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Abstract:

The incidence of obesity, type 2 diabetes and cardiovascular disease (CVD) is increasing at alarming rates worldwide. Obesity is associated with a chronic nutrient surplus that contributes to chronic low-grade inflammation, ectopic lipid deposition and insulin resistance. Insulin resistance is an important factor contributing to the development of both type 2 diabetes and CVD. Therefore, therapies that can address multiple aspects of cardio-metabolic diseases could have significant clinical utility to reduce morbidity and mortality associated with these conditions. Several distinct glucose lowering therapies have been developed, targeting unique molecular targets. Interestingly, three district glucose lowering therapies, metformin, canagliflozin and salsalate have been shown to potently activate the central energy regulating enzyme, AMP activated protein kinase (AMPK). Activation of AMPK has been shown to be important for regulating fatty acid and cholesterol synthesis, fatty acid oxidation, glucose homeostasis, inflammation and whole-body energy expenditure. Therefore, the objective of this thesis was to examine the effects of metformin, canagliflozin and salsalate, on obesity, atherosclerosis, hepatic lipid metabolism, and macrophage inflammatory signalling and to delineate the mechanism(s) by which these changes occur. In this thesis we show that metformin reduces obesity through a circulating hormone GDF15, and that AMPK is not required for metformin induced GDF15 secretion. Additionally, we show that canagliflozin reduces hepatic cholesterol synthesis and macrophage IL1-1β secretion through mechanisms requiring AMPKβ1. Lastly, we show that salsalate reduces atherosclerosis in a manner dependent on macrophage AMPK β1 and this is associated with reduced macrophage proliferation in vitro and in vivo. These insights into the mechanisms by which these glucose lowering therapies elicit beneficial effects on obesity and atherosclerosis further our understanding

of the potential use of these agents for treatments beyond improved glycemic control. Furthermore, this evidence can direct future drug development or drug combinations to more effectively treat multiple aspects of these common chronic diseases that affect over a billion people worldwide.

Thesis Publications:

Day, E. A., Ford, R. J., & Steinberg, G. R. *(*2017*).* AMPK as a therapeutic target for treating metabolic diseases. *Trends in Endocrinology & Metabolism, 28(8), 545-560.*

Day, E. A. *et al*. Metformin-induced increases in GDF15 are important for suppressing appetite and promoting weight loss. *Nature Metabolism* (2019) doi:10.1038/s42255-019-0146-4.

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Abbreviations:

ABC - ATP-binding cassette

ACC - Acetyl-CoA carboxylase activity

ACLY - ATP Citrate Lyase

ACSVL1 - Very long chain acyl-CoA synthase

ADP - Adenosine Diphosphate

AICAR - 5-Aminoimidazole-4-carboxamide ribonucleotide

AMP - Adenosine Monophosphate

AMPK - AMP activated protein kinase

ANOVA - Analysis of Variance

ApoB - Apolipoprotein B

ApoE - Apolipoprotein E

ATF4 - Activating transcription factor 4

ATGL - Adipose triglyceride lipase

ATMs - Adipose Tissue Macrophages

ATP- Adenosine Triphosphate

BAT - Brown Adipose Tissue

BBR - Berberine

BMDMs - Bone marrow derived macrophages

BMI - Body Mass Index

CHOP - C/EBP homology protein

CPT1 - Carnitine palmitoyl transferase 1

CRP - C Reactive Protein

CVD - Cardiovascular Disease

DNL - De Novo Lipogenesis

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

DPP - Diabetes prevention program

DPS - Diabetes Prevention Study

ER - Endoplasmic Reticulum

FAO - Fatty Acid Oxidation

G6Pase - Glucose-6-phosphatase

GDF15 - Growth Differentiation factor 15

GFRAL - GDNF family receptor α–like

GLP-1 - Glucagon-like peptide-1

GSKβ - Glycogen synthase kinase β

GTT - Glucose Tolerance Test

H & E - Hematoxylin and Eosin

HbA1C - Hemoglobin A1C

HCC - Hepatocellular carcinoma

HDAC - Histone deacetylases

HDL - High density lipoprotein

HFD – High Fat Diet

HGP - Hepatic Glucose Production

HMGR - HMG-CoA Reductase

HSL - Hormone Sensitive Lipase

IKKβ - IκB kinase beta

IL-1β - Interleukin 1 beta

IL-6 - Interleukin 6

IL-18 - Interleukin 18,

IL-23 - Interleukin 23

IMCL - Intramyocellular Lipid

JNK - c-Jun N-terminal kinases

KO - Knock Out

LDL - Low density lipoprotein

LDLr – Low density Lipoprotein receptor

LPS – Lipopolysaccharide

LXR - Liver X receptor

MFF - Mitochondrial fission factor

mGPD - Mitochondrial glycerol-3-phosphate

MKP1 - MAP kinase phosphatase-1

mRNA - Messenger RNA

mTOR - Mammalian target of rapamycin

mTORC1 - Mammalian target of rapamycin complex 1

NAFLD - Non-alcoholic fatty liver disease

NASH - Mon-alcoholic steatohepatitis

NFκB - Nuclear factor kappa-light-chain-enhancer of activated B cells

NLRP3 - NLR Family Pyrin Domain Containing 3

PBS - Phosphate buffered saline

PCOS - Poly-cystic ovary syndrome

PCSK9 - Proprotein convertase subtilisin/kexin type 9 i

PEPCK - Phosphoenolpyruvate kinase

PGC1α - PPAR-γ 1 co-activator

PIAS1 - Protein Inhibitor Of Activated STAT 1

PLIN2 - Perilipin-2

PPAR - Peroxisome proliferator-activated receptor

RALDH2 - Retin ALDH type 2

RER - Respiratory Exchange Ratio

RNA - Ribonucleic acid

RT-qPCR - Real-time quantitative PCR

SGLT2i - Sodium-glucose cotransporter 2 inhibitors

siRNA - Small interfering RNA

SIRT1 - Sirtuin 1

SREBP1 - Sterol regulatory element-binding transcription factor 1

STAT1 - Signal Transducer And Activator Of Transcription 1

STAT3 - Signal Transducer And Activator Of Transcription 3

T2D - Type 2 Diabetes

TG - Triglycerides

TGFβ - Transforming growth factor beta

TNFα - Tumor necrosis factor alpha

UKPDS - UK Prospective Diabetes Study

ULK1 - unc-51-like autophagy activating kinase 1

UCP-1 - Uncoupling protein 1

VLDL - Very low density lipoprotein

VSMC - Vascular smooth muscle

WT - Wildtype

1. Introduction:

1.1 Obesity, type 2 diabetes and cardiovascular disease

Obesity is a progressive chronic disease defined as body mass index (BMI, weight in kilograms divided by the square of height in meters) of >30. It is estimated that 2.88 billion adults are overweight and obese, which is an increase from 25.2% of the population to 38.9% of the population in the last 3 decades[1]. In Canada 29.4% of adults are obese while 34.8% are overweight (BMI 25-30)[1]. Obesity is a result of a chronic positive energy balance, meaning more calories consumed than calories expended. Factors contributing to positive energy balance include; increasingly sedentary lifestyles, with both leisure-time and occupational physical activities being replaced with sedentary activities, an increase in the availability of calorically dense food, served in larger potion sizes and an increase in per capita food supplies[2]. Other factors that are also contributing to the obesity epidemic include inadequate sleep, use of medicines that have weight gain as a side effect, prolonged lifespan as a result of reduced mortality from infectious diseases and genetics[2].

BMI generally increases slowly overtime with increases in adipose tissue mass contributing to most of the weight gain, however, ectopic lipid accretion also occurs in the liver, skeletal muscle and pancreas, where lipotoxicity impairs metabolic function of these organs which can result in insulin resistance[3]. Obesity is also associated with a state of chronic low-grade inflammation, potentially related to ectopic lipid accretion. Obesity induced inflammation can occur via several mechanisms. First, lipids can directly modulate immune responses. Specifically, the PPAR and LXR families of transcription factors are crucial for lipid modulation of immune function. Both of these receptor families downregulate NFκB and other inflammatory cytokines[4]. Second recruitment of monocytes and macrophages to adipose tissue during obesity is largely focused around necrotic

adipocytes suggesting that the death of adipocytes may lead to increased macrophage accumulation. These recruited macrophages then secrete inflammatory cytokines leading to the chronic inflammation and metabolic dysfunction seen with obesity[4]. Third, macrophages infiltrate other metabolic tissues including, liver, skeletal muscle and vasculature contributing to insulin resistance[5,6]. Given the systemic changes that occur in response to obesity, it is not surprising that obesity is a leading risk factor for development of type 2 diabetes (T2D) and cardiovascular disease (CVD)[7]

T2D is characterized by elevated levels of blood glucose due to either insufficient insulin supply from the pancreas or an inability of the body to respond to the insulin that is produced or a combination of these two deficiencies. Paralleling the rapid rise in rates of obesity, T2D is now one of the most common diseases worldwide, estimated to effect 422 million people, a prevalence of 8.5% of the global adult population[8]. Pre-diabetes is a practical term which refers to individuals at a high risk of developing diabetes and its complications, who have impaired fasting glucose, impaired glucose tolerance or an increased glycated hemoglobin A1C (HbA1C)[9]. In healthy individuals, following a meal, blood glucose rises due to the absorption of nutrients through the intestine. This increase in blood glucose is sensed by the β-cells of the pancreas, which respond by secreting insulin. Insulin acts to promote glucose uptake in the muscle, inhibit adipose tissue lipolysis and inhibit glucose production/secretion from the liver[10]. Insulin resistance in these metabolic tissues requires that the pancreas secrete more insulin in order to maintain glucose homeostasis[11].

The causes of insulin resistance are multifactorial and have been reviewed in detail previously therefore only a brief discussion of some of the potential mechanisms will be discussed here. Skeletal muscle insulin resistance has been shown to be closely associated

with intramyocellular lipid (IMCL) content which results in impaired glucose uptake[10]. Post-prandially, if muscle tissue is unable to take up glucose, it is used by the liver as a substrate for hepatic de novo lipogenesis (DNL)[12]. Increased hepatic lipid accumulation specifically, hepatic diacylglycerol content (an intermediate in triglyceride synthesis) is associated with insulin resistance[13–16].

With obesity adipose tissue also becomes resistant to insulin and this results in elevated lipolysis, leading to increased substrates (fatty acids and glycerol) for the liver that collectively contributes to sustained hepatic glucose production (HGP) post-prandially. Therefore, adipose tissue insulin resistance appears to effect whole body glucose metabolism not through impaired glucose uptake, because insulin stimulated glucose uptake in adipose tissue has a relatively small contribution to blood glucose, but through altered release of substrates and signalling molecules[10] which then alter liver and muscle metabolism. Adipose tissue insulin resistance, and therefore an inability to supress lipolysis has been closely linked with inflammation, and specifically increased adipose tissue macrophages (ATMs). Indeed, pro-inflammatory cytokines, including but not limited to TNFα, have been shown to directly impair adipose tissue insulin sensitivity[17,18]. In addition to secretion of cytokines, several inflammatory pathways have been shown to be upregulated in response to obesity and insulin resistance, including JNK and IKKβ, and inhibition of these pathways has been shown to improve insulin senstivity[19–21]. Collectively, insulin resistance and subsequent T2D is contributed to by deficits in insulin action from several tissues associated with improper lipid storage and inflammation.

One of the pathways contributing to obesity-induced insulin resistance that is activated by improper lipid storage and inflammation is ER-stress[22]. ER stress can result from both exogenous factors, such as pharmacological agents and toxins, as well as

endogenous factors, such as free fatty acids, reactive oxygen species or increased demand for secreted proteins[23]. ER stress activates the unfolded protein response (UPR) which has 3 distinct pathways, a) the ATF6 pathway, b) the IRE1/XBP1 pathway and c) the PERK/eIF2α pathway. Induction of the UPR serves to alleviate ER stress. Stimulation of the PERK/eIF2α pathway is notable because it results in global translational arrest and when overstimulated can lead to apoptosis[23]. Chronic stimulation of this pathway is associated with both increased hepatic glucose production and lipogenesis[24]. However, transient stimulation of this pathway has also been shown to induce several protective mechanisms including expression of the anti-obesogenic protein GDF15[25–27].

Importantly, individuals with T2D are 3-5x more likely to die of CVD than individuals without T2D[28]. Consistent with this, animals with chronic hyperglycemia develop worse atherosclerosis and demonstrate increased inflammatory markers than those who are normo-glycemic, independent of adiposity[29]. CVD is the leading cause of morbidity and mortality worldwide[30]. CVD results from the development of atherosclerotic plaques, which when disrupted can lead to thrombus formation and heart attack and/or stroke. Dyslipidemia is the leading risk factor for CVD and atherosclerosis[31]. The development of atherosclerosis begins with the recruitment of monocytes (and other immune cells) to the intima of blood vessels by endothelial cell expression of adhesion molecules and chemoattractant proteins. Once in the intima these monocytes differentiate to macrophages and uptake modified low density lipoprotein (LDL) such as oxidized LDL particles. If possible, these macrophages then efflux this cholesterol via ATP-binding cassette (ABC) transporters to HDL particles which are low in cholesterol. These lipid laden HDL particles can then leave the intima and unload this cholesterol to ApoB containing VLDL and LDL particles for clearance by the liver via LDL receptors (in its

entirety this process is known as reverse cholesterol transport). However, when these macrophages are unable to efflux cholesterol at an appropriate rate there is a buildup of lipids within these cells resulting in a foamy appearance. These early stages of atherosclerotic plaques are known as fatty streaks. These foam cells result in a positive feedback loop of pro-inflammatory cytokine release which results in the recruitment and local proliferation of macrophages as well as other immune cells including T cells[32,33].

However, macrophages often undergo cell death either by apoptosis, necrosis or necroptosis within plaques. Necrotic cell death is a common feature of atherosclerotic plaques, and results in unstable necrotic cores, or large areas of cellular death, usually associated with the presence of cholesterol crystals. These necrotic cores can be prone to rupture resulting in thrombosis. However, several cellular mechanisms have arisen to protect these necrotic areas, including the development of fibrous, collagen-rich caps that develop over atherosclerotic plaques to protect them from rupture. Collectively, this data demonstrates that several factors contribute to atherosclerotic CVD including dysglycemia, dyslipidemia and inflammation[34].

1.1.1 Current approved therapies for treatment of obesity, type 2 diabetes and cardiovascular disease

Obesity:

Currently, there are only two approved pharmacotherapies for weight management in Canada, Liraglutide and Orlistat[35–37]. Other therapies have been shown to be associated with modest weight loss include metformin, other GLP-1 receptor agonists and SGLT2 inhibitors. Importantly modest weight loss (≥5%) has been shown to be sufficient to improve glycemic parameters[37]. Liraglutide is a GLP-1 receptor agonist which signals through the brain to decrease hunger and increase satiety and has been approved for weight

management for individuals with and without T2D, however gall bladder disease and acute pancreatitis are rare potential complications. Orlistat, a pancreatic lipase inhibitor has been shown to be effective at promoting weight loss when coupled to lifestyle interventions. However, loose stools and gastrointestinal side effects are major issues for long term compliance with this medication. Bariatric surgery is also a therapeutic option for treatment of obesity, and has been shown to have wide ranging benefits including metabolic and psychological improvements and results in sustained weight loss, however, individuals must comply with lifelong medical surveillance[37].

Type 2 Diabetes:

Given pre-diabetes often exists for many years before the development of T2D preventative strategies have been tested. Lifestyle intervention has been proven effective in both the Finnish Diabetes Prevention Study (DPS)[38] and the Diabetes prevention program (DPP)[39]. Both interventions were promoting a low-calorie, high fibre and moderate-intensity physical activity program resulting in approximately 5-7% weight loss and demonstrated a reduction in risk for diabetes of 58% at the 5 year follow-up[40]. The DPP study also investigated pharmacotherapy as a means to prevent diabetes in comparison to lifestyle and placebo. Interestingly, metformin decreased progression of pre-diabetes to diabetes by 31% compared to placebo, with greater reductions in individuals with impaired fasting plasma glucose but no benefit in older populations (>60 years) or in individuals with less obesity (BMI<35kg/m^2)[39]. Overall conclusions from the DPP suggest that metformin may be an ideal strategy to prevent diabetes in individuals with impaired glucose tolerance and is more effective in younger individuals with significant obesity.

In addition to prevention, individuals with newly diagnosed T2D, metformin is the first-line therapy due to its favourable safety profile, low cost, and potential cardiovascular benefits[39–41]. Metformin's primary mechanism of action is reducing hepatic glucose output and increasing insulin sensitivity (reviewed here[42–44]). In addition to improving liver insulin sensitivity and reducing hepatic glucose output, metformin has been associated with decreased cardiovascular events[45], reductions in body weight[46], decreased cancer incidence[47], improved vascular/endothelial function[48] and haemostasis[49]. Metformin's effects on weight loss, reductions in inflammation, CVD and all-cause mortality in mice and humans[50,51]; are largely believed to be independent of its effects on lowering blood glucose[44]. Furthermore, metformin has also been shown to demonstrate CV disease benefit in individuals who are newly diagnosed with diabetes and are overweight in the UKPDS trial[45,49].

Three classes of second line therapeutics for type 2 diabetes are DPP-4 inhibitors, GLP-1 receptor agonists and SGLT2i's. DPP-4 inhibitors prevent the degradation of incretins such as GLP-1, while GLP-1 receptor agonists are synthetic mimics of GLP-1, which both act to inhibit glucagon secretion, promote glucose dependent insulin secretion, delay gastric emptying, and increase satiety, thereby reducing food intake and promoting weight loss[52]. Given these effects on body weight Liraglutide, a GLP-1 receptor agonist, has recently been approved for the treatment of obesity, however, these therapies are expensive, and long term safety effects are currently unknown, making them not-ideal for a preventative therapy[40]. SGLT2i's (i.e. Canagliflozin, Dapagliflozin and Empagliflozin) promote urinary glucose excretion by inhibiting glucose reabsorption in the kidneys to effectively reduce blood glucose[53,54], however they are also associated with increased risk of urinary tract infections. In addition to reducing blood glucose, they have been shown to

lower BMI, HbA1C, and HOMA-R, triglycerides, non-HDL cholesterol, and LDL-C [55]. Empagliflozin, canagliflozin and dapagliflozin also produce unexpectedly robust reductions in CVD mortalilty[56–58].

Most current therapies for the treatment for T2D directly target glucose metabolism or uptake however other strategies to combat insulin resistance have been proposed. For example, targeting inflammation to reduce insulin resistance and thereby effectively treat T2D has been studied in the Targeting Inflammation with Salsalate to treat Type 2 Diabetes (TINSAL-T2D) Study[59–61]. Salsalate is a prodrug of salicylate and is metabolized to two salicylate molecules in vivo. Salicylate is the active compound in aspirin and has been used to treat inflammation for years (for review see[62]). Thus far, salsalate has been shown to reduce circulating lipids in humans and improve dysglycemia in patients with T2D[59,60,63].

Atherosclerosis:

Statins are the first line of therapy for atherosclerotic CVD. Statins inhibit the rate limiting enzyme in cholesterol synthesis, HMG-CoA Reductase (HMGR) and effectively reduce dyslipidemia, specifically elevated LDL-c, in most individuals. However, recent evidence suggests that statins may increase the risk of T2D, which is a common comorbidity with CVD[64–66]. They may also cause muscle soreness which limits their use in some patients or prevents some patients from using doses to effectively lower LDL-c to the recommended range. This has prompted the use of other LDL lowering agents used in combination with statins, including ezetimibe, to reduce cholesterol absorption in the intestine[67], however some controversy still exists about the risk/benefit ratio of these therapies[67,68], and they do not address the issue of statin intolerance. Recently, PCSK9

inhibitors, have been approved for lowering LDL-c but these therapies are considered expensive compared to statins and require weekly injections which has largely limited their use to those with familial hypercholesterolemia[69]. Bempedoic acid, is a newly emerging treatment option which specifically targets the liver and inhibits ATP Citrate Lyase (ACLY) to reduce cholesterol synthesis[70–72]. Bempedoic acid has demonstrated efficacy in LDL-c lowering in statin intolerant patients[73] and is currently in phase 3 trials for cardiovascular outcomes(Clinical trial identifier NCT02993406).

In addition to lowering LDL-c, reducing inflammation may also inhibit atherosclerosis. For example a recent study has shown that the use of an IL1β antibody reduces cardiovascular events independently of changes in lipid levels[74]. In contrast the anti-inflammatory drug methotrexate was shown to be ineffective at reducing cardiovascular events, however this dose of methotrexate did not reduce IL-1β, CRP or IL-6[75]. Salsalate is another anti-inflammatory that has been shown to reduce circulating lipids and improve dysglycemia in people with T2D[59,61,63] , however a recent study has shown there was no regression of plaque size when added to statin therapy[76]. These data suggest that broadly targeting inflammation is not cardio protective but specifically targeting certain pro-inflammatory cytokines such as IL-1β[74] is an effective treatment for atherosclerosis.

Obesity, type 2 diabetes and atherosclerosis are commonly linked and cause significant morbidity and mortality. While significant progress has been made in treating these diseases new therapies that effectively target multiple aspects of these common chronic diseases are needed. One potential therapeutic target linking obesity, T2D and CVD is the AMP-activated protein kinase (AMPK).

1.2 AMP Activated Protein Kinase (AMPK)

The following section of the introduction is about AMPK as a therapeutic target for treating atherosclerosis and metabolic syndrome and is an excerpt from the following review article:

Day, E. A., Ford, R. J., & Steinberg, G. R. (2017). AMPK as a therapeutic target for treating metabolic diseases. Trends in Endocrinology & Metabolism, 28(8), 545-560.[77]

1.2.1 The Structure and Function of AMPK

The AMP activated protein kinase (AMPK) is a ubiquitously expressed serine-threonine kinase that is activated by low cellular energy status. Once activated, AMPK triggers catalytic processes to generate ATP while inhibiting anabolic processes that consume ATP in an attempt to restore cellular energy homeostasis [78–80]. The processes regulating activation of AMPK are dictated by its heterotrimeric structure, which consists of an α (α1, α2) catalytic subunit, a regulatory and structurally critical β (β1, β2) subunit, and a regulatory γ (γ1, γ2, γ3) subunit. These isoforms are encoded by distinct genes that are differentially expressed and have unique tissue specific expression profiles, creating the potential for the generation of 12 distinct heterotrimer combinations. The structure, function and regulation of these different isoforms have been reviewed in detail previously [78–80] and it is not within the scope of the current review to rigorously outline these further. However for the purposes of the discussion below, it is important to note that activation of AMPK leads to the phosphorylation of key metabolic substrates and transcriptional regulators that are linked to nearly all branches of cellular metabolism. In relation to metabolic diseases, the activation of AMPK has been shown to play a vital role in increasing glucose uptake, fatty acid oxidation (FAO), mitochondrial biogenesis and autophagy while suppressing the synthesis of fatty acids, cholesterol and protein [78–80]. In the subsequent sections of this review, we will discuss studies that have taken genetic modification approaches to uncover the role of AMPK in regulating these pathways in

immune cells, liver and adipose tissue as it relates to obesity, NAFLD, atherosclerosis, insulin resistance and type 2 diabetes.

1.2.2 The Role of AMPK in Immune Cells

Chronic low-grade inflammation is a defining characteristic of metabolic diseases including obesity, NAFLD, atherosclerosis, insulin resistance and type 2 diabetes. Chronic inflammation is both systemic and seen within adipose tissue, liver and the vasculature [81,82], and involves mobilization of macrophages as well as other immune cells including neutrophils, mast cells and t-cells [81,82]. AMPK activity is reduced in response to chronic low-grade inflammation induced by TNFα, lipopolysaccharide (LPS) or high-fat diet induced obesity in multiple tissues including skeletal muscle [83], liver [84,85], white adipose tissue [85,86], brown adipose tissue [87] and macrophages [86,88,89]. Consistent with these findings, germ-free mice or mice treated with antibiotics that have low levels of circulating LPS, have increased liver and adipose tissue AMPK activity [90]. Importantly, this relationship between chronic low-grade inflammation and reductions in AMPK activity is also observed in human adipose tissue [91], and is reversed following weight loss-induced by diet [92] or bariatric surgery [93,94]. The mechanisms by which AMPK activation is reduced with chronic inflammation and obesity is currently unclear, but may be related to increases in protein phosphatase 2C [83] and 2A [95], or by inhibitory phosphorylation of the AMPK α1/ α2 subunit at Thr479 by glycogen synthase kinase β (GSKβ) [96] or of AMPK α1/ α2 at Ser485/Ser491 by Protein kinase A [97,98], Protein Kinase D [99], Akt [100–103], or S6kinase [104].

In addition to being a downstream effector of chronic, low-grade inflammation in multiple tissues, AMPK is also an inhibitor of acute pro-inflammatory responses. Most studies have focussed on investigating the relationship between AMPK and inflammation in macrophages since metabolic activity and inflammatory status are directly linked in

these cells [81,105]. A number of *in vitro* studies using genetic models have shown that AMPK is important for switching macrophages from an M1, pro-inflammatory phenotype dependent on glycolysis, to an M2, anti-inflammatory phenotype dependent on oxidative metabolism[88,89,106,107]. Since mouse and human macrophages are primarily comprised of an AMPK α1, β1, γ1 heterotrimer, genetic models investigating this relationship have involved manipulation of AMPK α1 or β1 subunits. Reduced expression of AMPKα1 using dominant negative constructs or RNA interference accentuates pro-inflammatory cytokine production and NFκB signaling in macrophages [86,88], whereas constitutively active AMPK α1 expression or treatment with the non-specific AMPK activator AICAR produce the opposite effects [86,88]. Importantly, inactivation of macrophage AMPK α1 in macrophage-adipocyte co-culture also inhibits adipocyte insulin signaling and glucose uptake in these studies [86], demonstrating an important role for AMPK mediated macrophage inflammation in promoting host tissue insulin resistance. These findings are consistent with *in vivo* models, as mice lacking AMPKβ1 in hematopoietic cells (which results in loss of AMPK activity in macrophages) display an exacerbated accumulation of inflamed macrophages in white adipose tissue and liver, and accelerated development of insulin resistance when fed an obesity promoting high fat diet (HFD)[89]. Similar results are also observed in myeloid-deficient AMPKα1 KO mice fed a HFD [106], indicating a vital role for macrophage AMPK in regulating macrophage inflammation and insulin sensitivity. Interestingly, both AMPK α1 and β1 deficient mice exhibit low levels of inflammation when fed a standard chow diet, indicating that macrophage AMPK is only required under conditions of metabolic stress [89,106] such as nutrient overload (i.e. HFD) or during the resolution of inflammatory events such as muscle damage [107]. Together these data highlight an important role for AMPK as an advocate for anti-inflammatory status under conditions of pro-inflammatory stress.

In addition to insulin resistance, increases in inflammation mediated by M1 macrophages and the attenuation of M2-macrophages, is associated with the acceleration of atherosclerotic plaque progression [81,82]. Consistent with this, myeloid specific deletion of AMPKα1 accelerates atherosclerosis in mice on a low-density lipoprotein receptor deficient (LDLr KO) background [106]. However, the interpretation of these findings and whether this result is due to increases in macrophage inflammation is complicated by elevated plasma LDL and VLDL cholesterol in these mice, which could contribute to the accelerated atherosclerosis in this model. This increase in plasma lipids may be due to AMPK mediated regulation of macrophage cholesterol efflux, as mice lacking macrophage AMPK β1 have reductions in the expression of ATP binding cassette transporters, Abcg1 and Abca1 and reverse cholesterol transport *in vivo* [108]. However, surprisingly mice with a germline deficiency in both AMPK β1 and Apolipoprotein E ($ApoE^{-/-}$) do not have increases in plasma LDL, VLDL, inflammation or atherosclerosis development when fed a western diet high in cholesterol [70]. In contrast, mice lacking ApoE or LDLr and AMPK α2, an AMPK isoform expressed in skeletal muscle but not macrophages, do develop greater atherosclerosis when fed a HFD [109,110]. A number of germline models of AMPK deletion (α1 or α2) crossed with proatherogenic backgrounds (ApoE or LDLrKO) show exacerbated plaque development and refraction to improvements in atherogenic phenotypes when treated with AMPK activating compounds such as metformin or berberine [106,109–112]. However, it will still be important to establish tissue specific, inducible models of AMPK deletion along with bone marrow transplant experiments and AMPK specific activators to fully evaluate the role of AMPK in immune cells/macrophages versus that of other cells in the vascular wall such as vascular smooth muscle and the endothelium (as AMPK in these tissues also modulates atherogenesis), [112,113], in contrast to changes in

other whole body parameters that exist in whole body knockouts that also contribute to atherogenesis. This is particularly important for an enzyme like AMPK that is a modulator of many metabolic and immunological effectors and is expressed in all body tissues. Given the complexity and interrelationship between inflammation and lipid metabolism within the macrophages, liver and vasculature, future studies investigating the importance of macrophage AMPK in the development of atherosclerosis are warranted.

Consistent with heightened macrophage inflammation in the absence of AMPK, numerous activators of AMPK (i.e. AICAR, metformin, A769662, troglitazone, ENERGI-704, ETC-1002, canagliflozin, berberine, salicylate, resveratrol, genestein, palmitoleate, interleukin-10, Anti-miR-33) have been shown to reduce macrophage inflammation (**Table 1**). However, these pharmacological agents have only been tested using *in vivo* genetic models of immune cell AMPK deficiency in a few cases (outlined in Table 1). Use of genetic models will be important for establishing the specific role for AMPK in mediating the outcomes of these compounds since widely used indirect activators of AMPK such as AICAR, metformin and berberine, which have been shown to reduce insulin resistance and atherosclerosis, suppress macrophage inflammation through AMPK independent pathways [114–118]. However, several activators of AMPK including A-769662, anti-miR-33, or palmitoleate do appear to reduce macrophage inflammation through an AMPKβ1-dependent pathway [89,119], and therefore provide confirming evidence of the importance of AMPK in these events. Future studies investigating the role of macrophage AMPK in mediating the beneficial metabolic effects of AMPK activating therapeutics for treating insulin resistance and atherosclerosis will be important in order to establish whether macrophage targeted therapies may be a viable means to treat these chronic diseases.

The mechanism by which AMPK reduces inflammation in macrophages is still unclear, but is likely to be multifactorial; however, a consistent finding across multiple studies is the relationship between reduced FAO and inflammation [89,107,119]. This interaction may involve AMPK mediated regulation of multiple branches of cellular metabolism including reductions in acetyl-CoA carboxylase activity (ACC) [89]. AMPK phosphorylates ACC1 at Ser79 and ACC2 at Ser221 (212 in mice) to reduce the conversion of acetyl-CoA to malonyl-CoA(the first committed step in de novo lipogenesis), although macrophages only express ACC1 [89]. Malonyl-CoA is an inhibitor of carnitine palmitoyl transferase 1 (CPT1) which is required for uptake of fatty acyl-CoA into the mitochondria, thus reductions in malonyl-CoA stimulate FAO and inhibit lipogenesis. Reductions in AMPK content/activity are also accompanied by decreases in mitochondrial content [89], mitochondrial fission [120] and increased lipogenesis [108], which collectively could all contribute to reductions in FAO. Interestingly, the reduction in FAO appears to be important, as stimulating FAO reduces inflammation induced by saturated fatty acids [89], and may be important for preventing pro-inflammatory lipid accumulation [89] as well as increasing the expression of the enzyme retin ALDH type 2 (RALDH2) [119]. RALDH2 synthesizes retinoic acid which exerts anti-inflammatory effects in macrophages and is important for recruiting $FOXP3^+$ Tregs, which are T-cells that exert a protective role in both insulin resistance and atherosclerosis.

In addition to altering metabolic pathways, AMPK also phosphorylates critical proteins for controlling inflammatory pathways and cell proliferation. This includes inhibition of eNOS [121,122], CHOP (a key protein regulating endoplasmic reticulum (ER)-stress) [123,124] and JAK1 [125] while increasing the activity of the p38 and JNK inhibitor MAP kinase phosphatase-1 (MKP1)[126] and STAT1 inhibitor PIAS1 [112]. AMPK also inhibits

NFκB activity by phosphorylating FOXO [127] and increasing the activity of the deacetylase SIRT1 [128] and the transcriptional co-activator PGC1α [129] (**Figure 1**). Lastly, as deficits in lysosomal trafficking of lipids are associated with increased ER stress [130], inflammatory macrophage polarization [131] and worsening of advanced atherosclerotic plaques [132–134], an intriguing possibility is that AMPK may also regulate lipid content through control of lipophagy (the breakdown of lipid droplets via macro-autophagy), effects which may be mediated through AMPK inhibition of mTOR and activation of ULK1 [135–137].

In summary, AMPK inhibits inflammation through multiple mechanisms which involve metabolic reprogramming as well as direct inhibition of key inflammatory proteins. This multifaceted approach to targeting multiple branches of the inflammatory cascade makes AMPK an attractive target for treating chronic low-grade inflammatory diseases such as type 2 diabetes or atherosclerosis which do not result from a single cytokine or signaling cascade. Furthermore, since deficits due to the absence of AMPK only become evident under conditions of metabolic stress, activation of the pathway is unlikely to create an inability to mount appropriate challenges to pathogen infections. Given the intimate connection between inflammation, aging and numerous metabolic diseases, it will be important for future studies to investigate the effects of AMPK activators in mice lacking AMPK activity in macrophages and other immune cells to fully evaluate the role of immune cell AMPK in metabolic benefits and anti-aging effects .

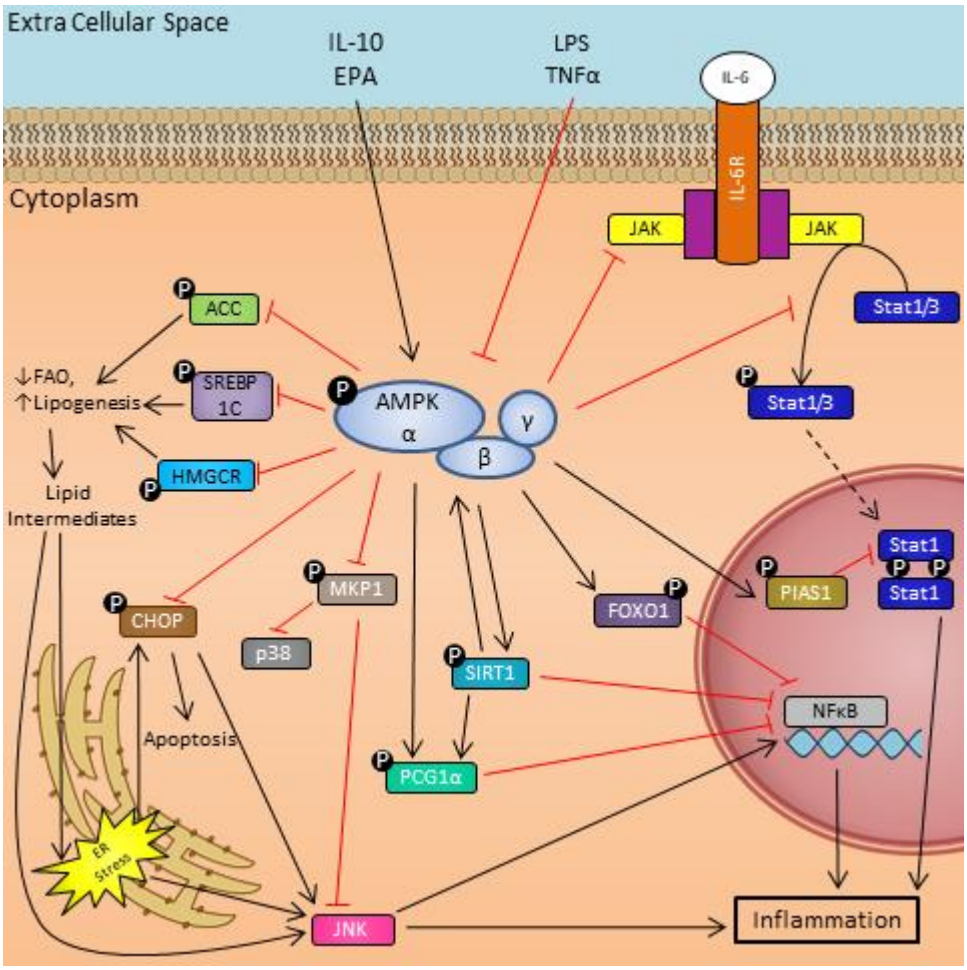

Figure 1: AMPK regulates inflammation through direct phosphorylation of inflammatory targets. AMPK inhibits fatty acid and cholesterol synthesis via inhibitory phosphorylation of HMGCR, SREBP1C, and ACC. Excessive accumulation of lipid intermediates can contribute to ER stress as well as JNK activation. Inhibitory phosphorylation of ER-stress protein CHOP by AMPK reduces apoptosis. Conversely, phosphorylation of MKP1, resulting in its degradation releases inhibition on JNK resulting in increased inflammation. Inhibitory phosphorylation of JAK1 at 2 residues has been shown to decrease STAT1 and STAT3 activity (transcription of inflammatory factors) and there is further control of STAT1 by AMPK by activating phosphorylation of Protein inhibitor of Activated Stat1 (PIAS1) which also reduces STAT1's transcriptional activity. Phosphorylation of PGC1α, SIRT1 and Fox01 reduces NFκB activity and transcription of inflammatory cytokines.

Table 1: AMPK Activators Shown to reduce Macrophage Inflammation

Citation	AMPK Activator	Cells	Controls	Experimental Results and Proposed Mechanism
[138]	Berberine (BBR)	RAW 264.7 macrophages	Compound C, DN-AMPKα1	AMPK activation inhibits MAPK signaling and decreases cellular ROS resulting in reduced IL-1β, IL-6, iNos, MCP1, Cox2 and MMP9 mRNA when stimulated with LPS
[86]	Aicar	RAW 264.7 macrophages	DN-AMPKα1, CA-AMPK	AMPK activation increases SIRT1 activity and expression resulting in decreased NF-κB activity therefore reduces TNFα mRNA and protein
[139]	Berberine (BBR)	RAW 264.7 macrophages	Compound C, DN-AMPKα1	AMPK activation causes increased transcription of Nrf2 antioxidant genes, resulting in decreased COX2 expression and increased NQO-1 and HO-1 expression in response to LPS
[140]	Berberine (BBR)	J774A.1 Macrophages	Compound C	AMPK activation induces autophagy which results in reduced MCP1α and RANTES expression and increased IL-10 expression in response to oxLDL treatment
[141]	Aicar, A769662, Metformin	J774.A1 macrophages	CA-AMPK, DN-AMPK, Compound C	AMPK increases ABCG1 expression, enhancing cholesterol efflux to HDL
[142]	Resveratrol	THP-1 monocytes	Compound C	AMPK activation causes an increase of GSH, preventing monocyte to macrophage differentiation, resulting in decreased IL-1β, TNFα and MCP1 expression and
[143]	Metformin	THP-1 monocytes	Aicar	AMPK reduces Stat3 phosphorylation, therefore decreasing monocyte to macrophage differentiation, causing decreased IL-1β, TNFα and MCP1 expression
[144]	Troglitazone and Δ2Troglitazone	THP-1 Macrophages	Compound C	AMPK activation increases de novo adiponectin synthesis reducing monocyte adhesion
[145]	Palmitoleate (PO)	Murine BMDMs	Compound C, AMPKβ1$^{-/-}$ BMDM	Mono-unsaturated fatty acid, cis PO can activate AMPK resulting in increased expression of Nos2 and IκBα

[146]	A769662	Primary Human Macrophages	siAMPKα1	AMPK increases expression of genes involved in fatty acid metabolism via PPARδ resulting in increased CPT1α
[108]	Salicylate, A769662	Murine BMDM	AMPK $\beta1^{-/-}$ BMDM	AMPK activation reduces lipogenesis of FA and sterols, decreases cholesterol uptake and increases cholesterol efflux to HDL and ApoA1 via increased ABCG1, ABCA1, LXRα and decreased SREBP2
[147]	A769662	THP-1 Human Monocytes	AMPKα1 KD	Activation of AMPK induces LXRα resulting in increased ABCA1 expression and cholesterol efflux
[148]	Metformin	Murine BMDM	Compound C	Activation of AMPK inhibits AGEs-induced TNFα, IL-1β and IL-6 signalling through suppression of RAGE/NFκB signaling. AMPK activation also increases CD206 and decreases CD86
[149]	Metformin	Diabetic Human monocyte-derived macrophages	Compound C, sh-AMPK	Metformin reduces IL-1β and IL-18 production through modulation of mitochondrial function and activation of AMPK. Decreased AMPK results in increased IL-18 and IL-1β production when stimulated with ATP or MSU
[150]	Aicar	J774 macrophages	DN-AMPK, CA-AMPKα2	AMPK activates TAK1 via p38 MAPK signalling leading to the expression of the COX2
[111]	Berberine (BBR)	$ApoE^{-/-}$ $AMPK\alpha2^{-/-}$ mice	$ApoE^{-/-}$ $AMPK\alpha2^{-/-}$ mice	Lack of AMPKα2 results in worse atherosclerosis. AMPK activation increases UCP2 expression suppressing oxidative stress and inflammation
[114]	Metformin	Murine BMDM	$AMPK\alpha1^{-/-}$ and $AMPK\beta1^{-/-}$ cells	Metformin reduces IL-1β production independent of AMPK and had no effect on TNFα
[151]	Aicar, Phenformin	Human Derived macrophages	A769662	AMPK interferes IL-4-induced human macrophage polarization by attenuating STAT3 activation, which diminishes ALOX15 expression
[152]	IL-10	BMDM	$AMPK\alpha1^{-/-}$ cells	IL-10 induction of LXRα, ApoE, ABCA1 and SOCS1 protein, and PI3K and AKT signalling are AMPK dependent. AMPK is required for IL-10 induced Jak1

				phosphorylation and activation of STAT3. IL-10 induced reductions in TNFα and IL6 are also AMPK dependent.
[153]	Aicar	Murine BMDM	CA-AMPK	AMPK activation increases autophagy in response to LPS or palmitate, decreasing inflammation and IL-1β production
[154]	Metformin, Heme	Human blood–derived macrophages, Murine BMDM	AMPKα1 siRNA, AMPK KO BMDM	Heme and Metformin activate ATF1 via AMPK to increase antioxidant gene transcription and LXR resulting in decreased foam cell formation, and increased cholesterol efflux.
[155]	Astragalus polysaccharide (APS)	RAW 264.7 macrophages	DN-AMPKα1, Compound C	APS activates AMPK resulting in increased IL-10 mRNA and protein expression and attenuated palmitate induced IL-1β expression
[156]	Genistein	RAW 264.7 macrophages	Aicar, Compound C	Genistein activates AMPK attenuating LPS-induced NFκB activity and TNFα and IL-6 expression
[119]	Anti-miR-33	Murine BMDM	AMPK $\beta1^{-/-}$ BMDM	*miR-33 and Anti-miR-33 act via AMPK to increase* IL-1β, IL-6, and Nos2 or decrease *Aldh1a2* respectively
[157]	ENERGI-704	RAW 264.7 macrophages	Compound C	ENERGI-704 activates AMPK therefore suppressing NF-κB signaling resulting in reduced LPS induced mRNA and protein of IL-6, IL-8, TNFα, PGE_2, iNOS, and Cox2

1.2.3 Hepatic AMPK

The liver is vital for controlling circulating glucose and lipid levels, and as such plays a central role in the development of type 2 diabetes and atherosclerosis when hepatic glucose production (HGP), insulin sensitivity and fat content become dysregulated. In the liver of rodents, AMPK β1 containing heterotrimers predominate (α1β1γ1 and α2β1γ1), however in humans AMPK β2 is more prevalent [158–160]. Consistent with these observations, genetic deletion of AMPK α1, α2 or β2 isoforms [161–163] have little effect on liver AMPK activity while in contrast germline deletion of AMPK β1 [159] leads to large (~95%)

reductions in liver AMPK activity (since the β subunit is obligatory for maintaining structural and functional integrity of the enzyme).

Chronic elevations in HGP mediated through increases in liver glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate kinase (PEPCK) are hallmark features of type 2 diabetes[164]. Initial studies using AICAR suggested that an important role for liver AMPK may be in limiting the expression and activity of these enzymes [165–167]. These finding were further supported by studies which found that in constitutively active AMPK α2 in the liver reduced blood glucose and gluconeogenic genes in wildtype and *ob/ob* diabetic mice [168], and that mice with liver specific knockout of AMPK α2 were hyperglycemic and had elevated HGP relative to controls [169]. However, mice lacking both AMPK α1 and α2 subunits in the liver (which led to no detectable liver AMPK activity) did not result in hyperglycemia or increases in HGP. In addition, AICAR continued to suppress HGP in liver-specific AMPKα1α2 KO mice suggesting that the action of AICAR here is independent of AMPK [170–173], an effect which may be due inhibition of fructose 1,6 biphosphatase by the cellular derivative of AICAR, ZMP(AICAR monophosphate)[174]. Furthermore, activation of liver AMPK using the AMPK β1-specific activator A769662 does not inhibit HGP in hepatocytes[170]. Collectively, these studies indicate that the liver AMPK is not essential to inhibit hepatic gluconeogenesis; however future studies using inducible liver-specific AMPK KO mice will be important to establish whether development adaptations due to the chronic absence of hepatic AMPK may have contributed to the upregulation of alternative pathways crucial for controlling HGP.

NAFLD is a global epidemic that is characterized by the ectopic accumulation of lipids within the liver that, if left unresolved, can contribute to the development of T2D, CVD, non-alcoholic steatohepatitis (NASH) and hepatic cellular carcinoma [175,176]. NAFLD

is the cumulative result of an over production of lipids from other carbon sources (de novo lipogenesis) or due to an inability to oxidize or clear lipids that are deposited in the liver. These effects may be countered by AMPK activation through multiple mechanisms. Reductions in liver AMPK activity in AMPK β1 KO hepatocytes reduces the phosphorylation of ACC and leads to reductions in FAO and increases in lipogenesis [159].

Overexpression of AMPK α1 in the liver reduces lipogenic gene expression, liver triglyceride content, and hepatic steatosis in hyperlipidemic, type 2 diabetic rats *in vivo* [177], and reduces triglyceride and intracellular lipid accumulation while increasing CPT1 expression (increasing fatty acid clearance via mitochondrial FAO) when overexpressed in hepatocytes *in vitro* [178]. Importantly, increases in fatty acid synthesis and reductions in FAO are also observed in hepatocytes isolated from mice with Ser-Ala knock-in mutations on the AMPK phosphorylation sites of ACC1 (S79A) and ACC2 (S212A) [179]. Consistent with these changes in hepatocyte fatty acid metabolism, young ACC knock-in mice fed a control chow diet rapidly develop NAFLD, liver insulin resistance that leads to whole body glucose intolerance, as well as early signs of liver fibrosis[179]. Recent data using a pharmacological ACC inhibitor also supports these findings, showing that a small molecule inhibitor of ACC (mimicking the effects of AMPK phosphorylation on ACC dimerization) reduces *de novo* lipogenesis while increasing FAO in isolated hepatocytes and rodents to alleviate NAFLD [180]. These data suggest that while AMPK is able to inhibit other enzymes and/or transcription factors critical for controlling lipid biosynthesis such as fatty acid synthase [181,182], SREBP1 [183] and SREBP2 [184], this is insufficient to prevent accelerated lipogenesis due to the loss of AMPK phosphorylation of ACC.

Metformin, the most commonly prescribed anti-diabetic drug in the world, exerts is beneficial metabolic affects in individuals with type 2 diabetes primarily by suppressing

HGP [43,185]. Although this effect was initially thought to involve the activation of AMPK [186,187], some studies in mice lacking liver AMPK have suggested this is unlikely to be the primary mechanism of action [170,171]. However, a notable concern with these studies is that AMPK-independent effects on HGP were established using phenformin or high concentrations of metformin outside of the clinically effective range observed in the portal vein (> 0.5mM). Both metformin and phenformin are known to potently inhibit complex-1 of the respiratory chain and increase AMP/ADP while reducing ATP, effects which may inhibit adenylate cyclase and enzymes involved in gluconeogenesis in addition to activating AMPK. In support of this idea, recent studies have indicated that at clinically relevant concentrations of metformin found within the portal vein which do not increase AMP (<100 μM), AMPK is still activated through a lysosomal pathway involving LKB1 and Axin [188] and that activation of AMPK by this mechanism is vital for metformin-induced suppression of HGP [97]. Alternatively, it has also been suggested that *in vivo,* metformin may inhibit HGP indirectly through a gut-brain-liver axis involving intestinal AMPK and GLP1 secretion [189,190]. Therefore, despite extensive study, the relative importance of AMPK in mediating the suppressive effects of metformin on HGP is still not fully resolved.

In addition to the acute suppression of HGP, metformin also improves liver insulin sensitivity[191,192]. The importance of this mechanism for lowering blood glucose is evident in that metformin has little effect in individuals with type 1 diabetes, where insulin secretion rather than insulin resistance plays a more prominent role in the disease pathology and progression[193,194]. Since NAFLD is an important cause of insulin resistance, metformin-induced reductions in liver lipid content potentially mediated through AMPK phosphorylation of ACC could be important for improvements in insulin sensitivity and reductions in HGP. Consistent with this hypothesis, metformin failed to suppress malonyl-

CoA levels, lipogenesis, and liver lipids in obese high-fat diet fed ACC KI mice, and these changes were associated with resistance to metformin-induced reductions in blood glucose and liver insulin resistance [179]. This response was specific for liver AMPK since both metformin and A769662 also failed to improve insulin sensitivity in primary hepatocytes generated from ACC KI and AMPK β1 KO mice [179]. These data suggest that under conditions where NAFLD and liver insulin resistance are present, a situation that would be common with most users of metformin, metformin-induced activation of AMPK and inhibition of ACC are important for improving insulin-induced suppression of HGP. Given the importance of lipogenesis in contributing to the development of NAFLD in humans[195–197], these data suggest that activators of AMPK may lower liver lipids and blood glucose through AMPK inhibition of ACC. Indeed, multiple activators of AMPK have been shown to inhibit hepatic lipogenesis, reduce liver lipids and improve insulin sensitivity, as has been reviewed in detail elsewhere [175]. However, AMPK-mediated inhibition of ACC does not appear to be important for liver insulin sensitization following high intensity exercise training [198] or salsalate therapy [199], and therefore the extent to which this mechanism is central to generating effects of other AMPK activators or activating conditions still needs to be explored.

In addition to the inhibition of ACC, AMPK may also regulate liver fatty acid metabolism through the control of total mitochondrial content, mitochondrial quality and mitochondrial function, effects that are mediated through the coordinated regulation of biogenesis, autophagy (mitophagy) and fission. The downstream mediators of AMPK effects on these pathways may be PPAR-γ 1 co-activator (PGC1α) and histone deacetylases (HDAC) (biogenesis)[200], the unc-51-like autophagy activating kinase 1 (ULK1)(mitophagy) [136] and mitochondrial fission factor (MFF) (fission) **(Figure 2)** [201,202].

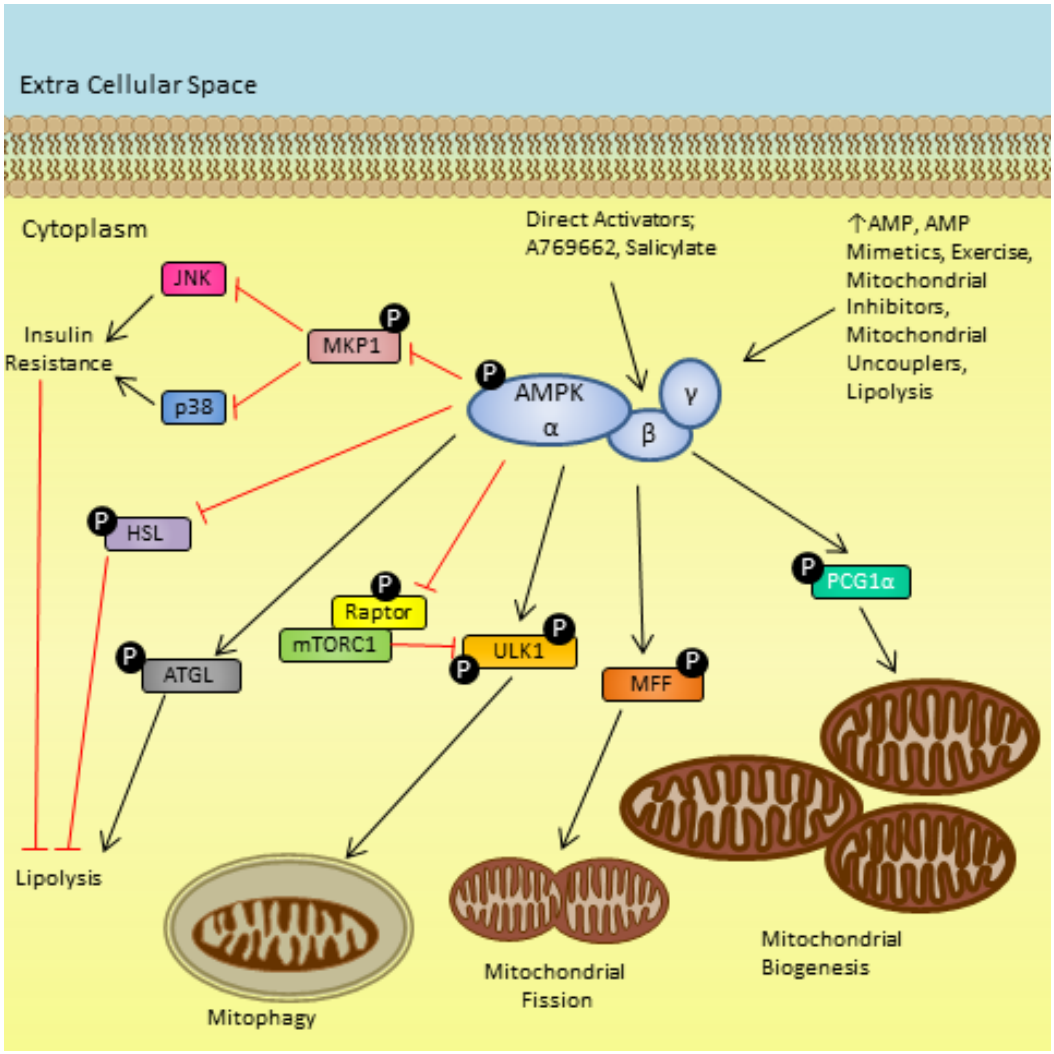

Figure 2: AMPK reduces NAFLD and increases lipolysis and thermogenesis. AMPK maintains mitochondrial number and quality via promotion of mitophagy and mitochondrial biogenesis. AMPK maintains mitophagy via inhibitory phosphorylation of Raptor, releasing mTORC1 inhibition on ULK1 as well as by activating phosphorylation of ULK1. AMPK promotes mitochondrial fission via phosphorylation of MFF and phosphorylates PGC1α to promote mitochondrial biogenesis. AMPK also promotes lipolysis via phosphorylation of ATGL and HSL. Furthermore; phosphorylation of MKP1 by AMPK targets MKP1 for degradation, therefore releasing the brake on JNK and p38 which leads to insulin resistance.

Recent studies have highlighted that dysregulation of these pathways results in increased NAFLD in both mice and humans [203–206]. Consistent with this concept, liver specific AMPK α1α2 null mice ($AMPK_{LS}\alpha1^{-/-}\alpha2^{-/-}$) have reduced mitochondrial content which may compromise their ability to oxidize fatty acids. Specifically, mitochondrial respiration was ~30% lower and mitochondrial oxidative capacity was reduced by 25% in KO animals, demonstrating that AMPK is required for maintenance hepatic of mitochondrial function [172]. Importantly, this appears to have functional consequences because during fasting, a time of heavy ATP demand due to gluconeogenesis, there are large reductions in ATP levels in $AMPK_{LS}\alpha1^{-/-}\alpha2^{-/-}$ mice[173]. In addition to reductions in mitochondrial content, this impairment in mitochondrial function may also be attributed to defects in mitophagy and mitochondrial fission; however, more detailed studies are required to evaluate this possibility. Defects in lipophagy have been shown to be an important cause of NAFLD (recently reviewed [207]), and recent work has demonstrated that AMPK (using compound C and A769662) may phosphorylate PLIN2 to trigger degradation by lipophagy [208]. Whether this process is altered in AMPK liver KO mice has not yet been investigated.

In conclusion, data from genetic mouse models support an important role for AMPK in inhibiting fatty acid synthesis and increasing FAO but not inhibiting hepatic glucose production. Furthermore, while acutely regulating lipid metabolism, AMPK is also crucial for maintenance of mitochondria content and quality. Therefore increasing hepatic AMPK activity would be expected to reduce NAFLD via multiple mechanisms which could have beneficial effects for improving glucose and lipid homeostasis, thereby reducing the risk of type 2 diabetes and atherosclerotic cardiovascular disease, in addition to potentially preventing or reversing NASH and possibly liver cirrhosis and hepatic cellular carcinoma.

[END OF EXCERPT]

1.3 Main Objective

AMPK is centrally poised to regulate inflammation, glucose homeostasis and lipid synthesis and oxidation. These processes have all been shown to be disrupted in obesity, T2D and atherosclerosis. Consequently, AMPK activating therapeutics may hold significant promise to effectively target multiple aspects of these common chronic diseases. Therefore, the aim of this thesis was to examine the effects of AMPK activating, glucose lowering therapeutics, metformin, canagliflozin and salsalate on several aspects of whole-body metabolism including; glucose homeostasis, lipid metabolism and inflammation. Specifically, this thesis examined the effects of these therapeutics on obesity, atherosclerosis, hepatic lipid metabolism, and macrophage inflammatory signalling and sought to delineate the mechanism(s) by which these changes occur to further our understanding of treatment of atherosclerosis, obesity and T2D.

2. Chapter 2

Metformin-induced increases in GDF15 are important for suppressing appetite and promoting weight loss.

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2.1 Preface and significance to thesis

Our lab has previously established that GDF15 was a novel biomarker of metformin treatment and that this association was independent of sex, body weight, glycemia and HBA1c in a large human population (8,401 participants (2,317 receiving metformin))[210]. However, the mechanisms and clinical significance of this association was unknown. This work demonstrates that metformin induces GDF15 secretion from hepatocytes through a pathway involving ATF4 and CHOP and that this is important for weight loss in humans and mice. Interestingly, these effects were independent of AMPK. This work demonstrates that metformin mediates some of its anti-obesogenic glucose normalizing effects through GDF15 and could potentially be leveraged as an anti-obesogenic therapy in individuals without T2D.

2.2 Author contribution

Emily was the primary contributor to data in Figure 2a, c-e, Figure 3a-h, and 4a-h, and extended data figure 1a, 2a-f, 3a-i, and 4a-e. Emily wrote the manuscript with Dr. Ford and Dr. Steinberg.

2.3 **Metformin-induced increases in GDF15 are important for suppressing appetite and promoting weight loss.**

Metformin-induced increases in GDF15 are important for suppressing appetite and promoting weight loss

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Abstract: Metformin is the most commonly prescribed type 2 diabetes medication due to its glucose lowering effects which are mediated through the suppression of hepatic glucose production (reviewed here[42–44]). However, in addition to its effects on the liver, metformin also reduces appetite and in preclinical models exerts beneficial effects on aging and a number of diverse diseases (e.g. cognitive disorders, cancer, cardiovascular disease) through mechanisms which are not fully understood[42–44]. Given the high concentration of metformin in the liver and its many beneficial effects beyond glycemic control we hypothesized that metformin may increase the secretion of a hepatocyte derived endocrine factor that would communicate with the central nervous system[211]. Here we show, using unbiased transcriptomics of mouse hepatocytes and analysis of proteins in human serum, that metformin induces expression and secretion of growth differentiating factor 15 (GDF15). In primary mouse hepatocytes, metformin stimulates the secretion of GDF15 by increasing the expression of ATF4 and CHOP. In wildtype mice fed a high-fat diet, oral administration of metformin increases serum GDF15 and reduces food intake, body mass, fasting insulin and glucose intolerance; effects which are eliminated in GDF15 null mice. An increase in serum GDF15 is also associated with weight loss in patients with type 2 diabetes taking metformin. Although, further studies will be required to determine the tissue source(s) of GDF15 in response to metformin treatment in vivo, our data indicate that the therapeutic benefits of metformin to reduce appetite, body mass and serum insulin depend on GDF15.

Metformin is one of the most widely used medications in the world. It is a strong base that at a physiological pH, exists in its protonated form, and therefore, does not pass easily through cellular membranes. In rodents, oral administration of metformin (250-300mg/kg) results in clinically relevant plasma concentrations of ~10-15 μM, however, concentrations in the liver are much higher (40-1000 μM) compared to other organs[212,213]. Similar tissue distributions[214,215] and serum concentrations[216] have been found in humans. This accumulation of metformin in the liver is important for the suppression of hepatic glucose production which involves the inhibition of fructose-1-6-bisphosphatase[217] and mitochondrial glycerol-3-phosphate (mGPD)[213,218] and the activation of AMP-activated protein kinase (AMPK) which improves insulin sensitivity through the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC)[179]. Metformin may also lower blood glucose by acting in the gastrointestinal tract where it alters the gut microbiome[219,220] and stimulates glucagon-like peptide-1 (GLP-1) release[189]; however increases in GLP1 are not required for metformin-induced glucose lowering[221].

In addition to lowering blood glucose, metformin consistently induces weight loss in people with or without type 2 diabetes[46,120,222–225]. This effect on weight loss is not due to increases in energy expenditure[222,226], but instead involves the suppression of appetite[223,227]. Many preclinical studies have also observed beneficial effects of metformin for slowing aging and treating a multitude of diseases including but not limited to cognitive disorders, multiple cancers and cardiovascular disease. These findings have laid the foundation for the initiation of many clinical trials but given the low concentrations of metformin outside the gastrointestinal tract and the liver, the mechanisms by which metformin suppresses appetite and elicits such a multitude of beneficial remains unclear. Given the emerging role of hepatokines in regulating metabolism, we hypothesized that

metformin may increase the secretion of a hepatocyte derived endocrine factor that would communicate with the central nervous system to elicit beneficial effects[211].

We began by examining transcriptional changes occurring with acute metformin treatment in primary mouse hepatocytes from wildtype C57Bl6J mice and found significant changes in 1403 transcripts (722 upregulated and 681 downregulated) **(Extended Data Figure 1A, Supplementary Table 1)**. To determine which of these transcripts could be secreted we cross referenced this list with the mouse secretome[228] and found 51 (33 upregulated and 18 downregulated) gene products altered by metformin treatment (**Supplementary Table 2)**. To determine which of these transcripts may be of clinical relevance we subsequently examined 900 proteins in the serum of 16 metformin naïve people who were randomized to metformin (N=10) or no metformin (continuing standard care-denoted as control) (N=6) for 8 weeks in the Remission Evaluation of Metabolic Interventions in Type 2 Diabetes (REMIT) pilot trial[229]. Subject characteristics are provided in **Supplementary Table 3**. Of the upregulated secreted gene products in hepatocytes (**Supplementary Table 2**), the most significantly upregulated protein in the serum of subjects taking metformin (+1.8 fold) relative to those who continued standard of care was growth differentiating factor 15 (GDF15) **(Figure 1A**).

GDF15 is a member of the transforming growth factor beta (TGFβ superfamily that is highly expressed in the liver[25]. Recent studies have indicated that recombinant GDF15 suppresses appetite and promotes weight loss through interactions with the GFRAL receptor in the hindbrain[230–233]. Consistent with these observations, increases in serum GDF15 were associated with weight loss in patients with type 2 diabetes taking metformin or those receiving standard of care (**Figure 1B**). These data indicate that metformin increases GDF15 mRNA expression in mouse hepatocytes and that increases in serum

GDF15 are associated with weight loss in people with type 2 diabetes, however, the primary tissue(s) contributing to this increase in GDF15 in vivo is unknown.

To examine potential mechanisms linking metformin to elevations in serum GDF15 we conducted studies in primary mouse hepatocytes. Metformin increased GDF15 expression by 55% **(Figure 2A)** and dose-dependently increased GDF15 release into the media **(Figure 2B).** Structurally similar biguanides, phenformin and buformin also increased GDF15 release from hepatocytes **(Extended Data Figure 1B and 1C)**. Metformin has been suggested to regulate multiple pathways secondary to the inhibition of mitochondrial complex I[42–44]. However, rotenone, a potent non-reversible complex I inhibitor, did not increase GDF15 release from hepatocytes **(Extended Data Figure 1D)**. Metformin increases AMPK and ACC phosphorylation[179], however, GDF15 secretion was not altered in hepatocytes genetically lacking AMPKβ1 (which reduces AMPK activity in hepatocytes by ~90%) or AMPK phosphorylation sites on ACC (ACC DKI) **(Extended Data Figure 1E)**. These data suggest that the inhibition of complex-1 or activation of AMPK is not required for metformin-stimulated GDF15 release from hepatocytes.

In multiple cell types a critical regulator of GDF15 transcription is the integrated stress response that culminates in interactions between the C/EBP homology protein (CHOP) and activating transcription factor 4 (ATF4) [25]. Consistent with previous reports showing that metformin acutely activates the integrated stress response[218,234], metformin increased ATF4 and CHOP expression **(Figure 2C-E).** To determine if ATF4 and CHOP were essential for metformin-induced GDF15 secretion, we utilized ATF4 siRNA. This siRNA did not reduce basal levels of ATF4, likely due to low ATF4 turnover in hepatocytes, however it prevented the increase in ATF4 seen with metformin treatment **(Figure 2F,G)**. Importantly, ATF4 siRNA blunted the increase of GDF15 in response to

metformin treatment **(Figure 2H)**. Hepatocytes generated from CHOP null mice were also refractory to metformin **(Figure 2I, J)**. These data demonstrate that metformin increases the secretion of GDF15 from hepatocytes through ATF4 and CHOP.

To examine the mechanism and potential physiological importance of metformin-induced increases in GDF15, we generated GDF15 null mice (GDF15 KO) and performed experiments using a single oral gavage of metformin. The dose of metformin was selected because it has been shown to elicit clinically equivalent serum concentrations of metformin in mice, and in agreement with previous studies[212], we found serum concentrations of metformin of approximately 150μM **(Extended Data Figure 2A**). On a control chow diet, treatment of wildtype (WT) mice with metformin increased serum GDF15 levels but this effect was not observed in GDF15 KO mice (**Figure 3A)**. In separate experiments mice were then treated with a single oral gavage of metformin while housed in metabolic cages to monitor food intake, physical activity levels, respiratory quotient and energy expenditure and metformin reduced food intake in both WT and GDF15 KO mice to a similar degree (**Figure 3B-D**). Experiments were then repeated in mice fed a high-fat diet (HFD). Metformin increased serum GDF15 in WT but not GDF15KO mice, but in contrast to mice fed a chow diet, this was accompanied by reduced food intake in WT but not GDF15 KO mice **(Figure 3F-H)**. Differences in food intake between WT and GDF15 KO mice following metformin treatment were unlikely due GLP-1 which was comparable between genotypes (**Extended Data Figure 2B)**. Consistent with differences between genotypes with respect to food intake, metformin reduced the respiratory exchange ratio in WT but not GDF15 KO mice (**Extended Data Figure 2C, D)**. There were no differences in other metabolic parameters including physical activity (beam breaks), or energy expenditure

(Extended Data Figure 2E, F). These data indicate that metformin acutely suppresses appetite in mice fed a HFD through GDF15.

We subsequently examined the potential chronic consequences of metformin exposure by treating WT and GDF15 KO mice fed a HFD with metformin in the drinking water. In WT mice, chronic metformin treatment lowered food intake and reduced weight gain over time (**Figure 4A, B, D**). In contrast, metformin did not suppress food intake or weight gain in GDF15 KO mice (**Figure 4A, C, D**). Importantly, metformin lowered fasting insulin **(Figure 4E)** and improved glucose tolerance in WT mice **(Figure 4F, G)**; effects which were eliminated in GDF15 KO mice (**Figure 4H, I)**. These data indicate that in mice fed a HFD, metformin suppresses appetite and induces weight loss through increases in GDF15 and this is important for reducing serum insulin and improving glucose tolerance.

To further examine the mechanisms contributing to this weight loss we assessed adiposity, physical activity, RER, energy expenditure and water consumption. Metformin tended to reduce adiposity in WT but not GDF15 KO mice without altering lean mass in either genotype (**Extended Data Figure 3A, B**). There were no differences in physical activity, RER or energy expenditure **(Extended Data Figure 3C-E)** even when corrected for body mass or lean mass **(Extended Data Figure 3F-I).** Reductions in feeding in WT mice treated with metformin were unlikely due to taste aversion as water intake was unchanged with metformin supplementation, and as expected resulted in a daily metformin dose of approximately 250mg/kg/day **(Extended Data Figure 4A, B)**. Consistent with previous literature[212], this dose of metformin delivered through the drinking water resulted in serum metformin concentrations of approximately 5μM and was not different between groups **(Extended Data Figure 4C)**. Lastly, we examined whether the effects of metformin

on serum GDF15 could be secondary to caloric restriction by matching the reduced food intake-induced by metformin to mice not receiving any metformin (**Extended Data Figure 4D**), however only metformin treated mice had increased serum GDF15(**Extended Data Figure 4E**). These data indicate that reductions in weight gain with metformin are due to GDF15 suppression of appetite.

Given the low systemic concentrations of metformin and wide-ranging beneficial effects on whole body parameters including the suppression of appetite, we hypothesized that metformin may induce the expression of a metformin-regulated endocrine factor. We find that metformin induces the expression of GDF15 in hepatocytes through a mechanism requiring ATF4 and CHOP. Furthermore, clinically relevant dosing of metformin increases serum GDF15 in mice and humans. Our findings showing that metformin acutely-increases serum GDF15 are consistent with findings by us[210] and others[235] showing that serum GDF15 is correlated with metformin dose but not the use of other glucose-lowering therapies in patients with insulin resistance or type 2 diabetes and importantly establish that increases in serum GDF15 are not secondary to other actions of metformin (i.e. insulin sensitization, glucose lowering, weight loss) And while our in vitro studies focused on the mechanisms by which metformin-stimulated GDF15 release from hepatocytes, the primary tissue(s) contributing to increases in serum GDF15 in vivo are not known. Given metformin accumulates at high levels in both the liver and the gastrointestinal tract future studies utilizing tissue-selective GDF15 null mice will be important to establish the relative contribution of these tissues to serum GDF15 levels and the regulation of appetite.

Our studies also establish the potential clinical significance of metformin-induced increases in GDF15 by demonstrating that this is important for reducing appetite and weight gain in mice fed a HFD. These GDF15 dependent effects of metformin to suppress

appetite and reduce weight gain in mice fed a HFD are consistent with findings that recombinant GDF15 induces weight loss through GDNF family receptor α–like (GFRAL) inhibition of appetite without altering energy expenditure[230–233]. Interestingly, metformin also reduced food intake in mice fed a control chow diet, however, this effect was not dependent on GDF15 suggesting distinct interactions between GDF15 and diet that require further investigation.

Our findings also raise a number of exciting avenues of research. There are currently over 1500 clinical trials registered testing the effects of metformin in different disease conditions including cancers, cardiovascular disease and even aging (clinicaltrials.gov). Mice overexpressing GDF15 have enhanced lifespan and are protected from atherosclerotic cardiovascular disease[236–241]. These phenotypes are remarkably similar to those induced by metformin, which also reduces cardiovascular disease and potentially improves lifespan[49,51]. Therefore, the potential that GDF15 is playing a causal role in multiple beneficial effects of metformin treatment warrant further investigation.

Figure 1

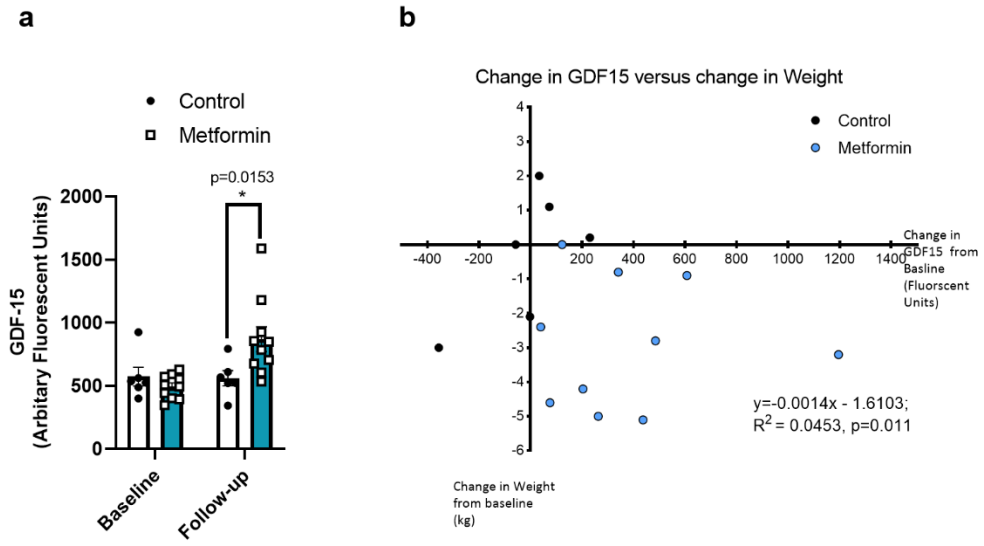


Figure 1: ***Metformin increases serum GDF15 and is associated with reductions in body mass in subjects with type 2 diabetes.*** **A)** Serum GDF15 measured at baseline and follow-up after randomization to standard care (control, n=6) or metformin (n=10) for 8 weeks. Data is represented as mean ± S.E.M. * indicates $p<0.05$ by repeated measure two-way ANOVA with Sidaks multiple comparison test. **B)** Change in body mass versus change in serum GDF15 in subjects receiving standard of care (control, n=6) or metformin (n=10) for 8 weeks. A linear model was constructed with a change in protein level from baseline to 8 weeks as the dependent variable and metformin status as the independent variable. Two-sided p-values are reported.

Figure 2

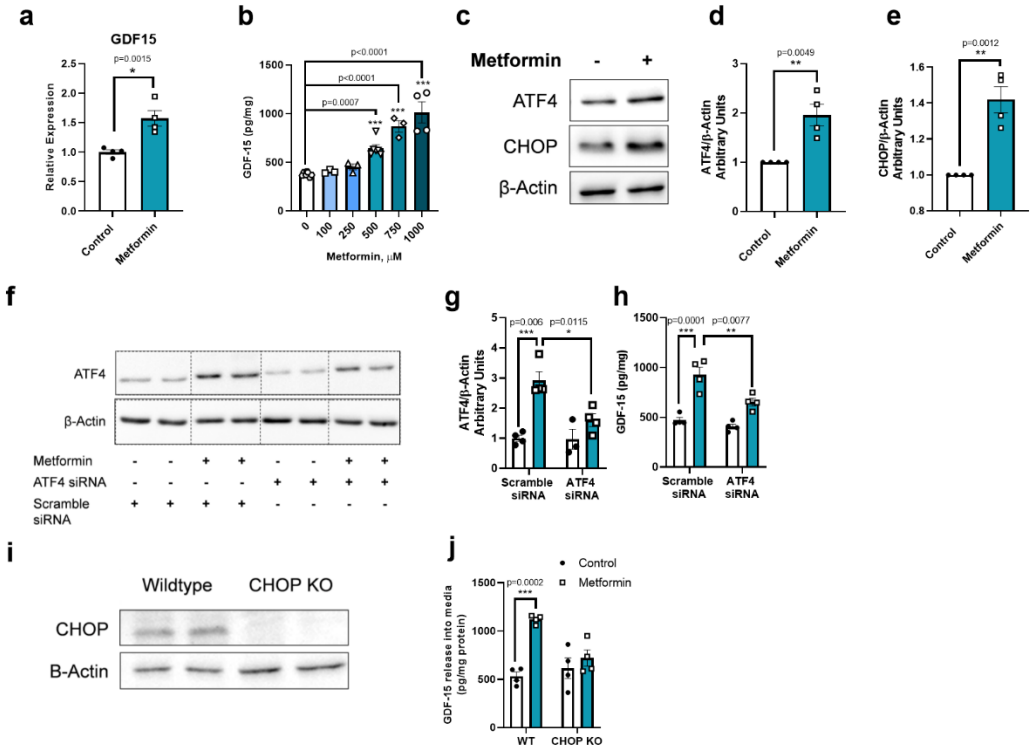


Figure 2: ***Metformin increases GDF15 release from hepatocytes through an integrated stress response pathway.*** A) Metformin increases GDF15 mRNA (n=4 per group) * indicates $p<0.05$ by t.test with 1000 permutations of the data. **B)** Metformin dose dependently increases GDF-15 release from primary hepatocytes over 24 hours of exposure at doses of 0μM (n=6), 100μM (n=3) 250μM (n=3), 500μM (n=6), 750μM (n=3) and 1000μM (n=4), n is of biologically independent samples that were averaged from triplicate or quadruplicate measurements. ** indicates $p<0.01$, *** indicates $p<0.001$by one-way ANOVA, with Sidak's multiple comparison test. **C-E)** Metformin increases protein expression of ATF4 (n=5 for control and metformin) and CHOP (n=4 for control and metformin)

relative to β-actin in hepatocytes over 24hrs. **F-H)** siRNA knockdown of ATF4 blunts metformin-induced ATF4 expression and GDF15 release (n=4 except for control ATF4 siRNA, n=3 independent samples). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, by 2-way ANOVA with sidak's multiple comparison test. **I-J)** Genetic deletion of CHOP prevents metformin-induced GDF15 secretion (n=4 independent samples) *** indicates $p<0.0001$, by 2-way ANOVA with sidak's multiple comparison test. Data is represented as mean ± S.E.M.

Figure 3

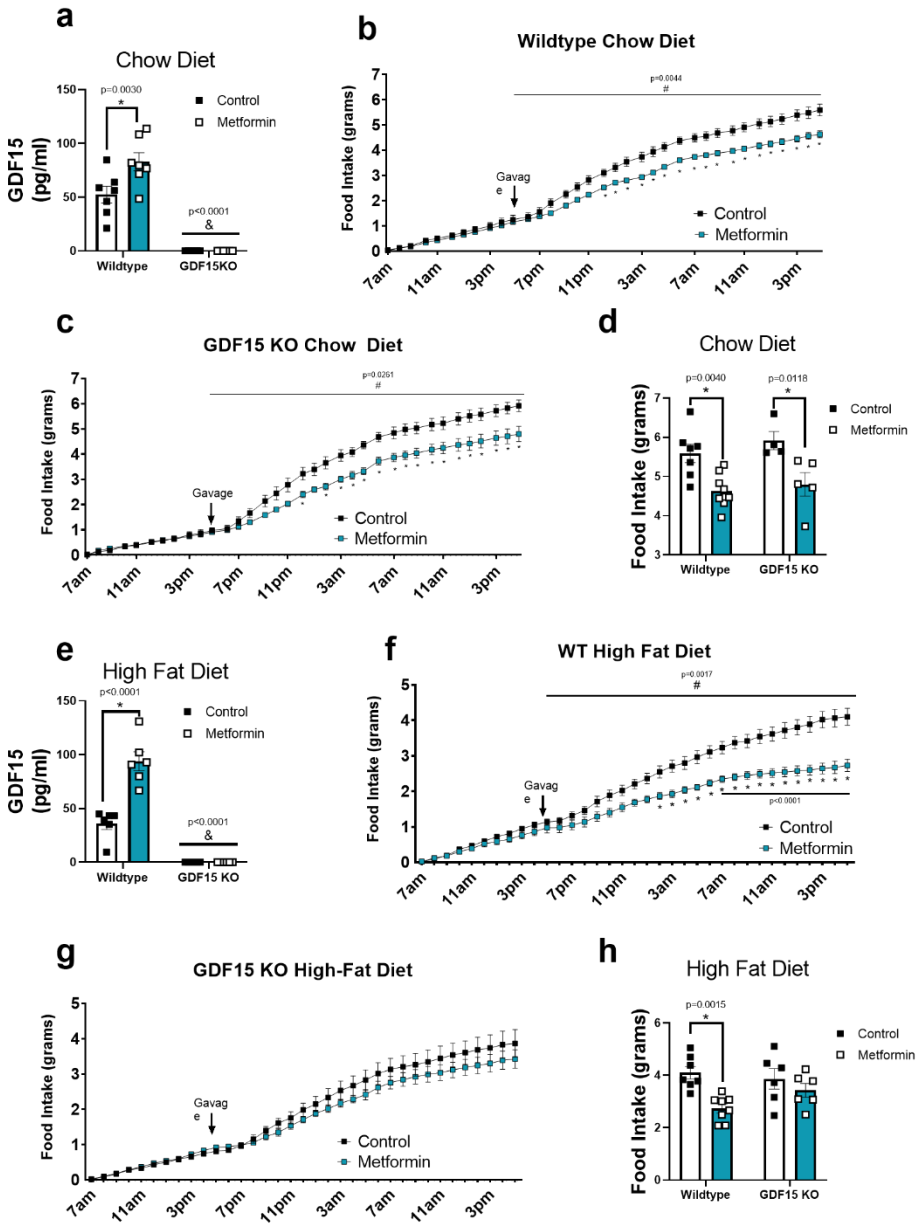


Figure 3: ***Metformin reduces food intake in mice fed a*** **HFD** ***through GDF15.***

A) Serum GDF15 in mice fed a control diet 6 hours after an oral gavage of metformin 250mg/kg body weight or equal volume of saline (control), Wildtype

control (n=7), Wildtype metformin (n=7) GDF15 KO control(n=6) and GDF15KO metformin (n=6). **B-D**) Food intake following gavage Wildtype control (n=7), Wildtype metformin (n=9), GDF15 KO control (n=4), GDF15 KO metformin (n=5). **E**) Serum GDF15 was measured 6 hours following single oral gavage of metformin 250 mg/kg body weight or appropriate volume of saline following 2-4 weeks of 45% HFD (n=6 animals per group). **F-H**) Mice on 45% HFD treated with metformin 250mg/kg body weight or appropriate volume of saline by gavage 2 hours prior to the onset of the dark period (indicated by arrow). Food intake was measured for 24 hours following gavage in wildtype control (n=7), wildtype metformin (n=8), GDF15KO control (n=6), GDF15KO metformin (n=6) treated animals. Data is represented as mean ± S.E.M. # indicates $p<0.05$ for overall effect of metformin, & indicates $p<0.05$ for overall effect of genotype * indicates $p<0.05$ by two-way ANOVA with Sidaks multiple comparison test.

Figure 4

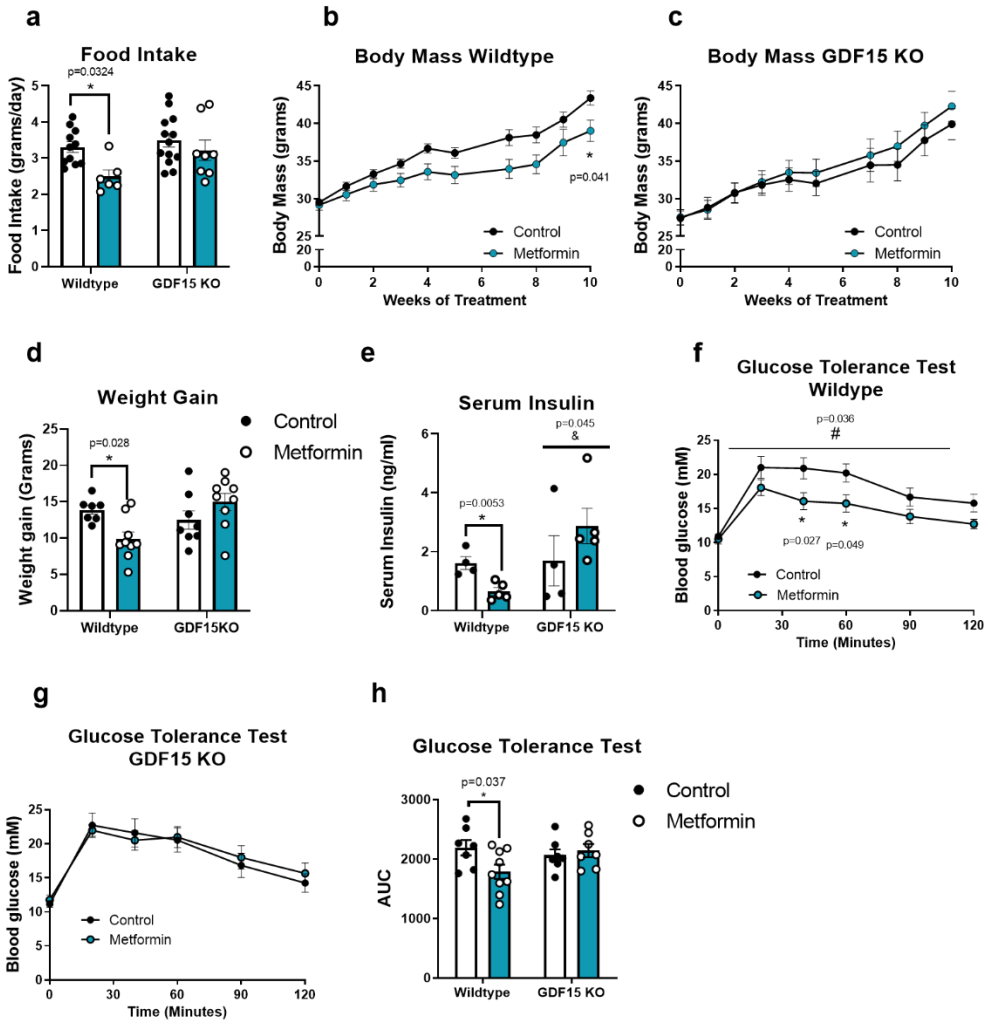
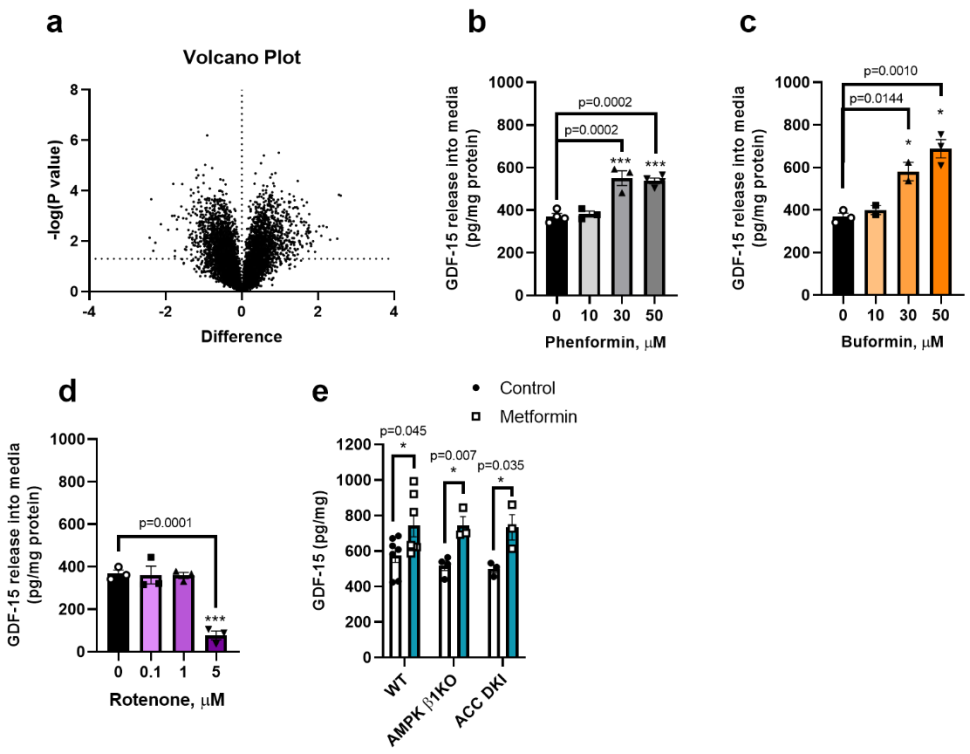


Figure 4: ***Metformin reduces body mass and serum insulin, and improves glucose tolerance through GDF15.*** Mice were fed a 45% HFD and provided ad libitum access to water (control) or water containing metformin. **A)** Food intake at week 7 of treatment (Wildtype control (n=11), Wt metformin (n=6), GDF15 KO control (n=13) and GDF15 KO metformin (n=8)). Body mass (**B)** in WT (control n=7, metformin n=9) (* indicates p <0.05 between control and metformin by repeated measures 2-way ANOVA with Sidak's multiple comparison test) and

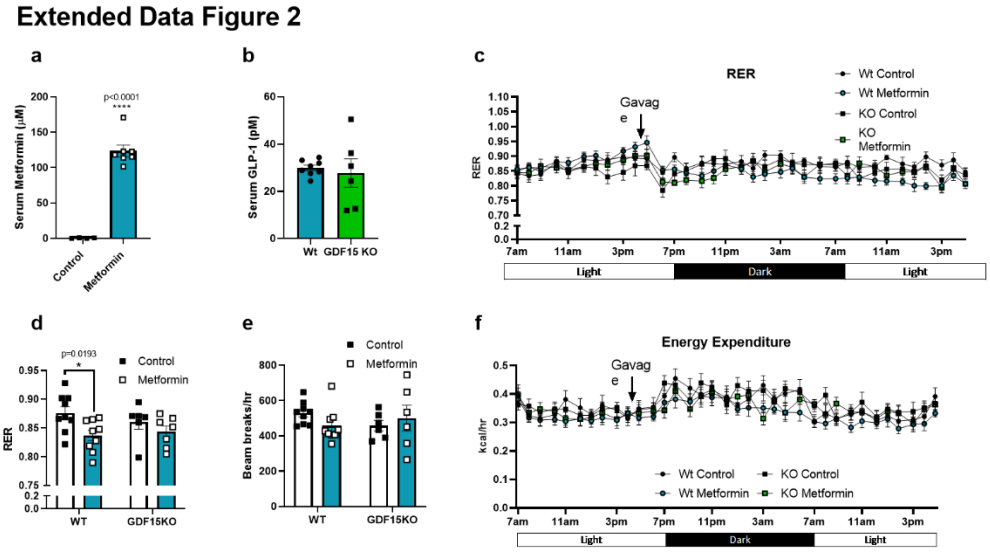
(**C)** GDF15 KO (control n=8, metformin n=8). (**D**) Total weight gain over 10 wks**.** **E)** 12hr fasting serum insulin (controls n=4, metformin n=5 per genotype), * indicates $p<0.05$ by t.test corrected by multiple comparisons using Bonferroni-Sidak method. **F-H)** Glucose tolerance was assessed following a 6 hour fast and bolus of glucose (1.2g/kg body weight) in WT (control n=7, metformin n=9) or GDF15 KO mice (control n=7, metformin n=7). Data is represented as mean ± S.E.M. For panels A, D, E, H & indicates $p<0.05$ for overall effect of genotype, * indicates $p<0.05$ between control and metformin by 2-way ANOVA with Sidak's multiple comparison test. For panels B, C, F, G, # indicates $p<0.05$ for overall effect of metformin, * indicates $p<0.05$ between control and metformin by repeated measures 2-way ANOVA with Sidak's multiple comparison test.

Extended Data Figure 1

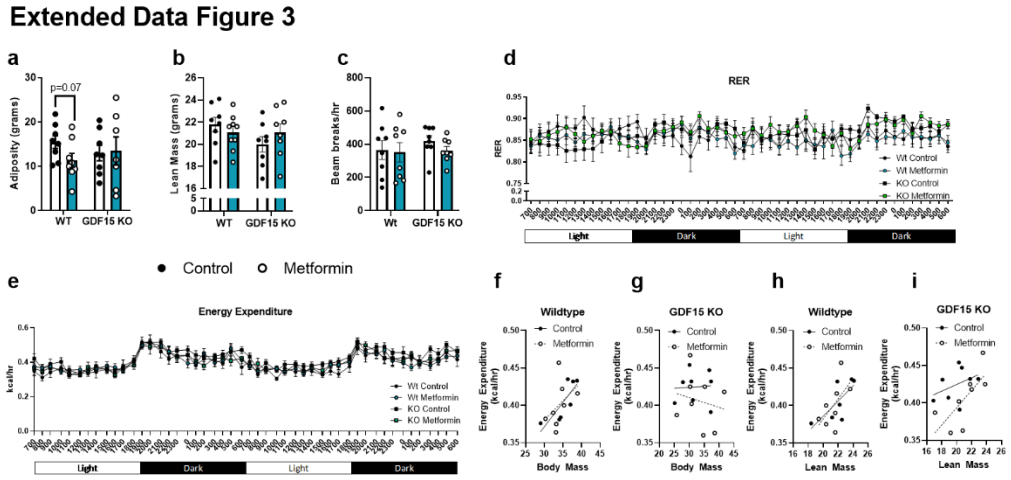


Extended Data Figure 1: M*etformin, phenformin and buformin increase GDF15 release independent of complex 1 inhibition or AMPK* A) Volcano plot showing differentially regulated genes following 24hours of metformin treatment (metformin-control, n=4 per group). Structurally similar biguanides to metformin, **B)** phenformin (0μM n=4, 10μM n=3, 30μM n=3 and 50μM n=4) and **C)** buformin (0μM n=3, 10μM n=2, 30μM n=2, and 50μM n=3) dose dependently stimulate GDF-15 release. **D)** Complex I inhibitor rotenone (0μM, 0.1μM, 1μM, 5μM n=3) does not increase GDF-15 release. **E)** Metformin increases GDF-15 release in primary hepatocytes from Wildtype (con n=7, met n=7) AMPK

β1KO(con n=4, met n=3) and ACC DKI (con n=3, met n=3) mice. Data is represented as mean ± S.E.M. For B-D * indicates $p<0.05$, *** indicates $p<0.001$ by one-way ANOVA with Sidak's multiple comparison test, for E * indicates $p<0.05$ by 2-way ANOVA with Sidak's multiple comparison test.

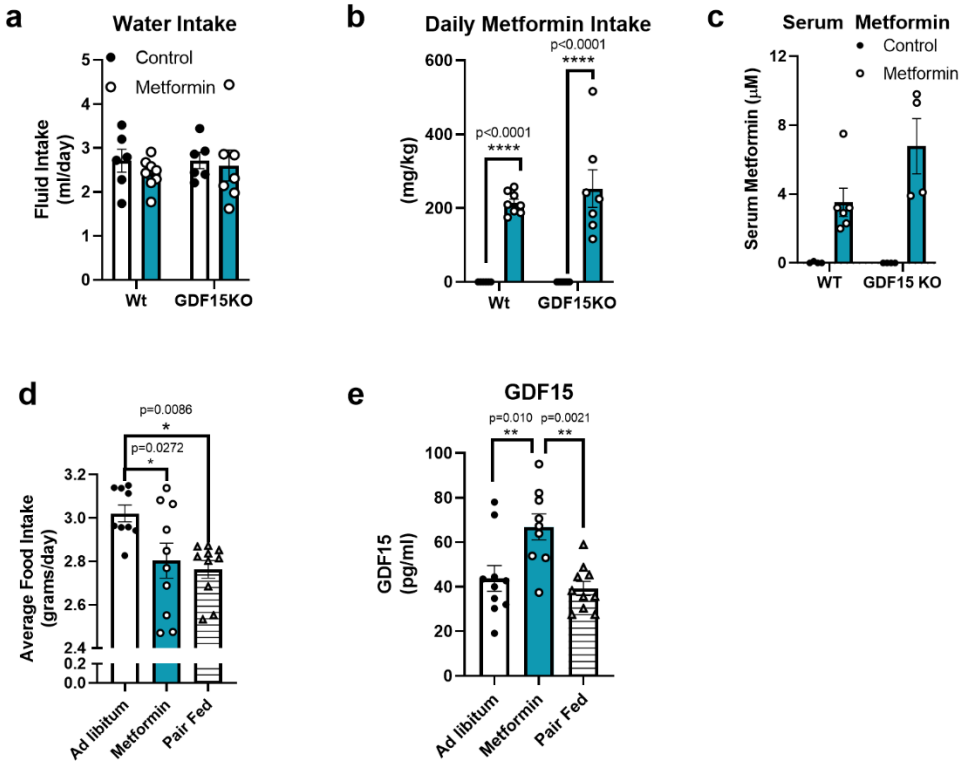


Extended Data Figure 2: ***Acute metformin treatment does not alter energy expenditure but reduces RER.*** **A)** Serum metformin 1hr following acute vehicle (n=4) or metformin gavage (n=7), * indicates $p<0.05$ by unpaired two-sided t.test. **B)** Serum GLP-1 10 minutes following acute metformin gavage in Wiltype (n=8) and GDF15 KO (n=6) mice fed a 45% HFD **C-F)** Wildtype(control n=9, metformin n=9) and GDF15 KO (control n=6, metformin n=6) mice were fed 45% HFD and put in metabolic cages and allowed to acclimatize for ~24hours prior to a single oral gavage of metformin 250mg/kg body weight or appropriate volume of saline 2 hours prior to the onset of the dark period, RER and beam breaks and energy expenditure were measured for 24 hours following gavage, data is represented as mean ± S.E.M. * indicates $p<0.05$ between control and metformin by 2-way ANOVA with Sidak's multiple comparison test.



Extended Data Figure 3: ***Chronic metformin treatment does not alter lean mass, RER or energy expenditure.*** Wildtype (control n=8, metformin n=9) and GDF15KO (control n=8, metformin n=8) mice were fed 45% HFD for 4 weeks prior to being switched to control (tap water) or metformin water (3g/L) for 10 weeks. **A,B)** Body composition was assessed at week 4 of treatment (wt con n=8, wt met n=9, KO con n=8, met =8). **C-I)** Wildtype (control n=7, metformin n=8), and GDF15KO (control n=8, metformin n=8) mice were put in metabolic cages and allowed to acclimatize for ~24hours food intake, activity, RER and energy expenditure were measured over 48 hours. Energy expenditure is shown **E)** uncorrected, **F,G**) corrected for body mass, and **H,I**) corrected for lean mass, data is represented as mean ± S.E.M.

Extended Data Figure 4



Extended Data Figure 4: ***Metformin in drinking water elicits clinically relevant serum metformin levels*****.** Wildtype and GDF15KO mice were fed 45% HFD for 4 weeks prior to being switched to control (tap water) or metformin water (3g/L) for 10 weeks. Wildtype (control n=6, metformin n=8) and GDF15KO (control n=6, metformin n-7) were put in metabolic cages and allowed to acclimatize for ~24hours water intake was measured over 48hrs (**A**). Metformin dose was calculated based on water intake and body mass (**B**). Serum metformin was measured following sacrifice at onset of the light period**(C). D, E)** Food intake of 45% HFD was monitored every 3-4 days in mice fed ad libitum (n=9), mice fed

ad libitum treated with metformin (n=10) mice pair fed to metformin treated animals (n=10), serum GDF15 was assessed at 3 weeks. Data is represented as mean ± S.E.M. * indicates $p < 0.05$, ** $p < 0.005$, ***$p < 0.001$ between control and metformin by 2-way ANOVA with Sidak's multiple comparison testing (**A-C**) and by 1-way ANOVA with Sidak's multiple comparison testing (**D-E**).

Competing Interests: SH, GP, HCG and GRS hold a patent titled; Growth differentiation factor 15 as biomarker for metformin (WO/2017/108941). SH and MK are employees of Sanofi. The other authors have no competing interests to declare.

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Author Contributions: E.A.D., R.J.F., B.K.S., N.M., S.H., G.P, H.C.G, and G.R.S. designed the experiments. E.A.D., R.J.F., B.K.S. P.M.S., M.R.M., R.L., and R.M.G performed the experiments and/or analyzed data. A.R.R. and A.G.M provided bioinformatics analysis and support. M.K. generated GDF15 KO mice. E.A.D., R.J.F., and G.R.S. wrote the manuscript. All authors edited the manuscript and provided comments.

3. Chapter 3

The SGLT2 inhibitor canagliflozin suppresses sterol synthesis and Interleukin-1 beta though AMPK

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Prepared for publication.

3.1 Preface and Significance to thesis

Our lab has previously demonstrated that canagliflozin is a potent AMPK activator in hepatocytes[242]. Clinically, canagliflozin has been shown to be an effective treatment for T2D and has produced unexpectedly robust reductions in CVD events in individuals with and without T2D. This work sought to examine the effects of canagliflozin in macrophages, specifically looking at AMPK activation and inflammation. Additionally, we sought to investigate the effects on atherosclerosis and dyslipidemia and whether they were mediated through AMPK. We found that canagliflozin activates macrophage AMPK and reduces macrophage and systemic inflammation as well as reducing hepatic de novo lipogenesis in an AMPK dependent manner. However, there were no effects on atherosclerosis which is consistent with literature suggesting the effects of canagliflozin on CVD are mediated through reduced heart failure which may be associated with changes in circulating IL-1β observed in this study.

3.2 Author contribution

Emily was the primary contributor to data in all figures of this manuscript. Emily wrote the manuscript with Dr. Steinberg.

3.3 **The SGLT2 inhibitor canagliflozin suppresses sterol synthesis and Interleukin-1 beta though AMPK**

Title: The SGLT2 inhibitor canagliflozin suppresses sterol synthesis and Interleukin-1 beta though AMPK

Authors: Emily A. Day[1], Rebecca J. Ford[1], Jessie H. Lu[1], Rachel Lu[1], Alex E. Green[1], Eric M. Desjardins[1], James S.V. Lally[1], Jonathan D. Schertzer[1,2], and Gregory R. Steinberg[1,2].

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Short Title: Canagliflozin supresses sterol synthesis and IL-1β

Abstract Word Count: 249

Number of Figure: 4 Main figures, 2 Supplemental Figures and 1 Supplemental Table

Keywords: Sodium glucose transporter 2 (SGLT2) inhibitors, AMP-activated protein kinase (AMPK), ApoE knockout mice, cardiovascular disease risk factors, Sterol regulatory element-binding protein 1, Atherosclerosis

Abbreviations: SGLT2is; sodium-glucose cotransporter 2 inhibitors, CVD; cardiovascular disease, AMPK; AMP-activated protein kinase, ACC; acetyl-coA carboxylase, MSCF; macrophage colony-stimulating factor, BAT; brown adipose tissue, WAT; white adipose tissue, DNL; *de novo* lipogenesis, BMDMs; bone marrow derived macrophages, IL-1β; Interleukin 1 beta, TNFα; Tumor necrosis factor alpha, , IL-12; Interleukin 12, IL-18; Interleukin 18, IL-23; Interleukin 23, RER: Respiratory Exchange Ratio, ApoE; Apolipoprotein E, ApoB; Apolipoprotein B, LDL; Low Density Lipoproteins, VLDL; Very Low Density Lipoprotein. HMGR; HMG CoA-reductase

ABSTRACT

Background: Sodium-glucose cotransporter 2 inhibitors (SGLT2is) such as canagliflozin, lower blood glucose in patients with type 2 diabetes but also have an unexpectedly large benefit on reducing cardiovascular events through mechanisms which are not fully understood. Canagliflozin has been shown to increase the activity of the AMP-activated protein kinase (AMPK), a metabolic enzyme important for increasing fatty acid oxidation and energy expenditure (through the browning of white fat) and suppressing fatty acid and cholesterol synthesis as well as inflammation. Therefore, we sought to determine if canagliflozin increases energy expenditure while reducing fatty acid and sterol synthesis, chronic low-grade inflammation and atherosclerosis through a mechanism involving activation of AMPK.

Methods: ApoE$^{-/-}$ and ApoE$^{-/-}$ AMPKβ1$^{-/-}$ were fed a western diet and treated with canagliflozin by daily gavage for six weeks. Subsequent studies were conducted in bone marrow derived macrophages treated with canagliflozin.

Findings: Contrary to our hypothesis, the metabolic effects of canagliflozin, including increased energy expenditure and reduced adiposity, blood glucose and liver fatty acid synthesis were independent of AMPK β1. Acutely canagliflozin lowered liver cholesterol synthesis through AMPK phosphorylation of HMG-CoA reductase. Canagliflozin also reduced circulating IL-1β, and studies in macrophages indicated that canagliflozin-induced activation of AMPKβ1 suppressed IL1β mRNA and secretion.

Interpretation: Canagliflozin reduces hepatic cholesterol synthesis and macrophage IL-1β secretion through activation of AMPK. These pathways may be important for the cardioprotective effects seen clinically with canagliflozin.

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Introduction:

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide[30]. Impairments in lipid metabolism and a maladaptive immune response can lead to a build-up of lipid-laden macrophages (foam cells) within the intima of large blood vessels a process known as atherosclerotic CVD[32]. Lowering LDL-cholesterol by inhibiting liver HMG-CoA reductase (HMGR) or ATP citrate lyase (ACLY) is associated with reduced atherosclerosis and death from cardiovascular events[70,180,243–246]. In addition to LDL-lowering, a recent clinical trial has shown that an IL-1β neutralizing antibody reduces cardiovascular events independently of changes in lipid levels[74]. Interestingly, these beneficial effects on cardiovascular events appear to be specific to Il-1β neutralization since the anti-inflammatory agent methotrexate had no discernable benefit[75]. Collectively, these data suggest that therapies that suppress LDL-cholesterol and/or inhibit IL-1β may be effective for reducing CVD, potentially through distinct mechanisms.

Canagliflozin is a sodium-glucose transporter 2 inhibitor (SGLT2i), that inhibits the reabsorption of glucose in the renal proximal convoluted tubules to promote urinary glucose excretion and thereby reduce blood glucose[53]. In addition to reducing blood glucose, canagliflozin treatment also lowers BMI as well as triglycerides and LDL-C in patients with type 2 diabetes[55], effects associated with increases in energy expenditure and the suppression of the respiratory exchange ratio (RER)[247,248]. Importantly, these changes in metabolism are associated with a reduction in cardiovascular events compared to placebo[57] and additional analysis has revealed that these effects are unlikely explained by

improved glycemic control[54,57,249–252]. Additionally, canagliflozin has been associated with improvement in NAFLD and liver function in patients with T2D or NAFLD respectively, however, whether these outcomes are mediated through glucose lowering alone is not known[253,254]. These data suggest that in addition to urinary glucose excretion, SGLT2i's may activate additional pathways that exert beneficial effects.

One of these additional pathways which may be activated by SGLT2i's is the AMP-activated protein kinase (AMPK)[242,255–257]. AMPK is a ubiquitously expressed, heterotrimeric (consisting of α,β,γ subunits), serine-threonine kinase, that is a central regulator of lipid metabolism and inflammation, and is an attractive target for therapeutics aimed at treating metabolic diseases including type 2 diabetes and CVD (reviewed here[77,258]). In mouse hepatocytes (which are composed of primarily an AMPK α1,β1,γ1 heterotrimer) the pharmacological activation of AMPKβ1 containing heterotrimers increases phosphorylation of its downstream substrates Acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR) resulting in reductions in fatty acid and cholesterol synthesis that lowers LDL cholesterol[259–261]. In white adipose tissue AMPKβ1 is the predominant subunit and is important for increasing the mitochondrial biogenesis, the browning of white fat and energy expenditure[87,262,263]. In macrophages, where inflammatory status and metabolic activity are directly linked[81,105], the activation of AMPK switches macrophages from an M1, pro-inflammatory phenotype, to an M2, anti-inflammatory phenotype[88,89,106,107]. Macrophages are primarily comprised of an AMPK α1,β1,γ1 heterotrimer and reduced expression of AMPKα1 or β1 isoforms (which eliminates nearly all AMPK activity) accentuates pro-inflammatory signaling (NFκB and JNK) and cytokine production (Il-1β, TNFα)[86,88,89]. These data suggest that pharmacologically targeting AMPK in the liver, white adipose tissue and macrophages may

exert positive effects on CVD and NAFLD by suppressing fatty acid and cholesterol synthesis and reducing inflammation.

Given the importance of AMPK in regulating liver lipid metabolism and macrophage inflammation, we hypothesized that some of the beneficial effects of canagliflozin seen clinically may be mediated through AMPK. We subsequently examined the effects of a clinically relevant dose of canagliflozin[264,265] delivered daily for 6 weeks to ApoE$^{-/-}$ mice (with normal AMPK expression) or ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice (low levels of AMPK activity in liver, white adipose tissue and macrophages[70]).

Methods:

Animal Experiments. All animal experiments were performed in accordance with McMaster Animal Care Committee guidelines (AUP #: 16-12-41, Hamilton, ON). The generation and characterization of AMPKβ1$^{-/-}$, ApoE$^{-/-}$ AMPKβ1$^{-/-}$ and NLRP3$^{-/-}$ mice have been described previously[70,159,266]. Specific Pathogen Free female mice were group housed in a temperature-controlled facility (22-23 ^{o}C) on a 12hr light/dark cycle where food and water was provided *ad libitum*. ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ were fed standard rodent chow diet (Envigo 8640) until 6-8 weeks of age, then switched to a high-fat, high-cholesterol (0.2%) "western diet" (TD.09821, Envigo Diet) and simultaneously treated daily by oral gavage for 6 weeks with vehicle or a clinically relevant dose of canagliflozin (30mg/kg) that has been shown to activate AMPK[242,264,265]. For tissue collection, a subset of mice were fasted for 6 hours then administered insulin (0.4U/kg) for 15 minutes before tissue harvesting from anaesthetized mice. Additional tissue collection was performed following in vivo lipogenesis described below from a separate cohort of mice.

Metabolic testing. For glucose tolerance test (GTT), all mice were fasted for 6 hours beginning at 7am. Blood glucose levels were assessed by a small tail vein nick using a handheld Aviva glucometer (Roche). Intraperitoneal injections of glucose (2.25g/kg body mass) were administered to initiate the GTT, and blood glucose measures obtained at the indicated time points. Body composition measures were obtained by TD-NMR (Bruker Corporation). Measurements of respiratory exchange ratio (RER), energy expenditure, ambient activity, food and water intake were assessed using a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments) as described[267]. Commercially available kits were used to measure Insulin (EMD Millipore).

Hepatic Lipogenesis. Hepatic lipogenesis was performed as described[199,268] with the following modifications; mice treated with canagliflozin for 6 weeks (as described above) were fasted for 12 hours overnight, followed by a 2 hour refeed to enhance lipogenesis. Mice were then treated with vehicle or canagliflozin (30mg/kg) by oral gavage for 1 hour before injection with [3H]-acetate (12μCi/mouse). Tissues were collected 1 hour after injection of acetate, and frozen in liquid nitrogen. Fatty acid and sterols fractions were extracted as previously described[259,87].

Cytokine Measurement. Cytokine analysis in plasma was done using the Bio-Plex Pro Mouse Cytokine 23-Plex Immunoassay (Bio-Rad) performed according to manufacturer's instructions, as described[269]. Liver IL-1β was measured using Mouse IL-1β DuoSet ELISA (R&D Systems), following homogenization of liver in 1% BSA (reagent diluent).

Immunoblotting, and Histological Analyses. For protein analyses tissue or cell lysates were diluted with Western sample buffer and loaded in SDS/PAGE gels as described previously[179]. Proteins were resolved by molecular mass and transferred to polyvinylidene difluoride membranes prior to blocking in 5% bovine serum albumin. All primary antibodies were obtained from Cell Signaling Technologies (P-Ser79/212-ACC Cat# 3661, ACC Cat# 3662, P-Thr172-AMPK Cat # 2531, AMPK panα Cat # 2532, β-actin Cat # 5125, except for UCP1 (Alpha Diagnostic International, Cat# UCP11-A) and SREBP1 (Santa Cruz Biotechnology Inc Cat # sc-13551). Antibodies were used at concentrations of 1:1000 except β-actin and UCP-1 which were used at 1:5000 and SREBP1 which was used at 1:250. For lesion histology, hearts with aortic roots intact were fixed in 10% neutral buffered formalin, cut transversely, and embedded in paraffin. Serial sections 4μM thick were cut beginning at the aortic root origin and mounted on slides for hematoxylin and eosin staining (sections were stained every 80μM to assess lesion size). Images were

captured using Nikon 90 Eclipse microscope (Nikon) and lesions were traced manually and measured using ImageJ. Lesion sizes were calculated from 5 sections of the aortic root at 80μM intervals from each animal.

Real-time quantitative PCR (RT-qPCR). RNA was isolated from cells using High Pure RNA Isolation Kit (Roche), and from tissues using TRIzol reagent (Invitrogen) and purified using the RNeasy kit (Qiagen). All Taqman primers were purchased from Invitrogen, and relative gene expression was calculated using (2-ΔCT) method. Values were normalized to housekeeping gene β-Actin and expressed relative to wildtype with LPS (cells) or wildtype vehicle (tissues).

Cell Culture Experiments. Primary hepatocytes were isolated following EGTA and collagenase perfusion. Cells were allowed to adhere overnight in Williams Media E (10% FBS, 1% antibiotic-antimycotic, 2mM L-glutamine). The following day media was replaced with serum free media with [3H]-acetate with the addition of 30uM Canagliflozin or vehicle (DMSO) for 4 hours. Cells were washed 3x with ice cold PBS and scraped in KOH. Sterol isolation was performed as previously described[259]. A portion of the sterol fraction was counted for radioactivity. Bone marrow derived macrophages (BMDMs) were isolated and differentiated for 7 days with conditioned media (from L929 cells, a source of macrophage colony-stimulating factor; MCSF)[89]. Macrophages were then reseeded in DMEM (10% FBS, 1% antibiotic-antimycotic) at 2×10^6 cells/mL for 6 and 12 well tissue-culture plates and allowed to adhere overnight. The following day, media was replaced with serum free media and cells were treated with the indicated drugs dissolved in DMSO (final concentration 0.1%). For protein signalling by immunoblotting, cells were treated with drugs for 90 minutes in serum free media then rapidly lysed in cell lysis buffer. For

mRNA analysis and cytokine release experiments, cells were treated with drug for 90 minutes followed by the addition of 10ng/ml LPS for 24hours.

Statistical Analyses. When comparing two factors, data was analyzed using 2-way ANOVAs to determine interaction and main effects, and Sidak's post-hoc tests used for comparisons between groups. One-way ANOVA with Sidak's post-hoc test was used when one variable was being compared. All data is represented as mean ± s.e.m., and $P < 0.05$ is considered statistical significance.

Results:

Canagliflozin reduces adiposity, increases energy expenditure and reduces blood glucose in ApoE null mice independently of AMPKβ1

Daily canagliflozin treatment did not affect body mass but did reduce adiposity and percent fluid mass (**Figure 1A-C).** Consistent with previous findings[159], ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice had a lower body mass and adiposity than control ApoE$^{-/-}$ mice (**Figure 1A,B).** As expected, canagliflozin reduced twelve hour fasted blood glucose and improved glucose tolerance in both ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice (**Figure 1D,E,F)**; an effect which occurred independently of reductions in fasting insulin levels (**Figure 1G)**.

Canagliflozin suppresses liver fatty acid synthesis by suppressing ACC and ACLY expression independently of AMPK β1.

To examine mechanisms which might contribute to the lower adiposity with canagliflozin treatment mice were housed in metabolic cages to assess energy expenditure, locomotor activity as well as food and water intake. Canagliflozin treatment increased energy expenditure (heat) as well as food and water intake, without affecting ambient locomotor activity in both ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice (**Supplemental Figure 1A-D**). In both ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice canagliflozin reduced RER **(Supplemental Figure 1E**). Calculations based off of the RER suggested canagliflozin increased rates of fatty oxidation **(Supplemental Figure 1F**) while tending to reduce carbohydrate oxidation (**Supplemental Figure 1G)**. In contrast to a previous report[270], canagliflozin did not increase the expression of *Ucp1 in* white or brown adipose tissue or alter the expression of other markers of adipose tissue browning (**Supplemental Figure 2A-G**). These data indicate that increases in fatty acid oxidation with canagliflozin occur

independently of AMPK β1 and consistent with previous studies suggest that this increase in fatty acid oxidation is likely secondary to reductions in blood glucose.

In lipogenic tissues such as the liver, blood glucose is also used for the synthesis of lipids through de novo lipogenesis (DNL). In addition to increases in fatty acid oxidation a reduction in DNL can also contribute to a reduction in RER[271]. We subsequently examined liver DNL and found that consistent with previous studies[159,179], AMPKβ1$^{-/-}$ mice had higher liver DNL compared to controls**,** but surprisingly, canagliflozin suppressed DNL independently of AMPKβ1 **(Figure 2A)**. AMPK primarily regulates DNL through phosphorylation of ACC[179,261], and consistent with the activation of AMPK, canagliflozin tended to increase the ratio of phosphorylated ACC (pACC) over total ACC in the liver of ApoE$^{-/-}$ mice but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice (**Figure 2B, C**). However, total ACC protein expression was significantly reduced in the absence of AMPK β1 and by canagliflozin (**Figure 2B, D**). Consistent with lower ACC there were also significant reductions in *Acc1* and *Acc2* mRNA (**Figure 2E,F**). In addition to ACC, the enzyme ACLY, which is upstream of ACC and generates acetyl-CoA from citrate, is also critical for regulating DNL. We found that consistent with reductions in ACC expression, there was a strong trend for a reduction in *Acly* expression with canagliflozin treatment (*p=0.058)* and that *Acly* expression was also reduced basally in ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice (**Figure 2G**). Consistent with reductions in *Acly* mRNA we found that ACLY protein expression was also reduced following treatment with canagliflozin in both genotypes (**Figure 2H, I**). Both ACLY and ACC expression are regulated by SREBP1c, therefore we examined protein levels of this transcription factor, and found that canagliflozin treatment reduced SREBP1c protein in the liver of both ApoE$^{-/-}$ and ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice. These data suggest that reductions in blood glucose elicited by canagliflozin leads to the inhibition of

DNL independently of AMPK β1 through transcriptional downregulation of key lipogenic enzymes ACLY and ACC.

Canagliflozin reduces liver cholesterol synthesis through phosphorylation of HMGR

Canagliflozin has been show to lower LDL-cholesterol in some but not all studies[272] . AMPK inhibits cholesterol synthesis through phosphorylation of HMGR[259–261]. Consistent with this, we found that canagliflozin reduced liver sterol synthesis in ApoE$^{-/-}$ controls but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice **(Figure 3A).** To examine whether this AMPK-dependent inhibition of sterol synthesis required the phosphorylation of HMGR we generated primary hepatocytes from wildtype mice, or mice in which the AMPK phosphorylation site on HMGR is mutated to prevent phosphorylation and inhibition (Ser782Ala- herein described as HMGR KI mice). We found that consistent with AMPK β1 dependent inhibition of sterol synthesis, canagliflozin suppressed sterol synthesis in hepatocytes from WT but not HMGR KI mice (**Figure 3B**). Consistent with reductions in liver sterol synthesis, plasma ApoB was reduced in ApoE$^{-/-}$ mice by canagliflozin, however, ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice, also had low levels of ApoB that did not decline further with canagliflozin (**Figure 3C**). This low level of ApoB in ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice may have been secondary to the reductions in liver ACLY which would be expected to lower acetyl-CoA availability for HMGR-mediated sterol synthesis. Despite lower levels of ApoB in ApoE$^{-/-}$ mice, surprisingly, we found no change in the size of atherosclerotic plaques in either genotype over the 6 week treatment period (**Figure 3D,E)**. These data indicate that *acutely* canagliflozin-induced activation of AMPK suppresses sterol synthesis through phosphorylation of HMGR but chronically, reductions in sterol synthesis are likely mediated through lower blood glucose and transcriptional inhibition of ACLY.

Canagliflozin decreases IL1β secretion from macrophages through a mechanism requiring AMPKβ1 but not the NLPR3 inflammasome.

In addition to lipids, inflammation is also a major driver of cardiovascular events[74]. The activation of AMPK reduces inflammation[89,273,274], therefore canagliflozin mediated AMPK activation could be important for the cardioprotective effects of canagliflozin. Canagliflozin reduced IL-1β in the plasma of ApoE$^{-/-}$ mice with the same trend present in the livers (p=0.069); an effect which blunted in ApoE-/- AMPKβ1$^{-/-}$ mice (**Figure 4A, B**). Other markers of inflammation in the plasma remained unchanged (**Supplemental Table 1**). Macrophages are an important cell type contributing to increases in plasma and liver IL-1β [275,276] and AMPK has been shown to reduce macrophage IL-1β expression[89]. To examine the potential mechanisms mediating the suppression of IL-1β, we treated bone marrow derived macrophages (BMDMs) with canagliflozin. The treatment of BMDMs with canagliflozin at 10μM increased the phosphorylation of AMPK (Thr172) and ACC (Ser79) in wildtype but not AMPK β1$^{-/-}$ macrophages (**Figure 4C-E**). Consistent with the activation of AMPK, we found that canagliflozin suppressed the secretion of IL-1β from wildtype but not AMPKβ1$^{-/-}$ macrophages (**Figure 4F**).

The secretion of IL-1β by macrophages requires cleavage of pro-IL-1β by the NLRP3 inflammasome, an effect which is inhibited by AMPK activation [277,278]. Therefore we hypothesized that AMPK inhibition of the NLRP3 inflammasome may be important for the effects of canagliflozin to lower circulating IL-1β however, canagliflozin reduced IL1β in both wildtype and NLRP3$^{-/-}$ macrophages (**Figure 4G)**. We looked upstream of the NLRP3 inflammasome at IL-1β mRNA, and found that canagliflozin reduced IL-1β mRNA in a dose dependent manner in wildtype but not AMPKβ1$^{-/-}$ macrophages (**Figure 4H**) These

data indicate that canagliflozin suppresses the expression and secretion of IL-1β from macrophages through a mechanisms involving AMPKβ1.

Discussion:

Dyslipidemia and inflammation are primary risk factors for CVD. The SGLT2 inhibitor canagliflozin reduces cardiovascular events[57,279,280] but the mechanisms mediating these beneficial effects are currently unclear. In the current study we find that canagliflozin exerts beneficial effects on metabolism through both AMPK dependent and independent pathways. Specifically, we find that canagliflozin lowers blood glucose, liver fatty acid synthesis and increases RER and energy expenditure independently of AMPK while the inhibition of liver sterol synthesis and secretion of IL-1β requires AMPK.

The defining feature of SGLT2is is that they lower blood glucose due to glycosuria. A reduction in blood glucose enhances rates of fatty acid oxidation due to a reduction in malonyl-CoA. Malonyl-CoA is an allosteric inhibitor of CPT-1 and also the first committed step in DNL. In contrast to other AMPK agonists such as metformin or direct AMPK activators, the effects of canagliflozin to lower fatty acid synthesis in vivo were independent of AMPK and were likely secondary to reductions in blood glucose, resulting in reduced SREBP1c and subsequent downregulation of ACLY and ACC expression. To the best of our knowledge this is the first evidence to shown that canagliflozin reduces the key lipogenic regulator, SREBP1. Genetic inhibition of ACC and ACLY leads to increases in fatty acid oxidation and reductions in fatty acid synthesis. The inhibition of ACC is currently under development for the treatment of NAFLD[180,281] and hepatocellular carcinoma (HCC)[268], however, increases in hypertriglyceridemia have been a potential complication of these therapies[281]. ACLY inhibitors also lower liver fat in preclinical animal models[70] and are associated with weight loss[282]. Our current findings cannot

determine if the effects seen here on ACC and ACLY expression are directly mediated by reduced blood glucose but it is clear the suppression of flux through this pathway occurs independently of AMPK.

In addition to suppressing liver fatty acid synthesis, canagliflozin also lowered sterol synthesis. This reduction in sterol synthesis was AMPK β1 dependent and was associated with reductions in phosphorylation of HMGR by AMPK. Canagliflozin also suppressed ACLY which reduces acetyl-CoA which is required for fatty acid synthesis as described above but also sterol synthesis. Interestingly, liver ACLY expression was reduced in ApoE AMPKβ1$^{-/-}$ compared to controls ApoE$^{-/-}$ controls and was not reduced further with canagliflozin treatment. These findings are consistent with previous studies which have established an important reciprocal link between AMPK and ACLY activity. Thus inhibitory phosphorylation of HMGR by AMPK as well as reductions in liver ACLY expression may be important for the effects of canagliflozin to lower LDL-C in clinical populations[55].

Anti-IL-1β therapies have been associated with reduced CV events, independent of changes in serum lipid levels[74]. Therefore, we sought to examine if canagliflozin reduced IL-1β, which could partially explain outcomes seen clinically. The relationship between canagliflozin and IL-1β is especially interesting because IL-1β has been directly implicated with heart failure[283,284], which seems to be the indication that canagliflozin is most protective against CVD mortality[279,280]. In the current study, we found that canagliflozin treatment reduced circulating IL-1β, while having no effect on other inflammatory cytokines measured. Subsequent studies in bone marrow derived macrophages demonstrated that canagliflozin activated AMPK and reduced IL-1β mRNA and secretion an effect we hypothesized was potentially mediated by AMPK inhibition of the NLRP3

inflammasome (recently reviewed here[285]). Increased NLRP3 inflammasome activity and the consequent increase in circulating IL-1β has been linked to a decrease in AMPK activity associated with obesity[286]. However, canagliflozin dose–dependently reduced IL-1β in both WT and NLRP3$^{-/-}$ macrophages suggesting, that these effects are independent of the activation of the canonical NLRP3 inflammasome. How AMPK suppresses IL-1β expression is not understood but is an area of active investigation.

Despite reductions in both ApoB and IL-1β we did not see any changes in atherosclerosis with canagliflozin. An important factor potentially contributing to null effects of canagliflozin on atherosclerosis is the increased food intake seen in this study. Both duration and cholesterol content of diet has been shown to worsen atherosclerosis in ApoE$^{-/-}$ mice[287], and dietary restriction has been shown to reduce atheroslceosis[288], therefore, increased food intake may be masking potential positive effects on atherosclerosis.

In conclusion, our study provides important insight into the potential mechanisms by which canagliflozin may exert beneficial effects on CVD. These data suggest that in addition to increasing glycosuria, canagliflozin also inhibits liver fatty and sterol synthesis and circulating IL1β, effects which may contribute to reductions in cardiovascular events in clinical populations. Canagliflozin lowered hepatic cholesterol synthesis, and macrophage IL-1β in an AMPK β1 dependent manner which is consistent with previous findings with AMPK activators in pre clinical models[108,259]. Future studies are now needed to evaluate whether these suppressive effects on both liver fatty acid and sterol synthesis and macrophage IL-1β may be important for the reduction in cardiovascular events seen clinically with canagliflozin treatment.

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Author Contributions: E.A.D., R.J.F., J.S.V.L, J.D.S. and G.R.S. designed the experiments. E.A.D., R.J.F., J.H.L., R.L., E.M.D., and A.E.G. performed the experiments and testing. E.A.D., R.J.F., and G.R.S. wrote the manuscript. All authors edited the manuscript and provided comments.

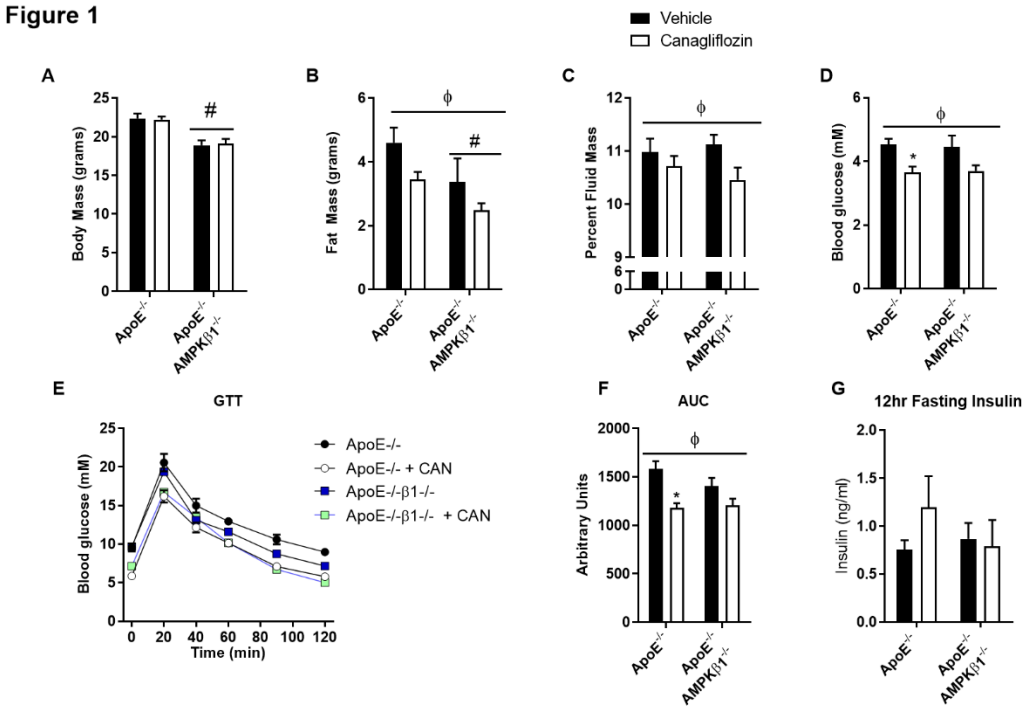


Figure 1. Canagliflozin reduces adiposity and blood glucose in ApoE null mice independently of AMPK β1 A) Body mass, **B)** adiposity, **C)** percent fluid mass, **D)** 12 hour fasted blood glucose, **E)** glucose and **F)** area under the curve analyses, **G)** 12 hour fasted insulin, of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice after 6 weeks of oral treatment with vehicle (white bars) or canagliflozin (black bars; 30mg/kg body weight). Data is expressed as mean ± s.e.m. n = 5-10 mice per group. Φ $p < 0.05$ for main effect of drug, # $p < 0.05$ for main genotype effect, *$p<0.05$ for canagliflozin versus control within a genotype.

Figure 2

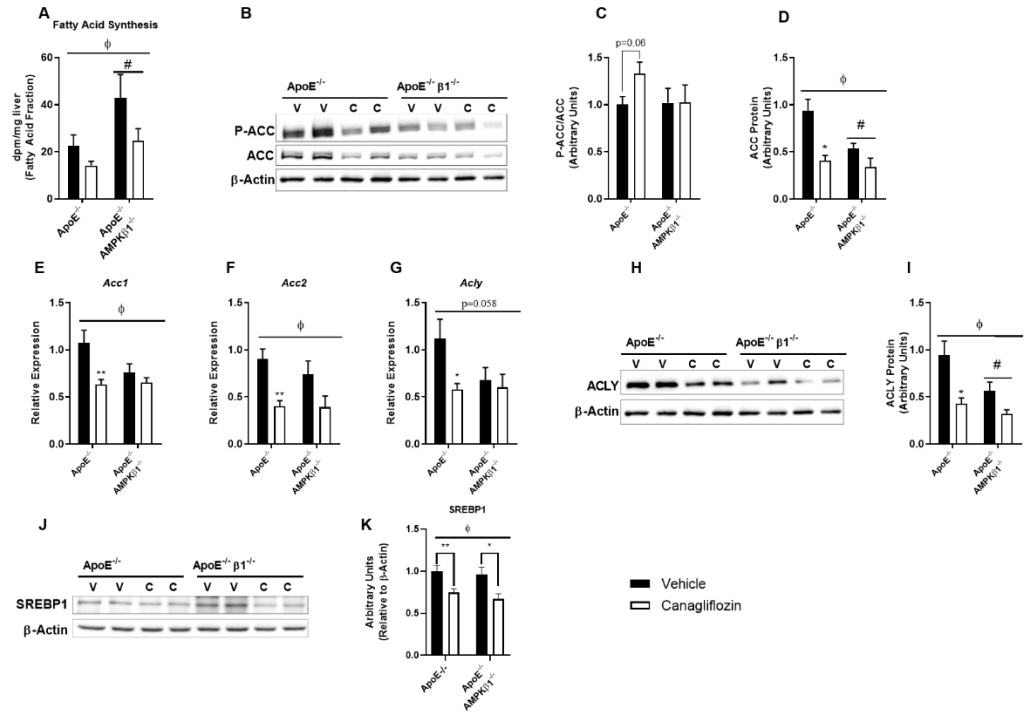


Figure 2. Canagliflozin reduces hepatic lipogenesis independently of AMPK by suppressing lipogenic gene expression. A) Fatty Acid Synthesis following canagliflozin treatment, **B,C,D)** Phosphorylation of acetyl-coA carboxylase (ACC) at serine 79 & 212 and total ACC protein content **E,F)** liver mRNA expression of *Acc1, Acc2,***G)** liver *Acyl* mRNA **H,I***)* ACLY protein content and **J,K**) SREBP1c protein, from ApoE and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice after 6 weeks of oral treatment with vehicle (white bars) or canagliflozin (black bars; 30mg/kg body weight). V, vehicle, C Canagliflozin, Data is expressed as mean ± s.e.m. n = 5-10 mice per group. Φ $P < 0.05$ for main effect of drug, # $P<0.05$ for main genotype effect, * $P<0.05$ relative to vehicle within genotype.

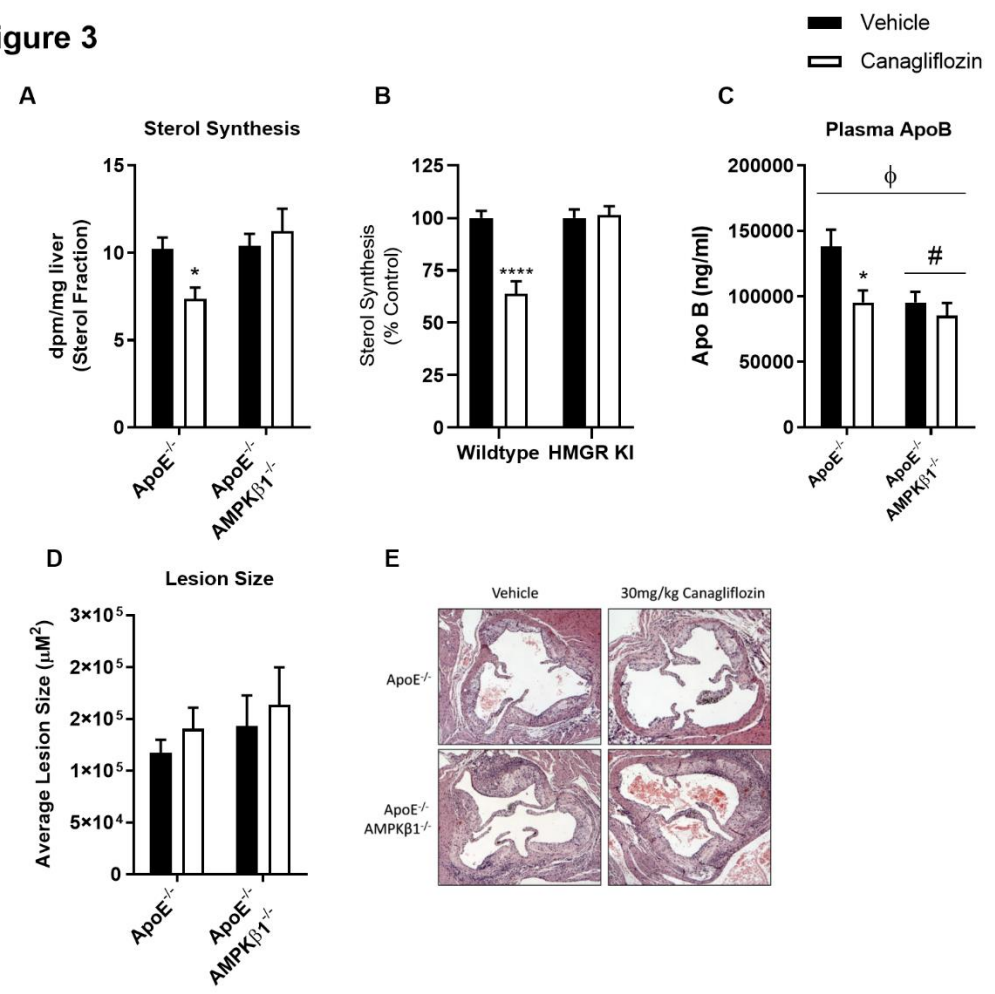


Figure 3. Canagliflozin reduces sterol synthesis in an ApoE$^{-/-}$ but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice. A. Sterol synthesis following canagliflozin treatment, **B)** Sterol synthesis of hypatocytes isolated from Wildtype and HMGRKI mice **,** , **C)** Plasma ApoB and **D)** Lesion area and **E)** representative images of H&E stained aortic roots of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice after 6 weeks of oral treatment with vehicle (white bars) or canagliflozin (black bars; 30mg/kg body weight). Data is expressed as mean ± s.e.m. n = 5-10 mice per group. Φ $P < 0.05$ for main effect of drug, # $P<0.05$ for main genotype effect, * $P<0.05$ relative to vehicle within genotype.

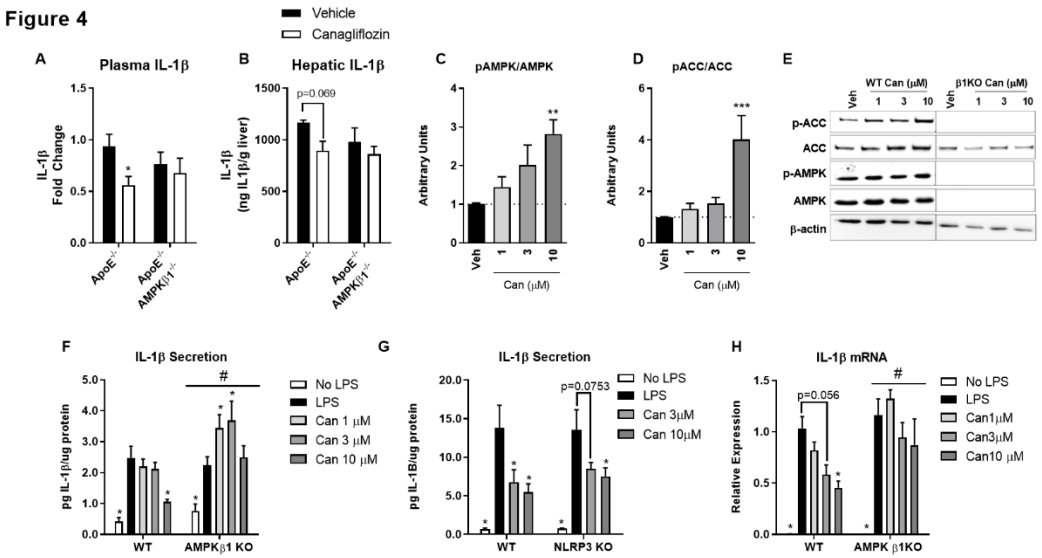
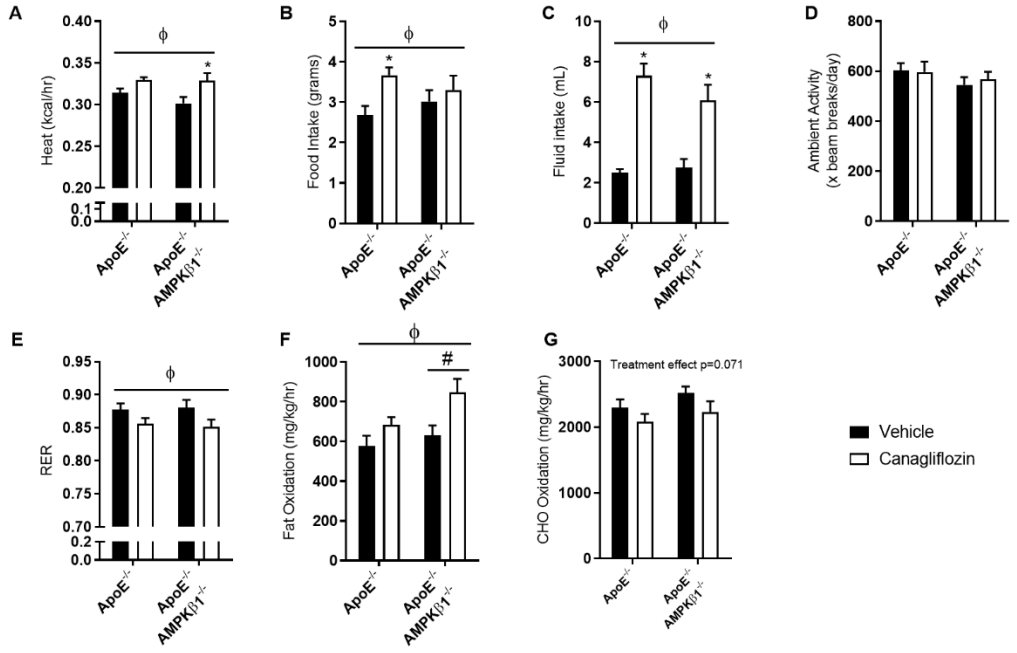


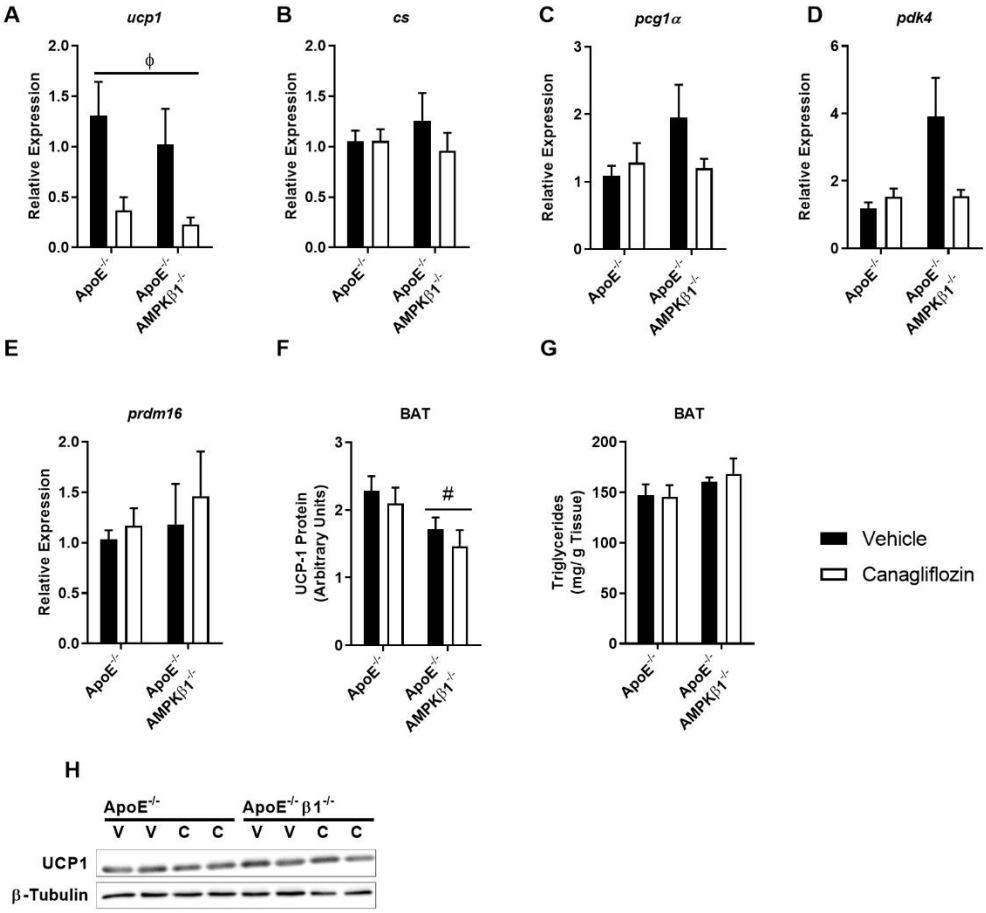
Figure 4. Canagliflozin decreases IL-1β. A) *Interleukin 1 beta* (IL-1β), in plasma and **B)** liver of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice after 6 weeks of oral treatment with vehicle (white bars) or canagliflozin (black bars; 30mg/kg body weight). **) C-E)** Phosphorylation of AMPK at threonine 172 and downstream targets acetyl-coA carboxylase (ACC) at serine 79 & 212 on in differentiated macrophages from wild type mice treated with varying concentrations of canagliflozin (Can), **F)**, **F)** IL1β protein in media measured from bone marrow derived macrophages derived from wildtype and AMPK β1$^{-/-}$ treated with canagliflozin at indicated concentrations and/or LPS (10ng/ml). **G)** IL-1β measured in media from BMDM from WT and NLRP3$^{-/-}$ mice following canagliflozin pre-treatment at indicated concentrations and LPS (10 ng/ml) stimulation *H) Il1b* mRNA expression from bone marrow derived macrophages derived from wildtype and AMPK β1$^{-/-}$ treated with canagliflozin at indicated concentrations and/or LPS (10ng/ml) Data is expressed as mean ± s.e.m., and represents 3 independent experiments performed in duplicate for immunoblotting or triplicate for ELISAs. * P < 0.05 vs vehicle or LPS if applicable, # P <0.05 for main genotype effect, Veh; vehicle.

Supplemental Figure 1



Supplemental Figure 1. Canagliflozin increases energy expenditure, food and fluid intake and decreases RER in ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice. A) Heat (Energy expenditure), **B)** food intake, **C)** fluid intake, **D)** ambient activity, **E)** RER, **F)** Fat oxidation **G)** carbohydrate oxidation of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$mice after 6 weeks of oral treatment with vehicle (White bars) or canagliflozin(black bars;30mg/kg body weight). Data is expressed as mean ± s.e.m. n = 5-7 mice per group. Φ $p < 0.05$ for main effect of drug, # $p < 0.05$ for main genotype effect *$p<0.05$ for canagliflozin versus control within a genotype.

Supplemental Figure 2



Supplemental Figure 2. Canagliflozin reduces UCP1 expression but does not change other markers of browning in iWAT or BAT. A-E) mRNA of browning markers uncoupling protein 1 (*ucp1), citrate synthase (cs), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (pgc1α), Pyruvate dehydrogenase lipoamide kinase isozyme 4 (pdk4) and prdm16* in inguinal white adipose tissue, **F,H)** UCP-1 protein and **G)** triglyceride content in brown adipose tissue of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$mice after 6 weeks of oral treatment with vehicle (White bars) or canagliflozin(black bars;30mg/kg body weight). Data is expressed as mean ± s.e.m. n = 5-10 mice per group. Φ $p < 0.05$ for main effect of drug, # $p < 0.05$ for main genotype effect.

Supplementary Table 1: Canagliflozin does not alter serum cytokines				
Cytokine pg/ml (S.E.M)	ApoE$^{-/-}$ Vehicle	ApoE$^{-/-}$ Canagliflozin	ApoE$^{-/-}$ AMPK β1$^{-/-}$ Vehicle	ApoE$^{-/-}$ AMPK β1$^{-/-}$ Canagliflozin
IL-1α	28.79 (1.30)	27.68 (2.06)	26.27 (1.83)	32.40 (14.36)
IL-2	18.81 (3.74)	24.15 (7.49)	18.26 (7.45)	20.32 (7.24)
IL-3	11.83 (2.56)	11.66 (2.14)	17.04 (1.37)	16.72 (10.12)
IL-5	13.82 (2.66)	11.26 (2.37)	10.00 (2.08)	12.82 (5.45)
IL-6	15.50 (1.06)	15.34 (1.66)	15.99 (1.55)	18.89 (9.20)
IL-10	89.93 (5.34)	87.27 (8.38)	88.38 (17.02)	66.54 (39.84)
IL-12 (p40)	321.02 (25.71)	303.70(15.80)	366.60 (41.34)	302.76 (147.24)
IL-12 (p70)	408.57 (37.41)	354.99 (63.99)	508.77 (124.61)	474.96 (245.75)
IL-13	227.24 (30.78)	213.58 (37.76)	165.58 (60.85)	313.60 (152.80)
IL-17	122.22 (11.77)	112.66 (18.59(	174.58 (55.49)	136.72 (70.19)
G-CSF	88.59 (5.99)	96.95 (10.01)	111.42 (17.07)	72.59 (31.01)
IFNγ	56.48 (5.46)	53.48 (4.32)	70.65 (10.60)	82.73 (43.40)
KC	101.49 (12.41)	83.32 (10.81)	106.79 (42.30)	91.98 (39.15)
MCP-1	273.28 (33.91)	202.54 (22.44)	244.04 (44.37)	265.51 (126.02)
MIP-1α	34.56 (6.84)	23.96 (2.71)	34.29 (4.46)	38.60 (15.85)
RANTES	26.75 (5.81)	26.36 (3.07)	27.31 (4.25)	21.60 (9.35)
TNFα	1038.51 (68.48)	892.17 (133.00)	1010.49 (248.35)	933.17 (466.23)

Table 1) Inflammatory markers in plasma of ApoE$^{-/-}$ (white bars) and ApoE$^{-/-}$AMPKβ1$^{-/-}$ (black bars) mice after 6 weeks of oral treatment with vehicle or canagliflozin (30mg/kg body weight). Data is expressed as mean ± s.e.m. n = 3-8 mice per group.

4. Chapter 4

Salsalate Reduces Aortic Plaque Size through activation of hematopoietic AMPK β1

Emily A. Day, Nicolas D. Leblond, Rebecca J. Ford, Brennan K. Smith, Vanessa P. Houde, Šarka Lhoták, Bernardo L. Trigatti, Geoff H. Werstuck, Richard C. Austin, Morgan D. Fullerton, Gregory R. Steinberg

Prepared for publication.

4.1 Preface and Significance to thesis

Salicylate has been used for centuries to treat pain and inflammation. Salicylate, the active compound in aspirin, has been shown to directly activate AMPK through a mechanism involving the allosteric activation site on the β1 subunit, Ser 108[19]. The β1 subunit is the primary isoform in human and mouse macrophages and in its absence 90% of AMPK activity is lost in these cells[20]. Genetic deletion of the AMPK β1subunit in macrophages promotes the development of insulin resistance and inflammation [20]. In 3 studies published from our laboratory we have found:1. Salicylate decreases lipogenesis and increases cholesterol efflux to HDL in macrophages in a manner dependent on AMPK β1[21]. 2. Salsalate, a dimer/prodrug of salicylate which is better tolerated in vivo, activates AMPK and inhibits liver lipogenesis while improving insulin sensitivity[22]. 3. Salicylate inhibits cancer cell proliferation through an AMPK dependent mechanism involving the suppression of de novo lipogenesis and mTor pathway[23]. Collectively these data indicate that salicylate/salsalate exerts anti-inflammatory, lipid lowering and anti-proliferative effects in a variety of cell types via a mechanism involving activation of AMPK β1 containing heterotrimers and may therefore be an ideal therapy for the treatment of atherosclerosis. Therefore, the aim of this work was to evaluate the effects of salsalate in a mouse model of atherosclerosis and evaluate the importance of AMPK β1 in mediating potential effects.

4.2 Author contribution

Emily was the primary contributor to all figures in this manuscript except for Figure 4. Emily wrote the manuscript with Dr. Steinberg.

4.3 **Salsalate Reduces Aortic Plaque Size through activation of hematopoietic AMPK β1**

Title: Salsalate reduces atherosclerosis in mice through AMPK β1

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Abstract:

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. CVD results from the development of atherosclerotic plaques which can be disrupted leading to thrombus, which can result in heart attack and/or stroke. Atherosclerosis is often a result of dyslipidemia and chronic low grade inflammation and worsened by dysglycemia. In pre-clinical models salicylate has been shown to modulate lipid metabolism and inflammation via the β1 subunit of the AMP activated protein kinase (AMPK). Additionally, hematopoietic AMPK β1 has been shown to be important in regulating inflammation as mice lacking hematopoietic AMPK β1 have increased systemic inflammation, macrophage lipid accumulation, and increased expression of inflammatory markers. Furthermore, salicylate has recently been shown to increase cholesterol efflux to HDL in macrophages in a manner dependent on AMPK β1. Clinically, salsalate (a pro-drug of salicylate) has been shown to improve dysglycemia and reduce circulating lipids in individuals with Type 2 Diabetes (T2D). Thus this study sought to determine the effects of salsalate on atherosclerosis *in vivo,* and the mechanisms and cell types targeted. Salsalate supplementation in the diet of ApoE$^{-/-}$ and ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice resulted in no changes in glucose tolerance or insulin sensitivity and no changes in circulating inflammatory markers. Salsalate treatment reduced plaque accumulation in the aortic root by ~30% compared to control treated animals in ApoE$^{-/-}$ but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice. These effects on atherosclerosis were shown to be specific to hematopoietic cells through bone marrow transfer of WT or AMPKβ1$^{-/-}$ marrow to LDLr$^{-/-}$ mice. Mechanistically, this was associated with a reduction in the number of proliferating cells within the plaques as indicated by Ki67 staining in ApoE$^{-/-}$ but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ animals with salsalate treatment. This is consistent with *in vitro* findings of decreased macrophage proliferation

in response to salicylate treatment in an AMPK β1 dependant manner. This data suggests that salsalate may be an effective treatment for atherosclerosis via hematopoietic AMPK activation.

Introduction:

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide[30]. CVD results from the development of atherosclerotic plaques, which when disrupted can lead to thrombus formation and heart attack and/or stroke. Dyslipidemia is the leading risk factor for CVD and atherosclerosis. Atherosclerosis is the result of a build-up of lipid laden macrophages (foam cells) within the intima of large blood vessels. These foam cells result in a positive feedback loop of pro-inflammatory cytokine release, classical M1 macrophage polarization and the recruitment and local proliferation of monocytes and macrophages as well as other immune cells including T cells[32,33]. Therefore, atherosclerosis can be viewed as an intersection of impaired lipid metabolism and maladaptive immune responses[32]. Consequently, therapies aimed at inhibiting macrophage proliferation and inflammation, and lipid accumulation are viewed as important therapeutic targets for the prevention and treatment of atherosclerosis.

Currently, statins are the most common treatment for CVD due to their lipid lowering properties. However, recent evidence suggests that statins may increase the risk of type 2 diabetes (T2D), which is a common comorbidity with CVD[64–66]. There is also a large portion of the population (~25%) who are statin intolerant which precludes the optimal use of high dose statins to achieve their LDL-target[289,290]. And while PCSK-9 inhibitors are effective at lowering LDL these therapies are not widely used for a variety of reasons. In addition to LDL lowering, targeting vascular inflammation, through anti-IL-1β antibodies, has also been shown to reduce cardiovascular events in patients taking

statins[74]. However, other anti-inflammatory therapies, such as low-dose methotrexate have not shown any benefit[75]. Collectively, these data suggest that new therapies that exert both LDL lowering and exert anti-inflammatory towards IL1β may be effective agents for treating CVD.

Salicylate, is the active metabolite of aspirin, and has been used for thousands of years to relieve pain and inflammation (for review see [62]). Salsalate, a pro-drug of salicylate, is rapidly converted into two salicylate molecules *in vivo.* Salsalate bas been used in clinical trials to treat T2D (TINSAL-T2D, where it has been shown to reduce circulating glucose and triglycerides [59,61,63] . More recently, salsalate has been tested in people with established atherosclerosis taking statins where it was found to have no effect on LDL-C, C-reactive protein or atherosclerotic plaques (TINSAL-CVD, Clinical Trial Identifier: NCT00624923) [76]. However, the effects of salsalate in statin naïve patients are currently unknown.

Mechanistically, salicylate has been shown to improve metabolic outcomes through a number of potentially interrelated mechanisms including the repression of inhibitor of nuclear factor κ-B kinase subunit β (IKK-β) [291–294], activation of AMPK [267] and mitochondrial uncoupling[199]. Clinical studies have demonstrated circulating concentrations of salicylate are approximately 1mM[59,61,63,295]. At these concentrations, activation of AMPK and mitochondrial uncoupling can occur [160,199,267] and this has been associated with improvements in insulin sensitivity, decreases in lipogenesis in liver and macrophages and increases in cholesterol efflux to HDL [108]. Recent studies have also indicated that AMPK phosphorylation of HMG-CoA reductase (HMGR) at Ser 782 is critical for reducing cholesterol synthesis[260]. Collectively these data indicate that salsalate exerts anti-inflammatory, insulin sensitizing and lipid lowering effects. However, whether

salsalate reduces atherosclerosis and the importance of AMPK in potentially mediating these effects has not been tested.

In the current study we hypothesized that salsalate would reduce atherosclerosis through modulation of hepatic and macrophage dyslipidemia and inflammation and that this would be mediated through activation of AMPK β1 containing heterotrimers. We find that salsalate reduces atherosclerosis in mice deficient in ApoE and that these effects are blunted in mice lacking AMPK β1. To further examine the primary tissue contributing to these effects subsequent studies using bone marrow chimeras were conducted and we find that the effects of salsalate are blunted in mice lacking hematopoietic AMPKβ1. These studies establish that salsalate may be effective for reducing atherosclerosis by targeting AMPKβ1 in immune cells.

Results:

Salsalate reduces plaque size in an AMPKβ1 dependent manner.

Salsalate did not result in changes in food intake, body mass or adiposity, and there were no differences in these parameters between genotypes (**Table 1**). Salsalate has been shown to improve glucose tolerance and insulin sensitivity in obese rodents fed a high-fat diet or individuals with dysglycemia, however, these ApoE mice were not obese (**Table 1**) and salsalate had no effect on glucose tolerance **(Supplementary Figure 1A-B)**. There were also no differences in glucose tolerance between genotypes (**Supplementary Figure 1A-B**). However, when we examined atherosclerotic burden in these mice we found that salsalate treatment resulted in an approximately 30% reduction in atherosclerotic plaque development in $ApoE^{-/-}$ mice while there was no effect on atherosclerosis in $ApoE^{-/-}$ $AMPK\beta1^{-/-}$ mice (**Figure 1A, B**). These data indicate that salsalate has minimal effects on

body composition and glucose homeostasis but does result in a significant reduction in atherosclerosis and this occurs through pathways requiring AMPK β1.

Salsalate reduces dyslipidemia independent of cholesterol synthesis and clearance

AMPKβ1 is predominately expressed in the liver and in macrophages. To examine the mechanisms contributing to reduced atherosclerosis with salsalate we examined whole body energy balance and substrate utilization using metabolic cages. Consistent with similar adiposity and body mass, there were no changes in ambient activity, VO2 or energy expenditure between groups (**Supplementary Figure 1C-F**), however, salsalate did lower RER in ApoE$^{-/-}$ but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice suggesting increases in fatty acid oxidation and/or reductions in de novo lipogenesis, through an AMPK dependent pathway (**Supplementary Figure 1G, H)**.

Increased hepatic and circulating lipid levels are known to be key drivers of atherosclerotic plaque development, and AMPK activation has been shown to reduce circulating lipids. Additionally, the supressed RER observed in ApoE$^{-/-}$ mice treated with salsalate suggests either increases in fatty acid oxidation or reductions in lipogenesis. Therefore, we examined both hepatic and plasma cholesterol and triglycerides. Salsalate reduced plasma cholesterol in ApoE$^{-/-}$ mice but not ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice **(Figure 2A)** without altering circulating triglycerides (**Figure 2B**). In the liver, salsalate lowered cholesterol but not triglycerides (**Figure 2C, D**). SREBP1c is key transcriptional regulator of cholesterol synthesis and clearance due to its regulation of the LDLr, and has been shown to be inhibited by AMPK, however there was no change in SREBP1c mRNA or LDLr suggesting effects on cholesterol were not being mediated through this pathway (**Figure 2E,F**). To directly examine the effects of salicylate on cholesterol synthesis we generated primary hepatocytes from WT, AMPK β1 null or mice lacking the AMPK phosphorylation

site on HMGR (HMGR KI). Consistent with observation in vivo indicating the cholesterol was reduced independently of AMPK β1 we found that salicylate (1mM) inhibited sterol synthesis independently of AMPKβ1 or AMPK phosphorylation of HMGR. These data indicate that salsalate-suppresses liver cholesterol levels through a pathway not requiring AMPK and given the discordance with plaque progression was unlikely to be the primary mechanism reducing atherosclerosis in ApoE but not ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice.

Salsalate/salicylate does not reduce markers of inflammation

Given the importance of inflammation in driving atherosclerotic burden and the anti-inflammatory effects of AMPK activation we next examined circulating (serum) inflammatory markers. Consistent with previous findings, mice lacking AMPKβ1 had significantly increased IL-1β and G-CSF (**Supplementary Figure 2A,B**) however, surprisingly, no markers of inflammation examined in the serum were altered with salsalate treatment **(Supplementary Figure 2C-I)**. Similarly, we found no changes in markers of adipose tissue inflammation (**Supplementary Figure 2J-L)**. As salicylate has been demonstrated to have a direct anti-inflammatory role in macrophages[296,297], we hypothesized that there may be subtle changes in macrophage inflammation in vivo that were undetectable in the serum or adipose tissue. We therefore conducted experiments in bone marrow derived macrophages(BMDMs) treated with LPS (10ng/ml) or LPS+salicylate (1mM, approximate serum concentration achieved in both mouse and human studies) and found that at this dose salicylate was effective in blunting LPS-induced reductions in AMPK activity, as measured by phosphorylation of its downstream target ACC and that this effect was blunted in BMDMs from AMPKβ1$^{-/-}$ mice (**Supplementary Figure 3A**). However, this restoration in AMPK activity was not associated with reductions in transcription factors critical for regulating inflammatory programs Stat1,

Stat3, IKKβ or NFκB (**Supplementary Figure 3B-E**). Collectively, these in vivo and in vitro data suggest that salsalate may not be mediating anti-atherosclerotic effects through reductions in macrophage inflammation.

Salicylate/Salsalate reduces macrophage proliferation

Given that there were no changes in systemic or macrophage inflammation, and that the effects on atherosclerosis were not consistently associated with changes in serum lipid levels with salsalate we began to examine alternative mechanisms that could contribute to the changes in atherosclerosis in both of our models. Salicylate has previously been shown to reduce cancer cell proliferation[298], and local macrophage proliferation has been shown to be a driver of atherosclerotic plaque development[33]. Therefore, we examined proliferating cells within the atherosclerotic plaques to determine if this was altered with salsalate treatment. Using an anti-body to Ki67, a marker of cell cycle shown to be very similar to BrdU staining[299], we found a reduction in Ki67+ cells in macrophage rich areas of the plaques (Mac-3 positive areas) in ApoE$^{-/-}$ mice treated with Salsalate and no differences in ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice (**Figure 3A,B**). Atherosclerotic plaques contain multiple cell types, therefore to examine effects on macrophages we used BMDMs and purified M-CSF to stimulate proliferation, and found that salicylate resulted in a 15% reduction in proliferation compared to control in wildtype cells, however salicylate had no effect on proliferation in AMPKβ1$^{-/-}$ cells (**Figure 3C**). These data suggest that salicylate reduces atherosclerosis through AMPKβ1-dependent suppression of macrophage proliferation.

Salsalate reduces plaque size through hematopoietic AMPKβ1

To more directly examine whether the effects of salsalate may be mediated through its effects on macrophage proliferation we conducted bone marrow transfer experiments where bone marrow from WT and whole body AMPKβ1$^{-/-}$ mice were transferred to LDLr$^{-/-}$ mice with normal levels of AMPK in all tissues including the liver. Consistent with our previous observations (Figure 1), salsalate reduced plaque size in mice receiving WT bone marrow, however in mice lacking AMPKβ1 in hematopoietic cells there was no reduction in plaque size (**Figure 4A**). Interestingly, mice receiving AMPKβ1$^{-/-}$ marrow had smaller atherosclerotic plaques than those receiving wildtype marrow, which is consistent with other models of AMPK deficiency[300]. These changes in plaque size were mirrored by reduced necrotic area in mice with AMPKβ1$^{-/-}$ marrow, and in WT mice receiving salsalate **(Figure 4B)**. We next examined serum lipid levels and found that salsalate reduced serum cholesterol and TG independent of hematopoietic AMPKβ1. These data indicae that salsalate reduces atherosclerosis through a direct effect on hematopoietic AMPK. Future experiments will examine Ki67 in the plaques from these mice, however given the age of the samples (data generated 8 years ago) this may not be possible

Discussion:

CVD is the leading cause of death worldwide[30]. Both dyslipidemia and inflammation have been shown to be causal in the progression of atherosclerosis. Statins are the standard of care for individuals with elevated LDL-c however, as many as 25% of the patients prescribed statins are unable to achieve LDL-c target lowering with statins alone.. For these patients, there is an urgent need for new therapeutic options to address this issue. Ezetimibe, is one option that when added to a statin can reduce LDL-c and allow for dose reduction of the statin[67,301], however this does not entirely solve the issue of statin intolerance. Furthermore, statins increase new incidence type 2 diabetes [65,66]. The incidence

on new on-set diabetes with statins is 1 in every 255 patients treated with statins, however, it is associated with risk factors that can be assessed prior to treatment including obesity, impaired fasting glucose, and elevated TGs[65]. This data suggests that individuals with these risk factors need therapeutic alternatives to effectively reduce cardiovascular disease while not causing new onset diabetes. Importantly, salsalate has been shown to reduce both circulating TG and improve fasting glucose in individuals with and without diabetes[60,61,302].

In the current study we found that salsalate reduced atherosclerosis in ApoE$^{-/-}$ but not ApoE, AMPKβ1$^{-/-}$ mice. This observation was discordant with the effects of salsalate on hepatic lipid levels which were reduced in both ApoE and AMPK β1 mice. To investigate the mechanisms for this effect subsequent studies were conducted in primary hepatocytes treated with salicylate at the concentration observed with salsalate intake and it was observed that salicylate suppressed cholesterol synthesis independently of AMPK β1 or AMPK phosphorylation of HMGR. It is likely that this AMPK independent effects on cholesterol synthesis is occurring due to the mitochondrial uncoupling effects of salicylate which would be expected to reduce acetyl-CoA thereby lowering substrate available for sterol synthesis. This would be consistent with our previous observations in hepatocytes where salicylate also lowered de novo lipogenesis independently of AMPK or phosphorylation of ACC due to its effects to uncouple mitochondria[199]. And while reductions in atherosclerosis did not track with changes in hepatic lipid content in the ApoE mouse model used in the current study this may be an added benefit of salsalate therapy if used in humans where serum cholesterol is more closely associated with disease profile.

In the current study we show that salsalate reduces atherosclerosis through reduced macrophage proliferation within the plaques as a result of activation of hematopoietic AMPK. For many years the recruitment of monocytes was thought to be critical for the

development of atherosclerotic plaques, however, more recent studies demonstrate that local macrophage proliferation accounts for more that 80% of macrophage accumulation over 1 month[33]. The mechanism of reduced macrophage proliferation with salsalate is currently unknown, however, in cancer cells, salicylate has been shown to reduce proliferation through reduced lipogenesis with limits lipids available for synthesis of new membranes[298]. Interestingly, statins have also been shown to reduce proliferating macrophages[303], and delivery of statins to atherosclerotic plaques (via HDL nano-particles) has been shown to reduce plaque size[304]. Interestingly, this work with nano-particle delivery of statins was the first to demonstrate that directly targeting macrophage proliferation was a valid therapeutic option. Understanding these effects about statins may indicate that salsalate therapy would not have additive effects to the effects of statins in humans. This is especially important to the interpretation of the TINSAL-CVD trial, given that inclusion criteria was a stable dose of statins. Additionally, the mechanistic insights in the study are especially important given that salsalate has long been held as an anti-inflammatory therapy. In the current study we saw no changes in circulating Il-1β, TNFα or any other circulating markers examined. This is consistent with clinical data that shows no change in circulating CRP (downstream of IL-1β signalling) with salsalate[60,61,63,76,302]. Furthermore, we found no direct effects on macrophage inflammation in vitro, including any changes in IKKβ or downstream mediator NFκB, suggesting that at clinically relevant concentrations of approximately 1mM salicylate, these mechanisms are not engaged.

In conclusion, this work shows, for the first time that salsalate is able to reduce atherosclerosis, and importantly that this is not due to changes in serum cholesterol or changes in systemic inflammation. Importantly this work shows that the reduction in atherosclerosis is through activation of hematopoietic AMPK. This work suggests that

salsalate may be a useful therapeutic option for individuals with elevated TG's and impaired fasting glucose that would be at high risk of new incident diabetes with statin therapy, or in individuals who are unable to tolerate effective statin doses due to statin intolerance.

Methods:

Mice: All animal experiments were approved by McMaster University Animal Research Ethics Board. Mice were group housed at conventional temperature (22-23°C) on a 12hr light-dark schedule with ad libitum access to food and water. Female C57Bl6 ApoE$^{-/-}$ mice were fed Western Diet ±2.5 g/kg salsalate starting at 6 weeks of age for 6 weeks. During the last week of treatment, a glucose tolerance test using 2g/kg glucose and insulin tolerance test using 0.4U/kg insulin were administered. For metabolic measurements, mice were placed into metabolic cages and allowed to acclimatize for approximately 24h. Food and fluid intake, ambient locomotor activity, O2 consumption (VO2), CO2 output (VCO2), RER and energy expenditure were measured using a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments). Mice were fasted 6 hours prior to harvesting tissues, and serum was collected for lipoprotein analysis. The heart and aortic root was dissected out, formalin fixed and paraffin embedded and 4μm thick sections of the aortic root were then collected and stained with H&E to measure plaque size at 80μm intervals, subsequent sections were used for IHC. IHC was done using appropriate primary and biotinylated secondary antibodies and detected using streptavidin peroxidase and Nova Red (all IHC reagents are from Vector Labs).

Bone Marrow Transfer: Whole body irradiation and bone morrow transfer was completed as previously described[89].

Cell Culture: Bone marrow derived macrophages (BMDMs) were isolated as previously described. Briefly, the tibia and femur were dissected from each leg, the ends cut off and marrow collected by centrifugation. Marrow was then suspended in DMEM supplemented with 10% FBS and 1% anti-anti. Cells were allowed to grow for 5 hours when L929 media (a source of M-CSF) was added and cells were plated in 10cm tissue culture dishes. Cells were allowed to differentiate for 7 days after which they were lifted by gentle scraping. For proliferation experiments cells were manually counted and seeded at 7000 cells per well in a 96 well dish. Cells were allowed to adhere for 24 hours prior to simultaneous drug treatment and M-CSF (2ng/ml) stimulation for 72 hours. Cells were then stained in with crystal violet, washed thoroughly and allowed to dry. The dye was solubilized in NaH_2PO_4 and absorbance read at 570nm. For inflammation experiments cells were seeded in 6 well culture dishes and allowed to adhere overnight. The following day, media was replaced, and salicylate (1mM) was added 30 minutes prior to the addition of LPS (10ng/ml) for 6 hours. Cells were then frozen snap frozen in liquid nitrogen.

Primary Hepatocytes: Primary hepatocytes were isolated from C57Bl6J, AMPKβ1$^{-/-}$ [159],HMGR KI[260] mice as previously described[209].

Immunoblotting: Cells were isolated in lysis buffer containing 50mM HEPES, 150mM NaCl, 100mM NaF, 10mM sodium pyrophosphate, 5mM EDTA, 250mM sucrose, 1mM dithiothreitol and 1mM sodium orthovanadate, with 1% Triton X and one tablet of cOmplete Protease Inhibitor Cocktail (Roche) per 50ml, then stored at −80 °C until analysis. Protein concentration was determined with the Pierce BCA Protein Assay kit (ThermoFisher). Lysates were then diluted with sample buffer and run on a polyacrylamide gel to separate proteins based on size. Next, samples were transferred to a polyvinylidene difluoride membrane and blocked in 5% BSA for 1h at room temperature. Membranes were

incubated with primary antibody (1:1,000, except β-actin 1:1,500) overnight at 4 °C. Appropriate secondary antibodies were used at a concentration of 1:10,000. Bound antibodies were detected using Clarity Western ECL Substrate (BioRad).

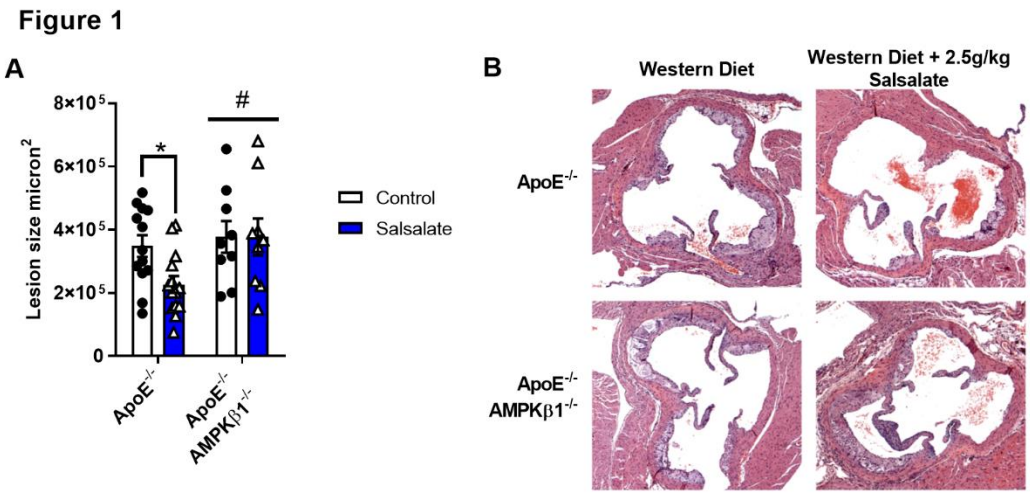


Figure 1: Salsalate reduced atherosclerosis in an AMPK β1 dependent manner. A) Lesion area and B) representative images of H&E stained aortic roots of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice after 6 weeks of Western Diet (white bars) or Western Diet + Salsalate (blue bars). Data is expressed as mean ± s.e.m. n = 9-14 mice per group. # P<0.05 for main genotype effect, * P<0.05 for salsalate versus control within genotype.

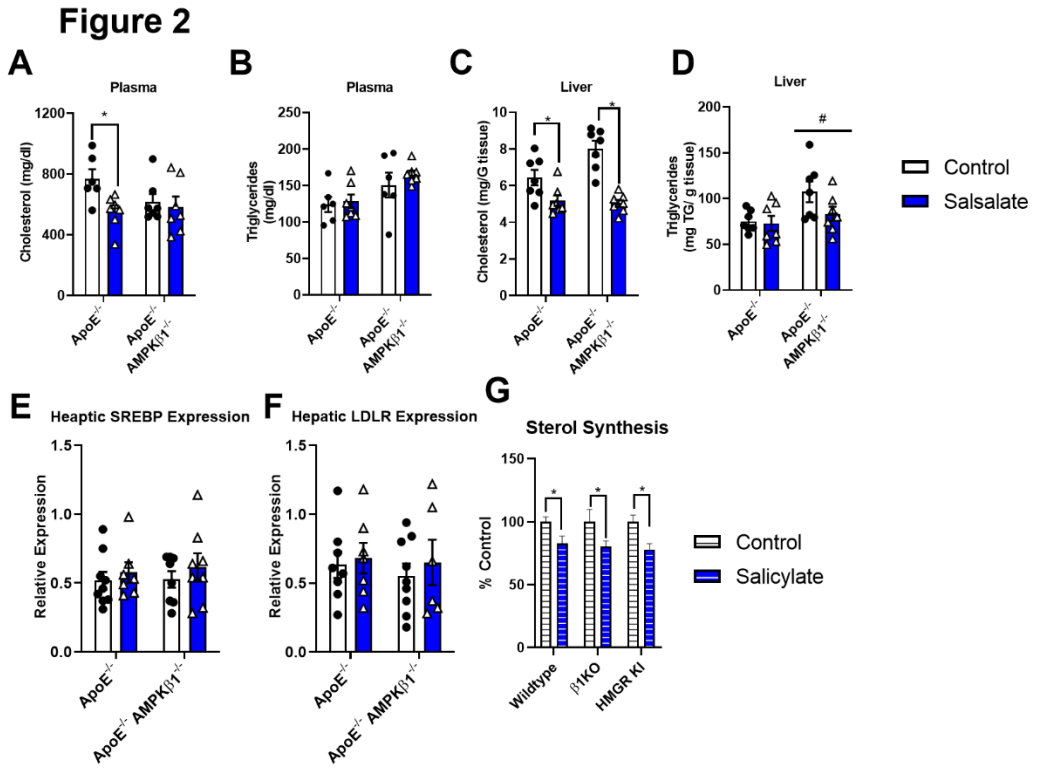


Figure 2: Salsalate reduces plasmas cholesterol via AMPK β1. Plasma **A**) Cholesterol, **B**) Triglycerides and hepatic **C**) Cholesterol and **D**) triglycerides. Hepatic SREPB1c and LDLr mRNA following 6 weeks of Western Diet (white bars) or Western Diet + Salsalate (blue bars) feeding in ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice. Cholesterol synthesis measured in hepatocytes from WT, AMPKβ1$^{-/-}$ and HMGRKI cells treated with 1mM salicylate for 4 hours. Data is expressed as mean ± s.e.m.. # P<0.05 for main genotype effect, * P<0.05 for salsalate versus control within genotype.

Figure 3

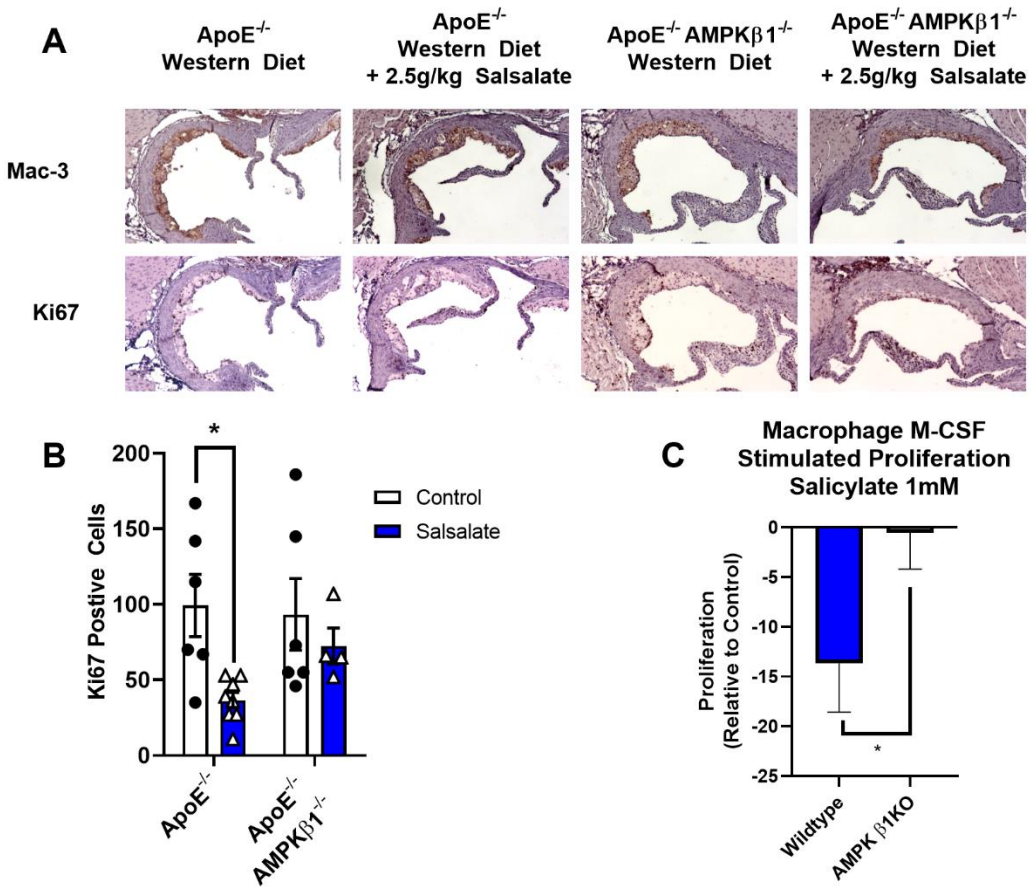


Figure 3: Salsalate reduces macrophage proliferation in vivo and in vitro. A) Representative Mac3 and Ki67 stained aortic roots from ApoE$^{-/-}$ and ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice after 6 weeks of Western Diet or Western Diet + Salsalate. **B**) Ki67 was quantified per section Data is expressed as mean ± s.e.m * $p<0.05$ for salsalate versus control within genotype. **C**) Wildtype and AMPKβ1$^{-/-}$ bone marrow derived macrophages were simultaneously treated with MCSF (2ng/ml) and salicylate (1mM) for 72 hours, data is expressed as mean ± s.e.m . from 7-8 individual experiments.

Figure 4

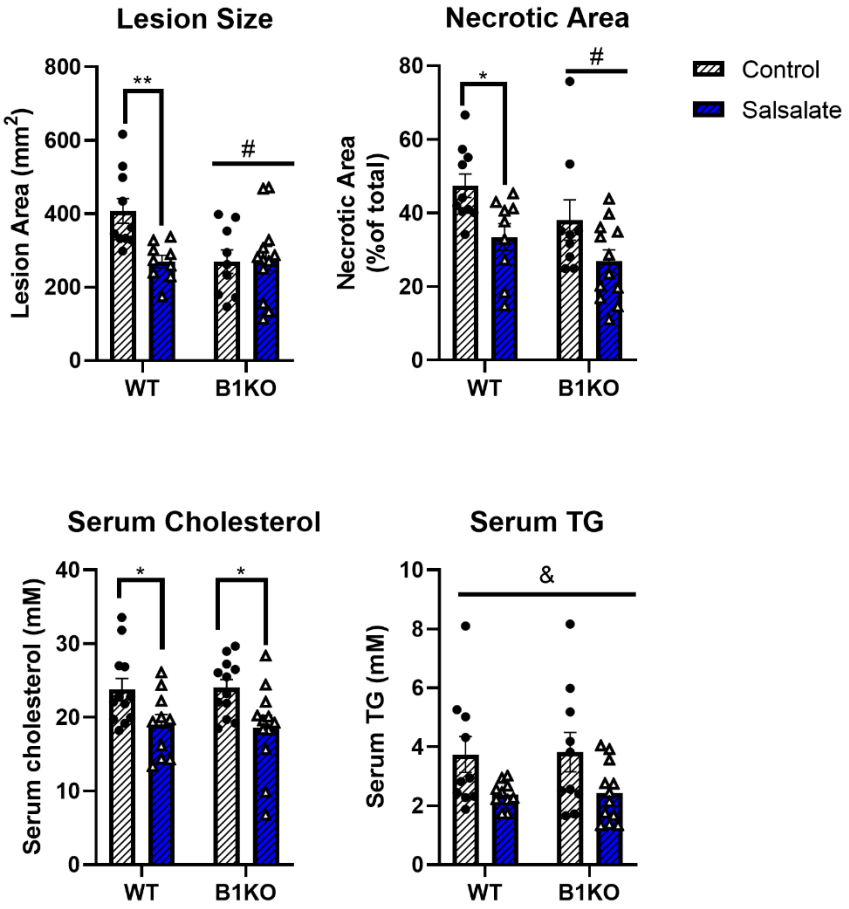
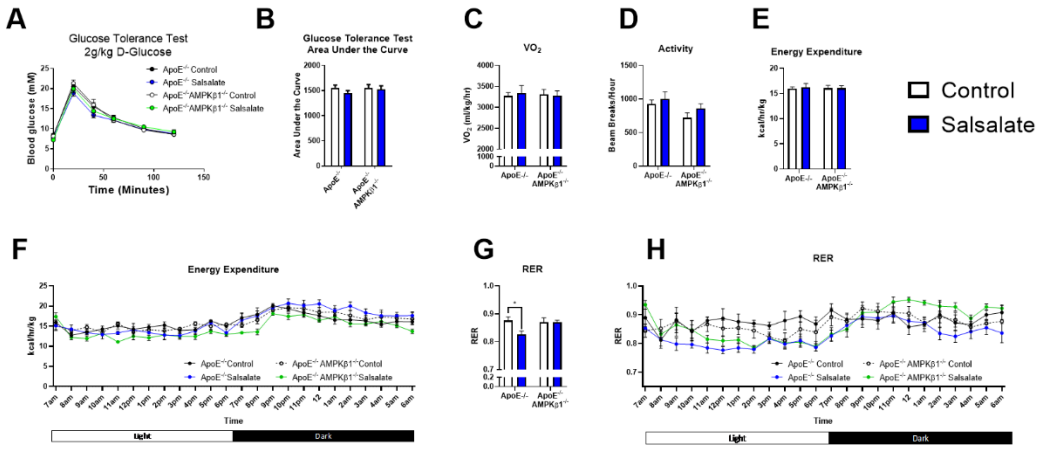
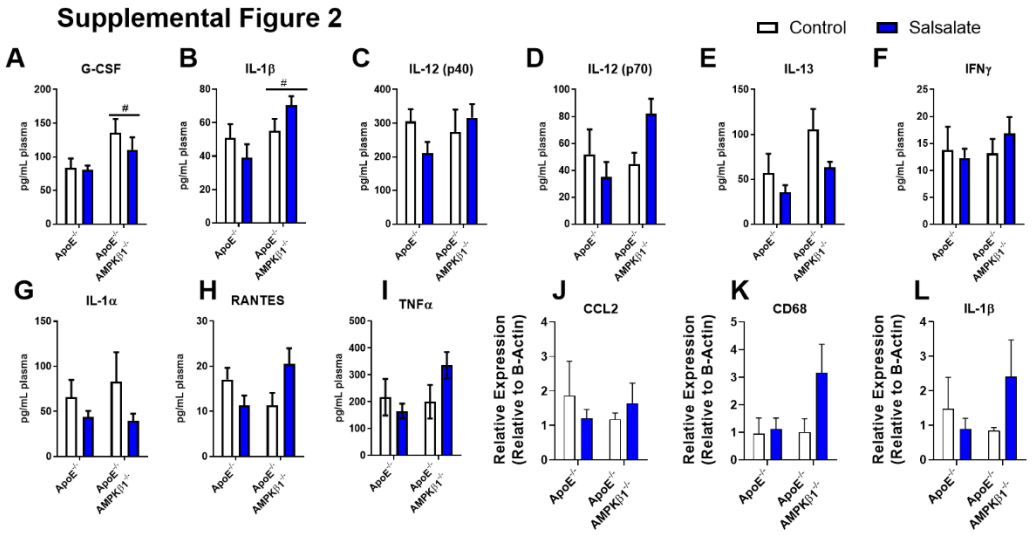


Figure 4: Salsalate reduced atherosclerosis through hematopoietic AMPK β1. A) Lesion area and **B**) Necrotic area for aortic root C, D) Serum Cholesterol and Triglycerides from LDLr $^{-/-}$ mice with WT or AMPKβ1$^{-/-}$ bone marrow engraftment fed Western Diet (white bars) or Western Diet + Salsalate (Blue bars). Data is expressed as mean ± s.e.m. # P<0.05 for main genotype effect, & P<0.05 for main treatment effect * P<0.05 for salsalate versus control within genotype.

Supplemental Figure 1

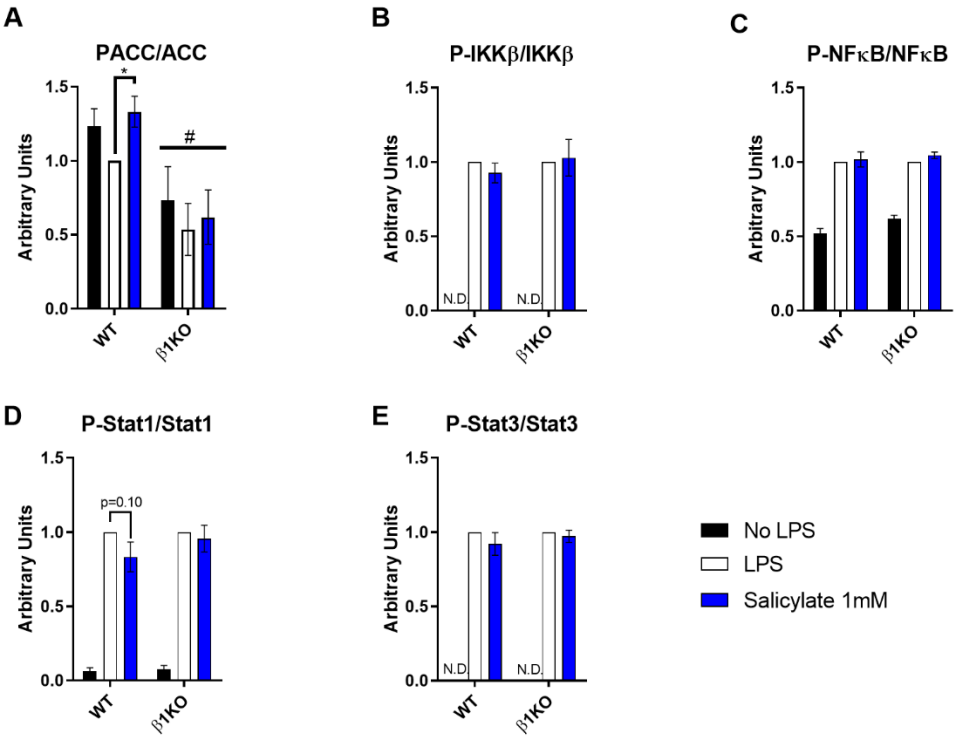


Supplemental Figure 1: Salsalate does not effect glucose tolerance, or energy expenditure but does reduce RER via AMPKβ1. A) Glucose tolerance test and **B**) Area under the curve following 6hour fast and injection with 2g/kg glucose. **C**) VO2, **D**) Activity, **E, F**) Energy expenditure, **G,H**) RER of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$mice after 6 weeks of Western Diet or Western Diet + Salsalate. Data is expressed as mean ± s.e.m. *p<0.05 for salsalate versus control within a genotype.



Supplemental Figure 2: Salsalate does not alter serum or adipose tissue markers of inflammation. A-I) Serum markers of inflammation assessed by bioplex assay, and **J-L)** adipose tissue inflammation assessed by qPCR **in** ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$mice after 6 weeks of Western Diet or Western Diet + Salsalate. Data is expressed as mean ± s.e.m. # $P<0.05$ for main genotype effect.

Supplemental Figure 3



Supplemental Figure 3: Salsalate does not reduce macrophage inflammation in vitro. Bone Marrow derived macrophages from Wildtype (WT) and AMPKβ1$^{-/-}$(β1KO) mice were treated with salicylate (1mM) for 30 minutes prior to 6 hour treatment with LPS (10ng/ml). **A**) PACC/ACC, **B**) P-IKKβ/ IKKβ, **C**) P-NFκB/NFκB, **D**) P-Stat1/Stat1 and **E**) P-Stat3/Stat3 were assessed by western blotting. Data is expressed as mean ± s.e.m.

Table 1: Salsalate does not alter body mass, food intake or adiposity

Parameter	ApoE$^{-/-}$ Control	ApoE$^{-/-}$ Salsalate	ApoE$^{-/-}$ AMPKβ1$^{-/-}$ Control	ApoE$^{-/-}$ AMPKβ1$^{-/-}$ Salsalate
Body Mass grams ±S.E.M. (n)	22.33 ±0.36 (9)	21.52 ±0.43(9)	22.46 ±0.79 (7)	21.57 ±0.84 (11)
Food Intake grams/day ±S.E.M (n)	2.79 ±0.13 (9)	2.57 ±0.12 (9)	2.51 ±0.18 (4)	2.54 ±0.09 (4)
Adiposity percent, ±S.E.M (n)	17.05 ±1.15 (9)	17.77 ±1.04 (9)	16.01 ±1.71 (7)	18.82 ±2.25 (11)

5. Chapter 5: Discussion

Despite intense interest, ongoing research and many pharmaceutical advances the number of individuals with obesity, diabetes and CVD are increasing in staggering numbers worldwide. Furthermore, CVD is the number one cause of mortality and morbidity world-wide. Importantly, individuals with diabetes are 2-5x more likely than individuals without diabetes to die of CVD for reasons that are not entirely understood[28]. This thesis focused on 3 distinct glucose lowering medications, metformin, canagliflozin, and salsalate which have all been shown to activate the cellular energy sensor AMPK[179,242,267].

Metformin was first used for adult onset diabetes in 1957 across Europe[305], while its mechanism of action was still unknown. The results of the UK Prospective Diabetes Study (UKPDS) in 1998 catapulted metformin to its widespread use today with the finding that metformin was cardio-protective in individuals with diabetes[45], and importantly, these results suggested that the cardio-protective effects were beyond that of improved glycemic control. Because of these results, as well as the efficacy and safety of metformin, it is now the front-line therapy for individuals diagnosed with diabetes. Currently, there is still much debate about the molecular mechanism(s) of action of metformin, however its pleotropic benefits are clear. Furthermore, investigation of metformin for other disease conditions is still rapidly occurring. Indications for PCOS, cancer prevention, cardiovascular protection, weight loss and even lifespan extension are only some of the currently researched topics[306]. Owing to the tissue specificity of metformin, it is unlikely that all of these effects are mediated by direct actions of the drug. Interestingly, the notion that hepatokines, chemical messengers from the liver, may communicate to other tissues and cell types is an intensely researched area[307]. This thesis examined one such hepatokine, or metokine (metformin

induced factor), GDF15. Interestingly, here we show that metformin induced GDF15 is associated with weight loss in humans and mice. One of the limitations of this study was the use of the mouse model. Interestingly, GDF15 is also strongly associated with the severity hyperemesis gravium[308] (extreme morning sickness), therefore it is possible that the reduced food intake seen with metformin via GDF15 is due to nausea, however, mice lack the emetic reflux[309], therefore to investigate this, other model systems will need to be used, such as the shrew which has an emetic reflux[309].

Both GDF15 and metformin have also been independently associated with changes in atherosclerosis[109,310] and lifespan extension[50], and therefore several new exciting research possibilities exist to examine if GDF15 is conferring other systemic benefits of metformin therapy. Association of metformin's effects on atherosclerosis and whether they are mediated by GDF15 is an active area of investigation within our lab. Importantly, this thesis provides strong evidence that metformin mediates some of its beneficial effects through secreted factors that travel systemically. Furthermore, this study identified several other transcripts upregulated by metformin treatment in hepatocytes which are known to produce secreted factors, therefore future work should examine these other metokines, to determine if they potentially confer some of metformin's beneficial effects.

Subsequent studies in this thesis examined the effects of glucose lowering therapies canagliflozin and salsalate on atherosclerosis and whether this was mediated through AMPKβ1. Interestingly, several studies have examined the effects of AMPK deletion (in several cell types) on atherosclerosis. Initially, studies in α2 whole body KO mice showed a protective role for AMPK in atherosclerosis[109–111]. Subsequent studies have shown protective roles for α1 in myeloid cells[106], while contradictory another study showed protective roles for whole body α1 and vascular smooth muscle (VSMC) α1 but not

myeloid α1 or whole-body α2[112]. However others still have shown a deleterious role for α1 and α2 in myeloid cells[300,311], suggesting that there are several complex processes contributing to atherosclerotic development. Interestingly, studies showing deleterious effects of AMPK on atherosclerosis attribute some of these effects to decreased monocyte differentiation and declines in monocyte/macrophage survival[300]. To date, only one study has examined the role of AMPKβ1 on atherosclerosis and shown that whole body deletion does not alter atherosclerotic development[70]. Interestingly, in the current study we similarly see no change in atherosclerosis with whole-body AMPKβ1deletion but do see lessened atherosclerosis with hematopoietic AMPKβ1 deletion, which could suggest a deleterious role of AMPK in atherosclerosis. This data is directly in contrast to the AMPK activation seen in this study that reduces atherosclerosis therefore demonstrating a protective role in AMPK activation. Thus, it is possible that if cells are unable to survive/differentiate there is reduced atherosclerosis, however in models where the activity of AMPK is only blunted (such is the case in humans with obesity), there is increased inflammation and lipogenesis therefore promoting atherosclerosis.

However, this thesis also goes beyond genetic modelling of deletion to use of clinically used pharmaceutical activators of AMPK and demonstrates that salsalate is able to reduce atherosclerosis through hematopoietic AMPK activation, an effect that was not seen clinically when added to statins[76]. Clinical data with salsalate examining atherosclerotic or cardiovascular outcomes is currently limited to individuals on stable doses of statins treated with salsalate in an short time frame (30 months)[76]. Future studies should examine the efficacy of salsalate on atherosclerosis in statin intolerant patients for a longer follow-up period. Additionally, retrospective studies of individuals with arthritis taking salsalate may also provide some evidence of cardiovascular benefit.

Use of the ApoE$^{-/-}$ model for most experiments completed in chapters 3 and 4 is a significant limitation on these studies. ApoE$^{-/-}$ mice are protected from the development of obesity and diabetes[312]. This provided interesting insight to the effects of these glucose lowering therapies that are independent from changes in body mass, and independent of changes in glycemic control in the case of salsalate (and only modest changes in glycemic control with canagliflozin). The lack of obesity seen in these models may provide support for use of the LDLr$^{-/-}$ model which is more prone to weight gain, however, the plaques formed in this model are less similar to the early stage fatty streak plaques seen in humans and ApoE$^{-/-}$ mice. Newer atherosclerotic models, such as the use of a PSCK9 adenovirus in obesity and insulin resistant prone C57Bl6 mice[313,314] could be used in future experiments to circumvent these issues. This model has normal expression of both ApoE and LDLr but is still hypercholesteremic and develops atherosclerosis with Western diet feeding and demonstrates more relevant reverse cholesterol transport[312]. This model is also especially attractive because the initiation of atherosclerosis can occur following the development of obesity. Furthermore, it allows for testing of other genetic models/background without the time it takes to backcross these strains to either the ApoE$^{-/-}$ or LDLr$^{-/-}$background[312]. This becomes especially important when using cre/flox systems and allows for the potential to study inducible deletions more readily, to circumvent some of the issues mentioned previously about cellular development and differentiation.

Since the advent of blockbuster drugs, such as metformin and statins, many have thought the epidemic of diabetes and cardiovascular mortality would be diminished, however, given the heterogenous nature of these conditions 'one-size fits all' pharmaceuticals have been unable to provide a solution. Indeed, many millions of individuals report metformin intolerance and statin intolerance. As many as 20% of

individuals report adverse gastrointestinal effects with metformin use in observational studies[315,316]. The mechanisms of metformin intolerance are very poorly understood. Given that the adverse events with metformin are generally gut specific it is likely that this is due to a direct effect of the drug, because it is given orally, however, it is also possible that metokines are mediating some of these adverse events. Furthermore, because of these adverse events, and insufficient glucose lowering on metformin alone, new classes of second line therapeutics have been developed.

The approval for new type 2 diabetes medications now requires additional safety trials to examine cardiovascular safety. This mechanism was implemented due to cardiovascular safety concerns with the thiazolindinedione, rosiglitazone, as well as the increased deaths in in the development program of the peroxisome proliferator-activated receptor (PPAR) agonist, muraglitazar, and increased mortality seen in the ACCORD trial[317]. These additional safety trials with canagliflozin treatment (CANVAS trial) demonstrated unexpectedly robust reductions in CVD mortality based on interim data, following which approval for treatment of type 2 diabetes was granted by the FDA, and subsequent studies were added (CANVAS-R) to maximize statistical power to detect cardiovascular benefit[57]. Similar to the development of metformin, the wide ranging systemic benefits of these therapies and the mediators of these effects were not entirely understood at the outset of development, but the effects on CVD are dramatic. Subsequent studies with canagliflozin have also investigated its effects for other diseases, including NAFLD[253]. Work in this thesis supports the notion the canagliflozin could be an effective treatment for NAFLD, through reductions in hepatic DNL and inflammation, or that at least canagliflozin may be superior to other glucose lowering therapies in individuals at high risk of developing NAFLD. Furthermore, mechanistically, this has been, in part, associated

with activation of hepatic AMPK, which is similar to the effects of metformin on hepatic lipid metabolism measures[179].

Unlike metformin intolerance, the root cause of statin intolerance is more thoroughly understood, largely being associated with on target effects (HMGR inhibition) in the muscle, resulting in myalgia[318]. Statin intolerance effects up to 20% of patients[289,290] and prevents them from reaching their LDL-c lowering targets due to discontinuation. This has fostered the development of newer pharmaceuticals to circumvent these issues. Bempedoic acid is one development that specifically addresses the issue of statin intolerance while effectively lowering LDL-c[71]. Bempedoic acid targets ACLY, which is upstream of HMGR in the cholesterol synthesis pathway but does so in a liver specific manner through requirement for the very long chain acyl-CoA synthease-1(ACSVL1), which is only expressed in the liver, for conversion to an active CoA form[70]. This specific targeting to the liver allows for similar LDL-c reduction as statins without the adverse myalgia. Bempedoic acid has also been shown to activate AMPK[319], however effects on atherosclerosis and LDL-c have been largely shown to be independent of AMPK[70]. Interestingly, statins have also been associated with reduced inflammation, however whether this is secondary to reduced LDL-c in unclear, however similarly, bempedoic acid has been shown to reduced CRP, suggesting that these effects are secondary to LDL-c lowering[71,320]. Lessons learned from both statin intolerant patients and commonalities between effective therapeutic options can direct future drug discovery. Ironically, this supports the use of one of the oldest known medications, salicylate, which is shown to improve glycemia and reduce circulating triglycerides and inflammation[60,61], and, based on work in this thesis, may be an effective treatment for atherosclerotic CVD. This treatment option would be especially useful for individuals who are statin intolerance and/or have

prediabetes, given the consistent glycemic benefits of salsalate in individuals with and without diabetes[59,61,63]. Furthermore, these studies also provide important, genetically validated, evidence that targeting hematopoietic AMPK could be an effective therapeutic option for the treatment of atherosclerosis, while also conferring positive glycemic and inflammatory effects. Moreover, this data provides evidence that newer generation AMPK activators, such as PF-06409577, may be able reduce atherosclerosis through similar hematopoietic AMPK activation with much lower dosing than salsalate. PF-06409577 has been shown to reduce NAFLD, and cholesterol synthesis in an AMPK dependent manner[259] and current work in the lab suggests that it has anti-atherosclerotic effects similar to salsalate, which further validates AMPK as an important molecular target for treatment of atherosclerotic CVD.

In conclusion, the work presented in this thesis presents important mechanistic insights of how glucose lowering therapies, metformin, canagliflozin and salsalate, elicit beneficial effects on obesity, insulin resistance and atherosclerosis and further our understanding of the potential use of these agents for treatments beyond improved glycemic control. Data presented in this thesis provides new evidence that; 1. metokines can be the causal mediators of metformin's systemic benefits and that GDF15 mediates metformin's effects on weight loss, 2. canagliflozin reduces IL-1β and hepatic DNL suggesting potential for indications for NAFLD, and insights into clinically observed reductions in CVD mortality, and 3. salsalate can reduce atherosclerosis through the activation of hematopoietic AMPK. Furthermore, this evidence can direct future drug development or drug combinations to more effectively treat multiple aspects of the common chronic diseases of obesity, diabetes and CVD that affect over a billion people worldwide.

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