Curtin Medical School

Epigenetics, cholesterol lowering and diabetes: a comparative study of statins and HDAC inhibitors

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Author's Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number # AEC_2016_17.

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Statement of contributors

This project was designed by myself and my supervisor, Associate Professor Cyril Mamotte. All experiments were conducted by myself with the following exceptions. The insulin assays were conducted by Dr Gaewyn Ellison with my assistance. Dr Gaewyn Ellison also extracted RNA for the 16 h treatment qPCR experiments. Wendy Northrop performed the HDAC activity assays on the mouse liver samples. The mouse liver samples were obtained from a previous animal study by our group, designed by Dr Gaewyn Ellison and overseen by Associate Professor Cyril Mamotte. Dr Thiruvarutchelvan Sabapathy assisted in the preparation of the mouse liver samples and prepared the insulin treatments for the insulin signalling experiments. All data analysis and visualisation were performed by myself. The draft thesis was written by myself and refined with suggestions from Associate Professor Cyril Mamotte, Dr Phillip Melton, Professor Philip Newsholme and Dr David Allen. Similarly, Dr Gaewyn Ellison, Associate Professor Cyril Mamotte, Dr Phillip Melton, Professor Philip Newsholme and Wendy Northrop contributed to the editing of the portions of peer-reviewed publications which appear in this thesis.

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Dedication

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Abstract

Cardiovascular disease (CVD) and type 2 diabetes (T2D) are two major causes of morbidity and mortality in the modern world, and both have been linked to altered cholesterol levels. In particular, dyslipidaemia, typically characterised by elevated levels of low-density lipoprotein (LDL) cholesterol and triglyceride and/or decreased levels of high-density lipoprotein (HDL) cholesterol, is a major risk factor for both conditions. HMG-CoA reductase (HMGCR) inhibitors, a class of LDL cholesterol (LDL-C) lowering drugs commonly known as statins, are widely used to reduce CVD risk by reducing cellular cholesterol biosynthesis. However, statins have been associated with an increased risk of developing T2D. Epigenetic modifications have also been associated with both CVD and T2D and there have been suggestions that statins have epigenetic effects such as inhibition of histone deacetylases (HDACs), enzymes that remove the epigenetic mark of histone acetylation. Established HDAC inhibitors, particularly the short-chain fatty acid butyrate, have been investigated in animal models of metabolic disease and reportedly not only lower serum cholesterol but also protect against the development and progression of diabetes in these animals. The project described in this thesis therefore set out to compare the effects of statins and established HDAC inhibitors on epigenetic modifications, cholesterol metabolism and T2D.

Studies were performed using a cell culture model, primarily using HepG2 hepatoma cells and BRIN-BD11 insulin secreting cells. THP-1 macrophages, due to the importance of macrophages in cholesterogenic atherosclerosis and CVD, and MDA-MB-231 breast carcinoma cells, as most previous studies reporting epigenetic effects of statins were conducted in the context of cancer, were used in some experiments. The livers of mice treated with statins from a previous animal study were also utilised. In most experiments, cells were treated with statins or HDAC inhibitors for 24 h in lipoprotein-deficient serum (LPDS). The activity of epigenetic modifying enzymes, the cellular cholesterol and triglyceride content and cellular cholesterol uptake were assessed using commercially available kits. Insulin secretion was quantified by sandwich enzyme-linked immunosorbent assay (ELISA). Global levels of modified histones and of proteins involved in cholesterol and glucose metabolism were assessed by immunoblotting. Gene expression of proteins involved in cholesterol and

glucose metabolism was assessed by quantitative real-time PCR (qPCR) following RNA extraction and reverse transcription. Histone modification in proximity to specific genomic regions was determined by chromatin immunoprecipitation-qPCR (ChIP-qPCR).

Both statins and HDAC inhibitors lowered cellular cholesterol in HepG2 and BRIN-BD11 cells. Lowering cholesterol as a result of HMGCR inhibition by statins resulted in activation of the SREBP-2 signalling pathway, leading to increased expression of HMGCR and the LDL receptor (LDLR) and subsequently increased cellular uptake of cholesterol. Conversely, HDAC inhibition reduced SREBP-2 signalling, providing a mechanism for cellular cholesterol lowering, and did not increase cholesterol uptake, calling into question whether HDAC inhibition could lower serum cholesterol in the same manner as statins. Both butyrate and atorvastatin altered levels of proteins involved in reverse cholesterol transport, or HDL cholesterol metabolism, but this was dependant on the cell type and the amount of lipoprotein in the media, making it difficult to draw conclusions about effects on reverse cholesterol transport *in vivo*.

Previous studies by the laboratory group of A/Prof Mamotte indicated that both increasing and decreasing cellular cholesterol, including by statins, impairs insulin secretion. Similarly, high dose 24 h HDAC inhibition impaired both chronic and acutely stimulated insulin secretion and decreased cellular insulin content. However, low dose (1 mM) butyrate for 24 h and acute high dose (5 mM) butyrate enhanced stimulated insulin secretion. Increased expression of hexokinase I, increasing glucose sensing at low concentrations, and of *TXNIP*, increasing oxidative stress, may account for HDAC inhibition induced impairment. Conversely, the statin induced impairment is most likely a direct result of cholesterol lowering, as all diabetes related genes altered by statin treatment are regulated by transcription factors whose activity is linked to cellular cholesterol levels. Butyrate also interfered with insulin signalling, decreasing AKT phosphorylation and increasing IRS-1 phosphorylation, possibly by increasing acetylation of these proteins.

In contrast to the literature, statins did not inhibit the activity of epigenetic modifying enzymes, namely HDACs, DNA methyltransferases (DNMTs) or histone acetyltransferases (HATs). HDAC activity was investigated in numerous cell types, in live cells and cell extracts and in the livers of statin treated mice. Atorvastatin also failed to alter global histone acetylation in multiple cell types. In all cell-based experiments, the established HDAC inhibitors butyrate and trichostatin A (TSA)

significantly inhibited HDAC activity and increased global histone acetylation. Despite having no effect on HDAC activity, atorvastatin tended to increase histone acetylation at SREBP-2 target genes, suggesting a mechanism by which statins may indirectly impact epigenetic modifications. The effect of butyrate on histone modifications in proximity to key genes implicated in cholesterol metabolism and T2D was dependant on the statistical methodology used to normalise ChIP-qPCR signals. Increased histone acetylation in proximity to the glucose transporter *SLC2A2* (GLUT2) was consistent across the different methods, suggesting that butyrate increases *SLC2A2* expression through HDAC inhibition.

Statins had much more limited effects on gene expression than HDAC inhibitors and all the noted effects of statins in this study are on-target effects of HMGCR inhibition and subsequent cellular cholesterol lowering. Conversely, HDAC inhibitors have more widespread effects on gene expression, altering the expression of many genes involved in cholesterol and glucose metabolism, in ways that would be expected to be beneficial and harmful for CVD and T2D.

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Allen SC, Mamotte CDS. Pleiotropic and adverse effects of statins: do epigenetics play a role? *J Pharmacol Exp Ther* 2017; 362(2): 319-326. doi: 10.1124/jpet.117.242081. (Review)

Bridgeman SC, Ellison GC, Melton PE, Newsholme P, Mamotte CDS. Epigenetic effects of metformin: From molecular mechanisms to clinical implications. *Diabetes Obes Metab* 2018; 20: 1553-1562. doi: 10.1111/dom.13262. (Review)

Bridgeman S, Northrop W, Ellison G, Sabapathy T, Melton PE, Newsholme P, Mamotte CDS. Statins do not directly inhibit the activity of major epigenetic modifying enzymes. *Cancers* 2019; 11(4): 516. doi: 10.3390/cancers11040516.

Bridgeman SC, Northrop W, Melton PE, Ellison GC, Newsholme P, Mamotte CDS. Butyrate, generated by gut microbiota, and its therapeutic role in metabolic syndrome. *Pharmacol Res* 2020; 160: 105174. doi: 10.1016/j.phrs.2020.105174. (Review)

Bridgeman S, Ellison G, Newsholme P, Mamotte, C. The HDAC inhibitor butyrate impairs β cell function and activates the disallowed gene hexokinase I. *Int J Mol Sci* 2021; 22(24): 13330. doi: 10.3390/ijms222413330.

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List of Abbreviations

ABCA1	ATP-binding cassette transporter sub-family A member 1
ABCG1	ATP-binding cassette transporter sub-family G member 1
ADP	Adenosine diphosphate
AKT	Protein kinase B
ANOVA	Analysis of variance
APO	Apolipoprotein
ATAC-seq	Assay of Transposase Accessible Chromatin sequencing
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCP	1-Bromo-3-chloropropane
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CoA	Coenzyme A
CREB	cAMP response element binding protein
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7 alpha-hydroxylase/Cytochrome P450 family 7 subfamily A member 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EWAS	Epigenome wide association study
EZH2	Enhancer of zeste homolog 2
FBS	Foetal bovine serum
FFAR	Free fatty acid G-coupled receptor
FOXO1	Forkhead box class O1
G6PC/G6Pase	Glucose 6-phosphatase
GLP-1	Glucagon-like peptide 1
GLUT2/SLC2A2	Glucose transporter 2
GLUT4/SLC2A4	Glucose transporter 4
GSIS	Glucose-stimulated insulin secretion
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HDL-C	HDL cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High fat diet
HLPS	High lipoprotein serum
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HMGCR	HMG-CoA reductase
HMT	Histone methyltransferase

HNF4α	Hepatocyte nuclear factor 4α
HRP	Horseradish peroxidase
IC50	Half maximal inhibitory concentration
IDL	Intermediate-density lipoprotein
INS	Insulin
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
K	Lysine
KRBB	Krebs-Ringer Bicarbonate buffer
LDL	Low-density lipoprotein
LDL-C	LDL cholesterol
LDLR	Low-density lipoprotein receptor
LDPS	Lipoprotein-deficient serum
LXR/NR1H3	Liver receptor X
MetS	Metabolic syndrome
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
ND	Normal diet
PBS	Phosphate-buffered saline
PDX1	Pancreatic and duodenal homeobox 1
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1α/PPARGC1A	PPARγ coactivator 1 alpha
PIC	Protease inhibitor cocktail

PPARγ/PPARG	Peroxisome proliferator-activated receptor gamma
PYY	Peptide YY
qPCR	Quantitative real-time polymerase chain reaction
R	Arginine
RCT	Reverse cholesterol transport
RIPA	Radioimmunoprecipitation assay (lysis buffer)
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (media)
RT	Room temperature
SAM	S-adenosylmethionine
SB	Sodium butyrate
SCFA	Short-chain fatty acid
SE	Standard error
SEM	Standard error of the mean
SIRT	Sirtuin
SRB1/SCARB1	Scavenger receptor class B type 1
SREBP/SREBF	Sterol regulatory element-binding protein/factor
T2D	Type 2 diabetes mellitus
TSBT	Tris-buffered saline with Tween 20
TG	Triglyceride
tRNA	Transfer RNA
TSA	Trichostatin A
TSS	Transcription start site
TXNIP	Thioredoxin-interacting protein

VLDL	Very low-density lipoprotein

Chapter 1. Introduction

This thesis explores the possible links between epigenetics, cholesterol lowering and type 2 diabetes (T2D). The rationale for this is that the widely used cholesterol lowering statin class of drugs have been shown to be associated with a small but significant increase in T2D and have been reported to have epigenetic effects, in particular inhibition of histone deacetylases (HDACs), but whether epigenetic effects could contribute to their diabetogenic effects has not been explored in detail. HDAC inhibitors, most notably the short-chain fatty acid (SCFA) butyrate, have also been shown to improve lipid profiles in animal models of metabolic disease, however, in contrast to statins, they have also been reported to be protective against diabetes in these animals. The work described in this thesis aimed to compare and contrast the effects of statins and established HDAC inhibitors, primarily butyrate, on cholesterol metabolism, glucose metabolism and epigenetic modifications.

1.1. Hypotheses

The objectives of this thesis are grounded in two hypotheses

1. Epigenetic modifications contribute to the diabetogenic effects of statins
2. Butyrate lowers cholesterol and protects against diabetes as a result inhibiting HDAC activity in cells such as hepatocytes and the insulin secreting β cells

1.2. Objectives

1. Investigate how statins and HDAC inhibitors affect cholesterol metabolism
2. Investigate how these treatments affect insulin secretion and signalling.
3. Investigate how these treatments influence epigenetic modifying enzymes and alter genome-wide epigenetics.

4a. Identify genes involved in cholesterol metabolism and implicated in T2D whose expression is altered by these treatments.

4b. Investigate if epigenetic modifications contribute to the altered expression of these target genes.

1.3. Significance

Cardiovascular disease (CVD) and T2D are two of the biggest causes of morbidity and mortality in the modern world and both are associated with altered cholesterol metabolism. As a result, the low-density lipoprotein (LDL) cholesterol lowering drugs known as statins are amongst the most prescribed medications in many advanced economies. However, statins are associated with a small but significant increased risk of developing T2D which, due to the widespread use of statins, may affect millions of users worldwide. Understanding the mechanisms by which statins alter diabetes risk may lead to identification of those most at risk and the development of mitigating strategies in high-risk individuals. In contrast to statins, epigenetic modifying agents, most notably HDAC inhibitors, have been reported to not only lower cholesterol but also protect against diabetes in animal models of metabolic disorders. If these results are translatable to humans and HDAC inhibitors are able to either replace or complement statin use, this could potentially benefit many millions of people at risk of CVD and T2D. This thesis makes a substantial contribution to the body of knowledge of how statins impact epigenetic modifications and of how HDAC inhibitors impact cholesterol and glucose metabolism.

1.4. Thesis overview

Chapter 2 provides the background to the problems explored in the subsequent chapters. It introduces the three major areas of exploration described in this thesis, epigenetics, cholesterol and T2D, and discusses the current understanding of links between the three. The effects of statins and the HDAC inhibitor butyrate on epigenetics, cholesterol and glucose metabolism are discussed, highlighting the gaps and inconsistencies in the current literature.

Chapter 3 details the methods and materials used throughout the thesis. A cell-culture based model was used, using cell types important to diabetes and cholesterol, most notably hepatic cells due to their importance in both cholesterol and glucose metabolism, pancreatic β cells due to the central role of insulin secretion in diabetes, and macrophages due to their role in the formation of cholesterol plaques in CVD. Despite the limitations of using immortalised cell lines and in extrapolating the results of *in vitro* studies to *in vivo* effects, this approach was chosen for several reasons. Firstly, many studies have explored the *in vivo* effects of statins and HDAC inhibitors

on cholesterol and glucose metabolism, in the case of statins the effect on humans *in vivo* is well-documented while with HDAC inhibitors most of the evidence comes from animal models. What these studies lack is exploration into the specific mechanisms governing these effects. The difficulty in exploring exacting mechanisms *in vivo* is that effects in one organ may have downstream effects on multiple tissues. For example, HDAC inhibitors such as butyrate have significant impact on intestinal cells, which has downstream effects on multiple cell types including hepatocytes and β cells. The work described in this thesis thus aimed to cover gaps in the literature by examining the possible mechanisms behind the observed *in vivo* effects without the compounding factors of whole-body effects.

While multiple statins and HDAC inhibitors were used in various experiments, atorvastatin and butyrate were chosen as the exemplar statin and HDAC inhibitor respectively for more detailed study. Curcumin was also used in preliminary experiments due to its reported direct inhibitory effects on multiple epigenetic modifying enzymes,[1] however experimentation revealed it was unsuitable for further study due to its lack of effect on cells *in vitro*, most likely due to its instability in cell culture media.[2] Atorvastatin was chosen as it was the most commonly prescribed statin in numerous advanced economies, including the United States,[3] England,[4] and Australia,[5] and, being lipophilic may be more representative of other statins such as simvastatin, fluvastatin, lovastatin and pitavastatin, while the two hydrophilic statins, rosuvastatin and pravastatin, vary greatly in potency and reported diabetes risk. Butyrate was chosen as it has been widely explored in animal studies, has been used in multiple human clinical trials for gastrointestinal disorders where it has shown a favourable safety and tolerance profile and has been used in small-scale clinical trials for diabetes and metabolic syndrome.[6,7]

Chapter 4 compares and contrasts the effects of statins and HDAC inhibitors on cellular cholesterol metabolism (Objective 1). In particular, this section of the thesis represents the most comprehensive study of the HDAC inhibitor butyrate on cholesterol metabolism. This includes looking at cellular cholesterol content in a range of cell types and conditions and the uptake and export of cholesterol from cells. Effects of treatments on proteins involved in cholesterol synthesis, uptake and reverse cholesterol transport are examined in a range of cell types and conditions, thus exploring the mechanisms by which statins and HDAC inhibitors lower cellular cholesterol and impact cholesterol transport. Key genes involved in cholesterol

metabolism whose expression is altered by statins or butyrate are identified (Objective 4a).

Chapter 5 describes the effects of statins and HDAC inhibitors on insulin secretion in pancreatic cells and insulin signalling in hepatic cells (Objective 2). Key proteins involved in glucose sensing are examined and key genes that have been implicated in T2D whose expression is altered by statins or butyrate are identified (Objective 4a), in order to examine the potential mechanisms by which statins and HDAC inhibitors impact insulin secretion and T2D.

Chapter 6 explores the effects of statins, HDAC inhibitors and curcumin on epigenetic modifications (Objective 3). In particular, extensive experimentation was conducted into effects on HDAC activity using multiple cell types and experimental protocols; this is the most comprehensive study on the effects of statins on HDAC activity. It also contains the most comprehensive study on the effects of statins on DNA methyltransferase (DNMT) activity as well as an examination of the effects of statins on histone acetyltransferase (HAT) activity, which has not been reported in the literature. This chapter also examines the effects of atorvastatin, sodium butyrate and curcumin on global histone modifications. Finally, the mechanism by which statins may indirectly impact epigenetic modifications are explored, as are the effects of atorvastatin and butyrate on histone acetylation in proximity to key genes altered by these treatments (Objective 4b).

Chapter 7 summarises the significant findings and implications of this thesis, addresses the limitations of the methodologies used and discusses ways in which future research can address these limitations and fill in remaining gaps in the literature. It also discussed the potential of using epigenetic modification in therapies for metabolic disorders.

Chapter 2. Literature Review

2.1. Introduction

CVD is the leading cause of mortality worldwide according to the World Health Organisation,[8] while T2D is also a major cause of morbidity and mortality in the modern world. The International Diabetes Foundation estimates that 463 million adults worldwide were living with diabetes,[9] and 4.2 million adults died of the disease in 2019.[10] Dyslipidaemia, typically characterised by elevated levels of LDL cholesterol (LDL-C) and triglycerides (TG) and/or decreased levels of high-density lipoprotein cholesterol (HDL-C), is a major risk factor for both CVD and T2D.[11] As over five million Australians have dyslipidaemia,[12] the burden of CVD and T2D may be significantly lessened by improving the population's cholesterol levels. As a result, HMG-CoA reductase (HMGCR) inhibitors, commonly known as statins, are widely used to lower LDL-C. Despite the proven ability of statins to reduce morbidity and mortality from CVD by approximately 25%,[13] meta-analyses of clinical trials have found statin use is associated with a small but significant increase in the risk of developing T2D.[14]

There has been much research linking epigenetics and developmental programming to dyslipidaemia,[15,16] insulin resistance and T2D,[17-20] and CVD.[21-23] As a result, there is interest in the use of epigenetic modifiers, in particular HDAC inhibitors, to prevent and treat these disorders.[24,25] Butyrate, a HDAC inhibitor produced by the gut microbiome, has shown promising results in pre-clinical experiments and has reached clinical trials for lipid lowering and T2D,[6,26,27] although there is still significant uncertainty over how HDAC inhibitors influence cholesterol and glucose metabolism.

There is also growing interest in how existing medications influence epigenetic processes. For example, statins have been reported to affect numerous epigenetic modifying enzymes and the subsequent epigenetic modifications have been linked to protection from CVD and cancer.[28] There has been little research into whether epigenetic modifications contribute to the adverse effects of statins, including T2D, or if statins alter epigenetic mechanisms regulating cholesterol metabolism genes. This chapter outlines cholesterol metabolism, glucose metabolism and epigenetics, and the current literature regarding statins and butyrate and their effects on epigenetic modifications, cholesterol metabolism and T2D.

2.2. Cholesterol

2.2.1. Cholesterol metabolism

Cholesterol is a lipid sterol (steroid precursor) that is essential to animal cells due to its vital role in plasma membranes. In addition, cholesterol is a precursor to steroid hormones, bile acids and vitamin D.[29] Excess cholesterol, however, is a contributory factor to some of the most common chronic diseases in the modern world, most notably CVD, but also non-alcoholic fatty liver disease (NAFLD)[30] and T2D.[31] Cholesterol is both synthesised by cells and sourced from the diet, and transported around the body in lipoproteins. Lipoproteins provide a means of transporting water insoluble lipids through the bloodstream by forming complexes containing amphipathic proteins known as apolipoproteins.[29]

Absorption of dietary cholesterol by enterocytes in the small intestine is regulated by Niemann-Pick-C1-like-1 protein (NPC1L1).[32] The cholesterol, together with TG, phospholipids and apolipoproteins, is packaged into chylomicrons, apo-B48 containing lipoproteins, for release into circulation. Chylomicrons deliver TG to peripheral cells such as adipose and muscle cells, leaving behind cholesterol-enriched chylomicron remnants, which are taken up by the liver.[33] Enterocytes also secrete cholesterol back into the intestinal lumen through ATP-binding cassette transporter sub-family G member 5 (ABCG5) and member G 8 (ABCG8).

In addition to dietary sources, cholesterol is synthesised by all nucleated cells. Cholesterol synthesis is a complicated process comprising of more than 30 chemical reactions.[32] To summarise, three molecules of acetyl-CoA are reversibly condensed to form 3-hydroxy-3-methylglutaryl-CoA coenzyme A (HMG-CoA). HMG-CoA is converted to mevalonate by HMGCR, the rate-limiting enzyme in cholesterol biosynthesis and the target of statins. Mevalonate is then converted to isopentenyl pyrophosphate, the precursor not only to cholesterol, but also many other sterol and non-sterol isoprenoids including vitamin K, heme and ubiquinone.[34] In the cholesterol pathway, isopentenyl pyrophosphate is converted to squalene, then lanosterol, and then cholesterol.

The liver packages cholesterol from multiple sources together with TG and apolipoproteins into very low-density lipoprotein (VLDL) which, similarly to chylomicrons, delivers TG to peripheral cells and leaves behind intermediate-density

lipoprotein (IDL).[35] IDL is either taken up by hepatic cells or further metabolised to form cholesterol-rich LDL, the primary source of plasma cholesterol in humans. Cholesterol is taken up by cells according to requirements through the LDL receptor (LDLR), which recognises the apolipoprotein apo-B100 in LDL.[36]

Cholesterol is removed from peripheral cells in a process known as reverse cholesterol transport (RCT).[32] In this process, ATP-binding cassette transporter sub-family A member 1 (ABCA1) mediates the efflux of cholesterol to apolipoprotein apo-A1 to form HDL. Mature HDL particles also take up cellular cholesterol through ATP-binding cassette transporter sub-family G member 1 (ABCG1) and scavenger receptor class B type 1 (SRB1). SRB1 can also take up cholesterol from HDL, particularly in the liver, where it can be recycled, converted to bile acids or excreted through bile as free cholesterol.[33]

The balance between cholesterol uptake, synthesis and efflux is regulated by transcription factors, including sterol regulatory element-binding proteins (SREBPs) and liver receptor X (LXR), that increase or decrease expression of cholesterol metabolism genes in response to altered cellular cholesterol levels. There are two SREBP genes encoding three proteins: SREBP-1a, SREBP-1c and SREBP-2. SREBP-2 activates cholesterol synthesis and uptake genes, while SREBP-1c regulates fatty acid synthesis and SREBP-1a is involved in both cholesterol and fatty acid metabolism.[37] In its inactive state, SREPB-2 is retained in the endoplasmic reticulum (ER) membrane, where it associates with SREBP cleavage activating protein (SCAP). When ER membrane sterol levels drops, the SCAP-SREBP-2 complex is released from the ER membrane and transported to the Golgi apparatus. Here, SREBP-2 is cleaved by two resident proteases, site-1 (S1P) and site-2 (S2P), releasing the active domain and allowing it to translocate to the nucleus, where it activates transcription of *LDLR* and genes in the cholesterol biosynthesis pathway, including *HMGCR*, to increase cholesterol uptake and synthesis, and thus increase cellular cholesterol.[32] Conversely, LXR is activated by oxysterols, which reflect cellular cholesterol levels, to promote cholesterol efflux and clearance. LXR target genes include ATP-binding cassette transporters *ABCA1*, *ABCG1*, *ABCG5* and *ABCG8* in addition to *CYP7A1*, which is the rate-limiting step in the conversion of cholesterol to bile acid, and genes involved in fatty acid synthesis and TG metabolism.[38] When oxysterols are present LXR activates these target genes, however, in the absence of oxysterols, LXR instead acts to repress target genes.[39]

Cholesterol transport pathways are summarised in Figure 2-1 and important proteins involved in cholesterol metabolism are summarised in Table 2.1.

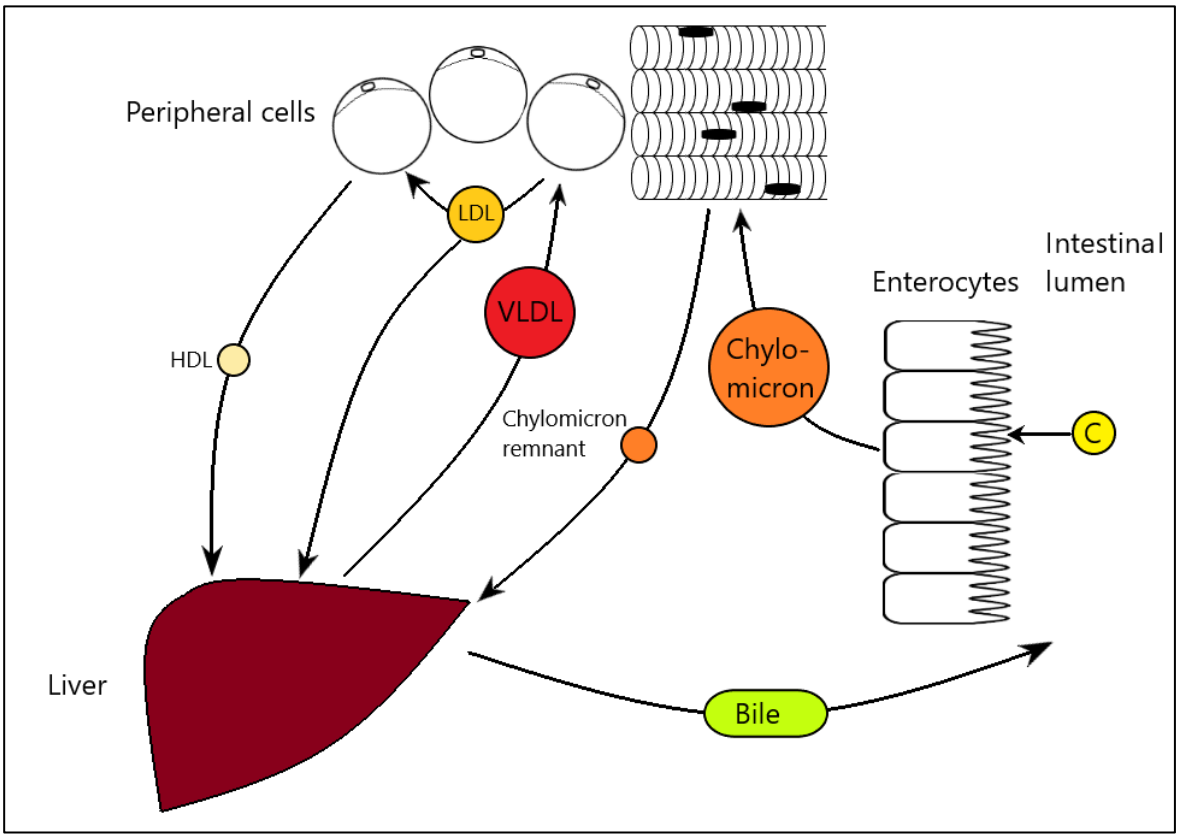

Figure 2-1 Lipoprotein metabolism

Dietary cholesterol (C) is taken up by enterocytes and packaged into chylomicrons together with other constituents including TG, phospholipids and apolipoproteins. The TG component is hydrolysed by lipoprotein lipase into monoacylglycerol and free fatty acids which can be taken up by peripheral tissues, particularly fat and muscle tissue, and the cholesterol-rich chylomicron remnant is delivered to the liver. The liver packages cholesterol and TG into very low-density lipoproteins (VLDL). TG from VLDL is hydrolysed and taken up by peripheral cells, resulting in low-density lipoproteins (LDL) which deliver cholesterol to cells. Cholesterol is exported from peripheral cells to the liver in high density lipoproteins (HDL). Cholesterol is eliminated from the body through bile.

Table 2.1 A selection of key genes involved in cholesterol metabolism

Gene	Protein	Function
NPC1L1	Niemann-Pick-C1-like-1 protein	Absorption of dietary cholesterol in intestine.
HMGCR	HMG-CoA reductase	Rate-limiting enzyme in cholesterol biosynthesis.

Gene	Protein	Function
LDLR	LDL receptor	Binds LDL and allows cellular uptake of cholesterol.
APOB	Apolipoprotein B-100 Apolipoprotein B-48	Component of VLDL and LDL, mediates binding of LDL to cell surface LDLR. Component of chylomicrons
APOA1	Apolipoprotein A1	Component of HDL, reverse cholesterol transport from peripheral cells to liver.
ABCA1	ATP binding cassette subfamily A member 1	Transport of cholesterol from cells to apo-A1.
ABCG1	ATP binding cassette subfamily G member 1	Transport of cholesterol, including within cells and export to HDL.
SCARB1	Scavenger receptor class B member 1 (SRB1)	HDL receptor, bidirectional of transfer of cholesterol between cells and HDL.
CYP7A1	Cytochrome P450 family 7 subfamily A member 1	Rate-limiting enzyme in bile acid synthesis, allows for cholesterol elimination.
SREBF2	Sterol regulatory element-binding protein 2 (SREBP-2)	Master transcription factor for proteins involved in cholesterol synthesis and cellular cholesterol uptake.
SREBF1	Sterol regulatory element-binding protein 1 (SREBP-1a or c)	Transcription factor for fatty acid synthesis (SREBP-1c) and/or cholesterol metabolism (SREBP-1a).
NR1H3	Liver X receptor (LXR)	Transcription factor for cholesterol transport and efflux.

2.2.2. Cholesterol and disease risk

Although all animals require cholesterol to function, excess levels of cholesterol and impaired cholesterol metabolism are linked to a number of common diseases. In fact, cholesterol was first discovered in 1769 as a component of gallstones,[40] and it is most notorious today for its link to CVD. In particular, high levels of LDL-C and low levels of HDL-C are strongly associated with mortality from coronary heart disease;[29] verified by the efficacy of statins in reducing cardiovascular events in proportion to

LDL-C lowering.[41] Although various models have been proposed, the initiation of atherosclerosis is classically associated with the accumulation of LDL in the subendothelial space of arteries.[42] Activation of endothelial cells may cause oxidation of LDL and the recruitment of monocytes, which then differentiate into pro-inflammatory macrophages. Uptake of LDL, especially oxidised LDL, and reduced cholesterol efflux to HDL by the macrophages may result in their transformation into cholesterol laden foam cells.[43] The macrophages may promote the infiltration and proliferation of smooth muscle cells, which can contribute to the formation of atherosclerotic plaques by producing a fibrous cap that surrounds a core of cholesterol laden foam cells, extracellular cholesterol and other debris from dead cells.[42] Rupture of atherosclerotic plaques can lead to thrombus formation and blockage of vessels causing myocardial infarction or stroke.

Cholesterol has also been implicated in numerous other diseases, including NAFLD, where the accumulation of free cholesterol is thought to be a major contributor to liver damage,[30] and neurodegenerative disorders such as Alzheimer's disease, Huntington's disease and Parkinson's disease.[44] Cholesterol is also linked to T2D, as discussed in detail in section 2.3.3.

2.3. Glucose and diabetes

Glucose is the primary energy source of mammalian cells and thus is vital for health, although elevated concentrations have detrimental effects on numerous organs. Thus, there are numerous pathways and hormones that act to maintain plasma glucose levels within a certain range.

2.3.1. Glucose metabolism

Following a meal, glucose is taken up by enterocytes primarily through the sodium-dependent glucose co-transporter SGLT1 and exported to the bloodstream by the glucose transporter GLUT2, which has a low affinity for glucose and thus is activated when glucose levels are high.[45] Circulating glucose is then taken up by pancreatic β cells and liver hepatocytes through GLUT2. The rise in glucose levels is recognised by these cells due to the glucose sensing protein glucokinase, also known as hexokinase IV, which also has a low affinity for glucose and thus phosphorylates glucose only when glucose levels are high.[46] In other cells types, glucose phosphorylation is carried out by hexokinases I, II and III, which respond to lower

levels of glucose and are usually silenced in β cells and hepatocytes to prevent insulin secretion and glycogenesis, respectively, when glucose levels are low.[47] β cells respond to increased glucose levels by secreting the hormone insulin in a process known as glucose-stimulated insulin secretion (GSIS). Insulin binds to insulin receptors in peripheral cells and initiates a series of phosphorylation events that results in skeletal muscle and adipocytes increasing their uptake of glucose through the insulin-responsive glucose transporter GLUT4. These cells subsequently use glucose for cellular energy production through glycolysis, the breakdown of glucose to produce pyruvate and ATP.[48] In the liver, insulin and glucose promote the production of glycogen, a storage form of glucose, in a process known as glycogenesis.[49] This process is enhanced by the fact that insulin also promotes the expression of glucokinase in the liver.[50]

In fasting conditions, the liver plays a crucial role in generating glucose for peripheral tissues through the breakdown of glycogen, known as glycogenolysis, and the *de novo* production of glucose, known as gluconeogenesis. These processes are promoted by the hormone glucagon, released by pancreatic α cells in low glucose and insulin conditions,[51] and inhibited by insulin in high glucose conditions.[52,53] Glycogenolysis is the primary glucose source during short-term fasting, such as overnight, and can provide fuel for approximately 30 hours, following which gluconeogenesis is required to provide energy for cells.[54] Gluconeogenesis produces glucose from non-carbohydrate substrates including amino acids (from proteins), glycerol (from lipids) and lactate (from metabolic processes) through enzymes including glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK).

Many other proteins play important roles in glucose metabolism including hormones, such as amylin from β cells and glucagon-like peptide-1 (GLP-1) from intestinal cells, and transcription factors such as pancreatic and duodenal homeobox 1 (PDX1), forkhead box class O1 (FOXO1), hepatocyte nuclear factor 4α (HNF4α) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α).[53,54] Glucose metabolism is summarised in Figure 2-2 and the key proteins involved summarised in Table 2.2.

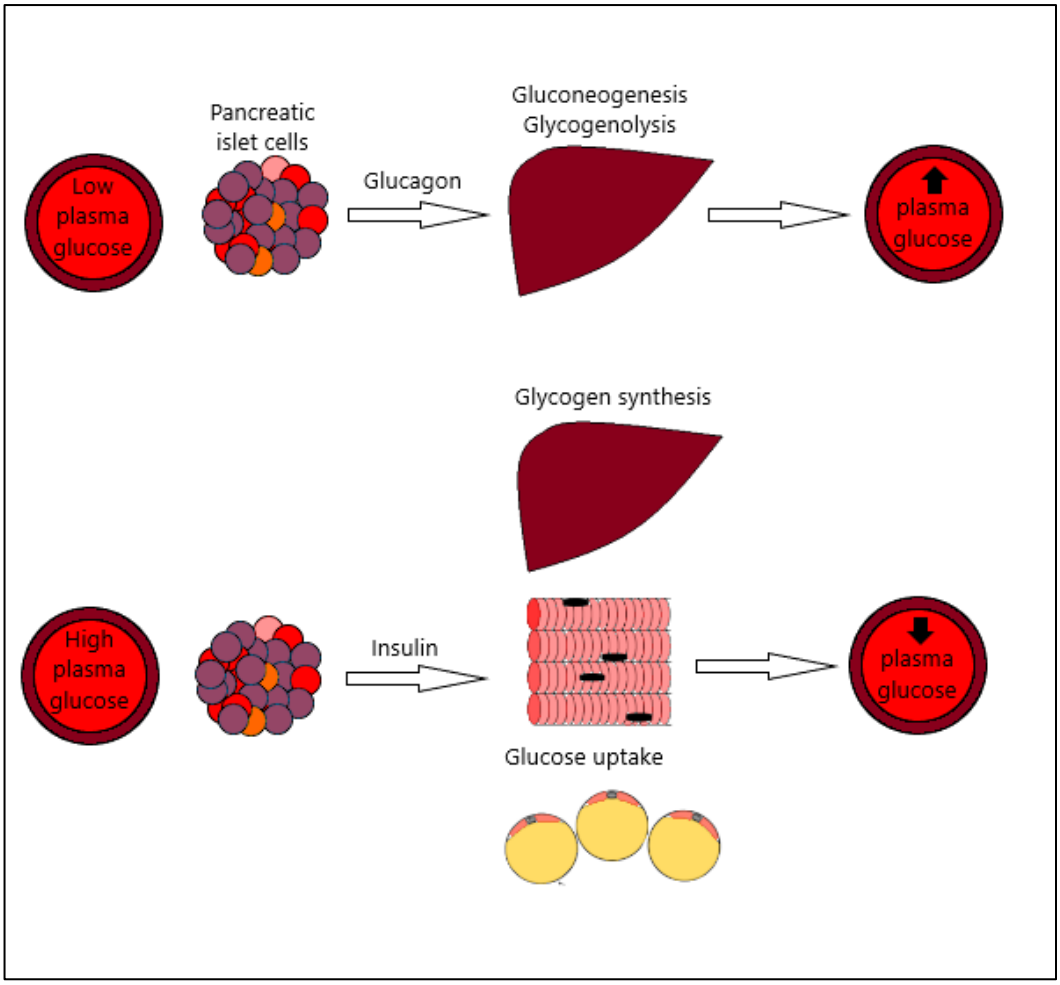

Figure 2-2 Glucose metabolism

For proper functioning of cells, it is important plasma glucose levels are maintained within a limited range (4.0-6.0 mM). Pancreatic islet cells play an important part in maintaining this balance. In conditions of fasting when plasma glucose levels are low, pancreatic α cells release glucagon, which promotes the liver to break down glycogen to release glucose, while also stimulating the liver to synthesise *de novo* glucose (gluconeogenesis), resulting in the release of glucose into the bloodstream for peripheral cells to utilise. When glucose levels rise post feeding, pancreatic β cells release insulin, which promotes glucose uptake in peripheral cells and in the liver promotes glucose storage in the form of glycogen, resulting in a decrease of circulating glucose.

Table 2.2 A selection of key genes involved in glucose metabolism

Gene	Protein	Function
GK	Glucokinase	Phosphorylates glucose in β cells and hepatocytes in high glucose conditions.
SLC2A2	Glucose transporter type 2 (GLUT2)	Transports glucose into β cells and hepatocytes in high glucose conditions.

Gene	Protein	Function
INS	Insulin	Released by β cells in response to high glucose, promotes glucose uptake and utilisation.
SLC2A4	Glucose transporter type 4 (GLUT4)	Transports glucose into adipocytes and myocytes in response to insulin.
GCG	Glucagon	Released by α cells in response to low insulin and glucose, promotes glycogenolysis and gluconeogenesis.
PEPCK	Phosphoenolpyruvate carboxykinase	Converts oxaloacetate into phosphoenolpyruvate in first committed step of gluconeogenesis.
G6PC	Glucose 6-phosphatase (G6Pase)	Hydrolyses glucose 6-phosphate in final step of gluconeogenesis.
PDX1	Pancreatic and duodenal homeobox 1	Transcription factor important in β cell development and function.
FOXO1	Forkhead box protein O1	Transcription factor important in gluconeogenesis, glycogenolysis, cell cycle regulation.
HNF4A	Hepatocyte nuclear factor 4α	Transcription factor important in hepatocyte and β cell development and function.
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α)	Transcription co-activator whose targets include LXR, HNF4, FOXO1 and gluconeogenic genes.

2.3.2. Diabetes

In diabetes, failure to properly regulate glucose levels results in chronically elevated blood glucose levels, known as hyperglycaemia. Type 1 diabetes mellitus (T1D), or insulin dependent diabetes, occurs when the insulin secreting β cells are destroyed in an auto-immune reaction and usually occurs in childhood. T2D usually occurs in adulthood when pancreatic β cells are unable to cope with the increased demand for insulin due to the insulin resistance of peripheral cells, resulting in chronic

hyperglycaemia. Insulin resistance is the failure of cells to respond properly to insulin, resulting in reduced glucose uptake, particularly in skeletal cells, and failure to suppress gluconeogenesis in the liver, further increasing blood glucose.[55] Insulin resistance has been shown to occur due to a reduction in the number of insulin receptors on the cell surface, resulting in reduced insulin binding, and/or the impairment of downstream phosphorylation events.[56]

As a result of insulin resistance, β cells typically initially increase insulin secretion to compensate for insulin resistance, this may be associated with an increase in β cell mass. Progression from insulin resistance to T2D arises when β cell dysfunction occurs, typically due to the accumulative exposure to high levels of glucose and fatty acids, known as glucolipotoxicity, and the action of proinflammatory cytokines associated with insulin resistance and obesity, leading to reduced insulin secretion and increased β cell death.[57]

The result is chronic hyperglycaemia, which damages blood vessels, leading to macrovascular (CVD) and microvascular (renal failure, blindness and neuropathy) complications.[58] Development of T2D depends on a number of risk factors including genetic disposition and lifestyle factors such as diet and lack of exercise.[59] Cholesterol and epigenetic modifications have also been linked to the development of T2D, as discussed in sections 2.3.3 and 2.4 respectively.

2.3.3. Cholesterol and diabetes

Dyslipidaemia is strongly linked to T2D; men with dyslipidaemia are at twice the risk of developing T2D, while for women the risk is tripled.[11] In particular, diabetic dyslipidaemia is classically associated with high levels of TG and small dense LDL particles, and low levels of HDL, thought to be a result of hepatic insulin resistance leading to uncontrolled secretion of apo-B containing lipoproteins and impaired clearance of lipids.[60]

Treating β cells with LDL[61,62] or cholesterol[63] has been found to reduce insulin secretion *in vitro*, effects that were prevented by HDL or cholesterol depletion, respectively. Reducing islet levels of several genes involved in cholesterol metabolism, namely LXR, ABCA1,[31,64] or ABCG1,[64] or increasing levels of SREBP-2,[65] all of which would increase cholesterol accumulation in β cells, has been

found to impair insulin secretion in animal models. Furthermore, several *ABCA1* variants have been linked with an increased risk of T2D in humans.[31,64]

In addition to impaired insulin secretion, dyslipidaemia may impair insulin signalling in peripheral tissues. Llanos et al.[66] reported increased cholesterol levels in muscle from mice fed a high fat diet (HFD). Depletion of cholesterol from these isolated muscle fibres with cyclodextrin resulted in increased glucose uptake, both in the presence and absence of insulin. Similarly, HDL and apo-A1 have been reported to increase glucose uptake in muscle cells and adipocytes, and, in a human trial, infusion of HDL acutely decreased blood glucose in diabetics, suggesting increased insulin sensitivity.[67,68]

Reduced cholesterol levels, however, have also been associated with T2D. In particular, the use of statins to lower LDL-C has been found to increase the risk of developing T2D, as later discussed in section 2.5.2. Furthermore, people with familial hypercholesterolaemia due to mutations in the *LDLR* gene or *APOB* gene have half the risk of developing T2D compared to unaffected relatives when adjusted for other risk factors,[69] and people with mutations in the *HMGCR* gene that result in lower plasma LDL-C levels have an increased risk of T2D.[70] Previous studies by our research group found that either increased or decreased cellular cholesterol impairs insulin binding in hepatic and muscle cells,[71] and insulin secretion from pancreatic cells.[72]

2.4. Epigenetics

Epigenetics is a rapidly growing field in medical research. Epigenetic modifications, changes to DNA structure that alter gene expression without altering the base nucleotide code, are crucial in the development of organisms and the differentiation and function of specific cell types. For example, epigenetic modifications permit the expression of the insulin gene in pancreatic β cells, while silencing it in other cells.[73] Epigenetic modifications have also been associated with numerous disorders, including T2D, CVD and cancer.[17,74] Epigenetic processes can be altered by environmental factors including diet, exercise and exposure to toxins, with the foetal environment playing a particularly important role in influencing epigenetic modifications that impact metabolism in adult life. Both maternal nutrient deficiency and maternal obesity have been linked to epigenetic changes and the development of T2D in offspring.[75] There is growing evidence that pharmaceuticals also alter

epigenetic processes, which may contribute to the beneficial and deleterious effects of widely-used medications.[76] This includes the widely used anti-diabetic drug metformin, as reviewed in Bridgeman et al.[77] Epigenetic modifications fall under two main categories: histone modifications and DNA methylation as summarised in

Figure 2-3. RNA mediated interference, such as microRNAs, are often included as a third category, but will not be discussed here.

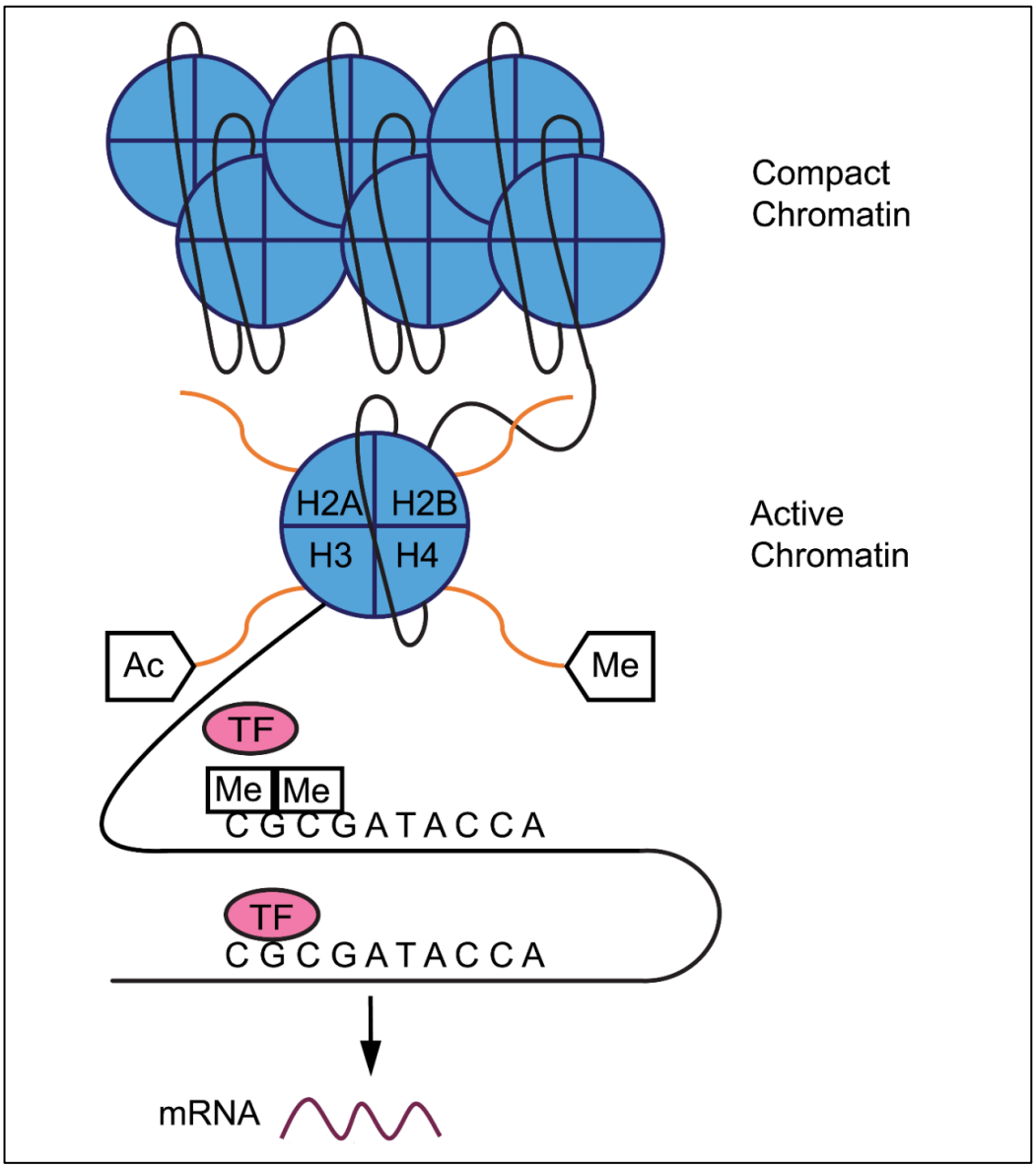

Figure 2-3 Epigenetic modifications

Chromatin consists of DNA wound around histone cores consisting of histone proteins H2A, H2B, H3 and H4. Compaction of chromatin reduces access of transcription factors and other proteins to DNA and is associated with transcriptionally silent genes. More relaxed, 'active' chromatin, mediated by modifications such as acetylation (Ac) and methylation (Me) of residues on histone

tails protruding from the nucleosome, allows easier access to DNA and is associated with active transcription. Methylation of cytosines in CpG dinucleotides represses mRNA transcription by blocking transcription factors (TF) and/or interacting with histones. Originally published in Allen and Mamotte.[28]

2.4.1. DNA methylation

DNA methylation occurs when methyl groups are transferred from S-adenosylmethionine (SAM) to nucleotides by DNMTs. In vertebrates, it is thought that methylation only occurs to cytosines in CpG dinucleotides.[78] CpG dinucleotides are rare throughout the genome, accounting for only 1% of cytosines, but occur at a high frequency in the majority (approximately 70%) of gene promoters.[79] These regions of CpG clusters are termed CpG islands. Most cytosines in CpG islands are unmethylated, with hypermethylation of CpG islands generally thought to result in transcriptional silencing due to decreased transcription factor binding and increased methyl-CpG binding protein binding which may initiate histone modifications that promote chromatin condensation.[78]

Four DNMTs with DNA methyltransferase activity have been identified, DNMT1, DNMT3a, DNMT3b and DNMT3c, while DNMT3L lacks methyltransferase activity and instead acts as a cofactor to *de novo* methyltransferases and DNMT2 methylates tRNA.[80] DNMT3a and DNMT3b are the *de novo* methyltransferases; they establish methylation in previously unmethylated cytosines, whereas DNMT1 maintains a state of methylation, for example in daughter cells following replication.[81] The newly described DNMT3c has been reported to silence retrotransposons in the germ cells of male mice.[82]

2.4.2. Histone modifications

DNA is wrapped around histone cores composed of two each of histone proteins H2A, H2B, H3 and H4; this structure is termed a nucleosome and comprises the fundamental unit of chromatin. DNA can be loosely packed and amenable to transcription as in euchromatin, or highly condensed and silenced as in heterochromatin. The state of chromatin condensation is determined by post-translational modifications to histone amino-terminal tails, most notably acetylation, phosphorylation and methylation, with the most common modifications summarised in Table 2.3.

Acetylation of histone lysine residues, particularly of H3 and H4, neutralises their positive charge and subsequently promotes an open chromatin structure.[83] Histone hyperacetylation is thus considered a signature of active transcription. Histone acetylation is highly dynamic and regulated by two opposing families of enzymes, HATs and HDACs. HATs add acetyl groups to lysine residues using acetyl-CoA as a cofactor. Of the two classes of HATs, Type-A HATs, including the CBP/p300 family, modify multiple sites in histone tails and act as transcriptional co-activators, whereas Type-B HATs, including HAT1, are cytoplasmic and acetylate newly formed histone proteins but not those already complexed to DNA.[83] HDACs are divided into four classes; class I HDACs, including HDAC 1, 2, 3 and 8, are nucleic whereas class II HDACs, including HDAC 4, 5, 6, 7, 9 and 10, move between the nucleus and cytoplasm.[24] Class III HDACs, also known as sirtuins, include SIRTs 1 to 7 and are associated with longevity and decreased disorders of aging, including T2D.[84] Unlike class I, II and IV HDACs, which are zinc dependent, sirtuins rely on NAD+ for their deacetylating activity. HDAC11 is the only class IV HDAC, of which little is known.

Similar to acetylation, histone phosphorylation neutralises the positive charge of the histone.[83] Serine, threonine and tyrosine residues serve as the phosphorylation and dephosphorylation sites for protein kinases and phosphatases respectively, in a process considered important for transcription, and therefore gene expression, as well as DNA repair, mitosis and apoptosis.[85]

Unlike acetylation and phosphorylation, methylation of histone lysine or arginine residues does not alter the net charge of the histone and can have varying influences on transcription depending on the specific residue methylated (denoted by the histone protein and the lysine [K] or arginine [R] that is methylated) and the degree of methylation (mono-, di- or trimethylation). Histone methylation involves the transfer of methyl groups from SAM to histone residues, catalysed by histone methyltransferase (HMT) enzymes. Methylation of histone lysines H3K4, H3K36, and H3K79 is generally associated with active transcription, whereas methylation of H3K9, H3K27, and H4K20 is more commonly found on transcriptionally silent genes.[86] Most HMTs are specific to a certain histone residue, for example enhancer of zeste homolog 2 (EZH2) trimethylates H3K27.[87] Histone methylation is not as dynamic as acetylation and was thought to be a stable event until the discovery of the first histone demethylase was reported in 2004.[88]

Numerous other post-translational modifications to histones have been discovered, including ubiquitination, sumoylation, O-GlcNAcylation, and ADP-ribosylation, although it is less clear how these modifications impact gene expression and chromatin configuration.

Table 2.3 Common histone modifications

Modification	Major histone residues	Associated effects
Acetylation	H3K9, H3K27, H4K5, H4K8	Activation of gene expression
Phosphorylation	H3S10, H3S28	DNA replication, chromatin condensation during mitosis and apoptosis
Methylation	H3K4, H3K36, H3K79	Activation of gene expression
Methylation	H3K9, H3K27, H4K20	Repression of gene expression

2.4.3. DNA methylation in cholesterol metabolism and T2D

There is an increasing body of evidence that epigenetic modifications are associated with dyslipidaemia and T2D. The majority of evidence in humans comes from epigenome wide DNA methylation association studies (EWAS) looking for associations between individual CpGs from peripheral blood cells and lipid levels or T2D. Multiple studies have identified that lower levels of HDL are associated with increased methylation of *ABCG1*, in particular the CpG cg06500161,[89-92] which has also been associated with increased TG levels and decreased expression of *ABCG1*.[92] Notably, the *ABCG1* CpG cg06500161 is also commonly reported to be hypermethylated in peripheral blood cells of people with T2D,[93-98] and methylation of *ABCG1* has been found to be a predictor of new onset T2D.[94,99] Another differentially methylated gene commonly reported in EWAS of T2D is thioredoxin-interacting protein (*TXNIP*), which is hypomethylated in the blood cells of people with diabetes.[93,96,98,100,101] TXNIP is associated with oxidative stress as it inhibits the antioxidant protein thioredoxin and has been found to induce β cell apoptosis.[102] Methylation of *TXNIP* was also found to be inversely correlated with triglyceride levels, but not LDL, HDL or total cholesterol levels.[91]

One study found increased methylation of *SREBF2*, the gene encoding SREBP-2, was associated with total cholesterol levels,[91] and *ABCA1* methylation was reported to inversely correlate with HDL levels in a study of familial hypercholesterolemia patients.[103] In a study of coronary heart disease patients, methylation of the glucokinase gene was found to correlate with LDL levels.[104] Increased glucokinase methylation has also been reported in the blood cells of men with diabetes.[105] The correlation of glucokinase methylation with LDL and of *ABCG1* methylation with diabetes thus highlights the relatedness of cholesterol and diabetes and shows that this relatedness is reflected in epigenetic modifications. Notable differentially methylated regions in the pancreatic islets of diabetics include the insulin gene, insulin receptor substrate 1 (*IRS1*), peroxisome proliferator-activated receptor gamma (*PPARG*) and PPARγ coactivator 1-alpha (*PPARGC1A*), the latter two of which are important regulatory factors in glucose and lipid metabolism.[106]

2.4.4. Histone acetylation in cholesterol metabolism and T2D

There is also evidence that histone acetylation impacts cholesterol metabolism and diabetes risk. HDAC3 has been found to negatively regulate cholesterol synthesis *in vitro* and in knockout mice,[16] while HDAC3 specific inhibition or knockout reportedly protected pancreatic β cells from cytokine-induced apoptosis and increased insulin secretion.[107-109] The HDAC inhibitor trichostatin A (TSA) downregulated a number of genes involved in cholesterol metabolism, including *SREBF2* and its target genes *HMGCR* and *LDLR*, in the hepatic HepG2 cell line.[25] TSA also downregulated SREBP-2 target genes in neuronal cells, while increasing *ABCA1* mRNA, with a resultant decrease in cellular cholesterol levels.[110] On the other hand, the HAT proteins p300 and CBP act as coactivators in SREBP-2 mediated activation of cholesterol genes and increase histone H3 acetylation in the promoters of *LDLR* and *HMGCR* in sterol depleted cells.[111] Furthermore, SREBPs themselves can be acetylated by p300 and CBP, increasing their stability,[112] and conversely can be deacetylated by the HDAC SIRT1, promoting their degradation.[113] p300 has also been shown to regulate gluconeogenesis by increasing *FOXO1* expression,[114] and FOXO1 subsequently recruits p300 and CBP to gluconeogenic genes *PEPCK* and G6Pase, promoting their expression.[115] Somewhat counterintuitively, HDAC inhibition has been found to decrease expression of *PEPCK* and *G6PC*.[116] In animal studies, HDAC inhibition with TSA[117] or sodium butyrate[118] has been reported to reduce serum

cholesterol and glucose in mice fed a HFD, however evidence is lacking in human trials.

2.5. Statins

HMGCR inhibitors, a class of cholesterol lowering drugs commonly known as statins, are the most commonly prescribed drugs in numerous advanced economies, with approximately one quarter of Americans over the age of 40 being prescribed statins.[119] The efficacy of statins in preventing morbidity and mortality from CVD is well established; a meta-analysis of 62 placebo-controlled trials reported reductions in major coronary events by 31% and overall mortality by 13% with statin treatment.[120]

2.5.1. Statins and cholesterol

Statins are competitive inhibitors of HMGCR, the rate-limiting enzyme in the mevalonate pathway of cholesterol biosynthesis. By reducing the rate of cholesterol biosynthesis, particularly in hepatocytes, statins lower cellular cholesterol and thus promote the activation of SREBP-2, as discussed in section 2.2.1. SREBP-2 activates expression of *LDLR* which results in the uptake of plasma LDL.[121] The continued inhibition of HMGCR by statins ensures that cholesterol biosynthesis does not increase despite the increased expression of *HMGCR* and other cholesterol synthesis genes as a result of SREBP-2 activation. Numerous clinical trials have demonstrated significant reductions of LDL-C with statin treatment, with atorvastatin and rosuvastatin possessing the most potent cholesterol lowering ability and pravastatin, which is associated with a more tolerable safety profile,[122] lowering cholesterol to a lesser degree.[123] Meta-analysis has shown that the degree of cholesterol lowering by statins is directly correlated with the reduced risk of major cardiovascular events.[124] Statins also have been found to increase levels of HDL-C, albeit to a lesser extent. A meta-analysis by Barter et al.[125] reported that different statins increase HDL-C to different extents independently of LDL-C reductions, with rosuvastatin and simvastatin increasing HDL-C to a greater degree than atorvastatin.

2.5.2. Statins and diabetes

Statins have been associated with small but statistically significant increased risk of T2D; a meta-analysis of 13 clinical trials reported a 9% increase in risk of T2D with statin use, although results were variable and some studies reported a possible

protective effect, especially for pravastatin.[14] *In vitro*, statins have been found to alter glucose uptake, utilisation and generation. In β cells, studies both in our group[72] and elsewhere,[126-129] have shown that statins impair GSIS. Additionally, atorvastatin and pravastatin reportedly decrease expression of GLUT2 in β cells.[129] Similarly, in myocytes and adipocytes, statins reportedly decrease expression of GLUT4,[129-131] the insulin-regulated glucose transporter, as well as other proteins involved in insulin signalling including the insulin receptor[132] and IRS-1.[133] Furthermore, lovastatin treatment was reported to reduce glucose uptake by adipocytes, skeletal myocytes and hepatocytes,[134] and atorvastatin decreased insulin-stimulated glucose uptake in adipocytes.[135] Finally, in hepatocytes, statins have been shown to decrease expression of glucokinase,[136] which phosphorylates glucose in the first step of glucose utilisation for both glycogen synthesis and glycolysis, and to increase expression of the gluconeogenic proteins G6Pase and PEPCK.[137] All these mechanisms may contribute to hyperglycaemia. Much like in clinical trials, variable results have been reported; Chen et al.[138] reported that atorvastatin treatment protected β cells from cholesterol-induced apoptosis *in vitro* and animal studies have reported improved insulin sensitivity with rosuvastatin[139,140] and lovastatin.[141]

Despite this research, the exacting mechanism by which statins may increase diabetes risk is not known, nor is why the risk seems to vary between difference statins. The possible protective effect of pravastatin has been attributed to its hydrophilic nature, whereas atorvastatin, simvastatin, fluvastatin and lovastatin are lipophilic and thus can enter numerous cell types readily, whereas hydrophilic statin are more hepatoselective.[142] However, rosuvastatin is also hydrophilic but is associated with a significant increase in diabetes risk, and meta-analysis has shown there is no difference in risk between hydrophilic and lipophilic statins.[14] The difference in risk may instead be directly related to the potency of LDL-C lowering, however, another meta-analysis by Navarese et al.[143] found that although diabetes risk was increased with higher statin doses, this was not influenced by the degree of LDL-C reduction. Numerous potential exacting mechanisms have been proposed, as reviewed by Paseban et al.[144] These include alterations in membrane lipids impacting Ca^{2+} channels in β cells and GLUT4 translocation in peripheral cells, and reduced production of other products of the mevalonate pathway, such as dolichol, which is involved in membrane receptor processing, and coenzyme Q10, which is involved in mitochondrial ATP generation.

2.5.3. Statins and epigenetics

There are several studies on the influences of statins on epigenetic modifications in the context of different pathologies, most notably in cancer. Statins may affect gene expression by modifying histone acetylation, reportedly by inhibiting HDACs. Studies of various cancer cell lines,[145,146] a macrophage line,[147] an epithelial line,[148] and a rabbit model of atherosclerosis[149] reported that statin treatment resulted in hyperacetylation of histones H3 and H4 and/or inhibition of HDAC activity or expression. Statin use has also been reported to increase the recruitment of the HAT p300 to specific loci.[147] Cooney[150] proposed that with less acetyl-CoA used in cholesterol biosynthesis, increased amounts are available to act as acetate donors to acetyltransferases, however this mechanism has not been investigated in the reported studies. Potential mechanisms by which statins may alter histone acetylation are summarised in Figure 2-4.

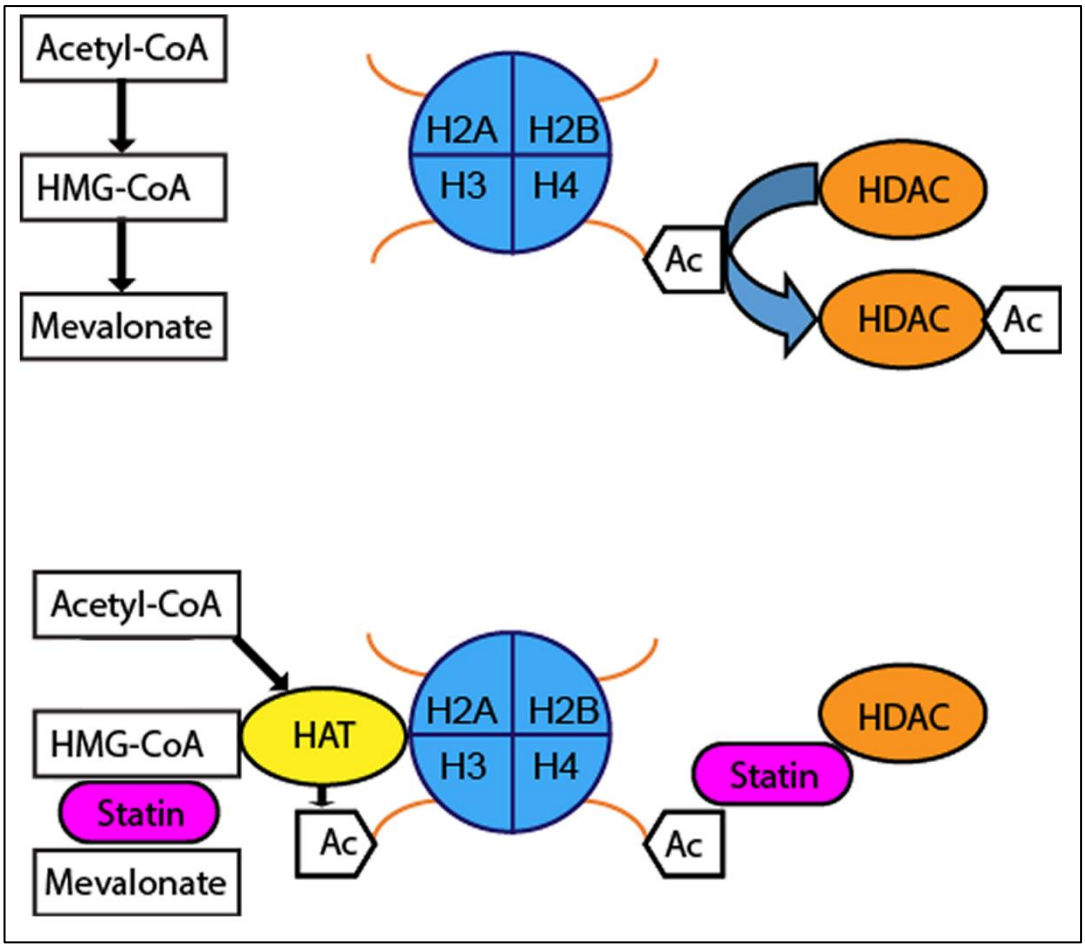

Figure 2-4 Postulated mechanisms by which statins may impact histone acetylation

Without statin treatment (top) acetyl-CoA is used in the mevalonate pathway in the synthesis of cholesterol and other biomolecules. Statins, HMG-CoA reductase inhibitors, block the reduction of HMG-CoA into mevalonate, potentially providing additional acetyl-CoA for histone acetyltransferases (HAT) to add acetyl groups (Ac) to histone tails (bottom). Statins may also increase the recruitment of HATs to target loci. Histone deacetylases remove acetyl groups from histone tails (top); statins may prevent this through HDAC inhibition (bottom). Originally published in Allen and Mamotte.[28]

Statins may also impact histone methylation. Ishikawa et al.[151] reported that statin treatment resulted in downregulation of mRNA and protein expression of EZH2, a HMT that methylates H3K27 and thus suppresses gene expression, in colorectal cancer cells, although this study did not investigate subsequent epigenetic modifications.

Statin treatment may increase expression of certain genes through reduced DNA methylation. Studies of statin treated cancer cell lines by Karlic et al.[146] and Kodach et al.[152] reported downregulated DNMT mRNA expression and protein activity, respectively. Statin treatment was also reported to reduce the methylation of promoters of certain genes and subsequently increase gene expression in cancer cells[152] and T cells.[153]

Studies on the epigenetic effects of statins in the context of cholesterol lowering and T2D are limited, despite being the primary goal of statin treatment and a significant adverse effect respectively. Recently, several studies have been published showing effects on DNA methylation in human statin users. In an EWAS of 8313 participants from five cohorts, differentially methylated CpGs in *DHCR24*, *SC4MOL*, and *ABCG1* were found in the blood cells of statin users, with increased methylation at one CpG, cg06500161, in *ABCG1* independently predicting incident T2D.[154] Methylation at this site was also associated with reduced *ABCG1* gene expression and with increased fasting glucose and insulin. Similarly, in a separate cohort of 2741 participants, and validated in an additional cohort of 2020 participants, statin use was associated with increased methylation of *ABCG1*.[155] This methylation was also associated with T2D, fasting glucose and insulin. In a published abstract of a cohort of 896 statin users and 943 non-users by Yao et al.,[156] statin use was associated with differentially methylated sites in *DHCR24*, *SC4MOL*, *ABCG1* and *NACA*. Importantly, the CpG at which statins increase methylation of *ABCG1*, cg06500161, is the same site associated with T2D, discussed in section 2.4.3. Both DHCR24 and SC4MOL are involved in cholesterol synthesis, while NACA binds to newly synthesised peptides that lack a signal peptide motif and does not appear to have been previously linked to cholesterol or diabetes. ClinicalTrials.gov shows a clinical trial (NCT02817230) investigating the effect of statin treatment on H3K4 trimethylation in the gene promoter regions of pro-inflammatory cytokines in hypercholesterolaemic patients, although no results or updates have been posted.

2.6. Butyrate

Butyrate is a SCFA produced by the bacterial fermentation of dietary fibre, in particular resistant starch, and is also present in some foods such as butter.[157] Butyrate is a known HDAC inhibitor with a reported IC50 ranging from 90 μM in HT-29 nuclear extracts to 1.13 mM in live HeLa cells.[158] Many of butyrate's reported effects

have been attributed to HDAC inhibition and subsequent increased histone acetylation and gene expression. Conversely, it has also been reported that butyrate may decrease acetylation and subsequent gene expression in some sites, particularly with prolonged exposure.[159] By altering histone acetylation, butyrate alters the expression of a significant number of genes; microarray analysis of nearly 20 000 genes in butyrate treated colonic epithelial cells found more than 10% of genes had a two-fold or greater change in expression compared to untreated controls, with 4% upregulated and 6% downregulated.[160] Butyrate may also influence metabolism through the binding and activation of free fatty acid G-coupled receptors, FFAR2 and FFAR3, particularly in the intestine. Binding of SCFAs to these receptors in intestinal enteroendocrine cells is thought to promote the release of GLP-1 and peptide YY (PYY),[161] the former promoting pancreatic insulin secretion and inhibiting glucagon secretion, and the latter reducing appetite and slowing gastric emptying.[162]

Butyrate's mechanisms of action are summarised in Figure 2-5.

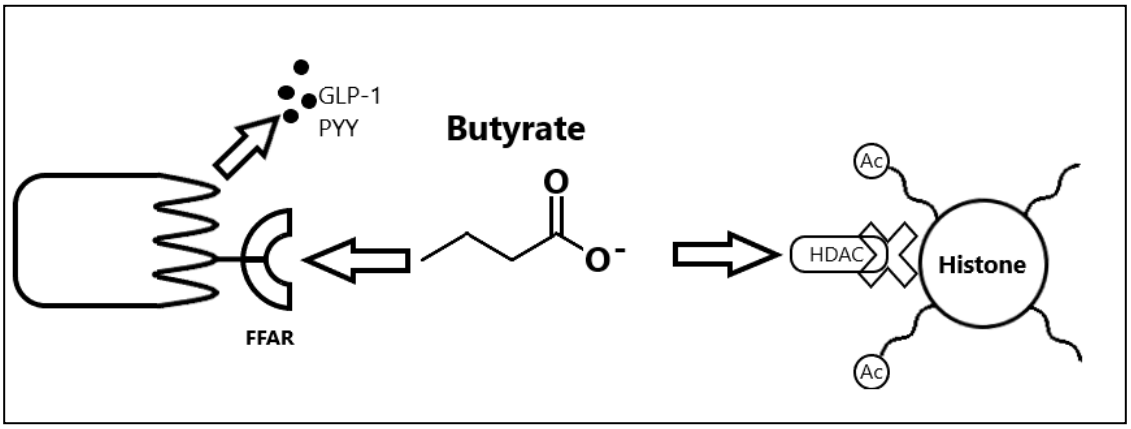

Figure 2-5 Butyrate's mechanisms of action

Butyrate activates free fatty acid receptors (FFAR), which in enteroendocrine cells may lead to the release of gut hormones such as GLP-1 and PYY. Butyrate also inhibits HDAC activity, increasing histone acetylation (Ac) and altering gene expression. Originally published in Bridgeman et al.[163]

In animal models of metabolic diseases, butyrate supplementation reportedly exerts numerous benefits, including lowering of serum triglyceride, cholesterol and glucose levels, reducing weight gain, improving glucose tolerance and protecting against cardiac dysfunction.[164,165] However, the few small-scale human trials conducted have lacked significant results; a randomised placebo-controlled clinical trial investigated the effect of six daily 100 mg sodium butyrate tablets for 45 days in type 2 diabetics.[6,7]

The study found butyrate did not significantly alter fasting blood glucose, fasting insulin, cholesterol or triglyceride levels, although it did increase GLP-1 levels and reduce diastolic blood pressure.[6] The lack of efficacy for diabetes in this study could be due to the small population (15 people per group). In a pilot study, four weeks of 4 g daily oral sodium butyrate improved peripheral and hepatic insulin sensitivity in lean males but not in males with metabolic syndrome (MetS).[166] This trial was not placebo-controlled and used small study groups (nine lean, ten MetS). Well-designed, large-scale clinical trials are needed to determine if there is any effect of butyrate on cholesterol and diabetes in humans.

2.6.1. Butyrate and cholesterol

Multiple studies have reported that butyrate lowers serum cholesterol in animals fed a HFD.[118,165,167,168] Reduced serum cholesterol could be due to alterations in cholesterol biosynthesis, cholesterol import/export from cells or dietary cholesterol uptake. *In vitro* studies suggest that butyrate may influence all three processes. Marcil et al.[169] reported that butyrate reduced cholesterol synthesis in Caco-2 enterocytes, associated with a reduction in HMGCR activity. Reductions in *HMGCR* mRNA expression have also been reported in Caco-2 cells, in addition to reduced HMG-CoA synthetase 2 expression.[170] Butyrate also decreased expression of genes in downstream steps of cholesterol synthesis, namely in the conversion of mevalonate to squalene (isopentenyl diphosphate isomerase, dimethylallyl/geranyl trans-transferase and farnesyl-diphosphatase franesyltransferase) and in the conversion of squalene to cholesterol (lanosterol 5-desaturase and methylsterol monooxygenase). These studies did not investigate if the altered expression was due to HDAC inhibition. Conversely, in hamsters on a high cholesterol diet, although butyrate decreased plasma total cholesterol, it increased hepatic *HMGCR* gene expression, in addition to *SREBF2* and *LDLR*, possibly as a compensatory measure in response to lowered cholesterol as is seen with statin treatment.[168] In this study, similar effects were found with acetate and propionate, suggesting this is an effect common to SCFAs and not a result of HDAC inhibition.

There is conflicting evidence for how butyrate influences cholesterol transport. Kaptein et al.[171] found increased secretion of cholesterol esters from HepG2 cells with 2 mM sodium butyrate. Conversely, Marcil et al.[169] found reduced secretion of cholesterol esters with 20 mM sodium butyrate in Caco-2 cells. Differences between

hepatic cells and intestinal cells and differences in butyrate concentration may contribute to these disparate effects. Marcil et al.[172] also reported reduced synthesis of apolipoproteins apoB-48 and apoA-I in butyrate-treated cells, whereas other studies found butyrate increased apolipoprotein expression and secretion. Nazih et al.[173] found butyrate increased apoA-IV mRNA expression and protein secretion in Caco-2 cells, and increases in apoA-1 and apoB-100 secretion have been reported in butyrate treated HepG2 cells.[174] Other proteins involved in cholesterol transport that may be upregulated by butyrate in HepG2 cells include phospholipid transfer protein, involved in the transfer of phospholipids and cholesterol amongst lipoproteins and lipids,[175] and cholesteryl-ester-transfer protein, which transfers lipids between VLDL or LDL and HDL.[176] Lecithin:cholesterol acyltransferase, which promotes the transfer of cholesterol from cell membranes to HDL, may be downregulated.[177] It was not investigated if changes in cholesterol transport were due to HDAC inhibition.

Finally, butyrate may reduce dietary cholesterol uptake by intestinal cells. In a study of Caco-2 cells, butyrate significantly reduced cholesterol uptake and reduced expression of *NPC1L1*.[178] The gene expression of *ABCG5* and *ABCG8*, involved in transporting cholesterol from intestinal cells into the intestinal lumen and thus away from the circulation, was increased. These effects were not seen in cells treated with acetate or propionate, indicating this is not a common effect of SCFAs.

2.6.2. Butyrate and diabetes

The ability of butyrate to induce insulin secretion in farmed animals has been known for decades, long before its ability to inhibit HDACs was discovered. In 1967, Manns et al.[179] reported that infusion of butyrate directly into a pancreatic artery of sheep resulted in a marked increase in plasma insulin within 3 min. Investigations into the effect of butyrate on insulin secretion *in vitro* have reported variable results, possibly as a result of the use of cell lines with defective GSIS. In pancreatic islets from mice fed an HFD with or without butyrate supplementation, butyrate increased GSIS by reducing basal (2.8 mM glucose) and increasing glucose-stimulated (16.7 mM) insulin secretion.[180] Conversely, in a study of BRIN-BD11 cells, three days of butyrate treatment reduced cell growth, insulin content and insulin secretion in both the presence and absence of glucose.[181] In RIN-m5F-2A cells, which are usually unresponsive to glucose, sodium butyrate increased insulin secretion, intracellular content and mRNA expression approximately three-fold in the presence and absence

of glucose and other secretagogues (glyceraldehyde and potassium), although GSIS remained impaired.[182] This was associated with increased activity of both hexokinase and glucokinase, indicating increased responsiveness to both low and high glucose concentrations, and increased GLUT2 expression. A similar study in RINm5F cells also reported increased insulin secretion with impaired GSIS, associated with increased hexokinase, but not glucokinase, gene expression and protein activity.[183]

Butyrate has been reported to improve insulin signalling, with multiple studies finding that butyrate increases phosphorylation of IRS-1 and/or AKT in animal models of diabetes.[118,165,167,184] Chriett et al.[185] showed that butyrate increased histone acetylation in proximity to the IRS-1 promoter, increasing its expression, in L6 myocytes exposed to palmitate in a model of insulin resistance, suggesting a direct mechanism by which butyrate can increase insulin sensitivity through HDAC inhibition. This increase in insulin signalling should lead to increased glucose uptake, and in porcine adipocytes an increase in glucose uptake has been reported with sodium butyrate treatment.[186] This was associated with an increase in expression of the glucose transporter GLUT4, which has also been reported in rat adipose tissue.[118] Similarly, butyrate has also been reported to increase GLUT2 expression in liver cells.[118]

Increased insulin signalling in the liver may also ameliorate hyperglycaemia through inhibition of gluconeogenesis. There is also evidence that butyrate inhibits gluconeogenesis through additional mechanisms. It has been shown that HDACs can deacetylate the gluconeogenic transcription factor FOXO1, leading to an alteration of its subcellular localisation and DNA binding.[187] HDAC inhibition may in this way decrease the expression of gluconeogenic enzymes such as PEPCK and G6Pase. In the livers of diabetic rats, butyrate treatment was shown to reduce nuclear FOXO1.[167] Furthermore, the same study found that sodium butyrate reduced the expression of glucagon, a hormone that promotes gluconeogenesis and glycogenolysis, in rat islets.

2.7. Conclusion

There is a growing body of evidence that epigenetic modifications are associated with the development and pathology of dyslipidaemia, insulin resistance and T2D. Furthermore, agents with reported epigenetic effects, namely butyrate and statins, have been shown to lower cholesterol levels and impact insulin sensitivity and the risk of developing T2D. However, there are significant gaps in the literature

concerning the underlying biological mechanisms. Few studies have linked specific epigenetic modifications to the observed phenotypic changes. In the case of statins, studies proposing epigenetic effects have been conducted primarily in the context of cancer and not in the context of cholesterol lowering or diabetes. For butyrate, *in vitro* studies have reported inconsistent results, while for *in vivo* animal studies, it is not known if the observed effects are a result of direct epigenetic changes in the involved tissues or indirect as a result of FFAR activation in intestinal cells and the subsequent release of hormones such as GLP-1. Further research is therefore needed, examining epigenetic changes in the vicinity of key genes involved in cholesterol and glucose metabolism in response to these agents.

Chapter 3. Methods and Materials

3.1. Creation of stock solutions

All statins were sourced from Cayman Chemical. Atorvastatin (calcium salt), rosuvastatin (calcium salt), simvastatin (sodium salt) and pravastatin (sodium salt), Trichostatin A (TSA) (Cayman Chemical) and curcumin and RG-108 (abcam) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a concentration of 10 mM and stored at -20°C or at -80°C for long-term use. Sodium butyrate (Selleck Chemicals) and sodium acetate (Toronto Research Chemicals) were dissolved in ultra-pure distilled water to a concentration of 50 mM and stored at -20°C.

3.1.1. Creation of lipoprotein-deficient serum and high-lipoprotein serum

The density of foetal bovine serum (Serana) was adjusted to 1.21 g/ml with sodium bromide. The solution was transferred to 11.5 ml polyallomer ultracrimp tubes and centrifuged for 20 h at 70000 g in a Sorvall WX ultracentrifuge. The base of the tube was pierced with a butterfly needle and 1 ml aliquots collected. Cholesterol and total protein concentrations of the aliquots were measured using the Amplex™ Red Cholesterol Assay Kit (Life Technologies) and the Pierce BCA Protein Assay Kit (ThermoFisher) respectively, to determine which aliquots were deficient in lipoproteins (lipoprotein-deficient serum; LPDS) and those enriched in lipoproteins (high-lipoprotein serum; HLPS). Salt was removed from the serum by centrifugation with Zeba™ desalting spin columns 7K MWCO (ThermoFisher) and serum was sterilised with a Millex 0.22 µm syringe filter prior to storage at -20°C.

3.2. Sample preparation

3.2.1. Cell culture and treatments

HepG2 human hepatocellular carcinoma cells were obtained from ATCC via Dr Ross Graham (Curtin University). MDA-MB-231 human breast carcinoma cells were obtained from ATCC via Professor Arunasalam Dharmarajan (Curtin University). BRIN-BD11 rat insulinoma cells were obtained from Professor Peter Flatt via Professor Philip Newsholme (Curtin University). THP-1 human leukemic monocytes

were obtained from Dr Hilary Warren (Canberra Hospital) via Professor Deirdre Coombe (Curtin University).

HepG2 human hepatocellular carcinoma cells were maintained in Dulbecco's Modified Eagle's medium: Ham's F-12 Nutrient Mixture (DMEM:F12 media) (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS). BRIN-BD11 rat insulin-secreting cells, MDA-MB-231 human breast cancer cells and THP-1 human monocytes were maintained in Roswell Park Memorial Institute (RPMI) media (Sigma-Aldrich) supplemented with 10% FBS. All cells were maintained in 25-cm^2 or 75-cm^2 tissue culture flasks at 37°C in a humidified incubator equilibrated with 5% CO_2. All cells tested negative for mycoplasma contamination. Cells were seeded in 6-well plates, 24-well plates, 96-well plates, 25-cm^2 vented tissue culture flasks or 75-cm^2 vented tissue culture flasks and allowed to recover overnight prior to treatment. Prior to treatment, THP-1 cells were differentiated into macrophages by the addition of 50 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) to the culture media. THP-1 cells were differentiated for 48 h, then allowed to recover for 24 h before treatment. All cells were treated in media supplemented with 10% LPDS unless otherwise indicated. Control cells were treated with media only or media containing DMSO as a vehicle control as indicated.

3.2.2. Animal Study

Liver samples were utilised from a previous animal study conducted by our group. In that study, eight-week-old male C57B1/6J mice from the Animal Resource Centre, Murdoch, Western Australia were delivered to the animal facility, Curtin University. Following acclimatisation, mice were randomly assigned to either normal diet (ND) (14% of energy from lipids, Meat Free Rat and Mouse Maintenance Diet, Specialty Feeds, Glen Forrest, WA, Australia) or high-fat diet (HFD) (59% of energy from lipids, 36% Fat Modification of AIN93G, Specialty Feeds). From week 4, mice were further divided into treatment groups to receive either 10 mg/kg/day of atorvastatin or vehicle (water) by gastric gavage for a further 12 weeks. Following completion of the treatment period, mice were starved for 6 h and anesthetised in an isoflurane chamber before being euthanised by cervical dislocation. Liver samples were removed from the carcass and collected in prechilled microcentrifuge tubes and snap-frozen either in dry ice or liquid nitrogen. Animal experiments were approved by

Curtin University's Animal Ethics Committee (AEC_2016_17, approval date 14/4/2016).

3.2.3. Nuclear extraction

Nuclear extraction was conducted using the abcam Nuclear Extraction Kit (ab113474) according to the manufacturer's instructions. For experiments on cultured cells, confluent cells were scraped and incubated on ice for 10 min in pre-extraction buffer containing dithiothreitol (DTT) and protease inhibitor cocktail (PIC), then centrifuged at 12 000 rpm for 1 min. The cytoplasm containing supernatant was removed, and the pellets incubated on ice for 15 min in nuclear extraction buffer containing DTT and PIC, then sonicated three times for 10 s in a Biorupter sonicating water bath. The extract was then centrifuged at 14 000 rpm for 10 min at 4°C and the supernatant removed for protein quantification using the Pierce Coomassie Protein Assay Kit (ThermoFisher). For experiments on murine liver from the animal study, samples were placed in a glass homogeniser containing pre-extraction buffer with DTT and PIC and homogenised manually. Homogenised samples were transferred to microcentrifuge tubes and incubated on ice for 15 min then centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was removed, and nuclear extraction continued as for cultured cells.

3.2.4. Whole cell protein extraction

Confluent cells were scraped then lysed on ice for 20 min in radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Sigma-Aldrich). The lysate was sonicated 10 times for 10 s then centrifuged at 14000 rpm for 10 min and the protein rich supernatant was removed for protein quantification by the bicinchoninic acid (BCA) method using the Pierce BCA Protein Assay Kit (ThermoFisher).

3.3. Viability assays

Viability was determined using alamarBlue® (ThermoFisher) as a measure of cellular metabolism. Upon entering living cells, resazurin is reduced to resorufin, a compound that is highly fluorescent. Following 22 h treatment in 96 well plates, 10 µL of alamarBlue® was added to the treatment media and cells were incubated for a further 2 h. Fluorescence was then measured using an EnSpire Multimode Plate Reader

(PerkinElmer) with an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

3.4. Lipid quantification

3.4.1. Lipid extraction

After 24 h treatment in 96 well plates, media was removed, and cells were washed twice with PBS. Lipids were extracted using 150 µL of a solution containing 3:2 hexane:isopropanol. Following 15 s incubation and mixing by pipetting, lipids were transferred to a 96 V well plate and allowed to dry in a fume hood at room temperature (RT) for at least 4 h or overnight.

3.4.2. Cellular cholesterol content

Cholesterol content was assessed using the Amplex Red Cholesterol Assay Kit (Life Technologies). Samples were dissolved in a 1:1 reaction buffer:isopropanol mixture and incubated with catalase for 15 min at 37°C to eliminate endogenous peroxidases which would otherwise interfere in this assay.[188] Amplex red working solution was added and following 30 min incubation at 37°C, fluorescence was read with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Cholesterol content of samples was determined using a standard curve based on a serial dilution of the cholesterol standard provided with the kit.

3.4.3. Cellular triglyceride content

TG content was assessed using the High Sensitivity Triglyceride Fluorometric Assay Kit (Sigma-Aldrich) as per the manufacturer's instructions. Samples were dissolved in 1:1 reaction buffer:isopropanol and incubated with lipase for 20 min at 37°C to hydrolyse TGs into glycerol and fatty acids. Samples were then incubated with the reaction master-mix containing the developer, probe and enzyme mix that reacts with the glycerol present. Following 30 min incubation at 37°C, fluorescence was measured with an excitation wavelength of 535 nm and an emission wavelength of 587 nm. TG content of samples was determined using a standard curve based on a serial dilution of the TG standard provided with the kit.

3.4.4. Cholesterol uptake and export assays

Cholesterol uptake and export was determined using the Cholesterol Uptake Cell-Based Assay Kit (Cayman Chemical). For cholesterol uptake assays, cells in 96-well plates were incubated in media containing 10% LPDS for 24 h prior to treatment containing 20 ug/ml of a fluorescently-tagged cholesterol (NBD cholesterol). Following 24 h treatment, cells were washed in PBS, assay buffer was added and fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The cholesterol transport inhibitor U-18666A was used as a positive control. For cholesterol export assays, cells in 96-well plates were incubated with media containing 10% LPDS and 20 ug/ml NBD cholesterol for 48 h prior to treatment in media containing 10% FBS. Following 24 h treatment, the media containing exported NBD cholesterol was transferred to fresh wells and fluorescence measured as for uptake assays.

3.5. Immunoblotting

Equal amounts of protein (20-30 µg depending on sample concentration and amount of protein of interest in sample) were denatured at 98°C for 10 min in solution containing Bolt Sample Reducing Agent (ThermoFisher) and SDS Sample Loading Buffer (Sigma Aldrich), then fractionated on Bolt™ 4-12% Bis-Tris Plus Gels (ThermoFisher). Proteins were transferred onto nitrocellulose membranes using the iBlot Gel Transfer Device (Invitrogen) and blocked with 3% bovine serum albumin (BSA) (Bovogen) in Tris-buffered saline with 0.1% Tween 20 (TBST) for at least 1 h. Membranes were incubated overnight with primary antibodies (Table 3.1) diluted in blocking buffer. Membranes were incubated with the secondary antibodies conjugated to horseradish peroxidase (HRP) (Table 3.1) in blocking buffer for at least 1 h then washed three times with TSBT. Immunodetection was performed using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and the Chemi-Doc™ Gel Imaging System (Bio-Rad). Band density was measured with Image Lab software (Bio-Rad) and normalised to the density of the housekeeping protein GAPDH and total protein as determined by Coomassie Blue staining in separate analyses.

Table 3.1 Antibodies used in immunoblotting

Target	Type	Product #	Company
GAPDH	Mouse monoclonal	ab8245	abcam
H3K9ac	Mouse monoclonal	sc56616	Santa Cruz Biotechnology
H4ac (S1, K5, K8 and K12)	Mouse monoclonal	sc377520	Santa Cruz Biotechnology
H3K9me3	Mouse monoclonal	sc130356	Santa Cruz Biotechnology
HDAC3	Mouse monoclonal	sc376957	Santa Cruz Biotechnology
ABCA1	Mouse monoclonal	ab66217	abcam
ABCG1	Rabbit monoclonal	ab52617	abcam
Caveolin-1	Rabbit polyclonal	ab18199	abcam
HMGCR	Rabbit monoclonal	ab174830	abcam
LDLR	Rabbit polyclonal	ab30532	abcam
SRB1	Rabbit monoclonal	ab52629	abcam
SREBP-2	Rabbit polyclonal	ab28482	abcam
APO-A1	Mouse monoclonal	sc376818	Santa Cruz Biotechnology
APO-B	Mouse monoclonal	sc-13538	Santa Cruz Biotechnology
Glucokinase	Rabbit polyclonal	ab37796	abcam
GLUT2	Rabbit polyclonal	ab54460	abcam
Hexokinase I	Rabbit monoclonal	2024	Cell Signalling
PEPCK	Mouse monoclonal	sc166778	Santa Cruz Biotechnology

Target	Type	Product #	Company
AKT (phospho-Ser473)	Rabbit monoclonal	ab81283	abcam
IRβ (phospho-Tyr1361)	Rabbit polyclonal	ab60946	abcam
IRS-1 (phospho-Tyr632)	Rabbit polyclonal	sc17196	Santa Cruz Biotechnology
Secondary antibodies			
Goat Anti-Mouse	IgG H&L (HRP)	ab6789	abcam
Goat Anti-Rabbit	IgG H&L (HRP)	ab6721	abcam

3.6. Gene expression

3.6.1. RNA extraction

RNA extraction was performed using the TRI reagent protocol as described by Rio et al.[189] using 1-bromo-3-chloropropane (BCP) in place of chloroform for phase separation.[190] Treated cells in 6-well plates were washed twice in PBS and 300 µL TRI reagent (Sigma-Aldrich) was added directly to cells. Following mixing, the solution was transferred to 1.5 ml tubes, BCP was added and tubes were vortexed and incubated at RT for 10 min. Tubes were centrifuged at 14000 rpm for 15 min at 4°C and the upper aqueous phase carefully transferred to fresh low adherence 1.5 ml tubes. An equal volume of isopropanol was then added, tubes were mixed by inversion and incubated at RT for 10 min. The tubes were then centrifuged at 14000 rpm for 15 min at 4°C, the supernatant removed, and the RNA pellet was washed twice with 75% ethanol and allowed to air dry. Pellets were dissolved in nuclease free water and allowed to equilibrate for 1 h at RT. RNA was quantified using the NanoDrop 1000 spectrophotometer (ThermoFisher), with purity considered sufficient if A_{260}/A_{280} measured between 1.8-2.0. Samples were stored at -80°C.

3.6.2. Reverse transcription

Reverse transcription was performed using the SensiFAST™ cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions. 1 µg of RNA was used per reaction in a 20 µl reaction mix containing reverse transcriptase and a blend of anchored oligo dT and random hexamer primers. Reverse transcription was

performed in a Veriti 96 Well Thermal Cycler (Applied Biosystems) with the following program: 25°C for 10 min (primer annealing), 42°C for 15 min (reverse transcription), 48°C for 15 min (reverse transcription of complex RNA), 85°C for 5 min (reverse transcriptase inactivation), 4°C hold (cooling). cDNA was diluted in 20 µl ultrapure water and stored at 4°C.

3.6.3. qPCR

Quantitative real-time PCR (qPCR) was performed using the DNA intercalating fluorescent dye, SYBR-Green.[191] Predesigned KiCqStart® SYBR® Green Primers (Sigma-Aldrich, sequences in Table 3.2, Table 3.3) were dissolved in ultrapure water to a concentration of 100 µM and stored at -80°C. Working primer mixes of 5 µM forward and reverse primers in ultrapure water were stored at 4°C. SensiFAST SYBR Lo-ROX Mix (Bioline) was used with 200 nM primer mix unless otherwise stated, 2 µl cDNA sample template and ultrapure water in a 10 µl reaction mix. qPCR was performed in a CFX Connect Real-Time System (Bio-Rad) with the following program: 95°C for 2 min (activation) followed by 40 cycles of 95°C for 15 sec (denaturation), 57°C-62°C for 15 sec (annealing) and 72°C for 1 min (extension). Melt curve analysis was then performed starting at 72°C for 30 sec, then increasing 0.5°C every 5 sec to a maximum of 95°C. The annealing temperature for each primer (Table 3.2, Table 3.3) was determined using a serial dilution of a mixed cDNA sample template and a temperature gradient, with a standard curve of Ct values plotted and efficiency calculated using $E = -1+10^{(-1/slope)}$. If efficiency was poor (<90%), the R^2 value of the standard curve was poor (<0.99) or multiple peaks were seen by melt curve analysis, additional optimisation was performed using different primer concentrations. Human *APOB* and *PPARG* and rat *Actb and Rpl13a* used 100 nM primers due to the presence of multiple peaks seen on melt curve analysis at 200 nM. Initial gene expression qPCR experiments included serial dilution to determine efficiency of primers, no template controls, no reverse transcriptase controls and three technical replicates of each sample, with two biological replicates of each treatment. Confirmatory experiments used two technical replicates and lacked serial dilutions to reduce costs. Gene expression was normalised to that of reference genes chosen by reviewing the literature for genes shown to be suitable for use in statin or HDAC treated HepG2 cells.[192-194] Human *GAPDH, RPL13A* and *YWHAZ* primers were used for HepG2 cells and rat *Actb* and *Rpl13a* primers were used for BRIN-BD11 cells.

Normalised gene expression ($\Delta\Delta C_q$) was calculated by Bio-rad CFX Manager 3.1 software.

Table 3.2 Human primers for qPCR experiments in HepG2 cells

Gene	Forward Primer	Reverse Primer	Annealing Temperature
GAPDH	ACAGTTGCCATGTAGACC	TTGAGCACGGGTACTTTA	57-60°
YWHAZ	AACTTGACATTGTGGACATC	AAAACTATTTGTGGGACAGC	57-63°
RPL13A	GTCTGAAGCCTACAAGAAAG	TGTCAATTTTCTTCTCCACG	61-63°
LDLR	GAGGACAAAGTATTTTGGACAG	GTAGGTTTTCAGCCAACAAG	57°
PPARG	AAAGAAGCCAACACTAAACC	TGGTCATTTCGTTAAAGGC	63°
SREBF2	CAGCAGGTCAATCATAAACTG	GGACATTCTGATTAAAGTCCTC	61.4°
NR1H3	CATGACCGACTGATGTTC	CAAACACTTGCTCTGAGTG	61.4°
APOA1	AGGAGTACACTAAGAAGCTC	AAACGTTTATTCTGAGCACC	62°
APOB	CTTACATCCTGAACATCAAGAG	AGTTTCCATACACGGTATCC	63°
GK	GTTCTTCTGAGATCTATGGC	AAATACACACTTATGGCCTG	60°
HMGCR	ACTTCGTGTTCATGACTTTC	GACATAATCATCTTGACCCTC	59°
PEPCK1	ATTCTGGGTATAACCAACCC	GTTGATGGCCCTTAAATGAC	59°
SLC2A2	AGAAGATTAGACTTGGACTCTC	GTGACCTTATCTTCTGTCATTG	59°
SREBF1	AATCTGGGTTTTGTGTCTTC	AAAAGTTGTGTACCTTGTGG	61.4°
CAV1	CAGGGACATCTCTACACC	TCAAAGTCAATCTTGACCAC	61.4°
CYP7A1	AAATCTACCCAGACCCTTTG	TTCCAGGACATATTGTAGCTC	58°
FOXO1	GTCAAGACAACGACACATAG	AAACTAAAAGGGAGTTGGTG	59°
INSR	GATCCAATCTCAGTGTCTAAC	CCTTTGAGGCAATAATCCAG	62°
ABCG1	GAGGTGAACCCTTTTCTTTG	CCTTTCTCAACCCCTTTAATC	60°
CEBPA	AGCCTTGTTTGTACTGTATG	AAAATGGTGGTTTAGCAGAG	59°
CPT1A	ACGGGGATTATAAGTCAAGG	CACAGCAAGTGAAAATCAAC	58°
HNF4A	AGTACATCCCAGCTTTCTG	AATGTAGTCATTGCCTAGGAG	61.4°
MVK	CATGTTGTCAGAAGTCCTAC	CTCAAGTTCAAGGATACAGC	59°
ABCA1	GTGTTTCTGGATGAACCC	TTCCATTGACCATGATTGC	62°
HK1	AGGTATGAGAAGATGATCAGTG	GAGAAACTTGGTCTCAAAGATG	61.4°
SCARB1	ACAAAAGCAACATCACCTTC	TGGGCTTATTCTCCATCATC	62°

Table 3.3 Rat primers for qPCR experiments in BRIN-BD11 cells

Gene	Forward Primer	Reverse Primer	Annealing Temperature
Actb	AAGACCTCTATGCCAACAC	TGATCTTCATGGTGCTAGG	57-61°
Rpl13a	GCACAAGACCAAAAGAGG	CGCTTTTTCTTGTCATAGGG	57-61°
Hk1	AAACTCTGGGAAACAAAGG	AAACTTGGTCTCAAAGATGC	57°
Hnf4a	TGTGTGAGTCTATGAAGGAG	ATGTAGTCATTGCCTAGGAG	61°
Pdx1	AAAGGTTACAAACTTGAGCG	AAACAGCTCCCTTTATTCTC	59°
Srebf1	AAACCTGAAGTGGTAGAAAC	TTATCCTCAAAGGCTGGG	59°
Ins1	AACGTGGTTTCTTCTACAC	TCTCCAGTTGGTAGAGGG	61°
Abcg1	GTTATGTTCTTTGATGAGCCC	CCTTGACTTAGGACATAAAG C	57°
Abca1	CATCTGAAAAACAGGTTTGG	GGGAGAGAATGCTGAATAT C	57°
Txnip	CGTCAATACTCCTGACTTAATG	AAATGTCATCACCTTCACAG	61°
Ldlr	CAGTGAAGATATTGACGAG	TCACTTACGTACCTCATGG	61°

3.7. Insulin secretion

3.7.1. Insulin secretion assay

Following 24 h treatment in 96-well plates, media was transferred to a fresh plate and stored at -80°C as 'chronic' insulin secretion samples, as opposed to stimulated insulin secretion following the addition of secretagogues such as glucose. Cells were washed in PBS and incubated for 40 min at 37°C in Krebs Ringer Bicarbonate Buffer (KRBB, 115 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 24 mM $NaHCO_3$, 0.1% HEPES (v/v), 0.1% BSA (w/v), pH 7.4) in order to allow the cells to metabolise any residual glucose in the media. A subset of previously untreated cells had 5 mM sodium butyrate in the KRBB for a 40 min treatment. Subsequently, cells were washed in PBS and incubated for 20 min at 37°C in stimulation media (10 mM alanine and 16.7 mM glucose in KRBB), a combination that robustly and reproducibly promotes insulin secretion.[195] A subset of previously untreated cells included 5 mM sodium butyrate in the stimulation media and a further subset used only KRBB ± 5 mM sodium butyrate to determine if butyrate can act as a secretagogue. Media was then transferred to a fresh plate at stored at -80°C for later insulin measurement. RIPA lysis buffer (Sigma-Aldrich) was added to cells for protein quantification using the Pierce BCA Protein Assay Kit (ThermoFisher).

3.7.2. Cellular insulin content

Following treatment as for insulin secretion, cells were washed with PBS and incubated with acid ethanol (1.5% HCl in 70% ethanol) overnight at 4°C. This solution was transferred to a fresh plate at stored at -80°C for later insulin quantification. RIPA lysis buffer was added to cells for protein quantification as for insulin secretion.

3.7.3. Insulin quantification

Insulin quantification assays were performed by Dr Gaewyn Ellison. Insulin was assayed by sandwich enzyme-linked immunosorbent assay (ELISA) using an ultrasensitive Rat Insulin ELISA kit (Mercodia) as per manufacturer's instructions. Briefly, samples and an enzyme-conjugated detection antibody were added to wells pre-coated with anti-insulin antibodies and incubated for 2 h at RT. Following washing with wash buffer, 3,3',5,5'-tetramethyl-benzidine (TMB) was added and colour was allowed to develop for 15 min. The reaction was stopped with 0.5 M H_2SO_4 and absorbance was measured with an absorbance wavelength of 450 nm.

3.8. Insulin signalling

206 µM human insulin stocks were prepared by Dr Thiruvarutchelvan Sabapathy. 12 mg recombinant human insulin powder (Sigma-Aldrich) was dissolved in 450 µl 0.02 M HCl. The solution was made up to 10 ml by the addition of 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) containing 0.1% BSA and the solution was sterilised with a Millex 0.22 um syringe filter prior to storage at -20°C. Following 24 h treatment in T25 flasks, 100 nM insulin was added directly to the treatment media. Cells were incubated for 7 min at 37°C prior to protein extraction as in section 3.2.4 and immunoblotting as in section 3.5.

3.9. Epigenetic modifying enzyme activity assays

3.9.1. HDAC activity

The *In Situ* Histone Deacetylase (HDAC) Activity Fluorometric Assay Kit (Sigma-Aldrich) was used to measure HDAC activity according to the manufacturer's instructions. The HDAC substrate was added to wells containing live cells directly to the treatment media after 23 h treatment with statins or HDAC inhibitors. For the

nuclear extract experiments, the HDAC substrate was added directly with the treatments and nuclear extracts in phosphate buffered saline (PBS). After 1 h incubation, the HDAC developer was added, the plate incubated for a further 30 min and fluorescence read with an excitation wavelength of 368 nm and an emission wavelength of 442 nm.

Confirmatory experiments were carried out with the Fluor De Lys® HDAC fluorometric activity assay kit (Enzo Life Sciences) according to the manufacturer's instructions. HeLa nuclear extract provided with the kit and HepG2 nuclear extract prepared as above were incubated with treatments and substrate for 30 min. Following incubation with developer for 10 min, fluorescence was measured with an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

3.9.2. HAT activity assays

HAT activity was measured using the EpiQuik™ HAT Activity/Inhibition Assay Kit (Epigentek) according to the manufacturer's instructions. The HAT substrate was captured in strip wells for 45 min. Following washing, strip wells were incubated with acetyl-CoA, HepG2 nuclear extracts and treatments for 90 min at 37°C. Following washing, wells were incubated sequentially with the capture antibody and detection antibody, followed by the addition of the developer solution. Once the solution in the control wells changed to a medium blue, the enzymatic reaction was stopped with the stop solution and the absorbance measured at 450 nm.

3.9.3. DNMT activity assays

DNMT activity was measured with the abcam DNMT Activity Assay Kit according to the manufacturer's instructions, with the following modification: whole cell extracts were used as opposed to nuclear extracts as experiments with nuclear extracts were not successful. For direct DNMT inhibition experiments, untreated cell extracts were incubated with treatments and the Adomet methyl donor at 37°C for 2 h in strip wells coated with DNMT substrate. For treated cells, 20 µg of total protein was incubated with Adomet. Following incubation and washing, wells were incubated sequentially with the capture antibody, detection antibody and enhancer solution, followed by the addition of the developer solution. Once the solution in the control wells changed to a medium blue, the enzymatic reaction was stopped with the stop

solution and the absorbance measured at 450 nm with a reference wavelength of 655 nm.

3.10. Chromatin immunoprecipitation

Histone modifications at specific genomic regions was determined by chromatin immunoprecipitation (ChIP) using the abcam ChIP Kit. Cross-linking with formaldehyde was used in pilot experiments (1% formaldehyde for 10 min at RT followed by addition of glycine to quench the formaldehyde) and was it was found that signals from immunoprecipitated samples were significantly enhanced when this step was skipped (Supplementary Figure S1). Cross-linking is not necessary in ChIP for histones modifications as histones are tightly linked to DNA.[196] Following treatment, cells were trypsinised and equal numbers were lysed in a series of buffers from the ChIP Kit according to the manufacturer's instructions. Chromatin was sheared by sonication in a buffer containing protease inhibitors for three times for 10 s following time course experiments that showed longer sonication times resulted in fragment sizes smaller than 200 bp. Chromatin was then incubated overnight at 4°C on a rotating rack with ChIP grade antibodies against H3 (positive control), H3K9ac, H4K8ac and H3K9me3 (Table 3.4), or frozen as input DNA. Bound chromatin was precipitated with the provided protein A sepharose beads for 1 h at 4°C on a rotating rack and beads were pelleted with centrifugation. Immunoprecipitated chromatin and input chromatin was then purified with the provided DNA purifying slurry and incubated at 98°C for 10 min. Proteinase K was then added and samples were incubated at 55°C for 30 min then 98°C for 10 min. The slurry was pelleted with centrifugation and the supernatant was used immediately for qPCR or frozen at -80°C.

Table 3.4 Antibodies used for ChIP

Target	Type	Product #	Company
H3	Rabbit polyclonal	ab1791	abcam
H3K9ac	Rabbit polyclonal	ab10812	abcam
H4K8ac	Rabbit polyclonal	ab15823	abcam
H3K9me3	Rabbit polyclonal	ab8898	abcam

3.10.1. ChIP qPCR

ChIP primers were designed using the protocol from Bridges Lab (http://bridgeslab.sph.umich.edu).[197] Genomic regions of interest were determined using data from the Encyclopedia of DNA Elements (ENCODE) project.[198] H3K9ac and SREBP-2 ChIP-seq data from HepG2 and other cell types from ENCODE were visualised in the UCSC Genome Browser, on the human Feb. 2009 (GRCh37/hg19) assembly (http://genome.ucsc.edu).[199] For SREBP-2 target genes, H3K9ac peaks located adjacent to SREBP-2 peaks were selected. For other genes, H3K9ac peaks with varying peak strength in HepG2 cells compared to other cell types were selected, typically upstream of the transcription start site (TSS). For GAPDH, used as a positive control, a region with strong H3K9ac peaks in multiple cell types was selected. DNA of the regions of interest was entered into Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) and human primers with a PCR product size of 70-150 bp were obtained. Primers were ordered through Sigma-Aldrich fully deprotected and desalted in de-ionised water at a concentration of 100 µM. ChIP primers are listed in Table 3.5.

qPCR was conducted as for gene expression (see section 3.6.3). Relative quantity (ΔC_q) was calculated by Bio-rad CFX Manager 3.1 software and percentage of input was calculated in Microsoft Excel.

Table 3.5 Primers used for ChIP qPCR

Genomic region	Forward Primer	Reverse Primer	Annealing Temperature
GAPDH exon 2	CCACATCGCTCAGACACCAT	CATACGACTGCAAAGACCCG	63°C
HMGCR ~500bp>TSS	TTCCCTCACGCTCCGATTCT	CTCAGTGCCTGACCCGTG	65°C
LDLR ~100bp>TSS	CACGGGTTAAAAAGCCGATGT	GGCCCACGTCATTTACAGCA	65°C
PPARG ~1000bp>TSS	ATCCGGGTCAACCTGACTAC	TCCTCCACAGCCCCTAAGAT	63°C
ABCA1 ~400bp>TSS	GGCACCAGTGGAATTTGCTT	CGTCCTGAGGGAGATTCAGC	65°C
ABCG1 ~600bp>TSS	AGCCACACCCACCTGTTTTG	ACAGTGGGGAAGTAAGGCAC	63°C
HK1 intron 1	AGGTCAAAAAGGTGAGCCCC	CTGCAGTCCAACTCGATGCT	63°C
SLC2A2 intron 1	AGACCCTACAGGGCACAGAT	CCTTGCTCCCTTATACGTGGT	63°C

>TSS- Upstream of transcription start site

3.11. Statistical analysis

At least three independent replicates were conducted of each experiment. Statistical significance was determined by analysis of variance (ANOVA) or Student's *t* test as indicated, with results considered significant if $p<0.05$. Analyses were conducted using GraphPad Prism software.

Chapter 4. Cholesterol metabolism

4.1. Abstract

Statins are widely used to reduce CVD risk by lowering plasma LDL-C, although they are associated with side effects, including increased risk of T2D. In animal studies, HDAC inhibitors such as butyrate have also been reported to reduce plasma cholesterol, while conferring protection from diabetes, but these studies lack investigation into the exacting mechanisms. This chapter compares the effects of statins and HDAC inhibitors on cholesterol metabolism in multiple cell lines, including hepatic cells due to the importance of the liver in cholesterol metabolism, insulin secreting cells due to the importance of cholesterol in insulin secretion and macrophages due to their role in cholesterogenic atherosclerosis. Cells were treated with statins and HDAC inhibitors and lipids were extracted and quantified. Cholesterol uptake was quantified using fluorescently tagged cholesterol. Expression of genes implicated in cholesterol synthesis and transport was assessed by immunoblotting and qPCR. Statins and the HDAC inhibitors sodium butyrate and TSA all reduced cellular cholesterol in hepatic and insulin secreting cells, while no treatment altered cholesterol in the macrophage cell line. Statins increased cholesterol uptake while butyrate had no effect. This is likely because statins increased SREBP-2 activity as a result of lowered cholesterol, increasing the expression of the LDLR receptor and genes involved in cholesterol synthesis. Conversely, butyrate inhibited SREBP-2 activity in a time-dependant manner. The effects of statins and butyrate on genes involved in reverse cholesterol transport, namely ABCA1 and SRB1, varied with cell type and levels of lipoprotein in the culture media. All genes with statin altered expression are controlled by transcription factors whose activity is influenced by cellular cholesterol levels, while butyrate altered expression of a significant proportion of genes involved in cholesterol synthesis and transport and thus may have unpredictable effects on lipid metabolism.

4.2. Objectives

1. Investigate how statins and HDAC inhibitors affect cholesterol metabolism.

4a. Identify genes involved in cholesterol metabolism or implicated in T2D whose expression is altered by these treatments.

4.3. Introduction

CVD is the leading cause of mortality worldwide.[8] Dyslipidaemia, usually characterised by high LDL-C, high TG and/or low HDL-C levels, is a major risk factor for CVD. As a result, HMGCR inhibitors, commonly known as statins, are widely used to lower LDL-C; it is estimated that there are over 30 million statins users in the United States.[200] By inhibiting HMGCR, the rate-limiting enzyme in the mevalonate pathway of cholesterol biosynthesis, statins decrease cellular cholesterol and subsequently increase the uptake of LDL-C from the bloodstream into cells, primarily hepatocytes. Despite the proven ability of statins to cut the morbidity and mortality from CVD by ~25%,[13] there has been some controversy in recent years over the widespread use of statins, in particular in people at low risk of CVD.[201] Statin use has been associated with adverse effects, including myopathy, rhabdomyolysis, T2D and liver damage.[13] Asymptomatic, transient elevations in aminotransferases are the most common liver complaint with statin use, although statins has also been associated with severe drug-induced liver injury. In particular, simvastatin and atorvastatin have each been implicated in over 60 published case reports of drug-induced liver injury and have both been associated with fatal liver injury.[202] As a result, there is interest in exploring alternative lipid-lowering agents with lower toxicity and diabetogenic risk.

Butyrate is a SCFA produced by the bacterial fermentation of dietary fibre and is an established HDAC inhibitor. HDACs regulate a variety of metabolic pathways and deregulation of HDACs has been associated with metabolic disorders including T2D and CVD.[203] In animal models of metabolic diseases, supplementation with sodium butyrate reportedly exerts numerous benefits, including reduced serum TG and cholesterol.[164,165] *In vitro*, butyrate has been found to lower cholesterol synthesis in the Caco-2 colon cancer cell line, associated with a decrease in HMGCR activity.[169] The effect of butyrate on cellular cholesterol levels in liver cells, the major site of cholesterol biosynthesis; macrophages, where cholesterol accumulation contributes to the development of foam cells and atherosclerotic plaques; or insulin-secreting beta cells, where altered cholesterol may impact insulin secretion and thus diabetes risk,[63] has not been explored.

This chapter therefore explores the effects of HDAC inhibition and statins on various cell types, including liver cells, due to the importance of the liver in cholesterol

metabolism, and macrophages, due to their key role in cholesterogenic atherosclerosis. Effects in insulin secreting cells were also examined due to our laboratory group's interest in the effects of altered cellular cholesterol on insulin secretion. In these cells, effects on cellular cholesterol content, the SREBP-2 signalling pathway and cholesterol import/export were explored. This chapter also includes preliminary work to establish dosage, ensuring the doses used are not toxic to cells, do not alter total protein content and are sufficient to lower cellular cholesterol.

4.4. Results

4.4.1. Effects of statins and sodium butyrate on cell viability and protein content

Statins decreased HepG2 viability, as determined by the reduction of resazurin using the Alamar Blue® assay, in a dose-dependent manner (Figure 4-1A). This was most apparent for the lipophilic statins, atorvastatin and simvastatin, reducing viability to 13% (± 5% SEM) and 3% (± 5% SEM) respectively at 500 µM. The hydrophilic pravastatin and rosuvastatin were less toxic, with viability remaining at 70% (± 6% SEM) and 83% (± 17% SEM) respectively at 500 µM. All statins except pravastatin significantly reduced viability at 50 µM, while pravastatin only reduced viability at 500 µM. Simvastatin reduced viability at all tested concentrations, however no other statin affected viability at 10 µM, a concentration commonly employed in statin studies, as such this concentration was used for subsequent experiments.

Conversely, sodium butyrate did not significantly reduce cell viability at any of the tested concentrations, up to 20 mM (Figure 4-1B). Additionally, neither 10 µM atorvastatin nor 1 mM or 5 mM sodium butyrate, concentrations used in subsequent experiments, altered the total protein content of HepG2 cells (Figure 4-2).

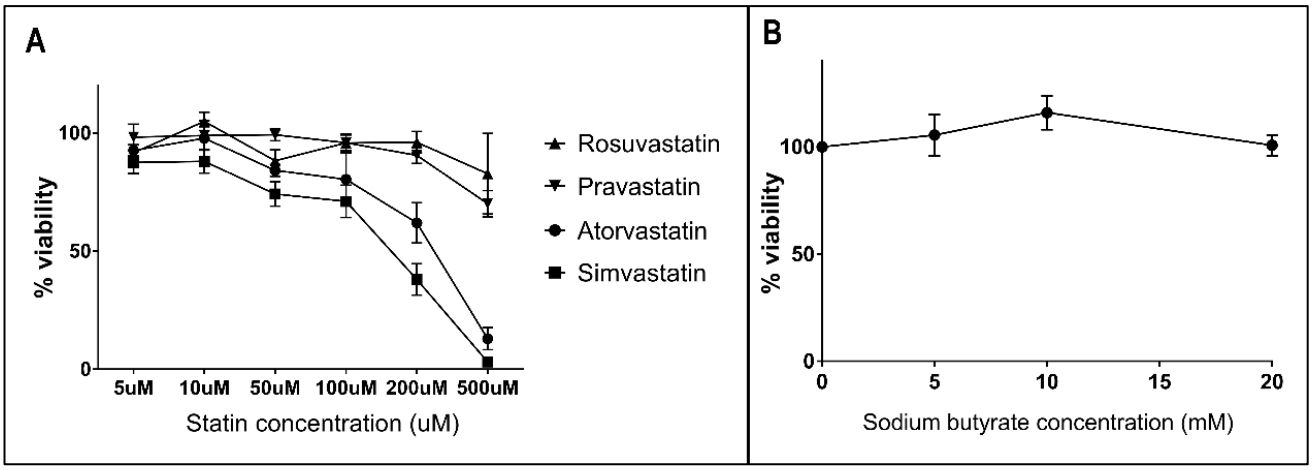

Figure 4-1 Influence of statins and butyrate on the viability of HepG2 cells

The effect of 24 h treatment with A) statins and B) sodium butyrate on HepG2 viability, measured using the AlamarBlue® assay as described in section 3.3. Results represent the mean from at least three independent experiments. Analysed by ANOVA. See Appendix 3 for full data tables.

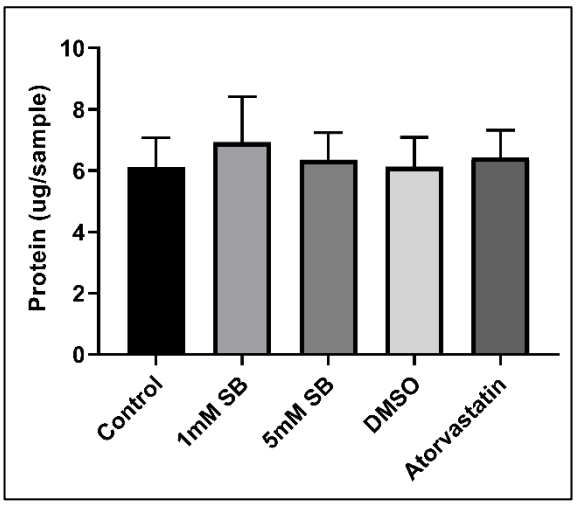

Figure 4-2 Influence of butyrate and atorvastatin on protein content in HepG2 cells

Cell protein content was measured using the BCA assay following RIPA protein extraction as described in section 3.2.4. Results represent the mean from three independent experiments, with error bars representing SEMs. Analysed by ANOVA.

4.4.2. Sodium butyrate reduces cellular cholesterol to a similar extent as statins

In HepG2 cells, 5 mM sodium butyrate decreased cellular cholesterol content to a similar degree as treatment with 10 µM statins (Figure 4-3A). After 24 h treatment in lipoprotein-deficient serum (LPDS), sodium butyrate treated cells showed a 31% (± 15% SEM) decrease in cholesterol content, comparable to the cholesterol lowering

effect of the hydrophilic statins rosuvastatin (44% ± 15% SEM) and pravastatin (31% ± 14% SEM), as well as atorvastatin (52% ± 4% SEM) and simvastatin (53% ± 8% SEM). Similar results were seen with BRIN-BD11 cells, although results were more variable and statistical significance differences were more marginal (Figure 4-3B). However, in THP-1 macrophages, which had significantly lower cholesterol content overall, neither statins nor butyrate affected cholesterol content after 24 h (Figure 4-3C). In a separate series of experiments, HepG2 cells were treated with both atorvastatin and sodium butyrate in order to determine if there were any additive or synergistic effects on cholesterol lowering. After 24 h treatment in LPDS, cells treated with both 5 mM sodium butyrate and 10 µM atorvastatin had comparable cholesterol content to cells treated with atorvastatin alone (p=0.78) (Figure 4-3D).

Similar results were observed with cells treated in media containing the high lipoprotein fraction of FBS (HLPS), rather than the lipoprotein-deficient FBS (LPDS) supplemented media used in the other experiments, although the magnitude of cholesterol lowering was lower than in LPDS, most likely due to increased uptake of cholesterol from the media. In HLPS, cellular cholesterol was lowered by 28% (± 6% SEM) by atorvastatin, 20% (± 3% SEM) by sodium butyrate and by 33% (± 15% SEM) by both atorvastatin and sodium butyrate (Figure 4-3E). There was no significance difference in cholesterol levels between cells treated by both atorvastatin and sodium butyrate and by atorvastatin alone (p=0.5).

In order to determine if the cholesterol lowering is a result of HDAC inhibition or FFAR activation, HepG2 cells were treated with TSA, a potent HDAC inhibitor, and sodium acetate, a SCFA and FFAR agonist but with no known effect on HDAC activity.[158] After 24 h treatment in cells treated with TSA, cellular cholesterol content was decreased by 20% (± 9% SEM), while cholesterol content in sodium acetate treated cells was unaffected (Figure 4-3F). Furthermore, there was no difference in cholesterol content between cells treated with a combination of TSA and sodium acetate compared to those treated with TSA alone (p=0.5), suggesting HDAC inhibition as the mechanism by which sodium butyrate lowers cellular cholesterol.

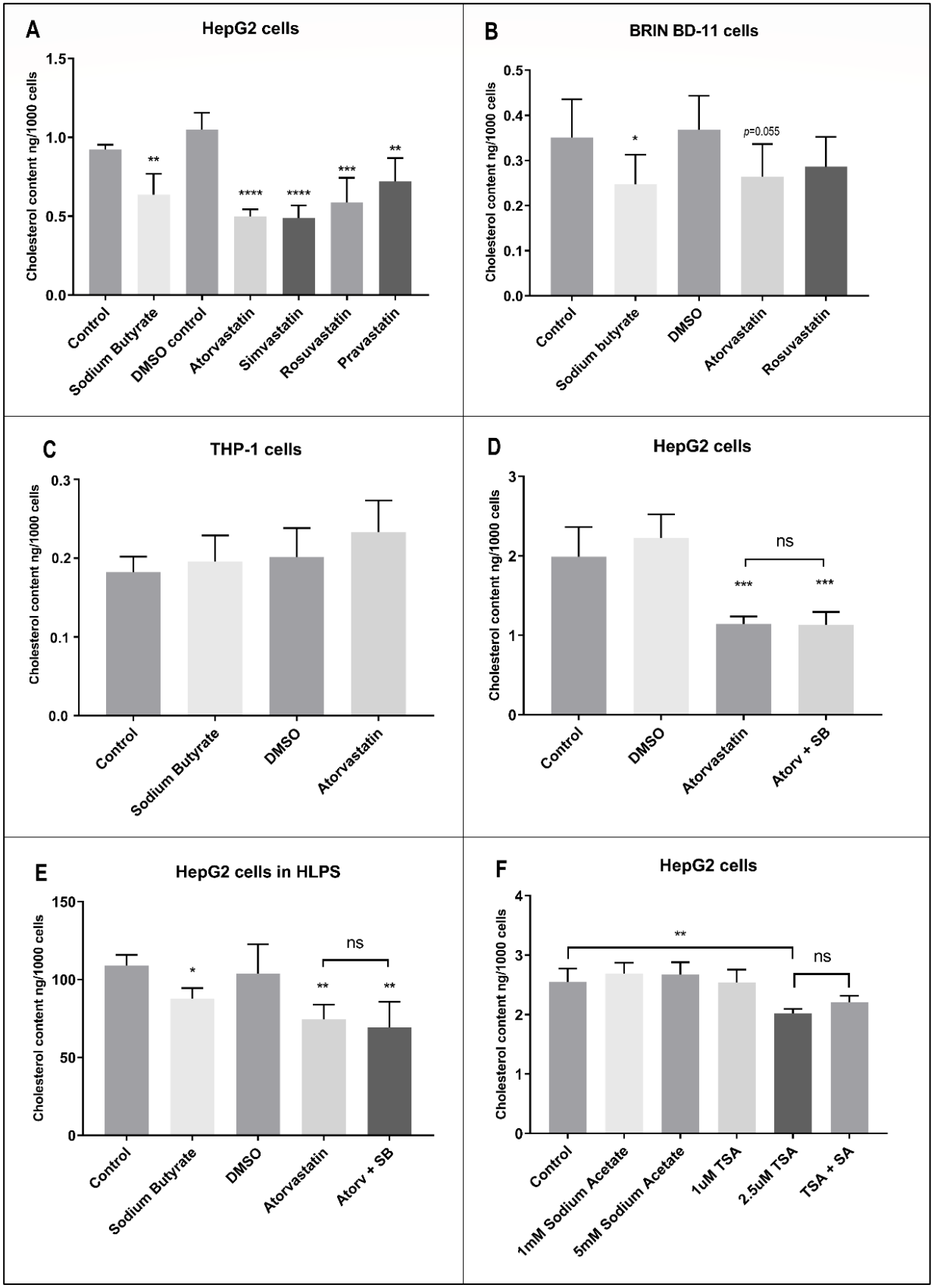

Figure 4-3 Influence of statins and butyrate on cellular cholesterol content in HepG2 cells, BRIN-BD11 cells and THP-1 cells

Cellular cholesterol content as measured by the Amplex Red Cholesterol Assay Kit (Life Technologies) as described in section 3.4; in A) HepG2, B) BRIN-BD11 and C) THP-1 cells treated with 10 µM statins or 5mM sodium butyrate (SB) for 24 h in media supplemented with lipoprotein-deficient serum (LPDS); D) in HepG2 cells treated with a combination of atorvastatin and sodium butyrate for 24 h in media containing LPDS; E) in HepG2 cells treated with statins or sodium butyrate for 24 h in media supplemented with high lipoprotein serum (HLPS); F) in HepG2 cells treated with sodium acetate (SA) or trichostatin A (TSA) for 24 h in media containing LPDS. Results represent the mean from at least three independent experiments, with error bars representing SEMs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns not significant. p values are in context of comparison to relevant controls, analysed by ANOVA.

4.4.3. Neither sodium butyrate nor statins alter cellular triglyceride content

In contrast to the effects on cellular cholesterol, neither sodium butyrate (Figure 4-4A), nor atorvastatin (Figure 4-4B) altered cellular TG content following 24 h treatment.

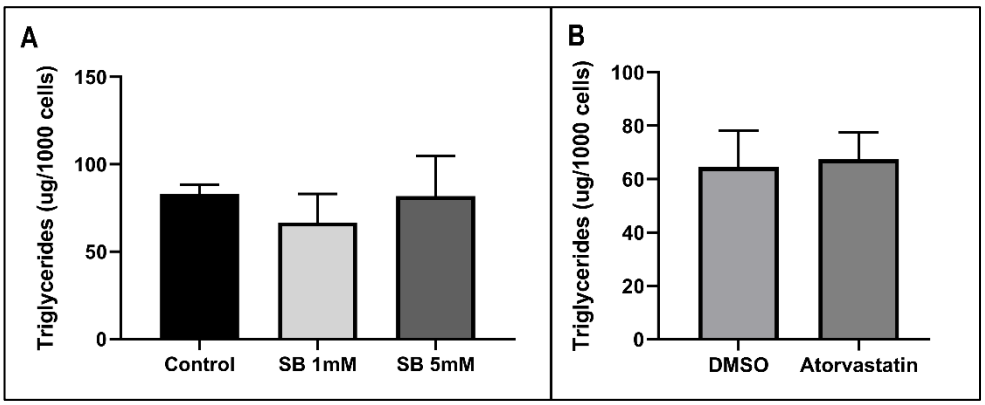

Figure 4-4 Influence of butyrate and atorvastatin on cellular triglyceride content in HepG2 cells

Cellular triglyceride content, as measured by the High Sensitivity Triglyceride Fluorometric Assay Kit (Sigma-Aldrich) as described in section 3.4.3, of HepG2 cells treated for 24 h with A) 1 mM or 5 mM sodium butyrate (SB); B) 10 µM atorvastatin. Results represent the mean combined from at least three independent experiments, with error bars representing SEMs. Analysed by ANOVA for butyrate and Student's *t* test for atorvastatin.

4.4.4. Statins increase cholesterol uptake; sodium butyrate affects neither cholesterol uptake nor export

The primary mechanism by which statins lower circulating LDL-C is thought to be increased LDLR mediated LDL-C uptake by hepatocytes to compensate for the

reduced cellular cholesterol biosynthesis due to HMGCR inhibition.[204] Fluorescently tagged cholesterol in the form of NBD-cholesterol was used to examine the effects of statins and sodium butyrate on cholesterol uptake in HepG2 cells (Figure 4-5A). As expected, 24 h treatment with atorvastatin increased the uptake of NBD-cholesterol, as did the included positive control treatment U-18666A, which inhibits cholesterol synthesis and intracellular cholesterol trafficking.[205] Sodium butyrate had no effect on NBD-cholesterol uptake.

NBD-cholesterol was also used to examine cholesterol efflux. In these experiments, HepG2 cells were incubated with NBD-cholesterol for 48 h prior to treatment and fluorescence of the media was measured to detect exported NBD-cholesterol. Neither atorvastatin nor sodium butyrate affected the levels of NBD-cholesterol in media after 24 h treatment, although there was no positive control treatment in these experiments (Figure 4-5B). Furthermore, neither atorvastatin nor sodium butyrate affected secretion of apo-A1 or apo-B, presumably as component of lipoproteins (e.g., HDL and LDL respectively), in the media of treated cells after 24 h treatment (Figure 4-6).

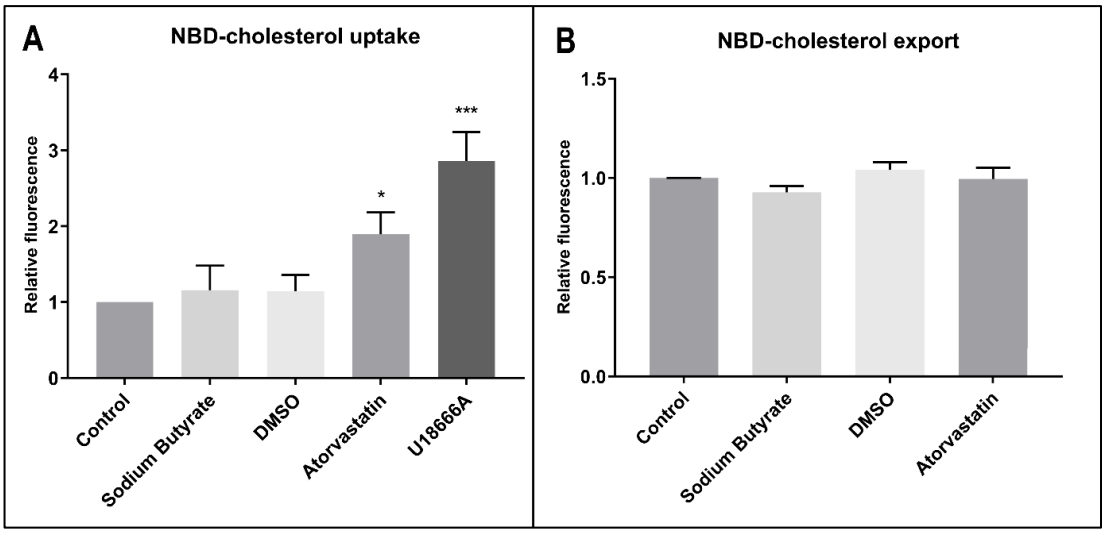

Figure 4-5 Influence of butyrate and atorvastatin on cholesterol uptake and export in HepG2 cells from HepG2 cells

A) Uptake of fluorescently tagged NBD-cholesterol as described in section 3.4.4, by HepG2 cells treated with atorvastatin or sodium butyrate after 24 h; B) export of fluorescently tagged NBD-cholesterol from HepG2 cells treated with 10 µM atorvastatin or 5 mM sodium butyrate after 24 h. Results represent the mean combined from at least three independent experiments, with error bars representing SEMs. * $p<0.05$, *** $p<0.001$, analysed by ANOVA.

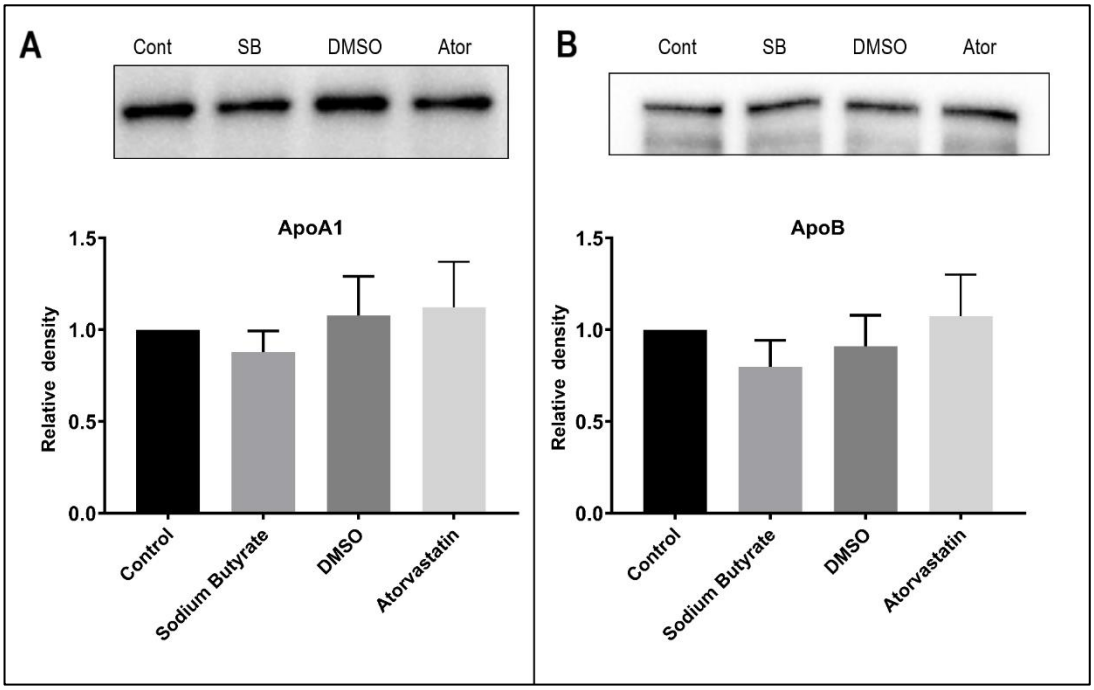

Figure 4-6 Influence of butyrate and atorvastatin on apoprotein secretion from HepG2 cells

Immunoblots were completed using the method described in section 3.5, of A) apo-A1 and B) apo-B in the media of HepG2 cells treated with 5 mM sodium butyrate and 10 µM atorvastatin for 24 h. The graphs represent the mean combined density readings from three independent experiments, with error bars representing SEMs. Analysed by ANOVA.

4.4.5. Sodium butyrate and statins have opposite effects on SREBP-2 signalling

SREBP-2 acts to increase intracellular cholesterol content by promoting the transcription of *LDLR* and cholesterol biosynthesis genes and is thought to be responsible for the increased LDL-C uptake seen in statin use.[121] When cellular cholesterol levels are low, SREBP-2 is cleaved, releasing the active subunit for entry into the nucleus and subsequent activation of gene transcription. In HepG2 cells, sodium butyrate lowered protein levels of the SREBP-2 target LDLR, while atorvastatin increased LDLR (Figure 4-7A). Neither treatment significantly altered protein levels of another SREBP-2 target, HMGCR, or of the cleaved SREBP-2 protein itself after 24 h treatment (Figure 4-7B, C). Similarly, in BRIN-BD11 insulin-secreting cells, sodium butyrate lowered LDLR levels while atorvastatin tended to increase them (p=0.06), and there was no significant effect on HMGCR levels (Supplementary Figure S2). In THP-1 macrophages, 5 mM sodium butyrate significantly lowered levels of cleaved SREBP-2 (Figure 4-7F), while also tending to

lower levels of SREBP-2 targets LDLR (Figure 4-7D) and HMGCR (Figure 4-7E) (significant using Student's *t* test but not ANOVA). Atorvastatin tended to increase SREBP-2 and SREBP-2 targets but this only reached statistical significance for LDLR.

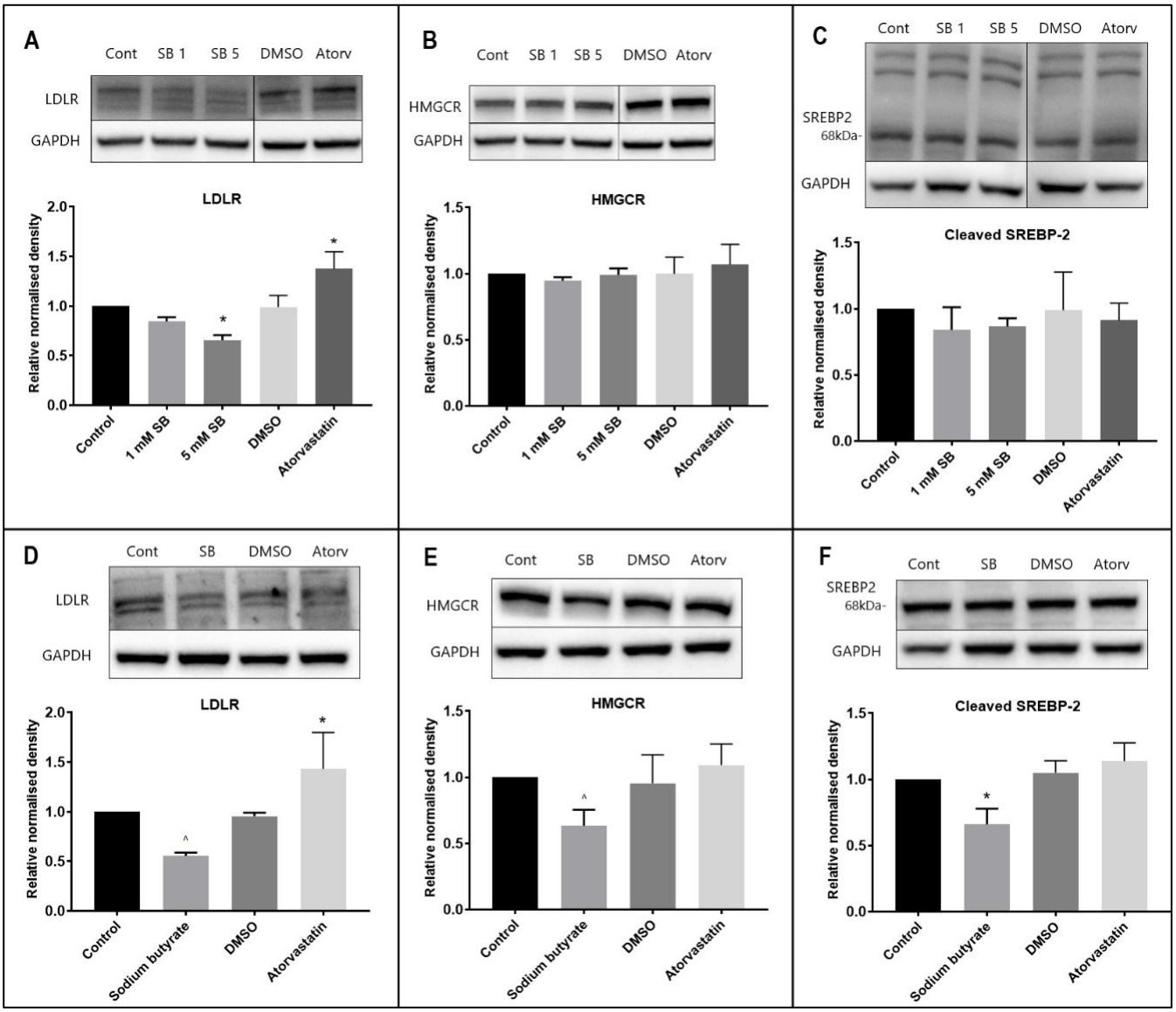

Figure 4-7 Influence of butyrate and atorvastatin on SREBP-2 pathway in HepG2 and THP-1 cells

Immunoblots were completed using the method described in section 3.5 for A) LDLR[1], B) HMGCR and C) active SREBP-2[2] in HepG2 cells and of D) LDLR, E) HMGCR and F) active SREBP-2 in THP-1 cells following treatment with 1 mM (SB1) or 5 mM (SB5 or SB) sodium butyrate or 10 µM atorvastatin for 24 h. The graphs represent the mean combined density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. * $p<0.05$ using ANOVA, ^ $p<0.05$ with two-tailed *t* test but not ANOVA.

1. LDLR contains multiple glycosylation sites which gives the appearance of multiple bands between 100-160 kDa (206)

2. SREBP-2 appears as two high molecular weight bands around 120 kDa representing the inactive form, and an additional band or bands around 68 kDa representing the cleaved form (207)

As sodium butyrate lowered active SREBP-2 without lowering cellular cholesterol in THP-1 macrophages, we theorised that butyrate may reduce SREBP-2 signalling at an earlier time point in HepG2 cells, lowering cholesterol and resulting in a compensatory increase of SREBP-2 signalling back to baseline levels. We therefore investigated SREBP-2 and HMGCR protein levels in HepG2 cells after 8 h and 16 h treatments. These experiments also included 16 h TSA treatment to determine if any effects seen are a likely result of HDAC inhibition. At 16 h, sodium butyrate and TSA significantly lowered HMGCR protein levels (

Figure 4-8A). Sodium butyrate (p=0.08 at 5 mM) and TSA also decreased active SREBP-2 levels at 16 h (

Figure 4-8B). qPCR was conducted on cells treated with 5 mM sodium butyrate for 16 h and showed a trend towards reduced *HMGCR* mRNA (p=0.0511) and no effect on *SREBF2* mRNA levels. This indicates that sodium butyrate likely alters SREBP-2 protein activity without altering its gene expression.

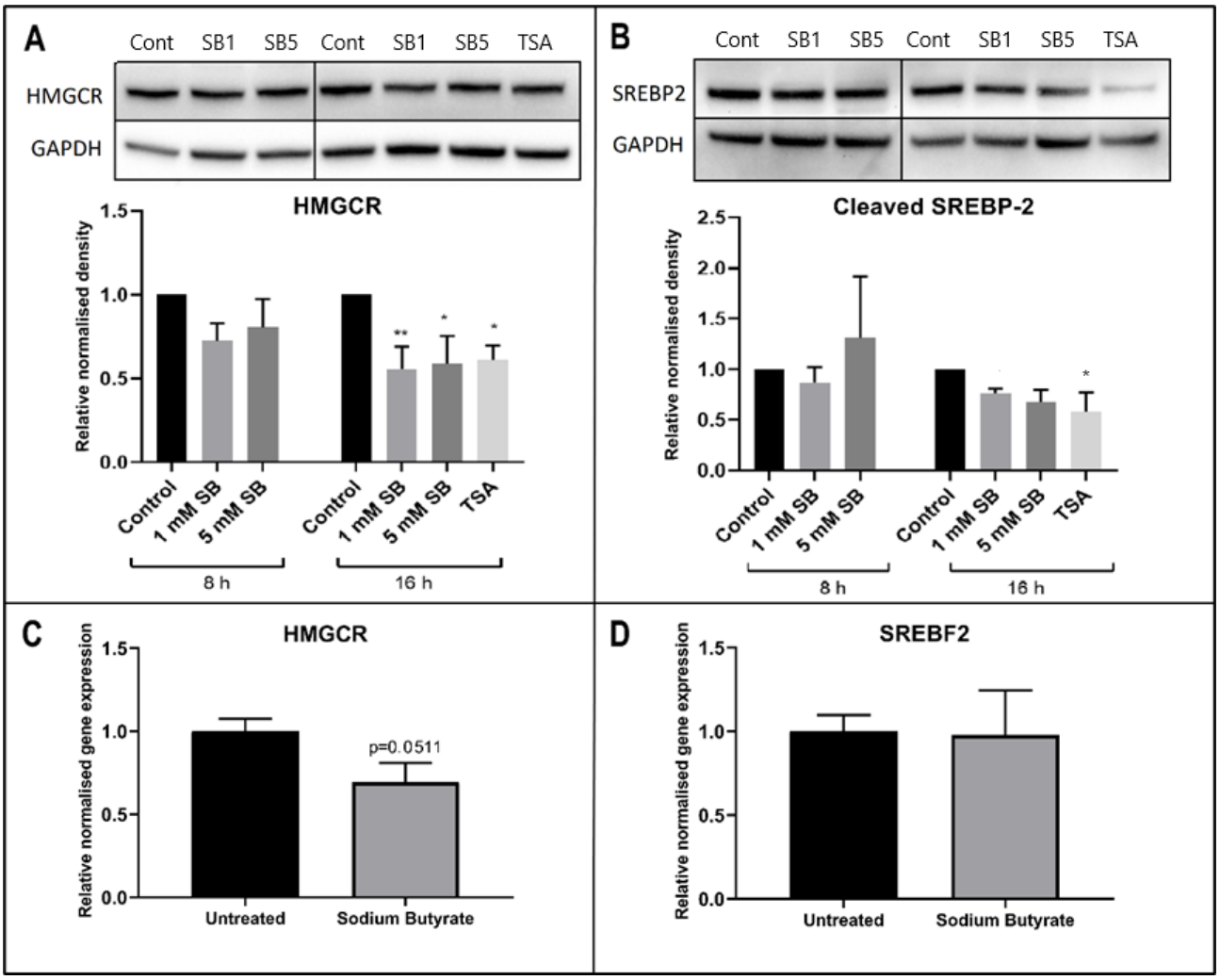

Figure 4-8 Influence of HDAC inhibition on the SREBP-2 pathway at 8 h and 16 h in HepG2 cells

Immunoblots were completed using the method described in section 3.5 for A) HMGCR and B) active SREBP-2 in HepG2 cells treated with 1 mM (SB1) and 5 mM (SB5) sodium butyrate for 8 h and 16 h or 2.5 µM TSA for 16 h compared to the untreated control, normalised to GAPDH. Reverse-transcription qPCR was conducted using the method described in section 3.6 for C) *HMGCR* and D) *SREBF2* in HepG2 cells treated with 5 mM sodium butyrate for 16 h compared to the untreated control, normalised to reference genes *GAPDH*, *YWHAZ* and *RPL13A*. Results represent the mean combined from at least three independent experiments, with error bars representing SEMs. * $p<0.05$, ** $p<0.01$. Analysed by ANOVA (butyrate immunoblots) and Student's *t* test (TSA immunoblots and butyrate qPCR).

4.4.6. The effect of sodium butyrate and statins on proteins involved in reverse cholesterol transport depends on cell type and media lipoproteins

Reverse cholesterol transport involves the efflux of cholesterol from peripheral cells to the liver via HDL particles. ABCA1 mediates the transfer of cholesterol to apolipoprotein apo-A1 to form HDL, while SRB1 can bind HDL and allows for bidirectional transfer of cholesterol between cells and HDL.[208] In HepG2 cells treated in LPDS supplemented media, sodium butyrate decreased protein levels of both ABCA1 and SRB1, while atorvastatin also decreased ABCA1 (Figure 4-9A, D). When HepG2 cells were treated in HLPS supplemented media, i.e., using the high lipoprotein fraction of FBS, atorvastatin increased ABCA1 and tended to decrease SRB1 (p=0.07), while sodium butyrate decreased ABCA1 and SRB1 as in LPDS (Figure 4-9B, E). In BRIN-BD11 cells in LPDS supplemented media, sodium butyrate increased, and atorvastatin tended to decrease ABCA1 levels (significant by Student's *t* test but not ANOVA) (Figure 4-9C). In THP-1 macrophages in LPDS supplemented media, sodium butyrate increased levels of SRB1 (Figure 4-9F), while ABCA1 was decreased by both sodium butyrate and atorvastatin (Supplementary Figure S3). The decrease in ABCA1 protein levels by sodium butyrate in HepG2 cells in LPDS supplemented media was evident from 8 h and was also observed with TSA, suggesting HDAC inhibition as the likely mechanism (Figure 4-10). The effect of sodium butyrate and statins on ABCA1 in different cell types and media lipoprotein conditions is summarised in Table 4.1.

Table 4.1 Influence of butyrate and statins on ABCA1 protein levels in different cell types

Treatment	HepG2 cells in LPDS	HepG2 cells in HLPS	THP-1 cells in LPDS	BRIN-BD11 cells in LPDS
Sodium butyrate	↓****	↓**	↓**	↑*
Atorvastatin	↓**	↑*	↓**	↓ (p=0.1)

** p<0.01, *** p<0.001, **** p<0.0001.

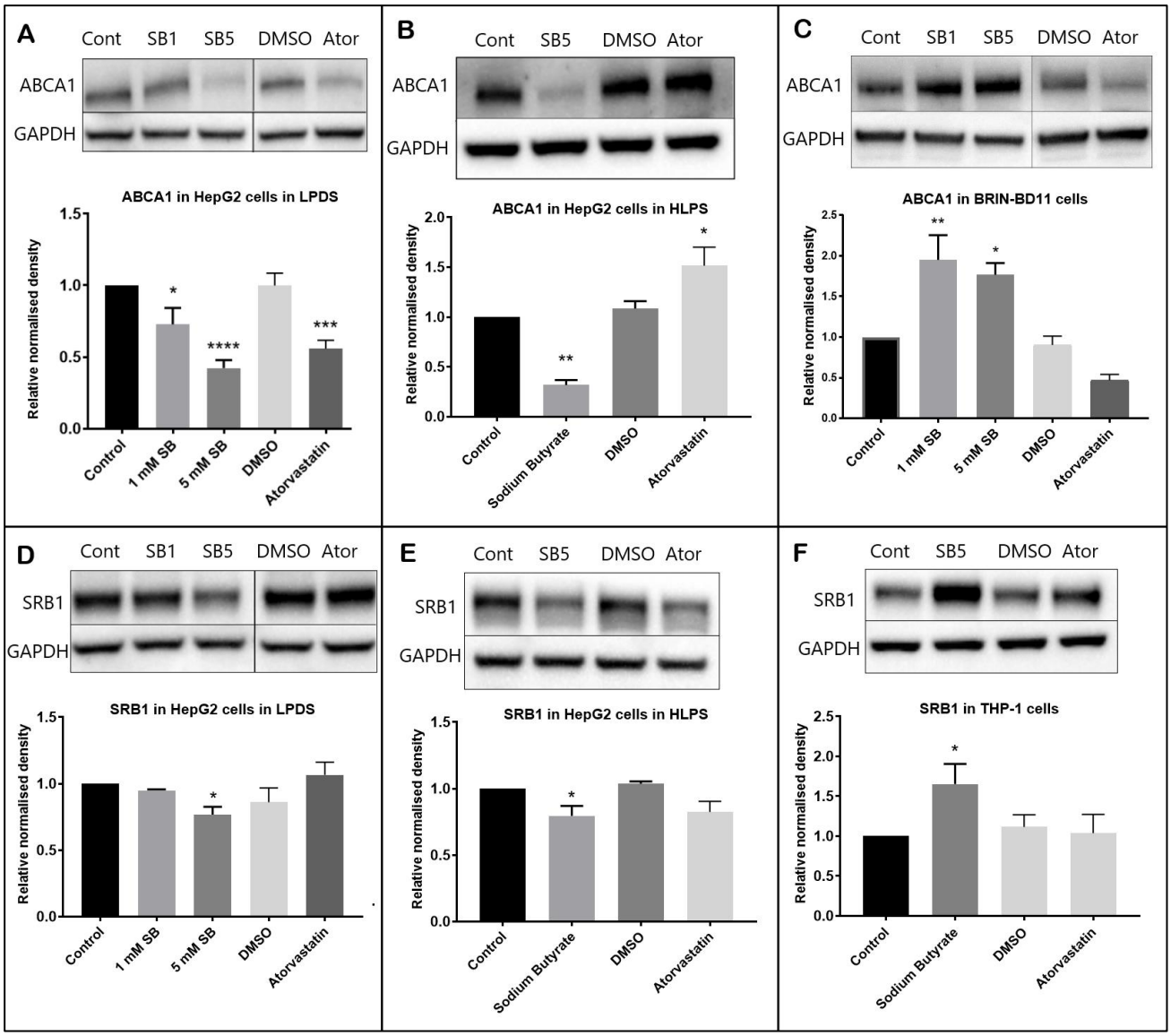

Figure 4-9 Influence of butyrate and atorvastatin on reverse cholesterol transport proteins in HepG2 cells, BRIN-BD11 cells and THP-1 cells

Immunoblots were completed using the method described in section 3.5 for ABCA1 in HepG2 cells treated in A) LPDS supplemented media and B) HLPS supplemented media, and in C) BRIN-BD11 cells treated in LPDS supplemented media, and of SRB1 in HepG2 cells treated in D) LPDS supplemented media and E) HLPS supplemented media, and in F) BRIN-BD11 cells treated in LPDS supplemented media. Cells were treated with 1 mM (SB1) or 5 mM (SB5) sodium butyrate, or 10 µM atorvastatin for 24 h. The graphs represent the mean density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to control. Analysed by ANOVA

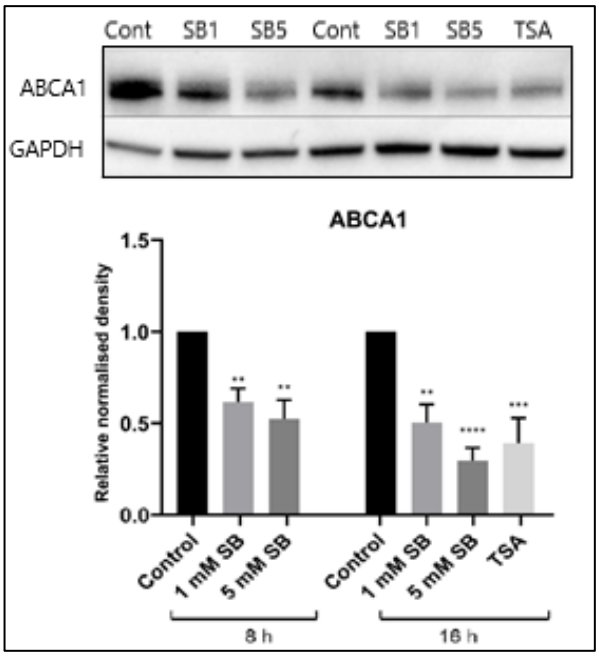

Figure 4-10 Influence of HDAC inhibition on ABCA1 at 8 h and 16 h in HepG2 cells

Immunoblots were completed using the method described in section 3.5 for ABCA1 in HepG2 cells treated with 1 mM (SB1) and 5 mM (SB5) sodium butyrate for 8 h and 16 h or TSA for 16 h. The graph represents the mean density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Analysed by ANOVA (butyrate) and Student's *t* test (TSA)

4.4.7. Sodium butyrate alters the expression of multiple cholesterol related genes, atorvastatin has more targeted effects on gene expression

qPCR was employed to examine the effects of sodium butyrate and atorvastatin on gene expression at the mRNA level. Sodium butyrate had a significant effect on mRNA levels of numerous cholesterol related genes. In particular, *ABCG1* was upregulated more than a hundred-fold in HepG2 cells, as was *CAV1*, encoding caveolin-1, the key protein in caveolae lipid rafts (Figure 4-11A). It should be noted that this robust upregulation did not result in a significant increase in protein levels of ABCG1 or caveolin-1 (Figure 4-12). *SREBF1, SCARB1*, encoding SRB1, and *APOA1* were also upregulated while *ABCA1* and *CYP7A1*, encoding the rate-limiting enzyme in bile acid production were downregulated. A limited number of cholesterol related genes were also examined in BRIN-BD11 cells. Sodium butyrate upregulated *ABCA1, ABCG1* and *SREBF1* and downregulated *LDLR* in BRIN-BD11 cells (Figure 4-11B).

Atorvastatin had more limited effects on gene expression. In HepG2 cells, atorvastatin significantly upregulated *HMGCR* expression and downregulated *SREBF1* (Figure 4-11C)

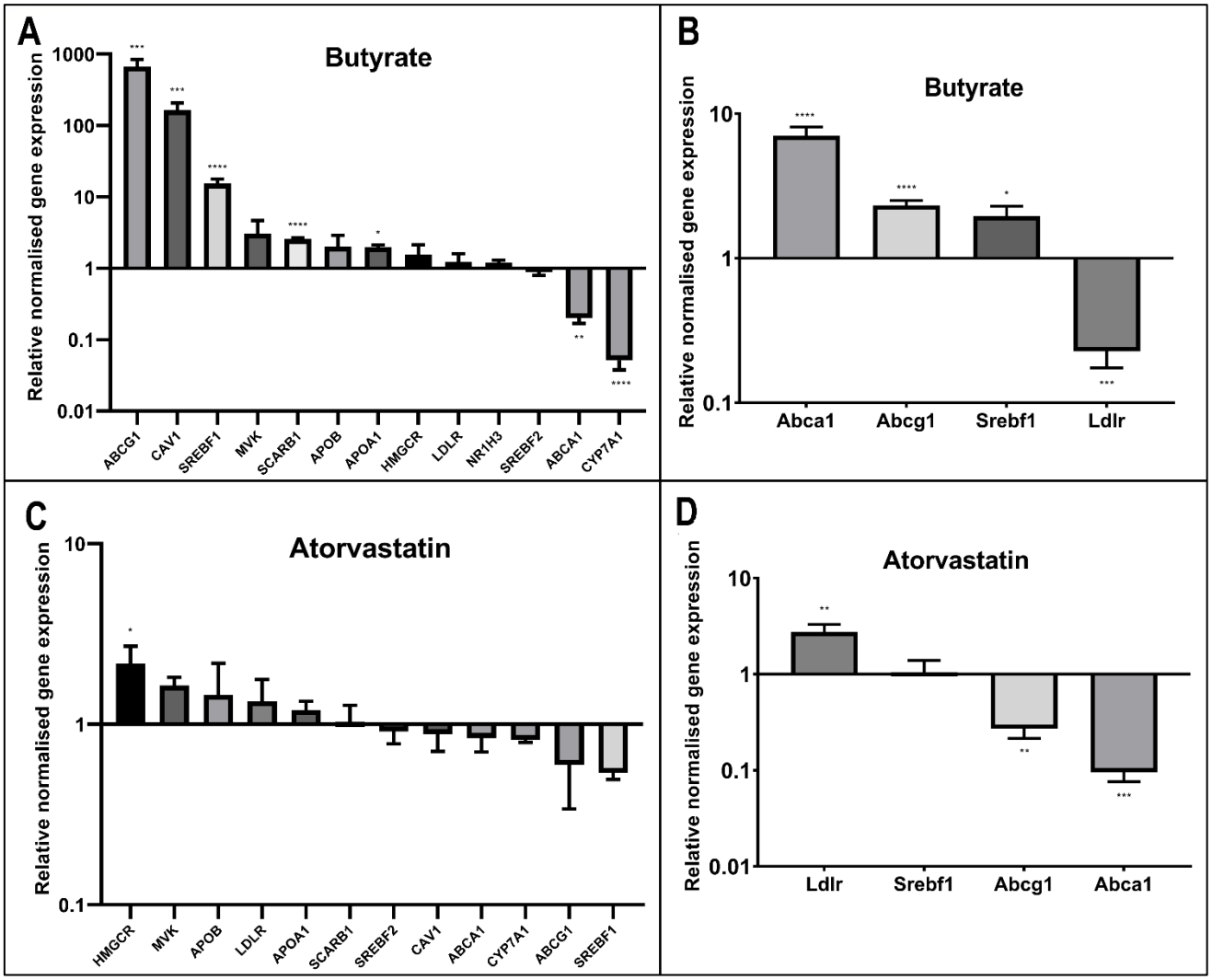

Figure 4-11 Influence of butyrate and atorvastatin on cholesterol-related gene expression in HepG2 cells and BRIN-BD11 cells

Reverse-transcription qPCR was conducted using the method described in section 3.6 for A) HepG2 and B) BRIN-BD11 cells treated 5 mM sodium butyrate and for C) HepG2 and D) BRIN-BD11 cell treated with 10 µM atorvastatin compared to the untreated control, normalised to reference genes *GAPDH, YWHAZ* and *RPL13A* for HepG2 cells and reference genes *Actb and Rpl13a* for BRIN-BD11 cells. Atorvastatin data is shown relative to the untreated control but statistical significance is compared to the DMSO vehicle control. Results represent the mean combined from at least three independent experiments, with error bars representing SEMs. * $p<0.05$ ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Analysed by ANOVA.

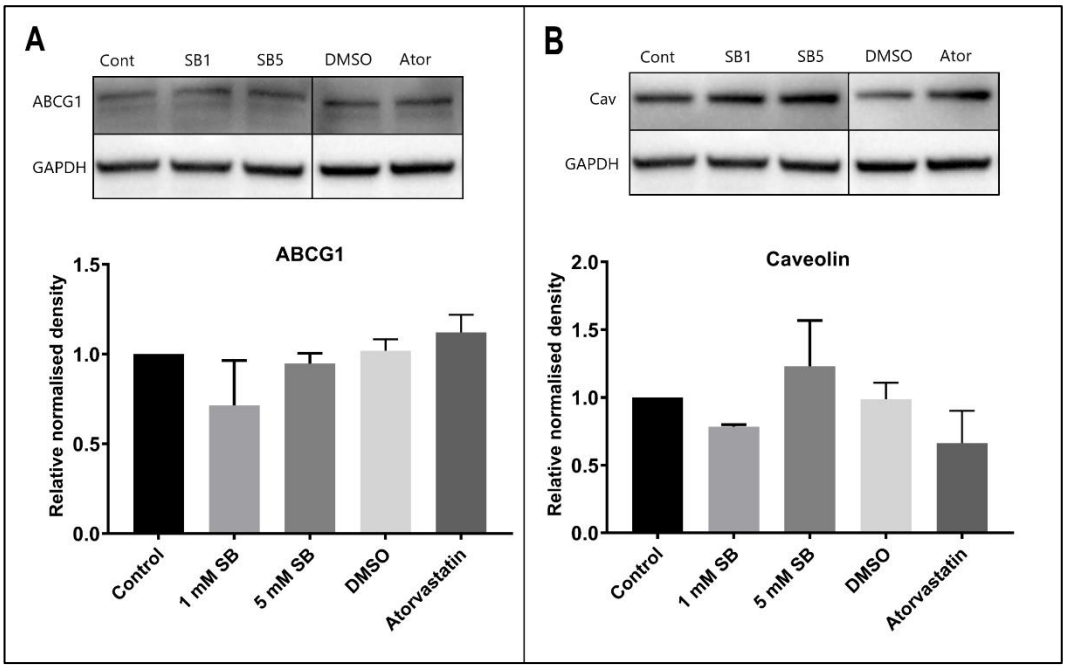

Figure 4-12 Influence of butyrate on protein levels of upregulated genes in HepG2 cells

Immunoblots were completed using the method described in section 3.5 for A) ABCG1 and B) caveolin-1 in HepG2 cells treated with 1 mM or 5 mM sodium butyrate or 10 µM atorvastatin for 24 h. The graphs represent the mean density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. Analysed by ANOVA.

4.5. Discussion

4.5.1. Mechanisms of cholesterol lowering by statins and butyrate

Statins are competitive inhibitors of HMGCR, the rate-limiting step in cholesterol biosynthesis. By inhibiting cholesterol biosynthesis and thus reducing cellular cholesterol, particularly in the liver, statins cause cells to increase cholesterol uptake from the bloodstream, lowering plasma LDL-C. This is mediated by SREBP-2, which is activated when cellular cholesterol levels drop, resulting in increased expression of LDLR.[121] Our experiments confirmed increased LDLR expression and cholesterol uptake with atorvastatin treatment in hepatic cells. Conversely, our results indicate that butyrate may inhibit SREBP-2 activity, resulting in reduced LDLR protein levels. Although cleaved (active) SREBP-2 or HMGCR protein levels were not altered after 24 h butyrate treatment in hepatic cells, both proteins were downregulated in macrophages despite cholesterol not being reduced by butyrate in these cells after

24 h. The macrophages had significantly lower cholesterol content than the HepG2 cells and the BRIN-BD11 cells; the lack of cholesterol lowering by the treatments may be because these macrophages do not synthesise as much cholesterol. Consequently, SREBP-2 and HMGCR protein levels were examined at earlier time points in butyrate treated hepatic cells and HMGCR was decreased after 16 h treatment, with a trend towards decreased levels of cleaved SREBP-2. TSA, a potent HDAC inhibitor, also decreased HMGCR and cleaved SREBP-2 protein levels at 16 h and cholesterol content at 24 h, while sodium acetate, a SCFA with no HDAC inhibitory activity, failed to impact cholesterol levels. Thus, it is likely that butyrate decreases cholesterol biosynthesis through HDAC inhibition and a decrease in SREBP-2 activity, lowering cellular cholesterol content and thereby causing a compensatory increase in SREBP-2 signalling back to baseline levels. While downregulation of HMGCR has been reported in butyrate treated intestinal cells, with studies finding reduced mRNA levels[170] and protein activity[169] in Caco-2 enterocytes, this time dependent decrease is a novel finding, as is the discovery that this is mediated via HDAC inhibition.

The qPCR results suggest that butyrate lowered *HMGCR* but not *SREBF2* mRNA after 16 h treatment, implying that butyrate alters SREBP-2 activity but not the expression of its gene. Similar results were seen in a study of TSA treated neuronal cells by Nunes et al.[110] which found that HDAC inhibition reduced SREBP-2 target gene expression and active SREBP-2 protein levels without significantly altering *SREBF2* gene expression. The SREBP-2 protein itself can be acetylated and this acetylation alters its localisation and activity.[112] In particular, the class III HDAC SIRT1 deacetylates SREBP-2, and SIRT1 inhibition increases levels of the active nuclear SREBP-2 protein and expression of SREBP-2 target genes.[209] Sodium butyrate does not inhibit class III HDACs,[210] and it has not been investigated what effect class I and II HDACs, inhibited by butyrate, have on the acetylation of SREBPs or whether inhibition of class I and II HDACs causes a compensatory increase in class III HDAC activity. Another possibility is that butyrate decreases *SREBF2* mRNA at an earlier timepoint than investigated in this study. Chittur et al.[25] reported that TSA downregulated *SREBF2* mRNA expression in HepG2 cells, with maximal repression occurring at 9 h treatment and levels normalising at 48 h.

While a decrease in SREBP-2 activity would explain the lowered cellular cholesterol, this calls into question whether butyrate could reduce plasma cholesterol and thus

CVD risk, especially as there was no effect of butyrate on hepatic cholesterol uptake. Although numerous rodent studies have reported reduced total plasma cholesterol with butyrate supplementation,[118,165,167,211] several studies found no effect on LDL-C levels.[167,212] In human clinical trials, one placebo-controlled study (15 individuals per treatment group) found 45 days of butyrate supplementation did not significantly alter plasma cholesterol,[6] while a study of nine healthy and ten metabolic syndrome participants reported that butyrate increased LDL-C levels in individuals with metabolic syndrome, although this study lacked a placebo group.[166] As HDL, as opposed to LDL, is the primary plasma cholesterol carrier in rodents,[213] reduced HDL due to the noted downregulation of ABCA1 could account for the reduced plasma cholesterol in rodent studies and biological differences could account for the lack of effect in the human study. More large-scale trials are clearly needed to determine if butyrate supplementation alters plasma LDL-C in humans. The lowering of hepatic cholesterol seen with butyrate may be beneficial in and of itself, for example in NAFLD, where the accumulation of free cholesterol is thought to be a contributor to liver damage.[30] Indeed, animal models have shown sodium butyrate to be protective against diet-induced liver damage in mice.[214,215]

4.5.2. Effects of atorvastatin and butyrate on reverse cholesterol transport proteins

The effect of sodium butyrate and atorvastatin on proteins involved in HDL metabolism, namely ABCA1 and SRB1, varied according to cell type and the presence or absence of lipoproteins in serum supplements. ABCA1 mediates the efflux of cholesterol and phospholipids to apolipoprotein apo-A1 to form HDL particles.[216] ABCA1 expression is regulated by LXR, a nuclear receptor activated by oxysterols, cholesterol metabolites that occur in proportion to total cholesterol.[39] In the absence of ligands, LXR acts to repress target genes. Thus, ABCA1 is expressed proportionally to cellular cholesterol, providing a mechanism by which the cholesterol lowering agents atorvastatin and sodium butyrate decreased ABCA1 levels in liver cells in low lipoprotein conditions. Paradoxically, in high-lipoprotein supplemented media, atorvastatin increased ABCA1 levels in hepatic cells, despite lowering cellular cholesterol in these conditions to a similar if not greater extent than sodium butyrate. Similar results have been reported in THP-1 macrophages, in which mevastatin reduced ABCA1 and ABCG1 expression in normal cells, with this effect reversed by cholesterol loading.[217] Atorvastatin may therefore increase LXR activity,

both to repress or activate its target genes depending on oxysterol levels. Statins have been shown to improve HDL-C in humans clinical trials,[218] an increase in LXR activity resulting in upregulated ABCA1 expression could account for this effect. Sodium butyrate affects ABCA1 levels in disparate ways, lowering it in all tested conditions except in BRIN-BD11 cells. The fact that the decrease in ABCA1 was apparent after only 8 h and was also observed in THP-1 macrophages despite the lack of cholesterol lowering seen in these cells suggests that butyrate can impact ABCA1 independent of cholesterol levels. As TSA also lowered ABCA1 protein from 8 h, HDAC inhibition is likely the contributing mechanism. It has been shown that LXR is acetylated and deacetylation by SIRT1 increases its activity;[219] HDAC inhibition could therefore decrease LXR activity and thus ABCA1 expression. However, ABCG1 and SREBP-1 are also LXR targets,[39] and their mRNA expression was increased by sodium butyrate in both HepG2 and BRIN-BD11 cells. It is unclear why ABCA1 is upregulated in BRIN-BD11 cells, especially as cellular cholesterol in these cells was reduced to a similar extent as in HepG2 cells. The downregulation of ABCA1 in macrophages could impair cholesterol efflux to HDL and thus increase cholesterol accumulation in these cells, while the upregulation of both ABCA1 and ABCG1 in insulin secreting cells suggests increased cholesterol efflux. It would be interesting to examine the effects of HDAC inhibition on ABCA1 in other peripheral cells, especially adipocytes and myocytes, while cholesterol import/export experiments, specifically using HDL-C, in multiple cell types could help elucidate how these alterations in ABCA1 impact cholesterol efflux and thus RCT.

SRB1 acts as an HDL receptor and thus facilitates bidirectional movement of cholesterol between cells and HDL.[220] There is uncertainty over the transcriptional regulation of SRB1. Its promoter contains potential SREBP-1a, SREBP-2,[221] and LXR[222] binding sites. In macrophages, SRB1 levels have been reported to inversely correlate with cholesterol levels, while neither expression of constitutively active SREBPs nor knockout of LXR affected SRB1 expression.[223] It is also unclear how the increased macrophage SRB1 observed with butyrate may affect overall cholesterol levels; Ji et al.[208] found that SRB1 contributed to both efflux and influx of cholesterol between mouse bone marrow-derived macrophages and HDL and as a result, there was no overall difference in cholesterol contents between wild type and SRB1 knockout cells. Conversely, evidence suggests that in hepatic cells, SRB1 mediated cholesterol uptake exceeds cholesterol efflux; liver specific SRB1 overexpressing mice have lower plasma HDL and increased hepatic uptake of

cholesterol esters from HDL,[224] while liver specific SRB1 knockout mice have increased plasma HDL due to reduced hepatic uptake.[225] The downregulation of hepatic SRB1 and ABCA1 seen with sodium butyrate may therefore result in impaired RCT, which could be examined by performing cholesterol uptake experiments using only HDL-C.

4.5.3. Effects of butyrate and atorvastatin on expression of cholesterol genes

Butyrate is a pan-HDAC inhibitor, inhibiting both Class I and Class II HDACs and thus increasing histone acetylation.[210] As histone acetylation is considered to be a sign of active gene expression, it is unsurprising that butyrate is associated with altered expression of a significant number of genes; as mentioned previously, it has been suggested that approximately 10% of genes are significantly altered by butyrate.[160] While we only examined at a small selection of cholesterol related genes, we found butyrate significantly altered the expression of seven out of 13 genes (five upregulated and two downregulated) in HepG2 cells. Upregulated genes include genes involved in cholesterol transport (*ABCG1, SCARB1*), cholesterol and fatty acid synthesis (*SREBF1*), lipid rafts (*CAV1*) and HDL-C (*APOA1*), while genes involved in bile acid synthesis (*CYP7A1*) and cholesterol efflux (*ABCA1*) were downregulated. In BRIN-BD11 cells, genes involved in cholesterol transport and efflux (*ABCA1, ABCG1*) and cholesterol and fatty acid synthesis (*SREBF1*) were upregulated while *LDLR*, involved in cholesterol uptake, was downregulated. As later discussed in Chapter 6, we found no significant changes to histone modifications detected in proximity to *ABCA1, ABCG1* or *LDLR* in butyrate treated HepG2 cells (see section 6.4.6), therefore suggesting that other pathways are more likely to be involved, such as via influences on non-histone acetylation of LXR and SREBP-2. It may also be that there are histone modifications in proximity to these genes that were not detected with the specific modifications and genomic regions examined.

Conversely atorvastatin, as an HMGCR inhibitor, has narrower effects on the expression of the examined genes, only significantly upregulating *HMGCR* and downregulating *SREBF1* in HepG2 cells, while *LDLR* was upregulated and *ABCA1* and *ABCG1* were downregulated in BRIN-BD11 cells. These same changes are also evident in the HepG2 cells, albeit below the threshold for statistical significance. These effects are associated with the mechanism of statins, i.e., inhibition of HMGCR and thus cholesterol biosynthesis. All the genes altered by atorvastatin treatment are

those activated by SREBP-2 (*LDLR*, *HMGCR*), or silenced by LXR (*ABCA1, ABCG1, SREBF1*) in response to low cellular cholesterol levels.[39] Similar results have been obtained with human samples using microarrays to interrogate thousands of genes. For example, Obeidat et al.[226] compared gene expression in the blood cells of statin users to that of non-users with a microarray containing probes for 28000 genes, and found 25 (<0.01%) with altered gene expression, including upregulation of *LDLR* and downregulation of *ABCG1*. These were significantly enriched in cholesterol pathways and 18 of these 25 genes were bound by SREBP-2.

Table 4.2 summarises the influence of butyrate and atorvastatin on gene expression, including mRNA and protein levels, and the roles of those genes on cholesterol metabolism. While it is tempting to look into the genes altered by butyrate and speculate how this could affect cholesterol metabolism, it should be noted that gene expression does not always reflect protein levels or activity. For example, although protein levels of SRB1, encoded by the *SCARB* gene, were decreased by butyrate in HepG2 cells, *SCARB* mRNA was increased, possibly as a compensatory mechanism. Furthermore, the 10-fold increase in *SREBF1* mRNA, encoding SREBP-1 which activates both fatty acid and cholesterol synthesis,[227] did not result in an increase in cellular triglyceride or cholesterol synthesis. Studies relying only on mRNA data should thus be treated with caution.

Table 4.2 Influence of butyrate and atorvastatin on a selection of genes involved in cholesterol metabolism.

Gene	**Butyrate**		**Atorvastatin**		**Role in cholesterol metabolism**
HepG2	qPCR	WB	qPCR	WB	
ABCA1	↓	↓	ns	↓	Efflux cholesterol from cells to HDL.
LDLR	ns	↓	ns	↑	Uptake of LDL-C into cells.
***SCARB1* (SRB1)**	↑	↓	ns	ns	Bidirectional transfer of cholesterol between cells and HDL.
SREBF2	ns	ns	ns	ns	Transcription factor, increases cholesterol synthesis and uptake.
***NR1H3* (LXR)**	ns		ns		Transcription factor, increases cholesterol efflux.
ABCG1	↑	ns	ns	ns	Transport of cholesterol, intracellular and to HDL.

Gene	Butyrate		Atorvastatin		Role in cholesterol metabolism
HMGCR	ns	ns	↑	ns	Cholesterol biosynthesis.
SREBF1	↑		↓		Transcription factor for fatty acid synthesis and cholesterol metabolism.
APOA1	↑	ns	ns	ns	Component of HDL.
CYP7A1	↓		ns		Bile acid synthesis.
MVK	ns		ns		Cholesterol biosynthesis.
CAV1	↑	ns	ns	ns	Component of lipid rafts in plasma membrane.
APOB	ns	ns	ns	ns	Component of LDL.
BRIN-BD11					
Abca1	↑	↑	↓	↓	Efflux cholesterol from cells to HDL.
Abcg1	↑		↓		Transport of cholesterol, intracellular and to HDL.
Ldlr	↓	↓	↑	↑	Uptake of LDL-C into cells.
Srebf1	↑		ns		Transcription factor for fatty acid synthesis and cholesterol metabolism.

4.6. Conclusion

This chapter describes the most comprehensive analysis of the effects of butyrate on cholesterol metabolism, in particular comparing and contrasting it with the commonly used LDL-C lowering drug atorvastatin. Previous animal studies have reported reduced serum cholesterol with butyrate supplementation, and it has been suggested that butyrate inhibits cholesterol biosynthesis *in vitro*. However, lowering of cellular cholesterol by butyrate has not previously been demonstrated. Furthermore, the results presented here suggest HDAC related influences, rather than FFAR activation, as the mechanism, with a time-dependent decrease in SREBP-2 signalling the most likely exacting mechanism. In contrast to statins, the cellular cholesterol lowering by butyrate does not increase uptake of cholesterol by hepatic cells and thus calls into question whether butyrate could lower serum LDL-C in a similar manner.

The effects of butyrate on proteins involved in RCT were also in contrast to statins, where noted effects were directly related to cholesterol levels. The effects of butyrate on these proteins differed according to cell type and as such it is difficult to draw

conclusions on how this may affect HDL metabolism and RCT at the organismal level. The effects of statins on gene expression are limited and directly tied to cellular cholesterol levels, whereas butyrate alters the expression of significantly more genes to a greater degree, reflecting their different mechanisms as HMGCR inhibitors and HDAC inhibitors respectively. Combined with the differences in cholesterol metabolism between humans and rodents, and the poor systemic availability of oral butyrate, there remains numerous doubts and questions over how butyrate may affect cholesterol metabolism in humans.

Chapter 5. Glucose metabolism

5.1. Abstract

The widely used cholesterol lowering drugs statins have been found to increase the risk of developing T2D in humans. Previous studies by our group have found that altering cellular cholesterol, whether by statins or other means, impairs insulin secretion and signalling. Conversely, HDAC inhibitors have been reported to reduce both cholesterol and diabetes risk in animal models of metabolic disease. This chapter explores the effects of statins and HDAC inhibitors on insulin secretion from pancreatic cells and on insulin signalling in hepatic cells, while also examining effects on the expression of genes implicated in T2D in these cells. Insulin secreting cells were treated with HDAC inhibitors and stimulated with glucose and alanine. Insulin was quantified from the 24 h treatment media and from the stimulation media. Hepatic cells were treated with statins and HDAC inhibitors and stimulated with insulin. Insulin stimulation was assessed by immunoblotting of proteins extracted from stimulated and non-stimulated cells. Gene expression was assessed by immunoblotting and qPCR. HDAC inhibition decreased chronic 24 h insulin secretion and the insulin content of cells. Robust HDAC inhibition with 5 mM butyrate or TSA for 24 h in insulin secreting cells decreased basal insulin secretion and content, as well as insulin secretion in response to acute stimulation. Treatment with butyrate also increased expression of the disallowed gene hexokinase I, possibly explaining the impairment to insulin secretion, and of TXNIP, which may increase oxidative stress and β cell apoptosis. In contrast to robust HDAC inhibition (>70% after 24 h), low dose and acute high dose treatment with butyrate enhanced nutrient stimulated insulin secretion. All the genes implicated in insulin secretion and diabetes altered by statins are controlled by cellular cholesterol, adding evidence to the theory that the diabetogenic effects of statins are a result of cholesterol lowering rather than an off-target effect. For insulin signalling, butyrate decreased AKT phosphorylation and tended to increase non-stimulated IRS1 phosphorylation, consistent with previous studies showing phosphorylation of these proteins is impacted by their acetylation. The *in vitro* effects of butyrate on insulin secretion and signalling contrast with the reported *in vivo* effects, which may be more reflective of effects on other tissues, such as GLP-1 secretion from intestinal cells.

5.2. Objectives

2. Investigate how statins and established epigenetic modifiers affect insulin secretion and signalling.

4a. Identify genes involved in cholesterol metabolism or implicated in T2D whose expression is altered by these treatments.

5.3. Introduction

Dyslipidaemia and T2D are closely linked,[11] and *in vitro*, increased cholesterol has been found to impair insulin secretion in β cells[63] and glucose uptake in muscle cells.[228] Reduced plasma LDL-C in humans, whether through the use of statins[14] or genetic mutations,[70] has also been associated with an increased risk of T2D. Conversely, although results in section 4.4.2 indicate HDAC inhibition also decreases cellular cholesterol, animal studies have suggested that HDAC inhibition may in fact be protective against diabetes.[165,167,180,229]

Previous studies in our group assessed the effects of altered cholesterol by statins and other means, namely using methyl-β-cyclodextrins to add or remove cholesterol from plasma membranes, on insulin secretion from β cells[72] and insulin binding and signalling in a number of cell types.[71] These studies found that both increasing and decreasing cellular cholesterol impaired insulin secretion and action. In this chapter, the effects of HDAC inhibition, another method of cholesterol depletion, on insulin secretion in β cells and insulin signalling in hepatic cells are examined and compared to those of statins. The mechanisms behind how statins and HDAC inhibition alter insulin secretion and potentially T2D risk were explored by examining the effects of the HDAC inhibitor sodium butyrate and the statin atorvastatin on numerous candidate genes implicated in T2D. In BRIN-BD11 cells this included genes involved in insulin secretion and other aspects of β cell function and identity, while the use of hepatic cells enabled the examination of genes involved in insulin signalling, glucose uptake, glucose sensing and gluconeogenesis.

5.4. Results

5.4.1. HDAC inhibition impairs insulin secretion and reduces insulin content

Previous investigation by our laboratory found that statins blunted insulin secretion stimulated by glucose and alanine in BRIN-BD11 cells without significant impact on 24 h secretion.[72] A series of experiments were conducted in order to determine if HDAC inhibition, which has a similar effect on cellular cholesterol, impacts insulin secretion in a similar manner. Treatment of BRIN-BD11 cells with 5 mM sodium butyrate (Figure 5-1A) and 2.5 μM TSA (Figure 5-1B) reduced chronic insulin secretion into culture media over the 24 h treatment period, conducted in the present work in media containing 11.1 mmol/L glucose as is standard.[230] Subsequent acute stimulation with 16.7 mM glucose and 10 mM alanine (a widely accepted positive control condition)[231] caused significant increases in insulin secretion in control cells and in cells treated for 24 h with 1 mM sodium butyrate, but this response was significantly blunted in cells treated with 5 mM sodium butyrate or 2.5 μM TSA for 24 h. The insulin content of these cells was also reduced (Figure 5-1C). The percentage of cellular insulin content secreted by cells acutely stimulated with glucose and alanine was also decreased by TSA (Figure 5-1D), thus the reduction in insulin secretion was not completely accounted for by a decrease in the insulin content of the cells. By contrast, 1 mM sodium butyrate significantly increased the percentage of insulin secreted.

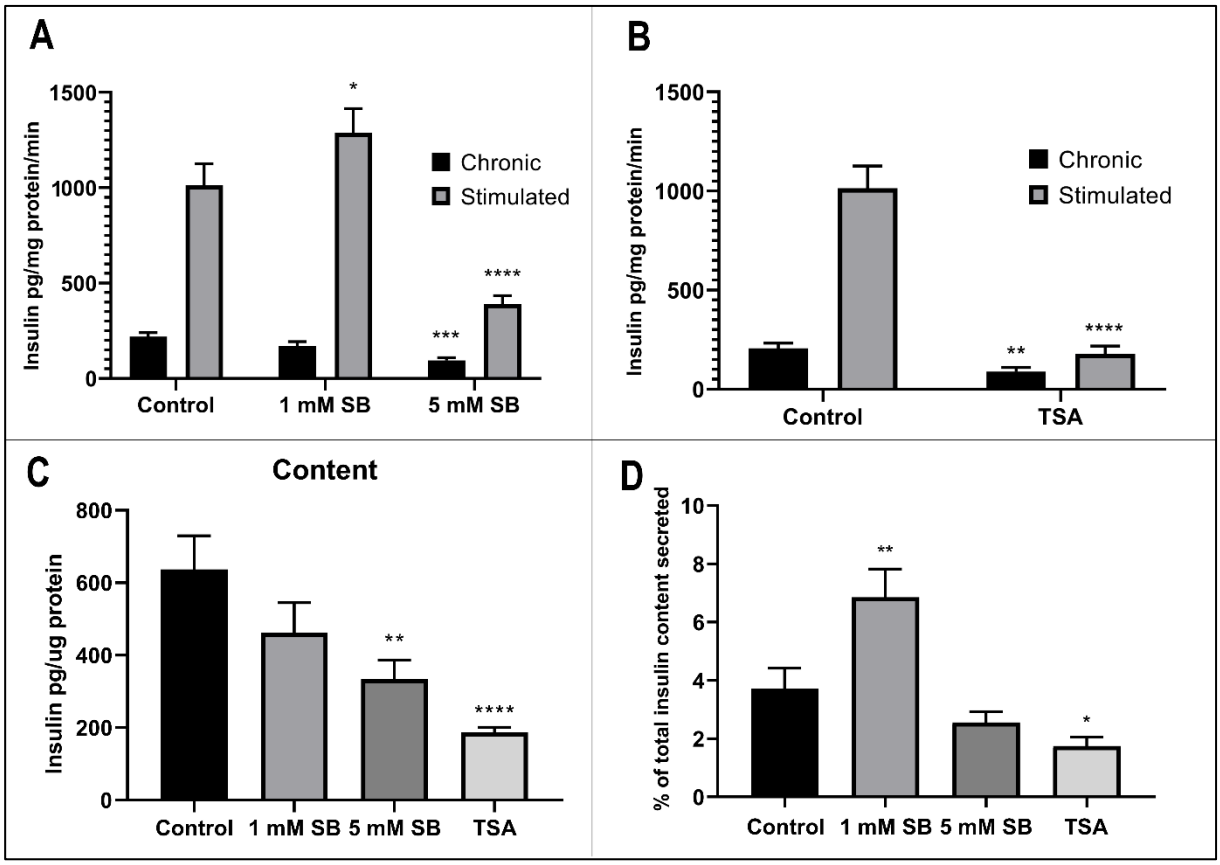

Figure 5-1 Influence of HDAC inhibition on insulin secretion

The effect of 24 h exposure to A) sodium butyrate (SB) and B) 2.5 µM trichostatin A (TSA) on 24 h (chronic) insulin secretion and in response to acute stimulation by 16.7 mM glucose and 10 mM alanine for 20 min (stimulated) using the method described in section 3.7. C) The effect of 24 h treatment of sodium butyrate and TSA on BRIN-BD11 cellular insulin content, determined using the method described in section 3.7.2, and D) the percentage of total insulin secreted in response to stimulation per the insulin content of unstimulated cells. Results are mean combined from at least three independent experiments + SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to the relative untreated control. Analysed by ANOVA.

5.4.2. Butyrate acts as an acute β cell secretagogue

The previous results demonstrated that 24 h treatment of BRIN-BD11 cells with 5 mM sodium butyrate impaired their ability to respond to acute stimulation by glucose and alanine. Conversely, in the absence of any prior treatment with butyrate, inclusion of 5 mM butyrate in the stimulation media enhanced the secretory response to alanine and glucose (Figure 5-2A). Butyrate also tended to increase insulin secretion in the absence of alanine and glucose, however this was not significant (p=0.09). To determine whether this may be due to effects of HDAC inhibition on gene expression, cells were treated with butyrate for 40 min, before addition of the

stimulation media without butyrate. This short-term butyrate treatment failed to significantly enhance stimulated insulin secretion (p=0.43) (Figure 5-2B). The lack of stimulation following the removal of butyrate suggests that the acute stimulatory effect is unlikely to be due to epigenetic effects and may instead be due to effects mediated via FFAR binding and activation, or due to metabolism of butyrate as an energy source.

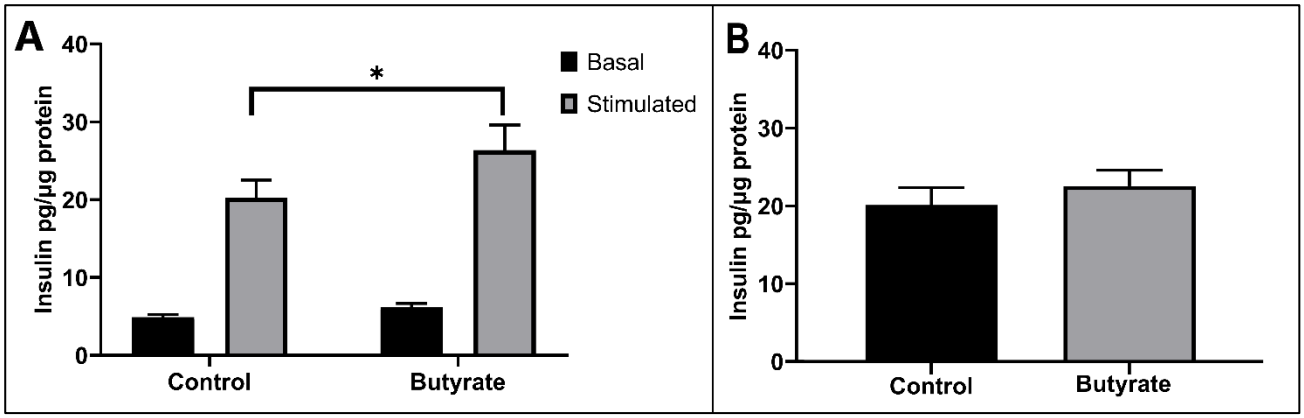

Figure 5-2 Influence of acute sodium butyrate treatment on insulin secretion

A) Direct effects of 5 mM sodium butyrate on BRIN-BD11 insulin secretion over 20 min, with (stimulated) or without (basal) 10 mM alanine and 16.7 mM glucose; B) effect of 40 min treatment with 5 mM sodium butyrate followed by 20 min stimulation with 10 mM alanine and 16.7 mM glucose on BRIN-BD11 insulin secretion. Results represent the mean combined from three independent experiments, with error bars representing SEMs. * p<0.05. Analysed by Student's t test.

5.4.3. Butyrate interferes with insulin signalling in hepatic cells

Reduced insulin sensitivity, or insulin resistance, usually precedes impaired insulin secretion in the development of T2D.[232] The effect of HDAC inhibitors and statins on insulin signalling was thus investigated in HepG2 hepatic cells with or without insulin stimulation (Figure 5-3). Sodium butyrate impaired insulin stimulated AKT phosphorylation and tended to decrease baseline (unstimulated) phosphorylation (p=0.2). On the other hand, butyrate tended to increase the baseline phosphorylation of insulin receptor substrate-1 (IRS-1) (p=0.066), an effect not seen in insulin stimulated cells.

Atorvastatin tended to increase insulin stimulated IRS-1 (p=0.09) but this was not statistically significant compared to the DMSO vehicle control.

In general, the quality of the blots was variable, and the error bars are often large which may have contributed to the lack of statistically significant results.

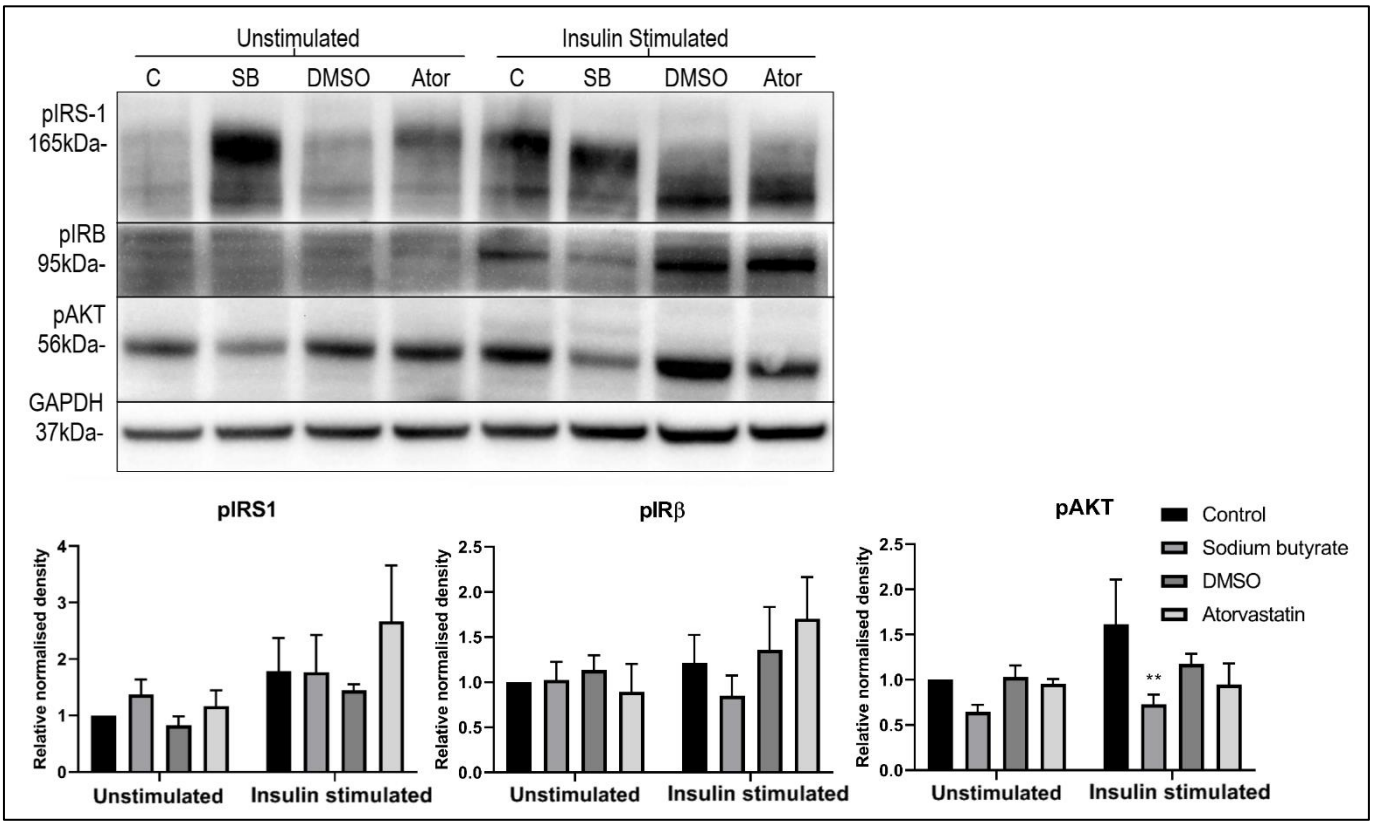

Figure 5-3 Influence of butyrate and atorvastatin on insulin signalling

Immunoblots were completed using the method described in section 3.5 for HepG2 cells treated with 5 mM sodium butyrate or 10 µM atorvastatin, showing the phosphorylation of IRS-1, IRβ and AKT with or without 7 min stimulation with 100 nM insulin as described in section 3.8. The graphs represent the mean combined density readings as normalised to GAPDH from at least three independent experiments, with error bars representing SEMs. ** $p<0.01$. Analysed by ANOVA.

5.4.4. Butyrate increases protein levels of the disallowed gene hexokinase I in HepG2 and BRIN-BD11 cells

Hexokinases phosphorylate glucose, and other hexoses, and for glucose this creates glucose 6-phosphate in the key first step of cellular glucose utilisation. Hexokinase I (HK1), which has a low Km, is usually silenced in β cells and liver cells in favour of glucokinase, which has a higher Km,[47] although some residual expression is a feature of BRIN-BD11 cells.[233] 24 h exposure to 5 mM butyrate caused a substantial increase in HK1 levels in both HepG2 (Figure 5-4A) and BRIN-BD11 cells (Figure 5-5A). Atorvastatin had no effect. Neither sodium butyrate nor atorvastatin significantly altered protein levels of glucokinase in either HepG2 (Figure 5-4B) or BRIN-BD11

cells (Figure 5-5B). Furthermore, neither sodium butyrate nor atorvastatin significantly altered protein levels of the glucose transporter GLUT2 (Figure 5-4C) or the gluconeogenic enzyme PEPCK (Figure 5-4D) in HepG2 cells.

Signs of increased expression of HK1 in HepG2 cells were apparent at 8 h of exposure to 5 mM butyrate and with 16 h treatment of TSA, although this was not statistically significant as the degree of upregulation varied between replicate experiments, reflected by the large SEMs (Figure 5-6).

Protein levels of cholesterol related genes which have also been implicated in diabetes were altered by both butyrate and atorvastatin; butyrate reduced protein levels of LDLR, SRB1 and ABCA1 in HepG2 cells and reduced LDLR but increased ABCA1 in BRIN-BD11 cells while atorvastatin increased LDLR and reduced ABCA1 in HepG2 and BRIN-BD11 cells (see section 4.4).

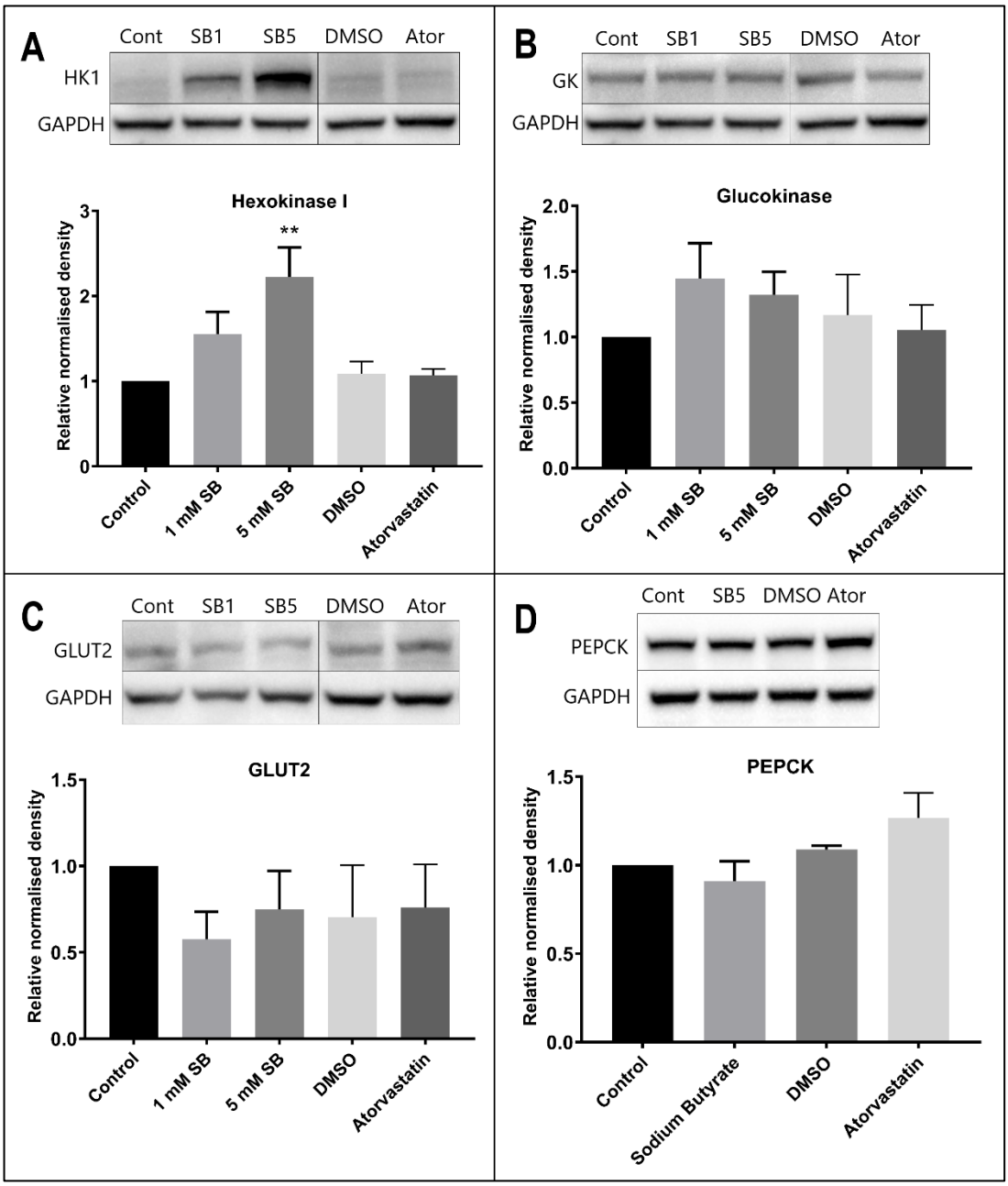

Figure 5-4 Influence of butyrate and atorvastatin on glucose metabolism proteins in HepG2 cells

Immunoblots were completed using the method described in section 3.5 for A) hexokinase I, B) glucokinase, C) GLUT2 and D) PEPCK in HepG2 cells following treatment with 1 mM (SB1) or 5 mM (SB5) sodium butyrate or 10 µM atorvastatin for 24 h. The graphs represent the mean combined density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. ** $p<0.01$. Analysed by ANOVA.

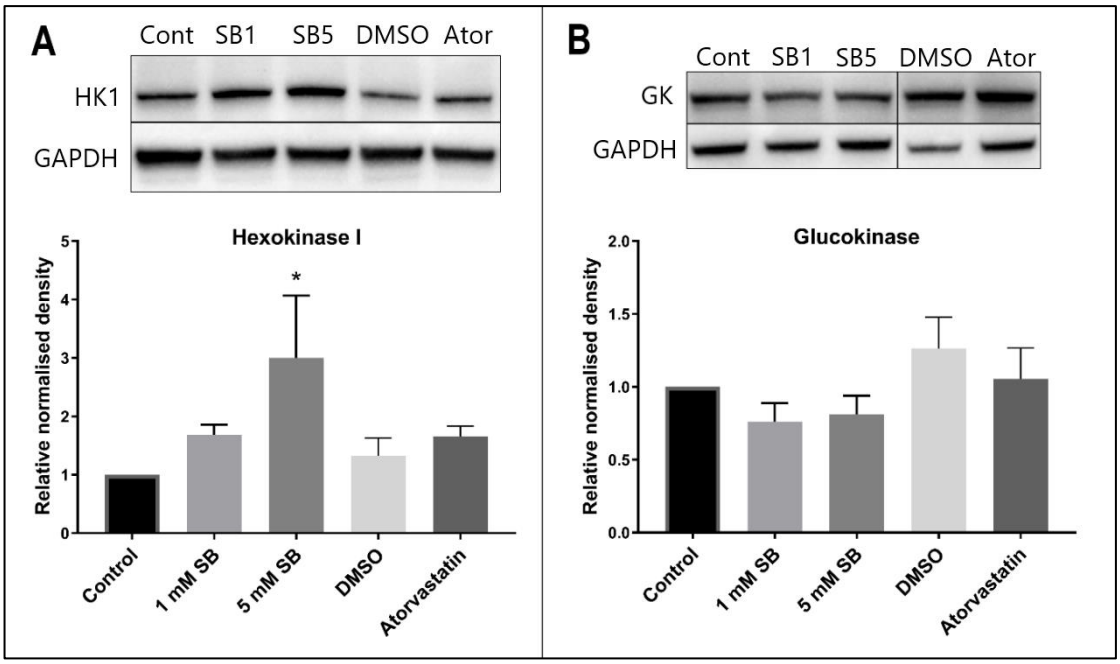

Figure 5-5 Influence of butyrate and atorvastatin on hexokinases in BRIN-BD11 cells

Immunoblots were completed using the method described in section 3.5 for A) hexokinase I and B) glucokinase in BRIN-BD11 cells following treatment with 1 mM (SB1) or 5 mM (SB5) sodium butyrate or 10 µM atorvastatin for 24 h. The graphs represent the mean combined density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. * $p<0.05$. Analysed by ANOVA.

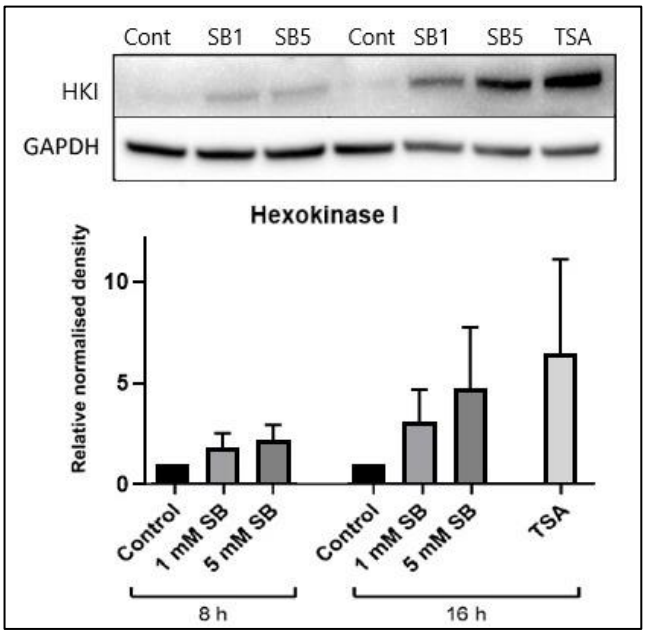

Figure 5-6 Influence of HDAC inhibition on hexokinase I in HepG2 cells at 8 h and 16 h

Immunoblots were completed using the method described in section 3.5 for hexokinase I in HepG2 cells following treatment with 1 mM (SB1) or 5 mM (SB5) sodium butyrate for 8 or 16 h or with

2.5 µM TSA for 16 h. The graph represents the mean combined density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. Analysed by ANOVA and Student's *t* test.

5.4.5. Butyrate significantly alters expression of numerous T2D candidate genes, atorvastatin has more limited effect on gene expression

5 mM sodium butyrate had significant effect on the mRNA expression of numerous genes that have been linked to T2D and/or glucose metabolism as determined by RT qPCR. Notably, hexokinase was upregulated more than 100-fold in HepG2 cells (Figure 5-7A) and was also upregulated in BRIN-BD11 cells, albeit to a lesser degree (Figure 5-7B). Furthermore, glucokinase was significantly downregulated by butyrate in HepG2 cells. *SLC2A2*, the gene encoding GLUT2, was upregulated by butyrate in HepG2 cells, as were the lipid related genes implicated in glucose metabolism, *ABCG1, CAV, SREBF1, SCARB1* and *APOA1*, as well as *CPT1A*, which regulates mitochondrial long-chain fatty acid oxidation. The lipid related genes *ABCA1* and *CYP7A1* were significantly downregulated by butyrate in HepG2 cells. In BRIN-BD11 cells, 5 mM sodium butyrate also significantly upregulated *Hnf4a, Txnip, Abca1, Abcg1, Srebf1,* and insulin (*Ins*) mRNA, all of which have been implicated in β cell function and insulin secretion. The important β cell identity gene *Pdx1* was marginally upregulated (p=0.06). *Ldlr* was downregulated.

Conversely, in HepG2 cells 10 µM atorvastatin only significantly altered expression of genes implicated in T2D but primarily involved in lipid metabolism, namely the transcription factor *PPARG* and the cholesterol synthesis gene *HMGCR* were upregulated, and *SREBF1* was downregulated (Figure 5-7C). Furthermore, atorvastatin upregulated *Ldlr* expression and downregulated *Abca1* and *Abcg1* expression in BRIN-BD11 cells, while having no significant effect on the other genes examined in this cell type (Figure 5-7D).

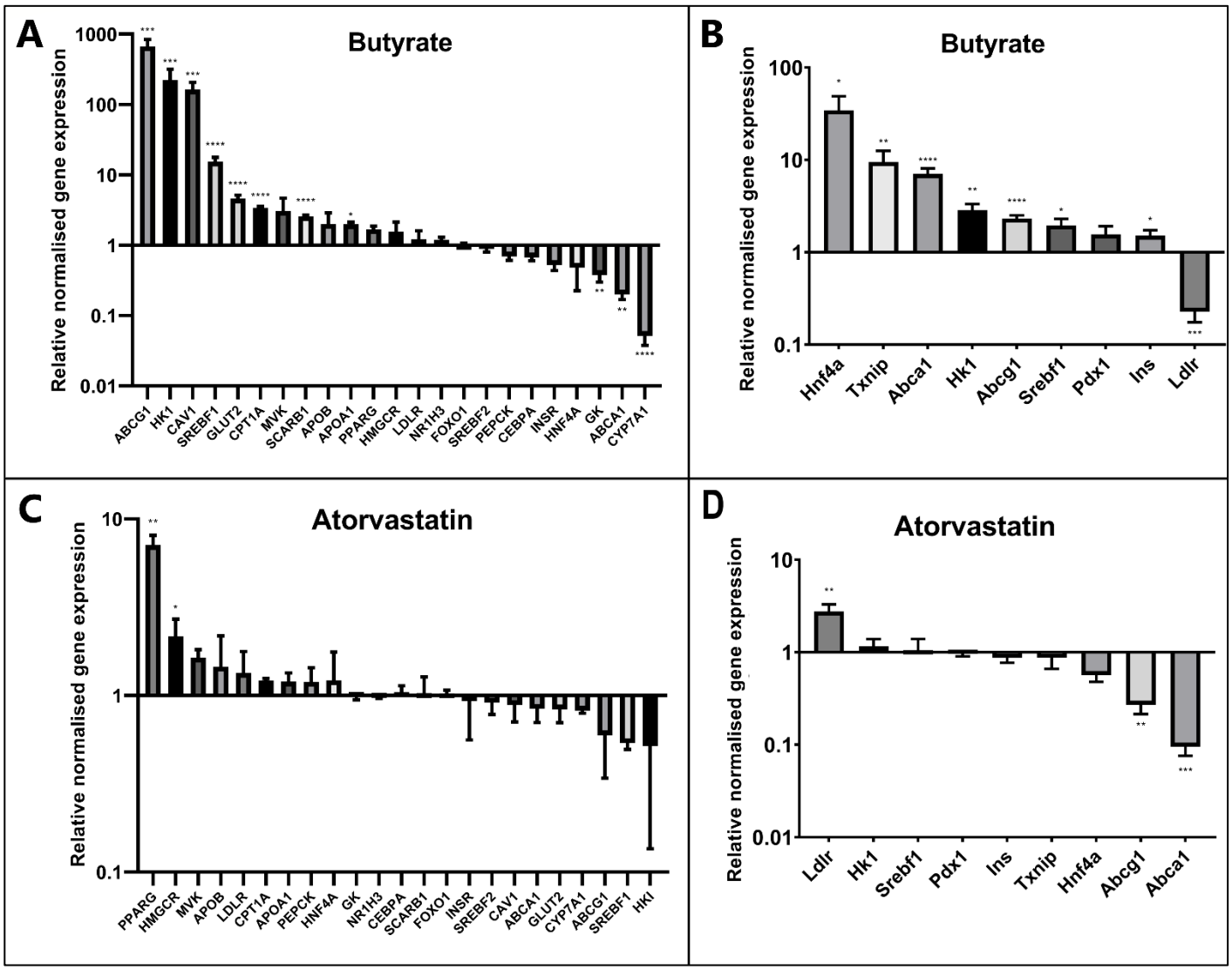

Figure 5-7 Influence of butyrate and atorvastatin on diabetes-related gene expression

Reverse-transcription qPCR was conducted as described in section 3.6 for A) HepG2 cells and B) BRIN-BD11 cells treated with 5 mM sodium butyrate; relative gene expression in C) HepG2 cells and D) BRIN-BD11 cells treated with 10 µM atorvastatin compared to the untreated control. HepG2 cell gene expression is normalised to reference genes *GAPDH, YWHAZ* and *RPL13A*. BRIN-BD11 gene expression is normalised to reference genes *Actb* and *Rpl13a*. Atorvastatin data is shown relative to the untreated control, but statistical significance is compared to the DMSO vehicle control. Results represent the mean combined from three independent experiments, with error bars representing SEMs. * $p<0.05$ ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Analysed by ANOVA.

5.5. Discussion

5.5.1. Effects of butyrate and statins on the expression of genes involved in diabetes and glucose metabolism

Sodium butyrate altered the expression of a significant number of genes that have been associated with glucose metabolism and T2D, while atorvastatin altered the expression of a smaller subset of genes. These are summarised in

Table 5.1. Of the 23 candidate genes analysed in HepG2 cells, butyrate significantly altered the mRNA expression of 11 (eight upregulated, three downregulated). In BRIN-BD11 cells, butyrate altered the mRNA expression of nine out of ten candidate genes (eight upregulated, one downregulated). Conversely, atorvastatin only altered the mRNA expression of three genes in HepG2 cells, increasing *HMGCR* and *PPARG* expression and decreasing *SREBF1* expression, and three genes in BRIN-BD11 cells, decreasing *Abcg1* and *Abca1* expression and increasing *Ldlr* expression, all of which have been implicated in T2D but are primarily involved in lipid metabolism. The disparity between butyrate and statins is likely due to their different mechanisms of action as discussed in Chapter 4 for cholesterol genes.

Also as discussed in Chapter 4, the effects on mRNA expression are not always linked to protein levels or phenotypic changes. For instance, many of the changes in gene expression by 5 mM sodium butyrate in BRIN-BD11 cells would be expected to increase β cell function and insulin secretion, however, the insulin secretion experiments indicate the opposite occurred.

Specific genes whose altered expression may contribute to observed effects on insulin secretion are discussed in the next two sections on HDAC inhibition and statins respectively.

Table 5.1 Influence of butyrate and atorvastatin on a selection of genes involved in glucose metabolism and T2D

Gene	Butyrate		Atorv		Role in glucose metabolism and T2D
HepG2	qPCR	WB	qPCR	WB	
ABCA1	↓	↓	ns	↓	Cholesterol efflux, downregulation impairs insulin secretion.[234]
LDLR	ns	↓	ns	↑	LDL receptor, may play a role in the diabetogenic effects of statins, mutations decrease T2D risk.[235]

Gene	Butyrate		Atorv		Role in glucose metabolism and T2D
***SCARB1* (SRB1)**	↑	↓	ns	ns	HDL receptor, upregulated in T2D,[236] polymorphisms alter T2D risk.[237]
HK1	↑	↑	ns	ns	High-affinity hexokinase, alters glucose sensing, repressed in healthy β cells and hepatocytes.[47]
PPARG	ns	ns	↑	ns	Increases insulin sensitivity in peripheral cells, increases glucose sensing in β cells and hepatocytes.[238]
ABCG1	↑	ns	ns	ns	Cholesterol transporter, reduced expression in T2D,[239] downregulation impairs insulin secretion.[234]
GK	↓	ns	ns	ns	Glucose sensor in β cells and hepatocytes, mutations cause diabetes.[240]
HMGCR	ns	ns	↑	ns	Cholesterol biosynthesis, mutations increase T2D risk.[241]
SREBF1	↑		↓		Transcription factor, downregulates gluconeogenesis and increases glycogen synthesis,[242] impairs insulin secretion.[243]
APOA1	↑	ns	ns	ns	Component of HDL, increases insulin secretion,[244] low levels increase T2D risk.[245]
CYP7A1	↓		ns		Bile acid sythesis, increased by glucose and insulin, overexpression protects mice from diabetes.[246]
SLC2A2	↑	ns	ns	ns	Glucose transporter in β cells and hepatocytes, important for GSIS.[247]
CAV1	↑	ns	ns	ns	Protein of plasma membrane caveolae which contain insulin receptors, important for insulin signalling.[248]
CPT1A	↑		ns		Controls hepatic mitochondrial fatty acid β-oxidation, inhibitors decrease gluconeogenesis.[249]
BRIN-BD11					
Ins1	↑		ns		Insulin gene.
Abca1	↑	↑	↓	↓	Cholesterol efflux, reduced in T2D,[250] downregulation impairs insulin secretion.[234]
	qPCR	WB	qPCR	WB	
Hk1	↑	↑	ns	ns	High-affinity hexokinase, alters glucose sensing, repressed in healthy β cells and hepatocytes.[47]
Pdx1	↑		ns		β cell specific transcription factor, required for β survival and function.[251]

Gene	Butyrate		Atorv		Role in glucose metabolism and T2D
Abcg1	↑		↓		Cholesterol transporter, reduced in T2D,[239] downregulation impairs insulin secretion.[234]
Ldlr	↓	↓	↑	↑	LDL receptor, may play a role in the diabetogenic effects of statins, mutations decrease T2D risk.[235]
Srebf1	↑		ns		Transcription factor, downregulates gluconeogenesis and increases glycogen synthesis,[242] impairs insulin secretion.[243]
Txnip	↑		ns		Inhibits thioredoxin which protects against oxidative stress, induced by glucose and promotes β cell apoptosis.[252]
Hnf4a	↑		ns		Transcription factor important in islet and liver function, mutations cause diabetes.[253]

5.5.2. Effects of HDAC inhibition on insulin secretion

The effect of statins and altered cellular cholesterol on insulin secretion were previously studied in-depth by our group.[72] Briefly, either reducing or increasing BRIN-BD11 cellular cholesterol content reduced stimulated insulin secretion. As butyrate and TSA both reduce cellular cholesterol (see section 4.4.2, note that the effect of TSA on the cellular cholesterol content of BRIN-BD11 cells was not examined), it is possible that the reduced insulin secretion observed here was also a result of reduced cellular cholesterol. Statins, however, did not impair chronic insulin secretion as HDAC inhibitors did, and the degree of reduced stimulated insulin secretion was greater with HDAC inhibitors than with statins despite comparable cholesterol lowering at the doses used. Thus, cholesterol lowering in and of itself does not account for the defects in insulin secretion seen with HDAC inhibition.

Potent inhibition of HDAC activity in BRIN-BD11 cells over 24 h with 5 mM sodium butyrate or 2.5 uM TSA significantly impaired both 24 h insulin secretion and secretion in response to acute (20 min) stimulation with well-established potent secretagogues. This is likely due to defects in both insulin production and secretion, since insulin content was decreased by 24 h HDAC inhibition, and the percentage of total insulin content secreted was also lower in cells subject to potent HDAC inhibition. It should be noted that insulin secretion was not increased, and in fact was decreased, during the treatment period, so the reduced content was not due to high

levels of insulin secretion depleting stores. As insulin mRNA was increased by 5 mM butyrate, it is unlikely that altered insulin gene expression contributed to the reduced cellular insulin content, although it is possible that expression was lowered at an earlier time point as seen with *HMGCR* in section 4.4.5. If not insulin gene expression, insulin content could be affected by influences on translation, processing or degradation. The insulin gene encodes preproinsulin, which is cleaved in the endoplasmic reticulum to form proinsulin, which is folded and transported to secretory vesicles where it is again cleaved to form mature insulin.[254] The insulin ELISA used in this study only recognises mature insulin and thus would not detect changes to the precursors preproinsulin and proinsulin. Many proteins are involved in the translation and processing of insulin, with glucose sensing upregulating the process.

The activation of hexokinase I expression, observed both in this study and in studies of butyrate treated RINm5F insulin-secreting cells,[182,183] may contribute to the detrimental effects of butyrate on both insulin content and GSIS. Furthermore, although protein levels of glucokinase, the 'glucose sensor' in β cells and hepatocytes,[255] were not significantly altered by butyrate treatment, glucokinase mRNA was significantly downregulated in HepG2 cells. Hexokinase I is silenced in healthy β cells and hepatocytes as it phosphorylates glucose at low concentrations, whereas glucokinase has a low affinity for glucose, as thus is only active when glucose concentrations are high.[47] As a result, hexokinase I expression would lead to insulin secretion and glycogen synthesis when blood glucose is low, thereby causing hypoglycaemia. Furthermore, unlike glucokinase, other hexokinases are inhibited by their product, thereby limiting their activity in high glucose environments. Therefore, hexokinase I expression would result in inappropriate response to both high and low glucose (Figure 5-8). Feeding hamsters high sucrose diets has been found to increase islet hexokinase I expression, leading to hyperinsulinaemia,[256] and hexokinase I expression was found to increase with age, obesity and diabetes development in rat islets.[257] While hexokinase I expression has been associated with β cell dedifferentiation,[258] the increased expression of transcription factors relevant to β cell identity, such as *Pdx1* and *Hnf4a*, as well as the insulin gene itself, suggest that more generalised dedifferentiation has not occurred.

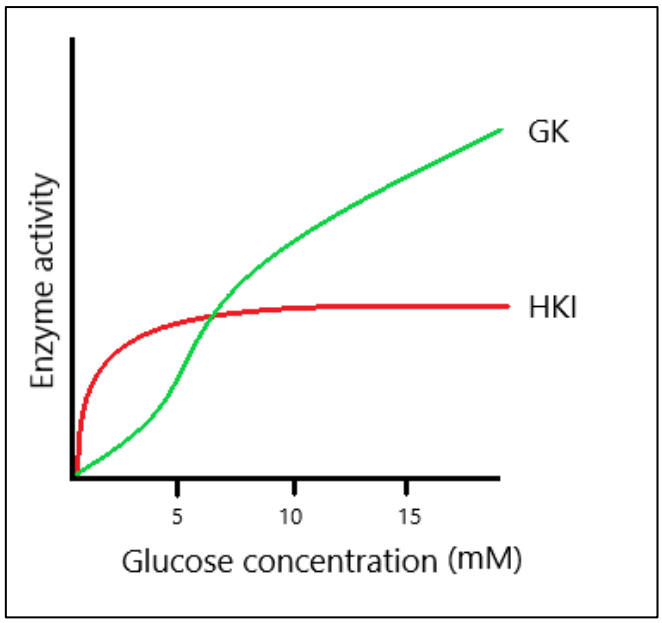

Figure 5-8 Hexokinase and glucokinase activity

Exposing islets to high glucose (27 mM) for 48 h has been shown to impair insulin secretion,[259] associated with an increase in the proinsulin:insulin ratio.[260] Although cellular insulin content is decreased by glucose overstimulation, this only partially explains the impaired insulin secretion. It seems plausible that increased glucose sensing due to hexokinase activity could have similar effects, especially as an increase in the proinsulin:insulin ratio would explain the increased the discord between increased insulin mRNA but decreased insulin content seen in butyrate treated cells. This is comparable to the glucotoxicity thought to occur in T2D, where prolonged hyperglycaemia and subsequent demand for insulin leads to a decline in β cell function and an eventual increase in β cell apoptosis, possibly due to oxidative stress.[261]

To explore the mechanism by which HDAC inhibitors increase hexokinase expression, ChIP experiments were conducted to examine histone acetylation in proximity to the *HK1* gene in HepG2 cells (see section 6.4.6). Using ENCODE data, an H3K9ac site in proximity to intron 1 of HK1 with low peak strength in HepG2 cells compared to other cell types was identified. ChIP experiments found no change in H3K9 or H4K8 acetylation in this region in butyrate treated HepG2 cells. Thus, the exact mechanism by which butyrate upregulates hexokinase I remains unknown. Interestingly, it has been shown that the repression of hexokinase I in healthy β cells is epigenetically controlled; Dhawan et al.[262] demonstrated increased DNA

methylation of the *Hk1* promoter in β cells from adult mice compared to immature β cells from neonatal mice, with increased binding of the DNA methyltransferase DNMT3A in this region, Histone modifications, namely decreased H3K9 acetylation and increased H3K27 methylation, have been found to play a role in the repression of two other genes disallowed in β cells, *Mct1* and *Ldha*.[263] ChIP-seq across the *Hk1* locus in both β cells and hepatocytes could elucidate the mechanism by which HDAC inhibition activates hexokinase I expression.

Butyrate treatment also increased expression of thioredoxin interacting protein (*Txnip*) in BRIN-BD11 cells. TXNIP inhibits thioredoxin, a thiol oxidoreductase that reduces oxidised proteins produced by reactive oxygen species.[102] The increase in *Txnip* mRNA thus provides further evidence that HDAC inhibition can increase oxidative stress in β cells; TSA has also been previously found to increase reactive oxygen species in insulin secreting cells.[264] In β cells, TXNIP is strongly upregulated by glucose and induces apoptosis. As a result, TXNIP has been implicated as the link between chronic hyperglycaemia and β cell death.[102] While the observed increase in *Txnip* mRNA could be due to increased glucose sensing as a result of hexokinase I expression, there is also evidence that *TXNIP* is epigenetically controlled. Glucose increases histone acetylation in proximity to the *TXNIP* gene and knockdown or inhibition of the histone acetyltransferase p300 ameliorates the glucose induced TXNIP upregulation.[265] The increased *Txnip* expression could therefore be a result of HDAC inhibition and subsequent increased histone acetylation; numerous studies have found various HDAC inhibitors increase TXNIP expression.[264,266-268]

The effect of chronic butyrate exposure on insulin secretion are summarised in Figure 5-9.

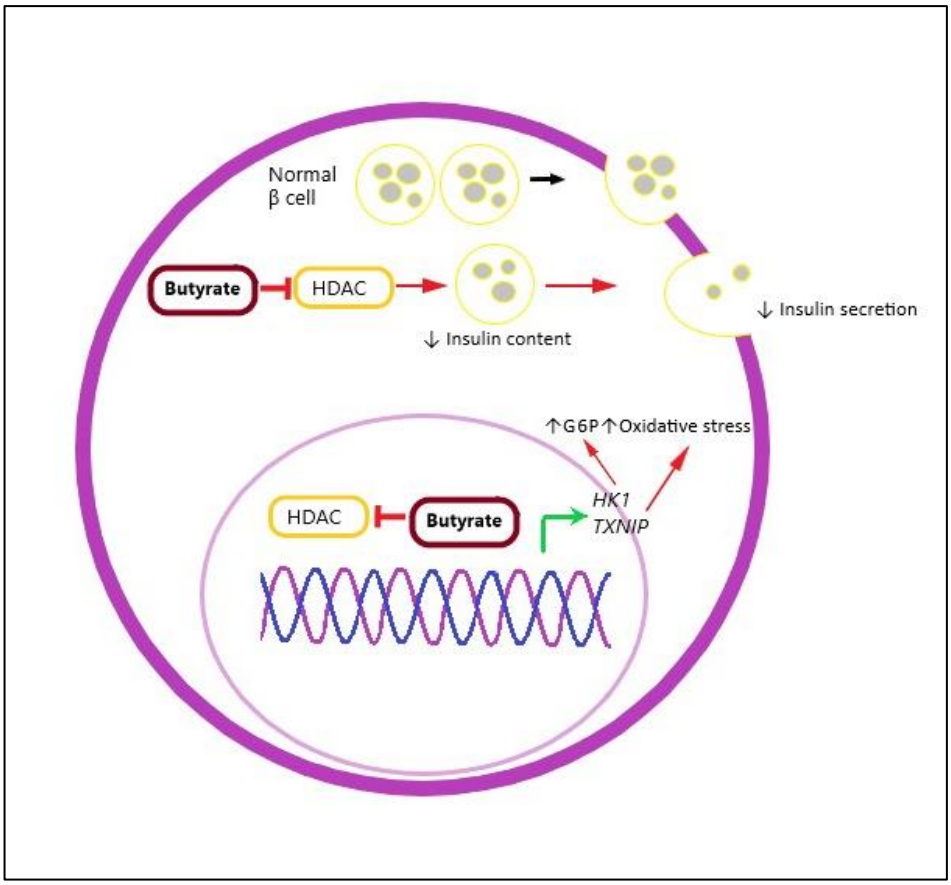

Figure 5-9 Summary of effects of chronic butyrate treatment on insulin secretion

24 h treatment with butyrate inhibits HDAC activity, which leads to both decreased insulin content and impaired insulin secretion. This may be due to the effects of HDAC inhibition on gene activity, namely upregulation of *HK1* and *TXNIP*, leading to increased glucose phosphorylation and increased oxidative stress respectively.

Although 24 h treatment with butyrate was detrimental to insulin secretion, acute exposure to butyrate enhanced the secretory response of BRIN-BD11 cells to alanine and glucose. In agreement with this, a historical study by Manns et al.[179] in 1967 reported that infusion of butyrate directly into the pancreatic artery of sheep resulted in a marked increase in plasma insulin within 3 min. More recently, Lin et al.[269] found increased plasma insulin 10 min after orally administering sodium butyrate to mice. This acute secretion is unlikely to be due to changes in gene expression, i.e., due to HDAC inhibition, as short term (40 min) butyrate treatment prior to stimulation failed to significantly enhance insulin secretion in our study. Instead, butyrate may act as a secretagogue through FFAR activation or through direct metabolism of butyrate as an

energy source. Butyrate is the primary energy source for colonocytes,[270] while, *in vitro*, butyric acid increased GSIS in MIN6 cells but not in FFAR2 knockdown cells, suggesting it may be the actions of butyrate as a FFAR agonist that can enhance insulin secretion.[271] Similar results with other SCFAs were reported by Pingitore et al.[272] and Priyadarshini et al.[273] however, other studies have suggested that FFAR activation impairs insulin secretion,[274,275] thus uncertainty remains regarding the role of FFARs in insulin secretion. It may be that the stimulatory effects of the 1 mM butyrate after 24 h are also a result of FFAR activation or butyrate metabolism, while the detrimental effects of HDAC inhibition are stronger at higher doses. Conversely, it may be that positive effects on gene expression, such as upregulation of *Pdx1, Hnfa* and insulin, have a greater impact on GSIS than the negative effects of upregulation of *Hk1* and *Txnip* at this lower dose.

Butyrate and TSA are known to have poor systemic availability and are rapidly metabolised.[276-278] Thus, the treatment times and doses used in this, and other *in vitro*, studies are unlikely to reflect the *in vivo* effects of oral treatment. Most notably, rather than impairing insulin secretion and β cell function, in high fat diet (HFD) fed rodents, butyrate supplementation reduces fasting glucose,[118,211,279] decreases β cell hyperplasia,[180,279] and reduces signs of inflammatory response in islets.[279] In an *ex vivo* study of pancreatic islets from mice fed a HFD with or without butyrate supplementation, butyrate increased GSIS by increasing glucose-stimulated (16.7 mM) insulin secretion while reducing basal (2.8 mM glucose) insulin secretion.[180] These *in vivo* and *ex vivo* effects may not be a direct effect of HDAC inhibition in the β cell, but rather the result of butyrate affecting other tissues. Butyrate stimulates the release of glucagon-like peptide 1 (GLP-1) from intestinal cells both *in vitro* and *in vivo*,[280,281] as also observed in a human trial,[6] and GLP-1 is an established enhancer of β cell function and insulin secretion.[282] It may also be that cells are exposed to low doses of butyrate for a short time period, and thus the low dose and acute butyrate treatments may be more reflective of *in vivo* effects than the supraphysiological 24 h treatments.

5.5.3. Cholesterol lowering by statins as a mechanism for impaired insulin secretion

The genes altered by atorvastatin treatment can all be linked to decreased cholesterol levels, as they are either activated by SREBP-2 (*LDLR, HMGCR, PPARG*),[283] or silenced by LXR (*ABCA1, ABCG1, SREBF1*) when cellular cholesterol levels are

low.[39] There were no changes in the examined diabetes related genes that cannot be explained by cholesterol lowering. There are multiple lines of evidence that suggest it is cholesterol lowering rather than an off-target effect that accounts for the diabetogenic effects of statins. Lowering cellular cholesterol with methyl-β-cyclodextrin, an oligosaccharide with a strong affinity for cholesterol that extracts cholesterol from cellular membranes,[284] impairs both insulin secretion[72] and signalling[71] *in vitro,* and mutations that increase[69] or decrease[70] plasma LDL-C decrease and increase the risk of T2D respectively. Furthermore, numerous studies have shown that silencing of the cholesterol transporters ABCA1[234,285-287] and ABCG1,[64,234] both of which were downregulated by atorvastatin in BRIN-BD11 cells, impairs β cell function and insulin secretion. The defects seen in the ABCA1 deficient cells were attributed to lack of cholesterol efflux leading to intracellular cholesterol accumulation; depleting the ABCA1 deficient cells of cholesterol corrected the impairment.[286] ABCA1 downregulation is thus unlikely to be the cause of impaired insulin secretion in statin treated BRIN-BD11 cells these cells had depleted cellular cholesterol (see section 4.4.2). Conversely, ABCG1 deficiency was found to not alter total cellular cholesterol nor cholesterol efflux, and addition of cholesterol to deficient cells rescued insulin secretion.[64] It was found that ABCG1 localised to insulin secretory granules and that ABGC1 deficient cells had reduced granule membrane cholesterol and altered granule morphology, namely an increase in cross-sectional area. The membranes of insulin secretory granules are approximately 35% cholesterol, similar to the plasma membrane,[288] and depleting secretory vesicles of cholesterol, by the use of MβCD, has been shown to reduce membrane fusion.[289] This may be because SNARE proteins, which facilitate the fusion of secretory vesicles with the plasma membrane, are found in cholesterol dependant clusters; cholesterol depletion by MβCD causes dispersion of these clusters and reduced exocytosis.[290] There is also evidence that ABCG1 deficiency and reduced insulin secretory granule cholesterol increases the lysosomal degradation of granules.[288] Reduced ABCG1 expression, and subsequent reduced granule cholesterol, may therefore contribute to the impairments in insulin secretion seen in statin treated cells and thus may contribute to the diabetogenic effects of statins.

Interestingly, DNA hypermethylation of *ABCG1* in blood cells has been associated with T2D,[93-97] with low levels of HDL-C,[89-92] and with statin use.[154-156] As DNA methylation was not examined in this thesis it cannot be ruled out that epigenetics contributes to the reduced *Abcg1* expression in statin treated BRIN-BD11 cells.

Similarly to histone modifications induced by SREBP-2 (see section 6.5.3), statins may induce epigenetic changes indirectly through LXR activity. Sandoval-Hernández et al.[291] found that the LXR agonist GW3965 reduced DNA methylation at numerous LXR target genes in mice, although they did examine effects on *ABCA1* or *ABCG1*. Similarly, Tristán-Flores et al.[292] found a differentially methylated region in the *Alu* retrotransposon in atherosclerotic plaques that contained an LXR binding site was hypermethylated by GW3965. Whether LXR activity alters DNA methylation of *ABCG1* does not appear to have been examined and would be an interesting area of further research.

There is also evidence that statins may alter *ABCG1* expression through histone modifications. In adipocytes, statins reduced *ABCG1* expression while increasing levels of HDAC9,[293] and HDAC9 deficient macrophages have increased *ABCG1* expression and H3K9 acetylation.[294] Figure 6-10 showed a trend towards decreased H3K9 acetylation in proximity to *ABCG1* in atorvastatin treated HepG2 cells but this was not significant. It should be noted that this was in HepG2 cells, where *ABCG1* expression was not significantly altered by atorvastatin, whereas ChIP was not performed in BRIN-BD11 cells due to budgetary constraints.

5.5.4. Effects of butyrate and statins on insulin signalling

Peripheral insulin resistance typically precedes impaired insulin secretion in the development of T2D. Some clinical trials have suggested a worsening of insulin sensitivity with statin treatment,[295,296] while other trials found either no effect[297,298] or an improvement in insulin sensitivity.[299,300] A previous study by our group found either increasing or decreasing cellular cholesterol using cyclodextrins decreased the binding of insulin to the insulin receptor in a number of cell lines, most likely due to changes in plasma membrane cholesterol.[71] Furthermore, reducing cellular cholesterol with cyclodextrins reduced the phosphorylation of AKT and IRβ in response to insulin. Atorvastatin, however, did not have the same effects on insulin binding or signalling. In fact, in HepG2 cells, but not the Chinese hamster ovary CHO cell line or differentiated myotubes, atorvastatin increased the phosphorylation of AKT and IRβ in response to insulin.[71] Conversely, results shown in this chapter did not find a significant effect of atorvastatin on insulin signalling, possibly due to a shorter treatment time (24 h v 48 h), although it did find a trend towards increased insulin-stimulated IRβ and IRS-1 phosphorylation but decreased AKT

phosphorylation. It should be noted AKT phosphorylation is not unique to insulin signalling and is involved in numerous other pathways involved in cell growth, metabolism and survival.[301] Regardless, the effects on statins on insulin signalling remain unclear.

The HDAC inhibitor sodium butyrate decreased AKT phosphorylation while tending to increase non-stimulated IRS-1 phosphorylation. This could be due to effects on non-histone lysine acetylation. In addition to impacting gene expression by reducing histone acetylation, HDACs can also remove acetyl marks from numerous other proteins, including those in insulin signalling pathways. In particular, IRS-1 has been found to associate with HDAC2 and HDAC inhibition with TSA increases lysine acetylation of IRS-1 and subsequently increases IRS-1 tyrosine phosphorylation.[302] AKT is also acetylated at lysine residues by HATs and deacetylated by the HDAC SIRT1.[303] Unlike IRS-1, acetylation of AKT reduces its ability to be phosphorylated, consistent with the results presented here. Multiple other proteins involved in insulin signalling have been shown to be acetylated, with acetylation again acting both to increase (Rictor and TRB3) and decrease (IRS-2 and PDK1) insulin signalling.[304] As a result, it is difficult to summarise the overall effect of HDAC inhibition on insulin sensitivity.

It is also unclear how HDAC inhibition affects insulin signalling *in vivo*. Zhang et al.[305] reported that sodium butyrate supplementation reduced liver AKT phosphorylation while increasing GSK3α and GSK3β phosphorylation in db/db mice. Increased IRS-1 phosphorylation has been reported in the liver[184] and skeletal muscle[165] of butyrate treated rodents. Increased AKT phosphorylation has also been reported in liver[118] and skeletal muscle[184] of butyrate treated rodents. Similarly to insulin secretion, *in vivo* effects on insulin signalling may not be due to direct effects on HDAC inhibitors on the genes and proteins involved in the insulin signalling pathway but may be downstream effects of changes to genes and proteins in other metabolic pathways, such as enhanced insulin secretion due to GLP-1 secretion.

5.6. Conclusion

Statin use has been linked to an increased risk of T2D, while HDAC inhibitors have been suggested to have positive effects on glucose metabolism in animal models of diabetes. The results presented here suggest that *in vitro*, statins and HDAC inhibitors

both impair insulin secretion and may interfere with insulin signalling. The effects of statins are most likely secondary to cholesterol lowering, as cholesterol depletion through other means has similar effects. In particular, the decrease in expression of ABCG1 in response to lowered cholesterol may impair insulin secretion, possibly by reducing the cholesterol content of insulin secretory granules. HDAC inhibition may impair β cell function and insulin secretion through activation of hexokinase I, causing cells to respond to low glucose and leading to β cell exhaustion, and TXNIP, increasing oxidative stress. Conversely, low concentrations of butyrate may increase stimulated insulin secretion and butyrate may directly act as a secretagogue to acutely increase secretion. Butyrate may interfere with insulin signalling by increasing acetylation of proteins involved in the insulin signalling cascade, which can either increase (IRS-1) or decrease (AKT) their ability to be phosphorylated. As the uptake of HDAC inhibitors such as butyrate into the systemic circulation is poor, these *in vitro* effects may not reflect *in vivo* effects, which may be more reflective of effects on intestinal cells, in particular increased secretion of GLP-1.

Chapter 6. Epigenetics

6.1. Abstract

Epigenetic modifications have been linked to dyslipidaemia, CVD and T2D. Statins have also been reported to have epigenetic effects, in particular inhibition of HDACs, however this has rarely been investigated in the context of diabetes or cholesterol lowering. HDAC inhibitors such as the SCFA butyrate have been reported to reduce cholesterol and diabetes risk in animal models, however the majority of studies do not report epigenetic modifications at target genes that may be involved. This chapter investigates the effect of statin drugs and established epigenetic modifiers on epigenetic modifications in multiple cell lines, including hepatic cells, insulin-secreting cells, macrophages and breast carcinoma cells, as well as liver extracts from statin-treated C57B1/6J mice. Cells or cell extracts were treated with statins and with established epigenetic modulators, and HDAC, HAT, and DNMT activities were quantified. Histone acetylation was assessed on a global level by immunoblotting and at specific genomic locations by ChIP-qPCR. Statins altered neither HDAC nor HAT activity. Accordingly, global acetylation of histones H3 and H4 was unchanged with statin treatment. Atorvastatin did tend to increase DNMT activity. Butyrate significantly inhibited HDAC activity and increased global histone acetylation in all experiments. Despite the lack of effect on HDACs, HATs and global histone acetylation, statins tended to increase histone acetylation in proximity to SREBP-2 target sites, significantly for HMGCR. The effects of butyrate on histone modifications at targeted genomic regions are unclear, as ChIP results were significantly impacted by the data normalisation method used. Increased H4K8 acetylation in proximity to the gene encoding the glucose transporter GLUT2 was consistent across all methods.

6.2. Objectives

3. Investigate how statins and established epigenetic modifiers influence epigenetic modifying enzymes and alter genome-wide epigenetics.

4b. Investigate if epigenetic modifications contribute to the altered expression of target genes.

6.3. Introduction

There has been considerable research linking epigenetics to dyslipidaemia,[15,16] insulin resistance and T2D,[17-20] and CVD.[21-23] As a result, there is interest in the use of epigenetic modifiers, in particular histone deacetylase (HDAC) inhibitors, to prevent and treat these disorders.[24,25] Butyrate, an HDAC inhibitor produced by the gut microbiome, has shown promising results in pre-clinical experiments and has reached clinical trials for lipid lowering and T2D.[6,26,27] Statins, the widely used cholesterol lowering drugs, also have reported epigenetic effects, as reviewed in Allen and Mamotte[28] with the majority of studies conducted in the context of cancer. The effect of statins on epigenetic enzyme activity and global histone modifications has not been investigated in the context of dyslipidaemia, the primary indication for statin use, or type 2 diabetes, which has been linked to statin use.[14] This chapter investigates the effects of statins and sodium butyrate on global and local histone modifications and on the activity of the major classes of enzymes that enact epigenetic changes in cell types implicated in the pathology of CVD and T2D, namely HepG2 hepatic cells, BRIN-BD11 insulin-secreting cells and THP-1 macrophages, with confirmatory experiments conducted in MDA-MB-231 breast cancer cells to compare to previous studies conducted in cancer cell lines.

6.4. Results

6.4.1. Statins do not inhibit HDAC activity

Sodium butyrate inhibited HDAC activity in live HepG2 cells with an IC_{50} of 445 µM (± 126 µM SE) (Figure 6-1A). Conversely, after 24 h treatment, no statin (at 10 µM) inhibited HDAC activity in live HepG2 cells (Figure 6-1B). The potent HDAC inhibitor TSA also significantly inhibited HDAC activity (p<0.0001). Atorvastatin, chosen as a model statin due to its potency and widespread use, was tested at a range of concentrations and did not inhibit HDAC activity at up to and including 50 µM (Figure 6-2), a concentration sufficiently high to impact cell viability (see section 4.4.1), and more than sufficient to reduce cellular cholesterol content (see section 4.4.2).

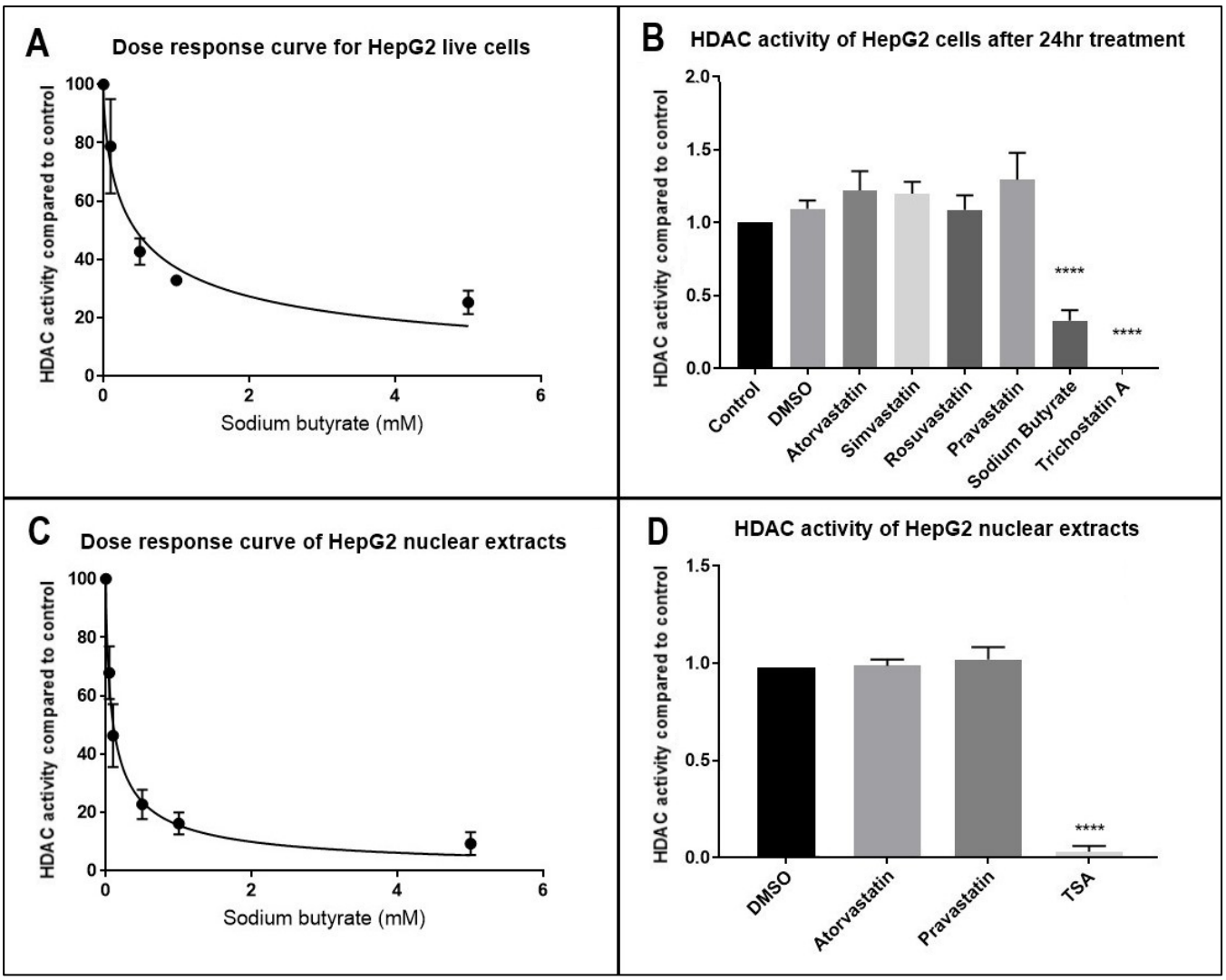

Figure 6-1 Influence of statins and butyrate on HDAC activity in HepG2 cells

HDAC activity was determined in live HepG2 cells as determined using the methods described in section 3.9.1 following 24 h treatment with A) varying concentrations of sodium butyrate and B) 10 µM statins, 5 mM sodium butyrate or 10 µM TSA. HDAC activity was also determined following direct application of C) varying concentrations of sodium butyrate and D) 100 µM statins or 10 µM TSA to HepG2 nuclear extracts. Results represent the mean combined from at least three independent experiments, with error bars representing SEMs. **** $p<0.0001$ compared to control. Analysed by non-linear regression (A and C) and ANOVA (B and D).

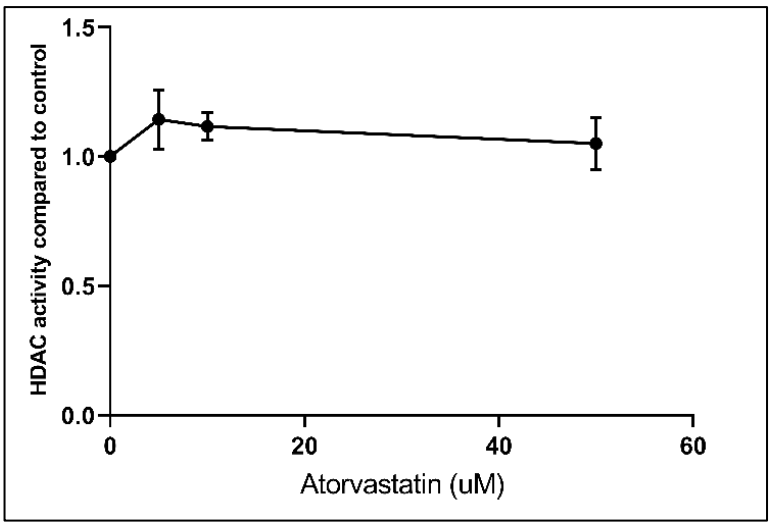

Figure 6-2 Influence of atorvastatin on HDAC activity in HepG2 cells

HDAC activity was determined in live HepG2 cells following 24 h treatment with varying concentrations of atorvastatin. Results represent the mean combined from three independent experiments, with error bars representing SEMs. Analysed by ANOVA.

As statins have been reported to directly inhibit HDAC activity in A549 lung carcinoma nuclear extracts,[145] we examined the direct effect of statins and sodium butyrate on HDAC activity in HepG2 nuclear extracts. Sodium butyrate inhibited HDAC activity with an IC_{50} of 104 µM (± 21 µM SE) (Figure 6-1C). Conversely, as in live cells, atorvastatin and pravastatin did not inhibit HDAC activity (Figure 6-1D). This was done with very high statin concentrations, 100 µM, a dose significantly higher than the 30 µM used by Lin et al.[145]

Furthermore, as previous studies of HDAC inhibition by statins were conducted primarily in the context of cancer, experiments were conducted using MDA-MB-231 breast carcinoma cells and found HDAC inhibition by sodium butyrate (IC_{50} of 403 µM) (Figure 6-3A). Experiments on live cells (Figure 6-3B) and on nuclear extracts showed no effect by statins (Figure 6-3C). Statins also failed to inhibit HDAC activity in live BRIN-BD11 insulin secreting cells, while sodium butyrate and TSA showed significant inhibition (Figure 6-3D).

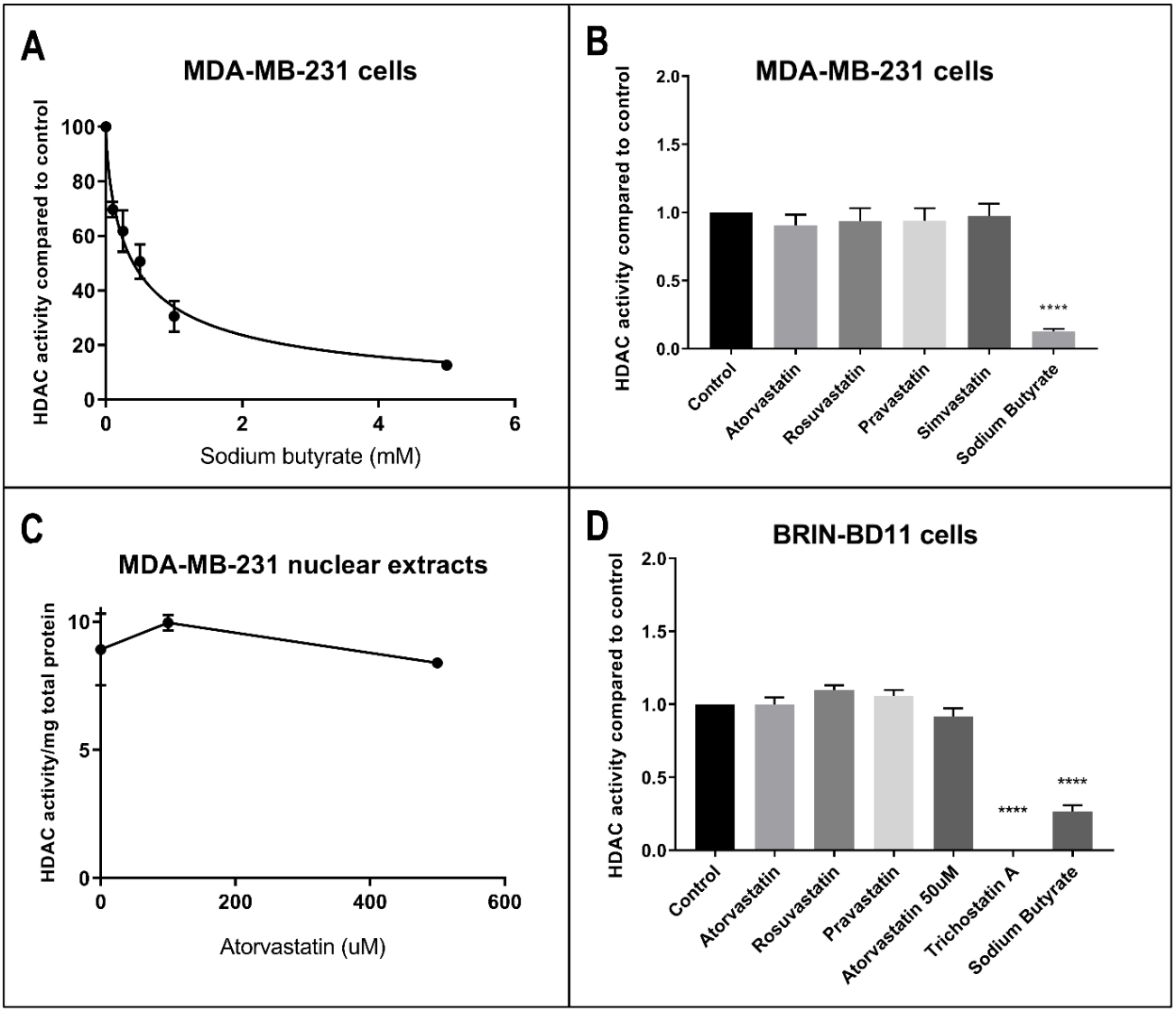

Figure 6-3 Influence of statins and butyrate on HDAC activity in MDA-MB-231 cells and BRIN-BD11 cells

HDAC activity was determined in A) live MDA-MB-231 cells following 24 h treatment with 10 µM statins or 5 mM sodium butyrate, B) live MDA-MB-231 cells following 24 h treatment with varying of sodium butyrate; C) MDA-MB-231 nuclear extracts exposed to varying concentrations of atorvastatin; D) BRIN-BD11 cells following 24 h treatment with 10 µM statins or trichostatin A, or 5 mM sodium butyrate. Results represent the mean combined from three independent experiments, with error bars representing SEMs. **** $p<0.0001$ compared to control. Analysed by ANOVA.

Additionally, experiments were conducted using the Fluor De Lys® HDAC fluorometric activity assay kit used in previous studies,[145,306,307] which again showed

HDAC inhibition with TSA and sodium butyrate but not statins in both HepG2 nuclear extracts and the HeLa nuclear extract provided with the kit (Figure 6-4).

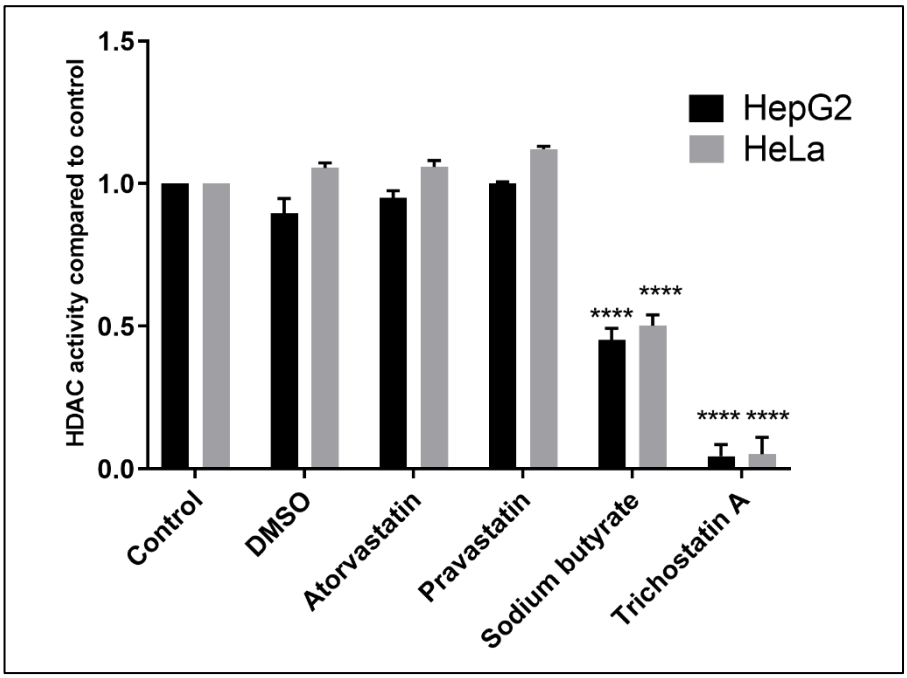

Figure 6-4 Influence of statins and HDAC inhibitors on HDAC activity using the Fluor De Lys HDAC activity kit

HDAC activity of HepG2 and HeLa nuclear extracts treated with 200 µM statins, 1 mM sodium butyrate or 4 µM TSA was determined using the Fluor De Lys HDAC activity kit (Enzo Life Sciences) as outlined in section 3.9.1. Results represent the mean combined from three independent experiments, with error bars representing SEMs. **** $p<0.0001$ compared to control. Analysed by ANOVA.

Finally, liver samples were utilised from a previous animal study in which male C57B1/6J mice were fed normal chow or high fat diet and treated with atorvastatin or water (vehicle control) for 12 weeks. HDAC activity was measured in nuclear extracts isolated from the liver of control animals fed a normal or HFD and from atorvastatin animals fed a normal or HFD. Neither diet nor treatment had a statistically significant effect on HDAC activity (Figure 6-5).

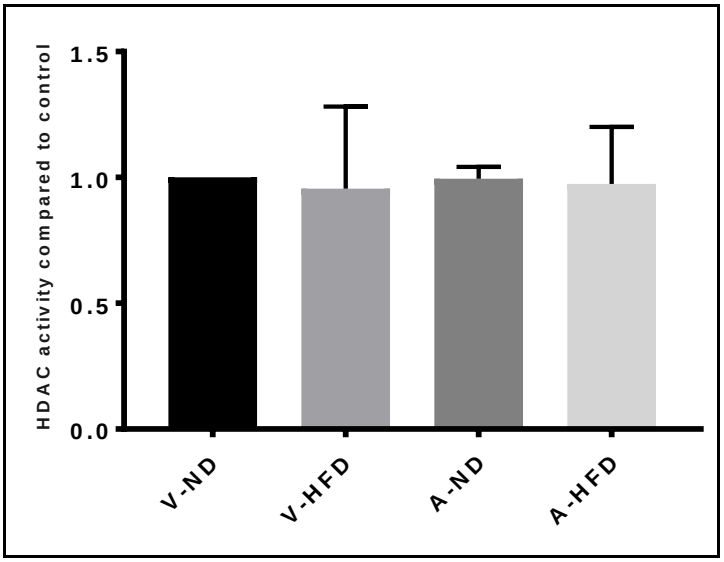

Figure 6-5 Influence of statin and diet on HDAC activity in mice livers

Mice were fed a normal diet (ND) or high fat diet (HFD) and treated with atorvastatin (A) or water (V) for 12 weeks (n=3 per group) as outlined in section 3.2.2 and HDAC activity was measured as described in section 3.9.1, with error bars representing SEMs. Analysed by ANOVA.

6.4.2. Statins do not inhibit HAT activity

In addition to HDAC inhibition, histone acetylation levels can be altered through influences on HATs. Curcumin was used as a control inhibitor and strongly inhibited the HAT activity of HepG2 nuclear extracts; 100 µM curcumin inhibited HAT activity by 70% (± 6% SE) (Figure 6-6). Conversely, high dose (100 µM) atorvastatin had no statistically significant effect on HAT activity.

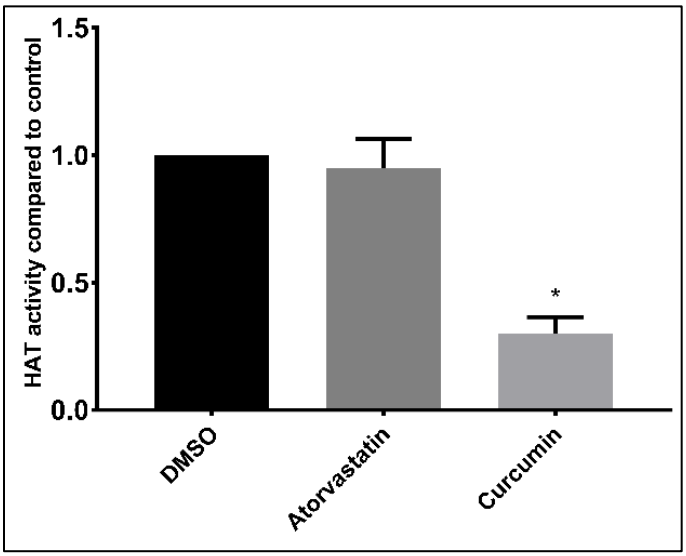

Figure 6-6 Influence of atorvastatin on HAT activity

100 µM curcumin, atorvastatin or DMSO vehicle control was applied to HepG2 nuclear extracts obtained using the method described in section 3.2.3, and HAT activity was measured using the

method described in section 3.9.2. Results represent the mean combined from three independent experiments, with error bars representing SEMs. * $p<0.05$. Analysed by ANOVA.

6.4.3. Sodium butyrate, but not statins, increase global histone acetylation

Global levels of histone modifications were determined by immunoblotting using antibodies against modified histones, namely against acetylated or trimethylated H3K9 and multiple acetylated residues of H4. H3K9 and H4 acetylation are commonly associated with open chromatin and gene expression,[308,309] while H3K9 trimethylation is associated with compact chromatin.[310] Protein levels of HDAC3 were also examined as this been implicated in the development of diabetes.[311] H3K9 and H4 acetylation was increased in a dose dependant manner by sodium butyrate treatment (Figure 6-7A, B). Conversely, acetylation levels were unchanged in cells treated with atorvastatin or curcumin. No treatment had significant effects on H3K9 trimethylation or on the levels of HDAC3 in HepG2 cells (Figure 6-7C, D). Similar results were seen in BRIN-BD11 cells (Supplementary Figure S4).

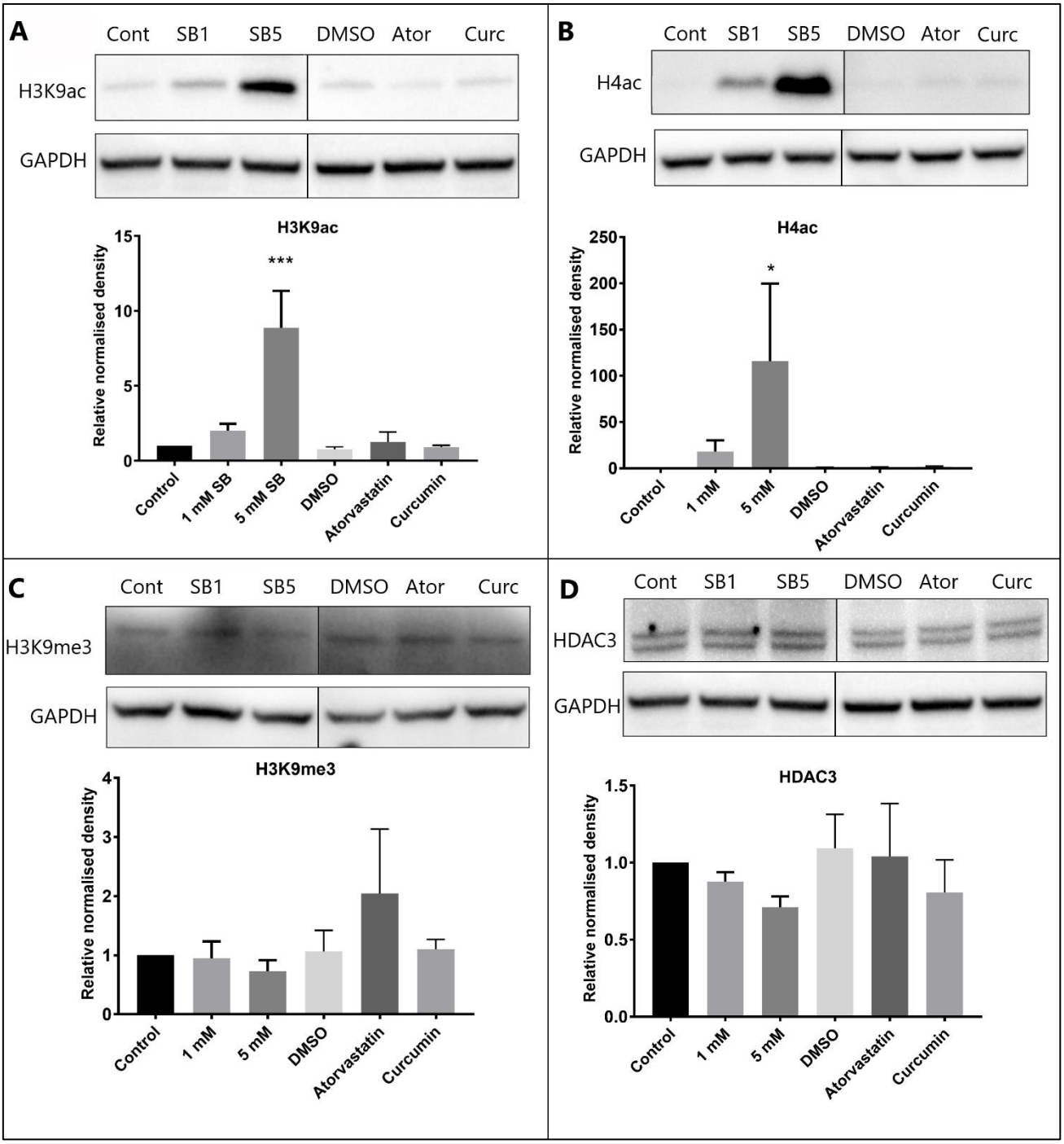

Figure 6-7 Influence of butyrate, atorvastatin and curcumin on global histone modifications in HepG2 cells

Relative protein levels of A) acetylated H3K9, B) acetylated H4, C) trimethylated H3K9 and D) HDAC3. Immunoblots obtained using the method described in section 3.5, using cell extracts obtained using RIPA buffer as described in section 3.2.4. The graph represents the mean combined density readings as normalised to GAPDH from at least three independent experiments, with error bars representing SEMs. * $p<0.05$, *** $p<0.001$. Analysed by ANOVA.

The bands for the acetylated histones were faint in control HepG2 and BRIN-BD11 cells. The untreated bands were denser from THP-1 macrophages, and this was further increased by sodium butyrate treatment (Figure 6-8). Atorvastatin did not alter histone acetylation in THP-1 macrophages.

In order to determine that the lack of lipoproteins in the serum did not affect results, H3K9 acetylation was also determined in cells treated in media containing high lipoprotein serum. As in LPDS, sodium butyrate significantly increased acetylation above baseline levels, whereas atorvastatin had no effect (Supplementary Figure S5).

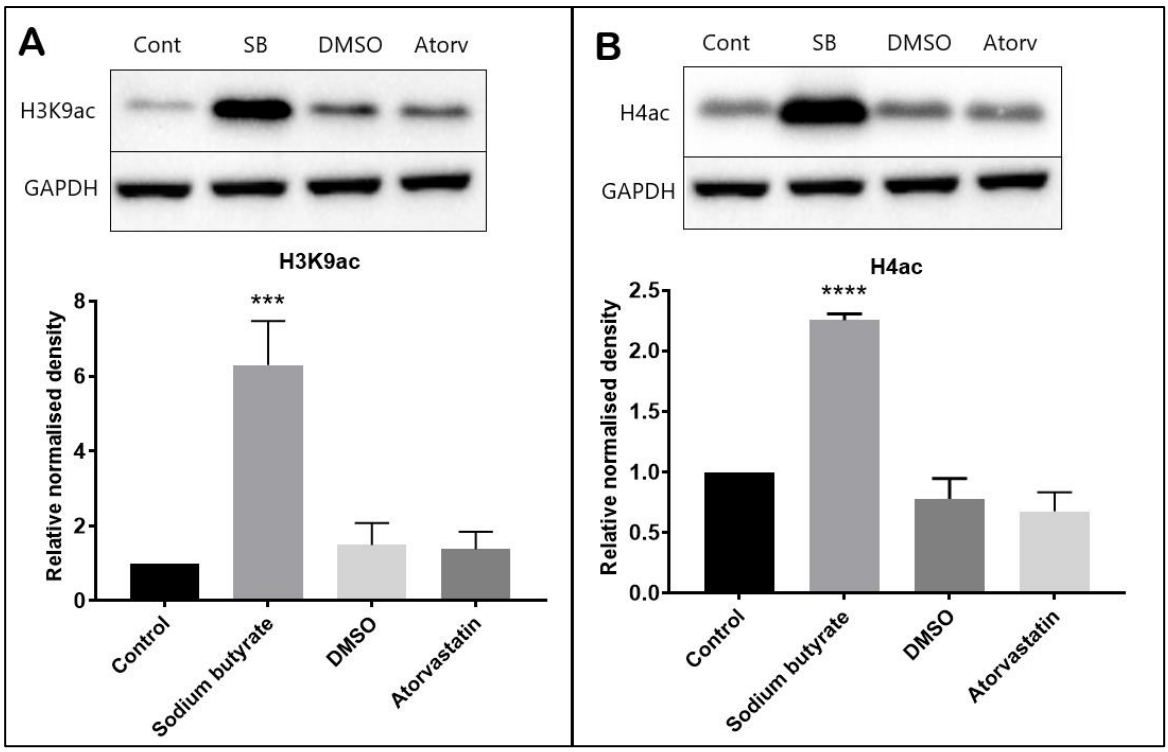

Figure 6-8 Influence of butyrate and atorvastatin on global histone modifications in THP-1 cells

Relative protein levels of A) acetylated H3K9 and B) acetylated H4. The graph represents the mean combined density readings as normalised to GAPDH from three independent experiments, with error bars representing SEMs. *** $p<0.001$, **** $p<0.0001$. Analysed by ANOVA.

6.4.4. Statins do not directly inhibit DNMT activity

Statins have also been reported to inhibit DNMT activity. When applied directly to whole cell extracts, the control DNMT inhibitor curcumin inhibited DNMT activity by 65% (± 8% SE) compared to the control (DMSO vehicle) (Figure 6-9A). This was significantly greater than an equal dose of the rationally designed DNMT inhibitor

RG108,[312] which inhibited DNMT activity by 25% (± 9% SE) and was nominally significant (p=0.057). 200 µM atorvastatin did not significantly inhibit, and in fact tended to increase, DNMT activity (p=0.064). Similar results were obtained with BRIN-BD11 cell extracts (Supplementary Figure S6A).

In a separate series of experiments, cells were treated for 24 h with atorvastatin or curcumin prior to extraction and subsequent measurement of DNMT activity. Activity was variable in these experiments but DNMT activity in atorvastatin cells was in fact higher than the DMSO vehicle control, although it did not differ significantly from untreated control cells (p=0.3) (Figure 6-9B). DNMT activity of cells treated with curcumin did not differ from those treated with DMSO. In BRIN-BD11 cells, there were no significant differences in DNMT activity between untreated, DMSO treated, atorvastatin treated and curcumin treated cells (Supplementary Figure S6B).

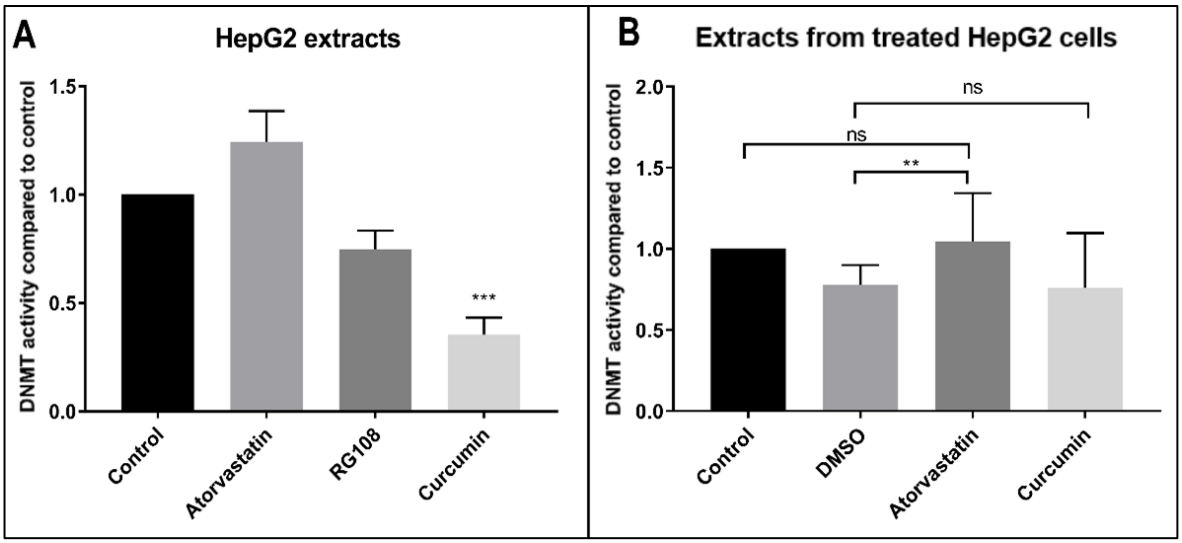

Figure 6-9 Influence of atorvastatin on DNMT activity in HepG2 cell extracts

A) DNMT activity in whole cell extracts exposed to 200 µM atorvastatin, RG108, curcumin or DMSO vehicle control; B) DNMT activity in whole cell extracts from cells treated for 24 h with 10 µM atorvastatin, curcumin or DMSO vehicle control. DNMT activity was measured using the method described in section 3.9.3, cell extracts were obtained using RIPA buffer as outlined in section 3.2.4. Results represent the mean combined from three independent experiments, with error bars representing SEMs. * p<0.05 *** p<0.001. Analysed by ANOVA.

6.4.5. Statins alter histone acetylation in specific genomic regions using ChIP-qPCR

Chromatin immunoprecipitation using antibodies against modified histones, namely acetylated and methylated H3K9 and acetylated H4K8, followed by qPCR was used to determine the effect of treatment on histone modifications in proximity to selected target genes. Data were normalised using the percentage of input method, where the qPCR signals are divided by signals from a portion of chromatin put aside before immunoprecipitation.[313] Treatment of HepG2 cells with 10 uM atorvastatin for 24 h significantly increased histone H4K8 acetylation upstream of the *HMGCR* transcription start site in proximity to a SREBP binding site, while non-significantly increasing H3K9 acetylation in this region (p=0.21) (Figure 6-10A). A similar pattern, though not statistically significant, was observed in proximity to fellow SREBP-2 targets, *LDLR* (p=0.10) (Figure 6-10B) and *PPARG* (p=0.21) (Figure 6-10C). When results from the three SREBP-2 target sites were combined, there was a significant increase in H4K8 acetylation (p=0.001) (Figure 6-10 Influence of atorvastatin on histone acetylation in proximity to specific genomic regionsF). There were no significant differences observed at the control gene *GAPDH* (Figure 6-10D), or in proximity to *ABCG1* (Figure 6-10E), a non-SREBP-2 target whose expression is decreased when cholesterol levels are low through LXR signalling. In these experiments, FBS was used instead of LPDS in both treated and control cells to reduce SREBP-2 signalling in control cells.

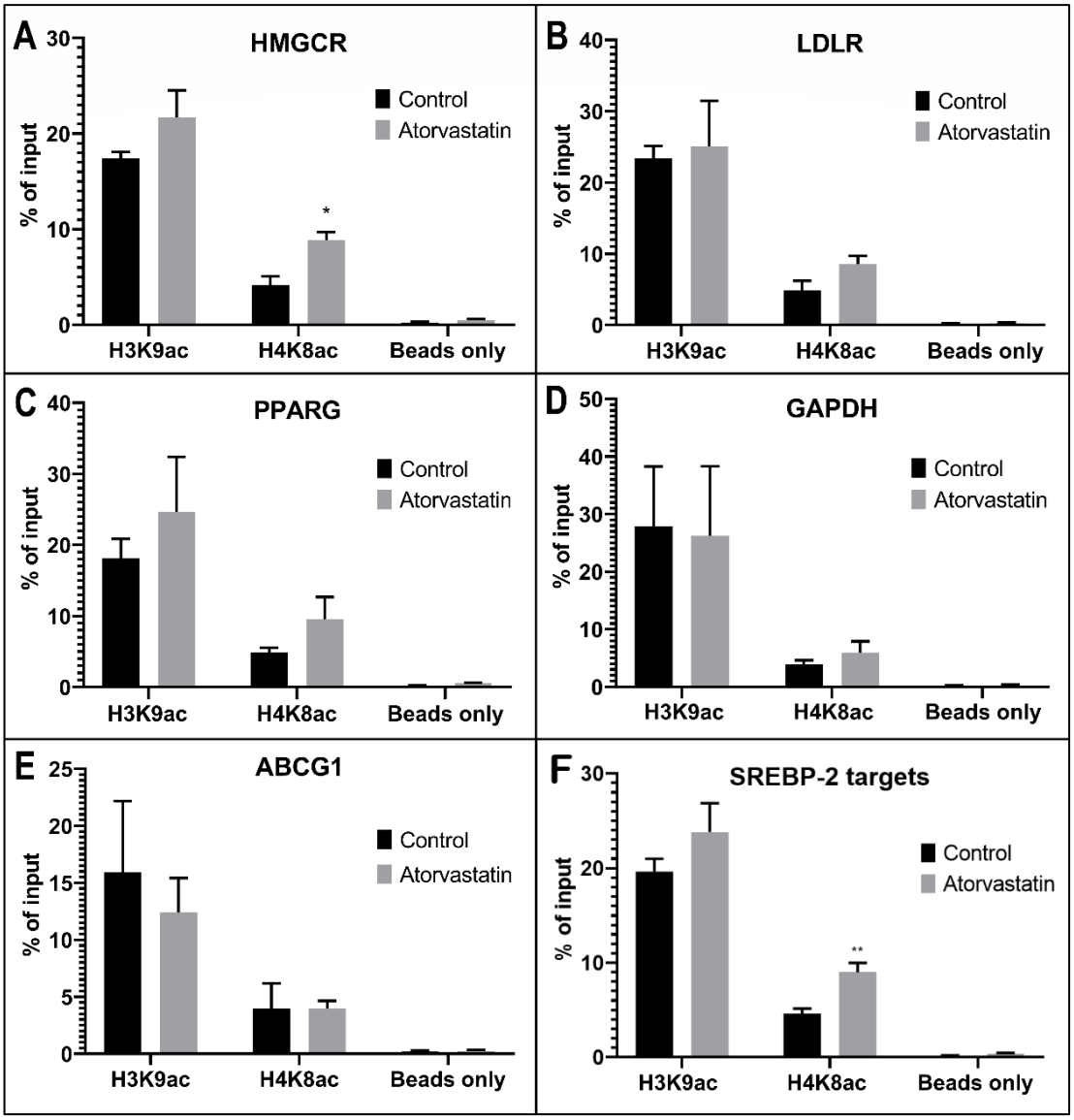

Figure 6-10 Influence of atorvastatin on histone acetylation in proximity to specific genomic regions

ChIP qPCR using the method described in section 3.10 from HepG2 cells treated with 10 μM atorvastatin or DMSO vehicle control for 24 h in media containing FBS in proximity to A) *HMGCR*, B) *LDLR*, C) *PPARG*, D) *GAPDH* or E) *ABCG1*, or F) the combined results from the SREBP-2 target sites in proximity to *HMGCR, LDLR* and *PPARG*. Results represent the mean combined from three independent experiments, with error bars representing SEMs. * $p<0.05$ Analysed by Student's *t* test.

There is no agreed upon method to normalise ChIP-qPCR data, although percentage of input DNA and fold enrichment are the most commonly cited.[313] Fold enrichment involves dividing signals by the background (beads only) signal, however the

background in these experiments was so low to be negligible. Other normalisation methods include relative to nucleosome density (total H3) or to a positive control sequence.[313] When normalised to either total H3 signal or to the positive control region of GAPDH (see section 6.4.6), the increase in H4K8 acetylation in proximity to *HMGCR* was still observed but no longer significant (p=0.29 and p=0.15 respectively), possibly due to increased standard error found using these methods (Supplementary Figure S7).

6.4.6. Butyrate increases ChIP signals and H4 acetylation at numerous genomic regions

Butyrate treated cells had higher histone H3 signals as a percentage of input DNA compared to control cells at all genomic regions examined (Figure 6-11A), including genes whose expression is upregulated, downregulated and unchanged by butyrate treatment (see section 5.4.5). This effect was not observed with atorvastatin treatment, where there were no significant differences between treated cells and DMSO control cells at any genomic region (Supplementary Figure S8) and was consistent across four independent experiments so is unlikely to be a result of technical error. As a result, signals for modified histones were similarly increased in butyrate treated cells when results from all genomic regions examined were combined (Figure 6-11B), a pattern that was also observed for each individual gene (Supplementary Figure S9). Similar results were observed with the raw data (Supplementary Figure S10).

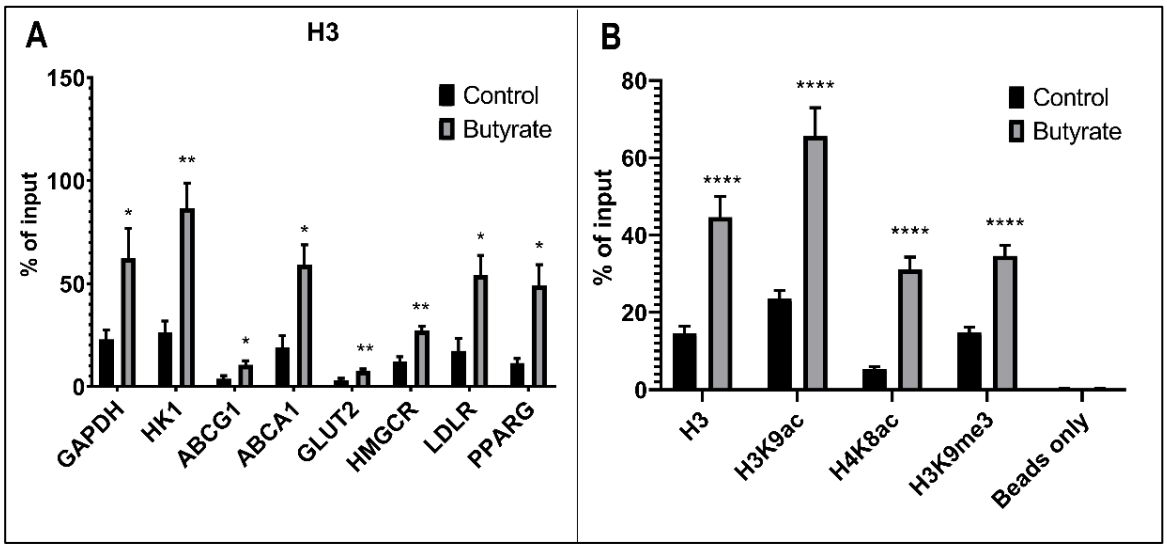

Figure 6-11 Influence of butyrate on histone signals in ChIP-qPCR

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS. A) ChIP qPCR H3 signals and B) combined ChIP qPCR signals from all genomic regions examined.

Results represent the mean combined from four independent experiments, with error bars representing SEMs. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. Analysed by Student's *t* test.

When signals were presented as a modified histone: total H3 ratio, only H4K8 acetylation remained elevated in butyrate treated cells when results from all genomic regions examined were combined (Figure 6-12). For individual genes, this increase in H4K8 acetylation was significant in proximity to *SLC2A2* (GLUT2) (Figure 6-13A) and *HMGCR* (Figure 6-13B). There were no statistically significant changes to histone modifications at any other genomic region when analysed in this manner (Supplementary Figure S11). The overall increase in H4K8 acetylation could be a real effect, as butyrate does increase global H4 acetylation as shown in section 6.4.3, but it could also be due to altered binding efficiency. Data were therefore also normalised to the average result from all regions examined. When analysed in this manner, the increase in H4K8 acetylation in proximity to *SLC2A2* (Figure 6-13C), but not *HMGCR* (Figure 6-13D), remained significant. There were no other statistically significant changes to histone modifications at any other genomic region when analysed in this manner (Supplementary Figure S12). Another method of normalisation is to use the signals of a positive control gene which is known to have high levels of histone acetylation.[313] A region of exon 2 of GAPDH was used for this purpose. When normalised to *GAPDH*, *SLC2A2* H4K8 acetylation was increased approximately three-fold in butyrate treated cells, however this was not significant due to interexperiment variability in the strength of the difference ($p=0.09$) (Supplementary Figure S13). No other histone modification at any genomic region examined had borderline significance ($p<0.2$). An increase in H4K8 acetylation could provide a mechanism for the increased *SLC2A2* expression observed in butyrate treated cells (see section 5.4.5).

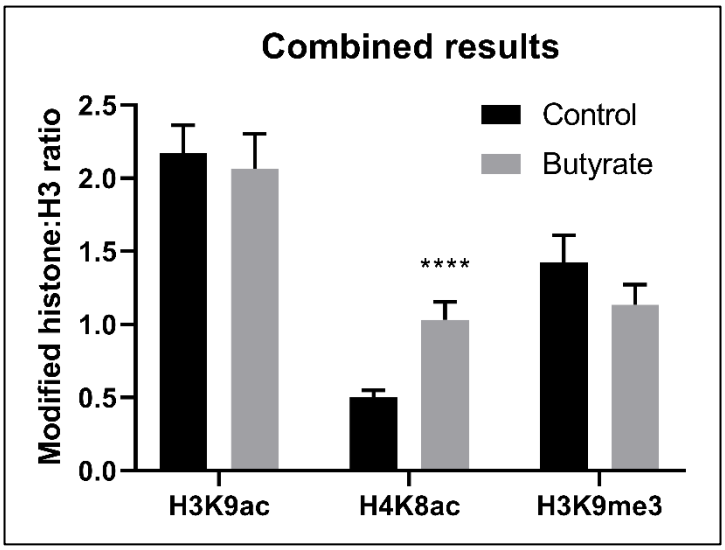

Figure 6-12 Influence of butyrate on histone signals in ChIP-qPCR relative to total H3

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS presented as a modified histone: total H3 ratio of combined ChIP qPCR signals from all genomic regions examined. Results represent the mean combined from four independent experiments, with error bars representing SEMs. **** $p<0.0001$. Analysed by Student's *t* test.

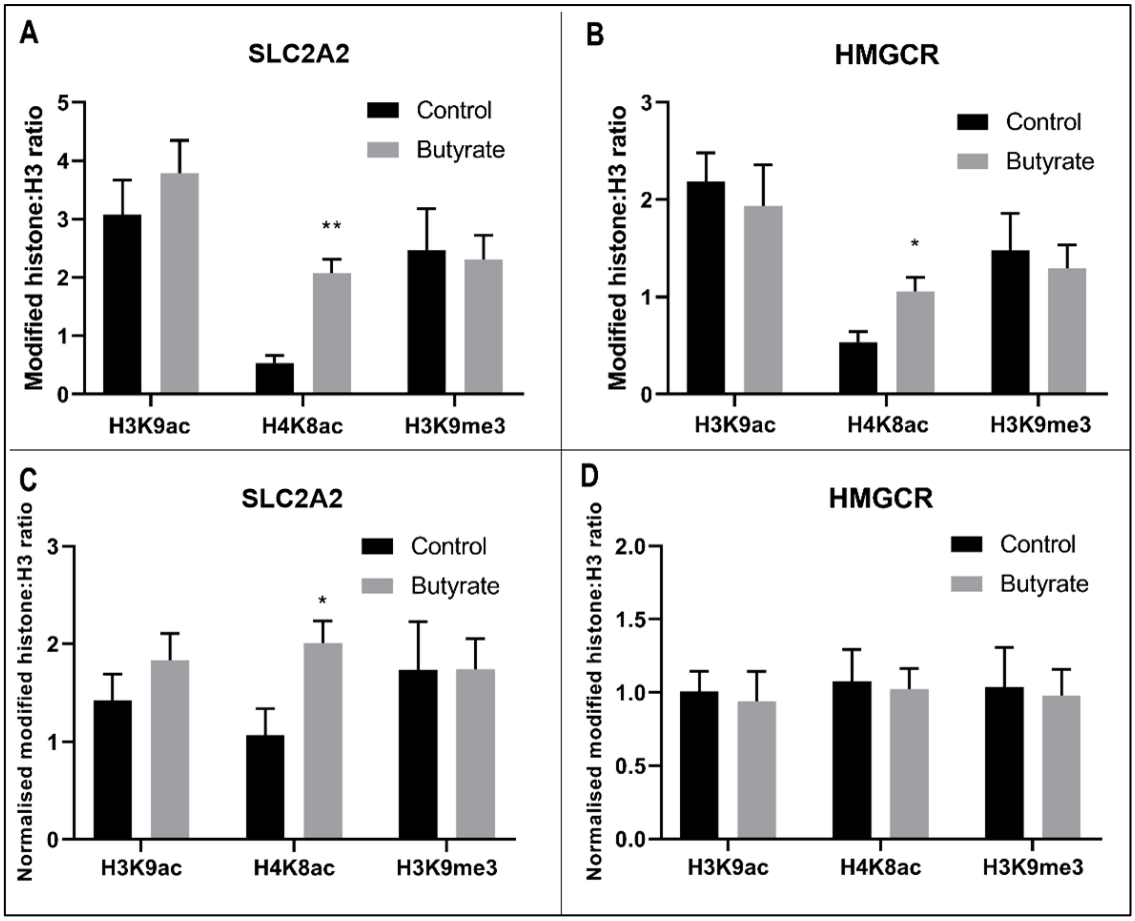

Figure 6-13 Influence of butyrate on histone acetylation in proximity to *SLC2A2* and *HMGCR*

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS presented as a modified histone: total H3 ratio of combined ChIP qPCR signals in proximity to A) *SLC2A2* (GLUT2) and B) *HMGCR*, and as normalised to average results from all genomic regions examined, in proximity to C) *SLC2A2* (GLUT2) and D) *HMGCR*. Results represent the mean combined from four independent experiments, with error bars representing SEMs. * $p<0.05$, ** $p<0.01$. Analysed by Student's *t* test.

6.5. Discussion

6.5.1. Sodium butyrate and curcumin inhibit epigenetic modifying enzymes

These results confirm the inhibitory effects of sodium butyrate and TSA on HDAC activity, and of curcumin on HAT and DNMT activity. Sodium butyrate is a well-established HDAC inhibitor with a reported IC_{50} values ranging from 90 µM in HT-29 nuclear extracts to 1.13 mM in live HeLa cells.[158] The results presented here fit into this range, with IC50s of ~100 µM in nuclear extracts and ~400 µM in live cells,

with the greater IC50 in live cells postulated to be a result of the cellular metabolism of butyrate over the 24 h treatment period.

Curcumin, the bright yellow compound in the spice turmeric, has purported benefits in a wide variety of disorders.[314] Research suggests that curcumin inhibits a variety of epigenetic modifying enzymes, including the HAT p300, HDACs 1, 4, 5, 6 and 8, and DNMTs 1, 3a and 3b.[1] Results confirmed a strong inhibitory effects on HAT and DNMT activity in cell extracts, but no significant change in DNMT activity in cells that had been treated with curcumin for 24 h. Furthermore, curcumin treatment did not result in a significant change in the acetylation of residues on histones H3 and H4 after 24 h. Curcumin is highly unstable in solution; Wang et al.[2] found 90% of curcumin degrades within 30 min in serum free media. This instability, combined with the poor bioavailability of curcumin in human trials; oral intake of 10 g or more results in serum concentrations of 50 ng/mL,[315] leads to uncertainty over the epigenetic effects of curcumin *in vivo*. Thus, curcumin was deemed unsuitable for use in experiments on cholesterol and glucose metabolism in cells.

6.5.2. Statins do not directly inhibit epigenetic modifying enzymes

As opposed to butyrate and curcumin, and contrary to the reported literature, statins failed to inhibit epigenetic enzymatic activity. Studies of various cell lines,[145-148] and a rabbit model of atherosclerosis[149] reported that statin treatment can cause hyperacetylation of histones H3 and/or H4. The literature suggests this may be due to inhibitory effects of statins on HDAC activity[306,307] and expression.[146] The results presented here on HDAC activity in live cells differ from those reported in the literature. This was demonstrated on a variety of different cells lines, namely HepG2, MDA-MB-231 and BRIN-BD11 cells, in addition to livers of mice treated with statins for 12 weeks, leading us to conclude that neither acute nor chronic statin treatment alters cellular HDAC activity. After preliminary experiments with live Hep-G2 and BRIN-BD11 cells failed to find HDAC inhibition, we hypothesised that in live cells, the statins may be unable to enter the nucleus, or some other element of cellular activity prevents them from interacting with HDAC proteins, as studies by other groups were conducted either using recombinant HDAC proteins[306] or cell extracts.[145,307] As a result, we conducted experiments using nuclear extracts, which again failed to show HDAC inhibition by statins. The negative results of these experiments are unlikely to be due to an inadequate dose as concentrations of up to

500 µM statins were used in the nuclear extract experiments. This concentration is significantly higher than the 10 µM doses reported to inhibit HDAC activity by Lin et al.[145] and well above the 1-15 nM serum concentration of statin users.[316] Furthermore, cholesterol assays (see section 4.4.2) showed that the statins did lower cellular cholesterol, indicating that the statins are able to enter the cells and inhibit HMG-CoA reductase. The difference could also be due to the different cell types (A549 lung carcinoma cells and primary glomerular mesangial cells) and HDAC substrates used (all reported studies used the Fluor-de-Lys™ HDAC Activity Assay Kit [Enzo Life Sciences]). Because the majority of studies reporting epigenetic effects have been conducted in the context of cancer, we used the aggressive breast cancer cell line MDA-MB-231 and found no effect in either live cells or nuclear extracts. We also obtained the Fluor-de-Lys™ assay kit and found no effect with statins on HDAC activity in HepG2 or HeLa nuclear extracts. The accuracy of using fluorogenic substrates to measure HDAC activity has been questioned and the Fluor-de-Lys™ substrate in particular tends to overestimate inhibition of HDAC1 and HDAC6 while failing to detect inhibition of HDAC8.[317] Similar experiments have not been reported for the cell permeable substrate used in the Sigma-Aldrich assay kit.

One potential contributor to the lack of an effect is the form of statins used. In the body, statins are present in both an active acid form and in a lactone form; in plasma, about 50% of atorvastatin and simvastatin are present in lactone form, whereas less than 10% of pravastatin, rosuvastatin and fluvastatin are present as lactones at any time.[318,319] Lactone statins are 1000-2000 times more lipophilic than their acid counterpoints and may be more important in side effects including muscle toxicity[318] and drug interactions.[320] The study by Lin et al.[145] did not state which form of statin they used. Acid salt statins were used in the experiments presented here as they are the active form, and of the statins used, only simvastatin is administered in the lactone form. Interestingly, in PBS and DMEM, used here in the nuclear extract and cell-based experiments respectively, it has been shown that lactone statins are largely converted to the acid form but not vice-versa.[321] The interconversion of statins by HepG2 cells does not appear to have been reported.

The lack of HDAC inhibition *in vitro* is supported by the immunoassay results showing no increase in acetylation of histone H3K9 or multiple residues of histone H4. In fact, despite reporting that lovastatin and atorvastatin directly inhibited the activity of recombinant HDAC1, HDAC2 and HDAC6 proteins with IC_{50} values less

than 30 µM, Chen et al.[306] failed to show increased histone H3 acetylation following 24 h treatment with 30-50 µM lovastatin, although Lin et al.[145] did report increased H3 acetylation following 16 h of treatment with 10 µM statins (lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin). Furthermore, although Lüthje et al.[148] reported increased H3 acetylation in telomerase-immortalised uroepithelial cells (TERT-NHUC) treated with 1 nM simvastatin for 24 h, this effect was marginal and there was no effect at 10 nM, nor with 1-10 nM atorvastatin or in simvastatin-treated HEKa keratinocytes.

Similarly, the results presented here failed to find an inhibitory effect of atorvastatin on DNMT activity, either in cells that had been treated for 24 h or when applied directly to cell extracts; in contrast, the opposite was found with an increase in DNMT activity in extracts from HepG2 cells treated with statins for 24 h. Conversely, Kodach et al.[152] reported reduced DNMT activity in extracts from lovastatin treated HCT116 colon cancer cells. Several differences in experimental design between our study and theirs may possibly account for the disparate results. Firstly, they used lovastatin, which we chose not to use. Instead, we chose to use atorvastatin as our exemplar statin as it was the most commonly prescribed drug in Australia.[5] Additionally, they used a different cell type and a longer treatment time, 48 h versus 24 h. As they did not investigate if lovastatin directly inhibited DNMT activity, it cannot be ruled out that the observed decrease in activity is a result of changed phenotype, as they did observe increased differentiation and reduced 'stemness' of the cancer cells. The differences between HepG2 hepatoma cells and HCT116 colon cancer cells may therefore account for the different results. Finally, different commercial kits were used, although both relied on similar ELISA type principles. Unfortunately, there is no current protocol to measure DNMT activity in live cells as there is for HDAC activity, a limitation of both studies as how enzymes behave in cell-free extracts may not reflect their actual activity in live cells.

The effect of statins on HAT activity has not been previously reported. As with HDAC and DNMT activity, results presented here failed to find a direct effect of atorvastatin on HAT activity in HepG2 cell extracts. Thus, the results indicate that statins are unlikely to directly inhibit the three major classes of epigenetic modifying enzymes.

6.5.3. Statins may impact epigenetic modifications in specific genomic regions

Despite having no direct effect on epigenetic modifying enzymes nor increasing global histone H3K9 or H4 acetylation, atorvastatin significantly increased histone H4K8 acetylation in proximity to the SREBP-2 binding site of the *HMGCR* gene. This is most likely not due to a direct epigenetic effect of atorvastatin, but an indirect effect of cholesterol lowering and subsequent SREBP-2 activation, as similar, though non-significant, effects were found in proximity to the SREBP-2 binding sites of *LDLR* and *PPARG*, while no effects were noted in proximity to non-SREBP targets *GAPDH* and *ABCG1*. Specifically, the literature suggests that SREBP-2 recruits the HATs p300 and CBP to SREBP-2 target sites in proximity to genes such as *HMGCR*, increasing histone acetylation at these sites and thereby activating gene expression (Figure 6-14). Oliner et al.[322] found that SREBP-2 interacts with p300 and CBP, and that CBP enhances the transcriptional activity of SREBP-2 on a reporter construct containing the *LDLR* promoter. Furthermore, in a study of Chinese hamster ovary CHO-7 cells incubated in media containing LPDS with or without cholesterol supplementation, cells in cholesterol deficient media displayed increased H3 acetylation in proximity to the *HMGCR* and *LDLR* promoters compared to the cholesterol supplemented cells.[111] Neither of these studies used statins to activate SREBP-2, this finding of an epigenetic effect of statin treatment is thus a novel finding.

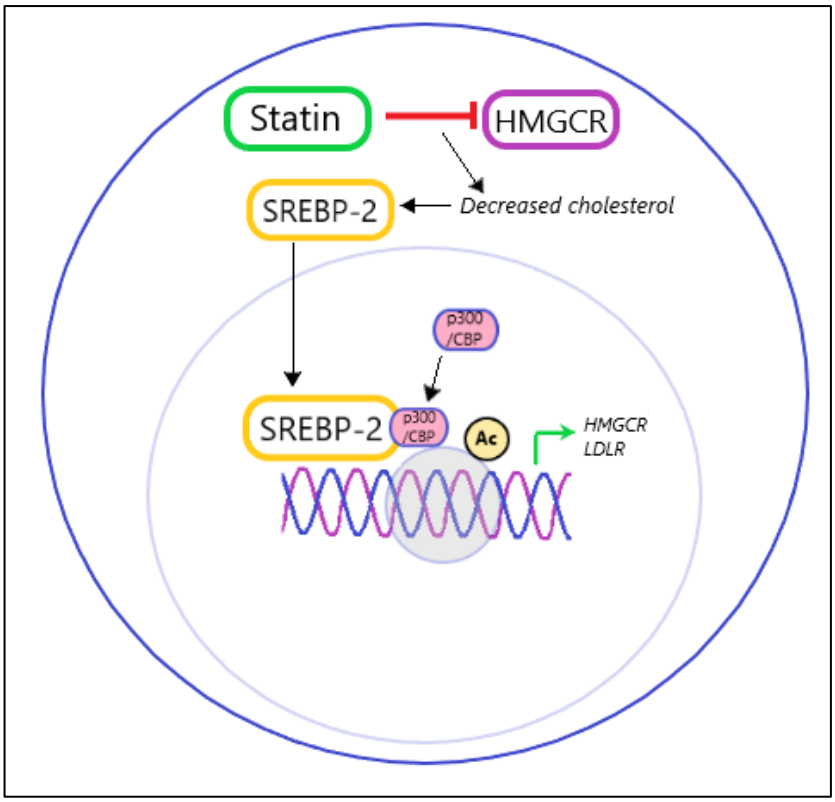

Figure 6-14 Postulated mechanism by which statins may impact histone acetylation

Statins inhibit HMGCR, leading to decreased cellular cholesterol. This leads to the activation of SREBP-2, which recruits p300/CBP to SREBP-2 target sites in the promoter regions of genes such as *HMGCR* and *LDLR*, increasing histone acetylation and activating gene expression.

6.5.4. Results of ChIP-qPCR experiments of butyrate treated cells depend on analysis and normalisation methods

The results of this study outline some of the difficulties in interpreting ChIP-qPCR data, particularly with treatments that may cause widespread changes to chromatin. The most widely used methods are the percentage input method and the fold enrichment method, with other methods including relative to nucleosome density and relative to control sequences.[313,323,324] The fold enrichment method divides the ChIP signal by the 'beads only' signal. As the 'beads only' signal was uniformly low (0.30% for control cells and 0.29% for butyrate treated cells), this method is unsuitable for this data as any small technical variation may result in large differences in the transformed data that would be unlikely to reflect real differences.[313] In the percentage input method (i.e. relative to total DNA), the signals of the immunoprecipitated samples are divided by the signals from a portion of chromatin put aside before the ChIP reaction and is used in the majority of ChIP-qPCR

experiments. In context of histone modifications this method assumes equal nucleosome density, which was the case for the atorvastatin ChIP experiments but not the butyrate experiments, which had uniformly higher H3 signals than the control, and subsequently higher signals of all modified histones. Similar results were found with the raw data. For this reason, ChIP signals were also normalised relative to nucleosome density (total H3). This method removed the differences between control and butyrate treatment for H3K9 acetylation and trimethylation, while H4K8 acetylation remained significantly higher for butyrate treated cells, significant for *SLC2A2* (GLUT2) and *HMGCR*. This may be a real effect, as butyrate does increase global H4 acetylation (see section 6.4.3), but it may be an effect of differential antibody binding efficiency. Another normalisation method is relative to a control gene, in this case *GAPDH*. An advantage of this technique is that it eliminates technical error, in that it directly compares the same sample whereas other methods compare different antibodies or controls which are handled separately following chromatin isolation.[313] This method assumes that the treatment has no effect on histone modifications at the control gene, which may not be the case with broad epigenetic modifiers, such as HDAC inhibitors, that drastically alter global histone acetylation. As a result, data were also normalised to average values, where the signal was divided by the average signal of that antibody across all genomic regions. With these methods, the increase in H4K8 acetylation at *SLC2A2* remained (p=0.09 and p=0.03 for the control sequence and average value method respectively), while there was no difference in H4K8 acetylation at *HMGCR* (p=0.45 and p=0.85). Thus, an increase in H4K8 acetylation in proximity to *SLC2A2* is the only histone modification observed consistently across different normalisation methods. Of course, the use of multiple normalisation methods does increase uncertainty and it's possible some real effects have been masked.

The H3 antibody used in these experiments (ab1791) is considered the gold standard for epigenomic experiments[325] and immunoblotting with this antibody found that butyrate had no effect on overall histone H3 levels (Supplementary Figure S14). Increased H3 occupancy is expected to correlate with inaccessible chromatin,[325] however, the increased H3 signal was seen in all genomic regions tested, including in the proximity of *HK1* and *ABCG1*, both of which were upregulated more than 100-fold by butyrate (see section 5.4.5), and thus is unlikely to be a result of chromatin compaction. It may be that instead butyrate relaxed chromatin compaction and thus increases the availability of histones to the antibodies. Genome-wide experimental

methods such as ChIP-seq and Assay of Transposase Accessible Chromatin sequencing (ATAC-seq), which maps accessible chromatin,[326] would be useful to determine if this increased H3 signal occurs across the genome and how it correlates with chromatin compaction.

The increase in H4K8 acetylation in proximity to *SLC2A2* with butyrate treatment is a novel finding. *SLC2A2* gene expression has previously been shown to be upregulated by butyrate in intestinal cells *in vitro*[327] and *in vivo*,[328] however, these studies did not examine histone modifications. Although the increase in *SLC2A2* gene expression here did not result in a noticeable increase in GLUT2 protein levels (see section 5.4.4), butyrate has been reported to increase GLUT2 protein levels in the livers of HFD fed rodents[118,211] and in insulin-resistant HepG2 cells.[118] Whether this epigenetic modification results in a phenotypic change, i.e., increased glucose uptake, remains an area of further research.

6.6. Conclusion

In contrast to the published literature, the results presented here indicate that statins are unlikely to inhibit the three major classes of epigenetic modifying enzymes, namely HDACs, HATs, and DNMTs, and in fact may increase DNMT activity. This represents the most comprehensive study of HDAC activity, using multiple cell types, in both live cells and nuclear extracts and using two different HDAC substrates to demonstrate no effect with statin treatment. While our *in vitro* studies indicate it is unlikely that statins directly inhibit these epigenetic enzymes, it is feasible that through effects on cell metabolism and/or phenotype, statins may have indirect effects on specific genomic regions. In fact, while the experiments on the livers of statin treated mice would also seem to rule out effects of prolonged treatment on HDAC activity, direct or otherwise, atorvastatin did increase H4K8 acetylation in proximity to the SREBP-2 binding site of the *HMGCR* gene. It is likely that while statins do not directly impact epigenetic changes, they instead indirectly impact histone modifications in proximity to SREBP-2 target genes as a result of their cholesterol lowering activity, the activation of SREBP-2 and the subsequent recruitment of HATs to SREBP-2 target sites.

Conversely, butyrate greatly inhibited HDAC activity and increased global H3K9 and H4 acetylation in all experiments. The noted differences in gene expression in Chapters 4 and 5 reflect these differences in epigenetic mechanisms, with butyrate

impacting the expression of many genes involved in different pathways, presumably due to direct effects of HDAC inhibition on the genes and proteins themselves, while all the noted effects of atorvastatin, including the epigenetic effect described in this chapter, seem to occur as a result of cellular cholesterol lowering, rather than a direct effect of the statin itself, and hence are more targeted towards cholesterol metabolism pathways. However, the specific effects of butyrate at the gene level remain unclear as ChIP results varied significantly with different normalisation methods. It is likely that butyrate increases *SLC2A2* expression by increasing H4K8 acetylation, presumably as a result of HDAC inhibition, a finding that has not been previously reported.

Chapter 7. Concluding remarks

This thesis is the first study to compare the effects of the statin class of cholesterol lowering drugs, which had previously been reported to inhibit HDACs, and HDAC inhibitors, which have been reported to alter cholesterol metabolism, on epigenetic modifications, cholesterol metabolism and glucose metabolism. This includes the most comprehensive examination of the epigenetic effects of statins yet conducted as well as significantly expanding on the existing literature on the effects of HDAC inhibitors on cholesterol and glucose metabolism *in vitro*.

The path taken by this thesis was driven by the data, firstly that in literature and then by the findings of preliminary experiments. As discussed in Allen and Mamotte[28] and in section 2.5.3, several studies had suggested statins have epigenetic effects, most notably HDAC inhibition, but none were conducted in the context of lipid disorders or diabetes, which are areas of interest for our laboratory group. Thus, the original plan was to explore how epigenetics may contribute to the diabetogenic effects of statins. Preliminary experiments showed that statins had no effect on HDAC activity, while butyrate, originally used as a control HDAC inhibitor, was shown to lower cellular cholesterol to a similar extent as the statins, leading us to examine the potential for butyrate to treat or prevent lipid disorders and diabetes, as reviewed in Bridgeman et al.[163] Metformin, a very widely used anti-diabetic drug, was also briefly investigated as it has been reported to have numerous epigenetic effects, as reviewed in Bridgeman et al.[77] Preliminary experiments with butyrate showed that the HDAC inhibitor had widespread effects worthy of further investigation and perhaps it would be more important to include another HDAC inhibitor, namely trichostatin A, rather than an unrelated extra treatment like metformin. The scope of the study was thus to compare and contrast the effects of statins and established HDAC inhibitors, primarily butyrate, on epigenetic modifications, cholesterol metabolism and insulin signalling and secretion. This is reflected in the literature review (Chapter 2), which evolved to include not only a thorough review of the epigenetic effects of statins, but contralaterally the potential beneficial effects of butyrate on lipid metabolism and the metabolic syndrome. The emphasis in the experimental chapters was thus on how butyrate influences cholesterol metabolism and how statins influence epigenetic modifications, as well as how both treatments may impact β cell function and potentially diabetes risk.

Possibly the most significant novel finding was that butyrate lowers cellular cholesterol content in both a hepatic and an insulin-secreting cell line and that this is most likely due to its actions as an HDAC inhibitor, since the potent HDAC inhibitor TSA also reduced cholesterol content, as outlined in Chapter 4. In particular, the data suggests that HDAC inhibition lowers cellular cholesterol by inhibiting SREBP-2 activity in a time-dependant manner, without altering *SREBF2* mRNA expression. The mechanism by which butyrate alters SREBP-2 activity is currently unknown and is under investigation by our group, specifically by looking at the localisation and acetylation of the SREBP-2 protein. As a result of the inhibition of SREBP-2 activity and subsequent reduced LDLR expression, butyrate did not increase cellular cholesterol uptake whereas statins did, and thus butyrate may not be able to lower plasma LDL-C *in vivo* as statins do. This chapter also explored how butyrate and atorvastatin affect proteins involved in reverse cholesterol transport (ABCA1, ABCG1, SRB1), with results differing according to cell type and amount of lipoprotein in the serum, making it difficult to speculate on how these treatments may impact reverse cholesterol transport *in vivo*.

An interesting finding regarding β cell insulin secretion, described in Chapter 5, is that substantial HDAC inhibition (5 mM butyrate or 2.5 μM TSA x 24 h) impairs insulin secretion while acute or low dose (1 mM) sodium butyrate enhances insulin secretion stimulated by high glucose and alanine. HDAC inhibition reduced both 24 h insulin secretion in cell media and the insulin content of cells; this was significant for 5 mM butyrate and TSA. The impaired β cell function could be explained by the noted upregulation of hexokinase I, which would interfere with glucose sensing at both low and high glucose, and of *TXNIP*, which would increase oxidative stress. At the same time, the data presented in this chapter suggests that statin-induced β cell dysfunction is a direct result of cellular cholesterol lowering rather than an off-target effect, as all genes linked to β cell function and diabetes with statin-altered expression are regulated directly by transcription factors whose activity is dependent on cellular cholesterol levels.

In addition to β cell insulin secretion, Chapter 5 also examined insulin signalling in hepatic cells, finding that butyrate reduced AKT phosphorylation while tending to increase unstimulated IRS-1 phosphorylation. Interestingly, both of these findings can be explained by increased acetylation of the respective proteins, as the literature suggests that acetylation of IRS-1 increases its phosphorylation while acetylation of

AKT inhibits its ability to be phosphorylated. Similarly, the literature suggests other proteins in the insulin signalling pathway can be acetylated and whether acetylation acts to increase or decrease insulin signalling varies from protein to protein. As a result, it is difficult to speculate how HDAC inhibition may impact insulin sensitivity.

Contrary to the published literature, summarised in Allen and Mamotte,[28] the findings in Chapter 6 demonstrate that statins do not directly inhibit the activity of epigenetic modifying enzymes, namely HDACs, HATs and DNMTs, or increase global histone acetylation. Statins may however alter histone acetylation at certain genomic sites, namely SREBP-2 target sites, through the activation of SREBP-2 and its subsequent recruitment of histone acetyltransferases, reflected in the finding that atorvastatin significantly increased H4K8 acetylation in proximity to the SREBP-2 target site in *HMGCR*. Unfortunately, the effects of butyrate on histone acetylation at target genes varied greatly depending on the normalisation method used. It does seem that butyrate upregulates *SLC2A2* (GLUT2) gene expression by increasing H4K8 acetylation in its vicinity, a finding that has not previously been reported despite other studies finding that butyrate upregulates GLUT2 expression and may consequently increase glucose uptake.

7.1. Future directions and perspectives

In research, there are always numerous further experiments that could be conducted as one set of results opens many rabbit holes that one could dive into. To start, experiments that ideally would have been included in this thesis but were not due to time and budgetary constraints include the use of primary cells rather than cell lines, ChIP analysis of BRIN-BD11 cells and bisulfite sequencing to examine changes in DNA methylation. Methods to assess the effect of HDAC inhibition on genome-wide chromatin changes such ChIP-seq and ATAC-seq would be ideal to resolve the uncertainty regarding the ChIP-qPCR results of butyrate treated cells. Repeating experiments with additional statins, particularly the hydrophilic rosuvastatin and pravastatin, would help elucidate if the findings with atorvastatin can apply to statins in general. Due to the often-opposing effects of atorvastatin and butyrate, for example on SREBP-2 signalling, it would also be interesting to conduct more experiments using HDAC inhibitor and statin co-treatments; this may have important clinical implications due to the high percentage of people with metabolic disorders already using statins.

Other experiments could be used to further support the hypotheses ascertained from the results. The hypothesis that statins indirectly alter histone acetylation through SREBP-2 recruitment of HATs to target genes could be explored by statin treatment of SREBP-2 knock-out cells and by co-treating with the p300/CBP HAT inhibitor A-485.[329] Whether butyrate alters non-histone acetylation of SREBP-2, IRS-1 and AKT could be determined using immunoprecipitation with antibodies against the protein in question followed by immunoblotting with anti-acetyl lysine antibodies.[112,302] The importance of ABCG1 in the impairment of insulin secretion by statins could be examined by statin treatment of ABCG1 knock-out and constitutively expressed cells and by co-treating with LXR agonists such as T0901317.[330]

Future animal models could be designed to ascertain whether the systemic effects of butyrate *in vivo* are a result of HDAC inhibition or effects on intestinal cells such as GLP-1 secretion. This could be accomplished by giving one group of HFD fed animals oral butyrate and another intravenous butyrate at a dose that gives comparable butyrate concentrations in peripheral circulation and comparing their metabolic parameters.

On a broader note, there is a need for well-designed placebo-controlled clinical trials for the use of butyrate for dyslipidaemia and T2D. The only studies reported so far lack statistically significant results, possibly due to their small sample sizes.[6,7,166] It may be that the uptake of butyrate is not sufficient to exert significant effects on non-intestinal tissues. For this reason, butyrate pro-drugs designed to improve the cellular uptake of butyrate have been developed. Of these, AN-9 (pivaloyloxymethyl butyrate, also known as Pivanex)[331] and tributyrin (a triglyceride with three esterified butyrate molecules)[332,333] have reached clinical trials, but in a very different context, namely for treatment of cancers. Interestingly, these trials reported hyperglycaemia as an adverse effect, although as they were not placebo-controlled it is unknown if this is an actual side effect. Nevertheless, it is in line with the results in section 5.4.1 suggesting that high-dose HDAC inhibition impairs β cell function. Further studies, including animal models, at appropriate doses are needed to ascertain if these pro-drugs may be useful in metabolic disorders.

Due to the wide-ranging effects of epigenetic modifying enzymes on gene expression across the genome, the future of altering epigenetic modifications for therapeutic reasons may not lie with altering the activity of these enzymes but instead in targeting epigenetic modifications at specific genomic regions. One approach is using a

modified CRISPR-Cas9 system, in which the Cas9 nuclease is deactivated (dCas9) and fused to an epigenetic modifying enzyme.[334] Hilton et al.[335] fused the HAT p300 to CRISPR-dCas9 to selectively activate numerous genes using appropriate guide RNAs in live cells and a similar approach using DNMT3a has been used to selectively silence genes.[336] While this technology has a long way to go, it has proven effective in animal models. In a mouse model, a transactivation complex designed to selectively target *Pdx1* increased histone acetylation at *Pdx1* and partially ameliorated STZ-induced diabetes.[337] The possibilities are intriguing. For example, could we see a future where statins users at risk of T2D have *ABCG1* epigenetically activated by CRISPR-dCas9 technology?

7.2. Limitations

Any research project will have its limitations, and the work in this thesis is no exception. In particular, the findings outlined in the thesis are limited by the fact that, with the exception of HDAC activity in the livers of statin-treated mice, all experiments were performed on cell lines either derived from human cancers (HepG2, THP-1, MDA-MB-231) or from the electrofusion of rat pancreatic islet cells and the insulinoma cell line RINm5F (BRIN-BD11).[338] Thus by definition these are not normal hepatocytes, macrophages or β cells. Ideally results would be validated by the use of additional cell lines or primary cells. It was decided that rather than using multiple cell types of the same origin tissue, we would obtain a wider picture by using cell types from different tissues that are important in dyslipidaemia and/or T2D.

Furthermore, like the vast majority of *in vitro* studies, the doses used in experiments are supraphysiological compared to concentrations reported in human serum. For butyrate, average concentrations of close to 100 μM have were reported in the portal vein following butyrate enemas;[277] the concentrations used in this study were 10 to 50-fold higher. For statins, the mean concentrations in serum of therapeutic doses is 1-15 nM,[316] around 1000 fold less than the concentrations used in this and most other *in vitro* studies. It is thus unknown how relevant the novel findings of this thesis are to the use of statins and butyrate as human therapeutics.

As mentioned in section 1.4, this study was designed to be mechanistic in nature and thus relied on *in vitro* experiments, as there have been numerous *in vivo* studies on the effects of butyrate and statins on metabolic disorders but the majority lack evidence on the exacting mechanisms. In particular, for butyrate it is unknown what

proportion of the noted phenotypic changes are the result of HDAC inhibition on the relevant tissues and how much is the result of FFAR activation in the intestine and the subsequent release of hormones such as GLP-1. While *in vitro* experiments enable controlled experiments to be conducted without the complications caused by effects on other organs, without confirming that the novel findings outlined earlier in this chapter also occur *in vivo*¸ their relevance is unknown.

7.3. Clinical relevance

Statins are very widely used to lower LDL-C, especially in advanced economies. However, they are associated with a small but significant increased risk of developing type 2 diabetes, although the benefits in reducing cardiovascular events greatly outweigh the risks.[339] Exploring the mechanism behind the diabetogenic effects of statins may help reduce these risks or identify people in which the risk is highest, enabling early detection and intervention. The findings of this project suggest that statins do not have direct epigenetic effects, and all noted effects of statins are directly explained by cellular cholesterol lowering. This adds further evidence that the increased risk of diabetes with statins is related to cholesterol lowering, rather than an off-target effect. In particular, the lowering of cholesterol in β cells and the downstream downregulation of ABCG1 may impair insulin secretion. People without significantly elevated cholesterol or with diabetes risk factors may reduce this risk by using a hydrophilic statin such as pravastatin which is likely to have lower uptake in non-hepatic tissues.[142]

HDAC inhibitors such as butyrate are of interest as therapeutics to protect against diabetes and the metabolic syndrome, however they are limited by their poor uptake.[163] The data in this study suggests that HDAC inhibitors have detrimental effects on β cell function and insulin secretion, and that attempts to increase butyrate uptake may have unfavourable consequences. Instead of focusing on effects of HDAC inhibitors in general on peripheral tissues, it may be best to focus on the action of butyrate on intestinal cells and GLP-1 secretion. It may indeed be as a GLP-1 secretagogue that butyrate may one day be used as a therapeutic to protect against T2D.

7.4. Conclusion

This project demonstrated that both statins and HDAC inhibitors reduce cellular cholesterol in numerous cell types and impair β cell insulin secretion, although they act through different mechanisms. All noted effects of statins can be linked directly to their primary mechanism of reducing cholesterol biosynthesis through HMGCR inhibition, resulting in the activation of SREBP-2 target genes and the repression of LXR target genes. HDAC inhibitors conversely have more widespread and varied effects on genes and proteins involved in cholesterol and glucose metabolism, most likely due to both histone and non-histone lysine acetylation. This does include downregulation of *HMGCR* expression, explaining the lowered cellular cholesterol. In contrast to statins, HDAC inhibition downregulates SREBP-2 activity and LDLR levels, thus may not reduce circulating LDL-C as statins do.

Similarly, both statins and HDAC inhibitors have been shown to impair insulin secretion from β cell lines. The results of this study suggest that the detrimental effects of statins are a direct result of cellular cholesterol lowering, with *ABCG1* downregulation potentially playing an important role. Conversely, HDAC inhibitors altered the expression of numerous genes involved in insulin secretion and β cell function, with the upregulation of *HK1* and *TXNIP* potentially leading to oxidative stress and β cell exhaustion.

The postulated mechanisms by which statins and butyrate alter cellular cholesterol and insulin secretion are summarised in Figure 7-1.

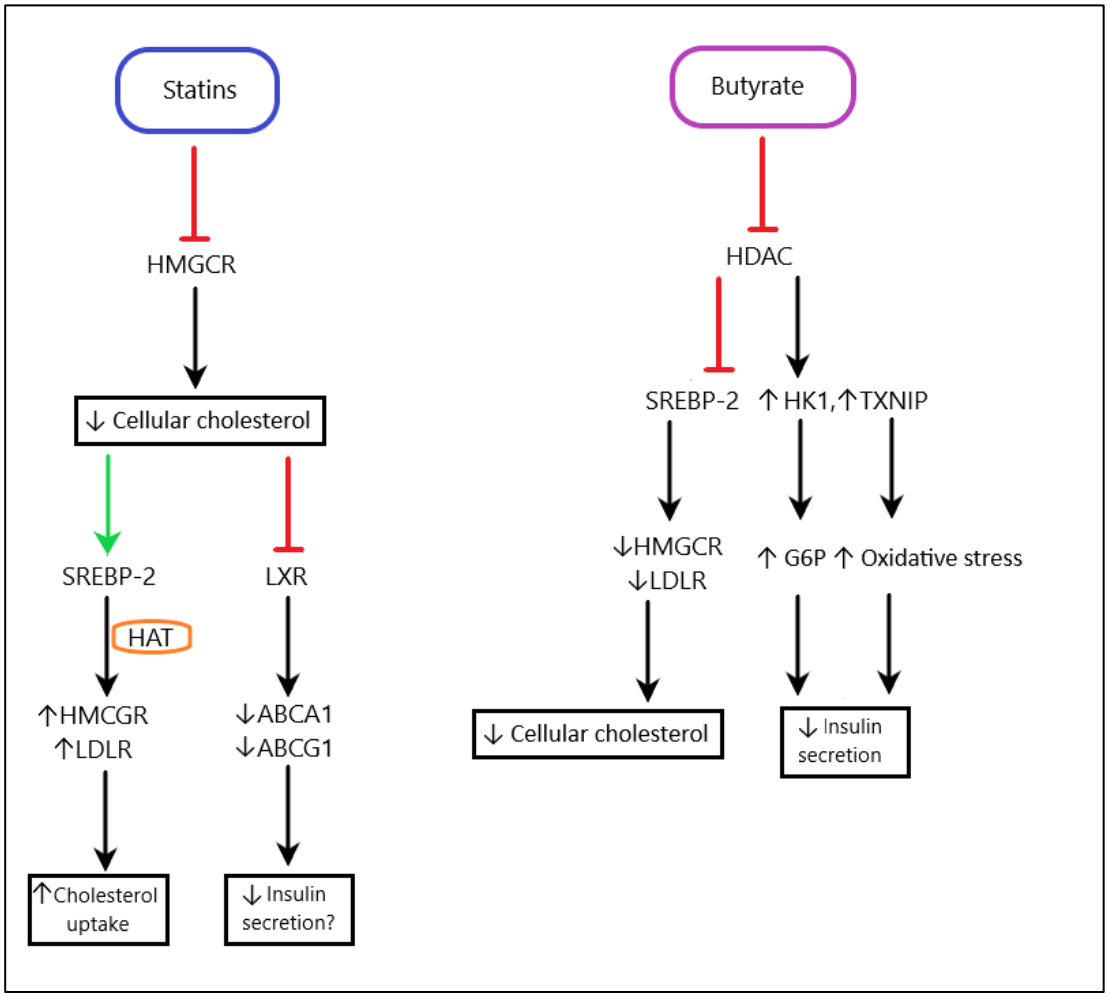

Figure 7-1 Postulate mechanisms by which statins and butyrate impact cellular cholesterol and insulin secretion

Statins inhibit HMGCR activity and thus reduce cellular cholesterol synthesis, and all noted effects can be explained by their lowering of cellular cholesterol. Low levels of cholesterol activate SREBP-2, enabling it to recruit HATs to HMGCR and LDLR, increasing their expression. Increased LDLR expression leads to increased cholesterol uptake. However, lowered cellular cholesterol results in the repression of ABCA1 and ABCG1, regulated by LXR. Decreased ABCG1 in particular may impair insulin secretion. Conversely, HDAC inhibition by butyrate inhibits SREBP-2 activity, leading to decreased HMGCR and LDLR expression and thus reduced cellular cholesterol. Butyrate also increases HK1 and TXNIP expression, leading to increased glucose sensing and increased oxidate stress respectively, both of which may result in impaired insulin secretion.

In stark contrast to the literature, the results presented here show that statins are unlikely to impact epigenetic modifications directly and noted epigenetic effects may be due to the downstream effects of cholesterol lowering and subsequent effects on

transcription factors, such as SREBP-2, which interact with epigenetic modifying enzymes.

HDAC inhibitors have been suggested as treatments for diabetes and other metabolic disorders due to the recent surge of research linking epigenetic modifications to these diseases and have shown promising results in animal models. This project highlights the danger of broad-spectrum epigenetic modifiers due to significant effects across the genome that may have detrimental and unpredictable consequences.

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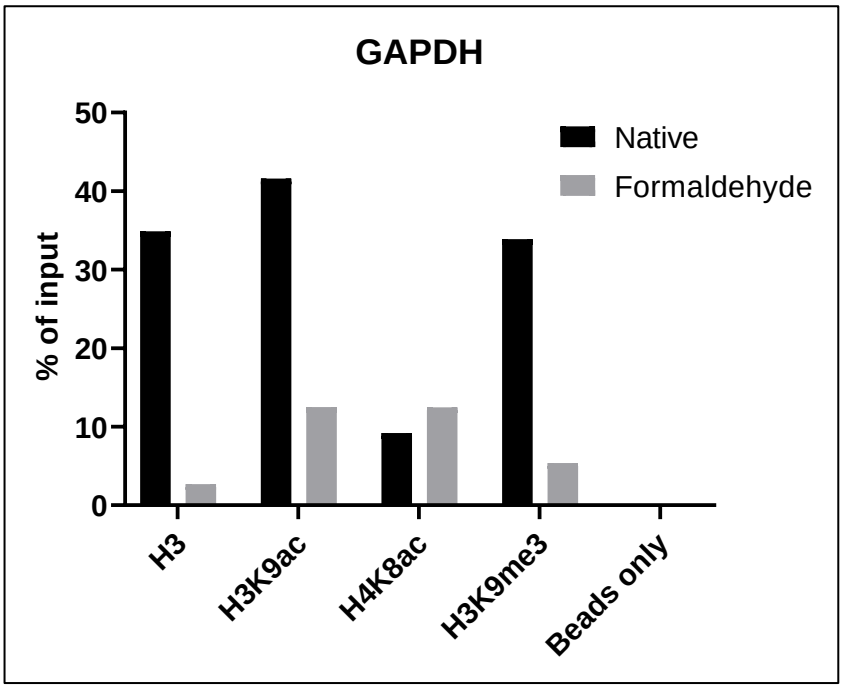
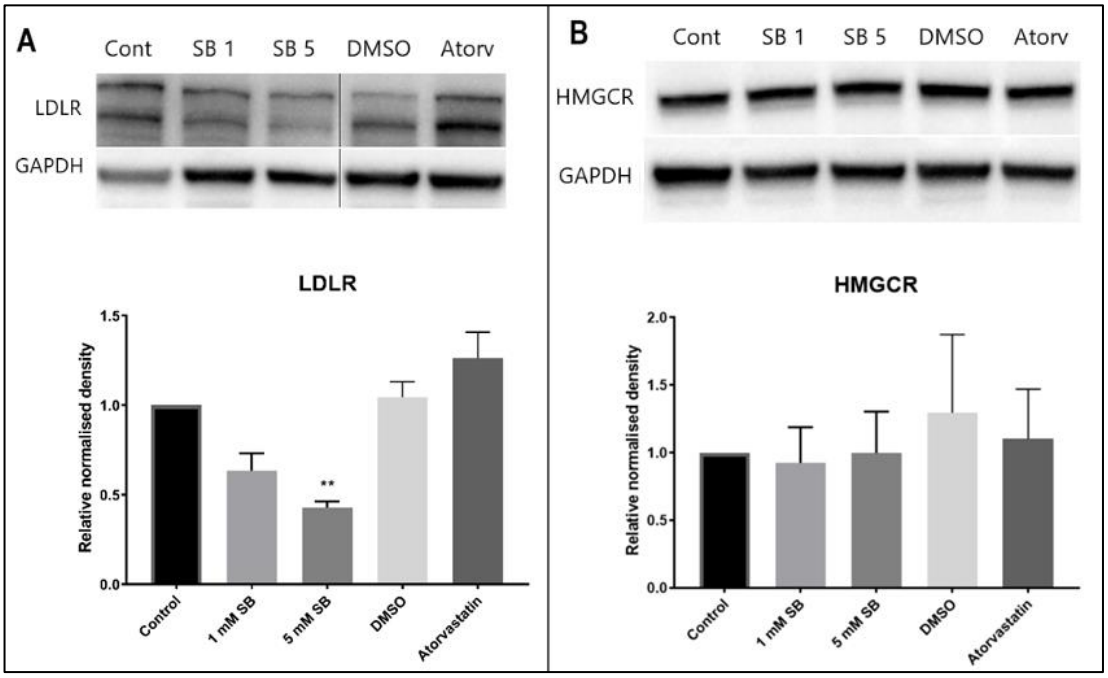
Appendix A: Supplementary figures

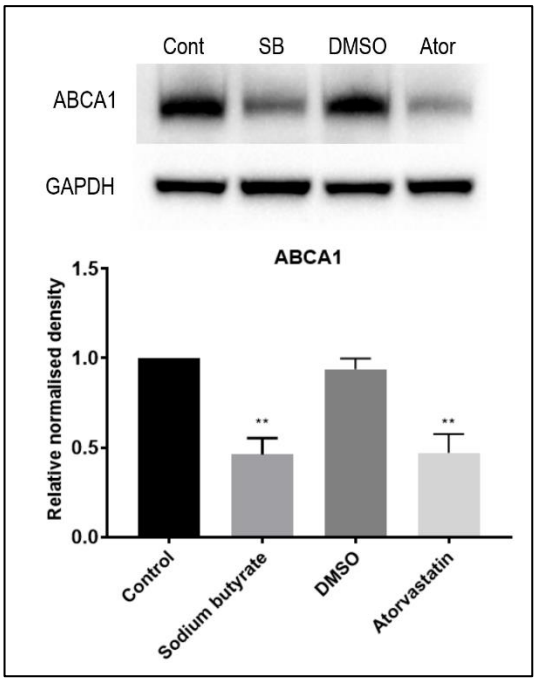


Supplementary Figure S1 Comparison of native and cross-linked ChIP qPCR of ChIP performed on HepG2 cells with and without formaldehyde cross-linking in proximity to GAPDH. Results are from a single experiment.



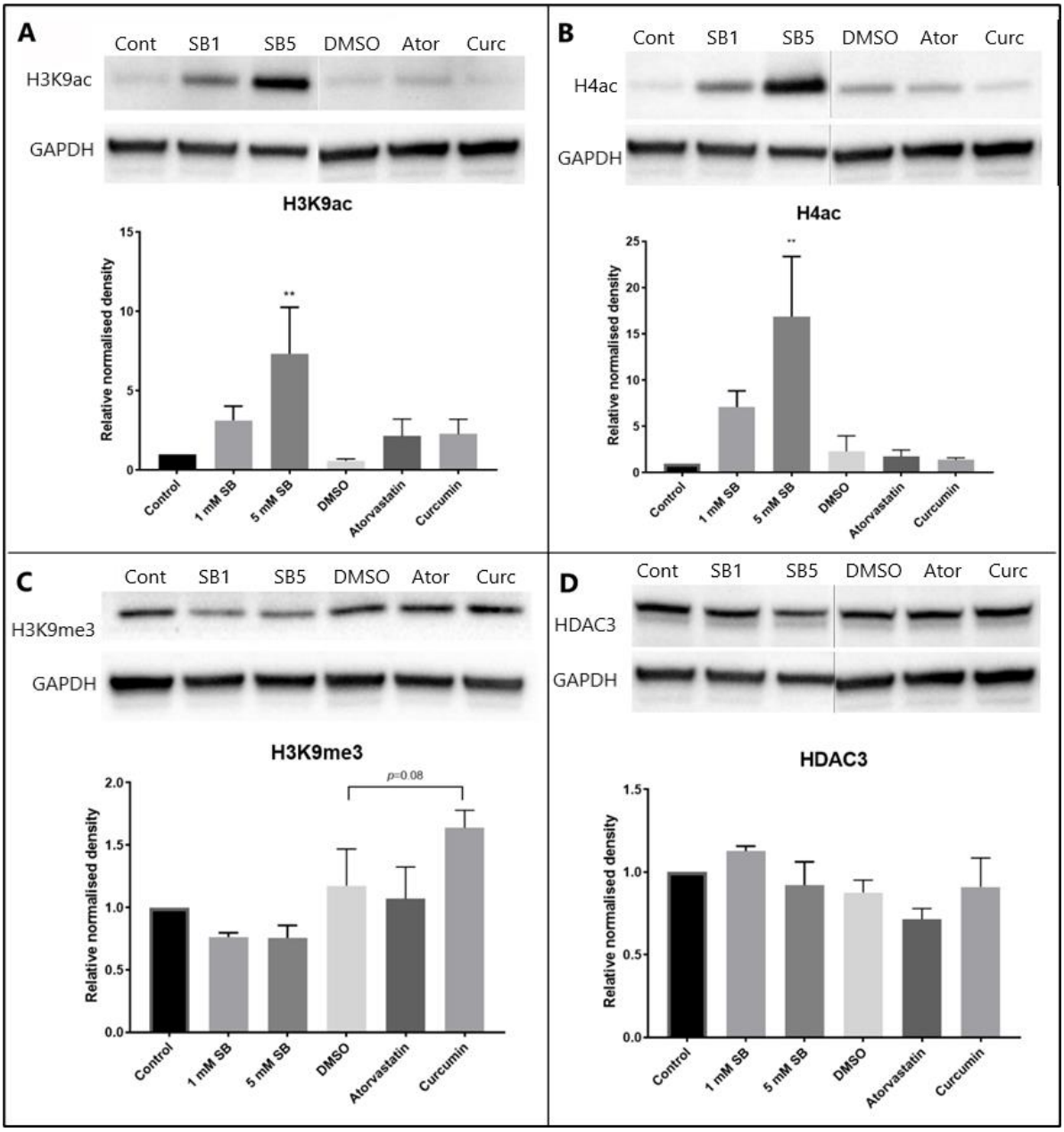
Supplementary Figure S2 Influence of butyrate and atorvastatin on SREBP-2 pathway in BRIN-BD11 cells

Relative protein levels of A) LDLR and B) in BRIN-BD11 cells following treatment with 1 mM or 5 mM sodium butyrate or 10 µM atorvastatin for 24 h. The graph represents the mean combined density readings as normalised to GAPDH from at least three independent experiments, with error bars representing SEMs. ** $p<0.01$.



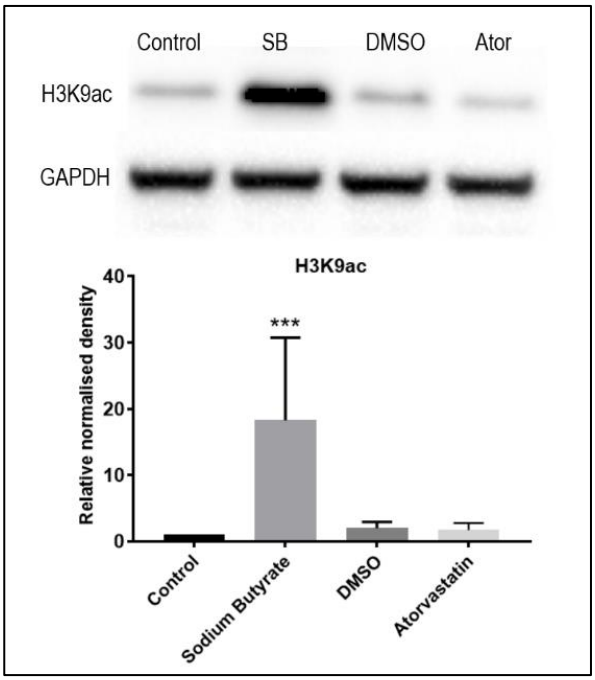
Supplementary Figure S3 Influence of butyrate and atorvastatin on ABCA1 in THP-1 cells

Relative protein levels of ABCA1 in THP-1 cells following treatment with 5 mM sodium butyrate or 10 µM atorvastatin for 24 h. The graph represents the mean combined density readings as normalised to GAPDH from three independent experiments with error bars representing SEMs. ** $p<0.01$.



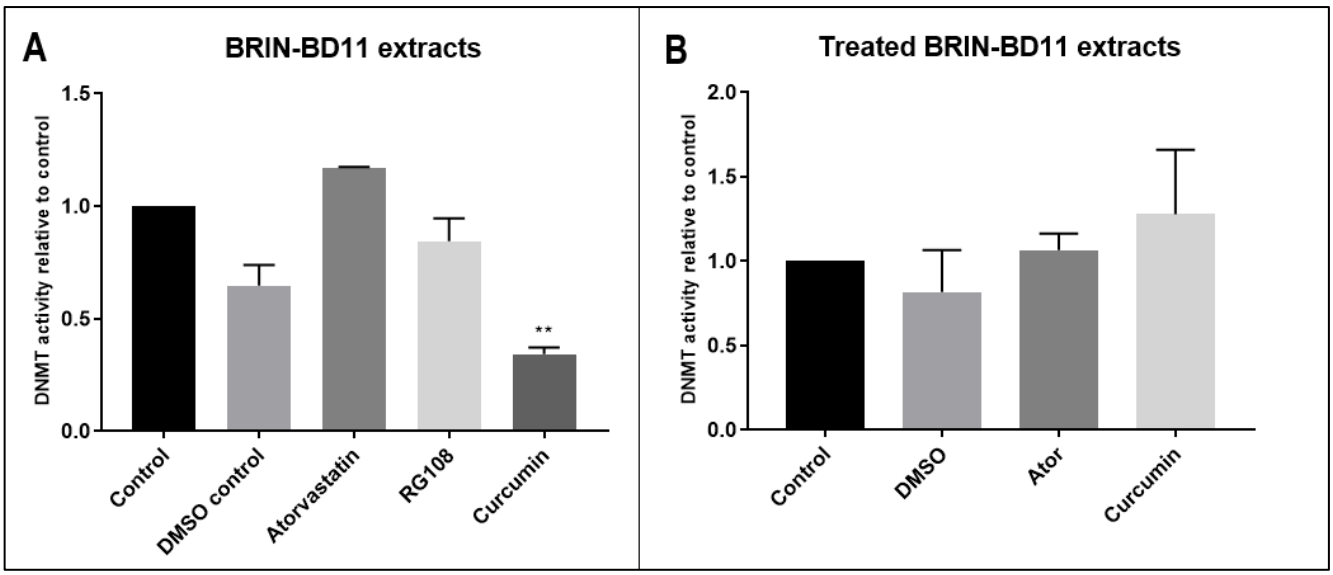
Supplementary Figure S4 Influence of butyrate, atorvastatin and curcumin on global histone modifications in BRIN-BD11 cells

Relative protein levels of a) acetylated H3K9, b) acetylated H4, c) trimethylated H3K9 and d) HDAC3. The graph represents the mean combined density readings as normalised to GAPDH from three independent experiments with error bars representing SEMs. ** $p<0.01$.



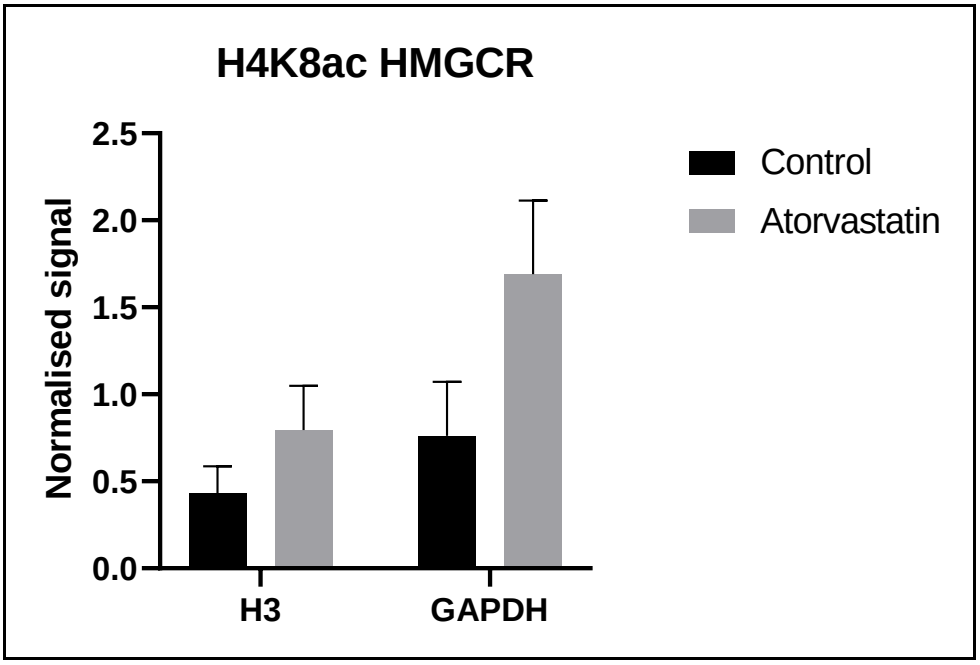
Supplementary Figure S5 Influence of butyrate and atorvastatin on global histone acetylation in HLPS

Relative protein levels of acetylated H3K9. The graph represents the mean combined density readings as normalised to GAPDH from three independent experiments with error bars representing SEMs. *** $p<0.001$.



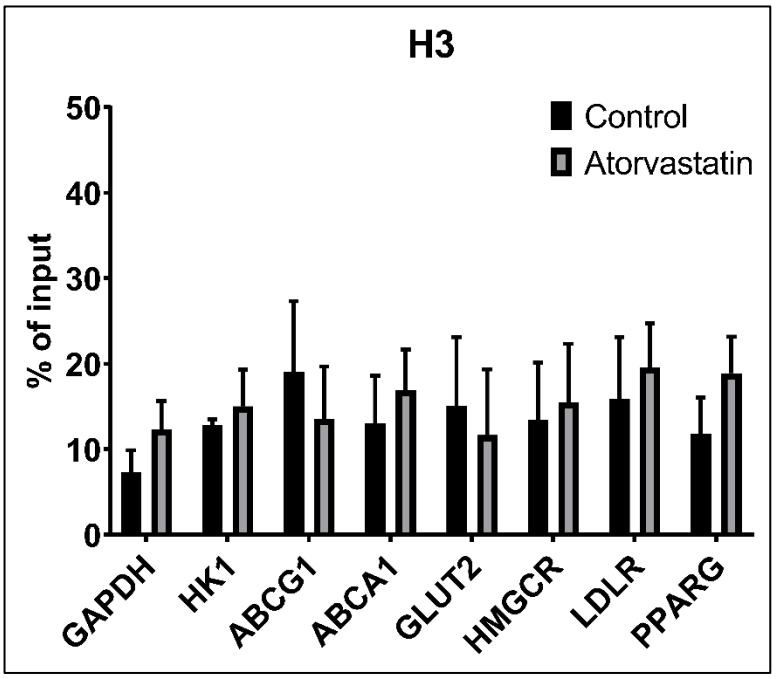
Supplementary Figure S6 Influence of atorvastatin on DNMT activity in BRIN-BD11 cell extracts

a) DNMT activity in whole cell extracts treated directly with 200 µM atorvastatin, RG108, curcumin or DMSO vehicle control. b) DNMT activity in whole cell extracts from cells treated for 24 h with 10 µM atorvastatin, curcumin or DMSO vehicle control. Results represent the mean combined from two independent experiments, with error bars representing SEMs. ** $p<0.01$.



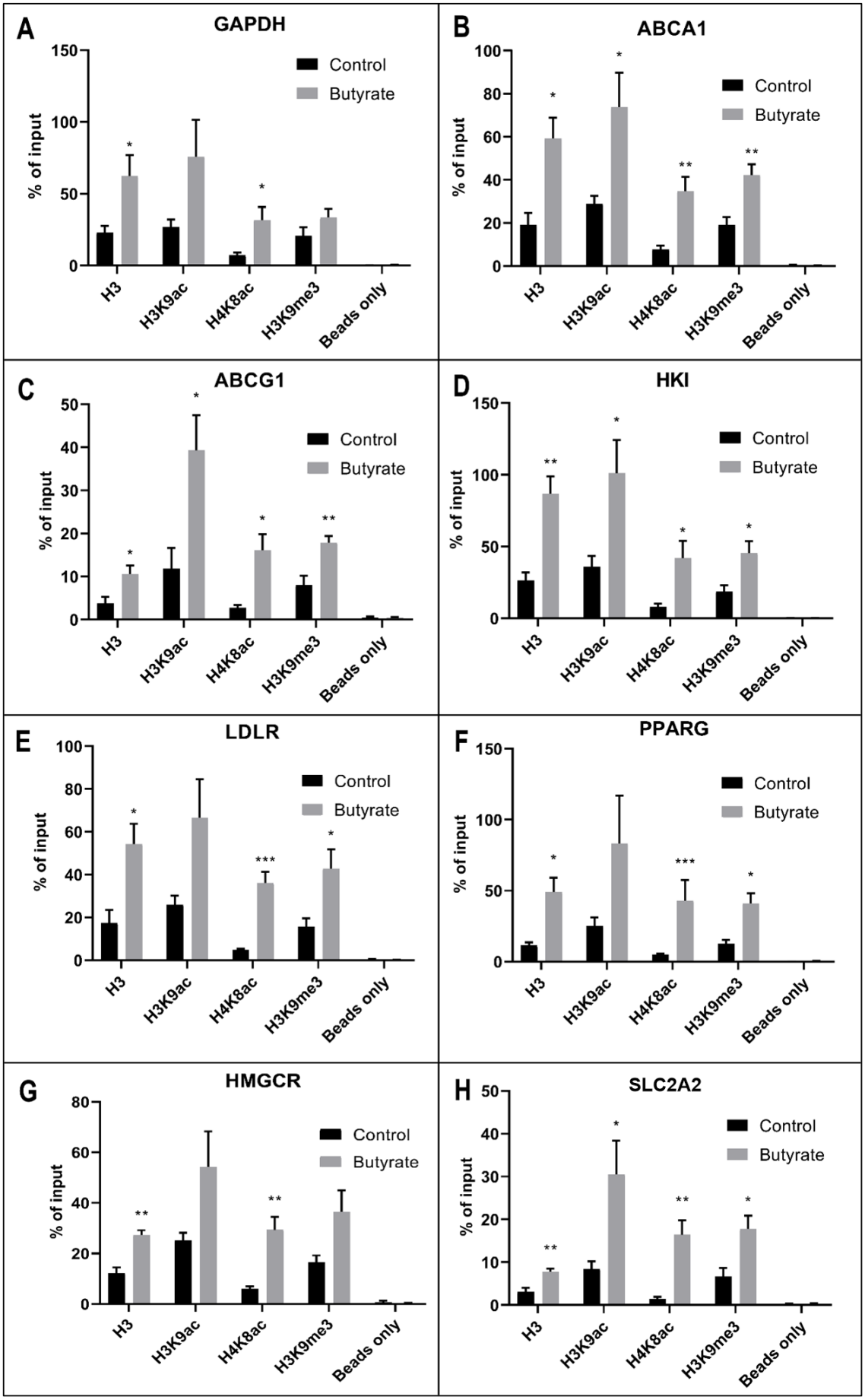
Supplementary Figure S7 Influence of atorvastatin on H4K9 acetylation in proximity to *HMGCR* using additional normalisation methods

ChIP qPCR H3 signals from HepG2 cells treated with 10 μM atorvastatin or DMSO vehicle control for 24 h in media containing FBS, normalised to nucleosome density (H3) or to positive control sequence (GAPDH). Results represent the mean combined from three independent experiments, with error bars representing SEMs.



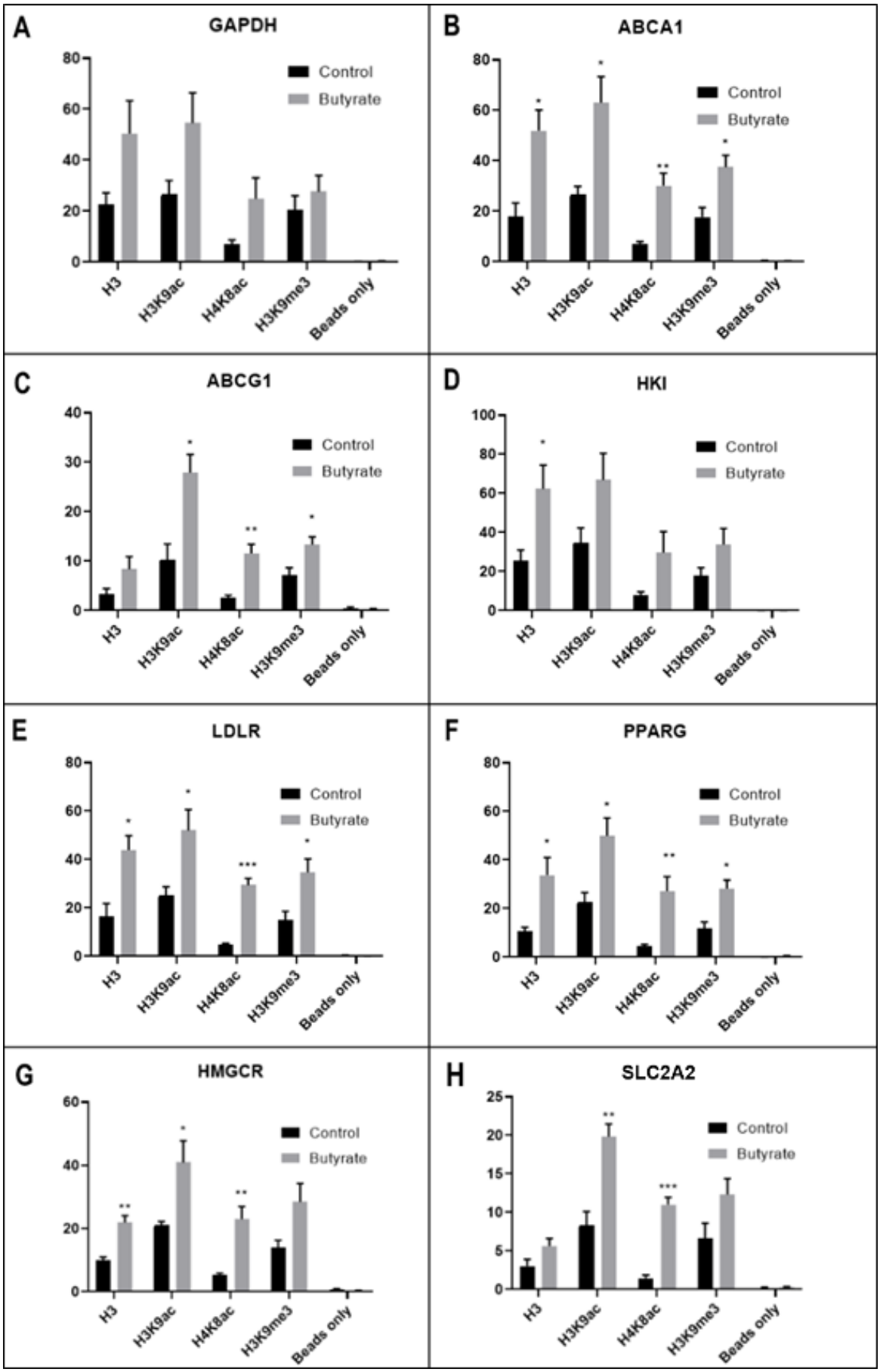
Supplementary Figure S8 Influence of atorvastatin on H3 signal in ChIP-qPCR

ChIP qPCR H3 signals from HepG2 cells treated with 10 μM atorvastatin or DMSO vehicle control for 24 h in media containing FBS. Results represent the mean combined from three independent experiments, with error bars representing SEMs.


A
GAPDH
% of input
Control
Butyrate
H3
H3K9ac
H4K8ac
H3K9me3
Beads only
B
ABCA1
C
ABCG1
D
HKI
E
LDLR
F
PPARG
G
HMGCR
H
SLC2A2

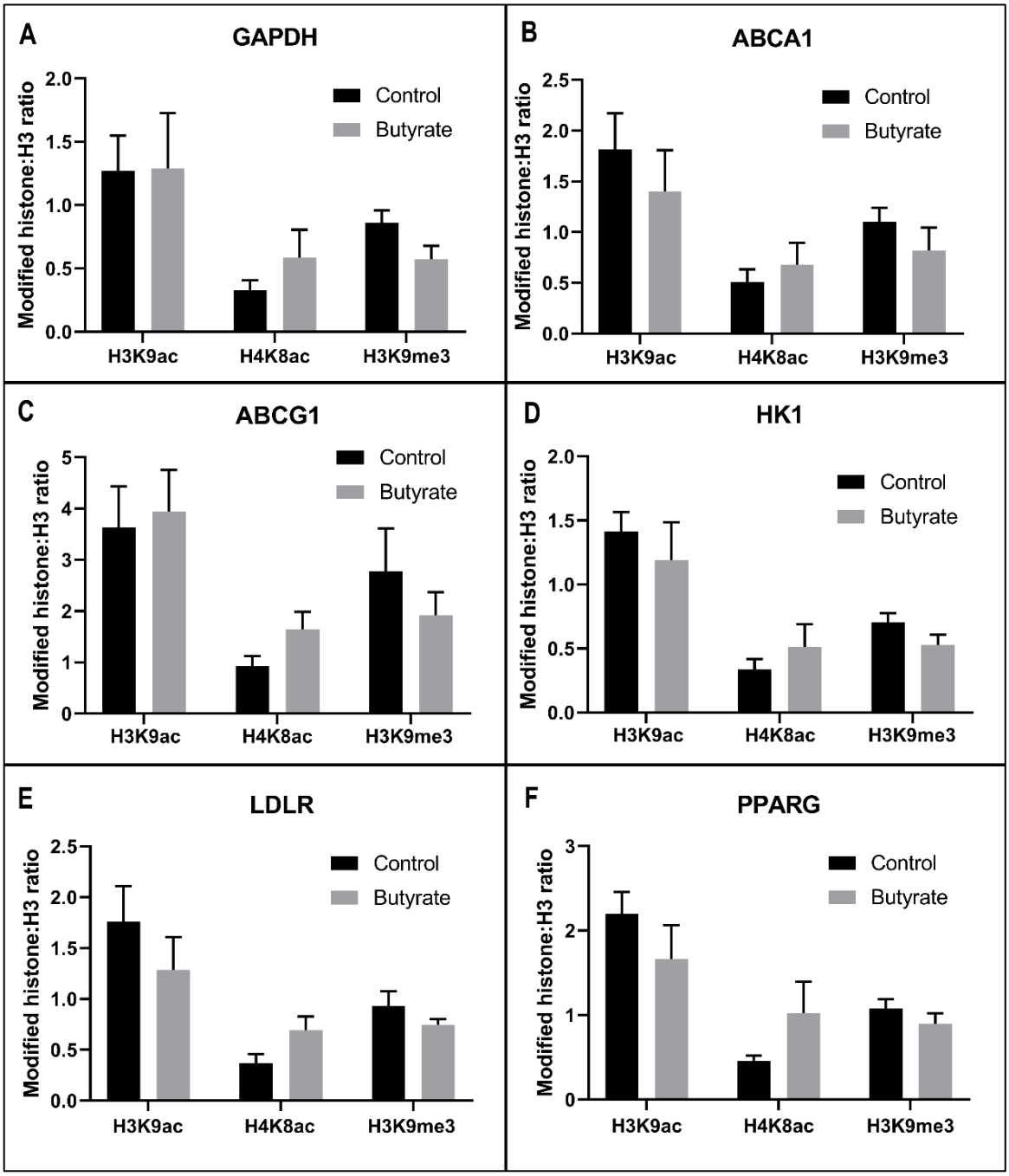
Supplementary Figure S9 ChIP qPCR of butyrate treated cells as percentage of input

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS presented as percentage of total chromatin input in proximity to a) *GAPDH*, b) *ABCA1*, c) *ABCG1*, d) *HK1*, e) *LDLR* f) *PPARG*, g) *HMGCR* and h) *SLC2A2* (GLUT2). Results represent the mean combined from four independent experiments, with error bars representing SEMs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.


A
GAPDH
Control
Butyrate
H3
H3K9ac
H4K8ac
H3K9me3
Beads only
B
ABCA1
C
ABCG1
D
HKI
E
LDLR
F
PPARG
G
HMGCR
H
SLC2A2

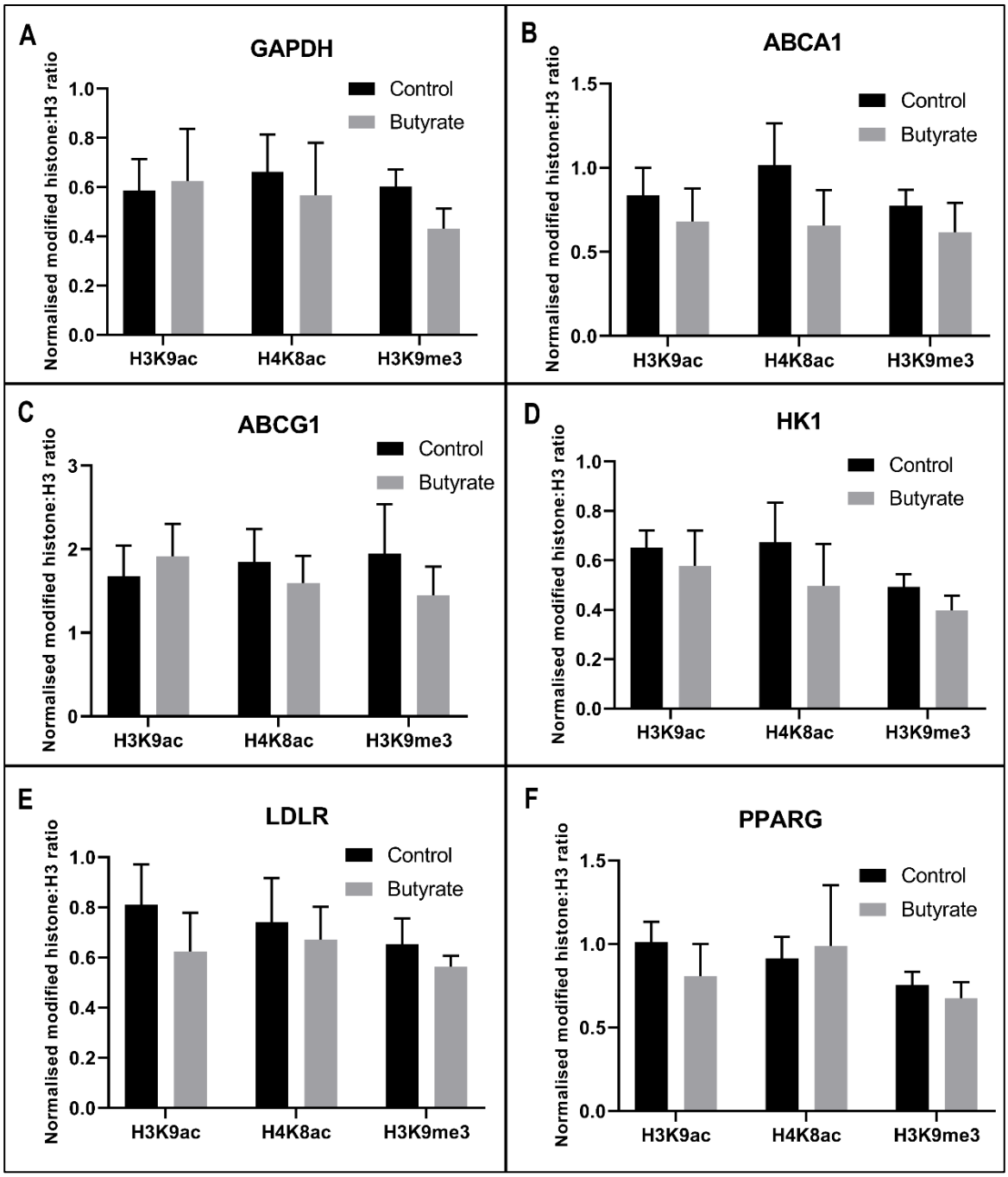
Supplementary Figure S10 ChIP qPCR of butyrate treated cells as raw data

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS as raw data in proximity to a) *GAPDH,* b) *ABCA1*, c) *ABCG1*, d) *HK1*, e) *LDLR* f) *PPARG*, g) *HMGCR* and h) *SLC2A2* (GLUT2). Results are mean combined from four independent experiments + SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.



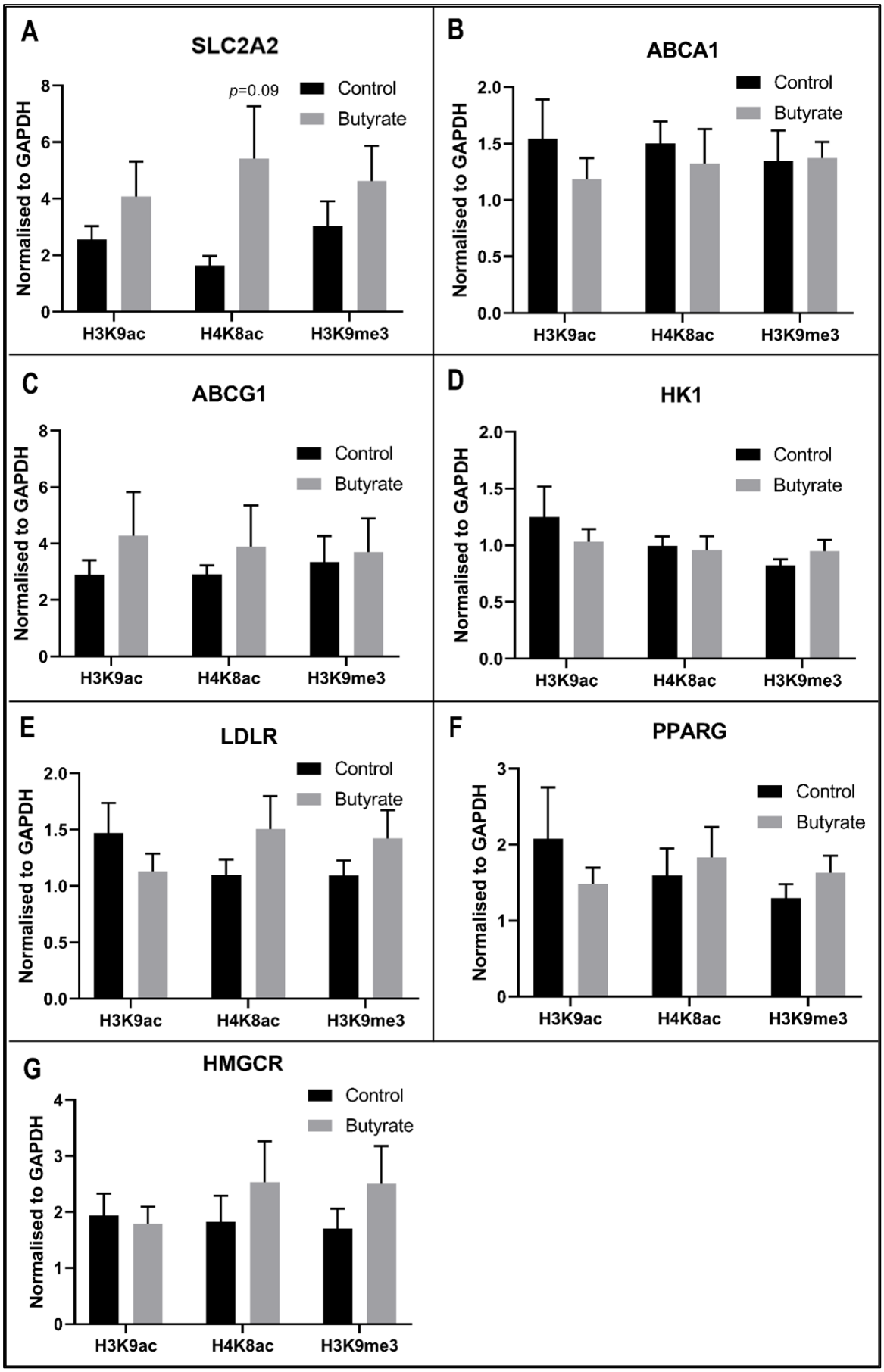
Supplementary Figure S11 ChIP qPCR of butyrate treated cells as modified histone:H3 ratio

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS presented as a modified histone: total H3 ratio of ChIP qPCR signals in proximity to a) *GAPDH*, b) *ABCA1*, c) *ABCG1*, d) *HK1*, e) *LDLR* and f) *PPARG*. Results are mean combined from four independent experiments + SEM.



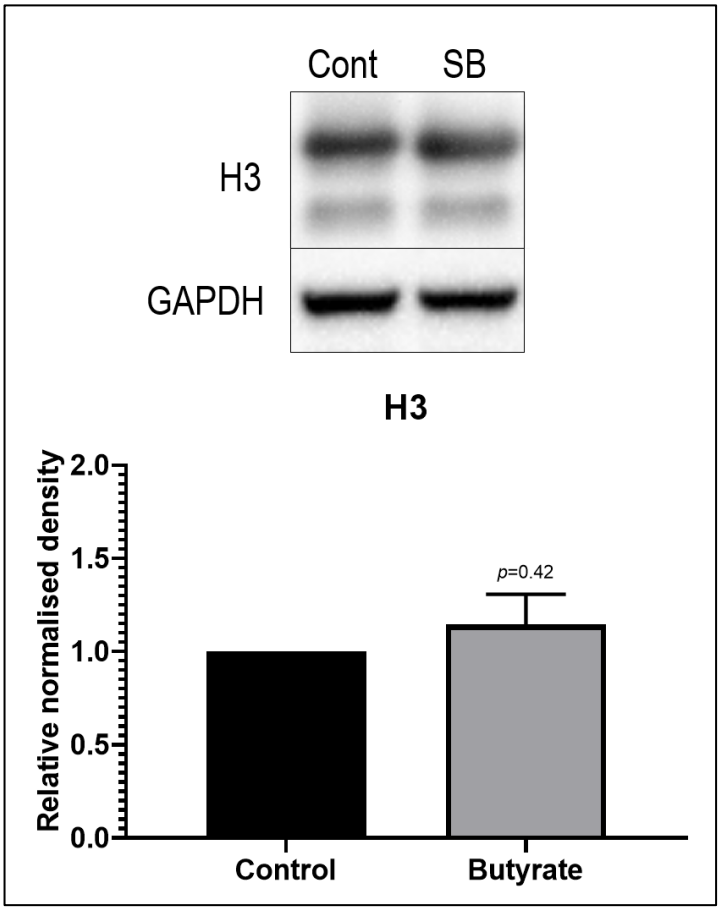
Supplementary Figure S12 ChIP qPCR of butyrate treated cells normalised to average results

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS normalised to average results from all genomic regions examined, in proximity to a) *GAPDH,* b) *ABCA1*, c) *ABCG1*, d) *HK1*, e) *LDLR* and f) *PPARG.* Results are mean combined from four independent experiments + SEM.


A
SLC2A2
p=0.09
Control
Butyrate
Normalised to GAPDH
H3K9ac
H4K8ac
H3K9me3
B
ABCA1
C
ABCG1
D
HK1
E
LDLR
F
PPARG
G
HMGCR

Supplementary Figure S13 ChIP qPCR of butyrate treated cells normalised to GAPDH

ChIP qPCR from HepG2 cells treated with 5 mM butyrate for 24 h in media containing LPDS normalised to *GAPDH* in proximity to a) *SLC2A2* (GLUT2), b) *ABCA1*, c) *ABCG1*, d) *HK1*, e) *LDLR* f) *PPARG* and g) *HMGCR*. Results are mean combined from four independent experiments + SEM.



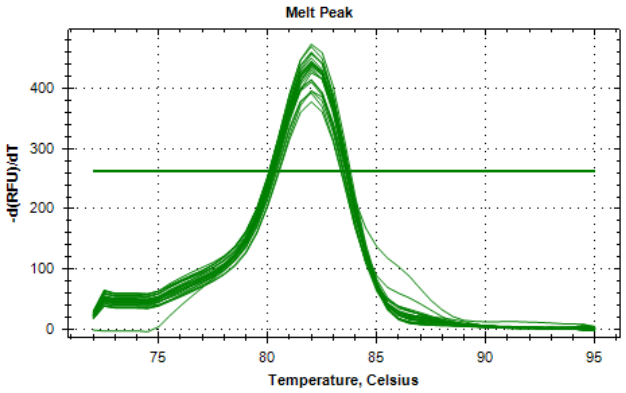
Supplementary Figure S14 Influence of butyrate on global H3

Relative protein levels of H3 in HepG2 cells treated with 5 mM sodium butyrate for 24 h. The graph represents the mean combined density readings as normalised to GAPDH from three independent experiments with error bars representing SEMs.

Appendix B: qPCR optimisation

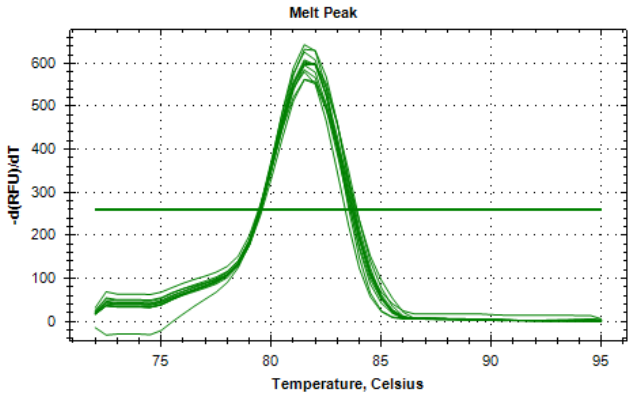
Human primers for RT-qPCR of HepG2 cells

GAPDH



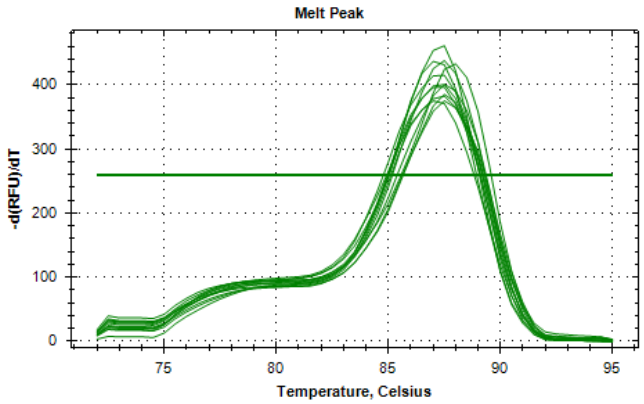
GAPDH	59.4	56.9	55	53.7
1	17.58	18.00	18.30	18.42
0.3	19.32	19.89	20.27	20.68
0.1	21.14	21.61	21.94	22.23
0.03	22.84	23.24	23.76	24.00
0.01	24.34	25.01	25.21	25.31
Efficiency	0.964113	0.9402481	0.9450484	0.9599434
R2	0.9989	0.9994	0.9987	0.9937

YWHAZ



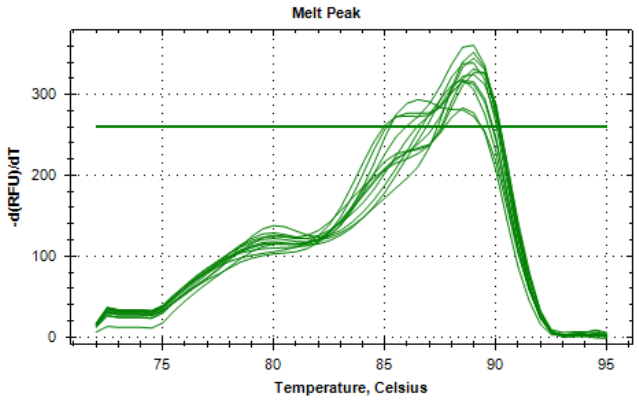
YWHAZ	61.4	59	57	55.7
1	20.36	20.32	20.27	20.26
0.3	22.53	22.25	22.14	22.05
0.1	23.90	24.00	23.72	24.19
0.03	25.71	25.73	25.55	25.59
0.01	27.09	26.86	27.03	27.19
Efficiency	0.9964699	1.003618	0.974712	0.9376052
R2	0.9957	0.9938	0.9995	0.9943

RPL13A



RPL13A	61.4	59	57	55.7
1	16.40	16.41	16.94	16.83
0.3	18.44	18.71	18.85	19.04
0.1	20.14	20.31	20.56	21.01
0.03	21.99	22.25	22.38	22.67
0.01	23.13	23.59	24.59	24.05
Efficiency	0.9672967	0.9013577	0.8436412	0.8908657
R2	0.9937	0.995	0.9973	0.9926

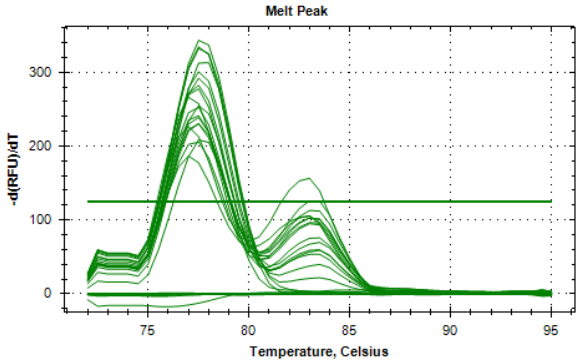
LDLR



LDLR	**61.4**	**59**	**57**	**55.7**
1	22.75	23.05	23.48	23.38
0.3	24.38	24.86	25.53	25.54
0.1	26.14	27.00	27.28	27.54
0.03	27.97	28.50	29.24	29.22
0.01	29.55	30.05	30.92	31.29
Efficiency	0.954899	0.9209507	0.8580468	0.8042045
R2	0.9994	0.9951	0.9997	0.998

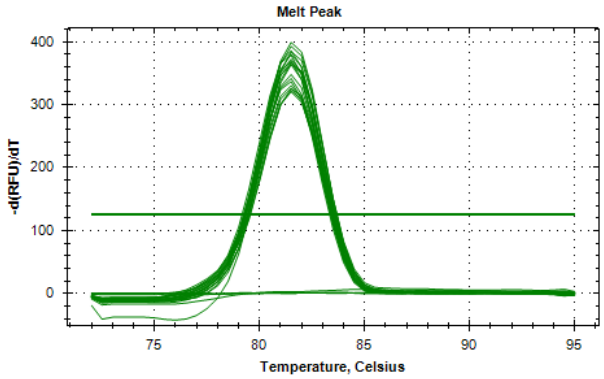
PPARG

200nM



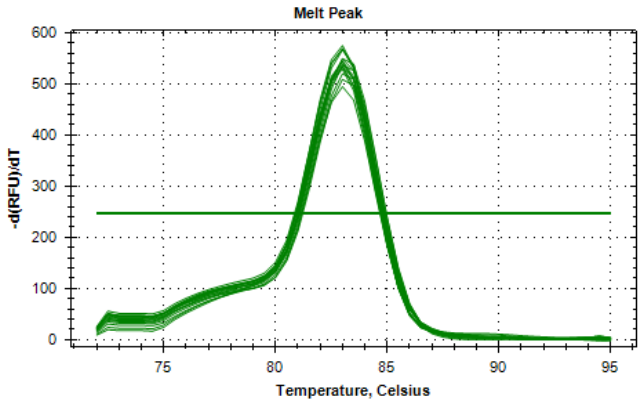
PPARG	61.4	59	57	55.7
1	25.14	24.97	25.14	25.13
0.3	26.41	26.80	26.53	26.60
0.1	28.47	28.42	28.42	28.42
0.03	30.12	30.22	30.07	29.89
0.01	31.56	31.44	31.83	31.50
Efficiency	1.005432	1.0199418	0.9751408	1.050543
R2	0.9943	0.9973	0.9969	0.9981

100nM



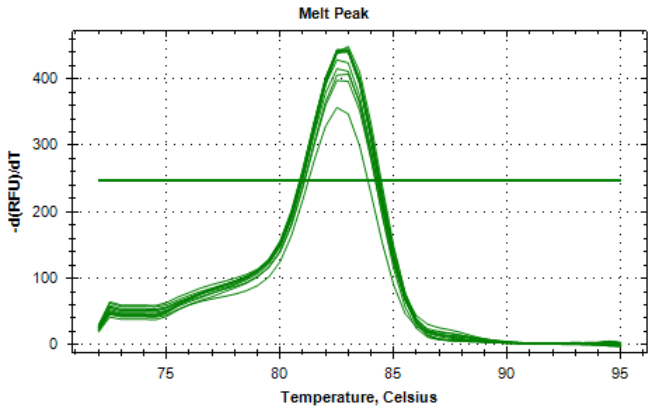
PPARG	63.4	61	59	57.7
1	26.8	25.66	24.74	
0.3	28.04	26.93	26.74	26.77
0.1	30.23	29.34	29.01	29.05
0.03	31.88	30.91	30.53	31.08
0.01		33.59	33.04	32.88
Efficiency	0.950548	0.788347	0.760757	0.750344
R2	0.9865	0.9841	0.9942	0.9968

SREBF2



SREBF2	**61.4**	**59**	**57**	**55.7**
1	21.88	21.81	22.19	22.21
0.3	23.73	24.06	24.33	23.86
0.1	25.43	25.81	26.54	26.77
0.03	27.34	27.72	28.24	28.90
0.01	29.04	29.63	30.43	30.33
Efficiency	0.8993008	0.8159885	0.7592014	0.7179961
R2	1.0000	0.9991	0.9976	0.9872

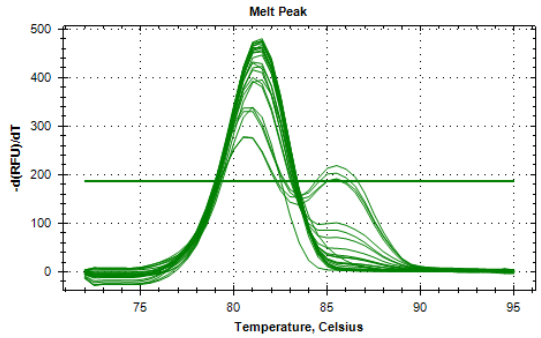
NR1H3



NR1H3	**61.4**	**59**	**57**	**55.7**
1	24.72	24.69	25.33	25.22
0.3	26.25	26.25	26.68	26.91
0.1	27.95	28.28	28.41	28.35
0.03	29.69	30.23	30.25	30.71
0.01	31.39	31.98	31.90	32.24
Efficiency	0.9857055	0.859746	0.9920203	0.9058429
R2	0.9989	0.9977	0.9968	0.9953

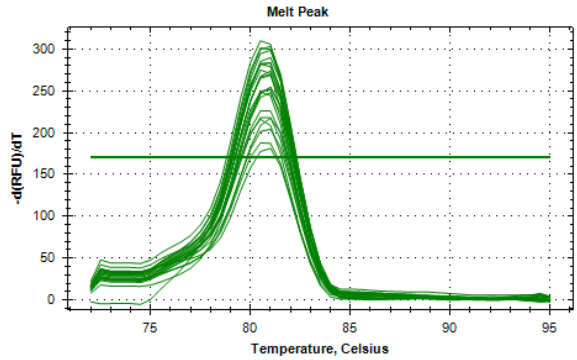
APOB

200nM



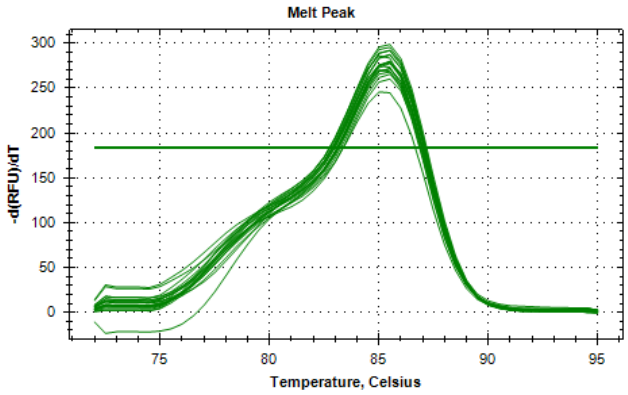
APOB	**61.4**	**59**	**57**	**55.7**
1	16.39	16.37	16.42	16.32
0.3	18.35	18.28	18.39	18.74
0.1	20.21	20.11	20.46	20.56
0.03	22.18	22.19	22.39	22.48
0.01	23.74	23.75	23.97	24.06
Efficiency	0.8609	0.8516278	0.8275951	0.8193501
R2	0.9992	0.9992	0.9982	0.996

100nM



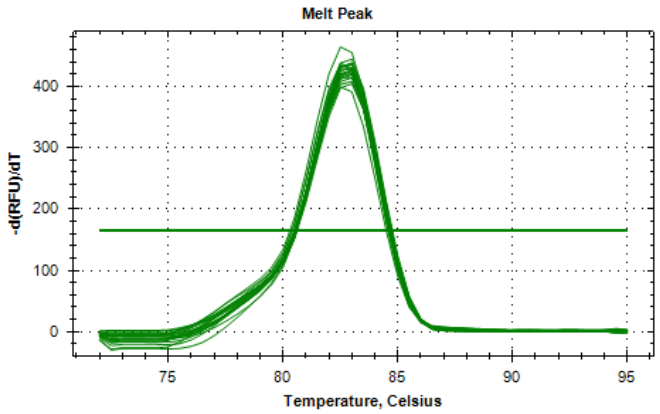
APOB	**63.4**	**61**	**59**	**57.7**
1	19.66	18.47	18.36	18.63
0.3	21.38	20.77	20.83	21.36
0.1	23.89	23.49	23.4	23.51
0.03	26.3	25.68	26.25	26.09
0.01	29.19	28.98	28.54	28.55
Efficiency	0.617119	0.559558	0.562846	0.597986
R2	0.991	0.9936	0.9994	0.9995

APOA1



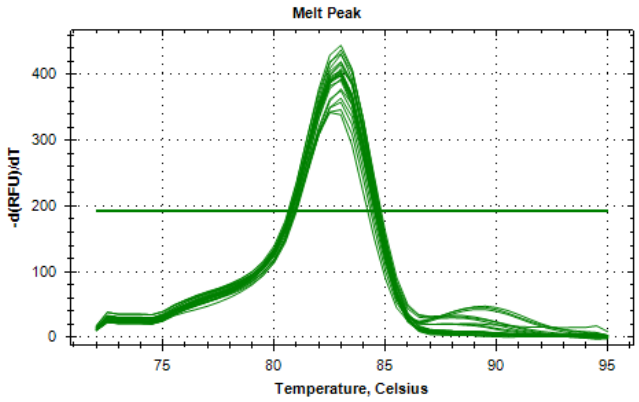
APOA1	62	58.4	55.5	53.5
1	16.57	16.68	16.70	16.78
0.3	18.28	18.45	18.49	18.76
0.1	20.22	20.08	20.34	20.34
0.03	22.03	21.85	22.12	22.23
0.01	23.57	23.49	23.79	23.96
Efficiency	0.913027	0.9663537	0.909281	0.9068332
R2	0.9987	1	0.9996	0.9997

GK



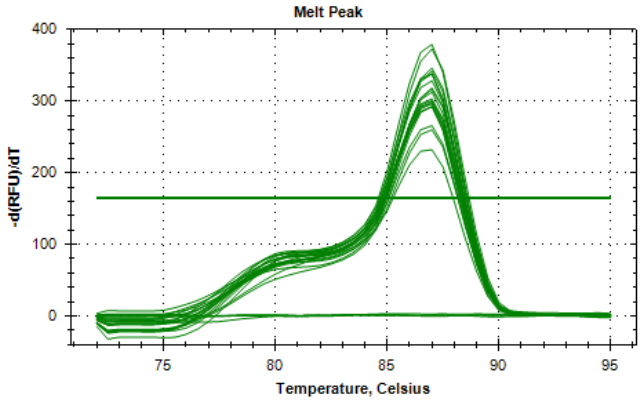
GK	61.4	59	57	55.7
1	20.82	20.94	20.99	20.76
0.3	22.55	22.62	22.60	22.79
0.1	24.36	24.25	24.31	24.43
0.03	26.25	26.31	26.35	26.61
0.01	27.69	27.68	28.10	28.05
Efficiency	0.9350129	0.9543705	0.8980633	0.8682931
R2	0.9988	0.9981	0.9982	0.9981

HMGCR



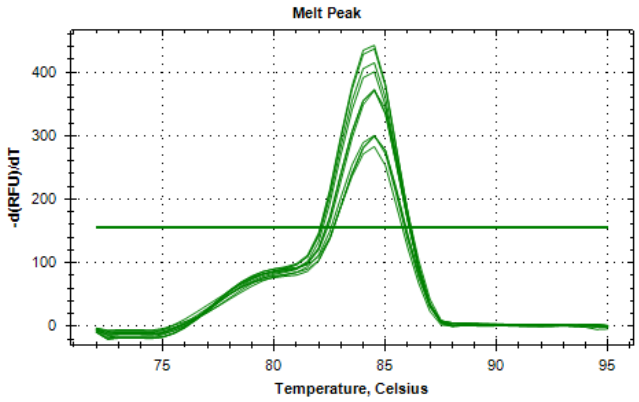
HMGCR	**61.4**	**59**	**57**	**55.7**
1	20.98	21.00	20.51	20.32
0.3	22.71	22.91	22.52	22.58
0.1	24.58	24.55	24.44	24.32
0.03	26.88	26.71	26.66	26.50
0.01	28.95	29.02	28.68	28.63
Efficiency	0.773543	0.7865357	0.7552778	0.7524436
R2	0.9966	0.9953	0.9996	0.9991

PEPCK1



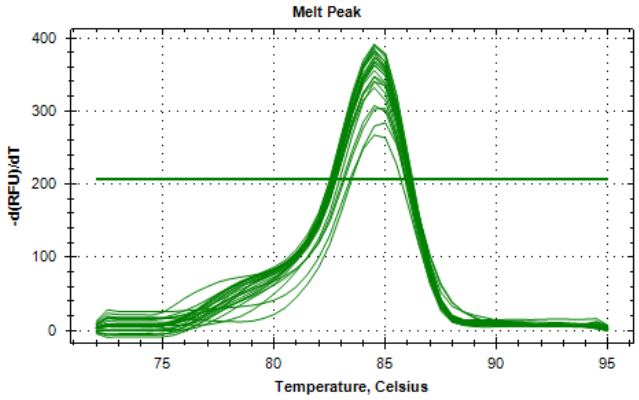
PEPCK1	**64.4**	**62**	**60**	**58.7**
1	24.93	24.57	24.27	24.61
0.3	26.89	26.42	26.28	26.82
0.1	28.31	28.19	28.29	28.47
0.03	30.55	29.93	30.26	30.31
0.01	32.39	32.07	31.80	31.65
Efficiency	0.8578761	0.8631919	0.8304793	0.9247788
R2	0.9973	0.9979	0.9982	0.9954

SLC2A2



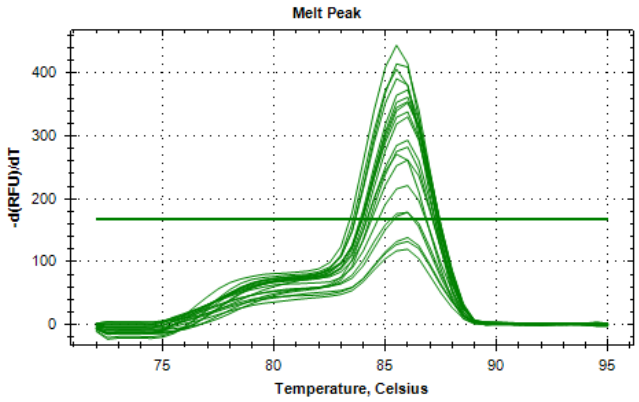
SLC2A2	61.4	59	57	55.7
1	30.11	29.75	29.67	29.93
0.3	32.42	31.55	31.59	31.4
0.1	34.68	32.87	33.05	33.03
0.03	35.26	35.4	36.4	35.2
0.01		35.77		37.78
Efficiency	0.929901	1.060796	0.70843	0.805656
R2	0.9434	0.9721	0.9708	0.984

SREBF1



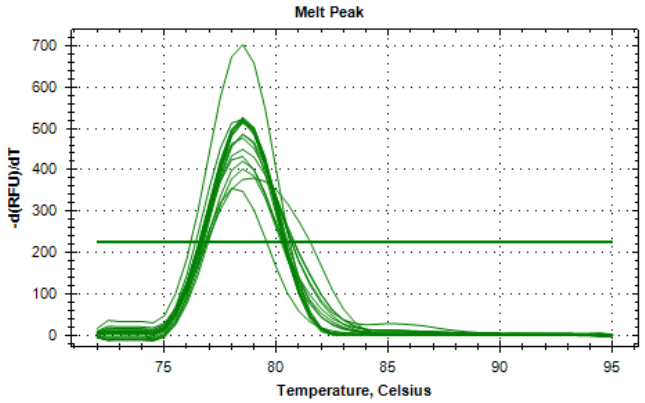
SREBF1	61.4	59	57	55.7
1	23.65	23.89	24.15	24.21
0.3	25.53	25.50	26.01	26.36
0.1	27.30	27.22	27.39	28.10
0.03	28.99	29.47	29.52	30.17
0.01	30.56	30.98	31.10	31.44
Efficiency	0.9468721	0.8850724	0.9365492	0.876142
R2	0.9991	0.9968	0.9979	0.9956

CAV1



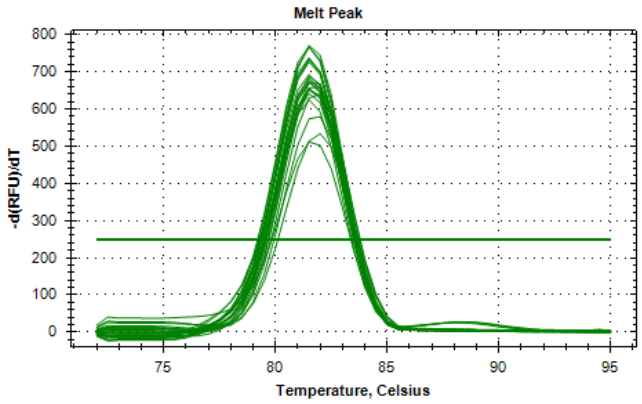
CAV1	61.4	59	57	55.7
1	27.99	27.68	27.08	27.28
0.3	29.89	29.72	29.76	30.62
0.1	30.85	31.10	31.52	31.16
0.03	33.04	32.35	34.16	34.66
0.01	34.82	33.52	34.21	35.10
Efficiency	0.9820708	1.2337783	0.8501961	0.7907112
R2	0.9907	0.9879	0.9495	0.9454

CYP7A1



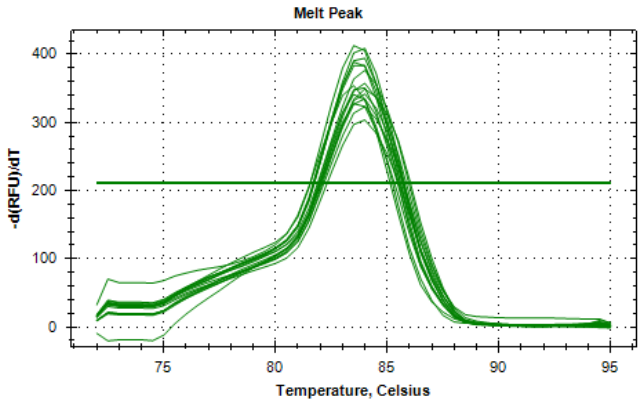
CYP7A1	62.4	60	58	56.7
1	29.62	29.81	29.85	29.85
0.3	31.45	31.65	31.16	31.89
0.1	33.44	33.49	33.41	33.24
0.03	37.50	35.33	34.84	36.16
0.01	36.03		36.90	
Efficiency	0.8362526	0.8823473	0.9127982	0.7720439
R2	0.8624	0.9997	0.9916	0.9822

FOXO1



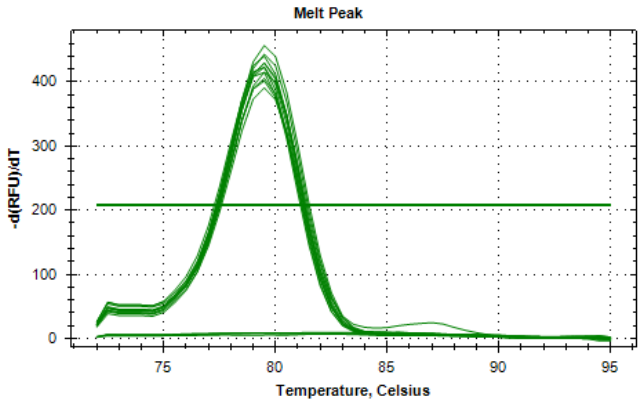
FOXO1	**61.4**	**59**	**57**	**55.7**
1	22.30	22.38	22.35	22.43
0.3	23.92	24.11	24.35	24.31
0.1	25.89	26.02	25.86	26.03
0.03	27.30	27.44	27.30	27.52
0.01	29.00	29.02	28.73	29.12
Efficiency	0.9868355	1.0001033	1.0819479	1.0014623
R2	0.9971	0.997	0.9957	0.9982

INSR



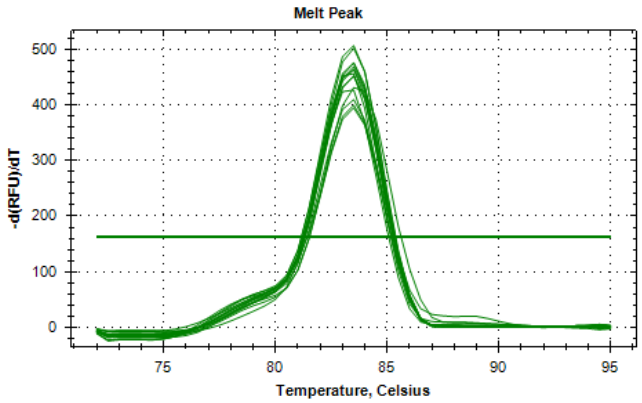
INSR	**64.5**	**60.9**	**58**	**56**
1	24.65	24.26	24.65	25.19
0.3	26.20	26.02	26.75	27.73
0.1	28.00	27.53	28.41	29.06
0.03	29.73	29.42	30.62	31.27
0.01	31.51	31.44	31.99	33.17
Efficiency	0.9499055	0.9122157	0.8603435	0.8044025
R2	0.9986	0.9971	0.9972	0.9954

ABCG1



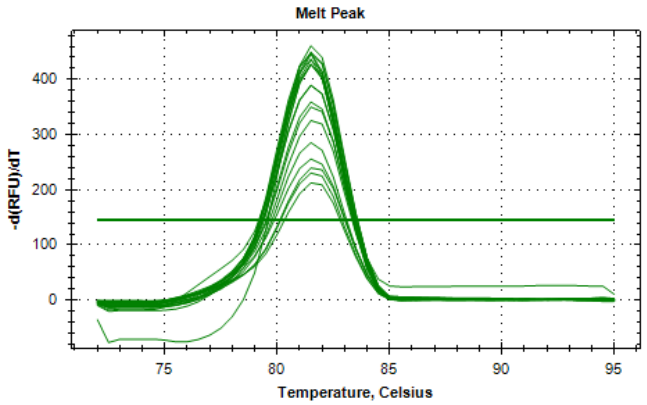
ABCG1	**64.4**	**62**	**60**	**58.7**
1	30.12	29.14	29.50	29.39
0.3	31.56	31.03	30.83	31.09
0.1	33.26	32.41	32.31	32.42
0.03	34.27	34.89	34.48	33.77
0.01	37.84	34.96	36.84	34.89
Efficiency	0.8889184	1.0979664	0.8753865	1.3194603
R2	0.9466	0.958	0.9821	0.9958

CEBPA



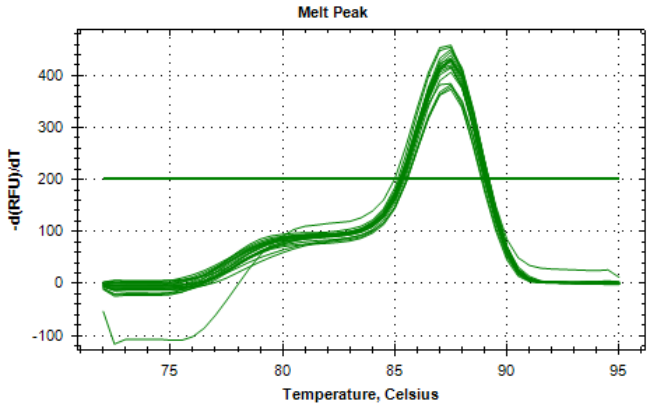
CEBPA	**61.4**	**59**	**57**
1	19.60	19.79	19.52
0.3	21.67	21.63	21.79
0.1	23.33	23.36	23.40
0.03	25.41	25.48	25.28
0.01	27.12	27.06	26.89
Efficiency	0.8455102	0.8696129	0.8795046
R2	0.9996	0.9992	0.9977

CPT1A



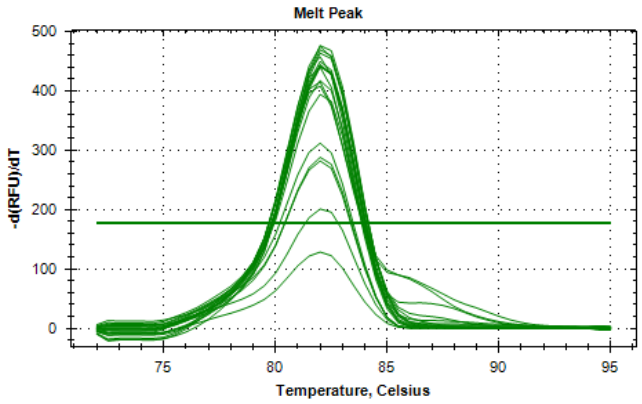
CPT1A	62.4	60	58	56.7
1	29.56	29.32	29.52	30.27
0.3	31.08	30.95	30.81	31.18
0.1	32.87	33.09	32.68	33.12
0.03	35.90	34.58	34.65	35.79
0.01	36.13	34.83	36.64	36.40
Efficiency	0.8964958	1.1924701	0.8908469	0.9786169
R2	0.96	0.95	0.9926	0.96

HNF4A



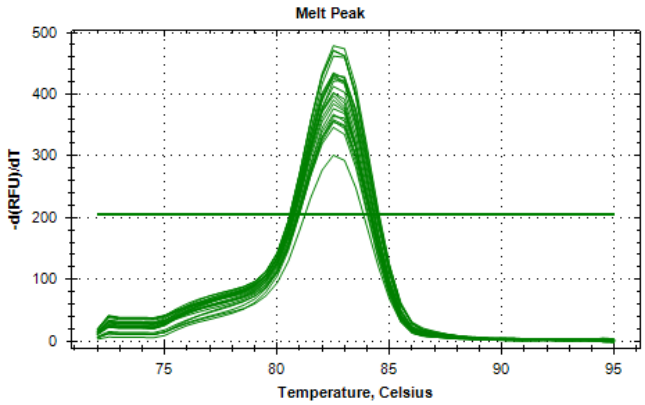
HNF4A	61.4	59	57	55.7
1	21.34	21.82	22.25	22.96
0.3	23.65	24.37	25.27	26.42
0.1	25.46	26.40	27.28	28.18
0.03	27.85	28.35	29.90	30.57
0.01	29.05	30.65	31.23	32.41
Efficiency	0.7966064	0.7026406	0.6640479	0.6463708
R2	0.9932	0.998	0.9889	0.9881

MVK



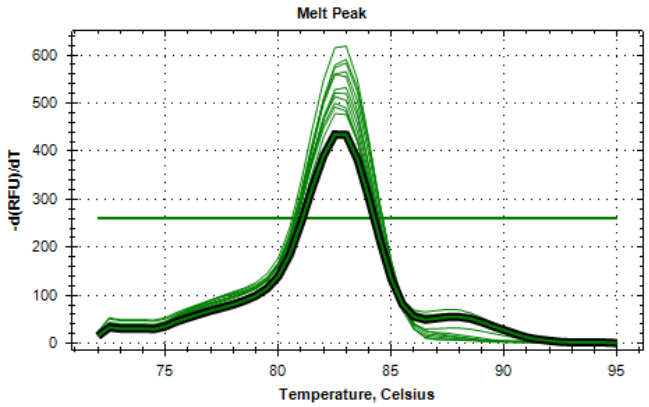
MVK	**59.4**	**56.9**	**55**	**53.7**
1	24.67	25.71	26.09	26.40
0.3	27.31	28.07	28.91	29.41
0.1	29.24	30.19	31.37	31.23
0.03	31.30	31.91	33.51	34.02
0.01	32.65	34.08	36.03	35.05
Efficiency	0.7795658	0.7493881	0.6009804	0.6899054
R2	0.9906	0.9973	0.998	0.9832

ABCA1



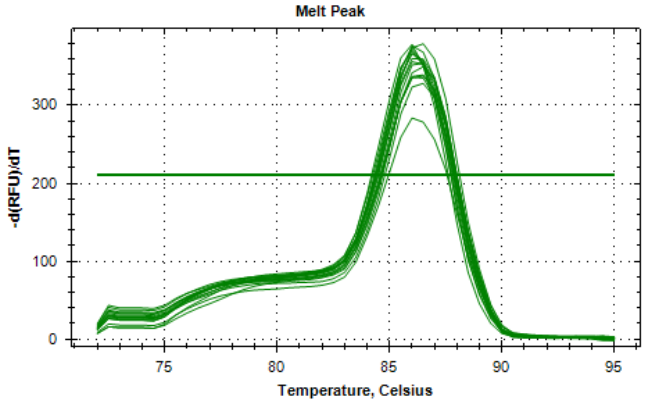
ABCA1	**63.4**	**61**	**59**	**57.7**
1	26.22	26.38	27.03	26.72
0.3	28.13	28.24	28.54	28.65
0.1	30.07	30.01	30.30	30.34
0.03	31.06	31.20	31.57	31.83
0.01	32.09	32.27	32.74	33.11
Efficiency	1.1906288	1.1826525	1.2182438	1.0555872
R2	0.9743	0.9851	0.9938	0.9947

HK1



HK1	62.4	60	58	56.7
1	26.37	26.41	26.04	26.13
0.3	28.02	28.37	27.59	27.72
0.1	30.32	29.86	29.61	29.21
0.03	32.00	31.60	31.74	31.24
0.01	33.06	34.04	32.57	33.04
Efficiency	0.9410328	0.865158	0.9519212	0.9412008
R2	0.9866	0.9921	0.9863	0.9968

SCARB1

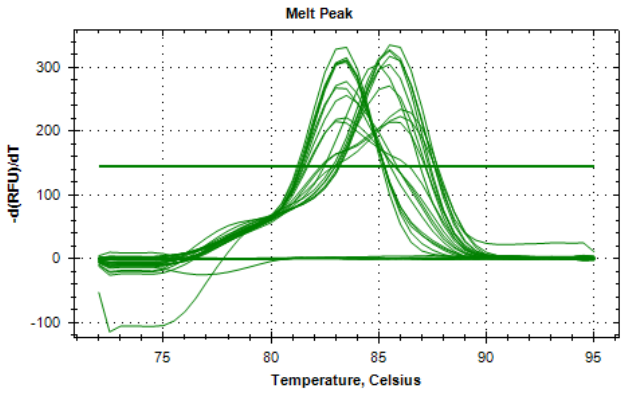


SCARB1	62.4	60	58	56.7
1	20.42	20.38	20.78	20.68
0.3	22.58	22.61	22.74	22.98
0.1	24.06	24.27	24.59	24.80
0.03	26.11	26.05	26.54	26.81
0.01	27.59	27.66	28.05	28.59
Efficiency	0.9046257	0.8963609	0.8720633	0.7953614
R2	0.9977	0.9974	0.9989	0.9990

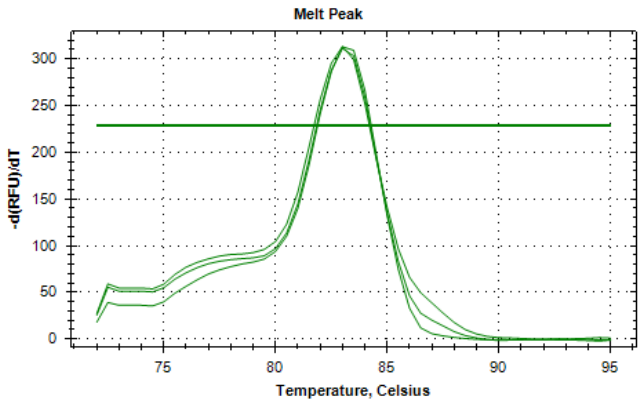
Rat primers for RT-qPCR of BRIN-BD11 cells

Actb

200nM



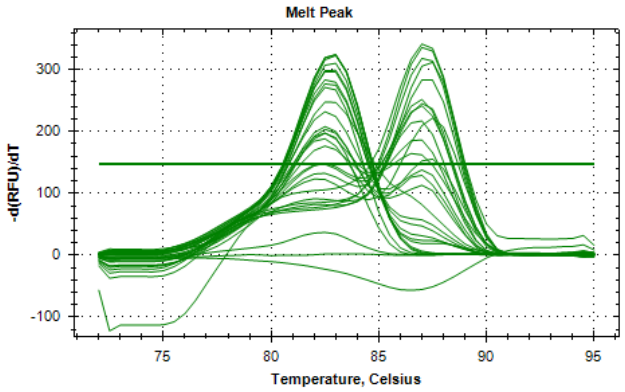
100nM



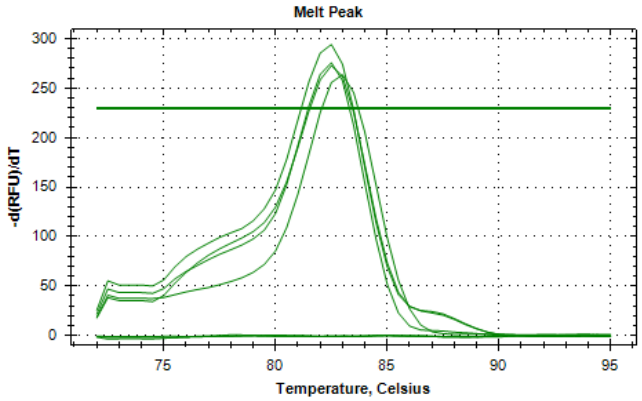
Actb	59	57	55.7	55
1	14.48	14.33	14.88	13.68
0.3	16.00	16.29	16.28	16.35
0.1	17.28	17.74	17.90	18.08
0.03	19.37	19.68	19.82	20.04
0.01	20.80	21.21	21.35	21.25
Efficiency	1.0527356	0.9564201	1.0135384	0.8425983
R2	0.9956	0.9991	0.9974	0.9866

Rpl13a

200nM

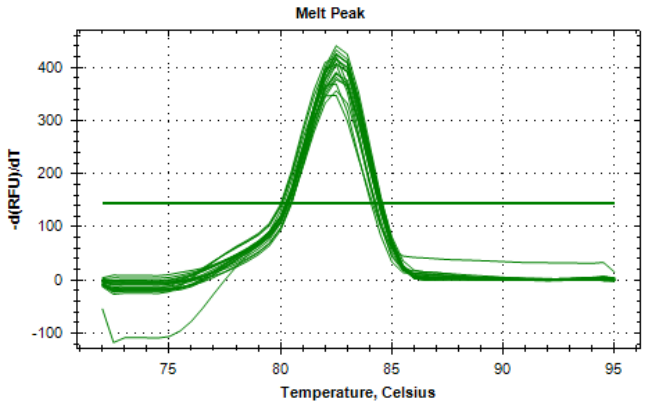


100nM



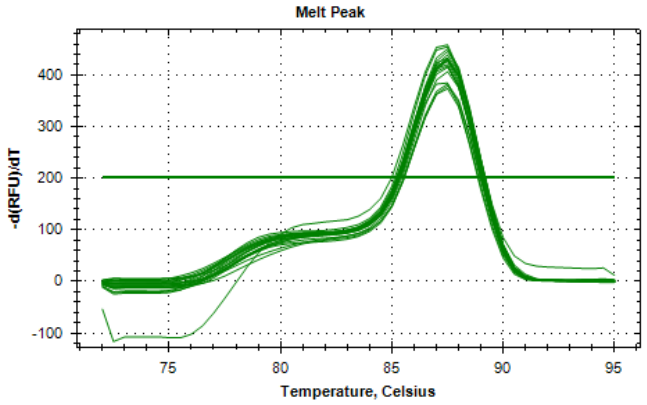
Rpl13a	63.3	61.4	59	57	55.7
1	16.42	16.15	15.98	16.18	16.84
0.3	17.60	17.51	17.70	18.03	18.22
0.1	18.95	18.49	18.63	19.07	19.40
0.03	20.83	20.43	20.50	21.14	21.33
0.01	21.97	21.81	21.86	22.45	22.75
Efficiency	1.2323495	1.2441613	1.2037336	1.0852234	1.1615314
R2	0.9939	0.9914	0.9945	0.9945	0.9949

Hk1



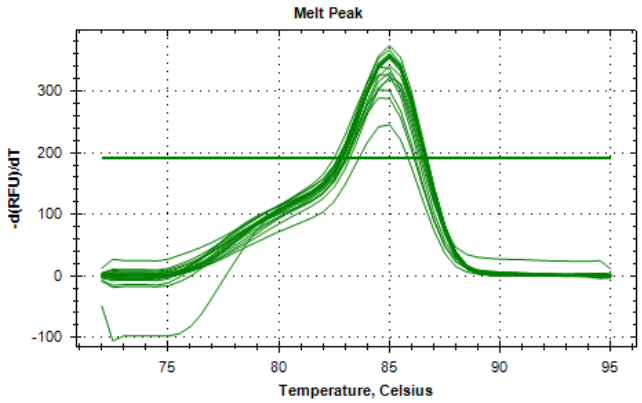
Hkl	61.4	59	57	55.7
1	25.28	21.98	21.59	22.06
0.3	24.37	23.52	23.08	23.37
0.1	25.74	24.67	24.46	24.47
0.03	27.57	26.61	26.22	26.13
0.01	29.06	27.86	27.77	27.63
Efficiency	1.9306367	1.1717975	1.1011962	1.2899785
R2	0.8058	0.9964	0.9986	0.9951

Hnf4a



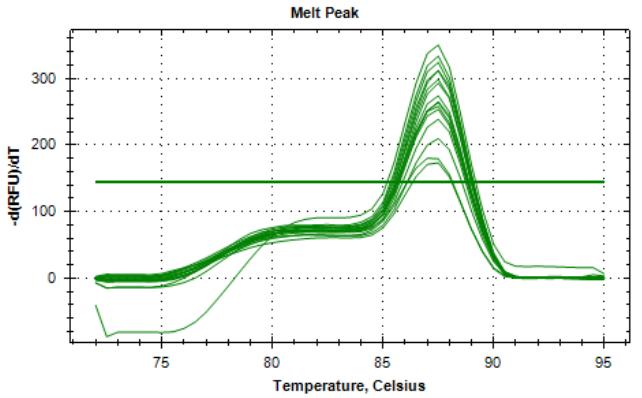
Hnf4a	61.4	59	57	55.7
1	31.23	32.04	32.37	33.19
0.3	34.23	34.77	34.96	35.55
0.1	35.33	35.63	37.41	39.31
0.03	36.91	39.34		39.58
0.01				
Efficiency	0.8935006	0.6623457	0.5794947	0.6639376
R2	0.9586	0.9573	0.999	0.9128

Pdx1



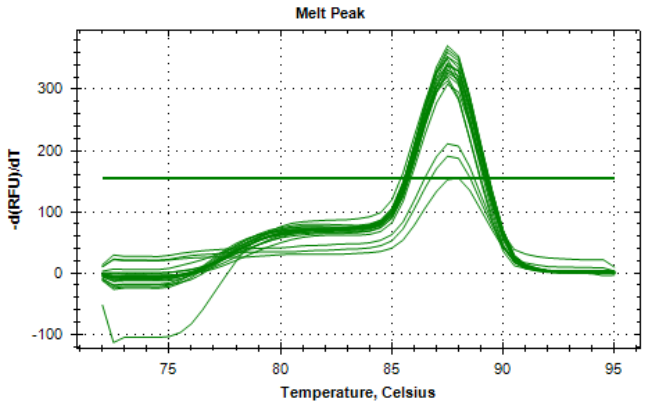
Pdx1	61.4	59	57	55.7
1	20.20	19.96	19.97	20.37
0.3	22.39	21.28	21.42	21.61
0.1	22.82	22.81	22.92	23.08
0.03	24.54	24.53	24.73	24.80
0.01	25.95	26.12	25.90	26.13
Efficiency	1.3212218	1.095499	1.1350287	1.1867001
R2	0.9762	0.9972	0.9976	0.9971

Srebf1



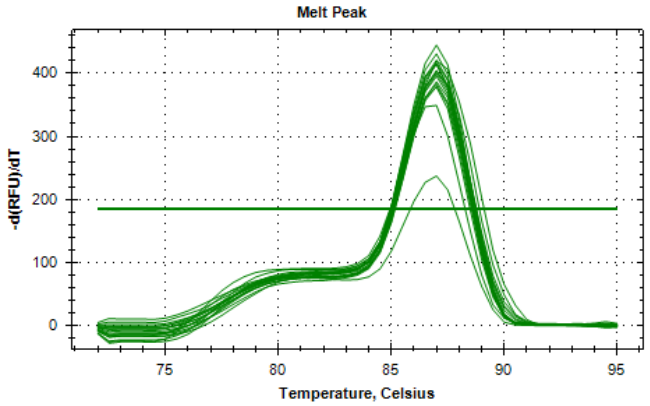
Srebf1	61.4	59	57	55.7
1	27.14	27.46	28.20	28.85
0.3	29.31	30.06	30.62	31.40
0.1	31.60	32.27	33.17	33.34
0.03	33.93	34.14	35.20	35.54
0.01	35.63	35.81	37.82	38.36
Efficiency	0.70331	0.739178	0.6215402	0.6445533
R2	0.998	0.9931	0.9979	0.9958

Ins1



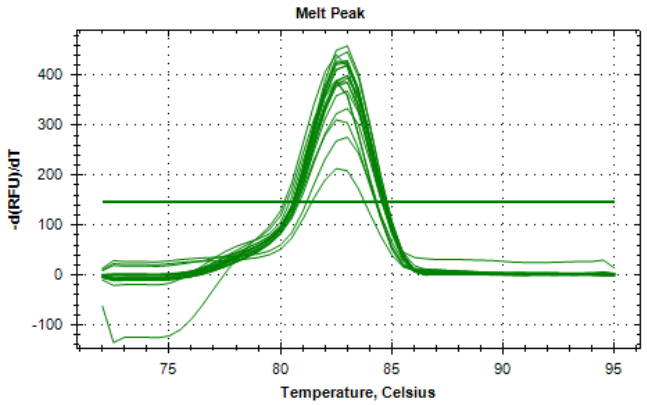
Ins1	64.5	60.9	58	56
1	17.67	15.33	16.02	17.20
0.3	17.55	17.45	18.38	19.42
0.1	20.03	19.20	20.76	21.58
0.03	22.56	21.38	23.22	24.07
0.01	23.39	23.09	24.36	25.95
Efficiency	1.0160538	0.8068622	0.7069122	0.6814312
R2	0.9246	0.9995	0.9888	0.9992

Abcg1



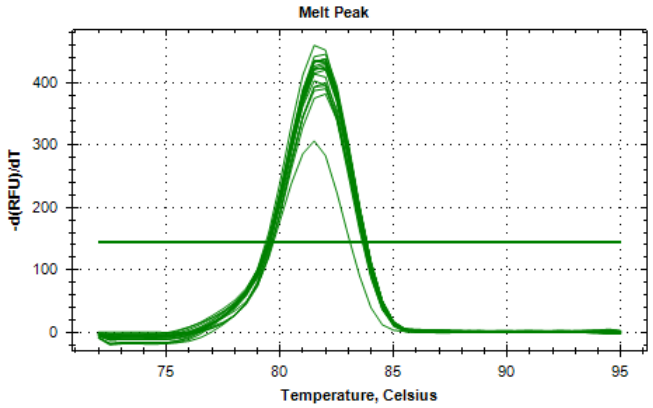
Abcg1	61.4	59	57	55.7
1	22.29	22.54		
0.3	24.17	24.37	24.55	24.65
0.1	25.51	25.93	26.26	26.11
0.03	27.71	27.62	28.28	28.14
0.01	29.06	28.79	29.59	29.47
Efficiency	0.9615034	1.0762592	0.9455305	0.9967133
R2	0.9967	0.9964	0.9957	0.9962

Abca1



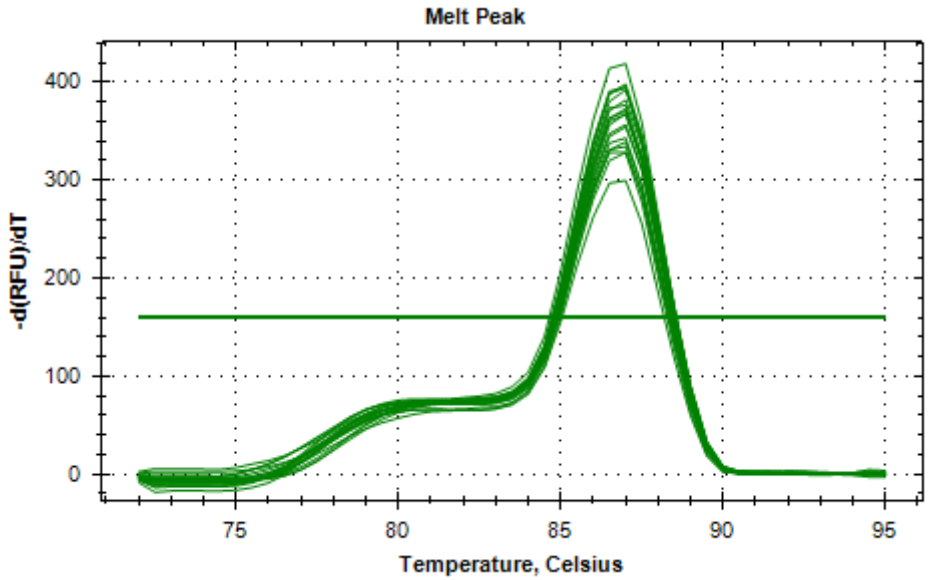
Abca1	61.4	59	57	55.7
1	25.25	24.45	22.88	23.05
0.3	25.45	25.50	24.64	25.02
0.1	27.45	26.13	26.27	26.39
0.03	28.40	28.04	28.40	28.19
0.01	30.06	29.18	29.36	29.27
Efficiency	1.5072278	1.6096618	0.9905646	1.0887878
R2	0.9534	0.9769	0.9925	0.9943

Txnip



Txnip	61.4	59	57	55.7
1	23.86	23.69	23.98	24.18
0.3	25.76	25.98	25.84	26.19
0.1	27.80	27.90	28.01	28.23
0.03	29.65	29.68	30.44	29.92
0.01	31.58	31.70	31.80	32.14
Efficiency	0.8146028	0.792771	0.7657868	0.7967935
R2	0.9993	0.9983	0.9948	0.9978

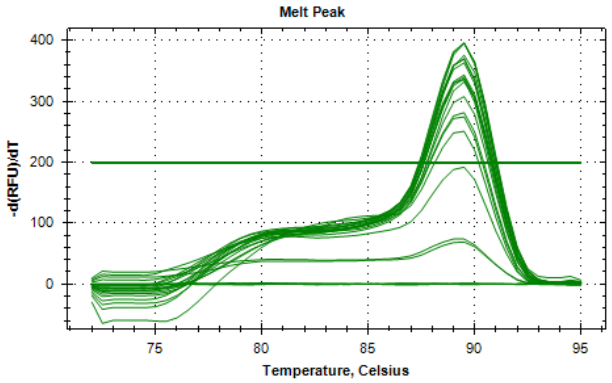
Ldlr



Ldlr	61.4	59	57	55.7
1	23.26	23.18	23.40	23.77
0.3	25.22	25.39	25.76	26.19
0.1	27.35	27.73	28.27	28.28
0.03	29.29	29.53	29.87	30.48
0.01	31.31	31.56	31.73	32.17
Efficiency	0.7709204	0.7352438	0.7411644	0.7262211
R2	0.9993	0.9976	0.9921	0.9977

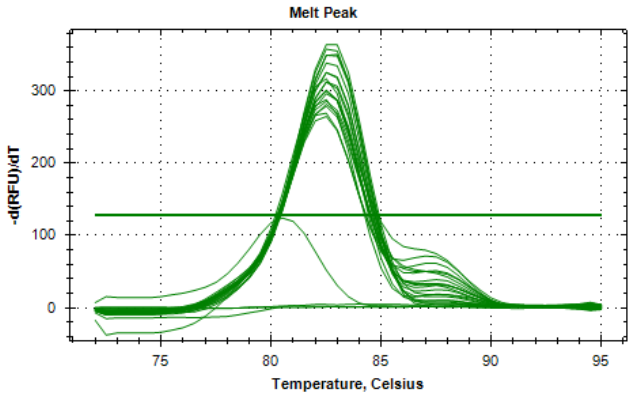
Human primers for ChIP-qPCR of HepG2 cells

GAPDH



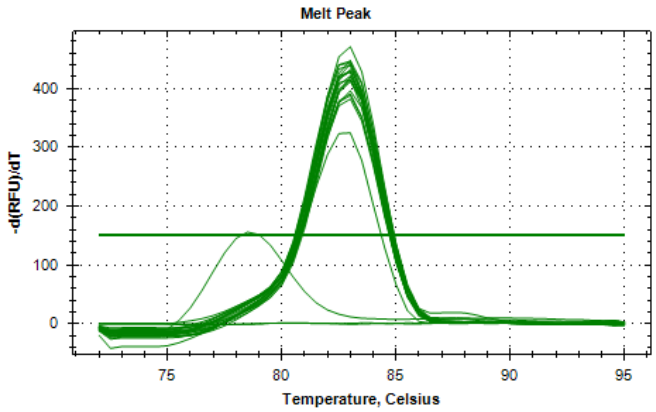
GAPDH	66.4	64	62	60.7	63
1	29.48	29.71	30.16	30.27	25.33
0.3	31.96	31.75	31.68	32.11	27.55
0.1	33.19	33.46	33.24	33.42	29.58
0.03	34.47	35.19	35.59	35.21	30.42
0.01	37.25	39.71	38.7	39.5	33.43
Efficiency	0.893041	0.636267	0.73255	0.708061	0.830286
R2	0.9756	0.9473	0.9716	0.9356	0.9746

HMGCR



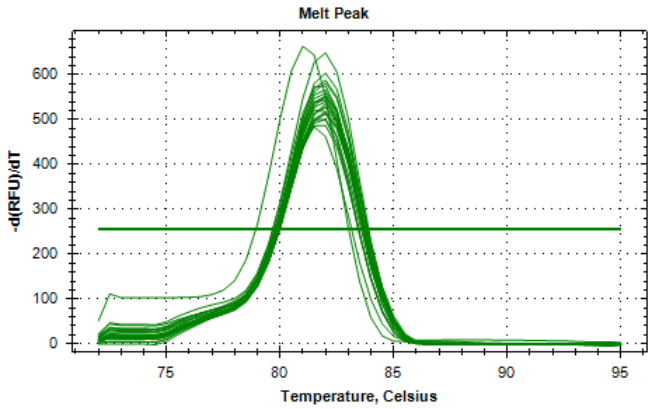
HMGCR	65.4	63	61	59.7
1	23.95	23.87	23.75	23.65
0.3	25.50	25.41	25.30	25.28
0.1	26.86	27.07	27.08	26.76
0.03	28.94	28.74	28.52	28.63
0.01	30.70	30.34	30.12	30.02
Efficiency	0.9743357	1.0301579	1.0569461	1.0438689
R2	1.00	1.00	1.00	1.00

LDLR



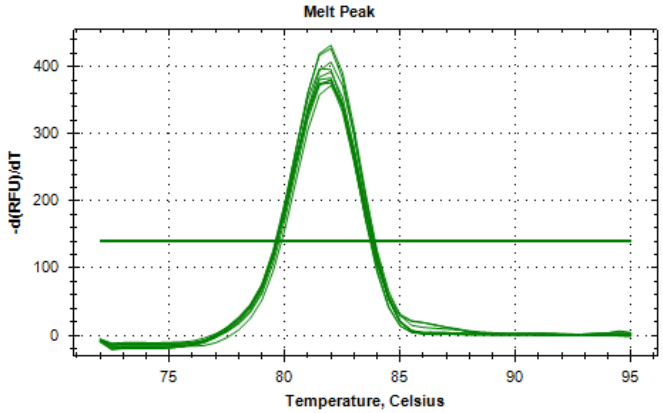
LDLR	65.4	63	61	59.7
1	23.52	23.44	23.53	23.64
0.3	25.13	25.47	25.19	25.30
0.1	26.98	26.69	26.80	26.67
0.03	28.50	28.60	29.06	28.70
0.01	30.02	30.02	30.17	29.82
Efficiency	1.0199225	1.0272733	0.955793	1.0746928
R2	1.00	1.00	0.99	1.00

PPARG



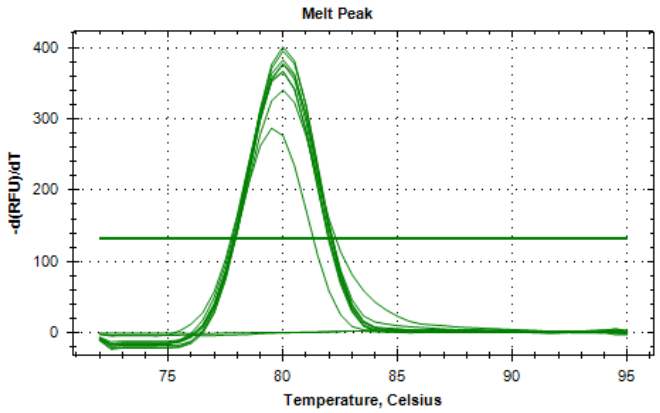
PPARG	65.4	63	61	59.7
1	23.57	23.51	23.52	23.81
0.3	25.15	25.36	25.23	25.41
0.1	26.87	26.80	26.83	27.03
0.03	28.53	28.81	28.60	28.49
0.01	29.78	30.25	30.15	30.04
Efficiency	1.0721559	0.972082	0.9969967	1.0989876
R2	1.00	1.00	1.00	1.00

ABCA1



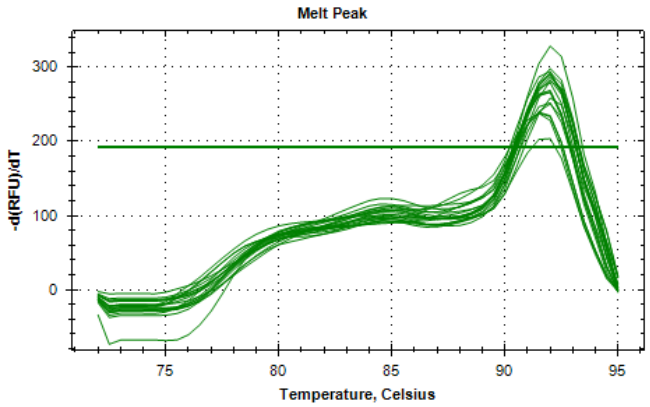
ABCA1	65.4	63	61	59.7
1	24.12	24.27	24.07	24.85
0.3	25.92	25.81	25.92	25.79
0.1	27.58	27.32	27.51	27.17
0.03	29.02	29.27	29.10	29.49
0.01	31.17	30.88	30.34	30.77
Efficiency	0.9534825	0.9937994	1.0775968	1.0984366
R2	1.00	1.00	1.00	0.98

ABCG1



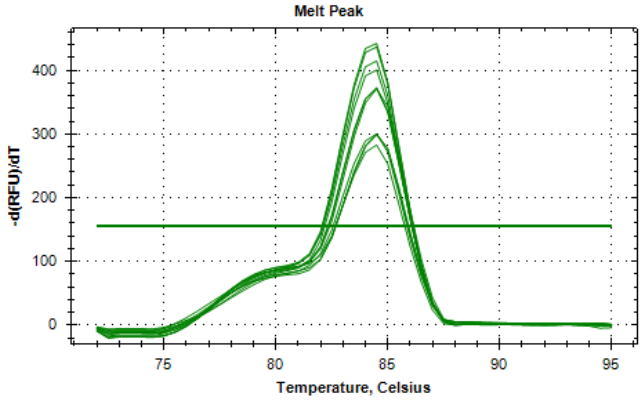
ABCG1	65.4	63	61	59.7
1	24.47	24.58	25.06	24.62
0.3	26.06	26.10	25.83	25.98
0.1	28.36	27.85	27.49	27.34
0.03	29.33	29.41	29.51	29.08
0.01	31.32	31.13	30.66	30.80
Efficiency	0.9716778	1.0161154	1.1702522	1.1085871
R2	0.99	1.00	0.98	1.00

HK1



HKI	66.4	64	62	60.7
1	26.08	25.73	25.38	25.05
0.3	27.53	27.64	26.26	26.36
0.1	29.17	28.88	28.74	27.76
0.03	31.14	31.37	30.50	30.20
0.01	33.82	33.59	32.70	32.24
Efficiency	0.829128	0.8073701	0.842305	0.8820033
R2	0.98	0.99	0.98	0.98

SLC2A2



SLC2A2	65.4	63	61	59.7
1	23.99	23.76	23.57	23.83
0.3	25.29	25.21	25.07	25.23
0.1	26.91	26.62	26.44	26.61
0.03	28.57	28.57	28.47	28.34
0.01	29.73	30.46	29.95	30.01
Efficiency	1.1805951	0.9873649	1.0401237	1.1048289
R2	1.00	0.99	1.00	1.00

Appendix C: Data tables

Table S. 1 Data table for Figure 4-1A

Concentration	Atorvastatin			Simvastatin			Rosuvastatin			Pravastatin		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
5	92.63	2.300	8	87.54	4.851	8	91.62	3.390	8	98.24	5.471	8
10	97.80	4.892	8	87.97	4.953	8	104.9	3.896	8	98.97	6.236	8
50	84.17	2.473	8	74.16	5.072	8	88.25	4.610	8	99.27	2.570	8
100	80.33	11.29	5	71.02	6.851	3	95.82	3.394	5	95.89	3.586	4
200	61.94	8.547	6	37.99	6.606	2	96.10	4.593	6	90.56	3.441	6
500	12.88	4.653	6	3.097	1.084	4	82.80	17.08	5	70.11	5.547	6

Table S. 2 Data table for Figure 4-1B

Concentration	Mean	SEM	N
0	100	1.375553	8
5	105.4508	9.61456	7
10	115.9366	7.958282	7
20	100.7191	4.811202	8

Table S. 3 Data table for Figure 4-2

Control			1mM SB			5mM SB			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
5.88	0.71	5	6.92	1.48	3	6.35	0.87	3	6.12	0.95	3	6.41	0.90	3

Table S. 4 Data table for Fig 4-3A

Control			DMSO control			Sodium Butyrate			Pravastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.923508	0.07685	6	1.084472	0.111021	5	0.635663	0.089083	6	0.72072	0.09910	6

Atorvastatin			Simvastatin			Rosuvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.498427	0.05554	6	0.48819	0.067417	6	0.587117	0.099725	6

Table S. 5 Data table for Figure 4-3B

Control			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.319149	0.056971	9	0.396359	0.077689	8	0.264036	0.049961	10
Rosuvastatin			Sodium butyrate					
Mean	SEM	N	Mean	SEM	N			
0.285854	0.047104	10	0.24715	0.046143	10			

Table S. 6 Data table for Figure 4-3C

Control			DMSO			Sodium Butyrate			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.1822	0.0141	9	0.2015	0.0200	9	0.1955	0.0238	9	0.2331	0.0217	9

Table S. 7 Data table for Figure 4-3D

Control			DMSO			Atorvastatin			Ator + SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.9304	0.2896	5	2.2283	0.2027	6	1.1825	0.0702	5	1.0743	0.1155	5

Table S. 8 Data table for Figure 4-3E

Control			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
108.9469	6.04404	6	104.0208	13.17387	6	74.55724	7.314398	6
Sodium Butyrate			Ator + SB					
Mean	SEM	N	Mean	SEM	N			
87.96054	4.24312	6	69.26368	13.10441	6			

Table S. 9 Data table for Figure 4-3F

Control			1mM Sodium Acetate			5mM Sodium Acetate		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
2.5473	0.146822	9	2.694133	0.091024	9	2.669854	0.193046	9
1uM TSA			2.5uM TSA			TSA + SA		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
2.537053	0.119734	9	2.024237	0.110603	9	2.249391	0.135274	6

Table S. 10 Data table for Figure 4-4A

Control			SB 1mM			SB 5mM		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
81.08217	13.23381	7	70.26502	16.63544	7	85.90672	20.05331	8

Table S. 11 Data table for Figure 4-4B

DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N
66.83944	9.400247	7	67.481	7.298949	6

Table S. 12 Data table for Figure 4-5A

Control			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.138594	7	1.16516	0.338658	7	1.959733	0.469753	8
Sodium Butyrate			U18666A					
Mean	SEM	N	Mean	SEM	N			
1.175793	0.461507	8	2.821978	0.296761	8			

Table S. 13 Data table for Figure 4-5B

Control			DMSO			Atorvastatin			Sodium Butyrate		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.05335	8	1.0306	0.0625	8	1.0082	0.0496	8	0.9339	0.0353	8

Table S. 14 Data table for Figure 4-6A

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.8787	0.1146	3	1.0780	0.21	3	1.122535	0.2474	3

Table S. 15 Data table for Figure 4-6B

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.7989	0.1435	3	0.9098	0.1686	3	1.0751	0.2264	3

Table S. 16 Data table for Figure 4-7A

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	5	0.84523	0.04152	3	0.655183	0.051189	5
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
0.989459	0.118174	4	1.373999	0.173825	5			

Table S. 17 Data table for Figure 4-7B

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	5	0.946458	0.026609	3	0.991058	0.048198	5
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
0.99866	0.125237	5	1.068916	0.152963	5			

Table S. 18 Data table for Figure 4-7C

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	4	0.841128	0.1708	3	0.867912	0.060888	4

DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N
0.989315	0.288271	4	0.913721	0.12886	4

Table S. 19 Data table for Figure 4-7D

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	4	0.594407	0.045973	4	1.053937	0.10377	4	1.433487	0.365412	3

Table S. 20 Data table for Figure 4-7E

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	4	0.659747	0.089939	4	1.110957	0.21698	4	1.574868	0.494242	4

Table S. 21 Data table for Figure 4-7F

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.660057	0.118032	3	1.050204	0.089883	3	1.138781	0.137285	3

Table S. 22 Data table for Figure 4-8A

	Control			1 mM SB			5 mM SB			TSA		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
8 h	1	0	3	0.72662	0.10130	3	0.80361	0.16853	3			
16 h	1	0	3	0.55585	0.13482	3	0.58905	0.16519	3	0.60962	0.0870	3

Table S. 23 Data table for Figure 4-8B

	Control			1 mM SB			5 mM SB			TSA		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
8 h	1	0	3	0.86875	0.15438	3	1.31019	0.60413	3			
16 h	1	0	3	0.76542	0.04133	3	0.67166	0.12489	3	0.58327	0.18924	3

Table S. 24 Data table for Figure 4-8C

Untreated			Sodium Butyrate		
Mean	SEM	N	Mean	SEM	N
1	0.076046	6	0.693246	0.11573	6

Table S. 25 Data table for Figure 4-8D

Untreated			Sodium Butyrate		
Mean	SEM	N	Mean	SEM	N
1	0.096883	6	0.976737	0.267836	5

Table S. 26 Data table for Figure 4-9A

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	5	0.727582	0.115316	3	0.422157	0.056063	5
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
0.999054	0.086362	4	0.558304	0.058791	5			

Table S. 27 Data table for Figure 4-9B

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.318403	0.048596	3	1.084409	0.073697	2	1.514569	0.18513	3

Table S. 28 Data table for Figure 4-9C

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	1.949224	0.303266	3	1.767324	0.144834	3
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
0.903413	0.107551	3	0.469336	0.071727	3			

Table S. 29 Data table for Figure 4-9D

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	5	0.94971	0.007517	3	0.767369	0.059091	5
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
0.86024	0.10773	5	1.065901	0.096204	5			

Table S. 30 Data table for Figure 4-9E

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.795773	0.07447	3	1.037112	0.01734	2	0.824361	0.07919	3

Table S. 31 Data table for Figure 4-9F

Control			Sodium Butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	1.654262	0.25269	3	1.115571	0.15223	3	1.038203	0.23389	3

Table S. 32 Data table for Figure 4-10

	Control			1 mM SB			5 mM SB			TSA		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
8 h	1	0	3	0.616607	0.07379	3	0.523082	0.10350	3			
16 h	1	0	3	0.504396	0.09811	3	0.296376	0.06969	3	0.39140	0.1374	3

Table S. 33 Data table for Figure 4-11A

ABCG1			CAV1			SREBF1			MVK		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
668.3042	171.426	3	163.329	43.3000	3	15.39177	2.3664	3	3.07924	1.58021	3
SCARB1			APOB			APOA1			HMGCR		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
2.575575	0.09559	4	1.997781	0.88145	3	1.984166	0.14157	3	1.56235	0.5659	3
LDLR			NR1H3			SREBF2			ABCA1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.217397	0.38314	4	1.194207	0.10248	3	0.887693	0.09144	4	0.19922	0.0303	4
CYP7A1											
Mean	SEM	N									
0.051334	0.01367	3									

Table S. 34 Data table for Figure 4-11B

HMGCR			MVK			APOB			LDLR		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
2.163681	0.54228	3	1.637362	0.18192	3	1.456659	0.7207	3	1.340921	0.43170	3
APOA1			SCARB1			SREBF2			CAV1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.197124	0.14569	3	1.031221	0.24284	3	0.912963	0.1326	3	0.882503	0.17505	3
ABCA1			CYP7A1			ABCG1			SREBF1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.839699	0.13737	3	0.819803	0.02644	3	0.594524	0.2546	3	0.537201	0.04215	3

Table S. 35 Data table for Figure 4-11C

Abca1			Abcg1			Srebf1			Ldlr		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
7.0290	1.0536	3	2.3227	0.1839	3	1.9528	0.3383	3	0.2267	0.0524	3

Table S. 36 Data table for Figure 4-11D

Ldlr			Srebf1			Abcg1			Abca1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
2.7587	0.5323	3	1.0508	0.3389	3	0.27059	0.0562	3	0.0952	0.0193	3

Table S. 37 Data table for Figure 4-12A

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.714237	0.249601	3	0.947624	0.056863	3
DMSO			Atorv					
Mean	SEM	N	Mean	SEM	N			
1.018796	0.063817	3	1.121307	0.097899	3			

Table S. 38 Data table for Figure 4-12B

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.7846	0.015367	3	1.228377	0.338997	3
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
0.987154	0.120969	3	0.662959	0.238234	3			

Table S. 39 Data table for Figure 5-1A

	Chronic			Stimulated		
	Mean	SEM	N	Mean	SEM	N
Control	218.3954	21.82505	9	1013.544	112.7708	9
1 mM SB	170.0649	22.69239	9	1288.458	126.3635	9
5 mM SB	94.55493	14.41834	9	390.4675	43.45239	9

Table S. 40 Data table for Figure 5-1B

	Chronic			Stimulated		
	Mean	SEM	N	Mean	SEM	N
Control	206.262	26.21946	9	1013.544	112.7708	9
TSA	88.61675	21.22867	9	177.4669	39.6835	9

Table S. 41 Data table for Figure 5-1C

Control			1 mM SB			5 mM SB			TSA		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
637.3141	92.3387	9	461.7518	83.4407	9	334.4585	52.4280	9	186.8659	14.4519	9

Table S. 42 Data table for Figure 5-1D

Control			1 mM SB			5 mM SB			TSA		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
3.716685	0.70238	9	6.850159	0.97249	9	2.56137	0.36144	9	1.745774	0.30346	9

Table S. 43 Data table for Figure 5-2A

	Basal			Stimulated		
	Mean	SEM	N	Mean	SEM	N
Control	4.902342	0.332749	9	20.27087	2.255416	9
5 mM SB	6.150682	0.504484	9	26.36796	3.230384	9

Table S. 44 Data table for Figure 5-2B

Control			5 mM SB		
Mean	SEM	N	Mean	SEM	N
20.27087	2.255416	9	22.7241	2.049333	9

Table S. 45 Data table for Figure 5-3

pIRS1	Control			Sodium butyrate			DMSO			Atorvastatin		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Unstimulated	1	0	4	1.3729	0.2634	4	0.8253	0.1640	4	1.1697	0.2739	4
Insulin stimulated	1.7829	0.5930	4	1.7669	0.6572	4	1.4467	0.1098	4	2.6732	0.9892	4
pIRB	Control			Sodium butyrate			DMSO			Atorvastatin		
Unstimulated	1	0	3	1.0251	0.1993	3	1.1349	0.1649	3	0.8930	0.3080	3
Insulin stimulated	1.2153	0.3081	3	0.8496	0.2245	3	1.3579	0.4763	3	1.7021	0.4609	3
pAKT	Control			Sodium butyrate			DMSO			Atorvastatin		
Unstimulated	1	0	3	0.6457	0.0774	3	1.0303	0.1289	3	0.9559	0.0539	3
Insulin stimulated	1.6149	0.4924	3	0.7272	0.1061	3	1.1741	0.1147	3	0.9469	0.2327	3

Table S. 46 Data table for Figure 4-4A

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	1.553009	0.260041	3	2.224221	0.346369	3
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
1.087269	0.144291	3	1.068166	0.074624	3			

Table S. 47 Data table for Figure 4-4B

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	1.44517	0.270056	3	1.320628	0.176685	3
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
1.167706	0.308929	3	1.053607	0.190113	3			

Table S. 48 Data table for Figure 4-4C

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.576051	0.158942	3	0.749055	0.222725	3
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
0.704479	0.300398	3	0.760322	0.249118	3			

Table S. 49 Data table for Figure 4-4D

Control			5 mM SB			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.91124	0.110143	3	1.088658	0.022506	2	1.26797	0.141355	3

Table S. 50 Data table for Figure 4-5A

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	1.680099	0.17753	3	3.001881	1.068331	3
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
1.324455	0.306324	3	1.652686	0.178387	3			

Table S. 51 Data table for Figure 4-5B

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.760989	0.126105	3	0.811917	0.128182	3
DMSO			Atorvastatin					
Mean	SEM	N	Mean	SEM	N			
1.262924	0.21487	3	1.054274	0.212314	3			

Table S. 52 Data table for Figure 4-6

	Control			1 mM SB			5 mM SB			TSA		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
8 h	1	0	3	1.804797	0.72871	3	2.198968	0.76037	3			
16 h	1	0	3	3.100068	1.59447	3	4.746619	3.02747	3	6.464769	4.6509	3

Table S. 53 Data table for Figure 5-7A

ABCG1			HK1			CAV1			SREBF1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
668.3042	171.426	3	222.415	93.9468	4	163.329	43.3000	3	15.39177	2.3664	3
GLUT2			CPT1A			MVK			SCARB1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
4.603193	0.53638	3	3.410493	0.14747	3	3.079249	1.58021	3	2.575575	0.0955	4
APOB			APOA1			PPARG			HMGCR		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.997781	0.88145	3	1.984166	0.14157	3	1.685258	0.17573	3	1.562352	0.5659	3
LDLR			NR1H3			FOXO1			SREBF2		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.217397	0.38314	4	1.194207	0.10248	3	1.011749	0.05941	2	0.887693	0.09144	4
PEPCK			CEBPA			INSR			HNF4A		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.69026	0.08299	3	0.669441	0.06776	3	0.522486	0.08699	3	0.47728	0.25262	3
GK			ABCA1			CYP7A1					
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N			
0.376628	0.07832	3	0.199221	0.03030	4	0.051334	0.01367	3			

Table S. 54 Data table for Figure 5-7B

Hnf4a			Txnip			Abca1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
34.41935	14.4156	3	9.493874	3.038801	3	7.029065	1.053698	3
Hk1			Abcg1			Srebf1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
2.861145	0.466113	3	2.322731	0.183997	3	1.952818	0.338361	3
Pdx1			Ins			Ldlr		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.560767	0.355013	3	1.515524	0.211544	3	0.226792	0.052465	3

Table S. 55 Data table for Figure 5-7C

PPARG			HMGCR			MVK			APOB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
7.115879	0.95227	3	2.163681	0.54228	3	1.637362	0.18192	3	1.456659	0.7207	3
LDLR			CPT1A			APOA1			PEPCK		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.340921	0.43170	3	1.213748	0.03436	3	1.197124	0.14569	3	1.19407	0.24131	3
HNF4A			GK			NR1H3			CEBPA		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.218385	0.54250	3	0.982249	0.03704	3	0.970094	0.00620	2	1.043452	0.09026	3
SCARB1			FOXO1			INSR			SREBF2		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.031221	0.24284	3	1.028486	0.04348	2	0.927445	0.36718	3	0.912963	0.13260	3
CAV1			ABCA1			GLUT2			CYP7A1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.882503	0.17505	3	0.839699	0.13737	3	0.834456	0.13281	3	0.819803	0.02644	3
ABCG1			SREBF1			HK1					
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N			
0.594524	0.2546	3	0.537201	0.04215	3	0.516951	0.38163	3			

Table S. 56 Data table for Figure 5-7D

Ldlr			Hk1			Srebf1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
2.758786	0.532392	3	1.160061	0.224709	3	1.050852	0.33897	3
Pdx1			Ins			Txnip		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.962424	0.059743	3	0.870342	0.100955	3	0.872894	0.211935	3
Hnf4a			Abcg1			Abca1		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.565406	0.086168	3	0.270599	0.056288	3	0.095209	0.01934	3

Table S. 57 Data table for Figure 6-1A

	Sodium Butyrate		
Concentration	Mean	SEM	N
0	100	0.961637	3
0.1	78.72977	16.19318	3
0.5	42.61836	4.544317	3
1	32.8029	1.065968	3
5	25.24123	4.056318	3

Table S. 58 Data table for Figure 5-1B

Control			DMSO			Atorvastatin			Simvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.03977	6	1.104573	0.05643	5	1.220747	0.08595	6	1.198252	0.05247	6
Rosuvastatin			Pravastatin			Sodium Butyrate			Trichostatin A		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.08882	0.06367	6	1.295094	0.14316	6	0.327732	0.04628	6	-0.01126	0.02055	6

Table S. 59 Data table for Figure 5-1C

	Sodium Butyrate		
Concentration	Mean	SEM	N
0	100	0	3
0.05	67.82084	9.012196	3
0.1	46.24424	10.80396	3
0.5	22.72317	4.996715	3
1	16.14158	3.794745	3
5	9.260247	3.908096	3

Table S. 60 Data table for Figure 5-1D

Control			Atorvastatin			Pravastatin			TSA		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.012439	6	0.985926	0.024931	6	1.018173	0.041494	6	0.036571	0.022419	5

Table S. 61 Data table for Figure 5-2

	Atorvastatin		
Concentration	Mean	SEM	N
0	1	0	3
5	1.143464	0.11431	5
10	1.116016	0.053551	6
50	1.050396	0.101692	6

Table S. 62 Data table for Figure 6-3A

	Sodium Butyrate		
Concentration	Mean	SEM	N
0	100	0	3
0.1	69.72656	2.732904	3
0.25	61.80136	7.56832	2
0.5	50.63009	6.328042	3
1	30.52603	5.587201	3
5	12.62711	1.864643	3

Table S. 63 Data table for Figure 6-3B

Control			Atorvastatin			Rosuvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.052643	8	0.905545	0.053925	6	0.972426	0.073523	5
Pravastatin			Simvastatin			Sodium Butyrate		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.971606	0.069404	5	0.928791	0.08354	4	0.125745	0.018713	3

Table S. 64 Data table for Figure 6-3C

	Atorvastatin		
Concentration	Mean	SEM	N
1	0.854076	0.037438	5
10	0.905545	0.053925	6
50	0.875636	0.072929	5

Table S. 65 Data table for Figure 6-3D

Control			Atorvastatin			Rosuvastatin			Pravastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.03296	10	0.997812	0.03693	6	1.098003	0.03306	6	1.055965	0.03041	6
Atorvastatin 50uM			Sodium Butyrate			Trichostatin A					
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N			
0.918106	0.03744	6	0.272226	0.04164	5	-0.00002	0.00290	5			

Table S. 66 Data table for Figure 6-4

	HepG2			HeLa		
	Mean	SEM	N	Mean	SEM	N
Control	1	0	3	1	0	3
DMSO	0.894935	0.053136	3	1.054556	0.018282	3
Atorvastatin	0.950889	0.024353	3	1.05843	0.022441	3
Pravastatin	1.000319	0.005347	3	1.120982	0.010564	3
Sodium butyrate	0.452774	0.039868	3	0.502634	0.037546	3
Trichostatin A	0.044317	0.040273	3	0.051373	0.059027	3

Table S. 67 Data table for Figure 6-5

V-ND			V-HFD			A-ND			A-HFD		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.955623	0.325894	3	0.99469	0.046384	3	0.974464	0.225863	3

Table S. 68 Data table for Figure 6-6

DMSO			Curcumin			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.182752	5	0.285028	0.056433	5	0.938461	0.14097	5

Table S. 69 Data table for Figure 6-7A

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	5	2.000413	0.462792	3	8.863314	2.468012	5
DMSO			Atorvastatin			Curcumin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.771394	0.149974	5	1.249411	0.658782	5	0.914562	0.11411	3

Table S. 70 Data table for Figure 6-7B

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	18.14247	12.12224	3	115.8061	84.04357	3
DMSO			Atorvastatin			Curcumin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
0.734829	0.300808	3	1.056317	0.510828	3	1.66294	0.809463	3

Table S. 71 Data table for Figure 6-7C

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.947585	0.288335	3	0.733673	0.182408	3
DMSO			Atorvastatin			Curcumin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.064864	0.35665	3	2.044735	1.09324	3	1.10677	0.160821	3

Table S. 72 Data table for Figure 6-7D

Control			1 mM SB			5 mM SB		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	0.877485	0.059914	3	0.710059	0.071135	3
DMSO			Atorvastatin			Curcumin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1.091229	0.221728	3	1.039188	0.344277	3	0.805363	0.211712	3

Table S. 73 Data table for Figure 6-8A

Control			Sodium butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	6.295907	1.172002	3	1.489678	0.582385	3	1.369342	0.471931	3

Table S. 74 Data table for Figure 6-8B

Control			Sodium butyrate			DMSO			Atorvastatin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	3	2.254542	0.051935	3	0.777882	0.168929	3	0.674292	0.157879	3

Table S. 75 Data table for Figure 6-9A

Control			Atorvastatin			RG108			Curcumin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0	4	1.243168	0.142191	4	0.74815	0.085685	4	0.35475	0.077943	4

Table S. 76 Data table for Figure 6-9B

Control			DMSO			Atorvastatin			Curcumin		
Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	0.022094	4	0.778467	0.120491	3	0.926802	0.242326	4	0.632198	0.270369	4

Table S. 77 Data table for Figure 6-10A

	Control			Atorvastatin		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	17.42667	0.646022	3	21.72333	2.807266	3
H4K8ac	4.17	0.912798	3	8.853333	0.834293	3
Beads only	0.196667	0.098206	3	0.463333	0.13776	3

Table S. 78 Data table for Figure 6-10B

	Control			Atorvastatin		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	23.41	1.718206	3	25.1	6.351795	3
H4K8ac	4.886667	1.309317	3	8.516667	1.190803	3
Beads only	0.17	0.045092	3	0.26	0.09609	3

Table S. 79 Data table for Figure 6-10C

	Control			Atorvastatin		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	18.11667	2.727088	3	24.62333	7.75242	3
H4K8ac	4.87	0.670149	3	9.566667	3.110757	3
Beads only	0.183333	0.061734	3	0.523333	0.078811	3

Table S. 80 Data table for Figure 6-10D

	Control			Atorvastatin		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	27.82333	10.42558	3	26.24333	12.07619	3
H4K8ac	3.863333	0.758339	3	5.896667	2.012008	3
Beads only	0.216667	0.056667	3	0.25	0.087369	3

Table S. 81 Data table for Figure 6-10E

	Control			Atorvastatin		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	15.91333	6.25713	3	12.38333	3.029331	3
H4K8ac	3.953333	2.242456	3	3.98	0.661085	3
Beads only	0.203333	0.088757	3	0.25	0.091652	3

Table S. 82 Data table for Figure 6-10F

	Control			Atorvastatin		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	19.65111	1.339233	9	23.81556	3.05049	9
H4K8ac	4.642222	0.513479	9	8.978889	1.003258	9
Beads only	0.183333	0.036132	9	0.415556	0.066752	9

Table S. 83 Data table for Figure 6-11A

	Control			Butyrate		
	Mean	SEM	N	Mean	SEM	N
GAPDH	22.9225	4.669926	4	62.2825	14.5707	4
HK1	26.31	5.510706	4	86.7425	12.08141	4
ABCG1	3.8075	1.476947	4	10.5625	2.040631	4
ABCA1	19.1475	5.476867	4	59.2325	9.625128	4
GLUT2	3.085	0.898429	4	7.7625	0.779694	4
HMGCR	12.18	2.290986	4	27.285	1.916148	4
LDLR	17.3575	6.124379	4	54.2725	9.440125	4
PPARG	11.4675	2.137886	4	49.0375	10.1554	4

Table S. 84 Data table for Figure 6-11B

	Control			Butyrate		
	Mean	SEM	N	Mean	SEM	N
H3	14.53469	1.901658	32	44.64719	5.38298	32
H3K9ac	23.52063	2.133185	32	65.59625	7.366463	32
H4K8ac	5.385938	0.565206	32	31.17688	3.124793	32
H3K9me3	14.75094	1.425563	32	34.64969	2.75697	32
Beads only	0.299375	0.09317	32	0.287813	0.046265	32

Table S. 85 Data table for Figure 6-12

	Control			Butyrate		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	2.170474	0.191635	32	2.062987	0.242709	32
H4K8ac	0.500303	0.048381	32	1.033261	0.120515	32
H3K9me3	1.425377	0.184921	32	1.135229	0.136204	32

Table S. 86 Data table for Figure 6-13A

	Control			Butyrate		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	3.082598	0.585862	4	3.783811	0.562843	4
H4K8ac	0.533706	0.134205	4	2.072116	0.236257	4
H3K9me3	2.472461	0.704868	4	2.308554	0.417101	4

Table S. 87 Data table for Figure 6-13B

	Control			Butyrate		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	2.187561	0.294915	4	1.937312	0.420533	4
H4K8ac	0.537339	0.109476	4	1.056104	0.1439	4
H3K9me3	1.478608	0.383547	4	1.297084	0.237913	4

Table S. 88 Data table for Figure 6-13C

	Control			Butyrate		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	2.560523	0.470258	4	4.078429	1.241096	4
H4K8ac	1.634905	0.341974	4	5.414223	1.846206	4
H3K9me3	3.031282	0.870822	4	4.625379	1.244075	4

Table S. 89 Data table for Figure 6-13D

	Control			Butyrate		
	Mean	SEM	N	Mean	SEM	N
H3K9ac	1.940387	0.386981	4	1.79002	0.304871	4
H4K8ac	1.823381	0.463461	4	2.53088	0.734037	4
H3K9me3	1.708368	0.353384	4	2.504702	0.672459	4

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