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ARTICLE

HLA-dependent autoantibodies against post-translationally modified collagen type II in type 1 diabetes mellitus

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Abstract
Aims/hypothesis In this study the involvement of oxidative stress in type 1 diabetes mellitus autoimmunity and the possible association with rheumatoid arthritis (RA) was investigated. We tested the hypothesis that oxidative stress induced by chronic hyperglycaemia triggers post-translational modifications and thus the formation of neo-antigens in type 1 diabetes, similar to the ones found in RA.
Methods Collagen type II (CII), a known autoantigen in RA, was treated with ribose and various reactive oxygen species (ROS). Levels of antibodies specific to native and ROS-modified CII (ROS-CII) were compared in type 1 diabetes, type 2 diabetes and healthy controls, and related to the HLA genotype.
Results Significantly higher binding to ROS-CII vs native CII was observed in type 1 diabetic patients possessing the *HLA-DRB1*04* allele irrespective of variables of glucose control (blood glucose or HbA_{1c}). Type 1 diabetic patients carrying a *DRB1*04* allele with the shared epitope showed the highest risk for ROS-CII autoimmunity, while the *DRB1*0301* allele was protective. Conversely, native CII autoimmunity was not associated with any specific *DRB1* allele. Positive and inverse seroconversion rates of response to ROS-CII were high in *DRB1*04*-positive type 1 diabetic patients.
Conclusion Hyperglycaemia and oxidative stress may trigger genetically controlled autoimmunity to ROS-CII and may explain the association between type 1 diabetes mellitus and RA.

Keywords Collagen type II · Post-translational modification · Reactive oxygen species · Rheumatoid arthritis

Abbreviations
ACPA Anti-citrullinated peptide antibodies

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CII	Collagen type II
HRP	Horseradish peroxidase
OD	Optical density
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
ROS-CII	CII modified by ROS
SE	Shared epitope
TPO	Thyroid peroxidase

Introduction

Type 1 diabetes mellitus is characterised by chronic insulin deficiency and hyperglycaemia due to extensive destruction of insulin-producing beta cells. The autoimmune nature of this process is supported by the presence of a pool of autoantibodies against beta cell antigens as well as the association with genes controlling immune homeostasis [1].

The major genetic risk for type 1 diabetes resides within the HLA complex region, which contains alleles that encode molecules involved in the recognition and presentation of peptide antigens to T cells. This risk is conferred by a combination of alleles of the HLA class II super-types *DRB1*03-DQB1*0201* and *DRB1*04-DQB1*0302* [2]. The *DQB1*0302-A1*0301 (DQ8)* haplotype defines the greatest risk for the disease, with an increased diabetogenic effect if the *DRB1*0401* allele is inherited as part of the haplotype. An effect predisposing to type 1 diabetes has also been found for other *DRB1*04* alleles, **0402*, **0404* and **0405* [3, 4]. Consistently, markers of beta cell autoimmunity such as GAD autoantibodies (GADA), tyrosine phosphatase autoantibodies (IA-2A) and insulin autoantibodies (IAA) are strongly associated with *HLA-DRB1*04* and/or *HLA-DRB1*03* not only in type 1 diabetes, but also in first-degree relatives of individuals with type 1 diabetes, as well as in the general population [5].

Hyperglycaemia is the hallmark of diabetes and is a major player in the chronic complications of the disease. Elevated blood glucose induces oxidative stress and changes in the cellular redox state. NADPH oxidase has been responsible for the formation of high levels of reactive oxygen species (ROS) in response to high glucose [6]. A second source of ROS production is the excessive production of AGEs. Two AGEs, namely carboxymethyl-lysine and pentosidine, are related to the severity of diabetic nephropathy and are known biomarkers for 'carbonyl stress' [7]. The main toxic effect of both ROS and AGEs is the induction of abnormal post-translational modifications of self-antigens and the generation of neo-antigens, thus bypassing immune tolerance and contributing to the development of autoimmune responses [8]. The involvement of oxidative stress in type 1 diabetes has been implied by the presence of autoantibodies against oxidised GAD [9–11]. In addition, experimental diabetes can be induced in rats by feeding with alloxan and streptozotocin, two substances that work by generating ROS and inducing selective damage to beta cells [12].

We have recently shown that post-translational modification by reactive oxidants results in the formation of neo-epitopes in rheumatoid arthritis (RA), an autoimmune disease where abnormal immune reactivity against a cartilage extracellular matrix protein, collagen type II (CII), is detected [13, 14]. We have demonstrated that ROS present in inflamed RA joints can generate CII neo-antigens, which stimulate autoimmunity to ROS-modified CII (ROS-CII), that have been detected in serum samples from individuals with RA [15]. Co-existence of type 1 diabetes and RA in the same individuals has been reported [16] and a large study of 3,093 individuals showed that type 1 diabetic patients are two to five times more likely than healthy individuals to develop RA [17]. Part of this association has been attributed to the presence of shared genetic susceptibility. Similarly to type 1 diabetes, the greatest risk for RA is restricted to *DRB1*04* alleles containing a shared epitope (SE), namely *DRB1*0401*, **0404* and **0405*, with an additional effect in the presence of the *DRB1*0101* and *DQB1*0302* alleles [18]. On the other hand, *DRB1*0402,* although conferring susceptibility to type 1 diabetes, is protective against RA [18].

The aim of this study was to assess the role of hyperglycaemia and oxidative stress in triggering neo-antigenicity to self-antigens in type 1 diabetes. We hypothesised that oxidative stress induced by chronic hyperglycaemia may trigger post-translational modifications, and thus the formation of neo-antigens in type 1 diabetes. Considering the existence of an association between type 1 diabetes and RA, we evaluated the presence of autoantibodies to ROS-CII, and the interplay with the HLA alleles conferring susceptibility in both diseases. Levels of native and ROS-CII antibodies were compared in type 1 diabetes, type 2 diabetes and healthy controls, and related to HLA genotype using both cross-sectional and longitudinal designs.

Methods

Participants Serum samples were obtained from participating centres of the Immunotherapy Diabetes (IMDIAB) group. Of the 151 individuals with type 1 diabetes, 81 were *HLA-DRB1*04* positive and 70 *HLA-DRB1*04* negative. Individuals with type 1 diabetes were selected according to the following criteria: (1) diagnosis of the disease according to American Diabetes Association criteria, with age at presentation less than 35 years—in most cases diagnosis was confirmed by positivity to glutamic acid decarboxylase, insulin or tyrosine phosphatase antibodies; (2) no other major chronic disease; and (3) less than 12 months' duration of clinical disease (since the beginning of

insulin therapy). The longitudinal study was conducted using sera from individuals recently diagnosed with type 1 diabetes (n=21 *HLA-DRB1*04* positive and n=13 *HLA-DRB1*04* negative) obtained within 6 months of diagnosis and 9–12 months after the first sampling. Sera from individuals with type 2 diabetes (n=40) and healthy individuals (n=85) were used as control groups. Within the control groups, HLA typing was obtained in individuals with type 2 diabetes (n=20) and healthy individuals (n=33). *DRB1*04* alleles were carried by 0% and 15% of type 2 diabetes and healthy control individuals, respectively; the most common *HLA-DRB1* types were *DRB1*11* and/or *DRB1*12*, *DRB1*15*, *DRB1*16* and *DRB1*03*. This project was approved by the Ethical Committee at University Campus Bio-Medico within the framework of the IMDIAB investigators type 1 study, with informed consent signed by patients or parents.

Chemical modification of CII and BSA Bovine CII was prepared from bovine cartilage and chemically modified as described [15]. Briefly, CII (2 mg/ml) in PBS was incubated overnight at 37°C with the following systems: (1) 2 mol/l ribose (Sigma, Gillingham, UK); (2) 1 mmol/l HOCl (BDH, Oxford, UK); (3) 1 mmol/l $CuCl_2$ (Sigma) and 2 mmol/l H_2O_2 (Sigma) which was used to produce hydroxyl radical (·OH) by the Fenton reaction; and (4) 2 mmol/l peroxynitrite ($ONOO^-$,Calbiochem, Beeston, UK). Modifications of CII were monitored by 8% SDS-PAGE and three-dimensional fluorescence, as described previously [15]. BSA was also modified as above.

HLA genotyping HLA typing and subtyping were performed by PCR using specific primers and hybridisation with sequence specific oligonucleotides, as previously described [19]. *DRB1*0401*, **0404* and **0405* were grouped together because they encode a conserved amino-acid sequence, an *HLA-DRB1* SE [20], depicting a genetic susceptibility shared by type 1 diabetes and RA [3, 4, 18].

ELISA An ELISA was performed using modified and native CII or BSA as targets as described previously [15]. Briefly, ELISA plates (Nunc, London, UK) were coated with 10 μg/ml of modified or native protein in PBS for incubation at 4°C overnight. Plates were then washed three times with PBS. After blocking for 1.5 h with 2% (wt/vol.) dry milk powder in PBS (Marvel-PBS), 100 μl of 1:100-diluted serum samples in 2% Marvel-PBS were added to each well, followed by 1.5 h incubation at 37°C. Plates were then washed with PBS plus 0.1% Tween, followed by three washes with PBS. Anti-human IgG-horseradish peroxidase (HRP)-conjugated antibodies (Sigma) were then added at 1:1,000 dilution in 2% Marvel-PBS for another 1.5 h incubation. The ELISA plates were washed, and 100 μg/ml 3,3′,5,5′-tetramethylbenzidine substrate (Sigma) in 100 mmol/l sodium acetate, pH6.0, were added. Subsequently, the reaction was stopped with 1 mol/l sulphuric acid. The optical density (OD) was measured at 450 nm using a GENios plate reader and Magellan software (Tecan, Reading, UK).

Western blotting For the western blots, 2 μg ROS-CII, native CII, ROS-BSA and native BSA were run on 8% denaturing SDS-PAGE and electroblotted onto a nitrocellulose membrane (BDH Chemicals). After blocking with 5% Marvel-PBS, membranes were incubated with a 1:100 dilution of serum samples in 5% Marvel-PBS for 1 h at room temperature, followed by incubation with 1:1,000 dilution of anti-human IgG-HRP (Sigma). Membranes were washed extensively with 0.1% Tween-PBS before development with enhanced chemiluminescence (Pharmacia, Milton Keynes, UK).

Statistical analysis Statistical analyses were performed using Prism Software (GraphPad, San Diego, CA, USA) and Stata 12 (StataCorp. 2011, Stata Statistical Software: Release 12, College Station, TX, USA). Differences in levels of autoantibodies between groups were tested by the Mann–Whitney test. Fisher's exact test was used to assess whether the reactivity toward ROS-CII or ROS-BSA was significantly different from native CII or native BSA. Levels of anti-CII and anti-BSA antibodies above the 95th percentile of the healthy individuals were defined as elevated. Pearson's correlation test was used to assess the association between reactivity to ROS-CII and native CII and metabolic control. ORs were calculated [21] and reported with 95% CIs to assess the association between genotype and presence of elevated CII or BSA autoantibodies. Longitudinal change in antibody binding was evaluated using a Wilcoxon matched-pairs signed rank test.

Results

Population study Features of the studied cohort are reported in Table 1. No differences were detected between *HLA-DRB1*04*-positive and *HLA-DRB1*04*-negative patients in term of sex, age or metabolic variables. Individuals with type 1 diabetes were younger than type 2 diabetes and healthy controls, and had shorter disease duration than type 2 diabetes patients.

Antibody binding to ROS-CII and ROS-BSA in type 1 diabetes and in control individuals Samples were grouped into type 1 diabetes *HLA-DRB1*04* positive and *HLA-DRB1*04* negative. Binding to native and ROS-CII was significantly higher in type 1 diabetes *HLA-DRB1*04*-positive compared with type 1 diabetes *HLA-DRB1*04*-negative samples, type 2 diabetes controls and healthy individuals (p<0.001,

Table 1 Clinical and biochemical features of the studied populations

Characteristic	T1D *DRB1*04*$^{+}$ (n=81)	T1D *DRB1*04*$^{-}$ (n=70)	T2D (n=40)	Healthy controls (n=85)
Male sex, n (%)	42 (52)	37 (53)	19 (48)	31 (36)
Age, years	12.09±0.79	14.02±0.95	56.88±1.86	40.12±1.3
BMI, kg/m^2	18.2±0.43	18.19±0.49	30.92±1.39	
Disease duration, years	≤1	≤1	6.52±1.19	
Blood glucose, mmol/l	8.46±0.47	8.26±0.40	8.14±0.27	
HbA_{1c}, % (mmol/mol)	6.61±0.18 (48.73±1.96)	7.05±0.34 (53.57±3.74)	6.87±0.19 (51.54±2.06)	

Data are mean±standard error

Sex, age and metabolic variables of type 1 diabetes *HLA-DRB1*04* positive and *HLA-DRB1*04* negative individuals were similar but they were younger than type 2 diabetic patients and healthy controls and had shorter disease duration than patients with type 2 diabetes

T1D, type 1 diabetes; T2D, type 2 diabetes

Fig. 1a). An increased percentage of binders to native and ROS-CII was observed in the *HLA-DRB1*04*-positive samples, 25.93% vs 62.96%, respectively (p<0.0001). Binding of the *HLA-DRB1*04*-negative group was generally low, with only 2.90% and 14.29% binders to native and ROS-CII, respectively. The lowest binding was seen in samples taken from individuals with type 2 diabetes, with 0% and 27.5% binders to native and ROS-CII, respectively. Nevertheless, in both type 1 diabetes (*HLA-DRB1*04* positive and negative) and type 2 diabetes groups, binding to ROS-CII was higher than to native CII (p<0.001, Fig. 1a). Binding to native BSA was higher in type 1 diabetes groups compared with healthy controls (p<0.001, Fig. 1b), but no significant differences occurred between *DRB1*04*-positive or *DRB1*04*-negative type 1 diabetes and type 2 diabetes. We observed 8% and 12% binders to native BSA and ROS-BSA in type 1 diabetes *DRB1*04* positive, respectively. Similarly in type 1 diabetes *DRB1*04* negative, 3% and 10% bound to native BSA and ROS-BSA, respectively. In both type 1 diabetes and type 2 diabetes, binding to ROS-BSA was weaker than to native BSA (p<0.001, Fig. 1b), except for peroxynitrite-modified BSA. Only 3.85% of healthy control individuals responded to either native or ROS-CII regardless of *HLA-DRB1*04* positivity, while 2.7% responded to BSA or ROS-BSA.

Analysis of serum reactivity to ROS-CII and ROS-BSA by western blotting Western blot analysis was performed on a range of serum samples with high or low binding to modified CII in ELISA. A correlation between ELISA binders and samples showing strong binding at the western blot analysis was found. A typical pattern of binding is shown in Fig. 2a for a *DRB1*04*-positive individual with type 1 diabetes and in Fig. 2b for a *DRB1*04*-negative individual. The *DRB1*04*-positive type 1 diabetes serum sample bound to native CII and ROS-CII as follows. For native CII, binding to a fragment in the region of 100 kDa, which correlates with the mobility of the CII α chain (lane 1). For glycated CII, binding was: (1) to a higher mobility fragment in the region of 250 kDa; (2) 100 kDa fragment corresponding to the α chain; (3) a slightly higher band that corresponds to CII with ribose condensation (shift base); and (4) fragmented CII in the region of 50–100 kDa (lane 2). A weaker pattern of binding to fragmented CII and high-molecular-weight aggregates was observed for CII modified by HOCl (lane 3). For CII modified by ·OH and modified by $ONOO^{-}$, there was strong binding to high-molecular-weight aggregates, with some stuck in the slots of the SDS-PAGE (lanes 4 and 5). Binding of the *DRB1*04*-negative serum sample was generally low or absent. No specific binding was found for native and ROS-BSA (data not shown).

Effect of HLA genotype on antibody binding to ROS-CII and ROS-BSA in type 1 diabetes The association between specific DRB1 genotype and reactivity to native or modified CII was analysed in 151 individuals by calculating the OR of responders vs non-responders for each genotype. Native CII and ROS-CII autoimmunity was positively associated with *DRB1*04* (OR 11.9 [95% CI 2.68, 52.88], p=0.0001) and (OR 10.2 [95% CI 4.55, 22.86], p<0.0001), respectively. *DRB1*03*, however, was protective against native and ROS-CII autoimmunity in the absence of *DRB1*04* (OR 0.04 [95% CI 0.003, 0.75], p=0.0006) and (OR 0.1 [95% CI 0.05, 0.37], p=0.0001), respectively (Table 2). Complete *HLA-DRB1* subtyping was available only for 57 individuals. Phenotype analysis showed that type 1 diabetic patients carrying SE-containing *DRB1*04* alleles, defined by the presence of at least one *DRB1*0401 *0404* or **0405*, had the highest risk for ROS-CII autoimmunity (OR 3.62 [95% CI 1.12, 11.74], p=0.027), while *DRB1*0301* provided protection from ROS-CII reactivity (OR 0.23 [95% CI 0.07, 0.75], p=0.013). However, other *DRB1* phenotypes, including the type 1 diabetes *DRB1*04* susceptibility allele **0402*, were not associated with anti-ROS-CII reactivity (OR 2.1 [95% CI 0.39, 11.23] p=0.378), suggesting a more selective relationship between *DBR1*04* and ROS-CII autoimmunity in type 1 diabetes. Native CII autoimmunity was, however, not associated with any specific *DRB1* phenotype (Table 2).

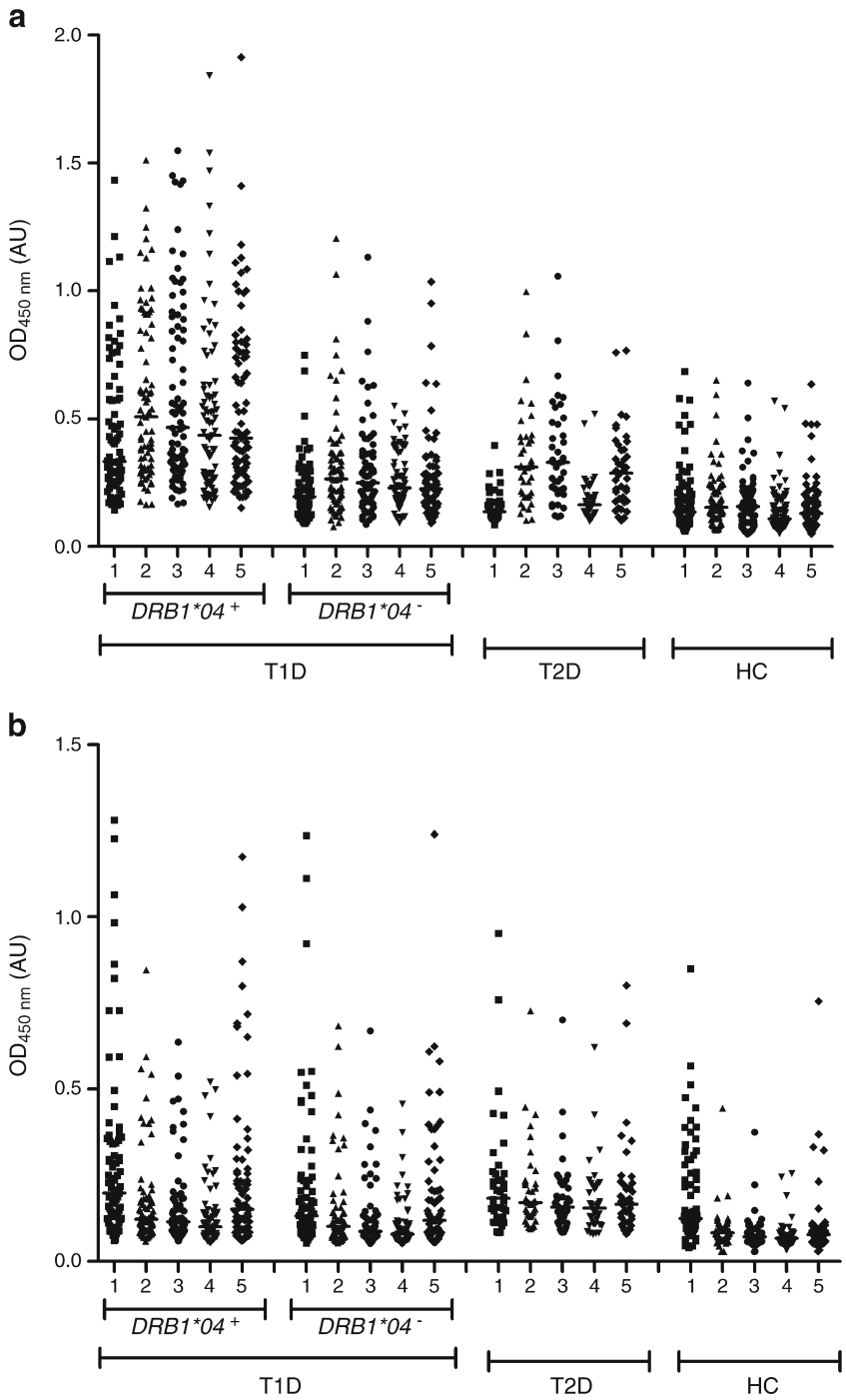

Fig. 1 Binding to CII (**a**) and BSA (**b**) by serum samples from patients with type 1 or type 2 diabetes and healthy controls. Binding was assessed by ELISA in individuals according to *HLA-DRB1*04* genotype. (**a**) Binding to native and ROS-CII was significantly higher in type 1 diabetes *DRB1*04*$^+$ compared with the other groups: type 1 diabetes *DRB1*04*$^-$, type 2 diabetes and healthy controls ($p<0.001$). A significant increase in the binding to ROS-CII was detected in type 1 and type 2 diabetes serum when compared with binding to native CII ($p<0.001$), except for CII modified by hydroxyl radical (·OH). Lane 1, native CII; lane 2, glycated CII; lane 3, HOCl-modified CII; lane 4, ·OH-modified CII; lane 5, peroxynitrite-modified CII. (**b**) In contrast, a significant decrease in binding to ROS-BSA was found in all individuals with type 1 diabetes ($p<0.001$). Lane 1, native BSA; lane 2, glycated BSA; lane 3, HOCl-modified BSA; lane 4, ·OH-modified BSA; lane 5, peroxynitrite-modified BSA. AU, arbitrary units; HC, healthy controls; T1D, type 1 diabetes; T2D, type 2 diabetes

To define the reciprocal effect of SE and *DRB1*0301* on CII autoimmunity, type 1 diabetic individuals were divided into four groups according to the presence/absence of SE and *DRB1*0301* on both alleles at the *DRB1* locus (SE$^+$/*DRB1*0301*$^+$; SE$^+$/*DRB1*0301*$^-$; SE$^-$/*DRB1*0301*$^+$; and SE$^-$/*DRB1*0301*$^-$). The strongest association was between *HLA-DRB1* SE and ROS-CII in the absence of *DRB1*0301*. This association, however, disappeared when SE and *DRB1*0301* coexisted in the same individual (Fig. 3). In addition, *DQB1* analysis, conducted on 98 individuals (data not shown), showed a positive association between *DQB1*0302* and ROS-CII reactivity (OR 3.73 [95% CI 1.28, 10.91]), as expected from the high degree of linkage disequilibrium with the *DRB1*04* alleles (*DQB1*0302* was carried by 100% of our *DRB1*04*-positive patients). On the other hand, native and ROS-BSA were not associated with any specific HLA super type or allele.

Absence of relationship between metabolic control and antibody binding to ROS-CII and ROS-BSA Binding to native

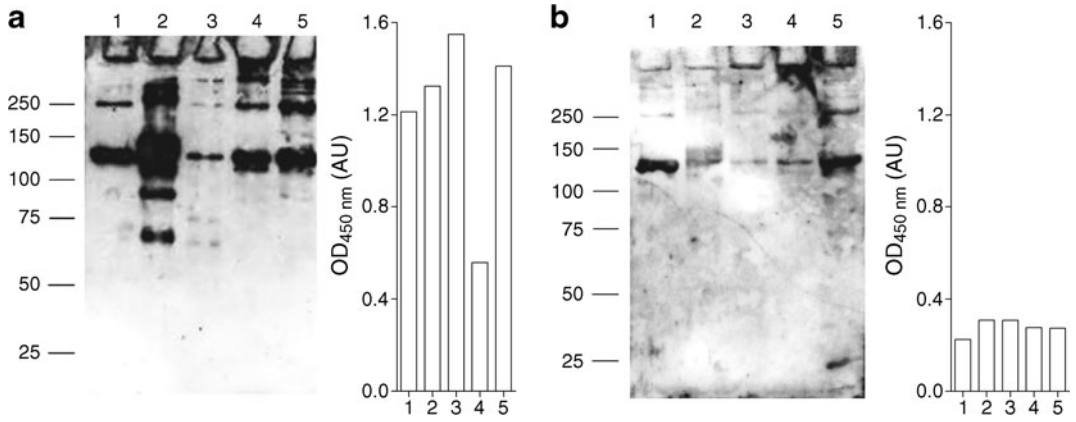

Fig. 2 Binding of type 1 diabetes serum samples to native CII and ROS-CII as determined by western blotting. Representative binding pattern to native CII and ROS-CII using two serum samples from type 1 diabetes-*DRB1*04*-positive (**a**) and *DRB1*04*-negative (**b**) individuals, respectively. (**a**) Type 1 diabetes-*DRB1*04*-positive sample showed binding to native CII and to a range of fragmented CII and high-molecular-weight aggregates following treatment with any of the systems for generating ROS. (**b**) Binding to native and ROS-CII was very low in the type 1 diabetes *DRB1*04*-negative sample. The position of molecular-weight markers (in kDa) is shown on the left. Lane 1, native CII; lane 2, glycated CII; lane 3, HOCl-modified CII; lane 4, ·OH-modified CII; lane 5, peroxynitrite-modified CII. AU, arbitrary units

CII and ROS-CII, as well as native BSA and ROS-BSA, were not correlated with indices of metabolic control, such as fasting plasma glucose and HbA_{1c} ($-0.22851 < \rho < 0.236$, Fig. 4), nor to fasting C-peptide or insulin requirement (not shown). Although not significantly related to any other clinically relevant variables, autoimmunity to ROS-CII tended to be more prevalent among females ($p=0.079$).

Longitudinal evaluation of antibody binding to ROS-CII and ROS-BSA in type 1 diabetes Change in binding to native and ROS-CII was evaluated in 34 recent-onset type 1 diabetes individuals (21 *DRB1*04* positive and 13 *DRB1*04* negative) over a time frame of 9 months. Positive and negative seroconversion rate to ROS-CII, but not to native CII, was high in *DRB1*04*-positive type 1 diabetic individuals, with significant changes in ROS-CII antibodies in 8/21 (38%) individuals. In the *DRB1*04*-positive group, 24% of individuals who were negative to ROS-CII at the first evaluation became strong binders after 9–12 months' follow-up, while 14% of those who were positive to ROS-CII became low binders; 19% of individuals remained positive to ROS-CII antibodies during the time frame and 43% remained negative. No relevant changes in ROS-CII reactivity were detected in the *DRB1*04*-negative group (Fig. 5). Change in binding was not related to any variable of metabolic control, such as blood glucose, HbA_{1c},

Table 2 *HLA-DRB1* genotypes and phenotypes associated with anti-CII reactivity

Genotype/phenotype	Native CII antibodies			ROS-CII antibodies		
	Positive *n*	OR (95% CI)	*p* value	Positive *n*	OR (95% CI)	*p* value
HLA-DRB1 genotypes, T1D, *n*=151						
*DRB1*04/DRB1*03* or *DRB1**X (*n*=81)	21	11.9 (2.68, 52.88)	0.0001	51	10.2 (4.55, 22.86)	<0.0001
*DRB1*03*/not *DRB1*04* (*n*=41)	0	0.04 (0.003, 0.75)	0.0006	5	0.1 (0.05, 0.37)	0.0001
DRX/DRX (*n*=29)	2	0.34 (0.075, 1.553)	0.25	5	0.35 (0.01, 0.98)	0.048
HLA-DRB1 phenotypes, T1D, *n*=57						
*DRB1*0301* (*n*=27)	2	0.22 (0.042, 1.15)	0.056	5	0.23 (0.07, 0.75)	0.013
SE (*n*=18)	4	1.57 (0.38, 6.44)	0.528	10	3.62 (1.12, 11.74)	0.027
*DRB1*X* (*n*=48)	10	5.18 (0.28, 96.5)	0.132	18	2.1 (0.39, 11.23)	0.378

The associated risk between autoreactivity to CII modified by reactive oxidants (ROS-CII) and *HLA-DRB1* genotypes and phenotypes was analysed

DRX represents all except *DRB1*04* and *DRB1*03*

SE refers to at least one *HLA-DRB1*0401*, **0404* or **0405*

*DRB1*X* equates to *HLA-DRB1* phenotypes other than those indicated in the table

T1D, type 1 diabetes; T2D, type 2 diabetes

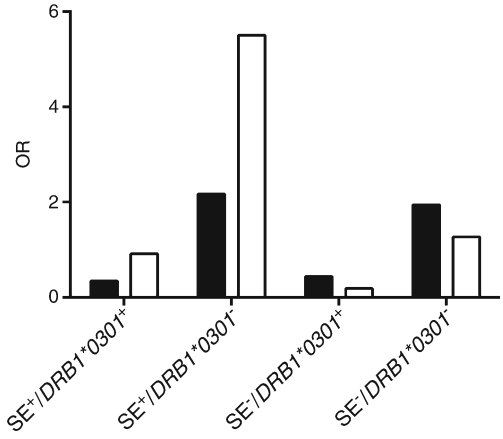

Fig. 3 Association between *HLA-DRB1* SE and *DRB1*0301* alleles with autoimmunity to native CII and ROS-CII in type 1 diabetic individuals. The association between *HLA-DRB1* SE and native CII autoimmunity was modest but became high when CII was chemically modified by reactive oxidants and in the absence of *DRB1*0301* (p= 0.01; OR 5.5 [95% CI 1.39, 21.64]). *HLA-DRB1*0301* contrasted the effect of SE (OR 0.92 [95% CI 0.15, 5.50]) for patients carrying *DRB1*0301* and SE, as well as protective against ROS-CII autoimmunity in the absence of SE (p=0.02; OR 0.18 [95% CI 0.05, 0.75]). SE refers to at least one *DRB1*0401*, **0404* or **0405* allele. Black bars, native CII; white bars, ROS-CII

C-peptide or insulin requirement. No significant changes in native or ROS-BSA reactivity were detected in the *DRB1*04*-positive and *DRB1*04*-negative groups, although both native BSA and ROS-BSA tended to be lower after 9 months in the *DRB1*04*-negative group (data not shown, p=0.08 for both).

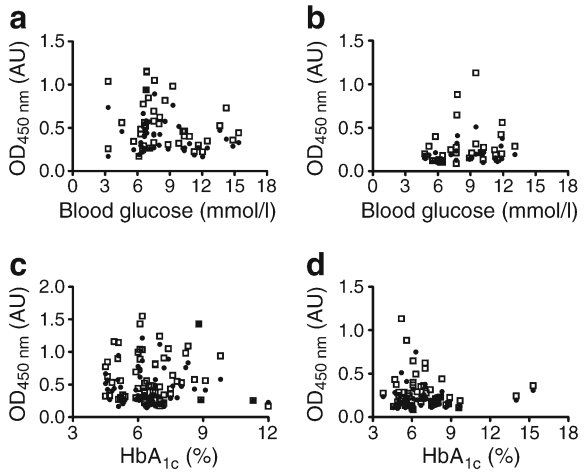

Fig. 4 Absence of relationship between metabolic control and antibody binding to ROS-CII in type 1 diabetes *DRB1*04*-positive (**a**, **c**) and *DRB1*04*-negative (**b**, **d**) individuals. Binding of samples from type 1 diabetic individuals to native CII and ROS-CII was not related to indices of metabolic control, such as fasting plasma glucose and HbA_{1c} ($-0.228<\rho<0.236$). White squares, ROS-CII; black circles, native CII; black square, overlapping of ROS-CII and native CII. AU, arbitrary units

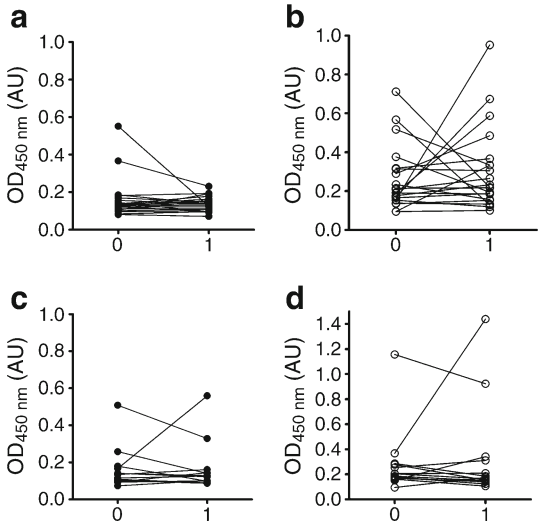

Fig. 5 Longitudinal evaluation of antibody binding to native- (**a**, **c**) and ROS-CII (**b**, **d**) in type 1 diabetes. Change in binding to native CII and ROS-CII was evaluated in 34 individuals with recent onset of type 1 diabetes (21 *DRB1*04* positive [**a**, **b**] and 13 *DRB1*04* negative [**c**, **d**]) within 6 months from the diagnosis (time 0) and 9–12 months after the first evaluation (time 1). In contrast to native CII (**a**), positive and negative seroconversion rates to ROS-CII (**b**) were high in *DRB1*04*-positive type 1 diabetic individuals. Of *DRB1*04*-positive individuals who were negative to ROS-CII at the first evaluation, 5/21 (24%) became strong binders after 9–12 months' follow-up, while 3/21 (14%) of those who were positive to ROS-CII became low binders. No relevant changes in native CII (**c**) and ROS-CII (**d**) reactivity were detected in the *DRB1*04*-negative group. AU, arbitrary units

Discussion

In the present study we reported on the combined effect of oxidative stress and HLA genotype in generating autoimmunity to ROS-CII in type 1 diabetes. We demonstrated for the first time that the exposure to reactive oxidants in diabetes can elicit autoimmune reactivity against ROS-CII specifically in type 1 diabetic patients, irrespective of clinical or biochemical features. In contrast to the specific association of ROS-CII autoreactivity with type 1 diabetes, antibodies to BSA were detected in both type 1 and type 2 diabetes regardless of the genotype, and hence were significantly reduced by ROS modification.

We found that autoimmunity to ROS-CII was positively associated with *DRB1*04*, while *DRB1*03* was protective against native CII and ROS-CII autoimmunity in the absence of *DRB1*04*. When autoimmune responses to CII and ROS-CII were analysed according to *HLA-DRB1* phenotype, we found that ROS-CII but not native CII autoimmunity was restricted to SE-containing *DRB1*04* alleles, known to confer the greatest risk for developing RA (OR 3–13) [20] and commonly associated with type 1 diabetes [1]. Besides the positive effect of the SE, we found that *HLA-DRB1*03* was negatively associated with the presence of reactivity against modified CII. The effect of each allele

was also nullified when the SE and *DRB1*0301* co-existed within the same individual.

Our study may imply that autoimmune reactivity in type 1 diabetes is ubiquitous and not necessarily directed to the diseased tissue, the pancreas. Autoantibodies to CII and ROS-CII, a specific extracellular matrix protein of the articular cartilage, were common in type 1 diabetes, even in the absence of RA. This phenomenon has been previously demonstrated by the presence of anti-thyroid autoantibodies, such as thyroid peroxidase (TPO) antibodies, that develop in over 20% of individuals with type 1 diabetes, even in absence of clinical thyroid disease [22–24]. TPO is an enzyme specifically produced in the thyroid and is a major target of autoimmune thyroid diseases. The same has been shown in RA: for example anti-citrullinated peptide antibodies (ACPA) directed against peptides modified by a post-translational modification of arginine residues to citrulline. ACPA is a known serological test for RA diagnosis, and is detected several years before the onset of RA [25]. It was demonstrated that this group of antibodies respond to filaggrin, an epithelial cell protein not present in the synovium, which plays a key role in joint inflammation during RA pathogenesis [26]. Therefore, the presence of anti-ROS-CII antibodies might be part of the autoimmune response in type 1 diabetes that does not necessarily imply that those individuals will develop RA. Nevertheless, the similar genetic constrain of the anti-ROS-CII of both diseases may lead to the hypothesis that the linkage is due to the anti-ROS-CII autoimmunity. This, however, needs to be established by further longitudinal studies.

Our results may suggest that the *DRB1*-dependent autoimmune reactivity is associated with post-translationally modified CII neo-epitopes rather than with native non-modified CII epitope. This was previously demonstrated in RA using CII epitope 263–270 that needed to be glycosylated to breach the T cell tolerance in *DRB1*0401* transgenic mice and RA patients carrying the *DRB1* SE alleles [27]. Similarly, *DRB1*04* alleles bind to insulin (A1–13 peptide) in individuals with type 1 diabetes, but they are able to trigger pathogenic T cells only when the presented peptide has been post-translationally modified [28]. Brorsson et al showed in type 1 diabetes that *DRB1*0401* is functionally related (co-expressed, interacting or structurally similar) to genes and proteins involved in antigen processing and proteasome activity [29] which are potentially involved in the regulation of post-translational modifications of proteins [30]. The negative association of *DRB1*0301* with the presence of reactivity against modified CII is reminiscent of recent findings by two large studies on RA patients, showing a contrasting effect of SE and *HLA-DRB1*03* [31, 32]. In an RA population, *HLA-DRB1*03* has been significantly associated with reduced titres of ACPA. These results highlight the complexity of the HLA system in controlling antibody production and immune response in general.

We did not find any correlation between antibodies to native CII or ROS-CII and levels of blood glucose or HbA_{1c}, nor was change in ROS-CII level associated with change in metabolic or clinical features. This is not completely unexpected and other studies assessing different ROS-modified proteins have yielded variable results. For example, Vay et al found that antibodies against glycated human albumin were not associated with metabolic control or diabetes duration [33]. On the other hand, levels of oxidised LDL (ox-LDL) antibodies have been correlated with HbA_{1c} levels and the presence of chronic complications [34]. The absence of a relationship between hyperglycaemia and ROS-autoimmunity in our study may be due to the fact that once the redox state has been unbalanced the oxidative reactions can continue for longer after the initial stimulus. In addition, it is possible that the relationship between antigen oxidation and autoantibody production is not continuous, rather it is an 'all-or-none' process. Accordingly, once hyperglycaemia and oxidative stress have triggered or amplified the immune response against ROS-CII, the presence of the native molecule might then be sufficient to sustain the immune response through a mechanism of epitope spreading [35]. Second, the majority of individuals with type 1 diabetes included in this study had relatively short disease duration presented with blood glucose control close to normal and a small inter-individual variation at the time of the analysis; therefore, it is possible that our sample size was too small for the detection of a significant association between dysglycaemia and antibody to ROS-CII. Finally, although hyperglycaemia leads to increased oxidative stress, neither blood glucose nor HbA_{1c} are reliable indices of oxidative stress.

The longitudinal assessment of ROS-CII antibody binding also did not show any relationship between clinical or biochemical features and ROS-CII autoimmunity, despite the presence of relevant changes in antibody binding during the time frame of 9 months soon after the diagnosis of type 1 diabetes. Changes of ROS-CII binding were variable, with high rates of positive and inverse seroconversion. This is generally different from what has been described for diabetes-related autoantibodies, which are already detectable before the diagnosis and rarely appear afterwards [36, 37]. Conversely, autoantibodies directed to other organs, such as anti-endomysium antibodies, which are not specific to diabetes, can appear also after the diagnosis [38]. This suggests the possibility that ROS-CII autoantibodies belong to the ubiquitous autoimmune response in type 1 diabetes, exacerbated after the onset, and possibly are not primarily involved in the pathogenesis of diabetes. The presence of such antibodies in the long term may result in RA in the presence of susceptible genes. This, however, needs to be studied longitudinally in a large diabetes cohort.

Larger longitudinal follow-up studies of people with impaired fasting glucose in which anti-ROS-CII autoreactivity will be measured together with markers of oxidative stress,

for example measuring levels of carboxymethyl-lysine and pentosidine in parallel, may better explain the link between oxidative stress and antibodies to ROS-modified autoantigen in type 1 diabetes. A similar approach was recently used to study the importance of carboxymethyl-lysine as an environmentally determined diabetes risk factor. Islet cell antibodies were measured in parallel to levels of carboxymethyl-lysine in a classic twin study that demonstrated that carboxymethyl-lysine is an environmentally determined diabetes risk factor, in addition to HLA genetic risk [39].

In conclusion, our finding supports the hypothesis that type 1 diabetes-related hyperglycaemia and oxidative stress might trigger neo-antigenicity to extrapancreatic self-antigens, as shown for CII in this study. This process is under the genetic control of the HLA system, highlighting the complex interplay between genetic susceptibility and non-genetic factors in the genesis of autoimmunity. However, larger genotyped cohorts and longer follow-up studies are required to establish this current proof of principle study for further understanding the role of oxidative stress in the pathogenesis of type 1 diabetes mellitus and the possible relevance of ROS-CII autoantibodies as a biomarker in type 1 diabetes mellitus and its association with the development of RA.

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Contribution statement RS was responsible for the conception and design of the study, acquisition of data, analysis and interpretation of data, writing the manuscript and revising it critically for important intellectual content. PR, MS, CH, FP, NN, AP and RB made substantial contributions to the acquisition of data and revised the manuscript critically for important intellectual content. RL made a substantial contribution to the analysis of the data and critical revision of the manuscript for important intellectual content. PP made a substantial contribution to the conception and design of the study and critical revision of the manuscript for important intellectual content. AN made substantial contributions to the conception and design of the study, analysis and interpretation of data, writing of the manuscript and revising the manuscript critically for important intellectual content. All authors approved the final version.
RS and AN are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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