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DOI:

[10.1152/ajpendo.00146.2017](https://doi.org/10.1152/ajpendo.00146.2017)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Miranda, ER, Somal, VS, Mey, JT, Blackburn, BK, Wang, E, Farabi, SS, Karstoft, K, Fealy, CE, Kashyap, SR, Kirwan, JP, Quinn, L, Solomon, TPJ & Haus, JM 2017, 'Circulating Soluble RAGE Isoforms are Attenuated in Obese, Impaired Glucose Tolerant Individuals and are Associated with the Development of Type 2 Diabetes', *American Journal of Physiology: Endocrinology and Metabolism*. <https://doi.org/10.1152/ajpendo.00146.2017>

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Publisher Rights Statement:

Checked for eligibility: 12/10/2017

DOI: 10.1152/ajpendo.00146.2017

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1 **Circulating Soluble RAGE Isoforms are Attenuated in Obese, Impaired Glucose**
2 **Tolerant Individuals and are Associated with the Development of Type 2 Diabetes**

3
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34
35 **Running Head:** Soluble RAGE isoforms and glucose tolerance status

47 **Abstract**

48
49 The soluble receptor for advanced glycation endproducts (sRAGE) may be protective
50 against inflammation associated with obesity and type 2 diabetes (T2DM). The aim of
51 this study was to determine the distribution of sRAGE isoforms, and whether sRAGE
52 isoforms are associated with risk of T2DM development in subjects spanning the
53 glucose tolerance continuum. In this retrospective analysis, circulating total sRAGE and
54 endogenous secretory RAGE (esRAGE) were quantified via ELISA and cleaved RAGE
55 (cRAGE) was calculated in 274 individuals stratified by glucose tolerance status (GTS)
56 and obesity. Group differences were probed by ANOVA and multivariate ordinal logistic
57 regression was used to test the association between sRAGE isoform concentrations
58 and the proportional odds of developing diabetes, versus normal glucose tolerance
59 (NGT) or impaired glucose tolerance (IGT). When stratified by GTS, total sRAGE,
60 cRAGE, and esRAGE were all lower with IGT and T2DM, while the ratio of cRAGE to
61 esRAGE (cRAGE:esRAGE) was only lower ($p<0.01$) with T2DM compared to NGT.
62 When stratified by GTS and obesity, cRAGE:esRAGE was higher with obesity and
63 lower with IGT ($p<0.0001$) compared to lean, NGT. In ordinal logistic regression models,
64 greater total sRAGE (odds ratio: 0.91; $p<0.01$) and cRAGE (odds ratio: 0.84; $p<0.01$)
65 were associated with lower proportional odds of developing T2DM. Reduced values of
66 sRAGE isoforms observed with both obesity and IGT are independently associated with
67 greater proportional odds of developing T2DM. The mechanisms by which each
68 respective isoform contributes to obesity and insulin resistance may reveal novel
69 treatment strategies for diabetes.

70

71 **Key words:** Receptor for Advanced Glycation End products, Type 2 Diabetes, obesity,
72 insulin resistance, glucose tolerance

73

74 **Abbreviations:**

75 ADAM10 – A Disintegrin And Metalloproteinase 10

76 AGE – Advanced Glycation End products

77 CAD – Coronary Artery Disease

78 cRAGE – Cleaved Receptor for Advanced Glycation End products

79 esRAGE – Endogenous Secretory Receptor for Advanced Glycation End products

80 GDR – Glucose Disposal Rate

81 GTS – Glucose Tolerance Status

82 hnRNPA1 – Heterogeneous Nuclear Ribonuclear Protein A1

83 hs-CRP – High Sensitivity C-Reactive Protein

84 IGT – Impaired Glucose Tolerance

85 NGT – Normal Glucose Tolerance

86 RAGE – Receptor for Advanced Glycation End products

87 sRAGE – Soluble Receptor for Advanced Glycation End products

88 T2DM – Type 2 Diabetes

89 TRA2 β - Transformer 2 Beta

90

91 **Introduction**

92 The study of advanced glycation end products (AGE) and their receptor (RAGE)
93 has maintained scientific interest over the past several decades given evidence
94 implicating them both as important contributors to the development, and progression of
95 complications associated with diabetes (8, 33, 45). Initiation of inflammation and
96 generation of reactive oxygen species as a consequence of RAGE activation is well
97 documented (39). Despite numerous attempts, targeting RAGE directly as a therapeutic
98 strategy has largely been unsuccessful (11). However, RAGE signaling can be
99 interrupted, *in vivo*, by directed proteolytic cleavage of the RAGE ectodomain (cleaved
100 RAGE: cRAGE) (16, 32), thus creating a soluble isoform of RAGE (sRAGE) that is
101 released from the cell and appears into the circulation (32). In addition, alternative
102 splicing of the RAGE gene at exon 9 produces a truncated c-terminus protein product
103 (endogenous secretory RAGE: esRAGE) that is expelled from the cell via exocytosis

104 (56). This heterogeneous pool of solubilized receptors, collectively termed total sRAGE,
105 serves to down-regulate the inflammatory response by absorbing excess RAGE ligands,
106 thus attenuating cell membrane RAGE signaling. The production of soluble receptors,
107 as a general concept, is regarded as a common feature of cytokine biology with
108 significant implications for inflammatory disease progression and therapy. Thus,
109 maintaining high levels of circulating sRAGE isoforms is apparently advantageous for
110 the organism (14, 17, 48). This is exemplified, in-part, by data demonstrating sRAGE
111 isoforms are decreased in inflammatory conditions such as type 2 diabetes mellitus
112 (T2DM), coronary artery disease (CAD), and neurodegenerative diseases (14, 48, 54),
113 while treatment with recombinant sRAGE (R-sRAGE), suppresses atherosclerosis and
114 vascular dysfunction in animal models of diabetic CAD (34) .

115 Given this evidence, efforts have been made to establish the efficacy of sRAGE
116 isoforms as biomarkers for diabetes and associated complications. However, existing
117 clinical data are equivocal, possibly due to low sample size, lack of metabolic control
118 measures and incomplete phenotyping. For example, several studies have
119 demonstrated no difference or even elevated total sRAGE levels in T2DM compared to
120 BMI-matched controls with no relationship to basic measures of insulin sensitivity such
121 as HOMA-IR (4, 18). Alternatively, attenuated total sRAGE has been independently
122 reported with obesity, pre-diabetes, and T2DM (5, 13, 40), and low total sRAGE was
123 associated with greater risk of developing T2DM and cardiovascular mortality of non-
124 diabetic individuals (40).

125 What these prior studies lack are normative values of sRAGE isoforms derived
126 from a population of young, lean, and physically active adults, which is generally

127 regarded as the ideal state of human health. Further, no studies have yet to examine
128 the independent effects of body composition or obesity on all sRAGE isoforms, nor have
129 sRAGE isoforms been comprehensively examined across the glucose tolerance
130 continuum, which underlies the natural history of T2DM. In addition, the relationships
131 between sRAGE isoforms and insulin sensitivity remains ambiguous, potentially due to
132 reliance on fasting indices of insulin sensitivity, such as HOMA-IR. Finally, cRAGE and
133 esRAGE data are seldom reported together and the ratio of cRAGE to esRAGE
134 (cRAGE:esRAGE) has yet to be explored as a potential index for insulin resistance or
135 risk of developing T2DM. The latter may be particularly insightful given the mechanistic
136 differences by which cRAGE and esRAGE are generated *in vivo*. Therefore, our aim
137 was to characterize total sRAGE, cRAGE, esRAGE and cRAGE:esRAGE in a young,
138 lean healthy reference group, as well as individuals stratified according to glucose
139 tolerance status (GTS), obesity or both. We hypothesized that sRAGE isoforms would
140 be reduced with impaired glucose tolerance (IGT) and T2DM and further reduced in the
141 presence of obesity in comparison to a lean healthy reference group. Further, we
142 assessed whether the circulating concentrations of sRAGE isoforms were associated
143 with greater odds of developing T2DM.

144 **Material and Methods**

145 **Study Design and Subjects**

146 This data set examines 274 individuals from whom we have quantified circulating
147 sRAGE isoform concentrations. Demographic and clinical data from some subjects
148 participating in this work have been published (25, 31, 41, 42, 53). However, this is the
149 first reporting of the sRAGE data in these subjects. Our intent was to examine sRAGE

150 isoforms and insulin sensitivity in a population of overweight and obese subjects that
151 spanned the glucose tolerance continuum (NGT, IGT, T2DM), and directly contrast
152 these observations with a group of young, lean healthy controls (LHC), who performed
153 at least 120 minutes of moderate intensity physical activity per week. We interpret the
154 LHC group to represent an optimal state of health and thus provide a benchmark of
155 “normal” sRAGE isoform concentrations. Potential participants underwent medical
156 screening to determine their eligibility for the study, which included a medical history
157 assessment, electrocardiogram, and blood chemistry screening. Evidence of prior or
158 current chronic pulmonary, hepatic, renal, gastrointestinal, or hematological disease,
159 weight loss (>2 kg within 6 months), smoking, and contraindication to an exercise test
160 were used as exclusion criteria. Blood glucose following a 2-hour oral glucose tolerance
161 test (OGTT) was used to stratify subjects by GTS according to the American Diabetes
162 Association (ADA) (2). However, T2DM stratification relied on ADA criteria, prior clinical
163 diagnosis, or use of prescription anti-diabetic medication. Body mass index (BMI) was
164 used to stratify subjects by obesity status (lean < 25 kg/m², overweight 25 – 29 kg/m²,
165 or obese > 29 kg/m²). Subjects were recruited by newspaper/radio advertisement from
166 the local municipal areas in Chicago, Illinois, Cleveland, Ohio, USA and Copenhagen,
167 Denmark. All subjects provided oral and written informed consent prior to participation,
168 and the methods were approved by local ethics committees at all locations (Institutional
169 Review Boards of the University of Illinois at Chicago and Cleveland Clinic and the
170 Scientific Ethics Committee of the Capital Region of Denmark).

171 **Pre-Test Control Period**

172 Tests took place in the Clinical Research Units of the University of Illinois at
173 Chicago and Cleveland Clinic, and at the Clinical Research Laboratory of the Centre of
174 Inflammation and Metabolism, Rigshospitalet, Denmark. Subjects being treated with
175 anti-diabetic drugs withheld their medications for at least 24 hours prior to metabolic
176 testing. Diet and physical activity records were taken in an outpatient setting and all
177 subjects were instructed to abstain from consuming alcohol 48 hours prior to their visit
178 and not to consume caffeine within 24 hours of their visit. Subjects also abstained from
179 structured exercise for at least 24 hours prior to metabolic testing.

180 **Clinical Procedures**

181 Height and weight were measured using standard techniques. Whole body
182 adiposity was estimated using dual-energy x-ray absorptiometry (Lunar iDXA, GE
183 Healthcare, Madison, WI, USA). Subjects performed an incremental treadmill exercise
184 test to determine their maximal oxygen consumption (VO_{2max}) as described previously
185 (43). The VO_{2max} test was conducted at least 48-hours prior to subsequent metabolic
186 assessments. On a separate day, following an 8-10 hour overnight fast, subjects came
187 to the laboratory and an antecubital venous cannula was placed for baseline blood
188 collection. Subjects ingested 75 g of anhydrous glucose dissolved in 300 mL water
189 (standard OGTT). Following glucose ingestion, regular venous blood samples were
190 collected for 2 hours. Blood was centrifuged at 2000 g for 15 min at room temperature
191 and respective serum/plasma was stored at $-80^{\circ}C$ until analysis. In addition, insulin
192 sensitivity was measured in 80 subjects via hyperinsulinemic ($40mU/m^2/min$)-
193 euglycemic (5 mmol/L) clamp. The methods of the hyperinsulinemic-euglycemic clamp
194 were described previously (31, 53).

195 **Blood Analyses**

196 Glucose concentrations were measured using a bed-side analyzer (YSI Stat,
197 Yellow Springs, USA; ABL, Radiometer, Denmark); insulin concentrations were
198 determined by electrochemiluminescence immunoassay (E-modular; Roche,
199 Switzerland) and radioimmunoassay (Millipore, Billerica, MA, USA); glycated
200 hemoglobin (HbA_{1c}) levels were determined by high performance liquid chromatography
201 (HPLC) (Tosoh G7 analyzer; San Francisco, CA, USA). High sensitive C-reactive
202 protein (hs-CRP) was determined via ELISA (Alpha Diagnostics International, San
203 Antonio, TX, USA). Total sRAGE concentrations were measured in plasma samples by
204 commercial ELISA (R&D Systems Inc., Minneapolis, MN, USA) as per the
205 manufacturer's protocol. This measure of total human sRAGE levels includes both the
206 cleaved (cRAGE) and spliced variants (esRAGE). A monoclonal antibody raised against
207 the N-terminal of the extracellular domain of RAGE, comprising amino acids 24-344,
208 was used to detect the sRAGE in the sample (R&D Systems Inc.). Plasma esRAGE
209 concentrations were measured separately by commercial ELISA (As One International,
210 Mountain View, CA, USA) as per the manufacturer's protocol. A monoclonal antibody
211 raised against human esRAGE, recognizing amino acids 332-347 was used to detect
212 esRAGE in the sample (B-Bridge International). Plasma cRAGE concentrations were
213 then determined by subtracting esRAGE from total sRAGE as previously described (47,
214 55). The sRAGE ratio (cRAGE:esRAGE) was derived by the quotient of cRAGE to
215 esRAGE and expressed in arbitrary units. All samples were analyzed in duplicate.

216 **Statistics**

217 All data was tested for normality using Shapiro-Wilk's test. Parametric or non-
218 parametric statistical tests were applied accordingly. Subject characteristics for each
219 group were compared using a one-way ANOVA. One-way ANOVA was also used to
220 compare mean sRAGE isoform data between groups. The effects of obesity (lean,
221 overweight, obese) and glucose tolerance status (NGT, IGT, T2DM) on sRAGE
222 isoforms were determined via two-way ANOVA. Bonferroni/Dunn post hoc tests were
223 used for multiple comparisons when appropriate. Multivariate ordinal regression
224 modeling was used to determine if sRAGE isoforms could predict risk of diabetes
225 progression using stratification by glucose tolerance status and adjustment for age, race
226 and obesity (proportional odds model) (52). Caucasian was used as the reference for
227 race, and lean was used as the reference for obesity status. Total sRAGE, esRAGE,
228 cRAGE and cRAGE:esRAGE were used to construct models. The values for total
229 sRAGE, cRAGE and esRAGE were multiplied by 100 before entering them into the
230 models. To avoid co-linearity, we did not generate a stepwise model that included all
231 sRAGE measures in the model. Homogeneity of the odds ratios was confirmed for all
232 variables prior to performing ordinal regression. Bivariate correlation analyses were
233 performed using Pearson or Spearman correlation coefficients. SPSS v24 (IBM,
234 Armonk, NY, USA) and SAS (Cary, NC, USA) were used to perform statistical analyses.
235 $p < 0.05$ was considered significant and data are presented as mean \pm SD.

236 **Results**

237 **Subject Characteristics**

238 Table 1 shows subject characteristics stratified by GTS. Markers of glycemic
239 control (HbA_{1c}, 2-h OGTT glucose and fasting glucose) were progressively increased

240 across the glucose tolerance continuum. The IGT and T2DM groups were of similar
241 age, BMI, and fitness level (VO_{2Max}) (Table 1; $p>0.05$). By design, compared to the IGT
242 and T2DM groups, the NGT group was younger, leaner (BMI), more fit (VO_{2Max}) and had
243 superior glycemic control apart from 2-h OGTT glucose iAUC, which was not different
244 from T2DM (Table 1). Further details of subject characteristics including gender and
245 race frequencies in each group are provided in Table 2.

246 **sRAGE Isoforms are Attenuated with Impaired Glucose Tolerance**

247 When stratified by GTS, NGT individuals had 33% (SD 37%) greater total
248 sRAGE compared to IGT individuals ($p<0.05$) and 31% (SD 29%) greater total sRAGE
249 compared to T2DM individuals ($p<0.05$; Figure 1A). cRAGE and esRAGE, which
250 comprise total sRAGE, were lower to a similar extent in IGT and T2DM compared to
251 NGT individuals ($p<0.05$; Figure 1B and C). However, cRAGE:esRAGE was only lower
252 in T2DM compared to NGT subjects pointing to a disproportionate lack of cRAGE in
253 T2DM individuals ($p<0.05$; Figure 1D). This observation is significant considering that
254 cRAGE made up 63% (SD 12.5%) of total sRAGE in subjects with T2DM.

255 **Increased circulating sRAGE Isoforms are associated with reduced** 256 **proportional odds of developing diabetes**

257 We had hypothesized that reduced sRAGE isoforms may underlie the natural
258 history of T2DM according to progression across the glucose tolerance continuum.
259 Using ordinal logistic regression analysis (Table 3), total sRAGE (Model 1), cRAGE
260 (Model 2), esRAGE (Model 3), and cRAGE:esRAGE (Model 4) were combined with
261 other independent variables (age, race, obesity) to form each respective model. As
262 expected, and shown previously, both age and race were associated with greater

263 proportional odds (Table 3) for the development of T2DM (19). For total sRAGE,
264 cRAGE, and cRAGE:esRAGE, each were independently associated with the
265 proportional odds for progression across the glucose tolerance continuum to T2DM,
266 whereas esRAGE was not. A 100 pg/mL increase in total sRAGE was associated with a
267 9% reduction in the proportional odds of developing T2DM, whereby the same increase
268 in cRAGE was associated with a 16% reduction (Table 3). Additionally, every 1 unit
269 increase in cRAGE:esRAGE predicted a 26% decreased risk of diabetes progression
270 (Table 3). The model demonstrating the greatest reduction in proportional odds was
271 Model 2 that included cRAGE isoforms (C-Statistic 0.805; Table 3).

272 **Relationships with sRAGE isoforms and Metabolic Variables**

273 Bivariate correlation analyses between sRAGE variables and metabolic variables
274 are presented in Table 4. Total sRAGE, cRAGE, and esRAGE negatively correlated
275 with BMI and percent body fat with esRAGE having the strongest relationships between
276 both variables. In addition, all sRAGE variables were positively correlated with
277 cardiorespiratory fitness (VO_{2Max}). Positive correlations between cRAGE:esRAGE,
278 VO_{2Max} and BMI again demonstrate that the proportion of cRAGE and esRAGE
279 isoforms, rather than just the independent quantity of each, is related to fitness level,
280 and body weight status.

281 Apart from 2-h OGTT iAUC, total sRAGE and cRAGE negatively correlated with
282 clinical markers of glycemic control (2-h OGTT glucose, HbA_{1c} , fasting glucose, fasting
283 insulin, and HOMA-IR). On the other hand, esRAGE negatively correlated with 2-h
284 OGTT iAUC, HbA_{1c} , and fasting glucose whereas sRAGE ratio positively correlated with
285 2-h OGTT iAUC, and negatively correlated with 2-h OGTT glucose, fasting glucose and

286 HOMA-IR. Finally, total sRAGE, esRAGE, and cRAGE all positively correlated with
287 Matsuda index; however, the strongest associations with insulin sensitivity were found
288 between clamp-derived glucose disposal rate (GDR) and total sRAGE ($\rho=0.472$,
289 $p<0.001$), cRAGE ($\rho=0.343$, $p=0.003$), and esRAGE ($\rho=0.594$, $p<0.001$). GDR also
290 negatively correlated with cRAGE:esRAGE ($\rho=-0.276$, $p=0.018$).

291 **sRAGE Isoforms are Reduced with Worsening Obesity Status**

292 Because the glucose tolerance groups were heterogeneous with regard to
293 obesity, we further stratified by obesity status to isolate the sRAGE phenotype of lean
294 NGT individuals. Because of low sample size in the overweight subgrouping, the IGT
295 group was combined with T2DM (IGT-T2DM) and overweight was combined with obese
296 (Overweight-Obese) (Figure 2). Using a 2-way (glucose tolerance x obesity) ANOVA,
297 obesity status displayed a group effect for esRAGE ($p=0.001$) and cRAGE:esRAGE
298 ($p<0.0001$). A group effect was also seen for GTS on total sRAGE ($p<0.0001$), cRAGE
299 ($p<0.0001$), esRAGE ($p=0.026$), and cRAGE:esRAGE ($p<0.0001$), and an interaction
300 effect was observed for total sRAGE ($p=0.002$), cRAGE ($p=0.001$), esRAGE ($p=0.048$),
301 and cRAGE:esRAGE ($p=0.032$).

302 Lean, NGT individuals displayed the highest concentration of total sRAGE
303 (Figure 2A), cRAGE (Figure 2B), and esRAGE (Figure 2C), compared to all other
304 subgroups. The largest deviation from lean, NGT individuals when examining cRAGE
305 was found in Lean, IGT-T2DM (61%, SD 16%). However, the largest deviation of
306 esRAGE from lean, NGT individuals was found in Overweight-Obese, IGT-T2DM
307 individuals (36%, SD 36%). Comparison of cRAGE:esRAGE between groups revealed
308 the largest ratio exists in the Overweight-Obese, NGT group (Figure 2D). This increase

309 in cRAGE:esRAGE ratio indicates a preferential decrease in esRAGE related to
310 worsening obesity status. Full analyses of the individual group stratifications and
311 alternative sub groupings were performed and statistically interrogated via ANOVA.
312 However, these analyses did not offer any insight beyond the results presented here.

313 We also analyzed the concentration of sRAGE isoforms across obesity status
314 alone by stratifying individuals into lean, overweight or obese groups. Individuals who
315 were overweight or obese had similar concentrations of sRAGE isoforms and 24-35%
316 lower concentrations of sRAGE isoforms compared to lean individuals ($p<0.05$). Being
317 that the NGT group was significantly younger than the IGT and T2DM groups (Table 2).
318 Lastly, we examined the effect of age on sRAGE isoforms by stratifying individuals into
319 young (18-35 y), middle-aged (36-64 y), and older (≥ 65 y) groups. Concentration of
320 sRAGE isoforms were similar between middle-aged and older individuals but were 25-
321 45% lower compared to young individuals ($p<0.05$). Interestingly, older individuals had a
322 lower cRAGE:esRAGE ratio compared to both young and middle-aged individuals
323 ($p<0.05$). Given this analysis demonstrated a significant effect of age on sRAGE
324 isoforms, we examined the effect of GTS on sRAGE measures while co-varying for age
325 as a continuous variable. The results of this analysis eliminated all significant effects of
326 GTS on esRAGE and cRAGE:esRAGE concentration ($p>0.05$). In addition, the
327 difference between total sRAGE and cRAGE in NGT compared to IGT groups that exist
328 in Figure 1 were also resolved. However, even after controlling for age, individuals with
329 T2DM still possess significantly lower total sRAGE and cRAGE compared to NGT
330 individuals. All sRAGE isoforms were also negatively correlated with age (Table 4).

331 Discussion

332 To our knowledge, the current study is the first to report circulating
333 concentrations of both major sRAGE isoforms (cRAGE and esRAGE) in the context of
334 obesity and T2DM. Our primary finding was that lean, NGT individuals possessed the
335 greatest concentration of sRAGE isoforms compared to states of obesity, IGT, T2DM or
336 both. These findings are in accord with previous reports of lower sRAGE with obesity (5,
337 13, 18) and impaired glucose tolerance (3, 12, 22, 46). Importantly, we also
338 demonstrate for the first time that reduced circulating concentrations of sRAGE isoforms
339 are associated with greater proportional odds for the development of T2DM.

340 To this end, we developed ordinal logistic regression models using the sRAGE
341 isoforms and cRAGE:esRAGE as independent variables to determine the proportional
342 odds ratio of progression across the glucose tolerance continuum to T2DM. GTS is
343 interpreted as having set thresholds along a range of possible outcomes according to
344 American Diabetes Association criteria for the diagnosis of T2DM, thus meeting the
345 assumption needed for ordinal regression (52). The application of this type of statistical
346 model allows for hypothesizing movement along a known continuum (using proportional
347 odds) without longitudinal follow-up. Importantly, our analyses revealed that a 100
348 pg/mL increase in total sRAGE and cRAGE resulted in a marked risk reduction for
349 progression across the glucose tolerance continuum. For calibration, 100 pg/mL
350 represents 12% of the cRAGE concentration in lean NGT individuals. Given our
351 regression model, the lower cRAGE observed in IGT subjects (276 pg/mL) equates to
352 ~44% increased proportional odds of progression towards T2DM. Our sample size was
353 relatively small and our sampling was cross-sectional so these data must be interpreted
354 with caution. However, Selvin et al. reported similar findings in a sample of 1,200

355 individuals without T2DM, whereby those in the lowest quartile of total sRAGE
356 concentration had an increased risk of developing T2DM 18 years later (hazard ratio
357 1.64; 95% CI: 1.10-2.44) (40). Further, the relationships between modulation of sRAGE
358 and health outcomes have been reported, whereby increased sRAGE, following a 12-
359 wk aerobic exercise intervention, was associated with reduced C-reactive protein and
360 improved aerobic fitness (9). Given the financial and time burden for longitudinal studies
361 such as the latter, the application of ordinal regression models has merit for
362 identification and characterization of novel targets such as sRAGE isoforms. Here, we
363 expand on previous observations by demonstrating cRAGE as the isoform with the
364 greatest ability to predict risk of progression across the glucose tolerance continuum
365 whereas esRAGE did not. These data suggest dichotomous roles for cRAGE and
366 esRAGE isoforms and their relevance to T2DM.

367 In line with this notion, we provide novel evidence of a disproportionate loss of
368 cRAGE and esRAGE in the case of T2DM and obesity respectively. Although both
369 cRAGE and esRAGE were significantly lower in IGT and T2DM compared to NGT
370 individuals, only the T2DM group possessed a significantly lower cRAGE:esRAGE ratio.
371 Additionally, when examining the effects of obesity and GTS on sRAGE measures,
372 there was a significant effect of GTS on cRAGE:esRAGE ratio whereby impaired
373 glucose tolerance tended to result in a lower ratio, implying a preferential loss of cRAGE
374 (Figure 2). The lean, IGT-T2DM group stratification also possessed the lowest
375 concentration of cRAGE compared to all other perturbations. Additionally, cRAGE
376 correlated with 2h OGTT glucose and HOMA-IR whereas esRAGE did not. Collectively,
377 these data suggest that loss of cRAGE is strongly influenced by IGT and T2DM.

378 The observed cRAGE phenotype may be mediated by a preferential attenuation
379 of cRAGE-producing mechanisms with IGT and T2DM, specifically the proteolytic
380 cleavage of the RAGE ectodomain via the enzyme A Disintegrin and Metalloproteinase
381 10 (ADAM10) or other matrix metalloproteinases (16, 32, 37). ADAM10 is the primary
382 enzyme responsible for cRAGE production (37). Retinoic acid receptor beta (RAR β)
383 positively regulates ADAM10 transcription by binding to its promoter site (28, 49).
384 Deacetylation of RAR β , is necessary for this action and is mediated by the deacetylase
385 activity of SIRT1 (10, 28). SIRT1 plays a role in beta cell insulin secretion and insulin
386 sensitivity in other tissues such as fat and skeletal muscle(27). Importantly, SIRT1
387 expression is reduced in T2DM and is also down regulated by RAGE signaling (21, 50).
388 Activation of RAGE signaling occurs via binding of its ligands such as AGEs. These
389 RAGE ligands are known to be elevated in the T2DM condition, and have been related
390 to insulin resistance (45). Specifically, exposure to the RAGE ligands reduces SIRT1
391 protein expression in the liver, skeletal muscle and adipose, resulting in the
392 development of insulin resistance in these tissues (7).

393 In the current study, cRAGE correlated to GDR ($r=0.343$, $p=0.003$) (Table 4) and
394 its reduction was strongly associated with the proportional odds for progression through
395 the glucose tolerance continuum (Table 3). GDR is the gold standard measure for
396 insulin-mediated glucose disposal which is largely dictated by the insulin sensitivity of
397 the skeletal muscle. Therefore, failure of the cRAGE producing mechanisms such as
398 RAR β , SIRT1, and ADAM10 in the skeletal muscle may allow for excessive RAGE
399 signaling to promote the development of insulin resistance in skeletal muscle. This may

400 help explain why higher cRAGE is strongly related to insulin sensitivity and lower
401 cRAGE is related to the progression toward T2DM.

402 Interestingly, we show that esRAGE is preferentially lost with obesity. We found a
403 significant group effect of obesity, whereby Overweight-Obese individuals possessed a
404 higher cRAGE:esRAGE ratio compared to Lean individuals suggesting a preferential
405 loss of esRAGE. Although we did not see any difference in cRAGE:esRAGE when
406 stratifying by obesity status alone, esRAGE was 35% lower in Obese compared to Lean
407 individuals whereas cRAGE was 24% lower in obese compared to Lean individuals. In
408 addition, the Overweight-Obese, IGT-T2DM group resulted in the lowest concentration
409 of esRAGE (Figure 2). Both BMI and body fat percentage displayed stronger
410 correlations with esRAGE compared to cRAGE (Table 4).

411 Production of esRAGE is regulated by the activity of two antagonistic splicing
412 factors, heterogeneous nuclear ribonuclear protein A1 (hnRNPA1) and transformer-2
413 beta (TRA2 β) (29). TRA2 β promotes esRAGE production whereas hnRNPA1
414 suppresses this activity. Both TRA2 β and hnRNPA1 are regulated by MAPK activity (1,
415 6, 51) which is well known to be activated by RAGE signaling and exacerbated in
416 obesity and insulin resistance (24). In addition, RAGE expression plays a critical role in
417 adipose differentiation, hypertrophy, and inflammation (15, 33, 44). Therefore, adipose
418 expansion and subsequent adipokine mediated inflammation may be suppressing the
419 splicing mechanisms that regulate esRAGE.

420 In support of this notion, TRA2 β is reduced in the liver and skeletal muscle of
421 obese, IGT-T2DM individuals (29, 35). In the current study, we found lower
422 concentrations of esRAGE in obese individuals compared to lean. Additionally, esRAGE

423 was correlated to GDR ($r=0.594$, $p<0.001$), and body fat percentage ($r=-0.311$,
424 $p<0.001$). These data suggest that both adipose, and skeletal muscle may be involved
425 in RAGE splicing, and that the mechanisms involved become dysfunctional with obesity
426 and insulin resistance. However, future studies are needed to identify the tissue- or,
427 cell-specific sources of sRAGE isoform production, and what mechanisms are
428 responsible for promoting and attenuating their release into the circulation.

429 These findings demonstrate that the study of sRAGE isoforms remains an
430 important area of research given both old and new data (30) reporting the potential role
431 of sRAGE to impart physiological benefit and protection from cardiovascular and
432 metabolic disease. It is evident that the mechanisms of sRAGE production are tightly
433 regulated and that relatively small changes in circulating concentrations are linked to the
434 natural history of T2DM. Herein, we are the first to characterize the circulating
435 concentrations of the two most prominent sRAGE isoforms across the glucose tolerance
436 continuum and demonstrate that total sRAGE, cRAGE, and cRAGE:esRAGE were
437 associated with the proportional odds for progression across the glucose tolerance
438 continuum using ordinal logistic regression. Our data are admittedly, limited by not
439 being age-matched across all groups, as others have demonstrated that chronological
440 age plays a significant role in sRAGE concentrations (36). However, juxtaposition of the
441 T2DM phenotype against a young, lean healthy phenotype, demonstrates the degree by
442 which circulating sRAGE isoforms, in obesity, states of impaired glucose tolerance, and
443 advanced age deviate from optimum health. To tease the effect of age away from these
444 other factors, we compared circulating sRAGE isoform concentrations across GTS while
445 covarying for age. This analysis revealed that sRAGE remained significantly reduced in

446 T2DM despite the age difference between the T2DM and LHC groups. However,
447 covarying for age did eliminate differences in total sRAGE and cRAGE between NGT
448 and IGT, as well as eliminate all differences previously observed for esRAGE and
449 cRAGE:esRAGE. This in addition to the inverse correlations between sRAGE measures
450 and age, implicate age to effect circulating sRAGE. However, given that differences
451 were still realized between T2DM and LHC individuals indicates that the T2DM
452 phenotype, regardless of age, is characterized in part by reduced total sRAGE and
453 cRAGE.

454 We also acknowledge that the limitations of stratifying our data by BMI are such
455 that BMI is less sensitive in detecting obesity than body fat percentage (38). However,
456 when we stratified by body fat percentage cutoffs as previously reported by Romero-
457 Corral et al, the findings were consistent with the data that is currently reported using
458 BMI (38).

459 We were also unable to genotype our participants due to limited sample. This
460 would have been an interesting addition to our data since multiple single nucleotide
461 polymorphisms have been identified for RAGE and have been implicated in the
462 development of obesity, and inflammation(23, 26). The SNP that involves glycine-serine
463 switch at codon 82 (G82S) occurs in the ligand binding domain of RAGE and enhances
464 its ability to promote RAGE activation (20). Koy et al demonstrated that lean and obese
465 individuals with the S/S genotype possessed lower sRAGE compared to those with the
466 G/S and G/G genotypes (26). Obese individuals in this cohort with the S/S genotype
467 also possessed higher BMI and greater circulating CRP compared to those with the G/S
468 and G/G genotypes (26). Our data demonstrate lower sRAGE isoform concentrations in

469 obese and T2DM individuals compared to lean healthy individuals. Unfortunately, we do
470 not know if the G82S SNP is partly responsible for these differences in our sample on
471 sRAGE. However, the frequency of glycine and serine alleles has not been previously
472 shown to be different in T2DM compared to healthy individuals (23). Nevertheless,
473 future studies should examine the effect of RAGE SNPs on the risk of obesity and
474 diabetes development, and if this risk is related to sRAGE concentrations. The
475 mechanism of how sRAGE concentrations are altered by SNPs in RAGE is also unclear
476 and warrant future study. In addition, Kim OY et al only examined the relationship
477 between the G82S SNP and total sRAGE concentrations and did not discriminate
478 between the esRAGE and cRAGE isoforms (26). We have demonstrated here that
479 dysregulation of these isoforms are associated with different phenotypes and therefore
480 are likely under parallel regulation.

481 In conclusion, the disproportionate reductions of cRAGE and esRAGE in T2DM
482 and obesity, respectively, require further mechanistic study as our data implicate
483 adiposity and insulin sensitivity, or both, to play a role in sRAGE biology. These finding
484 suggest the presence of a failure in the sRAGE producing mechanisms with the onset of
485 T2DM and obesity and requires further study. sRAGE was also strongly associated with
486 the proportional odds ratio for progression through the glucose tolerance continuum,
487 asserting sRAGE as a potential biomarker for T2DM. The long-term benefits for
488 reporting these data are: 1) to help direct research efforts toward elucidating failed
489 mechanisms underpinning the discrepancy in sRAGE isoform expression in T2DM and
490 obesity and 2) to determine the efficacy of targeting these mechanisms for treatment of
491 T2DM and obesity.

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498 **Acknowledgements**

499 The authors would like to thank Karia Coleman, Victoria Meyers, Alec Chaves, Giamila
500 Fantuzzi and Kelly Fuller of the University of Illinois at Chicago, for expert technical
501 assistance.

502 **Funding**

503 This work was supported by the American Diabetes Association-Junior Faculty Award,
504 1-14-JF-32 (JMH), CTSA grants UL1RR024989 and UL1RR029879, the Central Society
505 for Clinical and Translational Research, the University of Chicago Diabetes Research
506 Training Center (P30DK020595) and NIH grants R01AG12834 (JPK) R01NR007760
507 (LQ), and R01DK109948 (JMH). Additional funding from EFSD / Amylin (TPJS)
508 contributed to this work.

509 **Duality of Interests.** The author reports no potential conflicts of interest relevant to
510 this work.

511 **Author Contributions**

512 ERM conceived of and designed the study, acquired and analyzed data, and drafted
513 and reviewed the manuscript. VSS, JTM, BKB, SF, KK, CEF acquired and analyzed
514 data and reviewed the manuscript. EW conceived of and designed the study, analyzed

515 data, and reviewed the manuscript. SRK acquired and analyzed data, reviewed the
516 manuscript, obtained funding and supervised the study. JPK, LQ, and TPJS acquired
517 and analyzed data, drafted and reviewed the manuscript, obtained funding and
518 supervised the study. JMH conceived of and designed the study, acquired and analyzed
519 data, drafted and reviewed the manuscript, obtained funding and supervised the study.
520 JMH is the guarantor of this work and, as such, had full access to all the data in the
521 study and takes responsibility for the integrity of the data and the accuracy of the data
522 analysis.

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778 **Figure and Table Legends**

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780 **Figure 1** Soluble RAGE Isoforms According to Glucose Tolerance Status.

781 Subjects were stratified by glucose tolerance status NGT (n = 150): Normal Glucose
782 Tolerance, IGT (n = 30): Impaired Glucose Tolerance, T2DM (n = 94): Type Two
783 Diabetes Mellitus. Comparisons between groups were made for total sRAGE (A),
784 cRAGE (B), esRAGE (C) and cRAGE: esRAGE ratio (D). Differences between groups
785 were analyzed by one-way ANOVA and Bonferroni post hoc tests as necessary. Bars
786 represent MEAN (SD). *p < 0.05, **p < 0.01, and ***p < 0.0001 vs. NGT.

787

788 **Figure 2** Effects of Glucose Tolerance and BMI on sRAGE isoforms.

789 Subject groups were collapsed into NGT vs. IGT-T2DM designations and further
790 stratified by BMI (Lean vs. Overweight-Obese). Lean, NGT n = 74; Overweight-Obese,
791 NGT n = 76; Lean, IGT-T2DM n = 16; Overweight-Obese, IGT-T2DM n = 105. Group
792 comparisons were made for total sRAGE (A), cRAGE (B), esRAGE (C), and cRAGE:
793 esRAGE ratio (D) using two-way ANOVA and Bonferroni post hoc tests as necessary.
794 Bars represent MEAN (SD). *p < 0.05, **p < 0.01, and ***p < 0.0001 vs. NGT; #p <
795 0.05, ##p < 0.01, and ###p < 0.0001 vs. Lean.

796

797 **Table 1** Metabolic characteristics.

798 Data are presented as MEAN (SD). Normally distributed data were analyzed by one-
799 way ANOVA and Bonferroni adjustments for multiple comparisons. Non-normally
800 distributed variables (as indicated by ^) were analyzed using Kruskal-Wallis test and
801 Bonferroni adjustments for multiple comparisons. NGT (n = 150): Normal Glucose
802 Tolerance, IGT (n = 30): Impaired Glucose Tolerance, T2DM (n = 94): Type Two
803 Diabetes Mellitus. BMI: body mass index; VO_{2Max}: Maximal Aerobic Fitness; BF%: Body
804 Fat Percentage; Fat mass; 2-h OGTT Glucose iAUC: 2 Hour Oral Glucose Tolerance
805 Test Glucose Incremental Area Under the Curve; 2-h OGTT Glucose: Blood glucose at
806 2-h time point of OGTT; HbA_{1c}: Glycated Hemoglobin; HOMA-IR: Homeostatic Model

807 Assessment of Insulin Resistance; GDR: Hyperinsulinemic-Euglycemic Clamp Derived
808 Glucose Disposal Rate; hs-CRP: High Sensitivity C-Reactive Protein. ** $p < 0.01$, and
809 *** $p < 0.0001$ vs. NGT; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.0001$ vs. IGT.

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811 **Table 2** Descriptive Demographics.

812 Frequencies of demographic descriptors of individuals grouped by glucose tolerance
813 status. *** $p < 0.0001$ vs NGT.

814

815 **Table 3** Soluble RAGE isoforms and proportional odds for developing T2DM.

816 Total sRAGE, cRAGE, esRAGE and sRAGE Ratio (cRAGE: esRAGE) were used to
817 construct models 1, 2, 3 and 4 respectively. The values for total sRAGE, cRAGE and
818 esRAGE were multiplied by 100 before entering them into the models. Models were
819 corrected for age and race where Caucasian and lean were used as reference,
820 respectively. OR: Odds Ratio, CI: confidence interval, Other: Hispanic/Asian

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822 **Table 4** Correlations Between sRAGE Isoforms and Metabolic Characteristics.

823 Bivariate correlation analyses were used to examine relationships between sRAGE
824 isoforms and metabolic parameters. Pearson correlation coefficients were performed
825 unless denoted (^) which were analyzed by Spearman's Rho.

826 **Table 1 Metabolic characteristics.**

Variable, units	NGT	IGT	T2DM
Sex, M/F	79/71	10/20	47/47
Age, y	39 (SD 17)	61 ± (SD 10)***	57 ± (SD 9)***
BMI, kg/m ²	27.0 (SD 6.2)	34.8 (SD 4.8)***	32.6 ± (SD 7.3)***
VO _{2Max} , mL/kg/min	32.6 (SD 10.4)	23.3 (SD 6.4)***	26.3 (SD 6.6)***
BF, %	33.0 (SD 9.4)	43.2 ± (SD 8.1)***	36.0 (SD 9.5)##
Fat Mass, kg	29.0 (SD 12.9)	40.7 (SD 8.5)***	31.7 (SD 12.4)##
Lean Body Mass, kg	55.3 (SD 12.1)	54.2 (SD 12.0)	57.9 (SD 11.5)
2-h OGTT Glucose, mg/dL	114 (SD 22.5)	162 (SD 16.9)***	281 (SD 67.4)***###
2-h OGTT Glucose iAUC, AU	4201 (SD 2083)	7322 (SD 2639)**	5133 (SD 5567)#
HbA1C, %	5.4 (SD 0.46)	5.7 (SD 0.52)	7.1 (SD 1.6)***###
HbA1C, mmol/mol	35.7 (SD 4.98)	38.5 (SD 5.71)	54.5 (SD 17.7)***###
Fasting Glucose, mg/dL [^]	93 (SD 10.9)	97 (SD 11.7)	151 (SD 61.4)***###
Fasting Insulin, mU/L [^]	9.5 (SD 6.3)	15.9 (SD 11.6)**	13.5 (SD 6.6)***
HOMA-IR, AU [^]	2.2 (SD 1.6)	4.7 (SD 5.0)***	5.0 (SD 3.2)***
Matsuda Index, AU [^]	4.7 (SD 3.1)	2.5 (SD 1.5)***	3.1 (SD 1.9)***
GDR, mg/kg/min [^]	4.9 (SD 2.3)	2.9 (SD 1.2)**	2.6 (SD 0.96)**
hs-CRP, mg/L	2.2 (SD 1.9)	2.8 (SD 1.6)	2.6 (SD 2.6)

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828 **Table 2 Descriptive Demographics.**

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Variable	NGT (n=150)		IGT (n=30)		T2DM (n=94)	
	n	%	n	%	n	%
Age (y)	39 (SD 17)		61 (SD 10)***		57 (SD 9)***	
Young (18-35 y)	88	59	1	3	1	1
Middle age (36-64 y)	44	29	19	63	68	72
Old (\geq 65 y)	18	12	10	33	25	27
Gender						
Male	79	53	10	35	47	50
Female	71	47	20	67	47	50
Race						
White	107	71	21	70	63	67
Black	17	11	7	23	31	33
Hispanic	10	7	2	7	0	0
Asian	16	11	0	0	0	0
Obesity (kg/m ²)	27.0 (SD 6.2)		34.8 (SD 4.8)*		32.6 (SD 7.3)*	
Lean (18-24)	74	49	1	3	15	16
Overweight (25-29)	27	18	2	7	24	26
Obese (\geq 30)	49	33	27	90	55	59

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842 **Table 3 Soluble RAGE isoforms and proportional odds for developing T2DM.**

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Variable	Model 1			Model 2			Model 3			Model 4		
	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P	OR	95% CI	P
Age	1.06	1.03-1.08	<.001	1.06	1.03-1.08	<.001	1.07	1.05-1.10	<.001	1.06	1.04-1.09	<.001
Race												
Black	3.43	1.69-6.96	<.001	4.11	1.93-8.75	<.001	3.57	1.74-7.31	<.001	4.04	1.91-8.53	<.001
Other (Hispanic/Asian)	0.30	0.06-6.96	0.139	0.31	0.06-1.55	0.155	0.36	0.07-1.72	0.199	0.40	0.08-1.93	0.255
Obesity												
Overweight	1.39	0.58-3.35	0.459	1.58	0.64-3.87	0.322	1.29	0.53-3.16	0.576	1.79	0.72-4.45	0.208
Obese	1.08	0.46-2.50	0.864	1.34	0.57-3.15	0.505	1.06	0.45-2.53	0.890	1.68	0.69-4.07	0.253
Total sRAGE	0.91	0.85-0.97	0.003	-	-	-	-	-	-	-	-	-
cRAGE	-	-	-	0.84	0.77-0.92	<.001				-	-	-
esRAGE	-	-	-				0.93	0.78-1.10	0.374	-	-	-
cRAGE/esRAGE	-	-	-	-	-	-	-	-	-	0.74	0.58-0.96	0.022
C-statistics	0.782			0.805			0.773			0.784		

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859 **Table 4 Correlations Between sRAGE Isoforms and Metabolic Characteristics.**

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	<u>Total sRAGE (pg/ml)</u>		<u>cRAGE (pg/mL)</u>		<u>esRAGE (pg/mL)</u>		<u>cRAGE:esRAGE</u>	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Age (y)	-0.368	< 0.001	-0.387	< 0.001	-0.206	0.001	-0.254	<0.0001
VO ₂ Max (mL/kg/min)	0.231	0.002	0.291	< 0.001	0.156	0.039	0.202	0.007
BMI (kg/m ²)	-0.225	< 0.001	-0.158	0.010	-0.288	< 0.001	0.140	0.023
BF (%)	-0.288	< 0.001	-0.227	0.001	-0.311	< 0.001	-0.004	0.953
LBM (kg)	0.066	0.351	0.075	0.297	-0.058	0.414	0.136	0.058
Fat Mass (kg)	-0.211	0.003	-0.130	0.071	-0.312	< 0.001	0.101	0.158
2-h OGTT (mg/dL)	-0.233	0.002	-0.292	< 0.001	-0.075	0.332	-0.253	0.001
2-h OGTT iAUC (AU)	-0.068	0.185	0.078	0.300	-0.279	< 0.001	0.424	< 0.001
HbA1C (%)	-0.200	0.006	-0.183	0.013	-0.153	0.036	-0.001	0.989
FPG (mg/dL)	-0.292	< 0.001	-0.337	< 0.001	-0.134	0.046	-0.233	< 0.001
FPI (mU/L)	-0.184	0.006	-0.200	0.003	-0.107	0.116	-0.068	0.322
HOMA-IR (AU)	-0.255	< 0.001	-0.291	< 0.001	-0.121	0.075	-0.154	0.024
Matsuda Index (AU)	0.214	0.005	0.183	0.018	0.187	0.015	-0.007	0.928
GDR (mg/kg/min)	0.472	< 0.001	0.343	0.003	0.594	< 0.001	-0.276	0.018
CRP (mg/L)	-0.220	0.012	-0.138	0.119	-0.274	0.002	0.140	0.113

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Figure 1 Soluble RAGE Isoforms According to Glucose Tolerance Status.

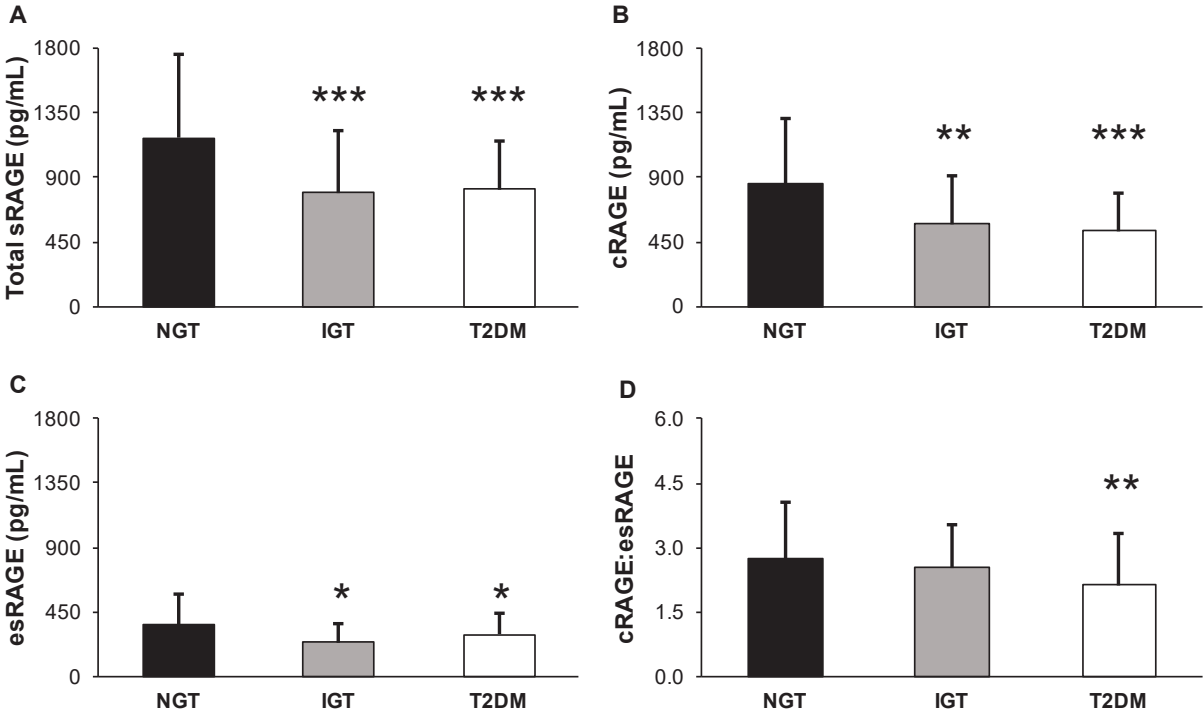


Figure 2 Effects of Glucose Tolerance and BMI on sRAGE isoforms.

