



Increased Neutrophil Elastase and Proteinase 3 and Augmented NETosis Are Closely Associated with β -cell Autoimmunity in Patients with Type 1 Diabetes

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1 Increased Neutrophil Elastase and Proteinase 3 and Augmented NETosis Are Closely
2 Associated with β -cell Autoimmunity in Patients with Type 1 Diabetes

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17

18 **Running title:** Neutrophil Serine Proteases, NETosis and T1D

19

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1 **ABSTRACT**

2 Type 1 diabetes (T1D) is an autoimmune disease resulting from self-destruction of
3 insulin-producing β cells. Reduced neutrophil counts have been observed in patients with T1D.
4 However, the pathological roles of neutrophils in the development of T1D remain unknown. Here
5 we show that circulating protein levels and enzymatic activities of neutrophil elastase (NE) and
6 proteinase 3 (PR3), both of which are neutrophil serine proteases (NSPs) stored in neutrophil
7 primary granules, were markedly elevated in patients with T1D, especially those with disease
8 duration of less than one year. Furthermore, circulating NE and PR3 levels increased progressively
9 with the increase of the positive numbers and titers of the autoantibodies against β -cell antigens. An
10 obvious elevation of NE and PR3 was detected even in those autoantibody-negative patients.
11 Increased NE and PR3 in T1D patients are closely associated with elevated formation of neutrophil
12 extracellular traps. By contrast, the circulating levels of α 1-antitrypsin (A1AT), an endogenous
13 inhibitor of NSPs, are decreased in T1D patients. These findings support **an early** role of neutrophil
14 activation and augmented NSPs activities in the pathogenesis of β -cell autoimmunity, and also
15 suggest that circulating NE and PR3 may serve as sensitive biomarkers for diagnosis of T1D.

16

17 (**194** words)

18

19 **Key words:** Autoimmune diabetes, neutrophil serine proteases, neutrophil elastase, proteinase 3,
20 α 1-antitrypsin, neutrophil extracellular traps, NETosis, biomarker

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1 INTRODUCTION

2 The global incidence of type 1 diabetes (T1D), an autoimmune disease caused by an interactive
3 combination of genetic and environmental factors, has more than doubled in the past two decades (1;
4 2). Although the triggering factors that are involved in the initiation of T1D remain unclear, it is
5 widely accepted that organ-specific autoimmune destruction of the insulin-producing β -cells in the
6 pancreatic islets of Langerhans is mediated primarily by autoreactive T cells, which is accompanied
7 by the production of different autoantibodies to β -cell antigens, including glutamic acid
8 decarboxylase autoantibody (GADA), insulinoma-associated protein 2 autoantibody (IA2A) and
9 zinc transporter-8 autoantibody (ZnT8A) (3-5). Although these autoantibodies have been proven to
10 be instrumental for prediction and diagnosis of T1D, they are deemed not to be pathogenic (6; 7). A
11 number of other immune cells, including dendritic cells (DCs), macrophages, B cells, neutrophils,
12 are also implicated in the development of insulinitis in T1D (8; 9).

13

14 Neutrophils, which are the most abundant (40-75%) type of white blood cells, have recently been
15 implicated in both the onset and progression of T1D (10; 11). The primary functions of neutrophils
16 are to eliminate extracellular pathogens by multiple strategies, including phagocytosis,
17 degranulation to release lytic enzymes and neutrophil extracellular traps (NETs), which are formed
18 through a unique cell death process clearly differentiated from both apoptosis and necrosis, termed
19 “NETosis” (12-14). On the other hand, improper activation of neutrophils may lead to tissue
20 damage during autoimmune or exaggerated inflammatory responses (15). Notably, circulating

1 neutrophil counts are reduced in patients with T1D as well as their non-diabetic first-degree
2 relatives, but not in patients with type 2 diabetes (11). In non-obese diabetic (NOD) mice (a
3 spontaneous model of T1D), neutrophil infiltration and NET formation in the islets were observed
4 as early as two weeks after birth, and blockage of neutrophil activities with an anti-Ly6G antibody
5 reduces the subsequent development of insulinitis and diabetes (9).

6

7 Neutrophil serine proteases, including neutrophil elastase (NE), proteinase 3 (PR3) and cathepsin G
8 (CG), are the major components of neutrophil azurophilic granules that participate in the
9 elimination of engulfed microorganisms (16). Neutrophil activation and degranulation can result in
10 the release of neutrophil serine proteases into the extracellular medium and circulation, where they
11 not only help to eliminate the invaded pathogens but also serve as the humoral regulators of the
12 immune responses during acute and chronic inflammation, modulating cellular signalling network
13 by processing chemokines and activating specific cell surface receptors (17-19). Abnormal
14 activities of neutrophil serine proteases have been implicated in the pathogenesis of several
15 inflammatory and autoimmune diseases, including chronic obstructive pulmonary disease, cystic
16 fibrosis, Wegener granulomatosis, Papillion-Lefèvre syndrome and small-vessel vasculitis (20).
17 However, their association with T1D has not been explored so far.

18

19 In this study, we measured circulating levels of two main types of neutrophil serine proteases (NE
20 and PR3) as well as their enzymatic activities in T1D patients with different disease duration,

1 together with age- and sex-matched healthy controls. Furthermore, we explored whether altered
2 NET formation and α 1-antitrypsin (A1AT, a major endogenous inhibitor of neutrophil serine
3 proteases) are associated with reduced neutrophil counts and markedly increased activities of
4 neutrophil serine proteases in patients with T1D. In addition, we measured the dynamic changes of
5 circulating NE/PR3 activities during the development of autoimmune diabetes in T1D.

6

7 RESEARCH DESIGN AND METHODS

8 Study cohort.

9 One hundred and forty-nine patients with T1D were randomly selected from children diagnosed at
10 the Diabetes Center, Second Xiangya Hospital of Central South University from October 2000 to
11 October 2013. Patients with T1D were diagnosed according to the criteria of the American Diabetes
12 Association (21). All patients were treated with insulin. The disease duration of T1D was 4.2
13 (1.7-7.1) years [median (interquartile range)].

14

15 A total of 77 age- and sex-matched healthy controls were recruited from children in the community
16 participating in health screening at the Children Health Center of the Second Xiangya Hospital,
17 Central South University, using the following inclusion criteria: (1) fasting plasma glucose less than
18 5.6 mmol/L and 2-h plasma glucose less than 7.8 mmol/L; (2) no family history of diabetes, and
19 other autoimmune or chronic diseases.

20

1 A total of 25 adults with type 2 diabetes (T2D) diagnosed within one year and 25 age- and
2 sex-matched healthy controls were recruited at the Diabetes Center, Second Xiangya Hospital of
3 Central South University, and the inclusion criteria was described in our previous study (22).

4

5 The study was approved by the Institutional Review Board of Second Xiangya Hospital of Central
6 South University, and written informed consent was obtained from the patients and healthy controls.

7

8 **Clinical and biochemical assessments.**

9 After overnight fasting, a venous blood specimen was collected in the morning (around 0800 am)
10 for analysis of various biochemical parameters. Plasma glucose was measured enzymatically on a
11 Hitachi 7170 analyzer (Boehringer Mannheim, Mannheim, Germany). HbA1c was measured by
12 automated liquid chromatography (Bio-Rad VARIANT II Hemoglobin Testing System, Hercules,
13 CA, USA). Serum levels of C-peptide and C-reactive protein were quantified using a
14 chemiluminescence immunoassay on a Bayer 180SE Automated Chemiluminescence Systems
15 (BayerAG Leverkusen, Germany), and an immunoturbidometric assay (Orion Diagnostica, Espoo,
16 Finland), respectively. The titers of GADA, IA2A and ZnT8A were determined by in-house
17 radioligand assays as previously described (22; 23).

18

19 Circulating protein levels of NE, PR3 and A1AT were measured using the enzyme-linked
20 immunosorbent assay (ELISA) kits established in our laboratory (Antibody and Immunoassay

1 Services, the University of Hong Kong). The limits of detection for NE, PR3 and A1AT ELISA kits
2 were 0.156 ng/ml. No cross reactivity among these proteins or with other proteins were detected.
3 The intra- and inter-assay variations were 4.5% and 5.1%, respectively for NE ELISA kit; 3.9% and
4 4.3%, respectively for PR3 ELISA kit; and 4.9% and 5.3%, respectively for A1AT ELISA kit.

5

6 The combined enzymatic activities of PR3 and NE in serum were determined with a
7 chromogen-based assay using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma-Aldrich,
8 St. Louis, MO, USA) as the substrate, which has a catalytic constant K_{cat}/K_m of $33915 \text{ M}^{-1}\text{s}^{-1}$ for NE
9 and $499 \text{ M}^{-1}\text{s}^{-1}$ for PR3 (24). Briefly, 20 μl of serum was incubated with 180 μl of 0.1 M Tris-HCl
10 buffer (pH 8.0) containing 0.5M NaCl and 1 mM substrate at 37°C for 24 hours. The amount of
11 p-nitroaniline released was measured spectrophotometrically at 405 nm. The enzymatic activities of
12 PR3 and NE were calculated according to the delta OD values before and after 24-hour incubation
13 with substrate and expressed as mU/ml serum, where one unit was defined as the amount of PR3
14 and NE that hydrolyze the substrate to yield 1 μmol of p-nitroaniline per minute at 37°C (25).

15

16 The levels of neutrophil NETosis were measured by quantifying the amount of circulating
17 myeloperoxidase (MPO)-DNA complexes, a well-established marker of NET formation as
18 previously described (26). Briefly, 5 $\mu\text{g}/\text{ml}$ of mouse anti-MPO monoclonal antibody (ABD Serotec,
19 Germany) was coated to 96-well microtiter plates overnight at 4°C. After blocking with 1% BSA,
20 serum samples were added per well in combination with the peroxidase-labeled anti-DNA

1 monoclonal antibody (component No.2 of the Cell Death Detection ELISA PLUS kit, Roche
2 Diagnostics, USA) according to the manufacturer's instructions. After two hours of incubation at
3 room temperature on a shaking device (300 rpm), the wells were washed three times and then
4 incubated with the peroxidase substrate at 37°C for 60 minutes. The optical density (OD) at
5 wavelength of 405 nm was measured using a μ Quant microplate reader (Biotek Instruments, USA).

6

7 **Animal studies.**

8 NOD/ShiLtJ breeder mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

9 BALB/c and C57BL/6N mice were obtained from the Animal Unit of the University of Hong Kong.

10 All mice were housed in a room under specific pathogen-free conditions and 12-hour light-dark

11 cycles at 22 to 24°C, with *ad libitum* access to water and standard chow (PicoLab Rodent Diet 20,

12 LabDiet). Blood was collected weekly from female mice from 2 to 30 weeks of age. Blood glucose

13 was monitored using an Accu-Chek Advantage glucose meter (Roche Diagnostics, USA) and

14 diabetes was defined as two consecutive readings above 11.1 mmol/L (9). Circulating NE/PR3

15 enzymatic activities were measured as described above. All experimental procedures were approved

16 by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong

17 Kong and were carried out in accordance with the Guide for the Care and Use of Laboratory

18 Animals.

19

20 **Statistical analysis.**

1 All analyses were performed with Statistical Package for Social Sciences version 16.0 (SPSS,
2 Chicago, IL). Normality was tested using the Kolmogorov-Smirnov test. Data that were not
3 normally distributed were logarithmically transformed before analysis. Differences between groups
4 were assessed by χ^2 or unpaired *t* test. Comparisons among groups were performed using one-way
5 ANOVA and independent *t*-tests. Correlations were analyzed using Pearson correlation or partial
6 correlation as appropriate. Data were expressed as mean \pm SD or median with interquartile range as
7 appropriate. In all statistical comparisons, a *p* value $<$ 0.05 was used to indicate a statistically
8 significant difference.

9

10 RESULTS

11 Subject characteristics.

12 The clinical characteristics of T1D patients and their healthy controls were described in Table 1.
13 T1D patients were further divided into three groups based on their disease duration, including
14 patients within 1 year from diagnosis (n=28), with a disease duration $>$ 1 and $<$ 5 years (n=59) and
15 with duration $>$ 5 years (n=62). Compared with healthy subjects, T1D patients had higher fasting
16 glucose and HbA1c, but lower fasting C-peptide levels. No significant differences in C-reactive
17 protein were found among these groups (Table 1). Consistent with the previous reports (10; 11), the
18 circulating neutrophils were moderately reduced in T1D patients diagnosed within 1 year compared
19 with the healthy controls [median (interquartile range) ($\times 10^6$ /ml), 2.27 (1.80-3.52) vs 3.63
20 (3.02-4.15), *p* $<$ 0.05], but not in T1D patients with a disease duration $>$ 1 and $<$ 5 years, or with

1 [duration >5 years \(Table 1\)](#).

2

3 **Circulating protein levels and enzymatic activities of NE and PR3 are dramatically increased**
4 **in T1D patients.**

5 In contrast to the mild reduction of peripheral neutrophils, we found that the circulating protein
6 levels of both NE and PR3 were dramatically increased in T1D patients compared to the healthy
7 controls [NE: 1594.7 (988.4-2284.6) vs 397.0 (262.2-468.8) ng/ml, $p < 0.001$; PR3: 295.3
8 (206.0-430.4) vs 107.4 (92.5-165.0) ng/ml, $p < 0.001$]. Notably, the magnitude of increases in protein
9 levels of NE and PR3 was significantly higher in T1D patients diagnosed within 1 year as compared
10 to the other two groups of patients with longer disease duration ([all \$p < 0.01\$, Fig. 1A and 1B](#)). There
11 were no differences in circulating protein levels of NE and PR3 between men and women in
12 patients or controls.

13

14 To further confirm the above findings, we measured the combined enzymatic activities of NE and
15 PR3 using a common substrate, N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (24). The
16 results showed that circulating NE/PR3 enzymatic activities in T1D patients were also substantially
17 higher than those in healthy individuals [0.69 (0.41-1.03) vs 0.14 (0.10-0.21) mU/ml, $p < 0.001$].

18 [Likewise, the most significant increase in NE/PR3 enzymatic activities was observed in T1D](#)
19 [patients within 1 year from diagnosis \(Fig. 1C\)](#). The correlation coefficient between NE/PR3
20 enzymatic activities and circulating protein levels was 0.915 ($p < 0.001$) for NE and 0.874 ($p < 0.001$)

1 for PR3.

2

3 **Circulating A1AT levels are decreased in T1D patients.**

4 The activities of plasma NE and PR3 are tightly controlled by their associated endogenous
5 inhibitors, especially A1AT, an archetype member of the serine protease inhibitor (SERPIN)
6 superfamily. Since our data showed that the amplitude of increases in NE/PR3 enzymatic activities
7 was higher than that of the circulating protein levels, we next investigated whether dysregulated
8 A1AT contributed to the increased enzymatic activities of NE and PR3 in T1D. In contrast to
9 elevated NE and PR3 levels, the circulating concentrations of A1AT in T1D patients diagnosed
10 within one year were significantly decreased compared to healthy subjects [1.37 (1.07-1.83) vs 1.80
11 (1.57-2.07) mg/ml, $p < 0.01$], whereas the decline in patients with disease duration for 1-5 years or
12 with disease duration >5 years did not reach statistical significance (Fig. 1D).

13

14 **Neutrophil NETosis is increased in T1D patients**

15 To explore the underlying mechanism responsible for the markedly elevated circulating NE and
16 PR3 levels, we examined the levels of neutrophil NETosis by quantifying the amount of circulating
17 MPO-DNA complexes, a well-established marker of NET formation (26). In line with the increased
18 NE and PR3 levels, a significant elevation of circulating MPO-DNA complexes was observed in
19 T1D patients, especially in T1D patients with the disease duration of less than one year, compared
20 to the healthy individuals [0.197 (0.049-0.412) vs 0.026 (0.011-0.058) (mean OD405), $p < 0.001$]

1 (Fig. 2A). Furthermore, the amount of MPO-DNA complexes in serum was significantly correlated
2 with the circulating protein levels of NE ($r=0.554$, $p<0.001$) and PR3 ($r=0.575$, $p<0.001$), as well as
3 NE/PR3 enzymatic activities ($r=0.527$, $p<0.001$) (Fig 2B-2D), suggesting that the increased
4 circulating NE and PR3 protein levels in T1D patients are at least in part attributed to enhanced
5 neutrophil NETosis.

6

7 **Circulating NE and PR3 are associated with the numbers and titers of autoantibodies in T1D** 8 **patients**

9 We next explored the relationship between circulating neutrophil serine proteases and the three
10 autoantibodies associated with β -cell autoimmunity in T1D patients, including GADA, IA2A and
11 ZnT8A. Among 149 T1D patients, 54 (36%) were autoantibody-negative, 61 (41%), 24 (16%), and
12 10 (7%) had one, two and three autoantibodies-positive, respectively. Notably, circulating levels of
13 both NE and PR3 proteins as well as their enzymatic activities were increased progressively with
14 increased numbers of the autoantibodies detected in these patients (Figure 3A-3C). Even for the
15 autoantibody-negative T1D patients, the circulating protein levels and enzymatic activities of both
16 NE and PR3 were much higher than those in healthy controls [protein levels: NE: 1154.90
17 (770.8-1749.5) vs 397.0 (262.2-468.8) ng/ml, $p<0.0001$; PR3: 237.4 (154.3-307.1) vs 107.4
18 (92.5-165.0) ng/ml, $p<0.0001$; enzymatic activities: 0.53 (0.37-0.79) vs 0.14 (0.10-0.21) mU/ml,
19 $p<0.001$] (Fig. 3A-3C). Furthermore, a strong correlation between the titers of GADA and the
20 circulating protein levels of NE ($r=0.296$, $p=0.011$) and PR3 ($r=0.270$, $p=0.021$) as well as NE/PR3

1 enzymatic activities ($r=0.275$, $p=0.019$) were detected in T1D patients with GADA-positive ($n=73$)
2 (Fig. 3D-3F). Likewise, the titers of IA2A in T1D patients with IA2A-positive ($n=44$) were also
3 positively associated with the protein levels of NE, PR3 and their enzymatic activities
4 (supplementary Fig. 1A-1C). After adjustment for disease duration, the circulating protein levels and
5 enzymatic activities of both NE and PR3 were still significantly correlated with the numbers and
6 titers of these autoantibodies (all $p<0.05$, supplementary Table 1). On the contrary, no significant
7 correlation between fasting blood glucose and circulating protein levels of NE ($r=-0.103$, $p=0.211$)
8 or PR3 ($r=-0.097$, $p=0.237$) or NE/PR3 enzymatic activities ($r=-0.078$, $p=0.342$) was observed in
9 the present study cohort. We further measured and compared the circulating protein levels and
10 enzymatic activities of NE and PR3 in 25 T2D patients within 1 year from diagnosis and 25 age-
11 and sex-matched healthy controls (supplementary Table 2). The results showed that there was no
12 significant difference in either protein levels or enzymatic activities of NE and PR3 or NETosis
13 between the two groups (supplementary Table 2). Taken together, these data suggested that elevated
14 NE and PR3 may be closely associated with β -cell autoimmunity, but not glycemic status in T1D
15 patients.

16
17 **Elevated NE/PR3 enzymatic activity is closely associated with the development of diabetes in**
18 **NOD mice.**

19 To further explore the relationship between neutrophil serine proteases and the development of T1D,
20 we determined the dynamic changes of PR3 and NE in NOD mice ($n=30$), a well-established

1 animal model for autoimmune diabetes from 2 to 30 weeks of age. These mice were then
2 retrospectively assigned to two groups: those that eventually developed diabetes (n=22, called
3 “diabetic”) and those that did not (n=8, called “non-diabetic”). In diabetic mice, the circulating
4 NE/PR3 enzymatic activities were markedly elevated by 4 folds as early as 4 weeks after birth
5 compared to those in 2-week-old mice, and such an elevation sustained for over 10 weeks before
6 the onset of diabetes. Afterwards, the NE/PR3 activities in diabetic mice were gradually decreased
7 to the baseline levels, presumably due to the termination of autoimmune responses as a result of
8 complete β -cell destruction (Fig. 4A). In non-diabetic mice, although there was a transient and
9 modest increase of circulating NE/PR3 activities between 4 and 5 weeks after birth (Fig. 4B), the
10 magnitude and duration of NE/PR3 elevation was substantially lower than in age-matched diabetic
11 mice (Fig. 4C). In BALB/c and C57BL/6N mice which do not develop insulinitis and autoimmune
12 diabetes, circulating NE/PR3 activities remained little changed throughout the 30-week observation
13 period (Supplementary Fig. 2A-2B).

14

15 **DISCUSSION**

16 In this study, we demonstrated that a modest reduction of neutrophil counts in patients with T1D at
17 onset is accompanied by a marked elevation of both protein levels and enzymatic activities of the
18 two major neutrophil serine proteases NE and PR3. Furthermore, these changes in T1D patients are
19 closely associated with increased neutrophil NETosis, as determined by quantification of
20 MPO-DNA complexes in the circulation. These findings suggest that the reduction of neutrophil

1 counts in T1D patients is attributed in part to augmented NETosis, which in turn leads to increased
2 NET formation and release of NE and PR3 into the blood stream.

3

4 We showed that the amplitude of elevation in circulating NE/PR3 enzymatic activities and NET
5 formation in patients with the disease duration of less than one year is substantially higher than
6 those with disease duration of more than one year. A significant reduction in neutrophil counts is
7 observed only in T1D patients with disease duration of less than one year. Consistent with our
8 findings, a previous study in Italy also found that neutrophil reduction is greatest in individuals with
9 the highest risk of developing T1D (11). After the disease onset, mild neutropenia persists for a few
10 years and then resolves at 5 years after diagnosis (as determined by a longitudinal analysis). In
11 NOD mice with spontaneous development of autoimmune diabetes, neutrophil infiltration and NET
12 formation in the islets are detected as early as two weeks after birth, well before the onset of overt
13 diabetes (9). Furthermore, neutrophil depletion at the early stage reduces subsequent development
14 of diabetes in NOD mice (9). Taken together, these data support an early role of neutrophil NETosis,
15 NET formation and augmented release of neutrophil serine proteases in the onset of β -cell
16 autoimmunity in T1D. Indeed, increased neutrophil NETosis and NET formation have been
17 implicated in a number of autoimmune diseases, including small vessel vasculitis (SVV), systemic
18 lupus erythematosus (SLE), and multiple sclerosis (26-28).

19

20 In SLE, NETs has been demonstrated to stimulate plasmacytoid DCs (pDCs) for releasing IFN- α .

1 which in turn augments the autoreactivity of both antigen-presenting and antibody-producing cells
2 (29; 30). NETosis leads to the release of intracellular proteins, including histones and high mobility
3 group protein B1, the latter of which is implicated in initiation and/or perpetuation of autoimmunity
4 in several types of autoimmune disorders, including T1D (30; 31). Furthermore, NETs is associated
5 with altered patterns of epigenetic and posttranslational modifications, such as methylation,
6 acetylation and citrullination, which may represent an important source of autoantigens promoting
7 the generation of autoantibodies (32). In particular, a growing body of evidence supports a
8 pathogenic role for citrullinated autoantigens in triggering autoimmune responses in SLE,
9 rheumatoid arthritis and multiple sclerosis (33). However, the pathophysiological roles of NETosis
10 and its associated changes in T1D remain elusive.

11

12 The current etiopathological diagnosis of autoimmune T1D heavily relies on the detection of the
13 autoantibodies against several β -cell antigens. However, in children these autoantibodies are rarely
14 detectable before six months of age (34). Moreover, the diagnostic sensitivity of the single
15 autoantibody measurement in T1D patients is as low as 59%-67% (35). To capture the therapeutic
16 window for this disease, it is critically important to identify new biomarkers for detection of early
17 immunological events that affect human islets. Our current study demonstrated approximately
18 4-fold increase of circulating protein levels and more than 5-fold elevation of enzymatic activities
19 of NE and PR3 in T1D patients. Furthermore, elevated NE and PR3 are significantly associated
20 with the positive numbers and titers of the autoantibodies detected in T1D patients. Even in those

1 autoantibody-negative patients, the circulating enzymatic activities of NE and PR3 are still
2 substantially higher than healthy controls. Using the animal model of T1D, we found that elevated
3 circulating NE/PR3 activities occur well before the onset of hyperglycemia and diabetes, and their
4 activities gradually decline after the development of overt diabetes. Taken together, our data suggest
5 that circulating NE and PR3 may serve as sensitive biomarkers for early detection of those
6 individuals with high risk of developing T1D. On the other hand, we found no significant
7 association between increased levels of NE and PR3 and the severity of hyperglycemia in T1D
8 patients. In fact, while hyperglycemia becomes more severe with the progression of T1D,
9 circulating levels of NE and PR3 exhibit opposite changes, suggesting that increased neutrophil
10 NETosis and augmented release of NE and PR3 are not the consequence of impaired glycaemic
11 controls, but are related to β -cell autoimmunity. Indeed, our observation that NETosis and NE/PR3
12 levels in T1D patients with longer disease duration are much lower than newly-onset patients (<1
13 year) may be attributed to the gradual attenuation of β -cell autoimmunity with the progression of
14 diabetes to an advanced stage. This is also in line with the fact that the number of autoantibodies in
15 newly-onset T1D patients was much higher than those with longer disease duration (36).

16

17 In addition to its classical roles for host defence against infection, neutrophil serine proteases are
18 important regulator of inflammation and innate immunity (17; 19; 37; 38). Both NE and PR3 are
19 involved in maturation and release of pro-inflammatory cytokines such as $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-18 ,
20 and also induces expression and activation of Toll-like receptors (39-42), all of which are important

1 mediator of insulinitis and β cell destruction (43; 44). Furthermore, NE and PR3 play an
2 indispensable role in recruiting neutrophils to the site of inflammation. Notably, neutrophil serine
3 proteases have recently been implicated in high fat diet-induced obesity, inflammation and
4 macrophage infiltration in adipose tissues in mice (45). Injection of recombinant PR3 alone is
5 sufficient to induce hyperglycemia in mice (46). By contrast, treatment with A1AT, a major
6 endogenous inhibitor of NE and PR3, decreases lymphocyte infiltration in the islets, and prevents β
7 cell loss and diabetes in rodent models of T1D (47; 48). These animal studies, in conjunction with
8 our clinical findings, suggest that elevated NE and PR3 may be the direct contributors to the
9 pathogenesis of autoimmune diabetes by early involvement of autoimmune inflammatory responses
10 in pancreatic islets.

11

12 A1AT, the most abundant circulating serpin secreted from hepatocytes, inhibits neutrophil serine
13 proteases by covalent binding to the enzymes (49). Deficiency of A1AT has been implicated in a
14 number of inflammatory disorders such as chronic obstructive pulmonary disease (50). Our present
15 study observed a modest, but significant reduction of circulating A1AT in patients with T1D,
16 suggesting that augmented circulating NE and PR3 activities may result from a combination of
17 increased release of these two enzymes from neutrophil NETosis and decreased production of their
18 endogenous inhibitor A1AT.

19

20 Our study has several limitations, including the relatively small sample size and the cross-sectional

1 design. In addition, since our samples were collected from Chinese only, whether or not the findings
2 are replicable in other ethnic group remains to be determined. Further large scale, longitudinal
3 studies on different ethnic groups are mandatory to clarify the roles of NE and PR3 in the initiation
4 and progression of β -cell autoimmunity, and to evaluate their clinical value for prediction and early
5 diagnosis of T1D.

6

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13 Y.W, Y.X, and L.Z conducted the experiments, analyzed data and wrote the manuscript. D.Y, J.Z
14 and Y.T conducted the experiments. S.R.B and K.S.L.L were involved in data analysis and edited
15 the manuscript. A.X and Z.Z contributed to experimental design, analyzed data, and wrote the
16 manuscript. A.X and Z.Z are the guarantors of this work and, as such, had full access to all the data
17 in the study and takes responsibility for the integrity of the data and the accuracy of the data
18 analysis.

19

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- 9

1 **Figure Legends**

2 **FIG.1.** Circulating protein levels of NE (A) and PR3 (B), NE/PR3 enzymatic activities (C), and
3 A1AT protein levels (D) in healthy controls (n=77), T1D patients within 1 year from diagnosis
4 (n=28), with a disease duration >1 and <5 years from diagnosis (n=59) and with duration >5 years
5 (n=62) from diagnosis are shown as box plots. The horizontal line in the middle of each box
6 indicates the median value; the top and bottom borders of the boxes represent the 75th and 25th
7 percentiles, respectively; the whiskers represent the 10th and 90th percentiles, and the dots
8 represent the outliers. ** p<0.01, *** p<0.001 vs Healthy controls; # p<0.05, ## p<0.01 vs T1D
9 patients within 1 year from diagnosis.

10

11 **FIG. 2.** Circulating levels of MPO-DNA complexes in healthy controls (n=77), T1D patients within
12 1 year from diagnosis (n=28), with a disease duration >1 and <5 years (n=59) and with duration >5
13 years (n=62) (A). Circulating MPO-DNA complexes were significantly correlated with circulating
14 NE (B) and PR3 (C) protein levels, and enzymatic activities of both NE and PR3 (D). ** p<0.01,
15 *** p<0.001 vs Healthy controls; ## p<0.01 vs T1D patients within 1 year from diagnosis.

16

17 **FIG. 3.** Circulating protein levels of NE (A) and PR3 (B), enzymatic activities of both NE and PR3
18 (C) in healthy controls (n=77), T1D patients with autoantibody negative (n=54), one
19 autoantibody-positive of GADA, IA2A or ZnT8A (n=61), two autoantibodies-positive of GADA,
20 IA2A or ZnT8A (n=24), or three autoantibodies-positive of GADA, IA2A and ZnT8A (n=10).

1 Circulating protein levels of NE (D) and PR3 (E), and enzymatic activities of both NE and PR3 (F)
2 were significantly correlated with the titers of GADA in T1D patients with GADA-positive (n=73).
3 *** $p < 0.001$ vs Healthy controls; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs T1D patients with autoantibody
4 negative; \$ $p < 0.05$ vs T1D patients with one autoantibody-positive.

5
6 FIG. 4. Dynamic changes in enzymatic activities of circulating NE/PR3 in NOD female mice.
7 Blood samples were collected weekly from 30 NOD female mice from 2 to 30 weeks after birth.
8 Circulating NE/PR3 enzymatic activities along with their blood glucose levels were measured in
9 mice that developed diabetes (n=22, A) and that remained non-diabetic (n=8, B) until 30 weeks
10 after birth. (C) Comparisons of NE/PR3 enzymatic activities between diabetic mice and
11 non-diabetic mice throughout the observation period. # $p < 0.05$ vs the NE/PR3 enzymatic activities
12 at 2 weeks of age; * $p < 0.05$ vs age-matched non-diabetic mice.

TABLE 1

Characteristics of healthy controls and T1D patients recruited for this study

	Healthy controls	T1D patients from diagnosis		
		< 1 year	1 - 5 years	> 5 years
n	77	28	59	62
Age (years)	13.3 ± 5.3	15.4 ± 6.9	12.9 ± 4.3	14.9 ± 3.8
Sex (men/women)	43/34	12/16	21/38	24/38
BMI (kg/m ²)	18.35 ± 2.70	17.56 ± 3.16	17.83 ± 3.77	18.46 ± 3.05
Duration of diabetes (years)	N/A	0.4 (0.2-0.7)	2.8 (1.9-3.9)	7.6 (6.4-9.3)
Fasting glucose (mmol/L) [§]	4.69 (4.41-4.89)	7.85 (6.20-11.93) ^a	8.4 (6.60-14.20) ^a	7.80 (5.68-11.83) ^a
HbA1c (%) [§]	5.00 (4.80-5.15)	8.05 (6.03-11.15) ^a	7.50 (6.70-10.10) ^a	7.40 (6.48-8.43) ^a
(mmol/mol) [§]	31 (29-33)	64 (42-99) ^a	58 (50-87) ^a	57 (47-69) ^a
Fasting C-peptide (pmol/L) [§]	445.4 (362.1-678.2)	55.35 (16.92-146.73) ^a	22.80 (5.50-92.95) ^{a,b}	5.50 (4.20-28.43) ^{a,b,c}
C reactive protein (mg/L) [§]	0.23 (0.13-0.61)	0.24 (0.11-0.51)	0.27 (0.13-0.75)	0.28 (0.15-1.12)
Blood cell counts				
Erythrocytes (x 10 ⁶ /ml) [§]	4.66 (4.30-4.94)	4.52 (4.06-5.12)	4.83 (4.54-5.17)	4.82 (4.50-5.03)
White blood cells (x 10 ⁶ /ml) [§]	6.85 (5.80-7.78)	4.70 (3.75-7.35) ^a	5.70 (4.95-6.90)	6.40 (5.20-6.97)
Lymphocytes (x 10 ⁶ /ml) [§]	2.18 (1.55-2.62)	1.74 (1.57-2.08)	1.98 (1.54-2.43)	1.84 (1.64-2.38)
Monocytes (x 10 ⁶ /ml) [§]	0.42 (0.34-0.49)	0.29 (0.17-0.37) ^a	0.29 (0.22-0.38) ^a	0.31 (0.23-0.39) ^a
Neutrophils (x 10 ⁶ /ml) [§]	3.63 (3.02-4.15)	2.27 (1.80-3.52) ^a	3.29 (2.75-4.15)	3.49 (3.02-4.14)
Eosinophils (x 10 ⁶ /ml) [§]	0.20 (0.12-0.34)	0.13 (0.08-0.20) ^a	0.15 (0.12-0.23)	0.15 (0.11-0.21)
Basophils (x 10 ⁶ /ml) [§]	0.05 (0.03-0.06)	0.03 (0.02-0.07)	0.07 (0.05-0.10) ^{a,b}	0.08 (0.06-0.14) ^{a,b}
Platelets (x 10 ⁶ /ml) [§]	256 (223-315)	244 (202-285) ^a	219 (193-265) ^a	250 (199-280)

Date are expressed as mean ± SD or median (interquartile range) as appropriate.

[§]Log transformed before analysis. ^aCompared with healthy controls, $p < 0.05$; ^bcompared with T1D patients from diagnosis < 1 years, $p < 0.05$; ^ccompared with T1D patients from diagnosis 1 - 5 years, $p < 0.05$.

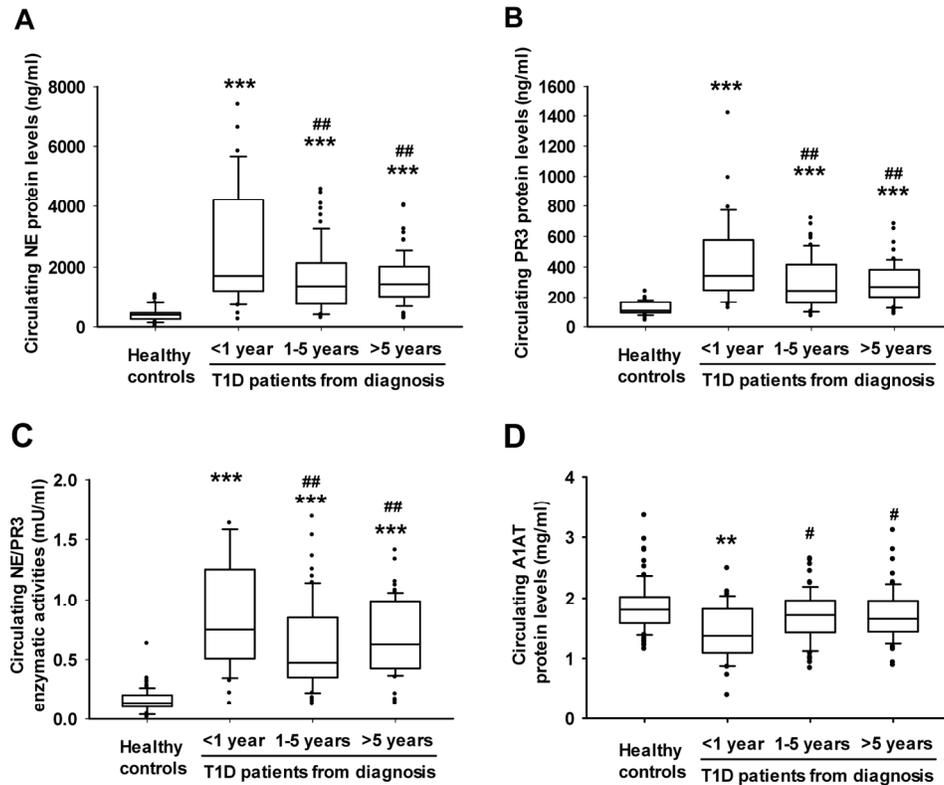


FIG.1. Circulating protein levels of NE (A) and PR3 (B), NE/PR3 enzymatic activities (C), and A1AT protein levels (D) in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years from diagnosis (n=59) and with duration >5 years (n=62) from diagnosis are shown as box plots. The horizontal line in the middle of each box indicates the median value; the top and bottom borders of the boxes represent the 75th and 25th percentiles, respectively; the whiskers represent the 10th and 90th percentiles, and the dots represent the outliers. ** p<0.01, *** p<0.001 vs Healthy controls; # p<0.05, ## p<0.01 vs T1D patients within 1 year from diagnosis.

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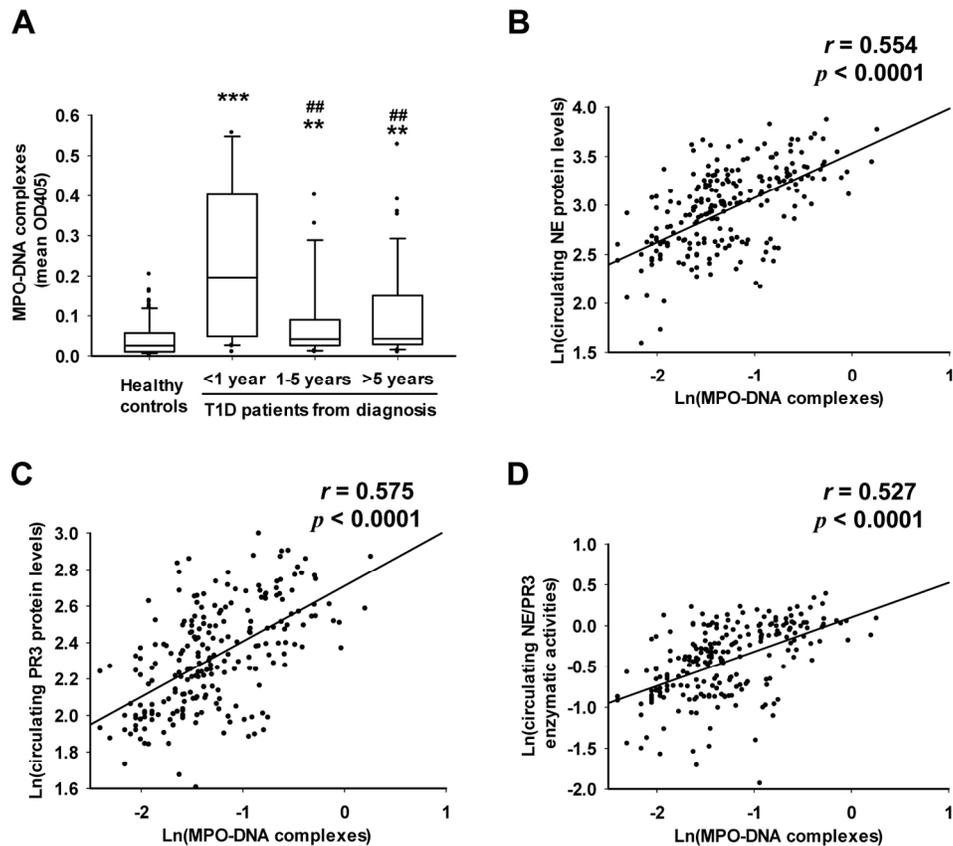


FIG. 2. Circulating levels of MPO-DNA complexes in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years (n=59) and with duration >5 years (n=62) (A). Circulating MPO-DNA complexes were significantly correlated with circulating NE (B) and PR3 (C) protein levels, and enzymatic activities of both NE and PR3 (D). ** p<0.01, *** p<0.001 vs Healthy controls; ## p<0.01 vs T1D patients within 1 year from diagnosis.

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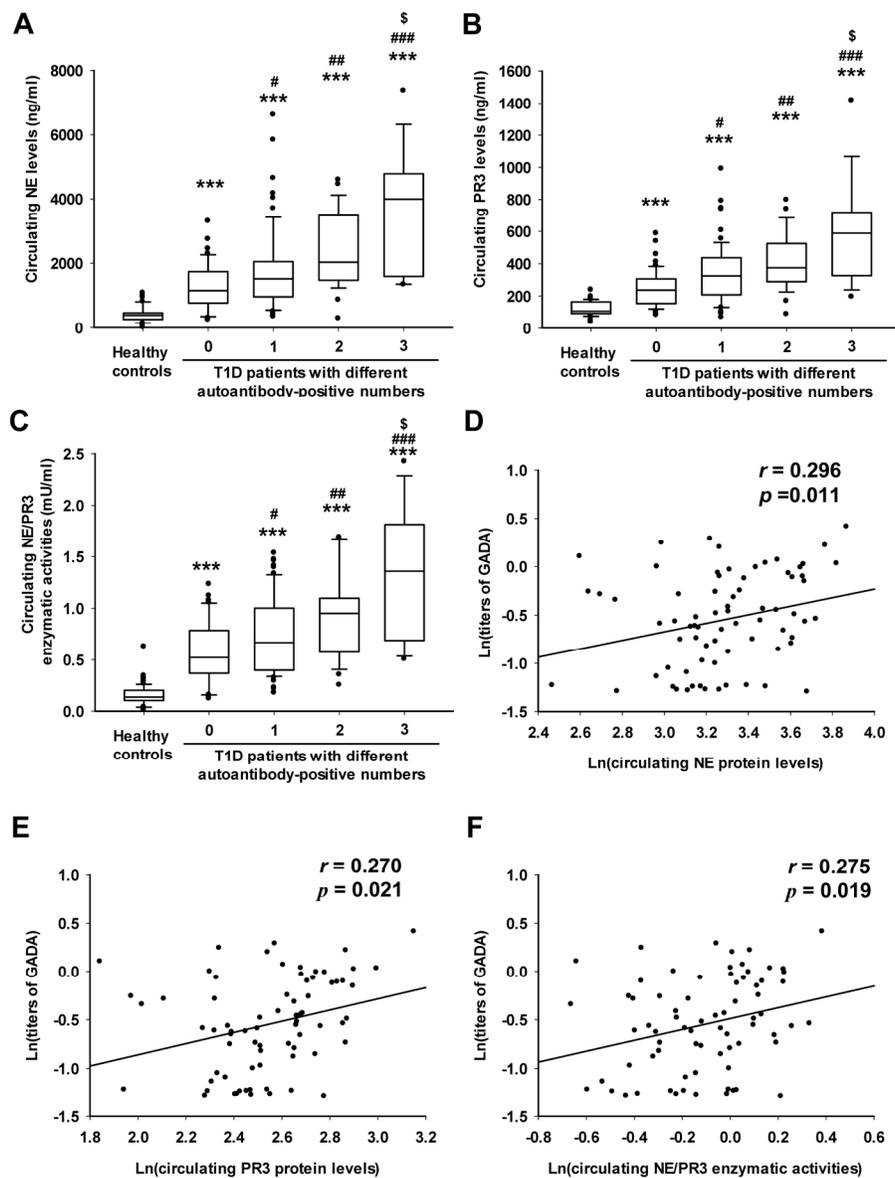


FIG. 3. Circulating protein levels of NE (A) and PR3 (B), enzymatic activities of both NE and PR3 (C) in healthy controls (n=77), T1D patients with autoantibody negative (n=54), one autoantibody-positive of GADA, IA2A or ZnT8A (n=61), two autoantibodies-positive of GADA, IA2A or ZnT8A (n=24), or three autoantibodies-positive of GADA, IA2A and ZnT8A (n=10). Circulating protein levels of NE (D) and PR3 (E), and enzymatic activities of both NE and PR3 (F) were significantly correlated with the titers of GADA in T1D patients with GADA-positive (n=73). *** $p < 0.001$ vs Healthy controls; # $p < 0.05$, ## $p < 0.01$, \$\$\$ $p < 0.001$ vs T1D patients with autoantibody negative; \$ $p < 0.05$ vs T1D patients with one autoantibody-positive.

152x204mm (300 x 300 DPI)

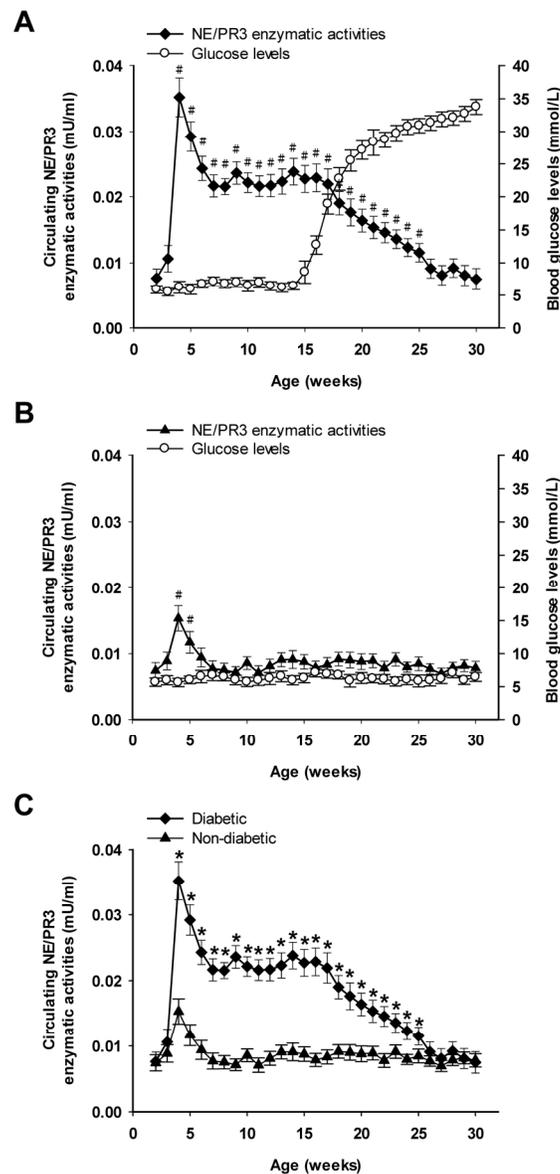


FIG. 4. Dynamic changes in enzymatic activities of circulating NE/PR3 in NOD female mice. Blood samples were collected weekly from 30 NOD female mice from 2 to 30 weeks after birth. Circulating NE/PR3 enzymatic activities along with their blood glucose levels were measured in mice that developed diabetes (n=22, A) and that remained non-diabetic (n=8, B) until 30 weeks after birth. (C) Comparisons of NE/PR3 enzymatic activities between diabetic mice and non-diabetic mice throughout the observation period. # $p < 0.05$ vs the NE/PR3 enzymatic activities at 2 weeks of age; * $p < 0.05$ vs age-matched non-diabetic mice. 113x201mm (300 x 300 DPI)

Supplementary Table 1

Correlations of circulating protein levels and enzymatic activities of NE and PR3 with clinical parameters in T1D patients

	Circulating NE protein levels		Circulating NE protein levels (Diabetes duration-adjusted)	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Diabetes duration (years)	-0.203	0.017	—	—
Fasting glucose (mmol/L)*	-0.103	0.211	-0.106	0.200
HbA1c (%)*	0.083	0.315	0.069	0.405
Fasting C-peptide (pmol/L)*	0.151	0.066	0.125	0.130
Neutrophils (x 10 ⁶ /ml)*	0.108	0.189	0.101	0.213
Numbers of autoantibodies-positive	0.427	<0.001	0.415	<0.001
GADA titers* ^a	0.296	0.011	0.313	0.007
IA2A titers* ^b	0.422	0.004	0.403	0.007
ZnT8A titers* ^c	0.205	0.360	0.201	0.380

	Circulating PR3 protein levels		Circulating PR3 protein levels (Diabetes duration-adjusted)	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Duration of diabetes (years)	-0.212	0.010	—	—
Fasting glucose (mmol/L)*	-0.097	0.237	-0.102	0.214
HbA1c (%)*	0.140	0.090	0.120	0.145
Fasting C-peptide (pmol/L)*	0.158	0.052	0.120	0.145
Neutrophils (x 10 ⁶ /ml)*	0.074	0.368	0.065	0.470
Numbers of autoantibodies-positive	0.428	<0.001	0.411	<0.001
GADA titers* ^a	0.270	0.021	0.279	0.018
IA2A titers* ^b	0.486	0.001	0.450	0.002
ZnT8A titers* ^c	0.288	0.194	0.285	0.209

	NE/PR3 enzymatic activities		NE/PR3 enzymatic activities (Diabetes duration-adjusted)	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Duration of diabetes (years)	-0.196	0.021	—	—
Fasting glucose (mmol/L)*	-0.078	0.342	-0.080	0.334
HbA1c (%)*	0.101	0.219	0.093	0.260
Fasting C-peptide (pmol/L)*	0.151	0.066	0.137	0.098
Neutrophils (x 10 ⁶ /ml)*	0.128	0.119	0.107	0.198
Numbers of autoantibodies-positive	0.403	<0.001	0.397	<0.001
GADA titers* ^a	0.275	0.019	0.280	0.017
IA2A titers* ^b	0.389	0.009	0.386	0.011
ZnT8A titers* ^c	0.193	0.390	0.190	0.409

*Log transformed before analysis;

a. include only subjects with GADA-positive (n = 73); b. include only subjects with IA2A-positive (n = 44); c. include only subjects with ZnT8A-positive (n = 22).

Supplementary Table 2

Circulating protein levels and enzymatic activities of NE and PR3, A1AT protein levels, and MPO-DNA complexes in T2D patients and their healthy controls

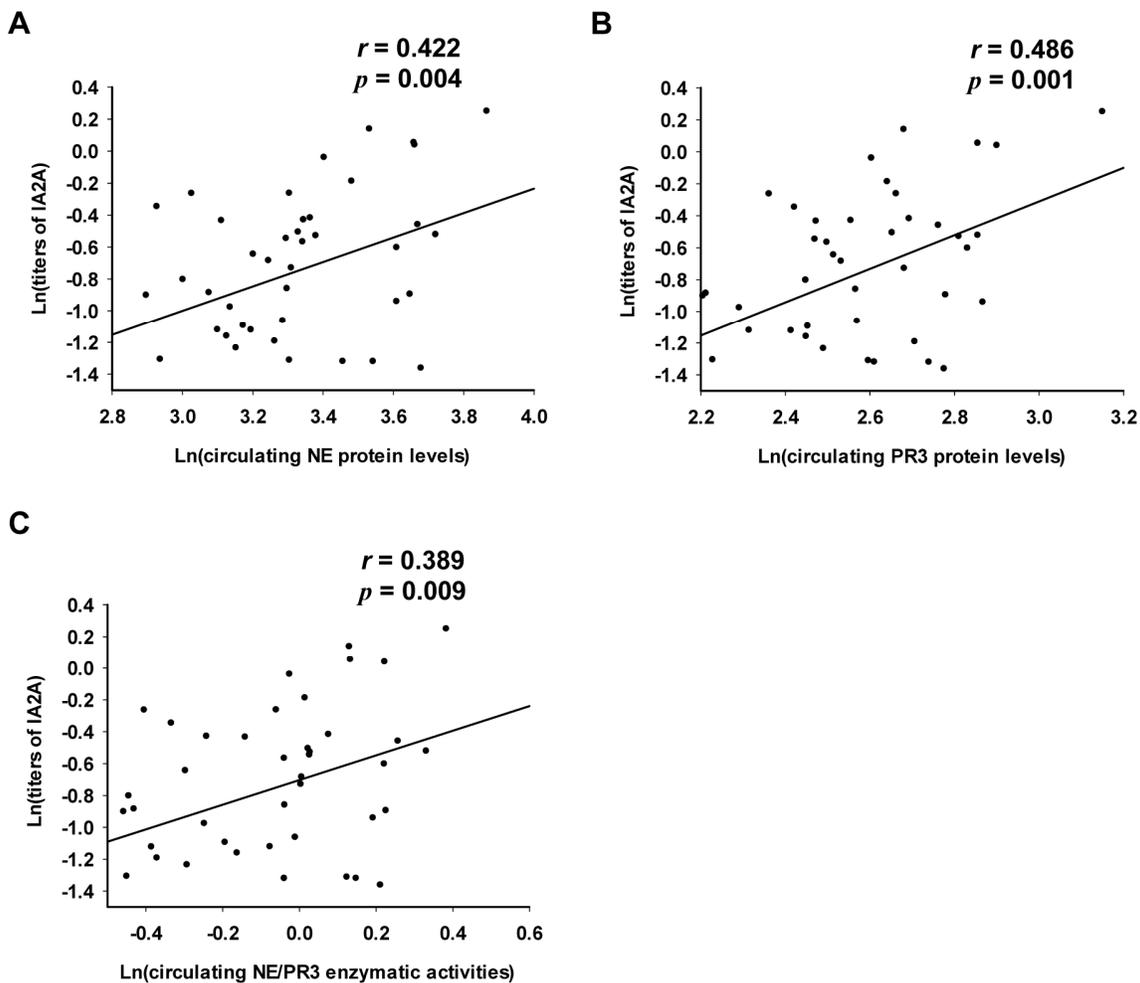
	Healthy controls	T2D patients	<i>p</i> value
n	25	25	-
Age (years)	47.8 ± 5.7	48.0 ± 6.0	0.815
Sex (men/women)	12/13	12/13	1.000
BMI (kg/m ²)	20.45 ± 2.57	26.37 ± 3.45 ^a	<0.001
Duration of diabetes (years)	N/A	0.4 (0.1-0.7)	-
Fasting glucose (mmol/L) [§]	4.86 (4.59-5.36)	7.62 (6.29-9.35) ^a	<0.001
HbA1c (%) [§]	5.24 (5.02-5.51)	7.45 (6.46-8.70) ^a	<0.001
(mmol/mol) [§]	34 (31-37)	58 (48-72) ^a	<0.001
C reactive protein (mg/L) [§]	0.47 (0.24-0.86)	1.82 (1.03-4.37) ^a	<0.001
Blood cell counts			
Erythrocytes (x 10 ⁶ /ml) [§]	4.72 (4.38-5.43)	4.79 (4.45-5.66)	0.877
White blood cells (x 10 ⁶ /ml) [§]	5.91 (5.23-7.46)	6.68 (4.62-7.93)	0.014
Lymphocytes (x 10 ⁶ /ml) [§]	2.11 (1.59-2.66)	3.08 (1.59-3.92) ^a	0.017
Monocytes (x 10 ⁶ /ml) [§]	0.38 (0.24-0.49)	0.39 (0.19-0.51)	0.796
Neutrophils (x 10 ⁶ /ml) [§]	3.42 (2.94-4.71)	3.54 (3.07-4.83)	0.487
Eosinophils (x 10 ⁶ /ml) [§]	0.15 (0.08-0.21)	0.16 (0.10-0.22)	0.864
Basophils (x 10 ⁶ /ml) [§]	0.03 (0.01-0.07)	0.04 (0.02-0.07)	0.763
Platelets (x 10 ⁶ /ml) [§]	225 (176-279)	228 (187-295)	0.824
Circulating NE protein levels (ng/ml)	403.7 (255.4-482.1)	411.5 (268.3-512.7)	0.377
Circulating PR3 protein levels (ng/ml)	109.3 (87.4-174.5)	113.7 (76.4-201.3)	0.413
Circulating NE/PR3 enzymatic activities (mU/ml)	0.16 (0.11-0.34)	0.18 (0.11-0.40)	0.428
Circulating A1AT protein levels (mg/ml)	1.70 (1.42-2.11)	1.63 (1.37-2.05)	0.275
MPO-DNA complexes (mean OD405)	0.030 (0.011-0.057)	0.033 (0.015-0.064)	0.439

Data are expressed as mean ± SD or median (interquartile range) as appropriate.

[§]Log transformed before analysis.

Supplementary Figure. 1.

Circulating protein levels of NE (A) and PR3 (B), and NE/PR3 enzymatic activities (C) were significantly correlated with the titers of IA2A in T1D patients with IA2A-positive (n=44).



Supplementary Figure. 2.

Dynamic changes of circulating NE/PR3 enzymatic activities along with their blood glucose levels in BALB/c (n=10, A) and C57BL/6 mice (n=10, B) from 2 to 30 weeks of age.

