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Neuropeptide Y autoantibodies in patients with long-term type 1 and type 2 diabetes and neuropathy.

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Abstract

Aims: The neurotransmitter Neuropeptide Y (NPY) was previously reported as a minor autoantigen in newly diagnosed type 1 diabetes (T1D) patients. The single nucleotide polymorphism at rs16139 (T1128C, L7P) in the NPY gene was associated with an increased risk for the development of type 2 diabetes (T2D). We aimed to develop a radiobinding assay for NPY-L (Leucine) and NPY-P (Proline) autoantibodies (A) to study the levels and the association with other islet autoantibodies and neuropathy. Methods: Autoantibodies against NPY-L, NPY-P, ZnT8, GAD65 and IA-2 were studied in T1D (n=48) and T2D (n=26) patients with duration up to 42 and 31 years. A subgroup of T1D (n=32) patients re-examined, 5-8 years after first visit, were tested for peripheral (Z-score) and autonomic neuropathy (E/I ratio). Results: NPY-LA and NPY-PA were detected in 23% and 19% in T1D ($p<0.001$), and 12% and 23% in T2D patients ($p<0.001$) compared to 2.5% controls (n=398). The levels of NPYA declined during follow-up in the T1D patients ($p<0.001$). The neuropathy was not related to the NPYA or the other islet autoantibodies. Conclusions: Regardless of the absence of an association between NPYA and neuropathy, NPY may contribute to the pathogenesis of T1D and T2D as a minor autoantigen.

Keywords

1. Minor autoantigen
2. NPY autoantibodies
3. Autoimmunity
4. Neuropathy
5. Long-term diabetes

1. Introduction

Type 1 diabetes (T1D) is strongly associated with autoantibodies (A) against zinc transporter 8 (ZnT8A), glutamic acid decarboxylase 65 (GADA), insulinoma-associated antigen-2 (IA-2A) and insulin (IAA) (reviewed in [1]). Together or alone, they are important not only for classification of diabetes at the time of clinical onset, but also for the prediction of T1D in subjects at risk [2] [3] [4]. More than 90% of patients with T1D have multiple autoantibodies, and less than 5% of T1D patients were found negative for any of these four islet autoantibodies at clinical onset [5]. However, it has been suggested that islet autoantibody negative individuals may be positive for yet other beta-cell autoantigen-specific autoantibodies [5]. Neuropeptide Y (NPY) was previously reported as a minor autoantigen in newly diagnosed T1D patients [6].

NPY is a neurotransmitter widely expressed within the central and peripheral nervous systems affecting vasoconstriction, insulin secretion and food intake [7] [8]. NPY has also been demonstrated in islet cells and pancreatic nerve fibers [9]. Furthermore, a single nucleotide polymorphism (SNP) at rs16139 (T1128C) identified an amino acid substitution from Leucine (L) to Proline (P) (L7P) in the signal peptide [10]. The minor allele (P) correlated with elevated NPY levels in plasma [11] perhaps due to an increased biosynthesis [12] [13]. It was suggested that the L7P affects NPY secretion as a consequence of a modified secondary and tertiary structure [12]. It is therefore of interest that the minor allele was associated with atherosclerosis [14], impaired glucose tolerance [15] as well as with a three- to four-fold increased risk for Type 2 diabetes (T2D) [15] [16]. More importantly, the minor allele also increased the risk for retinopathy [14] and nephropathy [17] in patients with T2D.

Autonomic neuropathy in patients with T1D was associated with impaired NPY responses to insulin-induced hypoglycaemia [18]. As T1D neuropathy was also associated with GADA [19] [20], we tested the hypothesis that NPY autoantibodies (NPYA) may be present in T1D patients with neuropathy and that NPYA would be related to the rs16139 polymorphism.

The aims of this study were to 1) develop a radiobinding assay (RBA) for NPY-L (Leucine) and NPY-P (Proline) autoantibodies (NPYA) in controls and in patients with long-term T1D and T2D, 2) relate both NPY-LA and NPY-PA with autoantibodies against ZnT8R (Arginine), ZnT8W (Tryptophan), ZnT8Q (Glutamine), GAD65 and IA-2; and 3) study these autoantibody levels during follow-up of T1D patients tested for neuropathy.

2. Materials and Methods

2.1. Patient and control samples

A total of 100 patients, 1.5-18.8 years of age, with T1D diagnosed in 1996-2001 were studied (Table 1). Serum samples from 48 long-term T1D and 26 long-term T2D patients were collected in 1998 at the Clinic of Diabetes at Skåne University Hospital in Malmö, Sweden (Table 2). A subgroup of 32 patients with long-term T1D, first seen in 1998, was followed-up in 2005 (Table 2). Sera for control samples were obtained from 398 healthy blood donors at 19-81 years of age (Table 2).

2.2. Nerve function tests during follow-up

The long-term T1D patients underwent peripheral and cardiac autonomic nerve function tests during the follow-up in 2005 (n=31; Table 2). The peripheral nerve function (peripheral neuropathy) was evaluated by measurement of conduction velocity (CV) and amplitude (AMP) of the sural and peroneal nerves in the lower extremities and calculated as a mean expressed in age-corrected values [Z-score in standard deviations (SD)]. A composite Z-score leg, reflecting the nerve function in the leg, was calculated: (Z-score CV peroneal nerve + Z-score CV sural nerve + Z-score AMP sural nerve)/3. The summation of nerve conduction attributes has been used elsewhere [21]. The cardiac autonomic nerve test (autonomic neuropathy) of the parasympathetic vagal nerve function (E/I ratio) was also tested [22]. The E/I ratio was calculated as the mean of the longest R-R interval during expiration (E) divided by the mean of the shortest R-R interval during inspiration (I) and expressed in age-corrected values [Z-scores in SD]. E/I Z-score values less than −1.64 SD (95% confidence interval, one-sided test) below the age-related reference values were considered as abnormal [22].

2.3. Subcloning of pJ201prepro-NPY

Prepro-Neuropeptide Y (NPY) (aa 1-97) cDNA was inserted in a de novo synthesised pJ201 vector (2759 bp) (DNA 2.0, Menlo Park, CA, USA) with both restriction cloning sites of Xho1 and Not1 at the 5´ end and 3´end of the insert. The pJ201NPY cDNA (2 µg) was cut with two restriction enzymes, Xho1 and Not1 (FastDigest™, Fermentas, Helsingborg, Sweden). The NPY insert was subcloned into the pTnT™ vector (2871 bp) (Promega, Madison, WI, USA) and transformed using DH5α E.coli competent cells (Invitrogen AB, Stockholm, Sweden). The

pThNPY plasmid DNA was extracted using the QiaPrep Spin Miniprep Kit (QIAGEN AB, Solna, Sweden) according to manufacturer's instructions followed by sequencing using (3700/3730 BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) at the Region Skånes Competence Centre (RSKC) in Malmö, Sweden.

2.4. Generation of pTnTprepro-NPY SNP *T1128C*-Proline variant

Both forward and reverse primers were designed according to manufacturer's instructions (DNA Technology, Risskov, Denmark). A Phusion™ site directed mutagenesis Kit (Thermo Fisher Scientific, Leicestershire, UK) was used to obtain Proline (P) in substitution for Leucine (L) (L7P) of the prepro-NPY insert. The pThNPY-P variant was transformed in DH5α E.coli competent cells prior plasmid DNA extraction and sequencing as previously described.

2.5. Radiolabeling of prepro-NPY protein products

Both pThNPY-L and pThNPY-P (1µg each) were subjected to *in vitro* coupled transcription translation to generate ^{35}S-methionine labeled human recombinant proteins (^{35}S-met, 500 uCi, PerkinElmer, Waltham, MA, USA) using the TNT® SP6 Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) in a 100 µL reaction mix for 90 min at 30 ^{0}C degrees during shaking (300 rpm). The translation mix was separated by gel filtration on Illustra™ NAP-5 Columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by adding antigen buffer (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 0.15%, Tween20, 0.1% BSA) in various volumes from 100-1500 µL in eleven steps. Prior gel filtration, columns were washed and equilibrated with antigen buffer. The amount of ^{35}S-met incorporated in both ^{35}S-met NPY-L and ^{35}S-met NPY-P was determined (1450 MicroBeta Counter, Perkin Elmer). The ^{35}S-met NPY-L and ^{35}S-met NPY-P protein products were stored up to one month at -80 ^{0}C degrees.

2.6. SDS-polyacrylamide gel electrophoresis

^{35}S-met NPY protein products were verified by 16% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (ClearPage™, C.B.S Scientific, Delmar, CA, USA) described elsewhere [23].

2.7. NPY autoantibody analysis

Serum samples were analyzed for autoantibodies against NPY-L and NPY-P using a standard RBA. In duplicates, 5 µL of serum sample was mixed with 60 µL of either ^{35}S-met NPY-L or ^{35}S-met NPY-P protein products to a final concentration of 425 ± 25 cpm/µL by dilution with antigen buffer in a 96-well plate. The reaction mix (antigen-antibody solution) was incubated over night at 4 °C with constant shaking. Cold washing buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 0.15% Tween 20) of 200 µL per plate were prepared on the first day. 200 µL of antigen buffer was added to each well of a 96-well filter plate and incubated at room temperature over night to block unspecific binding. The following day, the filter plates were emptied and 50 µL of the reaction mix was added to 20% v/v Protein A Sepharose (PAS, Invitrogen) in the filter plate and incubated for 1 h at 4 °C with constant shaking (Perkin Elmer, Waltham, MA, USA). The filter plate was washed eight times with washing buffer using a ELx50 Microplate Strip Washer (Biotek, Winooski, VT, USA). Antibody-bound radioactivity was analyzed in a β-counter (1450 MicroBeta Counter, Perkin Elmer). PAS-bound radioactivity was converted into in-house units (U) using a polyclonal rabbit IgG antibody against full length human NPY (Abcam, Cambridge Science Park, Cambridge, UK) in seven doubling dilution steps as a standard for both NPY antigens. The binding of both ^{35}S-met NPY-L and ^{35}S-met NPY-P to the standard IgG was displaced in a concentration-dependent manner by both cold NPY-L and NPY-P (Supplement Figure 1) generated by *in vitro* transcription translation as described [24]. Patient and control samples were analysed for autoantibodies in parallel on the same plate to reduce assay variances.

2.8. ZnT8, GAD65, IA-2 autoantibody analyses

The RBA for each of individual ZnT8 autoantibody (ZnT8RA, ZnT8WA, ZnT8QA) was carried out as described [25]. Autoantibodies against GAD65 and IA-2 were similarly analyzed as described previously [26].

2.9. Statistical analysis

Quantile-quanitle (Q-Q) analysis was used for the quantiles of the autoantibody distribution against the quantiles of a standard distribution. Pearson Chi-square (and two-sided Fisher´s exact test and Yate´s correction for continuity value when applied) was used to assess differences of autoantibody frequency in un-paired groups, and McNemar test in paired groups. Mann-Whitney U-test was used for calculation of differences in autoantibody levels and Wilcoxon signed ranks

test for change in autoantibody levels over time. Spearman two-tailed correlation test was used for correlation analysis. Agreement of islet autoantibodies was calculated with Kappa statistics. A value of one indicated perfect agreement, whereas a kappa of zero was what would be expected by chance and negative values a systematic disagreement between the observers. A p-value of <0.05 was considered significant. IBM SPSS Statistics 18.0 was used for the statistical calculations.

Interpretation of kappa agreement:
<0 Less than chance agreement
0.01-0.20 Slight agreement
0.21-0.40 Fair agreement
0.41-0.60 Moderate agreement
0.61-0.80 Substantial agreement
0.81-0.99 Almost perfect agreement

3. Results

3.1. NPY and islet autoantibodies in controls, type 1 and type 2 diabetes patients

In the newly diagnosed T1D patients (n=100) NPY-LA or NPY-PA showed a frequency of 29% for both autoantigens (Table 1). NPY-LA and NPY-PA double positives were found in 20% of the newly diagnosed patients.

The long-term T1D (n=48) patients were diagnosed at 15-26 years of age compared to the T2D (n=26) patients diagnosed at 31-50 years of age (Table 2). Duration of the disease was comparable between the two groups of patients. However, while all patients with long-term T1D had low level of C-peptide, the long-term T2D patients showed a marked variability (Table 2). A total of 32 T1D patients were re-examined after 21-50 years of disease (Table 2). The ^{35}S-met labeled NPY-L and NPY-P generated by *in vitro* transcription translation had the expected molecular mass of Mr 10.8 (Figure 1). NPY-LA and NPY-PA levels in serum from healthy controls were normally distributed. The cut-off levels at the 97.5th percentile were 44 U/mL for NPY-LA and 32 U/mL for NPY-PA (Figure 2, panels A-B). The Q-Q plots of the long-term 48 T1D patients for NPY-LA and NPY-PA, respectively, deviated from a normal distribution (Figure 2, panel C). The frequency at baseline in the long-term T1D patients was 23% for NPY-LA ($p<0.001$) and 19% for NPY-PA ($p<0.001$) compared to controls (Table 3). There were 6/48 (12.5%) NPY-LA and NPY-PA double positive T1D patients (Figure 2, panel D). The corresponding frequency in the patients with T2D were 12% for NPY-LA ($p=0.039$) and 23% for

NPY-PA (p< 0.001) as compared to the controls (Table 3). NPY-LA and NPY-PA double positive T2D were 3/26 (11.5%).

The median levels of the two NPYA were different from the controls in both the T1D (NPY-LA: p<0.001 and NPY-PA: p<0.001, respectively) and the T2D patients (NPY-LA: p=0.05 and NPY-PA: p<0.001) (Figure 3, panel a). The titers of the two NPYA variants correlated in both the T1D (R^2=0.547, p<0.001) (Figure 2, panel D) and in T2D patients (R^2=0.802, p<0.001) (data not shown).

Compared to the controls, the frequencies of the ZnT8A variants were 40% for ZnT8RA (p<0.001), 40% for ZnT8WA (p<0.001) and 35% for ZnT8QA (p<0.001) in the T1D and 15% (p=0.004), 12% (p=0.031) and 12% (p=0.031), respectively, in T2D patients (Table 3). The median levels of all ZnT8A variants (ZnT8RA, ZnT8WA, ZnT8QA) were higher in long-term patients with both T1D (ZnT8RA, p<0.001; ZnT8WA, p<0.001; ZnT8QA, p<0.001) and in T2D (ZnT8RA, p<0.001; ZnT8WA, p<0.001; ZnT8QA, p<0.001) compared to the controls (Figure 3, panel B).

GADA and IA-2A were detected in 52% (p<0.001) and in 31% (p<0.001), respectively, of the long-term T1D patients compared to the controls (Table 3) but were negative in all T2D patients. However, the median levels including also the samples below the cut-off of both GADA and IA-2A were higher in both patient groups compared to the controls (T1D: GADA p<0.001, IA-2A p<0.001; T2D; GADA p<0.001, IA-2A p=0.02) (Figure 3, panel C).

3.2. Combinations between NPYA and islet autoantibodies at baseline

We tested whether NPY-LA, NPY-PA, or both, occurred more often in patients with any of the ZnT8A variants, GADA or IA-2A. At baseline, neither of the single NPYA variants nor both of them taken together, appeared more often with ZnT8RA, ZnT8WA, ZnT8QA, GADA or IA-2A in patients with long-term T1D (Table 4). However, in 3/6 (50%) of T2D patients NPYA were double positive with both ZnT8RA (p=0.028) and ZnT8WA (p=0.008). NPY-PA showed a substantial agreement with ZnT8WA (kappa 0.61, p=0.001) and a moderate agreement with ZnT8RA (kappa 0.51, p=0.001) in the T2D patients (Table 4). Among the NPY-LA positive T1D patients 6/11 (55%) were also positive for NPY-PA (p=0.002). The two NPYA variants showed a moderate agreement (kappa 0.50, p=0.001) in T1D and a substantial agreement (kappa 0.61, p=0.001) in T2D patients (Table 4).

3.3. Number of islet autoantibodies at baseline

Among the autoantibody positive (41/48, 85%) patients with long-term T1D, there were 25/48 (52%) positive for at least one ZnT8A variant, and 12/48 (25%) positive for all three ZnT8A (Table 3). Most of the T1D patients, 13/48 (27%) had one autoantibody, although 9/48 (19%) were positive for four autoantibodies (Table 5). One patient with T1D had all seven autoantibodies whereas one had NPY-LA only. Most of the T2D patients (18/26, 69%) were negative for all autoantibodies.

3.4. NPY and islet autoantibodies during follow-up of long-term T1D patients

The subgroup of 32 patients with long-term T1D was analyzed after duration of 21-50 years and 5-8 years after baseline (Table 2). In general, 27/32 (84%) initially autoantibody positive T1D patients, 17/32 (53%) remained autoantibody positive ($p=0.002$) during follow-up. The NPY-LA frequency decreased from 9/32 (28%) to 0/32 (0%) and for NPY-PA from 5/32 (16%) to 0/32 (0%) (Table 6). The median levels of NPY-LA declined from 34 U/mL to 21 U/mL ($p<0.001$) (Figure 4, panel A) and of NPY-PA from 20 U/mL to 12 U/mL ($p<0.001$) (Figure 4, panel B). NPY-LA and NPY-PA levels correlated ($R^2=0.446$; $p=0.011$) at baseline, however this correlation was essentially lost ($R^2=0.348$; $p=0.051$) at follow-up.

In the same group of 32 patients, the frequency of ZnT8A decreased from 14/32 (44%) to 4/32 (13%). Also, the median levels of the ZnT8A variants were lower at the time of follow-up (ZnT8RA, $p<0.001$; ZnT8WA, $p<0.001$; ZnT8QA, $p<0.001$) compared to baseline (Figure 5, panels A-C).

The frequency of GADA ($p=0.687$) and IA-2A ($p=1.0$) did not change between baseline and follow-up (Table 6). The levels of GADA ($p=0.005$) and IA-2A ($p<0.001$) declined during follow-up (Figure 5, panels D-E).

It was also tested if NPY-LA and NPY-PA correlated to any of the islet autoantibodies in the 32 T1D patients. The levels of NPY-LA did not correlate with any of the ZnT8A variants (ZnT8RA, $R^2=0.341$, $p=0.056$; ZnT8WA, $R^2=0.307$, $p=0.087$; ZnT8QA, $R^2=0.329$, $p=0.066$) at baseline, although there were significant correlations at follow-up (ZnT8RA, $R^2=0.376$; $p=0.034$; ZnT8WA, $R^2=0.390$, $p=0.027$; ZnT8QA, $R^2=0.593$, $p<0.001$). The levels of NPY-PA correlated with the levels of ZnT8WA ($R^2=0.546$, $p=0.001$) and ZnT8QA ($R^2=0.524$, $p=0.002$), but not

with ZnT8RA (R^2=0.287, p=0.111) at baseline. At follow-up, the correlation between the NPY-PA levels with ZnT8WA (R^2=0.395, p=0.025) and ZnT8QA (R^2=0.364, p=0.040) variants remained (ZnT8RA, R^2=0.302, p=0.093). The levels of the two NPYA did not correlate with GADA or IA-2A at baseline or at follow-up.

3.5. NPYA and neuropathy in re-examined T1D patients

Peripheral neuropathy was related to cardiac autonomic neuropathy (R^2=0.328; p<0.001) in the long-term follow-up patients with T1D (Figure 6). It was observed that males (12/14; 86%), more often had an abnormal cardiac autonomic neuropathy (cut-off at -1.64) compared to females (2/14; 14%) (p=0.007). Taking these gender differences into account, we also addressed the question whether peripheral, autonomic, or both, neuropathies in the 32 T1D patients re-examined after 5-8 years were associated to any of the autoantibodies. Neither peripheral nor cardiac autonomic neuropathy was related to the presence or levels of the NPYA nor was there a correlation with ZnT8A, GADA or IA-2A.

4. Discussion

In this study, we established an autoantibody radiobinding assay to assess the levels and frequency of two NPY autoantibody variants (NPY-LA and NPY-PA) in patients with T1D and T2D compared to controls. The major findings were as follows. First, newly diagnosed T1D patients had 29% NPY-LA alone, 29% NPY-PA alone but 20% being double positive. Second, long duration T1D patients had 23% and 19% compared to 12% and 23%, respectively, in the patients with T2D using the control 97.5th percentile as cut-off. Previously, NPY-LA was reported in 9% of T1D patients newly diagnosed below the age of 19 years [6]. It was therefore of interest that the present group of patients not only were diagnosed as adults but also had disease duration of median 26 years at the time when the first samples were obtained (referred to as baseline). More remarkably, at follow-up the levels had decreased significantly. These findings were comparable to GADA, IA-2A and the ZnT8A, which showed decreased levels during follow-up despite the lack of correlation between the levels of any of the two NPYA with GADA and IA-2A. The loss of autoantibodies over time in the follow-up samples may be due to both storage and an unknown number of freezing and thawing cycles.

Other studies of newly diagnosed patients with T1D in active follow-up [27] will be needed to determine if NPYA will decrease rapidly after diagnosis such as IA-2A and ZnT8A or slowly similar to GADA [28]. It should also be noted that the present group of long-term T1D patients had frequencies of GADA, IA-2A and ZnT8A at baseline ranging between 31-52%.

Another major finding was the comparable frequencies of NPYA among the patients with T2D. These patients were also long-term and their duration amounted to an average of 23 years. The NPYA in this group of patients may be easily explained by the possibility that at least some of these patients might have had autoimmune diabetes (LADA) as opposed to T2D. In the event that LADA would be the classification, none of the patients would qualify as neither GADA nor IA-2A were found among the T2D patients. If the ZnT8A would allow these patients to be classified as LADA, 23% of our T2D patients would be classified with LADA. The frequency of ZnT8A in LADA patients has been reported to vary between 2% [29], 34% [30] and 42% [31]. Our observation that the NPY-PA variant correlated to ZnT8RA and ZnT8WA in the T2D patients (Table 4) is of interest for two reasons. The first is that ZnT8A in addition to GADA and IA-2A may also be a marker of autoimmune diabetes or LADA [32]. The second is that the co-occurrence of NPYA and ZnT8A would further support the notion that also long-term 56-73 year old T2D patients may have autoimmune diabetes [32]. The results of our study demonstrate that NPY is not necessarily a minor autoantigen to T1D, rather that it may be related to autoimmune diabetes. Further studies are needed to clarify to what extent NPYA and ZnT8A tend to occur together in newly diagnosed diabetes patients classified with either T1D or T2D.

To our knowledge this is the first study to investigate two different NPY autoantibody variants in patients with long-term T1D and T2D. Although the appearance of islet autoantibodies prior to the clinical onset of T1D have made it possible to predict disease, it is still of interest to establish to what extent islet autoimmunity may be related to late diabetes complications. It should be noted that the T1D patients in the present study were primarily investigated with a second hypothesis in mind namely to test whether NPYA are related to neuropathy. As a response to nerve injury, NPY increases in different regions of the nervous system [33] [34] [35]. Also, it has been reported that the NPY levels [36] and the release [37] are increased in the hypothalamic nuclei of streptozotocin-induced diabetic rats. However, in response to insulin-induced hypoglycemia the NPY expression is instead lost in patients with autonomic neuropathy [18]. On

the other hand, duration [38] and type [39] of diabetes or chronic hyperglycemia [40] have been suggested to be strongly related to the risk and degree of neuropathy in the lower extremities.

The appearances of islet antibodies GADA, IA-2A and ZnT8A predict beta-cell failure in T1D, while in T2D, where they are less common, have a higher preserved beta-cell function [41]. Yet, the possible relevance of autoantibodies in neuropathy is less known. It has been reported that increased GADA levels at onset were associated with retinopathy in patients with T1D [42]. In addition, higher GADA levels were found in T1D patients with retinopathy and nephropathy compared to T1D patients without complications, which may suggest GADA as a complication predictor [43]. Also, T1D patients with higher GADA levels was reported to have a slower motor nerve conduction velocity compared to T1D patients with lower GADA levels [19]. The ability to predict peripheral and autonomic neuropathy by studying the correlation with the islet autoantibody levels and frequency in patients with T1D was also investigated in the present study. Neither of the peripheral nor the autonomic neuropathy correlated to the presence of the autoantibodies in the patients. There was a clear difference of the abnormal autonomic neuropathy between genders, but this difference was not related to any of the islet autoantibodies, including NPY-LA or NPY-PA. This lack of association may be due to the small number of T1D patients (n=32). Thus, we cannot conclude that the presently used autoantibodies can predict presence of peripheral or autonomic neuropathy in T1D. We noted that 6/26 T2D patients (5/6 were overweight or obese, data not shown) had at least one of the NPYA variants. Similarly, ZnT8A were also positive in 5/6 overweight or obese T2D. Although there was no relation between NPYA (or ZnT8A) and neuropathy in the T1D patients, it cannot be excluded that there may be such a relation in T2D. Unfortunately, we did not have any neuropathy data of the T2D patients. Further studies are warranted to investigate this possibility.

It is known that the ZnT8 transporter is targeted by three ZnT8A variants dependent on the T2D associated SNP rs13266634 at the amino acid at position 325 [44] [45] [26]. Previously, it was indicated that the structure of the mature NPY protein is dependent on the amino acid residue at SNP rs16139 [12] resulting in an altered packaging and secretion of NPY [13]. The genetic polymorphisms of the patients in this study were not known. It would be of interest to determine if autoantibodies against NPY are related to the L7P genetic polymorphism. Our kappa analysis suggests that there was substantial agreement between the two NPYA variants, which suggest that NPY L7P polymorphisms may not be as strong as the ZnT8 R325W polymorphism [24] to

determine a crucial epitope. In order to detect specific serum autoreactivity against either NPY-P or NPY-L protein, displacement assays with a cold inhibitor was used. However, specific sera were rare and further analysis of large number of sera will be needed to search for possible isoform-specific sera. It cannot be excluded that individuals may have such autoantibodies.

5. Conclusions

Autoantibodies against both NPY-L and NPY-P were present in long-term T1D and T2D patients, and the NPYA levels declined during follow-up. Neither the frequency nor the titers of the NPYA variants were related to peripheral or cardiac autonomic neuropathy in the T1D patients. We conclude that NPY fulfills the criteria for a minor autoantigen in T1D. It cannot be excluded that NPY autoimmunity may contribute to the pathogenesis in long-term T1D, perhaps also in T2D.

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Conflict of interests

Declaration of competing interest: Nothing to declare.

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Table 1. Frequency of NPY-L (Leucine), NPY-P (Proline), ZnT8R (Arginine), ZnT8W (Tryptophan), ZnT8Q (Glutamine), GAD65 and IA-2 autoantibodies in newly diagnosed T1D (n=100) patients compared to controls (n=398).

	T1D n=100 (100%)	**Controls n=398 (%)**	***p***
Males, n (%)	58 (58)	66 (263)	
Age – median (range) years	N.A.	44 (19-81)	
Age at diagnosis – median (range) years	9.8 (1.5-18.8)	N.A.	
NPY-LA	29 (29)	10 (3)	p<0.001
NPY-PA	29 (29)	8 (2)	p<0.001
NPYA (1 ≥)	38 (38)	12 (3)	p<0.001
NPY-LA only	1 (1)	1 (0.03)	N.A.
NPY-PA only	0 (0)	1 (0.03)	N.A.
ZnT8RA	48 (48)	8 (2)	p<0.001
ZnT8WA	50 (50)	9 (2)	p<0.001
ZnT8QA	34 (34)	9 (2)	p<0.001
ZnT8A (1 ≥)	68 (68)	14 (4)	p<0.001
GADA	64 (64)	8 (2)	p<0.001
IA-2A	71 (71)	4 (1)	p<0.001
Autoantibody negative	4 (4)	367 (92)	p<0.001

Differences of autoantibody frequency between patients and controls were considered significant when p-value of <0.05 was applied.

Abbreviations: NPY-LA, Neuropeptide Y-Leucine autoantibodies; NPY-PA, Neuropeptide Y-Proline autoantibodies; N.A. is not applicable.

Table 2. Demographic data of patients with long-term Type 1 (n=48) diabetes (T1D) and Type 2 (n=26) diabetes (T2D) at baseline compared to healthy controls (n=398), and long-term T1D patients (n=32) at follow-up.

BASELINE	**T1D**	**T2D**	**Controls**
Subjects, n	48	26	398
Males, n (%)	29 (60)	24 (92)	263 (66)
Age – median (range) years	46 (30-67)	63 (56-73)	43 (19-81)
Age at onset – median (range) years	20 (15-26)	42 (31-50) [a]	N.A.
Duration – median (range) years	26 (13-42)	23 (10-31)	N.A.
HbA1c – median, range mmol/mol (%)	66, 42-117 (8.2, 6.0-12.9) [b]	N.A.	N.A.
C-peptide - median (range)	<0.10 (<0.10-0.18)	0.61 (0.11-1.32) [a]	N.A.
FOLLOW-UP			
Subjects, n	32	N.A.	398
Males, n (%)	19 (59)	N.A.	263 (66)
Age at onset – median (range) years	19 (15-26)	N.A.	N.A.
Duration – median (range) years	33 (21-50)	N.A.	N.A.
HbA1c – median, range mmol/mol (%)	67, 42-93 (8.3, 6.0-10.7)	N.A.	N.A.
C-peptide - median (range)	<0.1 (<0.1-0.18)	N.A.	N.A.
Composite Z-score leg – median (range)	-2.8 (-7.0- -0.5) [c]	N.A.	N.A.
E/I Z-score – median (range)	-1.5 (-2.5-07)	N.A.	N.A.
Abnormal E/I ratio (< -1.64), n (%)	14 (44)	N.A.	N.A.

The Z-score reflects the peripheral nerve function in the leg (peroneal and sural nerves) and expressed in age-corrected values. The E/I Z-score reflects the parasympathetic vagal nerve function and expressed in age-corrected values. E/I Z-score values less than −1.64 SD (95% confidence interval, one-sided test) below the age-related reference values were considered abnormal.

Abbreviations: T1D, Type 1 diabetes; T2D, Type 2 diabetes; N.A. is not applicable. Number of patients is indicated as followed; a=25; b=27; c=31.

Table 3. Frequency of NPY-L (Leucine), NPY-P (Proline), ZnT8R (Arginine), ZnT8W (Tryptophan), ZnT8Q (Glutamine), GAD65 and IA-2 autoantibodies in long-term T1D (n=48) and T2D (n=26) patients at baseline compared to the controls (n=398).

	T1D n=48 (%)	**T2D n=26 (%)**	**Controls n=398 (%)**	***p***
NPY-LA	11 (23)	3 (12)	10 (3)	*p<0.001 **p=0.039
NPY-PA	9 (19)	6 (23)	8 (2)	*p<0.001 **p<0.001
NPYA (1 ≥)	14 (29)	6 (23)	12 (3)	*p<0.001 **p<0.001
NPY-LA only	1 (2)	0 (0)	1 (0.03)	*N.A. **N.A.
NPY-PA only	0 (0)	3 (12)	1 (0.03)	*N.A. **N.A.
ZnT8RA	19 (40)	4 (15)	8 (2)	*p<0.001 **p=0.004
ZnT8WA	19 (40)	3 (12)	9 (2)	*p<0.001 **p=0.031
ZnT8QA	17 (35)	3 (12)	9 (2)	*p<0.001 **p=0.031
ZnT8A (1 ≥)	25 (52)	6 (23)	14 (4)	*p<0.001 **p<0.001
GADA	25 (52)	0 (0)	8 (2)	*p<0.001 **N.A.
IA-2A	15 (31)	0 (0)	4 (1)	*p<0.001 **N.A.
Autoantibody negative	7 (15)	18 (69)	367 (92)	*p<0.001 **p<0.001

P-values are shown as *T1D patients compared to controls and **T2D patients compared to controls. Differences of autoantibody frequency between patients and controls were considered significant when p-value of <0.05 was applied.

Abbreviations: NPY-LA, Neuropeptide Y-Leucine autoantibodies; NPY-PA, Neuropeptide Y-Proline autoantibodies; N.A. is not applicable.

Table 4. Agreement of NPY-L (Leucine) and NPY-P (Proline) autoantibodies with ZnT8, GAD65 and IA-2 islet autoantibodies to mark autoimmunity in long-term T1D and T2D patients at base-line.

T1D patients (n=48)				**T2D patients (n=26)**			
Antibody1-Antibody2	**Rank[a]**	**kappa**	***p***	**Antibody1-Antibody2**	**Rank[a]**	**kappa**	***p***
NPY-LA - NPY-PA	1	0.50	0.001	NPY-LA - NPY-PA	1	0.61	0.001
NPY-PA - ZnT8RA	2	0.23	0.065	NPY-PA - ZnT8WA	2	0.61	0.001
NPY-LA - ZnT8QA	3	0.21	0.131	NPY-PA - ZnT8RA	3	0.51	0.007
NPY-PA - ZnT8QA	4	0.19	0.161	NPY-PA - ZnT8QA	4	0.34	0.057
NPY-LA - ZnT8RA	5	0.16	0.248	NPY-LA - ZnT8WA	5	0.25	0.209
NPY-LA - ZnT8WA	5	0.16	0.248	NPY-LA - ZnT8QA	5	0.25	0.209
NPY-PA - ZnT8WA	7	0.14	0.277	NPY-LA - ZnT8RA	6	0.18	0.360
NPY-LA - IA-2A	8	0.06	0.677	NPY-LA -GADA		N.A.	
NPY-PA - IA-2A	9	0.02	0.881	NPY-LA - IA-2A		N.A.	
NPY-PA -GADA	10	-0.06	0.611	NPY-PA -GADA		N.A.	
NPY-LA -GADA	11	-0.06	0.616	NPY-PA - IA-2A		N.A.	

A P-value < 0.05 was considered significant, a= ranked by agreement to mark autoimmunity (kappa).

Abbreviations: NPY-LA, Neuropeptide Y-Leucine autoantibodies; NPY-PA, Neuropeptide Y-Proline autoantibodies; N.A. is not applicable.

Table 5. The number of autoantibodies in sera from long-term T1D (n=48), T2D patients (n=26) and in the healthy controls (n=398) detected by radioligand binding assay at baseline, respectively.

Subjects	Number of autoantibodies, n (%)								Total
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	
T1D	7 (15)	13 (27)	10 (21)	2 (4)	9 (19)	3 (6)	3 (6)	1 (2)	48 (100)
T2D	18 (69)	2 (8)	4 (15)	0 (0)	1 (4)	1 (4)	0 (0)	0 (0)	26 (100)
Controls	367 (92.2)	16 (4)	10 (2.5)	2 (0.5)	1 (0.3)	0 (0)	2 (0.5)	0 (0)	398 (100)

Table 6. The autoantibody frequency of the NPY-L (Leucine), NPY-P (Proline), ZnT8R (Arginine), ZnT8W (Tryptophan), ZnT8Q (Glutamine), GAD65 and IA-2 autoantibodies in the subgroup (n=32) of long-term T1D patients at baseline (duration range 13-42 years) and at follow-up 5-8 years after (duration range 21-50 years) baseline.

T1D patients, n (%)	**32 (100)**		
Autoantibodies n (%)	**Baseline**	**Follow-up**	***p***
NPY-LA	9 (28)	0 (0)	N.A.
NPY-PA	5 (16)	0 (0)	N.A.
ZnT8RA	13 (41)	3 (9)	p=0.002
ZnT8WA	14 (44)	3 (9)	p=0.001
ZnT8QA	13 (41)	1 (3)	p<0.001
GADA	14 (44)	12 (38)	p=0.687
IA-2A	9 (28)	8 (25)	p=1.0

Differences of autoantibody frequency between baseline and follow-up were considered significant when p-value of <0.05 was applied.

Abbreviations: NPY-LA, Neuropeptide Y-Leucine autoantibodies; NPY-PA, Neuropeptide Y-Proline autoantibodies; N.A. is not applicable.

Legends to Figures

Figure 1. ^{35}S-methionine labeled NPY-L (Leucine) and NPY-P (Proline) proteins (expected Mr 10.8) generated by *in vitro* transcription and translation as analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).

Figure 2. The healthy control (n=398) levels in quantile-quantile (Q-Q) plots showed that the cut-off at 97.5th percentile was 44 U/mL for NPY-L (Leucine) (panel A) and 32 U/mL for NPY-P (Proline) (panel B) autoantibodies. The cut-off levels (dotted line) were used to distinguish NPY-L (■) and NPY-P (●) autoantibody positive T1D patients (n=48) from negative T1D patients (panel C). At baseline, 6/48 (12.5%) long-term T1D patients were positive for both NPY autoantibody variants (filled symbol in the upper right square) in panel D). Also, the titers of the NPY autoantibodies correlated (R^2=0.547, p <0.001). A p-value of <0.05 was considered significant.

Figure 3. Box plots of autoantibody levels of NPY-L (Leucine) and NPY-P (Proline) (panel A), ZnT8R (Arginine), ZnT8W (Tryptophan) and ZnT8Q (Glutamine) (panel B) and GAD65 and IA-2 (panel C) in long-term 48 T1D (▩) and 26 T2D (■) patients compared to 398 healthy controls (□). A p-value of <0.05 was considered significant.

Figure 4. In the subgroup of long-term T1D patients (n=32) analyzed during follow-up (duration 21-50 years), 5-8 years after baseline, the median levels of NPY-LA (panel A) declined from 34 U/mL to 21 U/mL (p<0.001) and of NPY-PA (panel B) from 20 U/mL to 12 U/mL (p<0.001). A p-value of <0.05 was considered significant.

Figure 5. In the subgroup of long-term T1D patients (n=32) analyzed during follow-up (duration 21-50 years), 5-8 years after baseline, the median levels of ZnT8A, GADA and IA-2A declined demonstrated in panel A) ZnT8RA from 33 U/mL to 17 U/mL (p<0.001), panel B) ZnT8WA from 25 U/mL to 14 U/mL (p<0.001) and panel C) ZnT8QA from 52 U/mL to 28 U/mL (p<0.001). The median levels of GADA (panel D) declined from 33 U/mL to 20 U/mL (p=0.005) and of IA-2A (panel E) from 3 U/mL to 2 U/mL (p<0.001). A p-value of <0.05 was considered significant.

Figure 6. Correlation between cardiac autonomic Z-score for E/I ratio (mean of the longest R-R interval during expiration (E) divided by the mean of the shortest R-R interval during inspiration (I) expressed in age-corrected values) and peripheral (sural and peroneal function) composite Z-score were analyzed in the subgroup (n=32) of long-term T1D patients. The dotted line represents the cut-off (−1.64) for abnormal cardiac autonomic neuropathy calculated as E/I ratio. The solid line represents the correlation (R^2=0.328, p<0.001) between the E/I ratio and the peripheral Z-score composite. A p-value of <0.05 was considered significant.

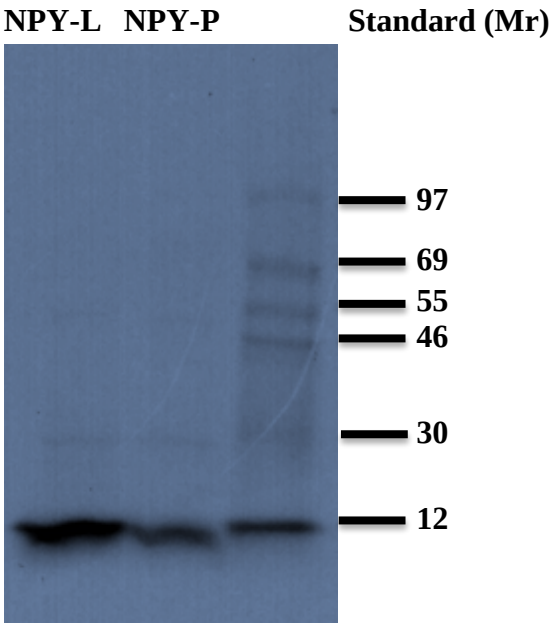

Figure 1.

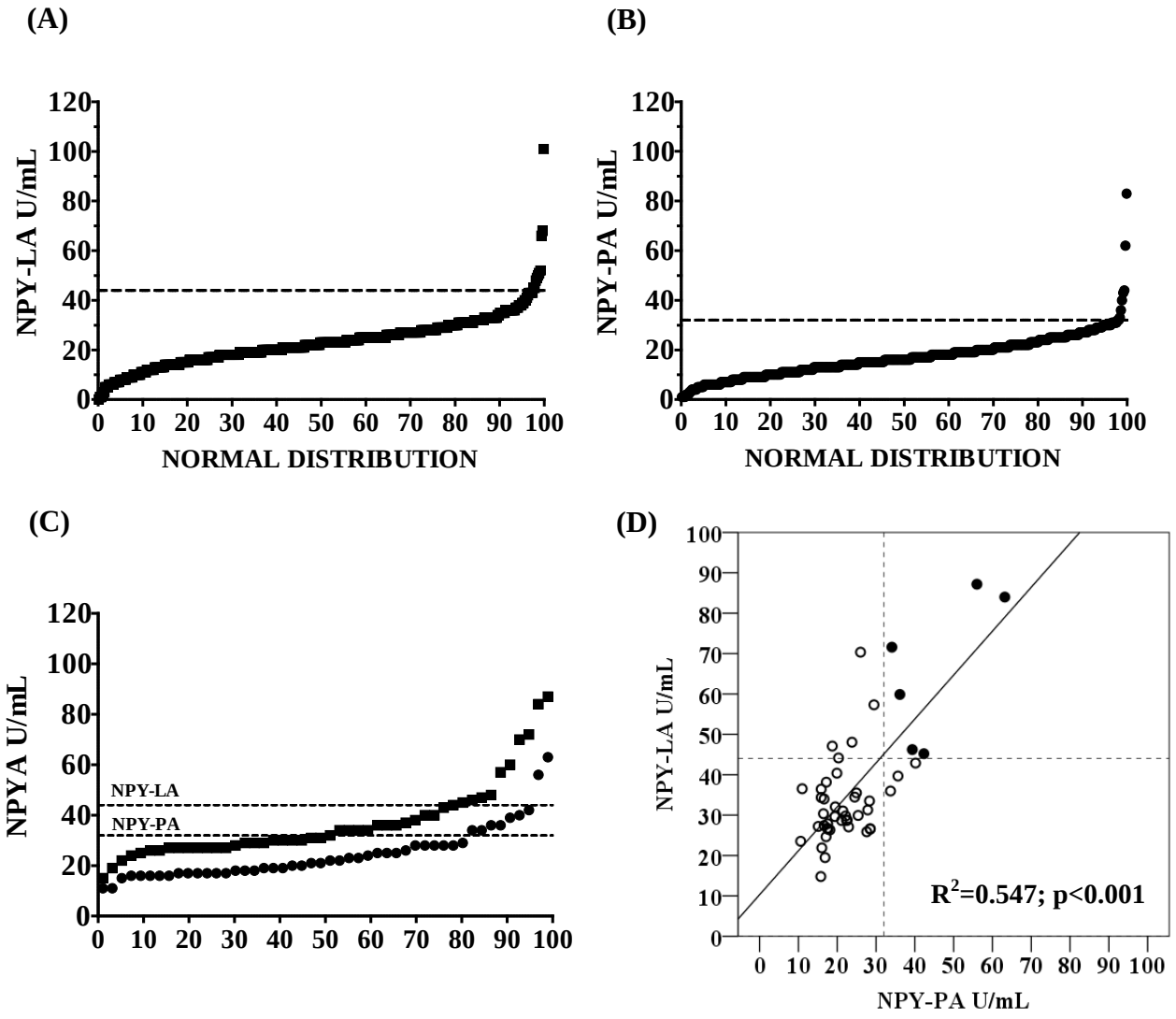
(A)
NPY-LA U/mL
NORMAL DISTRIBUTION
(B)
NPY-PA U/mL
NORMAL DISTRIBUTION
(C)
NPYA U/mL
NPY-LA
NPY-PA
(D)
NPY-LA U/mL
NPY-PA U/mL
R2=0.547; p<0.001

Figure 2.

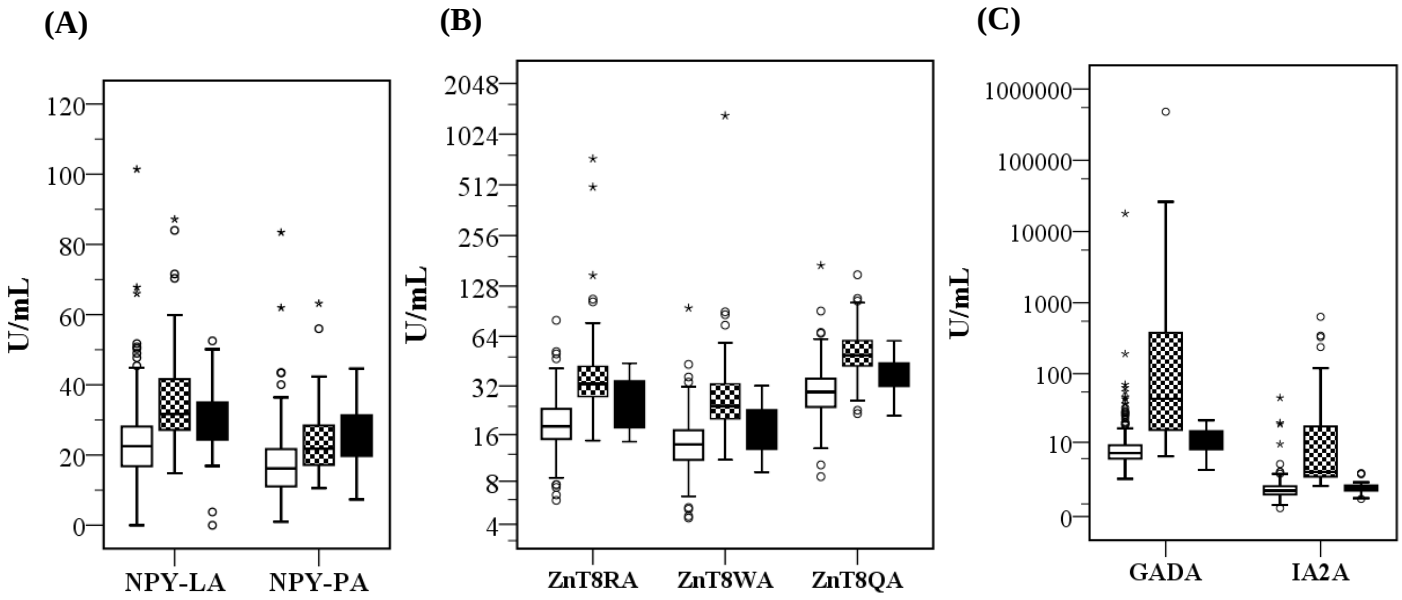
(A)
(B)
(C)
U/mL
120
100
80
60
40
20
0
NPY-LA
NPY-PA
2048
1024
512
256
128
64
32
16
8
4
ZnT8RA
ZnT8WA
ZnT8QA
1000000
100000
10000
1000
100
10
0
GADA
IA2A

Figure 3.

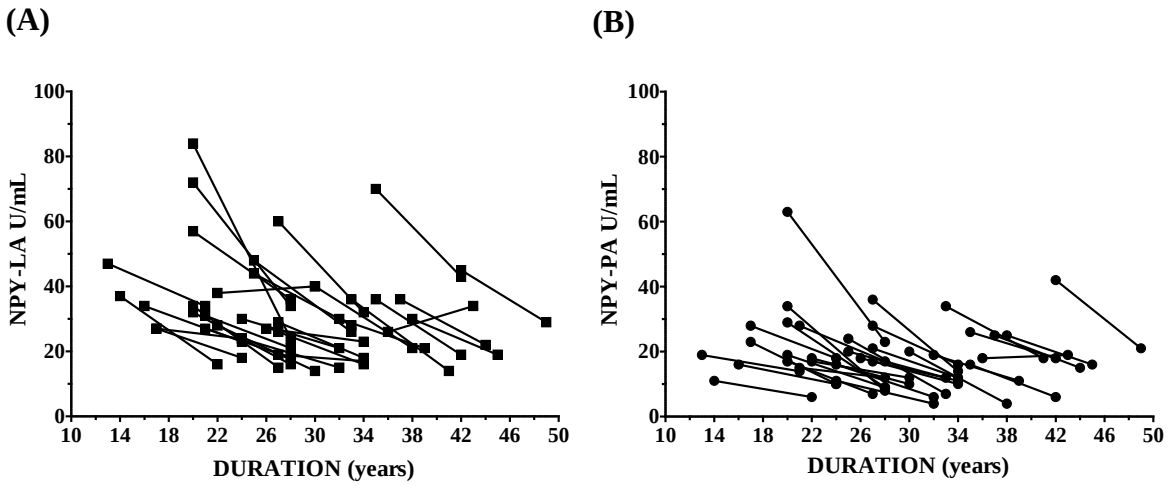
(A)
(B)
NPY-LA U/mL
NPY-PA U/mL
DURATION (years)
DURATION (years)
0
20
40
60
80
100
10
14
18
22
26
30
34
38
42
46
50

Figure 4.

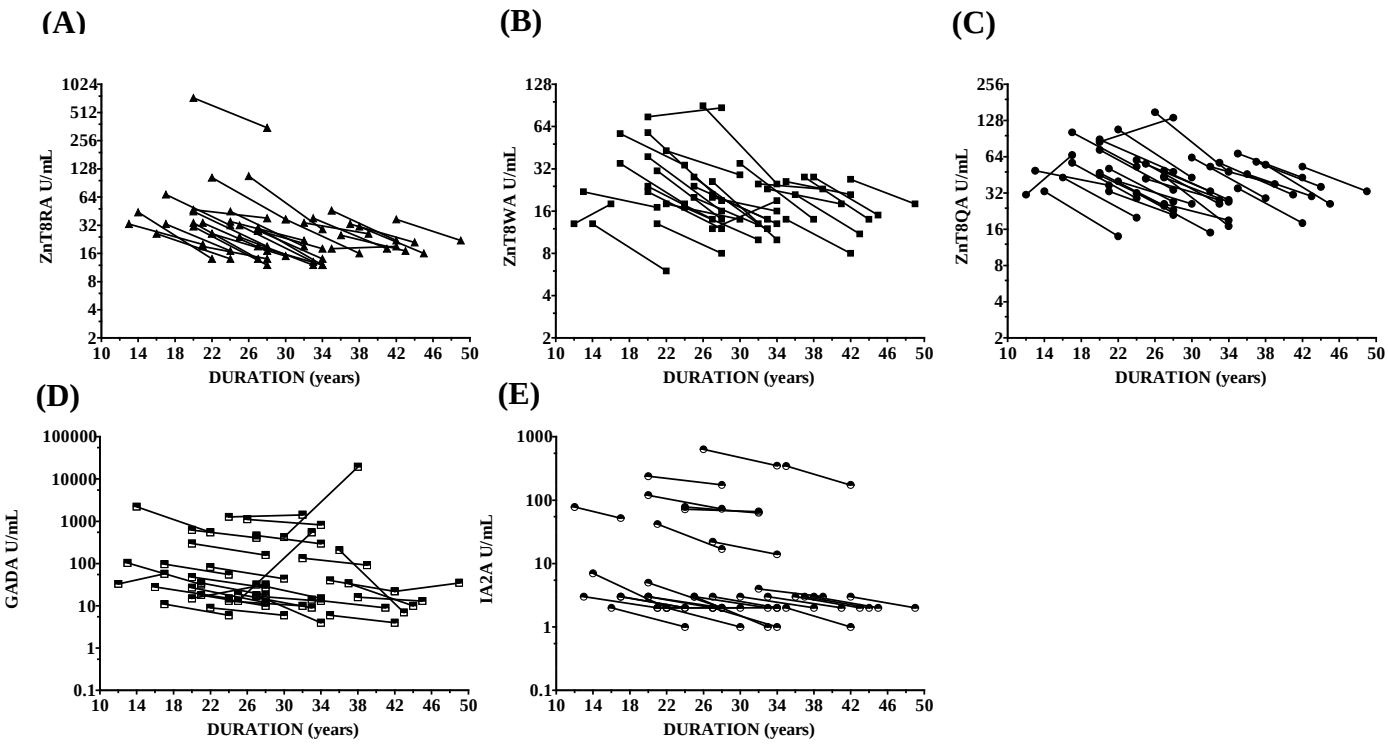
(A)
(B)
(C)
(D)
(E)
ZnT8RA U/mL
ZnT8WA U/mL
ZnT8QA U/mL
GADA U/mL
IA2A U/mL
DURATION (years)

Figure 5.

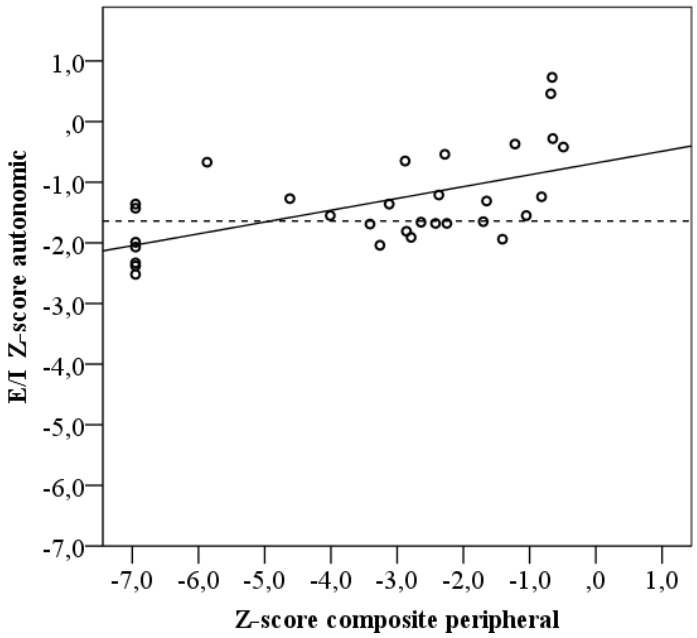
E/I Z-score autonomic
1,0
,0
-1,0
-2,0
-3,0
-4,0
-5,0
-6,0
-7,0
-7,0 -6,0 -5,0 -4,0 -3,0 -2,0 -1,0 ,0 1,0
Z-score composite peripheral

Figure 6.