Anti-Inflammatory Effects of Metformin Irrespective of Diabetes Status

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ABSTRACT

Rationale: The diabetes drug metformin is under investigation in cardiovascular disease but the molecular mechanisms underlying possible benefits are poorly understood.

Objective: Here we have studied anti-inflammatory effects of the drug and their relationship to anti-hyperglycaemic properties.

Methods and Results: In primary hepatocytes from healthy animals, metformin and the IKKβ inhibitor BI605906 both inhibited TNFα-dependent IκB degradation and expression of pro-inflammatory mediators IL-6, IL-1β, and CXCL1/2. Metformin suppressed IKKα/β activation, an effect which could be separated from some metabolic actions, in that BI605906 did not mimic effects of metformin on lipogenic gene expression, glucose production and AMPK activation. Equally AMPK was not required either for mitochondrial suppression of IκB degradation. Consistent with discrete anti-inflammatory actions, in macrophages metformin specifically blunted secretion of pro-inflammatory cytokines, without inhibiting M1/M2 differentiation or activation. In a large treatment naïve diabetes population cohort, we observed differences in the systemic inflammation marker, Neutrophil to Lymphocyte Ratio (NLR), following incident treatment with either metformin or sulfonylurea monotherapy. Compared to sulfonylurea exposure, metformin reduced the mean log-transformed NLR after 8-16 months by 0.09 units (95% CI=0.02-0.17, p=0.013), and increased the likelihood that NLR would be lower than baseline after 8-16 months (OR 1.83, 95% CI=1.22-2.75, p=0.00364). Following up these findings in a double blind placebo controlled trial in nondiabetic heart failure (trial registration: NCT00473876), metformin suppressed plasma cytokines including the ageing-associated cytokine CCL11.

Conclusion: We conclude that anti-inflammatory properties of metformin are exerted irrespective of diabetes status. This may accelerate investigation of drug utility in non-diabetic cardiovascular disease groups.

Name of the trial registry: Metformin in Insulin Resistant Left Ventricular (LV) Dysfunction (TAYSIDE Trial)
Registry's URL: https://clinicaltrials.gov/show/NCT00473876
Trial registration number: NCT00473876

Keywords:
Metformin, NF-κB, inflammation, heart failure, cardiovascular disease, metabolism, diabetes mellitus, pharmacology.

Nonstandard Abbreviations and Acronyms:

18S	18S ribosomal RNA
ACC	acetyl-CoA carboxylase
ACE	angiotensin-converting enzyme
AICAR	5-aminoimidazole-4-carboxamide riboside
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
BMDM	bone marrow derived macrophages
BMI	Body Mass Index
CCL11	C-C motif chemokine ligand 11
CCL22	C-C motif chemokine ligand 22
cDNA	complementary deoxyribonucleic acid
CHF	congestive heart failure
CVD	Cardiovascular Disease
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL2	chemokine (C-X-C motif) ligand 2

DM	Diabetes Mellitus
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FASN	fatty acid synthase
FBS	fetal bovine serum
FIRI	Fasting Insulin Resistance Index
GoDARTs	Genetics of Diabetes Audit and Research in Tayside Scotland
HRP	horseradish peroxidase
hsCRP	high sensitivity C-reactive protein
IFNγ	interferon gamma
IKK	inhibitor of kappa B kinase
IL-1	interleukin-1
IL-10	interleukin-10
IL-12p40	interleukin-12p40
IL-1β	interleukin-1β
IL-2	interleukin-2
IL-6	interleukin-6
IR	Insulin Resistant
KO	knock-out
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Neutrophil to Lymphocyte Ratio
NSAIDs	nonsteroidal anti-inflammatory drug
OR	Odds Ratio
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDI p	ropanediimidamide
PMSF	phenylmethylsulfonyl fluoride
PPARγ	peroxisome proliferator-activated receptor gamma
RNA	ribonucleic acid
SDF1αβ	stromal cell-derived factor 1
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard Error of Mean
SREBP-1c	sterol regulatory element-binding protein 1
T2D	Type 2 Diabetes
TAK1	transforming growth factor beta-activated kinase 1
TBP	TATA-binding protein
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
VE/VCO_2	minute ventilation/carbon dioxide production
VO_2	oxygen consumption

INTRODUCTION

Metformin is the first line drug in type 2 diabetes (T2D) because compared with other T2D treatments, in both clinical trials and in observational studies metformin monotherapy is associated with fewer adverse cardiovascular events (1; 2), and in some studies, a reduced risk of cancer (3). The reasons for this relative benefit are unclear and metformin's molecular action is a vigorous area of current research (4-7). Metformin's chemical properties include a strongly hydrophilic character, metal-binding properties and a pK_a within the physiological pH range (6-8). The key clinical hallmark of metformin's anti-hyperglycaemic action is suppression of hepatocyte gluconeogenesis (4; 5; 9). The most likely cellular effect underlying this response is inhibition of mitochondrial enzymes, including complex I in the electron transport chain (10; 11). More recently mitochondrial glycerophosphate dehydrogenase (mGPD) has been suggested as an alternative target (12). Mitochondrial inhibition activates AMPK (13) and recent work suggests that duodenal AMPK contributes towards effects of the drug on hepatic glucose production (14). Other studies indicate that metformin suppresses glucose production by AMPK-independent mechanisms (12; 15; 16) but more broadly, AMPK may still contribute to metformin-dependent regulation of other aspects of metabolic control, such as lipogenic gene expression (4).

The mechanism(s) underlying metformin's advantage in incidence of cardiovascular disease are unlikely to depend on effects of the drug on hyperglycaemia, which is controlled equally well by metformin and insulin secretagogues (2). In addition, in animals, metformin suppresses infarct size and adverse remodelling in diabetic and non-diabetic rodents (17-21) and retards heart failure progression in non-diabetic dogs (22). A better understanding of such glucose-independent properties might foster a more rational, less empirical exploitation of metformin in nondiabetic cardiovascular disease. Inflammation, including NF-κB signaling, is increasingly recognised as a significant contributing factor to diabetes and cardiovascular disease (23; 24) and several previous studies have found that metformin inhibits NF-κB signaling, including in vascular tissue (25) and recently in hepatocytes (26). In the current study, we have utilised multiple approaches including human studies, to define anti-inflammatory actions of metformin that may be separated from its anti-hyperglycaemic action.

METHODS

Animal and cell studies.

Metformin and rapamycin came from Calbiochem, AICAR and A769662 (Tocris), TNFα (e-bioscience), recombinant CINC1/CXCL1, CCL-11, IL-2, IL-4, SDF and CCL22 (R&D systems), mouse IL-6 (Sigma) and recombinant mouse IL-1β (Life Technologies). The phospho-acetyl-CoA carboxylase (ACC) Ser79 antibody was a generous gift from the DSTT (University of Dundee). The total ACC (Cat. number 3662), total AMPKα (2603), phospho-AMPKα Thr172 (2535), total S6 (2217), phospho-S6 Ser240/244 (2215), total p70 S6 kinase (2708), phospho-p70 S6 kinase Thr389 (9205), phospho-Raptor Ser 792 (2083), phospho IKKα/β Ser176/177 (2078), IKKα/β Ser176/180 (2697), total IκB, pNF-κB, total IKKα and total IKKβ (NF-κB sampler kit 9936) antibodies were from CST. Anti-sheep HRP (31480) and anti-rabbit HRP (31460) both came from Thermo and anti-mouse HRP was from Calbiochem (JA1200). BI605906 was generously gifted by Prof Sir Philip Cohen (Dundee).

Animal care.

C57BL/6 female mice (Charles River, 8-41 weeks) were maintained under a 12 hours:12 hours light:dark cycle (holding room lights on at 06:00; off at 18:00) at 22±1˚C and 50% humidity. Mice had *ad libitum* access to standard chow diet (7.5% fat, 75% carbohydrate and 17.5% protein by energy (RM1 diet; Special Diet Services) and water. All animal care protocols and procedures were performed in accordance with current regulations.

Cell culture and lysis for immunoblotting.
All cells were grown in an incubator at 37ºC and 5% CO_2. Primary mouse hepatocytes were extracted and maintained essentially as described previously (6; 15).

BMDMs were grown from mouse bone marrow in RPMI 1640 medium supplemented with 10% FBS (foetal bovine serum; Life Technologies) and 10ng/ml M-CSF (R&D systems). Cells were given fresh medium and growth factor on day 3 of culture. On day 6, BMDM cultures were supplemented with 100ng/ml IFNγ (for M1 differentiation; R&D systems), 20ng/ml IL-4 (for M2 differentiation; R&D systems), or 100ng/ml LPS (for activation; premium grade from Sigma, expected to activate TLR2 and TLR4) in the presence or absence of drug treatments for the final 24h.

Prior to SDS-PAGE, cells were lysed by scraping into buffer A: (50mM Tris acetate pH7.5, 1% (w/v) Triton X100, 1mM EDTA, 1mM EGTA, 0.27M sucrose, 50mM NaF, 1mM sodium orthovanadate, 10mM β-glycerophosphate, 5mM sodium pyrophosphate, 1mM benzamidine, 0.2mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% (v/v) β-mercaptoethanol) then prepared for SDS-PAGE as described in previous work (6). Immunoblot densitometry for each antibody was performed with Image Studio Lite version 5.2 (LI-COR). Each blot is representative of experiments carried out at least three times.

Glucose assay.
Treatment of cells for hepatocyte glucose production was performed essentially as described previously, using primary mouse hepatocytes plated in 12-well plates (1.25 x 10^5 cells/well) (6; 15; 27). Glucose production was determined after a 12 hour incubation period in glucose-free DMEM (11966; Life Technologies) supplemented with 1% pen/strep, lactate (Sigma)/pyruvate (Life Technologies) (10:1 mM) and 100nM dexamethasone (dex; Merck) with or without drugs/cytokines under investigation. At the end of the incubation period of 12 hours, 500µl of medium was collected and glucose concentration determined by GAGO assay (Sigma) by a modified protocol scaled down to a 96-well plate format. Each column consists of data from at least 12 wells of cells, six each from two mice.

RT-PCR.
Total RNA from primary mouse hepatocytes was extracted using QIAshredder (Qiagen) and Rneasy MINI KIT (Qiagen). cDNA was synthesized using RQ1 Rnase-Free Dnase kit (Promega) and ImProm-II Reverse Transcription System (Promega). Nucleospin RNA II Total RNA isolation kit (Macherey-Nagel) was used to isolate RNA from macrophages. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific). Real-time PCR was carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan 2x Universal PCR Master Mix (Applied Biosystems) and primer/probes mixes as stated (Applied Biosystems). Primer sets used were: IL-6 Mm00446190_ml; CXCL1 Mm04207460_m1; 18S Hs03003631_g1; IL-1β Mm00434228_m1; CXCL2 Mm00436450_m1; PPARγ Mm01184322_m1; FASN Mm00662319_m1; CCL22 Mm00436439_ml; CXCL12 Mm00445553_ml; TBP Mm01277042_m1 and SREBP-1c Mm00550338_m1. Cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression is expressed relative to 18s mRNA for hepatocytes and TBP for macrophages (Applied Biosystems) using the $2^{-\Delta\Delta Ct}$ method. Each column is composed of data from at least three separate experiments.

BMDM analysis.
BMDMs were harvested from culture plates using 4mM EDTA in PBS for 10min at 37°C. Cells were washed in flow cytometry buffer (PBS with 2% FBS and 1mM EDTA) and stained using the following antibodies (all BD Bioscience unless stated): F4/80 (BM8; e-bioscience), CD11c (HL3), CD206 (C068C2; Biolegend), CD69 (H1.2F3) and CD40 (3/23). Fc block (4.4G2) was included in all stains. Data were acquired on a LSR II flow cytometer (Becton Dickinson) and analysed using FlowJo software (TreeStar). BMDM culture supernatants were collected after 24 hours treatment with the differentiation or activation conditions. Levels of cytokines were quantified by standard sandwich ELISA using paired antibody kits (e-bioscience).

Validation in clinical patients.
We validated the animal study findings in clinical patients utilizing 2 approaches: A retrospective population cohort study and a randomized placebo controlled study of metformin. All patients provided written informed consent to participate in these clinical studies that were approved the local ethics committee.

Population cohort study: Diabetes patient Metformin exposure and Neutrophil-to-Lymphocyte Ratio (NLR).
In the population cohort study, we investigated whether or not the anti-inflammatory signature of metformin could be detected in humans with diabetes, using the GoDARTS (Genetics of Diabetes Audit and Research in Tayside Scotland) diabetes register (28). We compared the effect of metformin and sulfonylureas on the **N**eutrophil to **L**ymphocyte **R**atio (NLR), a marker of inflammation derived from a combination of haematological components of the systemic inflammatory response (29; 30) that has recently been found to be a predictor of all-cause mortality and cardiac events (31). We analysed data from T2D patients recruited in Tayside, Scotland, UK, between 1 October 1997 and 1 March 2010. Of the 9,205 subjects with diabetes within the GoDARTS study, we chose 3,575 treatment naïve patients who were either incident metformin users or incident sulfonylurea users (but not both), and non-insulin users. Incident use meant at least 6 months prior to first observed metformin/sulfonylurea prescription date during which they were observable for drugs. Of these 670 patients (mean (SD) age 65 (11) years, 54% males) had derived NLR values both at baseline (up to 120 days prior to first metformin/sulfonylurea prescription) and follow-up (8-16 months after baseline). NLR was calculated as the ratio of the neutrophil to lymphocyte count, both obtained from the same blood sample. 498 (74%) were treated with metformin and 172 (26%) with sulfonylurea. Multivariate linear and logistic regression models were run on the 8-16 month follow-up NLR against the treatment group, controlling for covariates including age, sex, and baseline NLR value.

Randomised placebo controlled study: Metformin exposure and cytokine levels in non-diabetic heart failure patients.
The anti-inflammatory effects of metformin were investigated in a randomly selected subset of patients who had participated in a double-blind, placebo-controlled study (www.clinicaltrials.gov: NCT00473876) that had evaluated the impact of metformin on IR and exercise capacity in non-diabetic patients with CHF (35). Every patient had provided written informed consent prior to participation in this study, which was approved by the East of Scotland Research Ethics Service. The subset of patients selected for this study involved 33 non-diabetic IR CHF patients (mean age, 63 ± 7.0 years; male, 85%; New York Heart Association class I/II/III/IV, 04/28/01/0) who were randomized to receive either 4 months of metformin (n = 20, 2 g/day) or matching placebo (n = 13). IR was defined by a fasting insulin resistance index (FIRI) ≥2.7. The effect of metformin on plasma inflammatory cytokines was examined by investigating changes from baseline to final visit after 4 months in the study.

Cytokine assay.
Human plasma was analysed using the Bio-Plex Pro Human Chemokine 40-Plex Panel (171-AK99MR2, Bio-Rad). The assay was performed following the manufacturer's instructions using the Bio-Plex 200 system (Bio-Rad). Freeze-thaw cycling of samples was avoided to prevent cytokine degradation and they were diluted 1:4 (12.5µl of plasma) for the assay.

Statistical analyses.
Results in bar graphs are expressed as mean ± SEM. Comparisons between groups were made by one-way ANOVA with Dunnett's or Tukey post-hoc test using Prism. Differences were considered statistically significant if *P* was less than 0.05. *** denotes $p<0.001$; ** denotes $p<0.01$ and * denotes $p<0.05$. For studies on the plasma, statistical analyses of data were performed using SPSS 14.1. ANOVA and Pearson correlation coefficients were calculated.

RESULTS

Metformin inhibits TNFα dependent NF-κB inflammatory signaling, comparably with the specific IKKβ inhibitor BI605906.

In primary mouse hepatocytes, the main target of metformin's anti-hyperglycaemic effects, we compared metformin with the specific IKKβ inhibitor BI605906 (32). Metformin treatment for 3 hours suppressed TNFα-induced degradation of the NF-κB negative regulator IκB, whilst modulating AMPK and mTOR signaling in a dose-dependent manner (Fig. 1a-c. All densitometry appears in online supplementary material). The magnitude of the effect on IκB was comparable with BI605906 (Fig. 1a,d). Unlike metformin, BI605906 did not suppress signaling downstream of mTOR, nor did it activate AMPK (Fig. 1e,f). We were unable to detect any effect of rapamycin on NF-κB signaling either (Fig. 1d), suggesting that the effect of metformin on NF-κB and mTOR occur independently. Consistent with these signaling results, TNFα-dependent expression of CINC-1/CXCL1, CXCL2, IL-1β and IL-6 was strongly inhibited by both metformin and BI605906 (Fig. 1g-j).

AMPK-independent regulation of NF−κB in primary hepatocytes.

To determine whether metformin directly regulated kinase activity that may mediate its effects on NF-κB signaling, a cell-free kinase profiling assay was performed. Metformin did not directly inhibit the upstream NF-κB regulator IKKβ and most other kinases exhibited little if any inhibition by metformin and none were inhibited >50% (data available on the profiling website http://www.kinase-screen.mrc.ac.uk/). These results suggest that metformin is unlikely to exert effects on NF-κB through direct IKKβ inhibition, or inhibition of other kinases. The lack of effect of metformin on kinase activity led us to explore the possibility that IκB regulation might occur as a consequence of AMPK activation (13) which occurs following mitochondrial inhibition by the drug (10; 11). In side-by-side experiments, we treated primary hepatocytes with AICAR (an AMP mimetic) and A769662, a direct AMPK activator. Compared with AICAR, which suppressed IκB degradation, there was little if any effect of A769662 on IκB degradation at the doses used (Fig. 2a) but both agents induced phosphorylation of the AMPK substrate acetyl CoA carboxylase (ACC, Fig. 2b). To investigate possible reason(s) for this difference, we investigated primary liver cells from AMPK catalytic subunit deficient mice (15). In these cells, AICAR still suppressed IκB degradation (Fig. 2c), suggesting the AICAR effect is AMPK-independent. Consistent with this, the effect of metformin on IκB signaling was similar in both genotypes (Fig. 2d).

Dissociation of anti-inflammatory responses from effects of metformin on hepatic glucose production and lipogenic gene expression.

Metformin's main anti-hyperglycaemic effect is to reduce hepatic glucose production. To determine whether metformin-regulated cytokines directly altered glucose production we incubated hepatocytes with and without metformin, IL-6, IL-1β, CXCL1 and TNFα. CXCL1 significantly increased glucose production (Fig. 3a). In all groups, metformin reduced glucose production to below control levels (basal) in the presence or absence of cytokine. Incubation of hepatocytes with BI605906 did not mimic the effect of metformin, nor was there any modulation of metformin's suppression of glucose production (Fig. 3b).

Next we compared the effect of metformin and BI605906 on lipogenesis, which is another metabolic response known to be regulated by metformin. Pro-inflammatory cytokines including TNFα are known to induce lipogenesis (33). This prompted us to study the effects of TNFα on lipogenic genes SREBP-1c, PPARγ and FASN, which are known to be regulated by metformin in hepatocytes (13; 34;

35). TNFα significantly increased FASN mRNA expression, with a trend towards increased expression of SREBP-1c and PPARγ (Fig. 3c-e). Metformin reduced mRNA expression of all three genes and prevented TNFα-induced increases. In contrast to the inflammatory genes, co-incubation of BI605906 and TNFα increased lipogenic gene expression (Fig. 3c-e). BI605906 alone did not alter SREBP-1c, FASN or PPARγ, however this compound significantly augmented TNFα-induced expression of each gene. This may be related to the existence of negative feedback loops in NF-κB signaling (32).

Direct anti-inflammatory effect of metformin on macrophage cytokine secretion.

Our evidence that metformin inhibits inflammatory responses in hepatocytes independently of some metabolic actions, prompted us to study inflammatory responses in extrahepatic tissues. Macrophages may undergo 'classical' pro-inflammatory M1 activation in response to cues including LPS and IFNγ. However, in response to agents including IL-13 and IL-4, they may become M2 cells, which are generally thought of as having anti-inflammatory or tissue repair actions (36). We studied the effects of metformin and another drug biguanide (BIG, structurally this drug is the same as metformin except that it lacks the two methyl groups present in metformin), which we have found previously acts similarly to metformin on hepatocytes (6). We investigated three aspects: macrophage differentiation, activation and secretion of cytokines. We measured effects on bone marrow derived macrophage (BMDM) differentiation into M1 and M2 macrophages, using expression of CD11c as a marker of M1 differentiation and CD206 as a measure of M2 differentiation. In addition, we investigated macrophage activation in response to LPS, which acts on the toll-like receptor TLR4, increasing expression of CD69 and CD40. There was no significant effect of the drugs on expression of any of these markers (Fig. 4a,b). As in hepatocytes, metformin suppressed IL-1β gene expression in macrophages (Online Figure IIIa), but somewhat reminiscent of the effect of BI605906 on lipogenic genes, metformin increased expression of the other cytokines we had studied in hepatocytes (Online Figure IIIb-d). We did however observe further drug-induced reductions when we measured cytokine secretion, to investigate macrophage activity and differentiation more directly. The three cytokines we studied were inflammatory cytokines IL-12p40, IL-6 and the anti-inflammatory cytokine IL-10 in these TLR-triggered cells. Both drugs reduced IL-12p40 and IL-6 secretion but were without effect on IL-10 secretion (Fig. 4c-e).

Chronic treatment of hepatocytes with low doses of metformin triggers anti-inflammatory signaling responses similar to those resulting from high-dose acute treatment.

Plasma levels of metformin in the clinical setting are understood to be in the low micromolar range (4; 9). Consequently, metformin-treated individuals may have lower intracellular concentrations of metformin than in our cell experiments but the duration of exposure will be much longer. Discrepancies in effective concentrations of metformin likely occur due to the length of exposure, as the drug must accumulate in active mitochondria over several hours (10; 37). In hepatocytes, long-term (24 hours) effects of the drug on NF-κB signaling occurred at concentrations close to the physiological range and this was unaffected by genotype (Fig. 5a-c).

To provide more insight into the site of metformin action, we investigated signaling further 'upstream' of IKK (Fig. 5a). We found that TNFα-induced phosphorylation of the upstream kinase (TAK1) site p176/177 (38) on IKKα/β was suppressed by metformin. In supporting studies, we found that PDI, a close structural analogue of metformin that we have found does not inhibit the mitochondria (6), does not inhibit IκB degradation, nor does it suppress phosphorylation of IKKα/β (Fig. 5a). Consistent with the notion that NF-κB signaling can respond to mitochondrial inhibition independently of AMPK, we found that the complex I inhibitor rotenone prevented TNFα-dependent IκB degradation in both genotypes (Fig. 5d).

Anti-inflammatory effects of metformin in a diabetes population cohort.

Next we compared the effect of metformin and sulfonylureas on the **N**eutrophil to **L**ymphocyte **R**atio (NLR), a marker of inflammation that has recently been found to be a predictor of all-cause mortality and cardiac events (31). To test the hypothesis that metformin reduces inflammation using the GoDARTS diabetic cohort, we chose individuals prescribed metformin alone (without sulfonylurea or insulin) or sulfonylurea alone (without metformin or insulin), and for whom NLR measurements were available in the 120 days prior to first metformin/sulfonylurea prescription (the baseline measure), and 12 months following the first prescription (within a 8-16 months window). There were 498 people in metformin group and 172 in sulfonylurea group. Baseline characteristics of the two groups are shown in Table 1a. Comparison of the two groups showed a significant effect of metformin exposure compared to sulfonylurea, with 12 month log-transformed NLR 0.09 lower in the metformin group, (95% CI = (0.02, 0.17), p=0.01), controlling for baseline values. This is equivalent to a 9%, (95% CI = (2%-15%)) lower geometric mean NLR. In addition, a logistic regression of 12 month NLR being lower than the baseline NLR gave an odds ratio of 1.83, (95% CI = (1.22, 2.75), p=0.0034) for the metformin group compared to the sulfonylurea group (Tables 1b,d). BMI both nearest baseline and follow up (4% missing) was not a significant variable (p=0.7), so was excluded. Inclusion of baseline HbA1c (19% missing) in the models resulted in similar effects. To examine the effect of metformin on high values on NLR, the models were rerun including only subjects with baseline NLR above the respective group median values (Tables 1 c,e). These showed a stronger metformin effect in the linear model, equivalent to a 15% (95% CI 5%-23%) lower geometric mean NLR, and an unchanged metformin effect for the logistic model, OR=1.91 (1.02-3.59). These results are summarised in Table 1f.

To control for the different characteristics of the metformin and sulfonylurea groups, further analyses following propensity score matching were performed. Nearest-neighbour one-to-one matching on DM duration, BMI, age and ACE exposure at baseline resulted in a reduced matched cohort of 318 (47% of original). Re-fitting the linear model using this cohort showed a similar effect of metformin exposure compared to sulfonylurea, with 12 month log-transformed NLR 0.10 lower in the metformin group (95% CI = (0.01, 0.20), p=0.03). The logistic model for 12 month NLR lower than the baseline NLR gave an odds ratio of 1.53 for the metformin group compared to the sulfonylurea group, however this effect was not statistically significant (95% CI = (0.93, 2.52), p=0.096).

Effect of metformin on inflammation in nondiabetic heart failure.

Given evidence that the anti-inflammatory effects of metformin may be dissociated from some metabolic responses in cells and from glycaemic responses in diabetes, we further investigated the anti-inflammatory effects of metformin in a placebo controlled clinical trial of metformin in a group of non-diabetic insulin resistant heart failure patients (39). In this study, compared with placebo, metformin significantly improved Fasting Insulin Resistance Index (FIRI) and resulted in a significant reduction in weight loss of 1.9 kg and body mass index (BMI). Metformin treatment also reduced the pre-specified secondary endpoint of the slope of the ratio of minute ventilation to carbon dioxide production (VE/VCO_2 slope) (39). We analysed plasma from 33 patients who took part in this study and carried out multivariate analysis of variance on all 40 cytokines with treatment (with and without metformin, 20 allocated to metformin and 13 to placebo) as the main factor and change in BMI as a covariate. This covariate analysis identified five cytokines that were significantly suppressed by metformin, after controlling for change in BMI (Table 2a). Investigating these cytokines further, we carried out Pearson correlations to identify cytokines significantly affected by metformin that correlated with a change in BMI. Amongst the five cytokines, correlations were observed for two out of the five cytokines, CCL22 and CXCL12 (Table 2b). Metformin improved insulin sensitivity as shown by significant reduction in FIRI ($t = 2.765$, $df = 30.762$) $p<0.01$ when an independent sample t-test (equal variances not assumed) is performed; however, there was no significant correlation between change in FIRI and any change in the cytokines in the panel using a Pearson correlation. When a second correction for change in FIRI was applied, in addition to change in BMI, four of the five original cytokines remained significantly different with treatment (Table 2a).

Most of the cytokines suppressed by metformin in plasma were not measurable in hepatocytes or macrophages and for those that could be measured, metformin did not inhibit their expression in these cell types (Online Figure IV). Similar to our earlier studies, these cytokines had little if any effect on inducing glucose production in hepatocytes and metformin could still suppress this parameter in their presence (Fig. 5e). All details of cytokine changes, metabolic, haemodynamic and other parameters of these patients are described in Online Tables I and II.

DISCUSSION

We have used pharmacological and genetic approaches to isolate anti-inflammatory effects of metformin from those on glucose in cells, plasma, patient records and in a placebo controlled study. Initiating the study in hepatocytes, we separated signaling effects of metformin on the metabolic regulator AMPK from effects on inflammatory signaling. Although the AMPK activator AICAR induced similar effects to metformin on IκB degradation, AMPK was not required for these effects. In long-term treatment, effects of metformin on NF-κB signaling occurred at concentrations towards the physiological range and in further studies, we found that PDI, a close structural analogue of metformin that does not inhibit the mitochondria (6), does not inhibit IκB degradation, nor does it increase phosphorylation of IKKα/β as is observed with metformin. Consistent with the possibility that NF-□B signaling can respond to mitochondrial inhibition independently of AMPK, we found that metformin and rotenone each prevented TNFα-dependent IκB degradation in an AMPK-independent manner. Considering information from these pharmacological and genetic experiments, our data indicates that metformin acts upstream of IKKα/β, through an AMPK-independent mechanism dependent on mitochondrial inhibition. This mechanism is fully consistent with our other observations that metformin does not directly inhibit IKK in vitro. These studies do not exclude the possibility of AMPK-dependent mechanisms contributing to anti-inflammatory actions of metformin in other ways. Effects of metformin on anti-inflammatory signaling pathways were separable from other metabolic responses to the drug. Inhibition of NF-κB signaling had little effect for example on glucose production or lipogenic gene expression, two key metabolic actions of metformin. Moreover, addition of cytokines suppressed by metformin either in hepatocytes, plasma or macrophages, did not block the effect of the drug on glucose production. Taken together, these results define a dual action of metformin, with anti-inflammatory effects occurring alongside known anti-hyperglycaemic and other metabolic effects. These two strands are both likely to be triggered by a mitochondrial target of the drug.

The evidence that metformin can suppress inflammatory signaling independently of some of its metabolic effects led us to investigate non-hepatic anti-inflammatory responses. Previous studies have suggested that inflammatory signaling on macrophages influences insulin sensitivity in other tissues. Loss of the LPS receptor TLR4 for example confers some protection from insulin resistance following a high-fat diet (40). In addition, M2 macrophages dominate in adipose tissue in lean mice, whilst M1 macrophages accumulate in adipose tissue during obesity, and are thought to contribute to systemic insulin resistance (41). At the level of gene expression there were some differences between the effect of metformin in hepatocytes and macrophages, although IL-1β was suppressed in both cell types. Studying cytokine secretion from macrophages, we found that metformin acted highly selectively to reduce pro-inflammatory cytokine secretion from activated macrophages, without affecting anti-inflammatory cytokine secretion and markers of macrophage differentiation and activation. This targeted mechanism may allow selective ablation of the ability of M1 macrophages to induce systemic insulin resistance in obesity. Taken together with the results in hepatocytes, this work suggests that metformin's anti-inflammatory actions are likely to be qualitatively different from conventional non-steroidal anti-inflammatory drugs (NSAIDs).

We wished to establish whether the effects of metformin could be detected in humans and we started with a diabetes cohort. Investigating the GoDARTS patient database, we found evidence of metformin reducing subclinical inflammation as measured by NLR in patients. It is noteworthy that our findings support previous reports that metformin is capable of suppressing markers of inflammation such as hsCRP in pre-diabetic individuals (42) and TNFα in insulin resistant individuals (43). NLR has recently been identified as a predictor of all-cause mortality and cardiovascular events (31) while previous studies demonstrated a substantial beneficial effect of metformin therapy on cardiovascular outcomes (2; 44; 45). Together, these results suggest that suppression of chronic inflammation by metformin might contribute to the difference in outcomes between these two treatment modalities.

Finally, given the evidence from cells that anti-inflammatory and metabolic effects of the drug can be separated, we studied a non-diabetic insulin-resistant heart failure cohort from a randomised controlled trial. Our research question was to determine whether or not metformin suppressed plasma cytokines. We observed a general trend of metformin treatment lowering cytokine concentrations. Correcting for change in BMI, five cytokines were significantly suppressed by metformin but only two of these, CCL22 and SDF 1αβ also correlated with change in BMI in follow-up analysis, suggesting that in individuals with established CVD, metformin exerts anti-inflammatory effects that are at least in part independent of BMI. Four of the five cytokines remained significant after additional correction for FIRI and there was no significant correlation between change in FIRI and any of the cytokines in the panel using a Pearson correlation, even though metformin did reduce FIRI. Together these data strongly suggest that metformin has effects above and beyond the known effects on BMI and insulin sensitivity. The identity of these five cytokines signpost ways in which anti-inflammatory effects of metformin could exert diabetes-independent therapeutic effects in CVD. One earlier cohort study for example found that a Thr/Ala substitution in the CCL11 gene increases risk of myocardial infarction independently of BMI and diabetes (46). Blockade of CCL11 can suppress aspects of age-related cellular dysfunction (47) and it is possible that observed effects of metformin on mammalian longevity (48; 49), where suppression of NF-κB is also observed (49), may owe at least in part to suppression of this cytokine. The other cytokines SDF 1αβ, IL-2 and IL-4 and CCL22 are each implicated in resolution of pancreatic beta cell inflammation (50-53) and SDF 1αβ, IL-2 and IL-4 are additionally upregulated in plasma from T2D individuals (54; 55). Further work will be required to determine how the effects on macrophages and hepatocytes that we have measured contribute to the changes in plasma cytokines observed. Changes in other inflammatory cell types, particularly neutrophils given the change in NLR, or in cell-cell interactions, may need to be taken into account. Altogether our results are consistent with metformin exerting a potentially cardioprotective anti-inflammatory effect in CVD patients, suppressing both age and metabolic inflammatory stress markers, independently of effects on BMI, insulin sensitivity and without the onset of frank diabetes.

We recognize the limitations that are inherent in retrospective, non-randomized, observational cohort data. It was impossible to account for all possible confounding influences that may have biased the observed differences between the groups considered. For example, the BMI of the two groups is different, consistent with historical prescribing patterns (Table 2). We have sought to minimize these as far as practicable by three different sensitivity analyses. First, by using a multivariate model adjusting for potential confounders; second, by performing a propensity score-matched analysis and thirdly, we detected an anti-inflammatory signal in a randomized double blinded placebo-controlled trial, providing definitive evidence of anti-inflammatory effects of metformin in this group of patients. The propensity score–matched analysis has been shown to eliminate as much as 90% of treatment bias in observational studies (56). Due to the small size of the clinical trial, this proof of concept study was designed and powered only to investigate the study-specific end point of peak oxygen uptake (VO_2) in patients with heart failure and not on clinical outcome. However, we have previously shown in a large population-based cohort study that patients with diabetes and heart failure who were treated with metformin alone or in combination with sulfonylureas were at significantly lower risk of all-cause mortality during 1 year and long-term follow-up than those who were treated with sulfonylurea alone (44). Our findings on metformin and inflammation will now similarly need to be confirmed in other patient cohorts.

In summary, cross-species evidence from cells, plasma, patient records and a randomized placebo controlled study strongly suggest that anti-inflammatory effects should be investigated further as a potentially important aspect of metformin's clinical pharmacology, that may particularly accelerate investigation of their utility in non-diabetic cohorts. There is overwhelming evidence that inflammation contributes to the development of cardiovascular disease (24) but counterbalancing this is evidence from meta-analysis of randomised control trials that existing NSAIDs tend to exacerbate risk of cardiovascular disease (57). If inflammation is to be targeted successfully in cardiovascular disease, new treatment paradigms will need to be established. It is likely for example that agents targeting only selected aspects of inflammation will need to be identified. Our work identifying discrete anti-inflammatory effects of metformin on cell signaling and plasma parameters independently of diabetes supports ongoing and prospective investigation into repurposing metformin in a broader spectrum of patients with cardiovascular disease.

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DISCLOSURES
No potential conflicts of interest relevant to this article were reported.

REFERENCES
1. Evans JMM, Ogston SA, Emslie-Smith A, Morris AD: Risk of mortality and adverse cardiovascular outcomes in type 2 diabetes: a comparison of patients treated with sulfonylureas and metformin. *Diabetologia* 2006;49:930-936
2. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group, *The Lancet* 1998;352:854-865
3. Evans JMM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD: Metformin and reduced risk of cancer in diabetic patients. *Brit Med J* 2005;330:1304-1305
4. Rena G, Pearson ER, Sakamoto K: Molecular Mechanism of Action of Metformin: Old or New Insights? *Diabetologia* 2013;56:1898-1906
5. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F: Cellular and molecular mechanisms of metformin: an overview. *Clin Sci* 2012;122:253-270
6. Logie L, Harthill J, Patel K, Bacon S, McDougall G, Hamilton DL, Macrae K, Wang H-H, Xue L, Jiang H, Sakamoto K, Prescott AR, Rena G: Cellular responses to the metal-binding properties of metformin. *Diabetes* 2012;61:1423-1433
7. Repiščák P, Erhardt S, Rena G, Paterson MJ: Biomolecular Mode of Action of Metformin in Relation to Its Copper Binding Properties. *Biochemistry* 2014;53:787-795

8. Quan X, Uddin R, Heiskanen A, Parmvi M, Nilson K, Donolato M, Hansen MF, Rena G, Boisen A: The copper binding properties of metformin - QCM-D, XPS and nanobead agglomeration. *Chemical Communications* 2015;51: 17313-17316
9. Rena G, Pearson ER, Sakamoto K: Molecular action and pharmacogenetics of metformin: current understanding of an old drug. *Diabetes Management* 2012;2:439-452
10. Owen MR, Doran E, Halestrap AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J* 2000;348:607-614
11. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X: Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* 2000;275:223 - 228
12. Madiraju AK, Erion DM, Rahimi Y, Zhang X-M, Braddock DT, Albright RA, Prigaro BJ, Wood JL, Bhanot S, MacDonald MJ, Jurczak MJ, Camporez J-P, Lee H-Y, Cline GW, Samuel VT, Kibbey RG, Shulman GI: Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* 2014;510:542-546
13. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyl-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 2001;108:1167-1174
14. Duca FA, Cote CD, Rasmussen BA, Zadeh-Tahmasebi M, Rutter GA, Filippi BM, Lam TK: Metformin activates a duodenal Ampk-dependent pathway to lower hepatic glucose production in rats. *Nat Med* 2015;21:506-511
15. Foretz M, Hébrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, Sakamoto K, Andreelli F, Viollet B: Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest* 2010;120:2355-2369
16. Miller RA, Chu Q, Xie J, Foretz M, Viollet B, Birnbaum MJ: Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* 2013;494:256-260
17. Calvert JW, Gundewar S, Jha S, Greer JJM, Bestermann WH, Tian R, Lefer DJ: Acute Metformin Therapy Confers Cardioprotection Against Myocardial Infarction Via AMPK-eNOS–Mediated Signaling. *Diabetes* 2008;57:696-705
18. Gundewar S, Calvert JW, Jha S, Toedt-Pingel I, Ji SY, Nunez D, Ramachandran A, Anaya-Cisneros M, Tian R, Lefer DJ: Activation of AMP-activated protein kinase by metformin improves left ventricular function and survival in heart failure. *Circ Res* 2009;104:403-411
19. Yin M, van der Horst IC, van Melle JP, Qian C, van Gilst WH, Sillje HH, de Boer RA: Metformin improves cardiac function in a nondiabetic rat model of post-MI heart failure. *Am J Physiol Heart Circ Physiol* 2011;301:H459-468
20. Xu X, Lu Z, Fassett J, Zhang P, Hu X, Liu X, Kwak D, Li J, Zhu G, Tao Y, Hou M, Wang H, Guo H, Viollet B, McFalls EO, Bache RJ, Chen Y: Metformin Protects Against Systolic Overload–Induced Heart Failure Independent of AMP-Activated Protein Kinase α2. *Hypertension* 2014;63:723-728
21. Cittadini A, Napoli R, Monti MG, Rea D, Longobardi S, Netti PA, Walser M, Samà M, Aimaretti G, Isgaard J, Saccà L: Metformin Prevents the Development of Chronic Heart Failure in the SHHF Rat Model. *Diabetes* 2012;61:944-953
22. Sasaki H, Asanuma H, Fujita M, Takahama H, Wakeno M, Ito S, Ogai A, Asakura M, Kim J, Minamino T, Takashima S, Sanada S, Sugimachi M, Komamura K, Mochizuki N, Kitakaze M: Metformin Prevents Progression of Heart Failure in Dogs: Role of AMP-Activated Protein Kinase. *Circulation* 2009;119:2568-2577
23. Hotamisligil GS: Inflammation and metabolic disorders. *Nature* 2006;444:860-867
24. Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO, Criqui M, Fadl YY, Fortmann SP, Hong Y, Myers GL, Rifai N, Smith SC, Taubert K, Tracy RP, Vinicor F: Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice: A Statement for Healthcare Professionals From the Centers for Disease Control and Prevention and the American Heart Association. *Circulation* 2003;107:499-511
25. Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, Schönbeck U, Libby P: Metformin Inhibits Proinflammatory Responses and Nuclear Factor-κB in Human Vascular Wall Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2006;26:611-617

26. Woo SL, Xu H, Li H, Zhao Y, Hu X, Zhao J, Guo X, Guo T, Botchlett R, Qi T, Pei Y, Zheng J, Xu Y, An X, Chen L, Chen L, Li Q, Xiao X, Huo Y, Wu C: Metformin ameliorates hepatic steatosis and inflammation without altering adipose phenotype in diet-induced obesity. *PLoS One* 2014;9:e91111
27. Patel K, Foretz M, Marion A, Campbell DG, Gourlay R, Boudaba N, Tournier E, Titchenell P, Peggie M, Deak M, Wan M, Kaestner KH, Goransson O, Viollet B, Gray NS, Birnbaum MJ, Sutherland C, Sakamoto K: The LKB1-salt-inducible kinase pathway functions as a key gluconeogenic suppressor in the liver. *Nature Commun* 2014;5:4535
28. Morris AD, Boyle DI, MacAlpine R, Emslie-Smith A, Jung RT, Newton RW, MacDonald TM: The diabetes audit and research in Tayside Scotland (DARTS) study: electronic record linkage to create a diabetes register. DARTS/MEMO Collaboration. *BMJ* 1997;315:524-528
29. Zahorec R: Ratio of neutrophil to lymphocyte counts: rapid and simple parameter of systemic inflammation and stress in critically ill. *Bratisl Lek Listy* 2001;102:5-14
30. Pinato DJ, Stavraka C, Flynn MJ, Forster MD, O'Cathail SM, Seckl MJ, Kristeleit RS, Olmos D, Turnbull SJ, Blagden SP: An Inflammation Based Score Can Optimize the Selection of Patients with Advanced Cancer Considered for Early Phase Clinical Trials. *PLoS ONE* 2014;9:e83279
31. Wang X, Zhang G, Jiang X, Zhu H, Lu Z, Xu L: Neutrophil to lymphocyte ratio in relation to risk of all-cause mortality and cardiovascular events among patients undergoing angiography or cardiac revascularization: A meta-analysis of observational studies. *Atherosclerosis* 2014;234:206-213
32. Clark K, Peggie M, Plater L, Sorcek RJ, Young ERR, Madwed JB, Hough J, McIver EG, Cohen P: Novel cross-talk within the IKK family controls innate immunity. *Biochem J* 2011;434:93-104
33. Grunfeld C, Dinarello CA, Feingold KR: Tumor necrosis factor-alpha, interleukin-1, and interferon alpha stimulate triglyceride synthesis in HepG2 cells. *Metabolism* 1991;40:894-898
34. Sozio MS, Lu C, Zeng Y, Liangpunsakul S, Crabb DW: Activated AMPK inhibits PPAR-alpha and PPAR-gamma transcriptional activity in hepatoma cells. *Am J Physiol Gastrointest Liver Physiol* 2011;301:G739-747
35. Bhalla K, Hwang BJ, Dewi RE, Twaddel W, Goloubeva OG, Wong K-K, Saxena NK, Biswal S, Girnun GD: Metformin Prevents Liver Tumorigenesis by Inhibiting Pathways Driving Hepatic Lipogenesis. *Cancer Prev Res* 2012;5:544-552
36. Martinez FO, Sica A, Mantovani A, Locati M: Macrophage activation and polarization. *Front Biosci* 2008;13:453-461
37. Bridges HR, Jones AJY, Pollak MN, Hirst J: Effect of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochem J* 2014;462:475-487
38. Zhang J, Clark K, Lawrence T, Peggie MW, Cohen P: An unexpected twist to the activation of IKKβ: TAK1 primes IKKβ for activation by autophosphorylation. *Biochem J* 2014;461:531-537
39. Wong AKF, Symon R, Alzadjali MA, Ang DSC, Ogston S, Choy AM, Petrie JR, Struthers AD, Lang CC, Wong AKF, Symon R, Alzadjali MA, Ang DSC, Ogston S, Choy AM, Petrie JR, Struthers AD, Lang CC: The effect of metformin on insulin resistance and exercise parameters in patients with heart failure. *European Journal of Heart Failure* 2012;14:1303-1310
40. Poggi M, Bastelica D, Gual P, Iglesias MA, Gremeaux T, Knauf C, Peiretti F, Verdier M, Juhan-Vague I, Tanti JF, Burcelin R, Alessi MC: C3H/HeJ mice carrying a toll-like receptor 4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet. *Diabetologia* 2007;50:1267-1276
41. Lumeng CN, Bodzin JL, Saltiel AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007;117:175-184
42. Goldberg RB, Temprosa MG, Mather KJ, Orchard TJ, Kitabchi AE, Watson KE: Lifestyle and metformin interventions have a durable effect to lower CRP and tPA levels in the diabetes prevention program except in those who develop diabetes. *Diabetes Care* 2014;37:2253-2260
43. Evia-Viscarra ML, Rodea-Montero ER, Apolinar-Jimenez E, Munoz-Noriega N, Garcia-Morales LM, Leanos-Perez C, Figueroa-Barron M, Sanchez-Fierros D, Reyes-Garcia JG: The effects of metformin on inflammatory mediators in obese adolescents with insulin resistance: controlled randomized clinical trial. *J Pediatr Endocrinol Metab* 2012;25:41-49
44. Evans JMM, Doney ASF, AlZadjali MA, Ogston SA, Petrie JR, Morris AD, Struthers AD, Wong AKF, Lang CC: Effect of Metformin on Mortality in Patients With Heart Failure and Type 2 Diabetes Mellitus. *Am J Cardiol* 2010;106:1006-1010

45. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HAW: 10-Year Follow-up of Intensive Glucose Control in Type 2 Diabetes. *New England Journal of Medicine* 2008;359:1577-1589
46. Zee RYL, Cook NR, Cheng S, Erlich HA, Lindpaintner K, Lee RT, Ridker PM: Threonine for alanine substitution in the eotaxin (CCL11) gene and the risk of incident myocardial infarction. *Atherosclerosis* 175:91-94
47. Villeda SA, Luo J, Mosher KI, Zou B, Britschgi M, Bieri G, Stan TM, Fainberg N, Ding Z, Eggel A, Lucin KM, Czirr E, Park J-S, Couillard-Després S, Aigner L, Li G, Peskind ER, Kaye JA, Quinn JF, Galasko DR, Xie XS, Rando TA, Wyss-Coray T: The aging systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* 2011;477:90-94
48. Bannister CA, Holden SE, Jenkins-Jones S, Morgan CL, Halcox JP, Schernthaner G, Mukherjee J, Currie CJ: Can people with type 2 diabetes live longer than those without? A comparison of mortality in people initiated with metformin or sulphonylurea monotherapy and matched, non-diabetic controls. *Diabetes, Obesity & Metabolism* 2014;16:1165-1173
49. Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, Gomes AP, Ward TM, Minor RK, Blouin M-J, Schwab M, Pollak M, Zhang Y, Yu Y, Becker KG, Bohr VA, Ingram DK, Sinclair DA, Wolf NS, Spindler SR, Bernier M, de Cabo R: Metformin improves healthspan and lifespan in mice. *Nature Communications* 2013;4:2192-2192
50. Hulme MA, Wasserfall CH, Atkinson MA, Brusko TM: Central Role for Interleukin-2 in Type 1 Diabetes. *Diabetes* 2012;61:14-22
51. Bischoff L, Alvarez S, Dai DL, Soukhatcheva G, Orban PC, Verchere CB: Cellular mechanisms of CCL22-mediated attenuation of autoimmune diabetes. *J. Immunol.* 2015;194:3054-3064
52. Cameron MJ, Arreaza GA, Zucker P, Chensue SW, Strieter RM, Chakrabarti S, Delovitch TL: IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function. *J Immunol.* 1997;159:4686-4692
53. Yano T, Liu Z, Donovan J, Thomas MK, Habener JF: Stromal Cell–Derived Factor-1 (SDF-1)/CXCL12 Attenuates Diabetes in Mice and Promotes Pancreatic β-Cell Survival by Activation of the Prosurvival Kinase Akt. *Diabetes* 2007;56:2946-2957
54. Ip B, Cilfone N, Zhu M, Kuchibhatla R, Azer M, McDonnell M, Apovian C, Lauffenburger D, Nikolajczyk B: An inflammatory T cell signature predicts obesity-associated type 2 diabetes (HUM3P.262). *J. Immunol.* 2015;194:121.122
55. Derakhshan R, Arababadi MK, Ahmadi Z, Karimabad MN, Salehabadi VA, Abedinzadeh M, Khorramdelazad H, Balaei P, Kennedy D, Hassanshahi G: Increased circulating levels of SDF-1 (CXCL12) in type 2 diabetic patients are correlated to disease state but are unrelated to polymorphism of the SDF-1beta gene in the Iranian population. *Inflammation* 2012;35:900-904
56. D'Agostino RB, Jr.: Propensity score methods for bias reduction in the comparison of a treatment to a non-randomized control group. *Statistics in medicine* 1998;17:2265-2281
57. Trelle S, Reichenbach S, Wandel S, Hildebrand P, Tschannen B, Villiger PM, Egger M, Jüni P: Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ* 2011;342

FIGURE LEGENDS

Figure 1. Effect of metformin on NF-κB signaling and gene expression.
(a-c) Primary hepatocytes were incubated in serum-free medium overnight and then stimulated for 3h with or without 0.5-2mM metformin. For the last 15 min cells were treated with or without 10ng/ml TNFα. Cells were lysed and prepared for immunoblotting using antibodies as described in the methods. In this figure and elsewhere, each blot is representative of experiments carried out at least three times. *(d-f)* Primary hepatocytes were incubated as in (a-c), prior to stimulation for 3h with or without 2mM metformin and TNFα. In addition, cells were incubated with/without 10μM BI605906 or 100nM rapamycin as shown, prior to lysis and immunoblotting as described in the methods. *(g-j)* Primary hepatocytes were treated with or without 10ng/ml TNFα, 2mM metformin or 10μM BI605906 for 8h followed by cell lysis, RNA extraction and preparation of cDNA for RTPCR using primer sets for individual genes shown as described in the methods.

Figure 2. Effect of AICAR and A769662 on NF-κB signalling.
(a-d) Primary wild type hepatocytes (a,b) and those taken from double-knockout (KO) AMPK animals or matched controls (WT) (c), were incubated in serum-free medium overnight, prior to stimulation for 3h with or without doses of A769662 and AICAR as shown. For the last 15 minutes cells were treated with or without 10ng/ml TNFα. *(d)* Hepatocytes from KO or WT animals treated with and without doses of metformin for 3h. For the last 15 minutes cells were treated with 10ng/ml TNFα. Cells were then lysed and immunoblots prepared as described in the methods and Fig. 1.

Figure 3. Effects of cytokines on glucose production and lipogenic gene expression in primary hepatocytes. *(a, b)* Primary hepatocytes were treated with/without metformin (2mM), IL-6 (5ng/ml), IL-1β (10ng/ml), CXCL1 (100ng/ml) and TNFα (10ng/ml) for 12h and glucose production was measured by GAGO assay as described in the Methods. *(c-e)* Primary hepatocytes were treated with or without 10ng/ml TNFα, 2mM metformin and 10μM BI605906 for 8h followed by cell lysis, RNA extraction and preparation of cDNA for RTPCR using primer sets for individual genes shown as described in the methods.

Figure 4. Effect of metformin and its analogue biguanide on bone marrow-derived macrophages: phenotypic markers and cytokine secretion.
(a) Macrophages were treated with/without metformin (2mM) or biguanide (BIG 2mM) to determine the effect on the M1 and M2 phenotypes of macrophages, which was measured by flow cytometry for CD11c and CD206 expression. The colours denote the following: Red – undifferentiated; Blue – differentiated, untreated; Orange – differentiated, Metformin; Green – differentiated, BIG. *(b)* Macrophages were treated with/without metformin (2mM) or biguanide (BIG, 2mM) to determine the effect on activation in response to 100ng/ml LPS, which was measured by studying CD69 and CD40 expression. Histograms are representative of N=4. The colours denote the following: Red – unactivated; Blue – activated, untreated; Orange – activated, Metformin; Green – activated, BIG. *(c-e)* Macrophages were treated with/without metformin (Met) or biguanide (BIG, 2mM) to determine the effect of these drugs on IL-6 *(c)*, IL-12p40 *(d)* and IL-10 *(e)* production. N=4.

Figure 5. Effect of long-term metformin treatment on NF-κB signaling responses in hepatocytes
(a,b) Primary hepatocytes were treated as in Fig. 1 with metformin or PDI at the doses indicated except that the treatment time was 24h. For the last 15 minutes cells were treated with 10ng/ml TNFα. In addition to antibodies used elsewhere, phosphorylation of IKKα/β was investigated using the phosphospecific antibodies indicated. After cell lysis, SDS-PAGE and immunoblotting was carried out as in Fig. 1. *(c,d)* Hepatocytes from wild-type (WT) and AMPK double knockout (KO) livers treated as in (a) or with doses of rotenone for 45 min prior to cell lysis, SDS-PAGE and immunoblotting. *(e)* Primary hepatocytes were treated in the presence or absence of the agents shown. Cells were treated with/without metformin (2mM), CCL-11 (5ng/ml), IL-2, IL-4, SDF and CCL22 (10ng/ml) for 12h and glucose production was measured by GAGO assay as described in the Methods.

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Novelty and Significance

What Is Known?

- Observational studies have repeatedly shown benefit of metformin in reducing incidence of cardiovascular disease (CVD) events in diabetes.

- Inflammation is understood to contribute to CVD aetiology but it has been difficult to harness anti-inflammatory effects for CVD therapy

What New Information Does This Article Contribute?

- Metformin exhibits an anti-inflammatory action in cells and patients, in addition to its known anti-hyperglycaemic effects.

- Anti-inflammatory effects of metformin are exerted irrespective of diabetes status, providing a non-empirical rationale for further testing of the drug in non-diabetic CVD

Inflammation is understood to contribute to cardiovascular disease (CVD) aetiology but existing NSAIDs have shown limited utility in CVD treatment. This suggests that other agents, with different anti-inflammatory mechanisms need to be identified for CVD. Observational studies have repeatedly shown benefit of metformin in reducing incidence of CVD events in diabetes, which do not seem to depend on anti-hyperglycaemic effects alone. In this study, we investigated anti-inflammatory effects of metformin, as these may contribute to the CVD benefit of this drug. We find that this drug acts by inhibiting the NF-κB signaling pathway upstream of IKKβ. In further work we find that anti-inflammatory effects of metformin are exerted irrespective of diabetes status, including suppression of the ageing-related cytokine CCL11 in a non-diabetic heart failure cohort. These results suggest that metformin suppresses chronic inflammation by a different mechanism to NSAIDs and provide a non-empirical rationale for further testing of the drug in non-diabetic CVD.

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Table 1a.
Baseline measurements of GoDARTs diabetes cohort

Variable	Metformin (N=498)	Sulfonylurea (N=172)	p-value
Age (years) (1)	65 (57-72)	69 (61-76)	0.042
Sex (% of males)	55	51.2	0.432
Diabetes duration (years) (1)	2.1 (0.2-5)	0.95 (0.1-4.2)	0.00193
Body mass index (kg/m^2) (1)	32 (29-35.98)	27.2 (24.4-31.05)	2.26E-14
HbA1c (%) (1)	8.2 (7.6-9.1)	8.4 (7.5-9.6)	0.471
Neutrophil to Lymphocyte Ratio (1)	1.94 (1.5-2.62)	2.56 (1.868-3.89)	2.47E-06
Neutrophils (x10^9/L) (1)	4.2 (3.3-5.5)	4.8 (3.775-6.225)	0.00103
Lymphocyes (x10^9/L) (1)	2.1 (1.7-2.8)	1.8 (1.4-2.3)	1.19E-05
Platelets (x10^9/L) (1)	236 (199-280)	251.5 (198-305.5)	0.133
C- reactive protein (mg/L) (1)	10 (5-16.75)	10 (4.1-20)	0.854
Creatinine (umol/L)	86 (75-98)	94.5 (78-120.8)	0.000172
Bilirubin (umol/L) (1)	9 (7-13)	9 (7-11.75)	0.069
Albumin (g/L) (1)	44 (42-45)	41 (38-43)	1.35E-10
Urea (mmol/L) (1)	5.8 (4.8-6.8)	6.6 (5.075-9.325)	0.000644
Medications			
Angiotension-converting enzyme inhibitors, n (%)	41	23.8	8.53E-05
Angiotensin receptor blockers, n (%)	11.8	7	0.0999
Beta-blocker, n (%)	32.1	28.5	0.428
Digoxin, n (%)	4.4	9.9	0.0143
Anticoagulants, n (%)	6	10.5	0.0758
Calcium channel blocker, n (%)	30.5	24.4	0.154
Antiplatelets, n (%)	37.3	31.4	0.19

(1) Median (Inter-quartile Range);
GoDARTS : Genetics of Diabetes Audit and Research in Tayside Scotland.

Table 1b. Regression coefficients of linear model (log-NLR)

	Estimate	Std. Error	95% CI	p-value
Sex=M	0.054775	0.031656	(-0.00727,0.117)	0.084038
Age	-0.035556	0.012157	(-0.0594,-0.0117)	0.003567
Age^2	0.000281	0.000095	(9.51e-05,0.000467)	0.003163
log(NLR) at baseline	0.498068	0.030933	(0.437,0.559)	$< 10^{-5}$
Group=Metformin	-0.093859	0.037702	(-0.168,-0.0200)	0.013037

Table 1c. Regression coefficients of linear model – Baseline NLR > Median

	Estimate	Std. Error	95% CI	p-value
Sex=M	0.1202	0.0427	(0.0364,0.204)	0.0052
Age	-0.0268	0.0149	(-0.0561,0.00241)	0.0730
Age^2	0.0002	0.0001	(-1.037e-05,0.000444)	0.0623
log(NLR) at baseline	0.2758	0.0559	(0.166,0.385)	$< 10^{-5}$
Group=Metformin	-0.1596	0.0551	(-0.268,-0.0517)	0.0040

Table 1d. Regression coefficients of logistic model (NLR_12 < NLR_0)

	Estimate	Std. Error	95% CI	p-value
Sex=M	-0.2280	0.1703	(-0.562,0.106)	0.1804
Age	0.1450	0.0669	(0.0139,0.276)	0.0302
Age^2	-0.0012	0.0005	(-0.00218,-0.000133)	0.0267
log(NLR) at baseline	1.9456	0.2128	(1.528,2.363)	$< 10^{-5}$
Group=Metformin	0.6054	0.2078	(0.198,1.013)	0.0036

Table 1e. Regression coefficients of logistic model - Baseline NLR > Median

	Estimate	Std. Error	95% CI	p-value
Sex=M	-0.4841	0.2419	(-0.958,-0.00992)	0.0454
Age	-0.0090	0.0104	(-0.0294,0.0114)	0.3869
log(NLR) at baseline	2.2827	0.4423	(1.416,3.15)	$< 10^{-5}$
Group=Metformin	0.6478	0.3219	(0.0168,1.279)	0.0442

Table 1f. Summary of GoDARTS analyses, comparing NLR in metformin and sulfonylurea groups

Group analysed	**12 month geometric mean NLR** **metformin versus sulfonylurea % difference (95% CI)**	**12 month NLR < 0 month NLR** **O.R., metformin versus sulfonylurea (95% CI)**
All subjects	-9% (2-15)	1.83 (1.22-2.75)
NLR above group median	-15% (5-23)	1.91 (1.02-3.59)

Table 2a ANOVA of heart failure cohort with treatment (without or with metformin) as main factor with covariate analysis.
Significance is taken as * $p < 0.01$. (n=27).

	Covariate	
Change in cytokine	**ΔBMI**	**ΔBMI & ΔFIRI**
Eotaxin/CCL11	$F(1,26) = 9.881$; $p = 0.004$*	$F(1,26) = 9.135$; $p = 0.006$*
IL-2	$F(1,26) = 9.089$; $p = 0.006$*	$F(1,26) = 8.078$; $p = 0.009$*
IL-4	$F(1,26) = 8.324$;$p = 0.008$*	$F(1,26) = 7.148$; $p = 0.014$
MDC/CCL22	$F(1,26) = 9.887$; $p = 0.004$*	$F(1,26) = 9.846$; $p = 0.005$*
SDF1αβ/CXCL12	$F(1,26) = 16.468$; $p = 0.000$*	$F(1,26) = 14.661$; $p = 0.001$*

Table 2b Correlation between change in BMI and the cytokines that were significantly affected by metformin treatment.
A Pearson correlation was used. Significance is only noted with drug treatment for those listed ($p < 0.01$).

	With metformin (n=20)		Without metformin (n=13)	
Change in cytokine	**Correlation coefficient**	**p-value**	**Correlation coefficient**	**p-value**
MDC/CCL22	-0.625	0.003*	0.077	0.804 ns
SDF1αβ/CXCL12	-0.620	0.004*	-0.103	0.738 ns

Figure 1

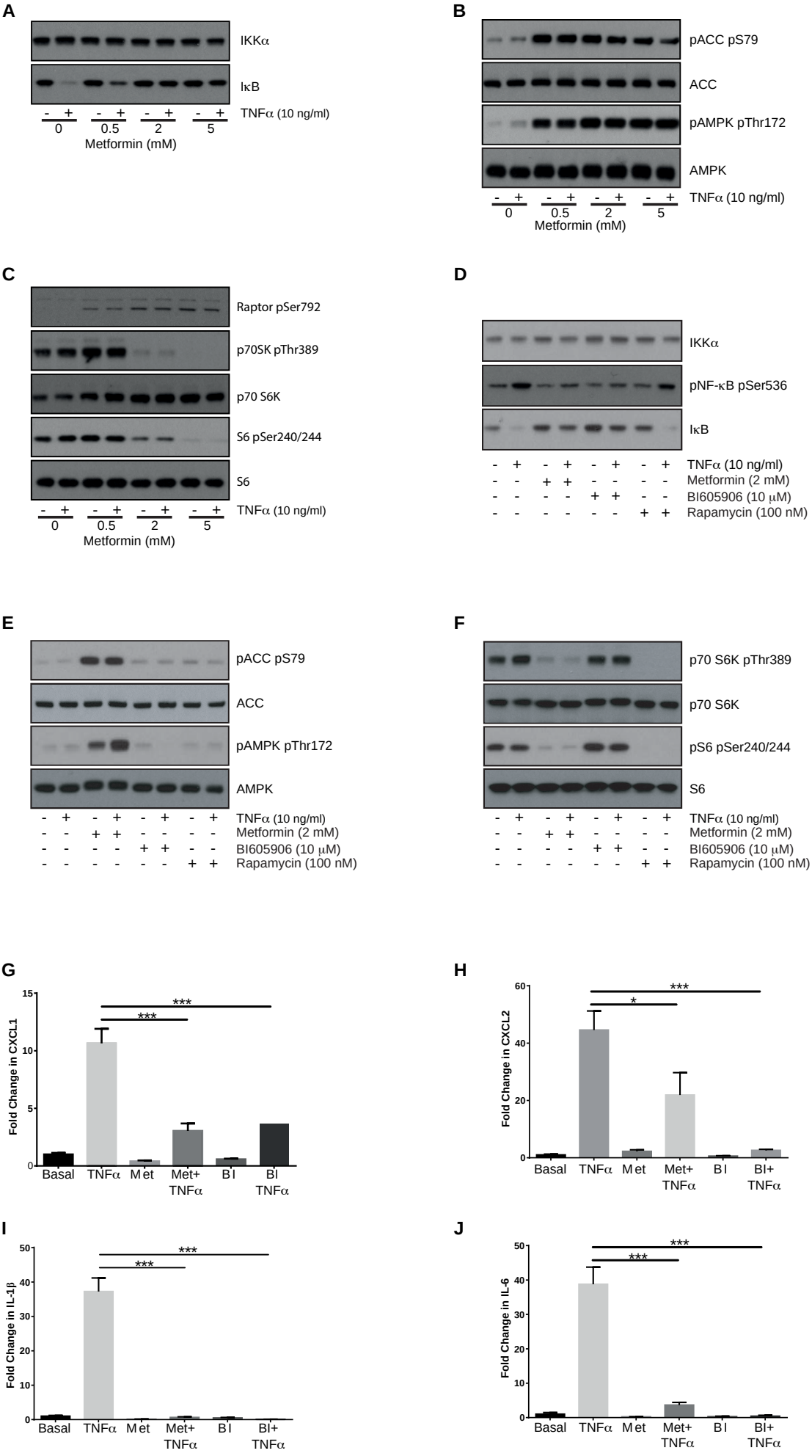


Figure 2

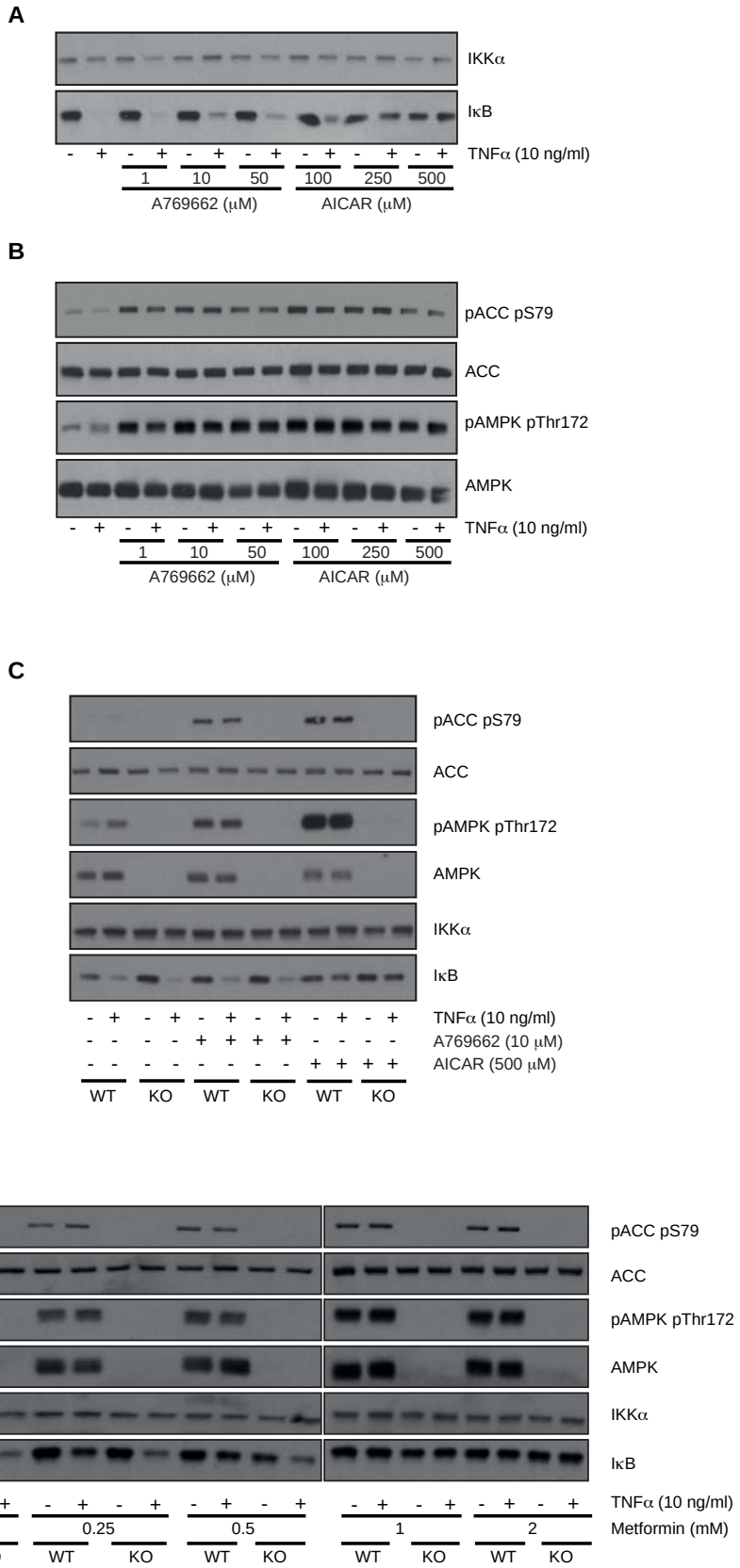


Figure 3

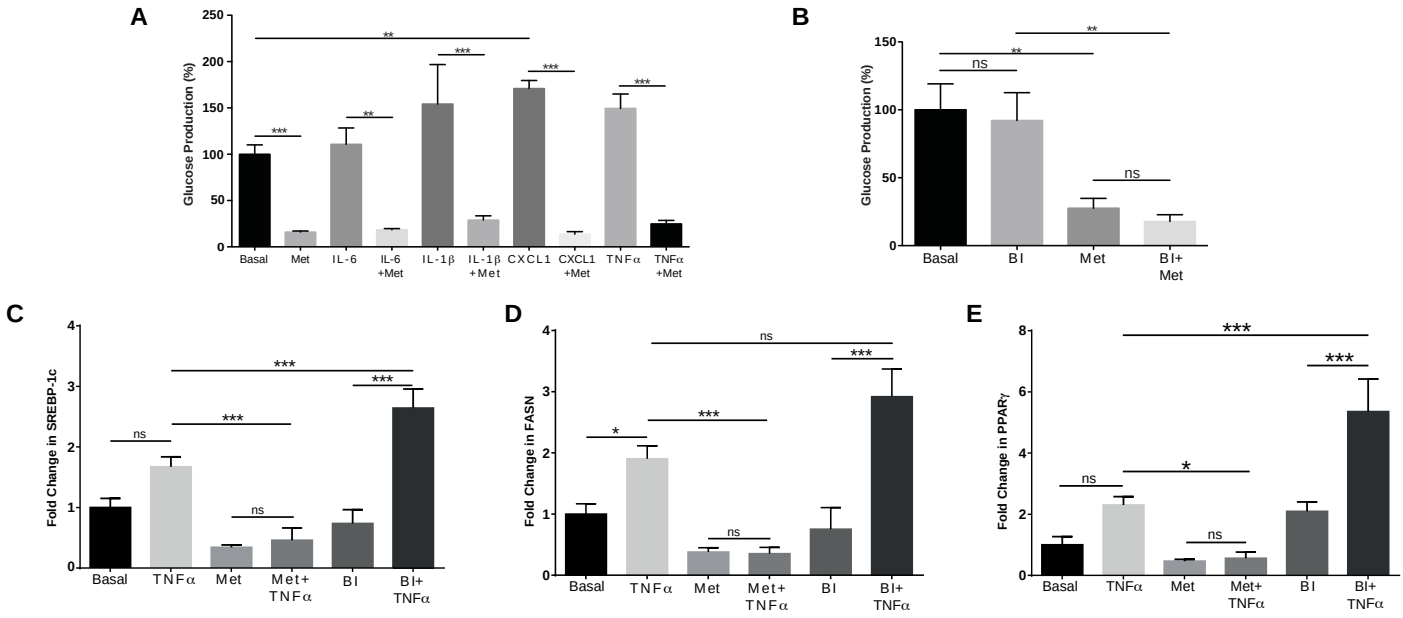


Figure 4

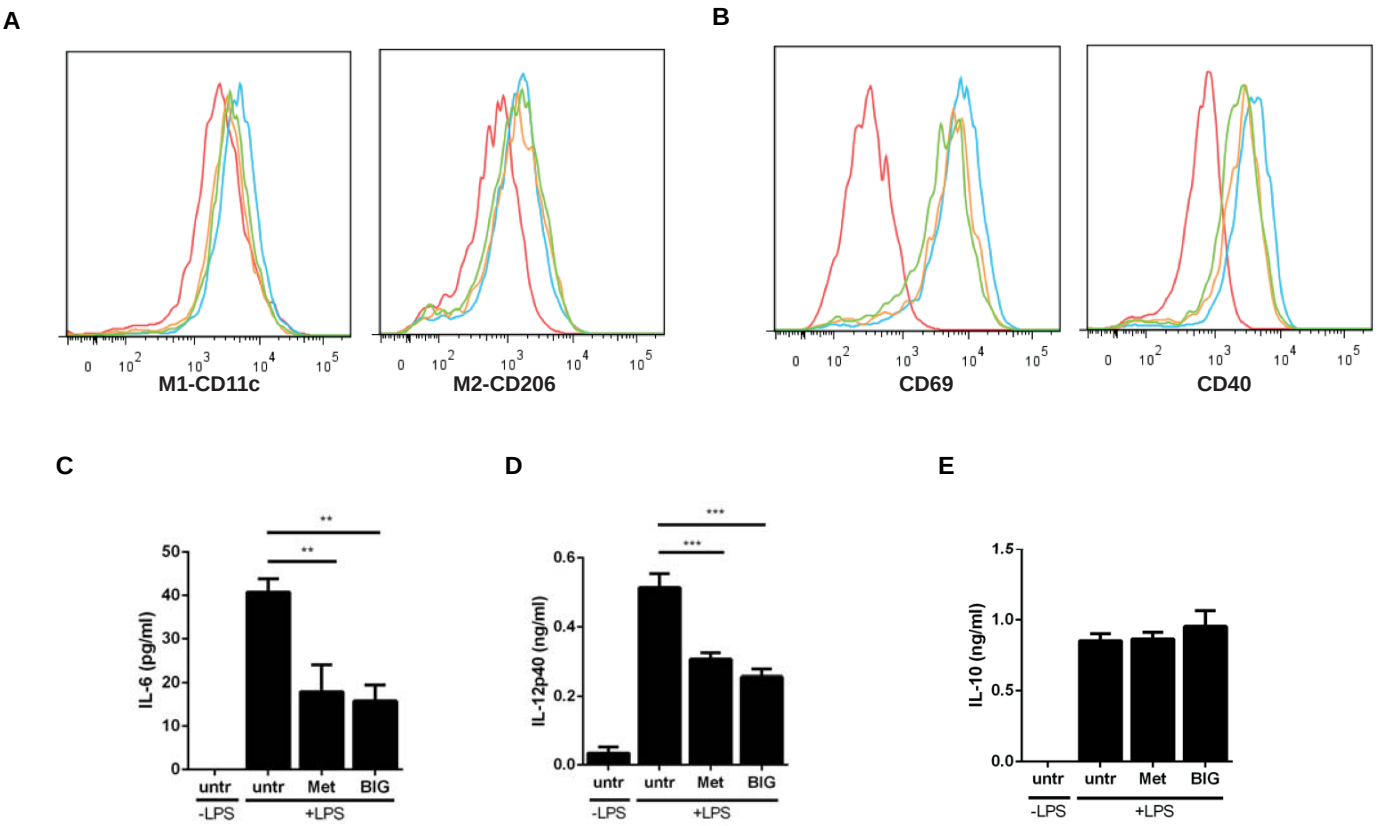
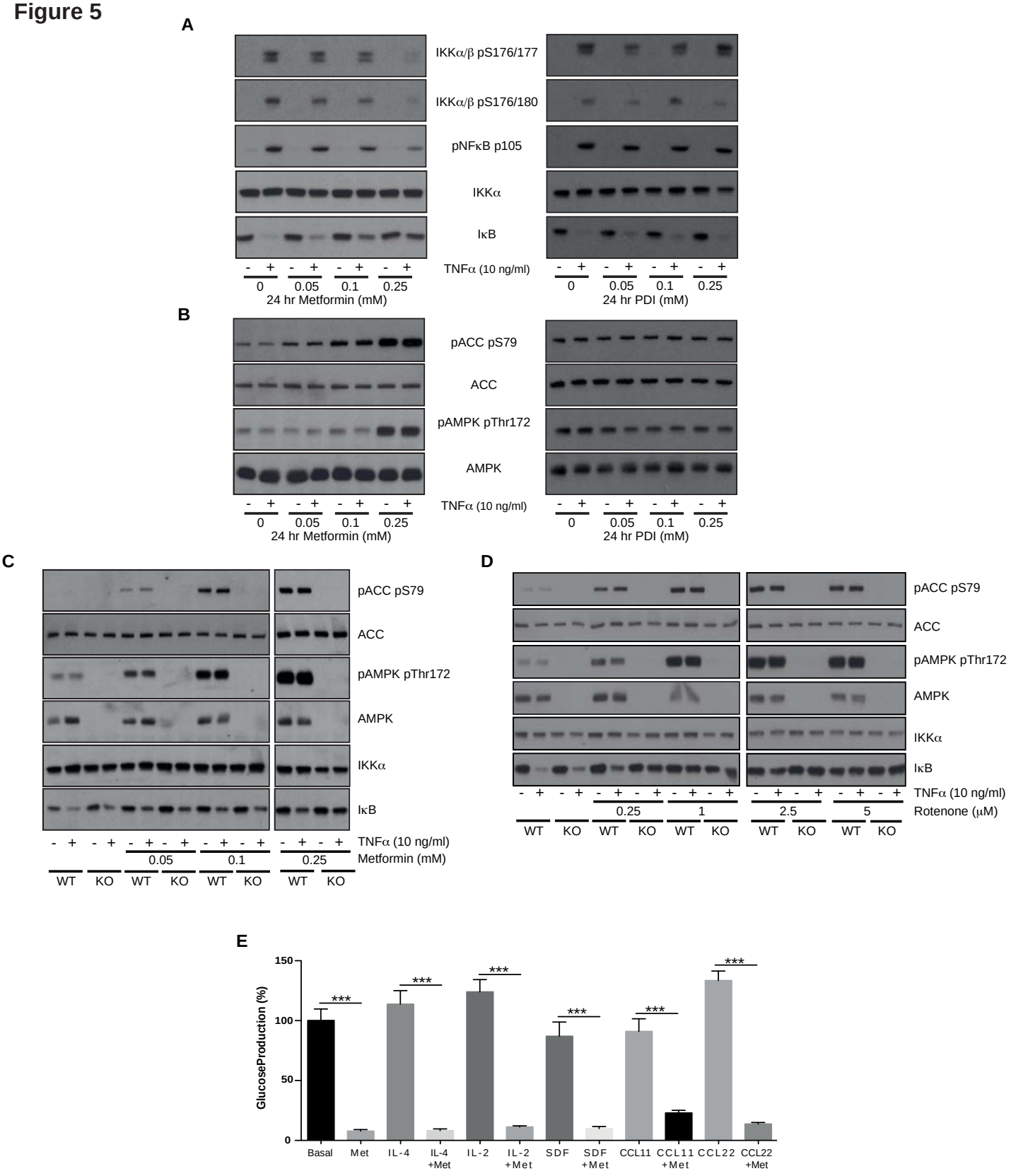


Figure 5



Circulation Research
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Anti-Inflammatory Effects of Metformin Irrespective of Diabetes Status

Amy R Cameron, Vicky Morrison, Daniel Levin, Mohapradeep Mohan, Calum Forteath, Craig Beall, Alison D McNeilly, David JK Balfour, Terhi Savinko, Aaron KF Wong, Benoit Viollet, Kei Sakamoto, Susanna C Fagerholm, Marc Foretz, Chim C Lang and Graham Rena

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Supplementary Materials

Expanded Methods

I. Animal and Cell Studies

Metformin and rapamycin came from Calbiochem, AICAR and A769662 (Tocris), TNFα (e-bioscience), recombinant CINC1/CXCL1, CCL-11, IL-2, IL-4, SDF and CCL22 (R&D systems), mouse IL-6 (Sigma) and recombinant mouse IL-1β (Life Technologies). The phospho-acetyl-CoA carboxylase (ACC) Ser79 antibody was a generous gift from the DSTT (University of Dundee). The total ACC (Cat. number 3662), total AMPKα (2603), phospho-AMPKα Thr172 (2535), total S6 (2217), phospho-S6 Ser240/244 (2215), total p70 S6 kinase (2708), phospho-p70 S6 kinase Thr389 (9205), phospho-Raptor Ser 792 (2083), phospho IKKα/β Ser176/177 (2078), IKKα/β Ser176/180 (2697), total IκB, pNF-κB, total IKKα and total IKKβ (NF-κB sampler kit 9936) antibodies were from CST. Anti-sheep HRP (31480) and anti-rabbit HRP (31460) both came from Thermo and anti-mouse HRP was from Calbiochem (JA1200). BI605906 was a generous gift from Prof Sir Philip Cohen.

Animal Care

C57BL/6 female mice (Charles River, 8-41 weeks) were maintained under a 12 hours:12hours light:dark cycle (holding room lights on at 06:00; off at 18:00) at 22±1°C and 50% humidity. Mice had *ad libitum* access to standard chow diet (7.5% fat, 75% carbohydrate and 17.5% protein by energy (RM1 diet; Special Diet Services) and water. All animal care protocols and procedures were performed in accordance with current regulations.

AMPKα1α2-null (AMPK KO) mice were maintained under a 12-hour light/12-hour dark cycle with free access to water and standard mouse diet (in terms of energy: 65% carbohydrate, 11% fat, 24% protein). These AMPK catalytic subunit deficient mice were generated as previously described (1). All procedures were performed in accordance with the principles and guidelines established in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (Council of Europe, ETS no. 123, 1991).

Hepatocyte Extraction

Mice were killed by cervical dislocation following guidelines set out by the Animals (Scientific Procedures) Act 1986. An incision into the abdomen was followed by dissection of the skin, abdominal cavity and diaphragm to expose the liver, kidney, inferior vena cava and portal vein. The superior vena cava was clamped to isolate the hepatic system and the inferior vena cava was cannulated just above the kidney with a 25G butterfly needle and clamped in place. Immediately after cannulation, the portal vein was cut. Successful cannulation was determined when the liver cleared quickly of blood and became pale throughout. The liver was perfused with 50ml pre-warmed perfusion buffer (137mM NaCl, 7mM KCl, 0.7mM Na_2HPO_4, 10mM HEPES pH 7.65 filter sterilized (0.2 µm) with 0.1% EDTA 0.5M pH 8 added just prior to use) at a rate of 5ml/min. After 10 min, the liver was perfused with 50ml digestion buffer (perfusion buffer without EDTA with 5.1mM $CaCl_2$ and 20mg collagenase (from Clostridium histolyticum type IV, Sigma)); added at a rate of 5ml/min. After digestion, the liver was excised from the abdominal cavity and transferred to a cell culture hood in a 10cm dish. The liver was resuspended in 10ml of plating media (440ml M199 + Glutamax (1x), Invitrogen; 5ml Pen/Strep (100x), Invitrogen; 6.7ml BSA (7.5%), Invitrogen; 50ml FBS (foetal bovine serum); 7.7µl Insulin Actrapid (100U/ml), Novo Nordisk; 100µl T3 (thyroid hormone 1mM stock), Sigma; 25µl Dexamethasone (10mM stock), Merck) and the hepatocytes isolated by gently agitating the liver. Cells were filtered through a 100 µm cell strainer and this process was repeated 4 more times until a final volume of 50ml was obtained. Hepatocytes were pelleted by centrifugation at 400 rpm for 5 min using no acceleration or braking. The supernatant was discarded and the cell pellet resuspended in 25ml plating media by gentle inversion. Cell viability was determined by 0.04% Trypan blue staining and the cell number determined using a haemocytometer. Cell viability of >90% was required for experimental use.

Cell Culture and Lysis for Immunoblotting

All cells were maintained in an incubator at 37ºC and 5% CO_2. For lysate and RT-PCR experiments, primary mouse hepatocytes were plated in 6-well plates (2.5×10^5 cells/well in 2ml media) while for glucose assay experiments, primary mouse hepatocytes were plated in 12-well plates (1.25×10^5 cells/well in 1ml media). After 4

hours, plating media was removed, cells were washed with warmed PBS and overnight media (500ml M199 + Glutamax (1x); 5ml Pen/Strep (100x); 25µl Dexamethasone (10mM stock)) was added at 2ml per well. Cells were incubated overnight and experiments were performed the following day.

BMDMs were grown from mouse bone marrow in RPMI 1640 medium supplemented with 10% FBS (Life Technologies) and 10ng/ml M-CSF (R&D systems). Cells were given fresh medium and growth factor on day 3 of culture. On day 6, BMDM cultures were supplemented with 100ng/ml IFNγ (for M1 differentiation; R&D systems), 20ng/ml IL-4 (for M2 differentiation; R&D systems), or 100ng/ml LPS (for activation; premium grade from Sigma, expected to activate TLR2 and TLR4) in the presence or absence of drug treatments for the final 24h.

Prior to SDS-PAGE, cells were lysed by scraping into buffer A: (50mM Tris acetate pH7.5, 1% (w/v) Triton X100, 1mM EDTA, 1mM EGTA, 0.27M sucrose, 50mM NaF, 1mM sodium orthovanadate, 10mM β-glycerophosphate, 5mM sodium pyrophosphate, 1mM benzamidine, 0.2mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% (v/v) β-mercaptoethanol) then prepared for SDS-PAGE as follows. The lysates were centrifuged at 13 000 g for 15 min, and the supernatants were removed. Protein concentration was determined by Bradford assay (Bio-Rad). The supernatant was loaded in equal amounts of protein and subjected to 4-20% gradient SDS-PAGE and subsequently were transferred to nitrocellulose membranes. Primary antibody incubations were performed at dilutions recommended by the manufacturer or determined by us in 5% milk TBS-T. All incubations were done at 4°C, overnight after a 1 hr block in 5% milk TBS-T. The secondary antibody was used at 1:5000 dilution for 1 hr at room temperature. Proteins were visualised using the enhanced chemiluminescence (ECL) system (Amersham) onto X-ray film (Kodak). Immunoblot densitometry for each antibody was performed with Image Studio Lite version 5.2 (LI-COR). Each blot is representative of experiments carried out at least three times.

Glucose Assay

Treatment of cells for hepatocyte glucose production was performed using primary mouse hepatocytes plated in 12-well plates (1.25 x 10^5 cells/well in 1ml media).

Glucose production was determined after a 12 hour incubation period in 750µl glucose-free DMEM (11966; Life Technologies) supplemented with 1% Pen/Strep, lactate (Sigma)/pyruvate (Life Technologies) (10:1 mM) and 100nM dexamethasone (dex; Merck) with or without drugs/cytokines under investigation. At the end of the incubation period of 12 hours, 500µl of medium was collected and glucose concentration determined by GAGO assay (GAGO-20; Sigma) by a modified protocol scaled down to a 96-well plate format. 50µl of sample medium followed by 100µl assay reagent was added to each well with no time delay. Following incubation at 37^{o}C for 30 minutes, 100µl 12N H_2SO_4 was added to each well and mixed using a multi-well pipette. Absorbance was measured at 540 nm. Each column consists of data from at least 12 wells of cells, six each from two mice.

RT-PCR

Primary mouse hepatocytes were incubated for 8 hours in 1ml glucose-free DMEM (11966; Life Technologies) supplemented with 1% pen/strep, lactate (Sigma)/pyruvate (Life Technologies) (10:1 mM) and 100nM dexamethasone (dex; Merck) with or without drugs under investigation. After this incubation period, media was removed and cells were washed once with warmed PBS. 350µl of Buffer RLT from the Rneasy MINI KIT (Qiagen) plus 10% β-mercaptoethanol was added to each well. Plates were then placed on ice for 10 min, followed by a cell harvest and samples were snap-frozen immediately in LN_2. Total RNA was extracted using QIAshredder (Qiagen) and Rneasy MINI KIT (Qiagen) as per the manufacturer's instructions. cDNA was synthesized from 1µg RNA using RQ1 Rnase-Free Dnase kit (Promega) and ImProm-II Reverse Transcription System (Promega). cDNA was diluted in nuclease-free water 1:10 prior to use.

Nucleospin RNA II Total RNA isolation kit (Macherey-Nagel) was used to isolate RNA from macrophages. cDNA was synthesized from 0.5µg RNA using High Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific). cDNA was diluted in nuclease-free water 1:2 prior to use.

Real-time PCR was carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan 2x Universal PCR Master Mix (Applied Biosystems) and primer/probes mixes as stated (Applied Biosystems). Primer sets

used were: IL-6 Mm00446190_ml; CXCL1 Mm04207460_m1; 18S Hs03003631_g1; IL-1β Mm00434228_m1; CXCL2 Mm00436450_m1; PPARγ Mm01184322_m1; FASN Mm00662319_m1; CCL22 Mm00436439_ml; CXCL12 Mm00445553_ml; TBP Mm01277042_m1 and SREBP-1c Mm00550338_m1. Cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression is expressed relative to 18s mRNA for hepatocytes and TBP for macrophages (Applied Biosystems) using the $2^{-\Delta\Delta Ct}$ method. Control samples were set at a value of 100% and results for all experimental samples were graphed as relative expression compared to control. Each column is composed of data from at least three separate experiments.

BMDM studies

BMDMs were harvested from culture plates using 4mM EDTA in PBS for 10min at 37°C. Cells were washed in flow cytometry buffer (PBS with 2% FBS and 1mM EDTA) and stained using the following antibodies (all BD Bioscience unless stated): F4/80 (BM8; e-bioscience), CD11c (HL3), CD206 (C068C2; Biolegend), CD69 (H1.2F3) and CD40 (3/23). Fc block (4.4G2) was included in all stains. Data were acquired on a LSR II flow cytometer (Becton Dickinson) and analysed using FlowJo software (TreeStar). BMDM culture supernatants were collected after 24 hours treatment with the differentiation or activation conditions. Levels of cytokines were quantified by standard sandwich ELISA using paired antibody kits (e-bioscience), according to the manufacturer's instructions.

Statistical Analyses

Results in bar graphs are expressed as mean ± SEM. Comparisons between groups were made by one-way ANOVA with Dunnett's or Tukey post-hoc test using Prism. Differences were considered statistically significant if *P* was less than 0.05. *** denotes $p<0.001$; ** denotes $p<0.01$ and * denotes $p<0.05$. For studies on the plasma, statistical analyses of data were performed using SPSS 14.1. ANOVA and Pearson correlation coefficients were calculated.

II. Validation in Clinical Patients

Population Cohort Study: Diabetes Patient Metformin Exposure and Neutrophil-to-Lymphocyte Ratio (NLR).

Sample Ascertainment

Patients were ascertained from the Diabetes Audit and Research in Tayside Scotland (DARTS) study, which has previously been described in detail (2). In brief, all the participants were linked through to the Health Informatics Centre Database to retrieve validated prescribing information, clinical information system, all haematological and biochemistry data and the Scottish Care Information–Diabetes Collaboration (SCI-DC) (REF) that provide additional clinical phenotypic data back to 1992. Prospective longitudinal data were also collected on these patients. The study was approved by the Tayside Regional Ethics Committee, and informed consent was obtained from all subjects since 1997 to DNA and serum collection as part of the Wellcome Trust United Kingdom Type 2 Diabetes Case Control Collection. Over 17,000 subjects have participated in this Genetics of DARTS (Go-DARTS) study till date, of whom over 9,000 have diabetes.

Hematological Measurements.

We analysed the electronically linked records of routine laboratory investigations of all the participants from the regional biochemistry and hematological database. The total and differential leucocyte counts (including the neutrophils and lymphocytes) were determined from peripheral venous blood samples using an automated Siemens' high-volume hematology analyzer, the ADVIA® 2120i System (peroxidase method) (3). NLR was calculated as the ratio between (percentage of) neutrophils and total lymphocyte counts in the study subjects.

Statistical Analysis

For the population cohort study, characteristics of patients with or without metformin therapy were compared by the chi-square test for categorical variables and by the t test or Mann-Whitney U test for continuous variables as appropriate. The effect of metformin therapy on NLR were examined together with the significant differences found at baseline using linear and logistic regression analysis. The following covariates were included: age, sex, HbA1c, BMI, duration of followup, prior

hospitalisation for COPD, Atrial Fibrilation or Hypertension, and baseline NLR. To minimize confounding influences, we performed two different sensitivity analysis. First, by using a multivariate model adjusting for potential confounders; second we determined a propensity score using a logistic regression model to control for the different characteristics of the metformin and sulfonylurea groups. A P-value of <0.05 was considered significant and all statistical analysis for this cohort study were performed using R for windows (v3.2.0).

Randomised Placebo Controlled Study

The effect of metformin on plasma inflammatory cytokines were further investigated in a subset of chronic heart failure (CHF) patients who had participated in a double-blind, placebo-controlled study of metformin, which has previously been described in detail (4). In brief, this study was designed and powered to evaluate the impact of metformin on IR and its effects on exercise capacity in non-diabetic IR patients with CHF. Every patient who participated in this study, provided written informed consent prior to participation in this study, which was approved by the East of Scotland Research Ethics Service (www.clinicaltrials.gov: NCT00473876). In this study we had shown that metformin treatment significantly improved IR but had no significant effect on the primary endpoint of exercise capacity, as measured by peak VO_2. However, metformin treatment did result in a significant improvement in the minute ventilation − carbon dioxide production relationship (VE/VCO_2 slope), a pre-specified secondary endpoint of this proof of concept study which is of prognostic significance in patients with CHF, and in some studies, it has outperformed peak VO_2 (5).

Cytokine Assay

We analysed plasma from 33 non-diabetic insulin resistant heart failure patients who took part in a placebo controlled clinical trial of metformin (4). The plasma was analysed using the Bio-Plex Pro Human Chemokine 40-Plex Panel (171-AK99MR2, Bio-Rad). The assay was performed following the manufacturer's instructions using the Bio-Plex 200 system (Bio-Rad). Freeze-thaw cycling of samples was avoided to prevent cytokine degradation and they were diluted 1:4 (12.5µl of plasma) for the assay.

References

(1) Foretz M, Hébrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, Sakamoto K, Andreelli F, Viollet B: Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest* 2010;120:2355-2369.
(2) Morris AD, Boyle DI, MacAlpine R, Emslie-Smith A, Jung RT, Newton RW, MacDonald TM: The diabetes audit and research in Tayside Scotland (DARTS) study: electronic record linkage to create a diabetes register. DARTS/MEMO Collaboration. *BMJ* 1997;315:524-528
(3) ADVIA 2120i Hematology System with Autoslide. SIEMENS Healthineers website. http://www.healthcare.siemens.com/hematology/systems/advia-2120-hematology-system-with-autoslide. Updated September 9, 2015. Accessed July 7, 2016.
(4) Wong AKF, Symon R, Alzadjali MA, Ang DSC, Ogston S, Choy AM, Petrie JR, Struthers AD, Lang CC: The effect of metformin on insulin resistance and exercise parameters in patients with heart failure. *European Journal of Heart Failure* 2012;14:1303-1310
(5) Arena R, Myers J, Aslam SS, Varughese EB, Peberdy MA. Peak VO2 and VE/VCO2 slope in patients with heart failure: a prognostic comparison. *Am Heart J* 2004; 147: 354–360.

Supplementary table I. Cytokine measurements

	Baseline			
	Placebo (n=13)		Metformin (n=20)	
Cytokine (pg/ml)	Average	SD	Average	SD
CCL21/6Ckine	5454.29	2067.61	5890.92	1853.73
BCA-1/CXCL13	28.52	9.54	35.02	13.22
CTACK/CCL27	1723.32	583.41	1973.63	815.37
ENA-78/CXCL5	642.91	213	781.07	321.26
Eotaxin/CCL11	51.4	13.67	60.8	17.69
Eotaxin-2/CCL24	685.11	518.81	640.79	402.68
Eotaxin-3/CCL26	56.27	18.48	74.93	60.97
Fractalkine/CX3CL1	172.05	61.95	237.93	93.3
GCP-2/CXCL6	20.34	8.37	26.27	14.34
GM-CSF	128.91	50.39	137.67	52.51
Gro-α/CXCL1	348.61	83.78	336.15	76.27
Gro-β/CXCL2	153.61	74.54	235.74	178.19
I-309/CCL1	83.83	17.2	92.43	18.49
IFNγ	64.35	20.88	75.98	27.42
IL-1β	7.53	3.66	9.33	3.84
IL-2	15.96	5.21	18.87	6.48
IL-4	32.19	7.45	33.4	9.48
IL-6	14.14	3.89	17.81	8.58
IL-8/CXCL8	14.19	4.16	16.9	4.72
IL-10	46.74	21.99	54.49	22.46
IL-16	329.73	120.54	364.8	130.41
IP-10/CXCL10	173.62	67.61	206.57	114.88
I-TAC/CXCL11	18.02	6.86	23.33	7.83
MCP-1/CCL2	69.92	33.76	86.03	52.74
MCP-2/CCL8	56.84	21.19	70.44	38.9
MCP-3/CCL7	126.6	35.08	146.64	53.26
MCP-4/CCL13	77.59	46.32	69.26	39.3
MDC/CCL22	1014.65	290	1151.86	505.54
MIF	6303.03	5822.99	5681.16	3323.86
MIG/CXCL9	296.66	136.95	404.71	277.22
MIP-1α/CCL3	10.5	2.02	12.22	3.69
MIP-1δ/CCL15	7487.28	3978.97	7716.92	4125.49
MIP-3α/CCL20	34.9	79.52	37.84	43.88
MIP-3β/CCL19	353.04	252.2	463.93	308.82
MPIF-1/CCL23	446.78	221.81	494.74	211.13
SCYB16/CXCL16	503.06	216.96	567.01	228.79
SDF-1α+β/CXCL12	1257.91	423.96	1487.04	500.84

TARC/CCL17	113.29	81.29	138.35	94.4		
TECK/CCL25	759.25	248.21	896.76	327.17		
TNFα	46.33	10.03	51.18	15.66		
	Change after 4 months metformin treatment					
	Placebo (n=13)		**Metformin (n=20)**		**BMI (p value)**	**BMI & FIRI (p value)**
Cytokine (pg/ml)	**Average**	**SD**	**Average**	**SD**		
CCL21/6Ckine	99.20	756.47	55.16	2595.81	0.857	0.8
BCA-1/CXCL13	3.92	7.29	2.34	14.28	0.036	0.029
CTACK/CCL27	54.09	401.40	-128.67	902.78	0.022	0.032
ENA-78/CXCL5	29.79	148.93	-60.30	375.81	0.02	0.025
Eotaxin/CCL11	5.18	8.94	-0.36	17.00	0.004 *	0.006 *
Eotaxin-2/CCL24	32.73	258.03	-21.23	210.01	0.309	0.356
Eotaxin-3/CCL26	6.54	13.67	-11.50	54.48	0.038	0.053
Fractalkine/CX3CL1	0.93	56.10	-25.89	107.44	0.085	0.081
GCP-2/CXCL6	0.91	6.29	1.05	13.11	0.169	0.216
GM-CSF	1.52	34.36	3.15	55.77	0.051	0.05
Gro-α/CXCL1	11.01	54.90	5.89	80.00	0.101	0.106
Gro-β/CXCL2	32.80	56.92	9.41	120.75	0.317	0.365
I-309/CCL1	6.16	10.89	-0.69	17.79	0.011	0.014
IFNγ	6.28	16.04	-1.85	29.38	0.019	0.025
IL-1β	0.16	2.90	-0.96	4.15	0.116	0.098
IL-2	1.47	3.17	-0.95	6.96	0.006 *	0.009 *
IL-4	1.33	4.55	-2.67	11.90	0.008 *	0.014
IL-6	0.86	3.43	2.75	14.94	0.022	0.027
IL-8/CXCL8	1.87	3.94	0.20	8.25	0.07	0.072
IL-10	6.79	14.29	-4.43	27.19	0.019	0.024
IL-16	32.62	82.96	-19.07	149.55	0.013	0.021
IP-10/CXCL10	-3.82	52.93	16.13	118.54	0.958	0.893
I-TAC/CXCL11	2.22	7.06	3.73	13.73	0.989	0.955
MCP-1/CCL2	12.78	26.27	-5.34	35.48	0.039	0.055
MCP-2/CCL8	9.01	16.58	-2.92	38.04	0.048	0.071
MCP-3/CCL7	13.07	31.37	-7.03	55.42	0.025	0.029
MCP-4/CCL13	-3.31	25.83	3.02	37.95	0.377	0.418
MDC/CCL22	93.18	244.23	-47.75	453.81	0.004 *	0.005 *
MIF	-1711.46	5475.63	-829.75	3572.37	0.436	0.464
MIG/CXCL9	52.40	128.26	-3.84	200.65	0.016	0.019
MIP-1α/CCL3	0.72	1.66	-0.52	2.67	0.013	0.013
MIP-1δ/CCL15	-300.03	2502.48	515.34	4045.71	0.505	0.368
MIP-3α/CCL20	-16.84	61.17	-4.44	45.65	0.832	0.991
MIP-3β/CCL19	97.36	212.33	-77.06	343.62	0.018	0.024
MPIF-1/CCL23	-16.10	95.08	-29.21	177.39	0.26	0.214
SCYB16/CXCL16	-11.35	114.84	30.26	203.08	0.083	0.11
SDF-1α+β/CXCL12	103.67	198.81	-49.25	409.72	0 *	0.001 *
TARC/CCL17	29.45	72.26	-20.89	96.02	0.022	0.03

TECK/CCL25	54.31	180.75	-57.17	354.85	0.019	0.026
TNFα	4.91	8.98	0.09	18.93	0.03	0.036

Significance taken as * $p < 0.01$.

Supplementary table II. Metabolic, haemodynamic and other characteristics of patients

	Baseline				
	Placebo (n=13)		**Metformin (n=20)**		**p value**
Sex	Male 12; Female 1		Male 16; Female 4		
	Average	**SD**	**Average**	**SD**	
Age	64.23	6.99	62.70	7.04	0.408
Metabolism Parameters					
Body Mass Index	29.37	5.11	30.19	5.23	0.319
Insulin (mU/L)	22.63	11.54	27.47	15.29	0.144
Glucose (mmol/L)	5.37	0.42	5.61	0.66	0.331
Fasting Insulin Resistance Index (log)	4.90	2.57	6.32	3.92	0.134
Severity of Heart Failure					
Brain Natriuretic Peptide (pg/ml)	116.27	131.98	139.09	197.95	0.750
Ejection Fraction (%)	28.82	8.29	37.29	7.93	0.016
Haemodynamic Conditions					
Resting Systolic Blood Pressure (mmHg)	116.54	21.05	107.60	9.68	0.101
Resting Diastolic Blood Pressure (mmHg)	75.15	10.38	69.35	7.15	0.178
Resting Heart Rate	70.08	21.22	68.60	14.44	0.992
Peak VO_2 (mL/kg/min)	18.02	5.94	19.72	4.75	0.776
VE/VCO_2 slope	30.65	5.31	31.64	6.03	0.244
Total exercise duration (s)	954.15	355.64	1063.80	204.51	0.469

	Change after 4 months metformin treatment				
	Placebo (n=13)		Metformin (n=20)		p value
	Average	SD	Average	SD	
Metabolism Parameters					
Body Mass Index	0.46	0.76	-1.16	1.08	0.000
Insulin (mU/L)	0.92	7.39	-6.39	9.19	0.044
Glucose (mmol/L)	-0.02	0.75	-0.29	0.50	0.042
Fasting Insulin Resistance Index (log)	0.26	1.77	-1.81	2.53	0.029
Severity of Heart Failure					
Brain Natriuretic Peptide (pg/ml)	17.26	122.33	-22.24	91.36	0.383
Ejection Fraction (%)	-0.89	2.52	-1.03	3.61	0.681
Haemodynamic Conditions					
Resting Systolic Blood Pressure (mmHg)	-7.15	16.18	0.80	14.63	0.049
Resting Diastolic Blood Pressure (mmHg)	-4.69	9.22	-0.45	7.84	0.177
Resting Heart Rate	1.00	11.73	2.70	9.95	0.451
Peak VO_2 (mL/kg/min)	1.25	4.59	-0.91	2.73	0.334
VE/VCO_2 slope	1.86	10.57	-4.52	5.37	0.029
Total exercise duration (s)	7.08	92.03	-22.75	99.45	0.671

Supplementary figure legends

Supplementary Figure I. Densitometry of blots in main figure 1
Densitometry was carried out as described in the methods to quantify data obtained in western blots. Bars significantly different from the respective control treatment (+/- TNFα) are shown, ***p<0.001, **p<0.01, *p<0.05. N=3 except for IκBa and pACC, N=4

Supplementary Figure II. Densitometry of blots in main figure 2
Densitometry was carried out as described in the methods to quantify data obtained in western blots. In experiments comparing wild-type (WT) and knockout (KO) genotype, black bars denote WT genotype, grey bars denote KO genotype. Bars significantly different from the respective control treatment (+/- TNFα) are shown,

***p<0.001, **p<0.01, *p<0.05, except in knockout experiments, where significance between genotypes is depicted. N=3

Supplementary Figure III. Effect of metformin on gene expression in macrophages: genes regulated in hepatocytes

Macrophages were treated with or without 100ng/ml LPS +/- 2mM metformin for 8h followed by cell lysis, RNA extraction and preparation of cDNA for RTPCR using primer sets for individual genes shown, as described in the methods. Bars significantly different from control treatment, or between two annotated bars are shown, ***p<0.001, *p<0.05

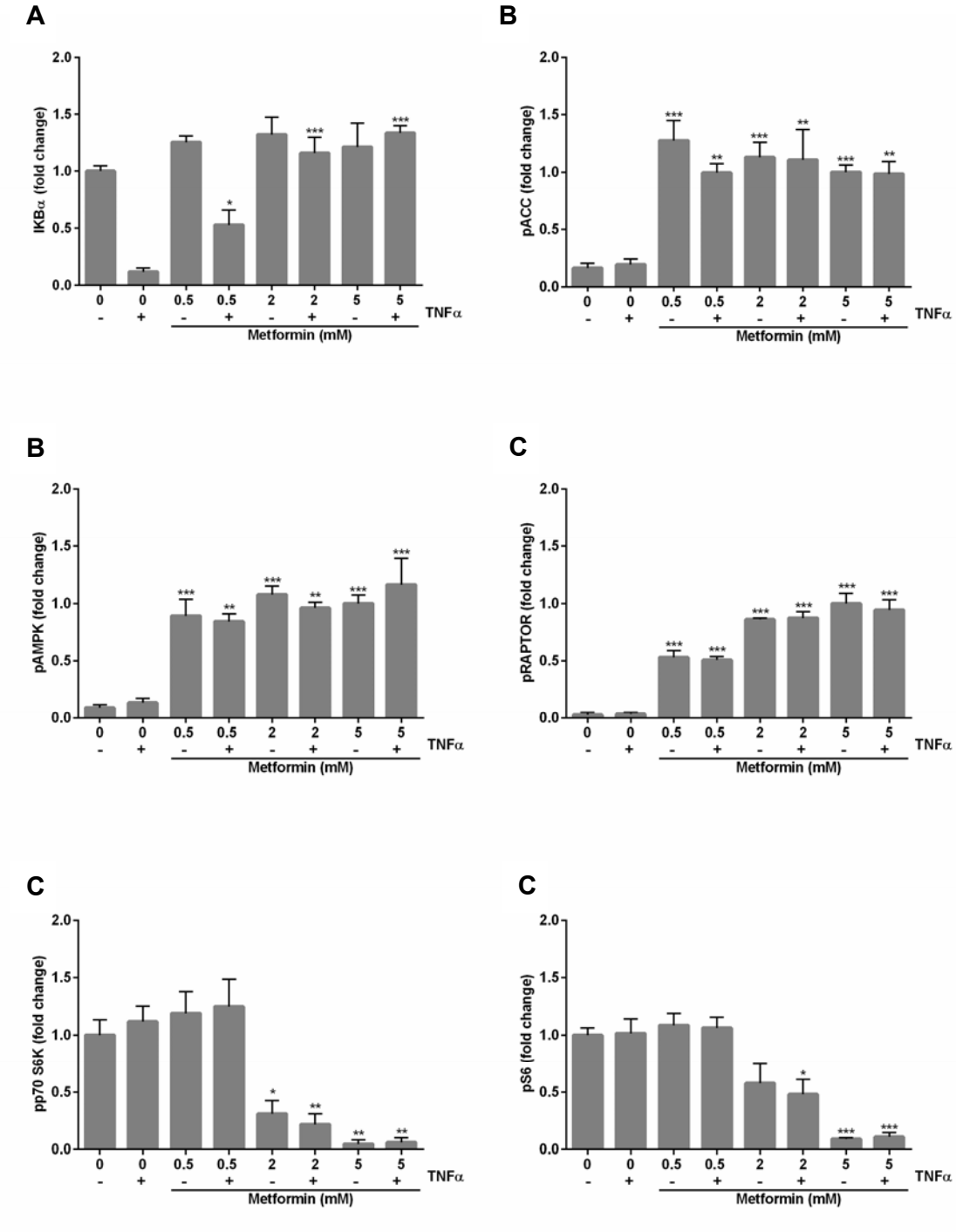
Supplementary Figure IV. Effect of metformin on gene expression in macrophages and hepatocytes: genes changed by metformin in human plasma

Macrophages *(a,b)* were treated with/without 100ng/ml LPS +/- 2mM metformin, while hepatocytes *(c)* were treated with/without 10ng/ml TNFα +/- 2mM metformin or 10mM BI605906 as shown, for 8h followed by cell lysis, RNA extraction and preparation of cDNA for RTPCR using primer sets for individual genes shown, as described in the methods. Bars significantly different from control treatment, or between two annotated bars are shown, ***p<0.001, **p<0.01, *p<0.05

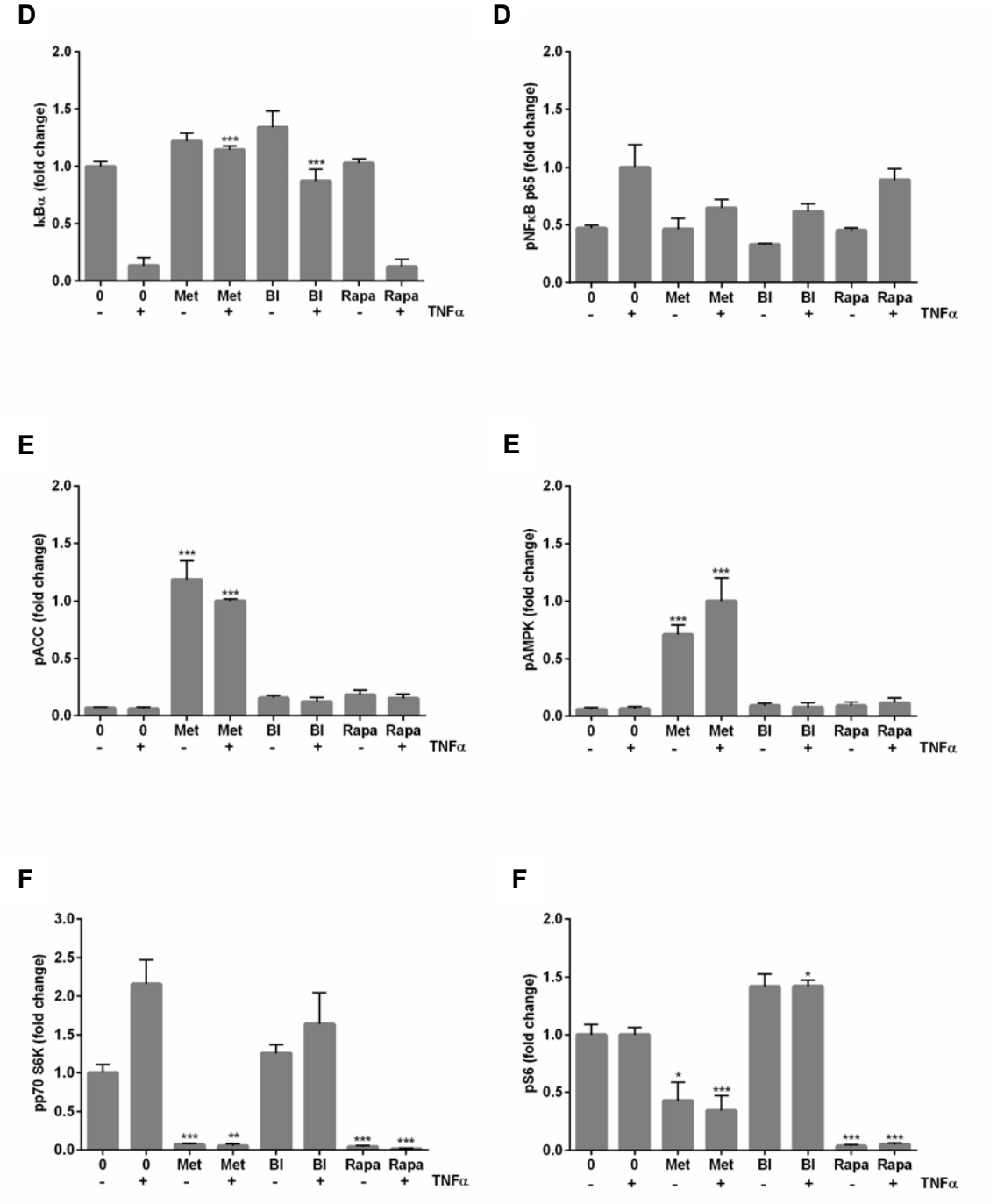
Supplementary Figure V. Densitometry of blots in main figure 5

Densitometry was carried out as described in the methods to quantify data obtained in western blots. In experiments comparing wild-type (WT) and knockout (KO) genotype, black bars denote WT genotype, grey bars denote KO genotype. Bars significantly different from the respective control treatment (+/- TNFα) are shown, ***p<0.001, **p<0.01, *p<0.05, except in knockout experiments, where significance between genotypes is depicted. N=3

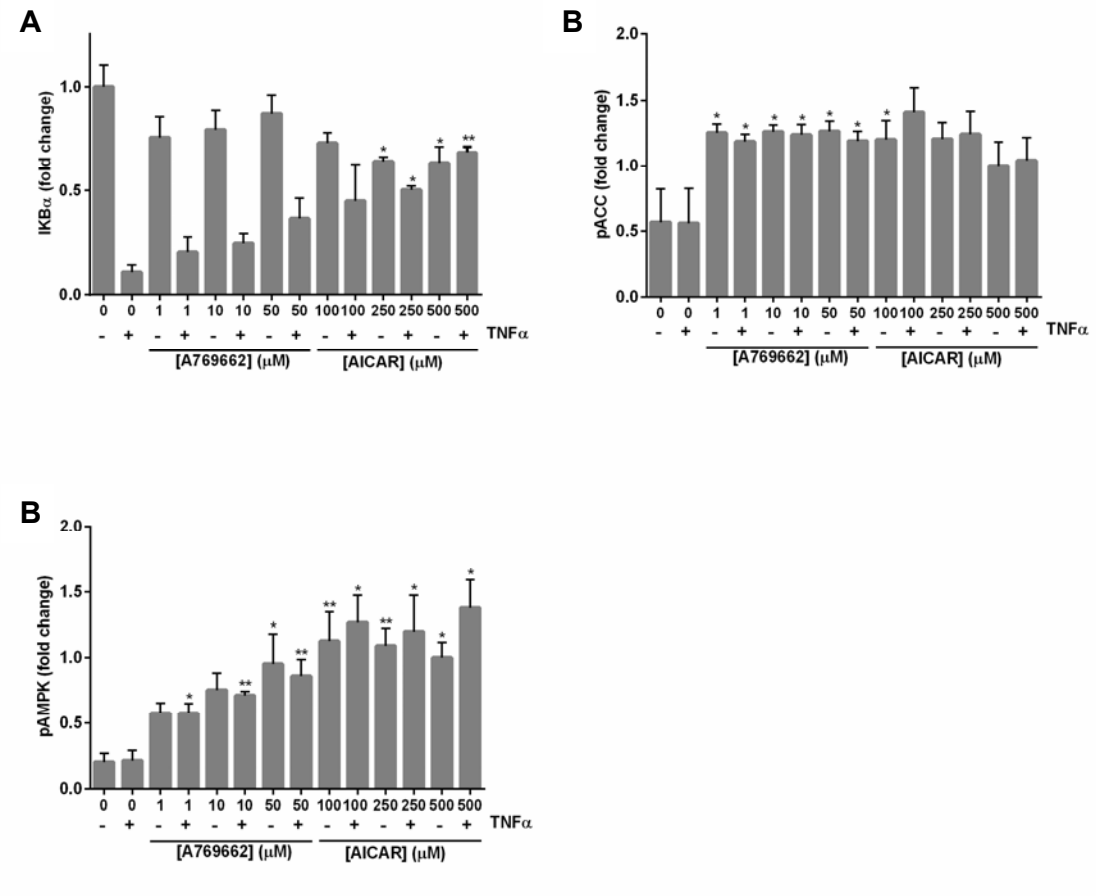
Supplementary Fig. I



Supplementary Fig. I



Supplementary Fig. II



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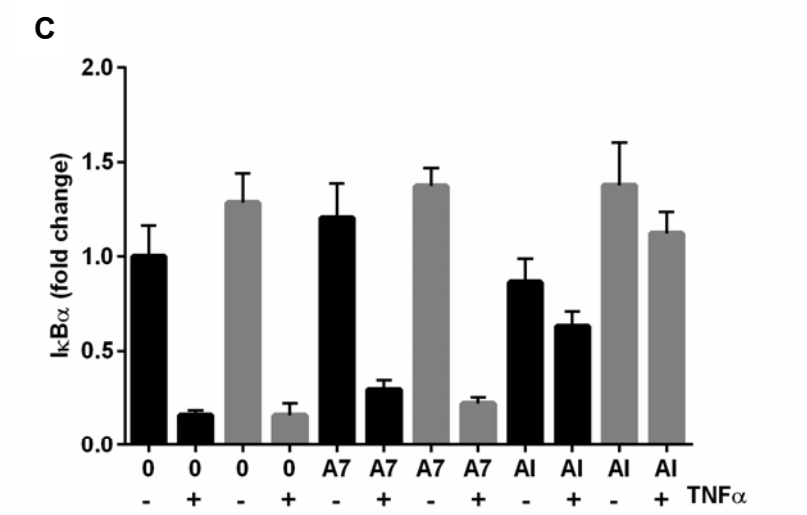

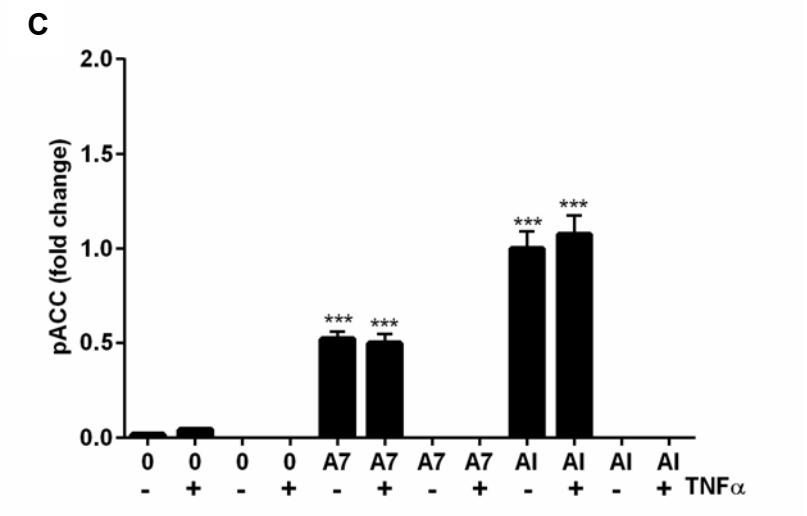

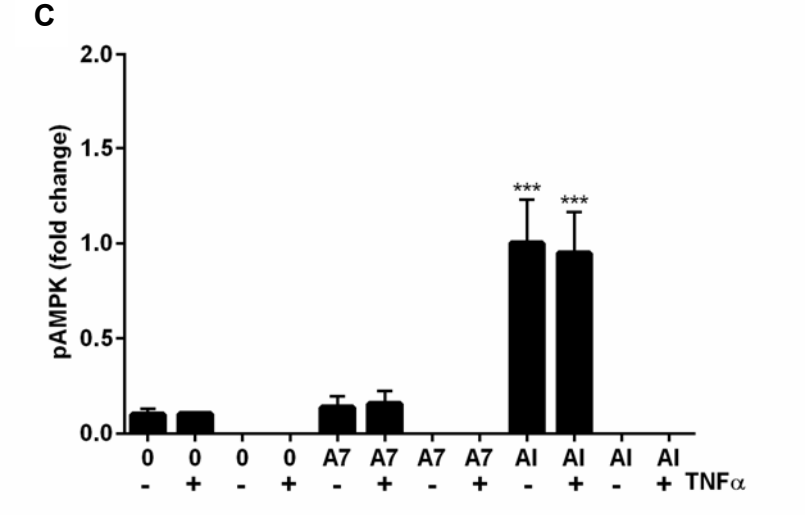

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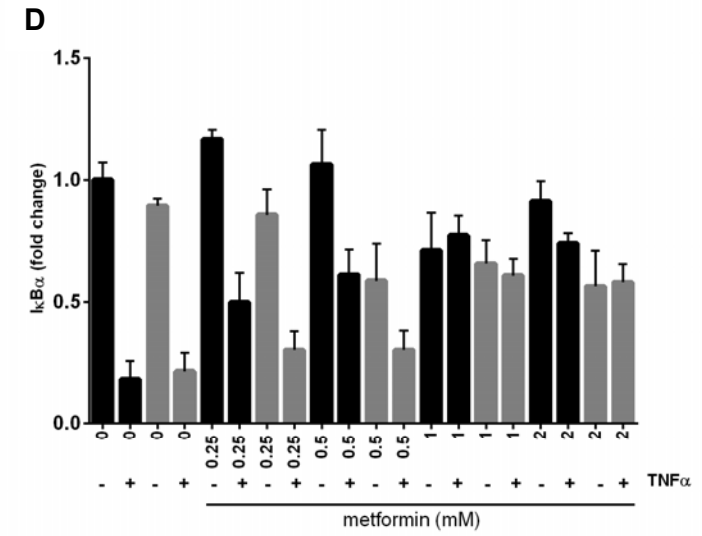

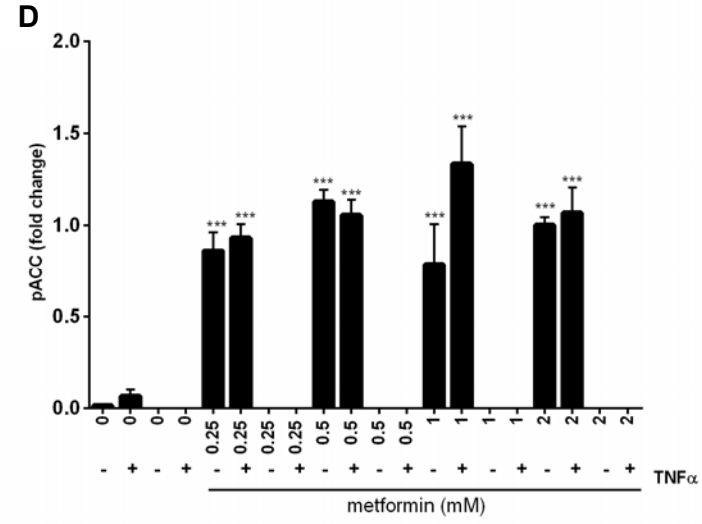

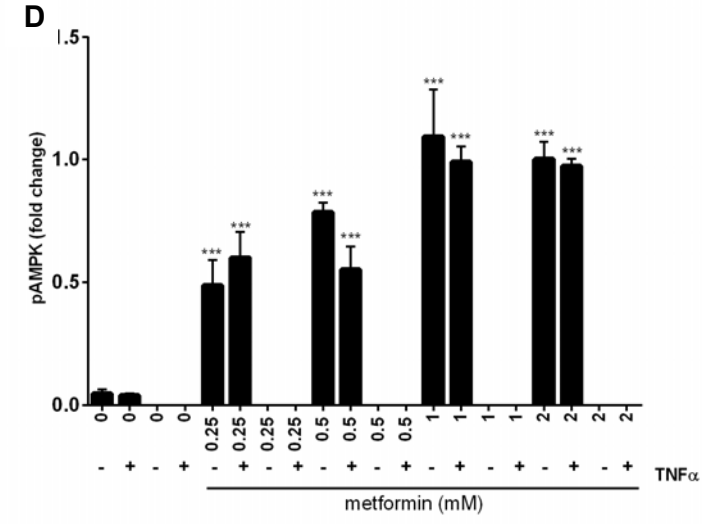

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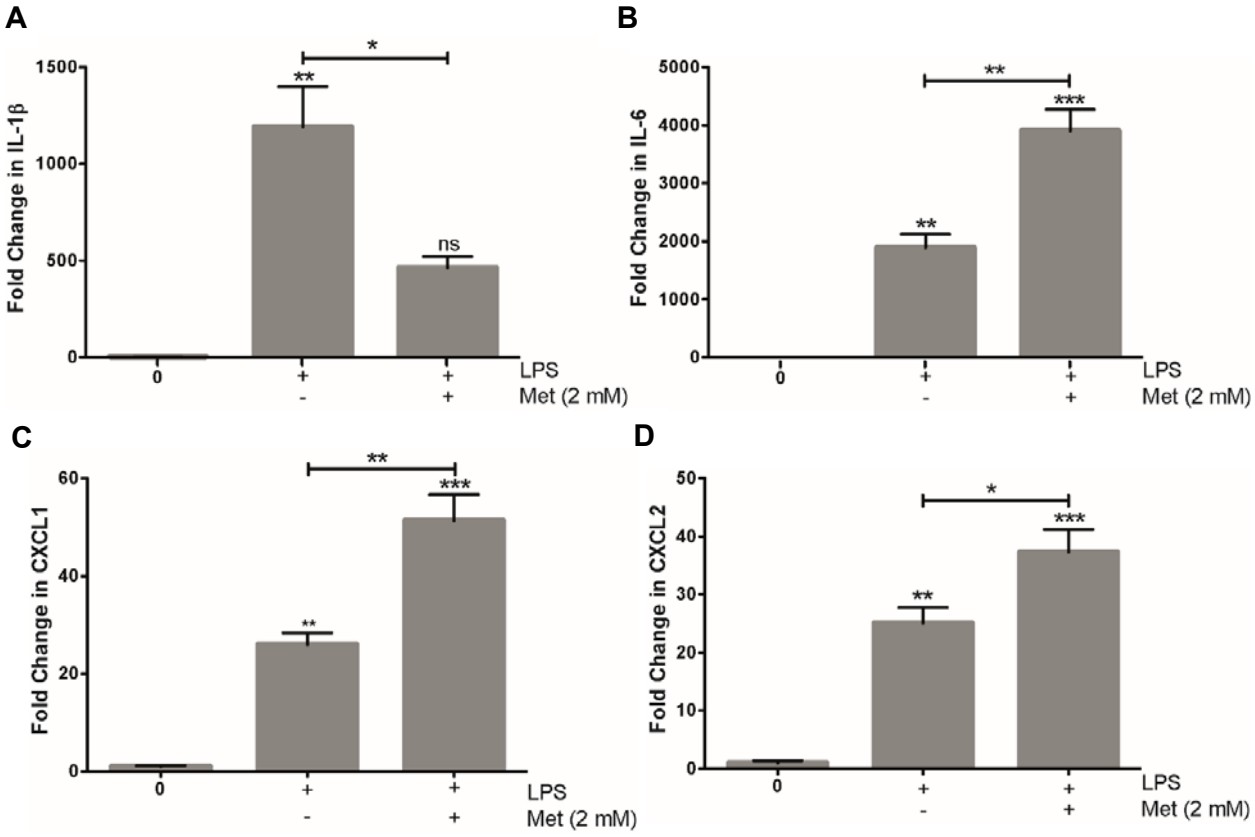
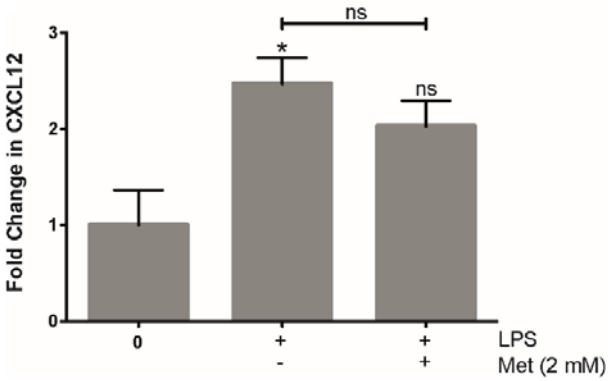


Supplementary Fig. IV

MACROPHAGES

A



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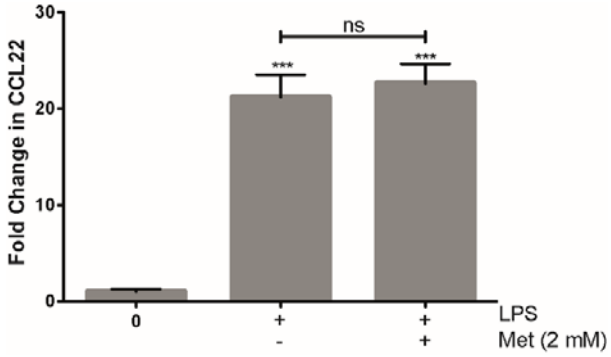
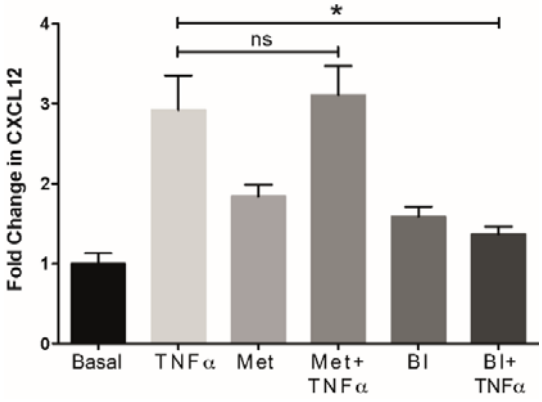


HEPATOCYTES

C



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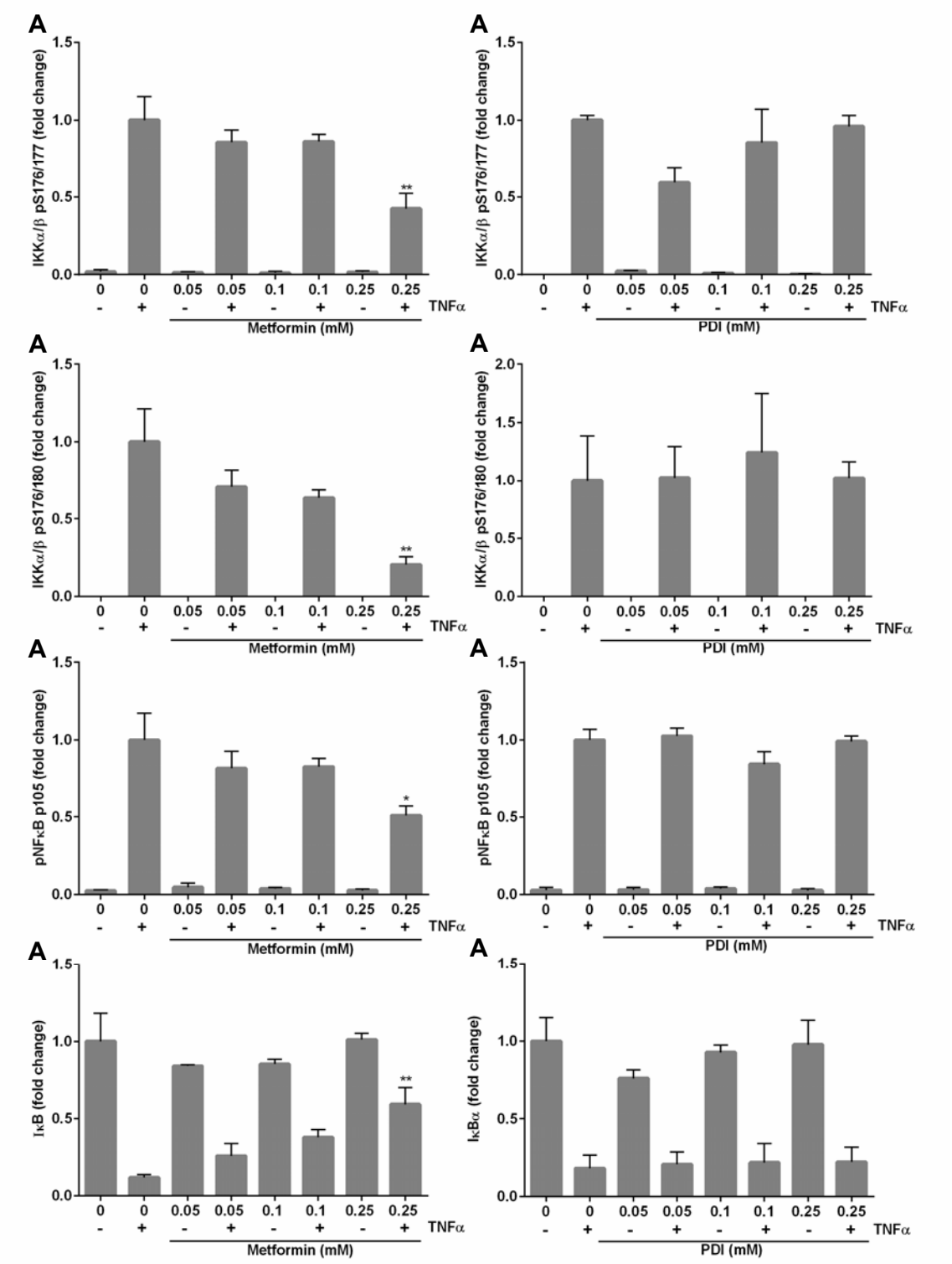
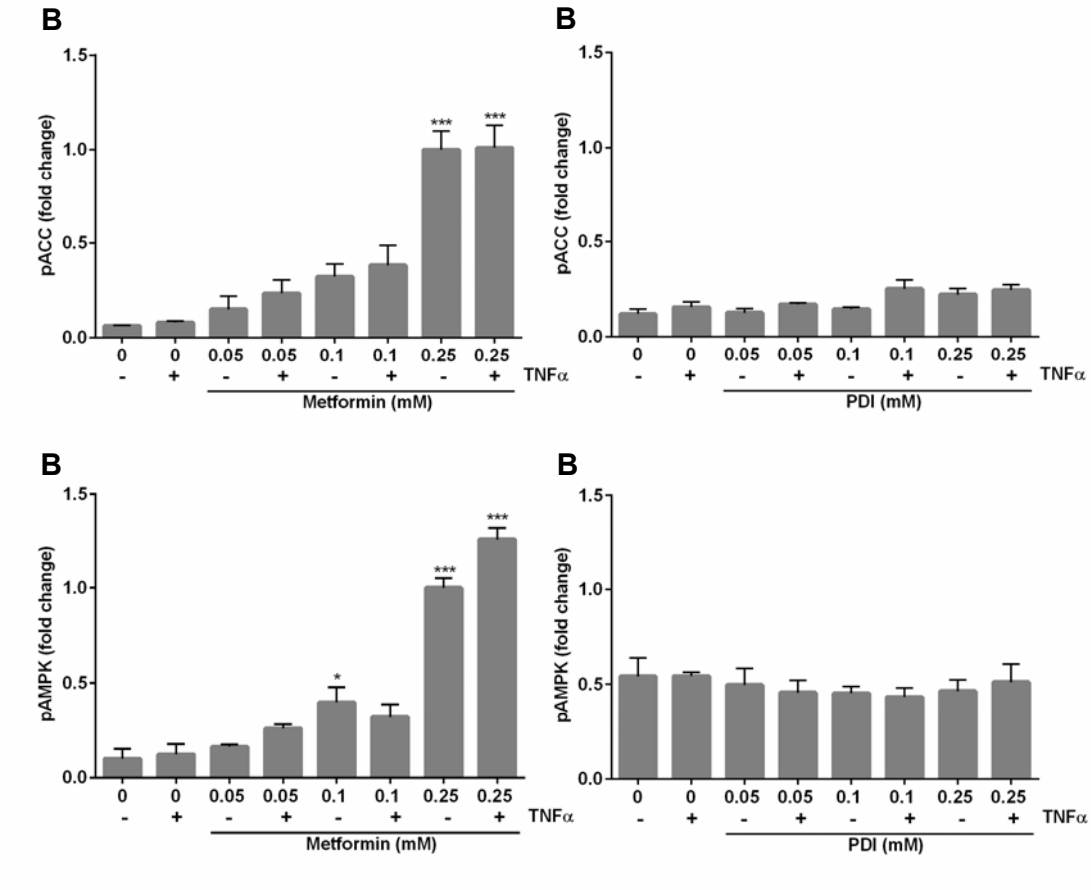


Supplementary Fig. V



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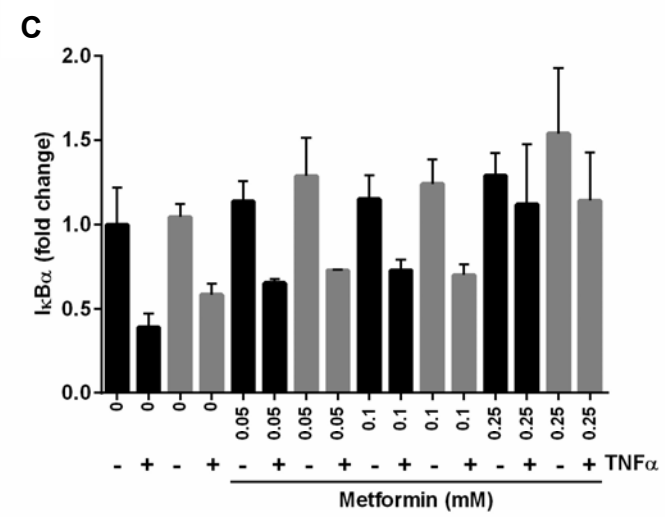

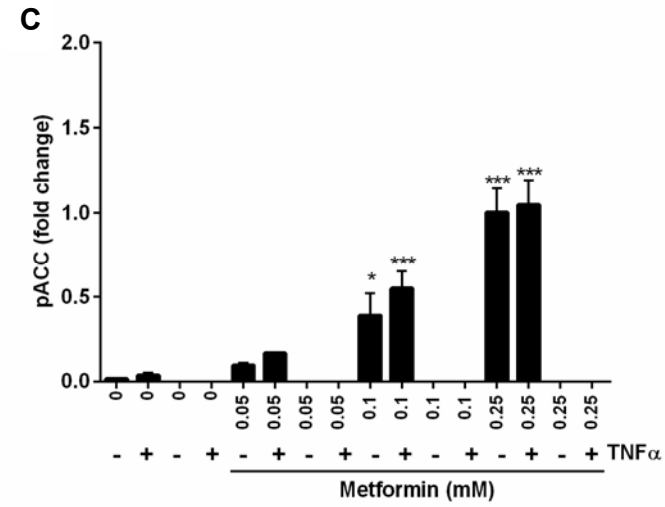

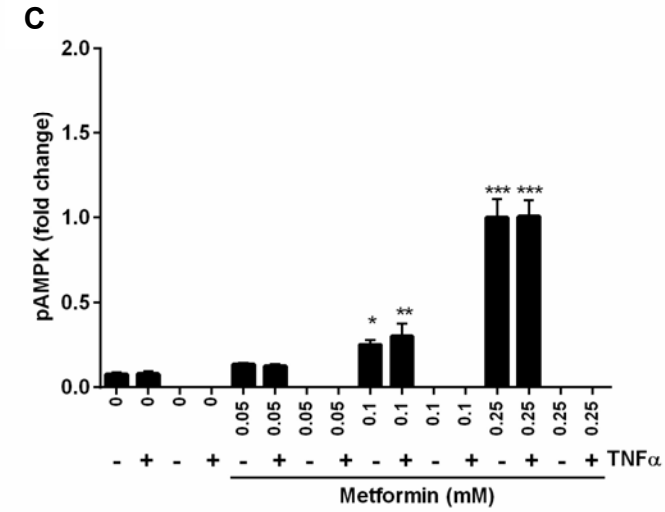

Supplementary Fig. V