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ARTICLE

# Receptor for advanced glycation end-products (RAGE) provides a link between genetic susceptibility and environmental factors in type 1 diabetes

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**Abstract**
*Aims/hypothesis* This group of studies examines human genetic susceptibility conferred by the receptor for advanced glycation end-products (RAGE) in type 1 diabetes and investigates how this may interact with a western environment.
*Methods* We analysed the *AGER* gene, using 13 tag SNPs, in 3,624 Finnish individuals from the FinnDiane study,

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followed by *AGER* associations with a high risk HLA genotype *(DR3)-DQA1*05-DQB1*02/DRB1*0401-DQB1*0302* ($n$=546; *HLA-DR3/DR4*), matched in healthy newborn infants from the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Study ($n$=373) using allelic analysis. We also studied islets and circulating RAGE in NOD*Lt* mice.

*Results* The rs2070600 and rs17493811 polymorphisms predicted increased risk of type 1 diabetes, whereas the rs9469089 SNP was related to decreased risk, on a high risk HLA background. Children from the DIPP study also showed a decline in circulating soluble RAGE levels, at seroconversion to positivity for type 1 diabetes-associated autoantibodies. Islet RAGE and circulating soluble RAGE levels in prediabetic NOD*Lt* mice decreased over time and were prevented by the AGE lowering therapy alagebrium chloride. Alagebrium chloride also decreased the incidence of autoimmune diabetes and restored islet RAGE levels.

*Conclusions/interpretation* These studies suggest that inherited *AGER* gene polymorphisms may confer susceptibility to environmental insults. Declining circulating levels of soluble RAGE, before the development of overt diabetes, may also be predictive of clinical disease in children with high to medium risk HLA II backgrounds and this possibility warrants further investigation in a larger cohort.

**Keywords** Advanced glycation · Alagebrium chloride · Autoimmune diabetes · Children · Insulin · NOD · Polymorphism

**Abbreviations**

| | |
|---|---|
| CML | Carboxymethyl lysine |
| DIPP | Diabetes Prediction and Prevention Study |
| HMGB1 | High mobility group protein B1 |
| HWE | Hardy–Weinberg equilibrium |
| MAF | Minor allele frequencies |
| RAGE | Receptor for advanced glycation end-products |
| SNP | Single nucleotide polymorphism |

## Introduction

Type 1 diabetes occurs as the result of a complex disease process where genetic and environmental factors lead to an autoimmune response which, to date, remains incompletely defined [1]. Although some major genetic determinants of type 1 diabetes, such as alleles of the major histocompatibility locus (HLA) at the *HLA-DRB1* and *DQB1* loci [2] and more recently the *HLA-B*39* locus [3], have been identified, these only account for some 40–50% of the familial clustering. In addition, approximately 70% of monozygotic twins are discordant for the development of type 1 diabetes [4], which indicates that environmental triggers are also likely to be important. Indeed, the risk of developing type 1 diabetes is still increasing by 3–4% annually in developed nations [5], which remains unexplained.

One such environmental contributor to type 1 diabetes may be AGEs. Western diets provide excesses of these non-enzymatic modifications [6], which contribute flavour and colour to foodstuffs (e.g. roasted meat or coffee), while more recently AGE modifications also add functional properties, such as improved emulsification. Dietary AGEs are absorbed intestinally and are a prominent contributor to the body's AGE pool [7], in addition to the natural ageing process [8], redox imbalances [9], non-diabetes related diseases including renal impairment [10] and hyperglycaemia [11]. There is increasing evidence to suggest that AGEs also promote beta cell dysfunction [12, 13]. Furthermore, a reduced incidence of diabetes in a mouse model of autoimmune diabetes, the NOD mouse (NOD*Lt*) [14], has been reported following dietary restriction of AGEs. However, the specific mechanisms whereby insulin secretory pathways are damaged by AGEs are not known.

AGEs can exert their biological effects via receptors such as the receptor for advanced glycation end-products (RAGE) [15], although these compounds may have greater effects via modulation of RAGE production per se [16]. RAGE is a multi-ligand receptor involved in host-pathogen defence, in addition to cellular apoptosis [17]. Thus, a recent study has identified a blockade of the late stages of adoptively transferred autoimmune diabetes with the 'decoy' soluble RAGE receptor [18]. In addition, a small molecule inhibitor of RAGE [19] or antibodies against another RAGE ligand, HMGB1 [20], have been shown to delay islet destruction in hyperglycaemic NOD*Lt* mice. Furthermore, the heritability of insulin resistance [21], another factor postulated to be contributing to the increasing rate of type 1 diabetes incidence [22], is associated with specific RAGE polymorphisms.

Genetic susceptibility to type 1 diabetes remains to be fully defined, in particular, the contribution of certain genotypes in the context of our increasingly Westernised environment. It is therefore unknown if a specific RAGE genotype may play a role in susceptibility to type 1 diabetes; we have now investigated this issue using a combination of human genetics, molecular biology and integrative pathophysiology.

## Methods

### Human study

*FinnDiane/DIPP design and recruitment* Characteristics of the 2,921 unrelated type 1 diabetic patients from the nationwide Finnish Diabetic Nephropathy (FinnDiane)

study group [23] are provided in Table 1. Age of diagnosis of 30 years or below was used as a criterion for inclusion in the analysis. A total of 703 unrelated control samples were obtained from the Finnish Red Cross Blood Service. These controls represent a healthy non-diabetic Finnish population of European descent given the strict criteria that the Red Cross Blood Service practices regarding blood donors. Notably, there was no population stratification or admixture applied to their selection which might influence the genetic results (see Electronic supplementary material [ESM]).

Since *AGER* is located within the HLA class III region [24], we further analysed these *AGER* associations outlined above on a matched high risk HLA background. To achieve this, newborn infants ($n$=373), who had the highest risk HLA genotype *(DR3)-DQA1*05-DQB1*02*/*DRB1*0401-DQB1*0302* (from $n$=546; *HLA-DR3*/*DR4* [25]), were matched with unaffected newborn infants with the identical HLA genotype, from the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Study ($n$=373) [26]. A subgroup of children was also used for circulating soluble RAGE analyses. We included all children with the high risk HLA genotype identified from the DIPP study as controls. However, unfortunately some participants with the high risk HLA background had to be excluded because it was not possible to extract sufficient quantities of DNA for the analyses from the miniscule blood samples available. All children studied were from Finland and no other children were excluded from the present study. The ethics committees of all participating centres approved the study protocol and all patients or guardians gave written consent. The studies follow the Declaration of Helsinki.

*DNA isolation and genotyping* DNA was isolated from whole blood with either a Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's protocol or with a phenol-chloroform protocol [27].

Tag SNPs for genotyping were selected from the HapMap Project's NCBI build 34 and 35 for the CEU (Utah, USA residents with ancestry from northern and western Europe populations; $n$=180), as defined by Gabriel et al. [28] to encompass the genetic variation of the entire *AGER* gene. Minor allele frequencies (MAFs) were required to be ≥0.05. Genotyping was performed with TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) with an ABI Prism 7900HT Sequence Detection System (Perkin-Elmer, Foster City, CA, USA) and the Sequenom multiplex platform (Sequenom, Hamburg, Germany) according to the manufacturers' instructions.

HLA genotyping for the two high risk haplotypes was performed with a sequence specific hybridisation method using oligonucleotide probes specific for *DQA1*05* and *DQB1*02* (DR3-DQ2 haplotype) and *DRB1*0401* and *DQB1*0302* (DR4-DQ8 haplotype) as described previously [25].

*Soluble RAGE analysis* A series of children from the DIPP study were analysed for plasma soluble RAGE. The group comprised 15 children (12 boys) who progressed to clinical type 1 diabetes during prospective observation (patients) and 15 autoantibody-negative controls (non-progressors) who remained non-diabetic and were matched with the patients for sex, date of birth and HLA genotype. Altogether 14 children carried the high risk genotype (*DQA1*05-DQB1*02*/*DQB1*0302*) and 16 patients carried moderate risk genotypes (*HLADQB1*0302*/*x*;×≠ *DQB1*02*, **0301*, **0602* or **0603*). All children provided three plasma samples. Among the patients, the first sample was obtained before seroconversion to autoantibody positivity at a mean age of 1.1 (range 0.3–3.0) years, the second sample soon after the seroconversion at a mean age of 2.4 (range 0.7–5.3) years and the third sample soon after the diagnosis of clinical type 1 diabetes at a mean age of 5.2 (range 2.0–8.9) years. In the control patients, the samples were obtained at the corresponding ages. Human plasma samples were assayed undiluted according to the manufacturer's instructions contained within the Human RAGE ELISA (R&D Systems, Minneapolis, MN, USA). The inter-assay CV was 7.8% while the intra-assay CV was 5.7%. The limit of sensitivity was 4.12 pg/ml.

*Statistical analysis* Hardy–Weinberg equilibrium (HWE) for SNPs was tested in the control individuals ($n$=703), and which were considered to be in HWE when $p$>0.05 using Haploview 4.0 (MIT/Harvard Broad Institute, Boston, MA, USA) [29]. Testing for allelic associations was carried out with logistic regression adjusted for sex in SPSS 15.0 (Chicago, IL, USA).

Due to the strong linkage disequilibrium between SNPs in the HLA region and the HLA class II loci, it was necessary to exclude an effect of the HLA on the associations. Therefore, we matched patients and references for the high risk HLA genotype *(DR3)-DQA1*05-*

**Table 1** Patient characteristics: patients with T1D taken from the FinnDiane study

| Characteristic | Patients | Controls[a] | DIPP[b] |
|---|---|---|---|
| *n* | 2,921 | 703 | 373 |
| Sex, male (%) | 52.2 | 48.9 | N/A |
| Age (years) | 40.3±9.9 | | N/A |
| Age at T1D diagnosis (years) | 12.8±7.6 | N/A | N/A |

[a] Finnish Red Cross Blood Donors

[b] Newborn infants from the Type 1 DIPP

T1D, Type 1 diabetes

*DQB1*02/DRB1*0401-DQB1*0302*. Logistic regression was used to evaluate allelic odds ratios.

The issue of sufficient power to detect association between *AGER* SNPs and type 1 diabetes was evaluated using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/; [30]) with the module of case–control for discrete traits. Bonferroni correction for multiple comparisons was applied to the FinnDiane cohort under the conditions of 13 SNPs, $\alpha$ level 0.05, and three performed tests. Human soluble RAGE concentrations were analysed by repeated measures ANOVA.

## Rodent study

We next studied islet and circulating RAGE production in a spontaneous experimental model which resembles type 1 diabetes, the NOD*Lt* mouse, since the genetic loci thought to be responsible for susceptibility to diabetes in these mice include the *AGER* gene sequence [31, 32]. NOD*Lt* or NOD*Scid* mice fed standard mouse chow (containing the AGE, carboxymethyl lysine (CML): 93.39 nmol [mol lysine]$^{-1}$ [100 mg]$^{-1}$) were studied prediabetes at 4, 8 and 12 weeks of age ($n$=6 per group). In addition, groups of 4-week-old NOD*Lt* mice were randomised to receive either no treatment (vehicle) or the AGE-lowering therapy alagebrium chloride (1 mg kg$^{-1}$ day$^{-1}$ i.p.) and followed for 6 weeks (prediabetes, $n$=10 per group). For incidence studies, littermate NOD*Lt* mice ($n$=20 per group) were studied until the diagnosis of diabetes was confirmed or until day 200 of age, with one group randomised to receive alagebrium chloride (1 mg kg$^{-1}$ day$^{-1}$ i.p.) for the study duration from week 4 of age. Mice were given ad libitum access to food and water and maintained on 12 h dark–light cycles. Plasma glucose was measured by autoanalyser (Beckman Coulter LX20PRO, Brea, CA, USA) and plasma insulin concentrations were measured by radioimmunoassay (Linco Research, MO, USA).

*Circulating RAGE and AGE ELISA* Soluble RAGE in mouse serum (RAGE sandwich ELISA kit, R&D systems, Minneapolis, MN, USA) was assayed undiluted according to the manufacturer's instructions. The inter-assay CV was 6.7% and the intra-assay CV was 5.9%. The limit of sensitivity of the assay was 1 pg/ml. The AGE, CML, was assessed in serum or plasma using an in-house ELISA. The limit of detection of the assay was 8.0 nmol/mol lysine. The inter-assay CV was 7.3%. The intra-assay coefficient of variation was 5.5%.

*Immunohistochemistry* Formalin fixed paraffin sections [33] were incubated with primary antibody (AGE [34], 1:1,000; goat anti-RAGE 1:2,500, Chemicon, Temecula, CA, USA) overnight at 4°C. Tissue sections were consecutively stained with biotinylated IgG for 10 min and avidin–biotin horseradish peroxidase complex for 15 min (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) before 3,3′-diaminobenzidine tetrahydrochloride treatment (DAB; Sigma Chemical Co., St Louis, MI, USA). Negative control sections omitted the primary antibody. Positive control tissues were also included. Quantitation of islet immunostaining was completed by computer-aided densitometry (Image Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA) where all islets were analysed (×100) and results expressed as proportional to the area of positive staining.

*Real-time reverse transcription-polymerase chain reaction* RNA obtained from pancreatic islet tissue collected immediately was later used to synthesise cDNA with the Superscript First strand synthesis system for RT-PCR (Gibco BRL, Grand Island, NY, USA). Gene expression for *AGER* was analysed by real-time quantitative RT-PCR performed with the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer) normalised to 18 S as previously [35]. For mouse *Ager* (*NM_007425*), the probe was 6-FAM CACAGCCCGGATTG-MGB, the forward primer was 5′-GCTGTAGCTGGTGGTCAGAACA-3′ and the reverse primer was 5′-CCCCTTACAGCTTAGCACAAGTG-3′.

*Islet extraction* Pancreatic islets were isolated as previously described [36]. Briefly, 10 ml of cold Hanks balanced salt solution (HBBS) containing 0.75 mg/ml collagenase type V (Sigma Chemical Co.) was injected into the bile duct. The pancreas was digested at 37°C for 10–20 min and then disrupted and filtered through a 500 μm mesh. The pancreatic islets were separated from exocrine tissue by histopaque density gradient, for which the islets were suspended in histopaque 1.119 g/l, followed by layering of histopaque 1.083 g/l and histopaque 1.077 g/l (Sigma). The hand-picked islets were then used for flow cytometry as outlined below and RNA extraction [37]. For flow cytometry, purified islets were washed (10 mM EDTA in Hanks' balanced salt solution) and stained using goat anti-RAGE (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by rabbit anti-goat IgG, F(ab′)$_2$, FITC conjugated (Chemicon International, Temecula, CA, USA).

*Statistical analysis* Mouse results are expressed as mean ± standard deviation unless otherwise specified. All analyses of rodent data with more than two groups were performed by ANOVA followed by post hoc analysis with Tukey's test. Student's $t$ test or Mann–Whitney analyses were used to compare two groups where appropriate and are labelled

as such. A logrank (Mantel–Cox) test was used to analyse diabetes incidence in NOD mice. A value of $p<0.05$ was considered to be statistically significant (GraphPad Prism; GraphPad Software, San Diego, CA, USA).

## Results

### *AGER* gene polymorphisms and type 1 diabetes susceptibility

*FinnDiane* Characteristics of individuals from the FinnDiane study have been previously described in detail [38] and are summarised in Table 1. We observed that all 13 SNPs for *AGER* were strongly associated with type 1 diabetes (Table 2), even after Bonferroni correction for multiple comparisons (cut-off $p=0.0013$). Regarding the HLA frequencies, 19% of the FinnDiane type 1 diabetic patients have the *(DR3)-DQA1*05-DQB1*02*/*DRB1*0401-DQB1*0302* genotype. This percentage of the high risk *DR3*/*DR4* genotype is small given that this is only one of several other HLA haplotypes/genotypes which are known risk factors for the development of type 1 diabetes [39]. Therefore, in the FinnDiane cohort, 72.8% of all patients belong to the genetic risk group, as described by Näntö-Salonen et al. [39]. HWE was tested in the controls and all SNPs were in HWE.

*DIPP* We performed allelic analyses, which identified that three SNPs (rs2070600, rs9469089 and rs17493811) were still associated with type 1 diabetes ($p=0.017$, $3.65\times10^{-5}$, and 0.031, respectively) in HLA-matched children from the DIPP (Table 2). In particular, the rs2070600 and rs17493811 polymorphisms predicted increased risk of type 1 diabetes (ORs 1.452 and 1.518, respectively) whereas rs9469089 was related to decreased risk (OR 0.423) in the high risk HLA setting. However, using less stringent HLA adjustment, i.e. analysing those individuals with the risk alleles *DQB1*02* and/or *DQB1*0302* (type 1 diabetes patients $n=2{,}660$ and controls $n=294$), all *AGER* SNPs apart from rs204993 remained associated with type 1 diabetes following HLA matching (data not shown).

### Circulating soluble RAGE concentrations

Children from the DIPP study who progressed to type 1 diabetes (diagnosis; 5.2±2.2 years) had a decline in

**Table 2** SNP associations between *AGER* and type 1 diabetes: analysis of genomic DNA from patients with T1D from the FinnDiane cohort and from newborn infants with the highest risk HLA genotype *(DR3)-DQA1*05-DQB1*02*/*DRB1*0401-DQB1*0302* obtained as part of the Finnish Type 1 DIPP

| Position NCBI35 Chr 6 (bp) | SNP (alleles) | Minor[a] allele | SNP location | The FinnDiane cohort | | HLA–*DR3/DR4* matched | | OR (95% CI)[b] |
|---|---|---|---|---|---|---|---|---|
| | | | | MAF controls/ patients | *p* value | MAF controls/ patients | *p* value | |
| Sample size (*n*) | | | | 703/2,921 | 3,624 | 373/546 | 924 | 924 |
| 32,269,408 | rs204990 (G/T) | T | 5′ | 0.148/0.231 | $1.82\times10^{-11}$ [c] | 0.468/0.45 | 0.465 | 0.932 (0.773–1.125) |
| 32,269,374 | rs3132940 (C/A) | A | 5′ | 0.143/0.043 | $1.45\times10^{-39}$ [c] | 0.011/0.015 | 0.436 | 1.404 (0.598–3.298) |
| 32,269,302 | rs12663103 (T/C) | C | 5′ | 0.025/0.005 | $6.30\times10^{-10}$ [c] | 0/0.002 | n/a | n/a |
| 32,263,559 | rs204993 (T/C) | C | 5′ | 0.18/0.318‡ | $1.27\times10^{-23}$ [c] | 0.445/0.425 | 0.380 | 1.088 (0.901–1.314) |
| 32,262,976 | rs204994 (G/A) | A | 5′ | 0.148/0.220 | $2.82\times10^{-9}$ [c] | 0.464/0.446 | 0.499 | 0.937 (0.777–1.131) |
| 32,260,420 | rs1800625 (T/C) | C | Promoter | 0.146/0.218 | $2.76\times10^{-9}$ [c] | 0.463/0.444 | 0.474 | 0.933 (0.772–1.128) |
| 32,260,365 | rs1800624 (T/A) | A | Promoter | 0.291/0.348 | $5.89\times10^{-5}$ [c] | 0.270/0.239 | 0.109 | 0.839 (0.677–1.040) |
| 32,259,421 | rs2070600 (G/A) | A | G82S | 0.074/0.118 | $2.71\times10^{-6}$ [c] | 0.091/0.127 | 0.017 | 1.452 (1.068–1.973) |
| 32,259,200 | rs1035798 (C/T) | T | Intronic | 0.295/0.348 | $1.53\times10^{-4}$ [c] | 0.268/0.239 | 0.132 | 0.848 (0.685–1.051) |
| 32,254,635 | rs9469089 (G/C) | C | 3′ | 0.234/0.142 | $3.03\times10^{-16}$ [c] | 0.084/0.037 | $3.65\times10^{-5}$ | 0.423 (0.282–0.637) |
| 32,253,685 | rs2269423 (G/T) | T | 3′ | 0.440/0.280 | $1.36\times10^{-30}$ [c] | 0.150/0.143 | 0.785 | 0.964 (0.741–1.254) |
| 32,253,377 | rs17493811 (C/G) | G | 3′ | 0.026/0.048 | $6.34\times10^{-4}$ [c] | 0.056/0.082 | 0.031 | 1.518 (1.038–2.220) |
| 32,253,183 | rs3134947 (G/A) | A | 3′ | 0.150/0.219 | $1.23\times10^{-8}$ [c] | 0.465/0.444 | 0.423 | 0.926 (0.767–1.118) |

In the FinnDiane cohort, the *p* values are from logistic regression analyses, adjusted for sex, and reflect the joint effect of *AGER* and HLA

The DIPP HLA *DR3*/*DR4* matched set show the effect of *AGER* conditional for HLA haplotype

[a] The minor allele is the opposite in the DR3/DR4 matched data

[b] The allelic ORs with 95% CIs are modelled for the respective minor allele

[c] SNPs in the FinnDiane cohort that survive the stringent Bonferroni correction for multiple testing nucleotide polymorphism

MAF, minor allele frequency

circulating soluble RAGE levels with concomitant seroconversion to positivity for type 1 diabetes-associated autoantibodies (islet cell antibodies, insulin autoantibodies, antibodies to glutamic acid decarboxylase and/or antibodies to islet antigen 2; Fig. 1).

### Islet RAGE and circulating soluble RAGE decline prediabetes in NOD*Lt* mice

We first identified that islet RAGE expression was decreased in NOD*Lt* mice and, to a significantly lesser extent, in NOD*Scid* mice by week 8, compared with 4-week-old NOD mice (Fig. 2 a,b and d). However, by week 12, islet RAGE expression was significantly higher in both NOD*Lt* and NOD*Scid* (Fig. 2c and d). In NOD*Lt* mice, the loss of islet RAGE expression between weeks 4 and 12 was prevented with the AGE-lowering therapy, alagebrium chloride (Fig. 2e). In addition, the islet expression of the gene encoding RAGE (*AGER*) declined over time and was below detectable levels by week 8 of age in NOD*Lt* mice (4 weeks, 1.14±0.63 fold induction; $n=4$ per group; $p<0.001$ 4 vs 8 or 12 weeks) as assessed by real-time RT-PCR [40]. There was also a significant decline in the levels of circulating soluble RAGE from 4 to 6 weeks of age in NOD*Lt* mice (Fig. 2f) that was not seen in NOD*Scid* mice (Fig. 2f). Alagebrium chloride also significantly improved circulating soluble RAGE concentrations at week 10 of age (Fig. 2g). There was a positive association between the loss of islet RAGE and declining circulating soluble RAGE in NOD*Lt* mice ($r=0.85$, $p<0.001$). In addition, serum concentrations of the AGE, CML, were elevated at both week 8 and 12 weeks of age in NOD*Lt* mice as compared with NOD*Scid* mice (Fig. 2h), which was also ameliorated by treatment to reduce AGE accumulation with alagebrium chloride at week 10 (NOD*Lt*, 4,826±12,61 nmol/mol lysine vs NOD*Lt* treated with alagebrium chloride, 2,059±589 nmol/mol lysine, $p=0.026$ Student's $t$ test). There was also a decline in plasma concentrations of the RAGE ligand HMGB1 seen at week 8 in NOD*Lt* mice when compared with NOD*Scid* mice (Fig. 2i). Furthermore, alagebrium chloride markedly decreased the incidence of autoimmune diabetes in NOD*Lt* mice from 50% to 10% (Fig. 2j).

Immunohistochemistry also demonstrated a decline in islet RAGE staining in NOD*Lt* mice at weeks 8 and 12 prediabetes, which was not seen in NOD*Scid* mice (see Fig. 3a–g). RAGE immunostaining in islets was localised primarily to alpha cells in both NOD*Lt* and NOD*Scid* mice (Fig. 3). Islet AGE immunohistochemistry demonstrated increased concentrations of CML at all prediabetic time points in NOD*Scid* mice (Fig. 3h), although only a small proportion of AGE staining in NOD*Scid* mice was cell surface bound as determined by flow cytometry compared with NOD*Lt* mice (Fig. 3i). There was also a significant, but transient, increase in plasma glucose concentrations seen at week 8 in NOD*Lt* (4 week NOD*Lt*, 2.7±0.5 mmol/l vs 8 week NOD*Lt*, 7.2±2.1 mmol/l, $p<0.05$; vs 12 week NOD*Lt*, 6.9±1.1 mmol/l, NS). There was no difference seen in plasma glucose (8 week NOD*Scid*, 7.4±4.2 mmol/l; 12 week NOD*Scid*, 6.1±2.6 mmol/l) or glycated haemoglobin concentrations (Table 3) between NOD*Lt* and NOD*Scid* mice at either 8 or 12 weeks of age. NOD*Lt* mice were heavier than NOD*Scid* mice by 12 weeks of the study (Table 3).

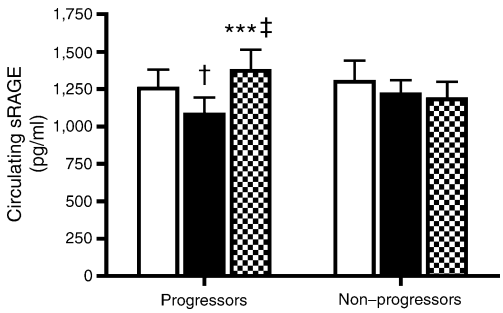


**Fig. 1** Human participants who progress to type 1 diabetes show a decline in soluble RAGE at seroconversion to autoantibody positivity. Serial plasma samples before seroconversion (white bars; mean age 1.1±0.6 years), following initial seroconversion (black bars; mean age 2.4±1.2 years) to positivity for diabetes-associated autoantibodies (islet cell antibodies, insulin autoantibodies, antibodies to glutamic acid decarboxylase, and/or antibodies to islet antigen 2) and at clinical diagnosis of type 1 diabetes (hatched bar; mean age 5.2±2.2 years), in the progressors ($n=15$) and samples obtained at corresponding ages in autoantibody-negative non-progressors (hatched bar; $n=15$), were analysed for soluble RAGE concentrations. $^{\dagger}p=0.025$ vs initial sample; $^{\ddagger}p=0.02$ vs initial sample; $^{***}p<0.001$ vs sample obtained at seroconversion to autoantibody positivity. sRAGE, soluble RAGE

## Discussion

Many chronic diseases are likely to be polygenic, including type 1 diabetes, where less than 50% of the familial clustering can be attributed to currently reported genotypes. This highlights the importance of discovering other candidate genes and environmental contributors. Within the present study, we have identified three polymorphisms in the *AGER* gene, which encodes for the proteins RAGE and soluble RAGE, that may partly explain the genetic susceptibility to type 1 diabetes in humans, even in individuals with a high risk HLA class II background. Of particular interest is the finding that a temporal decline in circulating concentrations of the protective isoform of RAGE (termed soluble RAGE) at seroconversion to produce autoantibodies, predicted the clinical onset of type 1 diabetes in patients within high to moderate risk class II HLA genotypes. These findings are reminiscent of changes

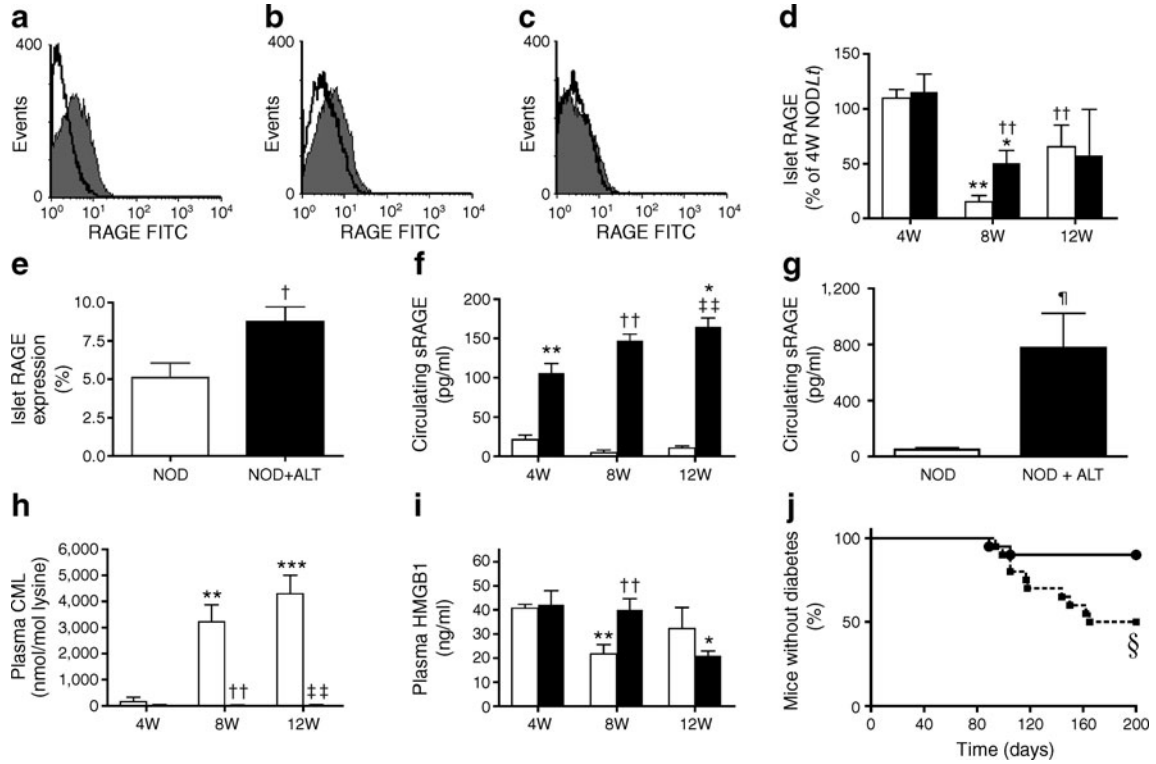


**Fig. 2** Prediabetic NOD mice have a deficiency in islet RAGE production. **a–e** Flow cytometry for islet RAGE levels; **a–c** representative histograms in prediabetic NOD*Lt* mouse islets ($n$=5 per group) at 4 weeks (**a**), 8 weeks (**b**) and 12 weeks (**c**). **d**,**e** Percentage of cell surface RAGE-positive islets in (**d**) NOD*Lt* (white bars) and NOD*Scid* (black bars) mice at weeks 4, 8 and 12 and (**e**) at week 10 ($n$=10 per group, after treatment with alagebrium chloride (ALT), an inhibitor of RAGE ligand (AGE) accumulation. **f**,**g** Plasma concentrations of circulating soluble RAGE by ELISA; (**f**) time course in prediabetic NOD*Lt* and NOD*Scid* mice (NOD*Lt*, white bars; NOD*Scid*, black bars) (**g**) At week 10 following ALT therapy. **h** Time-course plasma concentrations of CML in prediabetic NOD*Lt* and NOD*Scid* mice ($n$=5–8 per group) (NOD*Lt*, white bars; NOD*Scid*, black bars). **i** Plasma concentrations of the RAGE ligand, HMGB1, in NOD*Lt* and NOD*Scid* mice ($n$=10 per group) (NOD*Lt*, white bars; NOD*Scid*, black bars). **j** Diabetes incidence in untreated NOD*Lt* mice (square, dashed line) or in NOD*Lt* mice administered with ALT (circle, continuous line) ($n$=20 per group). $^{\dagger}p$=0.0283 vs NOD*Lt*, Student's $t$ test, two tailed; $^{**}p$<0.01 vs 4 W NOD*Lt*; $^{***}p$<0.001 vs 4 W NOD*Lt*, $^{\ddagger}p$=0.0238 vs 4 W, Mann–Whitney test; $^{\P}p$=0.0016 vs untreated NOD*Lt*, Student's $t$ test, two tailed; $^{\S}p$=0.009 vs NOD*Lt*+ ALT, logrank (Mantel–Cox) test; $^{\dagger\dagger}p$<0.01 vs 8 W NOD*Lt*; $^{\ddagger\ddagger}p$<0.01 vs 12 W NOD*Lt*, $^{*}p$<0.05 vs 4 W NOD*Scid*. sRAGE, soluble RAGE; W, week

in NOD*Lt* mice, in whom seroconversion appears concurrently with a decrease in circulating soluble RAGE concentrations [41]. There was also an inverse relationship between circulating CML (an AGE) and either soluble RAGE concentrations or islet RAGE expression seen in both NOD*Lt* and NOD*Scid* mice. Interestingly, despite elevated circulating AGE levels, islet AGE concentrations were also lower in NOD*Lt* mice compared with NOD*Scid* mice, suggesting that RAGE may be involved in the cellular uptake or intracellular production of AGEs in pancreatic islet cells.

A decline in circulating soluble RAGE has also been shown in children during active autoimmune disease, such as Kawasaki disease or systemic onset juvenile idiopathic arthritis [42]. Decreases in plasma soluble RAGE were also seen in samples from children who progressed to type 1 diabetes at seroconversion to autoantibodies, compared with samples obtained before seroconversion. At the diagnosis of diabetes, those individuals who progressed to type 1 diabetes in the DIPP study had an increase in plasma soluble RAGE, probably as the result of active inflammation, as has been seen previously in individuals with type 2 diabetes [43]. Therefore, although tantalising, the changes in soluble RAGE seen within the present study in children who progressed to type 1 diabetes need to be confirmed in larger patient cohorts. In addition, since soluble RAGE concentrations were not measured in the individuals from whom we have the *AGER* genotype data, Mendelian randomisation was not possible in the present populations.

The genetic loci thought to be responsible for susceptibility to autoimmune diabetes in NOD*Lt* mice include the *AGER* gene sequence encoding RAGE proteins [31, 32]. The presence of the serine residue within the *G82S* polymorphism, identified using *AGER* rs2070600 in humans, has previously been associated with lower [44] or elevated [43] circulating soluble RAGE concentrations.

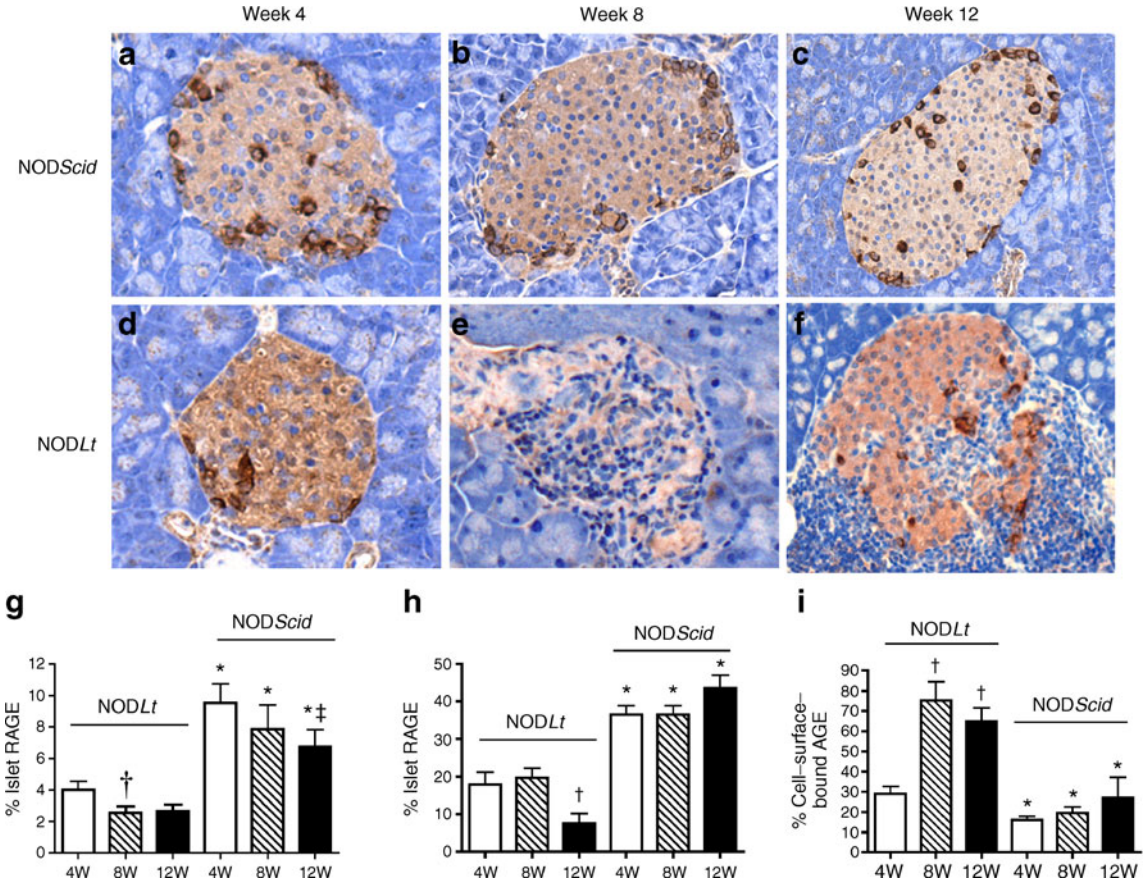


**Fig. 3** AGE and RAGE localisation in pancreatic islets in NOD mice. Photomicrographs of immunohistochemistry for RAGE in (**a–c**) NOD*Scid* mice at (**a**) week 4 (**b**) week 8 and (**c**) week 12; and (**d–f**) NOD*Lt* mice at (**d**) week 4 (**e**) week 8 and (**f**) week 12. **g** Quantification of islets. **h** Quantification of islet CML by immunohistochemistry. **i** Cell surface binding of AGEs to islets by flow cytometry. *$p<0.05$ NOD*Scid* vs NOD*Lt* within the same time point; †$p<0.05$ vs 4 W NOD*Lt*; ‡$p<0.05$ vs 4 W NOD*Scid*. W, week

**Table 3** Metabolic variables for NOD mouse groups

| Mouse group | GHb (%) | BW (g) |
|---|---|---|
| NOD*Lt* | | |
| 4 weeks | 2.6±0.1 | 18.3±1.3 |
| 8 weeks | 2.2±0.1 | 23.7±1.7* |
| 12 weeks | 2.4±0.1 | 27.7±1.0*† |
| NOD*Scid* | | |
| 4 weeks | 2.8±0.3 | 18.1±1.6 |
| 8 weeks | 3.1±0.1 | 23.4±1.6* |
| 12 weeks | 2.8±0.1 | 24.0±1.3*‡ |

Data are mean±SD

Glycated haemoglobin and final body weights at the study end-points

$n=10$ mice per group

*$p<0.05$ vs respective 4 week group; †$p=0.05$ vs 8 week NOD*Lt*, ‡$p=0.05$ at 12 weeks for NOD*Lt* vs NOD*Scid*

GHb, glycated haemoglobin; BW, body weight

Interestingly, this SNP was also associated with a higher risk of type 1 diabetes in our human cohorts in the present study. In our human studies, it is difficult to increase the numbers of patients and controls studied, given the key requirement to control for high risk HLA genotypes to delineate the association between *AGER* for type 1 diabetes. It is nevertheless intriguing that, even in such a small HLA-matched sample, there were clear genotypic differences and changes in circulating soluble RAGE concentrations between those with type 1 diabetes and those without. However, this finding now needs to be replicated in larger human populations.

One cannot discount, however, the possibility that changes in the proteases responsible for the cleavage of membrane RAGE to soluble RAGE [45] may also be altering the circulating soluble RAGE pool in our diabetic individuals and mice, independent of changes in the *AGER* gene. Indeed, this possibility should be further investigated in subsequent studies, in particular given the effects of AGEs on circulating RAGE concentrations in NOD*Lt* mice observed in the present study. Interestingly, prediabetic

NOD*Lt* mice in the present study also had a transient decline in islet RAGE expression at seroconversion to autoantibody positivity which was not seen in NOD*Scid* mice. Furthermore, we have determined that circulating soluble RAGE concentrations correlate with islet RAGE expression. In the future, it would be desirable to confirm these findings using a NOD*Lt* mouse with an islet specific knockout of the gene encoding RAGE (*AGER*). It would also have been highly desirable to confirm a loss of islet RAGE expression in human participants with type 1 diabetes, although we have shown that decreases in circulating soluble RAGE concentrations in children with medium to high risk HLA II genetic backgrounds may be predictive of this loss in islet RAGE expression. Therefore, at this stage we can only speculate as to the association of RAGE with type 1 diabetes.

Some previous studies in adoptively transferred autoimmune diabetes and syngeneic islet transplants in hyperglycaemic NOD*Lt* mice have shown benefits from the interruption of RAGE ligand binding with small molecule inhibitors of RAGE [19] or exogenous soluble RAGE [18]. In adoptively transferred diabetes [18], however, it could only be concluded that RAGE ligands may be involved in the differentiation of T cells to a mature phenotype. Indeed, NOD*Scid* mice also have neither T nor B cells, providing further evidence that RAGE expression on T cells, such as Th1 [18, 19], may also be important in the pathogenesis of diabetes, given that NOD*Scid* mice do not develop autoimmune diabetes.

Prediabetic NOD*Lt* mice in the present study showed increases in circulating RAGE ligands, AGEs, that were not seen with respect to another RAGE ligand, HMGB1. This was surprising given the previous association of HMGB1 to autoimmune diabetes in NOD*Lt* mice [20]. However, another ligand of RAGE is the amylin polypeptide, which is co-produced with insulin by the pancreatic beta cells in response to elevation of plasma glucose [46]. Moreover, there is speculation that insulin may also bind to RAGE, since there is evidence of amyloidosis composed of iatrogenic A-Ins type amyloid [47]. Indeed, other investigators have suggested that proinflammatory RAGE and HLA II DRB1 polymorphisms may synergise to activate the immune response in diabetes complications [48]. However, whether this affects the immune system during the development of autoimmune diabetes or type 1 diabetes remains to be determined.

Importantly, lowering AGEs using alagebrium chloride in the present study not only increased circulating soluble RAGE concentrations in NOD*Lt* mice at seroconversion to autoantibodies, but also reduced the incidence of autoimmune diabetes. Taken together, one could speculate that it is important therapeutically to prevent ligand-induced chronic modulation of RAGE to protect islet function. Also of interest was the fact that it is unlikely that increases in plasma glucose are driving the elevations in plasma AGEs seen within the present study, as is classically seen in diabetes complications [33, 49, 50], since elevations in circulating AGEs by 8 weeks of age were also evident in NOD*Scid* mice, which do not develop diabetes. These results raise the possibility that precipitation of diabetes could occur as the result of either genetic or environmental modulation of RAGE.

Another limitation of the present study is that so-called high risk HLA haplotypes are ‘losing power’ in the younger age groups developing type 1 diabetes [51]. However, FinnDiane is collecting data on adult type 1 diabetic patients, rather than paediatric patients, and therefore the mean duration of type 1 diabetes is relatively high (24.4 years, or median of 24.2 years), meaning that less than 10% of the patients studied were born in the late 1970s and 1980s, when the HLA trend mentioned above was primarily observed. Furthermore, given that the controls for the FinnDiane study were recruited through the Finnish blood bank, we have little epidemiological data relating to these individuals.

Thus, in summary, the data presented here show that inherited *AGER* gene polymorphisms should be considered as novel contributors to susceptibility to type 1 diabetes. This group of studies also suggests that excesses of environmental factors such as AGEs might modulate changes in islet and circulating RAGE expression, which may contribute to insulin secretory defects and ultimately the development of overt diabetes. In addition, declining circulating levels of soluble RAGE, which binds excesses of AGEs, at seroconversion before the development of overt diabetes, may warrant investigation in larger human cohorts as a predictor of clinical type 1 diabetes. Thus, we not only propose a novel mechanism for the development of diabetes, but also suggest a potential treatment, the AGE lowering therapy alagebrium chloride, currently under clinical investigation, albeit for other medical indications. Furthermore, we believe that these results challenge current thoughts in this area, by hypothesising that genetic or environmental declines in RAGE may be important for the development of type 1 diabetes. These findings are also likely to have implications for the pathogenesis of other chronic diseases in which this receptor is considered to play a pivotal role.

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