

## RESEARCH ARTICLE

# RBP4 increases lipolysis in human adipocytes and is associated with increased lipolysis and hepatic insulin resistance in obese women

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## Abstract

Retinol-binding protein-4 (RBP4) is elevated in serum and adipose tissue (AT) in obesity-induced insulin resistance and correlates inversely with insulin-stimulated glucose disposal. But its role in insulin-mediated suppression of lipolysis, free fatty acids (FFA), and endogenous glucose production (EGP) in humans is unknown. RBP4 mRNA or protein levels were higher in liver, subcutaneous adipose tissue (SAT), and visceral adipose tissue (VAT) in morbidly obese subjects undergoing Roux-en-Y gastric bypass surgery compared to lean controls undergoing elective laparoscopic cholecystectomy. RBP4 mRNA expression in SAT correlated with the expression of several macrophage and other inflammation markers. Serum RBP4 levels correlated inversely with glucose disposal and insulin-mediated suppression of lipolysis, FFA, and EGP. Mechanistically, RBP4 treatment of human adipocytes *in vitro* directly stimulated basal lipolysis. Treatment of adipocytes with conditioned media from RBP4-activated macrophages markedly increased basal lipolysis and impaired insulin-mediated lipolysis suppression. RBP4 treatment of macrophages increased TNF $\alpha$  production. These data suggest that elevated serum or adipose tissue RBP4 levels in morbidly obese subjects may cause hepatic and systemic insulin resistance by stimulating basal lipolysis and by activating macrophages in adipose tissue, resulting in release of pro-inflammatory cytokines that impair lipolysis suppression. While we have demonstrated this mechanism in human adipocytes *in vitro*, and correlations from our flux studies in humans strongly support this, further studies are needed

**Abbreviations:** ADA, adenosine deaminase; AT, adipose tissue; EGP, endogenous glucose production; FFA, free fatty acids; GLUT4, glucose transporter-4; KRB, Krebs-Ringer; PEPCCK, phosphoenolpyruvate carboxykinase; PI3K, phosphoinositide 3-kinase; PIA, phenylisopropyl adenosine; RBP4, retinol-binding protein-4; Rd, rate of disappearance; RYGB, Roux-en-Y gastric bypass; SAT, subcutaneous adipose tissue; SVF, stromal vascular fraction; T2DM, type 2 diabetes mellitus; VAT, visceral adipose tissue.

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to determine whether this mechanism explains RBP4-induced insulin resistance in humans.

#### KEYWORDS

endogenous glucose production, inflammation, insulin resistance, lipolysis, obesity, RBP4

## 1 | INTRODUCTION

Insulin resistance in obesity is a complex metabolic state consisting of increased endogenous glucose production (EGP), reduced muscle glucose uptake and increased lipolysis.<sup>1</sup> Many studies have now demonstrated low grade inflammation, systemically and in adipose tissue in the obese state<sup>1</sup> but the causal relationship between inflammation and insulin resistance in humans is still unclear. Inflammation in adipose tissue is associated with increased lipolysis which may be driven by proinflammatory cytokines.<sup>2</sup> This lipolysis can play a causal role in insulin resistance because the FFAs released can increase EGP and reduce muscle glucose uptake.<sup>3</sup>

Retinol-binding protein 4 (RBP4) is an adipose and liver secreted protein which is elevated in adipose tissue and serum of humans with obesity and/or type 2 diabetes (T2D).<sup>4,6</sup> Adipose expression and serum levels of RBP4 are associated with insulin resistance in most, but not all studies.<sup>4,7</sup> Data that support a causative role for RBP4 in insulin resistance/T2D include the fact that a gain-of-function polymorphism in the RBP4 promoter which increases adipose RBP4 expression<sup>8</sup> is associated with an 80% increased risk of T2D in humans.<sup>9</sup> Furthermore, obese, insulin-resistant women with breast cancer that were treated with the synthetic retinoid fenretinide which lowers circulating RBP4 levels, for 2 years had a sevenfold greater probability to normalize insulin sensitivity than women that were treated with tamoxifen or placebo.<sup>10</sup> While fenretinide has other effects in addition to lowering RBP4, these results are consistent with preclinical studies.<sup>4,11-13</sup>

Genetic or pharmacologic elevation of RBP4 in mice induces insulin resistance<sup>4</sup> and lowering RBP4 in obese mice improves insulin sensitivity.<sup>11-13</sup> Mechanistically, RBP4 elevation increases the expression of the major gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) in liver and impairs insulin signaling in skeletal muscle.<sup>4</sup> In

cultured hepatocytes, RBP4 increases basal EGP and reduces insulin-mediated EGP suppression,<sup>4</sup> but this remains to be investigated definitively in vivo in humans or rodents. RBP4 also induces adipose tissue inflammation in mice by activating both innate and adaptive immune responses<sup>14-16</sup> and blocking antigen presentation in RBP4 overexpressing mice reduces adipose tissue inflammation and improves insulin sensitivity.<sup>16</sup> Adipose-specific RBP4 overexpression in mice results in elevated plasma FFA and higher expression of lipolysis genes as well as increased adipose inflammation,<sup>17</sup> but lipolysis itself has not been measured. Clamp studies in humans have shown an inverse correlation between serum RBP4 and glucose disposal rates<sup>5,7</sup> but there is limited information in humans regarding whether RBP4 reduces insulin suppression of EGP or lipolysis. Because RBP4 levels in serum correlate with systemic low-grade inflammation<sup>18</sup> and subcutaneous adipose tissue (SAT) RBP4 expression correlates with inflammatory markers<sup>19</sup> in humans, it is possible that RBP4-induced inflammation could result in increased lipolysis and impaired suppression of EGP.

Therefore, in this study, we aimed to determine the relationship between circulating and tissue RBP4 levels, lipolysis, and hepatic insulin resistance in humans in vivo. We show that adipose tissue and hepatic RBP4 mRNA expression are both increased in obesity. Serum RBP4 is inversely correlated with insulin-mediated suppression of lipolysis and EGP as well as with peripheral insulin-mediated glucose disposal. In addition, adipose RBP4 expression correlates with inflammatory markers. Our mechanistic studies show that RBP4 directly stimulates basal lipolysis, and media from macrophages incubated with RBP4 augments isoproterenol- and 8-Br-cAMP-stimulated lipolysis and impairs insulin suppression of lipolysis ex vivo in adipocytes from obese subjects. The media from RBP4-treated macrophages has a marked increase in TNF $\alpha$  protein. Thus, RBP4 activation of macrophages increases lipolysis which may result at least in part, from proinflammatory cytokine release and this may

play an important role in RBP4-induced hepatic and systemic insulin resistance.

## 2 | MATERIAL AND METHODS

### 2.1 | Subjects

Twenty morbidly obese female subjects scheduled for laparoscopic Roux-en-Y gastric bypass (RYGB) surgery were studied. In these subjects, we performed a two-step hyperinsulinemic euglycemic clamp and during surgery, we collected liver, subcutaneous (SAT), and visceral AT (VAT) biopsies.<sup>20</sup> As controls for tissue RBP4, we included six lean and age-matched women undergoing elective laparoscopic cholecystectomy. Liver and adipose tissue biopsies were collected in the same way as in the obese subjects. To avoid confounding by impaired glucose tolerance, the control subjects needed to have a normal glucose tolerance test according to the criteria of the ADA.<sup>21</sup> Other eligibility criteria for the controls were an age older than 18 years, understanding the objective of the study, competent to give informed consent, and a stable body mass index (BMI) between 20 and 25 kg/m<sup>2</sup>. Exclusion criteria were performance of vigorous exercise, family history of T2DM, a recent history (6 months or less) of substantial alcohol or drug abuse and the use of any medication. For in vitro lipolysis experiments stromal vascular fraction (SVF) cells from six female subjects with a BMI of 32.5 ± 2.2 kg/m<sup>2</sup> were obtained from the Boston Nutrition and Obesity Research Center Adipocyte Core. The study was performed according to the guidelines of the Declaration of Helsinki (2008) and approved by the Medical Ethical Committees of the Academic Medical Center (AMC) of the University of Amsterdam and Boston University Medical Center. After a complete description of the study had been given, written informed consent was obtained.

### 2.2 | Two-step hyperinsulinemic euglycemic clamp

Glucose metabolism and lipolysis were assessed in the obese subjects only.<sup>20</sup> The subjects were admitted to the Metabolic Clinical Research Unit of the AMC at 08:00 AM after an overnight fast. A catheter was inserted into an antecubital vein for infusion of [6,6-<sup>2</sup>H<sub>2</sub>]glucose, [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol (>99% enriched; Cambridge Isotopes, Andover, MA, USA), glucose 20%, and insulin. Another catheter was inserted into a contralateral hand vein and kept in a thermoregulated (60°C) plexiglas box for sampling of arterialized venous blood. Saline was infused as NaCl 0.9% at a rate of 50 mL/h to sustain catheter patency. [6,6-<sup>2</sup>H<sub>2</sub>]-glucose and [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol were infused as tracers to study glucose

kinetics and lipolysis (total triacylglycerol hydrolysis), respectively. At 09:00 AM ( $T = -2$  hours), after drawing a blood sample for background enrichment of plasma glucose and glycerol, a primed-continuous infusion of [6,6-<sup>2</sup>H<sub>2</sub>]glucose and of [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol were started at a rate of 0.11 μmol/kg/min after a priming dose equivalent to 120-minute infusion. After 110, 115, and 120 minutes, blood samples were drawn for determination of glucose and glycerol enrichments, glucoregulatory hormones and FFA. Subsequently, at 11:05 AM ( $T = 0$ ), a continuous infusion of insulin (Actrapid 100 U/mL; Novo Nordisk Farma, Alphen a/d Rijn, the Netherlands) was started for 2 hours at a rate of 20 mU/m<sup>2</sup> body surface area/min. At  $T = 2$  hours, the infusion rate of insulin was increased to 60 mU/m<sup>2</sup> body surface area/min. Plasma glucose was measured every 10 minutes and glucose 20% (enriched with [6,6-<sup>2</sup>H<sub>2</sub>]glucose to approximate plasma enrichment) was infused at a variable rate to maintain plasma glucose at 5.0 mmol/L. At  $T = 2$  hours and  $T = 4$  hours, five blood samples with a 5-minute interval were drawn to measure glucose and glycerol enrichments and two samples were drawn to measure glucoregulatory hormones and FFA. During the study, the participants were allowed to drink water only.

### 2.3 | Biopsies

Roux-en-Y gastric bypass surgery was performed by experienced bariatric surgeons in two medical centers (Rijnstate Hospital, Arnhem and the former Slotervaart Hospital, Amsterdam, the Netherlands). The laparoscopic cholecystectomies were performed by an experienced surgeon in one medical center (Northwest Clinics, Alkmaar, the Netherlands). Before starting the RYGB procedure, tissue biopsies were taken from the liver and visceral and subcutaneous abdominal adipose tissue. Local hemostasis was checked directly after the biopsies and at the end of the surgical procedure. Tissue samples were obtained after a comparable fasting period (10-12 hours). The samples were immediately frozen in liquid nitrogen and stored at -80°C until further analyses.

### 2.4 | Gene expression

Total RNA was isolated using TRIzol reagent (Invitrogen, Breda, the Netherlands), followed by the NucleoSpin II extraction kit (Macherey & Nagel Gm bH, Duren, Germany) according to the manufacturer's recommendations. Briefly, 1 mL of TRIzol and glass beads (Biospec Products Inc., Bartlesville, OK, USA) were added to the tissue. After vigorous shaking using a Fast Prep-24 machine for 20 seconds. At 4.5 m/s (MP Biomedicals, Santa Ana, CA, USA), the

homogenate was centrifuged (10 minutes at 12 000×g at 4°C). The nonlipid containing fraction was transferred and 200 µL of chloroform was added. The mixture was subsequently vortexed and centrifuged (15 minutes at 16 100×g at 4°C). The aqueous phase was transferred to a new tube and an equal volume of 70% ethanol was added. Afterward, we continued with the NucleoSpin II extraction kit according to the manufacturer's instructions. RNA concentrations were measured using the Nanodrop Spectrophotometer 1 (Nanodrop Technologies, Wilmington, NC, USA). cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer's instructions. After synthesis, the cDNA was diluted 20×. mRNA expression of RBP4, GLUT4, and inflammatory markers was normalized to the housekeeping gene Acidic Ribosomal Phosphoprotein P0 (RPLP0).

## 2.5 | Protein isolation and detection of RBP4 in serum and tissue

Cell lysates were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris HCl pH 7.4, 2 mM EDTA, 0.5% deoxycholate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM NaF, 0.5% Triton X-100), supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland), and PMSF.

Serum was diluted 30× in a standard lysis buffer. Adipose tissue was lysed in standard lysis buffer. One microliter equivalent of human serum and 50 µg of protein from human adipose tissue were separated by 18% SDS-PAGE and transferred to nitrocellulose membranes. Human RBP4 proteins were detected using anti-human RBP4 polyclonal antisera (Dako, Glostrup, Denmark, catalog #A0040). For quantitation of serum RBP4 levels, a standard curve of purified human RBP4 was run on each gel.<sup>22</sup>

## 2.6 | Differentiation of human stromal vascular fraction (SVF) cells into adipocytes

Isolation, proliferation, and differentiation of human SVF cells were performed as described.<sup>23</sup> Briefly, AT biopsies were washed, minced, treated with collagenase, and cells were filtered and centrifuged. Cells were resuspended in growth media, counted and plated. After cells (preadipocytes) reached 70%-80% confluence, they were re-plated from 10-cm dish to 12-well plates, with density of 5000-15000 cells/cm<sup>2</sup>. Cells were grown in growth media (alpha-MEM with 10% FBS and 1% pen/strep antibiotics) and at 2 days after confluence cells were considered ready for differentiation. Growth media was changed to differentiation media (18.5 mM glucose DMEM/F12 media, 15 mM HEPES, 25 mM NaHCO<sub>3</sub>, 1% pen/strep antibiotics, 33 µM d-Biotin, 17 µM pantothenate, 100 nM dexamethasone, 100

nM insulin, 1 µM rosiglitazone, 0.5 mM IBMX (1-methyl-3-isobutylxanthine), 2 nM T<sub>3</sub>, 10 µg/mL transferrin). After 3 days of differentiation, differentiation media was replaced by maintenance media (18.5 mM glucose DMEM/F12 media, 15 mM HEPES, 25 mM NaHCO<sub>3</sub>, 1% pen/strep antibiotics, 33 µM d-Biotin, 17 µM pantothenate, 100 nM dexamethasone, 100 nM insulin) which was changed every second day. At day 12 after induction of differentiation, adipocytes were used for the experiment.

## 2.7 | Activation of macrophages (RAW 264.7 cells) by RBP4 to obtain conditioned media

RAW 264.7 cells were maintained in DMEM media (4.5 g/L glucose, L-glutamine, sodium pyruvate, 10% FBS, 1% pen/strep antibiotics). For the production of conditioned media, cells were treated with RBP4 (50 µg/mL) for 18-24 hours. Subsequently, media were harvested and any cell debris removed by centrifugation.

Differentiated human SVF-adipocytes at day 12 of differentiation were treated with RBP4 (50 µg/mL)/serum and insulin-free maintenance media or DMSO/serum and insulin-free maintenance media (control) for 3 or 18 hours. After treatment, cells were additionally serum and insulin starved for 1 hour followed by the lipolysis experiment.

Day 12 differentiated human SVF adipocytes were treated with conditioned media from RBP4-treated macrophages or conditioned media from untreated macrophages (25% conditioned media/75% serum and insulin free maintenance media) for 3 hours. After treatment, cells were additionally serum- and insulin-starved for 1 hour and the lipolysis experiment was performed.

## 2.8 | Basal and stimulated lipolysis in human adipocytes

The lipolysis experiment was performed as described previously.<sup>23</sup> After DMSO or RBP4 treatment and serum/insulin starvation, the lipolysis assay was performed using Krebs-Ringer (KRB) buffer with 5 mM glucose and 4% BSA, pH 7.4 with adenosine deaminase (ADA [1 U/mL]), and phenylisopropyl adenosine (PIA [20 nM]). For stimulated lipolysis, isoproterenol (10<sup>-8</sup>-10<sup>-6</sup> M) or 8-Br-cAMP (1 mM) was also added. Inhibition of lipolysis by insulin was measured under 8-Br-cAMP-stimulated conditions, with insulin at 0, 5, 10, 15, 30, and 600 pM concentrations.

Cells were incubated with KRB and reagents for basal, stimulated or insulin-suppressed lipolysis at 37°C for 2 hours. After incubation, cell culture plates were placed on ice to stop the reaction and media was saved for glycerol measurement and cells for cell number calculations.

Glycerol concentration in culture media was measured using a standard enzymatic fluorometric assay in neutralized perchloric acid extracts.<sup>24</sup> Cell number/double-stranded DNA was measured using Quant-iT PicoGreen ds DNA Assay Kit (ThermoFisher Scientific cat #P7589).

TNF $\alpha$  levels in media from the RAW macrophages that were treated with RBP4 (50  $\mu$ g/mL) were measured by Elisa as described.<sup>14</sup>

## 2.9 | Analytical procedures

In human plasma, glucose was measured using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barleben/Magdeburg, Germany). Insulin was determined using an Immulite 2000 system (Diagnostic Products Corporation, Los Angeles, CA, USA), with a chemiluminiscent immunometric assay (intra-assay variation 4%-5%; inter-assay variation 5%; detection limit 15 pmol/L). FFA were measured using an enzymatic method (NEFA-Cc, Wako Chemicals, Neuss, Germany; intra-assay variation 1%; inter-assay variation 4%-15%; detection limit 0.02 mmol/L). [6,6-<sup>2</sup>H<sub>2</sub>]glucose and [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol enrichment were measured as described previously.<sup>20</sup>

## 2.10 | Calculations

EGP, lipolysis rate, and peripheral glucose disposal (rate of disappearance [Rd]) were calculated using modified versions of the Steele equations as described previously.<sup>25,26</sup> EGP is expressed as  $\mu$ mol/(kg fat-free mass)/min. Lipolysis and Rd are both expressed as  $\mu$ mol/kg/min. Insulin-mediated suppression of lipolysis, FFA, and EGP is expressed as the % suppression of the basal values and was assessed during the first step of the hyperinsulinemic-euglycemic clamp.

## 2.11 | Statistical analysis

In vivo data are presented as median and range. Due to the small sample size and nonnormal distribution of study parameters, nonparametric tests were used. Between-group differences were tested using the Mann-Whitney *U* test. Correlations were calculated using the Spearman correlation test. For in vitro experiments, data are presented as means  $\pm$  SEM. Paired *t* test and two-way ANOVA were used to investigate differences between the groups. Subjects with missing data were excluded from analyses. A *P* value < 0.1 was considered a trend and a *P* value < .05 was considered statistically significant. All statistical analyses were run on IBM SPSS version 24 (SPSS, Chicago, IL, USA) and GraphPad PRISM 7 software (La Jolla, CA, USA).

## 3 | RESULTS

### 3.1 | Demographic and metabolic characteristics

Table 1 shows the clinical and metabolic characteristics. The subjects were all female and similar in age. Due to technical issues, the clamp was not completed in two of the obese subjects. In one lean control, visceral biopsies were not performed because it was considered not safe because of the low amount of visceral adipose tissue (VAT). The obese subjects were insulin-resistant reflected in higher fasting insulin levels.<sup>27</sup> Since reduced expression of GLUT4 specifically in adipocytes is a central feature of whole-body insulin resistance,<sup>28</sup> we measured GLUT4 mRNA expression in the adipose tissue depots of both groups. GLUT4 mRNA expression was lower in the obese subjects in both the subcutaneous (*P* < .001) and visceral (*P* = .019) adipose tissue depots (Figure 1). Subcutaneous and visceral adipose GLUT4 mRNA expression were both inversely correlated with BMI (*r*s = -0.601, *P* = .001; *r*s = -0.502, *P* = .011, respectively) and in the obese subjects subcutaneous GLUT4 mRNA expression correlated with Rd (*r*s = 0.591, *P* = .013), whereas there was no correlation between visceral GLUT4 mRNA expression and Rd (*r*s = 0.397, *P* = .115).

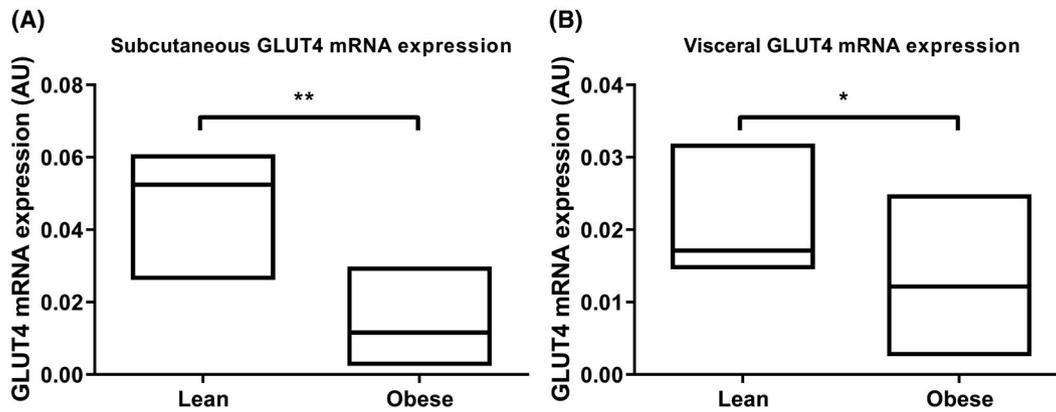
### 3.2 | Adipose and hepatic RBP4 mRNA expression is increased in obese women

Subcutaneous adipose RBP4 mRNA expression (*P* = .015) but not RBP4 protein expression (*P* = .570) was increased in the obese subjects. In VAT from obese subjects, RBP4 protein was increased (*P* = .009) and RBP4 mRNA expression tended to be increased (*P* = .062). Hepatic RBP4 mRNA expression was increased in the obese subjects compared to lean controls (*P* < .001) (Figure 2). Neither hepatic nor adipose mRNA expression of RBP4 correlated with serum RBP4 levels. A limitation of this study is that we were not able to measure RBP4 protein levels in liver due to insufficient sample size.

**TABLE 1** Demographic and metabolic characteristics

	Lean (N = 6)	Obese (N = 20)	<i>P</i>
Age (years)	37.5 [26-45]	41 [26-58]	.120
BMI (kg/m <sup>2</sup> )	22.1 [20-24.3]	42.9 [38.7-61.3]	<.001
Fasting glucose (mmol/L)	5.0 [4.5-5.6]	5.4 [4.4-8.8]	.200
Fasting insulin (pmol/L)	25 [14-63]	81 [20-142]	.002

Notes: Data are presented as median [range].



**FIGURE 1** Relative adipose tissue GLUT4 mRNA expression of lean versus obese subjects. Data presented as median (range).  $n = 6$  for panel A and  $n = 5$  for panel B lean and 20 obese women. \* $P < .05$ , \*\* $P < .001$

### 3.3 | Serum RBP4 levels correlate inversely with insulin sensitivity

Circulating RBP4 can interfere with insulin signaling in skeletal muscle and liver.<sup>4</sup> In line, in the obese subjects, serum RBP4 correlated inversely with Rd ( $rs = -0.551$ ,  $P = .022$ ). There was also a trend for an inverse correlation between SAT RBP4 protein and Rd ( $rs = -0.771$ ,  $P = .072$ ). In contrast, there was a strong inverse correlation between serum RBP4 levels and insulin-mediated suppression of lipolysis ( $rs = -0.635$ ,  $P = .008$ ), FFA ( $rs = -0.672$ ,  $P = .003$ ), and EGP ( $rs = -0.637$ ,  $P = .006$ ) (Figure 3). Serum RBP4 levels did not correlate with fasting lipolysis, FFA, or EGP.

### 3.4 | RBP4 mRNA expression correlates with expression of inflammatory markers in SAT, but not in VAT

Recent studies have shown that RBP4 activates pro-inflammatory pathways in cell lines and animal models, which may lead to insulin resistance.<sup>14-16</sup> To investigate the relationship between RBP4 and adipose inflammation, we measured the expression of several known inflammatory markers involved in adipose tissue inflammation. RBP4 mRNA expression was associated with mRNA expression of adipose macrophage (CD68, MRC1) and T cell (CD4, CD25) markers as well as with expression of members of the chemokine family (CCL18, CCR7) and TLR4 in SAT, but not VAT (Table 2 and Figure 4).

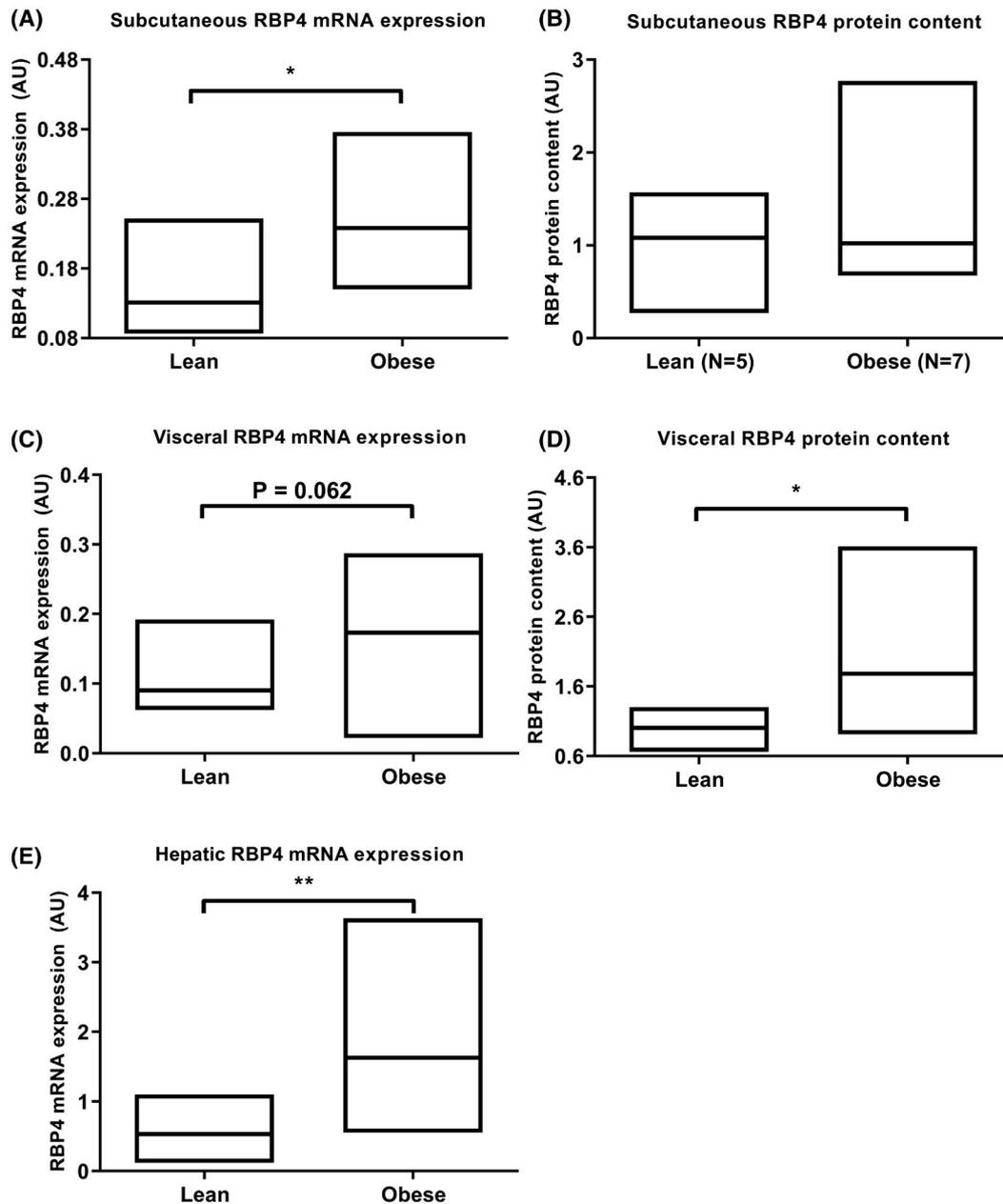
### 3.5 | Direct and indirect effects of RBP4 on lipolysis in human adipocytes

Direct treatment of human adipocytes with RBP4 increased basal lipolysis (Figure 5A) but did not impair the effect of

insulin to suppress lipolysis (Figures 5B,C). However, conditioned media from RBP4-treated macrophages markedly stimulated basal lipolysis, and augmented isoproterenol-stimulated and 8-Br-cAMP-stimulated lipolysis in human adipocytes (Figure 6A). This conditioned media also impaired insulin inhibition of lipolysis (Figure 6B). To explore the mechanisms by which RBP4 increases lipolysis, we measured TNF $\alpha$  protein levels in the conditioned media from macrophages treated with RBP4, as TNF $\alpha$  is known to increase lipolysis<sup>29</sup> and a previous study showed that RBP4 increased TNF $\alpha$  production from mouse and human macrophages.<sup>14</sup> Compared to the conditioned media of untreated macrophages, the conditioned media of macrophages treated with RBP4 had at least 40-fold higher TNF $\alpha$  protein levels (Figure 6C). Thus, treatment of adipocytes with conditioned media from RBP4-treated macrophages stimulates lipolysis and causes resistance to insulin-mediated inhibition of lipolysis. This could result from increased RBP4-induced secretion of pro-inflammatory cytokines such as TNF $\alpha$ .

## 4 | DISCUSSION

Obesity-induced insulin resistance is associated with increased lipolysis in adipose tissue.<sup>3</sup> Because increased RBP4 levels induce insulin resistance, we hypothesized that RBP4 may have a direct effect on basal lipolysis and/or the ability of insulin to suppress lipolysis and this could be an important mechanism by which elevated RBP4 levels result in whole body insulin resistance. We found a strong relationship between serum RBP4 levels and resistance to suppression of lipolysis by insulin in vivo in obese human subjects. In parallel, our mechanistic studies showed that in cultured human adipocytes, RBP4 directly stimulated lipolysis in the basal state but did not alter insulin suppression of lipolysis. Previous studies in human and rodent cells<sup>14</sup> and animal models have shown that RBP4 activates macrophages and other antigen-presenting cells. RBP4 activates both CD4-positive



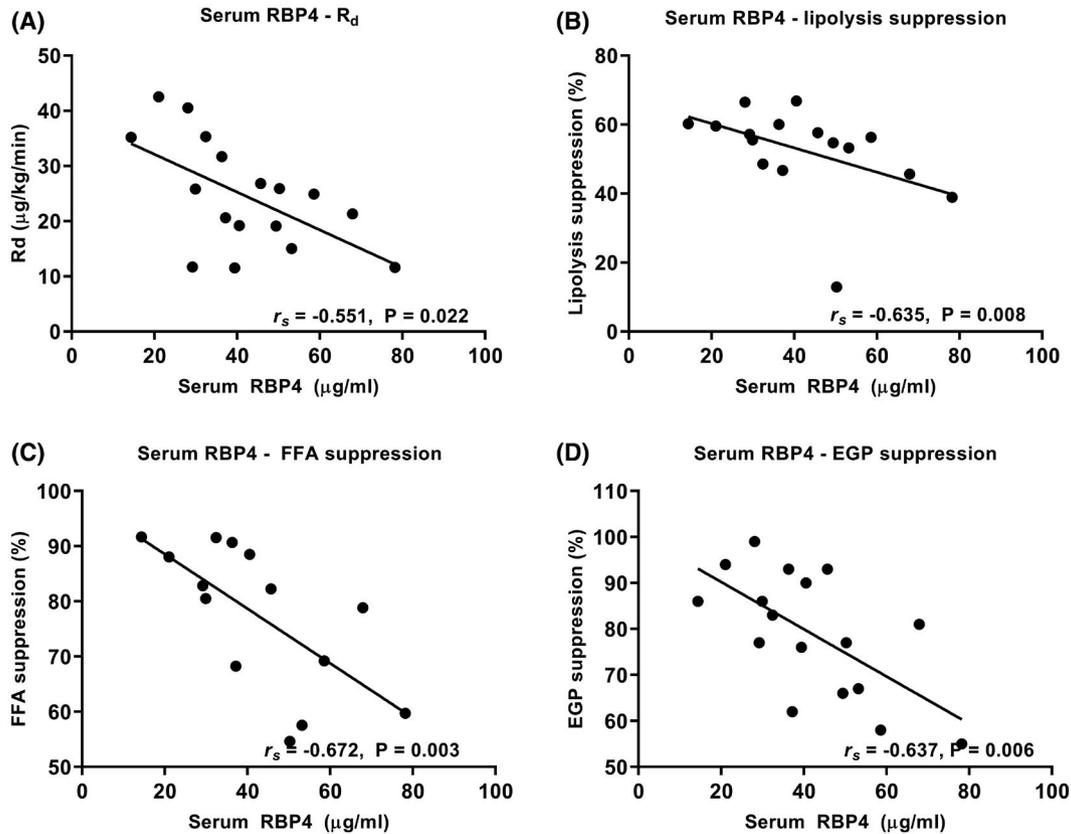
**FIGURE 2** Relative mRNA expression and protein content of RBP4 in subcutaneous AT (A and B), visceral AT (C and D), and relative RBP4 mRNA expression in liver (E) of lean versus obese subjects. Data presented as median (range). \* $P < .05$ , \*\* $P < .001$ .  $n = 6$  lean for panels A and E and  $n = 5$  lean for panels B-D.  $n = 7$  obese in panel B,  $n = 12$  obese in panel D,  $n = 20$  obese in panels A, C, and E

T cells and macrophages through TLR4- and JNK-dependent pathways, resulting in increased production of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6.<sup>14-17</sup> These cytokines have been shown to stimulate lipolysis and also to impair insulin suppression of lipolysis.<sup>29-32</sup> The possibility that RBP4 increases lipolysis is strongly supported by the observation that mice overexpressing human RBP4 selectively in adipocytes have increased mRNA expression of the lipolytic enzymes ATGL, HSL, and LPL in adipose tissues and elevated plasma FFA levels.<sup>17</sup>

We tested whether media from RBP4-activated macrophages could affect lipolysis. We found that conditioned

media from RBP4-activated macrophages augments basal-, isoproterenol-, and 8-Br-cAMP-stimulated lipolysis in adipocytes and causes resistance to insulin suppression of lipolysis. We hypothesized that the impaired suppression of lipolysis in vivo may result from increased adipose tissue inflammation and pro-inflammatory effects of RBP4 on macrophages,<sup>14-16</sup> since we find in this study that RBP4 mRNA expression correlates with several macrophage markers and other pro-inflammatory markers in SAT in obese humans.

RBP4 induces TNF $\alpha$  expression through a TLR4-dependent pathway in macrophages<sup>14</sup> and suppression of TLR4, either through a transgenic knockout or pharmacologically through



**FIGURE 3** Serum RBP4 levels correlate inversely with peripheral glucose disposal (A), and insulin-mediated suppression (% of basal) of lipolysis (B), free fatty acids (C), and endogenous glucose production (D) in the obese subjects

a TLR4 inhibitor, attenuates RBP4-dependent TNF $\alpha$  production.<sup>14,33,34</sup> Because RBP4 induces TNF $\alpha$  production, and TNF $\alpha$  stimulates lipolysis,<sup>29</sup> we measured TNF $\alpha$  protein levels in the media from macrophages treated with RBP4 which was used as conditioned media for the human adipocyte experiments. We found that the levels of TNF $\alpha$  protein were increased at least 40-fold in media from macrophages after RBP4 treatment. These results further support the hypothesis that RBP4 affects lipolysis by activating macrophages to produce pro-inflammatory cytokines which can stimulate lipolysis and also impair insulin signaling.

These results are consistent with our findings that elevation of RBP4 in adipose tissue inhibits insulin-stimulated phosphoinositide 3-kinase (PI3K) activity *in vivo*<sup>4</sup> through an indirect mechanism involving activation of antigen-presenting macrophages and dendritic cells and release of pro-inflammatory cytokines.<sup>14,15</sup> Since PI3K activity is a major pathway mediating the anti-lipolytic effect of insulin,<sup>35</sup> this could explain, at least in part, how RBP4 impairs insulin action on lipolysis in adipose tissue.

Surprisingly, adipose tissue RBP4 mRNA expression did not correlate with insulin-mediated suppression of lipolysis *in vivo*. It has been shown that the mRNA expression of RBP4 in adipocytes may not always correlate with serum levels.<sup>36</sup>

**TABLE 2** Correlation between RBP4 mRNA expression and inflammatory marker mRNA expression in white adipose tissue

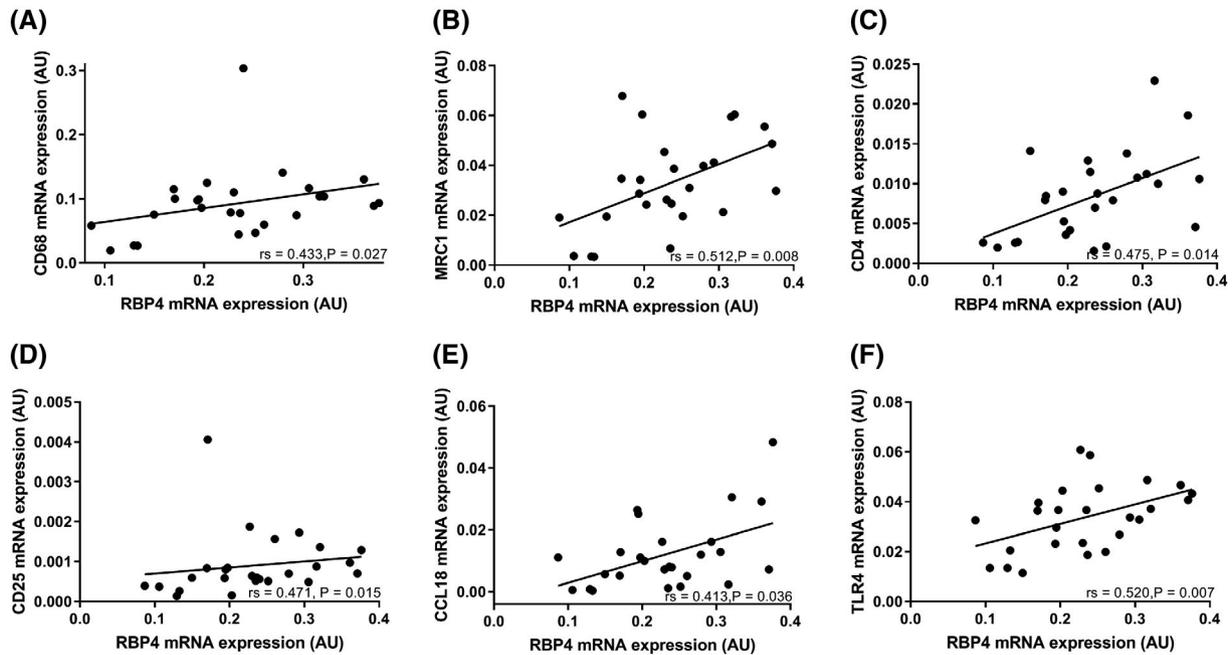
	SAT	VAT
CD68	0.433 <sup>a</sup>	0.168
CD163	0.281	0.220
MRC1	0.512 <sup>b</sup>	0.305
CCL18	0.413 <sup>a</sup>	0.254
CCR7	0.596 <sup>b</sup>	0.026
CCL4	0.346	-0.007
CCL2	0.294	-0.080
TLR4	0.520 <sup>b</sup>	0.283
IL10	0.520 <sup>b</sup>	0.129
CD200R	0.595 <sup>b</sup>	0.408 <sup>a</sup>
TNFA	0.139	0.065
CD11C	0.192	0.150
CD4	0.475 <sup>a</sup>	0.391
CD25	0.471 <sup>a</sup>	0.250
FOXP3	0.069	0.366

Correlations performed using Spearman correlation test.

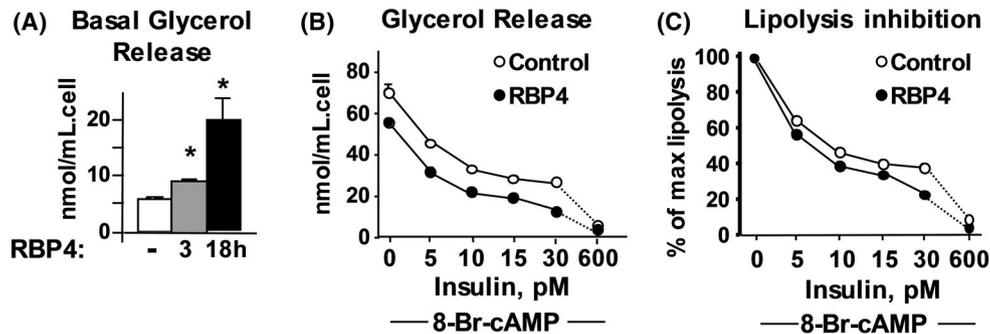
Abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

n = 23-26 for SAT. n = 21-25 for VAT.

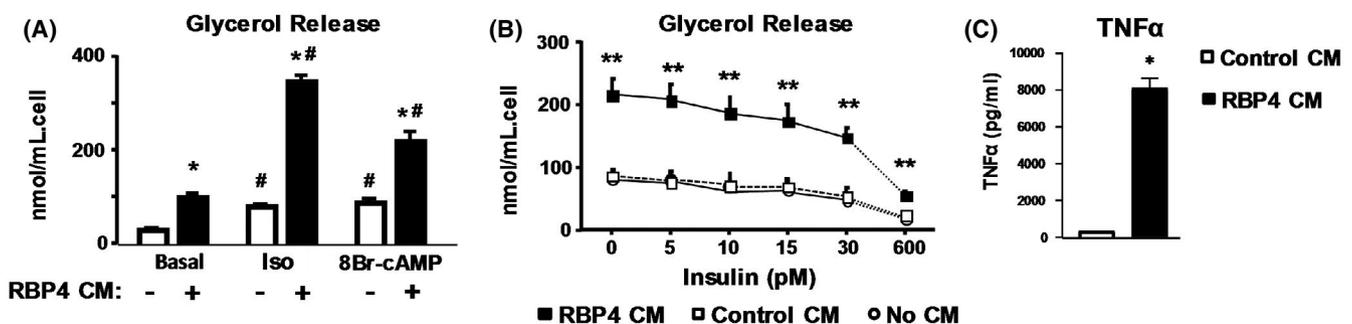
<sup>a</sup>P < .05, <sup>b</sup>P < .01.



**FIGURE 4** RBP4 mRNA expression correlates with the mRNA expression of CD68 (A), MRC1 (B), CD4 (C), CD25 (D), CCL18 (E), and TLR4 (F) in subcutaneous adipose tissue



**FIGURE 5** Direct effect of RBP4 on human SVF-derived adipocytes leads to increased basal lipolysis (A) but does not impair the ability of insulin to inhibit lipolysis (B, C). A, Human SVF-derived adipocytes were incubated with RBP4 (50  $\mu\text{g}/\text{mL}$ ) for 3 or 18 hours,  $*P < .01$  versus all other conditions. B, After RBP4 incubation, a dose-response to insulin of 8-Bromo-cAMP-activated lipolysis was performed. C, Results in panel B are expressed as % of maximal 8-Bromo-cAMP-activated lipolysis



**FIGURE 6** Indirect effect of RBP4 on human SVF-derived adipocytes (A). Media from RAW macrophages incubated with RBP4 (RBP4 CM) increases basal (–) and stimulated (+) lipolysis in human adipocytes.  $*P < .05$  versus (–) same condition,  $\#P < .05$  versus Basal same media condition. (B) Insulin inhibition of RBP4 CM-induced lipolysis has an ED50 over 30 pM compared to 10 pM for insulin inhibition of isoproterenol-induced lipolysis (data not shown). Control CM: media from RAW cells incubated with vehicle only without RBP4. No CM: adipocytes incubated without any conditioned media.  $**P < .05$  versus all other conditions. (C) Concentration of TNF $\alpha$  in media of RAW cells incubated without (open bar) or with RBP4 (50  $\mu\text{g}/\text{mL}$ ) (black bar).  $*P < .05$  versus conditioned media without RBP4

This is partly because circulating RBP4 can accumulate in adipose tissue where it can activate macrophages as demonstrated in mice overexpressing human RBP4 driven by a muscle promoter.<sup>15</sup> In this model, RBP4 is secreted from muscle and elevates circulating levels. Western blotting showed a greater than sixfold higher accumulation of human RBP4 in perigonadal adipose tissue compared to liver. Therefore, the RBP4 in adipose tissue may not be entirely derived from adipocytes but may originate in part from the circulation. Thus, mRNA expression may not reflect all the RBP4 present in adipose tissue.

In our study, in the obese subjects, hepatic RBP4 mRNA expression also did not correlate with serum RBP4 levels. Recently, a study in mice suggested that the main source of circulating RBP4 is from hepatocytes and that in liver-specific RBP4 knockout mice on a high-fat, high-sucrose diet, adipose tissue RBP4 expression was increased, while RBP4 was undetectable in serum.<sup>36</sup> However, another study showed that overexpression of human RBP4 selectively in adipocytes in mice resulted in increased adipocyte and serum RBP4 levels when mice were fed a high-fat diet.<sup>17</sup> The adipocytes were the source of the increased serum RBP4 since the human RBP4 protein was detected in the mouse serum and this could only be derived from the transgene. This suggests that under at least some obese/high-fat diet conditions, increased adipose secretion of RBP4 contributes to elevated serum levels. These studies underscore the complicated relationship between tissue levels of RBP4 and circulating RBP4 concentrations and explain the lack of a direct linear correlation.

Another novel finding is that serum RBP4 levels inversely correlate with insulin-mediated EGP suppression, that is, hepatic insulin sensitivity. One previous study showed that serum RBP4 levels inversely correlated with the absolute difference in hepatic glucose production between the basal state and upon insulin infusion in humans. However, in that study, after adjusting for sex, age, and body fat percentage, plasma RBP4 was not an independent predictor of EGP suppression.<sup>37</sup> In our study, serum RBP4 remained a negative predictor of EGP suppression after correction for both age and BMI (data not shown). Another study evaluated the correlation between plasma RBP4 levels and the hepatic insulin resistance index, a surrogate marker for hepatic insulin sensitivity, in Mexican Americans, ranging from lean to obese in weight and from normal glucose tolerant to T2DM. This study did not find a correlation.<sup>38</sup> However, a two step clamp was not performed and the insulin infusion rate seems too high to adequately assess hepatic insulin sensitivity. The results of the previous studies may differ from ours because of their inclusion of subjects of both sexes and subjects with both normal glucose tolerance and overt diabetes, which may have confounded the relationship between serum RBP4 levels and EGP suppression. The effect of RBP4 on hepatic insulin sensitivity might be through its stimulation of lipolysis, thus increasing

hepatic delivery of glycerol and acetyl CoA.<sup>39</sup> In support of this, adipose-specific overexpression of human RBP4 results in increased expression of lipolytic genes in white adipose tissue with increased fatty acid uptake in liver and hepatic steatosis.<sup>17</sup> It is possible that the effects of elevated RBP4 on insulin suppression of EGP may also have a direct component since liver PEPCK expression is increased in mice treated chronically with RBP4 and RBP4 impairs EGP suppression in cultured hepatocytes.<sup>4</sup> Thus, RBP4 might act through more than one mechanism including a retinol-dependent mechanism since retinoids induce the expression of some gluconeogenic enzymes such as PEPCK.<sup>40</sup>

Another finding in this study is that hepatic RBP4 mRNA expression was increased in obese subjects compared to lean controls. The causative factor is not known, but factors that increase RBP4 expression include retinol, glucagon, estrogen, and stimulation of cAMP.<sup>41-44</sup> There are mixed results in the literature in terms of hepatic RBP4 regulation in obesity and whether elevated liver RBP4 levels could contribute to nonalcoholic hepatic steatosis based on either mRNA or protein measurements. Studies have shown that hepatic RBP4 mRNA and protein expression is increased in obese rodents.<sup>45</sup> In humans, one study showed no difference in hepatic RBP4 mRNA expression between 45 obese women and 4 lean controls.<sup>46</sup> However, a recent study indicated that both mRNA and protein levels of RBP4 are increased in liver of humans with nonalcoholic fatty liver disease and RBP4 mRNA expression correlates with hepatic triglyceride content.<sup>47</sup> Adeno-associated viral overexpression of RBP4 in liver does not impair whole body glucose tolerance in mice but serum RBP4 levels were not elevated in that study and hepatic insulin sensitivity was not assessed.<sup>48</sup> Finally, adipocyte-selective overexpression of RBP4 and global overexpression of RBP4 both result in glucose intolerance and increased hepatic lipid accumulation even on a chow diet.<sup>17,47</sup>

In our study, in addition to the inverse correlation of serum RBP4 levels with suppression of lipolysis and hepatic glucose output, serum RBP4 levels correlated inversely with peripheral glucose disposal, which is in line with the effect of elevated RBP4 to impair insulin signaling in muscle *in vivo*.<sup>4</sup> We did not measure RBP4 protein levels in skeletal muscle or liver due to insufficient sample size. Therefore, we cannot exclude direct effects of tissue RBP4 protein on the measured metabolic fluxes.

Limitations of this study include the relatively small number of subjects for which we had tissue biopsies, the smaller number of lean controls compared to the obese subjects and the inclusion of female subjects only. Generalization to the general population needs to occur with caution as sexual dimorphism in the expression of RBP4 has been observed.<sup>6</sup>

We conclude that in morbid obesity, hepatic, visceral adipose and subcutaneous adipose RBP4 mRNA and/or protein levels are increased and circulating RBP4 levels

correlate inversely with insulin suppression of lipolysis and EGP, and with glucose disposal. Furthermore, RBP4 mRNA levels correlate with the mRNA levels of inflammation markers in WAT. RBP4 acts directly and indirectly through macrophage activation to augment lipolysis and induce resistance to insulin suppression of lipolysis in human adipocytes. Thus, RBP4-induced inflammation of adipose tissue increases lipolysis in vitro and our flux studies in humans in vivo show a corresponding correlation between RBP4 and resistance to insulin suppression of lipolysis. These results, combined with previous in vitro studies, suggest that elevated RBP4 levels in serum and/or adipose tissue may contribute to causing insulin resistance in humans by impairing lipolysis suppression. While we demonstrate a causative role in vitro in human adipocytes, our flux studies in vivo provide the basis for future experiments to test the potential causative role of RBP4 on increased lipolysis and the extent to which this contributes to insulin resistance in obese humans.

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## CONFLICT OF INTEREST

B.B.K. is an inventor on a patent on RBP4. There are no other potential conflicts of interest relevant to this article.

## AUTHOR CONTRIBUTIONS

M.J. Serlie and B.B. Kahn designed the research; M. Kilicarslan, B.A. de Weijer, and K. Simonytė Sjödin performed the research; M. Kilicarslan, M.J. Serlie, and B.B. Kahn analyzed data and wrote the paper; H. Cakir, I.M. Janssen, F.J. Berends, A.W. van de Laar, A.P. Houdijk collected the data; P. Aryal, K.W. ter Horst, M.T. Ackermans provided experimental technical support; and J.A. Romijn made revisions to the manuscript.

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