 3T3-L1 adipocytes were first washed once with serum-free DMEM then incubated with serum-free DMEM for 2 hours at 37 °C. The cells were transferred to a hot plate maintained at 37°C and washed twice with Krebs-Ringer-phosphate buffer (128 mM NaCl, 1.25 mM $CaCl_2$, 4.7 mM KCl, 5.0 mM NaH_2PO_4, 1.25 mM $MgSO_4$). Depending in the assay celles where either incubated with DMSO or AH7614 or cells were then incubated for 10 minutes or 30 minutes in KRP containing the agonist TUG891. After each incubation time, different insulin concentrations were added to the cells for 20 minutes. Thereafter, [$_3$H] deoxyglucose/deoxyglucose solution was added to achieve a final concentration 60 µM deoxyglucose and 0.30 µCi/well and quickly mixed. After 3 minutes the reaction was terminated by quickly flipping the plates to remove isotope and dipping the plates in ice-cold PBS three times to stop the

reaction. The plates were then allowed to dry for at least 30 minutes before the cells were solubilized overnight with 1% (v/v) Triton X-100. The solubilized material was then collected in scintillation vials with 5ml scintillation fluid vial. A Beckman Multi-Purpose scintillation counter LS6500 was used to measure 2-[^{3}H]-deoxy-D-glucose uptake.

2.8 Oil Red O stain.

3T3-L1 cells were grown and differentiated as described above (section 2.2.5). In preparation, fresh stain is prepared from stock isopropanol supplemented with 0.6% (w/v) Oil Red O, passed through a syringe filter to removed un-dissolved Oil Red O. The medium was removed from the cells, and they were washed three times with PBS. Cells were fixed in 10% formalin for 30 minutes at room temperature. Fixing solution was removed, and cells washed with twice with PBS followed by a rinse with 60% isopropanol. Freshly prepared stain was added to cells for 15 minutes at room temperature. Stain was discarded and cells rinsed with 60% isopropanol followed by a water rinse. After drying, the stained cells would show lipids in red. Images were taken using an inverted light microscope fitted with colour camera using Evos imaging system with colour camera at 200μM magnification. For quantitative, stained cells were washed twice with 60% isopropanol then oil red stain was dissolved in 500μl 100% isopropanol for each well in 12 well plates then incubated for 5 minutes. The isopropanol with extracted Oil red O was transferred to a clear 96 well plate to measure absorbance at 492nm using PHERAstar. Pluse, control wells was lodded with 100% isopropanol.

2.9 Lipolysis.

3T3-L1 cells were grown and differentiated as described above (section 2.2.5). On day 10 cells were washed wash twice with HBSS containing 25 mM glucose and 1% fatty acid-free bovine albumin serum, then cells were incubated with the same buffer either in the presence or absence of isoproterenol at 37°C for 1 h. After this time, 50 μl of cell supernatants was transferred to a 96-well plate. Glycerol concentration in the supernatants was measured by the addition of 50 μl/well free glycerol reagent (Sigma-Aldrich, 260mg/L), followed by

incubation at 37°C for 15 min before absorbance at 540 nm was measured using a Pherastar FS microplate reader. A stander glycerol curve was made along with experiment from the provided glycerol standard. The final concentrations for the Standard glycerol curve was ranged from (20, 15,10,8,6,4,2,1,.5,0) mg/ml.

2.10 Nuclear Stain and Cell Count

3T3-L1 cells were cultured in 24 well plate then a differentiation process started. In parallel, control undifferentiated cells were maintained in normal grown medium with NCS, which was change on the same days as the medium changes in the differentiation protocol occur in Section 2.2.1.2. At day 10, the medium was removed from the plate then washed twice in PBS. Cells were then fixed using 10% formalin at RT for 30 mins. Then the fixation solution was removed and cells were washed twice with PBS. 250 μl of staining solution (Hoechst 33342 at 1:1000 dilution in PBS.) was added to each well and incubated in the dark at RT for 15 mins. Then cells were washed twice in PBS and kept in fresh PBS for imaging on an EVOS FL Auto 2 system using 200μm objective and DAPI filters. Images were analysed using FIJI to determine cell counts by counting the intense dye in the nucleus. The counts were measured in counting the nucleus that have been dyed with blue florescent dye as a result of using Hoechst stain.

2.11 Data analysis

Statistical analyses were carried out using GraphPad Prism software. Using two-tailed unpaired student's t test (for two groups), one -way (ANOVA) to analyse how one factor affects a variable response and two-way (ANOVA) to compere 2 factors affects a variable response.

The two-tailed unpaired Student's t-test was employed in this thesis to compare the means of two independent groups and determine if there was a statistically significant difference between them. This test was chosen due to its ability to assess differences in means while considering the variability within the sample data. The null hypothesis stated that there was no significant difference between the means of the two groups, while the alternative hypothesis proposed the presence of a significant difference(Clarke et al., 2008). The test statistic

was calculated based on the means, standard deviations, and sample sizes of the two groups, and the resulting p-value was compared to a predetermined significance level(Livingston & Cassidy, 2005). If the p-value was lower than the significance level, it indicated a rejection of the null hypothesis, supporting the presence of a significant difference between the means of the two groups (Biau et al., 2010). The two-tailed unpaired Student's t-test served as an invaluable tool in this thesis to compare and evaluate the significance of observed differences between two distinct groups of interest.

Furthermore, one-way ANOVA and two-way ANOVA were used to analyze the effect of factors on a variable response (Armstrong et al., 2002). One-way ANOVA examined the influence of a single factor, comparing means across multiple groups. Two-way ANOVA assessed the combined effect of two factors and their interaction on the response variable(R. A. Armstrong et al., 2002). These analyses allowed for the identification of significant differences among groups or levels of a factor, as well as the examination of main effects and interaction effects. By using one-way ANOVA and two-way ANOVA, this thesis gained insights into how factors affected the variable response and drew meaningful conclusions from the data analysis.

For experiments with drugs concentrations Concentration–response curves obtained from functional assays were fitted according to a four-parameter logistic equation in GraphPad Prism 9 to determine EC50 for each compound. The collected data is subjected to curve fitting, which involves fitting a mathematical model to the data points. Common models used for curve fitting include the sigmoidal dose-response models in linear models (such as the linear regression) (Meddings et al., 1989). Once the curve is fitted to the data, the potency of the compound can be calculated. The potency is typically defined as the concentration or dose of the compound required to produce a specific response level (e.g., 50% of the maximum response). This value is often referred to as the EC50 (effective concentration 50%) or IC50(Kenakin & Williams, 2014; Sebaugh, 2011).Curve fitting and potency calculations are essential components in determining the potency of a compound and evaluating its activity. These techniques provide quantitative measures of the concentration-response relationship and help determine the potency values with statistical confidence.

For western blot data, Quantification of intensity of western blot bands was achieved by measuring using Image Studio Lite a LICOR-recommended software for blot analysis. Band intensity corresponding to the protein of interest was always normalised to total protein stain.

Chapter 3 Pharmacological evaluation of FFA4 agonist TUG-891 and FFA4 antagonist AH7614

3.1 Introduction

G-protein-coupled receptors are a major focus for drug companies because they control a wide range of human physiological processes, including growth, metabolism and homeostasis. According to a recent article in *Frontiers in Pharmacology*, 30%–50% of marketed drugs are thought to work by targeting GPCRs, and of the 219 new molecular entities approved by the US Food and Drug Administration between 2005 and 2014, 54 (25%) target GPCRs (Alimardani et al. 2021; Hauser et al. 2017). One of these is the FFA4 free fatty acid receptor, which has been reported as a drug target for the treatment of metabolic syndromes (Ichimura et al. 2014). This receptor has been demonstrated to be activated by long- and medium-chained FFAs such as omega-3 polyunsaturated fatty acids (n-3 PUFAs; Ichimura et al. 2014). However, these FFAs can also activate FFA1; thus, since there was a need for a novel agonist for FFA4, TUG-891 was identified as a selective agonist for the FFA4 receptor (Hudson et al. 2013). Although AH7614 is the only identified antagonist for FFA4, its mechanism of antagonism remains undetermined and requires further study. Having high-affinity and selective antagonists that can inhibit the actions of both endogenous and synthetic agonists for the FFA4 receptor is essential for the analysis of receptor function and support of potential therapeutic suggestions. For this reason, a set of pharmacological assays was designed to assay TUG-891 activation and AH7614 inactivation effects on the FFA4 receptor.

As it is frequently not feasible to measure these properties directly, the pharmacology of ligands is often characterised by means of functional assays in which a concentration–response curve is generated to determine a ligand's potency (EC50) and maximal response (Milligan et al. 2017); potency is related to affinity, whereas maximal response is related to intrinsic efficacy. However, due to the complexity of functional systems, both potency and maximal response vary depending on the assay system employed (Milligan et al. 2017). Therefore, it is impossible to directly compare the potency of two ligands at the same receptor without testing them under identical conditions (Milligan et al. 2017). For this reason, I tested both TUG-891 and AH7614 under identical assay conditions.

To test the efficacy and potency of TUG-891 and profiling AH7614 antagonism on mFFA4, a set of pharmacological assays was performed. These assays were performed in three heterologous systems that had been transfected with mFFA4 CHO Flp-In FFA4 cells, HEK293T cells and FLAG-mFFA4-eYFP Flp-In T-REx cells.

3.2 Aims

The main aim of this chapter is to establish the pharmacological properties of the key FFA4 ligands TUG-891 and AH-7614. To achieve this, I use three heterologous systems with mFFA4 CHO Flp-In FFA4 cells, HEK293T cells and FLAG-mFFA4-eYFP Flp-In T-REx cells. These cells are used in a set of pharmacological assays: extracellular signal regulated kinase 1 and 2 (ERK1/2) phosphorylation assay, Ca mobilisation, initialisation and ß-arrestin recruitment. In addition, a TRUPATH bioluminescence resonance energy transfer (BRET) assay is performed to assay G-coupled proteins.

Using this set of pharmacological assays, I aim to reveal the efficacy and potency of TUG-891 and profile AH7614 antagonism on mFFA4. Moreover, I hope to reveal more about the antagonist's properties because they have previously only been narrowly characterised. By determining the magnitude of responses in G-coupled assays, I hope to establish which G pathway is coupled more strongly with mFFA4 activation. As a final aim, I hope that this assessment of TUG-891 and AH7614 can help determine their use as a potential drug or as a tool in assaying more functions of the FFA4 receptor.

3.3 Results

3.3.1 FFA4 agonist induces the phosphorylation of extracellular signal regulated kinase

The phosphorylation of ERK1/2 was the first of the general assays to be examined to determine the pathways activated after inducing the FFA4 receptor with an agonist. The activation of FFA4 has previously been shown to stimulate ERK phosphorylation. To evaluate the ability of the FFA4 selective agonist to activate downstream signalling, two published FFA4 agonists, TUG 891 and TUG-1197, were tested in ERK phosphorylation assays using a CHO cell line engineered to express mFFA4 tagged at its C-terminal using eYFP. The two FFA4 agonists were evaluated by generating concentration-response curves for ERK phosphorylation after 5 min of agonist treatment. The FFA4 agonists tested produced clear concentration-dependant increases in ERK phosphorylation (Figure 3-1). The potency was 7.2 for TUG-891 and 6.2 for TUG-1197.

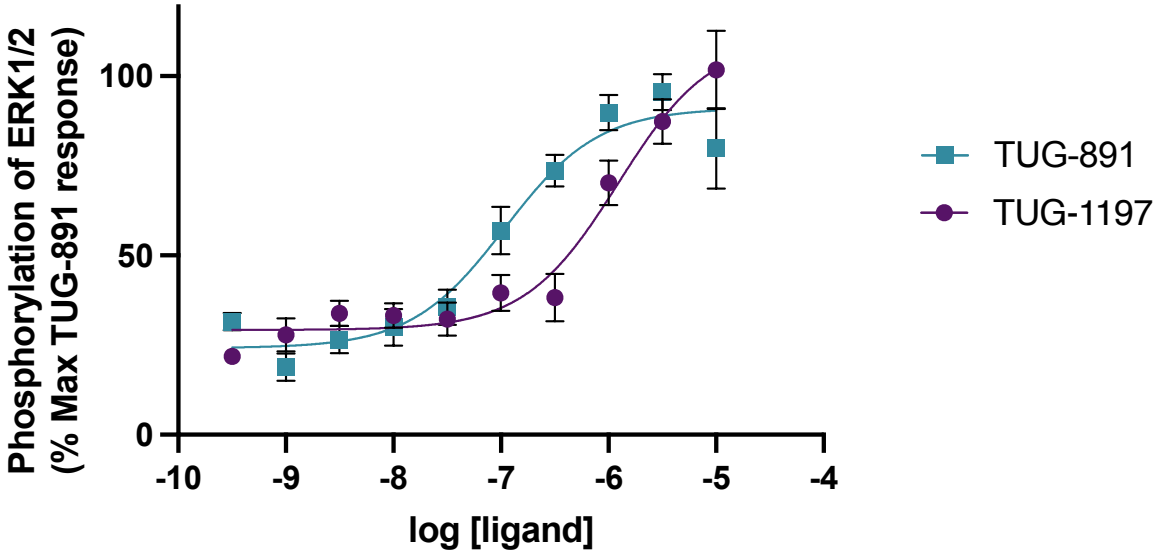


Figure 3-1 ERK phosphorylation assay using CHO FFA4 cells
Flp-In CHO cells stably expressing mFFA4 following treatment with FFA4 agonists TUG-891 or TUG-1197. Cells were treated at a range of concentrations for 5 min to produce a concentration–response curve. TUG-891 was used as the reference ligand. Results are shown as the mean ± standard error of three independent experiments (n = 3).

3.3.2 Activation of the FFA4 receptor leads to increases in intracellular Ca^{2+}

Reports have primarily described FFA4 as coupled with Gq/11 signalling (Hudson et al. 2013). I therefore next aimed to characterise the ability of FFA4 agonists to activate this pathway. Since intracellular Ca2+ is elevated when the Gq/11 pathway is activated (Rashid et al. 2007),

an assay was established to determine the ability of FFA4 agonists to stimulate Ca2+ mobilisation. To achieve this, a Flp-In T-REx 293 cell line generated to express murine FFA4 (mFFA4) tagged at its C terminal using eYFP was employed (Prihandoko et al. 2016). In these cells, mFFA4-eYFP expression is induced only following treatment with doxycycline (dox) to express the eYFP tag (Figure 3-2A).

To test the ability of both TUG-891 and an LCFA agonist of FFA4, α-linolenic acid (aLa), to stimulate Ca2+ mobilisation, mFFA4-eYFP cells were either treated with dox or left untreated and then tested in a Ca2+ assay using a range of agonist concentrations (Figure 3-4). Concentration-dependant increases in Ca2+ were observed for both TUG-891 and aLa. TUG-891 was significantly more potent ($P = 0.0344$), with a potency (6.51 ± 3.1) ~40-fold that observed for aLa (4.98 ± 1.4). By contrast, TUG-891 revealed no significant difference in efficacy compared with aLa ($p = 0.9821$). Notably, the responses to each ligand were mediated by mFFA4, as no responses were observed in cells that had not been treated with dox to induce mFFA4 expression (Figure 3-2B).

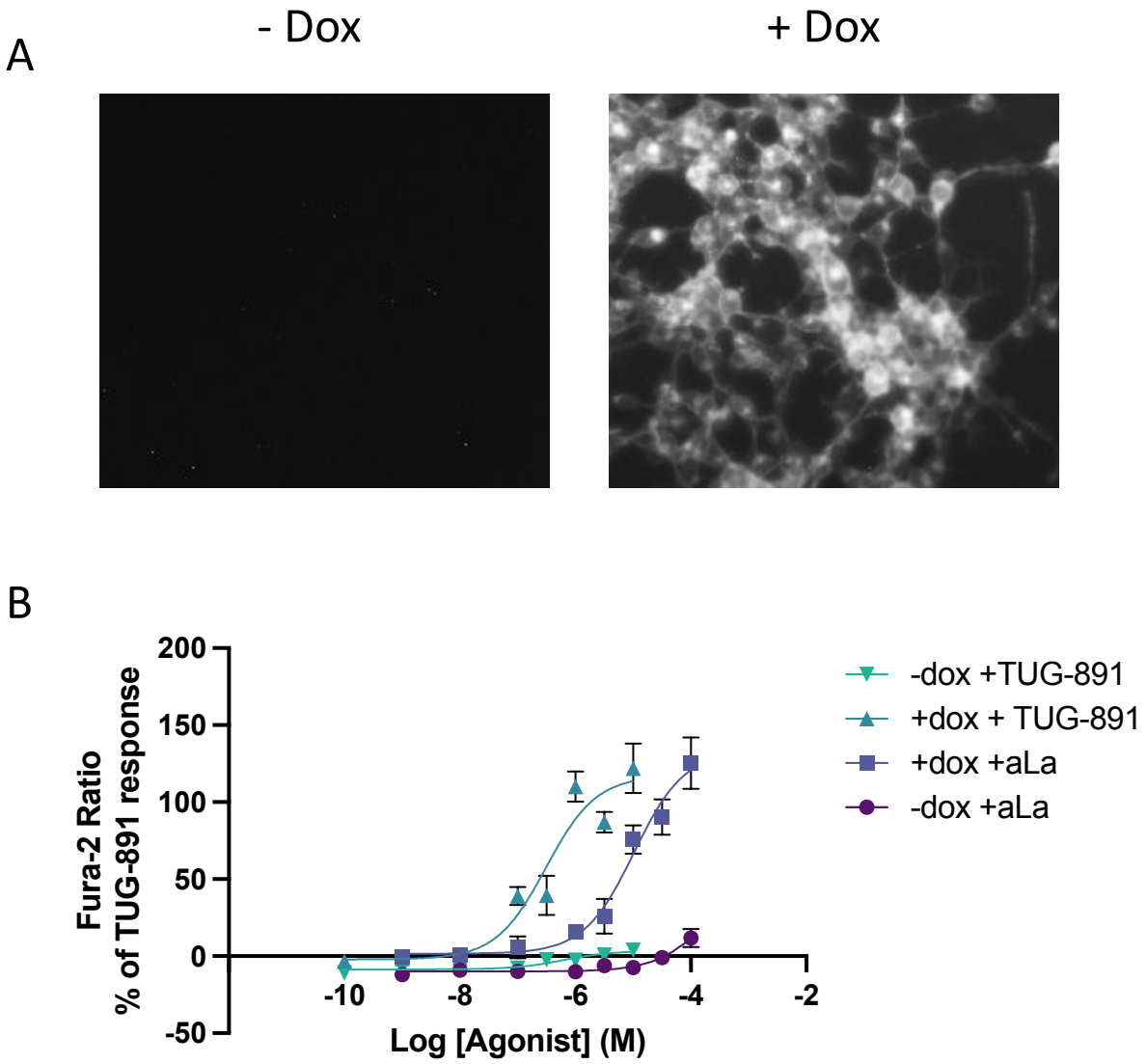


Figure 3-2 TUG-891 and α-linolenic acid (aLa) elevate intracellular calcium through mFFA4:

Flp-InTM T-RExTM 293 cells able to express mFFA4 were seeded into poly-D-lysine-coated black clear-bottomed 96-well plates. Expression of mFFA4 was induced by the antibiotic doxycycline (dox; 100 ng/ml). A) dox cells (+/−) showing eYFP expression + dox (scale = 1000 μM). Cells were labelled using the calcium-sensitive dye Fura-2 and were then exposed to varying concentrations of TUG-891 or aLa. Mobilisation of intracellular calcium was recorded in a FlexStation plate reader. Efficacy was normalised as the percentage of the TUG-891 maximum response. Data represent the mean ± standard error of three independent experiments.

3.3.3 Activation of mFFA4 coupled to Gi and Gq/11 G proteins

To establish more broadly which G proteins FFA4 may couple to, open-source BRET-based TRUPATH biosensors were next employed (Olsen et al. 2020). These sensors take advantage of the dissociation of Gα from G βγ in the G-protein activation step by tagging Gα with an Rluc BRET donor and Gγ with a GFP^2 BRET acceptor. When the G protein is activated and α dissociates from βγ, the BRET signal between Rluc and GFP^2 decreases.

TRUPATH sensors have been designed to measure the activation of up to 15 distinct Gα proteins (Olsen et al. 2020); however, I focused specifically on using these sensors to measure the coupling of FFA4 to Gαq, Gαi2 and Gα15. Before testing FFA4 in this assay, I first determined the viability of the assay using a positive control, the M1 muscarinic acetylcholine receptor, which has previously been shown to couple strongly with the Gq TRUPATH sensor (Brown et al. 2021)). HEK 293T cells were co-transfected with M1 as well as Rluc-tagged Gαq, Gß and GFP2-Gγ. When these cells were treated with acetylcholine, the endogenous agonist of the M1 receptor, a clear concentration-dependant reduction in BRET was observed, with a potency of 6.7. These data demonstrate that TRUPATH sensors are effective tools for measuring the activation of specific G proteins (Figure 3-3A).

Subsequently, I aimed to use TRUPATH sensors to assess G-protein coupling with mFFA4, starting with the Gq TRUPATH sensor. HEK 293T cells were co-transfected with mFFA4 as well as Rluc-tagged Gαq, Gß and GFP2-Gγ. When these cells were treated with the FFA4 receptor ligands, TUG-891 and aLa, a clear concentration-dependant reduction in BRET was observed, with a potency of 6.3 for TUG-891 and 4.4 for aLa (Figure 3-3B).

Gα15 was the next assay performed, with HEK 293T cells co-transfected with mFFA4 as well as Rluc-tagged Gα15, Gß and GFP2-Gγ. When these cells were stimulated with TUG-891 and aLa, the FFA4 receptor ligands, an obvious concentration-dependant decrease in BRET was observed, with a potency of 7.3 for TUG-891 and 4.1 for aLa (Figure 3-3C).

The final pathway to be assessed was Gαi2. HEK 293T cells were co-transfected with mFFA4 as well as Rluc-tagged Gαi2, Gß and GFP2-Gγ. When these cells were treated with TUG-891 and aLa, the FFA4 receptor ligands, an obvious concentration-dependant decrease in BRET was observed, with a potency of 6.2 for TUG-891 and 4.2 for aLa (Figure 3-5C). The pEC_{50} values are summarised in Table 3.1.

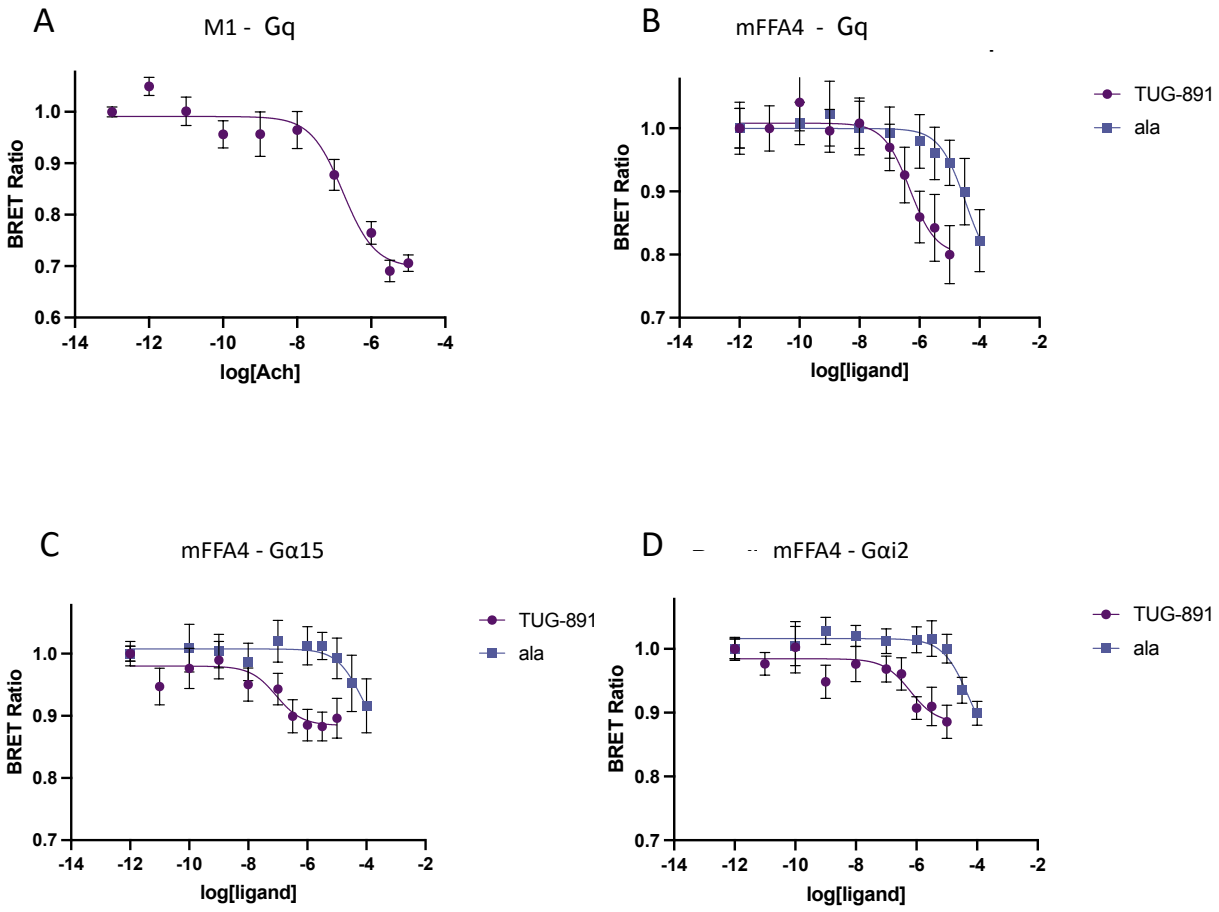


Figure 3-3 mFFA4 couples to Gq/11 and Gi/o G proteins
HEK cells transfected with the M1 receptor and Gαq subunits show a decrease in BRET signal after treatment with a range of acetylcholine concentrations. For FFA4, a range of TUG-891 or α-linolenic acid concentrations were used as a treatment. B) HEK cells transfected with mFFA4 and Gαq subunits reveal a reduction in BRET signal. C) HEK cells transfected with mFFA4 and Gα15 subunits show a reduction in BRET signal. D) HEK cells transfected with mFFA4 and Gαi2 subunits reveal a reduction in BRET signal. Results shown as a mean ± standard error of three independent experiments (n = 3).

Table 3-1 pEC50 potency values of agonists at mFFA4 in various Gα assays using BRET

G-coupled protein assayed	TUG-891	α-linolenic acid
Gαq	6.3	4.4
Gα15	7.1	4.1
Gαi2	6.2	4.2

3.3.4 Activation of the FFA4 receptor induces β-arrestin recruitment

FFA4 not only activates G-protein-mediated pathways but induces ß-arrestin recruitment (Hudson et al. 2013). The assay was performed using the BRET principle to measure this recruitment.

A BRET-based ß-arrestin recruitment assay was performed in HEK293T cells. HEK293T cells were co-transfected with untagged mFFA4 or human FFA4 (hFFA4), an mNeonGreen (mNG) fluorescent protein anchored to the cell membrane with the CAAX motif of 3:1 and ß-arrestin 2 tagged at its N terminal using nanoluciferase (Nluc). This format allowed for a bystander BRET-based arrestin assay that could measure the recruitment of ß-arrestin 2 following the activation of FFA4.

Two FFA4 agonists were used, TUG-891 and aLa, to stimulate ß-arrestin recruitment to the cell membrane. Both ligands were able to induce this recruitment following the activation of mFFA4 and hFFA4, as illustrated for TUG-891 in Figure 3-4 C and D and for aLa in Figure 3-5C and D. Clear concentration-dependant increases in BRET were observed in both the TUG-891 (Figure 3-4C and D) and aLa (Figure 3-5C and D) treatments.

Two negative controls were also included in this assay. First, to show that the BRET signal increase depends on the presence of the receptor in the transfection, the control was designed to replace the receptor with pcdna3 together with mNG-CAAX and Nluc-arrestin in both TUG-891 (Figure 3-4A) and aLa (Figure 3-5A).The other control aimed to demonstrate that the BRET assay indicated ß-arrestin recruitment; the transfection therefore replaced mNG-CAAX with pcdna3, the mFFA4 receptor and Nluc-arrestin (Figure 3-4B) for TUG-891 and (Figure 3-5B) for aLa.

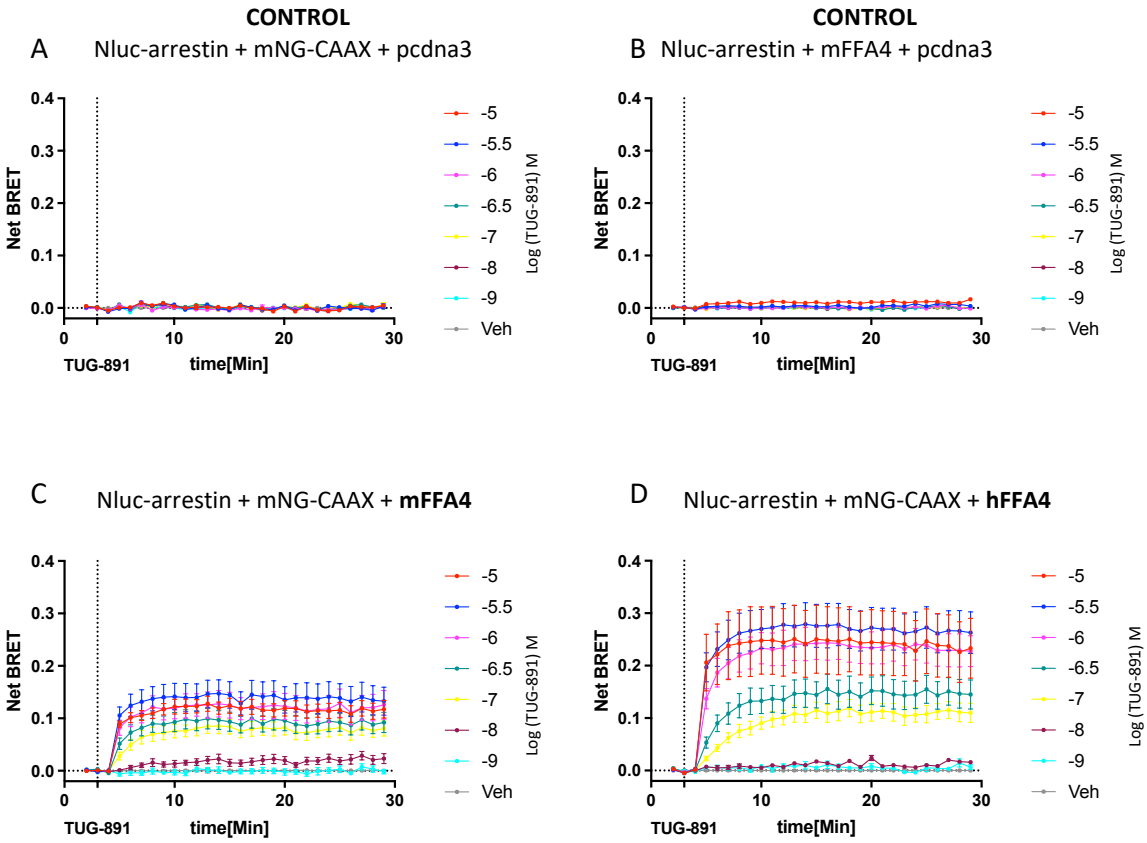


Figure 3-4 FFA4 receptor induced β-arrestin recruitment after stimulation by TUG-891
HEK cells were transfected with different plasmids as required and then seeded in 96-well plates pre-coated with poly-D-lysine. TUG-891 concentrations were added after measuring the BRET signal for 5 and 25 min. A) BRET signal in the absence of FFA4 receptor cells transfected with Nluc-arrestin and mNG-CAAX pcdna3. B) BRET signal in the absence of mNG-CAAX cells transfected with pcdna3, mFFA4 and Nluc-arrestin.C) BRET signal in the presence of mFFA4 receptor cells transfected with Nluc-arrestin and mNG-CAAX mFFA4. D) BRET signal in the presence of hFFA4 cells transfected with Nluc-arrestin and mNG-CAAX. Results shown as a mean ± standard error of three independent experiments (n = 3).

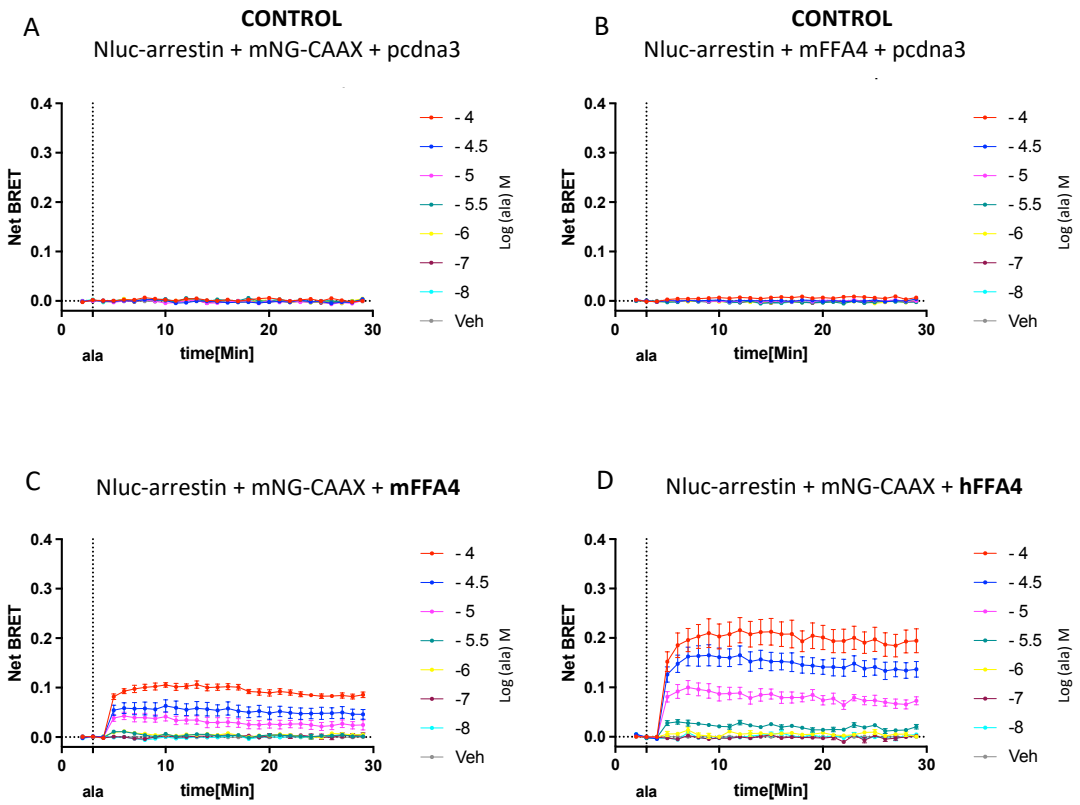


Figure 3-5 FFA4 receptor induced β-arrestin recruitment after stimulation with aLa
HEK cells were transfected with different plasmids as required and then seeded in 96-well plates pre-coated with poly-D-lysine. A range of aLa concentrations were added after measuring the BRET signal for 5 and 25 min. A) BRET signal in the absence of FFA4 receptor cells transfected with Nluc-arrestin and mNG-CAAX pcdna3. B) BRET signal in the absence of mNG-CAAX cells transfected with pcdna3, mFFA4 and Nluc-arrestin. C) BRET signal in the presence of mFFA4 receptor cells transfected with Nluc-arrestin and mNG-CAAX mFFA4. D) BRET signal when stimulated in the presence of hFFA4 cells transfected with Nluc-arrestin and mNG-CAAX. Results shown as a mean ± standard error of three independent experiments (n = 3).

These results demonstrate that mFFA4 can induce ß-arrestin recruitment in cells, indicating that both TUG-891 and aLa can induce the internalisation of the receptor, as reported by Hudson et al. (2013).

FFA4 can be internalised away from the plasma membrane following the ligand activation of GPCRs (Hirasawa et al. 2005; Watson et al. 2012).This was tested by Hudson et al. (2013) using confocal microscopy, with the internalisation of hFFA4-eYFP determined in the hFFA4 Flp-In T-REx 293 cell line. Prior to ligand treatment, hFFA4-eYFP was primarily localised at the cell surface. However, studies have observed an increase in intracellular hFFA4-eYFP expression with a punctate distribution 10 min after the addition of aLa. The amount of internalised hFFA4-eYFP increases over time, and by the end of a 45-min incubation period, very little hFFA4-eYFP is visible at the cell surface. TUG-891 produced a similar internalisation pattern. Thus, it was reasonable to perform an initialisation assay next.

3.3.5 AH7614 inhibits the agonist-promoted internalisation of mFFA4, visualised using confocal microscopy

The internalization of mFFA4 was evaluated in mFFA4-eYFP Flp-In T-REx cells. To establish a control, cells were exposed to TUG-891 alone (Figure 3-6A). In the treatment group, cells were treated with 10 μM of AH7614 for 5 minutes before the addition of TUG-891. The internalization process of mFFA4 in live cells was visualized using a Zeiss VivaTome spinning disk confocal microscopy system. Imaging was conducted prior to ligand addition and subsequently at one-minute intervals for a total duration of 45 minutes (Figure 3-6A and B).

Prior to the addition of TUG-891, the bulk of the eYFP tagged FFA4 receptors appeares to be located primarily at the cell surface. Within 10 min of the addition of TUG-891, there appears to be an increase in the amount FFA4-eYFP present in punctate intracellular spots, likely representing expression in endosomes. The fraction of mFFA4-eYFP appeared to increase over time, and after 45 min of incubation with TUG-891, little mFFA4-eYFP appeared to be present at the cell membrane (Figure 3-6A). In contrast, when cells were pre-treated with AH7614 prior to the addition of TUG-891, some mFFA4-eYFP expression appeared to remain at the cell surface, even after 45 min of agonist treatment, suggesting that AH7614 is inhibiting receptor internalisation. However, it is important to note that even in the presence of AH7614, TUG-891 treatment still appeared to increase the number of punctate spots of FFA4-eYFP expression, suggesting that this antagonist does not fully inhibit TUG-891 mediated internalisation of mFFA4-eYFP (Figure 3-6B).

These results suggest that AH7614 inhibits but does not completely block both ß-arrestin recruitment and internalisation of the mFFA4 receptor. This partial antagonism, is consistent with the idea that AH7614 acts as a negative allosteric modulator of the FFA4 receptor (Watterson et al. 2017) and confirms that this molecule is not a competitive antagonist of this receptor.

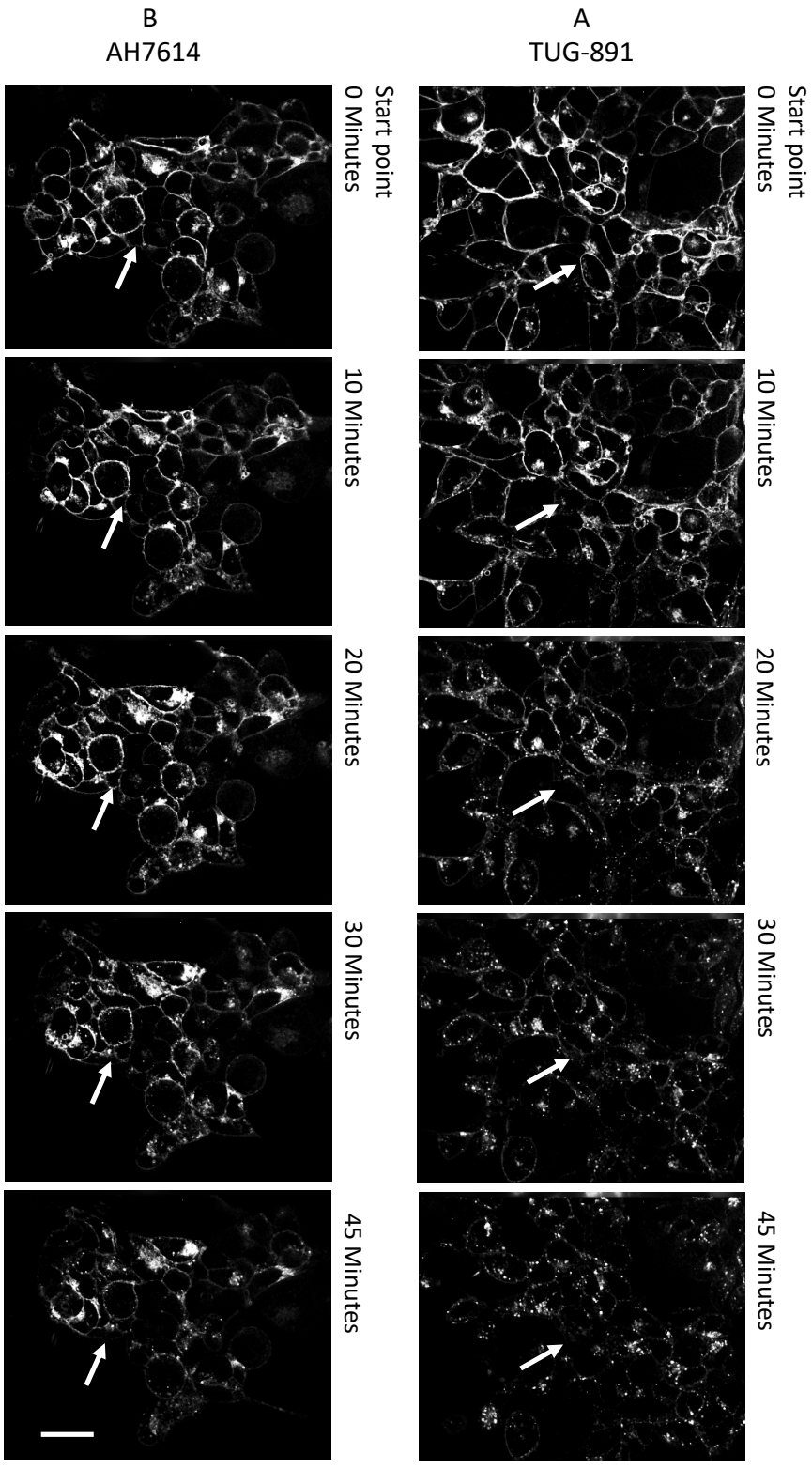


Figure 3-6 TUG-891 promotes the internalisation of mFFA4, as visualised by confocal microscopy

Flp-InTM T-RExTM 293 cells able to express FLAG-mFFA4-GYFP were placed on poly-D-lysine-coated chamber slides, and receptor expression was induced using doxycycline (100 ng/ml). The internalisation of mFFA4 in these cells was then imaged using a Zeiss VivaTome spinning disk confocal microscopy system. TUG-891 (10 μM) promoted the internalisation of FLAG-hFFA4-GYFP. Images were taken before the addition of TUG-891 and every minute after ligand addition for a total of 45 min. Scale = 20 μm. This process was repeated with AH7614. A) TUG-891 internalisation effect. B) AH7614 internalisation effect.

3.3.6 AH7614 inhibits TUG-891 and aLa action on intracellular Ca^{2+}

To assess the pharmacology of the FFA4 antagonist AH7614, a Ca^{2+} mobilisation assay was performed using mFFA4-eYFP Flp-In T-REx 293 cells.

With the exception of a few studies, AH7614 is a rarely characterised antagonist (Watterson et al. 2017). I therefore repeated the Ca^{2+} mobilisation assay to determine the influence of AH7614 on the ability of TUG-891 to increase Ca^{2+} mobilisation, as observed in Section 3.3.2.

I used the Flp-In T-REx 293 mFFA4-eYFP cell line. This time, the cells were pre-incubated with varying concentrations of AH7614 or dimethyl sulfoxide (DMSO) as a vehicle for 10 min. A Ca^{2+} mobilisation assay was then performed to test how the use of AH7614 affected the ability of TUG-891 and aLa to induce Ca^{2+} mobilisation using a range of TUG-891 and aLa concentrations. The results demonstrated that at the highest concentration, AH7614 was able to completely block both TUG-891 and aLa (Figure 3-7A and B). At lower concentrations of AH7614, the agonists were able to regain their ability to induce Ca^{2+} mobilisation (Figure 3-7A and B).

This result demonstrates that AH7614 acts on FFA4 because it was able to completely stop both TUG-891 and aLa action at its maximum concentration. These data also suggest that AH7614 blocks the pathway at different levels. Although a decrease in initialisation without agonist treatment was observed, the antagonist completely blocked the effect of Ca^{2+} mobilisation, indicating that AH7614 is an inverse agonist. I then performed a ß-arrestin assay to evaluate how AH7614 affected the trends observed in Section 3.3.3 and what that might reveal about AH7614 behaviour.

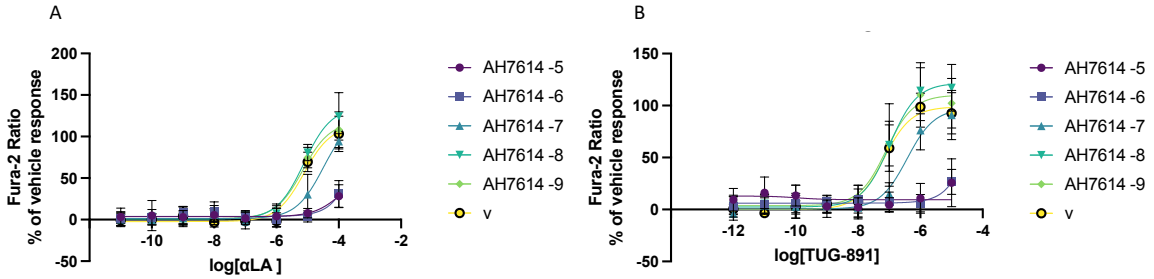


Figure 3-7 Mobilisation inhibition after the use of AH7614 at high concentrations. Flp-InTM T-RExTM 293 cells able to express mFFA4 were seeded into poly-D-lysine-coated black clear-bottomed 96-well plates. Expression of mFFA4 was induced by using the antibiotic doxycycline (100 ng/ml) for 24 h. Cells were labelled for 45 min with the calcium-sensitive dye Fura-2 and then exposed to varying concentrations of AH7614 for 15 min. Cells were then exposed to a range of concentrations of TUG-891 or aLa. Mobilisation of intracellular calcium was recorded in a Flex Station plate reader. Efficacy was normalised as the percentage of TUG- 891 maximum response. Data represent the mean ± standard error of three independent experiments.

3.3.7 Treatment with AH7614 decreases β-arrestin recruitment

The BRET-based ß-arrestin recruitment assay was performed in the same way as that for TUG-891 in HEK293T cells. HEK293T cells were co-transfected with untagged mFFA4, an mNG fluorescent protein anchored to the cell membrane with the CAAX motif of 3:1 and ß-arrestin 2 tagged at its N terminal with Nluc. This format allowed a bystander BRET-based arrestin assay to be performed that could measure the recruitment of ß-arrestin 2 following the activation of FFA4 in the presence of varying AH7614 concentrations. The cells were pre-incubated with AH7614 for 10 min before the start of the assay. TUG-891 was added after recording the basal BRET signal for 5 min. The BRET signal was also recorded in the vehicle (DMSO). The results revealed a strong BRET signal with the vehicle Figure (3-8A), which decreased slowly. The decrease in signal corresponded to the AH7614 concentration, which started at 100 nM (Figure 3-8B). As the concentration of AH7614 increased, BRET decreased until the highest concentration (10 μM) was reached and completely blook the activation (Figure 3-8F). This indicates that AH7614 decreases ß-arrestin recruitment, blocking it at higher concentrations.

To compare the data on trends obtained through the Ca^{2+} mobilisation assay, the data were plotted for the same time period, 10 min, for all the AH7614 treatments. The area under the curve was plotted, revealing that the behaviour

of AH7614 was similar to that demonstrated by the data for Ca^{2+} mobilisation, where higher concentrations stopped the action of ß-arrestin recruitment in a non-competitive antagonist (Figure 3-8G).

Finally, I explored the BRET signal before the addition of TUG-891 to assess if AH7613 changed the basal activation (Figure 3-8H). AH7615 was able to reduce the BRET signal even without agonist stimulation, indicating that FFA4 may be a constantly active receptor. This suggested that AH7614 may be an inverse agonist, which should be explored further; however, time constraints meant that there was no scope to do so in this study.

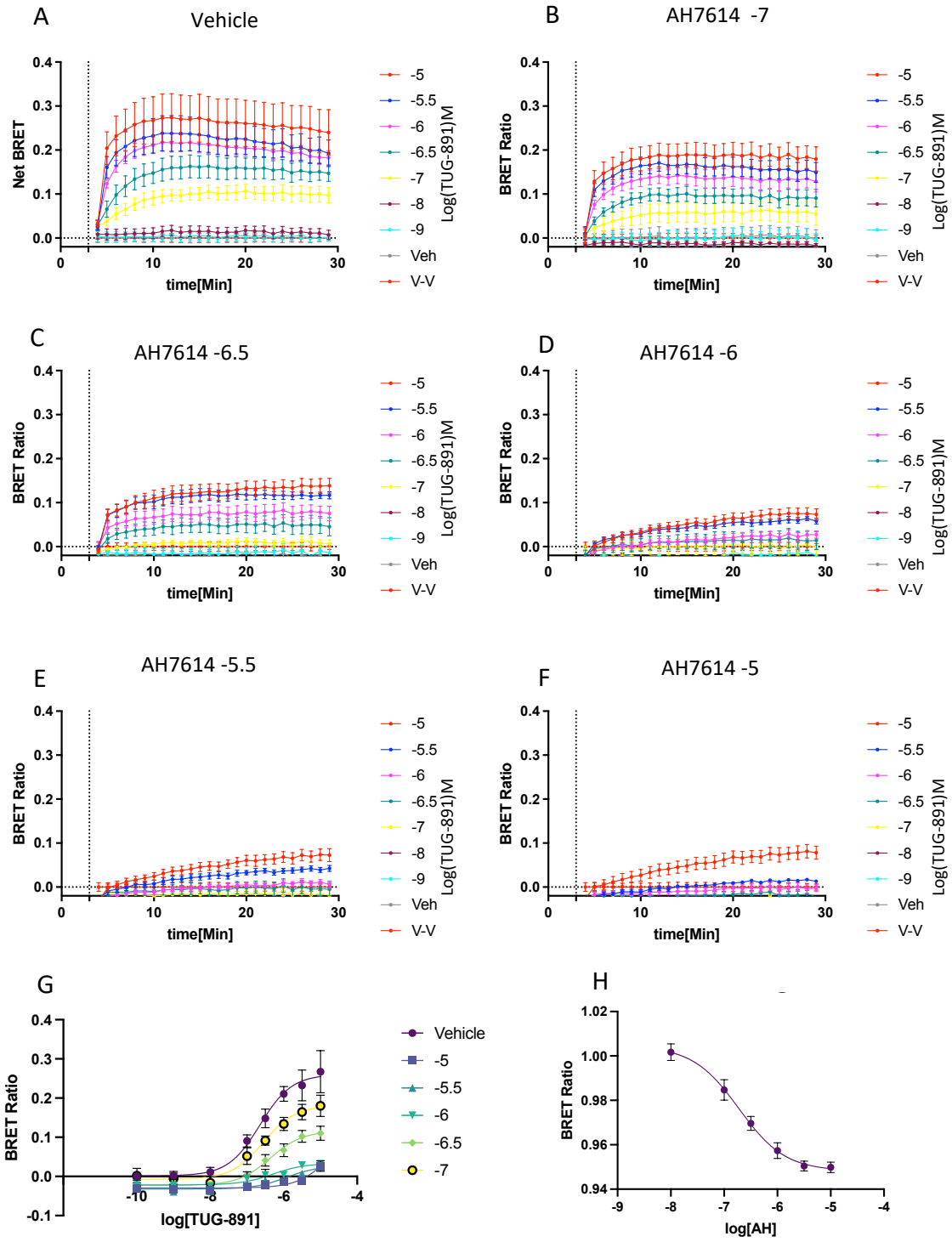


Figure 3-8 Inhibition of the TUG-891 promotion of β-arrestin recruitment to mFFA4
HEK cells were transfected with Nluc-arrestin and mNG-CAAX hFFA4 and then incubated with varying concentrations of AH7614; the log concentrations are stated for each experiment, and data are compared with baseline (V-V), with no AH7614 or TUG-891 added. After reading the basal signal for 5 min, TUG-891 was added and the read continued for 30 min. A) BRET signal with vehicle, B) BRET signal with 100 nM of AH7614, C) BRET signal with 1.5 μM of AH7614, D) BRET signal with 1 μM of AH7614, E) BRET signal with 15 μM of AH7614, F) BRET signal with 15 μM of AH7614. G) Plotted signal for the area under the curve at 10 min for a range of AH7614 concentrations stimulated by a range of TUG-891 concentrations. H) Basal BRET signal pre-stimulation after 4 min of the assay.

3.4 Discussion

TUG-891 is a commercially available tool compound that is commonly used in both in vitro and in vivo research. It was employed as a tool compound in this project to investigate the pharmacology and functional consequences of mFFA4 in cells. In this study, TUG-891 has been found to increase intracellular calcium levels, recruit arrestin 2 in a concentration-dependent manner and induce receptor internalisation. However, this project additionally used the FFA4 antagonist AH7614. In the same assays used for TUG-891, which included intracellular calcium mobilisation and ß-arrestin 2 recruitment, this drug demonstrated non-competitive antagonism as well as slow receptor internalisation in the presence of TUG-891 and showed an invers antagonism effect of FFA4 receptor.

To determine the pathways activated by FFA4 agonists, the phosphorylation of ERK1/2 was examined as a general assay. Previous research conducted by (Suzuki et al. 2008) aimed to assess various potential agonists for FFA4 and investigated the activation of ERK1/2 as an indicator of FFA4 activity. Similarly, (Hudson et al. 2013) specifically focused on the pharmacology of TUG-891 and confirmed the concentration-dependent activation of ERK phosphorylation using hFFA4-inducible Flp-In T-REx 293 cells. In this study, two FFA4 agonists, TUG-891 and TUG-1197, were evaluated to assess their ability to induce ERK phosphorylation in a CHO cell line expressing mFFA4. Concentration-response curves were generated, and both agonists demonstrated clear concentration-dependent increases in ERK phosphorylation, indicating the activation of this pathway. Despite the focus of the previous studies on hFFA4, their findings align with the results obtained in the present investigation using mFFA4. The potency values for TUG-891 and TUG-1197 were determined as 7.2 and 6.2, respectively, suggesting that TUG-891 exhibits superior efficacy in stimulating ERK phosphorylation in the mFFA4 receptor.

Then, the ability of FFA4 agonists (TUG-891 and aLa) to activate the Gq/11 signaling pathway was investigated by assessing intracellular Ca^{2+} mobilization. An assay was established using Flp-In T-REx 293 cells expressing mFFA4 tagged with eYFP. Concentration-dependent increases in Ca2+ were observed for both agonists,TUG-891 and aLa indicating the activation of the Gq/11 pathway.

Again, Hudson et al. (2013) assayed this function using Flp-In T-REx 293 cells, but it was genetically modified to express hFFA4. Ca^{2+} mobilisation was tested with a range of TUG-891 concentrations, with increases in intracellular Ca^{2+} also reported in a concentration-dependent manner.

To explore which G proteins are involved in FFA4 signalling, I employed TRUPATH G protein dissociation assays(Du et al., 2022) . These assays demonstrated that that mFFA4 couples to both Gq/11 and Gi/o family G proteins when the receptor is expressed HEK 293T cells. The observation that FFA4 couples to Gq/11 family G proteins is not surprising, given the widespread literature demonstrating the ability of this receptor to stimulate transient increases in intracellular Ca^{2+} (Prihandoko et al., 2016) as activation of Gq/11 G proteins results in Ca^{2+} mobilization (Takashima et al., 2006). Importantly, previously work has demonstrated that this FFA4 mediated increase in intracellular Ca^{2+} is completely inhibited by FR900359, a specific pharmacological inhibitor of Gq/11 family G proteins (Pfeil et al., 2020). Furthermore, the elimination of both Gq and G11 G proteins through CRISPR/Cas9 genome editing in HEK293 cells rendered FFA4 incapable of inducing an increase in intracellular Ca^{2+} or inositol phosphates (IPI) (Alvarez-Curto et al., 2016) , further highlighting the importance of the Gq/11 family of G proteins to FFA4 signalling.

It is interesting that in the TRUPATH assay FFA4 couples to a Gi/o family member in addition to the Gq/11 G proteins. Although in early studies FFA4 was not found to regulate cAMP when expressed in HEK 293 cell (Hirasawa et al., 2004) more recent studies have found FFA4 agonists do inhibit cAMP in these cells, albeit only at significantly higher concentrations than were require to activate FFA4-Gq/11 or β-arrestin signalling pathways(Satapati et al., 2017). Interestingly, when looking at FFA4 signalling outside of heterologous expression systems, several studies have found what appears to be FFA4-Gi/o coupling to control key FFA4 mediated physiological responses(Barella et al., 2021; Im, 2018). For example, pertussis toxin treatment, which inhibits Gi signalling (Mangmool & Kurose, 2011), abolished FFA4's ability to regulate the release of the satiety hormone ghrelin in mouse gastric cells (Engelstoft et al., 2013). Similarly, FFA4 mediated release of somatostatin from delta cells in pancreatic

islets was also inhibited by pertussis toxin (Stone et al., 2014). In 3T3-L1 adipocytes it has also been shown that FFA4 effectively recruits miniGi and miniGo protein to the plasma membrane (Husted et al., 2020), while activation of the recepetor in primarily cultured murine white adipocytes inhibits both accumulation of cAMP and lipolysis, following treatment with a ß-adrenoceptor agonist(Husted et al., 2020). Together these results indicate the complex and multifaceted nature of FFA4 signaling, involving the activation of different pathways and the potential involvement of Gi/o coupling in controlling various physiological responses.

The results of ß-arrestin recruitment experiments provide evidence of the ability of TUG-891 and aLa, both FFA4 agonists, to stimulate ß-arrestin recruitment to the cell membrane. This recruitment was observed upon activation of both mFFA4 and hFFA4 plasmids within the HEK cell system. Previous studies, such as the one conducted by (Hudson et al., 2013), have also shown that activation of hFFA4 leads to ß-arrestin recruitment and subsequent downstream signaling events. In particular, ß-arrestin has been implicated in facilitating the internalization of the FFA4 receptor, as demonstrated in the aforementioned study using HEK293T cells transfected with an eYFP-tagged form of hFFA4. The results of the current project align with these previous findings, as they reveal a concentration-dependent recruitment of ß-arrestin by TUG-891 and aLa, which are the same ligands utilized in the assay for ß-arrestin recruitment in this particular project. Using mFFA4 signaling appears to be similar, with the same pattern in ß-arrestin recruitment and internalization was observed.

This ß-arrestin-mediated signaling can lead to the activation of pathways such as phosphorylation. Prihandoko and colleagues (Prihandoko et al. 2016) have demonstrated that internalisation correlates with the agonist-induced interaction of the receptor with ß-arrestin, which is primarily defined by the phosphorylation status of a series of serine and threonine residues in the intracellular C-terminal tail of the receptor(Prihandoko et al. 2016). Moreover, (Butcher et al., 2014) findings demonstrate that the recruitment of arrestin 3 to mFFA4 in response to agonist activation is predominantly reliant on the phosphorylation of residues located at the C-terminal tail of the receptor. Remarkably, in this aspect, the behavior of mFFA4 closely resembles that of

human FFA4 (Butcher et al., 2014). Similar to human FFA4, the phosphoacceptor sites on mFFA4 were observed to be grouped into two clusters: cluster 1 consisting of Thr347, Thr349, and Ser350, and cluster 2 consisting of Ser357 and Ser361 (Prihandoko et al. 2016). Figure (3-9) show the snake plot diagram of hFFA4 that shows the c-terminal of the receptor where the phosphorylation sits residents.

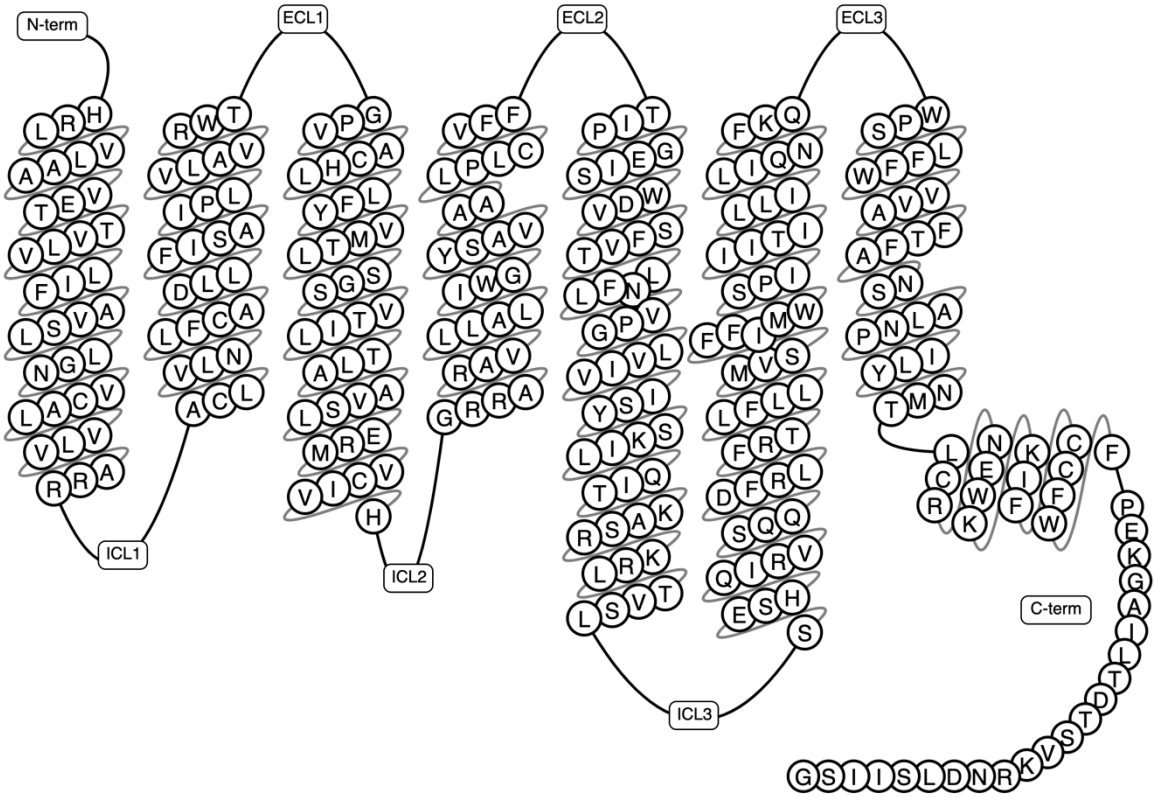


Figure 3-9 snake plot diagram of HFFA4
Sequence diagram of the human FFA4 was obtained from the GPCR database (GPCRdb.org)

When comparing the effects of ALa and TUG-891 on the FFA4 receptor, both ligands have been studied and evaluated for their effects. In terms of potency, TUG-891 has been found to be more potent than ALa. In my Results, the reported potency from both TRUPATH assay and Ca^{+2} mobilization has demonstrated that the concentration-dependent responses induced by TUG-891 were significantly stronger compared to those of ALa. This was also reported in ß-arrestin-2 recruitment assay, the rank-order potency at mFFA4 with TUG-891 being the most potent followed by GW9508, NCG21, and aLA (Hudson et al., 2013). This confirm that TUG-891 exhibits superior potency compared to ALa on the FFA4 receptor.

Using the same functional signalling assays employed for TUG-891, I conducted an analysis of the pharmacological properties of AH7614 to inhibit FFA4 signalling. At higher concentrations, AH7614 demonstrated complete inhibition of Ca^{2+} mobilization. Additionally, it exhibited an inhibitory effect on ß-arrestin and decelerated the internalization process of the FFA4 receptor.

The inhibitory effect that I I observed with AH7614 on Ala and TUG-891 response Ca^{+2} mobilization assay has been reported previously. In competition studies conducted using FFA4-expressing U2OS cells, AH7614 successfully inhibited the intracellular Ca^{2+} response induced by both Ala and GSK137647A (an agonist for FFA4). (Sparks et al., 2014). Moreover, In cells expressing green fluorescent protein-tagged human FFA4 receptors, arachidonic acid elicited an increase in intracellular calcium (Villegas-Comonfort et al., 2017). This effect was not hindered by cyclooxygenase or lipoxigenase inhibitors, but it was effectively inhibited by AH7614 (Villegas-Comonfort et al., 2017). Moreover, the findings from Watterson et al. (2017) also align with my own results, demonstrating that AH7614 effectively inhibits Ca^{2+} mobilization and diminishes the ability of ALa and TUG-891 to induce Ca^{2+} responses. Furthermore, the effect of AH7614 on FFA4 signalling impeded the recruitment of ß-arrestin on my study. The results of BRET assay in this chapter demonstrate that AH7614 decreases ß-arrestin-2 recruitment at the FFA4 receptor in a concentration-dependent manner. Moreover, it also effected the internalization of FFA4 receptor, the sequences event of ß-arrestin-2 recruitment. The inhibitory effects of AH7614 on ß-arrestin recruitment were consistent with the data obtained from Watterson et al. (2017) study on AH7614 produced results that the inhibition was observed in the ß-arrestin assay and slowed internalisation. Overall, these findings provide further evidence of the inhibitory actions of AH7614 on FFA4-mediated signaling pathways, encompassing both Ca^{2+} mobilization and ß-arrestin recruitment. These results contribute to the understanding of the pharmacological effects of AH7614 on FFA4 and its potential therapeutic applications in modulating FFA4 receptor activity.

The series of experiments conducted in this study have provided valuable insights into the pharmacology of AH7614 as a potential inverse agonist of the FFA4 receptor and highlighted a possible non-competitive manner.

Firstly, the identification of this ligand-independent receptor activity has led to the understanding that many ligands, previously believed to be antagonists without intrinsic activity, can actually inhibit this spontaneous activity, and agoinsts with this property are now described as 'inverse agonists' (Bond & Ijzerman, 2006; De Ligt et al., 2000; Y. Y. Zhang & Han, 1997) Based on my results presented in this chapter, it appears that FFA4 has some level of constitutive activity and that AH7614 acts as an inverse agonists-negative allosteric modulator of the receptor, inhibiting both ligand dependant and ligand independent FFA4-signalling.

One example of a GPCR where NAMs have been identified with inverse agonism properties is the CB1 cannabinoid receptor. Rimonabant, a well-known CB1 receptor antagonist, has been shown to not only block the effects of agonists but also reduce the basal activity of the receptor, resulting in inverse agonism(Xie et al., 2007). Rimonabant was initially developed as an anti-obesity drug due to its ability to suppress appetite by inhibiting the constitutive activity of CB1 receptors in the brain (Janero & Makriyannis, 2009). Similar to the CB1 receptor, the CB2 receptor can also exhibit inverse agonism by certain NAMs. For instance, AM630 has been identified as a CB2 receptor antagonist that displays inverse agonism by reducing the basal activity of the receptor (Bingham et al., 2007). Another example is NAMs of the D2 receptor that have been found to act as inverse agonists. For example, spiperone and raclopride have been shown to inhibit the constitutive activity of the D2 receptor below the basal level (Malmberg et al., 1998; B. Zhang et al., 2014).

Secondly, the Ca^{2+} mobilization assays in my study revealed that AH7614 effectively inhibited the intracellular Ca^{2+} response induced by agonists such as Ala and TUG-891 in a non-competitive mood of action. These findings are consistent with previous study (Watterson et al, 2017), which demonstrated the inhibitory effects of AH7614 on FFA4-mediated Ca^{2+} mobilization and suggested a non-competitive antagonism of AH7614.

To gain deeper insights into the function of AH7614 in relation to FFA4, future investigations aim to conduct additional pharmacological assays employing TUG-

891 and AH7614. These assays will provide further understanding of the specific interactions and effects of AH7614 on FFA4, expanding our knowledge of its pharmacological properties and its role in modulating FFA4 function. Notably, in my confocal microscopy experiments there appeared to be a disparity in the rate of internalization in response to TUG-891 stimulation in the absence and presence of AH7614. However, to date we have not been able to assess this in a quantitative way, so future experiments could us to more accurately study how the presence of AH7614 impacts agonist stimulated internalisation of the FFA4 receptor. This priceable was used in studies that have shown that antagonists of ß2-adrenergic receptor (ß2-AR), such as propranolol, can induce receptor internalization. The internalization process involves the recruitment of ß-arrestin proteins and subsequent endocytosis of the receptor(Ni et al., 2006). Also, Antagonists of the Dopamine 2 receptor, such as haloperidol, have been demonstrated to induce receptor internalization(Masri et al., 2008). The internalization of Dopamine 2 receptors is mediated by ß-arrestin proteins and plays a role in regulating receptor signaling and desensitization(Masri et al., 2008).

Another important question that remains unanswered about AH7614 is how it may affect trafficking of the FFA4 back to the cell surface after receptor activation. It is not uncommon for constitutively active receptors to have a significant proportion of the receptor expressed intracellularly in the basal state, and indeed for treatment with inverse agonists to cause re-localisation of this internalised receptors to the cell surface (El Buri et al., 2018; Hudson et al., 2010) .Currently nothing is known about what effect AH7614 may have on basal FFA4 localisation. To investigate the effect of AH7614 on the basal localization of FFA4, confocal microscopy can be a valuable tool. Confocal microscopy allows for high-resolution imaging of cellular structures and localization of specific molecules within cells. Different treatment conditions, including a control group without AH7614 and experimental groups with varying concentrations or durations of AH7614 treatment. It is important to establish a concentration range and time points based on prior knowledge or preliminary experiments. By comparing the control group with the AH7614-treated groups, you can assess any changes in the basal localization of FFA4 receptors induced by AH7614. This approach allows you to visualize and quantify the effects of AH7614 on FFA4

receptor localization, providing insights into its impact on the cellular distribution of FFA4. Moreover, performing BRET experiments with FYVE or CAAX tagged proteins, can assess the impact of AH7614 on the basal localization of FFA4. The BRET technique allows for real-time monitoring of protein-protein interactions or subcellular localization, providing insights into the localization changes induced by AH7614 treatment.

In summary, in this chapter the pharmacology of AH7614 and TUG-891 was investigated. TUG-891 increased Ca^{+2}, recruited arrestin , and induced receptor internalization. AH7614 acted as a non-competitive antagonist and acted as an inverse agonist on FFA4 receptor. These findings provide insights into the pharmacological properties of AH7614 and TUG-891, contributing to our understanding of their effects on FFA4 and give confident to use both compounds in the upcoming chapters.

Chapter 4 Acute treatment with FFA4 agonists and antagonists affects function of 3T3-L1 adipocytes

4.1 Introduction

3T3-L1 cells are a commonly used model of adipogenesis model that resembles WA. Over the past years, 3T3-L1 cells have been used extensively to evaluate the effects of compounds or nutrients on adipogenesis and to establish the molecular mechanisms underlying adipogenesis (Kang et al., 2016; Lai et al., 2016). Also, after differentiation, 3T3 L1 cells serve as an excellent in vitro adipocyte cell model and have proven tools for understanding glucose metabolism (Vishwanath et al., 2013). This is an important factor for my project, as T2D is one of the metabolic illnesses that this research aims to focus on.

Interestingly, the FFA4 receptor was reported as a possible novel therapeutic target for the treatment of (T2D) (Milligan, Alvarez-Curto, et al., 2017). This was supported by Hudson et al. (2013)'s work on 3T3-L1 adipocytes, which demonstrated that TUG-891 treatments enhanced glucose uptake in differentiated 3T3-L1 adipocytes. This fact can be directly linked to FFA4 activation that resulted in glucose transport being elevated in 3T3-L1 adipocytes.

These facts together led to the use of 3T3-L1 cells as the adipogenesis model for this project, which aims to use it as a platform for glucose uptake assays and lipolysis assay.

4.2 Aims

Although the FFA4 receptor attracted great interest as a target for diabetes and obesity, listed studies were performed only by using TUG-891 or Ko FFA4. Antagonism at the FFA4 receptor is still an area to be explored. In order to find a therapy or contribute to drug discovery for these metabolic disorders, an extensive study for agonists/antagonists at the FFA4 receptor and its concentrations should be explored.

In this chapter, I aim to establish the effects of acute treatment with either an FFA4 agonist, TUG-89, or FFA4 antagonist, AH7613 on 3T3-L1 adipocytes. First, I will measure the effect of the FFA4 ligands on glucose uptake. This will allow me to estimate the effect of FFA4 activation on glucose handling and on GLUT4 function on differentiated 3T3-L1. Second, I will evaluate the effects of agonists/antagonists of FFA4 to regulate adrenoceptor stimulation of lipolysis. Together, these studies aim to provide novel mechanistic insight into the potential role FFA4 may have in the treatment of T2D and obesity.

4.3 Results

4.1.1 FFA4 expression is upregulated during differentiation of 3T3-L1 adipocytes

In order to assess the suitability of 3T3-L1 adipocytes as a model to study FFA4 receptor function in adipocytes, qPCR was used to quantify levels of various FFA receptor family members. Expression of mRNA for all FFA receptors, including FFA1, FFA2, FFA3 and FFA4, was assessed by comparing expression in differentiated 3T3-L1 adipocytes with undifferentiated fibroblasts (Figure 4-1A). FFA4 mRNA was robustly increased ($p = 0.0004$; over 30,000-fold) in adipocytes compared to fibroblasts (Figure 4-1A). This suggests that FFA4 is expressed in 3T3-L1 adipocytes and supports our selection of this a model to investigate the effects of FFA4 receptor in functions in adipocyte. Moreover, these experiments demonstrate that 3T3-L1 cells downregulate FFA1 expression upon differentiation ($p < 0.0001$), as shown in (Figure 4-1B), and that in differentiated adipocytes, essentially no FFA1 mRNA could be detected, as supported by CT values compressions in (Table 4-1). In contrast, FFA2 mRNA was significantly ($p = 0.0078$) upregulated, 200-fold, when comparing 3T3-L1 fibroblasts to adipocytes (Figure 4-1C). FFA3 mRNA did not significantly change in the differentiation process ($p = 0.12$) (Figure 4.1D)., but CT values for this receptor suggest it was likely not expressed in either the fibroblasts or the adipocytes (Table 4-1).

Critically, the FFA1 receptor is not expressed, suggesting that, despite the fact that the 3T3-L1 cells are murine ((Hudson et al., 2013), the limited selectivity of some FFA4 ligands for mFFA4 over mFFA1 (Hudson et al., 2013) will not be a problem in this model.

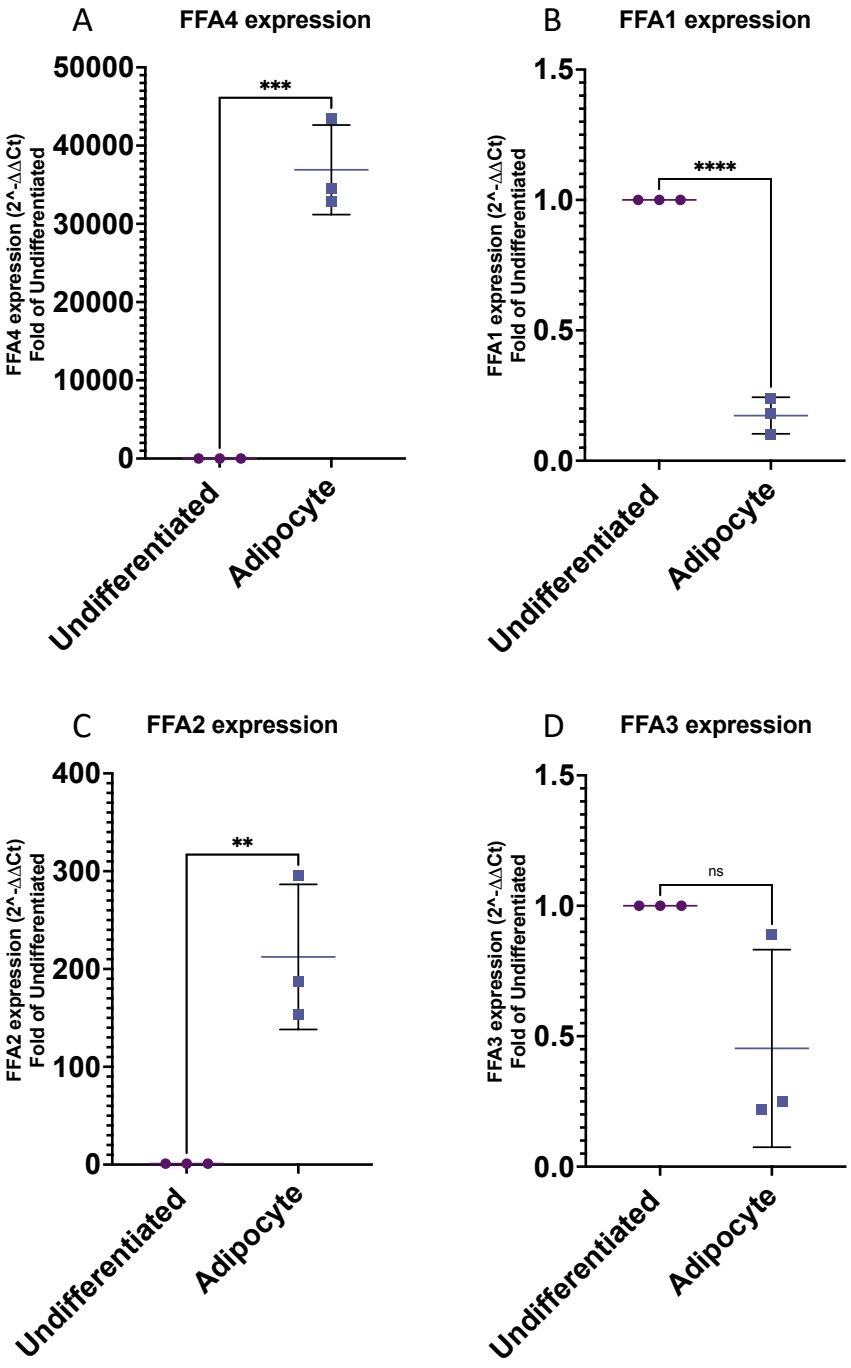


Figure 4-1 Expression of FFA4 and FFA2 mRNA is unregulated during 3T3-L1 differentiation mRNA was isolated in Day 9 of differentiation, and qPCR was carried out using primers for the murine orthologues of FFA4 (A), FFA1 (B), FFA2 (C) or FFA3(D). Data were analysed using the 2-ΔΔct method using primers for HPRT as the housekeeping control and using undifferentiated as the control condition. Results are presented from three individual experiments tested in in duplicate. **p < 0.1; ***p < 0.001; p < 0.0001

Table 4-1. CT values for FFAs in undifferentiated vs differentiated 3T3-L1.

Genes	Undifferentiated CT value			Adipocyte CT Value		
FFA4	35.2	34.6	38	20.5	20.7	20.2
FFA1	35.5	18.4	35.3	38.3	21.4	37.2
FFA2	38	37.9	37.9	25.5	30.2	30.1
FFA3	36	35.8	34.6	36.3	34.9	35.5

4.1.2 Adipogenesis marker upregulated during 3T3-L1 differentiation

As a confirmation of 3T3-L1 differentiation, I performed two assays to demonstrate the change from undifferentiated cells to differentiated 3T3-L1 adipocyte. First, Oil Red O staining was performed. Results showed that undifferentiated cells have only 5% of Oil Red staining on average in comparison to 3T3-L1 adipocyte (Figure 4-2 A). Second, qPCR was used to quantify levels of various adipogeneses markers. PPAR-γ expression sharply increased in 3T3-L1 adipocyte by increasing ($p = 0.0068$; over 10,000-fold) in adipocytes compared to fibroblasts (Figure 4-2 B). Also, Adiponectin expression showed upregulated levels in differentiated cells by ($p = 0.0006$; over 200,000-fold) (Figure 4-2C). The last marker to assay was Leptin, which followed the other adipogeneses markers and increased its expression in differentiated 3T3-L1 ($p = 0.0025$; over 150-fold) (Figure 4-2 D). As more support to understand the different expressions between adipogeneses markers in undifferentiated and differentiated cells, a CT values compression is provided in (Table 4-2).

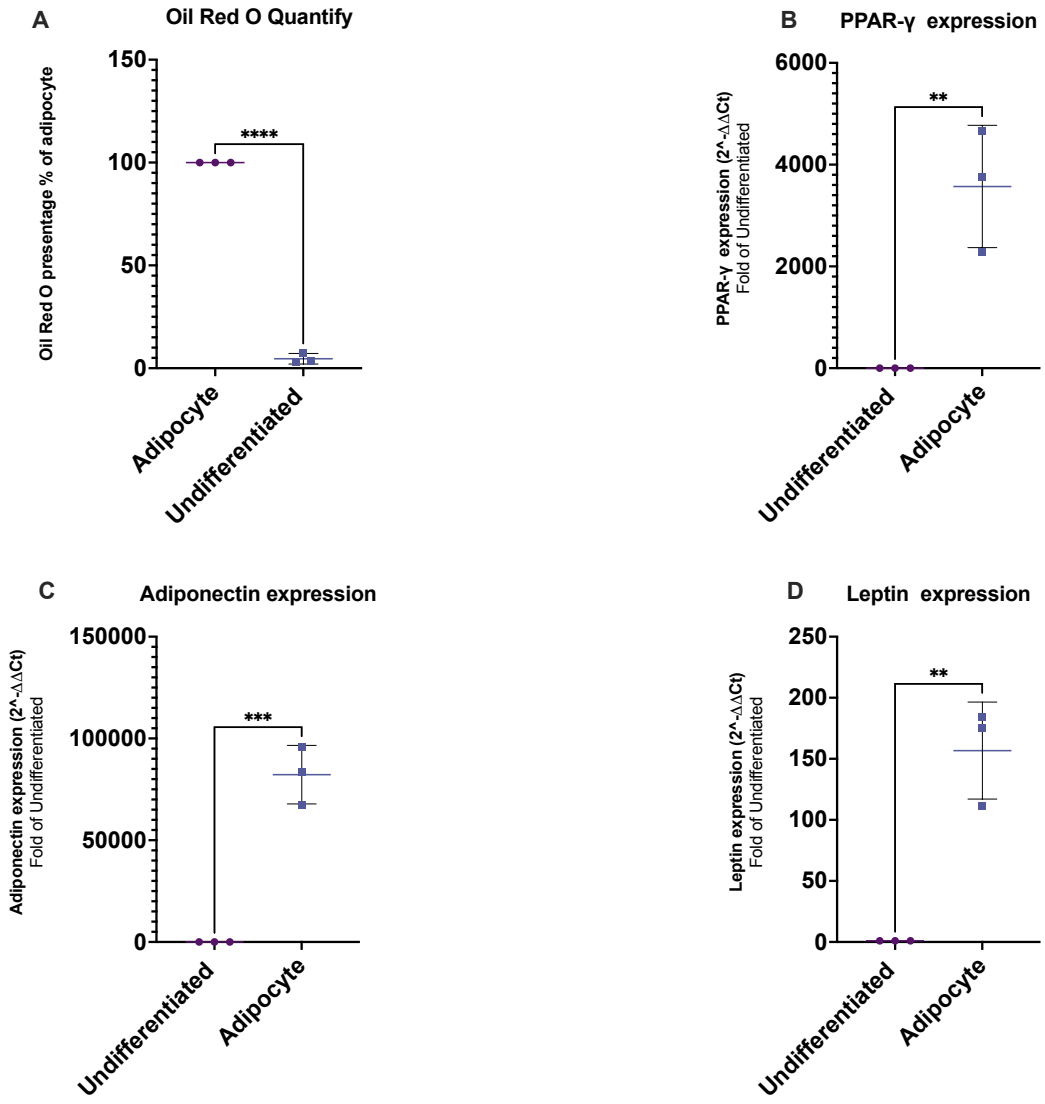


Figure 4-2 Adipogeneses conformed in 3T3-L1 by Oil Red O stain and adipogeneses marker expression via RT-qPCR.

3T3-L1 cells were differentiated; then, on Day 12, they were compared to undifferentiated cells. A) Oil Red O was subsequently dissolved in isopropanol and then quantified by measuring absorbance at 492 nm. Results are presented from three individual experiments tested in duplicate. ***p < 0.001; p < 0.0001. RT-qPCR B) PPAR-γ expression. C) Adiponectin expression. D) Leptin expression. Data were analysed using the 2-ΔΔct method using primers for HPRT as the housekeeping control and using undifferentiated as the control condition. Results are presented from three individual experiments tested in in duplicate. **p < 0.1; ***p < 0.001; p < 0.0001.

Table 4-2. CT values for adipogenesis markers that was assayed via RT-qPCR.

Gene	Undifferentiated CT value			Adipocyte CT Value		
PPAR-γ	40	37.8	37.8	24	23.6	24.6
Adiponectin	31.9	31.1	31.5	13.8	13.6	14.1
Leptin	35	34.4	41.7	24.8	25	26.3

4.1.3 Glucose transporter 4 (GLUT4) upregulated during differentiation of 3T3-L1 adipocytes

Since the focus for this chapter will be the interaction between FFA4 receptor and glucose uptake upon insulin simulation in 3T3-L1 adipose, an estimation of glucose transporters expressions was assayed using RT-qPCR method in those adipose. The same comparison between 3T3-L1 fibroblasts and adipocytes was performed on GLUT1 and GLUT4 gene expression to validate the differentiation process used. Consistent with previous data, GLUT4 shows a statistically significant robust increase in gene expression in differentiated 3T3-L1 cells (P = 0.0001), as presented in (Figure 4-3A). In contrast, GLUT1 mRNA levels fall upon differentiation from fibroblasts to adipocytes (Figure 4-3B). The reduction in gene expression was statistically significant (P = 0.0008). As more support to understand the different expressions between GLUT1 and GLUT4 in undifferentiated and differentiated cells, a CT values compression is provided in (Table 4-3).

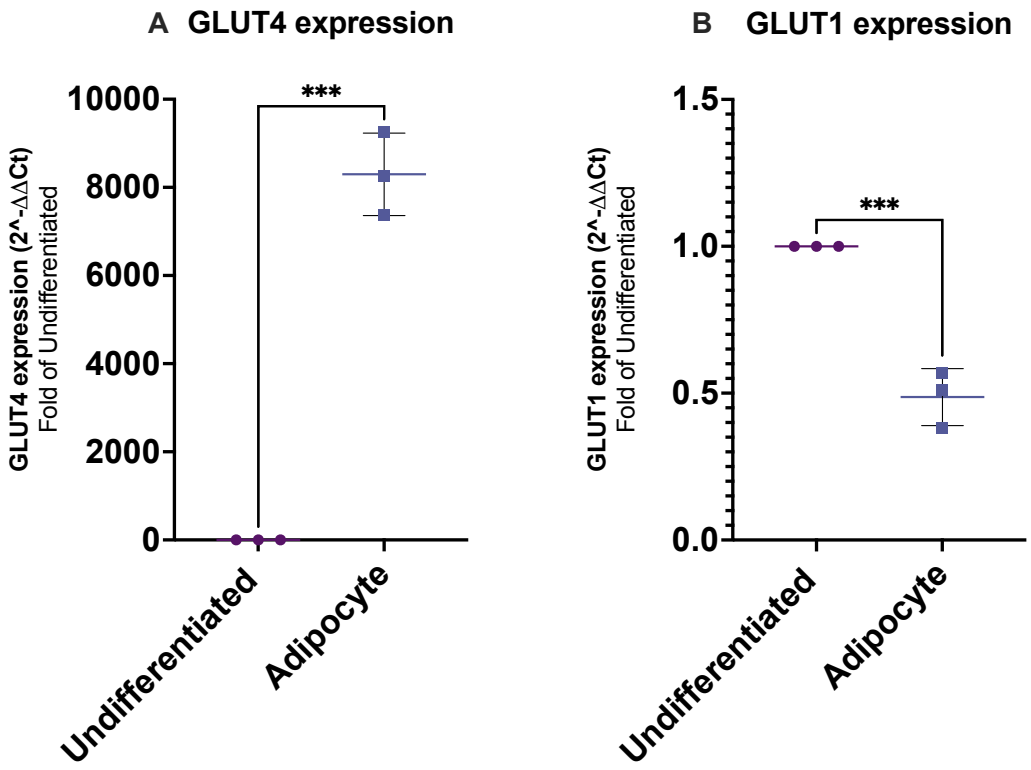


Figure 4-3 Expression of GLUT4 mRNA is unregulated during 3T3-L1 differentiation.

mRNA was isolated in Day 9 of differentiation, and qPCR was carried out using primers for the murine orthologues of GLUT4 (A), GLUT1 (B) Data were analysed using the $2^{-\Delta\Delta ct}$ method using primers for HPRT as the housekeeping control and using undifferentiated as the control condition. *Results are presented from three individual experiments tested in in duplicate. ***p < 0.001; p < 0.0001.*

Table 4-3. CT values for GLUT1 and GLUT4 in RT-qPCR for 3T3-L1 cell line (undifferentiated vs differentiated).

Genes	Undifferentiated CT value			Adipocyte CT Value		
GLUT1	18.2	18.4	20	17.1	19.7	20.7
GLUT4	33.5	34.1	31.3	17	18	18.5

4.1.4 Acute treatment with an FFA4 agonist, TUG-891, in various conditions does not affect basal or insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes

A [^{3}H] 2-deoxyglucose assay was used to assess the effects of FFA4 activation on glucose uptake in 3T3-L1 adipocytes. In this assay, because glucose can take several pathways once it is inside the cell (Hom et al., 1984), a modified 2-deoxyglucose that accumulates upon entering the cell as 2-deoxyglucose-6-phosphate (Hom et al., 1984) was used in this assay. This molecule is not farther metabolised, and so we can assay glucose transport by measuring accumulation of the radioactive [^{3}H]- 2-deoxyglucose in the cell.

To examine the ability of FFA4 to regulate glucose transport in 3T3-L1 cells, initially the effects of treatment with an FFA4 agonist, TUG-891, on insulin dependent glucose uptake were studied (Figure 4-4). Under those categories, we changed the time point for the addition of TUG891 and the incubation time for TUG891 with differentiated 3T3-L1 adipocytes. TUG-891 treatment was combined with increasing insulin concentrations (Figure 4-4). In parallel, cells were treated with 10 μM cytochalasin B to determine non-specific association of 2-[3H]-deoxyglucose. These studies showed that, although insulin produced a clear concentration dependant increase in glucose uptake (maximal response 19-fold; $p < 0.0001$), treatment with TUG-891 did not significantly impact uptake at any concentration of insulin tested. No significant change in [3H] deoxyglucose uptake upon the use of TUG891 as an agonist for FFA4 was indicated.

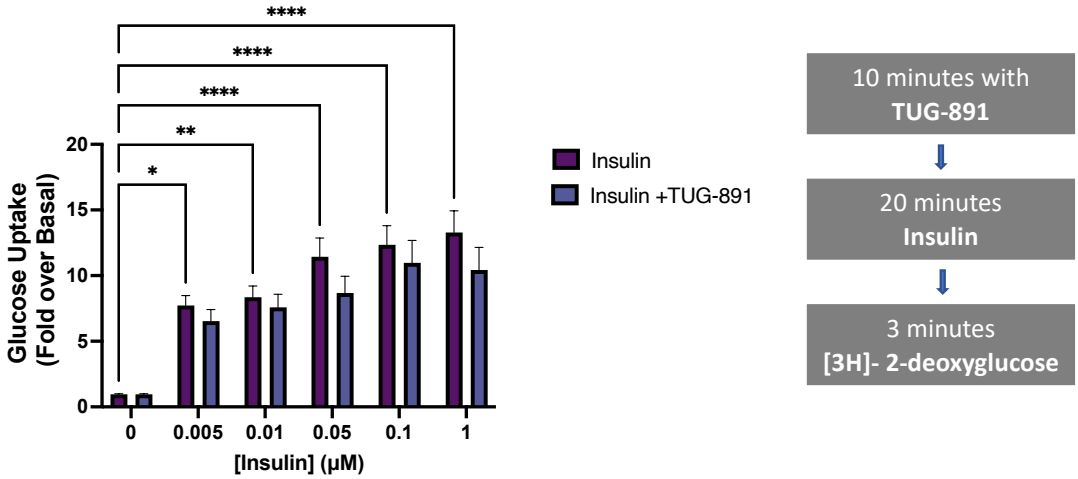


Figure 4-4 TUG-891 does not enhance insulin's responses glucose uptake in 3T3-L1 adipocytes.
3T3-L1 adipocytes were incubated with or without 10 μM TUG-891 for 30 min. Then, insulin was added for 20 min, followed by the addition of [^{3}H] 2-Deoxyglucose to measure glucose uptake over a three-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells not treated with either insulin or TUG-891. Data are expressed mean ± SEM from six independent experiments tested in in triplicates **p < 0.1; ***p < 0.001; p < 0.0001.

The no significant of TUG-891 effect, combined with previous work showing that FFA4 is rapidly desensitised through recruitment of ß-arrestin and internalisation from the cell membrane, as explained in Chapter 3, led me to examine if the long 30-min TUG-891 pre-treatment might be desensitising FFA4 in this assay.

In order to estimate the influence of activating FFA4 receptor at insulin response, an assay was performed to measure the influence of TUG-891 in accompanying with insulin in the same assay time. Insulin takes approximately 20 minutes to simulate translocation of GLUT4 in 3T3-L1 adipocytes (Proctor et al., 2006). Therefore, TUG-891 was incubated with the same total time that insulin was used to simulate glucose uptake. Cells were treated with insulin for 10 min, then TUG-891 was added and cells were incubated with both insulin and TUG-891 for a further 10 minutes before the [3H] 2-deoxyglucose uptake experiment was conducted (Figure 4-5). Moreover, insulin was added to 3T3-L1 adipose at two concentrations one that was 0.005 μM and the other was 0.1 μM. Based on the previous data (Figure 4-4), 0.005 μM was chosen, as it was the

lowest concentration that was examined with a significate (*p = 0.0126) to allow TUG-891 influence a chance to be examined. 1 μM was chosen also, as it is not the highest concentration used in the previous data, but it will also give a better chance of the TUG-891 effect being observed. Treatment of TUG-891 10 minutes in after the addition of insulin had no significant effect on insulin-stimulated glucose uptake at either the low or high concentration of insulin, as shown in (Figure 4-5) (p = 0.6821 and p = 0.5751, respectively).

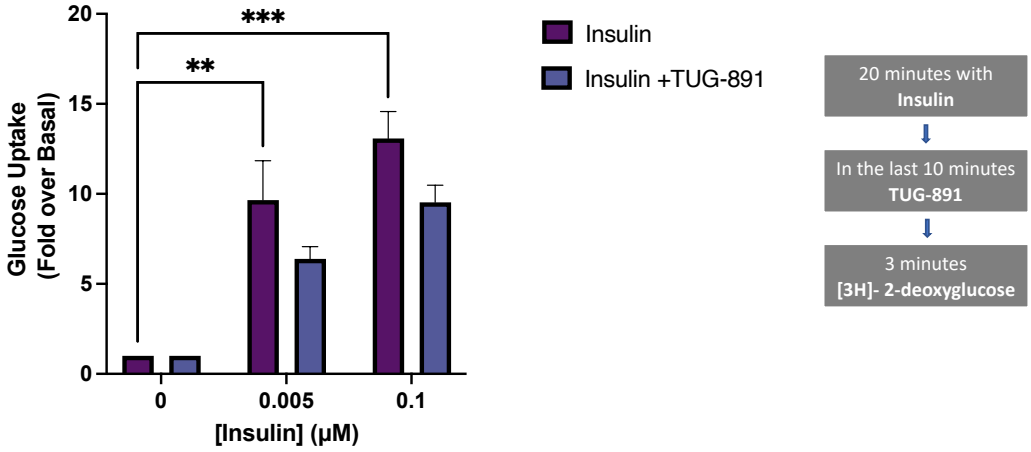


Figure 4-5 Adding TUG-891 within insulin incubation dose not enhance insulin response in glucose uptake assay in 3T3-L1 adipose.
3T3-L1 adipocytes were incubated with 0.005 μM or 0.1 μM of insulin for 20 minutes. After 10 minutes from incubation with insulin cells were treated with 10 μM TUG-891 for 10 minutes. Then [^{3}H] 2-Deoxyglucose was added to measure glucose uptake over a three-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells not treated with either insulin or TUG-891. Data are expressed mean ± SEM from three independent experiments tested in in triplicates. **p < 0.1; ***p < 0.001; p < 0.0001.

To measure the effect of TUG-891 on insulin-independent glucose uptake, increasing concentrations of TUG-891 were tested for their ability to directly affect [^{3}H] 2-deoxyglucose uptake in 3T3-L1 adipocytes (Figure 4-6A). A range of TUG-891 concentrations (0, 0.3, 1, 3, 10 and 30 μM) was added to 3T3-L1 adipocytes for 10 minutes before the assay was performed. The result showed that insulin-independent glucose uptake was not significantly affected by the addition of any TUG-891 up to 30 μM (p > 0.05). Alongside the experiment, a control experiment was performed to confirm that the cells used in the experiment were functional and did respond to insulin (Figure 4-6 B).

This result inspired the idea of performing the assay using an antagonist to FFA4. Since the activation for the receptor using TUG-891 did not affect glucose

uptake in 3T3-L1 adipocytes, the question is whether blocking FFA4 activation in the same cells will show an effect of AH7614 on glucose uptake.

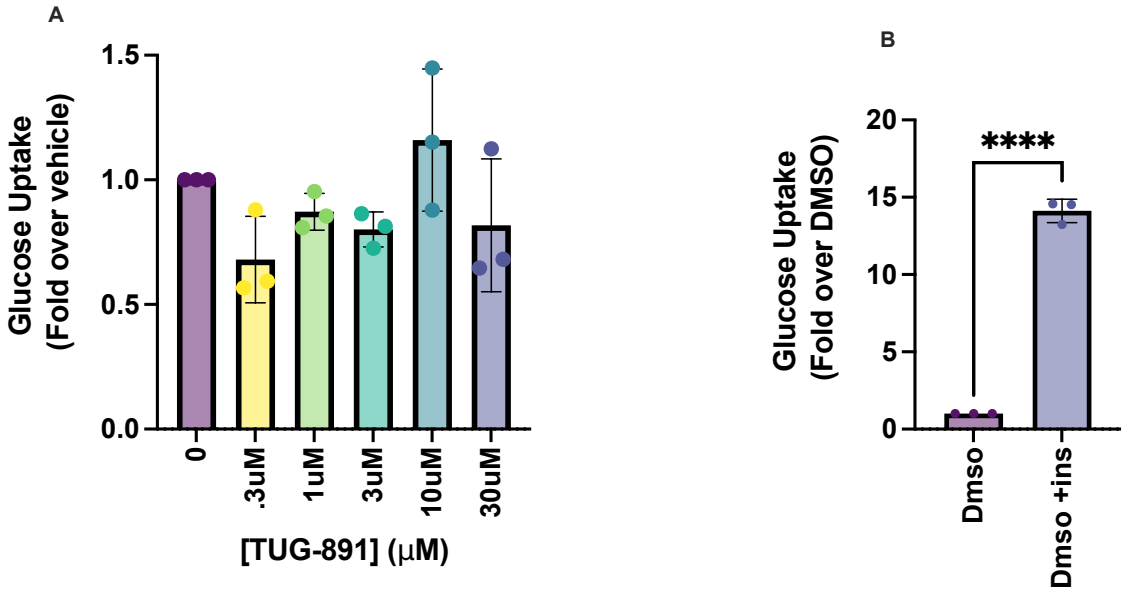


Figure 4-6 Acute treatment with a range of TUG-891 does not enhance glucose uptake in 3T3-L1.
A)3T3-L1 adipocytes were incubated with a range of TUG-891 concentration (0.0, 0.3, 1, 3, 10 and 30 µM) for 10 minutes. Then [^{3}H] 2-Deoxyglucose was added to measure glucose uptake over a five-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells not treated with TUG-891. B) Control was made by treating the 3T3-L1 adipocytes with 1µM or vehicle for 20 minutes; then, [^{3}H] 2-Deoxyglucose was added to measure glucose uptake over a five-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells treated with vehicle. Data are expressed mean ± SEM from three independent experiments tested in in triplicates. ****p < 0.0001.

4.1.5 Acute treatment of a range of concentrations of AH7614 decrease basal glucose uptake in 3T3-L1 adipose

In the interest of knowing the effect of blocking FFA4 receptor in glucose uptake function in 3T3-L1 adipocytes, a range of concentrations of AH7614 was put to the test to measure its effect on glucose uptake. 3T3-L1 cells were differentiated as described earlier, and on Day 9, glucose uptake assays were performed. As a first step, 3T3-L1 adipocytes were inculpated for 45 minutes with a range of concentrations of AH7614 (30, 10, 3, 1 and 0.3 µM). After that, [^{3}H] deoxyglucose was added to the cell for five minutes, then the glucose uptake inside the adipose cells were measured. The comparison was in the change in fold response comparing to the basal control. Just as in the previous

step, a conformation experiment was performed using insulin (1 μM) to assure the condition of the cell diffraction (Figure 4-7B).

The result showed that the use of AH7614 reduced glucose uptake in 3T3-L1 differentiated cells, as shown in (Figure 4-7A) at 3 μM. At 30 μM, a significant decrease in glucose uptake was measured ($p = 0.0102$ and $p = 0.0143$, respectively), which suggested that blocking FFA4 influences glucose uptake in 3T3-L1 adipocytes and, thus, that FFA4 has a role in glucose uptake and GLUT4 translocation.

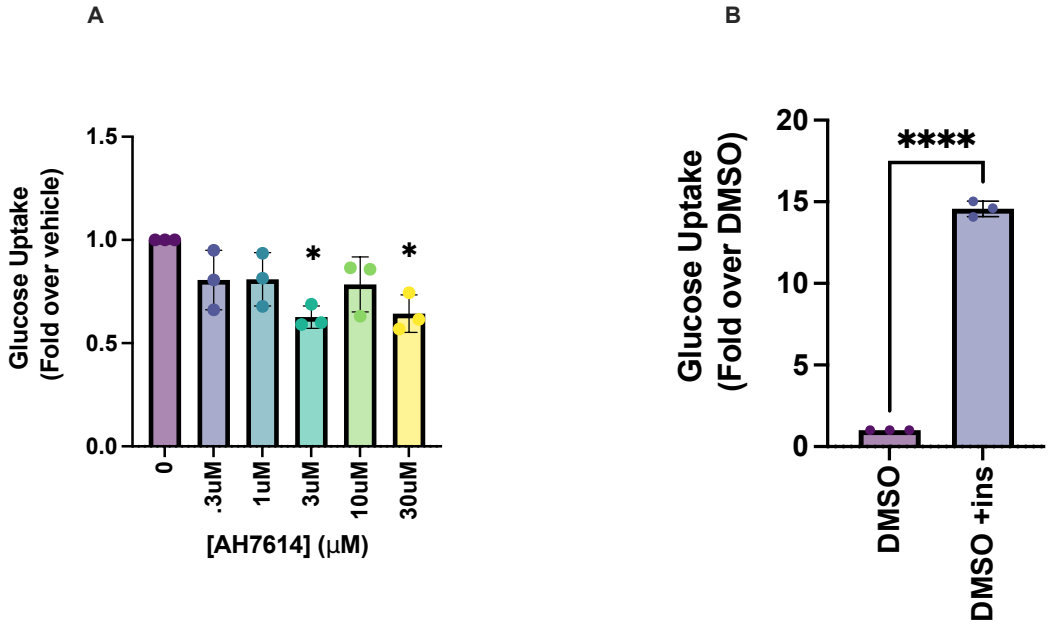


Figure 4-7 Acute treatment with a range of AH7614 decrease glucose uptake in 3T3-L1. A)3T3-L1 adipocytes were incubated with a range of AH7614 concentrations as shown for 45 minutes. Then [3H] 2-Deoxyglucose was added to measure glucose uptake over a five-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells not treated with AH7614. Data are expressed mean ± SEM from three independent experiments each tested in in triplicates (* $p > 0.05$) compared with the no-AH 7614 vehicle control. B) Control was made by treating the 3T3-L1 adipocytes with 1μM or vehicle for 20 minutes; then, [^{3}H] 2-Deoxyglucose was added to measure glucose uptake over a five-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells treated with vehicle. Data are expressed mean ± SEM from three independent experiments tested in in triplicates. ****$p < 0.0001$.

This outcome directs the next step to navigate the effect of AH7614 in the present of a range of insulin concentration. Glucose uptake in this specific step is designed to give information about GLUT4 translocation, so exploring the outcome from AH7614's effect on insulin response will be the consequence step.

4.1.6 AH7614 decrease insulin response in insulin-stimulated glucose uptake in 3T3-L1 adipocytes

To explore the role of FFA4 on insulin simulation response, a glucose uptake assay was designed to capture this effect. A fixed 10 μM of AH7614 were added to 3T3-L1 adipocytes on Day 9 for 30 minutes. Then, cells were simulated by a range of insulin concentrations (0.005, 0.01, 0.05, 0.1 and 1 μM) for 20 minutes; then, [^{3}H] deoxyglucose was added. By the same principle, glucose trapped inside the cells then was measured via liquid scintillation counting. The fold response was compared to the basal response for each insulin concentration. The result showed that AH7614 significantly decreased insulin simulation response in 3T3-L1 differentiated cells comparing to the vehicle, as shown in (Figure 4-8). Only on the lowest concentration (0.005 μM) of insulin did AH7614 not affect insulin response, although it followed the trend of decreasing the insulin response. Insulin concentration (0.01, 0.05 and 0.1 μM) experienced the same range of decreasing (p = 0.0349, p = 0.0064 and p = 0.0020, respectively), while 1 μM experienced the highest decrease (p = 0.0005).

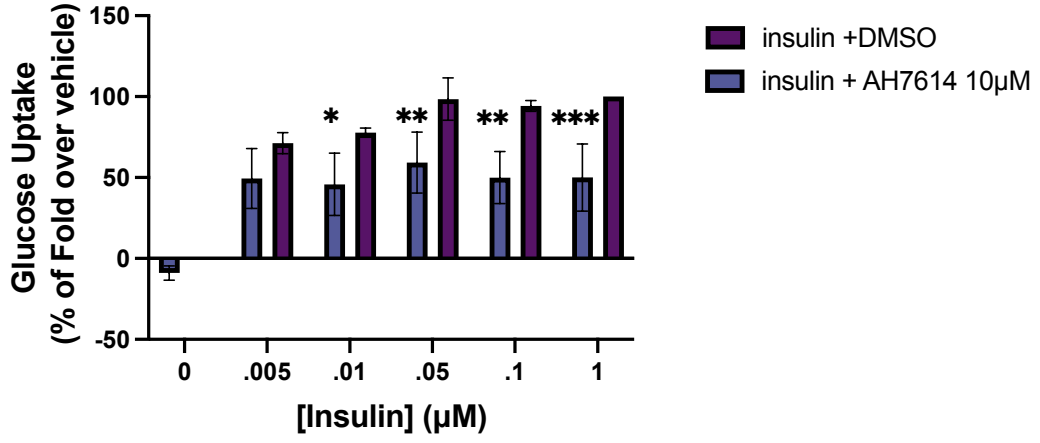


Figure 4-8 Acute treatment with AH7614 decrease insulin responded glucose uptake in 3T3-L1

3T3-L1 adipocytes were incubated with 10 μM AH7614 for 20 minutes. Then celles were simulated with insulin (0.005, 0.01, 0.05, 0.1 and 1 μM) for 20 minutes; then, [^{3}H] 2-Deoxyglucose was added to measure glucose uptake over a five-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells treated vehicle. Data are expressed mean ± SEM from three independent experiments tested in in triplicates (*p > 0.05, **p < 0.1; ***p < 0.001) compared to vehicle treatment at each insulin concentration.

This result highlights the effect of AH7614 on insulin response in a collection of concentrations and, therefore, GLUT4 translocation. This outcome drives the next step: to examine the effect of a range of AH7614 concentrations on insulin response itself.

4.1.7 A range of AH7614 concentration decrease the maximal insulin response in Differentiated 3T3-L1 Adipocyte

To measure the effect of AH7614 concentrations on insulin response, a glucose uptake was sited up to capture the change in glucose uptake level after treatment with a range of AH7614 concentrations. 3T3-L1 differentiated cells were inculpated for 10 minutes with 3 10 and 30 μM AH7614, and then cells were treated with a single insulin concentration (1 μM) for 20 minutes. 3T3-L1 adipose then were treated with [3H] Deoxyglucose for three minutes and then measured by liquid scintillation counting. Results were measured in a comparison between the basal fold increase of the insulin without the addition of AH7614. The outcome confirmed again that Ah7614 decreases insulin response in 3T3-L1 adipocytes. Insulin treatment after incubation with vehicle was able to simulate insulin's response in 3T3-L1 adipocyte with a sixfold increase ($p < 0.0001$). This response was decreased by the per-incubation with AH7614 concentrations. All the concentrations of AH7614 (3, 10 and 30 μM) that were used in this assay were able to significantly decrease the fold change in glucose uptake in 3T3-L1 adipocytes ($p = 0.0158$, $p = 0.0002$ and $p < 0.0001$, respectively) (Figure 4-9).

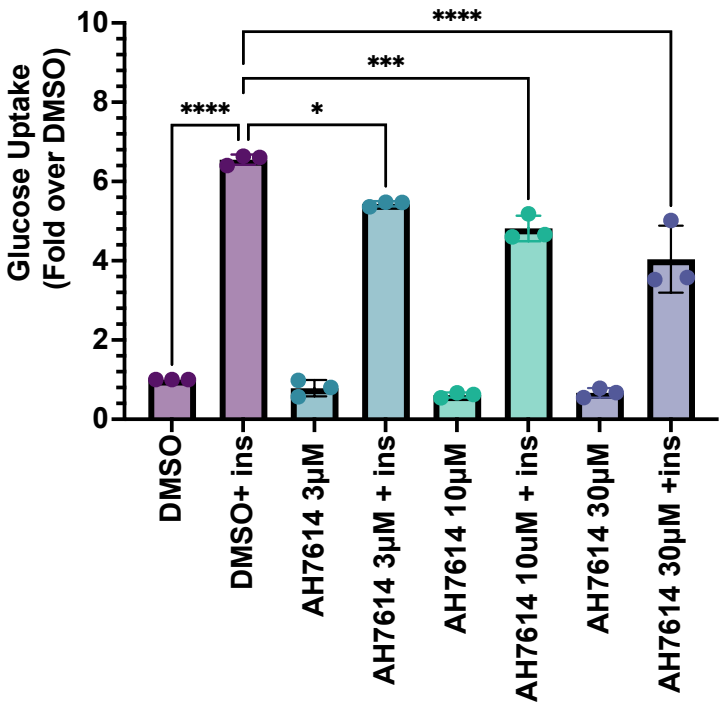


Figure 4-9 A pre-treatment with a range of AH7614 concentrations decrease the maximal insulin response in differentiated 3T3-L1 adipocyte.
3T3-L1 adipocytes were incubated with a range of AH7614 concentrations (0, 3, 10 and 30 µM) for 10 minutes. Then, 1 µM of insulin was added for 20 min, followed by the addition of [^{3}H] 2-Deoxyglucose to measure glucose uptake over a three-minute time period. Radioactivity was quantified and expressed as the fold over the signal obtained in cells treated with DMSO. Data are expressed mean ± SEM from three independent experiments tested in triplicates (*p > 0.05, **p < 0.1; ***p < 0.001, ****p < 0.0001.

This outcome confirms the effect of AH7614 on glucose transport on 3T3-L1 adipocytes, and, in this set of assay scenarios, we can conclude that AH7614 has a direct effect on GLUT4 translocation; but this translocation goes through specific pathways and phosphorating events. In order to locate the effect of AH7614 on GLUt4 translocation, a western blot was established to examine samples from this step of glucose uptake to assay p-Akt (one of the phosphorylation events that occur when GLUT4 translocate from plasma membrane).

4.1.8 AH7614 does not affect insulin-stimulated AKT phosphorylation in 3T3-L1 adipocytes

To identify the specific step at which AH7614 affects Glut4 translocation, protein samples were obtained from 3T3-L1 adipocytes and subjected to immunoblotting. The cells were treated with 10 μM AH7614 for 10 minutes, then stimulated or not with insulin (1 μM) for a further 20 min. Control samples were collected in the same manner, but DMSO was added instead of AH7614. The lysates obtained from these cells were then used for Western blot analysis, initially using a GLUT4 antibody (Figure 4-10A). These studies show the protein levels of GLUT4 in the lysates from each condition. In the immunoblot, GLUT4 was detected as a smear band (approximately 55-43 kDa) due to its high level of glycosylation (Cura, 2022; Gong et al., 2018). To control for gel loading, a total protein stain control was included (Figure 4-10B). Quantitative densitometry analysis indicated that there was no significant difference ($p > 0.05$) in GLUT4 protein expression among all the treatment conditions (Figure 4-10C).

The same lysates were also probed with a p-AKT antibody to assess activation of AKT (Figure 4-10D). Treatment with insulin resulted in an increase in phosphorylated AKT (approximately 66 kDa) regardless of the pre-treatment with AH7614. A protein staining control was included for comparison (Figure 4-10E). Quantitative densitometry analysis revealed that there was a significant increase ($p<0.05$) in p-AKT following insulin treatment, but that this increase was unaffected by pre-treatment with AH7614 ($p>0.05$)(Figure 4-10F). These results suggest that AH7614 may block insulin signalling downstream of AKT phosphorylation, thereby interrupting the translocation of GLUT4. This disruption ultimately leads to a decrease in glucose uptake, as discussed in Section 4.3.5.

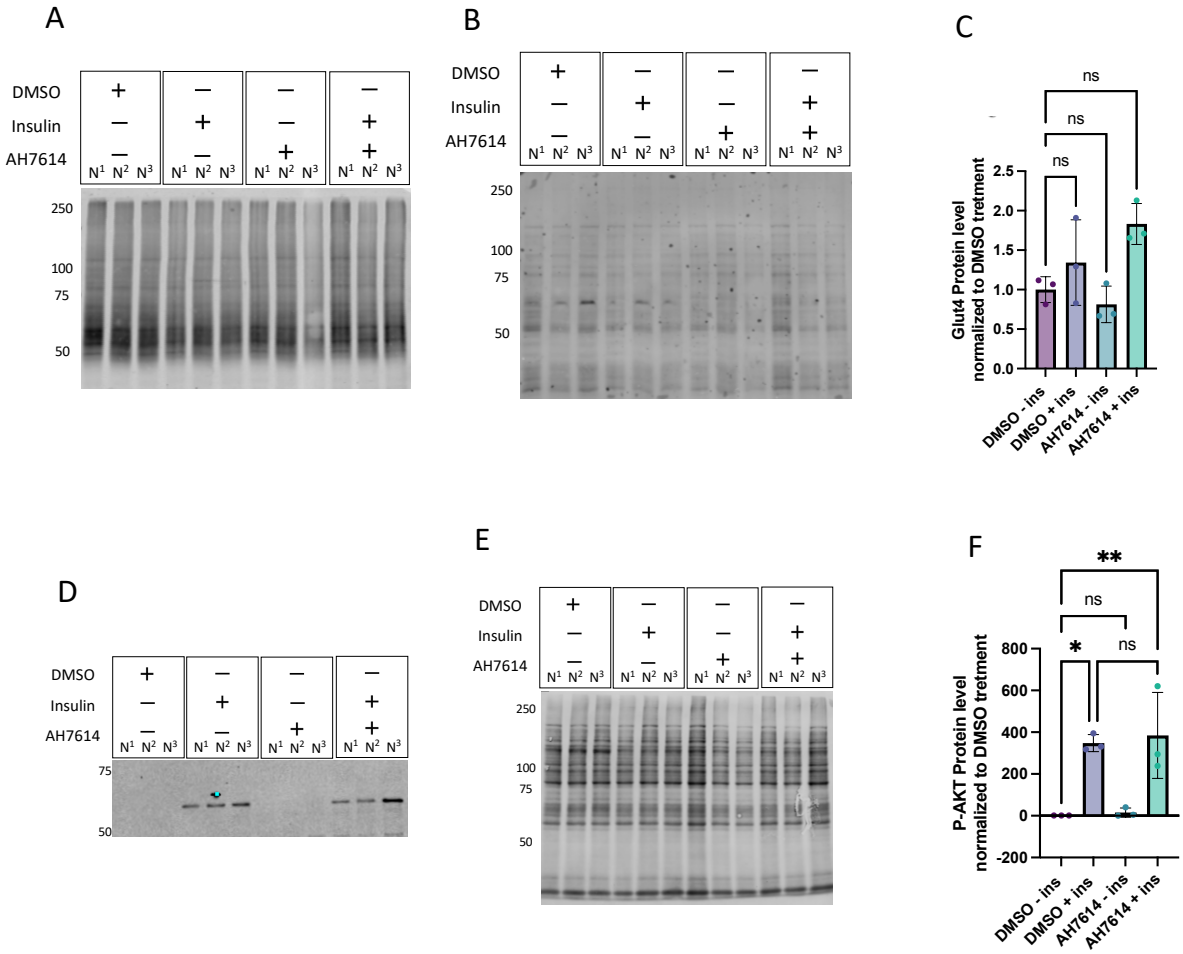


Figure 4-10 GLUT4 and P-AKT protein levels were not affected by short-term incubation with AH7614 in 3T3 adipocytes.
3T3-L1 adipocytes were treated with DMSO or 10 µM AH7614 for 10 minutes and then simulated by 1 µM insulin for 20 minutes, lysate (20 µg) as used in western blot.. A) Lysates from cells from each condition were prepared and immunoblotted for GLUT4 . B) Total protein staining for samples used in A. C) Quantification of GLUT4. The conditions repeated again. D) Lysates from cells from each condition were prepared and immunoblotted for P-AKT. E) total protein. F) Quantification of P-AKT. Data shown represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-way ANOVA; n.s. = not significant.

4.1.9 Treatment with TUG-891 inhibits lipolysis, while AH7614 treatment enhances lipolysis in 3T3-L1 adipocytes

After confirming that AH7614 has an effect on glucose uptake and GLUT4 translocation, another function of adipose was worth examining. Lipolysis was assessed in 3T3-L1 adipocytes by measuring glycerol release after simulation with the ß-adrenoceptor agonist isoprenaline (ISO). Cells were pre-treated with DMSO, TUG-891 (3, 10 and 30 µM) or AH7614 (3, 10 and 30 µM) for 45 minutes. Then, lipolysis was induced by treatment with ISO (Figure 4-11A). Conditions were that no additions of 1nM of isoprenaline were kept for each compound that was used to compare the basal lipolysis with the one initiated with isoprenaline

addition. Glycerol release was assayed by adding free glycerol reagent to the supernatants from each condition and inculpated for 10 minutes. Glycerol concentrations were detected by using a Pherastar FS microplate reader at 540 nm. By preparing a stander glycerol curve, the glycerol concentration release was measured in each condition.

Results showed that, in all conditions, the addition of isoprenaline increased glycerol release compared to the group that stayed without the addition of isoprenaline. Furthermore, in the condition where 3T3-L1 adipocytes were pre-treated with 10uM and 30μM TUG-891 before the start of the lipolysis, a significant decrease was observed ($p < 0.0001$) in both concentrations: a 7 mg/ml decrease in glycerol release at 10μM TUG-891 treatment and a 6 mg/ml decrease in glycerol release at 30 μM TUG-891 treatment, as shown in (Figure 4-11A). On the other hand, the groups that were treated with 10 μM and 30μM AH7614 showed a significant increase in glycerol release with p= 0.0088 at 10μM AH7614 treatment and p= 0.0002 at 30μM treatment (Figure 4-11B).
In other words, treatment with AH7614 increased the loss of lipids from the cell to the media in isoprenaline stimulation lipolysis. On the other hand, TUG-891 decreased lipolysis in the same cells with the same condition.

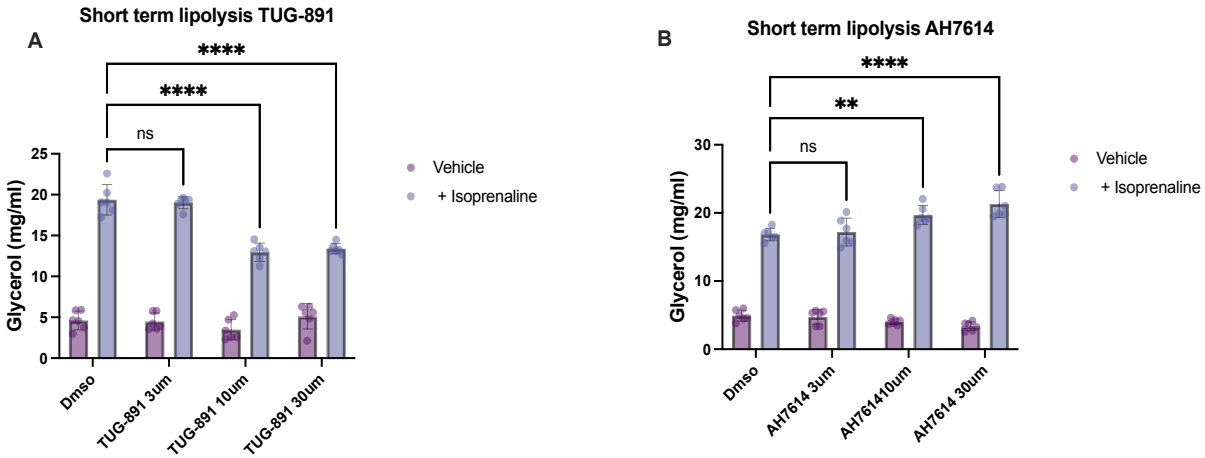


Figure 4-11 TUG-891 decreases lipolysis, while AH7614 increases lipolysis in 3T3-L1 adipocyte.
3T3-L1 cells were differentiated; then, on Day 10, lipolysis assay was performed. 3T3-L1 adipocyte was pre-treated for 45 minutes with a range of concentration of TUG-891 or AH7614; then, lipolysis was simulated by isoprenaline (1nM). Treatment with DMSO was the control for this assay. A) Glycerol release after treatment with (3,10,30 um) of TUG-891. B) Glycerol release after treatment with (3,10,30 um) of AH6714. Data shown represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-way ANOVA; n.s. = not significant.

4.4 Discussion

Prior to using 3T3-L1 cells to study FFA4 function in adipocytes, I first aimed to demonstrate which FFA receptors are expressed in these cells and which ones are upregulated during adipogenic differentiation. The results demonstrated that both FFA4 and FFA2 are upregulated through adipogeneses, while FFA1 is downregulated. No change in expression level was indicated in FFA3. This is consistent with previous studies examining FFA receptor expression in 3T3-L1 and other adipocyte models. According to Steneberg et al. (2005), FFA1 has not been detected in either human or mouse adipose tissue. Furthermore, in adipose tissue that was isolated from female mice, no FFAR3 mRNA or protein was detected (Hong et al., 2005).In addition, scanning the same isolated adipose tissue, expression level was high for FFA2 receptor (Y. H. Hong et al., 2005). In the same study, 3T3-L1 adipocytes were used to follow the level of FFA2 and FFA3 expression through differentiation. Results showed that FFA2 upregulated during the process of differentiation into adipocyte, while during adipocyte differentiation, no FFA3 mRNA was detected; this again confirms the results in this section of the thesis (Hong et al., 2005). A number of studies confirm the finding of upregulating FFA4 expression level in adipogenesis. FFA4 was discovered to be highly and widely expressed on the surface of mature mouse adipocytes in adipose tissues (Miyauchi et al., 2009). Additionally, in a study comparing adipocytes and stromal-vascular (S-V) cells, results showed that adipocytes had higher levels of FFA4 mRNA expression than S-V cells. Moreover, in isolated adipose tissue and mature adipocytes, 3T3-L1 FFA4 expression was high, while pre-differentiation 3T3-L1 showed no expression for FFA4 (Oh et al., 2010). Altogether, this gives me confidence in using 3T3-L1 when assaying adipose function and relating the results from the upcoming assays to FFA4 receptor.

Critically, low expression of FFA1 was observed in 3T3-L1 adipocytes, which is important for this study because FFA1 is activated by the same long-chain free FAs as FFA4 (Hidalgo et al., 2021). Not only that, FFA1 is activated by many of the same synthetic agonists as FFA4, including to a degree by TUG-891 (Hidalgo et al., 2021). Because of this, using a model that highly expresses FFA4 and with no expression for FFA1 gives more confidence that the effects of the FFA4 ligands used are mediated by FFA4 (Hudson et al., 2013).

To explore the influence of FFA4 activation on adipose functions, an examination of TUG-891, an agonist for FFA4, was conducted in several glucose uptake assay scenarios. The results concluded that, in all treatments and time courses explored in this study, TUG-891 did not result an enhancement of either insulin dependent or independent glucose uptake. This result stands in contrast to previous studies that showed FFA4's ability to enhance glucose uptake (Hudson et al., 2013). Using differentiated 3T3-L1 mouse adipocytes, the study found that treatment with aLA (100 μM) increased [3H] deoxyglucose uptake by these cells by a statistically significant 92%, A statistically significant increase in [3H] deoxyglucose uptake was also seen following treatment with TUG-891 (10 μM), albeit at only a lower 47% increase. Compared to the 557% increase seen in response to an insulin challenge (1 μM), the reactions to aLA and TUG-891 were very modest. In this case, it should be noted that TUG-891 produced a response that was only 8% of the maximum insulin response in glucose uptake assays, which will make the observation of such an effect challenging (Hudson et al., 2013). Moreover, in both primary adipose tissue cultures and 3T3-L1 adipocytes, (Oh et al., 2010) evaluated the effects of FFA4 stimulation on insulin sensitivity. Primary adipose tissue explants and 3T3-L1 adipocytes were pre-treated with GW9508 or DHA for 30 minutes; then, basal and insulin-stimulated GLUT4 translocation and deoxyglucose transport were measured (Oh et al., 2010). This study concluded that DHA's stimulatory effects are related to adipocyte glucose transport stimulatory actions (Oh et al., 2010). Although this finding is encouraging and one of the reasons that motivated the current project, the DHA's selectivity to FFA4 is not exclusive. DHA is one of the omega-3 FAs (Ajith and Jayakumar, 2019). Because this study relied solely on FAs as ligands, these investigations have been indirect (Hudson et al., 2013). The research needed to be in synthetic ligands with high potency and selectivity for FFA4 such as TUG-891 to completely confirm FFA4 as a therapeutic target (Hudson et al., 2013).

Although TUG-891 was used in this study as a well-defined selective agonist (Hudson et al., 2013), the outcome was not as anticipated in glucose uptake assay. Possible explanations for this can be identified by a deep look at the 3T3-L1 adipocytes as a model.

A factor that may cornubite in the different result that I had in glucose uptake after treatment with TUG-891 is the variety of protocols that are used to differentiate 3T3-L1 cells. (Zhao et al., 2019) provide a comparison study of the methods that are currently used to differentiate 3T3-L1 cells. The study compared several differentiations cocktail and compound. including comparing the use of three different insulin concentrations 1, 5, 10 μg. The results revealed that the adipogenic cocktail containing 0.5 mM IBMX, 1 μM DEX, and 10 μg/mL insulin was the most effective for 3T3-L1 cell differentiation, also the differentiation efficiencies of 3T3-L1 cells was different in all the methods that were used. Therefore, a difference between the level of differentiation in the current work compared to the previous studies may contribute in masking the enhancement of glucose uptake specially since the reported increase in glucose uptake in 3T3-L1 adipocytes after TUG-891 treatment was modest.

Another possible explanation for the lack of effect of the FFA4 agonist could be that FFA4 receptor in 3T3-L1 adipocytes is already activated. The FFA4 receptor is known to be activated by a wide range of LCFAs (Milligan, Shimpukade, et al., 2017). These LCFAs are present in 3T3-L1 adipocytes and are released by the cells into the culture media. This idea was explored by Pei et al. (2022). Using lipidomics and RNA sequencing, they identified specific changes in lipid composition and gene expression patterns during 3T3-L1 cell adipogenesis from undifferentiated and differentiated 3T3-L1 cells. Results using qRT-PCR showed that in differentiated 3T3-L1 cells, there were significant increases in the relative expression levels of acyl-Coenzyme A dehydrogenase medium chain (Acadm), acyl-CoA synthetase long-chain family member 1 (Acsl1), angiopoietin-like 4 (Angpt14), FA binding protein 5 (Fabp5), lipase hormone sensitive (Lipe) and patatin-like (Pei et al., 2022). This fact suggested that in 3T3-L1 adipocytes the FFA4 receptor may be activated independently without the addition of an agonist. Trigatti et al. (1991), which used photoaffinity labelling and FA permeation in 3T3-L1 adipocytes, stated that upon differentiation of 3T3-L1 cells from fibroblasts to adipocytes, the rate of oleate uptake increased by eight and a half times. Oleate is a type of LCFA that may activate FFA4. Previous research has demonstrated that activation of FFA4 by FAs stimulates both rapid phosphorylation and internalisation of the receptor (Burns and Moniri, 2010; Hirasawa et al., 2005). This release and uptake of LCFAs may repeatedly

activate FFA4 receptors, (Burns and Moniri, 2010) desensitising them to further activation by TUG-891. A similar effect has been reported in cells heterologous expressing FFA4 tagged with eYFP, where FFA4-eYFP expressing cells were desensitised to a TUG-891 Ca2+ response following FFA4 agonist treatment, suggests that receptor desensitisation has occurred (Hudson et al., 2013). The internalisation of FFA4-eYFP was examined in the same cell line. Prior to ligand administration, FFA4-eYFP was largely found at the cell surface; then, 10 minutes after the addition of 10 μM TUG-891, intracellular hFFA4-eYFP expression increased (Hudson et al., 2013).

These results explain FFA4 receptor internalisation as a first step after ligand activation. The study extends to assay FFA4 receptor desensitisation. As a follow-up, the study investigated whether TUG-891 enhanced receptor desensitisation. Ca2+ measurements on a single cell permit repeated ligand application and washing. After the ligand was withdrawn, intracellular Ca2+ recovered to baseline after a two-minute treatment with TUG-891 (3 μM). A second two-minute treatment with TUG-891 resulted in a Ca2+ response that was only 42% of the initial reaction, while a third two-minute treatment resulted in an additional drop to 34% of the initial response (Hudson et al., 2013). Also, S. J. Watson et al. (2012) indicated FFA4 internalisation. When HEK 293 cells were transfected with FLAG- or SNAP-tagged FFA4 receptors were stimulated with oleic acid, myristic acid or the agonist GW9508, intracellular calcium mobilisation was induced as well as robust internalisation of ß-arrestin2 recruitment. Moreover, in the experiment provided in this project's Section 3.4.6 using Flp-InTM T-RExTM 293, cells-mFFA4 also experienced an internalisation upon the simulation with TUG-891.

To expand more on this point, as described in Section 1.7.4, the development of a novel selective agonist to FFA4 receptor started with assaying PPAR-γ active compounds. PPAR-γ is the key transcription factor driving adipogenesis in 3T3-L1 adipocytes (Wagenaar et al., 1999). Moreover, PPAR-γ binds to a variety of natural ligands or lipophilic acids, including FAs (Marion-Letellier et al., 2016). Those same ligands can activate FFA4. The connection between those two receptors is not only the fact that PPAR ligands can activate FFA4 receptor but also the fact that their expressions were associated with each other. In a study,

during differentiation of 3T3-L1 cells, FFA4 mRNA expression coincided closely with that of the master adipogenic regulator PPAR. FFA4 suppression was found to reduce the mRNA levels of PPAR and FA binding protein 4 (FABP4), an adipogenic marker gene (Gotoh et al., 2007). These facts linked again to the idea that FFA4 is essentially activated in 3T3-L1 adipose primary without the addition of TUG-891 and suggest that the continued FFA4 activation led to receptor desensitisation and affected its activity, which may pose a significant challenge to using TUG-891 to unmask the effects of FFA4 in 3T3-L1 adipocytes.

Since all the pathways that were mentioned above were done in a theoretical cell system, a valid suggestion in this case is to study the same pathways assays in 3T3-L1 adipocytes, e.g. a Ca2+ mobilisation assay on 3T3-L1 adipocyte upon simulation with TUG-891, compared with AH7614 treatment. In addition, developing a 3T3-L1 cell line with tagged FFA4 to allow me to trace the receptor inside 3T3-L1 adipocyte and capture its translocation will be useful in here. This translocation can be followed by ß-arrestin2 assay using BRET technology or using confocal microscopy to trace the FFA4 receptor's translocation and internalisation. However, because of the timeframe, these experiments have yet to be done.

Indeed, also the FFA4 selectivity of the agonist used in this study, TUG-891, appears to be more limited for the mouse receptors than it is for human receptors. To determine if TUG-891 exhibited a comparable degree of selectivity for mFFA4 over mFFA1, two assays were performed: the -arrestin-2 experiment and Ca2+ mobilisation assay (Hudson et al., 2013). Using HEK293T cells transiently transfected with mFFA4, the ß-arrestin-2 experiment showed that TUG-891 exhibited a 61-fold greater selectivity for mFFA4 over mFFA1 in this experiment. In spite of this, using mFFA4 and µFFA1 Flp-In T-REx 293 stable inducible cells, Ca2+ mobilisation experiments showed that TUG-891 exhibited decreased activity against mFFA4. Moreover, in the Ca2+ experiment, the selectivity of TUG-891 for mFFA4 over mFFA1 decreased to barely threefold (Hudson et al., 2013). However, the limitations caused by this lowere selectivity for TUG-891 for mFFA4 are unlikely to affect the current work, given that 3T3-L1 adipocytes do not appear to express FFA1.

On the other hand, where FFA4 receptor was blocked by AH7614, a significant decrease was captured in glucose uptake by 3T3-L1 adipocytes. That confirms the fundamental part the FFA4 receptor plays in glucose uptake. In a study using siRNA technique to down-regulate the FFA4 expression in 3T3-L1 cell compared to the wild type 3T3-L1 (D. Liu et al., 2012), differentiated cells were incubated with palmitic acid, a long-chain FA. Results showed that gene expression level and protein level of IRS-1, PI3K and GLUT4 were significantly reduced in siRNA FFA4 3T3-L1 (D. Liu et al., 2012). The effect of AH7614 was also recorded on another cell model, Caco-2 cells. Castilla-Madrigal et al. (2018) reported that AH7614 inhibited the protective effect of eicosapentaenoic acid (EPA) against TNF-induced reductions in the cytokine on α-methyl-d-glucose (αMG) uptake and AMPK activation.

Continuing with the idea that blocking FFA4 activation may affect the function of insulin, one component of the insulin signalling cascade leading to GLUT4 translocation was assayed, namely, protein kinase B (AKT). Unexpectedly, my results showed that AH7614 treatment did not affect phosphorylation AKT.

GLUT4 plays an explicit role in glucose homeostasis regulation via translocation and activation, which is subsequently triggered by the insulin-dependent PI3K/phosphorylated protein kinase B (p-Akt) pathway (R. T. Watson et al., 2004). Several studies have shown the importance of P-AKT for GLUT4 translocation (Katome et al., 2003). Generated recombinant adenovirus encoding either constitutively active Akt or dominant negative Akt and examined the effect in 3T3-L1 through insulin actions, including GLUT4 translocation to plasma membrane and 2-deoxyglucose (2-DG) uptake. Mutations that constitutively active Akt remarkably enhanced Akt kinase activity in 3T3-L1 adipocytes (Katome et al., 2003). Moreover, the results showed that this same mutation was able to mimic the effects of insulin on GLUT4 translocation and 2-DG uptake (Katome et al., 2003). In the same study, a dominant negative Akt mutation was found to inhibited insulin-stimulated Akt kinase activity and both insulin-stimulated GLUT4 translocation and 2-DG uptake in 3T3-L1 adipocytes, which is an indicator of the importance of P-AKT in GLUT4 translocation. Yet the effect of insulin-stimulated GLUT4 and 2-DG uptake was not completely blocked, which suggests an additional pathway in 3T3-L1 adipocytes for insulin-stimulated

activity (Katome et al., 2003). This fact can be used to justify the fact that, although P-AKT occurred in 3T3-L1 adipocytes that were incubated with AH7614, the antagonist was able to reduce insulin simulation response, which suggests again that maybe AH7614 inhibits glucose uptake through a different pathway of P-AKT.

The last function that was assayed on this chapter is lipolysis. In more detail, adipocytes are storage cells that store FAs in the form of TAGs in lipid droplets. These lipids are mobilised during adipocyte lipolysis, which is the essential process of hydrolysing TAG into FAs for internal or systemic energy utilisation (Yang and Mottillo, 2020). While, in balanced circumstances, lipids provide energy to the body, the excess circulating FAs release during lipolysis may promote dysfunction (Yang and Mottillo, 2020). However, too many FAs can cause lipotoxicity, which is the buildup of toxic lipid metabolites (Yang and Mottillo, 2020). When too many FAs leak out of adipocytes and into other tissues, unhealthy outcomes occur, such as inflammation, insulin resistance and cell death. Because of this, adipocyte lipolysis needs to be carefully controlled by hormones with opposite effects at the physiological level (Yang and Mottillo, 2020).

The most straightforward way to evaluate lipolysis is by measuring the release of lipolytic products from adipocytes or fat explants (Schweiger et al., 2014). This method involves quantifying the levels of FAs and/or glycerol that is released into in the incubation medium. Most commonly, two distinct lipolytic states are assessed: the basal state and the ß-adrenoceptor-stimulated state (Schweiger et al., 2014). To accurately measure the stimulated lipolysis rate, it is crucial to account for the time-dependent nature of the process. The rate of stimulated lipolysis is highest and exhibits a nearly linear increase during the first and second hour of ß-adrenergic stimulation (Schweiger et al., 2014). Therefore, assess adrenergic stimulated lipolysis cells are typically incubated for 1 h with a beta adrenoceptor agonist (Schweiger et al., 2014).

With the importance of balanced lipid metabolism in mind, the effect of both TUG-891 and AH7614 were detected in lipolysis assay in 3T3-L1 adipocytes to capture their influence in glycerol relace after (ISO) simulation. TUG-891 reduced lipolysis in 3T3-L1 adipocytes, consistent with previous reports on FFA4

inhibition of lipolysis in white adipose tissue. Lean rodents were orally administered the FFA4 selective agonist Compound B, and plasma FFA and glycerol levels were measured(Satapati et al., 2017). At 0.5, 2 and 4 h after treatment of the FFA4 agonist, plasma FFA levels were substantially lower than in rats treated with a vehicle compared to those treated with a control group(Satapati et al., 2017). Furthermore, when primary cultures of mature white adipocytes from rats were prepared and treated with Compound B, with or without escalating concentrations of epinephrine to induce lipolysis (Satapati et al., 2017), treatment with Compound B concentration-dependently inhibited the release of glycerol and FFAs into culture medium (both with and without concomitant epinephrine), indicating that FFA4 activation inhibits lipolysis in rat adipocytes (Satapati et al., 2017). Furthermore, in response to variations in energy requirements and availability, lipolysis of adipose fat storage results in the generation of nonesterified long-chain FAs (LCFA)(Duncan et al., 2007). This idea inspired Kalderon et al. (2012), since the researchers used ß,ß'-Tetramethyl hexadecanedioic acid (MßB/EDICA16), which is a synthetic LCFA that, like natural LCFA, is not esterified into lipids or ß-oxidised. Therefore, it was used to block agonist-induced lipolysis. This study demonstrates that MßB inhibits isoproterenol-induced lipolysis in rats in vivo and in 3T3-L1 adipocytes (Kalderon et al., 2012). In contrast, AH7614 increased lipolysis in 3T3-L1 adipocytes. This result can be linked to lipid metabolism and energy balance, as explained in Section 4.1.3. An example of that link is the study of wild-type and KO FFA4 mice on a high-fat diet that compared the two groups' lipo-metabolism (Ichimura et al., 2012). Interestingly, results showed that plasma levels of low- and high-density lipoprotein cholesterol were significantly higher in HFD-fed FFA4-deficient animals, as were serum alanine aminotransferase levels, indicating aberrant cholesterol metabolism and liver function (Ichimura et al., 2012). Furthermore, these rodents exhibited liver steatosis and a marked increase in hepatic triglyceride content. This effect indicates that FFA4 receptor controls energy levels in rodents.

This fact can help in regain the control of FA metabolism by using FFA4 agonist and antagonist. For example, by storing FAs as TAGs in fat droplets, adipose tissue attenuates their harmful effects (Yang and Mottillo, 2020). FAs can accumulate in ectopic tissues such as skeletal muscle and liver when the storage

limit for TAGs is reached or when adipocyte lipolysis is dysregulated, leading to insulin insensitivity and T2D (Yang and Mottillo, 2020). This is best demonstrated by lipodystrophy and obesity, two clinical disorders. Patients with lipodystrophy lack adipose tissue due to genetic abnormalities that impair the formation of functioning adipocytes or the storage of FAs as TAGs (Yang and Mottillo, 2020). In obesity, adipocytes achieve their storage capacity for TAGs. FA leakage causes ectopic lipid buildup in peripheral organs and overt T2D in both illnesses (Yang and Mottillo, 2020).

With these facts in mind, it is important to explore the concept of adipose tissue as a therapeutic target with pharmacological models such as 3T3-L1 adipocytes, since improving adipocyte function by enhancing FAs storage in adipocytes is a key factor to suggest an idea to treat metabolic diseases.

To further validate the lipolysis assay results, an alternative method using Forskolin can be employed to induce lipolysis in adipocytes. This approach serves as a valuable confirmation of the observed findings since it triggers the lipolysis pathway independently of GPCR signaling (Yin et al., 2003).The lipolysis data in this chapter was obtained using isoprolipen, which activates GPCR signaling via the ß-adrenergic receptor and involves G protein signaling. By using Forskolin to stimulate lipolysis via cAMP, it will be possible to determine if the effects of AH7614 and TUG-891 on downstream target proteins occur through mechanisms other than G protein activation. Specifically, Forskolin stimulates the activation of adenylate cyclase in adipocytes, leading to an augmented production of cAMP (Yin et al., 2003). This elevation in cAMP effectively triggers the activation of lipolysis processes (X. Zhang et al., 2021). Forskolin activates adenylyl cyclase, the enzyme responsible for converting ATP into cAMP. By elevating cAMP l levels of cAMP serve as a second messenger. cAMP binds to the regulatory subunits of protein kinase A (PKA). (Schimmel, 1984; Yin et al., 2003)The released catalytic subunits of PKA phosphorylate various target proteins involved in lipolysis, including hormone-sensitive lipase (HSL) and perilipin(Schimmel, 1984; Yin et al., 2003). Activated HSL breaks down triglycerides stored within the lipid droplets into FFAs and glycerol which will be measured to assay lipolysis (Yin et al., 2003).

In summary, in this section, I was able to link FFA4 activation to glucose uptake and GLUT4 effect on this process. Blocking FFA4 receptor by using AH7614 has decreased glucose uptake in 3T3-L1 adipocyte. Moreover, AH7614 treatment was able to reduce the maximal insulin response on glucose uptake. In addition, the lipolysis assay has revealed another action for FFA4 receptor in maintain energy balance in adipocytes. TUG-891 treatment has decreased glycerol release while AH7614 increase the same action in 3T3-L1 adipocytes. This fact may serve as a gate to explore FFA4 receptor in energy control and adipocyte formation.

However, while the effect of blocking FFA4 by AH7614 treatment resulted in clear effects on glucose uptake, an effect upon treatment with TUG-891 was not observed. In this case, there are still some scenarios that can be applied at the differentiation process before the glucose uptake assay is performed to. Specifically, since FFA4 desensitisation by FFAs released from the cells during differentiation is the most plausible explanation for why TUG-891 had no effect, there are several ways this could be addressed. Washing of free FAs by changing the media more regularly throughout the differentiation process is one change that may reduce this level of desensitisation. Another possibility would be increasing the volume of medium used to culture the cells. The protocol I performed was by using 500 μl of media on each well in 12 well plates to differentiate the cell. I would increase the volume to 1 ml of media to see if, the resulting reduction on FFA levels in the medium would influence how the cell well behaved in glucose uptake assays upon TUG-891 treatment.

Another future idea that has captured my mind is to use a KO FFA4 3T3-L1 cell line that can be created by gene editing. CRISPR/Cas9 technology is a method that was used to demonstrate receptor influence in 3T3-L1 features (Bremner et al., 2022). By using the same technique with FFA4 receptor, 3T3-L1 that lacks FFA4 receptor could be generated. Then, KO FFA4 3T3-L1 and WT 3T3-L1 cells could be used in glucose uptake assay and lipolysis. In addition, the generated KO 3T3-L1 cells line could be used to assay key elements in glucose uptake and adipogeneses such as GLUT4 and PPAR-γ by RT- q-PCR for gene expression and western blotting for proteins regulation. This comparison between the generated KO 3T3-L1 cells and wild type would further confirm the finding in this section

and might provide new findings and contributions to the understanding of the FFA4 receptor's function in adipocyte.

All the results were assayed in vitro system with 3T3-L1 adipocyte. Another obvious future direction is to assay FFA4 receptor function in vivo or ex vivo. For example, TUG-891 and AH7614 can be assayed on primary adipose. Moreover, using KO FFA4 and WT mice models can be assayed as a basic comparison between the function in isolated adipose from the two modules. Alternatively, I could compare agonist and antagonist effects in both models, either in primary adipose differentiation or the effect of both compounds in isolated adipose tissue from KO/WT mice.

.

Chapter 5 Effects of agonism and antagonism of the FFA4 receptor on adipogenesis in 3T3-L1 adipocyte.

5.1 Introduction

Adipose tissue plays an important role in the body as a platform for metabolic activity. Moreover, adipose tissue is known to be an active organ that adds to several important physiological processes, such as lipid metabolism, systemic energy homeostasis, energy storage and whole-body insulin sensitivity (De Sá et al., 2017). Because of this, the fact that researchers reported that FFA4 is highly expressed in adipose tissue (Gotoh et al., 2007; Miyauchi et al., 2009; Quesada-López et al., 2019) resulted in further studies for this receptor in adipogenesis. Gotoh et al. (2007) discovered that knocking down FFA4 with siRNA dramatically reduced adipocyte markers and lipid accumulation in 3T3-L1 cells. Moreover, a beneficial effect of omega-3 polyunsaturated fatty acids was clearly observed in lipid metabolism (Scorletti & Byrne, 2013). Furthermore, another study reported that, in comparison to normal weight controls, obese people have significantly greater levels of GPR120 expression in adipose tissue. Obese individuals carrying a harmful non-synonymous mutation in GPR120 (p.R270H) in the gene's exons have their signalling abilities reduced (Ichimura et al., 2012). However, the precise role of GPR120 in adipose tissue has yet to be explored. The studies of FFA4 on adipogenesis are growing but are still limited. Our understanding of adipogenesis in terms of the use of FFA4 agonist and antagonist is not yet complete. Since FFA4 was suggested to have a role in obesity, using agonist and antagonist is a reasonable step in drug discovery.

These facts led to me to further explore the role of FFA4 on adipogenesis in 3T3-L1. During the differentiation of 3T3-L1 cells, TUG-891 and AH7614 were evaluated.

5.2 Aims

The main aim of this chapter is to track the changes in adipogenesis during 3T3_L1 cells' diffraction upon incubation with TUG-891 and AH-7614 throughout differentiation steps that were explained in Section 2.2.1.2. This was done to test and determine their ability to influence adipogenesis of 3T3-L1 cells. Oil Red O (ORO) was used to stain and quantify neutral lipid accumulation in lipid droplets during adipogenesis. RT-qPCR was used to evaluate changes in audiogenic gene expression. Adipocyte function was assessed using glucose uptake or isoprenaline-stimulated lipolysis assays.

By means of these assays, I aim to demonstrate the effect of FFA4 agonist and antagonist in adipogenesis and then reveal the changes in key adipogenesis markers in 3T3-L1 adipocyte following treatment with FFA4 agonist or antagonist. Finally, I will examine functional changes in 3T3-L1 adipocytes that were differentiated in the presence of TUG-891 or AH7614 to determine if they have an impact on the assays used.

5.3 Results

5.3.1 FFA4 agonist treatment does not affect lipid accumulation during 3T3-L1 differentiation, while antagonist treatment decreases lipid accumulation

To investigate the role of FFA4 in adipogenesis, 3T3-L1 cells were differentiated into adipocytes in the presence of either an FFA4 agonist, TUG-891, or an FFA4 antagonist, AH7614. Adipocyte differentiation was assessed using ORO staining, which labels the neutral lipid that accumulates in lipid droplets during adipogenesis (Du et al., 2023). As a control, undifferentiated 3T3-L1 cells were fixed and stained with ORO, revealing minimal neutral lipid staining prior to differentiation (Figure 5-1A). After 9 days of differentiation into adipocytes in the presence of a DMSO vehicle, clear oil red staining of lipid droplets was observed (Figure 5-1B). These lipid droplets were observed as multiple small droplets within each adipocyte, consistent with previously published adipogenesis studies using the 3T3-L1 model (Manickam et al., 2010). Similarly, when cells were differentiated in the presence of the FFA4 agonist, TUG-891, a pattern of ORO staining comparable to the DMSO vehicle treatment was observed (Figure 5-1C). In contrast, differentiation of 3T3-L1 cells in the presence of the FFA4 antagonist AH7614 resulted in a visible reduction in ORO staining (Figure 5-1D). This reduction was appeared to result from both a smaller number of differentiated cells as well as less ORO staining within the cells that had differentiated.

To quantitatively analyse the differences in neutral lipid content among the different treatments, the oil red stain was dissolved in absolute isopropyl alcohol after imaging the cells. The absorbance of this solution was then measured at 492 nm to quantify the amount of ORO staining in the cells (Figure 5-1E). These experiments demonstrated that treatment with TUG-891 did not significantly alter the amount of ORO staining compared to the control ($P = 0.9305$). However, consistent with the microscopy images (Figure 5-1D), cells treated with AH7614 showed a significant ($P < 0.0001$) 46% decrease in total lipid accumulation compared to the control DMSO treatment. These data indicate that antagonism of FFA4 has an inhibitory effect on the adipogenesis process of 3T3-L1 adipocytes.

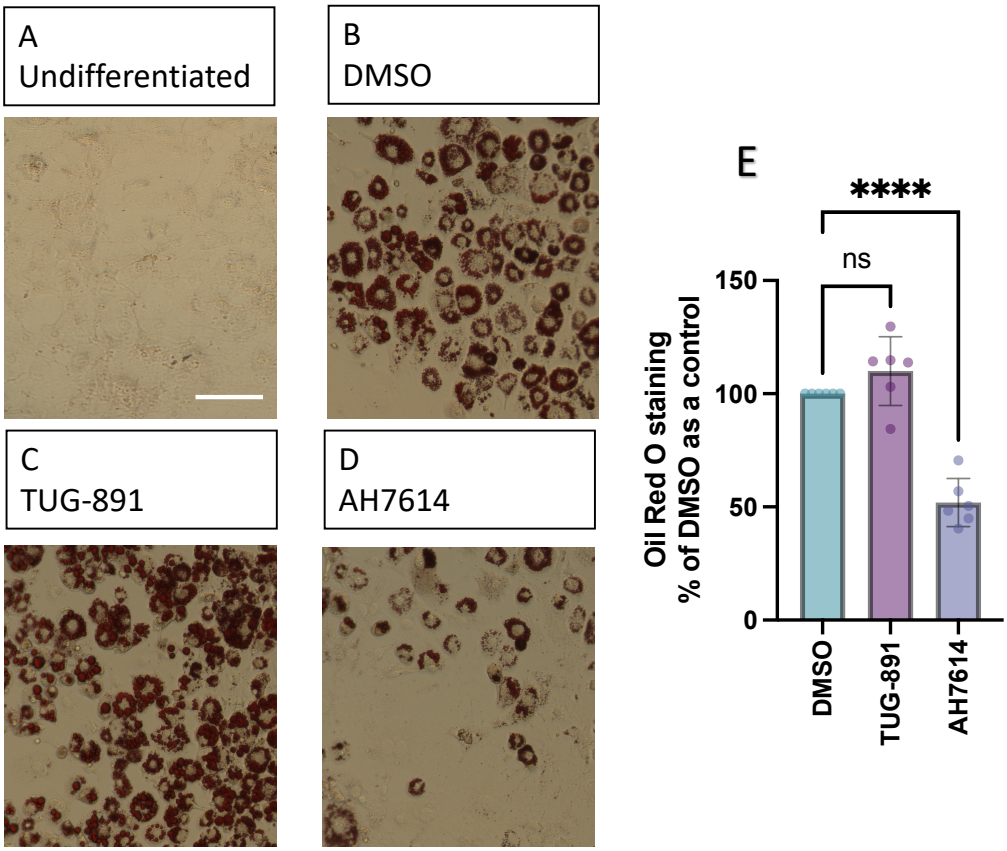


Figure 5-1 FFA4 antagonist treatment decreases adipogenesis in 3T3-L1 adipocytes. ORO neutral lipid stain is shown for undifferentiated 3T3-L1 cells (**A**) . (**B**) for 3T3-L1 differentiated into adipocytes and treated with DMSO vehicle (0.1%) or (**C**), 10 μM TUG-891 or (**D**) 10 μM AH7614 throughout differentiation. Images of differentiated cells were taken from cells that were fixed and stained on Day 9 of differentiation at 200um scale. E) Oil red staining was quantified by dissolving the dye in 100% isopropyl alcohol and measuring absorbance at 492 nm. Data are from four independent experiments with three technical replicates of absorbance measurements. Values are the means ± SD of four separate experiments. Fractions were compared using a one-way ANOVA. ns. = not significant, ****P < 0.0001 (scale bar = 200 μm).

To demonstrate that the treatments did not affect the cell number, cell counting and imaging were performed on Day 9 using Hoechst stain, which fluorescently labels the nucleus. This stain offers the advantage of being applicable to both living and fixed cells (Lahkar & Das, 2021). These results demonstrated that although all cells exposed to differentiation conditions had increased cell counts compared to cells that were not differentiated, there were no differences in cell number among the cells differentiated in the presence of DMSO, TUG-891 or AH7614 (Figure 5-2 A-E). This increase in cell number between the differentiated and undifferentiated cells is consistent with the well-established terminal cell division 3T3-L1 cells are known to undergo during the initial stages of differentiation (Moreno-Navarrete & Fernández-Real, 2017)

It was also notable that while the nuclear stain of the undifferentiated cells appeared to show larger nuclei with dimmer staining (Figure 5-2A), the nuclei of the cells differentiated in the presence of both DMSO (Figure 5-2B) and TUG-891 (Figure 5-2C) were smaller and more intensely stained, perhaps due to mechanical compression of the nuclei as a result to lipid accumulation (Loneker et al., 2023). In contrast, the AH7614 treated cells displayed a distinct pattern, with the nuclei appearing to include a combination of larger dimly labelled undifferentiated and cells, as well as some more intensely ladled nuclei consistent with the differentiated cells (Figure 5-2D). Importantly, in the AH7614 treated cells, the cells with intensely stained nuclei appeared to correlate with the cells that displayed ORO staining.

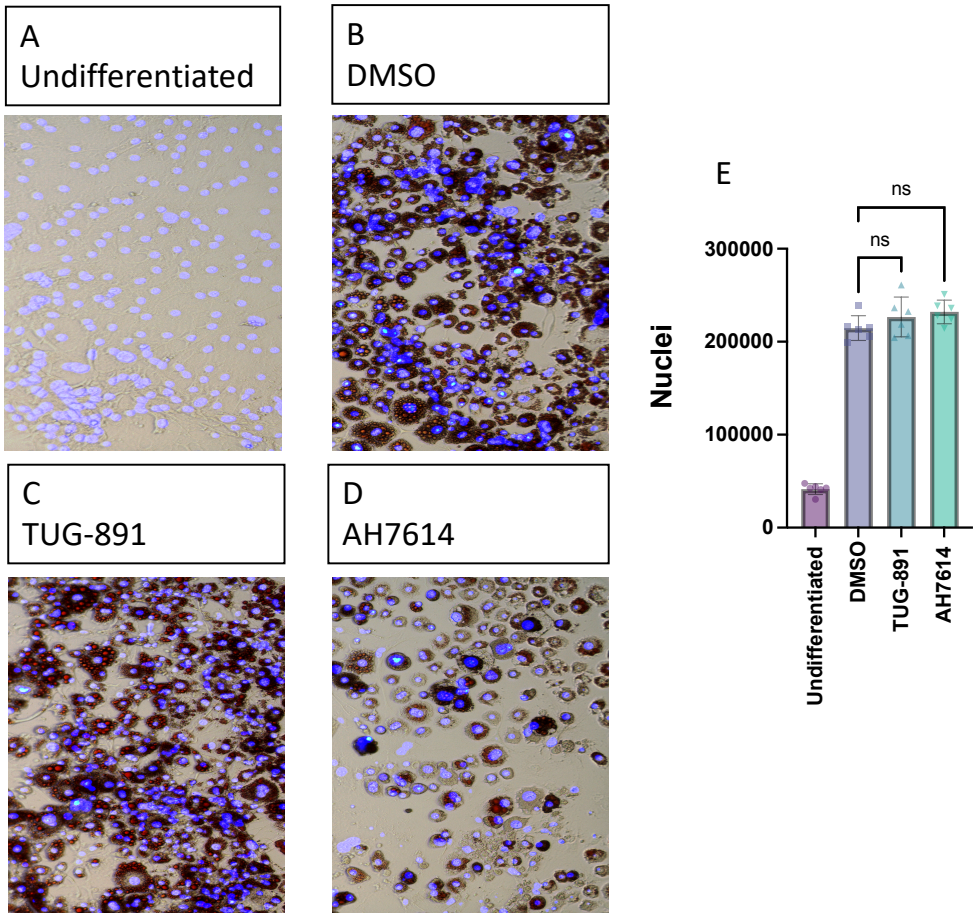


Figure 5-2 Treatment with AH7614 had no significant toxicity on cell growth in 3T3-L1 differentiated cells:
3T3-L1 was fixed at as undifferentiated cells and again at Day 9 after treatment with DMSO, TUG-891 and AH7614, then stained with ORO after that with Hoechst. 3T3-L1 cells were plated and differentiated into adipocytes for nine days before fixing and labelling nuclei with Hoschet. Images are shown of 3T3-L1 cells that were maintained for nine days without differentiating (A) and cells that were differentiated in the presence of DMSO vehicle (0.1%) (B), 10μM TUG-891 (C) or 10μM AH7614 (D). Quantified nucleus numbers obtained from three independent experiments in triplicate are shown in E. Values are the means ± SD of three separate experiments in triplicate. Fractions were compared using a one-way ANOVA. ns indicates $p > 0.05$ compared with DMSO treatment (scale bar = 200 μm).

These data suggest that there is no significant impact on cell growth and proliferation with any of the treatments. This finding supports the assumption that the observed effects on adipogenesis associated with the FFA4 antagonist (AH7614) are likely a result of blocking FFA4 activation during the differentiation of 3T3-L1 cells.

5.3.2 AH7614 affects 3T3-L1 adipogenesis as early as six days post differentiation

To identify the starting timepoint in which the FFA4 antagonist begins its effect, cells were fixed, stained and then imaged at each fundamental time point through differentiation, which are the dates on which I changed the media (Day 3, Day 6 and Day 9). These cells were treated with DMSO 0.1% at a control, the FFA4 agonist TUG-891 or the FFA4 antagonist AH7614. Over the differentiation procedure, cells were fixed and imaged to track each treatment over differentiation. following the ORO stain, the quantification for each treatment at each time point was measured by dissolving using absolute isopropyl alcohol; then, cells and the absorbance of this solution was measured at 492 nm at each time point for each treatment.

After ORO staining, undifferentiated 3T3-L1 cells at the start point (Day 0) were dissolved by absolute isopropyl alcohol; then, this was compared with 3T3-L1 adipocyte at Day 9. The quantified data from ORO staining emphasises the huge difference in oil droplets and the differentiated cells compared to the undifferentiated ones, with significant decrease of ORO staining ($P =< 0.0001$) (Figure 5-3A).

Cells that were treated with vehicle (DMSO 1%) were the control for this experiment. Results showed that both TUG-891 and AH7614 followed the same pattern of the control at the first time point (Day 3), as shown in (Figure 5-3 B1, B2 and B3). This also was confirmed by ORO quantitation data in (Figure 5-3 B4). Hence, the cells at Day 6 differ in their results. Cells that were treated with TUG-891 show more differentiated cells compared to vehicle-treated cells (Figure 5-3 C1 and C2); this was also confirmed in the quantification data, since treatment with TUG-891 showed a significant increase in ORO staining compared with the vehicle (Figure 5-3 C4). On the other hand, at the same time point (Day

6), cells treated with AH7614 experienced a decrease in ORO staining compared to the DMSO (1%) treatment (Figure 5-3 C3), and this fact was also confirmed with quantification data (Figure 5-3 C4), with a significate decrease compared with the vehicle. At the last time point, Day 9, there was no obvious difference in ORO staining between 3T3-L1 adipocytes in both DMSO control and TUG-891 treatment (Figure 5-3 D1 and D2); this was also confirmed in quantification data, since it showed no significant difference between the two treatments (Figure 5-3 D4). The effect of AH7614, the FFA4 antagonist, continued after Day 6, reaching its maximum effect on Day 9, as captured by ORO staining image (Figure 5-3 D3). This result was confirmed again using quantifying data that showed a significant decrease in ORO staining when quantified (Figure 5-3 D4).

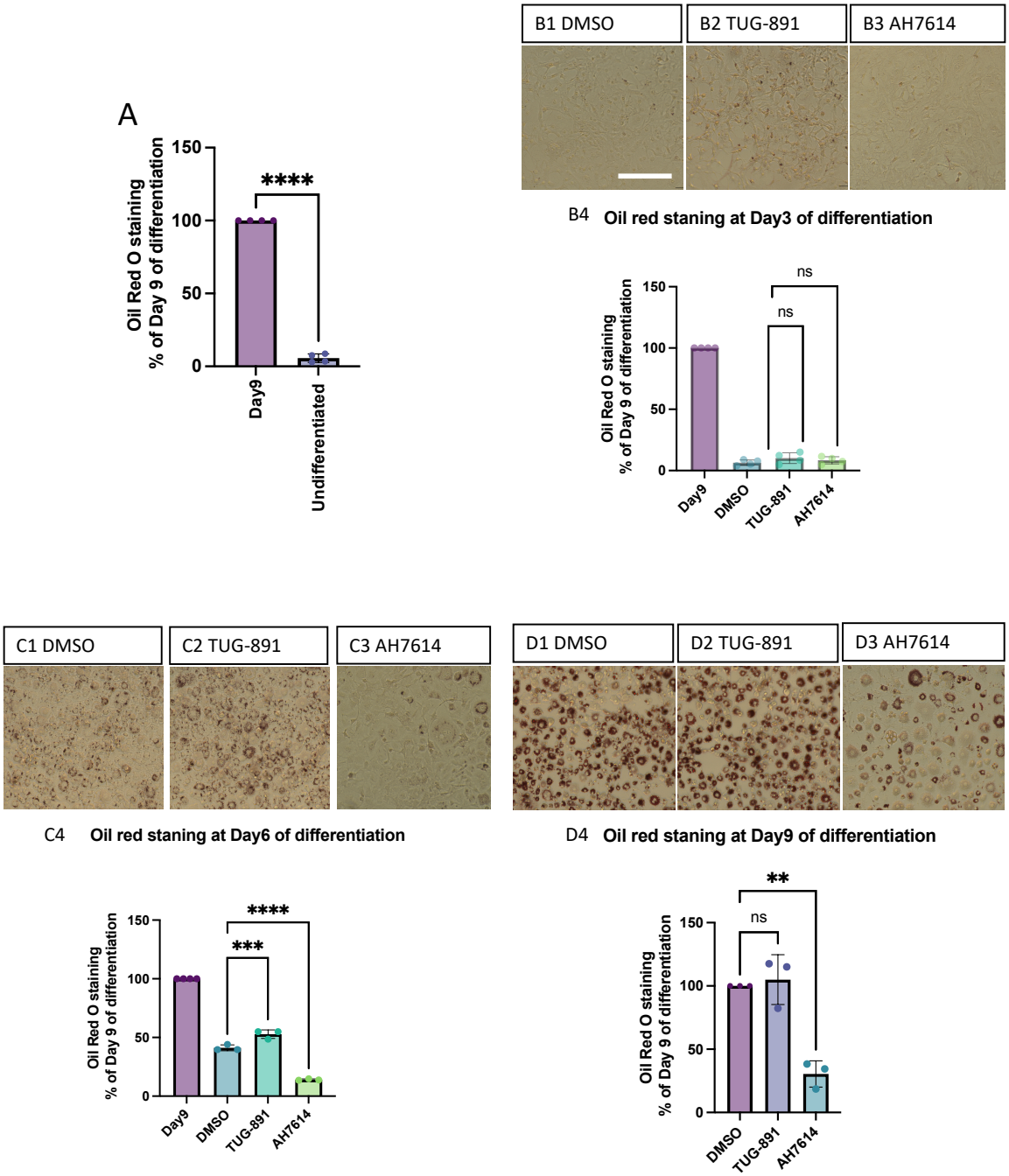


Figure 5-3 AH7614 affects adipogenesis byt Day 6 of 3T3-L1 differentiation.
ORO neutral lipid stain quantification is shown for undifferentiated 3T3-L1 cells vs 3T3-L1 adipocytes at Day 9 (A). Images of differentiated cells (Day 3–Day 6–Day 9) were fixed and stained then imaged at 200 um scale. At Day 3 (B1) DMSO vehicle (.1%), (B2) or 10 μM TUG-891 (B3) or 10 μM AH7614. By dissolving the oil red staining day in 100% isopropyl alcohol and measuring absorbance at 492 nm, the data was quantified (B4). On Day 6 (C1), DMSO vehicle (0.1%), (C2) or 10 μM TUG-891 (C3) or 10 μM AH7614. Oil red staining was quantified by dissolving the dye in 100% isopropyl alcohol and measuring absorbance at 492 nm (C4). On Day 9 (D1) DMSO vehicle (1%), (D2) or 10 μM TUG-891 (D3) or 10 μM AH7614. Oil red staining was quantified by dissolving the dye in 100% isopropyl alcohol and measuring absorbance at 492 nm (D4). Data are from three independent experiments with three technical replicates of absorbance measurements shown as an average of the three replicates. Values are the means ± SD of three separate experiments that were compared using a one-way ANOVA. ns. = not significant. ***P < 0.1 ****P < 0.0001. (scale bar = 200 μm).

To highlight the p value for each comparison, Table (5.1) was generated to summarise the value for each treatment (TUG-891 and AH7614) at each time point of differentiation (Day 3, Day 6 and Day 9) compared with the DMSO control.

Table 5-1: P value for the comparisons between treatments vs DMSO control at different time points for 3T3-L1 cells.

Day of 3T3-L1 differentiation	Treatment	P value	P value summary
Day 3	TUG-891	P = 0.3098	ns
	AH7614	P = 0.8335	ns
Day 6	TUG-891	0.0003	***
	AH7614	< 0.0001	****
Day 9	TUG-891	0.0565	ns
	AH7614	0.0014	**

This data suggests that blocking FFA4 receptor during differentiation significantly decrease adipogenesis at 3T3-L1 adipocyte as early as Day 6. At the same time point, using TUG-891 significantly enhanced adipogenesis, as captured in image and quantified data. This confirms that the FFA4 receptor influences the 3T3-L1 differentiation process and affects the 3T3-L1 adipocyte that will be formed. Still, at this point, I did not test AH7614 behaviour, which is what will be tested next.

5.3.3 FFA4 antagonist treatment decreased 3T3-L1 lipid accumulation in a concentration-dependent manner

After confirming the effect of AH7614, an FFA4 antagonist, on the adipogenesis process, cells were treated with a range of antagonist concentrations to determine its behaviour or trend. 3T3-L1 cells were differentiated using a variation of concentrations of AH7614 (1nM, 10nM, 100nM, 1μM and 10μm) throughout the differentiation days. Cells then were fixed and stained with the ORO technique, as in the previous steps. Images at Day 9 for each concentration showed that, with higher concentration of AH7614, fewer cells were adipose-like (Figure 5-4A). As the lowest concentration (1nM) of AH7614 appeared the same as the vehicle (Figure 5-4A), we can indicate the beginning of FFA4 antagonist influence on lipid accumulation starting with 10nM concentration in the image (Figure 5-4A). The images captured a decrease in ORO staining in the cells at 1μM (Figure 5-4A). Similarly, 100nM concentration showed the same trend

(Figure 5-4A). Finally, cells that were treated with the highest concentration (10uM) exhibited the clearest effect on lipid accumulation compared to the vehicle (Figure 5-4A).

In addition to this, data were quantified using absolute isopropyl alcohol to dissolve cells and dye from each concentration of AH7614 treatments and measure absorbance at 450nm. The ORO quantifications were used as observance to indicate the difference in lipid accumulation and then establish a plotted concentration curve. That curve conveyed the relationship between the effects on adipogenesis in 3T3-L1 differentiated cells and FFA4 antagonist (Figure 5-4B). This illustrated that AH7614, an FFA4 antagonist, acts in a concentration-dependent manner when it comes to its negative effect on adipogenesis in 3T3-L1 differentiated cells.

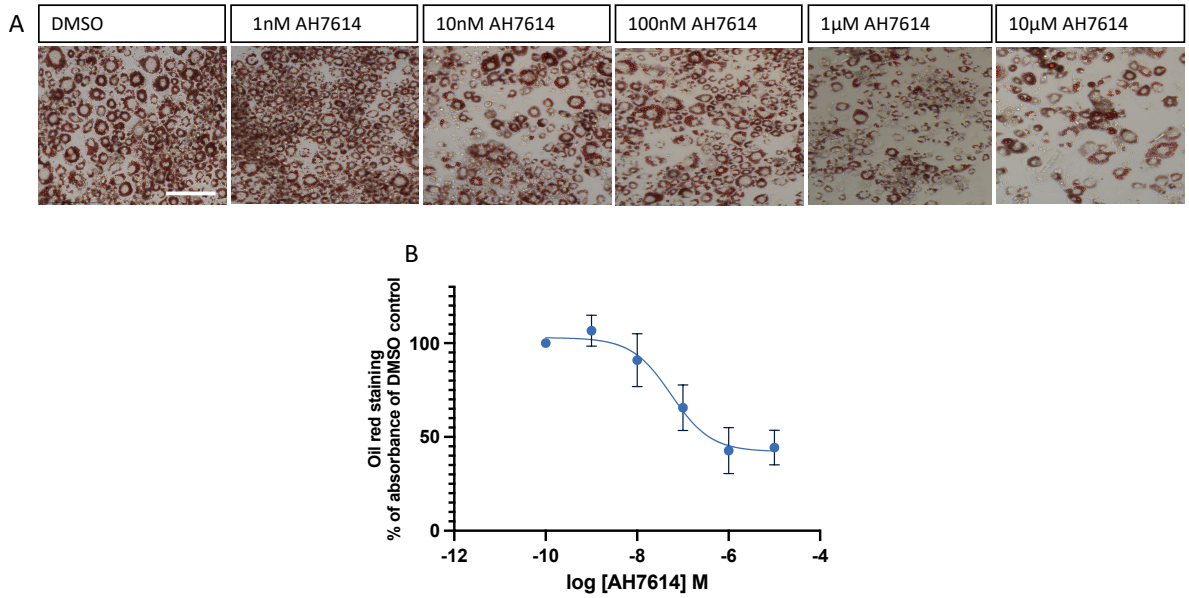


Figure 5-4 AH7614 has a conceration depond effact on 3T3-L1 adipocyte.
3T3-L1 cells were differentiated until Day 9, then fixed and stained, then imaged using ORO dye. A) ORO neutral lipid stain with DMSO 0.1% treatment as a control, 1nM AH7614, 10nM AH7614, 100nM AH7614, 1µM AH6714 and 10µM AH6714. B) Oil red staining was quantified by dissolving the dye in 100% isopropyl and measuring absorbance at 492 nm and piloted in a concentration curve. Data are from three independent experiments with three technical replicates of absorbance measurements blotted as an average for the three technical replicates in a nonlinear curve (scale bar = 200 µm).

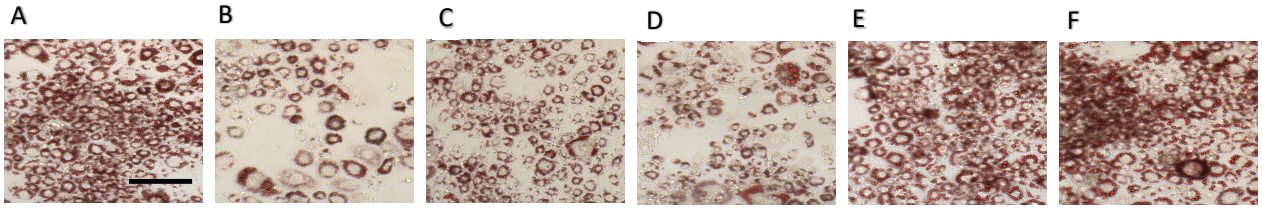
These results reveal that AH7614 manner in terms of concentration is a concentration-dependent effect, as the highest concentration has the maximum effect on lipid accumulation in tested concentration during 3T3-L1 differentiation. This fact led to more exploration of the role of FFA4 in adipogenesis throughout the differentiation process.

5.3.4 AH7614 has its strongest effect on 3T3-L1 adipogenesis at early stages of the differentiation

In pursuit of the investigation of FFA4 antagonist's impact on the 3T3-L1 adipogenesis process, AH7614 was added at different key time points during 3T3-L1 cells' differentiation. The AH7614 effect was tracked by adding it at different time points during the differentiation at Day 0, Day 3 and Day 6 in different patterns that will be explained.

In 3T3-L1 differentiation protocol, there are three important time points where the media is changed on the cells: Day 0, Day 3 and Day 6, as explained in Section 2.2.1.2. In light of this, 3T3-L1 cells were seeded and DMSO was added as a treatment at all time points (Day 0–Day 3–Day 6), then fixed and stained with ORO; this was used as a control (Figure 5-5A). AH7614 was also added at all time points (Day 0–Day 3–Day 6), which resulted in a significant decrease in lipid accumulation and the formation of lipid droplets when compared with DMSO (1%) vehicle (Figure 5-5B). Then, FFA4 antagonist was added at two time points only, either Days 0 and 3 or Days 3 and 6, and in both conditions, a decrease in lipid accumulation was indicated, but the impact was lower when compared to adding AH7614 at all time points (Day 0–Day 3–Day 6) (Figure 5-5C and D). Finally, AH7614 was added either on Day 0 only or Day 6 only. In both conditions, we did not observe a difference between those conditions and the control (Figure 5-5E and F).

In order to confirm the visualised observations that I made from ORO images, this step was followed by dissolving the cells and dye in absolute isopropyl alcohol for each treatment. Treatment was symbolised as in (Figure 5-5G). As a control, DMSO was added at all time points (Day 0–Day 3–Day 6) (Figure 5-5H), and each condition was compared to it. The same result appeared with a significant decrease in lipid accumulation when AH7614 was added on all differentiation days (Day 0–Day 3–Day 6) (Figure 5-5H). Moreover, a significant decrease was also confirmed with the ORO quantified data when AH7614 was added on Days 0 and 3. Again, a significant decrease was confirmed when AH7614 was added on Days 3 and 6. However, treatment with AH7614 at one time point (Day 0 or Day 6) did not affect lipid accumulation in 3T3-L1 adipocytes (Figure 5-5H).



G

Sample	Treatment at Day0 /Day3/ Day6
Control	DMSO/ DMSO / DMSO
D0+D3+D6	AH7614 / AH7614 / AH7614
D0+D3	AH7614 / AH7614 / DMSO
D3+D6	DMSO / AH7614 / AH7614
D0	AH7614 / DMSO/ DMSO
D6	DMSO / DMSO / AH7614

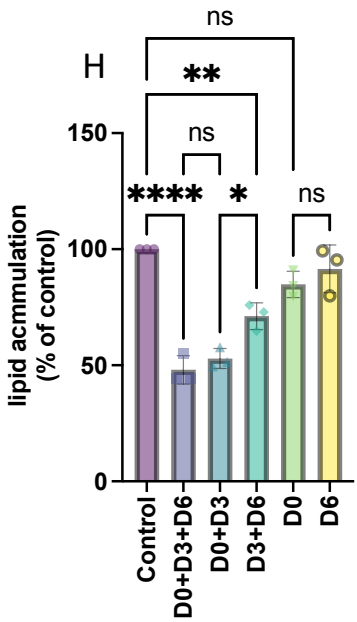


Figure 5-5 AH7614 maximum effect on 3T3-L1 adipogenesis reached when adding the antagonist at all differentiation timepoints.
3T3 cells were treated with 10μm AH7614 at different timepoint of the differentiation process. Oil red O neutral lipid stain was performed on differentiated 3T3-L1 cells on Day 9 at each treatment at 200uM scale. (A)0.1% DMSO was used as a control and added to all time points (Day 0/Day 3/Day 6). 10μm AH7614 was added at all time point (Day 0/Day 3/Day 6) at shown in (B). At (C) 10μm AH7614 was added at Day 0 and Day 3. At (D) 10μm AH7614 was added at two time points, Day 3 and Day 6. 10μm AH7614 was add only at Day 0 (E), while at (F) 10μm AH7614 was added at Day 6 only. The images are a resample of three independent experiments. A summary of the treatments symbols is shown in (G). (H) Oil red staining was quantified by dissolving the dye in 100% isopropyl alcohol and measuring absorbance at 492 nm for each treatment in (G). Data are from three independent experiments with three technical replicates of absorbance measurements. Values are the means ± SD of three separate experiments and were compared using a one-way ANOVA. ns. = not significant. *P > 0.05,**P < 0.1, P < 0.0001.****P < 0.0001 (scale bar = 200 μm).

To summarise the previous statements, FFA4 antagonists must be added at least two time points during differentiation. Moreover, adding AH7614 at early time points (Day 0 and Day 3) is more effective in influencing lipid accumulation inside 3T3-L1 differentiated cells than adding AH7614 on Days 3 and 6. A summary of all the comparisons between the different treatments' conditions is

provided in Table (5-2). The significant summary (*) was determined by one way ANOVA with Tukey.

Table 5-2: Summary of statistical comparisons when adding AH7614 at different timepoints during 3T3-L1 differentiation.

Condition 1 Day0/Day3/Day6	Condition 2 Day0/Day3/Day6	p*	Summary
DMSO/DMSO/DMSO	AH7614/AH7614/AH7614	<0.0001	****
DMSO/DMSO/DMSO	DMSO/AH7614/AH7614	<0.0001	****
DMSO/DMSO/DMSO	AH7614/AH7614/DMSO	0.0010	**
AH7614/AH7614/AH7614	DMSO/DMSO/AH7614	0.0001	***
AH7614/AH7614/AH7614	AH7614/AH7614/DMSO	0.0063	**
AH7614/AH7614/AH7614	AH7614/DMSO/DMSO	<0.0001	****
AH7614/AH7614/DMSO	AH7614/DMSO/DMSO	0.0004	***
AH7614/AH7614/DMSO	DMSO/AH7614/AH7614	0.0319	*
AH7614/AH7614/DMSO	DMSO/DMSO/AH7614	<0.0001	****
DMSO/AH7614/AH7614	DMSO/DMSO/AH7614	0.0156	*

These results confirm that treatment with AH7614 affected the differentiation process in the presence of FFA4 antagonist. Lipid formation in 3T3-L1 adipocyte was affected in a manner correlated with the time those cells were incubated with the antagonist.

5.3.5 AH7614 treatment downregulated adipogenesis markers and glucose transport machinery in gene expression in differentiated 3T3-L1

After capturing and confirming the noticeable effects in adipogenesis upon long-term treatment with FFA4 antagonist (AH7614) in both imaged and quantified lipid accumulation, an estimation for key genes in adipogenesis and glucose uptake machinery was assayed by using RT-qPCR. This step was done to estimate how deep the effect of FFA4 agonist and antagonist treatment on differentiated 3T3-L1 was.

Upon long-term treatment (adding compounds on Day 0, Day 3 and Day 6) with DMSO 1%, 10μM TUG-891 or 10μM AH7614, mRNA was isolated from cells on Day 9 from each treatment. Gene expressions were determined by comparing with HPRT (housekeeping gene) and again with undifferentiated 3T3-L1 cells as a reference of the changes through adipogenesis by using CT, as explained in Section 2.5. As a start, FFA4 expression was upregulated significantly when TUG-

891 was used during differentiation (P = 0.0181), whereas the 3T3-L1 differentiated cells in the presence of AH7614 downregulated FFA4 expression significantly (P = 0.0003). This decrease can be translated as a remarkable 300,000-fold decrease compared to the control group, which was treated with 0.1% DMSO (Figure 5-6A).

Since adipogenesis is the interest in this chapter and in this project, I then assayed a number of adipogenesis markers to see if those chosen were correlatedly effected by the downregulated expression that 3T3-L1 adipocytes experience when differentiated in the presence of FFA4 antagonist. This was, indeed, the case in all three adipogenesis markers that I assayed. The expression from 3T3-L1 adipocytes that were differentiated in the presence of DMSO were the control for this set of data. I started with PPAR-γ expression, which experienced a significant decrease in expression (Figure 5-6B) when AH6714 was used as treatment during 3T3-L1 differentiation (P = 0.0159) when compared to 3T3-L1 adipocytes that were differentiated in the presence of DMSO. However, differentiated 3T3-L1 in the presence of TUG-891 did not affect PPAR-γ expression (P = 0.1145) (Figure 5-6B) when compared to 3T3-L1 adipocytes that were differentiated in the presence of DMSO. Another adipogenesis marker was assayed, namely, adiponectin. Just like PPAR-γ, adiponectin showed a reduction in expression in 3T3-L1 adipocytes that were differentiated in the presence of AH7614 (P=0.0210) (Figure 5-6C). Yet again, treatment with TUG-891 during differentiation of 3T3-L1 did not affect adiponectin expression (P = 0.8722) when compared to 3T3-L1 adipocytes that were differentiated in the presence of DMSO (Figure 5-6C). The last adipogenesis marker that was assayed was leptin, which also showed the same trend. The addition of AH7614 during the differentiation of 3T3-L1 caused a significant decrease in leptin expression (P = 0.0354) when compared to 3T3-L1 adipocytes that were differentiated in the presence of DMSO (Figure 5-6D). On the other hand, differentiated 3T3-L1 cells in the presence of TUG-891 did not affect leptin expression when compared to 3T3-L1 adipocytes that were differentiated in the presence of DMSO (P = 0.1722) (Figure 5-6D).

The other focus in this project is the effect of FFA4 receptor in glucose uptake in adipocyte. In addition, because adipogenesis markers were assayed and all

experienced a decrease in expression in correlation with FFA4 downregulating in 3T3- L1 adipocytes that were differentiated in the presence of AH7614, I was encouraged by my results to assay GLUT4 and its machinery; I intended to explore whether those genes, too, were affected by FFA4 antagonist treatment during 3T3-L1 differentiation. The first gene to be assayed was GLUT1, which showed no change in expression between the two treatments (TUG-891 or AH7614) (P = 0.0990, P = 0.3934, respectively) (Figure 5-6E). In contract, GLUT4 experienced downregulating in gene expression in 3T3-L1 adipocytes that were differentiated in the presence of AH7614 (P = 0.0062) (Figure 5-6F). However, differentiated 3T3-L1 in the presence of TUG-891 showed no significant difference between it and 3T3-L1 adipocytes that were differentiated in the presence of DMSO as a control (P = 0.9991) (Figure 5-6F). Because of the decrease that was detected in GLUT4 expression upon adding FFA4 antagonist during 3T3-L1 differentiation, I assayed another two genes that colocalised with GLUT4, namely, Syntaxin 4, which has been linked to the fusion of GLUT4-containing vesicles with the plasma membrane in 3T3-L1 adipocytes (Black et al., 2022) and GLUT4, since GLUT4-containing vesicles contained more than 85% Syntaxin 6 (Perera et al., 2003). Data showed that Syntaxin 4 experienced a significate decrease in gene expression in 3T3-L1 adipocytes that were differentiated in the presence of AH7614 (P = 0.0027) (Figure 5-6G), while TUG-891 did not have an effect on Syntaxin 4 expression, since there was no significant difference between 3T3-L1 adipocytes that were differentiated in the presence of DMSO as a control and differentiated 3T3-L1 in the presence of TUG-891 (P = 0.8918) (Figure 5-6G). That difference was also true when Syntaxin 6 gene expression was assayed. 3T3-L1 adipocytes that had AH7614 added to the differentiation media during differentiation showed a significant reduction in Santaxin 6 gene expression (P = 0.0388) (Figure 5-6H) when compared to the control group. However, treatment with TUG-891 during the differentiation process of 3T3-L1 adipocytes did not change the expression of Syntaxin 6, since there was no significant difference between Santaxin 6 gene expression between TUG-891 treatment (P=0.8002) and the control group (Figure 5-6H). Completing with glucose transport machinery, gene expression for both insulin receptor (IRS) and (AKT) was assayed. 3T3-L1 adipocytes that were differentiated in the presence of AH7614 showed a decrease in gene expression for IRS (P = 0.0029) (Figure 5-6I), while the same treatment did not affect AKT gene expression,

since there was no significant difference between AH7614 treatment (P = 0.2244) and control treatment (Figure 5-6J). Treatment with TUG-891 during 3T3-L1 differentiation has no significant effect on IRS gene expression (P = 0.2244) (Figure 5.6 I) or gene expression of AKT (P = 0.9971) (Figure 5-6J).

These results strongly suggest that differentiation of 3T3-L1 in the presence of AH7614 affected not only the lipid accumulation and the formation of oil droplets inside the cell but also its gene profiling. This fact led to the investigation of two adipose functions, namely, glucose uptake and lipolysis.

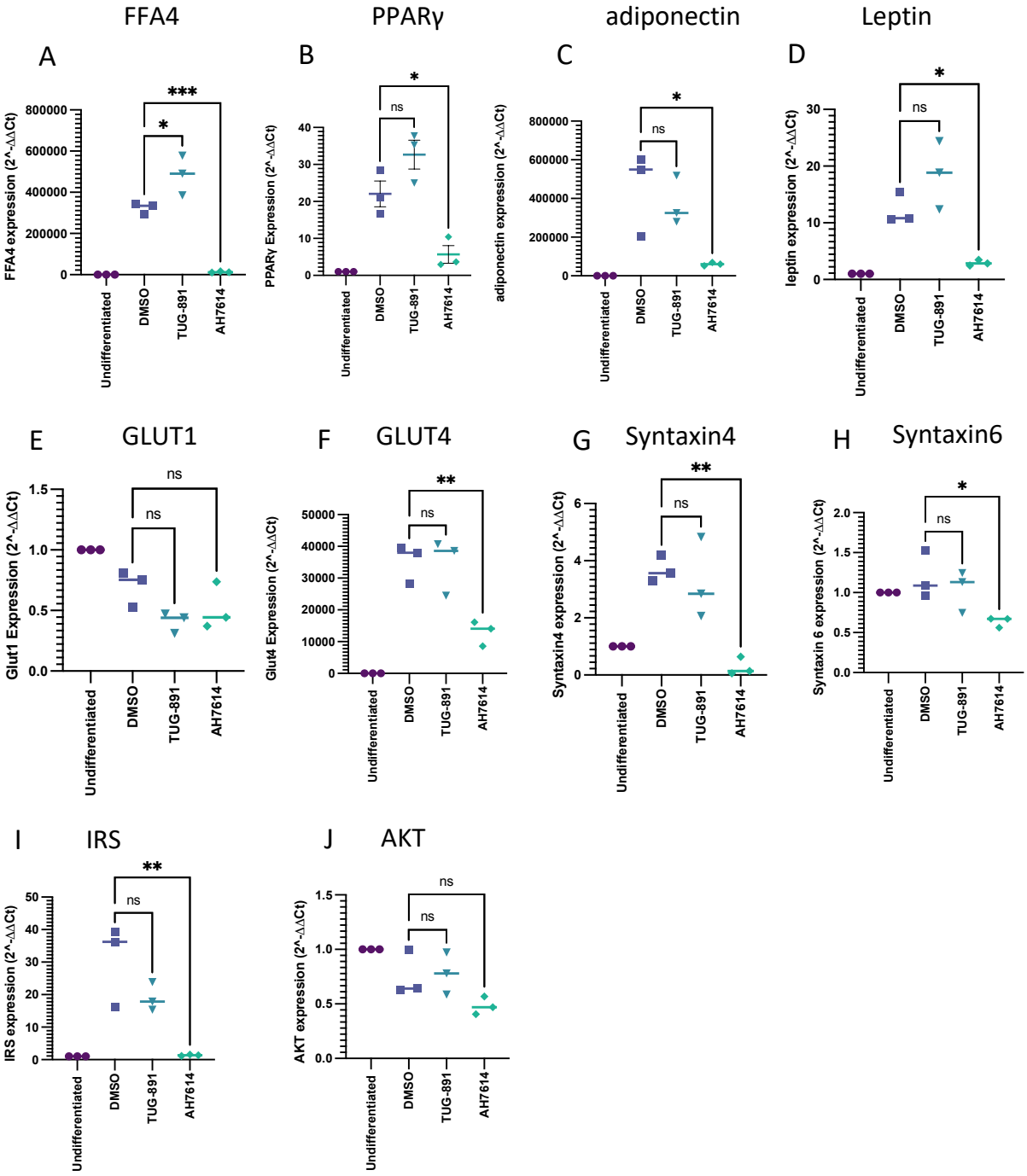


Figure 5-6 AH7614 treatment downregulate gene expression for both adipogenesis markers along with glucose transport machinery in 3T3-L1 adipocytes.
3T3-L1 cells were differentiated in the presence of DMSO 0.1%, TUG-891 or AH7614. mRNA was isolated at Day 9, and RT performed as described. qPCR using the primers shown in Table 2.6 was performed to quantify levels of expression of the indicated FFA4. A) FFA4 expression in 3T3-L1 fibroblasts and adipocytes treated with DMSO, TUG-891 and AH7614. B) PPAR-γ, C) adiponectin, D) leptin, E) GLUT1, F) GLUT4, G) Syntaxin 4, H) Syntaxin 6, I) IRS and J) AKT. Analysis was done compared to HPRT then with one-way ANOVA, * = $P < 0.05$, ** = $P < 0.01$, ***$P < 0.1$. to compere DMSO with TUG-891 and AH7614. Data is from three independent experiments; each data point is the average of one experiment.

5.3.6 Treatment with AH7614 during 3T3-L1 differentiation decreases glucose uptake

After confirming the changes in gene expression on GLUT4 machinery along with effects on adipogenesis, both in the ORO method and RT-qPCR, I started to explore FFA4 receptor in those cells in terms of function.

Since GLUT4 expression showed a reduction of expression by using qPCR on long-term treatment with a FFA4 antagonist, I then assayed the protein level of FFA4 in 3T3-L1 adipocytes after the same treatment. Samples were collected from 3T3-L1 adipocytes to use in immunoblotting at three different treatment and undifferentiated 3T3-L1 cells as a negative control. DMSO 0.1% were added to 3T3-L1 cells during differentiation, and samples were collected on Day 9 as a control group. Also, 3T3-L1 cells were treated with 10μM AH7614 or 10μM TUG-891 during differentiation process to assay the differences between treatments. Lysates of those cells were also collected on Day 9 and then used in Western blot by using GLUT4 antibody. Total protein loading stain control was used as a loading control (Figure 5-7A). All treatments detected GLUT4 as a smear band (≈55–43 kDa) in the immunoblot but at different levels (Figure 5-7B). Results revealed that the protein levels of GLUT4 in lysates from 3T3-L1 adipocyte that were differentiated in the presence of TUG-891 showed no difference in GLUT4 protein expression when compared to the control group that was differentiated in the presence of DMSO (Figure 5-7B). This was further confirmed by quantitative densitometry that indicated that there was no significant difference in GLUT4 protein expression between treatment with TUG-891 and DMSO 0.1% ($P = 0.1601$) (Figure 5-7C). On the other hand, treatment with AH7614 reduced GLUT4 protein level after differentiated 3T3-L1 cells in the presence of AH7614 when compared with DMSO treatment (Figure 5-7B). This was confirmed by quantitative densitometry that indicated that there was a significant difference in GLUT4 protein expression between treatment with AH7614 and DMSO ($P = 0.0126$) (Figure 5-7C).

To test if this reduction in both protein and gene expression level had an effect on 3T3-L1 adipocyte function, a glucose uptake assay was performed. This step was to see whether the noticed decrease in GLUT4 expression would change glucose transport in 3T3-L1 adipocytes. The assay was performed with 3T3-L1

differentiated cells on Day 9, after long-term treatments with 10 μM TUG-891 or 10 μM AH7614 throughout the differentiation process. 3T3-L1 cells were differentiated in the presence of DMSO 1% to use as a control. In addition, basal (B) 3T3-L1 cells differentiation without DMSO vehicle treatment were also included for comparison. Then, the differentiated cells' glucose uptake was assayed on Day 9. Basal glucose uptake without the addition of insulin or insulin-independent glucose uptake showed no significant differences between TUG-891 ($P = 0.9977$) and AH7614 ($P = 0.0803$) when compared with DMSO treatment (Figure 5-7D). Moreover, in all treatments, there was a significant increase in glucose uptake after insulin incubation or insulin-dependent glucose uptake, with $P =< 0.0001$ in all the different treatments (Figure 5-7D). Treatment with TUG-891 during differentiation of 3T3-L1 cells did not affect insulin-dependent glucose uptake, with no significant difference between TUG-891 treatment and DMSO control group ($P = 0.9935$) (Figure 5-7D). On the other hand, glucose transport was significantly decreased in 3T3-L1 adipocytes that were treated with AH7614 during differentiation in insulin-dependent glucose uptake when compared with DMSO 0.1% treatment ($P =< 0.0001$); this is an almost 50% reduction in glucose transport (Figure 5-7D).

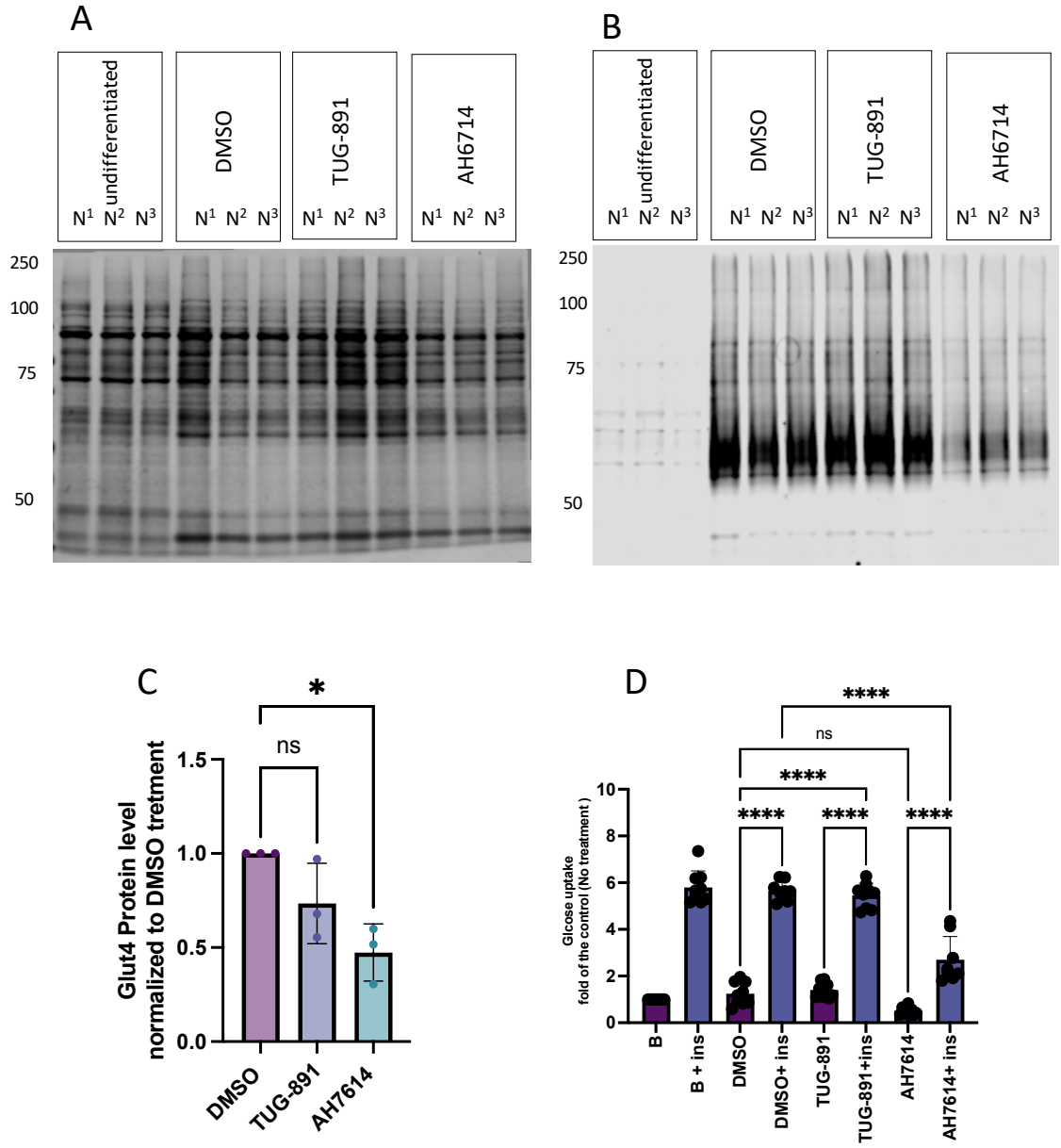


Figure 5-7 Glucose uptake and GLUT4 protein level both decreased in 3T3-L1 adipocytes that were differentiative in the presence of AH7614.
Adipocytes were incubated with 10 μM TUG891, 10 μM AH7614 and DMSO 0.1% during differentiation on Days 0, 3 and 6. Lysate was collected on Day 9 from each treatment (20 μg) as used in Western blot. A) total protein stanning for samples used in B. B) Lysates from cells from each condition were prepared and immunoblotted for GLUT4. C) Quantification of GLUT4. The conditions repeated again; then, on Day 9, 1 μM of insulin was added to all groups for 20 min, followed by the addition of 30 μl [3H] deoxyglucose for all groups for 3 min (D). Analysis was done with one-way ANOVA. * = P < 0.05, ** = P < 0.01, ***P < 0.1, ****P < 0.0001 to compare DMSO with TUG-891 and AH7614. Data is from three independent experiments that were performed in triplicate.

This result demonstrated that the adipogenesis inhibition that was observed after differentiated 3T3-L1 cells in the presence of AH7614, along with the reductions that were noticed in both GLUT4 machinery genes expression and protein level, altogether translated as a glucose uptake decrease in 3T3-L1 adipocytes that experienced differentiation in the presence of FFA4 antagonist.

5.3.7 Glycerol release upon Isoprenaline stimulation decreased in 3T3-L1 differentiated in the presence of AH7614

After verifying that incubation with AH7614 during differentiation affects the morphology and glucose transport of 3T3-L1 adipocytes, along with the decrease that was observed in adipogenesis marker genes in the same 3T3-L1 adipocytes that resulted from differentiation in the presence of AH7614 (Section 5.4.1 and Section 5.4.6), another function of adipose was tested, namely, lipolysis.
A lipolysis assay was performed on Day 9 upon differentiating 3T3-L1 cells in the presence of DMSO 0.1 %, 10μM TUG-891 or 10μM AH7614. Also, this assay was done on undifferentiated cells to demonstrate that no lipolysis occurred in this cell type (Figure 5-8). This assay aimed to measure the amount of glycerol released after stimulating adipocytes with 1nM isoprenaline. All 3T3-L1 differentiated cells under different treatments experienced a significant enhancement in glycerol release upon stimulation with isoprenaline ($P =< 0.0001$) (Figure 5-8).
Treatment with TUG-891 during differentiation of 3T3-L1 adipocytes did not affect or make a significant difference in the basal lipolysis ($P => 0.9999$) (Figure 5-8); nor did the isoprenaline-dependent glycerol release show a significant difference in glycerol release level ($P = 0.9721$) in both conditions when compared to DMSO treatment (Figure 5-8).
On the other hand, glycerol released levels were significantly decreased in the basal, glycerol release or independent isoprenaline glycerol release ($P=0.0259$) (Figure 5-8). In addition, a significant decrease was observed in isoprenaline-dependent glycerol release ($P = 0.0004$) when compared to the control group (Figure 5-8).
This result demonstrated that the morphology changes that I captured in 3T3-L1 adipocytes that were incubated with AH7614 during differentiation Section 5.4.1 translated to the lipolysis assay. I found that AH7614 decreases adipogenesis, hence lipid accumulation and the size of oil droplets in 3T3-L1 adipocytes. This results in a reduction in the amount of glycerol that is available inside the cell to be released.

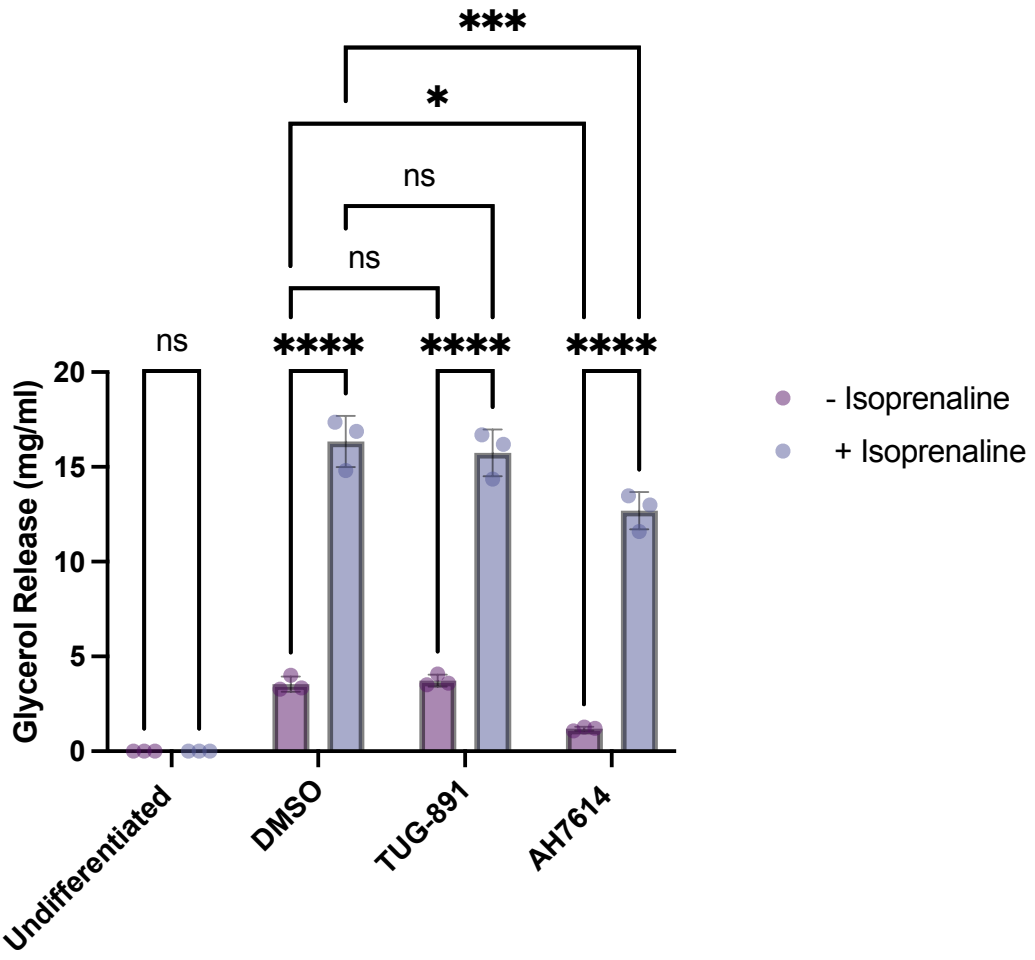


Figure 5-8 Glycerol release decreases in 3T3-L1 adipocytes that were differentiated in the presence of AH7614.
3T3-L1 cells were differentiated in the presence of DMSO vehicle (0.1%), TUG-891 or AH7614; then, on Day 10, lipolysis assay was performed. Lipolysis was stimulated by isoprenaline (1nM). Treatment with DMSO was the control for this assay. Glycerol release after treatment TUG-891 and AH6714. Data shown represent the mean ± SD of three independent experiments. n.s. = not significant, *P < 0.05,, ***P < 0.001****P < 0.0001 by two-way ANOVA.

5.4 Discussion

To study the functional changes in 3T3-L1 adipocyte in relation to FFA4 receptor, I first aimed to demonstrate the direct impact of the FFA4 receptor on adipogenesis. The results demonstrated that FFA4 antagonist was able to inhibit adipogenesis in 3T3-L1 adipocytes. This is consistent with previous studies examining FFA4 receptor in adipogenesis. Song et al. (2016) used lentiviruses to introduce specific genes into 3T3-L1 preadipocytes and generate FFA4 knockdown 3T3-L1 cells. To that end, cells were incubated overnight with lentiviruses and cultured in fresh media for another three days. The sh-FFA4 or sh-Scramble stable cells were then established by cultivating the cells in growth media containing 2 g/mL puromycin for four days (Aguilo et al., 2015; Song et al., 2016). Like the finding that FFA4 antagonist incubation treatment reduced adipogenesis in 3T3-L1 adipocyte when compared to lentivirus-mediated sh-Control 3T3-L1 cells, adipogenic capacity of sh-FFA4 cells was markedly suppressed (Song et al., 2016).

Moreover, using another knockdown strategy, namely, siRNA transfection (Gotoh et al., 2007), the siRNA-mediated knockdown of FFA4 prevented adipocyte differentiation in 3T3-L1 (Gotoh et al., 2007) can be linked to the effect of AH7614 antagonism on FFA4 receptor, which also results in inhibition of differentiation in 3T3-L1 cells. Using the same knockdown method, SiRNA-mediated decreased lipid droplets accumulated within differentiated 3T3-L1 cells (D. Liu et al., 2012). On another cell line, the result was also in agreement: a murine mesenchymal stem cell line C3H10T1/2 was used to study adipocyte differentiation (Watterson et al., 2017). C3H10T1/2 stem cells were differentiated into adipocyte, and, using ORO staining, it was possible to see how adipogenesis-related triglyceride deposits were growing (Watterson et al., 2017). When AH7614 was used during the differentiation process of C3H10T1/2 stem cells, this addition significantly decreased the ability to visually observe and quantify triglyceride stores as detected by ORO staining (Watterson et al., 2017). Furthermore, in another cell model, adipogenesis is suppressed in adipocytes derived from embryonic fibroblasts in FFA4-deficient mice (Ichimura et al., 2012).

The effect of FFA4 antagonism not only affected the morphology of 3T3-L1 adipocyte, which was recorded by virtualising by ORO staining and ORO quantification, but also affected the gene profiling of these adipocytes.

3T3-L1 adipocytes that were differentiated in the presence of AH7614 showed a decrease in FFA4 gene expression and reduced gene expression of a number of adipogenesis markers, PPARγ, adiponectin and leptin. This result was supported by several research findings. Liu et al. (2012) recorded that, according to the RT-PCR results, differentiated 3T3-L1 cells had significantly higher levels of FFA4 mRNA. On the other hand, transfection of FFA4-SiRNA dramatically reduced the expression of FFA4 at the gene and protein levels in differentiated 3T3-L1 cells (Liu et al., 2012), which can be linked or compared to the effect I found on differentiated 3T3-L1 cells in the presence of AH7614, which also shows a decrease in gene expression level of FFA4. Also, in Gotoh et al. (2007), 3T3-L1 adipocytes transfected with FFA4 siRNA (as opposed to those that received control siRNA, audiogenic genes PPAR-γ mRNAs) were drastically down-regulated (Gotoh et al., 2007). This also true for the RT-qPCR that was used by Song et al. (2016) to assay adipogenesis markers. In a similar vein is the finding that treatment with FFA4 antagonist in the 3T3-L1 differentiation process results in reducing PPAR-γ gene expression (Song et al., 2016). A further study that showed the same result that was detected when C3H10T1/2 stem cells were differentiated into adipocytes in the presence of AH7614 (Watterson et al., 2017). AH-7614 treatment reduced PPAR-γ mRNA expression level in C3H10T1/2 stem cells adipocytes (Watterson et al., 2017). Furthermore, mapping the connections between these genes showed that pathways related to adipocyte development were less active in HFD-fed GPR120-deficient mice, while pathways related to inflammation were more active. Quantitative real-time PCR (qRT–PCR) confirmed that an adipocyte differentiation marker gene (Fabp4) and a lipogenesis-related gene (Scd1) were downregulated in the epididymal fat of FFA4-deficient mice given (HFD) (Ichimura et al., 2012).

In addition, 3T3-L1 adipocytes that were differentiated in the presence of AH6714 experienced a reduction in GLUT4 machinery genes expressions. Results showed a decrease in gene expression of GLUT4, Santaxin4, Santaxin 6 and IRS. This result is supported by Liu et al. (2012), who recorded that a significant

reduction in the gene and protein levels of IRS-1 and GLUT4 were associated with the siRNA-mediated reduction of FFA4 (D. Liu et al., 2012). This finding was also in agreement with Ichimura et al. (2012)'s report when mapping the connections between these genes, which showed that pathways related to insulin signalling were less active in HFD-fed GPR120-deficient mice. qRT–PCR confirmed that insulin-signalling-related genes (Insr, Irs1 and Irs2) were downregulated in the epididymal fat of GPR120-deficient mice given a high-fat, high-sugar diet (HFD).

This inhibition in adipogenesis captured that AH6714 reduced the differentiation process for 3T3-L1 and may keep them at an undifferentiated stage. The same methods that I have used to demonstrate AH7614's effect was used by Miyauchi et al. (2009). Using RT-qPCR and ORO staining, the change in expression of GPR120 mRNA and the degree of differentiation were monitored. On Day 0, GPR120-positive cells were not detectable in undifferentiated 3T3-L1 cells, whereas the majority of differentiated 3T3-L1 cells were GPR120-positive on Day 10 (Miyauchi et al., 2009). This suggested that the effect I capture in AH7614 treatment for 3T3-L1 adipocytes may be because the FFA4 antagonist reduces the ability of 3T3-L1 for adipogenesis and keeps a majority of cells at an undifferentiated stage, as they were on Day 0 of differentiation. This also was noticed in human adipose tissue, since Gotoh et al. (2007) reported that FFA4 expression is significantly higher in human differentiated adipocytes than in human preadipocytes.

Since 3T3-L1 adipocytes that were incubated with AH7614 during differentiation and FFA4 knockout models have showed the same pattern in gene expression profiling, results from a study that used FFA4 knockout model can be a direct comparison to what occurred using FFA4 antagonist in 3T3-L1 adipogenesis process. The downregulating in GLUT4 gene and protein expression was translated to 3T3-L1 adipocytes that showed a proximately 50% decrease in glucose uptake when incubated with AH7614 during differentiation. This result is in line with Oh et al. (2010), who recorded a comparison between wild-type mice and FFA4 knockout. They reported that in wild-type mice, GPR120 can improve muscle and hepatic insulin sensitivity, raise glucose infusion rate,

stimulate hepatic lipid metabolism and reduce hepatic steatosis, but not in GPR120 knockout animals.

However, in vitro models differ in results reported from in vivo models in terms of FFA4 expression. Although reports from Rodriguez-Pacheco et al. (2014) showed that, when FFA4 levels were analysed in visceral adipose tissue (VAT) from lean and obese individuals, FFA4 mRNA and protein levels were lower in obese individuals, a contrary report from Ichimura et al. (2012) found that FFA4 expression in adipose tissue is considerably higher in obese individuals than in lean controls in humans, which is incoherent with the reduction I observed in FFA4 gene expression level in adipogenesis in 3T3-L1 that were differentiated in the presence of FFA4 antagonist; however, Ichimura et al. (2012) continued to find that, in obese subjects, FFA4 exon sequencing reveals a deleterious non-synonymous mutation (p.R270H) that inhibits GPR120 signalling activity. Moreover, reports also found that FFA4-deficient mice fed a high-fat diet developed obesity, which is the opposite of what I expected, since using AH7614 inhibits adipogenesis in lipid accumulation in 3T3-L1 adipocytes. This only led me to conclude that nutrition and free fatty acid consumption may contribute to developing obesity in FFA4-deficient mice. This assumption was supported by several studies. The fact is that, on a normal diet containing 13% fat, both GPR120-deficient and wild-type rodents had similar body weights; however, when FFA4-deficient mice were given a high-fat diet (HFD) containing 60% fat, their body weight gain was 10% greater than that of wild-type mice fed a high-fat diet (Ichimura et al., 2012). Likewise, high expression of FFA4 was found in four types of fat tissue, including subcutaneous, perinephric, mesenteric and epididymis tissue from mice that ate a high-fat diet (Gotoh et al., 2007). Not only food consumption but also changes in physical condition can induce FFA4 expression. Publications reported that high levels of FFA4 expression were seen in (BAT), along with the induction of protein levels in the BAT following exposure to cold. Additionally, it has been noted that activating FFA4 can encourage mice's white fat to turn brown (Quesada-López et al., 2016; Song et al., 2017). These findings support the idea that high lipid consumption in rodents and humans may cause the receptor to express itself, leading to differences in FFA4 expression from AH7614’s effect on FFA4 expression on 3T3-L1 adipocytes.

Lipolysis was the other adipocyte function that was performed to measure the glycerol release from 3T3-L1 adipocytes. It will help in understanding how fatty acids circulate in through lipolysis assay. Since the accumulation of lipids was interrupted through differentiation of 3T3-L1 cells with AH7614, and since that effect was captured in the reduction of the ORO image and quantification, this is coherent with the reduction of the basal glycerol release that was measured independently or prior to the addition of isoprenaline. This may suggest that FFAs in adipocytes treated with AH7614 is in constant FFAs lost flux from lipid droplets that possibility may contribute as a factor that AH7614 caused a reduce in oil droplet size in ORO imaging in Section 5.4.1. The reduction' in glycerol release was also recorded when lipolysis was induced with isoprenaline. This in important of place since regulation and the FFAs regulation in adipocyte tissue, is one of its main functions is to maintain energy balance in the body by storing FFAs or by mobilising them by lipolysis(Y. Li et al., 2022). This may be of interest as a drug target for regulating FFA circulation and demand by the body.

Another key point, the data obtained from this study could suggest the possibility of limited stability of the AH7614 drug under the experimental conditions. The observed decrease in lipid accumulation and lipid droplet formation when AH7614 was added at all time points (Day 0, Day 3, and Day 6) compared to the control treatment implies that the drug's efficacy may diminish over time. The findings indicate that adding AH7614 at the early time points (Day 0 and Day 3) resulted in a more pronounced effect on lipid accumulation compared to adding it on Days 3 and 6. However, when AH7614 was added at a single time point (Day 0 or Day 6), no significant difference in lipid accumulation was observed compared to the control. These results suggest a potential decline in the drug's stability or potency as the differentiation process progresses. Understanding the stability of a drug is crucial for its clinical application. If AH7614 exhibits limited stability, it may have implications for its therapeutic use in managing adipocyte-related disorders. For instance, the dosing strategy and treatment duration should be carefully considered to ensure sustained efficacy. It may be necessary to administer the drug at multiple time points during the course of treatment or explore alternative drug formulations that enhance stability. Furthermore, these findings raise questions about the pharmacokinetics and pharmacodynamics of AH7614 in vivo. Future studies

should investigate the drug's stability under physiological conditions and assess its bioavailability and clearance rates. This knowledge will be vital for designing appropriate dosing regimens and optimizing the clinical use of AH7614 or similar FFA4 antagonists.

While AH7614's effects on adipogenesis of 3T3-L1 were obvious, TUG-891 had no significant effect on 3T3-L1 adipogenesis. TUG-891 caused a significant increase in FFA4 gene expression level in 3T3-L1 adipocyte upon differentiating 3T3-L1 cells in the presence of TUG-891, yet this increase did not change the adipogenesis level or gene profiling of 3T3-L1 adipocytes. This leads back to the idea that FFA4 is already activated by free fatty acids in the media and that fact masked TUG-891's effect. This assumption can be supported by the fact that results from tracking TUG-891 during 3T3-L1 differentiation showed a significant increase in adipogenesis at Day 6 of differentiation (Figure 5-3). That significant increase faded by Day 9. When the cytoplasmic TG content was measured on Days 5, 8 and 12 of 3T3-L1 differentiation, results showed a significant increase in cytoplasmic TG when Day 5 was compared with Day 12 (Etesami et al., 2020). This fact supports the assumption that the reason the effect of TUG-891 was masked is the availability of free fatty acids in 3T3-L1 adipocytes.

Another explanation of the FFA4 agonist's limitation on inducing significant differences in adipogenesis is the fact that the potency of TUG-891 in 3T3-L1 adipocyte system decreased (Hudson et al., 2013), with a pEC50 of 5.86, which is lower than potency measurements obtained from cells heterologously expressing mFFA4 that showed a pEC^{50} of 7.29 (Hudson et al., 2013). This observation implies that TUG-891 may be a partial agonist, since if a 'full' synthetic agonist with the same potency as the endogenous ligand is used along with the endogenous ligand, there will be no change in the maximum reaction or potency, which has changed in this case (Milligan, Shimpukade, et al., 2017). Also, if a 'partial' agonist with less effect is used, the concentration of endogenous ligand will have to go up to get rid of the competing partial agonist. This causes a 'right' shift in the endogenous agonist concentration response, which was the observation in a number of pharmacological assays (Hudson et al., 2013) and Section 4.4.

In terms of AH7614 antagonist in concentration-response studies, it was demonstrated that the IC50 for AH7614 was 7.25, similar to its reported activity at FFA4 (Watterson et al., 2017). The effects of AH7614 that were recorded in 3T3-L1 adipocyte in the absence of agonist treatment suggested that FFA4 was in an activated state or constitutive activity in a state of agonist-independent activation (Milligan, Shimpukade, et al., 2017). Moreover, the effect of AH7614 that was observed implies that AH7614 may act as an inverse agonist, since AH7614 antagonist ligand was able to lower the amount of receptor's activation and transform this state into an inactive ground state (Milligan, 2003; Milligan, Shimpukade, et al., 2017). This observation was also made in the B-arristin assay in Section 3.5.

As a future step, I would suggest measuring FFAs replaced in the media from 3T3-L1 adipose at each treatment, determining its type (short, medium or long) and assessing whether that contributed to TUG-891's limitations in adipogenesis in 3T3-L1. Furthermore, this measurement will help to determine whether FFAs in differentiation media were affected by the treatment with TUG-891 and AH7614.

Using another cell line adipocyte model, such as OP9 from mouse stromal cell that can be differentiated in 72 hours only (Ruiz-Ojeda et al., 2016), to confirm and establish a compression to the finding in 3T3-L1 adipocyte; using primary adipose and differentiating them in the same method; and confirming flowing adipocyte in various different system models, such as wild-type and knockout mice, will help in understanding the sequence of using AH7614 in the adipogenesis process.

Furthermore, alternative methods should be employed to measure or estimate the toxicity levels when subjecting 3T3-L1 cells to AH7614 using a cytotoxicity assays. While Hoechst stain was utilized to label DNA in cells exposed to different treatments, confirming no alterations in cell number, viability and toxicity was not directly assessed. For example, the LDH (lactate dehydrogenase) assay and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay are both commonly used in cell biology to assess cell viability and cytotoxicity (Holder et al., 2012). However, they differ

in their principles and the information they provide. The LDH assay measures the release of the enzyme LDH into the cell culture medium. LDH is a cytoplasmic enzyme that is released into the extracellular space upon cell membrane damage or lysis. By quantifying LDH activity in the culture medium, the LDH assay provides an indirect measure of cell membrane integrity and cell death. An increase in LDH activity indicates cell damage or cytotoxicity (Riss et al., 2019).

On the other hand, the MTT assay measures the reduction of MTT, a yellow tetrazolium salt, by metabolically active cells. MTT is reduced to formazan crystals in the mitochondria of viable cells. The amount of formazan formed is proportional to the number of viable cells. After incubation with MTT, the formazan crystals are solubilized, and their absorbance is measured spectrophotometrically. A higher absorbance indicates a higher number of viable cells. (Khattak et al., 2006).

Considering the specific case of adipocytes and undifferentiated cells, it is important to evaluate the limitations of using the MTT assay in this context due to potential differences in mitochondrial activity between these cell types. Adipocytes, as differentiated cells specialized in lipid metabolism, may exhibit distinct mitochondrial functions compared to undifferentiated cells(Loneker et al., 2023). The metabolic activity and mitochondrial function can vary significantly between these cell types, particularly due to differences in lipid accumulation and energy metabolism(Aon et al., 2014; J. H. Lee et al., 2019). This discrepancy can impact the reduction of MTT to formazan crystals, leading to inaccurate measurements of cell viability and potential misinterpretation of the results. Furthermore, compounds or factors that affect adipocyte differentiation can also influence mitochondrial activity(De Pauw et al., 2009), even if they do not directly impact cell viability. These alterations in mitochondrial function can confound the MTT signal, resulting in unreliable or misleading data regarding cell viability. Considering these limitations, it is crucial to exercise caution when using the MTT assay as a sole indicator of cell viability in the context of adipocyte differentiation.

Also, AH7614 has reduced adipogenesis in 3T3-L1 adipocytes in a concentration depending on manner. I would like to assay each concentration in a glucose uptake assay, since, when I differentiated 3T3-L1 in the presence of 10μm AH7614, it resulted in a huge reduction in glucose uptake. This is why I think assaying glucose uptake in each concentration may reveal when adipogenesis will experience reduction and glucose transport would not be affected. This scenario may lead to and help with using FFA4 antagonist in adipogenesis inhibition without effected GLUT4 machinery.

Furthermore, I would use different concentrations or create a TUG-891 concentration curve associated with adipogenesis in differentiated 3T3-L1. This idea, led by a study of TUG-891 effect on adipogenesis in bone marrow mesenchymal stem cells BMMSCs, was established. This study was generated to investigate FFA4 role in osteoporosis (OP), which is characterised by a sharp change in the osteoblast/adipocyte ratio in the bone marrow cavity, along with a decline in bone mass and density (Gao et al., 2015; Rachner et al., 2011; Raisz, 2005). In summary, the abnormal osteoblast/adipocyte ratio is caused by an imbalance between BMMSCs decreased osteogenic and increased audiogenic differentiation tendencies (Idris et al., 2009). Since it has been reported that FFA4 mediates adipogenesis in lipid metabolism, TUG-891 was used in Gao et al. (2015) to demonstrate the effect of FFA4 activation on targeting the bi-potential differentiation of BMMSCs. For three days, cells underwent adipocyte differentiation in adipogenic medium containing varying concentrations of TUG-891 (0 (control), 0.1, 0.5, 1.5 μM). Intriguingly, we discovered that 0.5 M TUG-891 significantly increased the ability of adipogenesis, whereas μM TUG-891 significantly decreased the adipogenic potential in comparison to the controls, as determined by oil red staining and lipid quantification on BMMSCs. This result strongly suggests differentiating 3T3-L1 cells in the presence of a range of TUG-891 concentration and assaying the effect of the low and high TUG-891 concentration in 3T3-L1 adipogenesis process.

In summary, more understanding of adipogenesis and adipose formation would offer a great opportunity in the fight against the development of obesity and metabolic diseases (Q. Li et al., 2015; Song et al., 2017). The outcomes of this

chapter suggested the FFA4 receptor as a target for obesity treatment. The recorded reduction in adipogenesis on 3T3-L1 by incubation with AH6714 suggested this drug as potential treatment for obesity, since AH7614 was able to inhibit adipocyte differentiation, leading to suppression of adipogenesis. That means that it controls adipocyte cell formation and, therefore, body weight gain, which is a characteristic that defines obesity (Q. Li et al., 2015). This suggestion is made in light of using adipogenesis inhibition as one of the methods that under continues exploring as an obesity treatment (De Sá et al., 2017). However, adipogenesis inhibition in 3T3-L1 adipocytes by AH7614 led to glucose transport reduction in those cells. This fact complicates the suggestion of FFA4 as an obesity drug target, since one must consider the dawnside effect on glucose transport. This fact may benefit hypoglycemia patients who also suffer from obesity, since it was reported that obese individuals have higher rates of reactive hypoglycemia than other groups (Lv et al., 2020). Since glucose transport was reduced, not completely blocked, this provides the opportunity for more concentration depending on assays. Furthermore, the tracking of the outcomes on adipogenesis in the differentiation process results in different percentages of reduction under each treatment. The day of differentiation and the time of incubation causes different level of adipogenesis, which should be tested in glucose uptake and lipolysis to assess the effect each condition has on the functions. Because of this, suggesting FFA4 as a target for drug discovery in obesity is still a valid suggestion.

Chapter 6 Final discussion

Adipose tissue serves as a metabolic platform involved in regulating energy balance, lipid metabolism, and hormonal regulation. Its primary roles include storing and distributing energy in the form of triglycerides. Impairments in adipose tissue function contribute to metabolic disorders, including obesity, metabolic syndrome, non-alcoholic fatty liver disease, and T2DM. The principal mechanism underlying T2DM is the development of insulin resistance, which involves diminished insulin sensitivity in various metabolic tissues, including white adipose (Defronzo, 2009). Obesity, a prevalent metabolic disorder, is closely linked to the development of T2DM (Esser et al., 2014). It is projected that by the year 2025, the global population affected by T2DM attributable to obesity will exceed 300 million individuals (B. Zhou et al., 2016). Both of these metabolic disorders are linked to serious complication, including increased cardiovascular disease risk (Flint et al., 2010), complications associated with respiratory system (Zammit et al., 2011) and chronic kidney disease (McGill et al., 2022).

The existing treatments for obesity and T2DM encounter various challenges. The prescribed drugs for obesity are expensive and have associated side effects (Bessesen & Van Gaal, 2018), making them less desirable for patients. Additionally, these treatments often result in non-sustainable weight loss (Ryder et al., 2018) . Furthermore, weight loss surgery, which is another alternative for treating obesity, may not be a viable option for every obese patient. Moreover, the most commonly prescribed drug for T2DM has been associated with significant side effects reported with long-term use. The most commonly prescribed drug for T2DM is metformin(Rojas & Gomes, 2013). While metformin is generally well-tolerated, it can be associated with certain side effects, particularly with long-term use. Some of the reported side effects include(Drzewoski & Hanefeld, 2021; Sanchez-Rangel & Inzucchi, 2017). The most common side effects of metformin are gastrointestinal in nature. These may include diarrhea, nausea, vomiting, and abdominal discomfort (Bonnet & Scheen, 2017). Long-term use of metformin has been linked to decreased vitamin B12 absorption in the body (De Jager et al., 2010).Also, metformin use has been associated with elevated liver enzyme levels (Cone et al., 2010). This

highlights the importance of conducting research and advancing the development of enhanced pharmacological treatments for obesity and T2DM.

GPCRs constitute around 30% of all existing targets, owing to their remarkable capability to respond to a diverse range of stimuli and their involvement in numerous physiological processes.(R. Zhang & Xie, 2012). The primary focus of this project was directed towards a specific GPCR, namely FFA4, due to its role in metabolic regulation

FFA4 is predominantly expressed in metabolic tissues such as adipose tissue, pancreas, and the gastrointestinal tract (Hara et al., 2014; Ulven & Christiansen, 2015). it is activated by long-chain fatty acids and plays a crucial role in regulating various metabolic processes, including glucose homeostasis, lipid metabolism, and insulin secretion(L. Huang et al., 2021). The modulation of FFA4 activity has shown potential therapeutic benefits in the context of metabolic disorders, such as type 2 diabetes and obesity(Ulven & Christiansen, 2015). Also, FFA4 activation has been shown to have anti-inflammatory effects in various cell types and tissues(Hidalgo et al., 2021). It can suppress pro-inflammatory signaling pathways and cytokine production, thereby attenuating inflammation (Hidalgo et al., 2021; K. L. Liu et al., 2016). This anti-inflammatory property makes FFA4 an attractive target for the development of therapeutics targeting chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis(Di Petrillo et al., 2023; Scaioli et al., 2017). Moreover, FFA4 a potential role in satiety and food intake regulation. Activation of FFA4 has been implicated in the regulation of satiety and food intake (Freitas & Campos, 2021). Studies have shown that FFA4 activation can promote the release of appetite-suppressing hormones, such as peptide YY (PYY) and (GLP-1), leading to reduced food intake (Freitas & Campos, 2021; Ulven & Christiansen, 2015). This suggests that modulating FFA4 activity could have implications in the treatment of obesity and related metabolic disorders(Anandhakrishnan & Korbonits, 2016). Furthermore, FFA4 exhibits tissue-specific effects, which adds to its therapeutic potential. For example, FFA4 activation in adipose tissue has been associated with increased lipid storage and improved insulin sensitivity(Im, 2018; Smith & Kahn, 2016). In the

gut, FFA4 activation has been shown to enhance the release of incretin hormones, which are involved in glucose regulation(Gribble et al., 2017).

FFA4, with its tissue-specific effects, offers the potential for targeted modulation of specific metabolic pathways, making it an attractive candidate for drug development. Given its involvement in metabolic regulation, anti-inflammatory effects, and regulation of satiety and food intake, FFA4 emerges as a promising therapeutic target for addressing metabolic disorders such as obesity and type 2 diabetes. Extensive research conducted on FFA4 has provided valuable insights into its implications in these conditions, further supporting its therapeutic potential.

Key ligands for FFA4 include long chain free fatty acids, particularly the n-3 polyunsaturated fatty acid family (Davenport et al., 2013). The high expression of FFA4 in adipocytes (Gotoh et al., 2007), coupled with its crucial role in maintaining metabolic homeostasis (Liu et al., 2015), underlines the importance to explore FFA4 as a potential target for the discovery of drugs treating T2DM and obesity.

6.1 Key findings

In order to study the role of FFA4 in adipocyte function, I chose to employ a pharmacological approach to manipulate FFA4 activity in the murine derived 3T3-L1 adipocyte model. I aimed to use one FFA4 agonist, TUG-891 (Hudson et al., 2013), and one FFA4 antagonist, AH-7614 (Watterson et al., 2017). However, before these compounds could be used to characterise FFA4 function in 3T3-L1 cells, I first aimed to use a heterologous expression system to characterise the pharmacology of these ligands at the mouse ortholog of FFA4 (Chapter 3). In my investigation aimed at characterizing the pharmacological properties of TUG-891 and AH7614, I successfully demonstrated that TUG-891 activates downstream signaling pathways by inducing the phosphorylation of ERK. This activation indicates its potential to regulate various cellular responses. Additionally, TUG-891 stimulates intracellular calcium Ca^{2+} mobilization, further validating its ability to modulate Ca^{2+} levels. Using TRUPATH biosensors, I observed that TUG-891 couples with multiple G proteins, including Gαq, Gα15, and Gαi2, thereby

activating their respective signaling pathways. Moreover, TUG-891 induces the recruitment of ß-arrestin, suggesting its involvement in the ß-arrestin signaling pathway. To investigate receptor internalization, I employed confocal microscopy, which confirmed TUG-891's ability to induce the internalization of the receptor.

These findings elucidate the diverse effects of TUG-891 on ERK phosphorylation, Ca2+ mobilization, G protein coupling, and ß-arrestin recruitment. Thus, TUG-891 exhibits potent regulatory properties in FFA4-mediated signaling pathways, capable of modulating various downstream cellular responses.

In contrast, my Ca^{2+} mobilization assay demonstrated that AH7614 concentration-dependently inhibits the ability of TUG-891 to induce Ca^{2+} mobilization. This indicates that AH7614 specifically targets FFA4 signaling pathways, leading to the inhibition of downstream responses. Furthermore, employing a BRET assay, I observed that AH7614 reduces the recruitment of ß-arrestin following FFA4 activation. The extent of ß-arrestin recruitment inhibition correlates with the concentration of AH7614, with higher concentrations completely blocking the activation. These results provide further evidence that AH7614 acts as a concentration-dependent antagonist of ß-arrestin signaling, reinforcing its inhibitory role in FFA4-mediated pathways. Additionally, I assessed the effect of AH7614 on the agonist-promoted internalization of mFFA4 using confocal microscopy. The results indicated that AH7614 partially inhibits the internalization of the receptor, as TUG-891 still increased the number of punctate spots representing mFFA4 expression.

These findings are broadly consistent with previous studies on the pharmacology and signalling of mFFA4 (Hudson et al., 2013; Watterson et al., 2017; Villegas-Comonfort et al., 2017)

In addition to confirming TUG-891 and AH-7614 agonism and antagonism of mFFA4, respectively, my work has indicated that AH-7614 has inverse agonism properties on FFA4-arrestin recruitment. Inverse agonists are ligands or drugs that bind to a receptor and reduces its basal activity below its constitutive level (KENAKIN, 2001). Inverse agonism has not previously been reported for this

ligand, and the finding that it does have this prosperity may have implications in how future studies interpret the actions of this ligand (Strange, 2002) .In addition, the fact that AH-7614 showed inverse agonism by extension also demonstrates that in some experimental systems FFA4 is constitutively active. Constitutive activity has previously been described for other FFA receptors including FFA2(Hudson, Tikhonova, et al., 2012) and FFA1 (Stoddart & Milligan, 2010), but again has not been widely reported for FFA4. It is however important to consider that given fatty acids are common molecules found in all cells, it is difficult to fully rule out the possibility that the constitutive activation of FFA4, and associated inverse agonism by AH-7614, could be driven in part by fatty acids produced in the cells being tested, instead of by true ligand independent constitutive activity (Dussault et al., 2002; Kenakin, 2003).Regardless, my finding holds potential to stimulate future studies exploring what role FFA4 constitutive signalling may play in the function of this receptor.

The additional findings provide further insights into the role of AH7614 in adipocyte function, specifically its impact on insulin signaling, glucose uptake, GLUT4 translocation, and lipolysis in 3T3-L1 adipocytes. It is important to note that the treatment in (chapter 4) was acute, focusing on the immediate effects of AH7614 on these cellular processes. Results revealed a dose-dependent decrease in insulin response when AH7614 was present. Even at the lowest insulin concentration tested, AH7614 exhibited a trend of reducing insulin response. At higher insulin concentrations, AH7614 significantly decreased insulin-stimulated glucose uptake. These findings suggest that AH7614 interferes with the cellular mechanisms involved in glucose uptake and GLUT4 translocation (Huang et al., 2002).Building upon these initial findings, further investigated the effect of AH7614 on insulin response. The results demonstrated that AH7614 significantly reduced the maximal insulin response. This indicates that AH7614 directly impairs GLUT4 translocation, thereby hindering the ability of insulin to stimulate glucose uptake. The findings that AH7614 significantly reduces the maximal insulin response and impairs GLUT4 translocation have important implications for conditions such as type 2 diabetes (T2D) and obesity. Both T2D and obesity are characterized by insulin resistance, where cells become less responsive to the effects of insulin, leading to impaired glucose uptake(Kahn et al., 2006).

In terms of lipolysis, AH7614 demonstrated a significant enhancing effect and treatment with TUG-891 inhibits lipolysis. As an FFA4 receptor antagonist, AH7614 increased the release of stored lipids from adipocytes. This blockade of FFA4 receptor signaling led to a notable increase in lipolysis, highlighting the role of FFA4 receptor signaling in regulating this process. Increased breakdown of triglycerides and release of glycerol from adipocytes may contribute to a more efficient utilization of stored fat as an energy source(Morigny et al., 2021; Nye et al., 2008).These findings contribute to our understanding of the cellular processes affected by AH7614 and its potential implications in metabolic disorders and weight management. However, it is important to note that these results were obtained in an experimental setting using 3T3-L1 adipocytes, and further research is needed to validate these effects in relevant in vivo models and human studies.

Another key finding is the outcomes of my study that have unveiled a significant discovery, demonstrating the inhibitory effect of AH7614 on the adipogenesis process. The FFA4 antagonist, AH7614, significantly inhibits the long-term adipogenesis process in 3T3-L1 adipocytes. This suggests that FFA4 plays a role in promoting adipocyte differentiation and the accumulation of neutral lipids. Understanding the involvement of FFA4 in adipogenesis can contribute to our knowledge of adipose tissue development and potentially provide insights into the regulation of adipocyte function in conditions such as obesity and metabolic disorders(Hammarstedt et al., 2018). Results also provided that AH7614 exhibits a concentration-dependent effect on adipogenesis in 3T3-L1 differentiated cells and demonstrated that the IC_{50} for AH7614 was 7.25 similar to its reported activity at FFA4(Watterson et al., 2017). Moreover, AH7614 treatment significantly decreased expression of various adipogenic genes, including reductions in FFA4 PPARγ adiponectin , leptin and GLUT4 which is agreed of the data from FFA4 knockout Mice(D. Liu et al., 2012; Moniri, 2016). To explore the impact of AH7614 treatment during differentiation on the function of fully differentiated adipocytes, glucose uptake and lipolysis assays were conducted. Consistent with the observed effects on lipid uptake and gene expression, AH7614 treatment reduced both insulins stimulated glucose and isoprenaline stimulated lipolysis. All together highlights the potential of targeting FFA4 with

antagonists in drug design, offering a promising avenue for the development of new, effective, and safe anti-obesity compounds. This is particularly crucial considering the limited efficacy, potential side effects, and drug interactions associated with current anti-obesity treatments (Jakab et al., 2021).

However, it is important to note that AH7614 treatment also exhibited a negative aspect, as it reduced both GLUT4 expression and the responsiveness to insulin.

Overall, this thesis demonstrates that FFA4 is expressed in 3T3-L1 adipocytes and that the pharmacological manipulation of this receptor affects adipogenesis, glucose uptake, and lipolysis in these cells. A consistent finding throughout the study was that in the 3T3-L1 adipocyte model, antagonism of FFA4 tended to have more pronounced effects than agonism, likely suggesting that FFA4 is in naturally in a relatively activated stated, either constitutively or through the release of fatty acids, in these adipocytes. Taken together, these findings will provide important insight into future efforts to understand how the FFA4 receptor may be exploited for the treatment of metabolic disease including obesity and T2DM.

6.2 Limitations to this work

One limitation to this work is its reliance exclusively on the 3T3-L1 adipocyte model. While 3T3-L1 cells are by far the most commonly used in vitro model used to study adipocytes (Zebisch et al., 2012), they are known to have several limitations. These include differentiation variability since 3T3-L1 cells often exhibit heterogeneity in their ability to differentiate into mature adipocytes (Kassotis et al., 2021). This can result in differences in lipid accumulation and gene expression profiles among individual cell lines or experimental replicates (Kassotis et al., 2021). Also, simplified cell models such as 3T3-L1 adipocytes may not fully recapitulate the complexity of adipose tissue in vivo (Kassotis et al., 2021). They lack the heterogeneity and functional diversity seen in primary adipocytes or adipose tissue, limiting their ability to fully mimic physiological responses (Sadie-Van Gijsen, 2019). Moreover, 3T3-L1 cells are typically cultured in two-dimensional (2D) monolayer systems (Turner et al., 2014), which does not

fully replicate the three-dimensional (3D) architecture of adipose tissue (Bellas et al., 2013).

In order to address this, it will be important to explore FFA4 signalling and the effects of FFA4 agonism and antagonism in other adipocyte cell models. Several human adipocyte cell lines, such as Simpson-Golabi-Behmel syndrome (SGBS) cells and human preadipocyte cell lines (Kalkhof et al., 2020), serve as alternatives to 3T3-L1 cells. SGBS cells, in particular, have been widely used to study adipogenesis, insulin sensitivity, and lipid metabolism due to their ability to differentiate into functional adipocytes with similarities to human primary adipocytes (Tews et al., 2022; Yeo et al., 2017). Moreover, co-culture systems that combine adipocytes with other cell types, such as immune cells or endothelial cells, can offer a more comprehensive representation of the cellular interactions occurring within adipose tissue (Battiston et al., 2014; Nitta & Orlando, 2013). These systems can provide insights into paracrine signaling, immune cell modulation, and vascularization processes that influence adipocyte function. Furthermore, three-dimensional culture systems, including adipose tissue organoids or scaffold-based models, aim to mimic the complex architecture and cellular composition of adipose tissue(Baptista et al., 2023). These models provide a closer approximation of the in vivo microenvironment, allowing for a more accurate assessment of FFA4 signaling and its impact on adipocyte behaviour. Also, primary adipocytes derived from human or animal adipose tissue offer a more physiologically relevant model compared to cell lines (Poulos et al., 2010; Ruiz-Ojeda et al., 2016). These cells retain the heterogeneity and functionality of mature adipocytes in vivo, allowing for a closer examination of FFA4 signaling and its impact on adipogenesis, lipid metabolism, and gene expression. Primary adipocytes can be isolated from adipose tissue through enzymatic digestion and subsequent purification.

Another important limitation to this work comes from complexity of trying to measure signalling for a fatty acid receptor in cells that both store and release fatty acid themselves. This creates a number of difficulties, particularly when cells are cultured in vitro, where FFAs that may be released from the cells have the opportunity to accumulate in the culture medium. Recent reports have demonstrated that this is the case for 3T3-L1 cells, which do continuously

release FFAs into the culture medium (Pei et al., 2022). These FFAs have the potential to activate the FFA4 receptor, and this may contribute to constitutive FFA4 signalling that is inhibited by AH-7614, but also to the lack of response to the FFA4 agonist, TUG-891. Further, given that FFA4 is well known to requit arrestin, internalise and desensitise (Hudson et al., 2013), this constant activation by FFAs in the culture medium may ultimately lead to a downregulation of receptor signalling (Vinciguerra et al., 2008). This is presumably not a true reflection of what would happen in vivo, where FFAs released from adipocytes would enter the bloodstream and quickly be taken away from the local adipocyte microenvironment (Lafontan, 2014). To address this issue in future experiments, modifications may be made to the handling of the 3T3-L1 cell line, such as more regular medium changes or increasing the volume of culture medium, an effect that is already known to impact 3T3-L1 adipogenesis (Ariemma et al., 2016; Sheng et al., 2013). Within assays themselves, studies could also aim to optimise concentrations of BSA, so as to reduce the effective concentration of FFAs in the assay buffer available to bind to FFA4 (van der Vusse, 2009). However, it must also be noted that most FFA4 ligands, including TUG-891 are also known to bind to serum proteins (Chu et al., 2019), so finding the right balance to reduce basal activation while maintaining agonist stimulated activation will be difficult.

Lastly, the fact that this study relied exclusively on pharmacological approaches to characterise the role of FFA4 in 3T3-L1 cells is a potential limitation. There is currently only one class of antagonist described for the FFA4 receptor(Karmokar & Moniri, 2022). Further, the pharmacology of this ligand class is complex, where these compounds act as probe dependant negative allosteric modulators that typically do not fully inhibit FFA4 signalling (Watterson et al., 2017). In addition, the molecules are known to have chemical similarity to molecules with actions at other targets, and at times a molecule related to AH-7614 with no pharmacological activity at FFA4 known as TUG-1387 has been used as a control to confirm that AH-7614 effects are mediated by FFA4 (Watterson et al., 2017). In the future, it would be particularly useful to confirm the present results using additional FFA4 antagonists, either competitive orthosteric agonists, or allosteric antagonists from different chemical, if/when such ligands become available. Alternatively, future studies could aim to employ a genetic approach to

complement the pharmacological one used here. This may, for example, involve the knockout of FFA4 in 3T3-L1 cells using CRISPR/Cas9, an approach that has successfully been used to look at the role of other proteins in 3T3-L1 cells (Hilgendorf et al., 2019).

6.3 Future directions

Due to time constraints, the current study did not investigate two important aspects regarding the influence of FFA4 on adipocyte function. Firstly, the potential effects of FFA4 on the regulation of adipose inflammation were not explored. It has been observed that adipocytes exhibit inflammation and insulin resistance in obesity (Guillermier et al., 2017;Kim et al., 2014). In mouse models of genetic and high-fat-diet-induced obesity, increased expression of inflammation-related genes and macrophage infiltration has been observed in adipose tissue. To address this, an RT-qPCR analysis of genes involved in the inflammatory pathway could be conducted on 3T3-L1 adipocytes treated with TUG-891 or AH7614, enabling a comparison of changes in gene expression. Additionally, co-culture experiments involving 3T3-L1 adipocytes and inflammatory cells expressing FFA4, such as RAW 246.7 macrophages, could be performed to investigate how these interactions impact the effects of the FFA4 tool compounds.

Secondly, it would be beneficial to test AH7614 and TUG-891 in obese adipose tissue, which exhibits different levels of FFA4 expression compared to non-obese adipose tissue. To achieve this, adipose tissue could be obtained from mice fed a high-fat diet (HFD), High-Fat Diet-Induced Obesity Models, inducing obesity in animal models, typically through feeding a high-fat diet, can be used to study the impact of FFA4 signaling on adiposity, insulin resistance, and metabolic disorders. These models allow for the investigation of FFA4-related mechanisms in the context of obesity and associated metabolic dysregulation allowing for the examination of the effects of FFA4 agonism and antagonism on adipocyte function in an obese context. The assessment of the effects of AH7614 and TUG-891 on this model ,ay be exploted by other assays. For example, lipid metabolism via measuring the uptake, synthesis, and breakdown of lipids in adipocytes treated with AH7614 and TUG-891. Also, assessment of the

expression and activity of key enzymes involved in lipid metabolism, such as lipoprotein lipase, hormone-sensitive lipase, and fatty acid synthase could reveal new insight. Moreover, the impact of AH7614 and TUG-891 on inflammatory markers in adipose tissue should be done as a key initial exploration, such as quantification ofthe expression and secretion of pro-inflammatory cytokines (e.g., TNF-alpha, IL-6) and anti-inflammatory cytokines (e.g., adiponectin) using techniques like enzyme-linked immunosorbent assay (ELISA).

Another future direction that could help to understand the role of FFA4 in 3T3-L1 cell function would be the generation of a 3T3-L1 knockout for the FFA4 receptor. This approach holds promise for investigating the precise role and function of FFA4 in adipocyte biology. However, it is important to acknowledge the challenges associated with manipulating the genetics of 3T3-L1 cells. Typically, the generation of a 3T3-L1 knockout model for FFA4 would involve the utilization of gene-editing techniques such as CRISPR-Cas9, to knock out the receptor in undifferentiated 3T3-L1 fibroblasts (Hong et al., 2018). This approach has been widely used and employed to study a large number of other proteins expressed in the 3T3-L1 model (Ahmed et al., 2022; Ghosh et al., 2022; Lumaquin, 2022; Yu et al., 2023). It is, however, important to consider that given FFA4 is not expressed in 3T3-L1 fibroblasts, experimentally this approach will have some complexity in developing a suitable strategy to functionally confirm efficient knockout. Differentiation of this cell line to capture the result with ORO to compare the WT- FFA4 3T3-L1 to KO-FFA4 3T3-L1, if they differentiate, will give a more direct link to FFA4 and adipogenesis. Moreover, performing glucose uptake assay with the resulting adipocyte from KO-FFA4 3T3-L1 will also provide a specific link between FFA4 and insulin response.

To gain further insights into the role of FFA4 in obesity, employing various mouse models of obesity models could also offer valuable information. One such model is the monogenic obesity models, which involves mice with mutations in single genes associated with human obesity (Huszar et al., 1997). For instance, the Avy mouse model carries a mutation in the agouti signaling protein gene, resulting in obesity and a yellow coat colour (Huszar et al., 1997).By administering an FFA4 antagonist and monitoring body weight and adipose tissue mass before and after

treatment, it may be possible to assess the impact of FFA4 antagonism in these models. Additionally, employing mouse models of obesity could enable the examination of the effects of FFA4 antagonism not only on adipose tissue but also on other organs such as the liver. Since the liver plays a central role in lipid metabolism(Nguyen et al., 2008). It is responsible for various processes, including fatty acid synthesis, fatty acid oxidation(Kohjima et al., 2007), triglyceride synthesis, and lipoprotein metabolism(Alves-Bezerra & Cohen, 2017; Nguyen et al., 2008). FFA4 is involved in regulating lipolysis, the breakdown of stored triglycerides into fatty acids and glycerol (Husted et al., 2020). By examining the liver tissue one can assess how FFA4 activation or inhibition in adipose tissue affects the release of fatty acids and subsequent lipid accumulation or utilization in the liver. Another relevant model is the genetic obesity model, wherein genetically modified mice with mutations in genes regulating appetite, energy expenditure, or lipid metabolism are employed (Lutz & Woods, 2012). This model can establish connections or facilitate comparative studies with the genetic expression data presented in this project. For example, leptin-deficient (ob/ob) mice (Drel et al., 2006) or leptin receptor-deficient (db/db) mice (B. Wang et al., n.d.) can provide insights into the role of leptin, considering the study's findings that FFA4 antagonist downregulates leptin expression in 3T3-L1 adipocytes.

In addition to genetic models of obesity, The Diet-Induced Obesity (DIO) model, is another approached that can offer unique insights into the role of FFA4 in obesity (Bagnol et al., 2012).By employing the DIO model, will give the flexibility to adjust both the duration and composition of the diet, enabling the induction of varying degrees of obesity and metabolic abnormalities (Bortolin et al., 2017). This model could provide an opportunity to administer FFA4 agonists or antagonists at different time points, establishing a connection to the in vivo study. The in vitro study employing TUG-891 or AH7614 on 3T3-L1 cells at different stages of differentiation, and the DIO model allows for a similar approach by administering the FFA4 agonist or antagonist at different time points during the development of obesity. This can be measured via monitoring changes in body weight and composition, such as fat mass and lean mass, provides information on the effectiveness of the diet-induced obesity model and the impact of FFA4 modulation on body weight regulation. Moreover, assessing

food intake allows for the evaluation of the effect of FFA4 modulation on appetite regulation and energy balance. Changes in food intake can help understand the role of FFA4 in the regulation of feeding behaviour and satiety. Furthermore, measuring metabolic parameters, including glucose levels, insulin sensitivity, and lipid profiles, offers insights into the metabolic consequences of FFA4 modulation in the context of diet-induced obesity. These measurements can help assess the effects of FFA4 agonists or antagonists on glucose homeostasis and lipid metabolism. Also, examining the morphology of adipose tissue, including adipocyte size, can indicate the impact of FFA4 modulation on adipose tissue expansion and remodelling in the context of obesity. Additionally, assessing gene expression profiles in adipose tissue, liver, or other relevant tissues can reveal changes in the expression of genes involved in lipid metabolism, adipogenesis, inflammation, and signaling pathways related to FFA4 function.

Before introducing AH7614 into in vivo experiments, it is crucial to thoroughly characterize its pharmacological properties. The biggest challenge is that the Pharmacokinetics of AH7614 still unknown. Understanding the pharmacokinetics of a compound is crucial for determining the appropriate dosage, dosing intervals, and route of administration in preclinical and clinical studies. However, the pharmacokinetics of AH7614 are not known, which can be a significant limitation when considering its use in in vivo experiments. While AH7614 is known as an FFA4 agonist, it is important to consider the potential for off-target binding and effects on other receptors or pathways. These off-target effects can lead to undesired or unpredictable physiological responses and may confound the interpretation of experimental results. performing a large-scale selectivity screen would be an approach to assess the potential off-target effects of AH7614 (Morgens et al., 2017). A selectivity screen involves testing the compound against a panel of different receptors or molecular targets to evaluate its binding affinity and specificity (Walters & Namchuk, 2003). This experimental approach provides empirical data on the compound's selectivity and helps identify any potential off-target interactions. Then, Computational modelling can be employed to predict potential off-target binding sites at FFA4 receptor for AH7614, which will provide valuable information about its specific effects on the receptor(Chuong et al., 2016). The interaction between a drug

and its target at the binding site plays a pivotal role in defining the drug's mechanism of action, including effects such as enzymatic activity inhibition, modulation of receptor signaling pathways (Espinoza-Fonseca, 2006), or interference with protein-protein interactions (Sanders et al., 2012). Furthermore, comprehending the binding site can help identify potential cross-reactivity or unintended binding to non-target molecules, thereby enhancing the safety profile of the drug for subsequent primary studies (Halgren, 2007). Addressing these limitations would contribute to a more robust understanding of AH7614's efficacy, safety profile, and overall suitability for in vivo investigations.

It is crucial not only to characterize AH-7614, the currently known antagonist for FFA4, but also to explore and screen for new antagonists targeting FFA4. One approach is to conduct high-throughput screening or virtual screening of compound libraries to identify potential antagonist candidates (Zhang et al., 2008). High-throughput screening involves subjecting large collections of compounds to biochemical or cell-based assays against the receptor(Blay et al., 2020). On the other hand, virtual screening employs computational methods to search databases of chemical compounds and predict molecules that are likely to bind to the receptor (Jacob et al., 2008). Subsequently, computational techniques like molecular docking, molecular dynamics simulations, or quantitative structure-activity relationship modeling can be utilized to predict and analyze the binding interactions between the receptor and potential antagonist candidates (Naqvi et al., 2019). These computational methods play a crucial role in identifying compounds that exhibit favourable binding affinities and specific interactions with the receptor's binding site (Sliwoski et al., 2014).

By exploring these future directions, we can enhance our comprehension of how FFA4 impacts adipocyte function, inflammation, and obesity. This progress will lay the foundation for the development of innovative therapeutic approaches to address metabolic disorders.

In conclusion, the study of FFA4 and its pharmacological properties has provided valuable insights into its potential as a therapeutic target for metabolic disorders such as obesity and T2D. FFA4 plays a crucial role in regulating metabolic processes, including glucose homeostasis, lipid metabolism, and

insulin secretion. The findings of this study, including the characterization of FFA4 agonist TUG-891 and antagonist AH7614, contribute to our understanding of FFA4-mediated signaling pathways and provide a foundation for further research and the development of enhanced pharmacological treatments for metabolic disorders. Overall, FFA4 emerges as a promising avenue for future therapeutic interventions aimed at addressing the global burden of obesity and T2D.

List of References

Aguilo, F., Zhang, F., Sancho, A., Fidalgo, M., Di Cecilia, S., Vashisht, A., Lee, D. F., Chen, C. H., Rengasamy, M., Andino, B., Jahouh, F., Roman, A., Krig, S. R., Wang, R., Zhang, W., Wohlschlegel, J. A., Wang, J., & Walsh, M. J. (2015). Coordination of m6A mRNA Methylation and Gene Transcription by ZFP217 Regulates Pluripotency and Reprogramming. *Cell Stem Cell*, *17*(6), 689–704. https://doi.org/10.1016/J.STEM.2015.09.005

Ahmed, B., & Konje, J. C. (2023). The epidemiology of obesity in reproduction. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *89*, 102342. https://doi.org/10.1016/J.BPOBGYN.2023.102342

Ahmed, F., Hetty, S., Vranic, M., Fanni, G., Kullberg, J., Pereira, M. J., & Eriksson, J. W. (2022). ESR2 expression in subcutaneous adipose tissue is related to body fat distribution in women, and knockdown impairs preadipocyte differentiation. *Taylor & Francis*, *11*(1), 434–447. https://doi.org/10.1080/21623945.2022.2102116

Ahmed, K. A. A., Muniandy, S., & Ismail, I. S. (2010). *Type 2 diabetes and vascular complications: A pathophysiologic view*.

Ajith, T. A., & Jayakumar, T. G. (2019). Omega-3 fatty acids in coronary heart disease: Recent updates and future perspectives. *Clinical and Experimental Pharmacology and Physiology*, *46*(1), 11–18. https://doi.org/10.1111/1440-1681.13034

Alimardani, V., Farahavar, G., Salehi, S., Taghizadeh, S., Ghiasi, M. R., & Abolmaali, S. S. (2021). Gold nanocages in cancer diagnosis, therapy, and theranostics: A brief review. *Frontiers of Materials Science*, *15*(4), 494–511. https://doi.org/10.1007/S11706-021-0569-1

Altabas, V., & Zjačić-Rotkvić, V. (2015). Anti-ghrelin antibodies in appetite suppression: recent advances in obesity pharmacotherapy. *ImmunoTargets and Therapy*, 123. https://doi.org/10.2147/ITT.S60398

Alvarez-Curto, E., Inoue, A., Jenkins, L., Raihan, S. Z., Prihandoko, R., Tobin, A. B., & Milligan, G. (2016). Targeted Elimination of G Proteins and Arrestins Defines Their Specific Contributions to Both Intensity and Duration of G Protein-coupled Receptor Signaling. *The Journal of Biological Chemistry*, *291*(53), 27147–27159. https://doi.org/10.1074/JBC.M116.754887

Alves-Bezerra, M., & Cohen, D. E. (2017). Triglyceride metabolism in the liver. *Comprehensive Physiology*, *8*(1), 1. https://doi.org/10.1002/CPHY.C170012

Anandhakrishnan, A., & Korbonits, M. (2016). Glucagon-like peptide 1 in the pathophysiology and pharmacotherapy of clinical obesity. *World Journal of Diabetes*, *7*(20), 572. https://doi.org/10.4239/WJD.V7.I20.572

Aon, M. A., Bhatt, N., & Cortassa, S. (2014). Mitochondrial and cellular mechanisms for managing lipid excess. *Frontiers in Physiology*, *5 JUL*, 94873. https://doi.org/10.3389/FPHYS.2014.00282/BIBTEX

Ariemma, F., D'Esposito, V., Liguoro, D., Oriente, F., Cabaro, S., Liotti, A., Cimmino, I., Longo, M., Beguinot, F., Formisano, P., & Valentino, R. (2016). Low-Dose Bisphenol-A Impairs Adipogenesis and Generates Dysfunctional 3T3-L1 Adipocytes. *PLOS ONE*, *11*(3), e0150762. https://doi.org/10.1371/JOURNAL.PONE.0150762

Armani, A., Mammi, C., Marzolla, V., Calanchini, M., Antelmi, A., Rosano, G. M. C., Fabbri, A., & Caprio, M. (2010). Cellular models for understanding adipogenesis, adipose dysfunction, and obesity. *Journal of Cellular Biochemistry*, *110*(3), 564–572. https://doi.org/10.1002/JCB.22598

Armstrong, M. J., Gaunt, P., Aithal, G. P., Barton, D., Hull, D., Parker, R., Hazlehurst, J. M., Guo, K., Abouda, G., Aldersley, M. A., Stocken, D., Gough, S. C., Tomlinson, J. W., Brown, R. M., Hübscher, S. G., & Newsome, P. N. (2016). Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet (London, England)*, *387*(10019), 679–690. https://doi.org/10.1016/S0140-6736(15)00803-X

Armstrong, R. A., Eperjesi, F., & Gilmartin, B. (2002). The application of analysis of variance (ANOVA) to different experimental designs in optometry. *Ophthalmic and Physiological Optics*, *22*(3), 248–256. https://doi.org/10.1046/J.1475-1313.2002.00020.X

Arterburn, D. E., & Courcoulas, A. P. (2014). State of the Art Review: Bariatric surgery for obesity and metabolic conditions in adults. *The BMJ*, *349*. https://doi.org/10.1136/BMJ.G3961

Ashcroft, F. M., & Rorsman, P. (2012). Diabetes mellitus and the ß-cell: the Last Ten Years. *Cell*, *148*(6), 1160. https://doi.org/10.1016/J.CELL.2012.02.010

Azevedo, C. M. G., Watterson, K. R., Wargent, E. T., Hansen, S. V. F., Hudson, B. D., Kępczyńska, M. A., Dunlop, J., Shimpukade, B., Christiansen, E., Milligan, G., Stocker, C. J., & Ulven, T. (2016). Non-Acidic Free Fatty Acid Receptor 4 Agonists with Antidiabetic Activity. *Journal of Medicinal Chemistry*, *59*(19), 8868–8878. https://doi.org/10.1021/ACS.JMEDCHEM.6B00685

Bagnol, D., Al-Shamma, H. A., Behan, D., Whelan, K., & Grottick, A. J. (2012). Diet-Induced Models of Obesity (DIO) in Rodents. *Current Protocols in Neuroscience*, *59*(1), 9.38.1-9.38.13. https://doi.org/10.1002/0471142301.NS0938S59

Baptista, L. S., Silva, K. R., Jobeili, L., Guillot, L., & Sigaudo-Roussel, D. (2023). Unraveling White Adipose Tissue Heterogeneity and Obesity by Adipose Stem/Stromal Cell Biology and 3D Culture Models. *Cells 2023, Vol. 12, Page 1583*, *12*(12), 1583. https://doi.org/10.3390/CELLS12121583

Barella, L. F., Jain, S., Kimura, T., & Pydi, S. P. (2021). Metabolic roles of G protein-coupled receptor signaling in obesity and type 2 diabetes. *The FEBS Journal*, *288*(8), 2622–2644. https://doi.org/10.1111/FEBS.15800

Bargut, T. C. L., Souza-Mello, V., Aguila, M. B., & Mandarim-De-Lacerda, C. A. (2017). Browning of white adipose tissue: Lessons from experimental models. *Hormone Molecular*
Biology and Clinical Investigation, *31*(1). https://doi.org/10.1515/HMBCI-2016-0051/ASSET/GRAPHIC/J_HMBCI-2016-0051_FIG_002.JPG

Barkan, K. (2017). *An investigation into Glucagon receptor pharmacology*. http://webcat.warwick.ac.uk/record=b3152896~S15

Barroso, I., Luan, J., Middelberg, R. P. S., Harding, A. H., Franks, P. W., Jakes, R. W., Clayton, D., Schafer, A. J., O'Rahilly, S., & Wareham, N. J. (2003). Candidate Gene Association Study in Type 2 Diabetes Indicates a Role for Genes Involved in β-Cell Function as Well as Insulin Action. *PLOS Biology*, *1*(1), e20. https://doi.org/10.1371/JOURNAL.PBIO.0000020

Battiston, K. G., Cheung, J. W. C., Jain, D., & Santerre, J. P. (2014). Biomaterials in co-culture systems: Towards optimizing tissue integration and cell signaling within scaffolds. *Biomaterials*, *35*(15), 4465–4476. https://doi.org/10.1016/J.BIOMATERIALS.2014.02.023

Beck, B. (2006). Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *361*(1471), 1159. https://doi.org/10.1098/RSTB.2006.1855

Bellas, E., Marra, K. G., & Kaplan, D. L. (2013). Sustainable three-dimensional tissue model of human adipose tissue. *Tissue Engineering - Part C: Methods*, *19*(10), 745–754. https://doi.org/10.1089/TEN.TEC.2012.0620/ASSET/IMAGES/LARGE/FIGURE6.JPEG

Bessesen, D. H., & Van Gaal, L. F. (2018). Progress and challenges in anti-obesity pharmacotherapy. *The Lancet Diabetes & Endocrinology*, *6*(3), 237–248. https://doi.org/10.1016/S2213-8587(17)30236-X

Biau, D. J., Jolles, B. M., & Porcher, R. (2010). P value and the theory of hypothesis testing: An explanation for new researchers. *Clinical Orthopaedics and Related Research*, *468*(3), 885–892. https://doi.org/10.1007/S11999-009-1164-4/TABLES/3

Bielecka-Dabrowa, A., Lewek, J., Sakowicz, A., Paduszyńska, A., Dąbrowa, M., Orszulak-Michalak, D., & Banach, M. (2022). Effects of Implementing Personalized Health Education in Ambulatory Care on Cardiovascular Risk Factors, Compliance and Satisfaction with Treatment. *Journal of Personalized Medicine 2022, Vol. 12, Page 1583*, *12*(10), 1583. https://doi.org/10.3390/JPM12101583

Bingham, B., Jones, P. G., Uveges, A. J., Kotnis, S., Lu, P., Smith, V. A., Sun, S. C., Resnick, L., Chlenov, M., He, Y., Strassle, B. W., Cummons, T. A., Piesla, M. J., Harrison, J. E., Whiteside, G. T., & Kennedy, J. D. (2007). Species-specific in vitro pharmacological effects of the cannabinoid receptor 2 (CB2) selective ligand AM1241 and its resolved enantiomers. *British Journal of Pharmacology*, *151*(7), 1061–1070. https://doi.org/10.1038/SJ.BJP.0707303

Biomembranes: Molecular Structure and Function - Robert B. Gennis - Google Books. (n.d.). Retrieved November 1, 2023, from https://books.google.com.sa/books?hl=en&lr=&id=RJbkBwAAQBAJ&oi=fnd&pg=PR7&dq=.+The+polar+nature+of+glucose+arises+from+the+presence+of+hydroxyl+(-OH)+groups,+which+make+it+soluble+in+water.+Since+the+interior+of+the+lipid+membrane+is+nonpolar,+glucose+molecules+cannot+pass+through+it+without+the+help+of+specialized+transporters&ots=FYCt9d2dRb&sig=n4IfJC_PGPPx5HK57QeviD04Vl4&redir_esc=y#v=onepage&q&f=false

Black, H. L., Livingstone, R., Mastick, C. C., Tobi, M. Al, Taylor, H., Geiser, A., Stirrat, L., Kioumourtzoglou, D., Petrie, J. R., Boyle, J. G., Bryant, N. J., & Gould, G. W. (2022). Knockout of syntaxin-4 in 3T3-L1 adipocytes reveals new insight into GLUT4 trafficking and adiponectin secretion. *Journal of Cell Science*, *135*(1). https://doi.org/10.1242/JCS.258375

Blay, V., Tolani, B., Ho, S. P., & Arkin, M. R. (2020). High-Throughput Screening: today's biochemical and cell-based approaches. *Drug Discovery Today*, *25*(10), 1807–1821. https://doi.org/10.1016/J.DRUDIS.2020.07.024

Blum, R. A., Mair, S., & Duus, E. M. (2019). Appetite and food intake results from phase I studies of anamorelin. *Journal of Cachexia, Sarcopenia and Muscle*, *10*(5), 1027–1035. https://doi.org/10.1002/JCSM.12439

Bond, R. A., & Ijzerman, A. P. (2006). Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends in Pharmacological Sciences*, *27*(2), 92–96. https://doi.org/10.1016/j.tips.2005.12.007

Bonnet, F., & Scheen, A. (2017). Understanding and overcoming metformin gastrointestinal intolerance. *Diabetes, Obesity and Metabolism*, *19*(4), 473–481. https://doi.org/10.1111/DOM.12854

Bortolin, R. C., Vargas, A. R., Gasparotto, J., Chaves, P. R., Schnorr, C. E., Martinello, K. B., Silveira, A. K., Rabelo, T. K., Gelain, D. P., & Moreira, J. C. F. (2017). A new animal diet based on human Western diet is a robust diet-induced obesity model: comparison to high-fat and cafeteria diets in term of metabolic and gut microbiota disruption. *International Journal of Obesity 2018 42:3*, *42*(3), 525–534. https://doi.org/10.1038/ijo.2017.225

Boyers, D., Retat, L., Jacobsen, E., Avenell, A., Aveyard, P., Corbould, E., Jaccard, A., Cooper, D., Robertson, C., Aceves-Martins, M., Xu, B., Skea, Z., de Bruin, M., Jacobsen, E., Boyers, D., Cooper, D., Retat, L., Aveyard, P., Stewart, F., … Kemp, L. (2021). Cost-effectiveness of bariatric surgery and non-surgical weight management programmes for adults with severe obesity: a decision analysis model. *International Journal of Obesity 2021 45:10*, *45*(10), 2179–2190. https://doi.org/10.1038/s41366-021-00849-8

Brant, A. M., Gibbs, E. M., Gould, G. W., & Thomas, H. M. (1992). Immunological identification of five members of the human facultative glucose transporter

family. *Biochemical Society Transactions*, *20*(3), 236S-236S. https://doi.org/10.1042/BST020236S

Bray, G. A., Heisel, W. E., Afshin, A., Jensen, M. D., Dietz, W. H., Long, M., Kushner, R. F., Daniels, S. R., Wadden, T. A., Tsai, A. G., Hu, F. B., Jakicic, J. M., Ryan, D. H., Wolfe, B. M., & Inge, T. H. (2018). The Science of Obesity Management: An Endocrine Society Scientific Statement. *Endocrine Reviews*, *39*(2), 79. https://doi.org/10.1210/ER.2017-00253

Bremner, S. K., Al Shammari, W. S., Milligan, R. S., Hudson, B. D., Sutherland, C., Bryant, N. J., & Gould, G. W. (2022). Pleiotropic effects of Syntaxin16 identified by gene editing in cultured adipocytes. *Frontiers in Cell and Developmental Biology*, *10*, 2242. https://doi.org/10.3389/FCELL.2022.1033501/BIBTEX

Brennan, E. P., Mohan, M., Andrews, D., Bose, M., & Kantharidis, P. (2019). Specialized pro-resolving mediators in diabetes: novel therapeutic strategies. *Clinical Science*, *133*(21), 2121–2141. https://doi.org/10.1042/CS20190067

Briscoe, C. P., Peat, A. J., McKeown, S. C., Corbett, D. F., Goetz, A. S., Littleton, T. R., McCoy, D. C., Kenakin, T. P., Andrews, J. L., Ammala, C., Fornwald, J. A., Ignar, D. M., & Jenkinson, S. (2006). Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. *British Journal of Pharmacology*, *148*(5), 619–628. https://doi.org/10.1038/SJ.BJP.0706770

Briscoe, C. P., Tadayyon, M., Andrews, J. L., Benson, W. G., Chambers, J. K., Eilert, M. M., Ellis, C., Elshourbagy, N. A., Goetz, A. S., Minnick, D. T., Murdock, P. R., Sauls, H. R., Shabon, U., Spinage, L. D., Strum, J. C., Szekeres, P. G., Tan, K. B., Way, J. M., Ignar, D. M., … Muir, A. I. (2003). The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *Journal of Biological Chemistry*, *278*(13), 11303–11311. https://doi.org/10.1074/jbc.M211495200

Brown, A. J., Goldsworthy, S. M., Barnes, A. A., Eilert, M. M., Tcheang, L., Daniels, D., Muir, A. I., Wigglesworth, M. J., Kinghorn, I., Fraser, N. J., Pike, N. B., Strum, J. C., Steplewski, K. M., Murdock, P. R., Holder, J. C., Marshall, F. H., Szekeres, P. G., Wilson, S., Ignar, D. M., … Dowell, S. J. (2003). The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *The Journal of Biological Chemistry*, *278*(13), 11312–11319. https://doi.org/10.1074/JBC.M211609200

Brown, A. J. H., Bradley, S. J., Marshall, F. H., Brown, G. A., Bennett, K. A., Brown, J., Cansfield, J. E., Cross, D. M., de Graaf, C., Hudson, B. D., Dwomoh, L., Dias, J. M., Errey, J. C., Hurrell, E., Liptrot, J., Mattedi, G., Molloy, C., Nathan, P. J., Okrasa, K., … Tobin, A. B. (2021). From structure to clinic: Design of a muscarinic M1 receptor agonist with potential to treatment of Alzheimer's disease. *Cell*, *184*(24), 5886-5901.e22. https://doi.org/10.1016/J.CELL.2021.11.001

Burns, R. N., & Moniri, N. H. (2010). Agonism with the omega-3 fatty acids alpha-linolenic acid and docosahexaenoic acid mediates phosphorylation of both the short and long isoforms of the human GPR120 receptor. *Biochemical and*

Biophysical Research Communications, *396*(4), 1030–1035. https://doi.org/10.1016/J.BBRC.2010.05.057

Butcher, A. J., Hudson, B. D., Shimpukade, B., Alvarez-Curto, E., Prihandoko, R., Ulven, T., Milligan, G., & Tobin, A. B. (2014). Concomitant action of structural elements and receptor phosphorylation determines arrestin-3 interaction with the free fatty acid receptor FFA4. *The Journal of Biological Chemistry*, *289*(26), 18451–18465. https://doi.org/10.1074/JBC.M114.568816

Calder, P. C., & Yaqoob, P. (2009). Omega-3 polyunsaturated fatty acids and human health outcomes. *BioFactors*, *35*(3), 266–272. https://doi.org/10.1002/BIOF.42

Calderon-Dominguez, M., Mir, J. F., Fucho, R., Weber, M., Serra, D., & Herrero, L. (2016). Fatty acid metabolism and the basis of brown adipose tissue function. *Adipocyte*, *5*(2), 98–118. https://doi.org/10.1080/21623945.2015.1122857

Capanni, M., Calella, F., Biagini, M. R., Genise, S., Raimondi, L., Bedogni, G., Svegliati-Baroni, G., Sofi, F., Milani, S., Abbate, R., Surrenti, C., & Casini, A. (2006). Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study. *Alimentary Pharmacology & Therapeutics*, *23*(8), 1143–1151. https://doi.org/10.1111/J.1365-2036.2006.02885.X

Caprio, M., Fève, B., Claës, A., Viengchareun, S., Lombès, M., & Zennaro, M.-C. (2007). Pivotal role of the mineralocorticoid receptor in corticosteroid-induced adipogenesis. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *21*(9), 2185–2194. https://doi.org/10.1096/FJ.06-7970COM

Carmichael, R. E., Wilkinson, K. A., & Craig, T. J. (2019). Insulin-dependent GLUT4 trafficking is not regulated by protein SUMOylation in L6 myocytes. *Scientific Reports 2019 9:1*, *9*(1), 1–9. https://doi.org/10.1038/s41598-019-42574-3

Castilla-Madrigal, R., Barrenetxe, J., Moreno-Aliaga, M. J., & Lostao, M. P. (2018). EPA blocks TNF-α-induced inhibition of sugar uptake in Caco-2 cells via GPR120 and AMPK. *Journal of Cellular Physiology*, *233*(3), 2426–2433. https://doi.org/10.1002/JCP.26115

Castro, É., Silva, T. E. O., & Festuccia, W. T. (2017). Critical review of beige adipocyte thermogenic activation and contribution to whole-body energy expenditure. *Hormone Molecular Biology and Clinical Investigation*, *31*(2). https://doi.org/10.1515/HMBCI-2017-0042/MACHINEREADABLECITATION/RIS

Cersosimo, E., & DeFronzo, R. A. (2006). Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. *Diabetes/Metabolism Research and Reviews*, *22*(6), 423–436. https://doi.org/10.1002/DMRR.634

Chait, A., & den Hartigh, L. J. (2020). Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease. *Frontiers in Cardiovascular Medicine*, *7*, 22. https://doi.org/10.3389/FCVM.2020.00022

Chatterjee, S., Khunti, K., & Davies, M. J. (2017). Type 2 diabetes. *The Lancet*, *389*(10085), 2239–2251. https://doi.org/10.1016/S0140-6736(17)30058-2

Chen, K. Y., Muniyappa, R., Abel, B. S., Mullins, K. P., Staker, P., Brychta, R. J., Zhao, X., Ring, M., Psota, T. L., Cone, R. D., Panaro, B. L., Gottesdiener, K. M., Van Der Ploeg, L. H. T., Reitman, M. L., & Skarulis, M. C. (2015). RM-493, a Melanocortin-4 Receptor (MC4R) Agonist, Increases Resting Energy Expenditure in Obese Individuals. *The Journal of Clinical Endocrinology & Metabolism*, *100*(4), 1639–1645. https://doi.org/10.1210/JC.2014-4024

Chouchani, E. T., & Kajimura, S. (2019). Metabolic adaptation and maladaptation in adipose tissue. *Nature Metabolism 2019 1:2*, *1*(2), 189–200. https://doi.org/10.1038/s42255-018-0021-8

Chu, X., Zhou, Q., Xu, Y., Jiang, J., Li, Q., Zhou, Q., Wu, Q., Jin, M., Wang, H., Gu, Y., Wang, X., Wang, B., He, S., He, X., Wu, C., Zhang, F., & Zhang, Y. (2019). Aberrant fatty acid profile and FFAR4 signaling confer endocrine resistance in breast cancer. *Journal of Experimental and Clinical Cancer Research*, *38*(1), 1–18. https://doi.org/10.1186/S13046-019-1040-3/FIGURES/6

Chuong, E. B., Elde, N. C., & Feschotte, C. (2016). Regulatory activities of transposable elements: from conflicts to benefits. *Nature Reviews Genetics 2016 18:2*, *18*(2), 71–86. https://doi.org/10.1038/nrg.2016.139

Cichocki, F., van der Stegen, S. J. C., & Miller, J. S. (2023). Engineered and banked iPSCs for advanced NK- and T-cell immunotherapies. *Blood*, *141*(8), 846–855. https://doi.org/10.1182/BLOOD.2022016205

Cinti, S. (2012). The adipose organ at a glance. *Disease Models & Mechanisms*, *5*(5), 588. https://doi.org/10.1242/DMM.009662

Clarke, K. R., Somerfield, P. J., & Gorley, R. N. (2008). Testing of null hypotheses in exploratory community analyses: similarity profiles and biota-environment linkage. *Journal of Experimental Marine Biology and Ecology*, *366*(1–2), 56–69. https://doi.org/10.1016/J.JEMBE.2008.07.009

Colldén, G., Tschöp, M. H., & Müller, T. D. (2017). Therapeutic Potential of Targeting the Ghrelin Pathway. *International Journal of Molecular Sciences 2017, Vol. 18, Page 798*, *18*(4), 798. https://doi.org/10.3390/IJMS18040798

Collins, S. (2013). A heart–adipose tissue connection in the regulation of energy metabolism. *Nature Reviews Endocrinology 2013 10:3*, *10*(3), 157–163. https://doi.org/10.1038/nrendo.2013.234

Cone, C. J., Bachyrycz, A. M., & Murata, G. H. (2010). Hepatotoxicity Associated with Metformin Therapy in Treatment of Type 2 Diabetes Mellitus with Nonalcoholic Fatty Liver Disease. *Http://Dx.Doi.Org/10.1345/Aph.1P099*, *44*(10), 1655–1659. https://doi.org/10.1345/APH.1P099

Congreve, M., de Graaf, C., Swain, N. A., & Tate, C. G. (2020). Impact of GPCR Structures on Drug Discovery. *Cell*, *181*(1), 81–91. https://doi.org/10.1016/J.CELL.2020.03.003

Cook, K. S., Min, H. Y., Johnson, D., Chaplinsky, R. J., Flier, J. S., Hunt, C. R., & Spiegelman, B. M. (1987). Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. *Science (New York, N.Y.)*, *237*(4813), 402–405. https://doi.org/10.1126/SCIENCE.3299705

Couvineau, A., Voisin, T., Nicole, P., Gratio, V., Abad, C., & Tan, Y. V. (2019). Orexins as Novel Therapeutic Targets in Inflammatory and Neurodegenerative Diseases. *Frontiers in Endocrinology*, *10*, 488305. https://doi.org/10.3389/FENDO.2019.00709/BIBTEX

Cura, A. J. (2022). *Acute Modulation of Endothelial Cell Glucose Transport: A Dissertation*. https://doi.org/10.13028/JGZC-7722

Cypess, A. M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A. B., Kuo, F. C., Palmer, E. L., Tseng, Y.-H., Doria, A., Kolodny, G. M., & Kahn, C. R. (2009). Identification and importance of brown adipose tissue in adult humans. *The New England Journal of Medicine*, *360*(15), 1509–1517. https://doi.org/10.1056/NEJMOA0810780

Cypess, A. M., White, A. P., Vernochet, C., Schulz, T. J., Xue, R., Sass, C. A., Huang, T. L., Roberts-Toler, C., Weiner, L. S., Sze, C., Chacko, A. T., Deschamps, L. N., Herder, L. M., Truchan, N., Glasgow, A. L., Holman, A. R., Gavrila, A., Hasselgren, P. O., Mori, M. A., … Tseng, Y. H. (2013). Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nature Medicine 2013 19:5*, *19*(5), 635–639. https://doi.org/10.1038/nm.3112

da Silva Rosa, S. C., Nayak, N., Caymo, A. M., & Gordon, J. W. (2020). Mechanisms of muscle insulin resistance and the cross-talk with liver and adipose tissue. *Physiological Reports*, *8*(19), e14607. https://doi.org/10.14814/PHY2.14607

Davenport, A. P., Alexander, S. P. H., Sharman, J. L., Pawson, A. J., Benson, H. E., Monaghan, A. E., Liew, W. C., Mpamhanga, C. P., Bonner, T. I., Neubig, R. R., Pin, J. P., Spedding, M., & Harmar, A. J. (2013). International Union of Basic and Clinical Pharmacology. LXXXVIII. G protein-coupled receptor list: recommendations for new pairings with cognate ligands. *Pharmacological Reviews*, *65*(3), 967–986. https://doi.org/10.1124/PR.112.007179

Davenport, A. P., Scully, C. C. G., de Graaf, C., Brown, A. J. H., & Maguire, J. J. (2020). Advances in therapeutic peptides targeting G protein-coupled receptors. *Nature Reviews Drug Discovery 2020 19:6*, *19*(6), 389–413. https://doi.org/10.1038/s41573-020-0062-z

de Felice, A., Aureli, S., & Limongelli, V. (2021). Drug Repurposing on G Protein-Coupled Receptors Using a Computational Profiling Approach. *Frontiers in Molecular Biosciences*, *8*, 336. https://doi.org/10.3389/FMOLB.2021.673053/BIBTEX

De Jager, J., Kooy, A., Lehert, P., Wulffelé, M. G., Van Der Kolk, J., Bets, D., Verburg, J., Donker, A. J. M., & Stehouwer, C. D. A. (2010). Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-

12 deficiency: randomised placebo controlled trial. *BMJ*, *340*(7757), 1177. https://doi.org/10.1136/BMJ.C2181

de Laat, B., Goldberg, A., Shi, J., Tetrault, J. M., Nabulsi, N., Zheng, M. Q., Najafzadeh, S., Gao, H., Kapinos, M., Ropchan, J., O'Malley, S. S., Huang, Y., Morris, E. D., & Krishnan-Sarin, S. (2019). The Kappa Opioid Receptor Is Associated With Naltrexone-Induced Reduction of Drinking and Craving. *Biological Psychiatry*, *86*(11), 864–871. https://doi.org/10.1016/J.BIOPSYCH.2019.05.021

De Ligt, R. A. F., Kourounakis, A. P., & IJzerman, A. P. (2000). Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *British Journal of Pharmacology*, *130*(1), 1. https://doi.org/10.1038/SJ.BJP.0703311

De Pauw, A., Tejerina, S., Raes, M., Keijer, J., & Arnould, T. (2009). Mitochondrial (Dys)function in Adipocyte (De)differentiation and Systemic Metabolic Alterations. *The American Journal of Pathology*, *175*(3), 927–939. https://doi.org/10.2353/AJPATH.2009.081155

de sá, P. M., Richard, A. J., Hang, H., & Stephens, J. M. (2017). Transcriptional Regulation of Adipogenesis. *Comprehensive Physiology*, *7*(2), 635–674. https://doi.org/10.1002/CPHY.C160022

Deang, K. T., Sidi, H., Zakaria, H., Adam, R. L., Das, S., Hatta, N. H., Hatta, M. H., & Wee, K. W. (2017). The Novelty of Bupropion As a Dopaminergic Antidepressant for the Treatment of Adult Attention Deficit Hyperactive Disorder. *Current Drug Targets*, *20*(2), 210–219. https://doi.org/10.2174/1389450118666170511145628

Defronzo, R. A. (2009). From the Triumvirate to the Ominous Octet: A New Paradigm for the Treatment of Type 2 Diabetes Mellitus. *Diabetes*, *58*(4), 773–795. https://doi.org/10.2337/DB09-9028

Deng, X., Tavallaie, M. S., Sun, R., Wang, J., Cai, Q., Shen, J., Lei, S., Fu, L., & Jiang, F. (2020). Drug discovery approaches targeting the incretin pathway. *Bioorganic Chemistry*, *99*, 103810. https://doi.org/10.1016/J.BIOORG.2020.103810

Dhillon, S. (2022). Phentermine/Topiramate: Pediatric First Approval. *Paediatric Drugs*, *24*(6), 715–720. https://doi.org/10.1007/S40272-022-00532-Z

Di Petrillo, A., Kumar, A., Onali, S., Favale, A., & Fantini, M. C. (2023). GPR120/FFAR4: A Potential New Therapeutic Target for Inflammatory Bowel Disease. *Inflammatory Bowel Diseases*. https://doi.org/10.1093/IBD/IZAD161

Doblado, M., & Moley, K. H. (2009). Facilitative glucose transporter 9, a unique hexose and urate transporter. *Https://Doi.Org/10.1152/Ajpendo.00296.2009*, *297*(4), 831–835. https://doi.org/10.1152/AJPENDO.00296.2009

Donath, M. Y., & Shoelson, S. E. (2011). Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology 2011 11:2*, *11*(2), 98–107. https://doi.org/10.1038/nri2925

Doyle, M. E., & Egan, J. M. (2003). Pharmacological Agents That Directly Modulate Insulin Secretion. *Pharmacological Reviews*, *55*(1), 105–131. https://doi.org/10.1124/PR.55.1.7

Drel, V. R., Mashtalir, N., Ilnytska, O., Shin, J., Li, F., Lyzogubov, V. V., & Obrosova, I. G. (2006). The Leptin-Deficient (ob/ob) MouseA New Animal Model of Peripheral Neuropathy of Type 2 Diabetes and Obesity. *Diabetes*, *55*(12), 3335–3343. https://doi.org/10.2337/DB06-0885

Drzewoski, J., & Hanefeld, M. (2021). The Current and Potential Therapeutic Use of Metformin—The Good Old Drug. *Pharmaceuticals 2021, Vol. 14, Page 122*, *14*(2), 122. https://doi.org/10.3390/PH14020122

Du, Y. Q., Sha, X. Y., Cheng, J., Wang, J., Lin, J. Y., An, W. T., Pan, W., Zhang, L. J., Tao, X. N., Xu, Y. F., Jia, Y. L., Yang, Z., Xiao, P., Liu, M., Sun, J. P., & Yu, X. (2022). Endogenous Lipid-GPR120 Signaling Modulates Pancreatic Islet Homeostasis to Different Extents. *Diabetes*, *71*(7), 1454–1471. https://doi.org/10.2337/DB21-0794

Dubern, B., Mosbah, H., Pigeyre, M., Clément, K., & Poitou, C. (2022). Rare genetic causes of obesity: Diagnosis and management in clinical care. *Annales d'endocrinologie*, *83*(1), 63–72. https://doi.org/10.1016/J.ANDO.2021.12.003

Dufau, J. emy, Shen, J. X., Couchet, M., de Castro Barbosa, T., Mejhert, N., Massier, L., Griseti, E., Mouisel, E., Amri, E. Z., Lauschke, V. M., Ryden, M., & Langin, D. (2021). In vitro and ex vivo models of adipocytes. *American Journal of Physiology - Cell Physiology*, *320*(5), C822–C841. https://doi.org/10.1152/AJPCELL.00519.2020/ASSET/IMAGES/LARGE/AJPCELL.00519.2020_F004.JPEG

Duncan, R. E., Ahmadian, M., Jaworski, K., Sarkadi-Nagy, E., & Sul, H. S. (2007). Regulation of Lipolysis in Adipocytes. *Https://Doi.Org/10.1146/Annurev.Nutr.27.061406.093734*, *27*, 79–101. https://doi.org/10.1146/ANNUREV.NUTR.27.061406.093734

Dussault, I., Lin, M., Hollister, K., Fan, M., Termini, J., Sherman, M. A., & Forman, B. M. (2002). A Structural Model of the Constitutive Androstane Receptor Defines Novel Interactions That Mediate Ligand-Independent Activity. *Molecular and Cellular Biology*, *22*(15), 5270–5280. https://doi.org/10.1128/MCB.22.15.5270-5280.2002

Edfalk, S., Steneberg, P., & Edlund, H. (2008). Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion. *Diabetes*, *57*(9), 2280–2287. https://doi.org/10.2337/DB08-0307

El Buri, A., Adams, D. R., Smith, D., Tate, R. J., Mullin, M., Pyne, S., & Pyne, N. J. (2018). The sphingosine 1-phosphate receptor 2 is shed in exosomes from breast cancer cells and is N-terminally processed to a short constitutively active form

that promotes extracellular signal regulated kinase activation and DNA synthesis in fibroblasts. *Oncotarget*, *9*(50), 29453. https://doi.org/10.18632/ONCOTARGET.25658

El-Mesallamy, H. O., Kassem, D. H., El-Demerdash, E., & Amin, A. I. (2011). Vaspin and visfatin/Nampt are interesting interrelated adipokines playing a role in the pathogenesis of type 2 diabetes mellitus. *Metabolism*, *60*(1), 63–70. https://doi.org/10.1016/J.METABOL.2010.04.008

Engelstoft, M. S., Park, W. mee, Sakata, I., Kristensen, L. V., Husted, A. S., Osborne-Lawrence, S., Piper, P. K., Walker, A. K., Pedersen, M. H., Nøhr, M. K., Pan, J., Sinz, C. J., Carrington, P. E., Akiyama, T. E., Jones, R. M., Tang, C., Ahmed, K., Offermanns, S., Egerod, K. L., … Schwartz, T. W. (2013). Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. *Molecular Metabolism*, *2*(4), 376–392. https://doi.org/10.1016/J.MOLMET.2013.08.006

Enrique Martínez-Núñez, A., Gamboa-López, O. E., Bacardí-Gascón, M., & Jiménez-Cruz, A. (2017). Long-term complications and side effects of bariatric surgery: a systematic review Complicaciones a largo plazo y efectos secundarios de la cirugía bariátrica: una revisión sistemática. *JONNPR*, *2*(9), 410–415. https://doi.org/10.19230/jonnpr.1584

Eriksson, J., Taimela, S., Eriksson, K., Parviainen, S., Peltonen, J., & Kujala, U. (1997). Resistance training in the treatment of non-insulin-dependent diabetes mellitus. *International Journal of Sports Medicine*, *18*(4), 242–246. https://doi.org/10.1055/S-2007-972627

Espinoza-Fonseca, L. M. (2006). The benefits of the multi-target approach in drug design and discovery. *Bioorganic & Medicinal Chemistry*, *14*(4), 896–897. https://doi.org/10.1016/J.BMC.2005.09.011

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A. J., & Paquot, N. (2014). Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Research and Clinical Practice*, *105*(2), 141–150. https://doi.org/10.1016/J.DIABRES.2014.04.006

Etesami, B., Ghaseminezhad, S., Nowrouzi, A., Rashidipour, M., & RaziehYazdanparast. (2020). Investigation of 3T3-L1 Cell Differentiation to Adipocyte, Affected by Aqueous Seed Extract of Phoenix Dactylifera L. *Reports of Biochemistry & Molecular Biology*, *9*(1), 14. https://doi.org/10.29252/RBMB.9.1.14

Faselis, C., Katsimardou, A., Imprialos, K., Deligkaris, P., Kallistratos, M., & Dimitriadis, K. (2019). Microvascular Complications of Type 2 Diabetes Mellitus. *Current Vascular Pharmacology*, *18*(2), 117–124. https://doi.org/10.2174/1570161117666190502103733

Febriza, A., Ridwan, R., As'ad, S., Kasim, V. N., & Idrus, H. H. (2019). Adiponectin and Its Role in Inflammatory Process of Obesity. *Molecular and Cellular Biomedical Sciences*, *3*(2), 60–66. https://doi.org/10.21705/MCBS.V3I2.66

Figueiredo, L. M., Costa, E. B. O., Orellana, M. D., Picanço-Castro, V., & Covas, D. T. (2015). OP9 stromal cells proteins involved in hematoendothelial differentiation

from human embryonic stem cells. *Cellular Reprogramming*, *17*(5), 338–346. https://doi.org/10.1089/CELL.2015.0014/ASSET/IMAGES/LARGE/FIGURE4.JPEG

Filippatos, T. D., Derdemezis, C. S., Gazi, I. F., Nakou, E. S., Mikhailidis, D. P., & Elisaf, M. S. (2008). Orlistat-associated adverse effects and drug interactions: a critical review. *Drug Safety*, *31*(1), 53–65. https://doi.org/10.2165/00002018-200831010-00005

Fisk, H. L. (2021). *Adipose tissue inflammation in obesity and the influence of marine long chain polyunsaturated omega-3 fatty acids*.

Flachs, P. (2014). *The Effect of n-3 Fatty Acids on Glucose Homeostasis and Insulin Sensivity*. https://doi.org/10.33549/physiolres.932715

Flint, A. J., Hu, F. B., Glynn, R. J., Caspard, H., Manson, J. E., Willett, W. C., & Rimm, E. B. (2010). Excess weight and the risk of incident coronary heart disease among men and women. *Obesity (Silver Spring, Md.)*, *18*(2), 377–383. https://doi.org/10.1038/OBY.2009.223

Fowler, M. J. (2011). *Microvascular and Macrovascular Complications of Diabetes*. *29*(3). http://clinical.

Frayling, T. M. (2007). Genome-wide association studies provide new insights into type 2 diabetes aetiology. *Nature Reviews Genetics 2007 8:9*, *8*(9), 657–662. https://doi.org/10.1038/nrg2178

Freitas, R. D. S., & Campos, M. M. (2021). Understanding the appetite modulation pathways: The role of the FFA1 and FFA4 receptors. *Biochemical Pharmacology*, *186*, 114503. https://doi.org/10.1016/J.BCP.2021.114503

Fujiwara, K., Maekawa, F., & Yada, T. (2005). Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet β-cells: Mediation by PLC and L-type Ca2+ channel and link to insulin release. *American Journal of Physiology - Endocrinology and Metabolism*, *289*(4 52-4), 670–677. https://doi.org/10.1152/AJPENDO.00035.2005/ASSET/IMAGES/LARGE/ZH10100543150004.JPEG

Gadde, K. M., & Atkins, K. D. (2020). The limits and challenges of antiobesity pharmacotherapy. *Expert Opinion on Pharmacotherapy*, *21*(11), 1319. https://doi.org/10.1080/14656566.2020.1748599

Galindo, M. M., Voigt, N., Stein, J., Van lengerich, J., Raguse, J. D., Hofmann, T., Meyerhof, W., & Behrens, M. (2012). G Protein–Coupled Receptors in Human Fat Taste Perception. *Chemical Senses*, *37*(2), 123–139. https://doi.org/10.1093/CHEMSE/BJR069

Gandhi, G. R., Jothi, G., Antony, P. J., Balakrishna, K., Paulraj, M. G., Ignacimuthu, S., Stalin, A., & Al-Dhabi, N. A. (2014). Gallic acid attenuates high-fat diet fed-streptozotocin-induced insulin resistance via partial agonism of PPARγ in experimental type 2 diabetic rats and enhances glucose uptake through translocation and activation of GLUT4 in PI3K/p-Akt signaling pathway. *European Journal of Pharmacology*, *745*, 201–216. https://doi.org/10.1016/J.EJPHAR.2014.10.044

Gao, B., Huang, Q., Jie, Q., Lu, W. G., Wang, L., Li, X. J., Sun, Z., Hu, Y. Q., Chen, L., Liu, B. H., Liu, J., Yang, L., & Luo, Z. J. (2015). GPR120: A bi-potential mediator to modulate the osteogenic and adipogenic differentiation of BMMSCs. *Scientific Reports 2015 5:1*, *5*(1), 1–14. https://doi.org/10.1038/srep14080

García-Cantú, E. A., Alvarado-Saldaña, H. H., Támez-Pérez, H. E., & Rubio-Aguilar, G. (2014). Effects of liraglutide on weight reduction and metabolic parameters in obese patients with and without type 2 diabetes mellitus. *Medicina Universitaria*, *16*(63), 66–70. https://www.elsevier.es/en-revista-medicina-universitaria-304-articulo-effects-liraglutide-on-weight-reduction-X1665579614365466

Gasmi, A., Noor, S., Menzel, A., DoŦ#159;a, A., Pivina, L., & Bjørklund, G. (2020). Obesity and Insulin Resistance: Associations with Chronic Inflammation, Genetic and Epigenetic Factors. *Current Medicinal Chemistry*, *28*(4), 800–826. https://doi.org/10.2174/0929867327666200824112056

Gedebjerg, A., Almdal, T. P., Berencsi, K., Rungby, J., Nielsen, J. S., Witte, D. R., Friborg, S., Brandslund, I., Vaag, A., Beck-Nielsen, H., Sørensen, H. T., & Thomsen, R. W. (2018). Prevalence of micro- and macrovascular diabetes complications at time of type 2 diabetes diagnosis and associated clinical characteristics: A cross-sectional baseline study of 6958 patients in the Danish DD2 cohort. *Journal of Diabetes and Its Complications*, *32*(1), 34–40. https://doi.org/10.1016/J.JDIACOMP.2017.09.010

Gesta, S., & Kahn, C. R. (2017). White adipose tissue. *Adipose Tissue Biology: Second Edition*, 149–199. https://doi.org/10.1007/978-3-319-52031-5_5/FIGURES/3

Ghaben, A. L., & Scherer, P. E. (2019). Adipogenesis and metabolic health. *Nature Reviews Molecular Cell Biology*, *20*(4), 242–249. https://doi.org/10.1038/S41580-018-0093-Z

Ghosh, S., Ramadas, B., & Manna, D. (2022). Targeted protein degradation using the lysosomal pathway. *RSC Medicinal Chemistry*, *13*(12), 1476–1494. https://doi.org/10.1039/D2MD00273F

Godoy, A., Ulloa, V., Rodríguez, F., Reinicke, K., Yañez, A. J., De Los Angeles García, M., Medina, R. A., Carrasco, M., Barberis, S., Castro, T., Martínez, F., Koch, X., Vera, J. C., Poblete, M. T., Figueroa, C. D., Peruzzo, B., Pérez, F., & Nualart, F. (2006). Differential subcellular distribution of glucose transporters GLUT1–6 and GLUT9 in human cancer: Ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. *Journal of Cellular Physiology*, *207*(3), 614–627. https://doi.org/10.1002/JCP.20606

Gong, D., Chi, X., Ren, K., Huang, G., Zhou, G., Yan, N., Lei, J., & Zhou, Q. (2018). Structure of the human plasma membrane Ca2+-ATPase 1 in complex with its obligatory subunit neuroplastin. *Nature Communications 2018 9:1*, *9*(1), 1–9. https://doi.org/10.1038/s41467-018-06075-7

González-García, I., Tena-Sempere, M., & López, M. (2017). Estradiol regulation of brown adipose tissue thermogenesis. *Advances in Experimental Medicine and*

Biology, *1043*, 315–335. https://doi.org/10.1007/978-3-319-70178-3_15/FIGURES/1

Gotoh, C., Hong, Y. H., Iga, T., Hishikawa, D., Suzuki, Y., Song, S. H., Choi, K. C., Adachi, T., Hirasawa, A., Tsujimoto, G., Sasaki, S. ichi, & Roh, S. G. (2007). The regulation of adipogenesis through GPR120. *Biochemical and Biophysical Research Communications*, *354*(2), 591–597. https://doi.org/10.1016/J.BBRC.2007.01.028

Green, H., & Kehinde, O. (1976). Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell*, *7*(1), 105–113. https://doi.org/10.1016/0092-8674(76)90260-9

Green, H., & Kehinde, O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *Journal of Cellular Physiology*, *101*(1), 169–171. https://doi.org/10.1002/JCP.1041010119

Green, H., & Meuth, M. (1974). An established pre-adipose cell line and its differentiation in culture. *Cell*, *3*(2), 127–133. https://doi.org/10.1016/0092-8674(74)90116-0

Gregoire, F. M., Smas, C. M., & Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews*, *78*(3), 783–809. https://doi.org/10.1152/PHYSREV.1998.78.3.783/ASSET/IMAGES/LARGE/JNP.JY09F1.JPEG

Gribble, F. M., Diakogiannaki, E., & Reimann, F. (2017). Gut hormone regulation and secretion via FFA1 and FFA4. *Handbook of Experimental Pharmacology*, *236*, 181–203. https://doi.org/10.1007/164_2016_46/FIGURES/2

Grygiel-Górniak, B. (2014). Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications--a review. *Nutrition Journal*, *13*(1). https://doi.org/10.1186/1475-2891-13-17

Guillermier, C., Fazeli, P. K., Kim, S., Lun, M., Zuflacht, J. P., Milian, J., Lee, H., Francois-Saint-Cyr, H., Horreard, F., Larson, D., Rosen, E. D., Lee, R. T., Lechene, C. P., & Steinhauser, M. L. (2017). Imaging mass spectrometry demonstrates age-related decline in human adipose plasticity. *JCI Insight*, *2*(5). https://doi.org/10.1172/JCI.INSIGHT.90349

Gujral, U. P., Pradeepa, R., Weber, M. B., Narayan, K. M. V., & Mohan, V. (2013). Type 2 diabetes in South Asians: similarities and differences with white Caucasian and other populations. *Annals of the New York Academy of Sciences*, *1281*(1), 51–63. https://doi.org/10.1111/J.1749-6632.2012.06838.X

Guo, S. (2014). Insulin Signaling, Resistance, and the Metabolic Syndrome: Insights from Mouse Models to Disease Mechanisms. *The Journal of Endocrinology*, *220*(2), T1. https://doi.org/10.1530/JOE-13-0327

Guru, A., Issac, P. K., Velayutham, M., Saraswathi, N. T., Arshad, A., & Arockiaraj, J. (2020). Molecular mechanism of down-regulating adipogenic transcription factors in 3T3-L1 adipocyte cells by bioactive anti-adipogenic compounds.

Molecular Biology Reports 2020 48:1, *48*(1), 743–761. https://doi.org/10.1007/S11033-020-06036-8

Hajer, G. R., Van Haeften, T. W., & Visseren, F. L. J. (2008). Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *European Heart Journal*, *29*(24), 2959–2971. https://doi.org/10.1093/EURHEARTJ/EHN387

Halgren, T. (2007). New Method for Fast and Accurate Binding-site Identification and Analysis. *Chemical Biology & Drug Design*, *69*(2), 146–148. https://doi.org/10.1111/J.1747-0285.2007.00483.X

Hammarstedt, A., Gogg, S., Hedjazifar, S., Nerstedt, A., & Smith, U. (2018). Impaired adipogenesis and dysfunctional adipose tissue in human hypertrophic obesity. *Physiological Reviews*, *98*(4), 1911–1941. https://doi.org/10.1152/PHYSREV.00034.2017/ASSET/IMAGES/LARGE/Z9J003182820007.JPEG

Hampl, S. E., Hassink, S. G., Skinner, A. C., Armstrong, S. C., Barlow, S. E., Bolling, C. F., Avila Edwards, K. C., Eneli, I., Hamre, R., Joseph, M. M., Lunsford, D., Mendonca, E., Michalsky, M. P., Mirza, N., Ochoa, E. R., Sharifi, M., Staiano, A. E., Weedn, A. E., Flinn, S. K., … Okechukwu, K. (2023). Clinical Practice Guideline for the Evaluation and Treatment of Children and Adolescents With Obesity. *Pediatrics*, *151*(2). https://doi.org/10.1542/PEDS.2022-060640

Hansen, S. V. F., & Ulven, T. (2017). Pharmacological tool compounds for the free fatty acid receptor 4 (FFA4/GPR120). *Handbook of Experimental Pharmacology*, *236*, 33–56. https://doi.org/10.1007/164_2016_60/TABLES/3

Hara, T., Kashihara, D., Ichimura, A., Kimura, I., Tsujimoto, G., & Hirasawa, A. (2014). Role of free fatty acid receptors in the regulation of energy metabolism. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1841*(9), 1292–1300. https://doi.org/10.1016/J.BBALIP.2014.06.002

Hara, T., Kimura, I., Inoue, D., Ichimura, A., & Hirasawa, A. (2013). Free fatty acid receptors and their role in regulation of energy metabolism. *Reviews of Physiology, Biochemistry and Pharmacology*, *164*, 77–116. https://doi.org/10.1007/112_2013_13/FIGURES/00138

Harms, M., & Seale, P. (2013). Brown and beige fat: development, function and therapeutic potential. *Nature Medicine 2013 19:10*, *19*(10), 1252–1263. https://doi.org/10.1038/nm.3361

Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B., & Gloriam, D. E. (2017). Trends in GPCR drug discovery: new agents, targets and indications. *Nature Reviews Drug Discovery 2017 16:12*, *16*(12), 829–842. https://doi.org/10.1038/nrd.2017.178

Healthy Lives, Healthy People: A Call to Action on Obesity in England - GOV.UK. (n.d.). Retrieved October 30, 2023, from https://www.gov.uk/government/publications/healthy-lives-healthy-people-a-call-to-action-on-obesity-in-england

Heck, A. M., Yanovski, J. A., & Calis, K. A. (2000). Orlistat, a New Lipase Inhibitor for the Management of Obesity. *Pharmacotherapy*, *20*(3), 270. https://doi.org/10.1592/PHCO.20.4.270.34882

Hernández-Mosqueira, C., Velez-Delvalle, C., & Kuri-Harcuch, W. (2015). Tissue alkaline phosphatase is involved in lipid metabolism and gene expression and secretion of adipokines in adipocytes. *Biochimica et Biophysica Acta*, *1850*(12), 2485–2496. https://doi.org/10.1016/J.BBAGEN.2015.09.014

Hidalgo, M. A., Carretta, M. D., & Burgos, R. A. (2021). Long Chain Fatty Acids as Modulators of Immune Cells Function: Contribution of FFA1 and FFA4 Receptors. *Frontiers in Physiology*, *12*, 979. https://doi.org/10.3389/FPHYS.2021.668330/BIBTEX

Hilgendorf, K. I., Johnson, C. T., Mezger, A., Rice, S. L., Norris, A. M., Demeter, J., Greenleaf, W. J., Reiter, J. F., Kopinke, D., & Jackson, P. K. (2019). Omega-3 Fatty Acids Activate Ciliary FFAR4 to Control Adipogenesis. *Cell*, *179*(6), 1289-1305.e21. https://doi.org/10.1016/J.CELL.2019.11.005

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S., & Tsujimoto, G. (2004). Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nature Medicine 2004 11:1*, *11*(1), 90–94. https://doi.org/10.1038/nm1168

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S., & Tsujimoto, G. (2005). Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nature Medicine*, *11*(1), 90–94. https://doi.org/10.1038/NM1168

Hoffman, M. D., & Benoit, D. S. W. (2015). Agonism of Wnt-β-catenin signalling promotes mesenchymal stem cell (MSC) expansion. *Journal of Tissue Engineering and Regenerative Medicine*, *9*(11), E13–E26. https://doi.org/10.1002/TERM.1736

Holder, A. L., Goth-Goldstein, R., Lucas, D., & Koshland, C. P. (2012). Particle-induced artifacts in the MTT and LDH viability assays. *Chemical Research in Toxicology*, *25*(9), 1885. https://doi.org/10.1021/TX3001708

Hom, F. G., Goodner, C. J., & Berrie, M. A. (1984). A [3H]2-deoxyglucose method for comparing rates of glucose metabolism and insulin responses among rat tissues in vivo. Validation of the model and the absence of an insulin effect on brain. *Diabetes*, *33*(2), 141–152. https://doi.org/10.2337/DIAB.33.2.141

Hong, S., Song, W., Zushin, P. J. H., Liu, B., Jedrychowski, M. P., Mina, A. I., Deng, Z., Cabarkapa, D., Hall, J. A., Palmer, C. J., Aliakbarian, H., Szpyt, J., Gygi, S. P., Tavakkoli, A., Lynch, L., Perrimon, N., & Banks, A. S. (2018). Phosphorylation of Beta-3 adrenergic receptor at serine 247 by ERK MAP kinase drives lipolysis in obese adipocytes. *Molecular Metabolism*, *12*, 25–38. https://doi.org/10.1016/J.MOLMET.2018.03.012

Hong, Y. H., Nishimura, Y., Hishikawa, D., Tsuzuki, H., Miyahara, H., Gotoh, C., Choi, K. C., Feng, D. D., Chen, C., Lee, H. G., Katoh, K., Roh, S. G., & Sasaki, S. (2005). Acetate and Propionate Short Chain Fatty Acids Stimulate Adipogenesis

via GPCR43. *Endocrinology*, *146*(12), 5092–5099. https://doi.org/10.1210/EN.2005-0545

Hopkins, J. C. A., Blazeby, J. M., Rogers, C. A., & Welbourn, R. (2016). The use of adjustable gastric bands for management of severe and complex obesity. *British Medical Bulletin*, *118*(1), 64–72. https://doi.org/10.1093/BMB/LDW012

Hou, J. C., & Pessin, J. E. (2007). The Ins (endocytosis) and Outs (exocytosis) of GLUT4 Trafficking. *Current Opinion in Cell Biology*, *19*(4), 466. https://doi.org/10.1016/J.CEB.2007.04.018

Hou, W. L., Yin, J., Alimujiang, M., Yu, X. Y., Ai, L. G., Bao, Y. Q., Liu, F., & Jia, W. P. (2018). Inhibition of mitochondrial complex I improves glucose metabolism independently of AMPK activation. *Journal of Cellular and Molecular Medicine*, *22*(2), 1316–1328. https://doi.org/10.1111/JCMM.13432

Hsu, C. L., Lin, Y. J., Ho, C. T., & Yen, G. C. (2012). Inhibitory effects of garcinol and pterostilbene on cell proliferation and adipogenesis in 3T3-L1 cells. *Food & Function*, *3*(1), 49–57. https://doi.org/10.1039/C1FO10209E

Huang, B., Yuan, H. D., Kim, D. Y., Quan, H. Y., & Chung, S. H. (2011). Cinnamaldehyde prevents adipocyte differentiation and adipogenesis via regulation of peroxisome proliferator-activated receptor-γ (PPARγ) and AMP-activated protein kinase (AMPK) pathways. *Journal of Agricultural and Food Chemistry*, *59*(8), 3666–3673. https://doi.org/10.1021/JF104814T/ASSET/IMAGES/LARGE/JF-2010-04814T_0004.JPEG

Huang, C., Somwar, R., Patel, N., Niu, W., Török, D., & Klip, A. (2002). Sustained Exposure of L6 Myotubes to High Glucose and Insulin Decreases Insulin-Stimulated GLUT4 Translocation but Upregulates GLUT4 Activity. *Diabetes*, *51*(7), 2090–2098. https://doi.org/10.2337/DIABETES.51.7.2090

Huang, C., Zhang, Y., Gong, Z., Sheng, X., Li, Z., Zhang, W., & Qin, Y. (2006). Berberine inhibits 3T3-L1 adipocyte differentiation through the PPARγ pathway. *Biochemical and Biophysical Research Communications*, *348*(2), 571–578. https://doi.org/10.1016/J.BBRC.2006.07.095

Huang, L., Gao, L., & Chen, C. (2021). Role of Medium-Chain Fatty Acids in Healthy Metabolism: A Clinical Perspective. *Trends in Endocrinology & Metabolism*, *32*(6), 351–366. https://doi.org/10.1016/J.TEM.2021.03.002

Hudson, B. D., Christiansen, E., Tikhonova, I. G., Grundmann, M., Kostenis, E., Adams, D. R., Ulven, T., & Milligan, G. (2012). Chemically engineering ligand selectivity at the free fatty acid receptor 2 based on pharmacological variation between species orthologs. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *26*(12), 4951–4965. https://doi.org/10.1096/FJ.12-213314

Hudson, B. D., Hébert, T. E., & Kelly, M. E. M. (2010). Ligand- and Heterodimer-Directed Signaling of the CB1 Cannabinoid Receptor. *Molecular Pharmacology*, *77*(1), 1–9. https://doi.org/10.1124/MOL.109.060251

Hudson, B. D., Shimpukade, B., Mackenzie, A. E., Butcher, A. J., Pediani, J. D., Christiansen, E., Heathcote, H., Tobin, A. B., Ulven, T., & Milligan, G. (2013). The pharmacology of a potent and selective agonist, TUG-891, demonstrates both potential opportunity and possible challenges to therapeutic agonism of FFA4 (GPR120). *Mol. Pharmacol*, *84*, 710–725.

Hudson, B. D., Shimpukade, B., Milligan, G., & Ulven, T. (2014). The Molecular Basis of Ligand Interaction at Free Fatty Acid Receptor 4 (FFA4/GPR120). *The Journal of Biological Chemistry*, *289*(29), 20345. https://doi.org/10.1074/JBC.M114.561449

Hudson, B. D., Tikhonova, I. G., Pandey, S. K., Ulven, T., & Milligan, G. (2012). Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. *Journal of Biological Chemistry*, *287*(49), 41195–41209. https://doi.org/10.1074/jbc.M112.396259

Husted, A. S., Ekberg, J. H., Tripp, E., Nissen, T. A. D., Meijnikman, S., O'Brien, S. L., Ulven, T., Acherman, Y., Bruin, S. C., Nieuwdorp, M., Gerhart-Hines, Z., Calebiro, D., Dragsted, L. O., & Schwartz, T. W. (2020). Autocrine negative feedback regulation of lipolysis through sensing of NEFAs by FFAR4/GPR120 in WAT. *Molecular Metabolism*, *42*, 101103. https://doi.org/10.1016/J.MOLMET.2020.101103

Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P., & Frank, L. (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell*, *88*(1), 131–141. https://doi.org/10.1016/S0092-8674(00)81865-6

Ichimura, A., Hasegawa, S., Kasubuchi, M., & Kimura, I. (2014). Free fatty acid receptors as therapeutic targets for the treatment of diabetes. *Frontiers in Pharmacology*, *5*(NOV), 236. https://doi.org/10.3389/FPHAR.2014.00236/BIBTEX

Ichimura, A., Hirasawa, A., Poulain-Godefroy, O., Bonnefond, A., Hara, T., Yengo, L., Kimura, I., Leloire, A., Liu, N., Iida, K., Choquet, H., Besnard, P., Lecoeur, C., Vivequin, S., Ayukawa, K., Takeuchi, M., Ozawa, K., Tauber, M., Maffeis, C., … Froguel, P. (2012). Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. *Nature 2012 483:7389*, *483*(7389), 350–354. https://doi.org/10.1038/nature10798

Idris, A. I., Sophocleous, A., Landao-Bassonga, E., Canals, M., Milligan, G., Baker, D., van't Hof, R. J., & Ralston, S. H. (2009). Cannabinoid Receptor Type 1 Protects against Age- Related Osteoporosis by Regulating Osteoblast and Adipocyte Differentiation in Marrow Stromal Cells. *Cell Metabolism*, *10*(2), 139–147. https://doi.org/10.1016/J.CMET.2009.07.006

Im, D. S. (2013). Intercellular Lipid Mediators and GPCR Drug Discovery. *Biomolecules & Therapeutics*, *21*(6), 411. https://doi.org/10.4062/BIOMOLTHER.2013.080

Im, D. S. (2018). FFA4 (GPR120) as a fatty acid sensor involved in appetite control, insulin sensitivity and inflammation regulation. *Molecular Aspects of Medicine*, *64*, 92–108. https://doi.org/10.1016/J.MAM.2017.09.001

Itoh, Y., Kawamata, Y., Harada, M., Kobayashi, M., Fujii, R., Fukusumi, S., Ogi, K., Hosoya, M., Tanaka, Y., Uejima, H., Tanaka, H., Maruyama, M., Satoh, R., Okubo, S., Kizawa, H., Komatsu, H., Matsumura, F., Noguchi, Y., Shinohara, T., … Fujino, M. (2003). Free fatty acids regulate insulin secretion from pancreatic ß cells through GPR40. *Nature 2003 422:6928*, *422*(6928), 173–176. https://doi.org/10.1038/nature01478

Jacob, L., Hoffmann, B., Stoven, V., & Vert, J. P. (2008). Virtual screening of GPCRs: An in silico chemogenomics approach. *BMC Bioinformatics*, *9*(1), 1–16. https://doi.org/10.1186/1471-2105-9-363/TABLES/4

Jakab, J., Miškić, B., Mikšić, Š., Juranić, B., Ćosić, V., Schwarz, D., & Včev, A. (2021). Adipogenesis as a Potential Anti-Obesity Target: A Review of Pharmacological Treatment and Natural Products . *Diabetes, Metabolic Syndrome and Obesity*, *14*, 67–83. https://doi.org/10.2147/DMSO.S281186

Janero, D. R., & Makriyannis, A. (2009). Cannabinoid receptor antagonists: pharmacological opportunities, clinical experience, and translational prognosis. *Expert Opinion on Emerging Drugs*, *14*(1), 43–65. https://doi.org/10.1517/14728210902736568

Javeed, N., & Matveyenko, A. V. (2018). Circadian Etiology of Type 2 Diabetes Mellitus. *Physiology*, *33*(2), 138. https://doi.org/10.1152/PHYSIOL.00003.2018

Jebb, S. (1993). *is Head of Nutrition and Health Research at the Medical Research Council Human Nutrition Research Centre in Cambridge*.

Jing, X., & Sjöholm, Å. (n.d.). *Ion channel control of phasic insulin secretion*.

Juárez-Hernández, E., Chávez-Tapia, N. C., Uribe, M., & Barbero-Becerra, V. J. (2016). Role of bioactive fatty acids in nonalcoholic fatty liver disease. *Nutrition Journal*, *15*(1), 1–10. https://doi.org/10.1186/S12937-016-0191-8/FIGURES/3

Jung, U. J., & Choi, M. S. (2014). Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease. *International Journal of Molecular Sciences 2014, Vol. 15, Pages 6184-6223*, *15*(4), 6184–6223. https://doi.org/10.3390/IJMS15046184

Jyotsna, F., Ahmed, A., Kumar, K., Kaur, P., Chaudhary, H., Kumar, S., Khan, E., Khanam, B., Shah, S. U., Varrassi, G., Khatri, M., Kumar, S., Kakadiya, K. A., & Benazir, M. (2023). AMA School of Medicine, Makati, PHL 6. Dermatology, All India Institute of Medical Sciences. *National Tuberculosis Control Center*. https://doi.org/10.7759/cureus.43882

Kahn, S. E., Hull, R. L., & Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature 2006 444:7121*, *444*(7121), 840–846. https://doi.org/10.1038/nature05482

Kaikaew, K., Grefhorst, A., & Visser, J. A. (2021). Sex Differences in Brown Adipose Tissue Function: Sex Hormones, Glucocorticoids, and Their Crosstalk. *Frontiers in Endocrinology*, *12*, 652444. https://doi.org/10.3389/FENDO.2021.652444/BIBTEX

Kaisanlahti, A., & Glumoff, T. (2018). Browning of white fat: agents and implications for beige adipose tissue to type 2 diabetes. *Journal of Physiology and Biochemistry 2018 75:1*, *75*(1), 1–10. https://doi.org/10.1007/S13105-018-0658-5

Kaku, K., Araki, T., & Yoshinaka, R. (2013). Randomized, double-blind, dose-ranging study of TAK-875, a novel GPR40 agonist, in Japanese patients with inadequately controlled type 2 diabetes. *Diabetes Care*, *36*(2), 245–250. https://doi.org/10.2337/DC12-0872

Kaku, K., Enya, K., Nakaya, R., Ohira, T., & Matsuno, R. (2015). Efficacy and safety of fasiglifam (TAK-875), a G protein-coupled receptor 40 agonist, in Japanese patients with type 2 diabetes inadequately controlled by diet and exercise: a randomized, double-blind, placebo-controlled, phase III trial. *Diabetes, Obesity and Metabolism*, *17*(7), 675–681. https://doi.org/10.1111/DOM.12467

Kalderon, B., Azazmeh, N., Azulay, N., Vissler, N., Valitsky, M., & Bar-Tana, J. (2012). Suppression of adipose lipolysis by long-chain fatty acid analogs. *Journal of Lipid Research*, *53*(5), 868–878. https://doi.org/10.1194/jlr.M022673

Kalkhof, S., Büttner, P., Krieg, L., Wabitsch, M., Küntzel, C., Friebe, D., Landgraf, K., Hanschkow, M., Schubert, K., Kiess, W., Krohn, K., Blüher, M., von Bergen, M., & Körner, A. (2020). In Depth Quantitative Proteomic and Transcriptomic Characterization of Human Adipocyte Differentiation using the SGBS Cell Line. *PROTEOMICS*, *20*(15–16), 1900405. https://doi.org/10.1002/PMIC.201900405

Kang, M. C., Kang, N., Ko, S. C., Kim, Y. B., & Jeon, Y. J. (2016). Anti-obesity effects of seaweeds of Jeju Island on the differentiation of 3T3-L1 preadipocytes and obese mice fed a high-fat diet. *Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association*, *90*, 36–44. https://doi.org/10.1016/J.FCT.2016.01.023

Kapoor, R., & Huang, Y.-S. (2006). Gamma Linolenic Acid: An Antiinflammatory Omega-6 Fatty Acid. *Current Pharmaceutical Biotechnology*, *7*(6), 531–534. https://doi.org/10.2174/138920106779116874

Kargi, A. Y., & Iacobellis, G. (2014). Adipose tissue and adrenal glands: Novel pathophysiological mechanisms and clinical applications. *International Journal of Endocrinology*, *2014*. https://doi.org/10.1155/2014/614074

Karmokar, P. F., & Moniri, N. H. (2022). Oncogenic signaling of the free-fatty acid receptors FFA1 and FFA4 in human breast carcinoma cells. *Biochemical Pharmacology*, *206*, 115328. https://doi.org/10.1016/J.BCP.2022.115328

Kassotis, C. D., Hoffman, K., Völker, J., Pu, Y., Veiga-Lopez, A., Kim, S. M., Schlezinger, J. J., Bovolin, P., Cottone, E., Saraceni, A., Scandiffio, R., Atlas, E., Leingartner, K., Krager, S., Tischkau, S. A., Ermler, S., Legler, J., Chappell, V. A., Fenton, S. E., … Stapleton, H. M. (2021). Reproducibility of adipogenic

responses to metabolism disrupting chemicals in the 3T3-L1 pre-adipocyte model system: An interlaboratory study. *Toxicology*, *461*, 152900. https://doi.org/10.1016/J.TOX.2021.152900

Katome, T., Obata, T., Matsushima, R., Masuyama, N., Cantley, L. C., Gotoh, Y., Kishi, K., Shiota, H., & Ebina, Y. (2003). Use of RNA interference-mediated gene silencing and adenoviral overexpression to elucidate the roles of AKT/protein kinase B isoforms in insulin actions. *Journal of Biological Chemistry*, *278*(30), 28312–28323. https://doi.org/10.1074/jbc.M302094200

Katritch, V., Cherezov, V., & Stevens, R. C. (2013). Structure-Function of the G-protein-Coupled Receptor Superfamily. *Annual Review of Pharmacology and Toxicology*, *53*, 531. https://doi.org/10.1146/ANNUREV-PHARMTOX-032112-135923

Kautzky-Willer, A., Harreiter, J., & Pacini, G. (2016). Sex and Gender Differences in Risk, Pathophysiology and Complications of Type 2 Diabetes Mellitus. *Endocrine Reviews*, *37*(3), 278. https://doi.org/10.1210/ER.2015-1137

Kelly, D. M., & Jones, T. H. (2015). Testosterone and obesity. *Obesity Reviews : An Official Journal of the International Association for the Study of Obesity*, *16*(7), 581–606. https://doi.org/10.1111/OBR.12282

KENAKIN, T. (2001). Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *The FASEB Journal*, *15*(3), 598–611. https://doi.org/10.1096/FJ.00-0438REV

Kenakin, T. (2003). Ligand-selective receptor conformations revisited: The promise and the problem. *Trends in Pharmacological Sciences*, *24*(7), 346–354. https://doi.org/10.1016/S0165-6147(03)00167-6

Kenakin, T., & Williams, M. (2014). Defining and characterizing drug/compound function. *Biochemical Pharmacology*, *87*(1), 40–63. https://doi.org/10.1016/J.BCP.2013.07.033

Khatami, F., Mohajeri-Tehrani, M. R., & Tavangar, S. M. (2019). The Importance of Precision Medicine in Type 2 Diabetes Mellitus (T2DM): From Pharmacogenetic and Pharmacoepigenetic Aspects. *Endocrine, Metabolic & Immune Disorders - Drug Targets*, *19*(6), 719–731. https://doi.org/10.2174/1871530319666190228102212

Khattak, S. F., Spatara, M., Roberts, L., & Roberts, S. C. (2006). Application of colorimetric assays to assess viability, growth and metabolism of hydrogel-encapsulated cells. *Biotechnology Letters*, *28*(17), 1361–1370. https://doi.org/10.1007/S10529-006-9104-9/METRICS

Kim, G. W., Lin, J. E., Blomain, E. S., & Waldman, S. A. (2014). Antiobesity Pharmacotherapy: New Drugs and Emerging Targets. *Clinical Pharmacology & Therapeutics*, *95*(1), 53–66. https://doi.org/10.1038/CLPT.2013.204

Kim, J. M., Lee, K. P., Park, S. J., Kang, S., Huang, J., Lee, J. M., Sato, K., Chung, H. Y., Okajima, F., & Im, D. S. (2015). Omega-3 fatty acids induce Ca2+

mobilization responses in human colon epithelial cell lines endogenously expressing FFA4. *Acta Pharmacologica Sinica 2015 36:7*, *36*(7), 813–820. https://doi.org/10.1038/aps.2015.29

Kim, J., Okla, M., Erickson, A., Carr, T., Natarajan, S. K., & Chung, S. (2016). Eicosapentaenoic acid potentiates brown thermogenesis through FFAR4-dependent up-regulation of miR-30b and miR-378. *Journal of Biological Chemistry*, *291*(39), 20551–20562. https://doi.org/10.1074/jbc.M116.721480

Kim, N., Lee, J. O., Lee, H. J., Kim, H. I., Kim, J. K., Lee, Y. W., Lee, S. K., Kim, S. J., Park, S. H., & Kim, H. S. (2015). Endogenous ligand for GPR120, docosahexaenoic acid, exerts benign metabolic effects on the skeletal muscles via AMP-activated protein kinase pathway. *Journal of Biological Chemistry*, *290*(33), 20438–20447. https://doi.org/10.1074/jbc.M115.657379

Kim, S. M., Lun, M., Wang, M., Senyo, S. E., Guillermier, C., Patwari, P., & Steinhauser, M. L. (2014). Loss of White Adipose Hyperplastic Potential Is Associated with Enhanced Susceptibility to Insulin Resistance. *Cell Metabolism*, *20*(6), 1049–1058. https://doi.org/10.1016/J.CMET.2014.10.010

Kimura, I., Ichimura, A., Ohue-Kitano, R., & Igarashi, M. (2020). Free fatty acid receptors in health and disease. *Physiological Reviews*, *100*(1), 171–210. https://doi.org/10.1152/PHYSREV.00041.2018/ASSET/IMAGES/LARGE/Z9J0012029240008.JPEG

Klumpers, L. E., Fridberg, M., De Kam, M. L., Little, P. B., Jensen, N. O., Kleinloog, H. D., Elling, C. E., & Van Gerven, J. M. A. (2013). Peripheral selectivity of the novel cannabinoid receptor antagonist TM38837 in healthy subjects. *British Journal of Clinical Pharmacology*, *76*(6), 846–857. https://doi.org/10.1111/BCP.12141

Knebel, B., Fahlbusch, P., Poschmann, G., Dille, M., Wahlers, N., Stühler, K., Hartwig, S., Lehr, S., Schiller, M., Jacob, S., Kettel, U., Müller-Wieland, D., & Kotzka, J. (2019). Adipokinome Signatures in Obese Mouse Models Reflect Adipose Tissue Health and Are Associated with Serum Lipid Composition. *International Journal of Molecular Sciences*, *20*(10), 2559. https://doi.org/10.3390/IJMS20102559

Koh, E., Kim, B., & Choi, K. (2021). Torreya nucifera seed oil improves 3T3-L1 adipocyte differentiation. *BMC Complementary Medicine and Therapies*, *21*(1). https://doi.org/10.1186/S12906-021-03429-5

Kohjima, M., Enjoji, M., Higuchi, N., Kato, M., Kotoh, K., Yoshimoto, T., Fujino, T., Yada, M., Yada, R., Harada, N., Takayanagi, R., & Nakamuta, M. (2007). Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *International Journal of Molecular Medicine*, *20*(3), 351–358. https://doi.org/10.3892/IJMM.20.3.351/HTML

Kopelman, P. G. (1994). Hormones and obesity. *Bailliere's Clinical Endocrinology and Metabolism*, *8*(3), 549–575. https://doi.org/10.1016/S0950-351X(05)80286-1

Kopelman, P. G. (2000). Obesity as a medical problem. *Nature 2000 404:6778*, *404*(6778), 635–643. https://doi.org/10.1038/35007508

Kotarsky, K., Nilsson, N. E., Flodgren, E., Owman, C., & Olde, B. (2003). A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. *Biochemical and Biophysical Research Communications*, *301*(2), 406–410. https://doi.org/10.1016/S0006-291X(02)03064-4

Krentz, A. J., Clough, G., & Byrne, C. D. (2007). Interactions between microvascular and macrovascular disease in diabetes: pathophysiology and therapeutic implications. *Diabetes, Obesity and Metabolism*, *9*(6), 781–791. https://doi.org/10.1111/J.1463-1326.2007.00670.X

Kretzschmar, K., & Watt, F. M. (2012). Lineage tracing. *Cell*, *148*(1–2), 33–45. https://doi.org/10.1016/J.CELL.2012.01.002

Kropp, M., Golubnitschaja, O., Mazurakova, A., Koklesova, L., Sargheini, N., Vo, T. T. K. S., de Clerck, E., Polivka, J., Potuznik, P., Polivka, J., Stetkarova, I., Kubatka, P., & Thumann, G. (2023). Diabetic retinopathy as the leading cause of blindness and early predictor of cascading complications—risks and mitigation. *EPMA Journal 2023 14:1*, *14*(1), 21–42. https://doi.org/10.1007/S13167-023-00314-8

Kusminski, C. M., McTernan, P. G., & Kumar, S. (2005). Role of resistin in obesity, insulin resistance and Type II diabetes. *Clinical Science*, *109*(3), 243–256. https://doi.org/10.1042/CS20050078

Lafontan, M. (2014). Adipose tissue and adipocyte dysregulation. *Diabetes & Metabolism*, *40*(1), 16–28. https://doi.org/10.1016/J.DIABET.2013.08.002

Lai, C. S., Chen, Y. Y., Lee, P. S., Kalyanam, N., Ho, C. T., Liou, W. S., Yu, R. C., & Pan, M. H. (2016). Bisdemethoxycurcumin Inhibits Adipogenesis in 3T3-L1 Preadipocytes and Suppresses Obesity in High-Fat Diet-Fed C57BL/6 Mice. *Journal of Agricultural and Food Chemistry*, *64*(4), 821–830. https://doi.org/10.1021/ACS.JAFC.5B05577

Lamri, A., Bonnefond, A., Meyre, D., Balkau, B., Roussel, R., Marre, M., Froguel, P., & Fumeron, F. (2016). Interaction between GPR120 p.R270H loss-of-function variant and dietary fat intake on incident type 2 diabetes risk in the D.E.S.I.R. study. *Nutrition, Metabolism and Cardiovascular Diseases*, *26*(10), 931–936. https://doi.org/10.1016/J.NUMECD.2016.04.010

Lass, A., Zimmermann, R., Oberer, M., & Zechner, R. (2011). Lipolysis – A highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. *Progress in Lipid Research*, *50*(1–4), 14. https://doi.org/10.1016/J.PLIPRES.2010.10.004

Latif, W., Lambrinos, K. J., & Rodriguez, R. (2021). Compare And Contrast the Glucagon-like Peptide-1 Receptor Agonists (GLP1RAs). *StatPearls*. http://europepmc.org/books/NBK572151

Lee, J. H., Park, A., Oh, K. J., Lee, S. C., Kim, W. K., & Bae, K. H. (2019). The Role of Adipose Tissue Mitochondria: Regulation of Mitochondrial Function for the Treatment of Metabolic Diseases. *International Journal of Molecular Sciences 2019, Vol. 20, Page 4924*, *20*(19), 4924. https://doi.org/10.3390/IJMS20194924

Lee, S. R., Jeong, S. H., Mukae, M., Jeong, K. J., Kwun, H. J., & Hong, E. J. (2023). GLUT4 degradation by GLUTFOURINH® in mice resembles moderate-obese diabetes of human with hyperglycemia and low lipid accumulation. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1869*(5), 166668. https://doi.org/10.1016/J.BBADIS.2023.166668

Lefere, S., Vergote, K., De Bruyne, R., Provoost, V., & Satalkar, P. P. (2023). 'A radical operation' – a thematic analysis of newspaper framing of bariatric surgery in adolescents. *BMC Public Health*, *23*(1), 1–10. https://doi.org/10.1186/S12889-023-15366-8/TABLES/1

Leto, D., & Saltiel, A. R. (2012). Regulation of glucose transport by insulin: traffic control of GLUT4. *Nature Reviews Molecular Cell Biology 2012 13:6*, *13*(6), 383–396. https://doi.org/10.1038/nrm3351

Li, Q., Blume, S. W., Huang, J. C., Hammer, M., & Ganz, M. L. (2015). Prevalence and healthcare costs of obesity-related comorbidities: Evidence from an electronic medical records system in the United States. *Journal of Medical Economics*, *18*(12), 1020–1028. https://doi.org/10.3111/13696998.2015.1067623

Li, Q., Manolescu, A., Ritzel, M., Yao, S., Slugoski, M., Young, J. D., Chen, X. Z., & Cheeseman, C. I. (2004). Cloning and functional characterization of the human GLUT7 isoform SLC2A7 from the small intestine. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *287*(1 50-1), 236–242. https://doi.org/10.1152/AJPGI.00396.2003/ASSET/IMAGES/LARGE/ZH30070417291007.JPEG

Li, S., Pan, J., Hu, X., Zhang, Y., Gong, D., & Zhang, G. (2020). Kaempferol inhibits the activity of pancreatic lipase and its synergistic effect with orlistat. *Journal of Functional Foods*, *72*, 104041. https://doi.org/10.1016/J.JFF.2020.104041

Li, Y., Li, Z., Ngandiri, D. A., Llerins Perez, M., Wolf, A., & Wang, Y. (2022). The Molecular Brakes of Adipose Tissue Lipolysis. *Frontiers in Physiology*, *13*. https://doi.org/10.3389/FPHYS.2022.826314

Lin, X., & Li, H. (2021). Obesity: Epidemiology, Pathophysiology, and Therapeutics. *Frontiers in Endocrinology*, *12*, 1070. https://doi.org/10.3389/FENDO.2021.706978/BIBTEX

Liou, A. P., Lu, X., Sei, Y., Zhao, X., Pechhold, S., Carrero, R. J., Raybould, H. E., & Wank, S. (2011). The G-Protein–Coupled Receptor GPR40 Directly Mediates Long-Chain Fatty Acid–Induced Secretion of Cholecystokinin. *Gastroenterology*, *140*(3), 903-912.e4. https://doi.org/10.1053/J.GASTRO.2010.10.012

Liu, C., Zou, Y., & Qian, H. (2020). GLP-1R agonists for the treatment of obesity: a patent review (2015-present). *Expert Opinion on Therapeutic Patents*, *30*(10), 781–794. https://doi.org/10.1080/13543776.2020.1811851

Liu, D., Wang, L., Meng, Q., Kuang, H., & Liu 1?, A. X. (2012). G-protein coupled receptor 120 is involved in glucose metabolism in fat cells. *Cellular and Molecular Biology*, *58*(2). https://doi.org/10.1170/206

Liu, H. Da, Wang, W. B., Xu, Z. G., Liu, C. H., He, D. F., Du, L. P., Li, M. Y., Yu, X., & Sun, J. P. (2015). FFA4 receptor (GPR120): A hot target for the development of anti-diabetic therapies. *European Journal of Pharmacology*, *763*, 160–168. https://doi.org/10.1016/J.EJPHAR.2015.06.028

Liu, J., Ting, J. P., Al-azzam, S., Ding, Y., & Afshar, S. (2021). Therapeutic Advances in Diabetes, Autoimmune, and Neurological Diseases. *International Journal of Molecular Sciences 2021, Vol. 22, Page 2805*, *22*(6), 2805. https://doi.org/10.3390/IJMS22062805

Liu, K. L., Yang, Y. C., Yao, H. T., Chia, T. W., Lu, C. Y., Li, C. C., Tsai, H. J., Lii, C. K., & Chen, H. W. (2016). Docosahexaenoic acid inhibits inflammation via free fatty acid receptor FFA4, disruption of TAB2 interaction with TAK1/TAB1 and downregulation of ERK-dependent Egr-1 expression in EA.hy926 cells. *Molecular Nutrition & Food Research*, *60*(2), 430–443. https://doi.org/10.1002/MNFR.201500178

Liu, T., Ji, R. L., & Tao, Y. X. (2022). Naturally occurring mutations in G protein-coupled receptors associated with obesity and type 2 diabetes mellitus. *Pharmacology & Therapeutics*, *234*, 108044. https://doi.org/10.1016/J.PHARMTHERA.2021.108044

Livingston, E. H., & Cassidy, L. (2005). Statistical Power and Estimation of the Number of Required Subjects for a Study Based on the t-Test: A Surgeon's Primer. *Journal of Surgical Research*, *126*(2), 149–159. https://doi.org/10.1016/J.JSS.2004.12.013

Lizunov, V. A., Matsumoto, H., Zimmerberg, J., Cushman, S. W., & Frolov, V. A. (2005). Insulin stimulates the halting, tethering, and fusion of mobile GLUT4 vesicles in rat adipose cells. *The Journal of Cell Biology*, *169*(3), 481. https://doi.org/10.1083/JCB.200412069

Loneker, A. E., Alisafaei, F., Kant, A., Li, D., Janmey, P. A., Shenoy, V. B., & Wells, R. G. (2023). Lipid droplets are intracellular mechanical stressors that impair hepatocyte function. *Proceedings of the National Academy of Sciences of the United States of America*, *120*(16), e2216811120. https://doi.org/10.1073/PNAS.2216811120/SUPPL_FILE/PNAS.2216811120.SM02.AVI

Lopez Vicchi, F., Luque, G. M., Brie, B., Nogueira, J. P., Garcia Tornadu, I., & Becu-Villalobos, D. (2016). Dopaminergic drugs in type 2 diabetes and glucose homeostasis. *Pharmacological Research*, *109*, 74–80. https://doi.org/10.1016/J.PHRS.2015.12.029

Lumaquin, D. (2022). *LIPID DROPLETS ARE A METABOLIC VULNERABILITY IN MELANOMA*.

Lutz, T. A., & Woods, S. C. (2012). Overview of Animal Models of Obesity. *Current Protocols in Pharmacology / Editorial Board, S.J. Enna (Editor-in-Chief) ... [et Al.]*, *CHAPTER*(SUPPL.58), Unit5.61. https://doi.org/10.1002/0471141755.PH0561S58

Lv, X., Fang, K., Hao, W., Han, Y., Yang, N., & Yu, Q. (2020). Identification of Reactive Hypoglycemia with Different Basic BMI and Its Causes by Prolonged Oral Glucose Tolerance Test . *Diabetes, Metabolic Syndrome and Obesity*, *13*, 4717–4726. https://doi.org/10.2147/DMSO.S280084

Lyssenko, V., Jonsson, A., Almgren, P., Pulizzi, N., Isomaa, B., Tuomi, T., Berglund, G., Altshuler, D., Nilsson, P., & Groop, L. (2008). Clinical Risk Factors, DNA Variants, and the Development of Type 2 Diabetes. *New England Journal of Medicine*, *359*(21), 2220–2232. https://doi.org/10.1056/NEJMOA0801869/SUPPL_FILE/NEJM_LYSSENKO_2220SA1.PDF

MacDonald, P. E., Joseph, J. W., & Rorsman, P. (2005). Glucose-sensing mechanisms in pancreatic -cells. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*(1464), 2211–2225. https://doi.org/10.1098/RSTB.2005.1762

Madiraju, A. K., Erion, D. M., Rahimi, Y., Zhang, X. M., Braddock, D. T., Albright, R. A., Prigaro, B. J., Wood, J. L., Bhanot, S., MacDonald, M. J., Jurczak, M. J., Camporez, J. P., Lee, H. Y., Cline, G. W., Samuel, V. T., Kibbey, R. G., & Shulman, G. I. (2014). Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature 2014 510:7506*, *510*(7506), 542–546. https://doi.org/10.1038/nature13270

Mahler, R. J., & Adler, M. L. (1999). Type 2 Diabetes Mellitus: Update on Diagnosis, Pathophysiology, and Treatment. *The Journal of Clinical Endocrinology & Metabolism*, *84*(4), 1165–1171. https://doi.org/10.1210/JCEM.84.4.5612

Malmberg, Å., Mohell, N., Backlund Höök, B., Johansson, A. M., Hacksell, U., & Nordvall, G. (1998). Interactions of ligands with active and inactive conformations of the dopamine D2 receptor. *European Journal of Pharmacology*, *346*(2–3), 299–307. https://doi.org/10.1016/S0014-2999(98)00047-8

Mammi, C., Marzolla, V., Armani, A., Feraco, A., Antelmi, A., Maslak, E., Chlopicki, S., Cinti, F., Hunt, H., Fabbri, A., & Caprio, M. (2016). A novel combined glucocorticoid-mineralocorticoid receptor selective modulator markedly prevents weight gain and fat mass expansion in mice fed a high-fat diet. *International Journal of Obesity 2016 40:6*, *40*(6), 964–972. https://doi.org/10.1038/ijo.2016.13

Mangmool, S., & Kurose, H. (2011). Gi/o Protein-Dependent and -Independent Actions of Pertussis Toxin (PTX). *Toxins 2011, Vol. 3, Pages 884-899*, *3*(7), 884–899. https://doi.org/10.3390/TOXINS3070884

Manickam, E., Sinclair, A. J., & Cameron-Smith, D. (2010). Suppressive actions of eicosapentaenoic acid on lipid droplet formation in 3T3-L1 adipocytes. *Lipids in Health and Disease*, *9*(1), 1–8. https://doi.org/10.1186/1476-511X-9-57/TABLES/2

Mantas, I., Saarinen, M., Xu, Z. Q. D., & Svenningsson, P. (2021). Update on GPCR-based targets for the development of novel antidepressants. *Molecular Psychiatry 2021 27:1*, *27*(1), 534–558. https://doi.org/10.1038/s41380-021-01040-1

Marcelin, G., Silveira, A. L. M., Martins, L. B., Ferreira, A. V. M., & Clément, K. (2019). Deciphering the cellular interplays underlying obesity-induced adipose tissue fibrosis. *The Journal of Clinical Investigation*, *129*(10), 4032. https://doi.org/10.1172/JCI129192

Marion-Letellier, R., Savoye, G., & Ghosh, S. (2016). Fatty acids, eicosanoids and PPAR gamma. *European Journal of Pharmacology*, *785*, 44–49. https://doi.org/10.1016/J.EJPHAR.2015.11.004

Masri, B., Salahpour, A., Didriksen, M., Ghisi, V., Beaulieu, J. M., Gainetdinov, R. R., & Caron, M. G. (2008). Antagonism of dopamine D2 receptor/β-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(36), 13656–13661. https://doi.org/10.1073/PNAS.0803522105/SUPPL_FILE/0803522105SI.PDF

Mauer, Y., Parker, M., & Kashyap, S. R. (2021). Antiobesity drug therapy: An individualized and comprehensive approach. *Cleveland Clinic Journal of Medicine*, *88*(8), 440–448. https://doi.org/10.3949/CCJM.88A.20080

McGill, J. B., Haller, H., Roy-Chaudhury, P., Cherrington, A., Wada, T., Wanner, C., Ji, L., & Rossing, P. (2022). Making an impact on kidney disease in people with type 2 diabetes: the importance of screening for albuminuria. *BMJ Open Diabetes Research & Care*, *10*(4), 2806. https://doi.org/10.1136/BMJDRC-2022-002806

MD, J., DH, R., CM, A., JD, A., AG, C., KA, D., FB, H., VS, H., JM, J., RF, K., CM, L., BE, M., CA, N., FX, P.-S., J, S., VJ, S., TA, W., BM, W., SZ, Y., … GF, T. (2014). 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society. *Circulation*, *129*(25 Suppl 2). https://doi.org/10.1161/01.CIR.0000437739.71477.EE

Meddings, J. B., Scott, R. B., & Fick, G. H. (1989). Analysis and comparison of sigmoidal curves: application to dose-response data. *Https://Doi.Org/10.1152/Ajpgi.1989.257.6.G982*, *257*(6). https://doi.org/10.1152/AJPGI.1989.257.6.G982

Meier, U., & Gressner, A. M. (2004). Endocrine Regulation of Energy Metabolism: Review of Pathobiochemical and Clinical Chemical Aspects of Leptin, Ghrelin, Adiponectin, and Resistin. *Clinical Chemistry*, *50*(9), 1511–1525. https://doi.org/10.1373/CLINCHEM.2004.032482

Midlam, C. (2021). Status of Biologic Drugs in Modern Therapeutics-Targeted Therapies vs. Small Molecule Drugs. *Biologics, Biosimilars, and Biobetters: An*

Introduction for Pharmacists, Physicians, and Other Health Practitioners, 31–46. https://doi.org/10.1002/9781119564690.CH3

Milligan, G. (2003). Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Molecular Pharmacology*, *64*(6), 1271–1276. https://doi.org/10.1124/MOL.64.6.1271

Milligan, G., Alvarez-Curto, E., Hudson, B. D., Prihandoko, R., & Tobin, A. B. (2017). FFA4/GPR120: Pharmacology and Therapeutic Opportunities. *Trends in Pharmacological Sciences*, *38*(9), 809. https://doi.org/10.1016/J.TIPS.2017.06.006

Milligan, G., Shimpukade, B., Ulven, T., & Hudson, B. D. (2017). Complex pharmacology of free fatty acid receptors. *Chemical Reviews*, *117*(1), 67–110. https://doi.org/10.1021/ACS.CHEMREV.6B00056/ASSET/IMAGES/LARGE/CR-2016-00056N_0030.JPEG

Min, T., & Bain, S. C. (2021). The Role of Tirzepatide, Dual GIP and GLP-1 Receptor Agonist, in the Management of Type 2 Diabetes: The SURPASS Clinical Trials. *Diabetes Therapy*, *12*(1), 143–157. https://doi.org/10.1007/S13300-020-00981-0/TABLES/4

Miyauchi, S., Hirasawa, A., Iga, T., Liu, N., Itsubo, C., Sadakane, K., Hara, T., & Tsujimoto, G. (2009). Distribution and regulation of protein expression of the free fatty acid receptor GPR120. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *379*(4), 427–434. https://doi.org/10.1007/S00210-008-0390-8

Moniri, N. H. (2016). Free-fatty acid receptor-4 (GPR120): Cellular and molecular function and its role in metabolic disorders. *Biochemical Pharmacology*, *110-111*, 1–15. https://doi.org/10.1016/J.BCP.2016.01.021

Moodaley, R., Smith, D. M., Tough, I. R., Schindler, M., & Cox, H. M. (2017). Agonism of free fatty acid receptors 1 and 4 generates peptide YY-mediated inhibitory responses in mouse colon. *British Journal of Pharmacology*, *174*(23), 4508. https://doi.org/10.1111/BPH.14054

Moore, K., Zhang, Q., Murgolo, N., Hosted, T., & Duffy, R. (2009). Cloning, expression, and pharmacological characterization of the GPR120 free fatty acid receptor from cynomolgus monkey: comparison with human GPR120 splice variants. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *154*(4), 419–426. https://doi.org/10.1016/J.CBPB.2009.08.005

Moraes-Vieira, P. M., Saghatelian, A., & Kahn, B. B. (2016). GLUT4 Expression in Adipocytes Regulates De Novo Lipogenesis and Levels of a Novel Class of Lipids With Antidiabetic and Anti-inflammatory Effects. *Diabetes*, *65*(7), 1808–1815. https://doi.org/10.2337/DB16-0221

Morello, G., Imperatore, R., Palomba, L., Finelli, C., Labruna, G., Pasanisi, F., Sacchetti, L., Buono, L., Piscitelli, F., Orlando, P., Di Marzo, V., & Cristino, L. (2016). Orexin-A represses satiety-inducing POMC neurons and contributes to obesity via stimulation of endocannabinoid signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(17), 4759–

4764. https://doi.org/10.1073/PNAS.1521304113/SUPPL_FILE/PNAS.1521304113.SAPP.PDF

Moreno-Navarrete, J. M., & Fernández-Real, J. M. (2017). Adipocyte differentiation. *Adipose Tissue Biology: Second Edition*, 69–90. https://doi.org/10.1007/978-3-319-52031-5_3/FIGURES/2

Morgens, D. W., Wainberg, M., Boyle, E. A., Ursu, O., Araya, C. L., Kimberly Tsui, C., Haney, M. S., Hess, G. T., Han, K., Jeng, E. E., Li, A., Snyder, M. P., Greenleaf, W. J., Kundaje, A., & Bassik, M. C. (2017). Genome-scale measurement of off-target activity using Cas9 toxicity in high-throughput screens. *Nature Communications 2017 8:1*, *8*(1), 1–8. https://doi.org/10.1038/ncomms15178

Morigny, P., Boucher, J., Arner, P., & Langin, D. (2021). Lipid and glucose metabolism in white adipocytes: pathways, dysfunction and therapeutics. *Nature Reviews Endocrinology 2021 17:5*, *17*(5), 276–295. https://doi.org/10.1038/s41574-021-00471-8

Morrison, S., & McGee, S. L. (2015). 3T3-L1 adipocytes display phenotypic characteristics of multiple adipocyte lineages. *Adipocyte*, *4*(4), 295. https://doi.org/10.1080/21623945.2015.1040612

Mueckler, M., & Thorens, B. (2013). The SLC2 (GLUT) family of membrane transporters. *Molecular Aspects of Medicine*, *34*(2–3), 121–138. https://doi.org/10.1016/J.MAM.2012.07.001

Muoio, D. M., & Newgard, C. B. (2008). Molecular and metabolic mechanisms of insulin resistance and β-cell failure in type 2 diabetes. *Nature Reviews Molecular Cell Biology 2008 9:3*, *9*(3), 193–205. https://doi.org/10.1038/nrm2327

Naltrexone/bupropion for obesity. (2017). *Drug and Therapeutics Bulletin*, *55*(11), 126–129. https://doi.org/10.1136/DTB.2017.11.0550

Naqvi, A. A. T., Mohammad, T., Hasan, G. M., & Hassan, Md. I. (2019). Advancements in Docking and Molecular Dynamics Simulations Towards Ligand-receptor Interactions and Structure-function Relationships. *Current Topics in Medicinal Chemistry*, *18*(20), 1755–1768. https://doi.org/10.2174/1568026618666181025114157

Navale, A. M., & Paranjape, A. N. (2016). Glucose transporters: physiological and pathological roles. *Biophysical Reviews*, *8*(1), 5. https://doi.org/10.1007/S12551-015-0186-2

Nedergaard, J., Bengtsson, T., & Cannon, B. (2007). Unexpected evidence for active brown adipose tissue in adult humans. *American Journal of Physiology. Endocrinology and Metabolism*, *293*(2). https://doi.org/10.1152/AJPENDO.00691.2006

Nguyen, P., Leray, V., Diez, M., Serisier, S., Le Bloc'H, J., Siliart, B., & Dumon, H. (2008). Liver lipid metabolism. *Journal of Animal Physiology and Animal Nutrition*, *92*(3), 272–283. https://doi.org/10.1111/J.1439-0396.2007.00752.X

Ni, Y., Zhao, X., Bao, G., Zou, L., Teng, L., Wang, Z., Song, M., Xiong, J., Bai, Y., & Pei, G. (2006). Activation of ß2-adrenergic receptor stimulates γ-secretase activity and accelerates amyloid plaque formation. *Nature Medicine 2006 12:12*, *12*(12), 1390–1396. https://doi.org/10.1038/nm1485

Nieto Gutierrez, A., & McDonald, P. H. (2018). GPCRs: Emerging anti-cancer drug targets. *Cellular Signalling*, *41*, 65–74. https://doi.org/10.1016/J.CELLSIG.2017.09.005

Nilsson, N. E., Kotarsky, K., Owman, C., & Olde, B. (2003). Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. *Biochemical and Biophysical Research Communications*, *303*(4), 1047–1052. https://doi.org/10.1016/S0006-291X(03)00488-1

Nitta, C. F., & Orlando, R. A. (2013). Crosstalk between Immune Cells and Adipocytes Requires Both Paracrine Factors and Cell Contact to Modify Cytokine Secretion. *PLOS ONE*, *8*(10), e77306. https://doi.org/10.1371/JOURNAL.PONE.0077306

Nøhr, M. K., Pedersen, M. H., Gille, A., Egerod, K. L., Engelstoft, M. S., Husted, A. S., Sichlau, R. M., Grunddal, K. V., Poulsen, S. S., Han, S., Jones, R. M., Offermanns, S., & Schwartz, T. W. (2013). GPR41/FFAR3 and GPR43/FFAR2 as Cosensors for Short-Chain Fatty Acids in Enteroendocrine Cells vs FFAR3 in Enteric Neurons and FFAR2 in Enteric Leukocytes. *Endocrinology*, *154*(10), 3552–3564. https://doi.org/10.1210/EN.2013-1142

Norton, L., Shannon, C., Gastaldelli, A., & DeFronzo, R. A. (2022). Insulin: The master regulator of glucose metabolism. *Metabolism*, *129*, 155142. https://doi.org/10.1016/J.METABOL.2022.155142

Nye, C., Kim, J., Kalhan, S. C., & Hanson, R. W. (2008). Reassessing triglyceride synthesis in adipose tissue. *Trends in Endocrinology and Metabolism*, *19*(10), 356–361. https://doi.org/10.1016/j.tem.2008.08.003

Offermanns, S. (2014). Free Fatty Acid (FFA) and Hydroxy Carboxylic Acid (HCA) Receptors. *Https://Doi.Org/10.1146/Annurev-Pharmtox-011613-135945*, *54*, 407–434. https://doi.org/10.1146/ANNUREV-PHARMTOX-011613-135945

Oh, D. Y., Talukdar, S., Bae, E. J., Imamura, T., Morinaga, H., Fan, W. Q., Li, P., Lu, W. J., Watkins, S. M., & Olefsky, J. M. (2010). GPR120 is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-Inflammatory and Insulin Sensitizing Effects. *Cell*, *142*(5), 687. https://doi.org/10.1016/J.CELL.2010.07.041

Oh, D. Y., Walenta, E., Akiyama, T. E., Lagakos, W. S., Lackey, D., Pessentheiner, A. R., Sasik, R., Hah, N., Chi, T. J., Cox, J. M., Powels, M. A., Di Salvo, J., Sinz, C., Watkins, S. M., Armando, A. M., Chung, H., Evans, R. M., Quehenberger, O., McNelis, J., … Olefsky, J. M. (2014). A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. *Nature Medicine 2014 20:8*, *20*(8), 942–947. https://doi.org/10.1038/nm.3614

Olsen, R. H. J., DiBerto, J. F., English, J. G., Glaudin, A. M., Krumm, B. E., Slocum, S. T., Che, T., Gavin, A. C., McCorvy, J. D., Roth, B. L., & Strachan, R. T. (2020). TRUPATH, an open-source biosensor platform for interrogating the GPCR transducerome. *Nature Chemical Biology 2020 16:8*, *16*(8), 841–849. https://doi.org/10.1038/s41589-020-0535-8

Otto, T. C., & Lane, M. D. (2008). Adipose Development: From Stem Cell to Adipocyte. *Https://Doi.Org/10.1080/10409230591008189*, *40*(4), 229–242. https://doi.org/10.1080/10409230591008189

OWEN, M. R., DORAN, E., & HALESTRAP, A. P. (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal*, *348*(3), 607–614. https://doi.org/10.1042/BJ3480607

Padda, I. S., Mahtani, A. U., & Parmar, M. (2022). Sodium-Glucose Transport Protein 2 (SGLT2) Inhibitors. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK576405/

Park, A., Kim, W. K., & Bae, K.-H. (2014). Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. *World Journal of Stem Cells*, *6*(1), 33. https://doi.org/10.4252/WJSC.V6.I1.33

Pei, Y., Song, Y., Wang, B., Lin, C., Yang, Y., Li, H., & Feng, Z. (2022). Integrated lipidomics and RNA sequencing analysis reveal novel changes during 3T3- L1 cell adipogenesis. *PeerJ*, *10*, e13417. https://doi.org/10.7717/PEERJ.13417/SUPP-2

Perera, H. K. I., Clarke, M., Morris, N. J., Hong, W., Chamberlain, L. H., & Gould, G. W. (2003). Syntaxin 6 Regulates Glut4 Trafficking in 3T3-L1 Adipocytes. *Molecular Biology of the Cell*, *14*(7), 2946. https://doi.org/10.1091/MBC.E02-11-0722

Perry, R. J., Peng, L., Cline, G. W., Wang, Y., Rabin-Court, A., Song, J. D., Zhang, D., Zhang, X. M., Nozaki, Y., Dufour, S., Petersen, K. F., & Shulman, G. I. (2018). Mechanisms by which a Very-Low-Calorie Diet Reverses Hyperglycemia in a Rat Model of Type 2 Diabetes. *Cell Metabolism*, *27*(1), 210-217.e3. https://doi.org/10.1016/J.CMET.2017.10.004

Pessin, J. E., & Bell, G. I. (2003). Mammalian Facilitative Glucose Transporter Family: Structure and Molecular Regulation. *Https://Doi.Org/10.1146/Annurev.Ph.54.030192.004403*, *54*, 911–930. https://doi.org/10.1146/ANNUREV.PH.54.030192.004403

Pfeil, E. M., Brands, J., Merten, N., Inoue, A., Simon, K., & Kostenis, E. (2020). Heterotrimeric G Protein Subunit Gaq Is a Master Switch for Gbg-Mediated Calcium Mobilization by Gi-Coupled GPCRs Graphical Abstract Highlights d Newly delineated mechanism of Ca 2+ signaling by Gi-G-protein-coupled receptors d Paradigmatic Gi-Gbg-Ca 2+ signals require Ga q subunit in living cells d Relieving auto-inhibition of PLCb empowers Gbg-Ca 2+ signaling in the absence of Ga q Correspondence. *Molecular Cell*, *80*, 940-954.e6. https://doi.org/10.1016/j.molcel.2020.10.027

Pigeyre, M., Yazdi, F. T., Kaur, Y., & Meyre, D. (2016). Recent progress in genetics, epigenetics and metagenomics unveils the pathophysiology of human obesity. *Clinical Science (London, England : 1979)*, *130*(12), 943–986. https://doi.org/10.1042/CS20160136

Piper, N. B. C., Whitfield, E. A., Stewart, G. D., Xu, X., & Furness, S. G. B. (2022). Targeting appetite and satiety in diabetes and obesity, via G protein-coupled receptors. *Biochemical Pharmacology*, *202*, 115115. https://doi.org/10.1016/J.BCP.2022.115115

Pi-Sunyer, F. X., Becker, D. M., Bouchard, C., Carleton, R. A., Colditz, G. A., Dietz, W. H., Foreyt, J. P., Garrison, R. J., Grundy, S. M., Hansen, B. C., Higgins, M., Hill, J. O., Howard, B. V., Kuczmarski, R. J., Kumanyika, S., Legako, R. D., Prewitt, T. E., Rocchini, A. P., Smith, P. L., … Chiquette, E. (1998). Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: executive summary. Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults. *The American Journal of Clinical Nutrition*, *68*(4), 899–917. https://doi.org/10.1093/AJCN/68.4.899

Portha, B., Tourrel-Cuzin, C., & Movassat, J. (2011). Activation of the GLP-1 Receptor Signalling Pathway: A Relevant Strategy to Repair a Deficient Beta-Cell Mass. *Experimental Diabetes Research*, *2011*, 11. https://doi.org/10.1155/2011/376509

Poulos, S. P., Dodson, M. V., & Hausman, G. J. (2010). Cell line models for differentiation: preadipocytes and adipocytes. *Experimental Biology and Medicine (Maywood, N.J.)*, *235*(10), 1185–1193. https://doi.org/10.1258/EBM.2010.010063

Poulos, S. P., Dodson, M. V., & Hausman, G. J. (2010). Cell line models for differentiation: preadipocytes and adipocytes. *Https://Doi.Org/10.1258/Ebm.2010.010063*, *235*(10), 1185–1193. https://doi.org/10.1258/EBM.2010.010063

Pozo, M., & Claret, M. (2018). Hypothalamic Control of Systemic Glucose Homeostasis: The Pancreas Connection. *Trends in Endocrinology and Metabolism*, *29*(8), 581–594. https://doi.org/10.1016/j.tem.2018.05.001

Prihandoko, R., Alvarez-Curto, E., Hudson, B. D., Butcher, A. J., Ulven, T., Miller, A. M., Tobin, A. B., & Milligan, G. (2016). Distinct phosphorylation clusters determine the signaling outcome of free fatty acid receptor 4/g protein-coupled receptor 120. *Molecular Pharmacology*, *89*(5), 505–520. https://doi.org/10.1124/MOL.115.101949

Proctor, K. M., Miller, S. C. M., Bryant, N. J., & Gould, G. W. (2006). Syntaxin 16 controls the intracellular sequestration of GLUT4 in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications*, *347*(2), 433–438. https://doi.org/10.1016/J.BBRC.2006.06.135

Quesada-López, T., Cereijo, R., Turatsinze, J. V., Planavila, A., Cairó, M., Gavaldà-Navarro, A., Peyrou, M., Moure, R., Iglesias, R., Giralt, M., Eizirik, D. L., & Villarroya, F. (2016). The lipid sensor GPR120 promotes brown fat activation and

FGF21 release from adipocytes. *Nature Communications 2016 7:1*, *7*(1), 1–17. https://doi.org/10.1038/ncomms13479

Quesada-López, T., Gavalda-Navarro, A., Morón-Ros, S., Campderrós, L., Iglesias, R., Giralt, M., & Villarroya, F. (2019). GPR120 controls neonatal brown adipose tissue thermogenic induction. *American Journal of Physiology - Endocrinology and Metabolism*, *317*(5), E742–E750. https://doi.org/10.1152/AJPENDO.00081.2019/ASSET/IMAGES/LARGE/ZH100919081730005.JPEG

Quinn, C. E., Hamilton, P. K., Lockhart, C. J., & McVeigh, G. E. (2008). Thiazolidinediones: effects on insulin resistance and the cardiovascular system. *British Journal of Pharmacology*, *153*(4), 636–645. https://doi.org/10.1038/SJ.BJP.0707452

Rachner, T. D., Khosla, S., & Hofbauer, L. C. (2011). Osteoporosis: now and the future. *The Lancet*, *377*(9773), 1276–1287. https://doi.org/10.1016/S0140-6736(10)62349-5

Rahman, M. S., Hossain, K. S., Das, S., Kundu, S., Adegoke, E. O., Rahman, M. A., Hannan, M. A., Uddin, M. J., & Pang, M. G. (2021). Role of Insulin in Health and Disease: An Update. *International Journal of Molecular Sciences 2021, Vol. 22, Page 6403*, *22*(12), 6403. https://doi.org/10.3390/IJMS22126403

Raisz, L. G. (2005). Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *The Journal of Clinical Investigation*, *115*(12), 3318–3325. https://doi.org/10.1172/JCI27071

Rashid, A. J., O'Dowd, B. F., Verma, V., & George, S. R. (2007). Neuronal Gq/11-coupled dopamine receptors: an uncharted role for dopamine. *Trends in Pharmacological Sciences*, *28*(11), 551–555. https://doi.org/10.1016/J.TIPS.2007.10.001

Rasmussen, M. F. (2020). The development of oral semaglutide, an oral GLP-1 analog, for the treatment of type 2 diabetes. *Diabetology International*, *11*(2), 76–86. https://doi.org/10.1007/S13340-019-00423-8/FIGURES/4

Regnier, S. M., El-Hashani, E., Kamau, W., Zhang, X., Massad, N. L., & Sargis, R. M. (2015). Tributyltin differentially promotes development of a phenotypically distinct adipocyte. *Obesity*, *23*(9), 1864–1871. https://doi.org/10.1002/OBY.21174

Ren, Y., Zhao, H., Yin, C., Lan, X., Wu, L., Du, X., Griffiths, H. R., & Gao, D. (2022). Adipokines, Hepatokines and Myokines: Focus on Their Role and Molecular Mechanisms in Adipose Tissue Inflammation. *Frontiers in Endocrinology*, *13*, 873699. https://doi.org/10.3389/FENDO.2022.873699/BIBTEX

Reusch, J. E. B., Colton, L. A., & Klemm, D. J. (2000). CREB Activation Induces Adipogenesis in 3T3-L1 Cells. *Molecular and Cellular Biology*, *20*(3), 1008. https://doi.org/10.1128/MCB.20.3.1008-1020.2000

Rhythm Initiates Phase 2 Clinical Trial of RM-493 for Obesity | Fierce Biotech. (n.d.). Retrieved November 1, 2023, from https://www.fiercebiotech.com/biotech/rhythm-initiates-phase-2-clinical-trial-of-rm-493-for-obesity

Richard, A. J., White, U., Elks, C. M., & Stephens, J. M. (2020). Adipose Tissue: Physiology to Metabolic Dysfunction. *Endotext*. https://www.ncbi.nlm.nih.gov/books/NBK555602/

Riss, T., Niles, A., Moravec, R., Karassina, N., & Vidugiriene, J. (2019). Cytotoxicity Assays: In Vitro Methods to Measure Dead Cells. *Assay Guidance Manual*. https://www.ncbi.nlm.nih.gov/sites/books/NBK540958/

Ritter, K., Buning, C., Halland, N., Pöverlein, C., & Schwink, L. (2016). G Protein-Coupled Receptor 119 (GPR119) Agonists for the Treatment of Diabetes: Recent Progress and Prevailing Challenges. *Journal of Medicinal Chemistry*, *59*(8), 3579–3592. https://doi.org/10.1021/ACS.JMEDCHEM.5B01198/ASSET/IMAGES/LARGE/JM-2015-01198N_0003.JPEG

Roden, M., & Shulman, G. I. (2019). The integrative biology of type 2 diabetes. *Nature*, *576*(7785), 51–60. https://doi.org/10.1038/S41586-019-1797-8

Rodriguez-Pacheco, F., Garcia-Serrano, S., Garcia-Escobar, E., Gutierrez-Repiso, C., Garcia-Arnes, J., Valdes, S., Gonzalo, M., Soriguer, F., Moreno-Ruiz, F. J., Rodriguez-Cañete, A., Gallego-Perales, J. L., Martinez-Ferriz, A., Rojo-Martínez, G., & Garcia-Fuentes, E. (2014). Effects of obesity/fatty acids on the expression of GPR120. *Molecular Nutrition & Food Research*, *58*(9), 1852–1860. https://doi.org/10.1002/MNFR.201300666

Rojas, L. B. A., & Gomes, M. B. (2013). Metformin: an old but still the best treatment for type 2 diabetes. *Diabetology & Metabolic Syndrome 2013 5:1*, *5*(1), 1–15. https://doi.org/10.1186/1758-5996-5-6

Rosen, E. D., & Spiegelman, B. M. (2006). Adipocytes as regulators of energy balance and glucose homeostasis. *Nature*, *444*(7121), 847. https://doi.org/10.1038/NATURE05483

Ruiz-Ojeda, F. J., Rupérez, A. I., Gomez-Llorente, C., Gil, A., & Aguilera, C. M. (2016). Cell Models and Their Application for Studying Adipogenic Differentiation in Relation to Obesity: A Review. *International Journal of Molecular Sciences 2016, Vol. 17, Page 1040*, *17*(7), 1040. https://doi.org/10.3390/IJMS17071040

Ryder, J. R., Fox, C. K., & Kelly, A. S. (2018). Treatment Options for Severe Obesity in the Pediatric Population: Current Limitations and Future Opportunities. *Obesity*, *26*(6), 951–960. https://doi.org/10.1002/OBY.22196

Sadie-Van Gijsen, H. (2019). Adipocyte biology: It is time to upgrade to a new model. *Journal of Cellular Physiology*, *234*(3), 2399–2425. https://doi.org/10.1002/JCP.27266

Saikia, S., Bordoloi, M., & Sarmah, R. (2018). Established and In-trial GPCR Families in Clinical Trials: A Review for Target Selection. *Current Drug Targets*, *20*(5), 522–539. https://doi.org/10.2174/1389450120666181105152439

Saini, R. K., & Keum, Y. S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance — A review. *Life Sciences*, *203*, 255–267. https://doi.org/10.1016/J.LFS.2018.04.049

Saleh, J., Al-Wardy, N., Farhan, H., Al-Khanbashi, M., & Cianflone, K. (2011). Acylation stimulating protein: a female lipogenic factor? *Obesity Reviews : An Official Journal of the International Association for the Study of Obesity*, *12*(6), 440–448. https://doi.org/10.1111/J.1467-789X.2010.00832.X

Sampath, S. C., Sampath, S. C., Bredella, M. A., Cypess, A. M., & Torriani, M. (2016). Imaging of Brown Adipose Tissue: State of the Art. *Radiology*, *280*(1), 4. https://doi.org/10.1148/RADIOL.2016150390

Samuel, V. T., & Shulman, G. I. (2016). The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *The Journal of Clinical Investigation*, *126*(1), 12. https://doi.org/10.1172/JCI77812

Sanchez-Gurmaches, J., & Guertin, D. A. (2014). Adipocyte lineages: Tracing back the origins of fat. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1842*(3), 340–351. https://doi.org/10.1016/J.BBADIS.2013.05.027

Sanchez-Rangel, E., & Inzucchi, S. E. (2017). Metformin: clinical use in type 2 diabetes. *Diabetologia*, *60*(9), 1586–1593. https://doi.org/10.1007/S00125-017-4336-X/FIGURES/3

Sanders, M. P. A., McGuire, R., Roumen, L., De Esch, I. J. P., De Vlieg, J., Klomp, J. P. G., & De Graaf, C. (2012). From the protein's perspective: the benefits and challenges of protein structure-based pharmacophore modeling. *MedChemComm*, *3*(1), 28–38. https://doi.org/10.1039/C1MD00210D

Sasaki, T., Minoshima, S., Shiohama, A., Shintani, A., Shimizu, A., Asakawa, S., Kawasaki, K., & Shimizu, N. (2001). Molecular Cloning of a Member of the Facilitative Glucose Transporter Gene Family GLUT11 (SLC2A11) and Identification of Transcription Variants. *Biochemical and Biophysical Research Communications*, *289*(5), 1218–1224. https://doi.org/10.1006/BBRC.2001.6101

Satapati, S., Qian, Y., Wu, M. S., Petrov, A., Dai, G., Wang, S. P., Zhu, Y., Shen, X., Muise, E. S., Chen, Y., Zycband, E., Weinglass, A., Di Salvo, J., Debenham, J. S., Cox, J. M., Lan, P., Shah, V., Previs, S. F., Erion, M., … Shang, J. (2017). GPR120 suppresses adipose tissue lipolysis and synergizes with GPR40 in antidiabetic efficacy. *Journal of Lipid Research*, *58*(8), 1561–1578. https://doi.org/10.1194/jlr.M075044

Sawzdargo, M., George, S. R., Nguyen, T., Xu, S., Kolakowski, L. F., & O'dowd, B. F. (1997). A Cluster of Four Novel Human G Protein-Coupled Receptor Genes Occurring in Close Proximity to CD22 Gene on Chromosome 19q13.1. *Biochemical and Biophysical Research Communications*, *239*(2), 543–547. https://doi.org/10.1006/BBRC.1997.7513

Scaioli, E., Liverani, E., & Belluzzi, A. (2017). The Imbalance between n-6/n-3 Polyunsaturated Fatty Acids and Inflammatory Bowel Disease: A Comprehensive Review and Future Therapeutic Perspectives. *International Journal of Molecular Sciences 2017, Vol. 18, Page 2619*, *18*(12), 2619. https://doi.org/10.3390/IJMS18122619

Schalkwijk, C. G., & Stehouwer, C. D. A. (2005). Vascular complications in diabetes mellitus: the role of endothelial dysfunction. *Clinical Science*, *109*(2), 143–159. https://doi.org/10.1042/CS20050025

Schimmel, R. J. (1984). Stimulation of cAMP accumulation and lipolysis in hamster adipocytes with forskolin. *Https://Doi.Org/10.1152/Ajpcell.1984.246.1.C63*, *15*(1). https://doi.org/10.1152/AJPCELL.1984.246.1.C63

Schweiger, M., Eichmann, T. O., Taschler, U., Zimmermann, R., Zechner, R., & Lass, A. (2014). Measurement of Lipolysis. *Methods in Enzymology*, *538*, 171. https://doi.org/10.1016/B978-0-12-800280-3.00010-4

Scorletti, E., & Byrne, C. D. (2013). Omega-3 Fatty Acids, Hepatic Lipid Metabolism, and Nonalcoholic Fatty Liver Disease. *Https://Doi.Org/10.1146/Annurev-Nutr-071812-161230*, *33*, 231–248. https://doi.org/10.1146/ANNUREV-NUTR-071812-161230

Sebaugh, J. L. (2011). Guidelines for accurate EC50/IC50 estimation. *Pharmaceutical Statistics*, *10*(2), 128–134. https://doi.org/10.1002/PST.426

Shahbazian, H., & Rezaii, I. (2013). Diabetic kidney disease; review of the current knowledge. *Journal of Renal Injury Prevention*, *2*(2), 73. https://doi.org/10.12861/JRIP.2013.24

Shank, R. P., & Maryanoff, B. E. (2008). Molecular pharmacodynamics, clinical therapeutics, and pharmacokinetics of topiramate. *CNS Neuroscience and Therapeutics*, *14*(2), 120–142. https://doi.org/10.1111/J.1527-3458.2008.00041.X

Shao, S., Xu, Q. Q., Yu, X., Pan, R., & Chen, Y. (2020). Dipeptidyl peptidase 4 inhibitors and their potential immune modulatory functions. *Pharmacology & Therapeutics*, *209*, 107503. https://doi.org/10.1016/J.PHARMTHERA.2020.107503

Shavadia, J. S., Sharma, A., Gu, X., Neaton, J., DeLeve, L., Holmes, D., Home, P., Eckel, R. H., Watkins, P. B., & Granger, C. B. (2019). Determination of fasiglifam-induced liver toxicity: Insights from the data monitoring committee of the fasiglifam clinical trials program. *Clinical Trials (London, England)*, *16*(3), 253–262. https://doi.org/10.1177/1740774519836766

Sheena, A., Mohan, S. S., Haridas, N. P. A., & Anilkumar, G. (2011). Elucidation of the Glucose Transport Pathway in Glucose Transporter 4 via Steered Molecular Dynamics Simulations. *PLoS ONE*, *6*(10). https://doi.org/10.1371/JOURNAL.PONE.0025747

Sheng, X., Tucci, J., Malvar, J., & Mittelman, S. D. (2013). Adipocyte differentiation is affected by media height above the cell layer. *International Journal of Obesity 2014 38:2*, *38*(2), 315–320. https://doi.org/10.1038/ijo.2013.96

Shimpukade, B., Hudson, B. D., Hovgaard, C. K., Milligan, G., & Ulven, T. (2012). Discovery of a potent and selective GPR120 agonist. *Journal of Medicinal Chemistry*, *55*(9), 4511–4515. https://doi.org/10.1021/JM300215X

Shin, D. W., Kim, S. N., Lee, S. M., Lee, W., Song, M. J., Park, S. M., Lee, T. R., Baik, J. H., Kim, H. K., Hong, J. H., & Noh, M. (2009). (−)-Catechin promotes adipocyte differentiation in human bone marrow mesenchymal stem cells through PPARγ transactivation. *Biochemical Pharmacology*, *77*(1), 125–133. https://doi.org/10.1016/J.BCP.2008.09.033

Si, P., Ding, S., Yuan, J., Lou, X. W., & Kim, D. H. (2011). Hierarchically structured one-dimensional TiO2 for protein immobilization, direct electrochemistry, and mediator-free glucose sensing. *ACS Nano*, *5*(9), 7617–7626. https://doi.org/10.1021/NN202714C/SUPPL_FILE/NN202714C_SI_001.PDF

Singh, A. K., Yadav, D., Sharma, N., & Jin, J. O. (2021). Dipeptidyl Peptidase (DPP)-IV Inhibitors with Antioxidant Potential Isolated from Natural Sources: A Novel Approach for the Management of Diabetes. *Pharmaceuticals*, *14*(6). https://doi.org/10.3390/PH14060586

Sirdah, M. M., & Reading, N. S. (2020). Genetic predisposition in type 2 diabetes: A promising approach toward a personalized management of diabetes. *Clinical Genetics*, *98*(6), 525–547. https://doi.org/10.1111/CGE.13772

Sliwoski, G., Kothiwale, S., Meiler, J., & Lowe, E. W. (2014). Computational Methods in Drug Discovery. *Pharmacological Reviews*, *66*(1), 334–395. https://doi.org/10.1124/PR.112.007336

Smith, U., & Kahn, B. B. (2016). Adipose tissue regulates insulin sensitivity: role of adipogenesis, de novo lipogenesis and novel lipids. *Journal of Internal Medicine*, *280*(5), 465–475. https://doi.org/10.1111/JOIM.12540

Sommer, G., Garten, A., Petzold, S., Beck-Sickinger, A. G., Blüher, M., Stumvoll, M., & Fasshauer, M. (2008). Visfatin/PBEF/Nampt: structure, regulation and potential function of a novel adipokine. *Clinical Science*, *115*(1), 13–23. https://doi.org/10.1042/CS20070226

Song, T., Yang, Y., Zhou, Y., Wei, H., & Peng, J. (2017). GPR120: a critical role in adipogenesis, inflammation, and energy metabolism in adipose tissue. *Cellular and Molecular Life Sciences*, *74*(15), 2723–2733. https://doi.org/10.1007/S00018-017-2492-2/FIGURES/2

Song, T., Zhou, Y., Peng, J., Tao, Y. X., Yang, Y., Xu, T., Peng, J., Ren, J., Xiang, Q., & Wei, H. (2016). GPR120 promotes adipogenesis through intracellular calcium and extracellular signal-regulated kinase 1/2 signal pathway. *Molecular and Cellular Endocrinology*, *434*, 1–13. https://doi.org/10.1016/J.MCE.2016.06.009

Sparks, S. M., Chen, G., Collins, J. L., Danger, D., Dock, S. T., Jayawickreme, C., Jenkinson, S., Laudeman, C., Leesnitzer, M. A., Liang, X., Maloney, P., McCoy, D. C., Moncol, D., Rash, V., Rimele, T., Vulimiri, P., Way, J. M., & Ross, S. (2014). Identification of diarylsulfonamides as agonists of the free fatty acid receptor 4 (FFA4/GPR120). *Bioorganic & Medicinal Chemistry Letters*, *24*(14), 3100–3103. https://doi.org/10.1016/J.BMCL.2014.05.012

Steneberg, P., Rubins, N., Bartoov-Shifman, R., Walker, M. D., & Edlund, H. (2005). The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. *Cell Metabolism*, *1*(4), 245–258. https://doi.org/10.1016/J.CMET.2005.03.007

Stoddart, L. A., & Milligan, G. (2010). Constitutive Activity of GPR40/FFA1: Intrinsic or Assay Dependent? *Methods in Enzymology*, *484*(C), 569–590. https://doi.org/10.1016/B978-0-12-381298-8.00028-9

Stoddart, L. A., Smith, N. J., Jenkins, L., Brown, A. J., & Milligan, G. (2008). Conserved polar residues in transmembrane domains V, VI, and VII of free fatty acid receptor 2 and free fatty acid receptor 3 are required for the binding and function of short chain fatty acids. *Journal of Biological Chemistry*, *283*(47), 32913–32924. https://doi.org/10.1074/jbc.M805601200

Stolerman, E. S., & Florez, J. C. (2009). Genomics of type 2 diabetes mellitus: implications for the clinician. *Nature Reviews Endocrinology 2009 5:8*, *5*(8), 429–436. https://doi.org/10.1038/nrendo.2009.129

Stone, V. M., Dhayal, S., Brocklehurst, K. J., Lenaghan, C., Sörhede Winzell, M., Hammar, M., Xu, X., Smith, D. M., & Morgan, N. G. (2014). GPR120 (FFAR4) is preferentially expressed in pancreatic delta cells and regulates somatostatin secretion from murine islets of Langerhans. *Diabetologia*, *57*(6), 1182–1191. https://doi.org/10.1007/S00125-014-3213-0

Strange, P. G. (2002). Mechanisms of inverse agonism at G-protein-coupled receptors. *Trends in Pharmacological Sciences*, *23*(2), 89–95. https://doi.org/10.1016/S0165-6147(02)01993-4

Sun, J., Zhou, H., Deng, Y., Zhang, Y., Gu, P., Ge, S., & Fan, X. (2012). Conditioned Medium from Bone Marrow Mesenchymal Stem Cells Transiently Retards Osteoblast Differentiation by Downregulating Runx2. *Cells Tissues Organs*, *196*(6), 510–522. https://doi.org/10.1159/000339245

Suzuki, T., Igari, S. I., Hirasawa, A., Hata, M., Ishiguro, M., Fujieda, H., Itoh, Y., Hirano, T., Nakagawa, H., Ogura, M., Makishima, M., Tsujimoto, G., & Miyata, N. (2008). Identification of G protein-coupled receptor 120-selective agonists derived from PPARgamma agonists. *Journal of Medicinal Chemistry*, *51*(23), 7640–7644. https://doi.org/10.1021/JM800970B

Sykaras, A. G., Demenis, C., Case, R. M., McLaughlin, J. T., & Smith, C. P. (2012). Duodenal Enteroendocrine I-Cells Contain mRNA Transcripts Encoding Key Endocannabinoid and Fatty Acid Receptors. *PLOS ONE*, *7*(8), e42373. https://doi.org/10.1371/JOURNAL.PONE.0042373

Tak, Y. J., & Lee, S. Y. (2021). Long-Term Efficacy and Safety of Anti-Obesity Treatment: Where Do We Stand? *Current Obesity Reports*, *10*(1), 14–30. https://doi.org/10.1007/S13679-020-00422-W

Takashima, N., Fujioka, A., Hayasaka, N., Matsuo, A., Takasaki, J., & Shigeyoshi, Y. (2006). Gq/11-induced intracellular calcium mobilization mediates Per2 acute induction in Rat-1 fibroblasts. *Genes to Cells*, *11*(9), 1039–1049. https://doi.org/10.1111/J.1365-2443.2006.00999.X

Takemitsu, H., Mori, N., & Okada, Y. (2015). Identification of Free Fatty Acid Receptors GPR40/FFAR1 and GPR120/FFAR4 in a Domestic Cat. *Article in Asian Journal of Animal and Veterinary Advances*. https://doi.org/10.3923/ajava.2015

Takic, M., Pokimica, B., Petrovic-Oggiano, G., & Popovic, T. (2022). Effects of Dietary α-Linolenic Acid Treatment and the Efficiency of Its Conversion to Eicosapentaenoic and Docosahexaenoic Acids in Obesity and Related Diseases. *Molecules 2022, Vol. 27, Page 4471*, *27*(14), 4471. https://doi.org/10.3390/MOLECULES27144471

Tan, S. Y., Mei Wong, J. L., Sim, Y. J., Wong, S. S., Mohamed Elhassan, S. A., Tan, S. H., Ling Lim, G. P., Rong Tay, N. W., Annan, N. C., Bhattamisra, S. K., & Candasamy, M. (2019). Type 1 and 2 diabetes mellitus: A review on current treatment approach and gene therapy as potential intervention. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, *13*(1), 364–372. https://doi.org/10.1016/J.DSX.2018.10.008

Tang, T., Hartig, C., Chen, Q., Zhao, W., Kaiser, A., Zhang, X., Zhang, H., Qu, H., Yi, C., Ma, L., Han, S., Zhao, Q., Beck-Sickinger, A. G., & Wu, B. (2021). Structural basis for ligand recognition of the neuropeptide Y Y2 receptor. *Nature Communications 2021 12:1*, *12*(1), 1–9. https://doi.org/10.1038/s41467-021-21030-9

Teck, J. (2022). Diabetes-Associated Comorbidities. *Primary Care: Clinics in Office Practice*, *49*(2), 275–286. https://doi.org/10.1016/J.POP.2021.11.004

Tews, D., Brenner, R. E., Siebert, R., Debatin, K. M., Fischer-Posovszky, P., & Wabitsch, M. (2022). 20 Years with SGBS cells - a versatile in vitro model of human adipocyte biology. *International Journal of Obesity 2022 46:11*, *46*(11), 1939–1947. https://doi.org/10.1038/s41366-022-01199-9

Thong, F. S. L., Dugani, C. B., & Klip, A. (2005). Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. *Physiology*, *4*, 271–284. https://doi.org/10.1152/PHYSIOL.00017.2005/ASSET/IMAGES/LARGE/Y0017-5-03.JPEG

Tomita, T., Masuzaki, H., Iwakura, H., Fujikura, J., Noguchi, M., Tanaka, T., Ebihara, K., Kawamura, J., Komoto, I., Kawaguchi, Y., Fujimoto, K., Doi, R., Shimada, Y., Hosoda, K., Imamura, M., & Nakao, K. (2006). Expression of the gene for a membrane-bound fatty acid receptor in the pancreas and islet cell tumours in humans: Evidence for GPR40 expression in pancreatic beta cells and implications for insulin secretion. *Diabetologia*, *49*(5), 962–968. https://doi.org/10.1007/S00125-006-0193-8/FIGURES/3

Trigatti, B. L., Mangroo, D., & Gerber, G. E. (1991). Photoaffinity labeling and fatty acid permeation in 3T3-L1 adipocytes. *The Journal of Biological Chemistry*, *266*(33), 22621–22625. https://doi.org/10.1016/s0021-9258(18)54616-0

Turner, P. A., Harris, L. M., Purser, C. A., Baker, R. C., & Janorkar, A. V. (2014). A surface-tethered spheroid model for functional evaluation of 3T3-L1 adipocytes. *Biotechnology and Bioengineering*, *111*(1), 174–183. https://doi.org/10.1002/BIT.25099

Tutino, V., Orlando, A., Russo, F., & Notarnicola, M. (2016). Hydroxytyrosol Inhibits Cannabinoid CB1 Receptor Gene Expression in 3T3-L1 Preadipocyte Cell Line. *Journal of Cellular Physiology*, *231*(2), 483–489. https://doi.org/10.1002/JCP.25094

Tyagi, N. K., Puntheeranurak, T., Raja, M., Kumar, A., Wimmer, B., Neundlinger, I., Gruber, H., Hinterdorfer, P., & Kinne, R. K. H. (2011). A biophysical glance at the outer surface of the membrane transporter SGLT1. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1808*(1), 1–18. https://doi.org/10.1016/J.BBAMEM.2010.07.028

Ulven, T., & Christiansen, E. (2015). Dietary Fatty Acids and Their Potential for Controlling Metabolic Diseases Through Activation of FFA4/GPR120. *Https://Doi.Org/10.1146/Annurev-Nutr-071714-034410*, *35*(1), 239–263. https://doi.org/10.1146/ANNUREV-NUTR-071714-034410

van der Vusse, G. J. (2009). Albumin as Fatty Acid Transporter. *Drug Metabolism and Pharmacokinetics*, *24*(4), 300–307. https://doi.org/10.2133/DMPK.24.300

Vieira, W. A., Sadie-Van Gijsen, H., & Ferris, W. F. (2016). Free fatty acid G-protein coupled receptor signaling in M1 skewed white adipose tissue macrophages. *Cellular and Molecular Life Sciences 2016 73:19*, *73*(19), 3665–3676. https://doi.org/10.1007/S00018-016-2263-5

Villegas-Comonfort, S., Takei, Y., Tsujimoto, G., Hirasawa, A., & García-Sáinz, J. A. (2017). Effects of arachidonic acid on FFA4 receptor: Signaling, phosphorylation and internalization. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *117*, 1–10. https://doi.org/10.1016/j.plefa.2017.01.013

Vinciguerra, M., Veyrat-Durebex, C., Moukil, M. A., Rubbia-Brandt, L., Rohner-Jeanrenaud, F., & Foti, M. (2008). PTEN Down-Regulation by Unsaturated Fatty Acids Triggers Hepatic Steatosis via an NF-κBp65/mTOR-Dependent Mechanism. *Gastroenterology*, *134*(1), 268–280. https://doi.org/10.1053/J.GASTRO.2007.10.010

Vinik, A., Ullal, J., Parson, H. K., & Casellini, C. M. (2006). Diabetic neuropathies: clinical manifestations and current treatment options. *Nature Clinical Practice Endocrinology & Metabolism 2006 2:5*, *2*(5), 269–281. https://doi.org/10.1038/ncpendmet0142

Vishwanath, D., Srinivasan, H., Patil, M. S., Seetarama, S., Agrawal, S. K., Dixit, M. N., & Dhar, K. (2013). Novel method to differentiate 3T3 L1 cells in vitro to

produce highly sensitive adipocytes for a GLUT4 mediated glucose uptake using fluorescent glucose analog. *Journal of Cell Communication and Signaling*, *7*(2), 129–140. https://doi.org/10.1007/S12079-012-0188-9

Vodyanik, M. A., Bork, J. A., Thomson, J. A., & Slukvin, I. I. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood*, *105*(2), 617–626. https://doi.org/10.1182/BLOOD-2004-04-1649

Wagenaar, L. J., Kuck, E. M., & Hoekstra, J. B. L. (1999). Troglitazone. Is it all over? *The Netherlands Journal of Medicine*, *55*(1), 4–12. https://doi.org/10.1016/S0300-2977(99)00021-2

Walters, W. P., & Namchuk, M. (2003). Designing screens: how to make your hits a hit. *Nature Reviews Drug Discovery 2003 2:4*, *2*(4), 259–266. https://doi.org/10.1038/nrd1063

Wang, B., Charukeshi Chandrasekera, P., & J. Pippin, J. (n.d.). *Leptin- and Leptin Receptor-Deficient Rodent Models: Relevance for Human Type 2 Diabetes*.

Wang, J. Y., Wang, Q. W., Yang, X. Y., Yang, W., Li, D. R., Jin, J. Y., Zhang, H. C., & Zhang, X. F. (2023). GLP−1 receptor agonists for the treatment of obesity: Role as a promising approach. *Frontiers in Endocrinology*, *14*, 1085799. https://doi.org/10.3389/FENDO.2023.1085799/BIBTEX

Watson, R. T., Kanzaki, M., & Pessin, J. E. (2004). Regulated Membrane Trafficking of the Insulin-Responsive Glucose Transporter 4 in Adipocytes. *Endocrine Reviews*, *25*(2), 177–204. https://doi.org/10.1210/ER.2003-0011

Watson, S.-J., Brown, A. J., & Holliday, N. D. (2012). Differential signaling by splice variants of the human free fatty acid receptor GPR120. *Mol. Pharmacol*, *81*, 631–642.

Watterson, K. R., Hansen, S. V. F., Hudson, B. D., Alvarez-Curto, E., Raihan, S. Z., Azevedo, C. M. G., Martin, G., Dunlop, J., Yarwood, S. J., Ulven, T., & Milligan, G. (2017). Probe-Dependent Negative Allosteric Modulators of the Long-Chain Free Fatty Acid Receptor FFA4. *Molecular Pharmacology*, *91*(6), 630. https://doi.org/10.1124/MOL.116.107821

Watterson, K. R., Hudson, B. D., Ulven, T., & Milligan, G. (2014). Treatment of Type 2 Diabetes by Free Fatty Acid Receptor Agonists. *Frontiers in Endocrinology*, *5*(AUG). https://doi.org/10.3389/FENDO.2014.00137

Wells, J. C. K. (2007). Sexual dimorphism of body composition. *Best Practice & Research Clinical Endocrinology & Metabolism*, *21*(3), 415–430. https://doi.org/10.1016/J.BEEM.2007.04.007

Weyand, C. M., & Goronzy, J. J. (2016). Aging of the immune system: Mechanisms and therapeutic targets. *Annals of the American Thoracic Society*, *13*(Suppl 5), S422–S428. https://doi.org/10.1513/ANNALSATS.201602-095AW/SUPPL_FILE/DISCLOSURES.PDF

Wolins, N. E., Quaynor, B. K., Skinner, J. R., Tzekov, A., Park, C., Choi, K., & Bickel, P. E. (2006). OP9 mouse stromal cells rapidly differentiate into adipocytes: characterization of a useful new model of adipogenesis. *Journal of Lipid Research*, *47*(2), 450–460. https://doi.org/10.1194/JLR.D500037-JLR200

Wright, E. M., LOO, D. D. F. L., & Hirayama, B. A. (2011). Biology of human sodium glucose transporters. *Physiological Reviews*, *91*(2), 733–794. https://doi.org/10.1152/PHYSREV.00055.2009/ASSET/IMAGES/LARGE/Z9J0021125720040.JPEG

Wright, S. M., & Aronne, L. J. (2012). Causes of obesity. *Abdominal Imaging*, *37*(5), 730–732. https://doi.org/10.1007/S00261-012-9862-X/TABLES/1

Xie, S., Furjanic, M. A., Ferrara, J. J., McAndrew, N. R., Ardino, E. L., Ngondara, A., Bernstein, Y., Thomas, K. J., Kim, E., Walker, J. M., Nagar, S., Ward, S. J., & Raffa, R. B. (2007). The endocannabinoid system and rimonabant: a new drug with a novel mechanism of action involving cannabinoid CB1 receptor antagonism – or inverse agonism – as potential obesity treatment and other therapeutic use. *Journal of Clinical Pharmacy and Therapeutics*, *32*(3), 209–231. https://doi.org/10.1111/J.1365-2710.2007.00817.X

Xiong, Y., Swaminath, G., Cao, Q., Yang, L., Guo, Q., Salomonis, H., Lu, J., Houze, J. B., Dransfield, P. J., Wang, Y., Liu, J. J., Wong, S., Schwandner, R., Steger, F., Baribault, H., Liu, L., Coberly, S., Miao, L., Zhang, J., … Schwarz, M. (2013). Activation of FFA1 mediates GLP-1 secretion in mice. Evidence for allosterism at FFA1. *Molecular and Cellular Endocrinology*, *369*(1–2), 119–129. https://doi.org/10.1016/J.MCE.2013.01.009

Yamashita, J. K., Nishikawa, S., & Nishikawa, S. I. (2009). Potential of Embryonic and Induced Pluripotent Stem Cell Differentiation Culture for Vascular Biology. *Essentials of Stem Cell Biology, Second Edition*, 265–272. https://doi.org/10.1016/B978-0-12-374729-7.00032-9

Yan, Y., Jiang, W., Spinetti, T., Tardivel, A., Castillo, R., Bourquin, C., Guarda, G., Tian, Z., Tschopp, J., & Zhou, R. (2013). Omega-3 Fatty Acids Prevent Inflammation and Metabolic Disorder through Inhibition of NLRP3 Inflammasome Activation. *Immunity*, *38*(6), 1154–1163. https://doi.org/10.1016/J.IMMUNI.2013.05.015

Yang, A., & Mottillo, E. P. (2020). Adipocyte lipolysis: from molecular mechanisms of regulation to disease and therapeutics. *The Biochemical Journal*, *477*(5), 985–1008. https://doi.org/10.1042/BCJ20190468

Yang, N. J., & Hinner, M. J. (2015). Getting across the cell membrane: an overview for small molecules, peptides, and proteins. *Methods in Molecular Biology (Clifton, N.J.)*, *1266*, 29–53. https://doi.org/10.1007/978-1-4939-2272-7_3/FIGURES/2

Yeo, C. R., Agrawal, M., Hoon, S., Shabbir, A., Shrivastava, M. K., Huang, S., Khoo, C. M., Chhay, V., Yassin, M. S., Tai, E. S., Vidal-Puig, A., & Toh, S. A. (2017). SGBS cells as a model of human adipocyte browning: A comprehensive comparative study with primary human white subcutaneous adipocytes.

Scientific Reports 2017 7:1, *7*(1), 1–12. https://doi.org/10.1038/s41598-017-04369-2

Yin, W., Mu, J., & Birnbaum, M. J. (2003). Role of AMP-activated Protein Kinase in Cyclic AMP-dependent Lipolysis in 3T3-L1 Adipocytes. *Journal of Biological Chemistry*, *278*(44), 43074–43080. https://doi.org/10.1074/jbc.M308484200

Yu, W., Xie, D., Yamamoto, T., Koyama, H., & Cheng, J. (2023). Mechanistic insights of soluble uric acid-induced insulin resistance: Insulin signaling and beyond. *Reviews in Endocrine and Metabolic Disorders 2023 24:2*, *24*(2), 327–343. https://doi.org/10.1007/S11154-023-09787-4

Yuen, H., Hung, A., Yang, A. W. H., & Lenon, G. B. (2020). Mechanisms of Action of Cassiae Semen for Weight Management: A Computational Molecular Docking Study of Serotonin Receptor 5-HT2C. *International Journal of Molecular Sciences 2020, Vol. 21, Page 1326*, *21*(4), 1326. https://doi.org/10.3390/IJMS21041326

Zammit, C., Liddicoat, H., Moonsie, I., & Makker, H. (2011). Obesity and respiratory diseases. *American Journal of Clinical Hypnosis*, *53*(4), 335–343. https://doi.org/10.2147/IJGM.S11926

Zebisch, K., Voigt, V., Wabitsch, M., & Brandsch, M. (2012). Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Analytical Biochemistry*, *425*(1), 88–90. https://doi.org/10.1016/J.AB.2012.03.005

Zhang, B., Albaker, A., Plouffe, B., Lefebvre, C., & Tiberi, M. (2014). Constitutive Activities and Inverse Agonism in Dopamine Receptors. *Advances in Pharmacology*, *70*, 175–214. https://doi.org/10.1016/B978-0-12-417197-8.00007-9

Zhang, D., & Leung, P. S. (2014). Potential roles of GPR120 and its agonists in the management of diabetes. *Drug Design, Development and Therapy*, *8*, 1013–1027. https://doi.org/10.2147/DDDT.S53892

Zhang, L., Zheng, J., Ma, M., Zhao, Y., Song, J., Chen, X., Cao, W., He, X., Xue, C., & Tang, Q. (2021). Drug-guided screening for pancreatic lipase inhibitors in functional foods. *Food & Function*, *12*(10), 4644–4653. https://doi.org/10.1039/D0FO03366A

Zhang, R., & Xie, X. (2012). Tools for GPCR drug discovery. *Acta Pharmacologica Sinica*, *33*(3), 372. https://doi.org/10.1038/APS.2011.173

Zhang, S., Kumar, K., Jiang, X., Wallqvist, A., & Reifman, J. (2008). DOVIS: An implementation for high-throughput virtual screening using AutoDock. *BMC Bioinformatics*, *9*(1), 1–4. https://doi.org/10.1186/1471-2105-9-126/METRICS

Zhang, X., Gao, M., Chattha, S. A., Zhu, Y., Peng, B., & Ye, Y. (2021). Application of acidic protease in the pickling to simplify the pelt bating process. *Journal of Leather Science and Engineering*, *3*(1). https://doi.org/10.1186/S42825-021-00068-X

Zhang, Y. Y., & Han, Q. D. (1997). [Inverse agonist: a novel discovery in receptor research]. *Sheng Li Ke Xue Jin Zhan [Progress in Physiology]*, *28*(1), 9–13. https://europepmc.org/article/med/10921070

Zhao, X., Hu, H., Wang, C., Bai, L., Wang, Y., Wang, W., & Wang, J. (2019). A comparison of methods for effective differentiation of the frozen-thawed 3T3-L1 cells. *Analytical Biochemistry*, *568*, 57–64. https://doi.org/10.1016/J.AB.2018.12.020

Zhou, B., Lu, Y., Hajifathalian, K., Bentham, J., Di Cesare, M., Danaei, G., Bixby, H., Cowan, M. J., Ali, M. K., Taddei, C., Lo, W. C., Reis-Santos, B., Stevens, G. A., Riley, L. M., Miranda, J. J., Bjerregaard, P., Rivera, J. A., Fouad, H. M., Ma, G., … Cisneros, J. Z. (2016). Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4·4 million participants. *The Lancet*, *387*(10027), 1513–1530. https://doi.org/10.1016/S0140-6736(16)00618-8

Zhou, X. E., Melcher, K., & Xu, H. E. (2017). Understanding the GPCR biased signaling through G protein and arrestin complex structures. *Current Opinion in Structural Biology*, *45*, 150–159. https://doi.org/10.1016/J.SBI.2017.05.004

Zhu, F.-S., Liu, S., Chen, X.-M., Huang, Z.-G., Zhang, D.-W., & Taylor-Robinson, S. D. (2008). *Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia*. https://doi.org/10.3748/wjg.14.6395