CLINICAL STUDY

Receptor for advanced glycation end-products expression in subcutaneous adipose tissue is related to coronary artery disease

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Abstract

Objective: Obesity, a risk factor for coronary artery disease (CAD), is associated with inflammation and reactive oxygen species (ROS) production, while advanced glycation end-products, through their receptor (AGER or RAGE), play an important role on these processes. The aim of this study was to analyze the expression levels of RAGE, NADPH oxidase subunits, and catalase in adipose tissue in relation with CAD.

Design and methods: Patients undergoing heart surgery were included in two groups: with and without CAD. Epicardial adipose tissue (EAT) and subcutaneous adipose tissue (SAT) biopsies were analyzed for gene expression by RT-quantitative PCR, immunohistochemistry, or western blot.

Results: RAGE mRNA and protein expression in SAT from patients with CAD was lower than in patients without CAD. However, there was no change in EAT from patients with or without CAD. P22-PHOX and RAGE gene expression were higher in EAT than in SAT, whereas catalase mRNA levels were lower. NADPH oxidase subunits and catalase mRNA expression were not influenced by CAD. Whereas NADPH oxidase-dependent oxidative response of SAT and EAT to lipid circulating levels could be different; glycemic levels were not related with the analyzed genes expression.

Conclusions: This study demonstrates that RAGE expression in SAT, but not in EAT, is down-regulated in patients with CAD with respect to those without CAD. Although changes were not observed for NADPH oxidase subunits or catalase expression between CAD and non-CAD patients, a possible relationship between ROS production and RAGE expression in adipose tissues cannot be ruled out.

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Introduction

Obesity, associated with an increase in the size of adipocytes and endocrine function of adipose tissue (1), is related to cardiovascular disease and type 2 diabetes (2). Thus, increased epicardial, pericardial, and subcutaneous fat is associated with the presence and severity of coronary artery calcium (3). However, greater attention has focused on visceral adipose tissue in comparison with subcutaneous adipose tissue (SAT) in the last few years because it is an important risk factor for cardiovascular disease (4). In fact, in female coronary artery disease (CAD) patients, a strong decrease was observed in SAT of the legs in comparison to healthy controls, with no significant shift in SAT to the trunk (5), suggesting redistribution to visceral adipose tissue. Epicardial adipose tissue (EAT) is the visceral adipose tissue located in the atrioventricular and interventricular grooves, adjacent to adventitia of coronary arteries without fascia structures. This tissue is a source of pro-inflammatory cytokines (6–8) and contains low levels of adiponectin, an anti-inflammatory adipokine, in patients with CAD (9, 10). Moreover, adiponectin expression in EAT decreases in patients with hypertension (11); although its levels are not modified in diabetes (12). These findings suggest that EAT plays an important role in cardiovascular disease and some of its risk factors. Thus, the behavior of this fat pad seems to be different from SAT.

Recently, it was found that adipose tissue expresses the receptor for advanced glycation end-products (AGER or RAGE) (13). This is a multiligand receptor of the immunoglobulin superfamily (14) that binds advanced glycation end-products (AGE). AGE are modifications of
proteins or lipids that become non-enzymatically glycated and oxidized after contact with aldose sugars (15). AGE form in vivo in hyperglycemic environments and during aging, and they have been related with the initial steps of atherosclerosis and with inflammatory responses (16). Among the effects caused by these products, we can emphasize generation of reactive oxygen species (ROS), migration of cells, and expression of adhesion and inflammatory molecules (17). The AGE–RAGE engagement is widely related with cardiovascular disease and ROS generation, mainly mediated by NADPH oxidase (18). This enzyme consists in two membrane-bound subunits, the gp91-PHOX protein (NOX2) or some of its homologs (named NOX from 1 to 5) and p22-PHOX protein (19). Once activated, NADPH oxidase produces superoxide anions from oxygen and NADPH or NADH. Enhanced ROS production is an important factor associated with some cardiovascular diseases (20) such as CAD. Furthermore, we have previously observed that EAT presents higher oxidative stress than SAT (21).

Taking all this together, we can hypothesize that EAT, due to its physical location, its endocrinological activity, and its possible reactivity to AGE, could play an important role in CAD, a situation typically related with enhancement of both ROS production and inflammatory reactions. The aim of this study was to analyze the relationship between CAD and the expression of RAGE, NADPH oxidase subunits, and catalase in EAT and SAT of patients undergoing heart surgery.

Subjects and methods

Patients

Thirty-seven patients undergoing heart surgery with sternotomy were included in the study. Exclusion criteria were previous heart surgery or concomitant infective diseases. All participants gave their informed consent. The study protocol was approved by the local ethical committee and carried out according to the Declaration of Helsinki. Clinical data were obtained upon admission of patients without CAD.

Adipose tissue biopsies

EAT biopsy (0.1–1 g wet weight) was obtained from upper region of the right ventricle, and SAT biopsy (2 g wet weight) was obtained from the thorax. Samples were immediately split, when possible, into two pieces. One piece was frozen in liquid nitrogen before storage at −80°C until use, and the other one was formalin-fixed and paraffin-embedded.

mRNA extraction and real-time RT-PCR

mRNA was isolated from 50 to 100 mg of EAT and SAT with the oligotex mRNA Spin-column kit (Qiagen GmbH). The final concentration was 1 mg tissue/1 µl solution. Then, 4.14 µl of mRNA solution was transcribed using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp.) in 30 µl of a pH 8.4 solution containing 20 mM Tris–HCl, 50 mM KCl, 2.5 mM MgCl2, dNTPs (1 mM each), 20 U of RNase inhibitor, and random primers under the following conditions: 50 min at 37°C, 10 min 42°C, and 5 min at 95°C.

Comparative EAT and SAT gene expression levels with respect to β-actin were analyzed by real-time PCR (Chromo 4; MJ Research, Inc., Waltham, USA) in duplicated using 4 µl of cDNA, SYBR Green (Roche Diagnostics Corp., Inc.) as fluorochrome, and the primers sequence and amplification conditions shown in Table 1. during 40 cycles. Fluorescence curves were analyzed using Opticon Monitor software (MJ Research). Melting curves were tested in order to probe the correct amplicon. Gene expression levels were calculated with respect to β-actin expression and represented in arbitrary units (a.u.).

Immunohistochemistry

Paraffin-embedded tissue sections 3 µm-thick were deparaffined and rehydrated. Epitope retrieval was done in basic solution for 20 min at 97°C. Immunostaining was performed with anti-human RAGE antibody (1:500; Abcam, Cambridge, UK) for 30 min following the protocols. LSAB (Dako Diagnostics, Glostrup, Denmark) using 3,3-diaminobenzidine tetrahydrochloride was used for detection. Negative controls were carried out omitting the primary antibody. Results from different samples were analyzed for quantification of RAGE expression using the free ImageJ (1.37v) software from NIH, USA.

Protein extraction and western blot

Protein isolation from a series of SAT samples was performed as previously described (12). In brief, 100–150 mg of EAT and SAT were rinsed in 5 ml of phosphate saline solution containing 0.5 mM EDTA, 5 mM KCl, 10 mM HEPES acid, 2 mM MgCl2, 10 mM NaHCO3, 0.5 mM KH2PO4, 0.5 mM Na2HPO4, 10 mM glucose, 110 mM NaCl and 0.16 mM CaCl2 at pH 7.4, and then centrifuged at 300 g for 1 min to remove residual blood. Tissues were resuspended (weight/volume) in 600 µl lysis buffer (125 mM Tris pH 6.8, 10% glycerol, 2% SDS, 100 mM dithiothreitol, 1X anti-protease cocktail from Sigma–Aldrich) and sheared by homogenizer pestle using sample-grinding kit (GE Healthcare, Waukesha, WI, USA).

Prior to protein separation, immunoglobulin depletion from the samples was performed to allow
detection and analysis of low abundant proteins. Immunoglobulins were bound to protein A using PureProteome Protein A Magnetic Beads (Millipore, Billerica, MA, USA). From the protein solution, 20 ml were diluted with 100 ml of phosphate-buffered solution, and the mixture was incubated with protein A magnetic beads for 30 min under agitation at room temperature. Finally, magnetic beads binding immunoglobulins were separated, and the depleted sample was recovered. Proteins were concentrated 10 with centrifugal filter devices (Amicon Ultra-0.5, Millipore).

Protein separation (40 mg) was carried out in 12% SDS–PAGE gel and transferred to a polyvinylidene fluoride membrane for 45 min at 280 mA. The membranes were blocked for 2 h at room temperature with 5% milk in Tris-buffered saline–Tween-20 containing 20 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20. The membranes were then exposed with rabbit RAGE antibody (1:1000 dilution; Abcam) and mouse β-actin antibody (1:1000; Santa Cruz Biotechnology, Delaware, CA, USA) overnight, and then to peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (1:1000). Immunoreactive proteins were visualized using an ECL detection system (Amersham Pharmacia Ltd) and quantified by densitometry Image J software. RAGE protein levels were evaluated in duplicate and quantified with respect to β-actin.

Data analysis

Assumptions of normality of continuous variables were checked with Kolmogorov–Smirnov tests. Continuous variables were expressed as mean ± S.D. Differences between continuous variables were evaluated using Student’s t-test. Categorical variables were expressed as percentages and compared using the χ²-test. Correlations between variables were calculated by Pearson’s or Spearman’s tests according to the normality of the variable, and two-tailed P values are shown. Statistical significance was defined as P<0.05. All analyses were performed with the Statistical Package for Social Sciences (SPSS) version 15.0 for Windows (SPSS, Chicago, IL, USA).

Results

Clinical characteristics and main differences between patients with and without CAD

Thirty-seven patients undergoing heart surgery were included in the study and analyzed. Mean age was 71.5 ± 9.1 years; 62.2% were male, and mean body mass index was 28.3 ± 7.2 kg/m². All clinical characteristics are summarized in Table 2. Patients were classified as belonging to one of two groups: patients with CAD and patients without CAD. Differences in main clinical characteristics were not observed between groups, as shown in Table 2.

In this population, mRNA levels for AGER (RAGE), NOX (2, 4, and 5), p22-PHOX (CYBA), and catalase (CAT) were measured in EAT and SAT. Mean values of these parameters and the comparison between CAD and non-CAD groups of patients are shown in Table 3. From this comparison, only RAGE in SAT was expressed in a different manner between the two groups, being its levels significantly lower in CAD patients (P=0.002; Fig. 1).

To confirm these results, sections from paraffin-embedded adipose tissue from SAT or EAT were immunostained with anti-human RAGE. Quantification
of this immunostained area in the immunohistochemical preparations confirmed that in SAT from patients with CAD, RAGE expression was significantly reduced in comparison with expression in EAT of the same patient and with respect to SAT and EAT from patients without CAD (Fig. 2).

RAGE expression in SAT was also analyzed at protein level by western blot in a subset of patients (four CAD and three non-CAD). In these measurements, relative RAGE protein quantity, normalized with respect to β-actin, showed that in SAT, RAGE was lower in CAD patients than in patients without CAD (Fig. 3). These results agreed with the observation done at mRNA level, where RAGE expression in SAT was also lower in CAD patients with respect to non-CAD patients.

We also analyzed the difference in the gene expression between EAT and SAT. In this comparison, results showed that RAGE and p22-PHOX were highly expressed in EAT than in SAT, and NOX4 and catalase were highly expressed in SAT with respect to EAT (Table 4).

With regard to the possible influence of the co-morbidities of patients, the expression of the genes under study was analyzed for the possible influence of hypertension, hyperlipidemia, type 2 diabetes mellitus, and atrial fibrillation. We only found an interesting modification depending on diabetes mellitus: RAGE mRNA levels in both EAT and SAT were lower in patients with type 2 diabetes in comparison with non-diabetic patients (Table 5). These results suggest a down-regulating effect of diabetes on the RAGE expression of the adipose tissue. From the other genes analyzed, NOX2 mRNA levels in SAT were lower in non-diabetic patients with respect to diabetics and, on the contrary, NOX5 in SAT was lower in diabetic patients (Table 5).

### Relationship between variables

We analyzed the relationship between mRNA expression of NADPH oxidase subunits, catalase, and RAGE regarding to the adipose tissue source. In this sense, we observed that p22-PHOX levels in EAT positively correlated with levels in SAT in all patients ($r=0.766; P=0.001$). Although this correlation was maintained in the non-CAD group of patients ($r=0.809; P=0.028$), it disappeared in the CAD group ($r=0.430; P=0.336$; data not shown). If we accept that p22-PHOX could be an indicator of total NADPH oxidase expression in the tissue, total levels of NADPH oxidase would be directly related in EAT and SAT in the absence of CAD, but not in CAD patients.

Something similar happened with RAGE: its mRNA levels in EAT and in SAT were positively related ($r=0.425; P=0.014$; Fig. 4A) considering all patients. However, whereas in the non-CAD patients, this association became stronger ($r=0.652; P=0.002$; Fig. 4B), it disappeared in the CAD group of patients.
These results agree with a change in RAGE expression in SAT under CAD. Total cholesterol, low-density lipoprotein, and triglyceride levels were inversely related to \( r = 0.619; P = 0.032 \), \( r = 0.742; P = 0.035 \), and \( r = 0.587; P = 0.045 \) mRNA levels in EAT respectively; whereas mRNA levels of NOX4 in SAT correlated positively with serum levels of triglycerides \( r = 0.578; P = 0.049 \) and total cholesterol \( r = 0.633; P = 0.027 \). These data seem to indicate a different oxidative response of SAT and EAT to lipid circulating levels.

In our study, there was no significant relationship between glucose or HbA1c levels and the gene expressions analyzed. Only a significant inverse relationship was observed between HbA1c percentage and RAGE mRNA levels in EAT, but not in SAT \( r = -0.515; P = 0.041 \).

**Discussion**

For the first time, we have found that EAT from patients with cardiovascular disease who underwent heart surgery, expresses RAGE mRNA and protein. Moreover, this expression in EAT and in SAT was related to CAD.

Thus, RAGE expression levels were lower in SAT of patients with CAD in comparison with non-CAD patients. However, we have not found significant changes in EAT. On the contrary, NADPH oxidase subunits and catalase gene expression in EAT and SAT were not influenced by CAD, only the expression pattern of p22-PHOX in these tissues could be affected by CAD. Diabetes can also reduce, independent of CAD, RAGE expression in human adipose tissue.

Patients undergoing heart surgery were selected to participate in the study and divided into two groups with similar clinical characteristics and co-morbidities unless the presence of CAD. In these conditions, mRNA levels of RAGE, some NADPH oxidase subunits expressed in adipose tissue, and catalase were measured in SAT and EAT. Unless for RAGE, no statistical differences in mRNA levels of these genes were observed after comparing tissues between the groups of CAD and non-CAD patients. RAGE mRNA levels in SAT were

![Figure 1](http://dx.doi.org/10.1530/EJE-10-0904)

*Figure 1* Box plots for RAGE mRNA expression levels in EAT (A) and in SAT (B) in the groups of patients with CAD or without CAD. *P* < 0.05 with respect to non-CAD group of patients.

![Figure 2](http://dx.doi.org/10.1530/EJE-10-0904)

*Figure 2* Representative sections of paraffin-embedded epicardial (A and C) or subcutaneous (B and D) adipose tissue from patients with CAD (C and D) or without CAD (A and B) were immunostained with anti-RAGE. Positive immunostaining for RAGE was less pronounced in SAT from patients with CAD (D) than in patients without CAD (B) in adipocytes and in vessels (insets). RAGE expression in EAT was maintained to the same levels in non-CAD patients (A) and in CAD patients (C). Columns represent mean values (S.D. in vertical bars) of the percentage of RAGE-stained area in each type of preparation. *P* < 0.05 with respect to EAT. Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-10-0904.
significantly lower in patients with CAD than those without CAD; whereas no differences were observed between levels in EAT of the same groups. In fact, the positive association between RAGE mRNA levels in EAT and in SAT from patients without CAD disappeared in CAD patients. These results suggested that CAD induces down-regulation of RAGE expression in SAT, but not in EAT. This hypothesis was analyzed at protein level by immunohistochemistry and western blot, and lower presence of RAGE was evidenced in SAT of patients with CAD, while RAGE expression was maintained in EAT of these patients and in patients without CAD.

The physiological meaning of this finding cannot be inferred from our study. However, RAGE expression in tissues allows the engagement of its multiple ligands (AGE, S100/calgranulins, and others), and this binding can mediate pro-inflammatory responses (22), activate ROS production (18) and damage the cardiovascular system (23). Also, several variants of the RAGE gene have been implicated in the development of diabetes-associated atherothrombotic disorders. From these variants, the functional polymorphism Gly82Ser (database for SNP rs2070600) highlights. A significant association between the haplotype carrying the G82S polymorphism and an increased incidence of CAD and elevated serum CRP levels in the Chinese Han population has been described by Gao et al. (24). However, previous genetic epidemiological studies showed negative results from studies involving Caucasian subjects with cardiovascular disease (25). Further more, 82S allele seems to have a protective role in Korean CAD patients (26). Therefore, the pathophysiological role of these variants in CAD remains elusive, and further investigations must be performed.

It has been suggested that the diverse splicing variants of RAGE are possible in many tissues, and their products may influence the RAGE-mediated pathogenesis (27). Besides the RAGE expression variants, the tissue where they are expressed could have different

**Figure 3** RAGE protein levels in SAT from CAD and non-CAD patients analyzed by western blot. (A) Representative blots using antibodies against RAGE and β-actin of SAT from four patients, two without CAD and two with CAD. (B) Relative optical densities of RAGE with respect to β-actin signal from seven patients (four CAD and three non-CAD). a.u., arbitrary units relative to β-actin.

**Figure 4** Relationship between RAGE mRNA levels in SAT and in EAT considering all patients (A), considering the non-CAD group of patients (B), and considering the CAD group of patients (C). Straight lines represent total adjustment with the confidence interval represented as curved lines. \( r \) = Pearson’s correlation coefficient. Value of \( P \) is indicated in each case. \( P<0.05 \) was considered statistically significant.
expression of RAGE and Independent of CAD, the results revealed a higher that ROS production can be related to RAGE expression. catalase mRNA levels were not statistically related to expression levels in EAT under CAD. Proposed by other authors (29). In this context, altered gene expression that has been previously down-regulation of RAGE in SAT, evidencing a triangle change in SAT or EAT could be due in part to CAD. Thus, when considering only the group of patients with CAD. However, according to our results correlated to the levels in SAT when considering the total population of the study, but this correlation failed correlated to the levels in SAT when considering the total population of the study, but this correlation failed when considering only the group of patients with CAD. This result suggests that NADPH oxidase expression change in SAT or EAT could be due in part to CAD. Thus, ROS production changes could also be related to the down-regulation of RAGE in SAT, evidencing a triangle of interaction between RAGE, oxidative stress, and altered gene expression that has been previously proposed by other authors (29). In this context, oxidative stress has been reported to be higher in EAT than in SAT (21), and this could help to maintain RAGE expression levels in EAT under CAD.

Although in our study, NADPH oxidase subunits and catalase mRNA levels were not statistically related to RAGE expression, we could not discard the possibility that ROS production can be related to RAGE expression. Independent of CAD, the results revealed a higher expression of RAGE and p22-PHOX in EAT compared with SAT, while there was lower catalase expressed in EAT with respect to SAT. This could indicate a higher oxidative stress in EAT than in SAT, possibly mediated by RAGE (23) and NADPH oxidase (where p22-PHOX participate in almost all the isoforms). Further more, lower catalase expression in EAT, an antioxidant enzyme, also contributes to an oxidative state. This finding agrees with previous observations (21).

Intriguing relationships between circulating lipid levels and Nox isoforms expression in adipose tissue were observed. Whereas Nox isoform expressions in EAT were inversely related to lipid levels, the opposite occurred in SAT. It could be supposed that SAT exposed to lipids can be more sensitive to ROS production. However, the higher mRNA levels of p22-PHOX and the lower levels of catalase in EAT with respect to SAT indicated that ROS production in EAT could be higher than in SAT in patients with cardiovascular diseases, as previously shown (21). It is also known that visceral adipose tissue is an important contributor to oxidative stress (30). The opposite reaction of EAT and SAT to circulating lipid levels could help to explain the different response in RAGE expression observed in this work.

Although it was not the aim of this study, our results also suggested that diabetes mellitus can influence the levels of RAGE expression in the adipose tissue, since the expression of this receptor was lower in both EAT and SAT from diabetic patients with respect to non-diabetic patients. However, this finding seems to be independent of the association observed between RAGE expression in SAT and CAD, because the distribution of diabetic patients in the groups of CAD and non-CAD patients of our sample turned out to be exactly the same. This result is very interesting since AGE–RAGE axis can play a pivotal role in the setting of diabetes-derived vascular complications (31). We do not have a physiological explanation for RAGE-reduced expression in diabetic adipose tissue. Moreover, it is unknown which could be the real meaning of RAGE down-regulation in the setting of diabetes, since to block the AGE–RAGE axis has disparate effects in diabetic complications, with different clinical implications (32).

With regard to the limitations of this work, firstly, difficulty to include heart surgery patients made the pathophysiological roles in cardiovascular diseases. This is the reason because it is worth to test the expression of RAGE in adipose tissue and to analyze the differences between tissues from different locations.

In this observational work, we could not study the causes of the RAGE expression modification in SAT of patients with CAD. However, according to our results and previously reported data, we can suggest at least one factor that could be related with RAGE-altered expression. That is, down-regulation of RAGE in SAT could be related to changes in the ROS production of the tissue. One of the ways for studying the oxidative stress consists of measuring the enzymes involved in ROS generation, as NADPH oxidase. P22-PHOX is the unique subunit present in most of the NADPH oxidase complexes (28); so, it can be used as a marker of almost total NADPH oxidase content. The fact is that the mRNA levels of p22-PHOX in EAT were directly correlated to the levels in SAT when considering the total population of the study, but this correlation failed when considering only the group of patients with CAD. This result is very interesting since AGE–RAGE axis can play a pivotal role in the setting of diabetes-derived vascular complications (31). We do not have a physiological explanation for RAGE-reduced expression in diabetic adipose tissue. Moreover, it is unknown which could be the real meaning of RAGE down-regulation in the setting of diabetes, since to block the AGE–RAGE axis has disparate effects in diabetic complications, with different clinical implications (32).

With regard to the limitations of this work, firstly, difficulty to include heart surgery patients made the

<table>
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<th>Variables</th>
<th>EAT</th>
<th>SAT</th>
<th>P value</th>
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<td>NOX2</td>
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<td>6.13±0.41</td>
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<td>NOX4</td>
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<td>RAGE</td>
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P value for the comparison was obtained by Student’s t-test. Statistically significant (P<0.05) values are in boldface.

Table 5 | Variables | Non-diabetes | Diabetes | P value |
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<td>(n=28)</td>
<td>(n=9)</td>
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P value for the comparison was obtained by Student’s t-test. Statistically significant (P<0.05) values are in boldface.
final number of subjects of this study relatively low. Further research with a larger population could confirm our results. Second, the study was performed in patients undergoing heart surgery, so extrapolation of the results to other type of patients would need to be supported by appropriate data collection. Third, other than for RAGE, we have studied the mRNA levels, but not the protein content. The size of the adipose tissue sample obtained from heart surgery is always small, and it was impossible to combine mRNA and protein analysis for all genes.

In conclusion, this study demonstrated for the first time that RAGE expression in SAT, but not in EAT, is down-regulated in patients with CAD in comparison with non-CAD patients. Moreover, RAGE expression in diabetic patients was found to be lower than in non-diabetic patients both in EAT and in SAT. No changes were observed for NADPH oxidase subunits or catalase expression between CAD and non-CAD patients. However, a possible relationship between ROS production and RAGE expression in adipose tissues cannot be ruled out. Further mechanistic investigations would be necessary to explain these new relationships found between RAGE expression in adipose tissue and CAD or diabetes.

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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