Inverse relationship between organ-specific autoantibodies and systemic immune mediators in type 1 diabetes and type 2 diabetes. Action LADA 11

Nanette C. Schloot (MD)[1*], Minh N. Pham (PhD)[1,2,3*], Mohammed I. Hawa (PhD)[4*], Paolo Pozzilli (MD)[5], Werner A. Scherbaum (MD)[6], Matthias Schott (MD)[7], Hubert Kolb (PhD)[8], Steven Hunter (MD)[9], Guntram Schernthaner (MD)[10], Charles Thivolet (MD)[11], Jochen Seissler (MD)[§12], Richard David Leslie (MD)[4§], for the Action LADA group.

[1]Institute for Clinical Diabetology, German Diabetes Center, Leibniz Center for Diabetes Research at Heinrich-Heine University, Duesseldorf, [2]currrent address: Pacific Northwest Diabetes Research Institute, Seattle, Washington, USA, [3]Novo Nordisk Research Center, Seattle, Washington, USA [4]Blizard Institute, Queen Mary, London, United Kingdom, [5]Department of Endocrinology and Diabetes, University Campus Bio-Medico, Rome, Italy, [6]University of Duesseldorf, Medical Faculty, Duesseldorf, Germany, [7]University of Duesseldorf, Medical Faculty, Division for Specific Endocrinology, [8]West-German Centre of Diabetes and Health, Verbund Katholischer Kliniken Düsseldorf, Düsseldorf, Germany, [9]Regional Centre for Endocrinology and Diabetes, Royal Victoria Hospital, Belfast, Ireland; [10]Rudolfstiftung Hospital, Department of Medicine, Vienna, Austria. [11]Department of Endocrinology and Diabetes, Lyon-Sud Hospital, Hospices Civils de Lyon, Pierre Benite, France; Université Claude-Bernard Lyon, France,[12]Medizinische Klinik und Poliklinik IV, Diabetes Center, Ludwig-Maximillians-University, Munich, Germany.

*Equal contribution first author; § Equal contribution last author

Corresponding author:

Nanette C. Schloot

Institute for Clinical Diabetology

German Diabetes Center Leibniz Center for Diabetes Research

at Heinrich Heine University

Auf'm Hennekamp 65

40225 Düsseldorf

Tel. +49 152 227 20014

Fax +49 6172 2732726

Email nanette.schloot@web.de

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A**bstract (241 words)**

Objective We related organ-specific autoantibodies, including diabetes-associated antibodies (DAA) and non-diabetes-associated autoantibodies (non-DAA) to systemic cytokines/ chemokines in type 1 and type 2 diabetes.

Research Design and Methods From the European Action LADA cohort, patients with adult-onset type 1 diabetes (n=80, of which 50 had LADA and 30 had classic type 1 diabetes) and type 2 diabetes (n=626) were analysed for DAA (GADA, IA2A, ICA, ZnT8A), non-DAA (TGA, TPOA, PCA) and 10 immune mediator concentrations (measured by LUMINEX).

Results Type 1 diabetes patients (whether classic type 1 diabetes or LADA), apart from their clinical phenotype, could not be distinguished by either autoantibodies (both DAA and non-DAA) or immune mediators. In type 1 diabetes most immune mediators (9/10) were negatively correlated with DAA titres. Type 2 diabetes patients, by definition without DAA, had fewer non-DAA ($p<0.0005$), but higher levels of pro-inflammatory immune mediators, especially compared with type 1 diabetes patients with high GADA titre; IL-6 ($p<0.001$), sE-Selectin ($p<0.01$) and IL-1Ra ($p=0.052$, trend).

Conclusions Patients with type 1 diabetes had more DAA and non-DAA than type 2 diabetes, while the frequency and nature of these autoantibodies was broadly similar in classic type 1 diabetes and LADA. Systemic immune mediator levels, in the main, were negatively correlated with DAA titres, and, for some, higher in type 2 diabetes, especially when compared with high titre GADA patients. Differences in the clinical classification of diabetes are associated with graded differences in adaptive and innate immune reactivity.

Introduction

The definition of type 1 diabetes is clinically exclusive, encompassing patients with diabetes-associated autoantibodies (DAA) plus, when diagnosed outside a surveillance program, insulin dependence. Difficulty arises when patients with non-insulin requiring diabetes, clinically like type 2 diabetes, have immuno-genetic characteristics of type 1 diabetes. Such patients have been designated latent autoimmune diabetes in adults (LADA), also called slowly progressive insulin-dependent diabetes (SPIDDM) or type 1.5 diabetes (1). Adult-onset autoimmune type 1 diabetes (AID) is characterized by diabetes associated antibodies (DAA), usually autoantibodies against glutamic acid decarboxylase (GADA) (1,2) and encompasses patients with both classic type 1 diabetes, and LADA, of which the latter is most prevalent (2). Despite having different clinical presentations and phenotype, adults with classic type 1 diabetes and LADA resemble each other as regards their HLA and non-HLA genetic risk, the presence of DAA - predominantly GADA, the frequency of thyroid peroxides autoantibodies (TPOA), frequency of metabolic syndrome and levels in peripheral blood of systemic cytokines (IL-6, TNF-α, IL-1Ra, IL-10), chemokines (CCL2, CCL3, CCL4), adhesion molecules (sE-Selectin, sICAM-1 and sVCAM-1) and peripheral B-lymphocyte subsets (2-8). In contrast, patients with type 2 diabetes do not have an HLA genetic association, do not have DAA (1-4), have low frequency of TPOA (9) and substantially higher frequency of metabolic syndrome (10). Similarly, some systemic serum immune mediators are higher in type 2 diabetes than in type 1 diabetes including LADA, e.g. systemic cytokines (IL-6, TNF-α, IL-1Ra, IL-10), chemokines (CCL2, CCL3, CCL4) and adhesion molecules (sE-Selectin, sICAM-1 and sVCAM-1) (11,12).

In previous studies we defined a non-random association in type 1 diabetes between adaptive immune changes (the presence and number of DAA) and innate immune changes (the serum levels of systemic cytokines and chemokines) (11-15). Here, we extend and expand these

studies to include a range of adult-onset clinical diabetes (both forms of type 1 diabetes as well as type 2 diabetes, each analysed for both DAA and non-DAA antibodies as well as a panel of systemic immune mediators: classic type 1 diabetes cytokines, chemokines and adhesion molecules. Our hypothesis is that innate (immune mediators) and adaptive immune reactivity (antibodies) can occur in both the major types of diabetes; adult-onset type 1 diabetes is predominantly associated with adaptive immune changes, while type 2 diabetes is predominantly associated with innate immune changes. Here we show graded differences in adaptive and innate immune changes across the classification of diabetes.

Research Design and Methods

Patients The Action LADA multicentre study was performed to identify immune and clinical risk factors for adult-onset autoimmune diabetes (AID), including its epidemiology, genetic susceptibility, metabolic characteristics and clinical progression (16). The study population for the present study consisted of 706 individuals aged from 30 to 70 years; all had been diagnosed with diabetes within 5 years before entering this cross-sectional study from the Action LADA cohort (2). The cohort consisted of 50 subjects with LADA, 30 with type 1 diabetes and 626 subjects with type 2 diabetes. Patients with other diabetes forms were excluded.

Serum samples were randomly selected with stratification for age and significant serum samples left for analysis. Patients with classic type 1 diabetes were DAA-positive and received insulin treatment shortly after diabetes diagnosis. GADA-positive patients aged from 30 to 70 years who did not use insulin treatment for at least 6 months after diagnosis were termed LADA. The focus of anti-hyperglycaemic medication was on insulin, as the time to start of insulin treatment contributed to the definition of LADA. Antidiabetic medications, other than insulin, were not evaluated. GADA negative and other DAA negative patients who did not use insulin at least for the first year after diagnosis were defined as having type 2 diabetes.

Blood withdrawal from all participants was carried out in the fasting state. The local ethics committees of each study centre approved the study protocol, in accordance with the Declaration of Helsinki. All patients gave written informed consent for the study.

Antibody measurements DAA directed against islets (islet cell antibodies, ICA) (13), glutamic acid decarboxylase (GADA), insulinoma-associated antigen (IA2A) and zinc transporter T8 (ZnT8A) were determined (2).

Non-DAA directed against transglutaminase (TGA), parietal cells (PCA) and thyroid (TPOA) were measured as described. We chose to measure TPOA, PCA and TGA as they are known to be frequently positive in patients with autoimmune diabetes and are well-established gold standards to detect autoimmunity against thyroid, parietal cells and intestinal villous epithelial cells (17-21).

In brief, TPOA were examined by B.R.A.H.M.S TPO-Ab RIA® (B.R.A.H.M.S. AG) using native thyroid peroxidase as antigen, with results expressed in AU/ml. h and the functional assay sensitivity as 14 AU/ml; the upper limit of detection is 15,000 AU/ml, cut-off for positivity was 60 AU/ml.

Gastric H^+/K^+-ATPase IgG autoantibodies (PCA) were determined by ELISA (Euroimmun AG; Lübeck, Germany) according to the manufacturer´s instructions (cut-off 20 AU/ml).

Tissue transglutaminase antibodies (TGA) were measured by IgA-ELISA (Euroimmun AG); assay has an upper limit of detection of 200/AU/ml and a cut-off of 20 AU/ml. The intra- and inter-assay variation was 3.5% and 6.8%, respectively.

Systemic cytokines and chemokines Soluble immune mediators were measured as described (11, 12). Serum samples were obtained in a standardized form from freshly drawn blood samples from fasting subjects in the morning hours, and were stored at -80°C until the time of the assay without prior thawing. In brief, serum concentrations for sICAM-1, sVCAM-1, sE-Selectin, CCL2, CCL3 and CCL4 were determined with commercially available multiplex-bead technology kits (Fluorokine MAP; R&D Systems, Wiesbaden, Germany). Intra- and inter-assay coefficients of variations were <5% and <11%, respectively.

The detection limits of the assays were 13.8 ng/ml for sICAM-1, 69.0 ng/ml for sVCAM-1, 17.1 ng/ml for sE-Selectin, 40.3 pg/ml for CCL2, 2.4 pg/ml for CCL3 and 0.4 pg/ml for CCL4. At least 95% of serum concentrations were above the detection limit for all markers except for CCL3 levels, which were detectable in 72% of all samples. Determinations of immune mediators concentrations lower than the detection limit were assigned a value half of the detection limit as described (11, 22).

Serum cytokine concentrations of IL-1Ra, IL-6, TNF-α and IL-10 were measured by multiplex-bead technology using commercially available kits (Fluorokine MAP; R&D Systems, Wiesbaden, Germany). The detection limits of the assays were 9.56 pg/ml for IL-1RA, 0.1 pg/ml for IL-6, 0.08 pg/ml for TNF-α and 0.25 pg/ml for IL-10. For cytokine concentrations lower than the detection limit a value half of the detection limit was assigned (IL-6, n=46; IL-1Ra, n=0; TNF-α, n=0). Concentration of cytokine IL-10 was only detectable in 44% of the samples. Immunoassays showed inter-assay variations < 20% and intra-assay variations < 10%.

Statistical methods We performed the analyses in randomly selected serum samples from participants of Action LADA that were stratified by age. Analyses were performed using SAS Enterprise Guide version 4.2 (SAS Institute, Cary, NC, USA) and GraphPad Prism version 4 for Windows (GraphPad Software, La Jolla, California, USA). Continuous variables are presented as medians and range if not indicated otherwise. First, Gaussian distribution of data was assessed using the Kolmogorov–Smirnov test. The Kruskal–Wallis and Mann–Whitney tests were used to compare continuous variables. Fisher's exact test or the χ^2 test was performed to evaluate the differences in categorical data with two or more classes. Tests were not adjusted for multiple comparisons and are therefore descriptive if not indicated otherwise. Univariate correlations between organ specific antibodies titers, sex (men=1, women= 2), age,

BMI and diabetes duration were described by Spearman correlation (R). Kruskal-Wallis test was performed for the association analysis between systemic cytokines and multiple positivity for DAA and non-DAA. Associations of antibodies with cytokines upon adjustments for confounders including sex, age, BMI, and diabetes duration were carried out with multivariate regression analysis.

Results

Patients characteristics As expected adult onset autoimmune diabetes (AID) patients, including classic adult onset type 1 diabetes and LADA, and type 2 diabetes patients differed by age ($p < 0.0001$), BMI ($p < 0.0001$), diabetes duration ($p < 0.0001$) and family history for diabetes ($p < 0.01$). Classic type 1 diabetes patients were the youngest (median age 44.65 years), type 2 diabetes patients had the highest BMI (30.13 kg/m^2), and LADA cases had the longest diabetes duration (2.92 years) (**Table 1**).

Diabetes- Associated Antibodies Adult onset autoimmune diabetes type 1 diabetes patients (n=80) had by definition, one or more DAA (GADA, IA2A, ZnT8A, ICA) and within that group LADA (n=50) were, by definition, at least positive for GADA and did not start insulin therapy for at least 6 months post-diagnosis. Of classic type 1 diabetes patients, started on insulin close to diagnosis (n=30), 93.3% were GADA positive and their type of DAA did not differ from LADA cases (**Table 1**). Type 2 diabetes patients were, by definition, negative for DAA. Of all autoimmune diabetes patients (n=80), 30 (37.5%) were also positive for ICA, 16 had IA2A (20%) and 10 had ZnT8A (12.5%) **(Table 1**). Of adult onset autoimmune diabetes patients (n=80) only 5 had all four DAA (6.25%); 8 (10%) had three DAA and 23 (28.75%) had two DAA and 44 (55%) were single DAA positive (**Suppl. table 1**). Classic type 1 diabetes did not significantly differ from LADA in this comparison. Similarly, DAA titres did not differ among type 1 diabetes patients including LADA (data not shown). In adult onset autoimmune diabetes patients (n=80), 54 had high GADA titres, that is $\geq$ 200 WHO Units as defined by an inflection point in signal from the cohort previously described (2), and the remainder (n=26) were designated low GADA titres < 200 WHO Units. High GADA titre patients compared with low GADA titre patients did not differ in terms of the number of

DAA or non-DAA (data not shown), nor were the groups with high versus low GADA titres clinically different, including age, BMI and diabetes duration (data not shown).

Non-Diabetes Associated Autoantibodies

Non-Diabetes Associated Autoantibodies Non-DAA were detected more often in patients with adult onset autoimmune diabetes, whether LADA (19/50, 38%) or classic type 1 diabetes (15/30, 50%), when compared with type 2 diabetes (145/626, 23.2%) (p= 0.0004). TPOA were more frequent in adult onset autoimmune diabetes (26/80, 32.5%) than in type 2 diabetes (85/626 13.58%) (p<0.0001); while their frequency in LADA (15/50, 30%) did not differ from classic type 1 diabetes (11/30, 36.67%) (p=0.538) (**Table 1**). Positivity for both TPOA and PCA was marginally greater in adult onset autoimmune diabetes, both classic type 1 diabetes (3/30, 10%) and LADA (4/50; 8%), compared to type 2 diabetes patients (18/626 2.9%) (p=0.024). The clinical groups did not differ for TGA positivity or PCA positivity (**Table 1**). PCA outlier (defined as PCA >80 AU/ml) do associate with positive TPOA in LADA in 4 out 5 cases, but not in type 2 diabetesor type 1 diabetes. TGA outlier (defined as TGA > 30 AU/ml) did not correspond with other non-DAA (PCA and TPOA). TPOA outlier (defined as TPOA >300 AU/ml) associated with positive PCA in 1 out of 5 classic type 1 diabetes and in 4 out of 18 cases in type 2 diabetes. Overall, outliers of one non-DAA were not significantly associated with other non-DAA except for PCA and TPOA in LADA cases.

Non-Diabetes Associated Autoantibodies titres Among adult onset autoimmune diabetes patients, high and low titre GADA were not associated with either their classification as classic type 1 diabetes or LADA, the number or titre of non-DAA (data not shown) nor did TPOA, TGA or PCA titres differ between LADA and classic type 1 diabetes (**Figure 1**). In comparison with type 2 diabetes, adult onset autoimmune diabetes patients had lower mean TGA titres (Kruskal Wallis p=0.003, post hoc Dunn's comparison p< 0.05), while mean

TPOA titres in classic type 1 diabetes patients were higher (Kruskal Wallis p= 0.008, post hoc Dunn's comparison p< 0.05, **Figure 1**).

Association of DAA and non-DAA with clinical demographics There was no significant relationship between DAA concentrations and either sex, age, BMI or diabetes duration, with the exception that ZnT8A titres correlated positively with BMI (p=0.001, r= 0.548) in classic type 1 diabetes patients. Statistically significant associations were detected for non-DAA but did not impact our conclusions (**Suppl. table 2**). More specifically, sex was significantly related to PCA in type 1 diabetes in that PCA titres were increased in male, age was positively associated with PCA in LADA (r=0.29, p=0.038) and type 2 diabetes patients (r=0.15, p= 0.003). Age was negatively related with TPOA (r=-0.17, p= 0.001). BMI was positively associated with PCA (r=0.374, p= 0.03).

Associations of DAA and non-DAA with systemic immune-mediators

First, we investigated associations of antibodies with immune-mediators without adjustment for demographic effects. In patients with adult onset autoimmune diabetes (AID, n=80), we detected significant associations of systemic immune mediators and antibody titres in 14 combinations: 10 with DAA (9 negative, 1 positive) and 4 with non-DAA (TGA, TPOA) (2 negative, 2 positive) (table 2). Interestingly, no significant association of GADA with immune mediators was observed in this analysis.

As we detected some association of sex, age and BMI with non-DAA concentrations (**Suppl. table 2**), we adjusted for these parameters in further analysis. After adjustment for sex, age, BMI and diabetes duration, non-DAA associated significantly only for PCA and IL-10 (p=0.001, negative correlation). Two DAA were negatively correlated with immune mediators upon adjustments: IA-2A and TNF-α (p=0.0278), ICA and sVCAM-1 (p=0.0065),

sICAM-1 (p= 0.011) and TNF-α (p= 0.011). ZnT8A titre was associated with IL-6 (p=0.004, positive correlation). That means, of the 14 combinations found in the unadjusted analysis, 5 remained significant after adjustment and one association was newly significant (**Table 2**), most of them negatively correlated.

In patients with type 2 diabetes (n=626), without adjustment for demographic effects, we detected six associations of systemic immune mediators and non-DAA titres: all of them negative (PCA with IL-1Ra (r= -0.148, p = 0.025); IL-6 (r= -0.146, p= 0.027); IL-10 (r= -0.133, p= 0.044), and CCL2 (r= -0.133, p=0.043); TPOA with TNF-α (r= -0.157, p= 0.018) and sVCAM-1 (r= -0.198, 0.0026). TGA were excluded from these analyses, as so few patients were positive. After adjustment for sex, age, BMI and diabetes duration, non-DAA were not associated with any of the immune mediators in patients with type 2 diabetes (n=626).

Association of high GADA titres with cytokines

We compared adult onset autoimmune diabetes patients with high GADA (>200 U/ml), low GADA (<200 U/ml) and type 2 diabetes patients (all GADA negative). High titre GADA patients had lower IL-6 (p< 0.001), and sE-Selectin (p< 0.01) and a trend toward decreased IL-1Ra (p= 0.052), with a significant trend across the three diabetes cohorts for the former two (p<0.01) indicating an inverse association between an adaptive autoimmune response (GADA) and these systemic immune mediators (**Figure 2**). There were no significant differences for the other cytokines tested, including IL-10, TNF-α, CCL2, CCL3, CCL4, sICAM-1 and sVCAM-1.

Conclusions

We explored the relationship within the two major types of diabetes between adaptive immunity, represented by both DAA and non-DAA, and innate immunity, represented by 10 systemic immune mediators including cytokines, chemokines and adhesion molecules in the Action LADA cohort. It is well established that type 1 diabetes is associated with DAA irrespective of the clinical phenotype or age at diagnosis. In our study, neither DAA nor non-DAA nor immune mediators could distinguish classic type 1 diabetes from LADA.

As expected, we detected DAA in a large series of over 6,000 adult patients diagnosed with diabetes (2). We selected patients from that series with DAA based on their immediate need for insulin treatment (classic type 1 diabetes) and the lack of that need for at least six months (LADA) plus availability of sufficient sera from the same sample. The majority of these adult-onset cases had a single DAA and the dominant autoantibody was GADA. There was no difference in the frequency or titre of these DAA and the need for insulin therapy or initial clinical phenotype. Type 1 diabetes is associated with other autoimmune diseases including Pernicious Anaemia and Hashimoto's thyroiditis, respectively characterized by parietal cell autoantibodies (PCA) and thyroid peroxidase autoantibodies (TPOA) (25). The increased frequency of TPOA and PCA are well described in type 1 diabetes but also in LADA patients, e.g. the Italian NIRAD study and the Chinese LADA study (9,26–28). As expected, the frequency of non-DAA here was increased in type 1 diabetes compared with patients with type 2 diabetes irrespective of the initial need for insulin treatment, that is in both classic type 1 diabetes and in LADA; but that increase was due mainly to TPOA and to a lesser extent PCA but not to TGA. Whilst some non-DAA are a feature of type 1 diabetes, these autoantibodies, notably TPOA, were also found in a proportion of patients with type 2

diabetes. The percentage of non-DAA positive patients with LADA in our Action LADA cohort was similar to that from other cohorts (9, 28).

Importantly and unique to this study, we found for autoimmune diabetes, that significantly altered immune mediator levels were nearly all (9/10) negatively correlated with DAA titres and two of four were negatively associated with non-DAA. Serum concentrations of some immune mediators i.e. a cytokine and an adhesion molecule, were overall slightly increased in type 2 diabetes compared to adult onset autoimmune diabetes patients with high GADA titre. There was a graded increase in IL-6 and sE-Selectin across the main types of diabetes, with levels being highest in type 2 diabetes patients and lowest in those patients with classic type 1 diabetes, whilst LADA patients had intermediate levels without significant differences from classic type 1 diabetes. As in most studies, there was a considerable overlap of cytokine concentrations in classic type 1 diabetes, LADA and also type 2 diabetes and innate immune markers measured in serum are not sufficiently diagnostic to dissect out the different diabetes forms. This is not surprising as innate as well as adaptive immune alterations may have a role in all diabetes types including type 1 diabetes, LADA and type 2 diabetes (5-7,11,12,15,29). The amount of GADA titre was not associated with classic type 1 diabetes or LADA, and similarly, levels of the immune effector molecules were not different between these two clinically distinct forms of autoimmune diabetes and lower than in type 2 diabetes. It follows that there is an inverse relationship between adaptive immunity (antibodies, DAA, non-DAA) associated with type 1 diabetes and innate immune effector molecules (cytokines, chemokines, adhesion molecules) associated with type 2 diabetes, with a graded change across the clinical categories. Whether the observed inverse innate and adaptive immune responses are causal or merely associated cannot be addressed as our study was exploratory. However, one potential explanation for the inverse relationship between adaptive and innate

immune responses is that immune regulation of one component limits the potentially exaggerated, and, therefore harmful, response of the other component. Alternatively, since altered innate immune responses are common to both major types of diabetes but adaptive immune responses are a characteristic of only AID, it is the possible that their effect is additive and, therefore, the greater the adaptive immune effect the less the innate immune effect required to develop clinical disease.

Numerous studies have demonstrated clinical heterogeneity in autoimmune diabetes, especially adult-onset diabetes such that some patients present with severe insulin-dependent diabetes but others present with non-insulin requiring diabetes (1). Yet both these forms of diabetes are characterised by the presence of DAA, usually GADA, associated with genetic susceptibility through common Histocompatibility Lymphocyte Antigen (HLA) haplotypes. This clinical spectrum extends into childhood-onset autoimmune type 1 diabetes in which insulin-dependent patients have the same DAA, though not predominantly GADA, with a stronger HLA genetic susceptibility. That clinical heterogeneity is also reflected in striking variation in insulin secretion, illustrated by C-peptide levels, across the types of diabetes. Recent large studies have confirmed that there can be substantial serum C-peptide in adult-onset type 1 diabetes, suggesting that it is difficult to distinguish some forms of adult-onset type 1 diabetes from type 2 diabetes based on C-peptide alone (23,24). This present study confirms previous studies indicating that LADA and classic type 1 diabetes have, despite clinical differences, a similar cytokine profile with similar levels of serum IL-6 and sE-Selectin, with each being lower than their corresponding levels in type 2 diabetes. We have now analysed for the first time that relationship in more detail and found that there was an inverse relationship between DAA titres and the levels of four immune mediator molecules such that high titres of one, e.g. ICA, were associated with lower levels of the other e.g. sVCAM-1, sICAM-1 and TNF-**α**. It is unclear what pathogenetic mechanism accounts for the

relationship between these effector molecules and the two major types of diabetes, but that inverse relationship is even detected within autoimmune diabetes; the group with highest GADA showed decreased IL-6 and sE-Selectin. It will require further study to define the reason for this inverse relationship between adaptive and innate immune changes across a range of adult-onset forms of diabetes.

In summary, we present the first evidence for an effect in both adaptive and innate immunity across type 1 diabetes and type 2 diabetes. Since this graded effect was seen between type 1 diabetes and type 2 diabetes, and even within autoimmune diabetes, the change in immune effectors likely reflects some common pathogenetic mechanism between the major types of the disease.

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Members of the Action LADA Group:

Professor David Leslie, Mohammed I Hawa, Dr Huriya Beyan, Dr Stavroula A Paschou, Blizard Institute, Queen Mary University of London, London, UK

Professor Paolo Pozzilli MD, University Campus Bio-Medico, Rome

Professor Rhys Williams MD, Dr Sinead Brophy PhD and Ms H Davies MSc, Swansea University, Swansea

Professor Henning Beck-Nielsen MD and Dr Knud Yderstraede MD, University Hospital of Odense, Odense

Dr Steven Hunter, MD and Professor David Hadden* MD, Royal Victoria Hospital, Belfast.

*Prof David Hadden, Belfast passed away 2014.

Professor Raffaella Buzzetti MD, University La Sapienza, University of Rome

Professor Werner Scherbaum MD and Professor Hubert Kolb, PhD, University of Dusseldorf, Dusseldorf

Professor Nanette C. Schloot, MD, Institute for Clinical Diabetology, German Diabetes Centre, Leibniz Center of diabetes research, University of Duesseldorf, Duesseldorf and Department of Metabolic Diseases, University Clinics Düsseldorf, Heinrich-Heine University. N.C. Schloot is guest scientist at the German Diabetes Center and is currently employed by Lilly Deutschland, Bad Homburg, Germany

Professor Jochen Seissler, MD, Ludwig-Maximilians-University, Munich

Professor Guntram Schernthaner MD, Rudolfstiftung Hospital, Vienna

Professor Jaako Tuomilehto MD and Dr Cinzia Sarti MD, National Public Health Institute, Helsinki,

Professor Alberto De Leiva PhD, and Dr Eulalia Brugues MSc Universitat Autonoma de Barcelona, Barcelona

Dr Didac Mauricio MD, Hospital de Sant Pau, Barcelona

Professor Charles Thivolet, MD, Hopital Edouard Herriot, Lyon

Authors' conflict of interest statement

N.C.S. is employed at Lilly Germany, Bad Homburg, no conflict of interests is declared.

M.N.P. is employed at Novo Nordisk Research Center, Seattle, Washington. No conflict of interest is declared.

M.I.H., no conflict of interest is declared.

P.P., no conflict of interest is declared.

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C.T., no conflict of interest is declared.

J.S., no conflict of interest is declared.

R.D. L., no conflict of interest is declared.

Authors' contribution to the manuscript

N.C.S., M.N.P., M.I.H., R.D.L. measured, researched, analyzed the data and wrote the manuscript; J.S. and M.S. measured non-DAA, P.P., W.A.S, S.H., G.S., C.T., H.K. collected and/ or researched patient data and revised the manuscript.

Guarantor statement

Nanette Schloot is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

Figure 1

Levels of non-DAA PCA, TGA, TPOA, in type 2 diabetes, classic type 1 diabetes, LADA and adult onset autoimmune (AID, classic type 1 diabetes and LADA combined) . Cut-off for positivity was 20 AU/ml for PCA, 20 AU/ml for TGA, and 60 AU/ml for TPOA. Shown are all individual data and mean. p- values from Dunn's Multiple Comparison Test.

Figure 2

Concentrations (Box and whiskers) of cytokines, chemokines and soluble adhesion molecules in patients with adult autoimmune diabetes (AID) that were GADA high (>200 U/ml) or GADA low (<200 U/ml) and type 2 diabetes patients that were GADA negative. P-values from non-parametric Kruskal Wallis upper line, P-values from Dunn’s Multiple Comparison Test lower line.

	LADA **n=50**	**Classic Type 1 diabetes** **n=30**	**Type 2 diabetes** **n=626**	**p-value**
Demographic				
Sex m/f	25/25	17/13	361/265	n.s.
Age [years]	52.28 (31.87 – 69.10)	44.65 (33.56- 66.13)	56.08 (30.15 – 69.83)	**P<0.0001**
BMI [kg/m2]	25.96 (18.12 – 48.78)	23.83 (16.44 –53.15)	30.13 (18.00 – 71.94)	**P<0.0001**
Diabetes duration [yrs]	2.92 (0.05 – 6.63)	0.59 (0.02 – 5.36)	2.00 (0.0 – 5.79)	**P<0.0001**
Fam. hist diab pos/neg (% pos)	33/ 17 (66%)	9/21 (30%)	347/279 (55.4%)	**P= 0.0065**
Antibody status DAA n (%)				
GADA	50 (100%)	28 (93.33%)	0	n.s.
IA2A	9 (18.0%)	7 (23.3%)	0	n.s.
ZnT8A	6 (12%)	4 (13.33%)	0	n.s.
ICA	18 (36.0%)	12 (40%)	0	n.s.
Non-DAA n (%)				
TPOA	15 (30%)	11 (36.67%)	85 (13.58%)	**p< 0.0001** n.s.
PCA	6 (12%)	6 (20%)	75 (11.98%)	n.s. n.s.
TGA	1 (2%)	1 (3.3%)	5 (0.8%)	n.s. n.s.
TPOA and PCA	4 (8%)	3 (10%)	18 (2.9%)	**p=0.0245** n.s.

Table 1

Characteristics of the patients with LADA, classic type 1 diabetes, and type 2 diabetes. Data are shown as median (range) and numbers n (%). DAA, diabetes associated antibodies. P-values refer to the comparison of the three groups by non-parametric Kruskal Wallis testing or Fisher's exact test, two sided. DAA antibody positivity was compared between LADA and classic type 1 diabetes. Non-DAA antibody positivity was compared between LADA, classic

type 1 diabetes and type 2 diabetes (Kruskal Wallis test, p upper line), and between classic type 1 diabetes and LADA (p lower line). n.s. not significant

AID (n=80)						
	Non-DAA*		DAA			
	PCA	**TPOA**	**ICA**	**IA2**	**ZnT8A**	**GADA**
IL-1Ra	r value -0.01	0.09	-0.092	-0.20	0.12	0.13
	p value 0.93	0.36	0.34	0.03	0.34	0.30
IL-6	r value 0.06	-0.01	0.03	**-0.26**	**0.27***	-0.05
	p value 0.52	0.90	0.77	**0.006**	**0.0314**	0.67
IL-10	r value -0.05*	-0.02	0.14	0.08	-0.19	-0.20
	p value 0.64	0.85	0.14	0.40	0.14	0.10
CCL2	r value -0.04	0.06	0.14	-0.08	-0.14	0.07
	p value 0.69	0.53	0.14	0.43	0.27	0.56
CCL3	r value 0.01	**0.43**	0.13	-0.09	0.08	0.02
	p value 0.90	**<.0001**	0.16	0.38	0.54	0.90
CCL4	r value 0.037	0.12	-0.03	0.04	0.17	-0.02
	p value 0.71	0.20	0.72	0.65	0.17	0.89
TNF-α	r value -0.13	**-0.20**	**-0.38***	**-0.31***	0.03	-0.02
	p value 0.17	**0.04**	**<.0001**	**0.0009**	0.81	0.9
sICAM-1	r value 0.10	-0.09	**-0.34***	**-0.26**	0.08	0.13
	p value 0.28	0.33	**0.0004**	**0.007**	0.54	0.32
sVCAM-1	r value 0.05	**-0.24**	**-0.34***	-0.09	0.22	0.20
	p value 0.61	**0.01**	**0.0004**	0.35	0.08	0.10
sE-Selectin	r value **0.24**	0.002	**-0.20**	**-0.28**	-0.11	0.07
	p value **0.01**	0.98	**0.04**	**0.003**	0.38	0.60

Table 2

Correlation analysis of immune mediators and antibody titres in patients with autoimmune diabetes (AID, classic type 1 diabetes and LADA combined). Shown are r- and p- values from Spearman analysis. Significant correlations are in bold. TGA were not analysed as only 2 of 80 AID patients were positive for TGA. * Significant association after adjustment for sex, age, BMI, diabetes duration.

	LADA **n=50**	**Classic Type 1 diabetes** **n=30**	**Type 2 diabetes** **n=626**	**p-value**
Demographic				
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	AID (n=80)					
	Non-DAA		DAA			
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	p value 0.93	0.36	0.34	**0.03**	0.34	0.30
IL-6	r value 0.06	-0.01	0.03	**-0.26**	**0.27***	-0.05
	p value 0.52	0.90	0.77	**0.006**	**0.0314**	0.67
IL-10	r value -0.05*	-0.02	0.14	0.08	-0.19	-0.20
	p value 0.64	0.85	0.14	0.40	0.14	0.10
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	p value 0.69	0.53	0.14	0.43	0.27	0.56
CCL3	r value 0.01	**0.43**	0.13	-0.09	0.08	0.02
	p value 0.90	**<.0001**	0.16	0.38	0.54	0.90
CCL4	r value 0.037	0.12	-0.03	0.04	0.17	-0.02
	p value 0.71	0.20	0.72	0.65	0.17	0.89
TNF-α	r value -0.13	**-0.20**	**-0.38***	**-0.31***	0.03	-0.02
	p value 0.17	**0.04**	**<.0001**	**0.0009**	0.81	0.9
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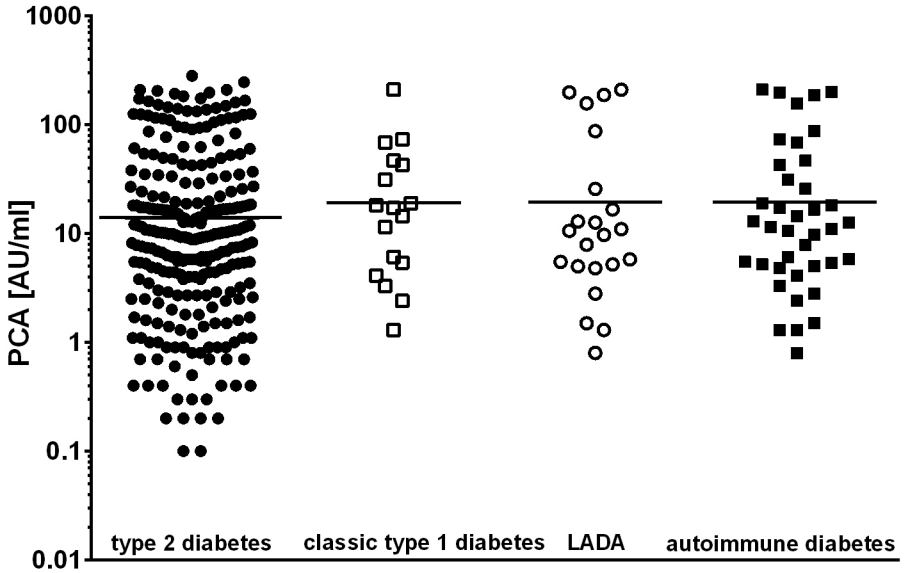
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0.1
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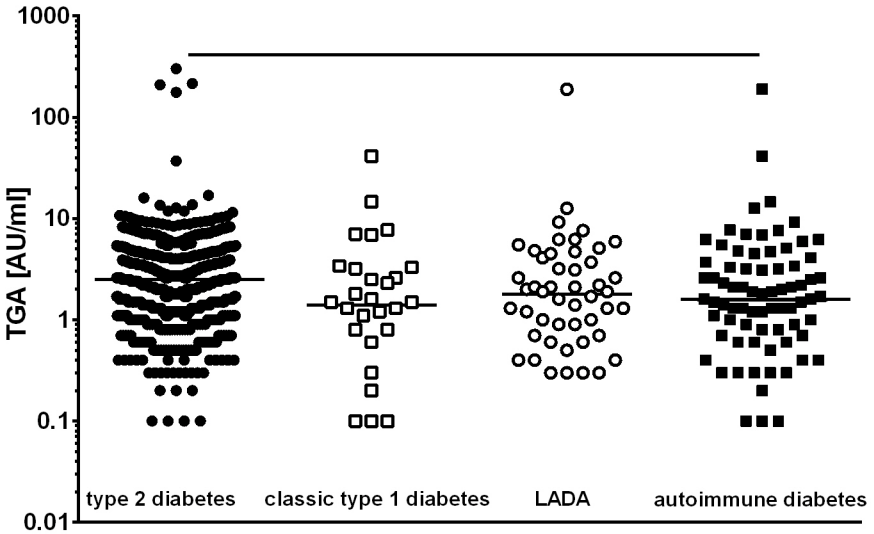
PCA [AU/ml]
type 2 diabetes
classic type 1 diabetes
LADA
autoimmune diabetes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TGA [AU/ml]
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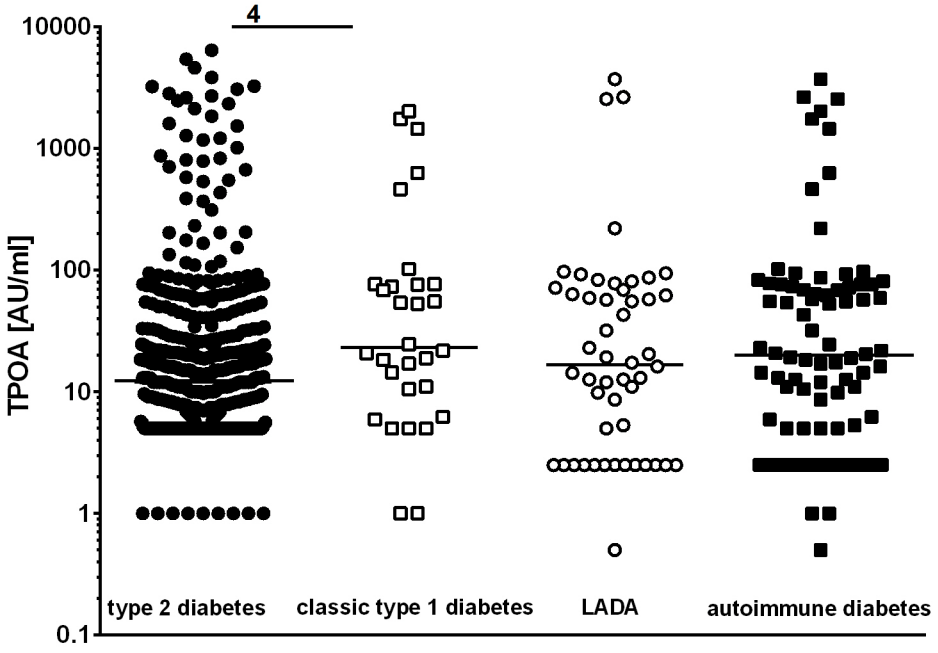
LADA
autoimmune diabetes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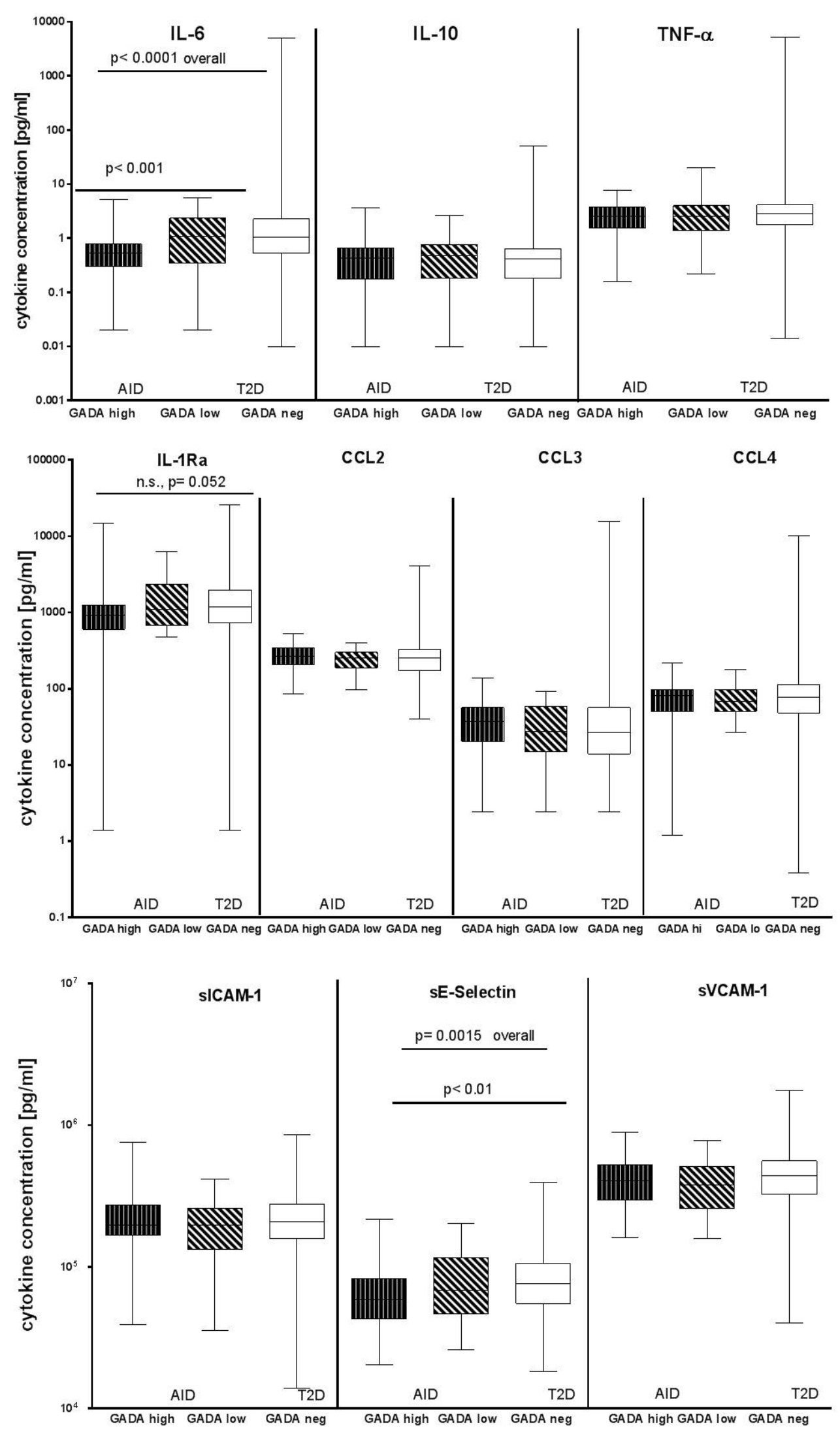
TPOA [AU/ml]
type 2 diabetes
classic type 1 diabetes
LADA
autoimmune diabetes


IL-6
p< 0.0001 overall
p< 0.001
IL-10
TNF-α
cytokine concentration [pg/ml]
10000
1000
100
10
1
0.1
0.01
0.001
AID
T2D
GADA high
GADA low
GADA neg
IL-1Ra
n.s., p= 0.052
CCL2
CCL3
CCL4
100000
GADA hi
GADA lo
sICAM-1
sE-Selectin
p= 0.0015 overall
p< 0.01
sVCAM-1
10^7
10^6
10^5
10^4

Positivity of islet directed antibodies	LADA n=50	Classic Type 1 diabetes n=30	AID n=80	p-value
Single ab positive	28/50 (56%)	16/30 (53.33%)	44/80 (55 %)	n.s.
Double ab positive	14/50 (28%)	9/30 (30%)	23/80 (28.75%)	n.s.
Triple ab positive	5/50 (10%)	3/30 (10%)	8/80 (10%)	**n.s.**
Quadruple ab positive	3/50 (6%)	2/30 (6.66%)	5/80 (6.25%)	**n.s.**

Supplement table 1

Data show the number and percentage of patients who are positive for one, two, three or four of the islet directed antibodies measured. LADA and classic type 1 diabetes patients did not differ statistically. ab, antibody; n.s., non-significant

Association with Sex	**LADA** N=50	**Classic Type 1 diabetes** N=30	**Type 2 diabetes** N=626
PCA	r = 0.16, p= 0.24	**r = - 0.47, p = 0.0056**	r= 0.09, p = 0.07
TPOA	r = 0.127, p = 0.37	r = - 0.09, p = 0.62	r = 0.09, p = 0.07
Age			
PCA	**r = 0.29, p = 0.038**	r = -0.13, p = 0.49	**r = 0.15, p = 0.0034**
TPOA	r = 0.100, p = 0.49	r = 0.15, p = 0.39	**r =-0.17, p = 0.0010**
BMI			
PCA	r = - 0.03, p = 0.82	**r = 0.374, p = 0.0318**	r = - 0.006, p = 0.91
TPOA	r = 0.08, P = 0.56	p = 0.16, p = 0.37	r = -0.06, p = 0.23

Supplement table 2

Association of sex, age and BMI with non-DAA concentrations. Shown are Spearman correlation R and respective p-values for BMI and age. Correlation analysis with sex was performed applying χ^2 test (R) analysis. Significant p-values in bold. P-values not corrected for multiple comparison. TGA were excluded from the analysis, as only very few patients were positive for TGA (1/50 LADA, 1/30 classic type 1 diabetes, 5/626 type 2 diabetes).