Published in final edited form as:

Vaccine. 2013 August 2; 31(35): 3603–3610. doi:10.1016/j.vaccine.2013.05.003.

Young and elderly patients with type 2 diabetes have optimal B cell responses to the seasonal influenza vaccine

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Abstract

We evaluated immune response to the seasonal influenza vaccine in young and elderly patients with type 2 diabetes (T2D). Immune measures included the in vivo serum response to the vaccine by hemagglutination inhibition (HAI) and ELISA in 22 patients (14 young, 8 elderly) and 65 healthy age-matched controls (37 young, 28 elderly). B cell-specific biomarkers of optimal vaccine response were measured ex vivo by switched memory B cells and plasmablasts and in vitro by activation-induced cytidine deaminase (AID) in stimulated cells. Markers of systemic and B cell-intrinsic inflammation were also measured. Results show that in vivo responses, as well as B cell-specific markers identified above, decrease by age in healthy individuals but not in T2D patients. This occurred despite high levels of B cell-intrinsic inflammation (TNF- α) in T2D patients, which was surprising as we had previously demonstrated this negatively impacts B cell function. These results altogether suggest that valid protection against influenza can be achieved in T2D patients and proposed mechanisms are discussed.

Keywords

Type 2 diabetes; Antibody responses; Inflammation; Influenza vaccine

1. Introduction

Type 2 diabetes (T2D) mellitus is a metabolic, inflammatory disease not associated with autoimmunity, but often characterized by obesity, hypertension, dyslipidemia, accelerated atherosclerosis and increased mortality [1,2]. It is a common disease affecting >15 million Americans and is dramatically increasing in incidence [1,2]. Inflammation in diabetes is thought to initiate in the visceral adipose tissue due to changes in fat metabolism occurring

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Conflict of interest: No potential conflicts of interest relevant to this article were reported.

Contributors: All authors reviewed, edited and approved the manuscript.

in individuals with abnormally large fat deposits consequent in most cases to hypernutrition [3,4]. The local inflammatory response results in higher levels of pro-inflammatory saturated fatty acids, which serve as ligands for the Toll-like receptors TLR2 and TLR4 [5,6]. TLRs activate the signaling pathways that culminate for example in activation of NF- κ B, its translocation into the nucleus and release of pro-inflammatory cytokines [7,8]. These cytokines, secreted by several immune and non-immune cells, are crucial regulators of the pathological inflammation that promotes and characterizes diabetes.

B cells are the first immune cells to infiltrate the expanding adipose tissues in response to hypernutrition, followed by T cells and macrophages [9]. B cells can be activated by products of altered lipolysis in the expanding adipose tissue to release proinflammatory cytokines, such as TNF- α and IL-6, or chemokines, such as IL-8, thus contributing in a significant way to systemic inflammation [2,8].

T2D patients are at risk for infections due to influenza or for complications related to it [10–12]. Therefore annual influenza vaccination is recommended [10]. Viral and bacterial infections and consequent diseases are associated with increased morbidity and mortality in T2D patients, causing loss of metabolic control leading to an increase of glycosylated serum proteins, ketoacidosis which may result in an increased hospitalization rate and mortality rate, and prolonged complications [13,14]. Studies assessing the clinical efficacy of influenza vaccination in T2D patients have provided incomplete and controversial results, which were attributed to the effects of confounding factors (age, duration of disease, comorbidities, treatments, nutritional status and vaccination history [15,16]). A few studies have shown clinical efficacy of influenza vaccination with reduction in health complications, hospitalization and deaths [17–20]. However, less is known on the effects of influenza vaccination on lymphocyte populations of T2D patients. The few studies addressing this issue have measured T cell function in vaccinated young [11] and elderly [21] T2D patients as compared to healthy controls [11,21] and have shown reduced [11] or similar [21] responses in patients versus controls. No studies have been conducted so far to evaluate the contribution of B cells to the influenza vaccine response in T2D patients.

In the present study we performed a detailed evaluation of B cell markers and function in T2D patients, vaccinated with influenza vaccine during the 2011–2012 season and determine the contribution of B cells and B cell-derived cytokines to the serum response in these individuals at risk for infections. It has previously been shown that elderly individuals have high levels of systemic inflammation [22–24] and we have shown that this negatively impacts the function of B cells [25]. We therefore hypothesized that the increased inflammation in T2D patients would also lead to decreased B cell function. Unexpectedly, we found normal in vivo and in vitro B cell responses, despite high levels of inflammation in these patients. We suggest that the innate immune system of T2D patients is beneficially hyperactivated, as we found elevated serum levels of bacterial lipopolysaccharide (LPS) and soluble (s)CD14, and we believe that these may not only counteract the negative effects of inflammation from increased TNF- α , IL-6 and CRP, but also to induce a direct stimulation of B cells. Moreover, T2D patients were all taking anti-inflammatory agents, such as Metformin, which blocks TNF- α signaling in all cells, including B cells.

2. Materials and methods

2.1. Subjects

Experiments were conducted using blood from T2D patients and healthy volunteers of different ages after appropriate signed informed consent and were approved with IRB protocol #20070481. In every experiment, 37 healthy young, 28 healthy elderly, 14 diabetic young and 8 diabetic elderly were evaluated. Young individuals are 20–59 years of age,

whereas elderly individuals are ≥ 60 years of age. All subjects at the time of enrollment were influenza-free, without symptoms associated with respiratory infections and did not contract flu-like symptoms within a 6-month follow-up period. Presence of relevant co-morbidities was recorded and T2D patients did not have cancer, major infectious or autoimmune diseases for at least 12 months prior to enrollment.

2.2. Influenza vaccination

The study was conducted during the 2011–2012 seasonal influenza vaccination. Two trivalent inactivated vaccines (TIV) were used: Novartis Fluvirin and GSK Fluarix. Fluvirin was given to 20 T2D patients whereas Fluarix was given to 2 T2D patients and healthy individuals). Blood samples were collected immediately before (t0), one week (t7) and 4–6 (t28) weeks post-vaccination.

2.3. Hemagglutination inhibition (HAI) assay

We evaluated the immunogenicity of the influenza vaccine by titer after vaccination (t7), as we have previously described [26,27]. The HAI test is useful for the measurement of antibody titers in serum and is the most established correlate with vaccine protectiveness [28,29]. Because the 2011–2012 season was the third consecutive one in which the H1N1 was the pandemic 2009 swine-origin strain, we saw an early serum response to the vaccine which peaked at t7 and only in a few cases (17% of the subjects) was sustained in the following 3 weeks.

2.4. PBMC cultures

PBMC were collected using Vacutainer CPT tubes (BD 362761). Cells were washed and cryopreserved (frozen) with 90% fetal bovine serum and 10% DMSO. Frozen PBMC (10×10^6) were stimulated 3 days with CpG (ODN 2006 InvivoGen) at the concentration of $1 \mu\text{g}/10^6$ cells, as described [27].

2.5. Flow cytometry

One hundred microliters of blood were stained for 20min at room temperature with the following antibodies: anti-CD19 (BD 555415), anti-CD27 (BD 555441), anti-IgD (BD 555778) to measure naïve (IgD+CD27–), IgM memory (IgD+CD27+), switched memory (IgD–CD27+) and late/exhausted memory B cells (IgD–CD27–). The relative percentages of these B cell subsets in freshly stained cells were the same as those previously published using IgG/IgA and CD27 antibodies [26,27,30]. After staining, red blood cells were lysed using the RBC Lysing Solution (BD 555899). Up to 10^5 events in the lymphocyte gate were acquired on an LSR-Fortessa (BD) and analyzed using FACS Diva (BD) software. Single color controls were included in every experiment for compensation.

To detect plasmablasts (CD3–CD19+CD20^{low}CD27^{bright}CD38^{bright}), PBMC were stained with anti-CD3 (BD 555339), anti-CD19 (BD 555415), anti-CD20 (BD 641396), anti-CD27 (BD 555441) and anti-CD38 (BD 551400), as described [26,27,31].

2.6. Intracellular staining of TNF- α

One hundred microliters of blood were stained with: anti-CD19 (BD 555415) and anti-CD14 (BD 555398). Then, cells were fixed, washed with 1X PBS/5% FCS, permeabilized with 1X PBS/0.2% Tween 20, followed by cytoplasmic staining with anti-TNF- α (BD 554512). Gates were set based on isotype control staining (BD 554879).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Vaccine-specific IgG response in serum before and after vaccination was evaluated using a human Ig quantitative ELISA kit (Bethyl Labs E80–104), with a modified protocol in which plates were coated with the vaccine at the concentration of 2 µg/ml.

Serum TNF-α, IL-6, CRP, Adiponectin, LPS and sCD14 were measured by the following ELISA kits: Life Technologies KHC3013, KHC0062, KHA0032, KHP0041; Lonza QCL-1000; and R&D Systems DC140, respectively.

2.8. RNA extraction and quantitative (q)PCR

mRNA was extracted from stimulated PBMC (10^6 cells, to measure AID), or from unstimulated B cells (10^4 cells, to measure TLR4), using the µMACS mRNA isolation kit (Miltenyi Biotec) and qPCR performed as described [26,27,32]. Primers were from Life Technologies.

2.9. Statistical analyses

Non-parametric tests were used for our statistical analyses. Mean comparisons between groups were performed by Mann-Whitney test (two-tailed), whereas correlation analyses were performed by Spearman test, using GraphPad Prism 5 software.

3. Results

3.1. Demographic characteristics and serological profile of the subjects in the study

Demographic characteristics and serological profile of the participants are in Table 1. We measured serum levels of the following markers of inflammation: TNF-α and IL-6, CRP, LPS, sCD14 and Adiponectin. Results show increased levels of serum IL-6, CRP, LPS and sCD14 in both young and elderly T2D patients as compared to healthy age-matched controls, and increased levels of TNF-α only in elderly T2D patients versus healthy controls. This can be due to the use of high doses of Metformin by young T2D patients. Metformin in some reports has been shown to lower plasma levels of TNF-α [33,34] and seems to have better effects in young patients in which TNF-α levels are not too high. Metformin is used to lower blood glucose, suppress hepatic glucose output and increase insulin-mediated glucose disposal [35].

LPS is the major component of Gram- bacterial cell walls and a potent immunostimulatory product whose serum levels indicate microbial translocation [36] following intestinal permeability occurring in obesity and T2D patients [37]. sCD14 is secreted by monocyte/macrophages and is a measure of activation. It binds to LPS and is correlated with in vivo LPS stimulation [38]. LPS and sCD14 were found significantly increased in young and old T2D patients, as compared to healthy controls.

Adiponectin is an immunoregulatory marker with anti-diabetic, anti-inflammatory and anti-atherosclerotic properties [39–41]. Adiponectin was significantly higher in elderly versus young healthy individuals and down regulated in T2D patients as compared to healthy controls.

3.2. Young and elderly T2D patients have serum influenza vaccine responses similar to healthy young controls

We analyzed the in vivo vaccine response by ELISA [27]. Results (Fig. 1A) show that aging significantly decreases the specific IgG response in serum of healthy individuals. Young T2D patients have a response similar to that of age-matched healthy controls. However,

elderly T2D patients show a response to the vaccine significantly higher than that of age-matched healthy controls and comparable to that of healthy young individuals.

We also tested the *in vivo* vaccine response by HAL. Results in Fig. 1B show similar titers after vaccination in young and elderly T2D patients and in healthy young controls. Healthy elderly had significantly reduced responses.

Results in Fig. 1C show that aging significantly decreases the plasmablast response at t7 in healthy individuals. Young T2D patients and their age-matched controls have a comparable plasmablast response, whereas elderly T2D patients have a plasmablast response significantly higher than that of their age-matched healthy controls and comparable to that of healthy young individuals at t7, which does not depend on higher levels of plasmablasts at t0 (Fig. 1 Cleft).

3.3. Young and elderly T2D patients have percentages of switched memory B cells similar to young healthy controls

The percentage of switched memory cells is a reliable biomarker to distinguish functional B cell activity in young and old healthy subjects [26,27]. These cells are up-regulated in young but not in elderly individuals after vaccination. Results in Fig. 2A show that switched memory B cells at t0 are comparable between young T2D patients and their healthy controls and in both groups this subset increases significantly after vaccination. Conversely, switched memory B cells at t0 are higher in elderly T2D patients as compared to elderly healthy controls and this subset also increases significantly after vaccination. Therefore, diabetes appears to have no effect on either the distribution of B cell subsets in blood or the response of switched memory B cells to the influenza vaccine. Moreover, we do not see a decrease in these parameters with age in T2D patients, in contrast with what we have seen in healthy individuals here and previously [30,32]. The percentages of naïve, IgM memory and late memory B cells are comparable between young T2D patients and their healthy controls as well as between elderly T2D patients and their healthy controls, either before or after vaccination (not shown).

Results in Fig. 2B show a significantly positive correlation between the *in vivo* response, measured by either ELISA (top) or HAI (bottom), and percentages of switched memory B cells at t0 in both T2D patients and healthy controls. These results confirm our previous findings that the subset of switched memory B cells can predict the response to the influenza vaccine [26,27]. Percentages of switched memory cells increased after vaccination in young and elderly T2D patients, similar to healthy young but not elderly individuals (percentages after vaccination were: 16 ± 2 in young, 18 ± 6 in young T2D, 4 ± 1 in elderly and 14 ± 4 in elderly T2D).

3.4. Young and elderly T2D patients have *in vitro* B cell AID mRNA expression in response to CpG similar to young healthy controls

AID is our chosen marker for optimal B cell function because we showed it completely correlates with the ability of human B cells to undergo class switch recombination (CSR) [32] and somatic hyper-mutation [42]. Results in Fig. 3 show that both young and elderly T2D patients have AID mRNA expression in response to CpG comparable to those in B cells from young healthy controls, whereas healthy elderly have significantly less CpG-induced AID response as compared to the younger adults, as we have shown (3–4-fold less) [26,27]. This occurs despite high levels of systemic and B cell-intrinsic (see below) inflammation, which negatively affects B cell function, as we have demonstrated [25]. The elderly T2D patients have a significantly higher response than their age-matched controls.

3.5. Both young and elderly T2D patients have high levels of intracellular TNF- α in both B cells and monocytes similar to elderly healthy controls

Results in Fig. 4A show that unstimulated, ex vivo stained B cells make detectable amounts of intracellular TNF- α and more in elderly and in T2D patients, either young or old, as compared to young healthy individuals. Unstimulated monocytes also make higher amounts of TNF- α as compared to B cells and, again, more in elderly and in T2D patients than in young healthy controls (Fig. 4B). The levels of TNF- α in unstimulated B cells and monocytes are significantly correlated in healthy individuals ($p < 0.0006$), but in T2D patients, although not statistically significant, a positive trend is shown ($p = 0.0505$) (Fig. 4C). These results indicate that B cells make detectable amounts of TNF- α , although lower than those in monocytes. We would predict that, at least in healthy individuals, this would be responsible for the reduced in vitro AID activation, as B cell-derived TNF- α inhibits in vitro AID in mouse B cells [43].

3.6. Both young and old T2D patients have high levels of systemic LPS and high B cell TLR4 mRNA expression as compared to age-matched healthy controls

T2D patients have normal responses to the influenza vaccine, despite high levels of inflammation. This could be due to the hyper-activation of the innate immune system, which can compensate the negative effects of circulating or B cell-derived TNF- α . To test this hypothesis, we measured the circulating levels of LPS and sCD14. High serum levels of LPS have been shown to be associated with metabolic syndromes such as diabetes [44]. It has also been shown that increased levels of sCD14 are positively correlated with both T and B cell activation [45]. LPS and sCD14 were found significantly increased in young and old T2D patients, as compared to healthy controls.

Moreover, to reveal what could be responsible for normal B cell activation in T2D patients, we measured levels of TLR4 mRNA, the receptor for LPS, in unstimulated B cells. Results in Fig. 5 show increased levels of TLR4 mRNA in unstimulated B cells from T2D patients, as compared to age-matched controls, whereas unstimulated B cells from healthy young and elderly individuals expressed comparable levels of both LPS/sCD14 and TLR4 mRNA. These results showing increased TLR4 expression on B cells and parallel increased serum levels of its ligand suggest an hyperactivated immune system in T2D patients as compared to healthy controls which may counteract the negative effects of intrinsic TNF- α on B cell function. At this point, we do not know the causes for increased TLR4 in T2D patients.

4. Discussion and conclusions

So far, no studies have been performed to determine the contribution of B cells to the influenza vaccine response in T2D patients. Our results herein show for the first time that young and elderly T2D patients have normal in vivo and in vitro B cell responses. Previous studies, designed to evaluate the effect of diabetes on influenza vaccine-specific T cell responses, have shown controversial results, as these responses can be either affected [11] or not [21] by the disease.

We have previously shown an age-related impairment in the ability of B cells from elderly individuals to undergo CSR, which is due to reduced expression of both AID and the E47 transcription factor. Results from 2 influenza vaccine studies, one with seasonal vaccine [26] and another with the 2009 pandemic vaccine [27], have shown that AID can accurately track optimal immune responses and therefore be considered a valid marker of humoral responses to the vaccine in humans. We show here that AID is also a good biomarker of antibody responses in T2D patients.

From our previous studies in mice [25], we expected that the increase in inflammation (TNF- α) in T2D patients would generate a decreased B cell function. Unexpectedly, the in vivo and in vitro B cell responses in T2D patients were not compromised. Possible reasons for this are the increase in serum LPS and sCD14 which together would stimulate B cells from T2D patients, in conjunction with the increase in TLR4 expression in B cells. In addition, the anti-inflammatory medications, e.g. Metformin, in these patients would prevent negative TNF- α signaling in B cells. As a consequence of Metformin treatment, although plasma TNF- α levels are not reduced in T2D patients, others have shown that there is a decreased signaling through the TNF- α receptors leading to reduced NF- κ B activation. The negative regulators of mRNA stability (e.g. tristetraprolin) induced by TNF- α [25] may be inhibited, leading to increased stability for E47 mRNA and increased AID. We are currently evaluating these molecular mechanisms. In addition, we hypothesize that all these and possibly other products derived from gut bacteria, viruses and fungi (RNA, DNA, flagellin, peptido-glycan), could help to explain the beneficial B cell response in T2D patients.

Acknowledgments

This study was supported by NIH AG-32576 (BBB).

We would like to express our gratitude to the people who participated in this study. We thank the personnel of the Department of Family Medicine and Common Health at the University of Miami Miller School of Medicine, in particular Dr. Robert Schwartz, chairman, Susie Batista (RN) for the recruitment of healthy volunteers and Isabel Alfonsin-Vittoria (LMHC) for the recruitment of patients with T2D; Dr. Sandra Chen-Walta, Employee Health Manager; and Sylvester Comprehensive Cancer Center Flow Cytometry Core Resource.

We are grateful to Dr. Savita Pawha, CFAR at the University of Miami Miller School of Medicine, for plasma LPS measurements.

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Fig. 1.

The serum influenza vaccine response in young T2D patients is comparable to age-matched controls, but in elderly T2D patients is higher than age-matched controls. (A) Sera isolated before (t0) and after vaccination (t7) were collected and evaluated in vaccine-specific IgG ELISA. The vaccine used in the ELISA was the same given to each individual in vivo. Both ELISA and HAI (see below) gave the same results when using a heterologous vaccine from a different company for assay. Data are expressed as OD at t7 minus OD at t0. All values here demonstrate a positive response. Mean comparisons between groups were performed by Mann-Whitney test (two-tailed), using GraphPad Prism 5 software. (B) Sera were also analyzed by HAI assay. Results are expressed as reciprocal of the titer after vaccination. A

titer of 1:40 indicates protection and a positive response. Only 6 out of 37 young, 2 out of 14 diabetic young, 4 out of 28 elderly and 1 out of 8 diabetic elderly individuals had a non-protective titer (below 1:40) at t0. Initial titers were $>1/40$ in almost all individuals in the study, indicating high levels of seroprotection. (C) Two million PBMC at t0 and t7 were stained as described in Section 2. Results are expressed as percentages of CD19+ cells, which do not change from t0 to t7. Therefore, both percentages and numbers of plasmablasts increase at t7.

Fig. 2.

The serum response after vaccination and the percentages of switched memory B cells at t0 are positively correlated in both T2D patients and healthy controls. (A) One hundred microliters of blood at t0 were stained as described in Section 2. *Left.* A dot plot from a representative experiment is shown. Data are from a young healthy individual. Switched memory B cells are CD27 + IgD⁻. *Right.* Switched memory B cells at t0, expressed as percentages of CD19⁺ B cells. The CD19⁺ cell percentages were: 13 ± 3 in young, 12 ± 2 in young T2D, 5 ± 2 in elderly and 13 ± 4 in elderly T2D. Mean comparisons between groups were performed by Mann-Whitney test (two-tailed), using GraphPad Prism 5 software. (B) ELISA and HAI were performed as described in Fig. 1. One hundred microliters of blood at

t0 were stained as described in Section 2. Correlations were performed by Spearman test, using GraphPad Prism 5 software. Correlation coefficients are: 0.54 (ELISA, Healthy), 0.51 (ELISA, T2D), 0.27 (HAI, Healthy), and 0.47 (HAI, T2D).

Fig. 3.

Young and elderly T2D patients have in vitro B cell AID mRNA expression in response to CpG similar to young healthy controls. Frozen PBMC (10^6 cells/ml), isolated from the peripheral blood at t0, were cultured with CpG, for 3 days. At the end of this time, cells were processed as described in Section 2. Results are expressed as raw qPCR values of AID mRNA normalized to GAPDH, as the individuals in this study have comparable percentages of B cells (see above, Fig. 2A). We have validated the use of frozen PBMC in preliminary experiments (not shown) by showing that in frozen PBMC cultures raw qPCR values are slightly reduced as compared to those in fresh cultures, but the differences between groups do not change (see Ref. [27]). Mean comparisons between groups were performed by Mann–Whitney test (two-tailed), using GraphPad Prism 5 software.

Fig. 4.

Both young and elderly T2D patients have high levels of intracellular TNF- α in both B cells and monocytes similar to elderly healthy controls. One hundred microliters of blood at t0 were stained as described in Section 2. *Left.* A histogram from a representative experiment is shown. Data are from a healthy elderly individual. Isotype controls are set up to be no more than 1% positive. *Right.* Results are expressed as percentages of CD19+ B cells (A) or CD14+ monocytes (B) expressing icTNF- α . Mean comparisons between groups were performed by Mann-Whitney test (two-tailed), using GraphPad Prism 5 software. (C) The levels of icTNF- α in CD19+ and CD14+ cells are positively correlated in all subjects. The correlations were performed by Spearman test, using GraphPad Prism 5 software.

Fig. 5.

Increased levels of B cell TLR4 mRNA expression in T2D patients as compared to age-matched healthy controls. B cells were isolated from frozen PBMC by magnetic sorting and mRNA extracted to evaluate TLR4 mRNA expression. Results are expressed as raw qPCR values of TLR4 mRNA normalized to GAPDH. Non-parametric tests were used for our statistical analyses, as indicated in Section 2.

Table 1

Demographic and serological characteristics of the individuals participating in the study.

	Healthy young	T2D ^e young	Healthy elderly	T2D ^e elderly
Participants (<i>n</i>)	37	14	28	8
Age (mean years $\pm$ SE)	42 $\pm$ 2	50 $\pm$ 3	66 $\pm$ 1	64 $\pm$ 2
Gender (M/F)	10/27	6/8	10/18	4/4
Race (W/B)	29/8	2/12	26/2	4/4
Ethnic categories				
Hispanic/Latino	8	3	9	3
Not Hispanic/Latino	29	11	19	5
Racial categories				
White	25	2	23	4
Black or African American	8	12	2	4
Asian	4	0	3	0
BMI(mean kg/m ² $\pm$ SE)	27 $\pm$ 1	36 $\pm$ 3 ^b	23 $\pm$ 1	31 $\pm$ 2 ^a
TNF- α (pg/ml)	6 $\pm$ 1	7 $\pm$ 4	13 $\pm$ 4 ^c	24 $\pm$ 16 ^a
IL-6 (pg/ml)	49 $\pm$ 22	63 $\pm$ 69 ^b	97 $\pm$ 15 ^d	170 $\pm$ 177 ^b
CRP (pg/ml)	648 $\pm$ 91	1678 $\pm$ 570 ^b	883 $\pm$ 63 ^d	1605 $\pm$ 114 ^b
LPS (pg/ml)	120 $\pm$ 3	143 $\pm$ 9 ^b	124 $\pm$ 6	151 $\pm$ 9 ^b
sCD14(μ g/ml)	0.8 $\pm$ 0.2	1.3 $\pm$ 0.1 ^a	0.9 $\pm$ 0.1	1.4 $\pm$ 0.2 ^b
Adiponectin (ng/ml)	9 $\pm$ 1	6 $\pm$ 1 ^b	13 $\pm$ 1 ^d	6 $\pm$ 1 ^a

Young: 20–59 years, elderly: >60 years.

p Values refer to either differences between patients with diabetes and age-matched controls (a, b) or differences between young and elderly healthy individuals (c, d).^a*p*<0.01.^b*p*<0.05.^c*p*<0.01.^d*p*<0.05.^ePatients with type 2 diabetes.