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ARTICLE

Luteolin reduces obesity-associated insulin resistance in mice by activating AMPKα1 signalling in adipose tissue macrophages

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Abstract
Aims/hypothesis Inflammatory polarisation of adipose tissue macrophages (ATMs) plays a critical role in the development of obesity-associated metabolic diseases such as insulin resistance and diabetes. Our previous study indicated that dietary luteolin (LU) could prevent the establishment of insulin resistance in mice fed a high-fat diet (HFD). Here, we further investigated the effects of LU, which is a natural flavonoid, on pre-established insulin resistance and obesity-associated ATM polarisation in mice.
Methods Five-week-old C57/BL6 mice were fed on a low-fat diet or HFD for 20 weeks, with some mice receiving supplementation with 0.01% LU from weeks 1 or 10 of the HFD to assess the actions of LU on insulin resistance and ATM polarisation. Furthermore, the role of LU in metabolic-dysfunction-associated macrophage phenotypes was investigated in vitro.
Results Dietary LU supplementation, either for 20 weeks or from weeks 10 to 20 of an HFD, significantly improved insulin resistance in HFD-fed mice. In addition, inflammatory macrophage infiltration and polarisation were suppressed in mouse epididymal adipose tissues. Furthermore, LU treatment directly reversed lipopolysaccharide-stimulated and metabolism-regulated molecules, and induced inflammatory polarisation in mouse RAW264.7 cells and peritoneal cavity resident macrophages. Finally, using the selective AMP-activated protein kinase (AMPK) inhibitor compound C and *Ampkα1* (also known as *Prkaa1*) silencing with siRNA, we found that LU activated AMPKα1 in macrophages to inhibit their inflammatory polarisation and enhanced insulin signals in adipocytes that were stimulated with macrophage-conditioned media.
Conclusions/interpretation Dietary LU ameliorated insulin resistance in diet-induced obese mice by promoting AMPKα1 signalling in ATMs.

Keywords Adipose tissue macrophage · AMPKα1 · Insulin resistance · Luteolin · Polarisation

Electronic supplementary material The online version of this article (doi:10.1007/s00125-016-4039-8) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Abbreviations

ABCA1	ATP-binding cassette sub-family A member 1
AICAR	5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMPK	AMP-activated protein kinase
ATM	Adipose tissue macrophage
CM	Conditioned media
DIO	Diet-induced obesity
EAT	Epididymal adipose tissue
HFD	High-fat diet
LFD	Low-fat diet
LPS	Lipopolysaccharide
LU	Luteolin
MMe	Metabolic activation macrophage
PCM	Peritoneal cavity resident macrophage
PIG	Palmitate insulin and glucose
siRNA	Small interfering RNA
SVF	Stromal vascular fraction

Introduction

Obesity, of which there is a worldwide epidemic, is fundamentally caused by a long-term energy imbalance. During the development of diet-induced obesity (DIO), overnutrition leads to adipocyte hypertrophy and progressive inflammatory cell infiltration into adipose tissues. Hypertrophic adipocytes and inflammatory cells secrete various proinflammatory cytokines and promote adipose tissue and systemic inflammation, finally resulting in insulin resistance and type 2 diabetes [1–3]. However, not every obese individual suffers from insulin resistance and type 2 diabetes, and not all body fat contributes equally to these common metabolic diseases. When responding to a high-fat diet (HFD), visceral adipose tissues undergo a greater degree of inflammatory cell recruitment and higher tissue inflammation compared with subcutaneous adipose tissues. Therefore, abdominal obesity and visceral fat, but not subcutaneous fat, are associated with a high risk of metabolic diseases [4–6].

Of all the immune cells, adipose tissue macrophages (ATMs) have been paid a great deal of attention and are regarded as critical to the development of obesity-associated inflammation and insulin resistance [7, 8]. Epididymal adipose tissue (EAT) has been reported to be the most commonly used visceral fat depot in mouse studies, and its macrophage infiltration is most severe during the development of DIO [9]. In the lean state, EAT macrophages are mainly alternatively activated (M2) macrophages, which are characterised by CD206 and arginase 1. M2 cells produce anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist, and maintain insulin sensitivity [10]. In contrast, classically activated (M1) macrophages express CD11c and nitric oxide synthase 2, and predominate in EATs of obese animals. These cells produce and secrete high levels of proinflammatory cytokines such as TNF-α and IL-6, resulting in insulin resistance [11, 12]. Although this switch of M2 to M1 macrophages has been considered to be responsible for obesity-associated metabolic complications [13], ATM polarisation is more complex in obese states. Recently, Kratz et al used a mixture of palmitate, insulin and glucose (PIG) to produce a ‘metabolic activation’ macrophage (MMe) and reported the existence of this phenotype in EATs of obese mice [14]. This macrophage phenotype overexpresses the proinflammatory cytokines TNF-α, IL-1β and IL-6, but not M1 macrophage surface markers [14].

Luteolin (LU) is a natural flavonoid that is found in many edible plants, including peppers, parsley, spinach, carrots and celery. We have previously reported that a low-dose dietary supplement of LU could block the establishment of insulin resistance in HFD-fed mice [15]. In addition, dietary LU reduced macrophage recruitment and the expression of proinflammatory cytokines in EATs in the mice. Moreover, several in vitro studies have reported that LU suppresses the production of the proinflammatory cytokines TNF-α and IL-6 in macrophages [16–18]. However, it is not clear whether LU affects pre-established insulin resistance and obesity-associated ATM polarisation in mice.

AMP-activated protein kinase (AMPK) is a member of the metabolite-sensing protein kinase family and a crucial metabolic and inflammatory regulator [19, 20]. The catalytic subunit AMPKα1 is mainly expressed in macrophages and suppresses associated proinflammatory responses and polarisation [21, 22]. Previous studies have reported that LU can enhance the phosphorylation of AMPK in HepG2 cells [23] and 3T3-L1 adipocytes [24]. Therefore, the functional correlation between LU and AMPKα1 signalling in ATM inflammation and polarisation should be assessed.

The aim of this study was to evaluate whether LU ameliorated pre-established insulin resistance by inhibiting obesity-associated ATM polarisation in mice.

Methods

Animals Three-week-old male C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China) and housed in ventilated cages within a pathogen-free barrier facility that maintained a 12 h light:12 h dark cycle. A total of 16 mice were fed an HFD from the age of 5 weeks. At 10 weeks, these mice were randomly divided into two groups: (1) an HFD group ($n = 8$), in which the animals were continued on the HFD; and (2) an HFD + LU10 group ($n = 8$), in which the animals were switched onto an HFD supplemented with 0.01% LU (Huayi Biotechnology, Shanghai, China). In addition, a further eight 5-week-old mice were fed on a low-fat diet (LFD group), and eight 5-week-old mice were fed on an HFD supplemented with 0.01% LU for 20 weeks (HFD + LU20 group). The detailed contents of all of the diets are shown in the electronic supplementary material (ESM) Table 1.

The mice were weighed every week. At 15 and 25 weeks of age, the mice were fasted overnight and a GTT and insulin tolerance test were performed as previously described [15]. At 25 weeks of age, all mice were killed with CO_2 and their blood and adipose tissues were harvested and stored at −80°C, as previously described [25]. All experimental procedures were approved by the Standing Committee on Animals of Hefei University of Technology.

Protein extraction and western blot analysis Protein extraction and immunoblots were performed as previously described [25]. See ESM Methods.

Total RNA isolation and quantitative real-time PCR Quantitative real-time PCR was used to determine the relative expression levels of mRNAs. See ESM Methods.

Immunohistochemistry and ELISA Immunohistochemistry stains and ELISA were prepared as previously described [15]. See ESM Methods.

EAT stromal vascular fraction (SVF) isolation Stromal vascular cells were isolated as previously described [26], with some minor modifications. See ESM Methods.

Flow cytometry analysis Flow cytometry analysis was conducted to determine macrophage polarisation. See ESM Methods.

Small interfering (si)RNA siRNA was used to silence *Ampkα1* (also known as *Prkaa1*). See ESM Methods.

Cell culture and treatment RAW264.7 macrophages were purchased from the Cell Culture Center of Peking Union Medical College (Beijing, China) and cultured in low D-glucose (1 g/l) DMEM supplemented with 10% FBS. Peritoneal cavity cells were harvested from 8-week-old male C57BL/6 mice. Using APC anti-mouse F4/80 antibody (BioLegend, San Diego, CA, USA), PCMs were sorted using a MoFlo XDP flow cytometer (Beckman Coulter, Fullerton, CA, USA) and cultured in low D-glucose (1 g/l) DMEM supplemented with 10% FBS.

RAW264.7 cells and PCMs were planted in 24-well plates and pretreated with vehicle, 20 μmol/l LU or 2 mmol/l of the AMPKα1 activator AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; Sigma-Aldrich, St. Louis, MO, USA) for 12 h. To examine the effects of LU on macrophage M1 polarisation, the cells were stimulated with 100 ng/ml LPS (Sigma-Aldrich) according to previously published reports [14, 25]. The MMe phenotype polarisation method was performed according to the procedures described by Kratz et al [14]. These cells were treated with PIG (100 μmol/l palmitate, 10 nmol/l insulin and 30 mmol/l glucose). In addition, compound C (1 μmol/l) was added as an AMPK inhibitor 30 min before LU or AICAR treatment.

Following the method of Ceppo et al [27], with minor modifications, a series of conditioned media (CM) were produced from LPS- and PIG-stimulated RAW264.7 cells and PCMs. Briefly, LU or/and AMPK inhibitor or siRNA was used to treat the macrophages, as described above. The pretreated macrophages were then stimulated with LPS or PIG. After 3 h, the macrophages were washed and incubated in fresh culture medium for 24 h.

3T3-L1 pre-adipocytes were purchased from the Cell Culture Center of Peking Union Medical College and differentiated into adipocytes as previously described [28]. The differentiated adipocytes were cultured in the above-mentioned macrophage CM for 24 h, followed by stimulation with 1 nmol/l insulin for 7 min. The cells were then lysed for insulin signalling analysis.

Statistical analysis All data are presented as means ± SEM. One-way ANOVAs with Duncan's post hoc tests were used for mouse assays. Two-tailed Student's *t* tests were used for cell assays. $p < 0.05$ was considered as statistically significant.

Results

Dietary LU not only prevented the establishment of diet-induced insulin resistance, but also reversed pre-established insulin resistance To confirm the effects of LU on the establishment of diet-induced insulin resistance, 5-week-old male mice were fed with an LFD, HFD or HFD supplemented with 0.01% LU. After 10 weeks, the mice fed on an HFD had gained significantly more body weight than those fed with an LFD (Fig. 1a). The GTT and insulin tolerance test revealed that HFD-fed mice had lower glucose tolerance and insulin sensitivity than LFD-fed mice (ESM Fig. 1), suggesting that diet-induced insulin resistance was established. Supporting our previous results, dietary LU remarkably reduced HFD-induced body weight gain (Fig. 1a) and insulin resistance (ESM Fig. 1) in mice.

To test whether dietary LU also reversed pre-established insulin resistance, the mice fed with an HFD were divided into an HFD group and an HFD + LU10 group. The remaining mice (i.e. those in the LFD and HFD + LU20 groups) continued on their respective diets. As expected, the HFD mice continued to gain body weight (Fig. 1a) and had greater adipose tissue weight (ESM Fig. 2a, b), lower glucose tolerance (Fig. 1b) and lower insulin sensitivity (Fig. 1c) compared with the LFD group. Furthermore, the body weight and insulin resistance of the HFD + LU20 group were significantly improved compared with those of the HFD group. Intriguingly, glucose homeostasis (Fig. 1b) and insulin sensitivity (Fig. 1c) were also ameliorated in the HFD + LU10 group, although the body weight (Fig. 1a) and adipose tissue weight (ESM Fig. 2a, b) of these animals were not affected by dietary LU. During 20 weeks of intervention, dietary LU did not affect food intake among the HFD-fed mice (ESM Fig. 2c).

Insulin can promote Akt phosphorylation to regulate adipose tissue glucose uptake and systemic glucose homeostasis. Along with improvements in insulin resistance, mice in the HFD + LU20 and HFD + LU10 groups had significantly more robust Akt phosphorylation in their EATs, similar to those in LFD group (Fig. 1d). Adipose tissue inflammation directly suppresses insulin signalling to develop insulin resistance. Therefore, we further looked at several important proinflammatory cytokines involved in obesity-associated insulin resistance, including TNF-α, IL-6 and monocyte chemoattractant protein 1. Dietary LU downregulated the EAT mRNA expression (Fig. 1e) and serum levels (Fig. 1f) of these cytokines in HFD-fed mice,

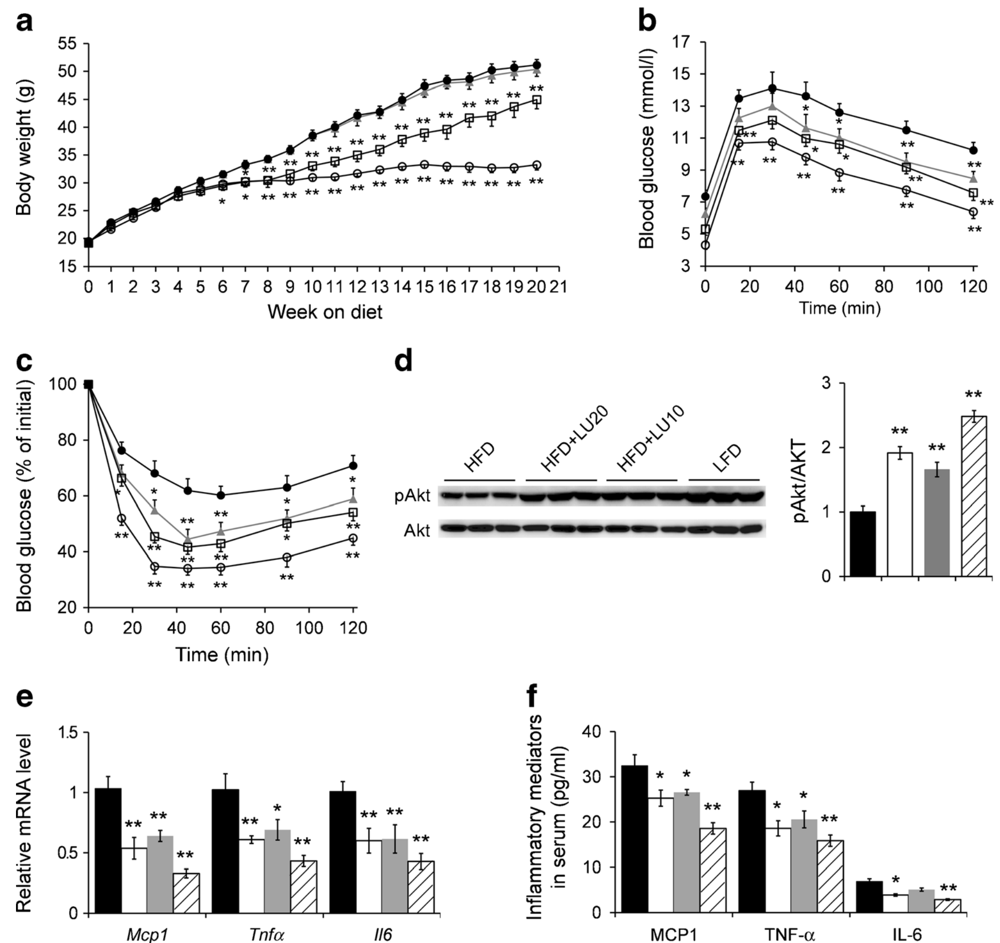

Fig. 1 Five-week-old mice were fed on an LFD (white circles and hatched bars) or HFD (black circles and black bars) for 20 weeks. In addition, mice of the same age were fed with an HFD supplemented with 0.01% LU during weeks 10–20 (HFD + LU10, grey triangles and grey bars) or weeks 1–20 (HFD + LU20, white squares and white bars) of the HFD. Body weight (**a**), and GTT (**b**) and insulin tolerance test (**c**) results at age 25 weeks. (**d**) Immunoblots for phospho-Akt (Ser473) and total Akt in EATs and quantification of phosphorylated Akt to total Akt proinflammatory cytokine mRNA levels in EATs (**e**) and proinflammatory mediators in serum (**f**). All data are presented as means ± SEM, $n = 8$ per group, one-way ANOVA with Duncan's post hoc test relative to HFD; $^*p < 0.05$, $^{**}p < 0.01$

suggesting a decline in adipose tissue and systemic inflammation. In conclusion, dietary LU not only resisted the establishment of diet-induced insulin resistance, but also ameliorated pre-established insulin resistance in HFD-fed mice.

Dietary LU abated HFD-induced macrophage infiltration into EATs Macrophages are the most abundant immune cells and the primary contributors to inflammation in adipose tissues in DIO mice. We used flow cytometry (Fig. 2a, ESM Fig. 3c) and macrophage-specific MAC-2 immunostaining (ESM Fig. 3a, b) to analyse EAT macrophage levels. The results indicated inhibition of macrophage infiltration by dietary LU.

Dietary LU reversed obesity-associated ATM polarisation Given ATM polarisation is important in obesity-associated adipose tissue inflammation and insulin resistance, we further investigated EAT macrophage subsets. Flow cytometry showed that an HFD elevated the percentages of CD11c$^+$F4/80$^+$ M1 macrophages and lowered those of CD206$^+$F4/80$^+$ M2 macrophages in EATs (Fig. 2a–d). Dietary LU markedly abolished M1 macrophage polarisation and enlarged the percentage of M2-type macrophages, resulting in a decline of the M1/M2 ratio in EATs in both the HFD + LU20 and HFD + LU10 groups (Fig. 2a–d). Recently, MMe was identified as a novel inflammatory macrophage phenotype in the adipose tissues of obese humans and mice. In this study, we also detected expression of the MMe markers *Cd36* and *Plin2* in EATs. An HFD resulted in the enhanced expression of these markers in EATs, while dietary LU abrogated the actions (Fig. 2e, f). These results suggest that dietary LU reverses both M1 and MMe polarisation in EATs in HFD-fed mice.

LU treatment directly inhibited M1 and MMe polarisation Since dietary LU inhibited obesity-associated ATM polarisation (Fig. 2), we attempted to investigate whether LU directly affects inflammatory macrophage polarisation, including LPS-stimulated M1 and PIG-induced MMe polarisation. As expected, the classic M1 mediator LPS and metabolic activation by PIG elevated the expression of the proinflammatory cytokine genes *Mcp1* (also known as *Ccl2*), *Tnf-a* (also known as *Tnf*) and *Il-6* in RAW264.7 cells and PCMs (ESM Fig. 4). LU (5–20 μmol/l) downregulated the expression of these genes in a dose-dependent manner (ESM Fig. 4).

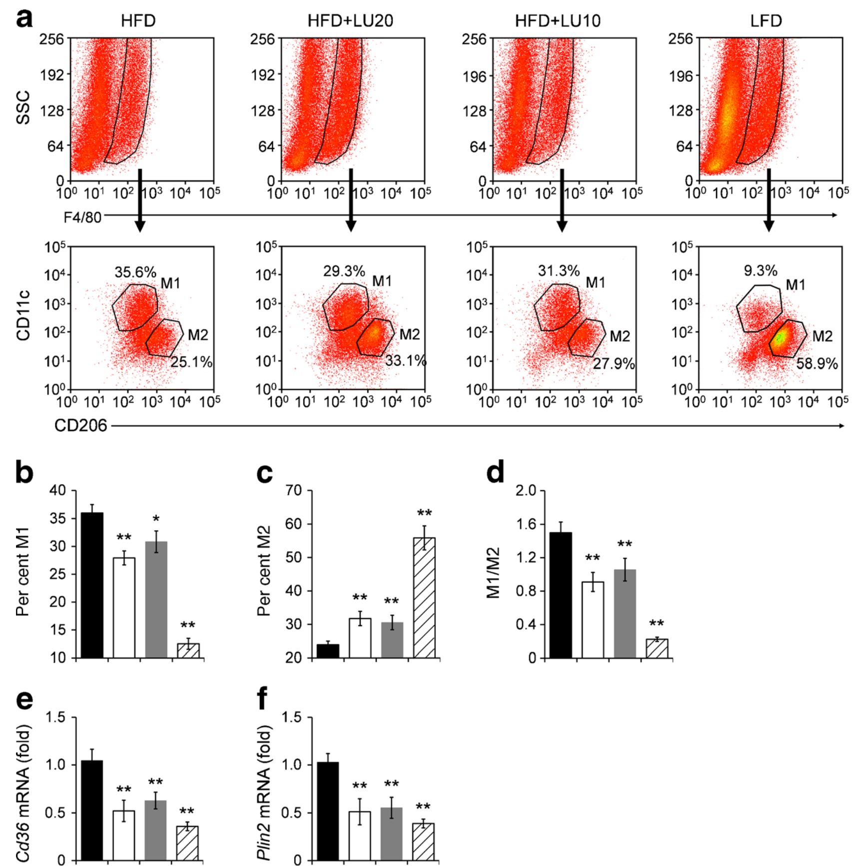

Fig. 2 Dietary LU inhibited obesity-associated ATM polarisation in EATs. (**a**) Using flow cytometry, F4/80$^+$CD11c$^+$ M1-type and F4/80$^+$CD206$^+$ M2-type macrophages from SVF cells in EATs were analysed. The percentage of M1- (**b**) and M2-type (**c**) macrophages in total SVF cells and the ratio of M1 to M2 cells (**d**). mRNA levels of the MMe markers *Cd36* (**e**) and *Plin2* (**f**) were quantified using quantitative real-time PCR. Black bars, HFD; white bars, HFD + LU20; grey bars, HFD + LU10; hatched bars, LFD. All data are presented as means ± SEM, $n = 8$ per group, one-way ANOVA with Duncan's post hoc test relative to HFD; $^*p < 0.05$, $^{**}p < 0.01$. SSC, side scatter

To explore whether LU treatment suppressed M1 macrophage polarisation, we further used 20 μmol/l LU to treat LPS-stimulated macrophages and determined the expressions of M1 and M2 subtype markers in the cells. Real-time PCR results showed that LPS enhanced the expression of the M1 marker *Nos2* and reduced the levels of the M2 marker *Arg1* in RAW264.7 cells (Fig. 3a, b) and PCMs (Fig. 3c, d). LU treatment significantly blocked these changes (Fig. 3a–d). Consistent with these results, flow cytometry showed that LU treatment also abolished LPS-induced overexpression of the M1 surface markers CD38 and CD274 in these cells (Fig. 3e–h).

Palmitate, insulin and glucose, as important regulated molecules during the development of metabolic diseases, can promote the MMe macrophage phenotype in vitro We used PIG to induce MMe polarisation in RAW264.7 cells and PCMs and assessed the effects of LU on MMe polarisation. Similar to studies in human monocyte-derived macrophages, the MMe surface markers CD36 and/or ABCA1 (ATP-binding cassette sub-family A member 1) were upregulated by PIG in RAW264.7 cells (Fig. 4a, b) and PCMs (Fig. 4c, d). LU treatment clearly restored the efficiencies (Fig. 4a–d). In addition, LU treatment also reduced the overexpression of the MMe-specific genes *Cd36* and *Plin2* in the macrophages (Fig. 4e–h).

LU activated AMPKα1 signalling to reduce inflammatory macrophage polarisation AMPK is an important nutrient sensor and inflammatory suppressor. In macrophages, AMPKα1 signalling critically regulates metabolism-related inflammation and polarisation. To explore the involvement of AMPKα1 signalling in LU-associated reductions in ATM polarisation and inflammation, we first detected phosphorylated AMPKα1 levels in mouse EATs. Compared with mice in the HFD group, those in the LFD, HFD + LU10 and HFD + LU20 groups had obviously higher AMPKα1 phosphorylation (Fig. 5a), implying that dietary LU might suppress inflammatory ATM polarisation by activating AMPKα1 signalling.

In LPS-stimulated (Fig. 5b) and PIG-stimulated (Fig. 5c) conditions, both LU and the AMPK activator AICAR significantly reversed the inhibitory effects of the proinflammatory reagents on AMPKα1 phosphorylation in RAW264.7 macrophages and PCMs. Similarly, the selective AMPK inhibitor compound C abated AMPKα1 activation by LU and AICAR (Fig. 5b, c). Furthermore, both LU and AICAR treatment also counteracted LPS- and PIG-induced overexpression of the proinflammatory cytokine genes *Mcp1*, *Tnf-a* and *Il-6* in RAW264.7 cells and PCMs (ESM Fig. 5). Polarisation-associated alterations in macrophage markers, including the M1 marker *Nos2*, the M2 marker *Arg1* (Fig. 6a–d) and the MMe markers *Cd36* and *Plin2*

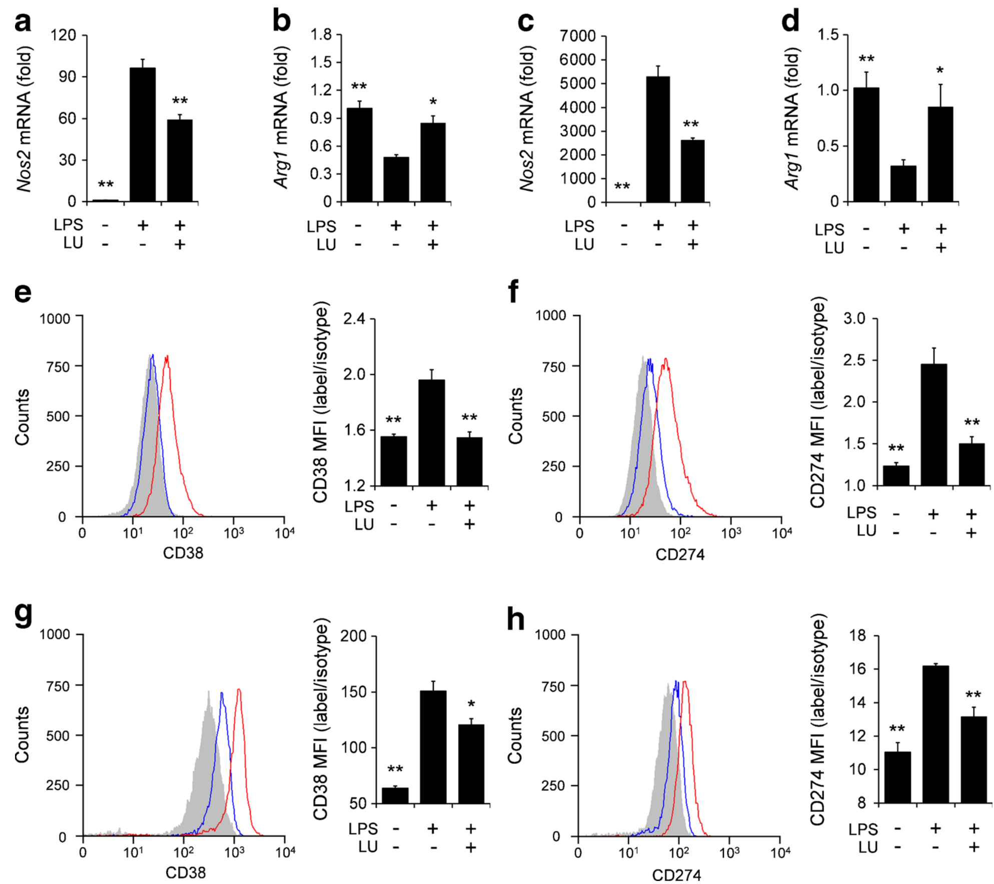

Fig. 3 LU repressed LPS-induced M1 macrophage polarisation. mRNA levels of the M1 marker *Nos2* and the M2 marker *Arg1* were detected in RAW264.7 cells (**a**, **b**) and PCMs (**c**, **d**). Expression of the M1-type macrophage markers CD38 and CD274 was detected using flow cytometry in RAW264.7 cells (**e**, **f**) and PCMs (**g**, **h**). Grey areas, control; red lines, LPS; blue lines, LPS + LU. All data are presented as means ± SEM, $n = 4$ per group, two-tailed Student's t test relative to LPS treatment; $*p < 0.05$, $**p < 0.01$

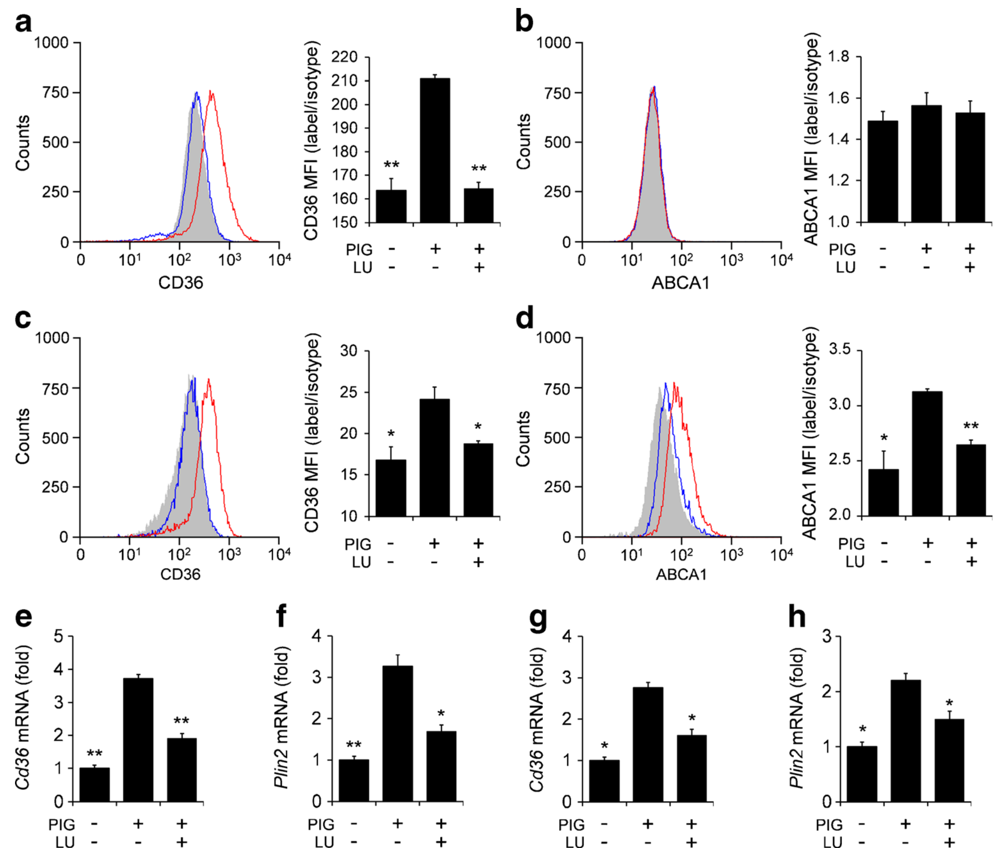

Fig. 4 LU suppressed PIG-induced MMe polarisation. Expression of the MMe markers CD36 and ABCA1 was detected by flow cytometry in RAW264.7 cells (**a**, **b**) and PCMs (**c**, **d**). mRNA levels of the MMe markers *Cd36* and *Plin2* in RAW264.7 cells (**e**, **f**) and PCMs (**g**, **h**) were quantified using quantitative real-time PCR. Grey areas, control; red lines, LPS; blue lines, LPS + LU. All data are presented as means ± SEM, $n = 4$ per group, two-tailed Student's t test relative to PIG treatment; $*p < 0.05$, $**p < 0.01$

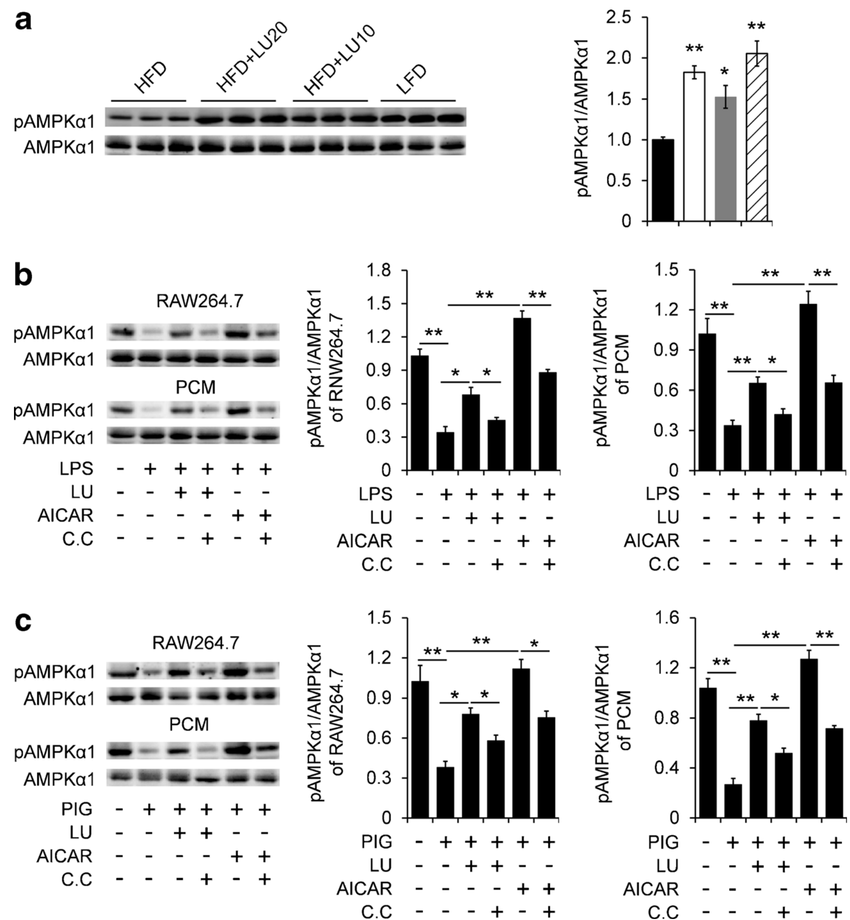

Fig. 5 LU activated AMPKα1 signalling in inflammatory macrophages. (**a**) Dietary LU increased AMPKα1 activity in EATs in HFD-fed mice. Black bars, HFD; white bars, HFD + LU20; grey bars, HFD + LU10; hatched bars, LFD. $n = 8$ per group, one-way ANOVA with Duncan's post hoc test relative to HFD; $*p < 0.05$, $**p < 0.01$. Immunoblots for phospho-AMPKα1 and total AMPKα1 and quantification of phosphorylated AMPKα1 to total AMPKα1 in LPS-treated (**b**) or PIG-treated (**c**) RAW264.7 cells and PCMs. C.C, compound C. Data are presented as means ± SEM, $n = 4$ per group, two-tailed Student's t test; $*p < 0.05$, $**p < 0.01$

(Fig. 6e–h), were also turned back by LU and AICAR treatment. Finally, compound C treatment (Fig. 6a–h, ESM Fig. 5) and *Ampkα1* silencing (Fig. 6i–p, ESM Figs 6, 7) could abrogate the actions of LU.

LU treatment ameliorated insulin sensitivity in CM-simulated 3T3-L1 adipocytes by activating AMPK signalling RAW264.7 and PCM CM significantly reduced the ratio of p-Akt to total Akt in differentiated 3T3-L1 adipocytes (Fig. 7). LU pretreatment of macrophages blunted the decline of Akt phosphorylation (Fig. 7). Furthermore, compound C or *Ampkα1* silencing abolished the actions of LU (Fig. 7).

Discussion

This study demonstrates that dietary LU can not only interfere with the establishment of HFD-induced insulin resistance, but also ameliorate pre-established insulin resistance and adipose tissue inflammation. Furthermore, LU antagonised M1 or MMe macrophage polarisation in EATs in HFD-fed mice. In addition, in vitro and in vivo studies showed that LU directly suppressed inflammatory macrophage polarisation by activating AMPKα1 signalling. These findings suggest that dietary LU reduces obesity-associated insulin resistance by activating AMPKα1 signalling in ATMs in HFD-fed mice.

LU possesses various bioactivities, including anti-inflammatory, anti-cancer and anti-oxidation activities [29, 30]. In rat models of streptozotocin-induced type 1 diabetes, LU administration has been reported to reduce blood glucose levels [31] and ameliorate diabetic cardiomyopathy [32] and nephropathy [31], suggesting its potency in improving glucose and lipid metabolism in animals. In addition, LU supplementation in mice fed with an HFD during the establishment of DIO has previously been reported to protect the animals from HFD-induced insulin resistance [15]. However, the effects of LU on pre-established insulin resistance in HFD-fed mice remain unclear. In the current study, we established insulin resistance with a 10-week HFD (ESM Fig. 1), and then supplemented the HFD with LU. Compared with the HFD group, the HFD + LU10 group showed significantly higher glucose tolerance and insulin sensitivity (Fig. 1b–d). Our results reveal that dietary LU not only resisted the establishment of HFD-induced insulin resistance, but also reversed pre-established insulin resistance.

Visceral adipose tissue inflammation critically promotes local and systemic insulin resistance [5, 33]. Various immune

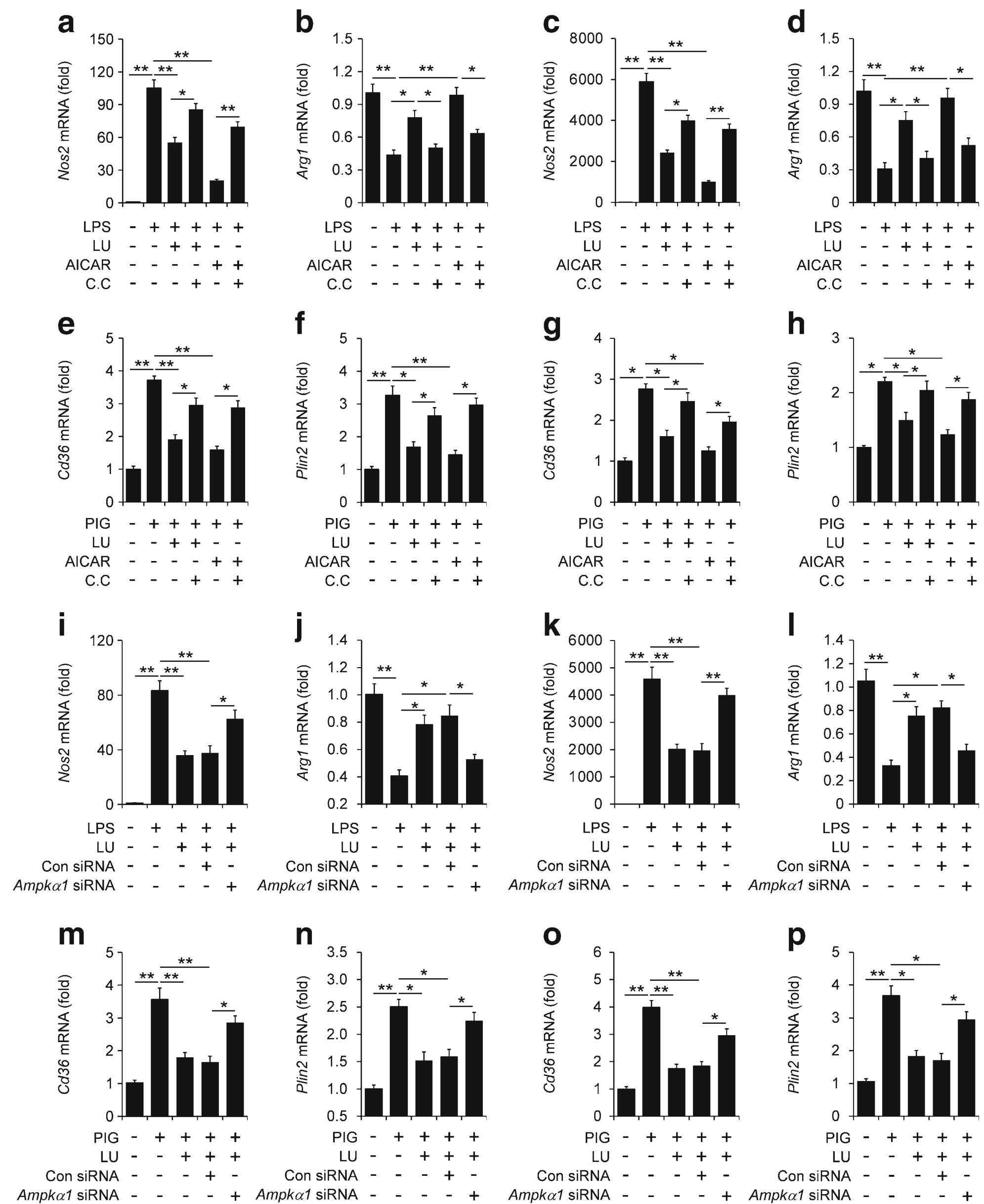

Fig. 6 LU blocked macrophage inflammatory polarisation through activating AMPKα1 signalling. mRNA levels of the M1 marker *Nos2* and the M2 marker *Arg1* were detected in LPS-stimulated RAW264.7 cells (**a**, **b**) and PCMs (**c**, **d**). mRNA levels of the MMe markers *Cd36* and *Plin2* in PIG-treated RAW264.7 cells (**e**, **f**) and PCMs (**g**, **h**). *Ampk* siRNA abrogated the actions of LU on the mRNA expression of *Nos2* and *Arg1* in LPS-stimulated RAW264.7 cells (**i**, **j**) and PCMs (**k**, **l**), and of MMe marker genes in PIG-treated RAW264.7 cells (**m**, **n**) and PCMs (**o**, **p**). All data are presented as means ± SEM, $n=4$ per group, two-tailed Student's t test; $*p<0.05$, $**p<0.01$. C.C, compound C. Con, control

cells, including macrophages, mast cells, neutrophils, eosinophils and B and T cells, have been implicated in inflammatory regulation of adipose tissues [5]. In the immune cells, macrophage infiltration and polarisation primarily contribute to adipose tissue inflammation and insulin resistance [7, 8]. Furthermore, other immune cells can affect adipose tissue inflammation by regulating macrophage polarisation [5, 34]. Moreover, in DIO mice, EATs undergo greater macrophage recruitment than other visceral and subcutaneous adipose tissues [9]. Therefore, we proposed that dietary LU should reduce macrophage inflammation and polarisation in EATs in HFD-fed mice.

Our results support this hypothesis. First, mice in the HFD + LU10 and HFD + LU20 groups had lower expression of inflammatory cytokines in EATs than mice in the HFD group (Fig. 1e). Second, dietary LU reduced macrophage recruitment into EATs in HFD-fed mice (Fig. 2a, ESM Fig. 3). Third, the M1/M2 ratio was modified in EATs in mice fed with an LU-containing HFD (Fig. 2a–d). Finally, dietary LU also downregulated expression of the MMe markers *Cd36* and *Plin2* in EATs (Fig. 2e, f). In conclusion, inflammatory macrophage polarisation, including M1 and MMe polarisation, was inhibited by dietary LU in EATs.

Since other immune cells can regulate macrophage polarisation, the direct effects of LU on metabolic dysfunction-associated macrophage polarisation should be investigated. The ATM phenotype undergoes intricate alterations during the development of obesity. LPS stimulation is a classical method of promoting M1 polarisation in vitro [35]. However, the surface markers of LPS-induced M1

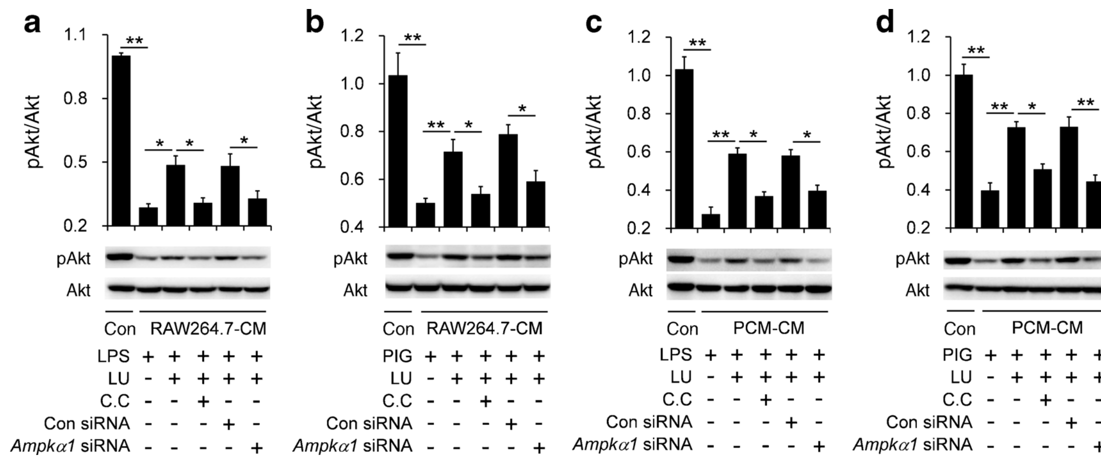

Fig. 7 LU enhanced insulin sensitivity in CM-stimulated 3T3-L1 adipocytes by activating macrophage AMPKα1 signalling. Immunoblots for phospho-Akt and total Akt were performed in 3T3-L1 adipocytes that were stimulated with CM from RAW264.7 cells (**a**, **b**) or PCMs (**c**, **d**). All data are presented as means ± SEM, $n = 4$ per group, two-tailed Student's t test; $*p < 0.05$, $**p < 0.01$. C.C, compound C; Con, control

macrophages are distinct from those of inflammatory ATMs in obese humans and mice [14]. Moreover, LPS stimulation also suppresses the expression of CD11c, which is the most common marker of M1 macrophages in adipose tissue [36]. Therefore, in addition to LPS-stimulated macrophages, we need to use other obesity-associated macrophage phenotypes. Relative to LPS, metabolic-disease-regulated molecules such as glucose, insulin and palmitate induce an inflammatory MMe phenotype. The markers of the MMe subtype, including CD36, ABCA1 and PLIN2, are more similar to ATMs in obese individuals [14]. Thus, we used LPS- and PIG-induced macrophages to further assess whether LU can directly regulate ATM polarisation. We established both LPS-induced (Fig. 3, ESM Fig. 4) and PIG-induced (Fig. 4, ESM Fig. 4) inflammatory macrophage phenotypes in the commercial RAW264.7 cell line and primary cultured PCMs. LU treatment remarkably inhibited inflammatory cytokine expression (ESM Fig. 4) and reversed alterations in M1, M2 and MMe markers in the cells (Figs 3, 4). These results indicate that LU treatment directly suppresses obesity-associated macrophage polarisation, and thereby maintains insulin sensitivity in HFD-fed mice.

AMPKα1 plays an important role in the suppression of proinflammatory responses and macrophage inflammatory polarisation [21, 22]. Accompanied by improvements in insulin resistance and ATM inflammation, mice in the HFD + LU10 and HFD + LU20 groups also showed increased EAT AMPKα1 signalling (Fig. 5a). Furthermore, LU treatment blocked LPS- and PIG-induced inactivation of AMPKα1 (Fig. 5b, c) and reserved the polarised alterations (Fig. 6) in RAW264.7 cells and PCMs. Moreover, both selective AMPK inhibitor compound C and *Ampkα1* silencing could abolish the effects of LU on inflammatory macrophage polarisation and associated insulin signalling in adipocytes (Figs 5, 6, 7, ESM Figs 5–7). These results suggest that AMPKα1 is critically involved in LU-reducing ATM polarisation and insulin resistance in HFD-fed mice, although the detailed molecular mechanisms behind macrophage AMPKα1 activation by LU need further investigation.

Although we found, in the current study, that LU treatment could abate inflammatory macrophage polarisation and reduce CM-induced insulin resistance by activating AMPKα1, the improvements in insulin resistance in HFD-fed mice might not be solely attributed to the inhibition of ATM polarisation and inflammation by LU. It has previously been reported that LU can activate AMPK signalling in differentiated 3T3-L1 adipocytes [24] and HepG2 hepatocytes [23]. In addition, AMPK, as an important nutrient sensor, can promote fatty acid oxidation [37] and energy expenditure [38]. Indeed, in the HFD + LU20 group, dietary LU not only suppressed EAT macrophage polarisation (Fig. 2), but also reduced body and subcutaneous adipose tissue weights (ESM Fig. 2). Therefore, LU might also enhance adipocyte AMPK signalling and associated fatty acid oxidation and energy expenditure in HFD-fed mice, a hypothesis that merits further investigation.

In conclusion, dietary LU inhibits ATM inflammatory polarisation in HFD-fed mice, suggesting its potential in remedying and intervening in common metabolic disorders.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement LZ and JL conceived and designed the study. LZ and YH acquired, analysed and interpreted data and revised the article critically for important intellectual content. XW and XZ analysed and interpreted data and revised the article critically for important intellectual content. BB and WQ interpreted data and revised the article critically for important intellectual content. LZ and JL analysed and interpreted data and drafted and revised the article. JL has primary responsibility for the final content. All authors read and approved the final manuscript.

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