Duodenal Mucosa of Patients With Type 1 Diabetes Shows Distinctive Inflammatory Profile and Microbiota

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Context: Increasing evidences suggest a correlation between gut and type 1 diabetes (T1D).

Objective: The objective of this study is to evaluate the gut inflammatory profile and microbiota in patients with T1D compared with healthy control (CTRL) subjects and patients with celiac disease (CD) as gut inflammatory disease controls.

Design/Setting/Participants: The inflammatory status and microbiome composition were evaluated in biopsies of the duodenal mucosa of patients with T1D (n = 19), in patients with CD (n = 19), and CTRL subjects (n = 16) recruited at San Raffaele Scientific Institute, in Milan, Italy, between 2009 and 2015.

Main Outcome Measures: Inflammation was evaluated by gene expression study and immunohistochemistry. Microbiome composition was analyzed by 16S ribosomal RNA gene sequencing.

Results: An increased expression of *CCL13*, *CCL19*, *CCL22*, *CCR2*, *COX2*, *IL4R*, *CD68*, *PTX3*, *TNFα*, and *VEGFA* was observed in patients with T1D compared with CTRL subjects and patients with CD. Immunohistochemical analysis confirmed T1D-specific inflammatory status compared with healthy and CD control tissues, mainly characterized by the increase of the monocyte/macrophage lineage infiltration. The T1D duodenal mucosal microbiome results were different from the other groups, with an increase in Firmicutes and Firmicutes/Bacteroidetes ratio and a reduction in Proteobacteria and Bacteroidetes. The expression of genes specific for T1D inflammation was associated with the abundance of specific bacteria in the duodenum.

Conclusions: This study shows that duodenal mucosa in T1D presents disease-specific abnormalities in the inflammatory profile and microbiota. Understanding the mechanisms underlying these features is critical to disentangle the complex pathogenesis of T1D and to gain new perspectives for future therapies targeting the intestine. **(*J Clin Endocrinol Metab* 102: 1468–1477, 2017)**

Recent observations indicate the presence of intestinal abnormalities associated with autoimmune diabetes in both experimental rodent models and patients (1), suggesting a potential role of the gut in the pathogenesis of type 1 diabetes (T1D) (2). Compared with healthy subjects, patients with T1D show increased intestinal permeability (3, 4), alteration of microvilli, leakiness of tight junctions (5, 6), and increased expression of human leukocyte antigen-antigen D related, human leukocyte antigen DP subregion, and intercellular adhesion molecule-1

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Abbreviations: CD, celiac disease; CTRL, control; GADA, glutamic acid decarboxylase autoantibodies; HbA1c, glycated hemoglobin; IA-2A, protein-tyrosine-phosphatase-2 autoantibodies; IAA, insulin autoantibodies; MPO, myeloperoxidase; PCR, polymerase chain reaction; T1D, type 1 diabetes; ZNT8, zinc transporter 8 autoantibodies.

in the villous epithelium and in the crypts, suggesting that antigen presentation by the epithelial cells of the small intestine may be enhanced (7, 8). Among the factors that may modify the intestinal barrier and affect its immune activation, the gut microbiota is at present the main suspect (9). Dysbiosis has previously been linked with predisposition to immune system activation that can trigger aberrant immune responses, resulting in inflammation in the intestine and other organs (10). Several articles have pointed out a substantial difference in the composition of the stool microflora in subjects with autoimmune diabetes (11–19). In particular, it has been reported that the stool bacterial number of Actinobacteria, Firmicutes, butyrate-producing bacteria (mainly Clostridia), and the Firmicutes/Bacteroidetes ratio are significantly decreased in patients with T1D compared with healthy subjects (11–17).

The study of the inflammatory status of gut mucosa and its interplay with resident microbiota in T1D is relevant to better understand the characteristics of the disease. A major advancement would come from the direct analysis of small intestine instead of studies on stool samples. In fact, because of their close functional and spatial relationship and shared blood supply, it is logical to consider the duodenum and the pancreas correlated. In healthy rats it was reported that different bacterial species, present in the human duodenum, instilled into the ductal system of the pancreas rapidly induce cellular infiltration, consisting of mainly neutrophil polymorphonuclear cells and monocytes/macrophages, which could trigger β-cell destruction (20). Morphological and functional alterations of duodenum have also been described in pancreatic diseases such as pancreatitis (21, 22). Until now, despite its importance, no study has analyzed the inflammatory milieu and microbiome in T1D directly on human duodenum gut mucosa.

We evaluated the inflammatory profile, the microbiome, and their correlation on the same duodenal biopsies of patients with T1D compared with healthy control (CTRL) subjects and patients with celiac disease (CD) as control of inflammatory disease of the gut. We found that duodenum mucosa in T1D presents a peculiar signature of inflammation (*i.e.*, a specific microbiota composition), and we discovered an association between some analyzed inflammatory markers and specific bacterial taxa. A better understanding of this relationship may lead to new therapeutic approaches for diabetes that are focused on the intestine.

Research Design and Methods

Study population

Study participants included 54 individuals who underwent gastroduodenal endoscopy and biopsy of the distal duodenum at the Gastroenterology Unit, San Raffaele Scientific Institute, Milan. Biopsy samples were taken between 18 December 2009 and 11 February 2015. Indications for the diagnostic procedure were based on suspicion of gastrointestinal disorders, including CD, or volunteer enrolment in the study. For the purpose of this investigation, participants were divided into 3 groups: (1) 19 individuals with already diagnosed T1D according to American Diabetes Association criteria and with either detectable glutamic acid decarboxylase antibodies (GADA), protein-tyrosine-phosphatase-2 autoantibodies (IA-2A), insulin autoantibodies (IAA), or zinc transporter 8 autoantibodies (ZnT8) (9 were volunteers and 10 underwent endoscopy based on suspicion of gastrointestinal pathologies but had a normal duodenal mucosa during endoscopy); (2) 19 individuals with CD diagnosed at the time of biopsy (all were on a gluten-containing diet and had suspected CD, with symptoms like recurrent diarrhea, abdominal bloating, or malabsorption and positivity to antitissue transglutaminase IgA antibodies or antitissue transglutaminase IgG antibodies and antiendomysial IgA antibodies, who underwent gastroscopy to confirm the diagnosis); (3) 16 healthy control individuals negative for GADA, IA-2A, IAA, ZnT8, antitissue transglutaminase IgA antibodies, antitissue transglutaminase IgG antibodies, and antiendomysial IgA antibodies, who underwent endoscopy based on suspicion of gastrointestinal pathologies that showed normal mucosa during endoscopy (Table 1). No members of the CD group were treated with drugs. There are no statistically significant differences in drug intake between the T1D and CTRL groups (Supplemental Table 1), with the exception of insulin ($P < 0.01$). Secondary complications associated with diabetes were present in subjects with T1D (7/19 retinopathy, 4/19 neuropathy, 3/19 gastroparesis). All patients enrolled, including those diagnosed with CD, were on an animal-based (not vegetarian) gluten-containing diet at the time of biopsy. Groups of study participants did not significantly differ in terms of delivery (vaginal birth or C-section), feeding (maternal or artificial milk), and sex. Age was not different between CTRL subjects and patients with T1D, whereas patients with CD were significantly younger ($P < 0.001$). Demographic and clinical characteristics of study participants are summarized in Table 1. The study protocol was approved by the Ethics Committee of the San Raffaele Scientific Institute and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. Written informed consent was obtained from all adult participants (or from children's parents) before biopsy collection.

Human inflammation Taqman low-density array

Fresh biopsy samples were collected in RNA (Invitrogen, Carlsbad, CA), transferred within 1 hour into lysis buffer (mirVana Isolation Kit, Ambion, Austin, TX), homogenized with Tissue Ruptor (Qiagen, Hilden, Germany), and stored at −80°C. Total RNA was extracted with a mirVana Kit and quantified by spectrophotometer lecture (Epoch, Gen5 software; BioTek, Winooski, VT). OD A260/A280 ratio ≥2.0 and *GAPDH* Ct<22 in Taqman single assay identified good quality RNA samples. For reverse transcription polymerase chain reaction (PCR), after DNAse treatment, 2 μg of RNA were retrotranscribed with SuperScript III RT (Invitrogen). Predesigned TaqMan Array Human Inflammation Panel (Applied Biosystems, Foster City, CA) was used for gene expression study. PCR runs were carried out in a 7900 Real-Time PCR System (Applied Biosystems). Results were expressed as fold changes over a mean of expression of the 3 reference genes *β-actin*, *β-2 Microglobulin*, and *GAPDH* (2^-ΔCt method). Good-quality RNA samples were available from 41 of the 54 study participants (11 CTRL, 15 T1D, and 15 CD).

Table 1. Characteristics of the Study Participants

Characteristic	Control Subjects	Individuals With T1D	Individuals With CD
No. of participants	16	19	19
Age, y	38 (10–56)	34 (6–65)	5 (1–19)
Male/female, n	7/9	10/9	6/13
Diabetes duration, y	—	20 (0–37)	—
Age at onset, y	—	23 (2–41)	—
HbA1c, %	—	8.2 (5.7–14)	—
HbA1c, mmol/mol	—	66 (39–130)	—
Autoimmunity, n			
GADA	0	13	0
IA-2A	0	3	0
IAA	0	10	0
ZNT8A	0	5	0
TGA	0	0	19
TGG	0	0	17
EMA	0	0	17
TPO	1	6	1
Reason for gastroscopy, n (male/female)			
Volunteer	0/16	9/19	0/19
Suspected celiac disease	1/16	1/19	19/19
Dyspepsia	4/16	4/19	0/19
Epigastralgia	2/16	0/19	0/19
Suspected gastroesophageal reflux	9/16	5/19	0/19

Data are median (range) unless otherwise indicated.

Abbreviations: EMA, endomysial antibodies; HbA1c, glycated hemoglobin; TGA, antitissue transglutaminase IgA antibodies; TGG, antitissue transglutaminase IgG antibodies; TPO, thyroid peroxidase antibody.

Histology

Formalin-fixed, paraffin-embedded biopsy samples (3-μm sections) were stained with anti-CD3 (clone LN-10, dilution 1:200; NOVOCASTRA, Newcastle upon Tyne, United Kingdom), anti-CD68 (clone KP-1, undiluted; Ventana, Tuscon, AZ), anti-myeloperoxidase (polyclonal, undiluted; Ventana), anti-*TNFα* (clone 4C6-H8, dilution 1:25; Neobiotechnologies, Union City, CA), and anti-*VEGFA* (clone SP28, dilution 1:50; Spring Bioscience, Pleasanton, CA) using an Ultravision Quanto Detection System HRP-DAB polymer (TL-125-QHL; Thermo, Waltham, MA) as detection system. Immunostained slides were digitalized with the Aperio Scanscope scanner (Leica, Wetzlar, Germany) and acquired with SpectrumTM Plus software. CD3, CD68, and myeloperoxidase (MPO) positivity was quantified using an *ad hoc* algorithm of Axio Vision 4.4 microscope software (Carl Zeiss, Germany Oberkochen, Germany) and reported as percentage of positive cells. Samples were available from 28 of the 54 study participants (9 CTRL, 9 T1D, 10 CD) for CD3 and CD68 and, 17 of the 54 study participants remained (6 CTRL, 6 T1D, 5 CD) for MPO staining. *TNFα* and *VEGFA* positivity was quantified in gut epithelium and lamina propria by 2 independent pathologists blind to disease history or laboratory findings. The following scores indicated the intensity of cell staining: 0 = negative cell, 1 = weak positivity, 2 = medium positivity, and 3 = strong positivity. Samples were available from 42 of the 54 study participants (12 CTRL, 12 T1D, 18 CD) for *VEGFA* and for 43 of the 54 study participants (12 CTRL, 13 T1D, 18 CD) for *TNFα* staining.

Microbiome

Purified RNA (500 ng per reaction) was used to reverse transcribe and amplify the 16S region using specific primers and the Titan One Tube RT-PCR System (Roche, Basal, Switzerland). A second amplification step was performed to amplify 16S V3-5 regions using barcoded sample-specific primers and AccuPrimeTaqPolimerase (Invitrogen). Amplicons were loaded on 1% agarose gel and purified with a QiaQuick Gel Extraction kit (Qiagen). Extracted amplicons were purified with AMPure XP beads (Beckman Coulter, Brea, CA) and used for emulsion-PCR and ultra-deep pyrosequencing following the manufacturer's instructions (454 GS Junior; Roche). After quality filtering, resulting sequences (>250 bp) were analyzed with the open-source software QIIME (1.9.0). Samples were available from 36 of the 54 study participants (9 CTRL, 15 T1D, 12 CD).

Statistics

Statistical analyses were performed using SPSS 13.0 for Windows and QIIME (1.9.0). Nonparametric tests (Kruskal-Wallis and Mann-Whitney *post hoc* test) were used to compare gene expression and microbiome composition within groups, and a 2-tailed P value <0.05 or 0.01 was considered significant. Categorical variables were compared using the χ^2 test or Fisher's exact test as appropriate. Correlation between gene expression levels and percentages of bacteria was evaluated by linear regression analysis (Pearson).

Results

Gene expression analysis shows a distinctive inflammatory profile of gut mucosa of patients with T1D

The expression of 91 genes related to inflammation (mainly cytokines, chemokines, and chemokine receptors) was measured and compared among patients with T1D, patients with CD, and CTRL subjects. Differentially expressed genes are reported in Fig. 1(A). Thirteen genes (*CCL7*, *CCL16*, *CCL18*, *CCR4*, *CXCL9*, *CXCL10*, *CXCR1*, *FCGR3B*, *IFNγ*, *IL8*, *IL10*, *IL17A*, and *NOS2A*)

were significantly more expressed in patients with CD compared with CTRL subjects, and 12 genes (*ALOX5*, *CCBP2*, *CD14*, *CD68*, *CSF1*, *CXCR7*, *CXCL12*, *IL1R1*, *IL2*, *PTX3*, *TNFα*, and *VEGFA*) were significantly less expressed. Four genes (*CCL20*, *CCR7*, *CCR8*, and *IL1β*) were more expressed both in patients with CD and in patients with T1D compared with CTRL subjects. Ten genes (*CCL13*, *CCL19*, *CCL22*, *CCR2*, *IL4R*, *CD68*, *COX2*, *PTX3*, *TNFα*, and *VEGFA*) were significantly more expressed in patients with T1D but not in patients with CD compared with CTRL subjects [Fig. 1(B)]. Among these genes, *CD68*, *PTX3*, *TNFα*, and *VEGFA* were also downregulated in patients with CD compared with CTRL subjects. In patients with T1D, we did not observe any correlation between gene expression and glycated hemoglobin (HbA1c) level, duration of diabetes, presence of secondary complications, or the condition that led to endoscopy, indicating that gene expression was not influenced by these variables.

Duodenal mucosa of patients with T1D show histological signs of a characteristic inflammatory status

The leukocytes infiltrating duodenal gut mucosa were evaluated and quantified in biopsies of the 3 groups of study participants (Fig. 2). Lymphocytes (CD3-positive cells) in the lamina propria were present in all the groups, and, as expected, their percentage was significantly higher in patients with CD compared with CTRL subjects and patients with T1D ($P < 0.05$ and $P < 0.01$, respectively). Interestingly, the analysis of the mono/macrophagic lineage cell infiltrate (CD68-positive cells) showed that this was more represented in biopsies of patients with T1D compared with CTRL subjects and patients with CD ($P < 0.01$). In T1D biopsies, CD68-positive cells were mainly present in the lamina propria, in the villous stroma, and in dense areas of inflammatory infiltrate. Instead, the analysis of neutrophil infiltration (MPO-positive cells) resulted in a low percentage of positive cells in all the biopsies (<10% positive cells) without any significant difference among the three groups. Immunohistochemical analysis of the proteic expression of the genes *TNFα* and *VEGFA* was performed on the same biopsies (Fig. 3). In patients with T1D, *TNFα* was mainly represented on the top of the villi, but it could be observed also in some inflammatory elements in the lamina propria; however, this kind of reactivity was not observed in patients with CD who did not show villous atrophy. *VEGFA* presented a more

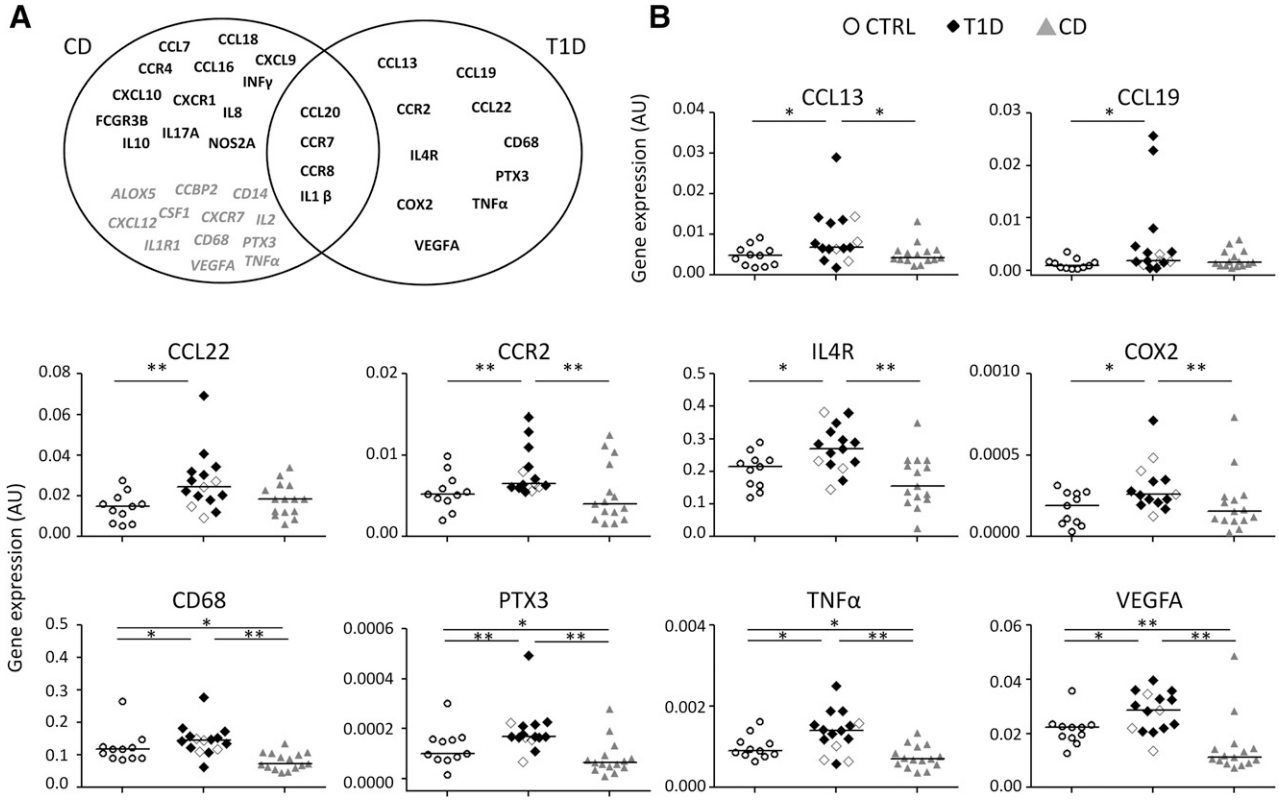

Figure 1. Differentially expressed genes in duodenal biopsies of patients with T1D and patients with CD compared with control subjects. (A) Venn diagram showing differentially expressed genes in T1D and patients with CD compared with control subjects, with overexpressed genes in black and underexpressed genes in gray. (B) Dot plots of genes significantly more expressed in patients with T1D compared with control subjects. Gene expression levels [arbitrary units (AU), 2^-ΔCt method] in the three groups are shown. Empty squares indicate patients with T1D at onset (<1 year from diagnosis). Black line indicates the median value. *$P < 0.05$; **$P < 0.01$.

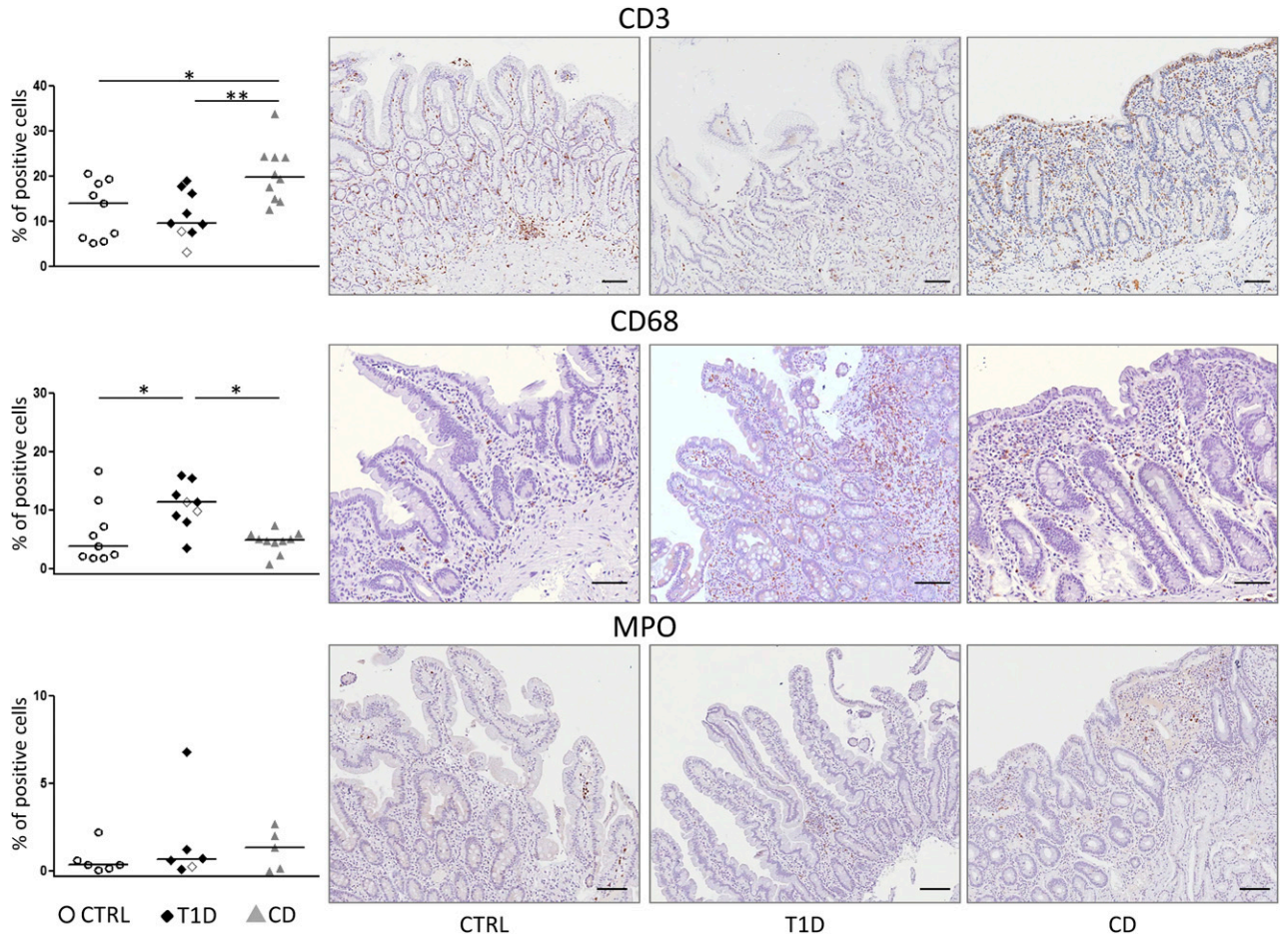

Figure 2. Inflammatory status of duodenal mucosa of control subjects, patients with T1D, and patients with CD. Quantification of staining was performed by automated nuclear counting and is reported as dot plots with line at median of the percentage of positive nuclei/total nuclei of counting area. Left: Empty squares indicate patients with T1D at onset (<1 year from diagnosis). *$P < 0.05$; **$P < 0.01$. Right: Representative images of lymphocytes (anti-CD3), macrophages (anti-CD68), and neutrophils (anti-MPO) staining in sections, counterstained with hematoxylin, of duodenal mucosa of control subjects, patients with T1D, and patients with CD. Scale bar, 50 μm (on the right).

diffuse positivity in all the groups, with strong expression particularly in the epithelial cell layer. A semiquantitative quantification at the microscope confirmed that *TNFα* in the lamina propria and *VEGFA* in the epithelium were more expressed in subjects with T1D in comparison with CTRL subjects and patients with CD.

Patients with T1D show a distinctive microbiota

The composition of bacterial populations was measured in patients with T1D, patients with CD, and CTRL subjects using ultra-deep pyrosequencing. The mean bacterial diversity, as estimated by Chao1 index from the equalized data sets, was not different among the groups (data not shown), although significant differences in phyla distribution were observed. Patients with T1D showed a reduction in the percentage of Proteobacteria and an increase in Firmicutes (Fig. 4). This result is particularly relevant because it seems specific for the T1D group (*i.e.*, patients with CD show the same levels as the CTRL group). On the other hand, the phylum of Bacteroidetes shows a trend to reduction in patients with T1D and patients with CD compared with CTRL subjects. Also, the ratio Firmicutes/Bacteroidetes, which is regarded to be of relevance in human gut microbiota composition (23), was significantly increased in T1D and CD groups. Going deep into the analysis of bacterial composition in terms of class, order, family, and genus, we recognized some interesting patterns (Supplemental Fig. 1). In the phylum of Firmicutes, the increase observed in T1D was mainly explained by an increase in Bacilli, and more specifically *Streptococcus*, which belongs to the family of Streptococcaceae and the order of Lacotbacillales. Moreover, we observed a substantial reduction in patients with T1D compared patients with CD in the order of Gemellales. The class of Clostridia, instead, resulted less present in T1D and CD, with a substantial reduction in the families of Lachnospiraceae and Peptosreptococcaceae. Regarding the phylum of Proteobacteria, the reduction observed specifically in T1D was mainly ascribable to the diminished presence of the class

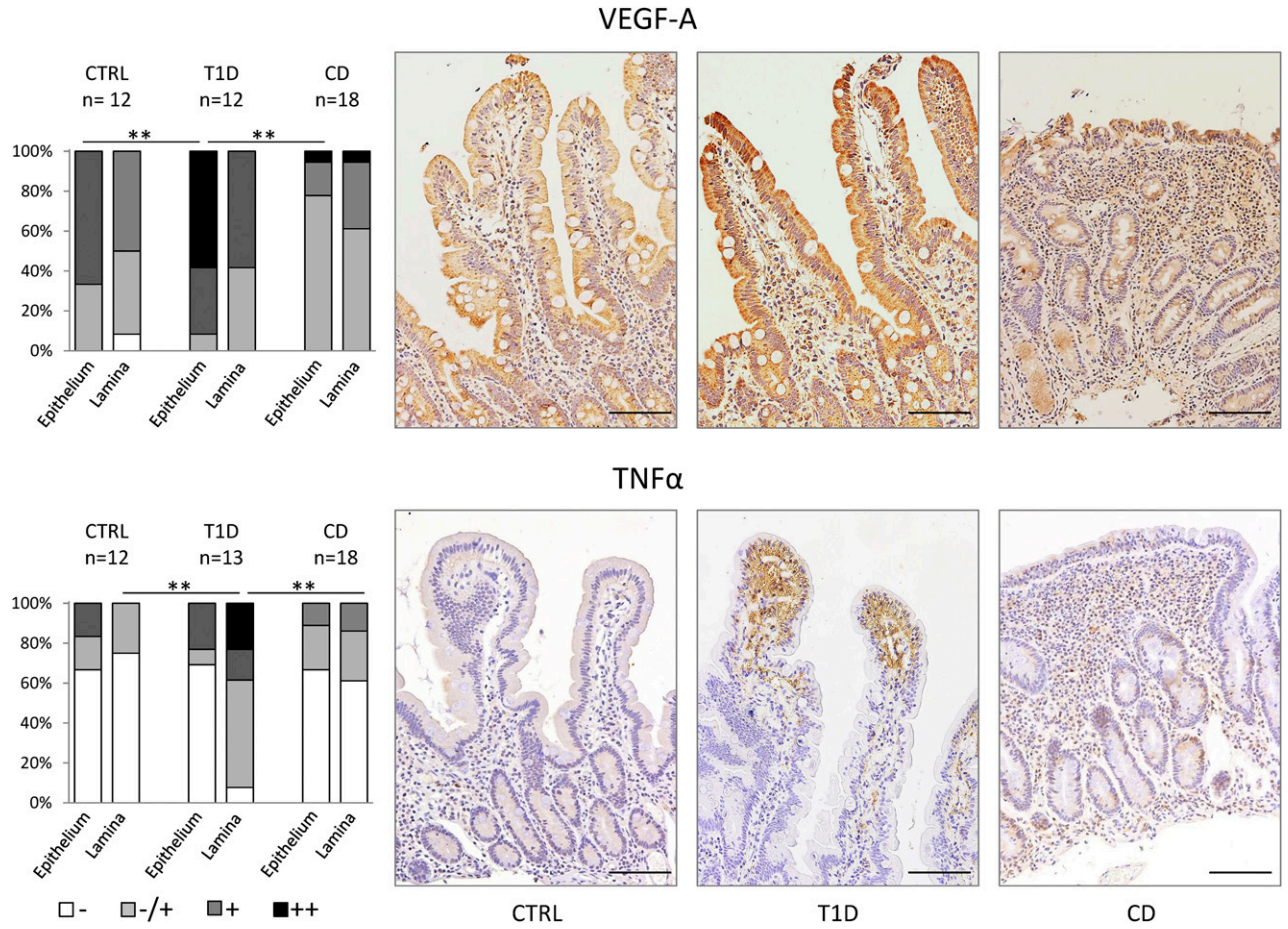

Figure 3. Protein expression of *TNFα* and *VEGFA* in duodenal mucosa of control subjects, patients with T1D, and patients with CD. Left: Semiquantitative analysis of *TNFα* and *VEGFA* staining in sections of duodenal mucosa of control subjects, patients with T1D, and patients with CD. Positivity score (0 = negative cell, 1 = weak positivity, 2 = medium positivity, and 3 = strong positivity) percentage distribution is shown as a histogram and analyzed using the χ^2 test. N indicates the number of cases available per group. $^{**}P < 0.01$. Right: Representative images of *TNFα* and *VEGFA* staining in the biopsies, counterstained with hematoxylin, of duodenal mucosa of control subjects, patients with T1D, and patients with CD. Scale bar, 50 μm.

Gammaproteobacteria and, in particular, to the family of Pasteurellaceae. Finally, in the phylum of Bacteroidetes, the class of Bacteroidia and the genus Prevotella, belonging to Prevotellaceae and Bacteroidales, specified the decrease observed in patients with T1D and patients with CD compared with CTRL subjects.

In patients with T1D, we did not observe any correlation between microbiome composition and HbA1c level, duration of diabetes, presence of secondary complications, or the condition that led to endoscopy.

Cytokines and bacteria differentially expressed in patients with T1D and CTRL subjects are correlated

To understand whether there is an association between the genes that were overexpressed exclusively in T1D gut mucosa and bacterial composition, the correlation between them was analyzed. Seven out of 10 overexpressed genes correlated with specific bacterial sequences in T1D ($P < 0.05$; $R^2 > 0.36$). Correlated taxa are reported in Table 2, and correlation plots are given in Supplemental Fig. 2.

In the CTRL group, Bacteroidetes, and in particular the class of Bacteroidia, the order of Bacteroidales and the genus Prevotella, was correlated with expression of CCL13. The same cytokine showed a substantial correlation with the phylum of Firmicutes, and in particular with the class of Clostridia and the order of Clostridiales and Gemellales. Also, the phylum Proteobacteria was correlated with the expression of CCL13. CCL20 expression was correlated only with the presence of the family of Lachnospiraceae, belonging to the phylum Firmicutes. CCR8 expression was correlated with Lachnospiraceae, and this cytokine was expressed more in mucosa where Bacteroidetes, and Bacteroidales in particular, were present. Finally, PTX3 expression was correlated with the presence of Gemellales.

In patients with T1D, the correlations were almost completely different, suggesting that the disease has an influence on or is influenced by a peculiar relationship between inflammation and microflora. In fact, CCL13

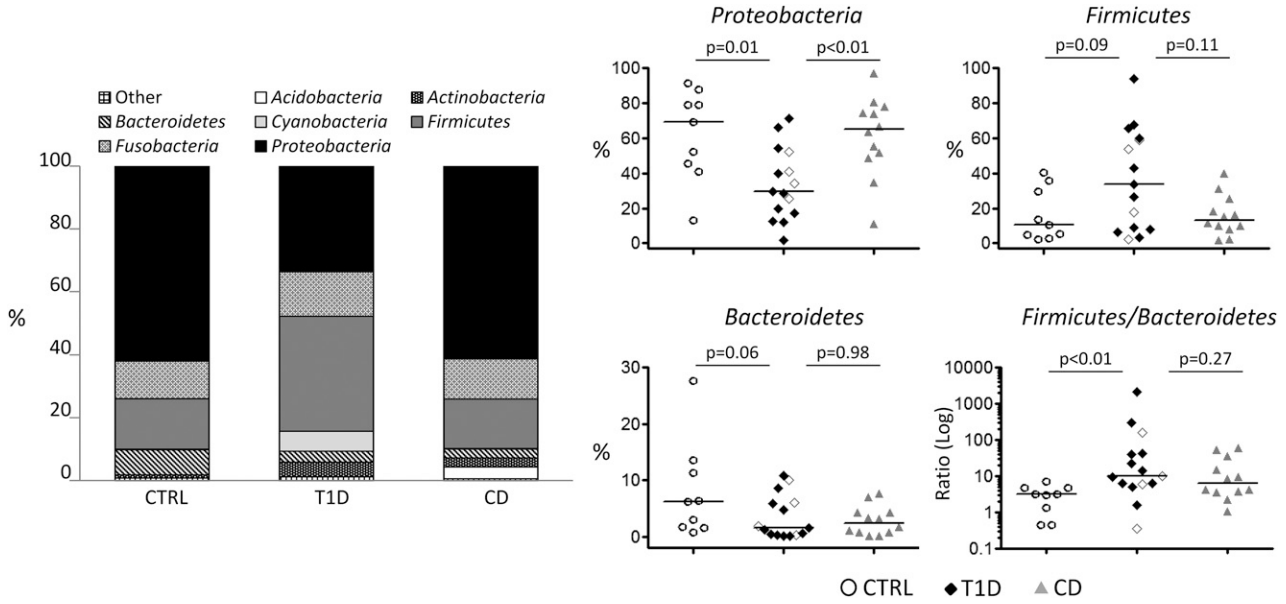

Figure 4. Phyla composition in duodenal biopsies of control subjects, patients with T1D, and patients with CD. Bar graph of percentages of phyla distribution in gut biopsies of control subjects, patients with T1D, and patients with CD (left) and dot plots of percentages of Proteobacteria, Firmicutes, Bacteroidetes, and Firmicutes/Bacteroidetes ratio in the three groups (right). Black line indicates the median value. Empty squares indicate patients with T1D at onset (<1 year from diagnosis). $*P < 0.05$; $**P < 0.01$.

was correlated only with Gemellaceae and PTX3 with Bacilli and Streptococcaceae. Finally, two cytokines showed a strong correlation only in T1D group: *VEGFA* expression was inversely correlated to the presence of Bacteroidetes and Bacteroidia, and *IL1β* expression was linked to Firmicutes, in particular to Gemellales, Gemellaceae and Lachnospiraceae.

Discussion

This study analyzed the inflammatory profile, the microbiome and their association on duodenal mucosa of patients with T1D, in comparison with patients with CD and healthy CTRL subjects. Few studies have been performed directly on the gut mucosa from patients with T1D due to the difficulty and complexity of collecting this bioptic samples. We found that duodenal mucosa in T1D shows a peculiar signature of inflammation and a specific microbiome composition. We also discovered an association between some analyzed inflammatory markers and specific taxa.

Our results showed the presence of a distinctive inflammatory profile of gut mucosa of patients with T1D. The literature reports several studies where cytokine profile in peripheral blood of patients with T1D was assessed (24), but very few works report the expression of inflammatory genes and proteins in human duodenal mucosa. Aware of the difficulties in handling gut samples for molecular analysis, we started from developing a fine-tuned technique for RNA preservation and extraction, and this allowed us to work on high-quality material. We used samples from patients with CD as controls of a well-characterized inflammatory disease of the intestine and found the upregulation of many inflammatory genes, as previously reported (7, 25, 26). Our cohort of patients with CD was significantly younger compared with the CTRL subjects and the patients with T1D; this is due to recruitment at onset of disease (before the beginning of the gluten-free diet), which occurs mostly in children (27). The analysis of T1D biopsies reported that some genes were upregulated in patients with T1D and in patients with CD, whereas others are specifically upregulated only in patients with T1D. The histological findings support the results obtained by the gene expression, and we also observed that the mucosa of subjects with T1D is abundantly infiltrated with macrophages, which could amplify the inflammatory cytokine response, as described for patients with inflammatory bowel diseases (28). These results highlight that duodenal mucosa shows signs of immune activation in mostly asymptomatic patients with T1D despite the evidence of a normal intestine at histopathological analysis. Accordingly, Savilahti *et al.* (8) found enhanced expressions of human leukocyte antigen-antigen D Related, human leukocyte antigen DP subregion, and intercellular adhesion molecule-1 in structurally normal intestine of patients with T1D, and Westerholm-Ormio *et al.* (7) reported that the small intestine in pediatric patients with T1D shows enhanced T immune activation, even in the presence of structurally normal intestine. It is possible that the increased infiltration of macrophages in gut mucosa may damage the barrier. It has been reported that the intestinal mucosa is

Table 2. Correlation of Cytokine Expression and Microbiota

	Phylum	Class	Order	Family	Genus	*CCL13*	*CCL20*	*CCR8*	*CCL19*	*PTX3*	*VEGFA*	*IL1β*
CTRL	Bacteroidetes					**0.54/0.012**		**0.45/0.023**				
	Firmicutes					**0.58/0.009**						
	Proteobacteria					**0.74/0.001**						
	Bacteroidetes	Bacteroidia				**0.54/0.013**		**0.47/0.021**				
	Bacteroidetes	Bacteroidia	Bacteroidales			**0.54/0.013**		**0.47/0.021**				
	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	**0.53/0.013**						
	Firmicutes	Bacilli	Gemellales			**0.65/0.004**				**0.39/0.036**		
	Firmicutes	Clostridia				**0.62/0.006**						
	Firmicutes	Clostridia	Clostridiales			**0.62/0.006**						
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae			**0.52/0.014**	**0.68/0.003**				
	Proteobacteria	Gammaproteobacteria	Pasturellales	Pasturellaceae	*Haemophilus*							
T1D	Bacteroidetes										**0.54/0.001**	
	Bacteroidetes	Bacteroidia									**0.38/0.007**	
	Firmicutes	Bacilli							**0.41/0.005**			
	Firmicutes	Bacilli	Gemellales									**0.59/0.000**
	Firmicutes	Bacilli	Gemellales	Gemellaceae		**0.38/0.007**						**0.60/0.000**
	Firmicutes	Bacilli	Lactobacilales	Streptococcaceae					**0.69/0.002**			
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae						**0.78/0.000**		**0.79/0.000**

Only correlations between bacteria and cytokines differentially represented in patients with T1D compared with CTRL subjects are reported. Bold cells indicate statistically significant correlation ($P < 0.05$). Numbers indicate the correlation coefficient R^2 (left) and P value (right).

more leaky before the clinical onset of T1D, as shown by higher lactulose/mannitol excretion rate (29) and, at the ultrastructural level, by altered microvilli and tight junctions (6) in rodent models (30, 31) and human subjects. This may result in increased antigenic load from intestinal lumen, causing altered immune activation and intestinal inflammation that could eventually contribute to the destruction of pancreatic β cells (32, 33). In our study, the absence of a correlation between inflammatory status and disease duration suggests that chronic hyperglycemia is not causative of inflammation and could be a pathogenetic feature of the disease. However, it is not possible to draw definitive conclusions about the correlation of these alterations with β cell autoimmunity. Further studies are needed on patients at risk for T1D to definitively support duodenal inflammation as a mechanism correlated to T1D pathogenesis.

In view of the fact that one of the major modulators of gut inflammatory status is the microbiota, we analyzed the composition of bacteria in our cohort of patients. Because dietary composition is known to be a major influence on microbiota composition (34), we made sure that our cohort of patients was homogeneous in terms of diet at the time of biopsy. For the analysis of microbiome, we analyzed complementary DNA from bacterial RNA coming directly from duodenal mucosa; thus, we were able to detect live bacteria on site. This is different from what was previously done. In fact, to date most human studies have used fecal samples and/or were conducted searching for bacterial DNA (11–13). It is recognized that microbiome composition may differ between fecal and duodenal samples of the same individual (35, 36). We assumed that, despite the difficulty in obtaining the bioptic material, the analysis of microflora in the original site was more appropriate and added value to the findings. As in previously published reports (37), we have found some substantial differences between microbiome in patients with T1D and in healthy control subjects. Patients with T1D, when compared with both healthy CTRL subjects and patients with CD, had a reduction in Proteobacteria and an increase in Firmicutes. This has not been described in humans, but it agrees with what was described in the composition of duodenal microbiome in rats with diabetes (38). Proteobacteria is a phylum that includes a wide variety of pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and many other well-studied genera. In our results, the reduction in Proteobacteria can be attributed to GammaProteobacteria and specifically to the family of Pasteurellaceae. In Firmicutes, the increase observed in patients with T1D was due to the presence of Bacilli and *Streptococcus*, but, at the same time, family comparison showed that healthy control subjects had significantly higher percentages of Peptostreptococcaceae and Lachnospiraceae (part of Clostridia class) when compared with gut biopsies of patients with T1D. It is known that Clostridia are mainly butyrate-producing and mucin-degrading bacteria, with immunomodulating properties, and are generally associated with a balanced status of gut mucosa; the reduction in Clostridia that we observed in patients with T1D agrees with other studies where similar findings were described (even if on fecal samples) (11, 14), and it could contribute to explain gut immune imbalance.

We were also able to describe important associations between the level of expression of inflammatory genes and the percentages of specific bacteria in the mucosa. Interestingly, the correlations found in the CTRL group were different from those observed in T1D cohort, suggesting that the correlation may be specific to a diseased status and can be added to the other alterations described.

In summary, we found signs of a chronic and latent inflammation and a specific microbial composition in the duodenal mucosa of subjects with T1D. Our findings

are a kind of "high-resolution snap-shot" of duodenal mucosa; this allows a very detailed description of mucosal environment at the time of biopsy, but we are unable to define a causative process that leads to the development of T1D, also because most patients with diabetes had a long-lasting disease. Our cohort of patients included few subjects at onset of diabetes, and even if their biological behavior did not diverge from patients with years of disease, their limited number does not allow us to draw conclusions. We suggest that there can be an "inflammatory signature" that characterizes T1D duodenal mucosa. Further research is needed to determine whether this inflammation is a cause or a consequence of the autoimmune process and how it is related to the alteration of microbiota or gut permeability. However, our findings support the hypothesis that a strong link exists between the duodenal inflammatory status and T1D.

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