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Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues

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Abstract. Arias de la Rosa I, Escudero-Contreras A, Rodríguez-Cuenca S, Ruiz-Ponce M, Jiménez-Gómez Y, Ruiz-Limón P, Pérez-Sánchez C, Ábalos-Aguilera MC, Cecchi I, Ortega R, Calvo J, Guzmán-Ruiz R, Malagón MM, Collantes-Estevez E, Vidal-Puig A, López-Pedrera C, Barbarroja N (IMIBIC/Reina Sofia Hospital/University of Cordoba, Spain; Center of Research of Immunopathology and Rare Diseases-Coordinating Center of Piemonte and Valle d'Aosta Network for Rare Diseases, Turin, Italy; Instituto de Salud Carlos III, Madrid, Spain; and University of Cambridge, Cambridge, UK). Defective glucose and lipid metabolism in rheumatoid arthritis is determined by chronic inflammation in metabolic tissues. *J Intern Med* 2018; https://doi.org/10.1111/joim.12743

Background. Rheumatoid arthritis (RA) patients are at increased risk of insulin resistance (IR); however, the specific mechanisms mediating this association are currently unknown.

Objective. To investigate whether the inflammatory activity associated with RA accounts for the observed defective glucose metabolism and lipid metabolism in these patients.

Methods. We followed two main strategies: (i) extensive metabolic profiling of a RA cohort of 100 patients and 50 healthy control subjects and (ii) mechanistic studies carried out in both a collagen-induced arthritis mouse model and 3T3-L1 adipocytes treated with conditioned serum from RA patients.

Results. Following the exclusion of obese and diabetic subjects, data from RA patients demonstrated a strong link between the degree of systemic inflammation and the development of IR. These results were strengthened by the observation that induction of arthritis in mice resulted in a global inflammatory state characterized by defective carbohydrate and lipid metabolism in different tissues. Adipose tissue was most susceptible to the RA-induced metabolic alterations. These metabolic effects were confirmed in adipocytes treated with serum from RA patients.

Conclusions. Our results show that the metabolic disturbances associated with RA depend on the degree of inflammation and identify inflammation of adipose tissue as the initial target leading to IR and the associated molecular disorders of carbohydrate and lipid homeostasis. Thus, we anticipate that therapeutic strategies based on tighter control of inflammation and flares could provide promising approaches to normalize and/or prevent metabolic alterations associated with RA.

Keywords: adipose tissue, inflammation, insulin resistance, molecular pathways, rheumatoid arthritis.

Introduction

Patients with rheumatoid arthritis (RA) have an increased risk of developing cardiovascular

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disease (CVD), and CVD is the leading cause of morbidity and mortality in these patients [1, 2]. The traditional risk factors do not fully account for the increased CVD risk in RA patients, suggesting that additional mechanisms may be pathogenically more relevant in these patients. Thus, the disease itself could constitute an independent risk factor for the development of CVD in patients with RA [3, 4].

Rheumatoid arthritis patients exhibit a cluster of CVD risk factors [5] including insulin resistance (IR), type 2 diabetes mellitus and dyslipidaemia, with an increased prevalence of metabolic syndrome (up to 40%) [6, 7]. The reverse relationship has also been observed: patients diagnosed with metabolic syndrome seem to have an increased risk of RA [6]. Furthermore, the prevalence of IR is increased in patients with RA in comparison with the general population. Recent studies indicate an association between IR and either increased body mass index (BMI) (probably due to inadequate physical activity) or prolonged glucocorticoid therapy in RA patients [7, 8].

Insulin resistance is associated with metabolic factors dysregulated in the context of overnutrition as well as with lipotoxicity (i.e. ectopic lipid accumulation in peripheral organs other than adipose tissue) and in many cases with an inflammatory component. Excessive and/or inappropriate accumulation of lipids can trigger inflammatory responses that contribute to the development of IR [9]. Conversely, it is conceivable that inflammation-induced IR may be exacerbated in individuals whose immune cells exhibit a relatively low threshold to respond to inflammatory triggers and/or a robust amplification of the inflammatory cascades [10, 11]. Thus, we hypothesized that inflammatory pathogenic mediators involved in RA may also contribute to facilitate the development of IR in these patients. The specific molecular mechanisms governing the dysfunction of the homeostatic processes controlling glucose metabolism and lipid metabolism in RA have not yet been elucidated. This is the first study in which the pathogenic effect of systemic inflammation to disturb insulin sensitivity and lipid metabolism in RA patients has been investigated. We explored this pathogenic process using multiple approaches *in vivo*, *ex vivo* and *in vitro*, combining the characterization of a cohort of RA patients, a mouse model of collagen-induced arthritis (CIA) and studies in murine 3T3-L1 adipocytes.

Methods

Patients

In total, 100 RA patients and 50 healthy control subjects matched for age, gender and BMI were included in this study. RA patients fulfilled at least four 1987 American College of Rheumatology (ACR) disease criteria and achieved a total score of ≥6 according to 2010 ACR classification [12, 13]. To avoid the effects of increased BMI and diabetes on IR, obese (BMI > 30 kg m^{-2}) and diabetic subjects (fasting blood glucose levels >126 mg dL^{-1}, haemoglobin A1c level >6.5% or antidiabetic medication) were excluded. Patients were receiving the following treatments: corticosteroids [low doses (5.0–7.5 mg), 94.5% deflazacort and 5.5% prednisone], antimalarials, nonsteroidal anti-inflammatory drugs (NSAIDs) and methotrexate. Tests were performed in all patients to determine the presence of anticyclic citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF). Disease activity score in 28 joints (DAS28) was determined following the guidelines of the ACR. Moderate–high disease activity was defined as DAS28 > 3.2 [14]. None of the healthy controls had a history of other autoimmune diseases, atherothrombosis or thrombosis.

All participants enrolled were Caucasian and recruited at the Department of Rheumatology, Reina Sofia University Hospital, Cordoba, Spain.

Metabolic features (lipid profile, BMI, glucose and insulin), disease activity and disease-modifying antirheumatic drug (DMARD) and glucocorticoid therapy were recorded (Table 1). DAS28 variables comprised erythrocyte sedimentation rate (ESR), swollen joint count (in 28 joints), tender joint count (in 28 joints) and patient assessment of disease activity (measured on a 0- to 100-mm visual analogue scale).

Blood samples collected from patients following fasting for 8 h were used for laboratory tests. The homeostasis model assessment (HOMA)-IR index was used to measure IR: [blood insulin concentration (mU L^{-1}) × blood glucose concentration (mg dL^{-1})]/405. HOMA-IR values >2.5 indicated IR [15, 16].

Table 1 *Clinical characteristics of the RA patients and healthy donors*

	RA patients (n = 100)	Healthy donors (n = 50)
Clinical parameters		
Female (n)/male (n)	75/25	38/12
Age (years)	54.93 ± 13.94	46.06 ± 10.08
Disease duration (years)	6.30 ± 5.75	–
RF positive (%)	55	–
ACPAs (%)	66	–
Tender joints (n)	2.5 ± 2.16	–
Swollen joints (n)	4.2 ± 7.9	–
DAS28	2.86 ± 0.96	–
Smoker (%)	25	22
BMI (kg m^{-2})	23.00 ± 2.93	24.36 ± 2.32
Comorbidities		
Hypertension (%)	18.0[a]	2.0
Insulin resistance (%)	15.0[a]	6.0
Metabolic syndrome (%)	7.0	5.0
Laboratory parameters		
Glucose (mg dL^{-1})	90.54 ± 19.96[a]	83.25 ± 9.40
Insulin (mg dL^{-1})	7.71 ± 3.91[a]	6.20 ± 3.39
Cholesterol (mg dL^{-1})	196.44 ± 31.16	125.53 ± 32.04
HDL cholesterol (mg dL^{-1})	59.75 ± 15.38	56.98 ± 14.41
LDL cholesterol (mg dL^{-1})	119.38 ± 23.54	125.53 ± 32.04
Triglycerides (mg dL^{-1})	90.92 ± 38.67	84.64 ± 44.64
ESR (mm h^{-1})	14.90 ± 9.83[a]	7.75 ± 4.33
CRP (mg dL^{-1})	12.33 ± 2.70[a]	1.30 ± 1.60
Treatments		
Corticosteroids (%)	37.0	–
Antimalarials (%)	41.0	–
NSAIDs (%)	68.0	–
Methotrexate (%)	60.0	–
Leflunomide (%)	32.0	–

Values are means ± SD, unless otherwise stated.
ACPAs, anticyclic citrullinated protein; BMI, body mass index; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NSAID, nonsteroidal anti-inflammatory drug; RA, rheumatoid arthritis; RF, rheumatoid factor.
[a]Significant difference vs. healthy donors ($P < 0.01$).

CIA mouse model

All animal experiments were carried out in accordance with the ARRIVE guidelines and with the UK Animals (Scientific Procedures) Act, 1986, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Twenty-five DBA1/J male mice (7–8 weeks old) were used in this study. Five mice were used as healthy controls, and 20 mice were injected subcutaneously with collagen/complete Freund's adjuvant emulsion (100 μg per mouse); on day 21, mice were boosted with a mixture of collagen solution and incomplete Freund's adjuvant emulsion (100 μg per mouse). Between days 22 and 42, macroscopic signs of arthritis were scored three times weekly, where each paw received a score: 0 = no visible effects of arthritis; 1 = oedema and/or erythema of one digit; 2 = oedema and/or erythema of two digits; 3 = oedema and/or erythema of more than two digits; and 4 = severe arthritis of entire paw and digits. The arthritic index (AI) was calculated by addition of individual paw scores (up to maximum of 16). Diseased mice were classified into two groups according to the AI score: low disease, 1–4; and moderate–severe disease, 5–16. Mice were weighed daily. The CIA mouse model was generated by Washington Biotechnology Inc. (Baltimore, MD, USA). Next, mice were killed, and gonadal adipose tissue, skeletal muscle, buffy coat and plasma were isolated and frozen at −80 °C and shipped to our laboratory in Spain for gene and protein analyses.

Culture, differentiation and treatment of 3T3-L1 pre-adipocytes

3T3-L1 cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured, tested for mycoplasma contamination and differentiated into adipocytes according to the protocol described by Guzman-Ruiz *et al.* [17]. Differentiated cells were used only when at least 90% showed an adipocyte phenotype by accumulation of lipid droplets by day 8. On day 8 of differentiation, 3T3-L1 adipocytes were treated for 24 h with medium containing 10% inactivated serum (incubated at 56 °C for 30 min) from 12 healthy donors [C-reactive protein (CRP) 0.58 ± 0.48 mg mL^{-1}] and 12 nonobese and non-diabetic RA patients with moderate–high disease activity (DAS28 > 3.2 and CRP >5 mg mL^{-1}). The clinical characteristics of this second cohort of participants are shown in Table 2. Subsequently, cells were collected for protein and mRNA analyses.

All participants enrolled were Caucasian and recruited at the Department of Rheumatology, Reina Sofia University Hospital, and gave their informed consent.

Serum levels of TNF-α and IL6

Serum levels of tumour necrosis factor alpha (TNF-α) and interleukin (IL)-6 in RA patients and healthy donors (for *in vitro* studies) were quantified by enzyme-linked immunosorbent assay, following the manufacturer's instructions (Bionova, Diaclone, Madrid, Spain).

Western blotting

Total protein from mice tissues and buffy coat or 3T3-L1 adipocytes was extracted using radioimmunoprecipitation assay buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mmol L^{-1} NaCl and 50 m mol L^{-1} Tris–HCl; pH 8.0) supplemented with protease inhibitors.

Proteins (25 μg) were subjected to Western blotting. Immunoblots were incubated with the following antibodies: AKT, phospho-AKT Ser473, IL1β, GAPDH, β-actin, ERK, phospho-ERK, STAT3, phospho-STAT3 and NFkB (Santa Cruz Biotechnology, Madrid, Spain), phospho-IRS Ser636/639, phospho-HSL Ser563 mTOR, Rictor, GβL and Raptor (Cell Signaling Technology, Inc., MA, USA), IRS, phospho-IRS Tyr 608 and HSL (Abcam, Cambridge, UK) and JNK and phospho-JNK (RD System, Minneapolis, MN).

Table 2 *Clinical characteristics of RA patients and healthy donors: second cohort for* in vitro *studies*

	RA patients (n = 12)	Healthy donors (n = 12)
Clinical parameters		
Female (n)/male (n)	9/3	7/5
Age (years)	53.25 ± 7.36	48.75 ± 7.62
Disease duration (years)	11.60 ± 2.53	–
RF positive (%)	50	–
ACPAs (%)	100	–
DAS28	5.05 ± 1.29	–
Smoker (%)	50.0	41.6
BMI (kg m^{-2})	21.61 ± 2.89	23.57 ± 0.96
Laboratory parameters		
Glucose (mg dL^{-1})	87.33 ± 6.33	83.66 ± 1.86
Insulin (mg dL^{-1})	8.40 ± 3.01	5.48 ± 0.69
Cholesterol (mg dL^{-1})	199.16 ± 12.07	189.00 ± 6.18
HDL cholesterol (mg dL^{-1})	51.16 ± 3.83	53.70 ± 4.46
LDL cholesterol (mg dL^{-1})	122.92 ± 10.13	121.80 ± 4.9
Triglycerides (mg dL^{-1})	93.67 ± 14.06	82.54 ± 4.19
CRP (mg dL^{-1})	22.00 ± 7[a]	0.58 ± 0.48
Treatments		
Corticosteroids (%)	75.0	–
Antimalarials (%)	16.6	–
NSAIDs (%)	75.0	–
Methotrexate (%)	75.0	–
Leflunomide (%)	50.0	–

Values are means ± SD, unless otherwise stated.
ACPAs, anti citrullinated protein; BMI, body mass index; CRP, C-reactive protein antibodies; DAS28, disease activity score in 28 joints; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NSAID, nonsteroidal anti-inflammatory drug; RA, rheumatoid arthritis; RF, rheumatoid factor.
[a]Significant difference vs. healthy donors ($P < 0.01$).

RT-PCR

RNA was extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the

manufacturer's instructions and reverse-transcribed into cDNA. Real-time PCR using SYBR green or TaqMan was performed according to the manufacturer's instructions (Thermo Fisher Scientific, Madrid, Spain). Expression of genes of interest was corrected by the geometrical average of 18s, β2m, β-actin and 36b4 using the BestKeeper tool [18].

The expression levels of genes involved in lipid metabolism [*DGAT1*/*DGAT2* (diacylglycerol O-acyltransferase 1/2), *PLIN1*/*PLIN2* (perilipin 1/2), *SREBP1a* (sterol regulatory element-binding transcription factor 1), *INSIG1* (insulin-induced gene 1), *ACC* (acetyl-CoA carboxylase), *ATGL* (adipose triglyceride lipase), *HSL* (hormone-sensitive lipase), *PPARα* (peroxisome proliferator-activated receptor alpha), *MCAD* (medium-chain acyl-CoA dehydrogenase), *PGC1α*/*PGC1β* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha/1-beta), *CD36* (cluster differentiation 36) and *LPL* (lipoprotein lipase)] and insulin signalling [*GLUT4* (glucose transporter type 4) and *IRS1*/*IRS2* (insulin receptor substrate ")] were analysed.

Adipocyte size

Histological sections of white adipose tissue stained with haematoxylin and eosin were prepared as described previously [19]. Adipocyte sizes were measured using Cell P (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Between 1000 and 3000 adipocytes per tissue section from each mouse were used to determine the mean cell area.

Statistical analysis

Student's unpaired *t*-test, ANOVA and Duncan's test were used for the statistical analysis. Spearman's correlation was calculated to estimate the linear correlations between variables ($P < 0.01$). Multiple linear regression analysis was performed to exclude the influence of potential confounding variables on the levels of IR. HOMA-IR was selected as the dependent variable. Different treatments (methotrexate, leflunomide, hydroxychloroquine, corticosteroids and NSAIDs) were selected as independent variables. As a positive control, IR was included as an independent variable. Statistical significance was set at $P < 0.05$.

Results

Comorbidities associated with RA: relationships between inflammation, disease activity and degree of IR

Our cohort of 100 nonobese, nondiabetic RA patients had an increased prevalence of IR compared to the age- and gender-matched control group, with significantly elevated levels of fasting blood glucose and insulin (Table 1). After classifying RA patients based on their degree of IR (insulin-resistant group: HOMA-IR >2.5; normoglycaemic group: HOMA-IR < 2.5), we found significant differences in parameters related to inflammation and disease activity. Thus, RA patients with IR had higher levels of CRP, ESR and DAS28 (Fig. 1a). However, we did not find any association between IR and levels of autoantibodies (ACPAs and RF) or disease duration (Fig. 1b). We observed a strong correlation between levels of DAS28 or CRP and HOMA-IR values in RA patients (Spearman's $\rho = 0.223$, $P = 0.011$; Spearman's $\rho = 0.367$, $P = 0.000$, respectively). Thus, RA patients with moderate–high disease activity (DAS28 > 3.2) had significantly elevated levels of HOMA-IR compared to patients in the low disease activity group (DAS28 < 3.2) (Fig. 1c). Additionally, patients with high levels of systemic inflammation (CRP > 5 mg L^{-1}) had higher levels of insulin and HOMA-IR compared to those with low levels of systemic inflammation (CRP < 5 mg L^{-1}) (Fig. 1d). In addition, low-dose corticosteroid therapy was not associated with high levels of fasting blood glucose and insulin (Fig. 1e). In multiple linear regression analysis in our cohort of RA patients, no treatment was a statistically significant confounding variable for HOMA-IR levels: methotrexate ($\beta = -0.201$, $P = 0.340$), hydroxychloroquine ($\beta = 0.251$, $P = 0.232$), leflunomide ($\beta = 0.151$, $P = 0.477$) and NSAIDs ($\beta = -0.239$, $P = 0.240$). Thus, corticosteroid therapy had no effect in HOMA-IR levels ($\beta = 0.246$, $P = 0.272$) (Fig. 1e and Table 3).

Effects of RA development on inflammation and glucose metabolism and lipid metabolism in peripheral blood and metabolic tissues of CIA mice

Systemic level: plasma and leucocytes

Rheumatoid arthritis development had no effect on the levels of fasting glucose in plasma of CIA mice (Fig. 2a); however, this group had significantly increased levels of insulin (Fig. 2b) which translated into an elevation of the HOMA-IR values

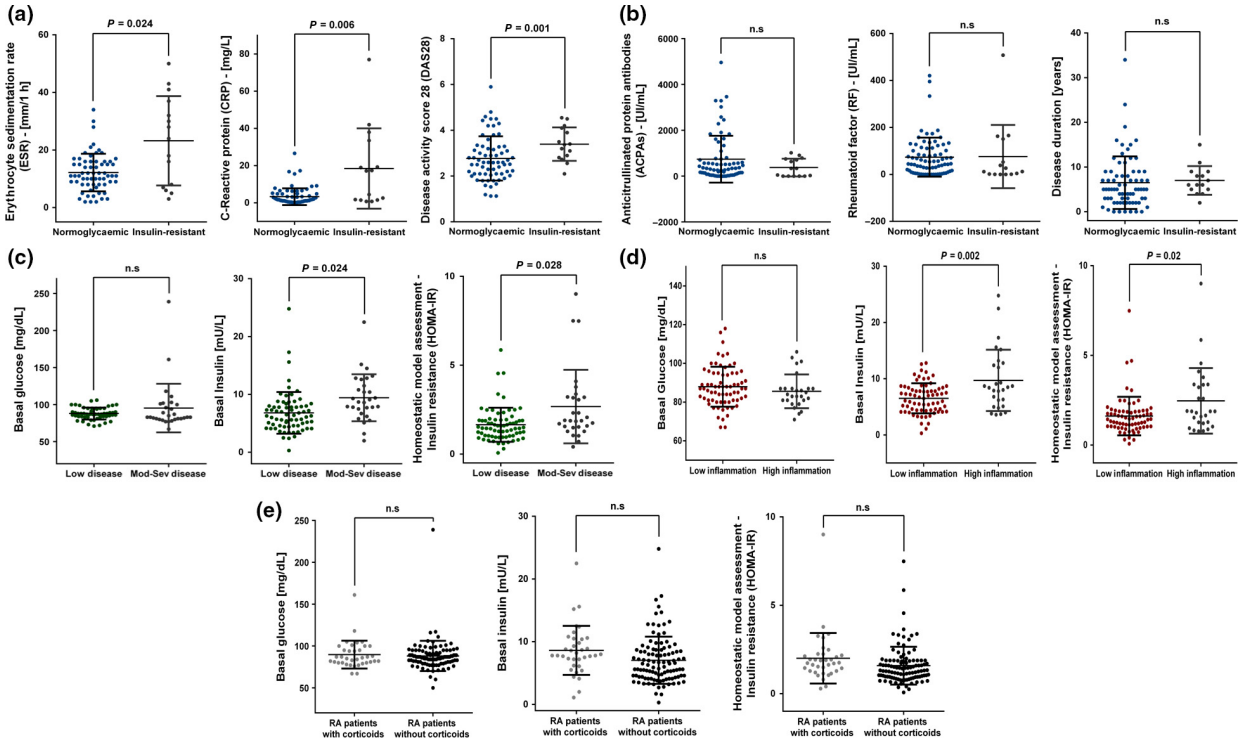

Fig. 1 *Association between inflammatory markers and disease activity with insulin resistance (IR). (a and b) Relationship between IR state and inflammation markers, such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and disease activity score in 28 joints (DAS28), and levels of anticitrullinated protein antibodies (ACPAs), rheumatoid factor (RF) and disease duration. (c) Association between moderate–severe disease activity and glucose, insulin and homeostasis model assessment (HOMA)-IR levels. (d) Relationships between high levels of inflammation in RA patients, insulin and HOMA-IR. (e) No association was found between corticosteroid therapy and blood glucose, insulin and HOMA-IR. Data for RA patients. Paired* t*-tests,* P < *0.05. NG, normoglycaemic; IR, insulin resistant; Low Dis, Low disease; Mod-Sev Dis, Moderate-severe disease; Low inf, low inflammation; High Inf, high inflammation.*

(Fig. 2c). Plasma levels of adiponectin were significantly decreased, together with an increase in the plasma levels of leptin, in CIA compared to non-arthritic mice (Fig. 2d,e).

As expected, TNF-α levels were elevated in plasma of CIA mice compared to the nondiseased control group (Fig. 2f). By contrast, a significant reduction in the plasma level of nonesterified fatty acids (NEFAs) was detected in CIA mice (Fig. 2g). Accordingly, IL1β protein expression was upregulated in leucocytes from CIA mice (Fig. 2h). Phosphorylation and expression levels of AKT were constitutively increased in CIA mice compared to the healthy control group (Fig. 2h).

Effects on adipose tissue

Disease progression in CIA mice was associated with a significant reduction in the expression of genes involved in lipogenesis (*INSIG1*, *SREBP1a* and *ACC*) (Fig. 3a) and lipid accumulation (*DGAT1*, *DGAT2*, *PLIN1* and *PLIN2*) (Fig. 3b), and this was evident in gonadal adipose tissue from the initial stages of the disease. We also observed a significant downregulation of genes involved in insulin signalling including *GLUT4*, *IRS1* and *IRS2* (Fig. 3c). In addition, a significant increase in the expression and phosphorylation of HSL, a key lipolytic enzyme, was observed in gonadal adipose tissue of CIA mice compared to nondiseased controls (Fig. 3a,d). A reduction in the size of the adipocytes was also noted in the CIA mice (Fig. 3e). Despite these changes, no significant effect on body weight was observed (data not shown).

Protein phosphorylation and expression levels of AKT were increased in adipose tissue from the CIA

Table 3 *Multiple linear regression analysis*

Independent variables	β	*P*	95% confidence intervals for β	
			Lower boundary	Upper boundary
HOMA-IR				
Model 1				
Methotrexate	–0.201	0.340	–0.620	0.217
Hydroxychloroquine	0.251	0.232	–0.164	0.665
Leflunomide	0.151	0.477	–0.270	0.571
NSAIDs	–0.239	0.240	–0.640	0.163
Corticosteroids	0.246	0.272	–0.197	0.689
IR	2.789	0.000	2.218	3.361
Model 2				
Methotrexate	–0.143	0.482	–0.548	0.261
Hydroxychloroquine	0.288	0.155	–0.112	0.688
NSAIDs	–0.259	0.185	–0.645	0.126
Corticosteroids	0.272	0.213	–0.159	0.703
IR	2.712	0.000	2.166	3.258
Model 3				
Hydroxychloroquine	0.300	0.166	–0.097	0.697
NSAIDs	–0.272	0.162	–0.655	0.111
Corticosteroids	0.307	0.159	–0.112	0.725
IR	2.731	0.000	2.190	3.272
Model 4				
Hydroxychloroquine	0.273	0.173	–0.122	0.668
Corticosteroids	0.274	0.194	–0.142	0.689
IR	2.729	0.000	2.188	3.271
Model 5				
Hydroxychloroquine	0.216	0.270	–0.171	0.603
IR	2.787	0.000	2.250	3.323

IR, insulin resistance; NSAID, nonsteroidal anti-inflammatory drug.

mice in comparison with the control animals (Fig. 3e). Next, we investigated the inflammation levels in gonadal adipose tissue and observed an upregulation of IL1β and mTOR complex 2 [(mTORC2); mTOR and Rictor] protein expression in moderate–severe CIA disease (Fig. 3e).

Effects on skeletal muscle

The mRNA expression of several genes involved in fatty acid oxidation (*CPT1B*, *PGC1*β and *MCAD*) was significantly reduced in skeletal muscle of CIA mice at a moderate–severe disease stage compared to nondiseased control animals (Fig. 4a). Similarly, genes involved in fatty acid uptake and lipid accumulation (*PPARα*, *DGAT1*, *DGAT2*, *PLIN2*, *CD36* and *LPL*) were also found to be significantly reduced in skeletal muscle of the CIA mice at a more severe disease stage (Fig. 4b). A significant reduction in mRNA expression of insulin signalling genes (*GLUT4*, *IRS1* and *IRS2*) was detected (Fig. 4c), and phosphorylation and protein expression levels of AKT were increased (Fig. 4d) in skeletal muscle of moderate–severe CIA mice compared to the control group. We further observed a significant elevation of IL1β, mTOR and Rictor (mTORC2) protein expression levels in CIA mice with moderate–severe disease (Fig. 4d).

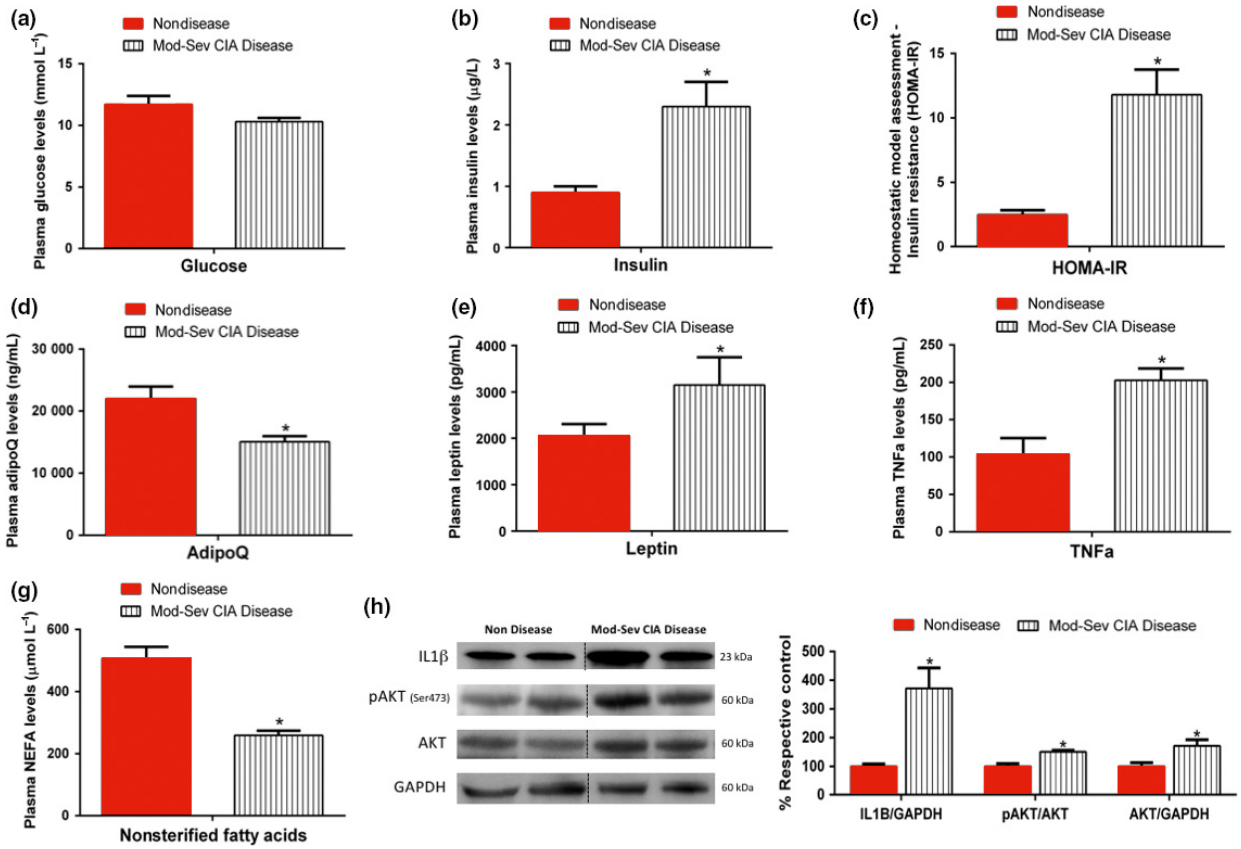

Fig. 2 *Effect of disease development in the collagen-induced arthritis (CIA) mouse model at the systemic level: plasma and leucocytes. Plasma levels of fasting glucose (a) and fasting insulin (b), homeostasis model assessment-insulin resistance (HOMA-IR) values (c) and plasma levels of adiponectin (AdipoQ) (d), leptin (e), tumour necrosis factor alpha (TNF-α) (f) and nonesterified fatty acid (NEFA) (g). (h) Phosphorylation and protein expression levels of protein kinase B (AKT) and protein expression of interleukin (IL)-1β. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *Significant differences vs. control mice (*P* < 0.05).*

Effect of in vitro treatment of 3T3-L1 adipocytes with serum from RA patients

Next, we performed *in vitro* studies in 3T3-L1 adipocytes exposed to serum from RA patients (RA serum) to evaluate the direct effects of inflammatory mediators present in the serum (enhanced circulating IL6 and TNF-α levels as compared to serum from healthy donors) on the metabolic changes observed in adipose tissue of CIA mice (Fig. 5a).

We evaluated the effect of the RA serum on inflammation, lipogenesis, lipolysis and insulin signalling. RA serum promoted a significant reduction in the expression of genes involved in lipogenesis and lipid accumulation (*SREBP1a*, *INSIG1*, *DGAT2*, *PLIN1* and *PLIN2*) compared with the serum from healthy donors (Fig. 5b,c). By contrast, genes involved in lipolysis showed a significant upregulation in adipocytes treated with RA serum (*HSL*) (Fig. 5c). At the protein level, after treatment with RA serum, phosphorylation and protein expression of HSL were significantly upregulated compared with 3T3-L1 adipocytes exposed to serum from healthy donors (Fig. 5e). Insulin signalling was also affected by RA serum through a significant reduction in GLUT4, IRS1 and IRS2 mRNA levels (Fig. 5d) and an increase in the Ser636/639 phosphorylated IRS combined with a reduction in the phosphorylation of IRS on Tyr608 (Fig. 5e). In addition, similar to the observations in adipose tissue and skeletal muscle of CIA mice, phosphorylation of AKT and the expression of both

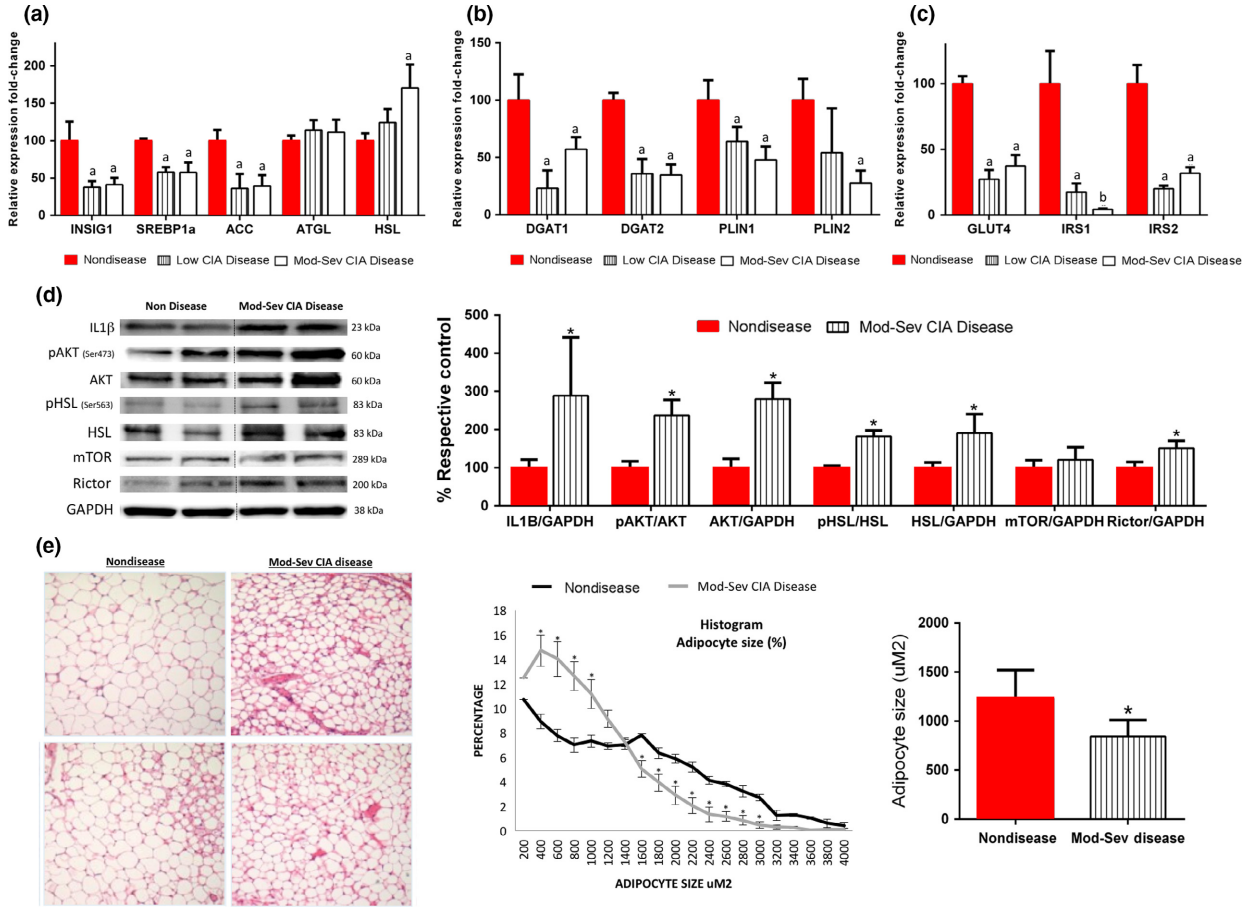

Fig. 3 *Effect of disease development on adipose tissue in the collagen-induced arthritis (CIA) mouse model. mRNA relative expression of genes involved in lipogenesis (a), lipolysis (b), lipid accumulation (c) and glucose and insulin signalling (d). Protein expression of interleukin (IL)-1β, mammalian target of rapamycin (mTOR) and rapamycin-insensitive companion of mTOR (Rictor). Phosphorylation and protein expression of AKT and hormone-sensitive lipase (HSL). (e) Adipocyte size in CIA compared to control mice. DGAT1/DGAT2, diacylglycerol O-acyltransferase 1/2; PLIN1/PLIN2, Perilipin 1/2; GLUT4, glucose transporter type 4; IRS1/IRS2, insulin receptor substrate 1/2; INSIG1, insulin-induced gene 1; SREBP1a, sterol regulatory element-binding transcription factor 1; ACC, acetyl-CoA carboxylase; ATGL, adipose triglyceride lipase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (a–c) Paired* t*-tests,* [a]*significant differences vs. nondiseased mice (*$P < 0.05$*),* [b]*significant differences vs. low CIA disease (*$P < 0.05$*). (d and e) Paired* t*-tests, *significant differences vs. nondiseased mice (*$P < 0.05$*).*

mTOR and Rictor were upregulated in adipocytes treated with RA serum (Fig. 5e). The levels of diverse inflammatory mediators were also elevated in adipocytes treated with RA serum, represented by increased levels of IL1β and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and elevated phosphorylation of JNK, ERK and STAT3 (Fig. 5e).

Correlation studies showed that in 3T3-L1 adipocytes treated with RA serum, the expression of several genes and proteins involved in inflammation, lipolysis and insulin signalling was correlated with several clinical parameters of these RA patients. Thus, CRP level and DAS28 score were strongly correlated with HSL expression levels in RA serum-treated adipocytes. These two clinical parameters were also correlated with inflammatory mediators such as IL1β and mTOR; moreover, CRP was correlated with the expression of NFκB and Rictor, and the phosphorylation of IRS on serine 636/639, AKT, ERK and JNK (Table 4).

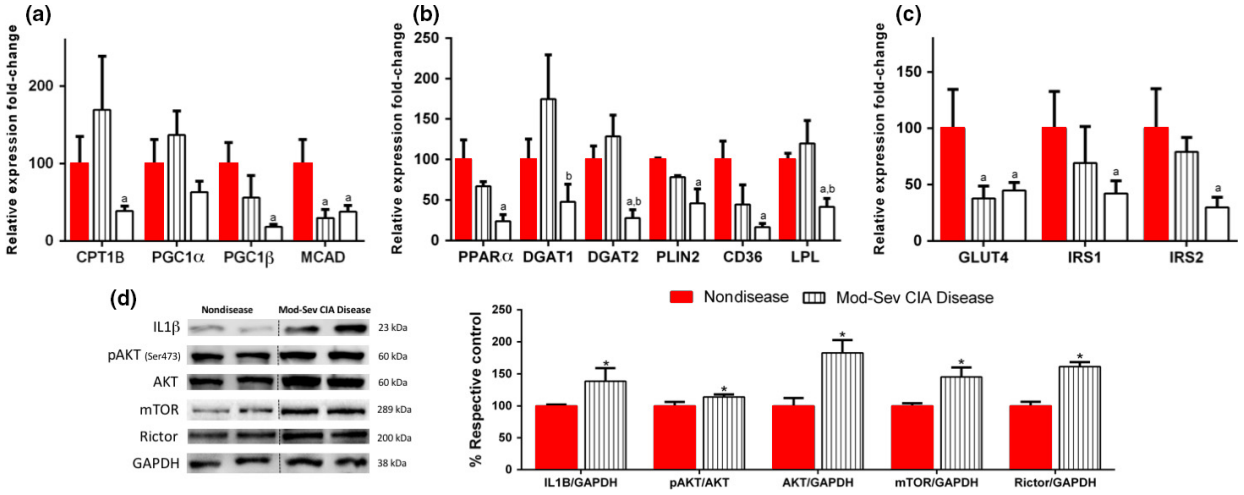

Fig. 4 *Effect of disease development on skeletal muscle in the collagen-induced arthritis (CIA) mouse model. mRNA relative expression levels of genes involved in fatty acid oxidation (a), lipid accumulation (b) and glucose and insulin signalling (c). (d) Protein expression levels of interleukin (IL)-1β, mammalian target of rapamycin (mTOR) and rapamycin-insensitive companion of mTOR (Rictor), and phosphorylation and protein expression levels of protein kinase B (AKT). CPT1B, carnitine palmitoyltransferase 1B; PGC1α/PGC1β, peroxisome proliferator-activated receptor gamma coactivator 1-alpha/1-beta; MCAD, medium-chain acyl-CoA dehydrogenase; PPARα, peroxisome proliferator-activated receptor alpha; DGAT1/DGAT2, diacylglycerol O-acyltransferase 1/2; PLIN2, perilipin 2; CD36, cluster of differentiation 36; LPL, lipoprotein lipase; GLUT4, glucose transporter type 4; IRS1/IRS2, insulin receptor substrate 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (a–c) Paired t-test, [a]Significant differences vs. nondiseased mice (P < 0.05), [b]significant differences vs. low CIA disease (P < 0.05). (d) Paired t-tests, *significant differences vs. nondiseased mice (P < 0.05).*

Of note, IL6 and TNF-α serum levels were strongly correlated with the activation and expression of HSL, AKT phosphorylation and the expression levels of IL1β and mTOR. In addition, phosphorylation of IRS (ser636/639) and ERK and expression of Rictor in 3T3-L1 adipocytes were correlated with the levels of IL6 present in the RA serum. Both inflammatory markers, IL6 and TNF-α, were negatively correlated with levels of genes involved in lipid accumulation and insulin signalling (Table 4). These data further support the notion that inflammatory mediators present in the serum from RA patients are closely linked to the metabolic and inflammatory changes observed in adipocytes including the activation of inflammatory pathways, promotion of IR, lipolysis and reduction in lipogenesis.

Discussion

To our knowledge, this is the first study in which the molecular mechanisms underlying the relationship between IR and RA have been evaluated simultaneously using human, animal and cellular

Fig. 5 *Effect of* in vitro *treatment with serum from patients with rheumatoid arthritis (RA serum) in 3T3-L1 adipocytes. (a) Interleukin (IL)-6 and tumour necrosis factor alpha (TNF-α) cytokine levels in serum from healthy donors and RA patients. mRNA relative expression levels of genes involved in lipid accumulation (b), lipogenesis and lipolysis (c) and glucose and insulin signalling (d). (e) Protein expression and phosphorylation levels. DGAT1/DGAT2, diacylglycerol O-acyltransferase 1/2; PLIN1/PLIN2, perilipin 1/2; GLUT4, glucose transporter type 4; SREBP1a, sterol regulatory element-binding transcription factor 1; INSIG1, insulin-induced gene 1; ACC, acetyl-CoA carboxylase; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase, IRS1/IRS2, insulin receptor substrate 1/2; AKT, protein kinase B; mTOR, mammalian target of rapamycin; GBL, G protein beta-subunit-like; Rictor, rapamycin-insensitive companion of mTOR; Raptor, regulatory-associated protein of mTOR; JNK, c-JUN N-terminal kinase; ERK, extracellular signal-regulated kinase; STAT3, signal transducer and activator of transcription 3; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells. Paired t-test, *significant differences vs. 3T3-L1 adipocytes treated with serum from healthy donors (HD serum) (P < 0.05).*

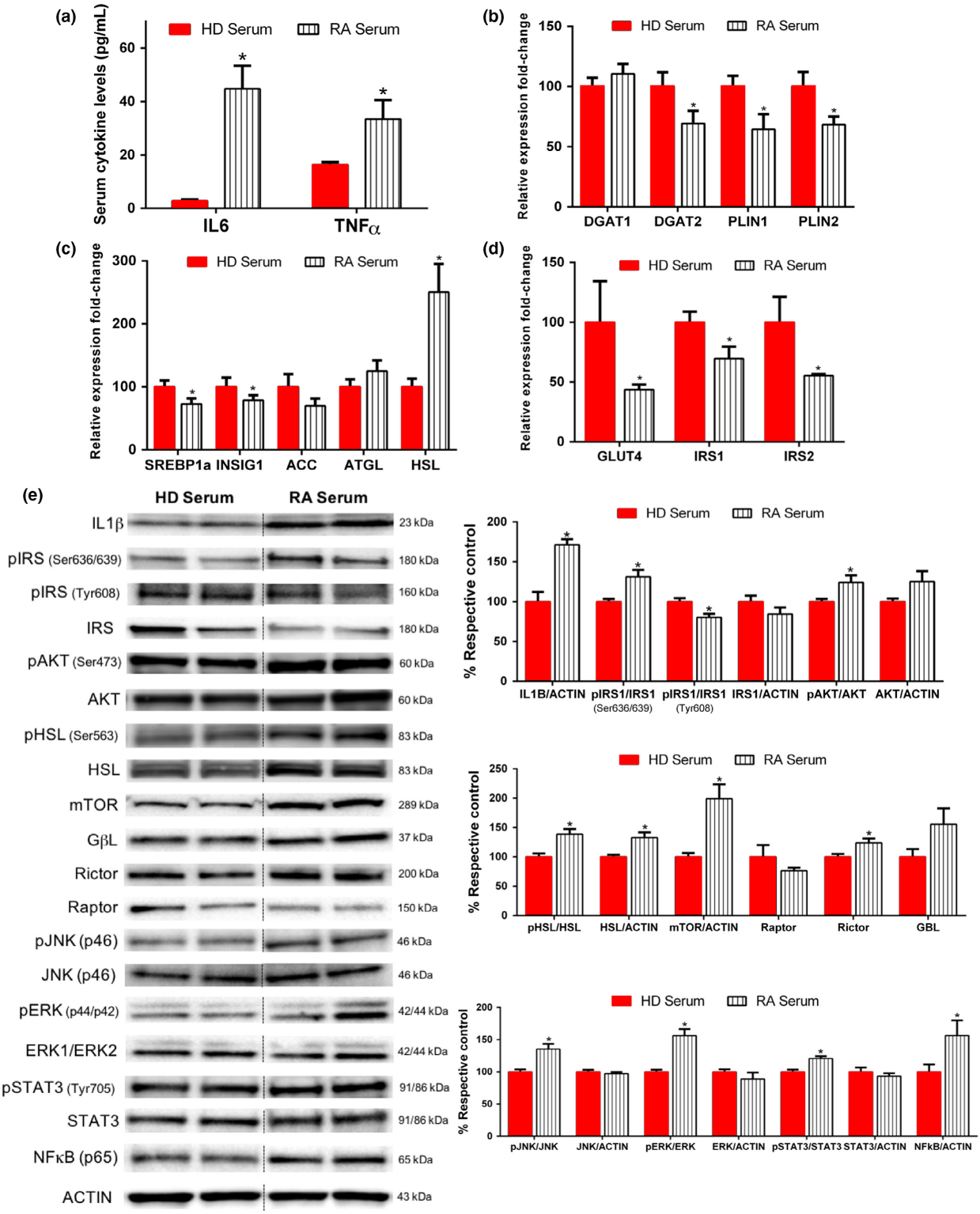
(a)
HD Serum
RA Serum
Serum cytokine levels (pg/mL)
IL6
TNFα
(b)
Relative expression fold-change
DGAT1
DGAT2
PLIN1
PLIN2
(c)
SREBP1a
INSIG1
ACC
ATGL
HSL
(d)
GLUT4
IRS1
IRS2
(e)
IL1β
pIRS (Ser636/639)
pIRS (Tyr608)
IRS
pAKT (Ser473)
AKT
pHSL (Ser563)
HSL
mTOR
GβL
Rictor
Raptor
pJNK (p46)
JNK (p46)
pERK (p44/p42)
ERK1/ERK2
pSTAT3 (Tyr705)
STAT3
NFκB (p65)
ACTIN
% Respective control
IL1B/ACTIN
pIRS1/IRS1 (Ser636/639)
pIRS1/IRS1 (Tyr608)
IRS1/ACTIN
pAKT/AKT
AKT/ACTIN
pHSL/HSL
HSL/ACTIN
mTOR/ACTIN
GBL
pJNK/JNK
JNK/ACTIN
pERK/ERK
ERK/ACTIN
pSTAT3/STAT3
STAT3/ACTIN
NFkB/ACTIN

Table 4 *Correlations between clinical and serological parameters of RA patients and gene and protein expression in 3T3-L1 adipocytes exposed to serum from such patients*

Parameter	mRNA	Correlation coefficient (r), P-value	Protein	Correlation coefficient (r), P-value
Clinical parameters				
CRP	HSL	$r = 0.513$, $P < 0.01$	IL1β/ACTIN	$r = 0.670$, $P < 0.01$
			pIRS1/IRS1 (Ser)	$r = 0.350$, $P = 0.04$
			pAKT/AKT	$r = 0.320$, $P = 0.10$
			AKT/ACTIN	$r = 0.310$, $P = 0.10$
			pHSL/HSL	$r = 0.580$, $P = 0.02$
			HSL/ACTIN	$r = 0.469$, $P = 0.01$
			mTOR/ACTIN	$r = 0.610$, $P < 0.01$
			Rictor/ACTIN	$r = 0.323$, $P = 0.05$
			pERK/ERK	$r = 0.596$, $P = 0.01$
			pJNK/JNK	$r = 0.425$, $P = 0.03$
			NFkB/ACTIN	$r = 0.378$, $P = 0.04$
DAS28	HSL	$r = 0.384$, $P = 0.09$	IL1β/ACTIN	$r = 0.448$, $P = 0.03$
			pHSL/HSL	$r = 0.715$, $P = 0.01$
			mTOR/ACTIN	$r = 0.612$, $P = 0.01$
Serological parameters				
IL6	HSL	$r = 0.506$, $P = 0.01$	IL1β/ACTIN	$r = 0.738$, $P = 0.00$
	DGAT2	$r = -0.408$, $P = 0.06$	pIRS1/IRS1 (Ser)	$r = 0.578$, $P = 0.01$
	PLIN1	$r = -0.539$, $P = 0.01$	pAKT/AKT	$r = 0.519$, $P = 0.03$
	GLUT4	$r = -0.391$, $P = 0.04$	pHSL/HSL	$r = 0.586$, $P = 0.03$
			HSL/ACTIN	$r = 0.721$, $P < 0.01$
			mTOR/ACTIN	$r = 0.585$, $P = 0.01$
			Rictor/ACTIN	$r = 0.645$, $P = 0.01$
			pERK/ERK	$r = 0.487$, $P = 0.02$
TNF-α	DGAT2	$r = -0.519$, $P = 0.04$	IL1β/ACTIN	$r = 0.531$, $P = 0.01$
			AKT/ACTIN	$r = 0.672$, $P < 0.01$
			pHSL/HSL	$r = 0.518$, $P = .02$
			HSL/ACTIN	$r = 0.458$, $P = 0.04$
			mTOR/ACTIN	$r = 0.445$, $P = 0.02$

AKT, protein kinase B; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; DGAT2, diacylglycerol O-acyltransferase 2; ERK, extracellular signal-regulated kinase; HSL, hormone-sensitive lipase; IL1β, interleukin-1 beta; IL6, interleukin-6; IRS1, insulin receptor substrate 1; JNK, c-JUN N-terminal kinase; mTOR, mammalian target of rapamycin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PLIN1, perilipin 1; Rictor, rapamycin-insensitive companion of mTOR; TNF-α, tumour necrosis factor alpha.

models to unravel the effects of inflammatory mediators present in RA on the physiology of metabolic tissues such as adipose tissue and skeletal muscle. Thus, our data show the close association between systemic inflammation, the activity of the disease and the development of IR in a cohort of 100 nonobese RA patients. To date, a number of studies have shown a higher prevalence of IR in RA patients, demonstrating a strong relationship between increased BMI and the development of IR, independent of disease duration and therapy received [7, 8, 10, 20, 21]. Of note, in the present study we investigated a cohort of RA patients and healthy donors characterized by similar BMI values (<30 kg m^{-2}) and with obese and diabetic subjects excluded. Despite these

exclusions, we confirmed and validated a higher prevalence of IR (15%) in our cohort of RA patients, even after correcting for any potential bias due to treatment with DMARDs and corticosteroids and short–medium duration of the disease. It is well recognized that the prolonged use of corticosteroid therapy affects glucose metabolism inducing IR. However, this effect might depend on the dosage and type of corticosteroid administered [22, 23]. Thus, in our RA patients, the dose of corticosteroids was low (<7.5 mg) and the main type of corticosteroid used was deflazacort, which has been shown to be less likely to cause hyperglycaemia compared to other agents [23].

Despite the large heterogeneity found in the different cohorts of RA patients in reported studies to date, it seems that there is some consensus regarding the association between systemic inflammation and IR [24]. In agreement, we have found a strong correlation between IR and both the activity and the inflammatory profile of the disease, with no influence of either the treatment administered or BMI, thus suggesting that inflammation *per se* may be the main determinant of IR in patients with RA.

The results from several studies have suggested that inflammatory mediators involved in RA such as TNF-α and IL6 are associated with the onset and development of IR and type 2 diabetes mellitus [11]. Beyond these associations, no previous studies have directly explored the effects of RA development on glucose metabolism and lipid metabolism at systemic and tissue-specific levels. We have demonstrated in the present study that the development of RA affects lipid metabolism and insulin signalling in adipose tissue and skeletal muscle in a CIA mouse model. At the systemic level, we observed a significant increase in TNF-α plasma levels and IL1β upregulation in leucocytes, demonstrating the inflammatory status induced by the development of RA. In addition, plasma insulin levels and HOMA-IR values were elevated in CIA mice. Adiponectin levels have been inversely related to IR [25], whilst high leptin levels have been associated with IR independently of BMI [26]. Lower plasma levels of adiponectin and higher levels of leptin together with higher HOMA-IR values suggest that arthritis induced a state of IR in these CIA mice.

At the tissue level, we also observed a significant increase in IL1β in adipose tissue and skeletal muscle. Several studies have demonstrated how inflammatory mediators such as TNF-α and IL1β, upregulated in immune cells and adipocytes in obesity, induce the development of IR through mechanisms including reduction in the expression of *GLUT4* and *IRS1* genes [27, 28]. Accordingly, in our study, RA-induced inflammation was accompanied by a significant reduction in the expression of *IRS1*, *IRS2* and *GLUT4* in adipose tissue and skeletal muscle. Of note, it has been demonstrated that the inhibition of these genes in animal models leads to IR states [29–31]. Moreover, it has been shown that normoglycaemic subjects with low levels of *IRS1* and *GLUT4* in adipose tissue developed IR later in life [32]. Our results also identified an increased phosphorylation of AKT in leucocytes, adipose tissue and skeletal muscle of CIA mice. Thus, it could be speculated that prolonged activation of AKT may lead to a negative feedback of insulin signalling. The acute effect of AKT activation would cause Thr308 phosphorylation, whereas prolonged stimulation would lead to Ser473 phosphorylation. It has been demonstrated that AKT phosphorylation in Ser473 acts as a negative regulator through phosphorylation of the insulin receptor β-subunit at threonine, thus causing decreased autophosphorylation of the receptor [33]. In addition, this activation might be caused by an inflammatory stimulus, as AKT dysfunction has been reported in diverse pathological settings such as cancer or CVD [34, 35]. This is in accordance with our results, where the development of RA induced prolonged Ser473 phosphorylation of AKT in leucocytes, adipose tissue and skeletal muscle, accompanied by increased IL1β levels and a reduction in the expression of *IRS1* and *IRS2*, thus promoting a defective insulin response which might lead to IR in these cells and tissues.

Furthermore, the development of RA promoted a reduction in lipid accumulation markers in adipose tissue of mice. This reduction was accompanied by an increase in the phosphorylation and expression of HSL, together with a reduction in adipocyte size compatible with an increase in lipolysis. In obesity, increased basal lipolysis has been reported in adipocytes, closely related to the development of IR [36]. In this regard, several *in vitro* studies have demonstrated that TNF-α can induce lipolysis in mouse and human adipocytes through the upregulation of HSL [37, 38]. We found that lipolysis was increased in adipose tissue of CIA mice, probably mediated by the inflammation-induced IR caused by the disease. Whereas the effects on insulin signalling and lipid metabolism in adipose tissue were evident from the early stages of arthritis,

similar effects in skeletal muscle were observed upon reaching a more severe stage reflected in significant changes in fatty acid metabolism. Thus, at moderate–severe disease stages, a marked reduction in the expression of genes involved in lipid accumulation, fatty acid oxidation and insulin signalling was also noted in skeletal muscle of CIA mice.

The reduced lipid accumulation in adipose tissue and skeletal muscle and the decreased levels of NEFA in plasma might suggest that lipids could be oxidized and/or could accumulate in the bloodstream or other tissues such as liver, contributing to the development of IR. Our results indicate that amongst all metabolic tissues affected by RA, inflammation of the adipose tissue appears to be an early target, which may be more susceptible to the metabolic changes caused by RA (observed from the initial stages of the disease). In more common metabolic disorders, it has also been suggested that IR is initiated in adipose tissue, playing a pivotal role in the subsequent induction of IR in other organs such as muscle and liver [38].

Interestingly, we were able to demonstrate that inflammatory mediators present in RA serum (e.g. TNF-α and IL6) also induce the alterations observed in adipose tissue. Treatment of 3T3-L1 adipocytes with RA serum containing high levels of these inflammatory mediators confirmed the results observed in adipose tissue of CIA mice, induction of lipolysis (increased phosphorylation and expression of HSL) and reduction in lipid accumulation (decreased expression of genes involved in lipid accumulation). RA serum induced a high inflammatory state in adipocytes through the activation of several intracellular kinases such as JNK, ERK and STAT3, and the elevated expression of IL1β, NFκB and mTORC2 (mTOR and Rictor). These kinases have been shown to be involved in the inactivation of IRS. IRS proteins are activated by tyrosine phosphorylation and inhibited by serine phosphorylation [39]. Thus, inflammation was associated with impaired insulin signalling, evidenced by the decreased mRNA expression of *IRS1/IRS2* and *GLUT4* and increased levels of serine phosphorylation of IRS, and a reduction in the tyrosine phosphorylation of IRS. Paradoxically, we also noted high levels of phosphorylated AKT at serine 473. AKT is a complex metabolic hub that can be modulated by a large number of proteins in response to a variety of nutritional and cellular stressors. One of these is the mTORC2. mTOR is a serine/threonine kinase that controls a wide spectrum of cellular processes, including growth, differentiation, inflammation and metabolism. mTOR exists in two functional complexes, mTORC1 and mTORC2. mTORC1 consists of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8) and two inhibitory subunits, proline-rich AKT substrate of 40 kDa (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR). mTORC2 contains mTOR, the rapamycin-insensitive companion of mTOR (Rictor), stress-activated map kinase (SAPK)-interacting 1 (SIN1), mLST8, proline-rich protein 5-like (PRR5L) and DEPTOR. These two complexes are involved in the activation of AKT in response to various stimuli including growth factors such as insulin, fatty acids and cytokines [40].

It has been reported that active mTORC2 is able to phosphorylate AKT and the loss of mTORC2 abrogates the activation of AKT [41]. In addition, mTORC2 is involved in IRS degradation and insulin receptor tyrosine phosphorylation [40]. There is also evidence that engagement of the AKT/mTOR pathway may account for TNF-α-mediated IR. Activation of the PI3K/AKT/mTOR cascade by TNF-α regulates the phosphorylation of IRS1 on serines 636/639 antagonizing its phosphorylation on tyrosine by the insulin receptor [42].

In the context of RA, we showed increases in mTORC2 (represented by high levels of mTOR and Rictor) and AKT in adipose tissue and skeletal muscle of CIA mice, as well as in adipocytes treated with RA serum, suggesting that activation of mTORC2 may be responsible *a priori* for the increased and decreased activation of AKT and IRS, respectively, in our CIA mouse model.

The relevance of these observations was further supported by our correlation studies of clinical parameters and the metabolic and inflammatory changes induced in the adipocytes by exposure to RA serum. These findings further support the notion that inflammatory mediators present in the serum may be responsible for the alterations.

Thus, we anticipate that inflammatory mediators such as TNF-α and IL6 would activate a complex repertoire of pathways that include mTORC2 and

other intracellular kinases such as ERK, JNK and STAT3 that would inactivate IRS through its phosphorylation on serines 636/639. mTORC2 containing mTOR, GBL and Rictor would also activate AKT, which together with JNK and ERK would be involved in the activation of NFκB, promoting the expression of inflammatory genes and further contributing to the inactivation of IRS and aggravating IR in peripheral tissues such as adipose tissue and skeletal muscle (Fig. 6).

Our results suggest that RA promotes a global inflammatory state (at systemic and tissue levels) that affects the adipose tissue, leading to IR, stimulating lipolysis and reducing lipid accumulation. This is followed at more advanced stages by a reduction in lipid content and exacerbated IR in skeletal muscle. Accordingly, the adipose tissue is an early target in this process.

Together, our results show a direct effect of RA-induced chronic inflammation mediating the alterations in glucose metabolism and lipid metabolism associated with this disorder. Thus, therapeutic strategies to inhibit inflammation, by targeting proinflammatory cytokines, might provide a novel approach to normalize the metabolic alterations associated with RA.

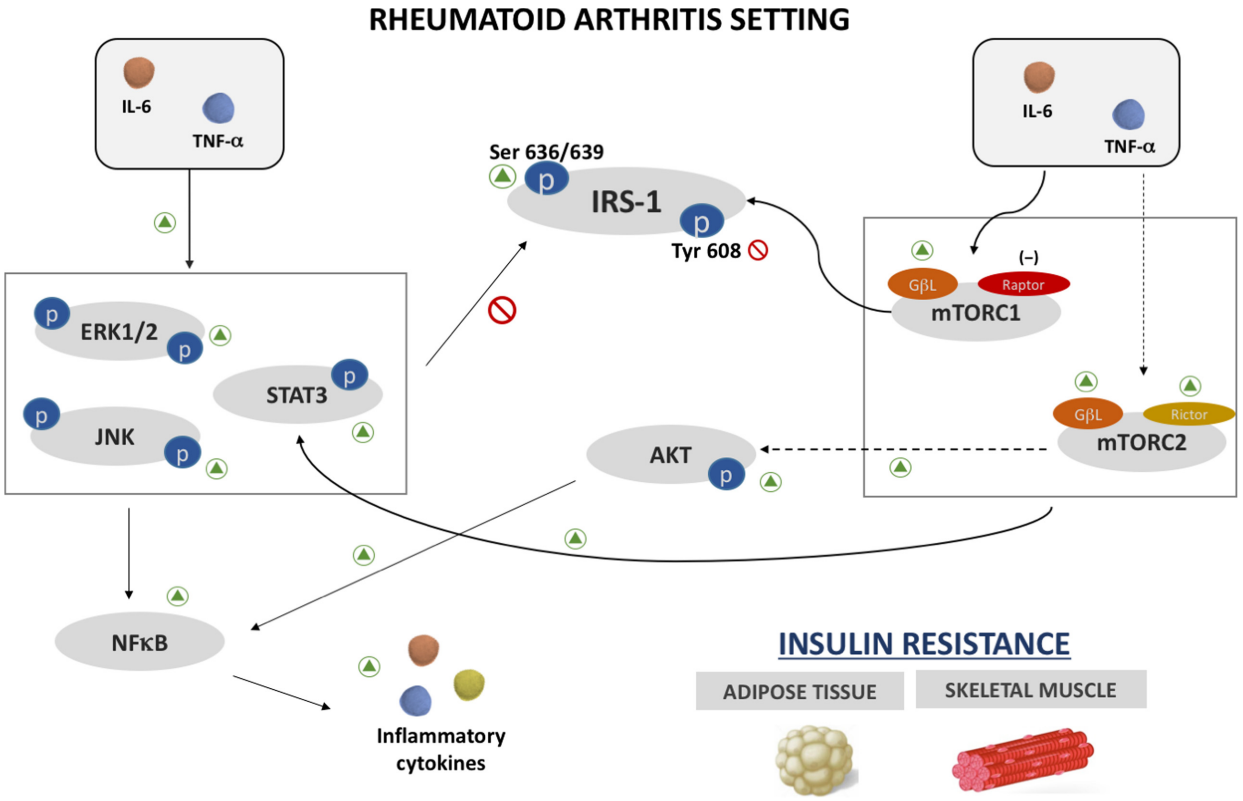

Fig. 6 *Proposed model of the molecular pathways involved in the development of insulin resistance in rheumatoid arthritis. In the setting of rheumatoid arthritis, inflammatory mediators such as interleukin (IL)-6 and tumour necrosis factor alpha (TNF-α) would activate a complex repertoire of pathways that include mTOR complex (mTORC)2 and other intracellular kinases such as extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK) and signal transducer and activator of transcription 3 (STAT3), which would inactivate insulin receptor substrate (IRS) through phosphorylation of serines 636/639. mTORC2 complex, containing mammalian target of rapamycin (mTOR), G protein beta-subunit-like (GBL) and rapamycin-insensitive companion of mTOR (Rictor), would also activate protein kinase B (AKT), which together with JNK and ERK would be involved in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), promoting the expression of inflammatory genes and further contributing to the inactivation of IRS and aggravating insulin resistance on peripheral tissues such as adipose tissue and skeletal muscle.*

Conflict of interest statement

No potential conflict of interests relevant to this article were reported. All authors have completed the ICMJE Disclosure Form for Potential Conflict of Interests.

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