Brief Communication

MOLECULAR METABOLISM

Salsalate reduces atherosclerosis through AMPKβ1 in mice

Emily A. Day [1,2], Rebecca J. Ford [1,2], Brennan K. Smith [1,2], Vanessa P. Houde [1,2], Stephanie Stypa [1,2], Sonia Rehal [1,2], Sarka Lhotak [2,3], Bruce E. Kemp [6,7], Bernardo L. Trigatti [1,4,5], Geoff H. Werstuck [1,4,5], Richard C. Austin [1,2,3], Morgan D. Fullerton [8], Gregory R. Steinberg [1,2,5,*]

ABSTRACT

Objective: Salsalate is a prodrug of salicylate that lowers blood glucose in people with type 2 diabetes. AMP-activated protein kinase (AMPK) is an αβγ heterotrimer which inhibits macrophage inflammation and the synthesis of fatty acids and cholesterol in the liver through phosphorylation of acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGCR), respectively. Salicylate binds to and activates AMPKβ1-containing heterotrimers that are highly expressed in both macrophages and liver, but the potential importance of AMPK and ability of salsalate to reduce atherosclerosis have not been evaluated.

Methods: ApoE$^{-/-}$ and LDLr$^{-/-}$ mice with or without (−/−) germline or bone marrow AMPKβ1, respectively, were treated with salsalate, and atherosclerotic plaque size was evaluated in serial sections of the aortic root. Studies examining the effects of salicylate on markers of inflammation, fatty acid and cholesterol synthesis and proliferation were conducted in bone marrow−derived macrophages (BMDMs) from wild-type mice or mice lacking AMPKβ1 or the key AMPK-inhibitory phosphorylation sites on ACC (ACC knock-in (KI)-ACC KI) or HMGCR (HMGCR-KI).

Results: Salsalate reduced atherosclerotic plaques in the aortic roots of ApoE$^{-/-}$ mice, but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice. Similarly, salsalate reduced atherosclerosis in LDLr$^{-/-}$ mice receiving wild-type but not AMPKβ1$^{-/-}$ bone marrow. Reductions in atherosclerosis by salsalate were associated with reduced macrophage proliferation, reduced plaque lipid content and reduced serum cholesterol. In BMDMs, this suppression of proliferation by salicylate required phosphorylation of HMGCR and the suppression of cholesterol synthesis.

Conclusions: These data indicate that salsalate suppresses macrophage proliferation and atherosclerosis through an AMPKβ1-dependent pathway, which may involve HMGCR phosphorylation and cholesterol synthesis. Since rapidly-proliferating macrophages are a hallmark of atherosclerosis, these data indicate further evaluation of salsalate as a potential therapeutic agent for treating atherosclerotic cardiovascular disease.

Keywords Sterol synthesis; Lipogenesis; Macrophage; Proliferation; Salicylate; Aspirin

1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide [1]. Atherosclerosis, the underlying cause of CVD, results from the development of atherosclerotic plaques, which when disrupted can lead to thrombus formation, heart attack and/or stroke. Atherosclerosis results from a buildup of lipid-laden cells (foam cells), which are predominantly macrophages, within the intima of large blood vessels. These foam cells propagate a positive feedback loop of pro-inflammatory cytokine release, classical M1 macrophage polarization and the recruitment and local proliferation of monocytes, macrophages and T cells [2−6]. Dyslipidemia is the leading risk factor for CVD and atherosclerosis. Currently, statins are the most common treatment for CVD due to their lipid-lowering properties; however, recent evidence suggests statins may increase the risk of type 2 diabetes, which is a common comorbidity with CVD [7−9]. Additionally, a significant portion of the population (~25%) are statin intolerant, which precludes the optimal use of statins in achieving their LDL-lowering target [10,11]. While Proprotein convertase subtilisin/kexin type 9 (PCSK-9) inhibitors lower LDL cholesterol effectively, these therapies are not widely used for a variety of reasons [12,13]. In addition to LDL-lowering therapies, targeting vascular inflammation through anti-IL-1β antibodies has also been shown to reduce cardiovascular events in patients taking statins [14]. Therefore, atherosclerosis can be viewed as impaired lipid metabolism overlaid with maladaptive immune response [2], and therapies aimed at inhibiting these pathways are viewed as important therapeutic strategies.

[1]Centre for Metabolism, Obesity and Diabetes Research, McMaster University, Canada [2]Department of Medicine, McMaster University, Canada [3]Hamilton Centre for Kidney Research, St. Joseph's Healthcare Hamilton, Canada [4]Thrombosis and Atherosclerosis Research Institute, McMaster University, Canada [5]Department of Biochemistry and Biomedical Sciences, McMaster University, Canada [6]St. Vincent's Institute of Medical Research and Department of Medicine, University of Melbourne, Fitzroy, Victoria, Australia [7]Mary MacKillop Institute for Health Research, Australian Catholic University, Fitzroy, VIC, Australia [8]Department of Biochemistry, Microbiology and Immunology, Centre for Infection, Immunity and Inflammation, Centre for Catalysis Research and Innovation, Faculty of Medicine, University of Ottawa, Canada

*Corresponding author. Centre for Metabolism, Obesity and Diabetes Research, HSC 4N63. McMaster University, 1280 Main St. West, Hamilton, Ontario, L8N 3Z5, Canada. E-mail: gsteinberg@mcmaster.ca (G.R. Steinberg).

Received July 4, 2021 • Revision received July 23, 2021 • Accepted August 10, 2021 • Available online 21 August 2021

https://doi.org/10.1016/j.molmet.2021.101321

Salicylate, an active metabolite of aspirin, has been used for thousands of years to relieve pain and inflammation (for review see [15,16]). Salsalate, a prodrug of salicylate, is rapidly cleaved into two salicylate molecules by carboxylesterases within the gastrointestinal tract (for review see [15,16]). But unlike aspirin, salsalate does not inhibit platelet function or cause gastrointestinal bleeding, and as a result can be taken at high doses (~4 g/day) that elicit serum salicylate concentrations of ~1 mM [17–20]. In addition to treating arthritis, salsalate has been evaluated in clinical trials in people with type 2 diabetes, where it has been shown to reduce circulating glucose and triglycerides [17–19]. Salsalate has also been tested in people with atherosclerosis taking statins, where it was reported to have no effect on LDL cholesterol, C-reactive protein or the progression of calcified atherosclerotic plaques (TINSAL-CVD, Clinical Trial Identifier: NCT00624923) [21]. However, it should be noted that this study had several important limitations. Firstly, the placebo group in this trial showed no atherosclerotic progression, so interpretation of these results is limited. Furthermore, the effect of salsalate in statin-naïve patients has not been assessed. Additionally, unlike the Odyssey and Fournier trials [22,23] or the CANTOS trial [14], this trial did not use a minimum LDL cholesterol level or CRP level as inclusion criteria to demonstrate efficacy in addition to that of statins.

Mechanistically, salicylate has been shown to reduce blood glucose through a number of potentially interrelated mechanisms, including the repression of an inhibitor of nuclear factor κ-B kinase subunit β (IKK-β) [24–27], mitochondrial uncoupling [28] and activation of the AMP-activated protein kinase (AMPK) [29]. AMPK is a heterotrimeric enzyme consisting of alpha catalytic and regulatory beta and gamma isoforms which have unique tissue-specific expression patterns. AMPK suppresses macrophage inflammation [30] as well as fatty acid and cholesterol synthesis in the liver through phosphorylation of acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGCR), respectively [30–32]. Previous studies have shown that salicylate directly binds to and activates AMPKβ1-containing heterotrimers [29], which are predominately expressed in hepatocytes and macrophages, and that this is important for increasing fatty acid oxidation and reducing fatty acid synthesis [30,33]. However, salsalate's reduction of atherosclerosis and the role of AMPK in mediating these effects have not been evaluated. In the current study, we investigate the effects of salsalate using two independent mouse models of atherosclerosis ($ApoE^{-/-}$ and $LDLr^{-/-}$) and find that salsalate reduces macrophage proliferation and atherosclerotic plaque development through a hematopoietic AMPKβ1-dependent pathway. Furthermore, salicylate suppresses macrophage cholesterol synthesis through phosphorylation of HMGCR, which is important for reducing macrophage proliferation.

2. MATERIAL AND METHODS

2.1. Animal ethics and models

All animal experiments were approved by the McMaster University Animal Research Ethics Board. The generation and characterization of $AMPK\beta1^{-/-}$ [34], $ApoE^{-/-}AMPK\beta1^{-/-}$ [35], HMGCR Ser871Ala knock-in (HMGR KI) [36] and ACC1/ACC2 Ser79/212Ala Double Knock-in (ACC DKI) [32] mice have been described previously. Mice were group-housed at the conventional temperature (22–23 °C) on a 12-hour light–dark schedule with *ad libitum* access to food and water. $ApoE^{-/-}$ and $ApoE^{-/-}$ $AMPK\beta1^{-/-}$ mice were fed a western diet (TD.09821, Envigo) or western diet containing 2.5 g/kg salsalate (TD.130,258, Envigo, Salsalate from Cayman Chemicals) for 6 weeks, starting at 6 weeks of age. During week 5 of treatment, mice were fasted for 6 h (beginning at 7 am) and a glucose tolerance test was performed using intraperitoneal injection of 2 g/kg D-glucose. Blood glucose was measured at the indicated time points using an Accu-Chek Performa blood glucose meter. After 6 weeks, mice were anaesthetised with Ketamine (75 mg/kg) and Xylazine (10 mg/kg) before tissues were harvested. A subset of mice was injected with BrdU (Invitrogen) 2 h prior to sacrifice, as described [37].

2.2. Bone marrow transfer

Whole-body irradiation and bone marrow transfer were completed as previously described [30]. Briefly, bone marrow was collected from wild-type or $AMPK\beta1^{-/-}$ mice. Recipient 8-week-old $LDLr^{-/-}$ mice purchased from JAX (stock: 002207) were irradiated using two doses of 550 Rads 3–4 h apart. Bone marrow was collected from femur and tibia of wild-type and $AMPK\beta1^{-/-}$ mice through centrifugation. Cells were resuspended in PBS and intravenously injected in recipients at 5×10^6 cells per mouse at a volume of 200 μL. Mice were allowed to recover for 6 weeks, at which point engraftment has been shown to be established [30], followed by 8 weeks of western diet when they were assigned to either continue receiving western diet or western diet ±2.5 g/kg salsalate for another 8 weeks. Engraftment was confirmed using genomic DNA isolation from whole blood using the Fast Blood/Cell PCR Genotyping Kit (EZ Bioresearch, G1002-10) per manufacturer's instructions.

2.3. Atherosclerotic measurements

The heart and aortic root were removed, formalin fixed and paraffin embedded. 4-μm thick sections of the aortic root were then collected and stained with H&E to measure plaque size at 80-μm intervals; subsequent sections were used for immunohistochemistry (IHC) as previously described [38,39]. Images were captured using a Nikon 90 Eclipse microscope (Nikon). Atherosclerotic plaque size was determined from 5 sections per mouse, and the area was calculated by manually outlining the plaques on ImageJ software by an individual who was blinded to the groups. Lesion area is reported as an average of the 5 sections per mouse. IHC was done using primary antibodies to Mac-3 (1:1000, BD-Pharmingen 553,322), Ki67 (1:100, Abcam, ab16667) and BrdU (1:500 Abcam ab1893)) and biotinylated secondary antibodies (1:500, MJS BioLynx, VECTBA1000, VECTBA4001, VECTBA6000) and detected using streptavidin peroxidase and Nova Red (all IHC reagents are from Vector Labs/MJS BioLynx). Lipid content was measured by a group-blinded individual using ImageJ to outline plaques and colour threshold 8-bit images for white areas, and is reported as an average of 3 sections per mouse.

2.4. Cell culture

Bone marrow–derived macrophages (BMDMs) were generated by isolating marrow from the tibia and femur of each leg, followed by centrifugation as described [33]. Marrow was then suspended in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. After 5 h, L929 media (a source of M-CSF) was added and cells were plated in 10 cm tissue culture dishes. Cells were then allowed to differentiate for 7 days before reseeding for experiments as described.

2.4.1. Proliferation

Following differentiation, cells were scraped from 10-cm dishes, manually counted and seeded at 7000 cells per well in a 96-well plate. Cells were plated in a 96-well plate (100 μL/well) in DMEM (10% FBS, 1% antibiotic-antimycotic) overnight. The following morning, media was replaced with DMEM +/− M-CSF (2 ng/mL) and + salicylate (1 mM) as indicated for 72 h. For add-back experiments, as previously described [40], 1% BSA (sterile, fatty acid free,

low endotoxin, Sigma A8806) + mevalonate 100 μM (79,849, Sigma) and oleate 50 μM (O7501, Sigma) were added as indicated. After 72 h, cells were formalin fixed, stained with crystal violet, washed thoroughly, and allowed to dry. The dye was solubilized in NaH_2PO_4 and absorbance read at 570 nm. Data is expressed relative to M-CSF stimulated samples.

2.4.2. De novo lipogenesis

Cells were reseeded in 12-well plates at 2×10^6 cells/mL and allowed to adhere overnight. The following day, media was replaced with serum-free DMEM with [3H]-acetate and salicylate (1 mM). Lipid extraction was performed as previously described [41,42], and a portion of the sterol and fatty acid fractions were counted for radioactivity.

2.4.3. In vitro cytokine analysis

Cells were seeded in 6-well culture dishes at 2×10^6 cells/mL and allowed to adhere overnight. The following day, media was replaced and salicylate (1 mM) was added 30 min prior to the addition of LPS (100 ng/mL). After 6 h, media and/or cells were frozen at -80 °C until future analysis.

2.5. Analytical methods

2.5.1. ELISAs

Cytokines were measured in media using Duo-Set ELISA (all from R&D) for TNFα (Catalogue: DY410), IL-1β (Catalogue: DY401), IL-6 (Catalogue: DY406) and IL-10 (Catalogue: DY:417) according to manufacturer protocols.

2.5.2. RT-q-PCR

RNA was isolated using RNEasy Columns (Qiagen) with on-column DNase treatment. RT-q-PCR was performed as previously described [41]. All Taqman primers were purchased from Invitrogen, and relative gene expression was calculated using ($2^{-\Delta CT}$) method. Values were normalized to housekeeping gene β-actin of the wild-type control.

2.5.3. Serum salicylate levels

Serum Salicylate levels were measured in blood (collected from a small nick in the tail vein at indicated time points) using Salicylates (RTU) Forensic Kit (133,619, Neogen).

2.5.4. Plasma triglycerides and cholesterol

Plasma lipids were measured from mice fasted for 6 h prior to sacrifice. Blood was collected retro-orbitally through a heparinized capillary tube into an Eppendorf containing EDTA. Samples were diluted 10-fold and cholesterol was measured using the Infinity cholesterol kit (TR13421, Thermo Scientific) according to manufacturer's instructions. Triglycerides were measured in samples diluted 2-fold using the Triglyceride Colorimetric Assay Kit (10010303, Cayman Chemicals) according to manufacturer's instructions.

2.5.5. Serum cytokine analysis

Serum cytokines were assessed by Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad laboratories), according to manufacturer's instructions, using a 4-fold dilution of serum.

2.5.6. Cytotoxicity assay

Cytotoxicity was assessed using CytoTox 96® Non-Radioactive Cytotoxicity Assay (G1780, Promega) according to manufacturer instructions. Briefly, fresh supernatants were collected from BMDMs treated with indicated doses of salicylate for 6 h and mixed 1:1 with assay reagent for 30 min at room temperature, at which point stop solution was added. Absorbance was read at 490 nm. Data is expressed as a percent of the completely lysed well.

2.6. Statistical analysis

All statistical analysis was performed using Graphpad Prism version 8. Data was tested for normality using the Shapiro–Wilk test. Unless otherwise noted, data were normally distributed and analysed with a two-way ANOVA, with post-hoc analysis performed with Sidak's multiple comparison correction. Non-normal data were analysed with a non-parametric One-way ANOVA, non-parametric t-test or Kruskal–Wallis test with Dunn's multiple comparison as appropriate (indicated in the figure legends). All data is presented as mean + S.E.M. Statistical outliers were determined using two standard deviations from the mean.

3. RESULTS

3.1. Salsalate reduces atherosclerosis in ApoE$^{-/-}$ but not ApoE$^{-/-}$/AMPKβ1$^{-/-}$ mice

For 6 weeks starting at 6 weeks of age, ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice were fed a western diet (control) or western diet supplemented with salsalate. The dose of salsalate (**2.5 g salsalate/kg diet**) was selected to generate a sustained serum salicylate concentration of ~900 μM (Figure 1A) throughout the day, consistent with previous rodent studies [28], and is aligned with serum concentrations of salicylate in clinical trials of patients with T2D or CVD treated with 3–4 g/day of salsalate [17–20]. Food intake between groups was comparable (Figure 1B) and there were no differences in body mass (Figure 1C) or glucose tolerance between treatments or genotypes (Figure 1D,E). Despite similar body mass, and glucose homeostasis, salsalate reduced atherosclerotic plaques in ApoE$^{-/-}$ mice by approximately 30%, an effect which was not observed in ApoE$^{-/-}$/AMPKβ1$^{-/-}$ mice (Figure 1F,G). Necrotic area within the atherosclerotic plaque is an important marker of plaque stability that can contribute to poor outcomes clinically. In this model, we saw very little plaque necrosis (~2%), and there were no differences between groups (Figure 1H). Total plaque lipid area, which includes necrotic areas, cholesterol crystals and intracellular lipid droplets, was dramatically reduced in ApoE$^{-/-}$ but not ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice treated with salsalate (Figure 1I). This effect was discordant with plasma triglycerides, which were elevated in ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice but not altered by salsalate in either genotype (Figure 1J). However, mirroring reductions in plaque size and plaque lipid content, salsalate lowered plasma cholesterol in ApoE$^{-/-}$ but not ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice (Figure 1K). Plasma cholesterol was not different between genotypes of mice (Figure 1K). These data indicate that salsalate reduces atherosclerosis through a mechanism requiring AMPKβ1.

3.2. Salsalate reduces atherosclerosis in LDLr^{WT} but not LDLr^{β1KO} mice

ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice show large reductions in AMPK activity in both liver and macrophages [35,41], both of which could contribute to reductions in atherosclerosis. Salicylate elicits anti-inflammatory effects in macrophages [43,44], so we hypothesized that AMPKβ1 may be critical for mediating reductions in atherosclerosis in macrophages. To directly examine this hypothesis, we conducted bone marrow transfers from WT and whole-body AMPKβ1$^{-/-}$ mice into LDLr$^{-/-}$ mice to generate LDLr^{WT} and LDLr^{β1KO} mice. Genotyping of whole-blood genomic DNA confirmed successful reconstitution of the LDLr$^{-/-}$ mice with WT or β1KO bone marrow (Figure 2A). There were

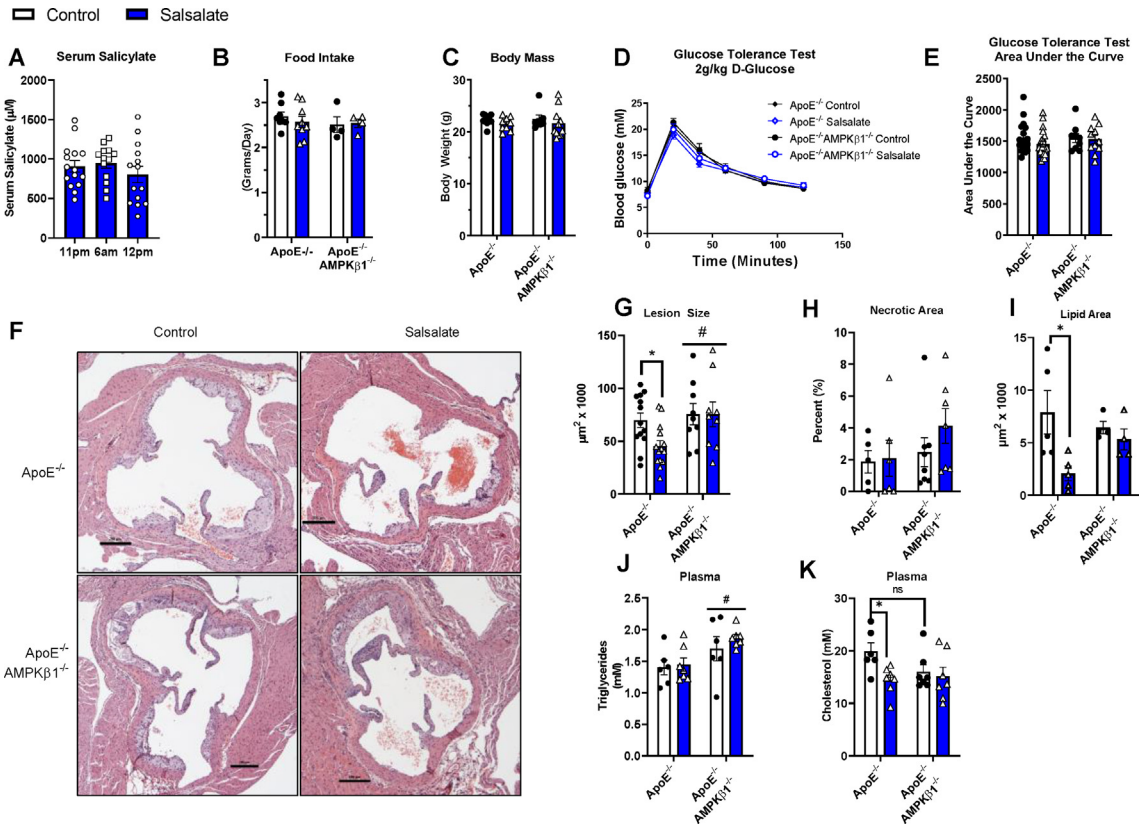

Figure 1: Salsalate reduces atherosclerosis through AMPKβ1. A) Serum salicylate levels on western diet +2.5 g/kg salsalate. B) Food intake, C) body mass, D) glucose tolerance and E) GTT Area under the curve of ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ fed a western diet or western diet containing + salsalate. F) Representative images of H&E—stained aortic roots from control or salsalate-treated ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice. G) Average plaque size, H) plaque necrotic area, I) plaque lipid area, J) plasma triglyceride and K) plasma cholesterol. Data is expressed as mean ± S.E.M. # indicates significant effect of genotype, * indicates $p < 0.05$ when compared to control. Scale bar is 200 μm.

no differences in body mass between groups (Figure 2B). Consistent with observations in the ApoE$^{-/-}$ mice, salsalate reduced atherosclerotic lesions by approximately 30% in LDLr^{WT} but not LDLr$^{\beta 1KO}$ mice. However, complicating the interpretation of this finding, atherosclerotic lesions were smaller in LDLr$^{\beta 1KO}$ compared to LDLr^{WT} mice (Figure 2C,D), a finding consistent with a previous study that showed that irradiated mice reconstituted with bone marrow from AMPKα1$^{-/-}$ (the primary α subunit in hematopoietic cells) mice also show reductions in atherosclerosis [45]. Similarly, percent necrotic area of the plaques was smaller in LDLr$^{\beta 1KO}$ plaques than LDLr^{WT} mice, and salsalate reduced necrotic area in LDLr^{WT} but not LDLr $^{\beta 1KO}$ plaques (Figure 2E). The same trend ($p = 0.062$) was seen in lipid area of the plaques, which includes necrotic area, cholesterol crystals and intracellular lipids (Figure 2F). Importantly, in contrast to the ApoE$^{-/-}$ mice but consistent with normal levels of AMPKβ1 in the liver, serum cholesterol levels were reduced by salsalate in both LDLr^{WT} and LDLr$^{\beta 1KO}$ mice (Figure 2G). Further, salsalate tended to reduce serum triglycerides in both LDLr^{WT} and LDLr$^{\beta 1KO}$ mice (Figure 2H). In contrast to the ApoE$^{-/-}$ model, LDLr $^{\beta 1KO}$ mice did not have increased serum triglycerides relative to the LDLr^{WT} mice (Figure 2H). These data indicate that salsalate reduces atherosclerosis and that haematopoietic AMPKβ1 is an important factor contributing to the mechanism.

3.3. Salsalate reduces Ki67 and BrdU incorporation in plaques of ApoE$^{-/-}$ but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice

While multiple haematopoietic cell types can contribute to the development of atherosclerosis, macrophage accumulation in the artery wall is well documented underlying the pathogenesis of disease development [4—6]. Given the important role of macrophage inflammation in the development of atherosclerosis [46], we examined plasma inflammatory markers IL-1α, IL-1β, IL-12 (p40), IL-12 (p70), IL-13, G-CSF, IFNγ, RANTES, TNFα and MCP-1 and found that, surprisingly, salsalate had no effect (Supplemental Figs. 1A—J). As salicylate has been demonstrated to reduce inflammation in macrophages [43,44], we hypothesized that there may be subtle changes in this cell type that are not represented by levels in the plasma. To directly evaluate this hypothesis, we cultured bone marrow—derived macrophages (BMDMs) with LPS (100 ng/mL) or LPS + salicylate (1 mM, approximate serum concentration achieved in both mouse and human studies). Consistent with our *in vivo* data, salicylate had no effect on the levels of IL-1β, TNFα, IL6 or anti-inflammatory IL-10 transcript or secretion following LPS stimulation (Supplementary Figs. 2A—H). Additionally, we confirmed that this dose of salicylate did not induce cytotoxicity (Supplementary Fig. 2I). These data suggest that when treated with a therapeutically achievable concentration of salicylate, LPS-induced inflammation is not supressed in mouse primary macrophages.

Given that there were no changes in markers of inflammation, we examined alternative mechanisms by which salsalate-induced activation of AMPKβ1 complexes might reduce atherosclerosis. Recent studies have demonstrated that local macrophage proliferation is a critical driver of disease development [3]; therefore, we examined proliferating cells within atherosclerotic plaques to determine if this was altered by salsalate treatment. In ApoE$^{-/-}$ but not ApoE$^{-/-}$

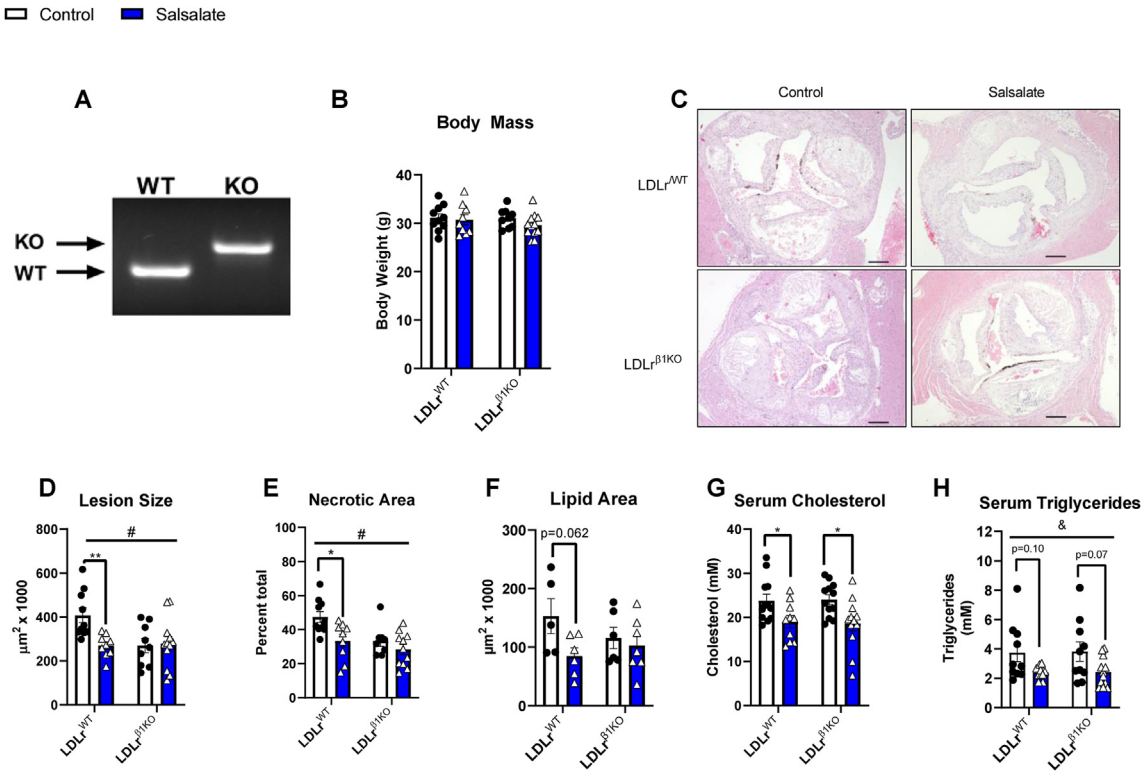

Figure 2: Salsalate reduces atherosclerosis through hematopoietic AMPKβ1. A) Representative image of genotyping from blood of LDLr^{WT} and LDLr ^{β1KO} mice. B) Body mass of LDLr^{WT} and LDLr ^{β1KO} mice fed a western diet or western diet + salsalate. C) Representative images of H&E—stained aortic roots from control or salsalate-treated LDLr^{WT} and LDLr ^{β1KO} mice. D) Average plaque size, E) plaque necrotic area, F) plaque lipid area, G) serum cholesterol and H) serum triglycerides. Data is expressed as mean ± S.E.M. # indicates significant effect of genotype, & indicates significant effect of salsalate, and * indicates $p < 0.05$ when compared to control. Scale bar is 200 μm.

AMPKβ1$^{-/-}$ mice, salsalate reduced staining of the proliferation marker Ki67 within macrophage-rich areas of the plaques (Mac-3 positive areas) (Figure 3A,B). Given that Ki67 is expressed in a cell cycle—dependant manner, and that cell cycle arrest could alter Ki67 levels, we examined a separate cohort of mice injected with BrdU, thus allowing for accurate measurements of *in situ* macrophage proliferation [37]. Importantly, mice were sacrificed 2 h after BrdU injection to eliminate effects from bone marrow niche or progenitor subsets in this analysis. As with Ki67, we found that salsalate reduced BrdU incorporation in ApoE$^{-/-}$ but not ApoE$^{-/-}$ AMPKβ1$^{-/-}$ mice, although there was a trend for decreased BrdU staining in the ApoE$^{-/-}$ AMPKβ1$^{-/-}$ relative to ApoE$^{-/-}$ (Figure 3A,C). These data suggest that salsalate reduces atherosclerosis by suppressing local macrophage proliferation and that this effect requires AMPKβ1.

3.4. Salicylate suppresses proliferation of bone marrow—derived macrophages through phosphorylation of HMGCoA reductase

To directly test the effect of salicylate on macrophage proliferation, studies were conducted in BMDMs from WT and AMPKβ1$^{-/-}$ mice. In agreement with previous studies [30,33,41], macrophages from AMPKβ1$^{-/-}$ mice had no detectable AMPK alpha expression (Figure 4A) and dramatic reductions in the phosphorylation of acetyl-CoA carboxylase (a marker of cellular AMPK activity which takes into account allosteric activation elicited through direct β1-activators like salicylate) compared to WT mice. Importantly, salicylate increased ACC phosphorylation in macrophages from WT but not AMPKβ1$^{-/-}$ mice (Figure 4A,B). Macrophages proliferate very slowly in the absence of stimulation, and *in vitro* and *in vivo*, M-CSF is critical for stimulating macrophage proliferation [47,48]. Therefore, we measured *in vitro* macrophage proliferation over 72 h when stimulated with M-CSF (2 ng/mL) + salicylate. Consistent with the activation of AMPK, we found that salicylate suppressed the proliferation of macrophages from WT but not AMPKβ1$^{-/-}$ mice (Figure 4C). AMPK exerts effects on multiple pathways critical for regulating cell growth and proliferation [49]; however, cell division and proliferation are ultimately dependent on the generation of new cellular membranes, which requires *de novo* synthesis of fatty acids and cholesterol. This regulation of fatty acid and cholesterol synthesis has been shown to be important for dividing cancer cells [50,51] but also many immune cell types, including macrophages, T cells and platelets [52—54]. Previous studies conducted in cancer cells have shown that salicylate-induced suppression of cellular proliferation requires the inhibition of fatty acid and cholesterol synthesis [40]; therefore, we hypothesized that a similar mechanism of action may be important for mediating the anti-proliferative actions of salicylate in macrophages. To examine the dependency of macrophage proliferation on cholesterol or fatty acid synthesis, the culture media was supplemented with mevalonate or oleate, respectively. We found that supplementation with mevalonate but not oleate blocked the effects of salicylate and reduced proliferation (Figure 4D), which is consistent with the hypothesis that salicylate induces AMPKβ1-dependent suppression of cholesterol synthesis, resulting in reduced macrophage proliferation.

AMPK inhibits fatty acid and cholesterol synthesis through the phosphorylation of ACC and HMGCR, respectively. Therefore, to directly test the hypothesis that salicylate activation of AMPK suppresses cholesterol synthesis, we generated BMDMs (in addition to AMPKβ1$^{-/-}$

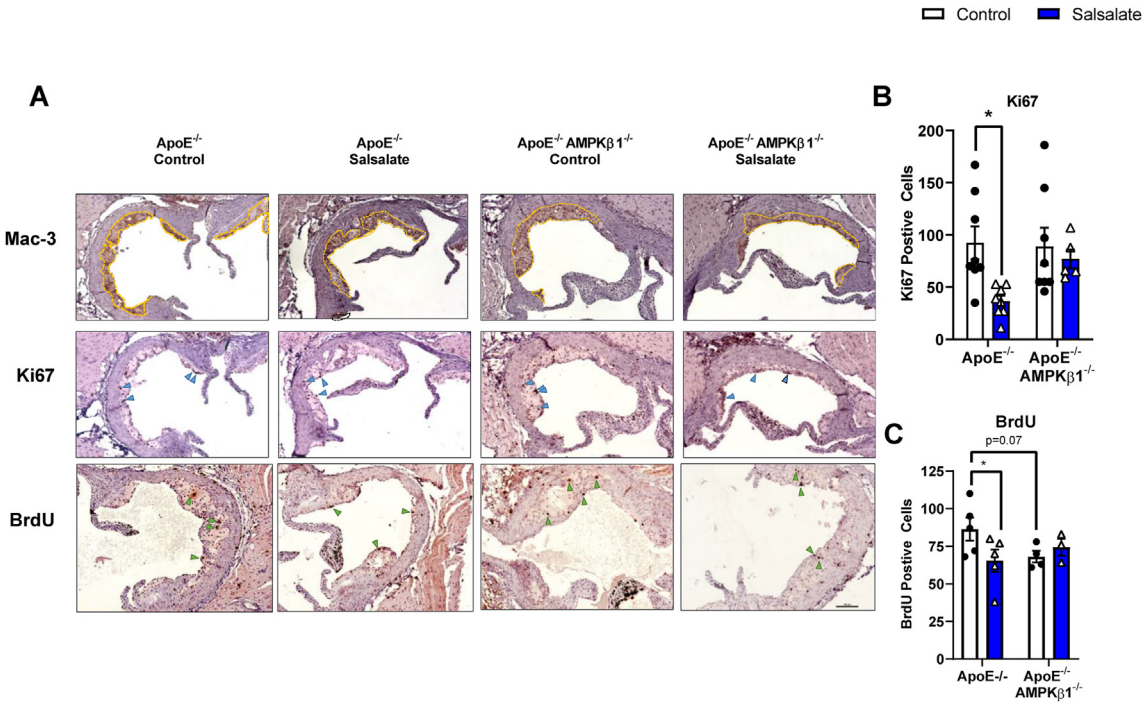

Figure 3: Salsalate reduces macrophage proliferation *in vivo*. A) Representative Mac3- (yellow dashed lines), Ki67- (blue triangles) and BrdU- (green triangles) stained aortic roots from ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice after 6 weeks of western diet or western diet + salsalate. B, C) Ki67-and BrdU-positive cells within the atherosclerotic plaques. Data is expressed as mean ± S.E.M. *p < 0.05 for salsalate versus control within genotype. Scale bar is 100 μm.

macrophages) from knock-in (KI) mouse models, where the AMPK phosphorylation site(s) on HMGCR (HMGCR-KI) [36] and ACC (ACC-DKI) [32] are mutated from a serine to an alanine, blocking the inhibitory effects of AMPK on cholesterol and fatty acid synthesis, respectively. As anticipated, salicylate induced suppression of cholesterol synthesis in wild-type BMDM, but this effect was impaired in AMPKβ1$^{-/-}$ and HMGCR KI but not ACC DKI macrophages (Figure 4E). In contrast, but consistent with previous studies [28], salicylate suppressed fatty acid synthesis in wild-type, AMPKβ1$^{-/-}$, HMGCR KI and ACC DKI macrophages (Figure 4F). These data indicate that salicylate-induced suppression of cholesterol synthesis is mediated through HMGCR, but that the suppression of fatty acid synthesis is mediated through AMPK-ACC—independent pathways. Consistent with these findings, we found that the ability of salicylate to reduce proliferation was blunted in macrophages from the HMGCR KI but not the ACC DKI mice (Figure 4G). These data indicate that AMPKβ1-dependent phosphorylation of HMGCR and suppression of the mevalonate pathway, which ultimately leads to cholesterol synthesis, is required for salicylate-induced reductions in macrophage proliferation.

4. DISCUSSION

We found that there were no differences in the size of atherosclerotic lesions between ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ (Figure 1), consistent with our previous studies using this mouse line [35,41]. In contrast, in LDLr$^{-/-}$ mice, reconstitution of bone marrow from AMPKβ1$^{-/-}$ mice to generate LDLr ^{β1KO} mice resulted in smaller plaques compared to LDLr^{WT} mice (Figure 2). This finding is consistent with a previous study that showed that irradiated mice reconstituted with bone marrow from AMPKα1$^{-/-}$ (the primary α subunit in hematopoietic cells) mice also show reductions in atherosclerosis [45].

One limitation of this study is that atherosclerosis was only assessed at one site (aortic sinus), where lesions develop first. Site-specific differences in atherosclerosis, while uncommon, could also present in these models [55]. The mechanisms contributing to this reduction in atherosclerosis in the absence of hematopoietic AMPKβ1 but not germline ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice are not fully understood, but may be related to differences between ApoE and LDLr null mice. Alternatively, they may involve compensatory adaptations present in the AMPKβ1 germline bone marrow niche that were not developed in the more acute setting following bone marrow transfer. Another possibility is that comparable plaque sizes in ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ may be due to the loss of AMPKβ1 in other tissues, such as the liver, which may drive atherosclerotic plaque development and mask the effects of AMPKβ1 deletion in hematopoietic cells.

We found that salsalate reduced atherosclerosis in two distinct mouse models (ApoE$^{-/-}$ and LDLr$^{-/-}$). In ApoE$^{-/-}$ mice, this reduction in atherosclerosis required AMPKβ1 and was associated with reductions in plasma cholesterol, plaque lipid content and macrophage proliferation. Atherosclerosis involves interactions with multiple cell types and cellular pathways, but most notably hepatocytes and macrophages, tissues which predominately express the AMPKβ1 isoform in mice. To investigate the importance of macrophages in potentially mediating the beneficial effects of salsalate, experiments were then conducted in LDLr$^{-/-}$ mice reconstituted with bone marrow from WT mice (LDLr^{WT}) or AMPKβ1$^{-/-}$ mice. Again, we observed reductions in plaque size, an effect that was eliminated in LDLr ^{β1KO} mice. Importantly, in contrast to ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice, this occurred despite comparable reductions in serum cholesterol in LDLr^{WT} mice, suggesting that hematopoietic AMPKβ1 is important for reducing atherosclerosis. To further investigate the mechanisms contributing to reductions in plaque size in separate experiments, ApoE$^{-/-}$ and ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice were injected

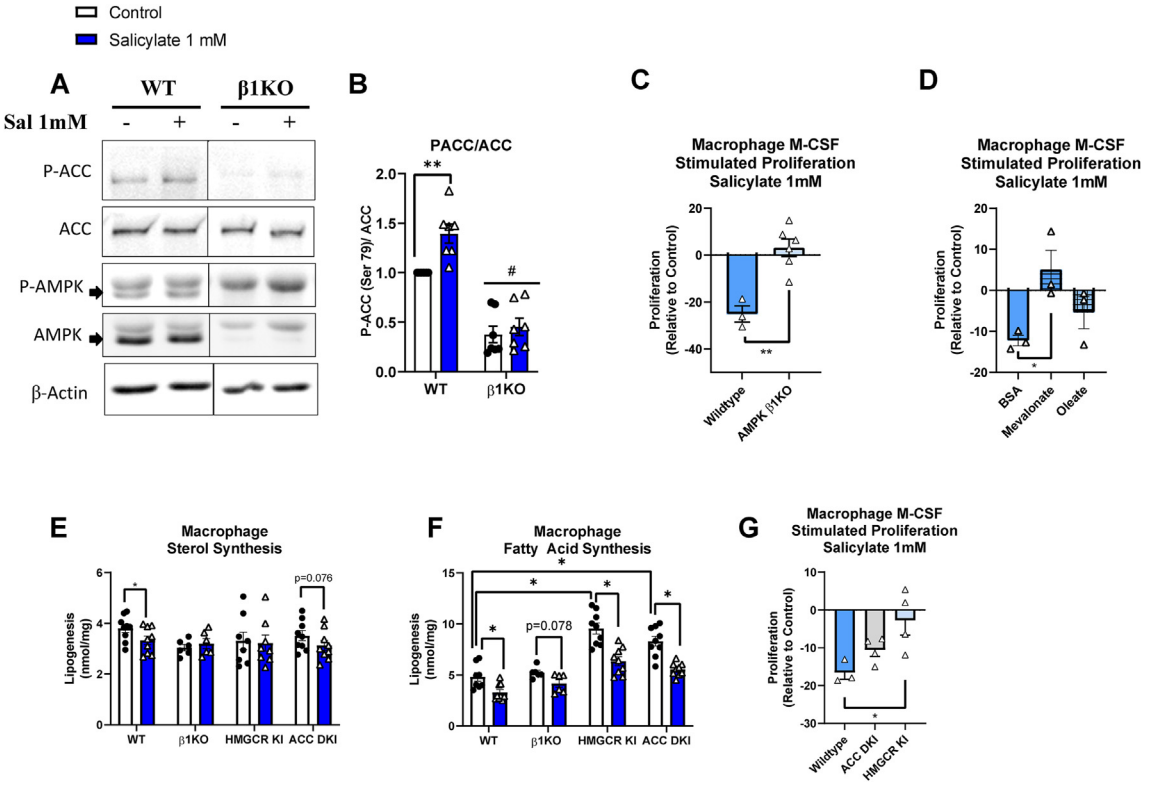

Figure 4: AMPK phosphorylation of HMGCR is required for the decrease in macrophage proliferation with salicylate. A, B) Representative western blots and quantification of wild-type and AMPKβ1$^{-/-}$ bone marrow–derived macrophages were treated with 1 mM salicylate for 90 min (Statistical test; One-Way ANOVA). C) Proliferation relative to control (MCSF only) of wild-type and AMPKβ1$^{-/-}$, simultaneously treated with MCSF (2 ng/mL) and salicylate (1 mM) for 72 h (Statistical test non-parametric t-test) D) Wild-type bone marrow–derived macrophages were simultaneously treated with MCSF (2 ng/mL), salicylate (1 mM) ± mevalonate or oleate where indicated for 72 h (Statistical Test Kruskal–Wallis) E, F) Incorporation of radiolabelled acetate into sterol and fatty acid fractions in wild-type, AMPKβ1$^{-/-}$ ACC DKI and HMGCR KI bone marrow–derived macrophages over 4 h. G) Proliferation relative to control (MCSF only) of wild-type, ACC DKI and HMGCR KI bone marrow–derived macrophages simultaneously treated with MCSF (2 ng/mL) and salicylate (1 mM) for 72 h (Statistical Test, Kruskal–Wallis with Dunn's multiple comparison). Data is expressed as mean ± S.E.M. from 3 to 6 individual experiments. *p < 0.05 for comparison indicated.

with BrdU. Consistent with reduced plaque size and staining of the proliferative marker Ki67, ApoE$^{-/-}$ but not ApoE$^{-/-}$AMPKβ1$^{-/-}$ mice had reduced incorporation of BrdU, indicating reduced proliferation of macrophages within the atherosclerotic plaque. These data indicate that salsalate reduces macrophage proliferation and that this effect requires AMPKβ1. Here, we focused on the effects of salsalate on macrophage proliferation within the plaque as a mechanism for reduced atherosclerosis, as local proliferation has been shown to account for over 85% of macrophage content [3]; however, there could also be effects on hematopoietic stem cell (HSC) and monocyte proliferation, seen with other AMPK activators [56]. Reductions in HSC proliferation would be consistent with reduced white blood cell, lymphocyte, monocyte and neutrophil counts seen clinically with salsalate treatment [21]; however, this would not explain the reduced number of BrdU-positive cells within the plaques 2 h after BrdU injection. Therefore, our data suggest that *in situ* macrophage proliferation is reduced with salsalate via AMPKβ1, but potential effects on HSCs and monocytes cannot be excluded and warrant further investigation.

As reductions in serum cholesterol are important for driving proliferation of macrophages, we subsequently conducted experiments in cultured BMDMs so that this variable could be controlled. Using a clinically relevant concentration of salicylate that did not affect viability, we observed reduced proliferation in BMDMs from WT but not AMPKβ1$^{-/-}$ mice. Subsequent experiments established that these anti-proliferative effects of salicylate on BMDMs were blunted in media supplemented with mevalonate and in mice lacking inhibitory AMPK phosphorylation sites on HMGCR. While it remains feasible that reductions in atherosclerosis mediated by salsalate *in vivo* involve reductions in serum cholesterol, the alignment of the *in vitro* with reductions in macrophage proliferation observed *in vivo* support a direct effect of salicylate in suppressing local macrophage proliferation. The demonstration of the anti-proliferative effects of salicylate involves suppression of HMGCR and cholesterol synthesis and provides potential insight into why salsalate had no effect on atherosclerotic plaque development in the TINSAL-CVD trial, as the inclusion criteria was a stable dose of statins [21]. While the inhibition of HMGCR in the liver and the subsequent upregulation of the LDLr is vital for the lipid-lowering effects of statins, multiple studies have also documented lipid-independent effects of statins, suggesting that additional mechanisms may be important. Consistent with the concept, very recent studies have indicated that statins also potently reduce local macrophage proliferation, which is critical for plaque regression in APOE*3Leiden.Cholesteryl ester transfer protein (CETP) mice, which model human-like lipid changes in response to oral statin treatment [57]. These novel data, with respect to the mechanisms of statins in combination with our observations with salsalate, support a common mechanism mediating reductions in atherosclerosis, giving a potential reason for why no beneficial effects of salsalate were observed in the TINSAL-CVD trial.

We have demonstrated for the first time that salsalate reduces atherosclerosis, requiring AMPKβ1, while also describing a novel mechanism by which salicylate and AMPK inhibit macrophage proliferation. However, it should be noted that like many effective type 2 diabetes medications that exert cardioprotective effects, such as metformin, SGLT2 inhibitors and GLPR1 agonists, multiple overlapping mechanisms may be involved in the beneficial effects of salsalate on atherosclerosis, including effects on the liver, serum lipids and macrophage metabolism. Future studies examining these mechanisms and whether salsalate is a useful therapeutic option for patients with impaired fasting glucose, at high risk of new incident diabetes with statin therapy or with an inability to tolerate effective statin doses due to statin intolerance are warranted.

AUTHOR CONTRIBUTIONS

E.A.D., R.J.F., B.K.S, V.P.H, S.R., S.S., S.L. and M.D.F. performed the experiments and analysis. E.A.D. and G.R.S. wrote the manuscript. All authors edited the manuscript and provided comments. G.R.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ACKNOWLEDGEMENTS

We thank Dr. Thomas J. Hawke for allowing microscope access and Kevin P. Foley for technical assistance. G.R.S. is supported by a Canada Research Chair and the J. Bruce Duncan Chair in Metabolic Diseases, as well as research grants from the Heart and Stroke Foundation, the Canadian Institutes of Health Research (201709FDN-CEBA-116200 to GS) and Diabetes Canada (DI-5-17-5302-GS). BEK is supported by the National Health and Medical Research Council of Australia and the Australian Research Council (NHMRC, APP1085460 & ARC DP170101196).

CONFLICT OF INTEREST

G.R.S. has received research funding from Esperion Therapeutics, Espervita Therapeutics, Poxel Pharmaceuticals and Novo Nordisk, honoraria and/or consulting fees from Astra Zeneca, Eli-Lilly, Esperion Therapeutics, Poxel Pharmaceuticals and Merck and is a founder and shareholder of Espervita Therapeutics.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101321.

REFERENCES

[1] Rached, F.H., Chapman, M.J., Kontush, a, 2014 Jul. An overview of the new frontiers in the treatment of atherogenic dyslipidemias. Clinical Pharmacology & Therapeutics 96(1):57—63.

[2] Libby, P., Ridker, P.M., Hansson, G.K., 2011 May. Progress and challenges in translating the biology of atherosclerosis. Nature 473(7347):317—325.

[3] Robbins, C.S., Hilgendorf, I., Weber, G.F., Theurl, I., Iwamoto, Y., Figueiredo, J.-L., et al., 2013 Sep. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. Nature Medicine 19(9):1166—1172.

[4] Koelwyn, G.J., Corr, E.M., Erbay, E., Moore, K.J., 2018. Regulation of macrophage immunometabolism in atherosclerosis. In: Nature immunology, vol. 19. Nature Publishing Group. p. 526—37.

[5] Moore, K.J., Sheedy, F.J., Fisher, E.A., 2013. Macrophages in atherosclerosis: a dynamic balance. Nature Reviews Immunology vol. 13:709—721. Nat Rev Immunol.

[6] Goldberg, I.J., Sharma, G., Fisher, E.A., 2020. Atherosclerosis: making a U turn [internet]. In: Annual review of medicine, vol. 71. Annual Reviews Inc.. p. 191—201

[7] Rajpathak, S.N., Kumbhani, D.J., Crandall, J., Barzilai, N., Alderman, M., Ridker, P.M., 2009 Oct. Statin therapy and risk of developing type 2 diabetes: a meta-analysis. Diabetes Care 32(10):1924—1929.

[8] Sattar, N., Preiss, D., Murray, H.M., Welsh, P., Buckley, B.M., de Craen, A.J., et al., 2010. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. Lancet 375(9716):735—742.

[9] Preiss, D., Seshasai, S.R.K., Welsh, P., Murphy, S.A., Ho, J.E., Waters, D.D., et al., 2011 Jun. Risk of incident diabetes with intensive-dose compared with moderate-dose statin therapy. Journal of the American Medical Association 305(24):2556.

[10] Maningat, P., Breslow, J.L., 2011. Needed: pragmatic clinical trials for statin-intolerant patients. In: New england journal of medicine, vol. 365. Massachussetts Medical Society. p. 2250—1.

[11] Mampuya, W.M., Frid, D., Rocco, M., Huang, J., Brennan, D.M., Hazen, S.L., et al., 2013 Sep. Treatment strategies in patients with statin intolerance: the Cleveland Clinic experience. American Heart Journal 166(3):597—603.

[12] Cohen, J.D., Cziraky, M.J., Jacobson, T.A., Maki, K.C., Karalis, D.G., 2017 Jul 1. Barriers to PCSK9 inhibitor prescriptions for patients with high cardiovascular risk: results of a healthcare provider survey conducted by the National Lipid Association. J Clin Lipidol 11(4):891—900.

[13] Baum, S.J., Toth, P.P., Underberg, J.A., Jellinger, P., Ross, J., Wilemon, K., 2017 Apr 22. scp>PCSK9</scp> inhibitor access barriers—issues and recommendations: improving the access process for patients, clinicians and payers. Clinical Cardiology 40(4):243—254.

[14] Ridker, P.M., Everett, B.M., Thuren, T., MacFadyen, J.G., Chang, W.H., Ballantyne, C., et al., 2017 Sep 21. Antiinflammatory therapy with canakinumab for atherosclerotic disease. New England Journal of Medicine 377(12):1119—1131.

[15] Steinberg, G.R., Dandapani, M., Hardie, D.G., 2013. AMPK: mediating the metabolic effects of salicylate-based drugs? Trends in Endocrinology and Metabolism 24(10):481—487.

[16] Shoelson, S.E., Lee, J., Goldfine, A.B., 2006. Inflammation and insulin resistance. Journal of Clinical Investigation 116:1793, 801.

[17] Fleischman, A., Shoelson, S.E., Bernier, R., Goldfine, A.B., 2008 Feb. Salsalate improves glycemia and inflammatory parameters in obese young adults. Diabetes Care 31(2):289—294.

[18] Goldfine, A.B., Silver, R., Aldhahi, W., Cai, D., Tatro, E., Lee, J., et al., 2008 May. Use of salsalate to target inflammation in the treatment of insulin resistance and type 2 diabetes. Clinical Transl Science 1(1):36—43.

[19] Goldfine, A.B., 2010. The effects of salsalate on glycemic control in patients with type 2 diabetes. Annals of Internal Medicine 152(6):346.

[20] Barzilay, J.I., Jablonski, K.A., Fonseca, V., Shoelson, S.E., Goldfine, A.B., Strauch, C., et al., 2014. The impact of salsalate treatment on serum levels of advanced glycation end products in type 2 diabetes. Diabetes Care 37(4):1083—1091.

[21] Hauser, T.H., Salastekar, N., Schaefer, E.J., Desai, T., Goldfine, H.L., Fowler, K.M., et al., 2016 Jul 1. Effect of targeting inflammation with salsalate. JAMA Cardiol 1(4):413.

[22] Schwartz, G.G., Steg, P.G., Szarek, M., Bhatt, D.L., Bittner, V.A., Diaz, R., et al., 2018 Nov 29. Alirocumab and cardiovascular outcomes after acute coronary syndrome. New England Journal of Medicine 379(22):2097—2107.

[23] Sabatine, M.S., Giugliano, R.P., Keech, A.C., Honarpour, N., Wiviott, S.D., Murphy, S.A., et al., 2017 May 4. Evolocumab and clinical outcomes in patients with cardiovascular disease. New England Journal of Medicine 376(18):1713—1722.

[24] Kim, J.K., Kim, Y.J., Fillmore, J.J., Chen, Y., Moore, I., Lee, J., et al., 2001. Prevention of fat-induced insulin resistance by salicylate. Journal of Clinical Investigation 108(3):437—446.

[25] Yin, M.J., Yamamoto, Y., Gaynor, R.B., 1998 Nov 5. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. Nature 396(6706):77—80.

[26] Alpert, D., Vilček, J., 2000 Apr 14. Inhibition of IκB kinase activity by sodium salicylate in vitro does not reflect its inhibitory mechanism in intact cells. Journal of Biological Chemistry 275(15):10925—10929.

[27] Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z.W., Karin, M., et al., 2001 Aug 31. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkβ. Science 293(5535):1673—1677, 80-.

[28] Smith, B.K., Ford, R.J., Desjardins, E.M., Green, A.E., Hughes, M.C., Houde, V.P., et al., 2016. Salsalate (salicylate) uncouples mitochondria, improves glucose homeostasis, and reduces liver lipids independent of AMPK-β1. Diabetes 65(11).

[29] Hawley, S.A., Fullerton, M.D., Ross, F.A., Schertzer, J.D., Chevtzoff, C., Walker, K.J., et al., 2012 May. The ancient drug salicylate directly activates AMP-activated protein kinase. Science 336(6083):918—922.

[30] Galic, S., Fullerton, M., Schertzer, J., 2011. Hematopoietic AMPK beta1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. D-J Series 121(12):4903—4915.

[31] Carling, D., Clarke, P.R., Zammit, V.A., Hardie, D.G., 1989 Dec 8. Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. European Journal of Biochemistry 186(1—2):129—136.

[32] Fullerton, M.D., Galic, S., Marcinko, K., Sikkema, S., Pulinilkunnil, T., Chen, Z.-P., et al., 2013 Nov. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nature Medicine 19(12):1649—1654.

[33] Fullerton, M.D., Ford, R.J., McGregor, C.P., LeBlond, N.D., Snider, S.A., Stypa, S.A., et al., 2015 May. Salicylate improves macrophage cholesterol homeostasis via activation of Ampk. The Journal of Lipid Research 56(5):1025—1033.

[34] Dzamko, N., van Denderen, B.J.W., Hevener, A.L., Jørgensen, S.B., Honeyman, J., Galic, S., et al., 2010 Jan. AMPK beta1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. Journal of Biological Chemistry 285(1):115—122.

[35] Pinkosky, S.L., Newton, R.S., Day, E.A., Ford, R.J., Lhotak, S., Austin, R.C., et al., 2016 Nov. Liver-specific ATP-citrate lyase inhibition by bempedoic acid decreases LDL-C and attenuates atherosclerosis. Nature Communications 7:13457.

[36] Loh, K., Tam, S., Murray-Segal, L., Huynh, K., Meikle, P.J., Scott, J.W., et al., 2019. Inhibition of adenosine monophosphate—activated protein kinase—3-hydroxy-3-methylglutaryl coenzyme A reductase signaling leads to hypercholesterolemia and promotes hepatic steatosis and insulin resistance. Hepatol Commun 3(1):84.

[37] Š, Lhoták, Gyulay, G., Cutz, J.-C., Al-Hashimi, A., Trigatti, B.L., Richards, C.D., et al., 2016 Aug 15. Characterization of proliferating lesion-resident cells during all stages of atherosclerotic growth. J Am Heart Assoc 5(8).

[38] Venegas-Pino, D.E., Banko, N., Khan, M.I., Shi, Y., Werstuck, G.H., 2013. Quantitative analysis and characterization of atherosclerotic lesions in the murine aortic sinus. Journal of Visualized Experiments 82:50933.

[39] Š, Lhoták, Zhou, J., Austin, R.C., 2011. Immunohistochemical detection of the unfolded protein response in atherosclerotic plaques. Methods in enzymology. Academic Press Inc.. p. 23—46

[40] O'Brien, A.J., Villani, L.A., Broadfield, L.A., Houde, V.P., Galic, S., Blandino, G., et al., 2015 Jul. Salicylate activates AMPK and synergizes with metformin to reduce the survival of prostate and lung cancer cells ex vivo through inhibition of de novo lipogenesis. Biochemical Journal 469(2):177—187.

[41] Day, E.A., Ford, R.J., Lu, J.H., Lu, R., Lundenberg, L., Desjardins, E.M., et al., 2020 Jun 1. The SGLT2 inhibitor canagliflozin suppresses lipid synthesis and interleukin-1 beta in ApoE deficient mice. Biochemical Journal 477(12):2347—2361.

[42] Esquejo, R.M., Salatto, C.T., Delmore, J., Albuquerque, B., Reyes, A., Shi, Y., et al., 2018 Apr 8. Activation of liver AMPK with PF-06409577 corrects NAFLD and lowers cholesterol in rodent and primate preclinical models. EBioMedicine, 0(0).

[43] Xu, X.M., Sansores-Garcia, L., Chen, X.M., Matijevic-Aleksic, N., Du, M., Wu, K.K., 1999 Apr 27. Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. Proceedings of the National Academy of Sciences of the U S A 96(9):5292—5297.

[44] Liu, Y., Fang, S., Li, X., Feng, J., Du, J., Guo, L., et al., 2017. Aspirin inhibits LPS-induced macrophage activation via the NF-κB pathway. Scientific Reports 7(1):11549.

[45] Zhang, M., Zhu, H., Ding, Y., Liu, Z., Cai, Z., Zou, M.-H., 2017. AMP-activated protein kinase α1 promotes atherogenesis by increasing monocyte-to-macrophage differentiation. Journal of Biological Chemistry 292(19):7888—7903.

[46] Libby, P., 2012. Inflammation in atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology 32(9).

[47] Otero, K., Turnbull, I.R., Poliani, P.L., Vermi, W., Cerutti, E., Aoshi, T., et al., 2009. Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and β-catenin. Nature Immunology 10(7):734—743.

[48] Asakura, E., Tojo, N., Tanabe, T., 1999. Monocyte proliferation induced by modified serum is associated with endogenous M-CSF production: evidence for involvement of a signalling pathway via scavenger receptors. Cell Proliferation 32(4):185—194.

[49] Steinberg, G.R., Carling, D., 2019 Mar 13. AMP-activated protein kinase: the current landscape for drug development. Nature Reviews Drug Discovery, 1.

[50] DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G., Thompson, C.B., 2008. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. In: Cell metabolism, vol. 7. Cell Press. p. 11—20.

[51] Chen, M., Huang, J., 2019 Oct 1. The expanded role of fatty acid metabolism in cancer: new aspects and targets. Precis Clin Med 2(3):183—191.

[52] Howie, D., Bokum, A Ten, Necula, A.S., Cobbold, S.P., Waldmann, H., 2018. The role of lipid metabolism in T lymphocyte differentiation and survival. In: Frontiers in immunology, vol. 8. Frontiers Media S.A.. p. 1949

[53] Kelly, K.L., Reagan, W.J., Sonnenberg, G.E., Clasquin, M., Hales, K., Asano, S., et al., 2020 Oct 1. De novo lipogenesis is essential for platelet production in humans. Nat Metab 2(10):1163—1178.

[54] Ecker, J., Liebisch, G., Englmaier, M., Grandl, M., Robenek, H., Schmitz, G., 2010 Apr 27. Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. Proceedings of the National Academy of Sciences of the U S A 107(17):7817—7822.

[55] Vanderlaan, P.A., Reardon, C.A., Getz, G.S., 2004. Site specificity of atherosclerosis site-selective responses to atherosclerotic modulators.

[56] Seneviratne, A., Cave, L., Hyde, G., Moestrup, S.K., Carling, D., Mason, J.C., et al., 2020 Jun 25. Metformin directly suppresses atherosclerosis in normoglycaemic mice via haematopoietic adenosine monophosphate-activated protein kinase. Cardiovascular Research.

[57] Härdtner, C., Kornemann, J., Krebs, K., Ehlert, C.A., Jander, A., Zou, J., et al., 2020 Dec 1. Inhibition of macrophage proliferation dominates plaque regression in response to cholesterol lowering. Basic Research in Cardiology 115(6):78.

