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Reduced GLP-1 response to a meal is associated with the *CTLA4* rs3087243 *G/G* genotype

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Abstract

Although insulitis is the characteristic main feature of type 1 diabetes mellitus (T1DM), many aspects of β cell loss still remain elusive. Immune dysregulation and alterations in the dipeptidyl-peptidase-4-incretin system might have a role in disease development, but their connection is poorly understood. We assessed the associations of a few selected, immunologically relevant single nucleotide gene variants with the DPP-4-incretin system in individuals with T1DM and in healthy controls. Prandial plasma (total, active) GLP-1 levels, serum DPP-4 activity, CD25 and CTLA-4 expression of T cells and DPP4 rs6741949, CTLA4 rs3087243, CD25 rs61839660 and PTPN2 rs2476601 SNPs were assessed in 33 T1DM patients and 34 age-, gender-, BMI-matched non-diabetic controls without a family history of T1DM. CTLA-4 expression was lower in the Foxp3$^+$CD25$^+$ regulatory T cells from individuals homozygous for the CTLA4 rs3087243-G variant compared to those who carry an A allele. Prandial plasma total GLP-1 levels 45 min after a standardized meal were reduced in individuals homozygous for the CTLA4 rs3087243 G major allele compared to A allele carriers both in the entire study population (with statistical power over 90%) and within the T1DM group. Here we report for the first time a reduced total prandial GLP-1 plasma concentration in individuals with the CTLA4 rs3087243 G/G genotype. One may speculate that immune response-related L cell damage might possibly explain this novel association.

Key words: *type 1 diabetes, GLP-1, autoimmune, CTLA4, incretin response, genetic, association study, SNPs, genetic susceptibility.*

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Introduction

Although autoimmune insulitis as the pathophysiological basis of type 1 diabetes mellitus (T1DM) has been known for decades [1, 2], many aspects of the disease development still remain elusive. The degree of insulitis in different islets is not completely simultaneous and affected and unaffected islets may even coexist [3]. Recent results also suggest that insulitis is characterized by a highly variable grade of destruction and also the inflammatory lesion is open in terms of cell influx and leukocyte turnover [4]. Insulitis might coexist with still functioning β cells. As a clinical evidence of this observation, the Joslin Medalist Study indicates that endogenous insulin production may be present in a significant proportion of individuals with long-standing T1DM [5]. However, the majority of factors including non-HLA gene polymorphisms that could determine which subset of type 1 diabetic individuals may present with endogenous insulin production even after 50 years are still largely unknown.

Autoimmune diseases commonly coexist in clinical practice, possibly as a consequence of the fact that they often share common genetic risk alleles. Both autoreactive T cells and autoantibodies were detectable from the peripheral blood of healthy individuals when assessed with highly sensitive methods [6, 7], establishing the role for disturbed immunoregulation in autoimmune disease development. The susceptibility to a progressive clinical course of autoantibody positive pediatric individuals that will decide who may eventually progress to clinical T1DM in three years or who may remain diabetes-free for up to ten years may likely be determined by non-HLA genotypes including polymorphisms of genes

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Table 1. Gene variants selected for assessment in our study according to their immunologic relevance

Reported gene	Region	SNP	Mapped gene	Context	Pathophysiological relevance of gene product in T1DM	Odds ratio for T1DM	Other pathologic conditions related to reported gene	Physiologic parameters associated with known SNPs of the gene	Ref.
IL2RA	10p15.1	rs61839660	IL2RA	Intron	α-chain of the IL2 receptor, marker of active Treg cells, activation marker of CD4 T cells	1.6	Alopecia areata, multiple sclerosis, vitiligo, rheumatoid arthritis	circulating soluble CD25 levels, CD25 expression on naive and memory T cells	[30-32]
CTLA4	2q33.2	rs3087243	CTLA4	Near gene	Analogue of the T cell costimulatory molecule CD28, inhibits T cell responses via downregulation of CD80/86 complexes on antigen presenting cells	NR (1.17-calculated)	Celiac disease, alopecia areata, rheumatoid arthritis, Graves' disease, hypothyroidism, autoantibodies in T1DM	CTLA-4 expression in T cells, Treg frequency in peripheral blood	[25, 30, 33, 34]
PTPN2	18p11.21	rs2542151	PSMG2-PTPN2	Intergenic	Protein tyrosine phosphatase, reported to inhibit β cell apoptosis, inhibits STAT5 signaling that is crucial for Treg induction	NR	Crohn's disease, rheumatoid arthritis, celiac disease, esophageal cancer	IL2RA signaling (according to STAT5 phosphorylation), FOXP3 expression in T cells	[30, 35]
DPP4	2q24.2	rs6741949	DPP4	Intron	Although not identified as a candidate gene, the product cleaves GLP-1 and numerous cytokines involved in insulitis		Rheumatoid arthritis, schizophrenia, hippocampal volume, educational attainment	sensitivity to pharmacologic inhibition, plasma DPP-4 levels in patients with coronary disease	[30, 36]

implicated in interleukin (IL)-2 signal transduction [8]. Polymorphisms of the *CTLA4* (cytotoxic T-lymphocyte-associated protein 4) gene were found to be associated with a higher risk for T1DM [9], celiac disease [10] and other autoimmune disorders including rheumatoid arthritis and Graves' disease [11, 12] in genome-wide association studies (GWA). This extensive association with autoimmune diseases may likely be explained by the *CTLA4* gene product function on regulatory T cells (Treg) that is characterized by the downregulation of B7 costimulatory complexes (CD80, 86) on the surface of antigenpresenting cells with the subsequent inhibition of effector T cell responses [13].

In recent years the protective effect of incretin hormones on β cells was raised based on observations that described alterations of the incretin system (GLP-1-DPP-4) in patients with T1DM and also on pilot studies in T1DM using therapeutic approaches acting on the incretin axis [14-18]. Due to the fact that these investigations, i.e. the genetic association studies and the research on the potential role of the incretin effect in T1DM, were running on different strands we applied a novel approach and assessed a few selected gene polymorphisms (*DPP4, CTLA4, CD25, PTPN2*; Tables 1 and 2) and in parallel we measured the protein expression of a few important molecules in immune regulation and also assessed the prandial (peak) incretin response (active, inactive and total GLP-1 plasma concentrations) and the fasting serum DPP-4 enzymatic activity in healthy volunteers and patients with T1DM.

Material and methods

Patients and study setup

The study protocol was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics and all participating individuals gave written informed consent. Thirty-three patients with T1DM (F/M = 17/16, mean age: 33.4 years, mean disease duration of 14.3 years) treated at our outpatient clinics in Budapest and 34 age- and gender-matched non-diabetic control subjects (F/M = 18/16, mean age: 32.6 years) without a family history of T1DM were enrolled on a voluntary basis between November, 2013 and March, 2014. All participants were Caucasian. T1DM patients were diagnosed according to international standard criteria [19]. Patients on systemic immunomodulatory medication, or with off-label use of drugs acting on the incretin axis (i.e. DPP-4-inhibitors), were excluded from the study group. Control subjects were free of known autoimmune diseases, endocrine disorders and any other significant chronic or acute diseases. Comorbidities and the most important metabolic characteristics are shown in Tables 3 and 4. T1DM subjects with extreme disturbances of carbohydrate metabolism (HbA_{1C} > 12% and/or fasting plasma glucose > 20 mmol/l) were also excluded.

Table 2. Genotype distributions for selected SNPs in study participants

Gene	SNP identified	Distribution of individuals according to genotypes (% within study group)						Minor allele frequency			HWE fulfilled
CTLA4	rs3087243	*AA*		*AG*		*GG*		Alleles: *G/A*			Both study groups
		T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	1000 Genomes (EUR)	
		9.1	14.7	45.5	44.1	45.5	41.2	0.32 (*A*)	0.36 (*A*)	0.47 (*A*)	
DPP4	rs6741949	*CC*		*CG*		*GG*		Alleles: *C/G*			Both study groups
		T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	1000 Genomes (EUR)	
		9.1	20.6	42.4	58.8	48.5	20.6	0.30 (*C*)	0.50 (*C*)	0.41 (*C*)	
PTPN2	rs2542151	*GG*		*GT*		*TT*		Alleles: *G/T*			both study groups
		T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	1000 Genomes (EUR)	
		6.1	2.9	30.3	17.6	63.6	79.4	0.21 (*G*)	0.12 (*G*)	0.14 (*G*)	
CD25	rs61839660	*TT*		*TC*		*CC*		Alleles: *T/C*			T1DM study group
		T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	T1DM	CNTRL	1000 Genomes (EUR)	
		0	2.9	12.1	11.8	87.9	85.3	0.11 (*T*)	0.15 (*T*)	0.07 (*T*)	

Table 3. Clinical characteristics in patients with type 1 diabetes mellitus and in control individuals

Characteristics	T1DM (*n* = 33)	CNTRL (*n* = 34)
Gender (F/M)	17/16	18/16
Age (years)	33.35 (95% CI: 27-34.71)	32.6 (95% CI: 29.89-35.92)
BMI (kg/m^2)	23.4 (95% CI: 21.94-24.86)	23.82 (95% CI: 22.53-25.12)
Fasting plasma glucose (mmol/l)	8.65 (95% CI: 7.39-9.9)*	4.93 (95% CI: 4.81-5.07)
C-peptide positivity	8/33 (24.2%)	Not measured
Age at diagnosis (years)	18.67 (95% CI: 15.7-21.57)	Not applicable
Disease duration (years)	14.34 (95% CI: 11.53-17.15)	Not applicable
HbA_{1C} (%) (mmol/mol)	7.25 (95% CI: 6.79-7.7) 55.8 (95% CI: 50.86-60.73)	Not measured

* *type 1 diabetes mellitus*

Assessment of serum DPP-4 enzymatic activity and plasma GLP-1 levels

Fasting serum DPP-4 activity was determined in a continuous monitoring assay in a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA, USA) at 405 nm, 37°C for 30 min, using 9.4 µl of serum and 115.6 µl of assay buffer (10 mM Tris-HCl, pH 7.6) containing 2 mmol/l Gly-Pro-paranitroanilide tosylate substrate (Bachem, Bubendorf, Switzerland) in each microplate well. Enzyme activity is expressed in nmol/ml/min (U/l) of pNA hydrolyzed. Active (GLP-1$^{7-36}$amide) and total GLP-1 (GLP-1$^{7-36}$amide and GLP-1$^{9-36}$amide) were assessed from prandial plasma samples taken 45 minutes after having a standardized test meal in the morning containing 50 grams of carbohydrate, 22 grams of protein and 9 grams of fat. Patients with T1DM received their regular doses of short/rapid acting insulin calculated for the morning test meal. Measurements were made using specific ELISA kits according to the manufacturer's recommendations (EMD Millipore, Billerica, MA, USA). Plasma samples for all GLP-1 assessments were treated with DPP-4 inhibitor (sitagliptin) and a protease inhibitor cocktail (P8340, Sigma Aldrich, Saint Luis, MO, USA). All measurements were run in duplicate. Plasma GLP-1 levels were expressed in pmol/l.

Flow cytometric analysis

Fasting, EDTA-anticoagulated blood samples were collected. The flow cytometric analysis was performed as described in detail in our prior publication [20] using the following flow cytometric antibodies: CD3-PE-Cy7, CD8-PerCP, CD25-AF700 (Biolegend, San Diego, CA, USA), Foxp3-PE-CF594 and CTLA-4-APC (BD Biosciences, San Jose, CA, USA). A Beckman Coulter Navios flow cytometer and version 1.2 of Beckman Coulter Kaluza software (Brea, CA, USA) were used for quantitative analysis. CTLA-4 expression levels were assessed intracellularly; median fluorescence intensity values (MFI) are indicated. Our gating strategy is described in Figure 1.

Table 4. Comorbidities, target organ damage and drug use in patients with type 1 diabetes mellitus

Autoimmune comorbidities	
Hashimoto-thyreoiditis	6/33 (18.2%)
Autoimmune gastritis	2/33 (6.1%)
Celiac disease	1/33 (3%)
Vitiligo	1/33 (3%)
Crohn's disease	1/33 (3%)
Target organ damage	
Diabetic retinopathy	8/33 (24.2%)
Diabetic neuropathy	5/33 (15.2%)
Diabetic nephropathy	3/33 (9.9%)
Major cardiovascular event	0/39
Medication used	
Analogue insulin users	24/33 (72.73%)
ACEI/ARB treatment	7/33 (21.2%)
Statin treatment	2/33 (6.1%)
Aspirin treatment	0/38

Assessment of genotypes

Genomic DNA was isolated by a magnetic bead (Mag Maxi Kit, LGC Genomic Solutions, Berlin, Germany) based method using a Hamilton MagNA STAR automated robotized system (Hamilton Robotics, Bonaduz, Switzerland). Subsequently 500 ng of genomic DNA (in 5 µl volume, 100 ng/µl) was used in predesigned (TaqMan) genotyping assays (Thermo Fisher Scientific, Waltham, MA USA) with pre-mixed master-mix (FastStart Essential DNA Probes Master, Roche, Basel, Switzerland) supplemented with ROX with cycle conditions suggested by the manufacturer in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Genotype distributions were assessed using the Hardy-Weinberg equilibrium (HWE) test. The Kolmogorov-Smirnov test was used to assess normality for the continuous parameters measured, including 45 min prandial plasma GLP-1 concentrations (nmol/l) and fasting serum DPP-4 (U/l) enzymatic activity. Statistica software (version 12, StatSoft, Tulsa, OK, USA) was used. In situations of normal distributions an unpaired two-tailed *t*-test (Welch *t*-test for comparison of groups with significantly different variances), and in situations of non-normal distribution the Mann-Whitney *U*-test (MWU) was used to assess the difference in central tendency between study groups. The Wilcoxon test was used to compare dependent variables. One-way ANOVA with Scheffé post-hoc test was used to compare means of independent variables. Spearman rank order (SRO) and Pearson tests were used to assess correlations. The χ^2 test (CST) was used to assess gender proportion and allele distribution similarity.

We performed power calculation using the mean values and the sample size numbers and the population sigma values. A sample size calculation was also performed under the dominant genetic model (*CTLA4* rs3087243-*A* allele carriers vs. non-carriers) using the measured effect sizes in GLP-1 response to meals (45 min total plasma GLP-1 concentrations) as there were no reported clinical studies prior to our observation that could have had been used for effect size estimations and subsequent sample size calculations.

Results

Association of the rs3087243-*G/A* polymorphism SNP with CTLA-4 expression in Foxp3$^+$CD25$^+$ Treg cells

The distributions of *G/A* alleles of the *CTLA4* rs3087243 SNP in our study population and the European population are shown in Table 2. CTLA-4 expression levels were higher in the Foxp3$^+$CD25$^+$Treg cells isolated from individuals who carry an *A* allele of the rs3087243 SNP of the *CTLA4* gene compared to individuals homozygous for the *G* allele (MFI; rs3087243-*A* allele carriers: 2.91 [95% CI: 2.74-3.08] vs. *G/G* homozygous individuals: 2.52 [95% CI: 2.23-2.82], $p = 0.017$, *t*-test, statistical power = 65.84%, Figs. 1 and 2).

Association between the *CTLA4* genotypes (rs3087243 SNP) and the peak plasma total GLP-1 levels

The 45 min prandial plasma total GLP-1 levels were significantly lower in the peripheral blood of individuals with the *CTLA4* rs3087243-*G/G* genotype both in the entire study population (45 min total plasma GLP-1 cc. in individuals with rs3087243-*G/G* genotype: 12.5 pmol/l [95% CI: 11.52-13.48] vs. *A* allele carriers: 15.62 pmol/l [95% CI: 14.33-16.9], $p = 0.0008$, Welch *t*-test, statistical power = 91.92%; Fig. 3) and the T1DM subjects (in patients with rs3087243-*G/G* genotype: 12.77 pmol/l [95% CI: 11.34-14.2] vs. *A* allele carriers: 15.15 pmol/l [95% CI: 13.28-17.02], $p = 0.0464$, statistical power = 48.16%, *t*-test; Fig. 3). No difference could be detected between the heterozygous and homozygous carriers of the rs3087243-*A* allele in the total study population (individuals with one rs3087243-*A* allele: 15.47 pmol/l [95% CI: 14.08-16.86] vs. homozygous rs3087243-*A* allele carriers: 16.19 [95% CI: 12.19-20.19], ANOVA $p < 0.05$, Scheffé post-hoc test p = n.s.; Fig. 3). The Scheffé post-hoc test

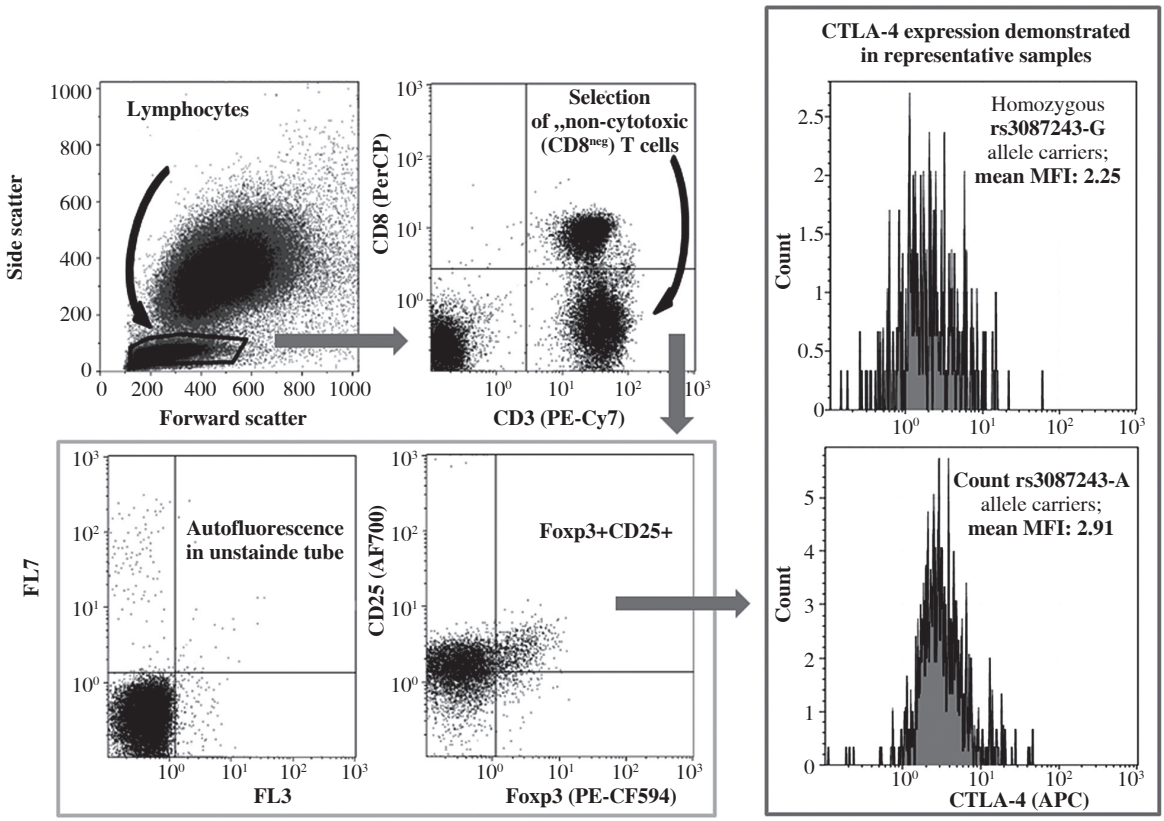

Fig. 1. Lymphocytes were gated according to the forward and side scatters. T lymphocytes were gated according to their CD3 expression and $CD8^-$ non-cytotoxic (Tnc) cells were also gated. Treg cells were defined as $Foxp3^+$ cells within the Tnc lymphocyte population. We gated the $CD25^+$ subpopulation within the Treg cells. Representative samples are shown indicating that the CTLA-4 expression of $Foxp3^+CD25^+$ Treg cells was higher in participants with at least one rs3087243-*A* allele than in homozygous *G* allele carriers

also demonstrated a significant ($p = 0.006$) difference in the postprandial total GLP-1 plasma levels between individuals with *CTLA4* rs3087243-*G/G* genotype and heterozygous *A* allele carriers (Fig. 3). Carrying the *A* allele of rs3087243 was related to a 3.11 pmol/l increase in the prandial total GLP-1 plasma levels ($p = 0.002$) in the entire study population. The calculated 45 min prandial plasma cleaved $GLP\text{-}1^{9\text{-}36}$ levels were also significantly lower in the peripheral blood of individuals with the *CTLA4* rs3087243-*G/G* genotype in the entire study population (in individuals with rs3087243-*G/G* genotype: 7.96 pmol/l [95% CI: 6.97-8.94] vs. *A* allele carriers: 10.57 pmol/l [95% CI: 9.31-11.82], $p = 0.0026$, *t*-test, statistical power = 81.81%).

The sample size calculation using the measured effect sizes in GLP-1 response to meals in *CTLA4* rs3087243-*A* allele carriers vs. non-carriers gave the result that 25-25 carrier and non-carrier individuals were needed in order to reach the level of statistical significance with a statistical power over 80%.

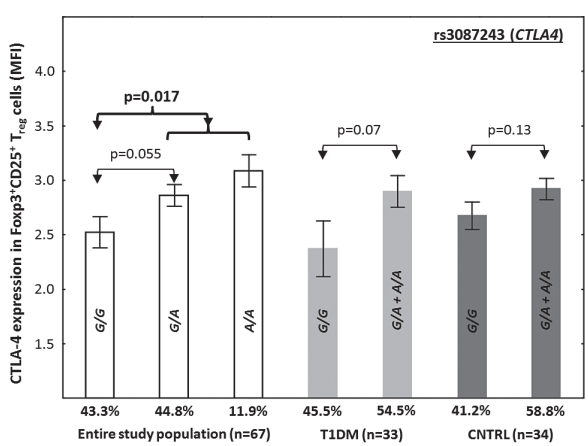

Fig. 2. We found higher CTLA-4 expression (MFI) in the $Foxp3^+CD25^+$ Treg cells of participants carrying an rs3087243-*A* allele than in rs3087243-*G* homozygous individuals in the entire study population. Columns represent means. The 95% confidence intervals are also shown

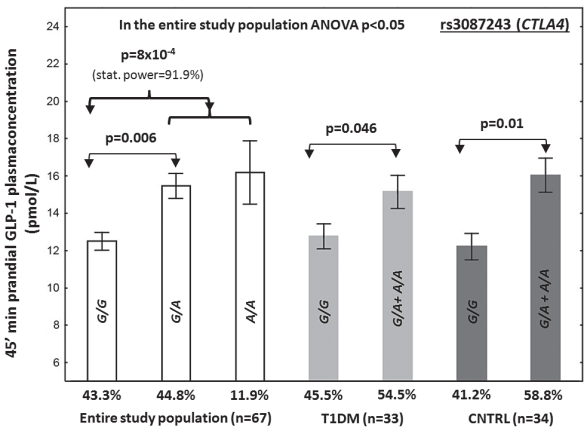

Fig. 3. The postprandial plasma total GLP-1 levels were lower in homozygous rs3087243-*G* carriers than in individuals who carry at least one *A* allele in the entire study population and also in both study groups separately. No difference could be detected between the heterozygous and homozygous carriers of the rs3087243-*A* allele, but the peak plasma total GLP-1 levels were also higher in the heterozygous group than in homozygous *G* allele carriers. Group means and the 95% confidence intervals are indicated

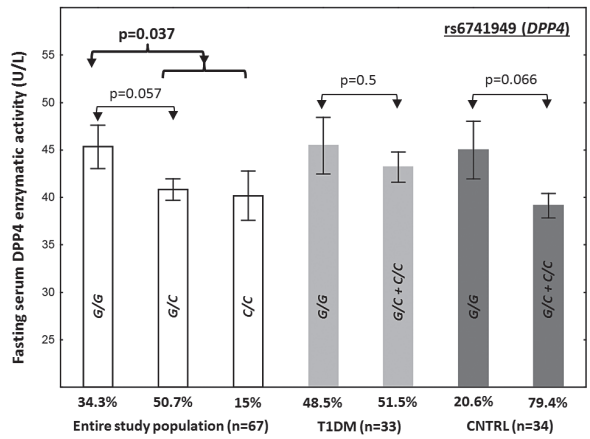

Fig. 4. Fasting serum DPP-4 enzymatic activity was higher in individuals with the rs6741949-*G/G* genotype than in those who carry at least one *C* allele. Group means and the 95% confidence intervals are indicated

We could not detect any further difference in the active GLP-1^{7-36} amide 45 min prandial plasma concentrations or in fasting serum DPP-4 enzymatic activity according to different *CTLA4* rs3087243 genotypes and also other genotypes studied. Adjustment of the plasma GLP-1 levels to BMI in the total study population and also to HbA_{1c} and total daily insulin requirement in the T1DM group did not significantly alter our results.

Associations with other parameters

Earlier disease onset characterized those T1DM individuals who did not carry any *A* allele of the rs3087243 polymorphism compared to *A* allele carriers with borderline significance (individuals with rs3087243-*G/G* genotype: 15.8 years [95% CI: 11.98-19.62] vs. *A*-allele carriers: 21 years [95% CI: 16.65-25.35]; $p = 0.0713$). Plasma total GLP-1 levels did not differ between patients with detectable plasma C-peptide levels and C-peptide negative patients, although only 8 patients had residual detectable β cell function and the mean disease duration of the two groups was different (7.96 years vs. 16.33 years). Prandial plasma total GLP-1 levels tended to decrease with age in T1DM patients ($r = –0.27$) and the r value was more pronounced in subjects with rs3087243 *G/G* genotype ($r = –0.41$), but the p values were non-significant ($p = 0.13$, Pearson test). Fasting serum DPP-4 enzymatic activity was higher in individuals with the rs6741949 *G/G DPP4* genotype than in those who carry at least one *C* allele (45.34 U/l [95% CI: 40.57-50.11] vs. 40.7 [95% CI: 38.6-42.79] respectively, $p = 0.0372$, Welch t-test, statistical power = 54.41%, Fig. 4). Carrying the *C* allele of rs6741949 was related to a 4.64 U/l decrease in serum DPP-4 enzymatic activity in the entire study population with borderline statistical significance ($p = 0.037$).

Discussion and conclusions

Here we report for the first time that the *CTLA4* rs3087243 *G/G* genotype was significantly associated with lower total plasma 45 min GLP-1 concentration in our study participants, independently of the presence of T1DM. Although the rs3087243 *CTLA4* gene variant was reported to be only moderately (OR of 1.2) associated with the risk of developing T1DM [9], our results might suggest that the rs3087243 gene variant may play an important role in qualitative traits related to the reduced incretin response to meals, possibly through previously unexpected immune-mediated pathology in the GI tract.

According to our knowledge, this is the first report of an association between a common gene variant and the total prandial GLP-1 plasma levels. The *CTLA4* rs3087243 variant is located downstream from the *CTLA4* gene within a regulatory region and the *G/G* genotype is relatively common (43.3% in our study population, 39.7% worldwide and 28.1% in the European population according to the 1000 Genomes database [21]).

In contrast, rs4664447 *C/C* (fwd strnd) of the *GCG* gene, which was – to our knowledge – the only previously reported risk genotype to be associated with a reduced GLP-1 response, occurs only in approximately 4 out of 10 000 individuals with European origin (Ensemble genomic database – Human – GRCh38.p13) [22, 23].

The decreased regulatory capacity in complex immune responses might likely be the common pathophysiological hallmark of autoimmune disorders. Autoimmune diseases frequently share common genetic susceptibility loci and polymorphisms as a common background for disease development in different target organs [24]. In addition, *CTLA4* rs3087243 was identified as a shared candidate gene variant for celiac disease and T1DM [25]. We may therefore speculate that our findings might be related to immune-mediated damage of L cells that results in impaired incretin (GLP-1) secretion after a meal and this is indicated by the lower total prandial plasma GLP-1 concentrations in genetically susceptible individuals. The higher Treg CTLA-4 expression we found in *CTLA4* rs3087243-*A* carriers might possibly refer to an altered suppressive capacity of Treg cells, consistently with our theory. The intestinal immune dysregulation and the disruption of mucosal immune tolerance are likely to be involved in the pathogenesis of T1DM [26, 27].

Blaslov *et al.* found the largest reduction in total prandial GLP-1 levels in T1DM patients who concurrently had metabolic syndrome (MS) [28]. In the view of the results presented here, one might also speculate about the potential of an inverse event sequence, namely that the reduction in GLP-1 response in susceptible individuals might have contributed to the development of MS [29]. However, our results were unlikely influenced by MS as both the T1DM and control groups were matched and presented with normal mean BMI and no difference in GLP-1 levels was found after adjustment for BMI.

In summary, we report for the first time the association between *CTLA4* rs3087243 *G/G* genotype and reduced total prandial plasma GLP-1 concentrations independent of T1DM in our cohort. Based on its prevalence in the population the *CTLA4* rs3087243 might be potentially considered as a candidate gene variant for future trials tailoring the incretin response/therapy.

Limitations

These findings about the association between *CTLA4* rs3087243 risk genotype and the total plasma GLP-1 levels after a meal should also be confirmed in replication studies with at least similar sample sizes. Higher sample sizes would likely be required to assess the clinical significance of this association between total prandial GLP-1 levels and *CTLA4* genotypes.

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The authors declare no conflict of interest.

References

1. Opie EL (1901): On the relation of chronic interstitial pancreatitis to the islands of Langerhans and to diabetes mellitus. J Exp Med 5: 397-428.
2. Lecompte PM (1958): Insulitis in early juvenile diabetes. AMA Arch Pathol 66: 450-457.
3. Willcox A, Richardson SJ, Bone AJ, et al. (2009): Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol 155: 173-181.
4. Magnuson AM, Thurber GM, Kohler RH, et al. (2015): Population dynamics of islet-infiltrating cells in autoimmune diabetes. Proc Natl Acad Sci U S A 112: 1511-1516.
5. Keenan HA, Sun JK, Levine J, et al. (2010): Residual Insulin Production and Pancreatic β-Cell Turnover After 50 Years of Diabetes: Joslin Medalist Study. Diabetes 59: 2846-2853.
6. Danke NA, Koelle DM, Yee C, et al. (2004): Autoreactive T cells in healthy individuals. J Immunol 172: 5967-5972.
7. Oak S, Radtke J, Törnt C, et al. (2011): Immunoglobulin Subclass Profiles of Anti-idiotypic Antibodies to GAD65Ab Differ Between Type 1 Diabetes Patients and Healthy Individuals. Scand J Immunol 74: 363-367.
8. Achenbach P, Hummel M, Thümer L, et al. (2013): Characteristics of rapid vs slow progression to type 1 diabetes in multiple islet autoantibody-positive children. Diabetologia 56: 1615-1622.
9. Plagnol V, Howson JMM, Smyth DJ, et al. (2011): Genome-Wide Association Analysis of Autoantibody Positivity in Type 1 Diabetes Cases. PLoS Genet 7: e1002216.
10. Dubois PCA, Trynka G, Franke L, et al. (2010): Multiple common variants for celiac disease influencing immune gene expression. Nat Genet 42: 295-302.
11. Chu X, Pan C-M, Zhao S-X, et al. (2011): A genome-wide association study identifies two new risk loci for Graves' disease. Nat Genet 43: 897-901.
12. Okada Y, Wu D, Trynka G, et al. (2014): Genetics of rheumatoid arthritis contributes to biology and drug discovery. Nature 506: 376-381.
13. Wing K, Onishi Y, Prieto-Martin P, et al. (2008): CTLA-4 Control over Foxp3+ Regulatory T Cell Function. Science 322: 271-275.
14. Kielgast U, Holst JJ, Madsbad S (2011): Antidiabetic actions of endogenous and exogenous GLP-1 in type 1 diabetic patients with and without residual β-cell function. Diabetes 60: 1599-1607.
15. Kielgast U, Krarup T, Holst JJ, Madsbad S (2011): Four weeks of treatment with liraglutide reduces insulin dose without loss of glycemic control in type 1 diabetic patients with and without residual beta-cell function. Diabetes Care 34: 1463-1468.
16. Varga T, Somogyi A, Barna G, et al. (2011): Higher serum DPP-4 enzyme activity and decreased lymphocyte CD26 expression in type 1 diabetes. Pathol Oncol Res 17: 925-930.
17. Kaas A, Andersen ML, Fredheim S, et al. (2012): Proinsulin, GLP-1, and glucagon are associated with partial remission in

children and adolescents with newly diagnosed type 1 diabetes. Pediatr Diabetes 13: 51-58.
18. Hari Kumar KV, Shaikh A, Prusty P (2013): Addition of exenatide or sitagliptin to insulin in new onset type 1 diabetes: a randomized, open label study. Diabetes Res Clin Pract 100: e55-58.
19. American Diabetes Association (2014): Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 37: S81-S90.
20. Zóka A, Barna G, Somogyi A, et al. (2015): Extension of the CD4(+)Foxp3(+)CD25(-/low) regulatory T-cell subpopulation in type 1 diabetes mellitus. Autoimmunity: 48: 289-297.
21. 1000 Genomes. A Deep Catalog of Human Genetic Variation. https://www.internationalgenome.org/
22. Torekov SS, Ma L, Grarup N, et al. (2011): Homozygous carriers of the G allele of rs4664447 of the glucagon gene (GCG) are characterised by decreased fasting and stimulated levels of insulin, glucagon and glucagon-like peptide (GLP)-1. Diabetologia 54: 2820-2831.
23. Institute E-EatWTS (2017): Ensemble genomic database, Release 98 (September, 2019) – Human – GRCh38.p13, available at the website: https://www.ensembl.org/Homo_sapiens/Info/Index?db=core
24. Cotsapas C, Voight BF, Rossin E, et al. (2011): Pervasive Sharing of Genetic Effects in Autoimmune Disease. PLoS Genet 7: e1002254.
25. Smyth DJ, Plagnol V, Walker NM, et al. (2008): Shared and distinct genetic variants in type 1 diabetes and celiac disease. N Engl J Med 359: 2767-2777.
26. Vaarala O, Atkinson MA, Neu J (2008): The "Perfect Storm" for Type 1 Diabetes The Complex Interplay Between Intestinal Microbiota, Gut Permeability, and Mucosal Immunity. Diabetes 57: 2555-2562.
27. Pellegrini S, Sordi V, Bolla AM, et al. (2017): Duodenal Mucosa of Patients With Type 1 Diabetes Shows Distinctive Inflammatory Profile and Microbiota. J Clin Endocrinol Metab 102: 1468-1477.
28. Blaslov K, Bulum T, Zibar K, Duvnjak L (2015): Relationship between metabolic syndrome and meal-induced glucagon like peptide-1 response in type 1 diabetic patients. J Diabetes 7: 340-346.
29. Firneisz G (2014): Non-alcoholic fatty liver disease and type 2 diabetes mellitus: the liver disease of our age? World J Gastroenterol 20: 9072-9089.
30. EMBL-EBI (2017): Catalog of Published Genome-Wide Association Studies. https://wwwebiacuk/gwas/ (accessed: 18.05.2017).
31. Lowe CE, Cooper JD, Brusko T, et al. (2007): Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nat Genet 39: 1074-1082.
32. Dendrou CA, Plagnol V, Fung E, et al. (2009): Cell-specific protein phenotypes for the autoimmune locus IL2RA using a genotype-selectable human bioresource. Nat Genet 41: 1011-1015.
33. Atabani SF, Thio CL, Divanovic S, et al. (2005): Association of CTLA4 polymorphism with regulatory T cell frequency. Eur J Immunol 35: 2157-2162.
34. Karabon L, Kosmaczewska A, Bilinska M, et al. (2009): The CTLA-4 gene polymorphisms are associated with CTLA-4 protein expression levels in multiple sclerosis patients and with susceptibility to disease. Immunology 128: e787-e796.
35. Long SA, Cerosaletti K, Bollyky PL, et al. (2010): Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4(+)CD25(+) regulatory T-cells of type 1 diabetic subjects. Diabetes 59: 407-415.
36. Aghili N, Devaney JM, Alderman LO, et al. (2012): Polymorphisms in dipeptidyl peptidase IV gene are associated with the risk of myocardial infarction in patients with atherosclerosis. Neuropeptides 46: 367-371.