Reduced circulating neutrophils precedes and accompanies type 1 diabetes

Short running title: mild neutropenia and type 1 diabetes

Andrea Valle\textsuperscript{1,2,*}, Gian Maria Giamporcaro\textsuperscript{1*}, Marina Scavini\textsuperscript{1,3*}, Angela Stabilini\textsuperscript{1}, Pauline Grogan\textsuperscript{3,4}, Eleonora Bianconi\textsuperscript{3,4}, Guido Sebastiani\textsuperscript{5}, Matilde Masini\textsuperscript{6}, Norma Maugeri\textsuperscript{7}, Laura Porre\textsuperscript{8}, Riccardo Bonfanti\textsuperscript{1,9}, Franco Meschi\textsuperscript{9}, Maurizio De Pellegrin\textsuperscript{10}, Arianna Lesma\textsuperscript{11}, Silvano Rossini\textsuperscript{12,§}, Lorenzo Piemonti\textsuperscript{1}, Piero Marchetti\textsuperscript{13}, Francesco Dotta\textsuperscript{5}, Emanuele Bosi\textsuperscript{1,2,4}, Manuela Battaglia\textsuperscript{1,4}

\begin{itemize}
\item \textsuperscript{1} San Raffaele Scientific Institute, Diabetes Research Institute. Milan, Italy.
\item \textsuperscript{2} Vita-Salute San Raffaele University. Milan, Italy.
\item \textsuperscript{3} San Raffaele Hospital, Internal Medicine Department. Milan, Italy.
\item \textsuperscript{4} TrialNet Clinical Center, San Raffaele Hospital Milan, Italy.
\item \textsuperscript{5} Diabetes Unit, Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry, University of Siena; Fondazione Umberto Di Mario ONLUS c/o Toscana Life Science, Siena, Italy.
\item \textsuperscript{6} Department of Experimental Pathology, University of Pisa, Pisa, Italy.
\item \textsuperscript{7} San Raffaele Scientific Institute, Division of Regenerative Medicine, Stem Cells & Gene Therapy. Milan, Italy.
\item \textsuperscript{8} Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, Interdep. Center of Cytometry. Milan, Italy.
\item \textsuperscript{9} San Raffaele Hospital, Paediatric and Neonatology Department. Milan, Italy.
\item \textsuperscript{10} San Raffaele Hospital, Paediatric Ortopaedics Department. Milan, Italy.
\item \textsuperscript{11} San Raffaele Hospital, Urology Department. Milan, Italy.
\item \textsuperscript{12} San Raffaele Hospital, Immunohematology and Transfusion Medicine Department. Milan, Italy.
\item \textsuperscript{13} Department of Endocrinology and Metabolism, University of Pisa and Unit of Endocrinology and Metabolism of Transplantation, AOUP, Pisa, Italy.
\end{itemize}

\* Equal contributors

\textsuperscript{§} current address: Niguarda Ca' Granda Hospital, Dept. of Transfusion Medicine, Milan, Italy

**Correspondence to:**

Manuela Battaglia
San Raffaele Diabetes Research Institute.
Via Olgettina 60. 20132-MILANO (Italy)
Phone: +39-02-26431
E-mail: battaglia.manuela@hsr.it

Word count: 2000
Number of Table and Figures: 4
ABSTRACT

Human type 1 diabetes (T1D) is an autoimmune disease associated with MHC polymorphisms, β-cell autoantibodies and autoreactive T-cells. However, there is increasing evidence that innate cells may also play critical roles in T1D. We aimed at monitoring peripheral immune cells in early stages of T1D (i.e., in healthy autoantibody positive subjects) and in more advanced phases of the disease (i.e., at disease onset and years after diagnosis). We found a mild, but significant and reproducible, peripheral neutropenia that both precedes and accompanies T1D onset. This reduction was not due to peripheral neutrophil cell-death, impaired differentiation or the presence of anti-neutrophil antibodies. Neutrophils were observed by electron microscopy and immunohistochemical analysis in the exocrine pancreas of multi-organ donors with T1D (both at onset and at later stages of the disease) and not in that of donors with T2D or non-diabetic donors. These pancreas-infiltrating neutrophils mainly localized at the level of very small blood vessels. Our findings suggest the existence of a hitherto unrecognized clinical phenotype that might reflect yet unexplored pathogenic pathways underlying T1D.
Type 1 diabetes (T1D) is an autoimmune disease that is associated with, and predicted by, β-cell autoantibodies (autoAb) (1), where insulin-producing β-cells are thought to be destroyed by autoreactive T cells (2). These findings, together with the recognized MHC restricted genetic susceptibility (3), suggest a prominent role of adaptive immunity in the pathogenesis of T1D. However, there is increasing evidence that innate cells play critical roles in T1D (4; 5).

RESEARCH DESIGN AND METHODS

Subjects and data collection. The study was approved by the San Raffaele Hospital Ethics Committee (protocol: DRI-002). Cell blood counts (CBC) performed by the automated haematology analyser Sysmex XE-2100 (6) at the San Raffaele Hospital were retrospectively collected from pediatric (age 4 to 17 years) and adult individuals (age ≥18 years) in the groups described below and in Table 1. **Pediatric patients with type 1 diabetes (T1D) at onset:** using the Pediatric Department Registry, we identified children diagnosed with T1D between August 2006 and December 2011, and hospitalized. Patients with at least one islet-specific autoantibody (ICA, IA2, GAD, or ZnT8) were 89.4%. CBC closest to hospital discharge and the first CBC obtained at each time point during clinical follow-up after T1D diagnosis and up to March 2012 were included in the analysis. **Healthy pediatric controls:** using the Orthopaedic Pediatric Surgery Registry we identified all children with no concomitant diseases who had elective orthopedic surgery between August 2006 and February 2012. CBC prior to surgery were included in the analysis. **Adult patients with T1D at onset:** using the San Raffaele Hospital electronic records we identified all patients newly diagnosed with T1D and who were admitted to the Department of Internal Medicine between May 2006 and October 2011 to start insulin therapy. The diagnosis of T1D was made on sustained hyperglycemia (documented by repeated glucose measurements and measurement of HbA1c), fasting C-peptide levels <1.0 ng/ml, or the presence of at least one islet-specific autoantibody. Patients with at least one islet-specific autoantibody were 71.4%. CBC closest to hospital discharge were included in the analysis. **Adult patients with long-standing T1D and T2D:** patients with T1D and duration of disease ≥5 years and patients with T2D (median disease duration: 10 years; IQR: 5-15) were recruited from the Diabetes Clinics of the San Raffaele Hospital between April and August 2011. The CBC obtained during a
scheduled follow-up visit at the clinic in the absence of acute conditions were included in the analysis. **Healthy adult controls:** the list of all active blood donors between July 2006 and December 2010 was obtained from the blood bank of the San Raffaele Hospital (ABZero) (n=7903). From this pool of donors we randomly selected sex- and age-matched controls for patients with T1D or T2D. CBC obtained prior to the first blood donation were included in the analysis. **Relatives of patients with TID:** first degree relatives (age 1 – 45 years) and second and third degree relatives (age 1 – 20 years) of patients with T1D were enrolled in the Type 1 Diabetes TrialNet Pathway to Prevention Trial (TN01 trial, former TrialNet Natural History Study) (7). The overall objective of this study is to perform baseline and repeated assessments over time of the immunologic and metabolic status of individuals at risk for T1D. The complete protocol is available online (8). Our local study was approved by the TrialNet Ancillary Studies Subcommittee and we started to collect blood in February 2012. AutoAb detection was performed at a central core laboratory. Subjects with at least two positive tests for any one of the five islet-specific autoAb (GADA, ICA512A, ICA, mIAA, and ZnT8) were defined as autoAb positive (autoAb^{POS}); subjects with all autoAb tests negative or unconfirmed (*i.e.*, one positivity never confirmed in subsequent analyses) were defined as autoAb negative (autoAb^{NEG}). AutoAb^{POS} subjects have a higher risk of developing T1D than do autoAb^{NEG} individuals (7). The individuals enrolled in the TN01 trial and included in our ancillary study were further classified as relatives of T1D patients at low risk (*i.e.*, subjects with 1 autoAb^{+} and normal OGTT, n=15) who have a 5-year diabetes risk < 2.5%, and relatives at high risk (*i.e.*, subjects with ≥2 autoAb^{+} or an history of at least one abnormal OGTT, n=10) who have a 5-year diabetes risk of 32% (9).

**Phenotype and apoptosis analysis by flow cytometry.** Fresh whole blood was stained with specific mAbs (all from BD Pharmingen, NJ, USA) and samples were analyzed with the BD FACSCanto-II flow cytometer (Becton Dickinson, BD, Franklin Lakes, NJ). Rainbow calibration particles (Spherotech Inc, IL, USA) were used to calibrate and normalize acquisition settings in each experiment. Apoptosis was defined by staining of fresh whole blood with Annexin V mAb and 7-AAD (from BD Pharmingen), and analyzed within 1 hour with the BD FACSCanto-II flow cytometer (Becton Dickinson). Analysis of flow cytometry data was performed with FCS Express 4.0 software (De Novo Software, CA, USA).

**Immunohistochemical and Electron Microscopical Studies of pancreata from organ donors.** Whole
pancreases were obtained from 3 multi-organ donors with T1D, 3 with T2D, and 6 caucasoid multi-organ donors (age: 33.2 ± 14.4 yrs; gender: 3M/3F; body mass index: 24.9 ± 1.3kg/m²) with no family history of T1D or T2D. Multi-organ donor characteristics are described in Table 1. Length of stay in intensive care unit, perfusion protocol, and cold ischemia time were not different among the 3 groups of donors analyzed. Pancreatic specimens were formalin-fixed and paraffin-embedded for immunohistochemical investigations. Immunoperoxidase staining for neutrophils on pancreatic paraffin sections was performed using rabbit anti-human myeloperoxidase mAb (Abcam, Cambridge Sciences Park, Cambridge, UK) followed by HRP-conjugated swine anti-rabbit secondary antibody (Dako Corporation, Carpinteria, CA, USA). For electron microscopy, pancreatic samples were fixed dehydrated, transferred to propylene oxide, and embedded in Epon-Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife.

**Statistical analysis.** Comparisons of general characteristics and different blood cell counts between groups were conducted using analysis of variance (ANOVA) after rank transformation when necessary. Pairwise comparisons were conducted using the Tukey WSD test when the ANOVA showed a significant difference between at least two group means. Comparisons of neutrophil and platelet counts in pediatric patients at T1D onset and one year (±60 days) after diagnosis was conducted using the Mann-Whitney U-test. Neutrophil and platelets counts obtained during the clinical follow-up of pediatric patients after T1D diagnosis was analyzed using a mixed-effect model, with time as the fixed effect. An unstructured variance-covariance matrix was used to model the correlation between repeated measurements within each patient. Data management and statistical analysis were conducted with the statistical software Stata, version 11.0 (Stata Corp., College Station, TX).

**RESULTS**

A retrospective analysis of cell blood counts (CBC) performed in pediatric patients newly diagnosed with T1D demonstrated that circulating neutrophils and platelets were significantly lower than those in healthy control subjects (Table 2). The same phenotype was confirmed in a subset of 123 patients with T1D at onset tested 1 year (±60 days) after diagnosis (data not shown). Given that the reduced neutrophil and platelet counts might derive from the metabolic derangements that often characterize patients with T1D, we analyzed CBC
performed in the subjects enrolled in the Path to Prevention Clinical Trial (TN01, TrialNet) (7). Both neutrophils and platelets were already significantly reduced in healthy pediatric autoAb\textsuperscript{POS} relatives of T1D patients but not in autoAb\textsuperscript{NEG} relatives (Figure 1A). Neutrophil and platelet reductions were proportionate to the risk of developing T1D \textit{(i.e., the more pronounced the neutrophil decline the higher the risk of developing T1D)} (see Research Design and Methods section for risk-assessment) (Figure 1B). All CBC obtained after T1D diagnosis were then analyzed and neutrophils appeared to return to normal levels at 5 years from diagnosis, while platelets remained low (Figure 1C).

A similar reduced level of circulating neutrophils was also confirmed in a group of adult patients newly diagnosed with T1D and not in those with T2D, while platelets, in contrast with the findings for pediatric subjects, were not reduced. As in the pediatric populations, neutrophils were normal in adult patients with \( \geq 5 \) years from diagnosis (Figure 1D). Similar results were observed in an independent group of adult patients followed in the Outpatient Diabetes Care Unit at Siena University Hospital (Suppl. Figure 1).

Reduced circulating neutrophils can be caused by one or more of the following: (i) impairment in neutrophil output from the bone marrow and/or in their differentiation; (ii) increase in peripheral consumption/destruction; (iii) tissue sequestration. A bone marrow defect and/or an impaired peripheral differentiation are unlikely, since the phenomenon is mild, transitory (from preclinical phase to a few years after clinical onset) and there was no evidence of peripheral accumulation of immature forms, such as banded cells (Suppl. Figure 2A). Alternatively, an increase in peripheral neutrophil consumption/destruction might be caused by augmented neutrophil apoptosis (10) or by anti-neutrophil specific antibodies (11). Both hypotheses were tested and neither of the two were supported by experimental evidence. Neutrophil apoptosis, as determined by 7AAD and annexin V stainings, was very low in all donors tested \textit{(i.e., 11 autoAb\textsuperscript{NEG} relatives, 3 autoAb\textsuperscript{POS} relatives, 3 patients with T1D at onset and 2 T1D patients long-term)} (data not shown). In addition, the expression levels of CD11b and CD16 in neutrophils were similar in all cohorts analyzed, a finding that further confirms the lack of any specific neutrophil activation, which might specifically account for increased neutrophil death (Suppl. Figure 2B). Anti human-neutrophil antibodies directed towards cytoplasmic (both pANCA and cANCA) or surface neutrophil antigens (HNA) were not consistently observed in T1D patients and relatives.
(supplementary Table 1). Finally, the hypothesis of tissue sequestration at pancreatic level was tested in pancreatic tissue specimens from adult organ donors with T1D, T2D, and from non-diabetic donors (12-14) (donor characteristics described in Table 1). Increased numbers of neutrophils were specifically detected in the exocrine pancreas of the 3 T1D patients analyzed but not in that of T2D and non-diabetic donors. These pancreas-infiltrating neutrophils mainly localize at the level of very small blood vessels and, to a lesser extent, adjacently to acinar cells (Figure 2A-B). These data, albeit generated in a limited number of donors, were consistent between individuals and independent of the disease stage (from onset to long-standing) (Figure 2C). A low number of neutrophils was observed close to beta islets, but only in the recently diagnosed T1D patient (Figure 2D).

DISCUSSION

These findings demonstrate an association between human T1D and a hitherto unrecognized neutropenia; though minor, said neutropenia is not negligible (with neutrophil reduction varying from 7% to 27%). This abnormality manifests from the preclinical phase of the disease to onset and persists for some years prior to long-term resolution. During the preclinical phase, neutrophil reduction is greatest in the subjects with the highest risk of developing T1D, a finding that possibly reflects the severity of the underlying autoimmune process. After disease onset, the persistence of mild neutropenia for a few years, and its subsequent resolution, seems to mirror the continuing destruction of β-cell mass for the time of its residual survival. Hence, reduced circulating neutrophils seems to be a phenotype that accompanies the phase of active, ongoing, destructive β-cell specific autoimmunity. Conversely, the lack of this phenotype in patients with T2D and the lack of correlation between glucose levels/HbA1c and neutrophil counts in both pediatric and adult subjects (data not shown) excludes hyperglycemia and the associated metabolic abnormalities as the cause of this reduction in T1D.

Platelet levels seem to be reduced exclusively in patients with disease onset during childhood, and to differ in trend from neutrophil levels (i.e., the former remain low beyond 5 years from diagnosis). However, this difference might be ascribed to the small group of adult individuals analyzed as compared to the pediatric one.
Future longitudinal studies including more adult subjects and with a well-designed sampling time frame should allow drawing definitive conclusion about neutrophil and platelet counts in type 1 diabetes.

A specific reduction in circulating neutrophils in T1D might be an indirect evidence of a chronic viral infection, which has been long suspected as a trigger in susceptible hosts. However, despite decades of research, the body of evidence supporting a relationship between viral infections and initiation or acceleration of islet autoimmunity remains largely circumstantial (15).

The occurrence of neutrophils in inflamed tissues targeted by an autoimmune process is not surprising from an immunological point of view (16), but, to our knowledge, this finding in humans has been reported only once (several years ago) (17) and has never been confirmed in subsequent studies (18). However, we recognize that our data were generated in a limited amount of donors and are far from being conclusive.

While neutrophils play a crucial role in several autoimmune diseases (e.g., SLE, RA) (19) and also, as recently demonstrated, in a murine model of T1D (4), their function in human T1D has to date been ignored. There are several likely reasons for this neglect: the perception of neutrophils as terminally differentiated, short-lived immune cells; the lack of appropriate methods of molecular manipulation of neutrophils; and the inability to test the role of such cells in a given disease mechanism. However, more recent studies, indicate that neutrophils are capable of performing a large number of functions that are critical for the autoimmune disease process, including: antigen presentation, regulation of the activity of other cell types (20; 21), and direct tissue damage (16). Our new evidence suggests that neutrophils might be key also in the pathogenesis of T1D.
AUTHOR CONTRIBUTIONS

A.V., G.M.G., M.S. conceived the study, designed the experiments, analyzed and interpreted data, and wrote the manuscript. A.S. processed samples and performed in vitro experiments. P.G. and E.Bi. collected samples. G.S. and M.M. performed analyses on pancreas sections. N.M. contributed to experiment design, analyzed and interpreted data. L.Po. performed experiments. A.L. recruited pediatric patients and performed anti-neutrophil antibodies assays. R.B. and F.M. recruited pediatric patients with T1D. M.De P. and A. L. recruited pediatric non diabetic individuals. S.R. recruited adult non diabetic individuals. L.Pi. contributed to experiment design and interpreted data. P.M. collected pancreas from multi-organ donors. F.D. contributed to experiment design and interpreted data. E.Bo. analyzed and interpreted data, and wrote the manuscript. M.B. conceived the study, designed the experiments, analyzed and interpreted data, and wrote the manuscript. M.B. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ACKNOWLEDGMENTS

M. Battaglia is supported by the Juvenile Diabetes Research Foundation, the Italian Ministry of Health and the European Union. F. Dotta is supported by the European Union [Collaborative Projects NAIMIT and PEVNET in the Framework Program 7 (FP7)] and by the Italian Ministry of Research.

Type 1 Diabetes TrialNet is a clinical trials network funded by the National Institutes of Health (NIH) through the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the National Institute of Allergy and Infectious Diseases (NIAID), the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), the National Center for Research Resources (NCRR), the Juvenile Diabetes Research Foundation International (JDRF), and the American Diabetes Association (ADA).

The TrialNet TN01 study at the San Raffaele Hospital is supported by a grant from the Juvenile Diabetes Research Foundation (JDRF# 6-2012-18) to E. Bosi.

Andrea Valle conducted this study as partial fulfillment of his PhD in Molecular Medicine, Cellular and Molecular Biology Program, San Raffaele University, Milan, Italy.

The authors thank: all Manuela Battaglia’s lab (especially Georgia Fousteri and Cristina Morsiani, for excellent technical help/support); all nurses from the pediatric department at the San Raffaele Hospital for blood collection; Sonny Michael Assennato (Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico) and Elena Bazzigalupi (Laboraf) for performing the anti-neutrophil Ab tests; Giulio Frontino and Andrea Rigamonti (Paediatric and Neonatology Department at the San Raffaele Hospital) and Andrea Laurenzi (Internal Medicine Department at the San Raffaele Hospital) for patient recruitment; Isabella Spagnuolo and Aurora Patti (University of Siena) for excellent contribution to morphometric counting of neutrophils on human pancreatic sections; Gaetano Di Terlizzi, Ferruccio Ceriotti, Massimo Locatelli (Laboraf) for technical support; Davide Di Napoli (San Raffaele Hospital) for providing patient information; and Luca G. Guidotti and Angelo Manfredi (San Raffaele Scientific Institute) for suggestions and critical discussion.

All authors declare to have no conflicting financial interests.
REFERENCES

| **TABLES**  
Table 1. Characteristics of the subjects recruited at the San Raffaele Hospital and of the multi-organ donors included in the study. |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PEDIATRIC POPULATION</strong></td>
<td><strong>Healthy controls</strong></td>
<td><strong>Relatives autoAb&lt;sup&gt;NEG&lt;/sup&gt;</strong></td>
<td><strong>Relatives autoAb&lt;sup&gt;POS&lt;/sup&gt;</strong></td>
<td><strong>T1D at onset</strong></td>
</tr>
<tr>
<td>n</td>
<td>198</td>
<td>138</td>
<td>25</td>
<td>238</td>
</tr>
<tr>
<td>Age (years)</td>
<td>12 (9-14)</td>
<td>11 (8-14)</td>
<td>9 (5-14)</td>
<td>10 (7-13) §</td>
</tr>
<tr>
<td>Sex, n females (%)</td>
<td>73 (37)</td>
<td>63 (46)</td>
<td>12 (48)</td>
<td>113 (47)</td>
</tr>
<tr>
<td>Glucose (mg/dL)*</td>
<td>n.a.</td>
<td>n.a.</td>
<td>89 (85 – 92)</td>
<td>359 (238-477)</td>
</tr>
<tr>
<td>HbA1c (%) (mmol/mol)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>4.9 (4.7-5.0)</td>
<td>30 (28 – 31)</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.27 (0.2-0.57)</td>
</tr>
<tr>
<td><strong>ADULT POPULATION</strong></td>
<td><strong>Healthy controls</strong></td>
<td><strong>T1D at onset</strong></td>
<td><strong>T1D long-standing</strong></td>
<td><strong>T2D</strong></td>
</tr>
<tr>
<td>n</td>
<td>511</td>
<td>45</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44 (28-60)</td>
<td>26 (20-31) §</td>
<td>41 (34-51)</td>
<td>65 (56.5-69) §</td>
</tr>
<tr>
<td>Sex, n females (%)</td>
<td>178 (35)</td>
<td>17 (38)</td>
<td>30 (45)</td>
<td>14 (23)</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>n.a.</td>
<td>176 (145-266)</td>
<td>145 (108-202)</td>
<td>128 (111-156)</td>
</tr>
<tr>
<td>HbA1c (%) (mmol/mol)</td>
<td>n.a.</td>
<td>12 (10.9-13.4)</td>
<td>7.8 (7.4-8.6)</td>
<td>7.3 (6.5-7.7)</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>n.a.</td>
<td>0.47 (0.32-0.86)</td>
<td>0.09 (0.01-0.37)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>-</td>
<td>-</td>
<td>17 (11-29)</td>
<td>10 (5-15)</td>
</tr>
<tr>
<td><strong>MULTI-ORGAN DONORS</strong></td>
<td><strong>Age (years)</strong></td>
<td><strong>Sex</strong></td>
<td><strong>Diabetes duration</strong></td>
<td><strong>β-cell loss</strong></td>
</tr>
<tr>
<td>Non-diabetic donors* (n=6)</td>
<td>39.5 (24-47)</td>
<td>3 M; 3 F</td>
<td>-</td>
<td>no</td>
</tr>
<tr>
<td>T1D #1</td>
<td>25</td>
<td>M</td>
<td>10 weeks</td>
<td>partial</td>
</tr>
<tr>
<td>T1D #2</td>
<td>19</td>
<td>F</td>
<td>7 years</td>
<td>yes</td>
</tr>
<tr>
<td>T1D #3</td>
<td>45</td>
<td>F</td>
<td>17 years</td>
<td>yes</td>
</tr>
<tr>
<td>T2D #1</td>
<td>47</td>
<td>M</td>
<td>3 years</td>
<td>no</td>
</tr>
<tr>
<td>T2D #2</td>
<td>45</td>
<td>F</td>
<td>6 years</td>
<td>no</td>
</tr>
<tr>
<td>T2D #3</td>
<td>60</td>
<td>M</td>
<td>11 years</td>
<td>partial</td>
</tr>
</tbody>
</table>
The pediatric population includes individuals ≥4 and ≤17 years old. The adult population includes individuals ≥18 years old. For the pediatric and adult populations continuous variables are presented as median with interquartile range (IQR) in parentheses; categorical variables as frequency and percent in parentheses.

For Multi-organ donors individual data are presented.

T1D = type 1 diabetes; T2D = type 2 diabetes; HbA1c = glycated hemoglobin. §Significantly different from controls. n.a. = not available. *Laboratory glucose measurement upon hospital admission.
Table 2. *Cell Blood Counts (x10^3/µl)*

Data are expressed as median with interquartile range (25th percentile – 75th percentile) in parentheses.  
* lower limit for determination of precision profiles of eosinophils >0.1 x10^3/µl (6). Only samples with eosinophil counts >0.1 x10^3/µl were included in the analysis (*i.e.*, controls n=123 and T1D at onset n=128).  
§ too low to be counted with high precision levels with the automated haematology analyser Sysmex XE-2100 (6).

<table>
<thead>
<tr>
<th>PEDIATRIC POPULATION</th>
<th>controls (n=198)</th>
<th>T1D at onset (n= 238)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Blood Cells</td>
<td>6.9 (5.8-8.3)</td>
<td>6.4 (5.5-7.8)</td>
<td>0.062</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.5 (2.1-2.9)</td>
<td>2.7 (2.1-3.3)</td>
<td>0.192</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.6 (0.5-0.7)</td>
<td>0.5 (0.4-0.7)</td>
<td>0.951</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.4 (2.6-4.4)</td>
<td>2.6 (2.1-3.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Eosinophils*</td>
<td>0.3 (0.2-0.4)</td>
<td>0.2 (0.2-0.3)</td>
<td>0.201</td>
</tr>
<tr>
<td>Basophils§</td>
<td>//</td>
<td>//</td>
<td>-</td>
</tr>
<tr>
<td>Red Blood Cells</td>
<td>4.8 (4.5-5.0)</td>
<td>4.7 (4.4-4.9)</td>
<td>0.899</td>
</tr>
<tr>
<td>Platelets</td>
<td>259 (225-306)</td>
<td>241 (207-287)</td>
<td>0.0046</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. (A) Neutrophil and platelet counts in pediatric healthy controls (n=198), autoAb\(^{-}\)NEG (n=138) and autoAb\(^{\text{POS}}\) (n=25) relatives of patients with T1D and patients with T1D at onset (n=238); *** p<0.0001, ** p<0.005 vs healthy controls, ANOVA and Tukey WSD test. The lower normal laboratory range is shown by the dotted line. (B) Neutrophil and platelet counts in pediatric autoAb\(^{-}\)NEG relatives (n=138), autoAb\(^{\text{POS}}\) relatives at low (n=15) and high risk (n=10) as defined in the Research Design and Methods section. The non parametric test for trend was significant for both neutrophil (p<0.001) and platelet (p=0.023) counts. Means and standard deviations of neutrophil and platelet counts for each group are also reported. (C) Neutrophil and platelet counts in pediatric patients studied at onset of T1D and measured during clinical follow-up. Dots represent mean values and bars 95% confidence intervals. Numbers in parentheses on the x axis represent the number of patients analyzed. (D) Neutrophil and platelet counts in adult healthy controls (n=511), patients with T1D at onset (n=45), individuals with ≥ 5 years of T1D (n=67), and T2D individuals (n=60) aged ≥18 years; ** p<0.005 vs healthy controls, ANOVA and Tukey WSD test. The lower normal laboratory range is shown by the dotted line.

Figure 2. (A) Neutrophils were detected by immunoperoxidase in the exocrine pancreas. Representative neutrophil stainings of sections from donor T1D\#1 (I-II), donor T1D\#3 (III), and a non-diabetic donor (IV) are shown. These pancreas-infiltrating neutrophils mainly localized at the level of very small blood vessels (panels I and III) and, to a lesser extent, adjacent to acinar cells (panel II). (B) Representative sections analyzed at the electron microscopy of the pancreas collected from donor T1D\#1 showing a granulocyte (GN) and a lymphocyte (L) in a microvessel of the exocrine tissue (left panel), and a granulocyte (GN) adjacent to acinar cells (right panel). In both panels, red stars identify pancreatic acinar cells. (C) The frequency of pancreatic neutrophils was determined by immunoperoxidase on pancreatic paraffin sections. The numbers of myeloperoxidase positive cells \(/\text{mm}^2\) in the pancreas (left panel) and within the small blood vessels (right panel) are shown. At least 20 fields/donor have been examined. (D) A representative section of the pancreas collected from donor T1D\#1, analyzed at electron microscopy, showing granulocytes (GN) adjacent to beta cells (β).
Figure 1

(A) Box plots showing the distribution of neutrophils and platelets (x10^3/µl) in healthy controls, relatives autoAb^NEG, relatives autoAb^PO, and T1D new onset.

(B) Box plots showing the distribution of neutrophils and platelets (x10^3/µl) in relatives autoAb^NEG, relatives autoAb^PO, and T1D new onset grouped by LOW and HIGH risk.

(C) Line graphs showing the change in neutrophils and platelets (x10^3/µl) over years after T1D diagnosis (number of patients indicated).

Diabetes

Valle A, Giamporcaro G, Scavini M et al.
Figure 1

Neutrophils ($\times 10^3/\mu l$)

- Healthy controls
- T1D new onset
- T1D long-standing
- T2D

Platelets ($\times 10^3/\mu l$)

- Healthy controls
- T1D new onset
- T1D long-standing
- T2D

Valle A, Giamporcaro G, Scavini M et al.
Figure 2

A

B

C

D
Supplementary Figure 1. The neutrophil counts were determined by the automated haematology analyzer at the Siena University Hospital on blood collected from: patients within 1 week from diagnosis of T1D (all patients were positive for at least one T1D-associated autoantibody: anti-GAD, anti-IA-2, anti-ZnT8); patients with T1D and a disease duration >1 and <5 years; patients with T1D and a disease duration >5 years; and non-diabetic control individuals with no family history for T1D recruited among blood donors at Immunohematology Unit of the Siena University Hospital. Patient characteristics are described in the table below. Stars indicate values significantly different as compared to those in healthy controls (** p=0.0003; *** p<0.0001; * p=0.03).

Characteristics of subjects included in the analysis.
*Age is presented as median with interquartile range (25th percentile – 75th percentile) in parentheses

<table>
<thead>
<tr>
<th>ADULT POPULATION</th>
<th>T1D at onset (n=32)</th>
<th>T1D 1-5 yrs from diagnosis (n=24)</th>
<th>T1D &gt;5 yrs from diagnosis (n=78)</th>
<th>healthy controls (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years*</td>
<td>30 (25-35)</td>
<td>33 (28-37)</td>
<td>42 (24-51)</td>
<td>34 (27-39)</td>
</tr>
<tr>
<td>Sex (% females)</td>
<td>34%</td>
<td>54%</td>
<td>51%</td>
<td>47%</td>
</tr>
</tbody>
</table>
Supplementary Figure 2. (A) The frequency of immature neutrophils (i.e., banded cells CD10−) was determined by flow-cytometry on fresh total blood upon gating on CD3−CD14−CD11b+CD16+CD49d− cells. (B) Mean fluorescence intensity of CD11b and CD16 on mature neutrophils (i.e., segmented cells CD10+) was determined by flow-cytometry on fresh total blood upon gating on CD3−CD14−CD11b+CD16+CD49d− cells. Each dot represents one donor.
**Supplementary Table 1.** *Anti human-neutrophils Ab directed towards cytoplasmic (both pANCA and cANCA) or surface neutrophil antigens (anti-HNA)*

The number of donors tested are listed and the samples positive are shown in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>healthy controls</th>
<th>relatives autoAb&lt;sup&gt;NEG&lt;/sup&gt;</th>
<th>relatives autoAb&lt;sup&gt;POS&lt;/sup&gt;</th>
<th>T1D at onset</th>
<th>T1D 1 year</th>
<th>T1D long-term</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-ANCA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tested (pos)</td>
<td>23 (0)</td>
<td>17 (0)</td>
<td>12 (0)</td>
<td>23 (1)</td>
<td>17 (1)</td>
<td>13 (0)</td>
</tr>
<tr>
<td><strong>Anti-HNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tested (pos)</td>
<td>5 (0)</td>
<td>14 (2)</td>
<td>5 (0)</td>
<td>6 (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**SUPPLEMENTARY TABLES**

**Supplementary Table 1.** *Anti human-neutrophils Ab directed towards anti-neutrophil cytoplasmic antibodies (both pANCA and cANCA) or surface neutrophil antigens (anti-HNA).*

The number of donors tested are listed and the samples positive are shown in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>healthy controls</th>
<th>relatives autoAb&lt;sup&gt;NEG&lt;/sup&gt;</th>
<th>relatives autoAb&lt;sup&gt;POS&lt;/sup&gt;</th>
<th>T1D at onset</th>
<th>T1D 1 year</th>
<th>T1D long-term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ANCA tested (pos)</td>
<td>23 (0)</td>
<td>17 (0)</td>
<td>12 (0)</td>
<td>23 (1)</td>
<td>17 (1)</td>
<td>13 (0)</td>
</tr>
<tr>
<td>Anti-HNA tested (pos)</td>
<td>5 (0)</td>
<td>14 (2)</td>
<td>5 (0)</td>
<td>6 (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>