The Association Between Soluble Receptor for Advanced Glycation End-Products and Cardiovascular Dysfunction Explained by Non-Alcoholic Fatty Liver Disease; A 7-year Follow-up of the CODAM Study

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Abstract

Background. The risk for cardiovascular events and cardiovascular death in diabetic patients is twice as high compared to non-diabetic controls. Glucose-lowering therapy aimed at relieving these complications have led to minimally altered cardiovascular morbidity and mortality, implicating that other pathways may be involved in micro- and macrovascular diseases in diabetic patients. Experimental studies suggest that interaction of ligands and receptor for advanced glycation end products (RAGEs) play an important role in cardiovascular disease (CVD). Associations between non-alcoholic fatty liver disease (NAFLD) and CVD have been reported previously. Moreover, RAGE-dependent inflammation in steatotic livers may play a pivotal role in the pathogenesis of NAFLD. Thus, we hypothesize that signaling via RAGE contributes to the initiation and progression of cardiovascular dysfunction and disease, and this relation is partly the result of and effects of RAGE signaling on the development of NAFLD. **Objective.** We investigated the association between the soluble form of RAGE (sRAGE) and (incidence of) cardiovascular disease (CVD). Furthermore we investigated whether NAFLD may be an intermediate step to causing CVD. **Methods.** In the current analysis, we used data from 574 Caucasian participants from the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study. Plasma biomarkers for endothelial dysfunction and liver enzyme markers were measured. These biomarkers were subsequently converted into averaged standardized scores, ultimately creating one integrated measure for the liver function, *i.e* the LE score and for endothelial dysfunction, *i.e* the ED score. Liver fat percentage was estimated

using a validated equation. CVD and cardiovascular events (CVE) were also identified. Atherosclerosis was identified using measures of carotid intima media thickness (cIMT) and ankle-arm-index (AAIx). For continuous outcomes multiple linear regression was performed, whereas for dichotomous outcomes binomial logistic regression analysis was performed. All analysis included adjustments for age, gender, glucose metabolism status, smoking, energy intake, alcohol consumption, physical activity, use of medication, and waist circumference. **Results.** Higher plasma sRAGE levels were associated with a higher score for endothelial dysfunction, However, no cross-sectional association was found with markers of atherosclerosis (cIMT, AAIx) or prevalence of CVD. NAFLD was positively associated with endothelial dysfunction, atherosclerosis, and CVD *[β:* 0.338; 95% *CI: 0.251; 0.424]*, *[β: 0.017; 95% CI: 0.001; 0.033]*, and *[OR: 1.*442; 95% *CI:* 1.023; 2.032]. Mediation analysis suggests that FLD accounts for 2.4% of the coefficient in the association between sRAGE and endothelial dysfunction. Next, prospective analysis indicated no association between sRAGE and CVD, CVE, or endothelial function. Furthermore, the prospective analysis indicated a strong association between LE markers and changes in endothelial dysfunction [*β*: -0.079; 95% CI: -0.116; -0.043], but not with CVD or CVE. **Conclusion.** These data suggest that cross-sectionally sRAGE can potentially contribute to progression of CVD, through its action on endothelial function. Additionally, the mediating role of NAFLD between sRAGE and endothelial dysfunction is negligible. However, prospective analysis indicated that only NAFLD, but not sRAGE, contributes to cardiovascular complications after 7 year follow-up.

Keywords
Diabetes, sRAGE, RAGE, NAFLD, CVD, CODAM, atherosclerosis, endothelial dysfunction.

Introduction

Diabetes is a worldwide epidemic currently affecting more than 347 million people, and the number of deaths associated with diabetes is expected to rise by more than 50% in the upcoming 10 years (1). Microvascular and macrovascular diseases are the culprit for increased morbidity and mortality associated with diabetes. This is supported by studies indicating that risk for cardiovascular events and cardiovascular death in diabetic patients is twice as high compared to non-diabetic controls (2). Regardless of the strong association of T2D with cardiovascular complications, glucose-lowering therapy aimed at relieving these complications have been unsatisfying; cardiovascular morbidity and mortality are minimally altered when using glucose-lowering medication (3). This implicates that there are other (path) ways in which hyperglycemia is able to influence the micro- and microvasculature.

In the past decade there has been growing interest in the mechanisms leading the micro- and macrovascular diseases. Advanced glycation end-products (AGEs) are well-known for their role in increasing oxidative stress, inflammation, and more importantly in micro- and macrovascular diseases. AGEs exert their effect in many ways: glycation of intracellular proteins lead to disruption of cell function; glycation of extracellular matrix proteins leads to abnormal interaction between the cell and proteins; and lastly interaction with the receptor for advanced glycation end-products (RAGE) on different cell types leading to activation of intracellular signaling pathways.

The RAGE belongs to the superfamily of immunoglobulins, and was initially identified as a receptor that can bind AGEs. There are many RAGE isoforms known, with the most important being the full-length RAGE (fl-RAGE) and soluble RAGE (s-RAGE). The soluble form can be further subdivided into an endogenously secreted RAGE (esRAGE) and a cleaved RAGE (cRAGE). Both isoforms of sRAGE function as a so-termed decoy receptor by intercepting with the ligand-fl-RAGE interaction at the cell surface. However, the protective role is not physiologically possible in humans, as serum sRAGE concentrations are too low to counteract AGE levels (4). In the current study, serum sRAGE concentrations were used as a biomarker that reflects RAGE signaling on the target cells.

Although it is evident that ligand-RAGE interactions play an important role in the development of atherosclerosis and cardiovascular complications, the precise role of sRAGE in cardiovascular disease (CVD) in human studies has still to be elucidated (5).

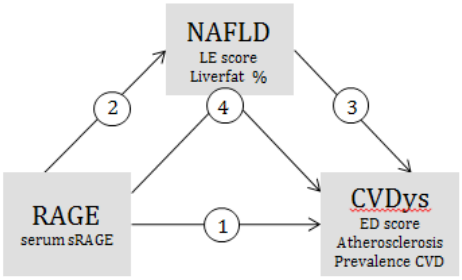

Figure 1. Illustration showing an overview of the main associations analyzed in this study. LE score and Liverfat% were measures of NAFLD. ED score, Atherosclerosis, and Prevalence CVD were measures of cardiovascular dysfunction. Total plasma sRAGE was a measure of RAGE.

Associations between non-alcoholic fatty liver disease (NAFLD) and CVD have been reported previously. Moreover, RAGE-dependent inflammation in steatotic livers may play a pivotal role in the pathogenesis of NAFLD. From these observations, it is speculated that there is a potential interaction between sRAGE and NAFLD that may subsequently result cardiovascular complications.

In view of the described associations of sRAGE and NAFLD with CVD, we hypothesized that signaling via the RAGE contributes to the initiation and progression of cardiovascular dysfunction and disease, and this relation is partly the result of and effects of RAGE signaling on the development of non-alcoholic fatty liver disease. In the current analysis, data obtained from the CODAM study was utilized to answer (1) the association between sRAGE and CVD, (2) and if NAFLD could have a mediating roel in the association between sRAGE and CVD (Figure 1).

For CV dysfuction (CVDys), three different read-outs were distinguished throughout this study, *i.e* endothelial dysfunction, atherosclerosis, and CVD. Moreover, two different read-outs, *i.e* the LE markers and equation for liver fat percentage, were used as measures for hepatocellular injury and NAFLD, respectively.

Material and methods

Subjects

The Cohort study of Diabetes and Atherosclerosis Maastricht (CODAM) study started in 1999-2000 and was intended to investigate the effects of several parameters, including blood lipid profile, lifestyle, glucose metabolism, and genetic factors, on the morbidity and mortality of cardiovascular diseases (CODAM1). Baseline measurements included characterizing the individuals with regard to their lifestyle, cardiovascular, and metabolic profile during two visits to the metabolic research unit of Maastricht University.

In the current analysis, 574 Caucasian subjects participating in the CODAM study were included. After exclusions, a total of 512 patients were included for cross-sectional analysis. For prospective analysis, a total of 495 patients were included at follow-up. Hereafter, patients were excluded if the outcome variable used was not measured.

Measures of NAFLD

The liver enzyme (LE) score and an estimation of the liver fat content (eLF%) were calculated to represent hepatocellular injury in NAFLD. Liver fat percentage was estimated by a validated (against proton magnetic resonance spectroscopy) equation allowing the identification of the liver fat percentage using easy-to-perform clinical and laboratory tests. The LE score was constructed using a combination of three liver enzymes, including AST, ALT, gamma glutamyltransferase (GGT).

Measures of endothelial dysfunction, atherosclerosis, CVD, and CVE

Endothelial dysfunction was estimated by integrating four markers associated with endothelial activation (sICAM1, sVCAM1, vWf, and sE-Sel), all measured by a multiarray detection system based on electrochemiluminescence technology in EDTA plasma. Ankle-arm index (AAIx) and carotid intima media thickness (cIMT) were used as measures of atherosclerosis. The AAIx was calculated as the ratio of the systolic blood pressure at the level of the ankle to that at the level of the arm. The cIMT was measured with the aid of an ultrasound (US) scanner (Ultramark 4+, Advance Technology Laboratories, Bothel, WA, USA). Cardiovascular events were identified as having (I) coronary events (CE) and/or (II) a stroke, whereas the prevalence of CVD was defined as the presence of (I) coronary heart disease (CHD) and/or (II) stroke and/or (III) peripheral arterial disease (PAD).

Statistical Analysis

Variables with a skewed distribution were $\log_e$ transformed prior to further analysis. General characteristics of the study population were compared between tertiles of sRAGE using the Chi Square test and ANOVA test for discrete and parametric variables, respectively. We used two different statistical analyses to examine the associations between NAFLD, sRAGE, vascular dysfunction, atherosclerosis and CVD. For continuous outcomes multiple linear regression analysis was performed, whereas for dichotomous outcomes binomial logistic regression analysis was performed. The parameters for endothelial dysfunction and LE score were composed of averaged standardized scores of different blood markers. The stepwise analyses included three models with potential confounders that were adjusted for. First, the association of the outcome variable and the main independent variable was calculated, followed by the adjustment for age, gender, and glucose metabolism status (model 1), smoking, energy intake, alcohol consumption, physical activity, and use of medication (model 2), and lastly waist circumference (model 3). All statistical analyses were carried out using the Statistical Package for Social Sciences (v. 21).

Results

Subjects

The study population consisted of subjects with an average age of 60, mostly consisting of male participants (60%). Subjects in the lowest tertile had a higher prevalence of T2D, increased fasting plasma glucose levels, and a higher HOMA2-IR. Nonetheless, no significant differences were observed between groups concerning HbA1c values, partly due to the patients either recently being diagnosed with T2D, or highly regulating the disease.

Cross-sectional Association of sRAGE with CVDys

First, we investigated the association of sRAGE with CV dysfunction (Figure 1). Plasma sRAGE levels were strongly positively associated with endothelial dysfunction in CODAM 1 (*β*: 0.162; 95% *CI*: 0.040;0.212). The data should be interpreted as follows: 1 SD increase in ln sRAGE leads to a 0.162 increase in the ED score. However, no association between sRAGE and atherosclerosis or CVD was observed.

Cross-sectional Association of sRAGE with NAFLD

Next, we investigated the association of sRAGE with NAFLD (Figure 1). Inverse correlations were observed between sRAGE and LE score (*β*:-0.060; 95% *CI*:-0.145;0.025) or liver fat percentage (*β*:-0.062; 95% CI: -0.133;0.009), when only crude associations were analyzed. This inverse trend remained as the association between LE score or liver fat percentage and sRAGE was adjusted for the remaining confounders. However, this association was not statistically significant.

Cross-sectional Association of NAFLD with CVDys

Third, we investigated the association of NAFLD with CV dysfunction (*β*: 0.338; 95% *CI*: 0.251; 0.424) (Figure 1). The LE score, as a measure of hepatocellular damage, was strongly associated with endothelial dysfunction. The LE score was also positively, and independent of confounders, associated to cIMT (*β*: 0.017; 95% *CI*: 0.001; 0.033). Of note, is that severe atherosclerosis is reflected by high cIMT. The other measure of NAFLD, *i.e* liver fat percentage, did show positive, crude associations with endothelial dysfunction, cIMT and CVD [(*β*: 0.432; 95% *CI*: 0.308; 0.555),(*β*: 0.022; 95% *CI*: -0.001; 0.044), and (*OR*: 1.442; 95% *CI*: 1.023; 2.032), respectively].

Mediation of NAFLD in the Association of sRAGE with Endothelial Dysfunction

Fourth, we investigated whether the association between sRAGE and endothelial dysfunction could be mediated by NAFLD (Figure 1). The LE score and liver fat percentage, mediated the association between sRAGE and endothelial dysfunction (from *β*: 0.162; 95% *CI*: 0.040;0.212 to *β*: 0.166; 95% *CI*: 0.088;0.245).

Prospective Association of sRAGE with Endothelial Dysfunction, CVD and CVE

We next investigated the association between sRAGE and change in ("Δ" or "delta") endothelial dysfunction. Our results suggest that sRAGE is not associated with changes in endothelial dysfunction within the 7 years. Note that the latter was in contrast to the strong cross-sectional association found between sRAGE and endothelial dysfunction.

No association was found between plasma sRAGE levels and CVD or CVE at follow-up. The prospective association between sRAGE and CVD was negligible (*OR*: 1.043; 95% *CI*: 0.733-1.485) even when adjusted for all models. The data can be interpreted as follows: per 1 SD chance in ln sRAGE, there is a 4.3% increased chance to develop CVD. A similar weak-to-no association was observed between sRAGE and CVE (*OR*: 1.013; 95% *CI*: 0.642-1.599). This finding further emphasizes the lack of association between sRAGE and CVD observed at baseline.

Prospective Association of NAFLD with Endothelial Dysfunction, CVD and CVE

The LE score was not associated with the CVD or CVE at follow-up. However, a strong association was found between the LE score and change in endothelial dysfunction after all model adjustments (β: -0.079; 95% *CI*: -0.116; -0.043). The latter indicates a strong association between NAFLD and endothelial dysfunction. Again, results acquired when performing the same analysis for each liver enzyme separately, were in line with results from the LE score (data not shown). Of interest is that an inverse association was observed between the LE score and endothelial dysfunction prospectively, whereas a positive association was observed cross-sectionally.

Discussion/Conclusion

The strong association of sRAGE and endothelial dysfunction is in line with previously reported data (6). However, the lack of association found between sRAGE and atherosclerosis was not expected. A desintigrin and metalloprotease 10 (ADAM10) is a novel mediator of vascular endothelial growth factor (VEGF)-induced endothelial cell function in angiogenesis, which was reported to be expressed in human atherosclerotic lesions (7). Interestingly, ADAM10 is the same metalloprotease that has been identified to cleave off the membrane-bound fl-RAGE isoform. Thus, one would expect that the presence of ADAM10 in atherosclerosis, would lead to increased concentrations of sRAGE.

Earlier reports have linked endothelial dysfunction and atherosclerosis to an increased CVD risk in diabetic patients (8). The finding that no association was found between sRAGE and CVD contrasted our expectations. A reciprocal role between esRAGE and cRAGE has been suggested by the literature (9). However, as Schalkwijk and Stehouwer (4) mentioned before, the possibility of sRAGE to function as a decoy receptor for AGEs is rather small, given that the plasma concentration of AGEs are a thousand-fold greater than that of sRAGE. For this reason we rule out the possibility of esRAGE having a protective role in humans. With sRAGE being strongly associated with endothelial dysfunction, and

excluding the reciprocal effect of esRAGE and cRAGE, our data suggests sRAGE to influence the progression of CVD.

Prior to investigating the mediating role of markers of NAFLD in the association between sRAGE and endothelial dysfunction, it was essential to first analyze if sRAGE was associated with these markers of NAFLD. Assuming that sRAGE is a measure for RAGE expression, it was expected that an increased sRAGE concentration would lead to increased hepatocellular injury. The present results show that there is no association between sRAGE and NAFLD. The inverse correlation indicates that as the concentration of sRAGE increases, the markers of NAFLD (LE score and liver fat percentage) decrease, indicating decreased hepatocellular injury. However, irrespective of the significance, the inverse association observed in the results has been reported elsewhere (10). The exact mechanism underlying this inverse association has still to be elucidated. It should be noted, that we used indirect measures of NAFLD, *i.e* liver fat percentage and the LE score, which could be a potential reason for this deviating finding.

In the present study, a strong cross-sectional association between markers of NAFLD and endothelial dysfunction was observed, a known factor to be present prior to developing subclinical atherosclerosis in patients with NAFLD (11, 12). Literature suggests that endothelial vasculature is important in maintaining the vascular homeostasis and the normal functioning of the endothelium (8). Disruption of the endothelial function is an early sign in the onset of CVD, even before this becomes clinically evident (13). However, no significant association between markers of NAFLD and CVD was observed, as opposed to numerous studies showing that the risk to CVD is increased in patients with NAFLD (11). Furthermore, a strong cross-sectional association was found between cIMT and the LE score. This indicates that patients with an increased LE score, had a greater cIMT, which is a measure used to characterize CVD risk in patients with NAFLD (12). Ultimately, the strong association observed between markers of NAFLD with endothelial dysfunction and cIMT, implicates that an increased risk to CVD was present despite the association with CVD not being statistically significant.

Markers of NAFLD do not possess a mediating role in the association between sRAGE and endothelial dysfunction. The mediating role is almost negligible as only 2.4% change in the coefficient is observed. The inverse correlation could be explained by the fact that there already was an inverse correlation found between sRAGE and NAFLD, despite it being statistically insignificant.

Our prospective analyses showed that the LE score, but not sRAGE, was significantly and positively associated with changes in endothelial dysfunction, as opposed to cross-sectional results. The positive association translates to: a greater sRAGE, as a measure of RAGE expression, implies an aggravation of endothelial function. Interestingly, a strong inverse association was found between the LE score and change in endothelial dysfunction. This contrasted our expectations, as markers of NAFLD have previously been associated with worsening of the endothelial function (12). This discrepancy could be explained by the lack of adjustments during the prospective linear regression analysis. Note that adjustments performed for the prospective analysis were identical to those for the cross-sectional analysis. However, there is a 7-year gap in between the measurements for markers of endothelial function, and it should not be ruled out that possible attenuations in the patients' medication use could have occurred, thereby influencing the patients' endothelial function.

There are some limitations inherent to the current study that should be mentioned. First, we think that measuring serum cRAGE, rather than total sRAGE, would have been a more precise measure for the expression of RAGE, despite cRAGE being the predominant subtype in serum. Currently, there are no assays on the market specifically for cRAGE, thus we suggest measuring both total sRAGE and esRAGE in future studies. Second, sRAGE values were not measured at follow-up, thus we could not confirm that the cross-sectional association between sRAGE and endothelial dysfunction observed in CODAM 1, was also present in CODAM 2. Nonetheless, our results suggest that sRAGE can be used as a biomarker, yet not as a predictive tool for endothelial dysfunction. Third, we did not include adjustments for covariates measured in CODAM2 when performing the prospective analyses. Lastly, causality is very difficult to interpret in a cross-sectional study design. One can only argue about associations between the risk factors and the main outcome variable.

Altogether, our cross-sectional results indicate RAGE being a potential contributor to progression of CVD, through its action on endothelial function and atherosclerosis. Additionally, the action of RAGE on endothelial function is not mediated by markers of NAFLD. However, prospectively RAGE shows not to have any effect on endothelial dysfunction, atherosclerosis, or CVD. Future studies should focus on optimizing the prospective analysis to maximally profit from the available data.

Role of the student

Johnny de Nóbrega Teixeira was a motivated third year biomedical student that was working on an extracurricular project under the supervision of Marleen van Greevenbroek. The topic was primarily proposed by both Marleen van Greevenbroek and her colleague Kristiaan Wouters. Later on this project became the subject for the writing of this report.

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