TNFRSF14 deficiency protects against ovariectomy-induced adipose tissue inflammation

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Abstract

To elucidate the role of tumor necrosis factor receptor superfamily member 14 (TNFRSF14) in metabolic disturbance due to loss of ovarian function, ovariectomy (OVX) was performed in TNFRSF 14-knockout mice. OVX increased fat mass and infiltration of highly inflammatory CD11c cells in the adipose tissue (AT), which was analyzed by flow cytometry, and resulted in disturbance of glucose metabolism, whereas TNFRSF14 deficiency attenuated these effects. TNFRSF14 deficiency decreased recruitment of CD11c-expressing cells in AT and reduced the polarization of bone marrow-derived macrophages to M1. Upon engagement of LIGHT, a TNFRSF14 ligand, TNFRSF14 enhanced the expression of CD11c via generation of reactive oxygen species, suggesting a role of TNFRSF14 as a redox modulator. TNFRSF14 participated in OVX-induced AT inflammation via upregulation of CD11c, resulting in metabolic perturbation. TNFRSF14 could be used as a therapeutic target for the treatment of postmenopausal syndrome by reducing AT inflammation.

Key Words
- adipose tissue
- inflammatory diseases
- ovarian function
- menopause
- glucose metabolism

Introduction

Menopause increases the incidence of metabolic diseases. The loss of ovarian function is associated with increased visceral fat along with metabolic pathologies due to chronic inflammation (Shoelson et al. 2007). The ovariectomy (OVX) model in mice is commonly accepted to reflect human menopause. OVX increased body weight and chronic inflammation (Rogers et al. 2009), indicating that increased fat is associated with inflammation, leading to the metabolic complications observed in OVX. Increased fat leads to the accumulation of adipose tissue macrophage (ATM), which plays a critical role in chronic inflammation and development of insulin resistance (IR) (Shoelson et al. 2007). Exacerbation of insulin sensitivity is related to the increased ATM (Kanda et al. 2006). Inversely, decreased ATM reduces IR caused by diet-induced obesity (Lesniewski et al. 2007). The local metabolic micro-environment affects phenotypes of predominant cells, leading to heterogeneous ATM. Macrophages are classified into two populations, one of which expresses CD11c, an M1 macrophage marker specifically recruited to adipose tissue (AT) in obesity (Lumeng et al. 2007). M1 ATM produces proinflammatory cytokines such as tumor necrosis factor α (TNFa), interleukin 6 (IL6), and monocyte chemoattractant protein 1, which contribute to the induction of IR, indicating a connection between CD11c expression and IR. M2 ATMs have different expression patterns, with a high

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level of CD163, arginase-1, or IL10 that are mainly associated with tissue repair (Gordon & Taylor 2003).

TNF receptor superfamily 14 (TNFRSF14) is a TNF receptor family member and was originally identified as the receptor for herpes simplex virus 1 (Montgomery et al. 1996). TNFRSF14 was initially identified to act as a co-stimulator in T-cells, and cross-linking of LIGHT, a TNF family member, to TNFRSF14 stimulates T-cells and accelerates proliferation and cytokine production (Tamada et al. 2000). TNFRSF14 is expressed constitutively on myeloid lineage cells and is also strongly expressed on nonimmune cells such as endothelial cells and adipocytes (Bassols et al. 2010a), suggesting that TNFRSF14 participates in metabolism as well as immune modulation. TNFRSF14 transmits a signal that leads to the activation of NF-κB (Marsters et al. 1997), a transcriptional regulator of inflammatory genes, suggesting a link between TNFRSF14 and inflammatory metabolic diseases. LIGHT, which is released in platelets, has been demonstrated to induce an inflammatory response in monocytes (Otterdal et al. 2006) and to mediate upregulation of matrix metalloproteinases (MMPs) and intercellular adhesion molecule 1 in monocytes, resulting in aggravation of rheumatic arthritis (Kang et al. 2007). Cross-linking of TNFRSF14 and its ligand is also involved in the development of inflammatory diseases by recruiting immune cells and releasing inflammatory cytokines (Kim et al. 2011a). Lack of TNFRSF14 reduces high-fat diet (HFD)-induced IR (Kim et al. 2011b). LIGHT is also associated with hyperglycemia in obese subjects (Bassols et al. 2010b).

In this study, we investigated the role of TNFRSF14 in OVX-induced AT inflammation to cause metabolic perturbation in mice.

Materials and methods

Animals and study design

TNFRSF14−/− (TNFRSF14-knockout (KO)) mice with a C57BL/6 genetic background were prepared as described previously (Kim et al. 2011c) and were provided by the Immunomodulation Research Center (IRC), University of Ulsan. The genotypes of offspring were determined by Southern blot analysis of DNA from tail biopsies. All mice were housed in the specific pathogen-free animal facility of the IRC and were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the IRC. The standards were approved by that Committee (2008-033). Six-week-old female TNFRSF14+/+ (WT) and TNFRSF14−/− (KO) mice were subjected to OVX or sham operation. Food intake and body weight were monitored daily and weekly respectively. After 12 weeks, mice were fasted for 6 h and killed by cervical dislocation. Blood was collected by cardiac puncture, and tissues were immediately harvested. Blood glucose was measured with a commercially available enzyme assay kit (Asan Pharmacology, Hwa-Seong, Korea). Glucose and insulin tolerance tests were performed on 6 h-fasted mice. For glucose tolerance, animals were injected i.p. with glucose (1 mg/kg), whereas for insulin tolerance, 0.75 m-units/kg of recombinant human regular insulin (Eli Lilly) was injected i.p. Blood samples were drawn at 0, 15, 30, 60, 90, and 120 min after glucose injection or 0, 15, 30, 60, 90, and 120 min after insulin injection. Serum H2O2 was determined by Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen).

Stromal vascular cell isolation

Visceral fat pads were weighed, rinsed three times in PBS, and minced with fluorescence-activated cell sorter (FACS) buffer (PBS with 1% BSA). Tissue suspensions were centrifuged at 500 g for 5 min and then treated with type 2 collagenase (1 mg/ml; Sigma Chemical) for 90 min at 37 °C by shaking. Cell suspensions were filtered through a 100 μm filter and centrifuged at 500 g for 5 min. Stromal vascular cell (SVC) pellets were incubated with RBC lysis buffer (eBioscience, San Diego, CA, USA) for 5 min, centrifuged at 300 g for 5 min, and resuspended in FACS buffer. SVC were incubated with Fc blocker for 20 min at 4 °C before staining fluorescently labeled antibodies against CD11b, CD11c, F4/80, CD4, and CD8 (eBioscience). Cells were gently washed twice, resuspended in FACS buffer, and analyzed using a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA).

Cell preparation

Femora and tibiae were removed aseptically and dissected free of adherent soft tissue. The bone ends were cut and the marrow cavity was flushed out with α-MEM (minimum essential media) from one end of the bone, using a sterile 21-gauge needle. The bone marrow was further agitated using a Pasteur pipette to obtain a single-cell suspension, which was washed twice and incubated on plates with macrophage colony-stimulating factor (M-CSF) (20 ng/ml; R&D Systems, Minneapolis, MN, USA) for 16 h. Non-adherent cells were then harvested, layered on a Ficoll–Hypaque gradient, and cultured for two more days, by which time large populations of adherent monocyte-/macrophage-like cells had formed.
on the bottoms of the culture plates as previously described (Kim et al. 2012). The few non-adherent cells were removed by washing the dishes with PBS, and the adherent cells (bone marrow-derived macrophages (BMMs)) were harvested and seeded on plates. The adherent cells were analyzed by FACS and found to be negative for CD3 and CD45R and positive for CD11b. The absence of contaminating stromal cells was confirmed by lack of cell growth in the absence of M-CSF. Additional medium with M-CSF was added and later refreshed on day 3. After incubation for the recommended times, the cells were analyzed using a FACSCanto II flow cytometer. BMM was transfected with siRNA against p47phox (sip47phox), or scrambled siRNA (scRNA) (Santa Cruz Biotechnology), using Lipofectamine RNAiMAX (Invitrogen) and further analyzed.

Quantitative PCR

Total RNA from BMMs incubated with M-CSF for the indicated time period was extracted using TRIzol solution (Gibco, Life Technologies) and reverse-transcribed with oligo-dT and Superscript I enzyme (Invitrogen). Quantitative PCR (qPCR) was carried out using SYBR Green 1 Engine Opticon Continuous Fluorescence Detection System (MJ Research, Inc., Quebec, Canada). The specificity of each primer pair was confirmed by melting curve analysis and agarose-gel electrophoresis. GAPDH was amplified in parallel with genes of interest. Relative copy numbers were calculated using 2^(-ΔΔCt). The primer sequences used were as follows: 5'-ctggatatgcctttctctgtc-3' and 5'-gcaaccttctgctg-3' (CD11c); 5'-cagctgggctgtacaaacctt-3' and 5'-acccagaagactgtggatgg-3' (iNOS); 5'-tcgctcagggtcacaagaaa-3' and 5'-cgca-tgcttaggacatgtg-3' (IL1); 5'-gctcttactgactggcatgag-3' and 5'-ctccaagccaaagtccttaga-3' and GAPDH, 5'-accagaagactgtggatgg-3' and 5'-cacattgggggtaggaacac-3'.

Intracellular reactive oxygen species detection

The intracellular formation of reactive oxygen species (ROS) was detected using the fluorescence probe, 2',7'-dichloro-fluorescein diacetate (H2DCF-DA; Molecular Probes, Eugene, OR, USA). After BMM was cultured under the different experimental conditions for 2 days, the cells were washed, trypsinized, suspended in PBS, loaded with H2DCF-DA, and incubated at 37 °C for 30 min. The measurement of intracellular ROS was performed using flow cytometry with a FACS (FACSCanto II).

Statistical analysis

Values are expressed as mean ± S.E.M. Student’s t-test was used to evaluate differences between samples of interest and the corresponding controls. Differences between groups were assessed by one- or two-way ANOVA, followed by Bonferroni post-hoc tests. A P value <0.05 was considered statistically significant.

Results

TNFRSF14 deficiency decreases fat mass and metabolic perturbation induced by OVX

To investigate whether TNFRSF14 contributes to OVX-induced metabolic dysfunction, we analyzed TNFRSF14-KO mice compared with WT mice. The body weight increase in TNFRSF14-KO mice was slightly lower than that of WT mice at 12 weeks after sham surgery but not significantly (Table 1). No significant difference was found in visceral fat or subcutaneous fat between the two groups after sham surgery (Table 1), demonstrating that TNFRSF14 did not affect fat mass after sham surgery. Then, we performed OVX to assess the role of TNFRSF14 in metabolic disorder due to loss of ovarian function. Along with body weight, the fat masses of visceral and subcutaneous AT were significantly reduced in TNFRSF14-KO mice compared with WT mice after OVX (Table 1). However, neither OVX nor TNFRSF14 induced a significant difference in daily food intake (Table 1), suggesting that an altered metabolic rate may be responsible for the increased fat mass and body weight induced by OVX. Previously, we demonstrated that OVX induced oxidative stress (Phan et al. 2013), and the absence of TNFRSF14 decreases ROS levels in vitro (Kim et al. 2012). Elevated serum levels of H2O2 after OVX were significantly decreased in the absence of TNFRSF14 in vivo (Table 1). TNFRSF14 deficiency also reduced elevated levels of blood glucose and serum insulin induced by OVX (Table 1). To confirm that the absence of TNFRSF14 improved glucose tolerance aggravated by OVX, we determined glucose clearance following an i.p. injection of glucose. In OVX TNFRSF14-KO mice, the glucose level decreased significantly after glucose injection, and the area under the curve for glucose tolerance was also decreased compared with that of OVX WT mice (Fig. 1A). However, TNFRSF14 did not show any difference in sham mice. Next, after i.p. injected insulin was given to determine insulin sensitivity, glucose was cleared more effectively
in OVX TNFRSF14-KO mice compared with OVX WT mice (Fig. 1B), indicating that the lack of TNFRSF14 improved insulin sensitivity upon OVX.

**TNFRSF14 deficiency decreases M1 macrophages in AT induced by OVX**

The findings that ATM accumulated during obesity result in IR (Xu et al. 2003) and TNFRSF14 mediates macrophage recruitment in tissues (Kim et al. 2011a) prompted us to further link the cause of improved glucose clearance observed in OVX TNFRSF14-KO mice to attenuated ATM. To assess AT inflammation induced by OVX, we evaluated SVC of the visceral fat pad by flow cytometry. A profound increase in ATM was found in OVX WT mice, whereas the level was not completely rescued to that observed in OVX TNFRSF14-KO mice to attenuated ATM. As shown in Fig. 2A, serum H2O2 increased cell numbers of CD11cF4/80, CD4, and CD8 in WT mice, whereas TNFRSF14 deficiency significantly reduced all numbers, suggesting that TNFRSF14 plays a role in increasing immune cells in AT upon OVX.

As the M1 macrophage expressing CD11c is responsible for chronic inflammation and insulin sensitivity (Patsouris et al. 2008), we focused on investigating the precise mechanism of how TNFRSF14 acts to increase CD11c macrophages in AT. First, we sought to determine whether this increase is due to an increased influx of CD11c-expressing cells in AT and/or increased activation of bone marrow-derived precursors into CD11c-expressing M1 macrophages in residential tissues. Using CD11b as a marker of monocytes, we classified blood monocytes as CD11c⁺ or CD11c⁻ cells. Significant increase in the fraction of CD11c cells among total leukocytes was observed in OVX mice compared with sham mice (Fig. 3A). TNFRSF14 deficiency reduced blood CD11c cells upon OVX, indicating that TNFRSF14 plays a role in the recruitment of CD11c cells in AT. Next, to assess whether TNFRSF14 affects phenotypes of macrophage in residential tissues, bone marrow-derived precursors were exposed to M-CSF. After a 4-day exposure of M-CSF to BMM, ~20% of total cells expressed CD11c and F4/80, and the lack of TNFRSF14 reduced this

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT</th>
<th>OVX</th>
<th>TNFRSF14-KO</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased body weight (g)</td>
<td>5.886 ± 0.3965</td>
<td>8.871 ± 0.4144</td>
<td>4.968 ± 0.2149</td>
<td>6.467 ± 0.6182**</td>
</tr>
<tr>
<td>Subcutaneous fat (mg)</td>
<td>288 ± 55.89</td>
<td>1029 ± 198.8</td>
<td>315 ± 66.64</td>
<td>633.3 ± 128.3**</td>
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<tr>
<td>Visceral fat (mg)</td>
<td>327 ± 9.46</td>
<td>1213 ± 155.1</td>
<td>337 ± 57.2</td>
<td>777.6 ± 220.1**</td>
</tr>
<tr>
<td>Serum H2O2 (mmol/ml)</td>
<td>49.87 ± 1.602</td>
<td>66.62 ± 3.545</td>
<td>45.09 ± 1.730</td>
<td>55.67 ± 1.641**</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>92.20 ± 3.261</td>
<td>125.75 ± 5.878</td>
<td>102.3 ± 6.968</td>
<td>107.4 ± 5.626*</td>
</tr>
<tr>
<td>Serum insulin (ng/ml)</td>
<td>0.6030 ± 0.04380</td>
<td>0.8499 ± 0.05070</td>
<td>0.5454 ± 0.07600</td>
<td>0.6828 ± 0.03790*</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>4.340 ± 0.2300</td>
<td>4.230 ± 0.2800</td>
<td>4.020 ± 0.2300</td>
<td>4.260 ± 0.1800</td>
</tr>
</tbody>
</table>

Differences between groups were analyzed by two-way ANOVA, followed by Bonferroni post-hoc tests to compare the effect of TNFRSF14-KO deficiency (increased body weight, subcutaneous fat, visceral fat, serum H2O2, and serum insulin; *P < 0.01, serum glucose; **P < 0.01, effect of surgery. Increased body weight and serum insulin; *P < 0.05, serum H2O2; **P < 0.01, effect of TNFRSF14). WT OVX vs TNFRSF14-KO OVX; *P < 0.05, **P < 0.01.
TNFRSF14 induces CD11c expression via generation of ROS

As TNFRSF14 deficiency blunted M-CSF-induced CD11c expression in BMM, we investigated a mediator generated by TNFRSF14 to upregulate the expression of CD11c. It is known that oxidative stress induced by OVX was attenuated in the absence of TNFRSF14, and ROS contributes to increased CD11c expression (Moon et al. 2006). Thus, we assessed the association of ROS in CD11c expression with TNFRSF14-involved signaling. Exogenous H₂O₂ elevated CD11c in WT cells and completely recovered the decreased level of CD11c observed in TNFRSF14-KO cells (Fig. 4A). Decreased ROS levels due to percentage significantly (Fig. 3B). To analyze other characteristics of M-CSF-stimulated cells, we compared the transcript levels of specific markers. The absence of TNFRSF14 resulted in a decreased level of CD11c, IL1β, and iNOS and increased levels of arginase-1 and IL10 compared with WT cells (Fig. 3C), demonstrating that the absence of TNFRSF14 reduced the ability of BMM to express M1 specific characteristics. To confirm that the decreased CD11c expression observed in TNFRSF14-KO BMMs was due to lack of a signal through TNFRSF14, we examined the effect of LIGHT on CD11c expression. LIGHT was constitutively expressed in BMMs as shown previously (Kim et al. 2012). Soluble LIGHT (sLIGHT) significantly increased CD11c expression in WT cells but had no effect on TNFRSF14-KO cells (Fig. 3B), suggesting that LIGHT transmits a signal through TNFRSF14 leading to CD11c expression. To provide further evidence that a signal through TNFRSF14 is responsible for CD11c expression, we attempted to compete out membrane-bound TNFRSF14 with soluble TNFRSF14 (sTNFRSF14). sTNFRSF14 significantly reduced the expression of CD11c in WT cells but had no effect in the TNFRSF14-KO cells (Fig. 3B). These results demonstrated that the LIGHT–TNFRSF14 interaction is a positive regulator of CD11c expression via a signal through TNFRSF14.
diphenylene iodonium (NADPH oxidase inhibitor) or exposure to N-acetylcysteine also decreased the expression level of CD11c in WT BMM, but no further decrease was observed in the absence of TNFRSF14 (Fig. 4A). These results suggested that ROS play a positive role in the elevation of CD11c in BMM upon M-CSF stimulation, and that TNFRSF14 is associated with ROS generation to upregulate CD11c expression. Next, we confirmed that the LIGHT–TNFRSF14 interaction is involved in M-CSF-induced ROS levels. M-CSF resulted in ROS production in WT BMM, but the absence of TNFRSF14 reduced this production (Fig. 4B). sLIGHT increased ROS while sTNFRSF14 decreased ROS in WT cells, but neither had an effect on ROS in TNFRSF14-KO cells (Fig. 4B), indicating that sLIGHT sends a signal through TNFRSF14 to upregulate ROS level upon M-CSF stimulation. To confirm that TNFRSF14 mediates signaling to express CD11c via generation of ROS, sip47phox or...
scRNA was transfected to BMM. Downregulation of p47phox (Fig. 4C) decreased ROS levels as well as the fraction of M-CSF-induced CD11c cells in WT BMM, whereas no further decreases were observed in TNFRSF14-KO cells (Fig. 4D and E).

**Discussion**

We have demonstrated that the absence of TNFRSF14 attenuated AT inflammation by reducing CD11c-expressing cells (Fig. 5) and improved metabolic disturbance induced by OVX. However, TNFRSF14 did not affect these factors in mice that received sham surgery, indicating that TNFRSF14 may mediate chronic inflammation induced by loss of ovarian function. In this study, OVX increased fat mass and CD11c-expressing ATM as well as CD11bF4/80, CD4, and CD8 T-cells. CD11c-expressing cells show proinflammatory characteristics of classically activated M1 macrophages (Lumeng et al. 2007) and generate proinflammatory cytokines, contributing to obesity-induced AT inflammation (Wu et al. 2010). Depletion of CD11c leads to a dramatic decrease in both local and systemic inflammatory markers in obese mice (Patsouris et al. 2008), supporting a role for CD11c in inflammation. Increased fat results in an increase in ATM (Shoelson et al. 2007), which is responsible for generating proinflammatory cytokines to develop IR. Development of IR has been promoted by proinflammatory cytokines (Stienstra et al. 2010), but attenuated by anti-inflammatory cytokines (Hong et al. 2009). To develop IR, the importance of macrophages has been implicated by the findings. The adipocyte-specific overexpression of heme oxygenase 1 (HO1) does not protect against HFD-induced obesity or IR (Huang et al. 2013), whereas myeloid HO1 haploinsufficiency attenuates these conditions (Huang et al. 2012). Our study also showed that the number of M1 macrophages in AT and the expression of CD11c, IL1β, and iNOS in BMM were decreased in the absence of TNFRSF14. In contrast, the expression of IL10 and arginase-1 was elevated in TNFRSF14 deficiency. These findings suggested that TNFRSF14 plays a role in the disrupted glucose metabolism due to loss of ovarian function at least in part through the polarization of macrophages to M1.

Next, we demonstrated how TNFRSF14 was involved in the upregulation of CD11c expression in AT upon OVX. The lack of TNFRSF14 attenuated the influx of blood CD11c cells as well as CD11b cells, which were significantly elevated by OVX. We cannot exclude the contribution of blood neutrophils to the elevated level of CD11bCD11c upon OVX, because blood neutrophils also express CD11bCD11c. However, their infiltration in AT

**Figure 5**

Schematic presentation for OVX-induced infiltration of M1 macrophages in AT. Upon LIGHT stimulation, TNFRSF14 on macrophages acts as a potent stimulator of M-CSF/c-Fms signaling pathway for ROS production, resulting in elevated CD11c expression.
has been reported to be negligible compared with F4/80-expressing cells (~50-folds lower) when serum insulin level starts to rise with exhibiting IR (Komori et al. 2013). In addition to CD11c-expressing macrophages, TNFRSF14 deficiency reduced the infiltration of immune cells including CD11bF4/80, CD4, and CD8 T-cells in AT upon OVX, which induced them significantly, suggesting a role of TNFRSF14 in immune cell infiltration in AT. TNFRSF14 has been demonstrated to participate in the recruitment of macrophages and T-cells in vitro (Kim et al. 2011a). Accordingly, increased CD11c in AT upon OVX could be due to polarization of macrophages in AT. OVX increased serum level of M-CSF (Ke et al. 2012), which may promote the elevation of CD11c in AT. Decrease in CD11c cells is observed in mice lacking functional M-CSF (MacDonald et al. 2005), indicating that M-CSF is required for signaling in CD11c expression in vivo. Our data showed that M-CSF-induced CD11c expression was attenuated in the absence of TNFRSF14. sLIGHT increased CD11c and sTNFRSF14 decreased it in WT BMM, suggesting that LIGHT transmits the signal through TNFRSF14 to upregulate CD11c expression.

Loss of ovarian function significantly increased serum ROS in mice (Phan et al. 2013), and menopause causes oxidative stress in humans (Sanchez-Rodriguez et al. 2012). Elevation of ROS contributes to development of diabetes (Li et al. 2012) as well as upregulation of CD11c expression (Moon et al. 2006), suggesting that oxidative stress induced by OVX contributes to the elevation of CD11c expression. We showed that exogenous H2O2 increased CD11c expression in BMM upon M-CSF stimulation and rescued the decrease induced by TNFRSF14 deficiency. Knockdown of p47phox attenuated the differences of CD11c expression as well as ROS level due to the presence of TNFRSF14, indicating that ROS generation is mediated by TNFRSF14 to upregulate CD11c. In vivo TNFRSF14 deficiency reduced elevated serum ROS levels induced by OVX, suggesting that TNFRSF14 may be a mediator of increased redox level caused by loss of ovarian function. Consistently, our previous finding demonstrated that lack of TNFRSF14 reduced ROS levels upon RANKL stimulation in vitro (Kim et al. 2012).

Taken together, we showed that engagement of TNFRSF14 upregulated CD11c expression in BMM via ROS generation, resulting in chronic inflammation which contributes to impaired glucose metabolism due to loss of ovarian function. Understanding the role of TNFRSF14 in OVX-induced metabolic disturbances could aid in the design of a novel therapy for postmenopausal syndrome.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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