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Abnormal neutrophil signature in the blood and pancreas of presymptomatic and symptomatic type 1 diabetes

Federica Vecchio,[1] Nicola Lo Buono,[1] Angela Stabilini,[1] Laura Nigi,[2] Matthew J. Dufort,[3] Susan Geyer,[4] Paola Maria Rancoita,[5] Federica Cugnata,[5] Alessandra Mandelli,[1] Andrea Valle,[1] Pia Leete,[6] Francesca Mancarella,[2] Peter S. Linsley,[3] Lars Krogvold,[7] Kevan C. Herold,[8] Helena Elding Larsson,[9] Sarah J. Richardson,[6] Noel G. Morgan,[6] Knut Dahl-Jørgensen,[7] Guido Sebastiani,[2] Francesco Dotta,[2] Emanuele Bosi,[1,10,11] the DRI_Biorepository Group,[12] the Type 1 Diabetes TrialNet Study Group,[13] and Manuela Battaglia[1,11]

[1]Diabetes Research Institute, IRCCS San Raffaele Scientific Institute, Milan, Italy. [2]Diabetes Unit, Department of Medicine, Surgery and Neuroscience, University of Siena, and Fondazione Umberto Di Mario ONLUS c/o Toscana Life Science, Siena, Italy. [3]Systems Immunology Division, Benaroya Research Institute, Seattle, Washington, USA. [4]University of South Florida, TNCC, Tampa, Florida, USA. [5]Centre of Statistics for Biomedical Sciences (CUSSB), Vita-Salute San Raffaele University, Milan, Italy. [6]Institute of Biomedical and Clinical Sciences, University of Exeter Medical School, RILD Building Barrack Road, Exeter, Devon, United Kingdom. [7]Pediatric Department, Oslo University Hospital HF, Oslo, Norway; Faculty of Medicine, University of Oslo, Oslo, Norway. [8]Departments of Immunobiology and Internal Medicine, Yale University, New Haven, Connecticut, USA. [9]Department of Clinical Sciences, Lund University/CRC, Skane University Hospital SUS, Malmo, Sweden. [10]Vita-Salute San Raffaele University, Milan, Italy, and the Department of Internal Medicine, IRCCS San Raffaele Hospital, Milan, Italy. [11]TrialNet Clinical Center, IRCCS San Raffaele Hospital, Milan, Italy. [12]The DRI_Biorepository Group is detailed in the Supplemental Acknowledgments. [13]The Type 1 Diabetes TrialNet Study Group is detailed in the Supplemental Acknowledgments.

BACKGROUND. Neutrophils and their inflammatory mediators are key pathogenic components in multiple autoimmune diseases, while their role in human type 1 diabetes (T1D), a disease that progresses sequentially through identifiable stages prior to the clinical onset, is not well understood. We previously reported that the number of circulating neutrophils is reduced in patients with T1D and in presymptomatic at-risk subjects. The aim of the present work was to identify possible changes in circulating and pancreas-residing neutrophils throughout the disease course to better elucidate neutrophil involvement in human T1D.

METHODS. Data collected from 389 subjects at risk of developing T1D, and enrolled in 4 distinct studies performed by TrialNet, were analyzed with comprehensive statistical approaches to determine whether the number of circulating neutrophils correlates with pancreas function. To obtain a broad analysis of pancreas-infiltrating neutrophils throughout all disease stages, pancreas sections collected worldwide from 4 different cohorts (i.e., nPOD, DiViD, Siena, and Exeter) were analyzed by immunohistochemistry and immunofluorescence. Finally, circulating neutrophils were purified from unrelated nondiabetic subjects and donors at various T1D stages and their transcriptomic signature was determined by RNA sequencing.

RESULTS. Here, we show that the decline in β cell function is greatest in individuals with the lowest peripheral neutrophil numbers. Neutrophils infiltrate the pancreas prior to the onset of symptoms and they continue to do so as the disease progresses. Of interest, a fraction of these pancreas-infiltrating neutrophils also extrudes neutrophil extracellular traps (NETs), suggesting a tissue-specific pathogenic role. Whole-transcriptome analysis of purified blood neutrophils revealed a unique molecular signature that is distinguished by an overabundance of IFN-associated genes; despite being healthy, said signature is already present in T1D-autoantibody-negative at-risk subjects.

CONCLUSIONS. These results reveal an unexpected abnormality in neutrophil disposition both in the circulation and in the pancreas of presymptomatic and symptomatic T1D subjects, implying that targeting neutrophils might represent a previously unrecognized therapeutic modality.

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Introduction

Neutrophils are key cells in the recognition and elimination of pathogens, but they also sense self components, including products of sterile tissue damage. Normally, self-recognition contributes to tissue repair but, in some circumstances, it can also lead to the release of highly immunogenic factors that can trigger and/or amplify autoimmune pathogenic loops. Neutrophils therefore can contribute to the clearance of acute inflammation, but they may also play a harmful role in chronic inflammatory and autoimmune diseases (1). Recent experimental data indicate that neutrophils play a pivotal role in both the immunization and the effector phases in systemic lupus erythematosus (SLE) (2, 3), rheumatoid arthritis (4, 5), and autoimmune vasculitis (6).

Type 1 diabetes (T1D) is an autoimmune disease characterized by loss of insulin production and reliance on exogenous insulin for survival. Development of islet autoantibodies (autoAbs) occurs before clinical diagnosis, making T1D a predictable disease in an individual with 2 or more autoAbs (7). T1D is mainly considered to be a T cell–centered disease and the role of neutrophils (the most abundant circulating immune cell type and the first immune cells to respond to inflammation) in the etiology of this disease in humans has mostly been ignored (8). The contribution of neutrophils to the pathogenesis of T1D was demonstrated for the first time in the nonobese diabetic (NOD) mouse model, in which self-DNA–anti-DNA IgG immune complexes formed in the pancreas trigger neutrophil extracellular trap (NET) formation and the release of the cathelicidin peptide CRAMP. These complexes activate plasmacytoid dendritic cells to release IFN-α, which is believed to trigger further T cell–mediated autoimmune processes (9). In humans we have previously reported that the number of circulating neutrophils is reduced in patients with T1D as well as in presymptomatic at-risk subjects (10) (see supplemental materials for detailed T1D staging; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.122146DS1). This reduction was subsequently confirmed independently (11–14). We also reported that neutrophils infiltrate the pancreas of donors with T1D and are not observed in the pancreas of nondiabetic or T2D donors (10), raising the notion that these cells may be playing a pathologic role. The aim of the present work was to identify possible changes in circulating and pancreas-residing neutrophils in T1D throughout the disease course to better elucidate neutrophil involvement also in humans. To perform this study, data and samples were collected from various donor cohorts and details of the study design can be found in Table 1 and in the Methods.

Results

Presymptomatic T1D subjects with reduced circulating neutrophil numbers have the poorest β cell function. To test whether the number of circulating neutrophils correlates with pancreas function, we collected data from 3 clinical prevention studies performed by TrialNet (see supplemental materials for detailed study descriptions). The studies involved 289 presymptomatic at-risk subjects (Supplemental Tables 1 and 2 for study and donor characteristics). Several metabolic measures (single and composite) collected in a 2-hour standard oral glucose tolerance test (OGTT) at study screening (i.e., before any therapeutic intervention) were evaluated in relation to the number of circulating neutrophils measured by complete blood counts (CBCs) (Supplemental Table 3). In these at-risk individuals the number of circulating neutrophils significantly correlated with fasting C-peptide, early C-peptide response (30 minute – basal C-peptide), and stimulated C-peptide (mean C-peptide AUC) as well as with the composite metabolic measures HOMA-β and Index60 but not with any of the remaining measures (Figure 1A and Supplemental Table 4). These correlations were neutrophil specific since significant meaningful associations were not observed in relation to lymphocyte counts except for a modest correlation with fasting C-peptide and glucose (Figure 1A and Supplemental Table 4).

The number of circulating neutrophils was significantly influenced by age, sex, and BMI percentile (Supplemental Table 5). These parameters were therefore evaluated in multivariable models with the metabolic markers of interest and we found that fasting and stimulated C-peptide remained significantly associated with peripheral neutrophil counts after adjustment for these factors (Supplemental Table 6). Further evaluation of the metabolic markers adjusting for these parameters (as well as interactions with them), were used in the model-building approaches. Significant interactions emerged between fasting C-peptide and age, as well as between stimulated C-peptide and BMI in relation to neutrophil counts. Thus, the influence of fasting C-peptide on neutrophil counts was more strongly correlated and influential in older subjects, while that of stimulated C-peptide was more strongly associated and influential in those subjects who are overweight or obese (Figure 1B; see Supplemental Tables 7 and 8 for estimated model descriptions).

Table 1. Study design

Study Objective	Data|Sample origin	Donor characteristics	n	Reference Figure|Table|Video
Correlation between pancreas function and CBC measures	TN10, TN18, TN20	AutoAb pos	Subjects 289, Observations 289	Figure 1, A and B, Tables S1–S8[A]
	TN01 – Milan Site	AutoAb neg&pos	Subjects 109, Observations 303	Figure 1C, Tables S9–S12
Neutrophil localization and characterization in the pancreas	nPOD	nondiabetic	Subjects 6, Sections 14	
		AutoAb pos	Subjects 4, Sections 10	
		T1D	Subjects 4, Sections 10	
	SIENA	T1D	Subject 1, Sections 3	Figures 2 and 3, Figures S1–S3, Tables S12 and S13, Videos S1–S4
	DiViD	T1D	Subjects 6, Sections 12	
	EXETER	T1D	Subjects 5, Sections 5	
Transcriptomic signature of circulating (blood-derived) neutrophils	Local Cohort	nondiabetic	Subjects 16	
		T1D	Subjects 5	Figures 4–6, Figures S4–S6, Table S14
	TN01	AutoAb neg	Subjects 13	
		AutoAb pos	Subjects 8	

[A]S indicates Supplemental. TN, TrialNet; AutoAb, T1D autoantibody; pos, positive; neg, negative; CBC, complete blood count.

We emphasize that these data originate from intervention studies in which CBCs were measured at differing clinical sites and where donor selection was determined by relevant study inclusion criteria (listed in Supplemental Table 1). To address this potential bias, we collected analogous data from our local (Milan, Italy) TN01 (TrialNet Pathway to Prevention Study) cohort in whom CBC measurements were performed at a single clinical site and no inclusion criteria (other than having a relative with T1D) were applied (see supplemental materials pertinent study details). A total of 109 presymptomatic subjects with 303 overall observations were included in the analysis (see Supplemental Tables 9 and 10 for donor and observation characteristics). Given the presence of repeated measures and the familial relationships between some of the donors, linear mixed-effects models were applied. On the basis of previous analysis of the TN-intervention cohort, we focused on fasting and stimulated C-peptide and considered the same covariates. In both models, neutrophil counts significantly increased with BMI. Fasting C-peptide also had a significantly positive effect on neutrophil counts and this effect increased with age. Stimulated C-peptide had a similar effect on neutrophil counts, but the size effect depended both on age (bigger effects were seen in older people) and on sex (males had lower circulating neutrophils) (Figure 1C; see Supplemental Tables 11 and 12 for estimated model descriptions).

Overall, these data gathered from 398 presymptomatic at-risk TrialNet donors (for a total of 592 observations), upon careful and comprehensive statistical analyses that considered several potentially confounding factors, indicate that reduced β cell function (as measured by fasting and stimulated C-peptide production after a standard 2-hour OGTT) consistently and significantly correlates with reduced circulating neutrophil counts.
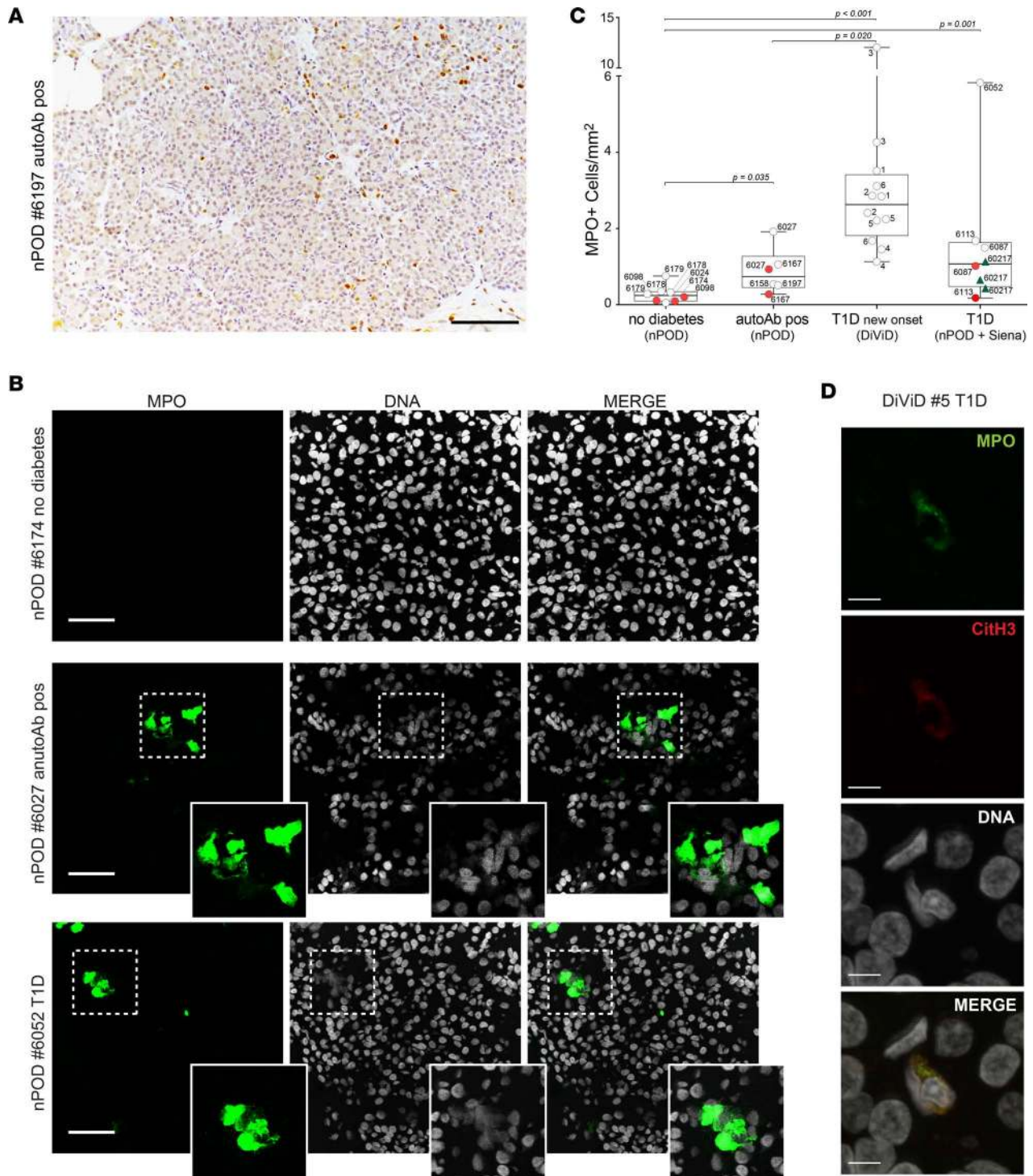
Neutrophils releasing NETs infiltrate the pancreas of presymptomatic and symptomatic T1D subjects. We know, from our previous work, that neutrophils infiltrate the pancreas of organ donors with T1D (10) but it remains unclear whether this is limited to the cohort originally examined and whether neutrophils also infiltrate the pancreas of presymptomatic individuals. We therefore analyzed 4 independent pancreas cohorts collected worldwide: nPOD (15), DiViD (16), Exeter (17), and Siena (18) (see Supplemental Tables 13 and 14 for donor and pancreas section characteristics). Immunohistochemical analysis demonstrated, for the first time to our knowledge, that pancreas-residing neutrophils can be found in presymptomatic autoAb-positive donors (Figure 2A) and confirmed that these cells infiltrate the pancreas of subjects with T1D (Supplemental Figure 1A). To increase our understanding of tissue-resident neutrophil morphology, we performed immunofluorescence analysis upon DNA and myeloperoxidase (MPO) staining in 2 independent laboratories on frozen, optimal cutting temperature compound–embedded (OCT-embedded) (Figure 2B and Supplemental Videos 1 and 2) and on formalin-fixed, parrafin-embedded (FFPE) sections (Supplemental Figure 1B). Quantitative analysis of MPO-positive cells confirmed that pancreas-residing neutrophils are more frequent in presymptomatic autoAb-positive and T1D donors than in nondiabetic
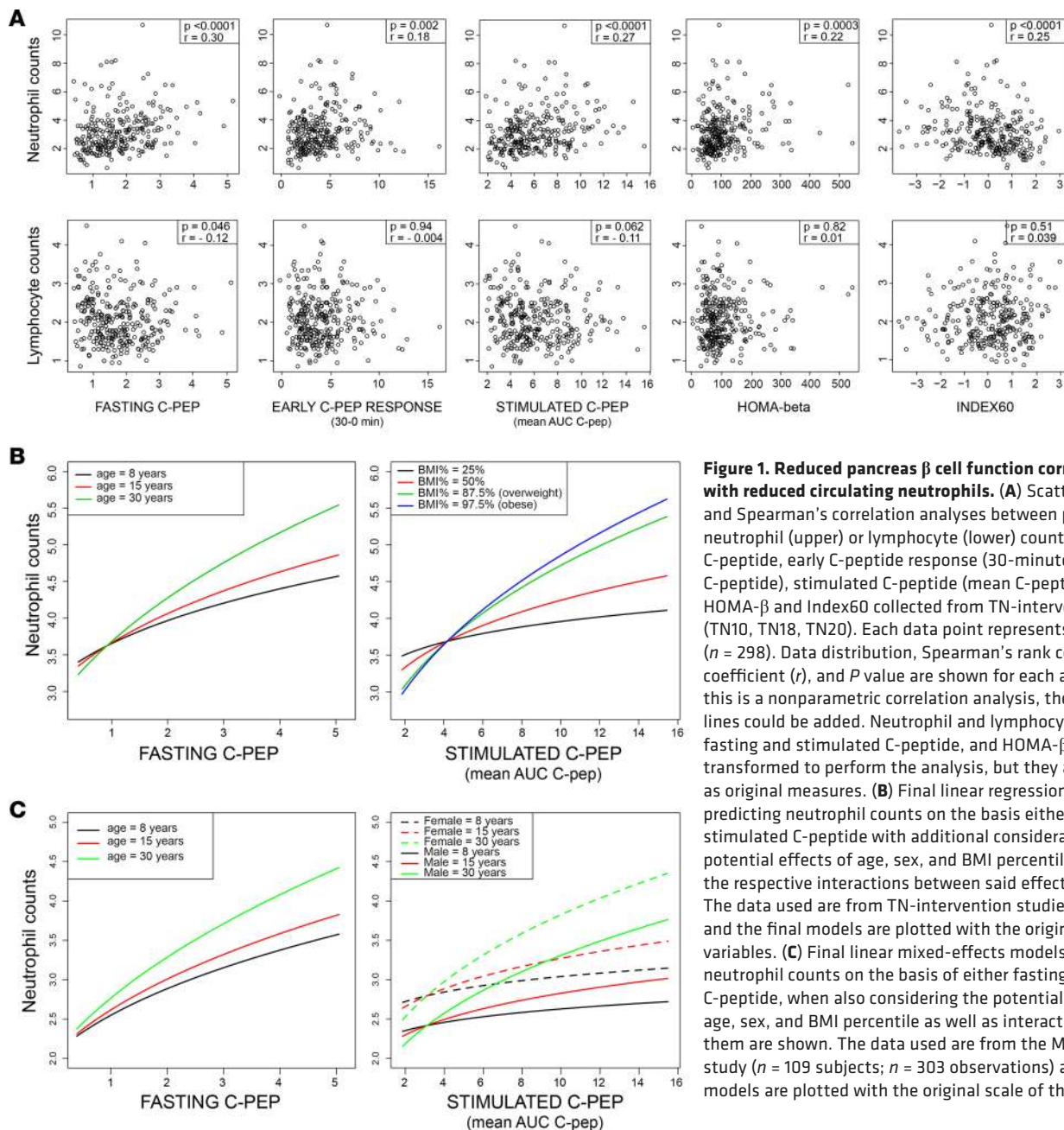
Figure 1. Reduced pancreas β cell function correlates with reduced circulating neutrophils. (A) Scatterplot and Spearman's correlation analyses between peripheral neutrophil (upper) or lymphocyte (lower) counts and fasting C-peptide, early C-peptide response (30-minute – 0-minute C-peptide), stimulated C-peptide (mean C-peptide AUC) HOMA-β and Index60 collected from TN-intervention studies (TN10, TN18, TN20). Each data point represents 1 donor (n = 298). Data distribution, Spearman's rank correlation coefficient (r), and P value are shown for each analysis. As this is a nonparametric correlation analysis, the regression lines could be added. Neutrophil and lymphocyte counts, fasting and stimulated C-peptide, and HOMA-β were log transformed to perform the analysis, but they are shown as original measures. (**B**) Final linear regression models for predicting neutrophil counts on the basis either of fasting or stimulated C-peptide with additional consideration for the potential effects of age, sex, and BMI percentile as well as the respective interactions between said effects are shown. The data used are from TN-intervention studies (n = 298) and the final models are plotted with the original scale of the variables. (**C**) Final linear mixed-effects models for predicting neutrophil counts on the basis of either fasting or stimulated C-peptide, when also considering the potential effects of age, sex, and BMI percentile as well as interactions with them are shown. The data used are from the Milan-TN01 study (n = 109 subjects; n = 303 observations) and the final models are plotted with the original scale of the variables.

donors (Figure 2C). Examination of pancreatic sections from the Exeter cohort was more complex, likely because of the tissue origin and preservation. The Exeter Archival Diabetes Biobank contains samples of autopsy pancreas having variable degrees of autolysis (due to nonstandardized conditions of fixation and postmortem processing), which can lead to interpatient variation when studying labile antigens (17). However, immunofluorescence analysis of pancreatic autopsies from 5 T1D subjects all showed neutrophil infiltration (Supplemental Figure 2).

The occurrence of decondensed DNA colocalized with MPO in both frozen OCT-embedded and FFPE sections (as highlighted in Figure 2B and Supplemental Figure 1B) suggests the presence of pancreas-residing NETting neutrophils (i.e., releasing NETs). Additional proof of tissue-residing NETting neutrophils was also obtained by the identification of decondensed DNA decorated with MPO and citrullinated histones. Staining with anti–human citrullinated histone antibodies is known to be cumbersome and highly dependent on organ preservation (19, 20). This staining was therefore only possible in a proportion of the available pancreas sections (i.e., 21 sections from 13 subjects across the 4 cohorts). Nevertheless, this

Figure 2. Intact and NETting neutrophils infiltrate the pancreas of presymptomatic and symptomatic T1D donors. (**A**) One representative image of an immunohistochemical (IHC) analysis of a formalin-fixed, paraffin-embedded (FFPE) section (out of 12 total sections analyzed) from an autoAb-positive nPOD donor (no. 6197) stained with hematoxylin and anti-MPO (neutrophil-specific) antibody (brown staining). Scale bar: 100 μm. (**B**) Representative images of immunofluorescence (IF) analyses of frozen OCT-embedded nPOD sections (out of 13 total sections analyzed) stained with anti-MPO antibody (green, left column) and Hoechst 33342 for DNA detection (white, middle column). Expression signals were then merged (right column). Donor ID and characteristics are shown on the left. Images are represented as Z-stacked following projection. Insets were cut (dotted square) and magnified ×1.5 at the bottom of each panel and they highlight the occurrence of decondensed DNA colocalized with MPO, which in turn suggests the presence of pancreas-residing NETting neutrophils. Scale bars: 20 μm. (**C**) Quantification of MPO-positive cells on FFPE pancreatic sections analyzed by IHC (empty dots, and green triangles for the Siena cohort) and IF (red dots) are shown in box-and-whisker plots as number of cells per squared millimeter. Numbers indicate donor ID. Each symbol represents 1 section analyzed (*n* = 26 donors; *n* = 34 sections). Comparisons between groups were performed with a linear mixed-effects model followed by post hoc analysis (see supplemental methods for details). (**D**) One representative image of an IF analysis of an FFPE section (out of 22 total sections analyzed) from one DiViD T1D donor (no. 5) stained with anti-MPO (green), anti-citrullinated histone H3 (red) antibodies and Hoechst 33342 for DNA detection (white). Scale bars: 10 μm.
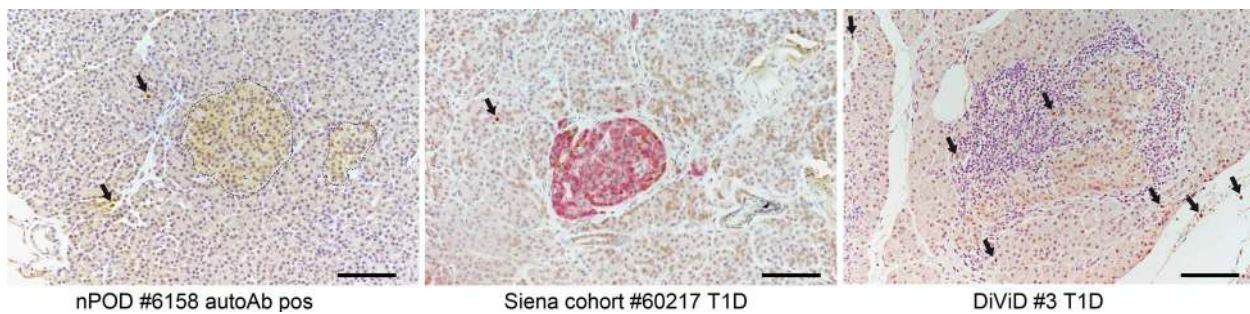
Figure 3. Neutrophils infiltrating the pancreas of presymptomatic and symptomatic T1D donors are not merely localized close to β cells. Representative images of IHC analyses of FFPE sections stained with hematoxylin and anti-MPO antibody (out of 12 total sections analyzed). Donor ID and characteristics are shown at the bottom. Arrows indicate MPO-positive cells (brown), while islets are highlighted by dotted lines (left panel), glucagon staining (middle panel), or immune cell infiltration (right panel). Scale bars: 10 μm.

analysis clearly showed that a fraction of neutrophils infiltrating the pancreas had undergone NETtosis (i.e., 54% ± 10% and 50% ± 17% [mean ± SD] of neutrophils were releasing NETs in autoAb-positive and T1D donors, respectively) (Figure 2D and Supplemental Videos 3 and 4).

As we previously reported with regard to T1D donors (10), we hereby noted that neutrophils do not specifically localize in close proximity to the β cells even in presymptomatic autoAb-positive subjects, but that they are uniformly distributed within vessels and, more significantly, throughout the pancreas (Figure 3 and Supplemental Figure 3).

Thus, the data we generated in 2 distinct labs and on sections from 4 different cohorts collected worldwide, including living and brain-dead organ donors, indicate that regions of the pancreas of presymptomatic and symptomatic T1D subjects are infiltrated by neutrophils to a varying degree and that a proportion of these cells undergoes NETtosis.

Blood neutrophils of T1D subjects at all disease stages have a unique molecular signature that is distinguished by an overabundance of IFN-associated genes. Overall, our data indicate that, as T1D develops, neutrophils are attracted into the pancreas and that the ensuing reduction in circulating numbers correlates with reduced β cell function. To test whether this abnormal T1D neutrophil disposition is confined to the pancreas or whether it also applies to the periphery, we purified circulating neutrophils from the blood of donors at various disease stages (see Supplemental Table 15 for donor characteristics) and generated genome-wide transcriptional profiles. Differential gene expression analysis revealed a similarity in the transcriptional profiles between autoAb-negative, autoAb-positive, and new-onset T1D subjects (Figure 4). Notably, this similarity recurred when we applied unsupervised hierarchical clustering of subjects on the bases of the subjects' transcriptional profiles. Unrelated nondiabetic controls all clustered together (except for 1 outlier: ctrl-110) while autoAb-negative, autoAb-positive, and new-onset T1D patients formed a single and distinct large cluster (Supplemental Figure 4A). Given that age range was not identical between the groups of donors analyzed and that neutrophil purity is key for transcriptomic data (21), both factors were taken into account. Of note, the fundamental trend remained unchanged even after accounting for patient age and/or purity of neutrophil samples (Supplemental Figure 4B).

Pairwise analyses of differential expression among all the patient groups revealed a large number of genes that differentiated unrelated nondiabetic controls from autoAb-negative, autoAb-positive, and new-onset T1D patients. By contrast, only a very limited number of genes differentiated the at-risk and diabetic subject groups from each other (Table 2), but it is likely that more differences between the 3 donor groups would be detected if larger sized sample groups were analyzed. These data show that the transcriptional signature in purified blood neutrophils is abnormal in T1D subjects and that it is found even before seroconversion. At-risk and T1D donors notably shared several of the genes differentially expressed between each of them and nondiabetic controls, suggesting a high degree of neutrophil transcriptome similarities at all disease stages (Supplemental Figure 4C).

In view of the fact that there was a high degree of similarity between the gene expression signatures in neutrophils isolated from at-risk subjects (independent of autoAb status), we considered that it would be appropriate to combine the 2 donor categories (autoAb-negative and -positive subjects) as a single at-risk category for subsequent analysis. To identify functional components of the transcriptional
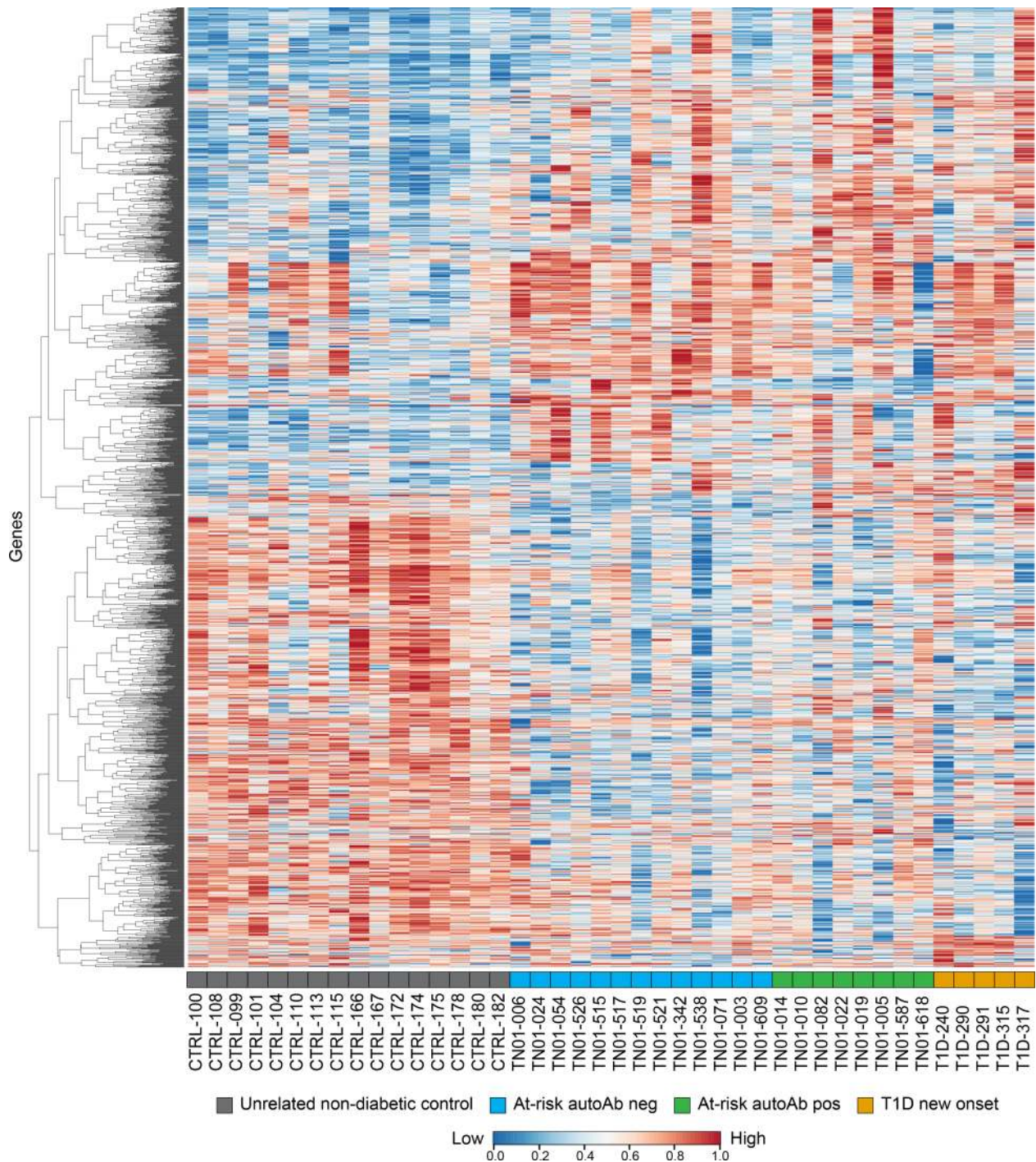
Figure 4. Peripheral neutrophil transcriptomic signature from at-risk autoAb-negative, autoAb-positive, and new-onset T1D patients form a single large cluster distinct from that of unrelated nondiabetic controls. Heatmap showing 2,058 genes differentially expressed in neutrophils freshly isolated from pediatric nondiabetic controls without relatives with T1D (unrelated nondiabetic controls, gray, *n* = 16), autoAb-negative relatives of patients with T1D (at-risk autoAb nega-tive, blue, *n* = 13), autoAb-positive relatives of patients with T1D (at-risk autoAb positive, green, *n* = 8), and patients with T1D at disease onset (T1D new onset, orange, *n* = 5). Scale bar represents the relative gene expression and ranges from the minimum log expression (0.0) to the maximum log expression (1.0).

response during disease development, gene ontology (GO) enrichment analysis was performed on the genes differentially expressed between the unrelated nondiabetic controls and the at-risk donors. The transcriptional signature recovered in T1D was enriched in genes associated with IFN signaling path-ways and related immunological responses (Figure 5A). Genes downstream of both type I IFN-α and type II IFN-γ signaling were significantly upregulated in neutrophils purified from T1D at all stages

Table 2. Number of deregulated genes among donor groups

	At-risk autoAb neg	At-risk autoAb pos	T1D new onset
Unrelated nondiabetic controls	1,309	829	981
At-risk autoAb neg		9	1
At-risk autoAb pos			1
T1D new onset			

(Figure 5B). All differentially expressed IFN-associated genes were more highly expressed in at-risk and T1D patients (Figure 5C), even after the removal of 3 subjects with extremely high values (Supplemental Figure 5A). These genes were distributed across numerous steps in the IFN signaling pathway, as well as downstream of signaling (Figure 6).

An IFN-inducible transcriptional signature associated with disease has been demonstrated in patients with rheumatoid arthritis at onset and before disease development (22) and in patients with SLE where neutrophils were the major IFN sensors (3). Of note, a similar signature was also shown in whole blood of subjects at risk of developing T1D already before the development of autoAbs and throughout disease onset (23, 24). We compared these published modular IFN signatures with our neutrophil-specific transcriptional signature. Our set of IFN-responsive genes found in purified neutrophils showed greater overlap with those generated in children before T1D seroconversion (24) than with those generated from purified neutrophils from patients with SLE (Supplemental Figure 5B). This analysis further supports the concept that genetically predisposed subjects have an abnormal IFN signature before T1D seroconversion and that neutrophils may be the bearers of this signature. Our data agree with the belief that a proinflammatory state affects all T1D family members (regardless of the presence of autoAbs), and that this effect is likely to be due to underlying genetic risk factors (25). To understand whether this unique neutrophil signature is driven by a common HLA-predisposing genetic background, we performed HLA genotype analysis on all subjects. The HLA T1D–associated risk alleles were more represented in T1D donors at all disease stages than in nondiabetic controls (Supplemental Figure 6). However, there was no strong correlation between T1D high-risk HLA carriers and deregulated IFN gene sets (data not shown), thus suggesting that either the aberrant neutrophil signature we observed is not genetically imprinted or that other non-HLA T1D susceptibility risk-conferring alleles influence gene expression, as shown in a comprehensive genome-wide association study that tested 1 million genetic variants for association with red cell, white cell, and platelet properties in more than 100,000 participants (26). Our sample size was, however, insufficient to address the latter.

Discussion

This study suggests that neutrophils may play a previously undisclosed role in human T1D. In presymptomatic individuals at risk of developing T1D, we observed that lower peripheral neutrophil counts were associated with worsening β cell function. Histological analysis of sections of human pancreases collected worldwide from 4 different cohorts revealed that neutrophils accumulate in the pancreas of T1D donors at all disease stages — including in autoAb-positive subjects at risk of the disease. We also found that human pancreas–infiltrating neutrophils can extrude NETs. Finally, transcriptomic analysis of purified circulating neutrophils from T1D donors throughout the disease course uncovered a signature distinct from that of nondiabetic donors and characterized by altered IFN-responsive genes.

The role of neutrophils in human T1D — a disease that has been considered as mainly T cell mediated (27) — has not been explored extensively. Nevertheless, we have reported previously that mild neutropenia occurs in patients with T1D as well as in presymptomatic subjects (10) and this is now considered a hallmark of human T1D (11–14). Our new findings demonstrate that this peripheral reduction in neutrophils is associated with poor endocrine pancreatic function. It remains to be clarified whether neutrophils act directly on the endocrine pancreas to exert a negative impact on activity or whether their circulating numbers are influenced by other, currently unknown, pathological mechanisms associated with the early stages of T1D. Nevertheless, our demonstration that neutrophils accumulate in the pancreas early during disease development and that
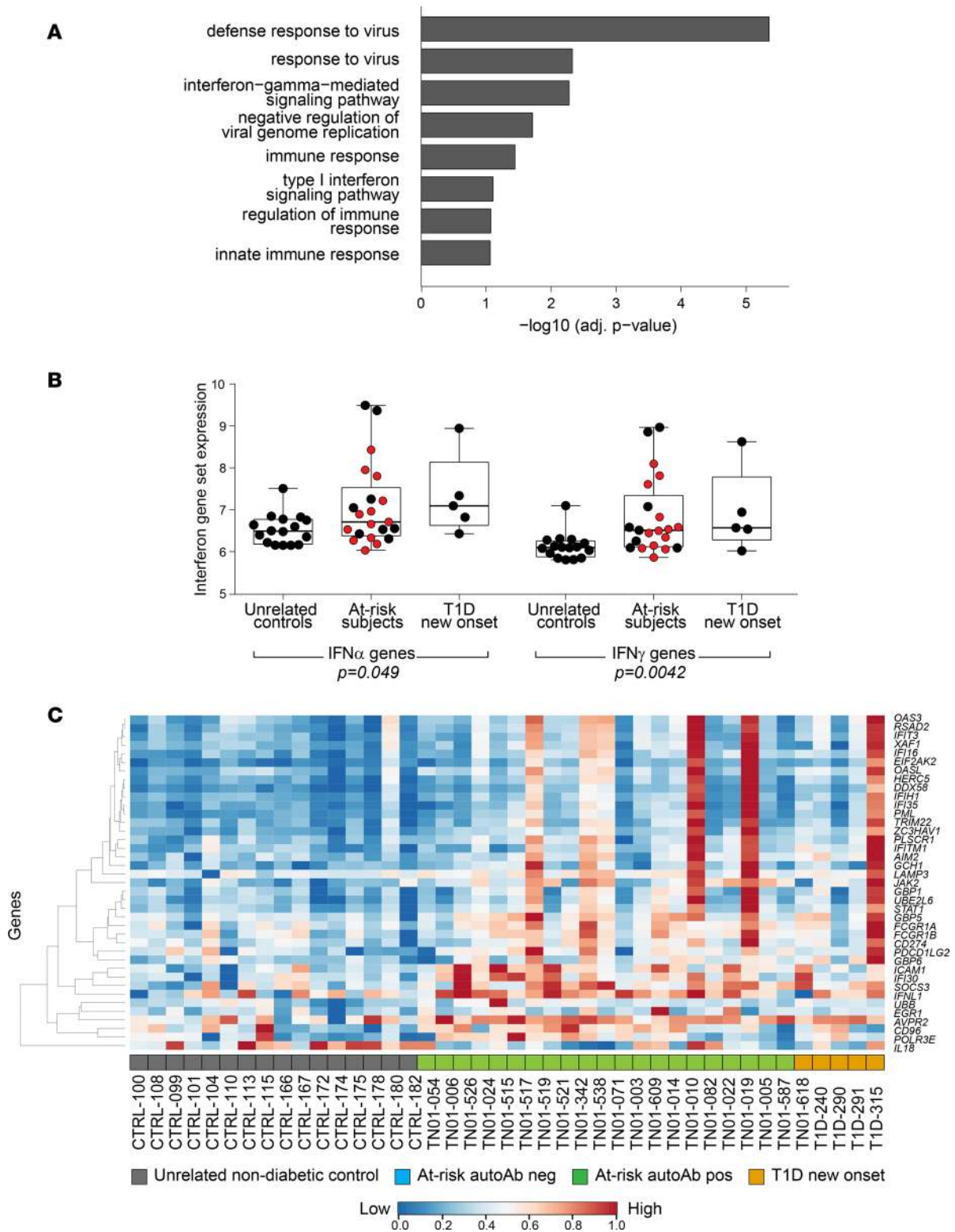
Figure 5. IFN-inducible gene expression signature characterizes circulating neutrophils during all stages of T1D. (**A**) The most highly enriched terms from Gene Ontology Biological Processes in genes differentially expressed between at-risk subjects and unrelated nondiabetic controls are shown (terms shown have significant enrichment threshold of 0.1 for the Benjamini-Hochberg–adjusted P value). (**B**) Median expression of the Molecular Signatures Database (MSigDB) hallmark gene sets for response to IFN-α and IFN-γ, by donor group; points show values for individual donor (in the at-risk donor category: red dots represent autoAb-negative subjects, black dots autoAb-positive subjects). To test variation between the 3 groups, Kruskal-Wallis test was applied, and results are shown below each IFN set. To test differences between groups the Mann-Whitney test was applied (IFN-α: at-risk subjects vs. unrelated nondiabetic controls $P = 0.059$, T1D new onset vs. unrelated nondiabetic controls $P = 0.032$, T1D new onset vs. at-risk subjects $P = 0.41$; IFN-γ: at-risk subjects vs. unrelated nondiabetic controls $P = 0.0015$, T1D new onset vs. unrelated

nondiabetic controls *P* = 0.032, T1D new onset vs. at-risk subjects *P* = 0.71). (**C**) Heatmap showing the 39 IFN-related genes differentially expressed in neutrophils freshly isolated from pediatric nondiabetic controls without relatives with T1D (unrelated nondiabetic controls, gray, *n* = 16) versus relatives of patients with T1D who are either autoAb negative or positive (at risk, green, *n* = 21). Patients with T1D at disease onset are shown for comparison (T1D new onset, orange, *n* = 5). Scale bar represents the relative gene expression and ranges from the minimum log expression (0.0) to the maximum log expression (1.0).

they extrude NETs suggests a direct pathogenic role. This is consistent with evidence that neutrophils and NETs also play a pathogenic role in a murine model of autoimmune diabetes (9). In humans, peripheral neutrophils are more susceptible to NETosis in diabetic subjects and NETs are known to impair wound healing in diabetes (28). However, to our knowledge, this is the first time that NETs have been found in the pancreas of T1D-autoAb-positive donors and in patients with T1D.

As we reported previously (10), we find that pancreas-residing neutrophils lack a specific peri-islet localization and it is of interest that a similar conclusion was reached for infiltrating pancreatic CD8+ T cells by others (29). The pathogenic significance of this distribution remains to be determined, but we hypothesize that the immune cells may infiltrate the entire pancreas, thus creating a generalized state of pancreatic inflammation (in which neutrophils play a central role) and that an intrinsic β cell vulnerability or fragility may then underpin disease pathogenicity (30).

Many data sets suggest that type I IFNs contribute to the development of T1D in mouse models (31, 32) as well as in humans (33–35). Our new data are in accord with this and provide additional evidence that neutrophils might represent very effective circulating IFN sensors. To confirm that neutrophils were sensing the presence of an IFN-enriched environment, we attempted to measure type I and type II IFNs in the plasma of those subjects whose neutrophils were used to generate the transcriptomic data. Unfortunately, however, due to rapid clearance, detection of IFNs in the circulation can prove challenging (36) and conclusive results were not obtained (data not shown). Nevertheless, we note that Hayday and colleagues have demonstrated that the presence of neutralizing self-reactive antibodies specific for type I IFNs is associated with protection against T1D in patients with AIRE mutations and immunopositivity to GAD (37), thereby supporting an important pathogenic role for IFNs in human T1D.

Another interesting conclusion from our transcriptomic data concerns the presence of an altered neutrophil-specific signature in T1D family-related autoAb-negative subjects — of whom only a minority will develop T1D (~3%–5%). This finding confirms data reported by Hessner and colleagues who discovered that plasma of low-risk subjects (i.e., family-related subjects without high-risk HLA) leads to the most robust induction of proinflammatory transcripts and the lowest induction of genes associated with regulatory activity (25). Similarly, 2 independent studies reported that a type I IFN signature is temporally increased in whole blood of susceptible children prior to the development of autoAbs (23, 24). These studies — together with our new data — provide a unique insight into the preclinical stages of T1D before seroconversion and also have important experimental implications; autoAb-negative donors from T1D families should not be considered nondiabetic controls in immunological and metabolic studies, as they appear to be distinct from nondiabetic unrelated subjects.

Overall, our data provide evidence that infiltrating neutrophils may contribute to reduced pancreatic function and tissue damage in human T1D and that they serve as sensors of an IFN-rich environment, which is known to be highly pathogenic for β cells. Importantly, in addition to generating new mechanistic hypotheses, these data suggest that targeting of neutrophils might represent a previously unrecognized therapeutic modality for T1D.

Inevitably, our study also has limitations that should be addressed in further work. For example, it remains unclear whether the subjects with the lowest peripheral neutrophil counts are those with the highest extent of pancreatic neutrophil infiltration. Verification of this proposition remains challenging, as it is currently difficult to collect pancreas biopsies and blood neutrophil counts from the same subjects. Another important issue is to understand how NETs are formed in the pancreas and to confirm whether and how they contribute to tissue damage. Studies are currently ongoing to characterize the proteome of pancreatic NETs to determine the nature of the externalized proteins and immunostimulatory molecules that may promote aberrant immune responses and the pathogenesis of human T1D, as happens in other autoimmune diseases (3, 4). Finally, our observation that autoAb-negative subjects already display a distinct neutrophil signature suggests that certain risk-conferring alleles may influence gene expression to create a fertile field on which additional hits may then promote disease development. This is an interesting hypothesis that will require confirmation via a dedicated genotype/phenotype study employing a larger data set.
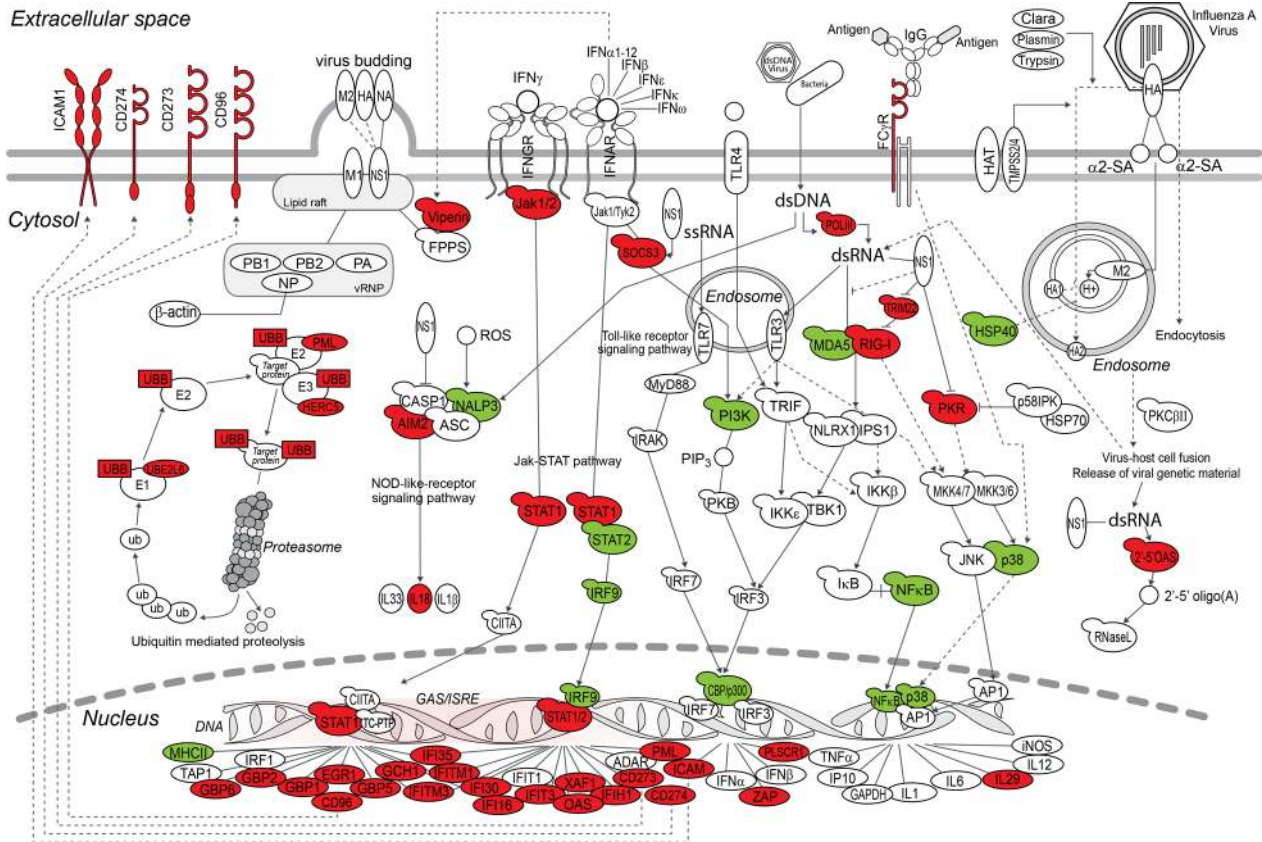
Figure 6. The IFN pathway is active during all stages of T1D. Pathway diagram was constructed from the "Influenza A pathway" (KEGG PATHWAY Database hsa05164) and literature on IFN-related genes (described in detail in the supplemental methods section). Dysregulated transcripts are shaded in green (influenza pathway) or in red (IFN-related genes).

All in all, on the basis of the current evidence, our data should encourage additional studies to clarify further the role of neutrophils in human T1D pathogenesis.

Methods

Study design

An overview of the study design is presented in Table 1.

Sample sizes: β cell function and CBC measures analysis with data of TrialNet-intervention Studies (TN10, TN18, and TN20). This analysis utilized participants in 3 separate prevention trials in at-risk subjects for T1D. The overall evaluable number of subjects available for our analyses was $n = 289$, and for those analyses that included BMI percentile, which was only available on 2 trials, the sample size was $n = 210$. Our primary models to analyze the impact of metabolic factors on neutrophil or lymphocyte counts were based on multivariable generalized linear regression models. Even for the more restricted models with $n = 210$ subjects and adjusting for 3 clinical covariates in the model, we had 80% power to detect a multiple squared partial correlation coefficient ($\rho2$) for the metabolic marker of interest that is approximately 0.13 over the null-hypothesis value, corresponding to a moderate effect size based on Cohen's criteria. This also accounted for a 0.05 type I error constraint. Pairwise analyses of correlation coefficients between neutrophil or lymphocyte counts and metabolic markers of interest also were conducted. Here with $n = 289$ subjects and a type I error constraint of 0.05, we had at least 80% power to detect a correlation coefficient of 0.16 versus the null hypothesis of 0 using a 2-sided test.

β Cell function and CBC measures analysis with data of Milan-TN01 Study. The sample includes all patients with available information on the variables considered in the analysis. The data consist of 303 observations gathered from 109 subjects. We performed a power analysis for all the tests of the coefficients in the final models and for almost all tests we obtained a power higher than 0.9.

RNA sequencing data analysis. For RNA sequencing (RNA-seq) data analyses, samples from 42 individuals were obtained and included 16 nondiabetic healthy controls, 13 autoAb-negative and 8 autoAb-positive relatives of T1D patients, and 5 recently diagnosed T1D patients.

Data exclusion: β cell function and CBC measures analysis with data of TrialNet-intervention Studies. In the cohort of participants in the 3 prevention trials, as noted in this study, we excluded 30 subjects who had enrolled in 1 of the 3 included trials. Of these 30 excluded subjects, 20 had CBC measures that were flagged for review and were currently undergoing quality control, and 10 subjects did not yet have their baseline CBC measures submitted. Thus, of the 319 enrolled participants across the trials, 289 (90.6%) were included in the analyses presented in this study.

β Cell function and CBC measures analysis with data of Milan-TN01 Study. In the analysis of the Milan-TN01 cohort, few observations were removed for the estimation of the mixed models, because they were outliers for the model.

RNA-seq data analysis. One sample was removed because genetic data indicated that it was incorrectly identified. Three additional samples showed anomalous values — analyses were run with and without those samples to determine sensitivity of results to their inclusion.

Replication

β Cell function and CBC measures analysis. In the estimation of the models with the data of the Milan-TN01 cohort, we enhanced the robustness of the results by removing from the analysis few observations that were outliers for the models.

RNA-seq data analysis. RNA-seq results have not been replicated. Insufficient samples were available to split the data into training and test sets. A number of tests were conducted to determine sensitivity of results to outlier samples and/or analytical assumptions — these are described in the main text and the Methods.

Randomization

The study of the Milan-TN01 data and RNA-seq data is retrospective and not all the analyses regard a comparison among groups. When this was the case, the groups were defined based on disease state and family history of disease, so randomization was not applicable. Covariates that could have influenced the response variable (such as patient age and sex and, for RNA-seq data, the purity of samples) were evaluated in the analysis (details in main text and Methods). For the data associated with the prevention TrialNet trials, these are randomized trials, but since they are ongoing and still in follow-up for their endpoints, our focus was only on the cross-sectional pretreatment data on these subjects. As such, randomization approaches and assignments were not applicable to these analyses.

Blinding

Regarding the collection of RNA-seq data, this is not predictably influenced by the person collecting the data; hence, blinding during the data collection was not necessary. For clinical data, groups were present only in the data of pancreas images and the blindness of the group was not possible in the collection of the data. In both cases, data analysis involved comparison of groups, which required knowledge of the group identities.

Composite measures of β cell function and T1D disease risk

HOMA-β. The homeostatic model assessment (HOMA) is a method for assessing β cell function and insulin resistance from basal (fasting) glucose and insulin or C-peptide concentrations. The equation for the HOMA-β model is, β cell function (%) = 20 × fasting insulin (μIU/ml)/(fasting glucose [mmol/l] − 3.5). This model was built up assuming that normal-weight subjects aged less than 35 years have 100% β cell function (38).

Index60. The T1D diagnostic Index60 — a metabolic index — was developed from 2-hour OGTT using the log fasting C-peptide, 60-minute C-peptide, and 60-minute glucose: Index60 = 0.3695 × (log fasting C-peptide) + 0.0165 × (60-minute glucose) – 0.3644 × (60-minute C-peptide). An OGTT with an Index60 of 2.0 represents a point of transition from prediction to a virtual indication of T1D development in autoAb-positive relatives of patients with T1D (the higher the Index60, the higher the risk of disease development) (39).

The human pancreas samples

Pancreas sections were collected from 4 distinct cohorts.

nPOD cohort. Following acquisition of informed research consent, human pancreases were obtained from deceased organ donors in the USA and shipped to the Network for Pancreatic Organ Donors with Diabetes (nPOD) program at the University of Florida for processing, following standardized procedures. Pancreas donors used in this study included T1D patients, nondiabetic autoAb-positive subjects, and nondiabetic controls. Pancreas recovery and transport meet transplant-grade criteria. Frozen OCT-embedded and FFPE tissues were used (15).

Siena cohort. Whole pancreas was obtained from 1 multiorgan donor with T1D and it was processed according to nPOD standard operating procedures and with the approval of the Tuscany region transplantation network based on the Institutional Review Board (IRB) of the University of Pisa (18). FFPE sections were used for immunohistochemical investigations.

DiViD cohort. Adults with newly diagnosed T1D were invited to participate in the DiViD study. The study was approved by the Government's Regional Ethics Committee (16). After oral and written information from the diabetologist and the surgeon separately, they consented to participate in the trial. Resection of the pancreatic tail was performed 3–9 weeks after diagnosis of T1D. The biopsy samples were processed under sterile conditions and immediately divided into multiple smaller pieces; FFPE tissue blocks were prepared simultaneously.

Exeter Archival Diabetes Biobank. This comprises a large collection of pancreas samples from across the United Kingdom originally compiled by Foulis (40) but now held at the University of Exeter (17). Pancreases were from patients with recent-onset T1D and were mainly recovered postmortem. They represent a historical collection dating from the 1960s to the 1980s and were processed and fixed by various methods according to the location. All samples were used with ethical permission from the West of Scotland Research Ethics Committee (ref: 15/WS/0258).

Details of pancreas donors in each cohort can be found in Supplemental Tables 13 and 14.

Pancreas immunohistochemical and immunofluorescence analysis

FFPE pancreatic sections (5 μm thickness) were analyzed as briefly follows. After deparaffinization and rehydration, sections were incubated with Tris-buffered saline (TBS, Sigma-Aldrich) supplemented with 3% H_2O_2 to block endogenous peroxidases (only for immunohistochemical experiments) and with TBS supplemented with 3% bovine serum albumin (BSA, Sigma-Aldrich) to reduce nonspecific reactions. Antigen retrieval was performed with 10 mM citrate buffer, pH 6.0.

For immunohistochemical analysis, sections were incubated with rabbit polyclonal anti–human MPO (Abcam, ab45977) and swine polyclonal anti-rabbit IgG-HRP (DAKO, P0217) as secondary antibody. MPO signal was detected with 3,3′-diaminobenzidine (DAB Quanto, ThermoFisher Scientific, TA-060-HDX). Sections were then incubated with Mayer's hematoxylin solution (Sigma-Aldrich) to counterstain nuclei, dehydrated, and mounted with Eukitt (Bio-Optica). Sections from the Siena cohort were also incubated with mouse monoclonal anti–human glucagon (R&D Systems, clone 181402, MAB1249) and goat anti-mouse IgG–alkaline phosphatase (ThermoFisher Scientific, 31320) as secondary antibody. Glucagon signal was detected with Liquid Fast Red (ThermoFisher Scientific, TA-060-AL) supplemented with levamisole endogenous alkaline phosphatase inhibitor (DAKO, Agilent Technologies, X3021).

For immunofluorescence analysis, sections were incubated with rabbit polyclonal anti–human MPO (Abcam) and mouse anti–citrullinated histone H3 (anti-CitH3; citrulline R2 + R8 + R17, clone 7C10, LifeSpan Biosciences, LS-C144555) antibodies and with goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, ThermoFisher Scientific, A11034) and goat anti-mouse IgG Alexa Fluor 594 (Molecular Probes, ThermoFisher Scientific, A11032) as secondary antibodies. nPOD sections were also stained with polyclonal guinea pig anti-glucagon antibody (LifeSpan Biosciences, LS-C20275) and goat anti-guinea pig IgG Alexa Fluor 647 (Molecular Probes, ThermoFisher Scientific, A21450) as secondary antibody. DNA was counterstained with Hoechst 33342 (ThermoFisher Scientific, 62249). Finally, sections were mounted using Vectashield (Vector Laboratories, H1000) mounting medium.

OCT-embedded tissues (5 μm thickness) were methanol/acetone (1:1, –20°C) fixed, then blocked (PBS supplemented with 1% denatured BSA) and incubated with primary antibodies: mouse monoclonal anti-MPO (Bio-Rad, clone 4A4, 0400-0002) and polyclonal rabbit anti-CitH3 (citrulline R2 + R8 + R17, Abcam, ab5103). Sections were washed and incubated with the proper secondary antibodies: goat anti-mouse IgG

(H+L) Alexa Fluor 488 (Jackson ImmunoResearch, 115-545-003) and goat anti-rabbit IgG (H+L) Alexa Fluor 546 (ThermoFisher Scientific, A11035). DNA was counterstained with Hoechst 33342. Sections were mounted on slides with homemade Mowiol mounting medium (glycerol, G5516; Mowiol 4-88, 81381; and Dabco 33-LV, 290734, Sigma-Aldrich).

Library preparation and RNA-seq

The cDNA library was prepared using a NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) following the manufacturer's instructions. Samples were then purified (AMPure XP Beads, Beckman Coulter) and enriched with PCR to create the final cDNA library. The quality of the cDNA libraries was assessed using an Agilent Technologies 2100 Bioanalyzer. The libraries were hybridized to the flow cell and cluster was generated by bridge amplification (TruSeq SBS Kit v3-HS, Illumina). Single-end sequencing was performed using the Illumina Hiseq-2500 platform (41). RNA-seq data analysis is explained in detail in the supplemental materials and methods section.

Statistics

Pancreas function and CBC measures analysis. All measures were evaluated using graphical and analytical exploratory analyses to assess distribution patterns and identify the need for transformations. Metabolic markers that required a log-based transformation were transformed by taking the log of the marker plus the constant value of 1 [i.e., $\log(x + 1)$]. Neutrophil and lymphocyte counts were also log transformed. In all the analyses, BMI percentiles were calculated using the R package childsds (https://github.com/mvogel178/childsds/wiki). For the TN prevention studies, the US CDC reference values were used (cdc.ref), and for the Italian subjects an Italian reference cohort was used (italian.ref). BMI percentiles and age were considered in the analyses only as continuous variables.

For the cross-sectional baseline data in the 3 TN prevention trials, data were summarized across and within studies, and χ^2 tests and Kruskal-Wallis tests were used to assess differential distributions of categorical and continuous measures, respectively, between studies. Scatterplots were used to evaluate relationships between continuous measures and correlations were assessed using nonparametric Spearman's rank correlation coefficients and tests. Multivariable generalized linear regression models were used to evaluate relationships of each of the clinical and metabolic markers on neutrophil and lymphocyte counts. Multivariable models were evaluated for each metabolic marker and adjusted for age, sex, and BMI percentile along with interactions of each of these factors with the metabolic marker. Variable selection was done using a composite approach of backward selection as well as Furnival and Wilson's leaps and bounds method for all subsets regression analyses (R package leaps). In the longitudinal cohort of Italian subjects followed through the TN01 PTP study, multivariable analyses were performed using linear mixed-effects models, since the data consist of repeated measurements of the same subjects with some subjects belonging to the same family. Random effects were accordingly defined nested by family and by subject. As in the analysis of the other cohort, the effect of each metabolic marker on the neutrophil counts was evaluated separately and accounting for the possible effects of age, sex, BMI percentile and the interactions between these variables with the metabolic marker. Final models were obtained using a backward selection procedure. All statistical analyses were performed using R 3.4.0 and R 3.4.1 (http://www.R-project.org/). Differences were considered significant with $P < 0.05$.

Pancreas images analysis. Comparisons were carried out using linear mixed-effects model followed by a post hoc analysis. *P* values were adjusted for multiple comparisons with Bonferroni's correction. All statistical analyses were performed using R 3.4.0 and R 3.4.1. Differences were considered significant with $P < 0.05$.

Data and materials availability

RNA-seq data were deposited in the NCBI's Gene Expression Omnibus (GEO) database under accession number GSE110914 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110914).

Custom R codes used to analyze RNA-seq data are available at GitHub (https://github.com/mjdufort/Battaglia_T1D_neutrophils).

Study approval

The protocols and consent documents were approved by appropriate independent ethics committees or IRBs. All participants (or parents) provided written, informed consent; in addition to their parents providing consent, participants younger than 18 years of age signed a study assent form.

Author contributions

FV and NLB designed and performed the experiments and contributed to data analysis, scientific discussion, and manuscript writing. AS designed and performed the experiments and contributed to data analysis. LN performed immunohistochemical studies on some of the pancreas sections and contributed to pancreas data analysis and discussion. MJD analyzed and quality-checked RNA-seq data, contributed to RNA-seq data discussion, scientific discussion, and manuscript writing. SG analyzed and quality-checked data collected for the TN-intervention studies, performed statistical analyses, contributed to data discussion and to manuscript writing. PMR and FC analyzed and quality-checked data collected for the Milan-TN01 study, performed statistical analyses, contributed to data discussion and to manuscript writing. AV designed, performed, and contributed to analysis of RNA-seq data, and contributed to scientific discussion. AM designed and performed the experiments and contributed to data analysis. PL performed neutrophil and NET stainings on pancreas sections from the Exeter cohort and contributed to data analysis. LK shared pancreas samples from the DiV-iD cohort and contributed to scientific discussion. FM assisted in performing immunohistochemical studies on some of the pancreas sections and contributed to data analysis. KCH and HEL contributed to data analysis and scientific discussion of TN-intervention studies. SJR supervised data generation and analysis on pancreas sections from Exeter and contributed to data analysis and discussion. NGM shared pancreas samples from the Exeter cohort, supervised data generation and analysis on pancreas sections from Exeter, contributed to scientific discussion and to manuscript writing. KDJ shared pancreas samples from the DiViD cohort. PSL contributed to RNA-seq data discussion, scientific discussion, and manuscript preparation. GS supervised data generation and analysis on the pancreas images and contributed to scientific discussion. FD shared the pancreas sample from the Siena cohort, supervised data generation and analysis on the pancreas images, and contributed to scientific discussion. EB contributed to data and scientific discussion and to manuscript writing. The DRI Biorepository Network informed the donors, collected biological samples and donor data, and contributed to scientific discussion. The Type 1 Diabetes TrialNet Study Group performed TN clinical trials, collected and shared data. MB supported, designed and supervised the study; coordinated sample/data collections; analyzed and discussed the data; coordinated scientific discussion and wrote the manuscript.

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Address correspondence to: Manuela Battaglia, Via Olgettina 58 – 20132 Milano, Italy. Phone: 39.02.2643.3197; Email: battaglia.manuela@hsr.it.

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