Immune Response to Rotavirus and Gluten Sensitivity

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1. Introduction

Nonceliac gluten sensitivity (NCGS) can be defined as a nonallergic condition in which the consumption of gluten can lead to symptoms similar to those observed in celiac disease (CD). NCGS is characterized by the absence of celiac specific antibodies (against tissue transglutaminase, endomysium, and/or deamidated gliadin peptide) and absence of classical enteropathy (Marsh 0-1) although an increased density of CD3+ intraepithelial lymphocytes can be observed in duodenal biopsies. Patients with NCGS may have variable HLA status, and positivity for HLA-DQ2 and/or DQ8 has been found in roughly 50% of patients with NCGS. Serological analyses of NCGS patients revealed a high prevalence (40–50%) of first generation antigliadin IgG antibodies. NCGS is characterized by symptoms that usually occur soon after gluten ingestion and disappear or improve with gluten withdrawal but relapse following gluten challenge. The clinical presentation of NCGS may be a combination of gastrointestinal symptoms, including abdominal pain, bloating, bowel habit abnormalities (diarrhoea or constipation), and systemic manifestations, that is “foggy mind,” fatigue, muscle and joint pain, leg or arm numbness, eczema and skin rash, depression, and anemia. Similarly to patients with CD, subjects with clinical manifestations compatible with NCGS should start a gluten-free diet. Since it is still not clear whether NCGS is a permanent or transient condition, reintroduction of gluten after 1-2 years on a gluten-free diet can be considered [1, 2].
Rotavirus is a double-stranded RNA virus belonging to the family of Reoviridae.

The virus is transmitted by the faecal-oral route and infects intestinal cells causing gastroenteritis. Rotaviruses are the main cause of severe acute diarrhoea in children less than 5 years of age worldwide [3]. They are responsible for 453,000 deaths worldwide each year, which in most cases (85%) occur in developing countries [3]. The virus particle is composed of six viral proteins (VPs) called VP1, VP2, VP3, VP4, VP6, and VP7. Among these, the glycoprotein VP7 is located on the outer surface of the virus determining the specific G-type of the strain and plays a role in the development of immunity to infection [4].

We have previously described the presence, in active celiac disease (CD), of a subset of antitransglutaminase IgA antibodies that recognizes the viral protein VP-7 and is able to increase intestinal permeability and induce monocyte activation [5]. We then showed that the rotavirus VP7 antibodies may be even detected before the CD onset and the detection of antitissue transglutaminase (tTG) and antiendothymus antibodies, showing a predictive role [6]. In addition, we observed that these antibodies were able to induce in human T84 intestinal cell line the modulation of genes involved in biological processes that represents typical features of CD [6]. Taken together, our data seem to provide a link between rotavirus infection and CD.

In this paper, we aim at clarifying some aspects of the pathogenesis of NCGS by a gene-array approach. In particular, we plan at verifying the possibility of the involvement of an autoimmune mechanism in the disease. In addition, we also aim at investigating a possible involvement of rotavirus infection in the development of NCGS. For this purpose, we compared the global panel of modulated genes in NCGS to the dataset of human T84 intestinal cells treated with antirotavirus VP7 antibodies, described in our previous work [6], and to a dataset of acute phase of rotavirus infection, downloaded from the GEO (Gene Expression Omnibus) database, searching for transcriptional profiles that may be associated to viral infection.

2. Materials and Methods

2.1. Patients. We studied a cohort of 16 patients (6 males and 10 females, mean age: 27.3 years) affected by NCGS, attending the Unit of Autoimmune Diseases and the Immunology Unit and Child Neuropsychiatry Unit at the University Hospital of Verona, Italy.

All the enrolled subjects were recruited after informed consent. Main symptoms were headache, dermatitis, chronic urticaria, muscle and joint pain, bloating, abdominal pain, diarrhoea, alternating bowel movements, and fatigue in a variable combination.

Diagnosis of NCGS was established when all the following criteria were met: (1) exclusion of wheat allergy by clinical history and determination of specific IgE; (2) exclusion of celiac disease by absence of celiac-specific antibodies tissue transglutaminase (tTG), endomysium (EMA), and/or deamidated gliadin peptides (DGP); (3) duodenal biopsy with a histological damage grade 0 to 1, according to Marsh’s classification; (4) significant improvement of symptoms on strict gluten-free diet and relapse of symptoms after gluten reintroduction.

2.2. Detection of Anti-VP7 Peptide Antibodies. The ELISA test for antibody binding to the synthetic peptides has been carried out as already described elsewhere with minor modifications [7]. The synthetic peptides were used at a concentration of 20 μM in PBS to coat polystyrene plates (Immulon 2HB, Thermo). For the detection of antirotavirus VP7 peptide IgA antibodies, only the sera whose OD readings were higher than the mean plus three standard deviations of each serum dilution of the control group were considered positive. OD values higher than 0.140 were considered positive.

2.3. Gene Array. Peripheral blood cells were collected for analysis of gene expression profiles on a gluten-containing diet. PAxgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) were used for blood collection and total RNA was extracted according to the protocol supplied by the manufacturer. Preparation of cRNA hybridization and scanning of arrays for each samples were performed following the manufacturer instructions (Affymetrix, Santa Clara, CA, USA) by Cogentech Affymetrix microarray unit (Campus IFOM IEO, Milan, Italy) using the Human Genome U133A 2.0 GeneChip (Affymetrix). The gene expression profiles were analysed using the GeneSpring software version 12.1 (Agilent Technologies, Santa Clara, CA, USA) that calculated a robust multiarray average of background-adjusted, normalized, and log-transformed intensity values applying the robust multiarray average algorithm (RMA). The normalized data were transformed to the log2 scale. The unpaired t-test was performed to determine which genes were modulated at a significant level (p ≤ 0.01), and p values were corrected for multiple testing by using Bonferroni correction. Finally, statistically significant genes were chosen for final consideration when their expression was at least 1.5-fold different in the test sample versus control sample. Genes that passed both the p value and the FC restriction were submitted to functional and pathway enrichment analysis according to the Gene Ontology (GO) annotations employing the Panther expression analysis tools (http://pantherdb.org/).

2.4. Protein-Protein Interaction (PPI) Network Construction and Network Modular Analysis. All the possible interactions among the protein products of DEGs were analysed with Search Tool for the Retrieval of Interacting Genes (STRING version 1.0; http://string-db.org/) a web-based database that includes experimental as well as predicted interaction information and covers more than 1100 sequenced organisms. Only protein-protein interaction (PPI) pairs that were confirmed by experimental studies were selected, and a score of ≥0.7 for each PPI pair was used to build a PPI network.

Cytoscape software [8] was used to define the topology of the built network, and the Molecular Complex Detection (MCODE) [9] was used to find densely connected region (modules) of the network that could be involved in the
modulation of biological processes that are relevant for the disease pathogenesis. To find locally dense regions of a graph, MCODE applies a vertex-weighting scheme based on a clustering coefficient that is a measure of the degree to which nodes in a graph tend to cluster together.

The following settings in MCODE were used: degree cutoff = 2, K-core = 3, and max. depth = 100. Functional enrichment for a given module was assessed quantitatively using the Panther tool.

2.5. Analysis of the Association between DEGs and Human Diseases. We used the software Ingenuity Pathway Analysis (IPA, Ingenuity Systems) to evaluate diseases and disorders that could be statistically significantly associated to gene modulation observed in NCGS samples. The statistical significance of gene-disease associations was calculated in IPA by the Fisher’s exact test (p ≤ 0.0001).

2.6. Detection of Soluble Mediators in GS Sera. Serum levels of sCTLA-4, s PD-1, and sgp130/IL6ST were detected before and after gluten-free diet using commercially available ELISA kits according to the manufacturer’s instructions. ELISA kits were purchased from Bender MedSystems (Milano, Italy) (sCTLA-4), from R&D Systems (Minneapolis, United States) (sgp130), and from EMELCA Bioscience (Clinge, Netherlands) (sPD-1).

2.7. FACS Analysis. Cells collected from patients and normal controls were cultured at a concentration of 1 × 10⁶ cells/mL in 2 mL tubes containing 1 mL of RPMI 1640 + FCS 10% (Lonza, Basel, CH). Cells were stimulated overnight with Dynabeads Human T-Activator CD3/CD28 (Life Technologies, Carlsbad, CA, USA). The detection of IL-17 production was analysed using the IL-17 Secretion Assay (Miltenyi Biotechnologies, Carlsbad, CA, USA). The detection of IL-17 production was assessed quantitatively with 10 μL of cold buffer and resuspended in 90 μL of cold medium. Cells were then incubated with 10 μL of IL-17 Catch Reagent for 5 minutes in ice and cultured in 1 mL of warm medium at 37°C for 45 minutes under slow continuous rotation. Cells were then washed with cold buffer and resuspended in 75 μL of cold buffer; 10 μL of IL-17 Detection Antibody APC, 10 μL of anti-CD3 PerCP (Becton Dickinson, Franklin Lakes, NJ, USA), and 5 μL of anti-CD4 APC-H7 (Becton Dickinson) monoclonal antibodies were added. Incubation was carried out in ice for 10 minutes. Finally, cells were washed and resuspended in an appropriate volume of PBS and acquired on a FACSCanto II cytometer (Becton Dickinson). Analysis was performed with FlowJo 9.3.3 software (Tree Star, Ashland, OR, USA).

2.8. Statistical Analysis. Data obtained from the analysis of the soluble mediators CTLA-4, gpl30, and PD-1 and from the detection of antigliadin antibodies were submitted to statistical testing using the Wilcoxon nonparametric statistical hypothesis test for paired samples. Data obtained from the ELISA test for the detection of antitropanovirus VP7 peptide antibodies were submitted to statistical testing using the Mann–Whitney nonparametric test. Statistical analysis was performed using GraphPad Prism Software version 5.00 (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com).

3. Results and Discussion

Many aspects of NCGS are still unknown; in particular, it is still not clear whether the disease is permanent or transient or whether the disease has features of autoimmunity. The pathogenesis of NCGS is also unclear and data obtained so far suggest a prevalent activation of innate immune responses [2].

We aimed at clarifying some aspects of NCGS pathogenesis using a gene array approach which we successfully used in the study of many immune-mediated diseases [6, 10–12].

In order to identify specific gene signatures typically associated with NCGS, we compared the gene expression profiles of 8 PBC samples obtained from individual NCGS patients with 10 PBC samples obtained from healthy age- and sex-matched donors. We observed that the disease has a profound impact on gene expression profiles since a large number of differentially expressed genes (DEGs) (1293, represented by 1521 modulated probe sets) complied with the Bonferroni-corrected p value criterion (p < 0.01) and the fold change criterion (FC > 1.5) showing robust and statistically significant variation between healthy controls and NCGS samples. In particular, 695 and 598 genes resulted to be up- and downregulated, respectively (Additional Table 1).

DEGs were submitted to functional enrichment analysis according to terms of the Gene Ontology (GO) biological processes (BP) and canonical pathways. The most enriched biological process was “immune system,” followed by “intracellular signal transduction” (Table 1). In addition, several enriched terms were related to the immune response gene category, including “leukocyte differentiation,” “leukocyte activation involved in immune response,” “T cell differentiation,” “neutrophil degranulation,” “adaptive immune response,” and “defense response.” Interestingly, we observed an enrichment in “cellular response to organic substance,” “cellular response to endogenous stimulus,” and “viral process.” The BP named “viral process” is defined by the Gene Ontology Consortium as a “multi-organism process in which a virus is a participant and the other participant is the host.” This term is related to the infection of a host cell, the replication of the viral genome, the viral transcription, and the assembly of progeny virus particles.

Pathway enrichment analysis showed that the most enriched signaling pathways were “inflammation mediated by chemokine and cytokine,” “apoptosis,” and “angiogenesis,” followed by “T cell activation” and “B cell activation” (Table 1). Other enriched pathways were: “integrin signaling,” “EGF receptor signaling,” “Toll-like receptor signaling,” “PI3 kinase,” “interleukin signaling,” and JAK/STAT signaling. Since the majority of the top-enriched functional classes and pathways were related to the immune system, we selected, within the entire data set, all modulated genes associated to the “Immune response” GO term to better characterize the immunological processes that are involved in NCGS pathogenesis. Although both innate and adaptive immunity play a crucial role in the development of CD,
Table 1: Biological processes and pathways that were enriched in the NCGS dataset.

| Biological processes                                      | p value* |
|-----------------------------------------------------------|----------|
| Immune system process                                     | 6.3 × 10^{-20} |
| Intracellular signal transduction                          | 4.6 × 10^{-16} |
| Cellular response to organic substance                     | 1.5 × 10^{-13} |
| Cell surface receptor signaling pathway                    | 8.2 × 10^{-10} |
| Leukocyte differentiation                                  | 6.3 × 10^{-9} |
| Viral process                                              | 7.7 × 10^{-9} |
| Leukocyte activation involved in immune response           | 8.0 × 10^{-8} |
| Apoptotic process                                          | 2.2 × 10^{-6} |
| Cellular response to endogenous stimulus                   | 3.0 × 10^{-6} |
| T cell differentiation                                     | 5.6 × 10^{-5} |
| Neutrophil degranulation                                   | 5.6 × 10^{-5} |
| Adaptive immune response                                   | 6.5 × 10^{-5} |
| Defense response                                           | 6.8 × 10^{-5} |

Pathways

| Biological processes                                      | p value* |
|-----------------------------------------------------------|----------|
| Inflammation mediated by chemokine and cytokine signaling pathway | 2.1 × 10^{-7} |
| Apoptosis signaling pathway                               | 1.6 × 10^{-4} |
| Angiogenesis                                              | 4.1 × 10^{-4} |
| T cell activation                                         | 5.3 × 10^{-4} |
| B cell activation                                         | 5.7 × 10^{-4} |
| Integrin signaling pathway                                | 7.8 × 10^{-4} |
| EGF receptor signaling pathway                            | 4.0 × 10^{-3} |
| Toll like receptor signaling pathway                       | 4.6 × 10^{-3} |
| PI3 kinase pathway                                        | 7.6 × 10^{-3} |
| Interleukin signaling pathway                             | 8.1 × 10^{-3} |
| JAK/STAT signaling pathway                                | 1.6 × 10^{-2} |

* Bonferroni corrected.

NCGS has been mainly associated with activation of the innate immune response [2].

It is therefore surprising to notice that both transcripts involved in the innate immune response as well as genes of the adaptive immune response were well represented in our dataset (Table 2).

In this regard, 14 genes involved in NK activity were modulated in NCGS samples (i.e., IL1R1A1, IL1R2A2, CLEC2D, and KLRC4). Moreover, several genes involved in macrophage activation were modulated in NCGS including TNFRSF10B, the ligand of the death receptors TRAIL that play important roles in set up both innate and adaptive immune responses against pathogens [13], and the scavenger receptors MRC1/CD206 [14] and MARCO, a member of the class A scavenger receptor family strongly upregulated in MΦs by various microbial stimuli in a TLR-dependent manner [15].

Noteworthy, 38 genes prevalently related to B cell activity (i.e., IL2RG, IL6R, KLF12, and CD27) were also modulated, indicating an important role for this cell subset in NCGS, 20 genes involved in T cell activation were upregulated in NCGS samples (i.e., CD28, CD3E, CD3G, and CTLA-4). Remarkably, Th17-lymphocyte-related genes and transcripts that can modulate Th17 cell development and functions were overexpressed including IL4R, IL2RG, IL6ST, IL1B, IL7R, STAT6, STAT5B, SOCS3, and CXCL2.

DEGs indicate therefore active involvement of both arms of the adaptive immune response (i.e., T and B cells response) and a prevalent upregulation of several Th17-related genes in the T cell response category. It is well known that Th17 cells play an important role in autoimmunity and have been implicated in the pathogenesis of psoriasis and in the amplification of inflammation in rheumatoid synovitis and in lupus nephritis [16–18].

In the NCGS dataset, 6 type I interferon inducible genes (IFIG) were upregulated (IFNA17, IFRF5, IFRF3, STAT2, STAT1, and LY9), thus indicating the presence of an IFN type I signature, typically associated with autoimmune disease such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Crohn’s disease, and Sjogren syndrome [19–25].

In this respect, it is well known that Th17 cells and related cytokines are crucial in promoting autoimmunity, in particular, when they act in synergy with type I IFN-driven inflammation. In the presence of IFN type I signature, CCR6+ memory T-helper cells producing IL-17A, IL-17F, IL-21, and/or IL-22 are increased in SLE, [26] indicating that, in the pathogenesis of systemic autoimmune diseases, IFN type I signature coacts with Th17 cells and related cytokines.

In order to further confirm our gene expression data on overexpression of IFIG and Th17 pathways, we analysed the presence of IL-17-producing CD4+ T cells and found a significantly (p = 0.0159) increased percentage of these cells in PBMC of patients with NCGS compared with normal subjects (Figure 1).

The analysis of genes modulated in gluten sensitivity was paralleled by the detection of some of the corresponding soluble mediators in the sera of NCGS patients. We analysed selected molecules that are widely recognized to be associated to an autoimmune response, including sCTLA-4, sPD-1, and sgp130/IL6ST. Figure 2 shows the concentration of these molecules in the sera of NCGS patients before and after gluten-free diet. The serum levels of all the molecules tested were significantly higher in NCGS before GFD than after GFD.

In order to gain further insights into the molecular mechanisms relevant in NCGS pathogenesis, we constructed a protein-protein interaction (PPI) network starting from all the 1293 DEGs. The resulted PPI network contained 853 nodes and 3512 edges (Figure 3). By performing a modular analysis of the constructed PPI network, we were able to identify clusters of the most densely interconnected nodes (modules) and to extrapolate 15 main modules of genes displaying the highest degree of connection. Figure 4 shows a graphical representation of such modules, where the nodes represent proteins and the edges indicate their relations.

All modules were submitted to enrichment analysis to find enriched GO biological processes and pathways.

Among the 15 modules in particular, five (module 1, 3, 7, 10, and 14) showed a prevalent enrichment in BP and
Table 2: Genes modulated in NCGS patients that are involved in immune response and molecular signalings.

| Probe set ID | p value | Gene symbol | Gene title | FC | Representative public ID |
|--------------|---------|-------------|------------|----|--------------------------|
| T cell activation | | | | | |
| 203809_s_at | <0.001 | AKT2 | v-akt murine thymoma viral oncogene homolog 2 | 2.36 | NM_001190720 |
| 211861_x_at | <0.001 | CD28 | CD28 molecule | 2.75 | AF222343 |
| 205456_at | <0.001 | CD3E | CD3e molecule, epsilon (CD3-TCR complex) | 2.67 | NM_000733 |
| 206804_at | <0.001 | CD3G | CD3g molecule, gamma (CD3-TCR complex) | 2.13 | NM_000073 |
| 211027_s_at | <0.001 | IKBKB | Inhibitor of kappa light polyp. gene enhancer in B cells, kinase β | 2.69 | NM_00190720 |
| 213281_at | 0.007 | JUN | jun proto-oncogene | 3.07 | NM_002228.3 |
| 204890_s_at | <0.001 | LCK | Lymphocyte-specific protein tyrosine kinase | 2.05 | U07236 |
| 213490_s_at | <0.001 | MAP2K2 | Mitogen-activated protein kinase kinase 2 | 1.83 | NM_030662 |
| 214786_at | 0.013 | MAP3K1 | Mitogen-activated protein kinase kinase 1 | 1.52 | NM_005921 |
| 210671_x_at | <0.001 | MAPK8 | Mitogen-activated protein kinase 8 | 2.33 | NM_001278548 |
| 211230_s_at | <0.001 | PIK3CD | Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic sub. Δ | 1.88 | U57843 |
| 212249_at | 0.004 | PIK3R1 | Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) | 2.76 | NM_181523 |
| 216551_x_at | 0.001 | PLCG1 | Phospholipase C, gamma 1 | 1.53 | NM_002660 |
| 208640_at | <0.001 | RAC1 | Rho family, small GTP-binding protein Rac1 | −1.92 | NM_006908 |
| 207419_s_at | <0.001 | RAC2 | Rho family, small GTP-binding protein Rac2 | 2.31 | NM_002872 |
| 217576_x_at | 0.002 | SOS2 | Son of sevenless homolog 2 | 1.90 | NM_006939 |
| 216042_at | <0.001 | TNFRSF25 | Tumor necrosis factor receptor superfamily, member 25 | 2.40 | NM_148965 |
| 221331_x_at | <0.001 | CTLA4 | Cytotoxic T-lymphocyte-associated protein 4 | 2.26 | NM_005214 |
| 206569_at | <0.001 | IL24 | Interleukin 24 | 2.84 | NM_006850 |
| 203828_s_at | 0.003 | IL32 | Interleukin 32 | 2.10 | NM_004221 |
| B cell mediated immune response | | | | | |
| 211027_s_at | <0.001 | IKBKB | Inhibitor of kappa light polyp. gene enhancer in B cells, kinase β | 2.69 | NM_00190720 |
| 213281_at | 0.007 | JUN | jun proto-oncogene | 3.07 | NM_002228.3 |
| 202626_s_at | 0.004 | LYN | v-yes-1 Yamaguchi sarcoma viral related oncogene homolog | −2.22 | AI356412 |
| 213490_s_at | <0.001 | MAP2K2 | Mitogen-activated protein kinase kinase 2 | 1.83 | NM_030662 |
| 210671_x_at | <0.001 | MAPK8 | Mitogen-activated protein kinase 8 | 2.33 | NM_001278548 |
| 211230_s_at | <0.001 | PIK3CD | Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic sub. Δ | 1.88 | U57843 |
| 32540_at | <0.001 | PPP3CC | Protein phosphatase 3, catalytic subunit, gamma isoform | 2.00 | NM_001243975 |
| 208640_at | <0.001 | RAC1 | Rho family, small GTP-binding protein Rac1 | −1.92 | NM_006908 |
| 207419_s_at | <0.001 | RAC2 | Rho family, small GTP-binding protein Rac2 | 2.31 | NM_002872 |
| 217576_x_at | 0.002 | SOS2 | Son of sevenless homolog 2 | 1.90 | NM_006939 |
| 207540_s_at | 0.008 | SYK | Spleen tyrosine kinase | −1.92 | NM_003177 |
| 207224_s_at | 0.003 | SIGLEC7 | Sialic acid-binding Ig-like lectin 7 | −2.21 | NM_016543 |
| 206150_at | 0.004 | CD27 | CD27 molecule | 1.96 | NM_001242 |
Table 2: Continued.

| Probe set ID   | p value | Gene symbol | Gene title                                                                 | FC  | Representative public ID |
|---------------|---------|-------------|----------------------------------------------------------------------------|-----|--------------------------|
| 214477_at     | 0.005   | ETS1        | v-ets erythroblastosis virus E26 oncogene homolog 1                        | 2.25| NM_005238                |
| 201328_at     | 0.012   | ETS2        | v-ets erythroblastosis virus E26 oncogene homolog 2                       | −1.83| NM_005239                |
| 212420_at     | 0.001   | ELF1        | E74-like factor 1 (ets domain transcription factor)                       | 1.94| KJ896761                 |
| 211825_s_at   | <0.001  | FLI1        | Friend leukemia virus integration 1                                       | 2.28| AF327066                 |
| 215967_s_at   | <0.001  | LY9         | Lymphocyte antigen 9                                                      | 2.08| NM_002348                |
| 210690_at     | 0.001   | KLRC4       | Killer cell lectin-like receptor subfamily C, member 4                    | 2.23| U96845                   |
| 204116_at     | <0.001  | IL2RG       | Interleukin 2 receptor, gamma                                            | 1.84| NM_000206                |
| 217489_s_at   | <0.001  | IL6R        | Interleukin 6 receptor                                                    | 1.79| S72848                   |
| 204863_s_at   | <0.001  | IL6ST       | Interleukin 6 signal transducer (gp130, oncostatin M receptor)           | 4.52| NM_002184                |
| 206966_s_at   | <0.001  | KLF12       | Kruppel-like factor 12                                                   | 1.83| AH10423                  |
| 219878_s_at   | <0.001  | KLF13       | Kruppel-like factor 13                                                   | 1.89| NM_015995                |
| 219386_s_at   | <0.001  | SLAMF8      | SLAM family member 8                                                     | −2.27| NM_020125                |
| 210405_s_at   | 0.003   | TNFRSF10B   | Tumor necrosis factor receptor superfamily, member 10b                   | 1.50| NM_003842                |
| 219386_s_at   | <0.001  | SLAMF8      | SLAM family member 8                                                     | −2.27| NM_021025                |
| 210405_s_at   | 0.003   | TNFRSF10B   | Tumor necrosis factor receptor superfamily, member 10b                   | 1.50| NM_003842                |
| 203508_at     | 0.005   | TNFRSF11B   | Tumor necrosis factor receptor superfamily, member 1B                    | −2.06| NM_001066                |
| 216042_at     | <0.001  | TNFRSF25    | Tumor necrosis factor receptor superfamily, member 25                     | 2.40| NM_148965                |
| 218856_at     | 0.008   | TNFRSF21    | Tumor necrosis factor receptor superfamily, member 21                     | −1.68| NM_014452                |
| 206181_at     | 0.003   | SLAMF1      | Signaling lymphocytic activation molecule family member 1                | 1.65| NM_003037                |
| 210796_s_at   | <0.001  | SIGLEC6     | Sialic acid-binding Ig-like lectin 6                                     | 1.58| D86359                   |
| 211192_s_at   | <0.001  | CD84        | CD84 molecule                                                             | 2.55| AF054818                 |
| 220132_s_at   | <0.001  | CLEC2D      | C-type lectin domain family 2, member D                                   | 3.18| NM_013269                |
| 204773_at     | 0.005   | IL11RA      | Interleukin 11 receptor, alpha                                            | 1.56| AY532110                 |
| 210850_s_at   | <0.001  | ELK1        | ELK1, member of ETS oncogene family                                       | 1.60| AF00672                  |
| 209894_at     | 0.002   | LEPR        | Leptin receptor                                                           | −2.10| U50748                   |
| 203005_at     | 0.006   | LTBR        | Lymphotoxin beta receptor (TNFR superfamily, member 3)                    | −1.92| NM_002342                |

**NK cell activation**

| Probe set ID   | p value | Gene symbol | Gene title                                                                 | FC  | Representative public ID |
|---------------|---------|-------------|----------------------------------------------------------------------------|-----|--------------------------|
| 220132_s_at   | <0.001  | CLEC2D      | C-type lectin domain family 2, member D                                   | 3.18| NM_013269                |
| 203233_at     | 0.014   | IL4R        | Interleukin 4 receptor                                                    | 1.50| NM_000418                |
| 210152_at     | 0.007   | LILRB4      | Leukocyte immunoglobulin-like receptor, subfamily B, member 4             | −1.83| NM_001278426             |
| 210784_s_at   | 0.012   | LILRA6      | Leukocyte immunoglobulin-like receptor, subfamily A, member 6             | −1.74| NM_024318                |
| 211405_s_at   | 0.003   | IFNA17      | Interferon, alpha 17                                                     | 1.59| NM_021268                |
| 210660_at     | 0.008   | LILRA1      | Leukocyte immunoglobulin-like receptor, subfamily A, member 1             | −2.81| NM_001278319             |
| 207857_at     | 0.016   | LILRA2      | Leukocyte immunoglobulin-like receptor, subfamily A, member 2             | −2.35| NM_006866                |
| 210690_at     | 0.001   | KLRC4       | Killer cell lectin-like receptor subfamily C, member 4                    | 2.23| U96845                   |
| Probe set ID     | p value | Gene symbol | Gene title                                                                 | FC   | Representative public ID |
|-----------------|---------|-------------|-----------------------------------------------------------------------------|------|--------------------------|
| 206881_s_at     | 0.013   | LILRA3      | Leukocyte immunoglobulin-like receptor, subfamily A, member 3               | -2.96| NM_006865                |
| 210313_at       | 0.003   | LILRA4      | Leukocyte immunoglobulin-like receptor, subfamily A, member 4               | -1.83| NM_012276                |
| 215838_at       | 0.012   | LILRA5      | Leukocyte immunoglobulin-like receptor, subfamily A, member 5               | -3.15| NM_181985                |
| 210146_x_at     | 0.004   | LILRB2      | Leukocyte immunoglobulin-like receptor, subfamily B, member 2               | -3.41| AF004231                 |
| 208982_at       | 0.008   | PECAM1      | Platelet/endothelial cell adhesion molecule 1                               | -1.73| M37780                   |
| 203828_s_at     | 0.003   | IL32        | Interleukin 32                                                              | 2.10  | NM_004221                |
| **Macrophage activation** |         |             |                                                                             |      |                          |
| 210405_x_at     | 0.003   | TNFRSF10B   | Tumor necrosis factor receptor superfamily, member 10b                      | 1.50  | NM_003842                |
| 221900_at       | 0.003   | COL8A2      | Collagen, type VIII, alpha 2                                                | -1.62 | NM_005202                |
| 205819_at       | <0.001  | MARCO       | Macrophage receptor with collagenous structure                             | -3.01 | NM_006770                |
| 208602_x_at     | <0.001  | CD6         | CD6 molecule                                                                | 3.67  | NM_006725                |
| 207540_s_at     | 0.008   | SYK         | Spleen tyrosine kinase                                                      | -1.92 | NM_003177                |
| 203508_at       | 0.005   | TNFRSF1B    | Tumor necrosis factor receptor superfamily, member 1B                       | -2.06 | NM_001066                |
| 204438_at       | 0.012   | MRC1        | Mannose receptor, C type 1                                                  | -2.04 | NM_002438                |
| 202269_x_at     | 0.006   | GBP1        | Guanylate binding protein 1, interferon-inducible                          | -1.77 | NM_002053                |
| 208982_at       | 0.008   | PECAM1      | Platelet/endothelial cell adhesion molecule 1                               | -1.73 | M37780                   |
| **Complement activation** |         |             |                                                                             |      |                          |
| 205500_at       | 0.006   | C5          | Complement component 5                                                     | -1.60 | NM_001735                |
| 206244_at       | 0.003   | CR1         | Complement component (3b/4b) receptor 1 (Knops blood group)                | -1.79 | NM_000573                |
| **Response to gamma interferon** |         |             |                                                                             |      |                          |
| 205831_at       | 0.001   | CD2         | CD2 molecule                                                                | 2.00  | NM_001767                |
| 205468_s_at     | 0.015   | IRF5        | Interferon regulatory factor 5                                              | 1.52  | NM_003243                |
| 219386_s_at     | <0.001  | SLAMF8      | SLAM family member 8                                                        | -2.27 | NM_020125                |
| 211192_s_at     | <0.001  | CD84        | CD84 molecule                                                               | 2.55  | AF054818                 |
| 202269_x_at     | 0.006   | GBP1        | Guanylate-binding protein 1, interferon-inducible                          | -1.77 | NM_002053                |
| 202621_at       | <0.001  | IRF3        | Interferon regulatory factor 3                                              | 1.67  | NM_001571                |
| 33148_at        | <0.001  | ZFR         | Zinc finger RNA binding protein                                            | 2.19  | NM_016107                |
| 206181_at       | 0.003   | SLAMF1      | Signaling lymphocytic activation molecule family member 1                  | 1.65  | NM_003037                |
| 215967_s_at     | <0.001  | LY9         | Lymphocyte antigen 9                                                       | 2.08  | NM_002348                |
| 201461_s_at     | 0.011   | MAPKAPK2    | Mitogen-activated protein kinase-activated protein kinase 2                | 1.85  | NM_004759                |
| 216450_x_at     | <0.001  | HSP90B1     | Heat shock protein 90 kDa beta (Grp94), member 1                            | 3.76  | AK025862                 |
| 214370_at       | <0.001  | S100A8      | S100 calcium-binding protein A8                                            | 3.65  | AW238654                 |
| **Antigen processing and presentation** |         |             |                                                                             |      |                          |
| 206050_s_at     | 0.010   | RNH1        | Ribonuclease/angiogenin inhibitor 1                                         | -1.53 | NM_002939                |
| 204770_at       | <0.001  | TAP2        | Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)                | 1.86  | NM_000544                |
Table 2: Continued.

| Probe set ID  | p value | Gene symbol | Gene title                                      | FC  | Representative public ID |
|---------------|---------|-------------|-------------------------------------------------|-----|--------------------------|
| **TH17 related genes**                         |         |             |                                                 |     |                          |
| 203233_at     | 0.014   | IL4R        | Interleukin 4 receptor                          | 1.50| NM_000418                |
| 204116_at     | <0.001  | IL2RG       | Interleukin 2 receptor, gamma                   | 1.84| NM_000206                |
| 204863_s_at   | <0.001  | IL6ST       | Interleukin 6 signal transducer (gp130, oncostatin M receptor) | 4.52| NM_002184                |
| 205067_at     | 0.015   | IL1B        | Interleukin 1, beta                             | 1.52| NM_000576                |
| 205798_at     | 0.011   | IL7R        | Interleukin 7 receptor                          | 1.55| NM_002185                |
| 201332_s_at   | 0.013   | STAT6       | Signal transducer and activator of transcription 6 | 1.54| AH006951                 |
| 205026_at     | <0.001  | STAT5B      | Signal transducer and activator of transcription 5B | 1.60| NM_012448                |
| 206360_s_at   | <0.001  | SOCS3       | Suppressor of cytokine signaling 3              | 1.83| NM_003955                |
| 209774_x_at   | 0.015   | CXCL2       | Chemokine (C-X-C motif) ligand 2                | 1.53| M57731                   |
| **Type I interferon signaling**                |         |             |                                                 |     |                          |
| 211405_x_at   | 0.003   | IFNA17      | Interferon, alpha 17                            | 1.59| NM_021268                |
| 205468_s_at   | 0.015   | IRF5        | Interferon regulatory factor 5                  | 1.52| NM_032643                |
| 202621_at     | <0.001  | IRF3        | Interferon regulatory factor 3                  | 1.67| NM_001571                |
| 217199_s_at   | <0.001  | STAT2       | Signal transducer and activator of transcription 2, 113kDa | 1.59| S81491                   |
| M97935_5_at   | <0.001  | STAT1       | Signal transducer and activator of transcription 1, 91kDa | 2.73| NM_007315                |
| 210370_s_at   | <0.001  | LY9         | Lymphocyte antigen 9                            | 2.05| NM_002348                |
pathways associated to the activation of T cells. Similarly, “B cell activation” pathways were significantly enriched in modules 1, 9, 10, and 14. Interestingly, in modules 3, 10, and 11, we observed an enrichment in the JAK-STAT signaling pathway, which is highly relevant to human autoimmunity [27] and plays a role in the intestinal mucosal immune homeostasis as well as in intestinal epithelial repair and regeneration [28]. We also observed that module 11 contained several genes involved in Th-17 cell functions (i.e., IL2RG, IL4R, IL6ST, IL7R, SOCS3, STAT5B, and STAT6) and several IFIG, including IFNA17, STAT1, and STAT2. Other IFIG genes were ascribed to module 9 which also shows an enrichment in BPs associated to type I interferon signaling, including positive regulation of type I interferon production, positive regulation of interferon-beta production, and type I interferon biosynthetic process (Table 3).

Loss of the intestinal barrier integrity is a typical feature of CD and represents an important mechanism of autoimmunization through the passage of antigens across the intestinal epithelium [29]. However, Sapone et al. [29] have shown that NCGS patients have normal intestinal permeability when compared to CD patients, as assessed by the lactulose-mannitol test.

Indeed, in module 13, in which the most enriched BP was “adherent junction assembly,” we observed a reduced expression of molecules involved in cell adhesion including CDH1 (epithelial cadherin), CTNNA1, VCL, and CTNN, a molecule expressed on the apical surface of the polarized epithelium. In the same module, we also observed underexpression of Rac1, a critical regulator of intestinal epithelial barrier functions [30] and EGF, known to protect intestinal barrier integrity by stabilizing the microtubule cytoskeleton [31] and upregulation of FYN and PIK3R1, both involved in the signaling pathway by which IFNγ increases intestinal permeability [32].

The gene expression data would therefore indicate deregulation of adherent junctions and altered intestinal permeability also in NCGS, which seems to be in contrast with the data of Sapone et al. Nevertheless, it is important to point out that the lactulose-mannitol test may not be sensitive enough to detect mild alterations of the intestinal barrier function in patients with NCGS.

In module 12, the most enriched pathway was “inflammation mediated by chemokine and cytokine signaling”; this pathway was also enriched in modules 9, 10, and 11, which is consistent with inflammatory/autoimmune origin of NCGS.

Moreover, modules 1, 2, 7, and 10 were enriched in BPs related to viral infection including “viral process,” “viral gene expression,” “intracellular transport of virus,” and “regulation of defense response to virus.”

In addition, we observed that modules 10 and 11 showed enrichments in the gamma interferon pathways typically associated to the innate response to viruses [33].

Therefore, to further clarify the relationship between viral infections and NCGS, we searched in the IPA software database to find all diseases that are most likely to be statistically significantly associated to the genes modulated in the NCGS dataset. We found that, in the resulting list of most significantly associated diseases, “Infectious diseases” ranked first and, among these, “Viral infection” showed the best statistical p value (Figure 5(a)). Moreover, we could find a cluster of 134 DEGs that, in our NCGS dataset, showed a modulation that was consistent with a process of viral infection (Figure 5(b)). Based on these data, we aimed at investigating whether rotavirus, known to be linked to CD, [5, 6, 34] could also play a role in NCGS.

In the second part of our study, we made a comparison between the dataset obtained from our previous analysis of intestinal human T84 cells treated with anti-VP7 antibodies (that we indicate in this paper as “T84 dataset”) and genes modulated in NCGS. We found that 529 genes modulated in NCGS (accounting for the 41% of genes modulated in this dataset) were also modulated in treated T84 cells. Interestingly, several DEGs that were shared by the two datasets are involved in BP that may be related to the pathogenesis of celiac disease, including apoptosis, inflammatory and immune response, cell proliferation, cell differentiation, cell junctions, matrix metalloproteases, receptors and signal transducers, cytoskeleton components, ion transport and exchange, and EGF receptor pathway. Table 4 shows a selection of genes ascribed to the above-mentioned functional classes. As a whole in NCGS dataset, the modulation of genes ascribed to the abovementioned categories indicated an upregulation of apoptotic genes accompanied by a downregulation of genes involved in cell differentiation and an increased transcription of proliferative genes. All these observation are in agreement with what we described on human T84 cells treated with anti-rotavirus Vp7 peptide antibodies and are related to the typical features of celiac disease. Indeed in CD, an increased apoptosis is the main cause of villous atrophy that is also sustained by a dysregulation of cell differentiation [35]. Moreover, it has been observed that the increase of intestinal cell proliferation leads to crypt hyperplasia seen in celiac disease [35]. Other aspects of CD previously observed in our T84 treated cells, that are paralleled by the
Figure 2: Serum levels of selected soluble mediators in NCGS patients and in normal subject sera. The histograms represent the mean of the results obtained in 20 healthy donors and in 16 NCGS patients. \( p \) values calculated with the Wilcoxon nonparametric statistical test for paired samples were: \( p < 0.0001 \) for sCTLA-4, \( p < 0.001 \) for sPD-1, and \( p < 0.05 \) for sgp130.

Figure 3: Protein-protein interaction (PPI) network of DEGs in NCGS patients.
Figure 4: Modules originated from the network analysis of DEGs in NCGS patients.
| Biological processes | \( p \) value | Pathways | \( p \) value |
|----------------------|---------------|----------|---------------|
| **M0**               |               |          |               |
| Exocytosis           | <0.001        | None     |               |
| Secretion by cell    | <0.001        |          |               |
| Secretion            | <0.001        |          |               |
| Vesicle-mediated transport | 0.0018     |          |               |
| Single-organism transport | 0.0220    |          |               |
| Single-organism localization | 0.0308  |          |               |
| **M1**               |               |          |               |
| T cell receptor signaling pathway | <0.001     | T cell activation | <0.001 |
| Transmembrane receptor protein tyrosine kinase signaling pathway | <0.001     | B cell activation | 0.0012 |
| T cell costimulation | <0.001        | Cadherin signaling pathway | 0.0056 |
| Viral process        | <0.001        | Integrin signaling pathway | 0.0081 |
| Fc-gamma receptor signaling pathway involved in phagocytosis | <0.001     |          |               |
| Peptidyl-tyrosine modification | 0.0016      |          |               |
| Adaptive immune response | 0.0017      |          |               |
| Positive regulation of antigen receptor-mediated signaling pathway | 0.0029 |          |               |
| Positive regulation of alpha-beta T cell proliferation | 0.0038 |          |               |
| Phosphatidylinositol phosphorylation | 0.0060     |          |               |
| Phosphatidylinositol-mediated signaling | 0.0162     |          |               |
| Positive regulation of calcium-mediated signaling | 0.0192 |          |               |
| T cell selection     | 0.0244        |          |               |
| Leukocyte migration  | 0.0303        |          |               |
| Interleukin-2-mediated signaling pathway | 0.0324  |          |               |
| MAPK cascade         | 0.0371        |          |               |
| Positive regulation of immune effector process | 0.0466  |          |               |
| Positive regulation of defense response | 0.0485     |          |               |
| **M2**               |               |          |               |
| mRNA export from nucleus | <0.001     | None     |               |
| Spliceosomal complex assembly | <0.001   |          |               |
| Termination of RNA polymerase II transcription | <0.001    |          |               |
| Regulation of mRNA splicing, via spliceosome | <0.001  |          |               |
| Positive regulation of RNA splicing | <0.001     |          |               |
| mRNA 3′-end processing | <0.001      |          |               |
| Regulation of gene silencing by miRNA | <0.001    |          |               |
| tRNA export from nucleus | 0.0010     |          |               |
| Viral gene expression | 0.0054        |          |               |
| Intracellular transport of virus | 0.0078      |          |               |
| Protein sumoylation  | 0.0294        |          |               |
| Regulation of cellular response to heat | 0.0310     |          |               |
| Fibroblast growth factor receptor signaling pathway | 0.0414  |          |               |
| **M3**               |               |          |               |
| Positive regulation of T cell activation | 0.0035     | T cell activation | <0.001 |
| Interleukin-2-mediated signaling pathway | 0.0065     | Interleukin signaling pathway | <0.001 |
| Interleukin-4-mediated signaling pathway | 0.0065      | PDGF signaling pathway | 0.0010  |
| Protein phosphorylation | 0.0349     | Integrin signaling pathway | 0.0017  |
| Biological processes                                                                 | $p$ value | Pathways                                                                 | $p$ value |
|-------------------------------------------------------------------------------------|-----------|--------------------------------------------------------------------------|-----------|
| JAK/STAT signaling pathway                                                            | 0.0057    |                                                                           |           |
| Hypoxia response via HIF activation                                                   | 0.0110    |                                                                           |           |
| Insulin/IGF pathway-protein kinase B signaling cascade                                 | 0.0136    |                                                                           |           |
| p53 pathway feedback loops 2                                                          | 0.0176    |                                                                           |           |
| PI3 kinase pathway                                                                    | 0.0182    |                                                                           |           |
| VEGF signaling pathway                                                                | 0.0238    |                                                                           |           |
| Endothelin signaling pathway                                                          | 0.0284    |                                                                           |           |
| p53 pathway                                                                          | 0.0290    |                                                                           |           |
| M4                                   |            | Pospholipase C-activating G-protein-coupled receptor signaling pathway     | <0.001    |
| G-protein coupled acetylcholine receptor signaling pathway                            | <0.001    | Heterotrimeric G-protein signal. pathway-Gq α and Go α med. pathway       | <0.001    |
| Activation of phospholipase C activity                                               | <0.001    | PI3 kinase pathway                                                        | <0.001    |
| Positive regulation of cytosolic calcium ion concentration                            | <0.001    | Endothelin signaling pathway                                              | 0.0013    |
| Adenylyate cyclase-modulating G-protein-coupled receptor signaling pathway            | 0.0048    | Wnt signaling pathway                                                     | 0.0015    |
| M5                                   |            | Translational initiation                                                  | <0.001    |
| Nuclear-transcibed mRNA catabolic process, nonsense mediated decay                    | <0.001    | None                                                                     |           |
| SRP-dependent cotranslational protein targeting to membrane                           | <0.001    |                                                                           |           |
| rRNA processing                                                                      | <0.001    |                                                                           |           |
| Ribosomal small subunit assembly                                                      | 0.0083    |                                                                           |           |
| M6                                   |            | Regulation of small GTPase-mediated signal transduction                   | <0.001    |
| Positive regulation of GTPase activity                                               | <0.001    | None                                                                     |           |
| Small GTPase-mediated signal transduction                                             | <0.001    |                                                                           |           |
| Actin cytoskeleton organization                                                      | 0.0108    |                                                                           |           |
| M7                                   |            | T cell costimulation                                                      | <0.001    |
| Phosphatidylinositol-mediated signaling                                              | <0.001    | T cell activation                                                         | <0.001    |
| T cell receptor signaling pathway                                                     | <0.001    | Inteigrin signaling pathway                                               | 0.0041    |
| Phosphatidylinositol phosphorylation                                                 | <0.001    |                                                                           |           |
| Transmembrane receptor protein tyrosine kinase signaling pathway                     | 0.0016    |                                                                           |           |
| Peptidyl-tyrosine autophosphorylation                                                | 0.0033    |                                                                           |           |
| Viral process                                                                        | 0.0035    |                                                                           |           |
| Fc receptor signaling pathway                                                        | 0.0050    |                                                                           |           |
| Regulation of apoptotic process                                                      | 0.0055    |                                                                           |           |
| Leukocyte differentiation                                                             | 0.0122    |                                                                           |           |
| Leukocyte migration                                                                  | 0.0232    |                                                                           |           |
| Lymphocyte activation                                                                | 0.0237    |                                                                           |           |
| B cell receptor signaling pathway                                                     | 0.0256    |                                                                           |           |
| Positive regulation of defense response                                              | 0.0340    |                                                                           |           |
Table 3: Continued.

| Biological processes                                           | P value | Pathways                               | P value |
|----------------------------------------------------------------|---------|----------------------------------------|---------|
| *M8*                                                          |         |                                        |         |
| Response to unfolded protein                                   | <0.001  | None                                   |         |
| Response to topologically incorrect protein                    | <0.001  |                                        |         |
| Chaperone-mediated protein complex assembly                    | <0.001  |                                        |         |
| Protein folding                                                | <0.001  |                                        |         |
| Protein transmembrane transport                                | <0.001  |                                        |         |
| Response to stress                                             | <0.001  |                                        |         |
| *M9*                                                          |         |                                        |         |
| Activation of innate immune response                           | <0.001  | Toll-like receptor signaling pathway    | <0.001  |
| Positive regulation of innate immune response                  | <0.001  | Ras pathway                            | <0.001  |
| Toll-like receptor signaling pathway                           | <0.001  | Apoptosis signaling pathway             | <0.001  |
| Fc-epsilon receptor signaling pathway                          | 0.0020  | T cell activation                      | <0.001  |
| MAPK cascade                                                   | 0.0026  | p38 MAPK pathway                       | <0.001  |
| Positive regulation of type I interferon production            | 0.0029  | Oxidative stress response               | <0.001  |
| Positive regulation of cytokine production                    | 0.0035  | Angiogenesis                            | <0.001  |
| TRIF-dependent toll-like receptor signaling pathway            | 0.0136  | B cell activation                      | <0.001  |
| Positive regulation of interferon-beta production              | 0.0202  | FGF signaling pathway                   | <0.001  |
| Response to lipopolysaccharide                                 | 0.0268  | EGF receptor signaling pathway          | <0.001  |
| Type I interferon biosynthetic process                         | 0.0419  | Integrin signaling pathway              | 0.0024  |
| Inflammation mediated by chemokine and cytokine signaling path |         |                                        | 0.0079  |
| *M10*                                                         |         |                                        |         |
| T cell receptor signaling pathway                              | <0.001  | T cell activation                      | <0.001  |
| T cell costimulation                                           | <0.001  | EGF receptor signaling pathway          | <0.001  |
| Fc-epsilon receptor signaling pathway                          | <0.001  | Integrin signaling pathway              | <0.001  |
| Phosphatidylinositol phosphorylation                          | <0.001  | p38 pathway feedback loops 2           | <0.001  |
| Peptidyl-tyrosine autophosphorylation                         | <0.001  | VEGF signaling pathway                  | <0.001  |
| Fc-gamma receptor signaling pathway involved in phagocytosis   | <0.001  | B cell activation                      | <0.001  |
| Leukocyte migration                                            | <0.001  | Ras pathway                            | <0.001  |
| Growth hormone receptor signaling pathway                     | <0.001  | Angiogenesis                            | <0.001  |
| Regulation of defense response to virus                       | <0.001  |                                        |         |
| Innate immune response                                         | <0.001  |                                        |         |
| Positive regulation of MAP kinase activity                    | <0.001  | Inflammation mediated by chemokine and cytokine signaling pathway | <0.001  |
| T cell differentiation                                         | <0.001  |                                        |         |
| Regulation of apoptotic process                               | <0.001  | PI3 kinase pathway                      | <0.001  |
| JAK–STAT cascade                                              | 0.0011  | p53 pathway                            | <0.001  |
| Positive regulation of immune effector process                 | 0.0031  | Interferon-gamma signaling pathway      | <0.001  |
| MAPK cascade                                                   | 0.0056  | FGF signaling pathway                   | <0.001  |
| Adaptive immune response                                      | 0.0088  | Endothelin signaling pathway            | 0.0101  |
| B cell receptor signaling pathway                              | 0.0121  | JAK/STAT signaling pathway              | 0.0176  |
| Phosphatidylinositol 3-kinase signaling                        | 0.0214  |                                        |         |
| Stimulatory C-type lectin receptor signaling pathway           | 0.0363  |                                        |         |
| Innate immune response activ. cell surface receptor signal. pathway | 0.0387  |                                        |         |
| Biological processes | \( p \) value | Pathways                             | \( p \) value |
|----------------------|---------------|-------------------------------------|---------------|
| **M11**              |               |                                     |               |
| Cellular response to cytokine stimulus | \(<0.001\)    | JAK/STAT signaling pathway          | \(<0.001\)    |
| JAK–STAT cascade involved in growth hormone signaling pathway | \(<0.001\)    | Interleukin signaling pathway       | \(<0.001\)    |
| Positive regulation of cytokine production | \(<0.001\)    | PDGF signaling pathway              | \(<0.001\)    |
| Response to interleukin-2 | \(<0.001\)    | Interferon-gamma signaling pathway  | \(<0.001\)    |
| Positive regulation of T cell differentiation | \(<0.001\)    | EGF receptor signaling pathway      | \(<0.001\)    |
| Positive regulation of tyrosine phosphorylation of STAT protein | \(<0.001\)    | Integrin signaling pathway          | \(<0.001\)    |
| Regulation of interferon-gamma-mediated signaling pathway | \(<0.001\)    | Inflammation mediated by chemokine and cytokine signaling pathway | \(<0.001\)    |
| MAPK cascade         | \(<0.001\)    | p33 pathway feedback loops 2        | 0.0025        |
| Adaptive immune response | \(<0.001\)    | PI3 kinase pathway                  | 0.0027        |
| Innate immune response | 0.0014        | VEGF signaling pathway              | 0.0045        |
| Positive regulation of T cell proliferation | 0.0022        | B cell activation                   | 0.0045        |
| Positive regulation of inflammatory response | 0.0025        | Ras pathway                         | 0.0050        |
| Antigen receptor-mediated signaling pathway | 0.0072        | T cell activation                   | 0.0078        |
| T cell differentiation | 0.0085        | Cadherin signaling pathway          | 0.0201        |
| Inflammatory response | 0.0194        |                                      |               |
| Positive regulation of antigen receptor-mediated signaling pathway | 0.0227        |                                      |               |
| Transcription factor import into nucleus | 0.0313        |                                      |               |
| T cell costimulation | 0.0396        |                                      |               |
| **M12**              |               |                                     |               |
| G-protein-coupled receptor signaling pathway | \(<0.001\)    | Inflammation mediated by chemokine and cytokine signaling pathway | \(<0.001\)    |
| Chemokine-mediated signaling pathway | \(<0.001\)    | Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha-mediated pathway | 0.0473        |
| Positive regulation of cytosolic calcium ion concentration | \(<0.001\)    |                                      |               |
| Inflammatory response | \(<0.001\)    |                                      |               |
| Cell chemotaxis      | \(<0.001\)    |                                      |               |
| Positive regulation of neutrophil chemotaxis | 0.0136        |                                      |               |
| Response to lipopolysaccharide | 0.0268        |                                      |               |
| **M13**              |               |                                     |               |
| Adherens junction assembly | \(<0.001\)    | Integrin signaling pathway          | \(<0.001\)    |
| Phosphatidylinositol phosphorylation | 0.0015        | Cadherin signaling pathway          | \(<0.001\)    |
| Vesicle-mediated transport | 0.0026        |                                      |               |
| Positive regulation of protein localization to nucleus | 0.0043        |                                      |               |
| Actin cytoskeleton organization | 0.0105        |                                      |               |
| Cell differentiation | 0.0308        |                                      |               |
| **M14**              |               |                                     |               |
| Positive regulation of T cell activation | 0.0035        | T cell activation                   | \(<0.001\)    |
| Interleukin-2-mediated signaling pathway | 0.0065        | Integrin signaling pathway          | \(<0.001\)    |
| Interleukin-4-mediated signaling pathway | 0.0065        | Angiogenesis                        | 0.0032        |
| Regulation of immune response | 0.0430        | Toll like receptor signaling pathway | 0.0269        |
|                                      |                | VEGF signaling pathway              | 0.0387        |
|                                      |                | B cell activation                   | 0.0387        |
|                                      |                | Ras pathway                         | 0.0431        |
gene modulated observed in NCGS are the upregulation of members of the epidermal growth factor receptor (EGFR) signaling pathway and the concomitant downregulation of cell adhesion molecules beside a deregulation of ion transport. Noteworthy, the activation of EGFR signaling has been already observed in CD [36], and dysfunction of cell adhesion and transport are typical features of epithelial cells from active CD [37].

In this regard, it is worthwhile mentioning that patients with NCGS have normal to mildly inflamed mucosa (Marsh 0-1), while partial or subtotal villous atrophy and crypt hyperplasia are hallmarks of CD. Nevertheless, we cannot

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**Figure 5:** (a) List of diseases which are most likely to be statistically significantly associated and compatible with the transcriptional profile observed in NCGS. (b) DEGs in NCGS showing a modulation consistent with a viral infection process. (c) Detection of antibodies directed against the rotavirus VP7 peptide in the sera of patients with NCGS. Each circle represents a measurement for one patient, and the dashed horizontal line indicates the threshold for positivity (O.D. 0.140). The statistical p value was calculated with the Mann–Whitney test (p < 0.0001).
| Gene symbol | Accession number | Gene title | FC NCGS PBCs | FC T84 treated cells |
|-------------|-----------------|------------|--------------|----------------------|
| **Apoptosis** | | | | |
| SOCS3 | NM_003955 | Suppressor of cytokine signaling 3 | 1.83 | 2.75 |
| ANXA6 | NM_001155 | Annexin A6 | 1.57 | 2.72 |
| SOS2 | NM_006939 | Son of sevenless homolog 2 (Drosophila) | 1.90 | 1.75 |
| DEDD | AF064605 | Death effector domain containing | 1.78 | 1.47 |
| **Immune response** | | | | |
| IFNA17 | NM_021268 | Interferon, alpha 17 | 1.59 | 1.56 |
| IL6R | S72848 | Interleukin 6 receptor | 1.79 | 2.76 |
| IRF5 | NM_03264335 | Interferon regulatory factor 5 | 1.52 | 1.52 |
| CD84 | AF054818 | CD84 molecule | 2.55 | 3.40 |
| **Inflammatory response** | | | | |
| IL1B | NM_000576 | Interleukin 1, beta | 1.52 | 1.80 |
| IL24 | NM_006850 | Interleukin 24 | 2.84 | 2.19 |
| IL2RA | K03122 | Interleukin 2 receptor, alpha | 1.86 | 1.48 |
| S100A8 | AW238654 | S100 calcium-binding protein A8 | 3.65 | 1.86 |
| **Cell proliferation** | | | | |
| FGFR2 | NM_022975 | Fibroblast growth factor receptor 2 | 1.56 | 2.89 |
| RAC2 | NM_002872 | Ras-related C3 botulinum toxin substrate 2 | 2.31 | 1.53 |
| CDK2 | AB012305 | Cyclin-dependent kinase 2 | 1.63 | 1.78 |
| DLG1 | AL121981 | Discs, large homolog 1 (Drosophila) | 1.57 | 1.74 |
| **Cell differentiation** | | | | |
| GAS7 | BC006454 | Growth arrest-specific 7 | −2.03 | −1.90 |
| SRD5A1 | NM_001047 | Steroid-5-alpha-reductase, alpha polypeptide 1 | −2.53 | −1.54 |
| VAMP5 | NM_006634 | Vesicle-associated membrane protein 5 | −1.71 | −1.58 |
| ZAK | NM_016653 | Sterile alpha motif and leucine zipper containing kinase AZK | −2.02 | −1.71 |
| **Cell–cell junctions** | | | | |
| VCL | NM_014000 | Vinculin | −1.68 | −1.56 |
| CTNND1 | NM_001331 | Catenin (cadherin-associated protein), delta 1 | −2.33 | −1.75 |
| CTNNA1 | NM_001903 | Catenin (cadherin-associated protein), alpha 1, 102 kDa | −2.49 | −1.57 |
| COL8A2 | NM_005202 | Collagen, type VIII, alpha 2 | −1.62 | −1.64 |
| **Metalloproteases** | | | | |
| ADAM8 | AI814527 | ADAM metallopeptidase domain 8 | 1.94 | 1.57 |
| ADAM9 | NM_003816 | ADAM metallopeptidase domain 9 | 2.81 | 1.48 |
| ADAM17 | AI797833 | ADAM metallopeptidase domain 17 | 1.51 | 1.56 |
| **Receptors and signal transduction** | | | | |
| IL2RA | K03122 | Interleukin 2 receptor, alpha | 1.86 | 1.48 |
| IRF5 | NM_03264335 | Interferon regulatory factor 5 | 1.52 | 1.52 |
| IL6R | S72848 | Interleukin 6 receptor | 1.79 | 2.76 |
| **Cytoskeleton** | | | | |
| FGD6 | NM_008351 | FYVE, RhoGEF, and pH domain containing 6 | −2.40 | −1.48 |
| ABLIM3 | NM_014945 | Actin-binding LIM protein family, member 3 | 1.86 | 1.49 |
| PFN2 | NM_002628 | Profilin 2 | 1.51 | 1.47 |
| **Ion transport** | | | | |
| SLC2A4 | AF096132 | Solute carrier family 24 (Na/K/Ca exchanger), member 1 | 1.59 | 1.95 |
| SLC30A1 | AI972416 | Solute carrier family 30 (zinc transporter), member 1 | 1.94 | 1.55 |
| SLC4A4 | AF069510 | Solute carrier family 4, NaHCO 3 cotransporter, member 4 | 1.52 | 1.92 |
| **EGFR signaling pathway** | | | | |
| AKT2 | NM_001626 | v-akt murine thymoma viral oncogene homolog 2 | 2.36 | 2.19 |
| PIK3R1 | NM_181523 | Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) | 2.76 | 1.54 |
| PTPN12 | S69182 | Protein tyrosine phosphatase, nonreceptor type 12 | 2.27 | 1.50 |
Table 5: Genes modulated in the three datasets playing a role in selected GO BPs related to the viral infection process.

| Dataset       | GO BP                        | Gene symbol | Gene title                                              | FC   |
|---------------|------------------------------|-------------|---------------------------------------------------------|------|
| NCGS dataset  | Viral transcription/gene expression | RANBP2      | RAN-binding protein 2                                   | 1.98 |
|               |                              | RPL27A      | Ribosomal protein L27a                                   | 3.22 |
|               |                              | RPL37A      | Ribosomal protein L37a                                   | 2.94 |
|               |                              | RPLP2       | Ribosomal protein, large, P2                            | 2.15 |
|               |                              | RPS10       | Ribosomal protein S10                                    | 2.66 |
|               |                              | RPS11       | Ribosomal protein S11                                    | 2.49 |
|               |                              | TPR         | Translocated promoter region, nuclear basket protein     | 4.40 |
|               | Response to virus            | RELA        | v-rel reticuloendotheliosis viral oncogene homolog A (avian) | 1.54 |
|               |                              | IKBKB       | Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta | 2.69 |
|               |                              | IRF5        | Interferon regulatory factor 5                           | 1.52 |
|               |                              | IFNA17      | Interferon, alpha 16                                     | 1.59 |
|               |                              | DDX3X       | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked       | 3.22 |
|               |                              | STAT2       | Signal transducer and activator of transcription 2, 113 kDa | 1.59 |
|               |                              | STAT1       | Signal transducer and activator of transcription 1, 91 kDa | 2.73 |
|               |                              | IRF3        | Interferon regulatory factor 3                           | 1.67 |
|               |                              | DDX17       | DEAD (Asp-Glu-Ala-Asp) box helicase 17                   | 4.75 |
|               | Viral life cycle             | TPR         | Translocated promoter region, nuclear basket protein     | 4.40 |
|               |                              | ATG16L1     | Autophagy-related 16-like 1 (S. cerevisiae)               | 1.87 |
|               |                              | HSP90AB1    | Heat shock protein 90 kDa alpha (cytosolic), class B member 1 | 1.87 |
|               |                              | RANBP2      | RAN-binding protein 2                                    | 1.98 |
|               |                              | DPP4        | Dipeptidyl-peptidase 4                                   | 1.61 |
|               |                              | DDX6        | DEAD (Asp-Glu-Ala-Asp) box helicase 6                    | 4.70 |
|               |                              | HTATSF1     | HIV-1 Tat specific factor 1                              | 2.23 |
|               |                              | SLAMF1      | Signaling lymphocytic activation molecule family member 1 | 1.65 |
| T84 dataset   | Viral transcription/gene expression | RPL27A      | Ribosomal protein L27a                                   | 1.68 |
|               |                              | RPS2        | Ribosomal protein S2                                     | 1.99 |
|               |                              | RPS6        | Ribosomal protein S6                                     | 1.51 |
|               | Response to virus            | IFIH1       | Interferon induced with helicase C domain 1              | 1.52 |
|               |                              | IFNA7       | Interferon, alpha 7                                      | 1.53 |
|               |                              | IFIT3       | Interferon-induced protein with tetratricopeptide repeats 3 | 1.46 |
|               |                              | IFNA4       | Interferon, alpha 4                                      | 1.73 |
|               |                              | IFI44       | Interferon-induced protein 44                            | 1.46 |
|               |                              | IFNGR1      | Interferon gamma receptor 1                              | 1.67 |
|               |                              | IFNA17      | Interferon, alpha 17                                     | 1.56 |
|               | Viral life cycle             | CTBP1       | C-terminal-binding protein 1                             | 1.58 |
|               |                              | ADRBK1      | Adrenergic, beta, receptor kinase 1                      | 1.46 |
|               |                              | HCRP1       | Hepatocellular carcinoma-related HCRP1                   | 1.61 |
|               |                              | C9orf28     | Chromosome 9 open reading frame 28                       | 1.56 |
exclude that some NCGS patients, especially those positive for HLA-DQ2 and/or DQ8, may switch to classical CD in the course of the follow-up.

Since a large part of DEGs in the NCGS paralleled the modulation of genes seen in human T84 cells treated with antivotavirus Vp7 peptide antibodies, we next aimed at identifying the presence of such antibodies in sera of NCGS patients. We therefore tested in ELISA assay the sera from 16 NCGS patients and 20 healthy subjects for the detection of antirotavirus VP7 peptide antibodies. We found that these antibodies were present in 6 out of 16 (37%) NCGS patients while were not detected in the sera of healthy subjects. Figure 5(c) shows that the levels of such antibodies are significantly different in the two set of tested samples (p < 0.0001). The detection of these antibodies in NCGS patients may be relevant to the pathogenesis of the NCGS given their ability to modulate sets of genes in intestinal epithelial cells as we previously demonstrated [6].

Taken together, the modulation of highly connected genes associated to the viral infection process and the presence of anti-VP7 antibodies in the sera of some NCGS patients may suggest that a link also exists between immune response to rotavirus infection and NCGS.

In this perspective, since anti-VP7 rotavirus antibodies are often present before the onset of CD, preceding the detection of celiac specific autoantibodies, [6] it is tempting to speculate that NCGS patients with CD genetic predisposition (DQ2/DQ8) and presence of anti-VP7 antibodies may develop CD in the course of the follow-up.

Therefore, to further clarify the relationship between rotavirus infection and NCGS, we decided to perform an integrative bioinformatics analysis using the dataset GSE50628 downloaded from GEO (Gene Expression Omnibus) database (http://www.ncbi.nlm.nih.gov/geo/) that included samples of peripheral blood cells from patients affected by acute rotavirus infection (named in the paper “Rotavirus infection dataset”). This dataset was analysed to detect significantly modulated genes (Additional Table 2), and a comprehensive GO analysis was carried out on all datasets including NCGS, Rotavirus infection, and T84 datasets that we analysed in our previous work [6].

We then searched on the four datasets for the presence of genes associated to GO terms containing the words “virus” and/or “viral” and we found in all datasets a great number of such terms to which modulated genes were connected/related.

The searched terms explored a wide range of biological processes associated to viral infection from the entry of virus in the host cell, viral transcription and gene expression, modulation of host physiology by virus to cellular response to virus.

All the GO terms retrieved in the three datasets are listed in Additional Table 3.

| Gene symbol | Gene title | FC |
|-------------|------------|----|
| Rotavirus infection dataset | **Viral transcription/gene expression** | |
| NUP58 | Nucleoporin 58 kDa | 6.38 |
| RPS16 | Ribosomal protein S16 | 2.10 |
| DENR | Density-regulated protein | 2.11 |
| **Response to virus** | | |
| XPR1 | Xenotropic and polytropic retrovirus receptor 1 | 1.72 |
| CNOT7 | CCR4-NOT transcription complex subunit 7 | 3.54 |
| CD40 | CD40 molecule, TNF receptor superfamily member 5 | 2.72 |
| ITCH | Itch E3 ubiquitin protein ligase | 2.26 |
| ARF1 | ADP-ribosylation factor 1 | 1.91 |
| BCL2L11 | BCL2-like 11 (apoptosis facilitator) | 3.21 |
| BCL2L1 | BCL2-like 1 | 3.37 |
| IKBKE | Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase ε | 1.50 |
| DDX17 | DEAD (Asp-Glu-Ala-Asp) box helicase 17 | 2.13 |
| **Viral life cycle** | | |
| NUP153 | Nucleoporin 153 kDa | 2.01 |
| VPS37A | Vacuolar protein sorting 37 homolog A (S. cerevisiae) | 1.90 |
| XPR1 | Xenotropic and polytropic retrovirus receptor 1 | 1.72 |
| UBB | Ubiquitin B | 1.75 |
| ITCH | Itch E3 ubiquitin protein ligase | 2.26 |
| NUP58 | Nucleoporin 58 kDa | 6.38 |
| TNFRSF4 | Tumor necrosis factor receptor superfamily, member 4 | 1.94 |
| SCARB2 | Scavenger receptor class B, member 2 | 1.96 |
Table 6: Selection of genes modulated in human T84 cells after stimulation with anti-VP7 rotavirus peptide antibodies, involved in immune response and in molecular signaling related to the viral infection process.

| Gene symbol | Gene title | FC |
|-------------|------------|----|
| **Immune response** | | |
| CCR2 | Chemokine (C-C motif) receptor 2 | −1.48 |
| CXCL1 | Chemokine (C-X-C motif) ligand 1 | 1.81 |
| CXCL13 | Chemokine (C-X-C motif) ligand 13 | −5.52 |
| GATA3 | GATA-binding protein 3 | −6.62 |
| TROVE2 | TROVE domain family, member 2 | −1.64 |
| ICOSLG | Inducible T cell costimulator ligand | 2.51 |
| FCGR1A | Fc fragment of IgG, high affinity Ia, receptor (CD64) | 2.00 |
| FOXP3 | Forkhead box P3 | 1.49 |
| ULBP1 | UL16-binding protein 1 | −1.77 |
| ITGA4 | Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor) | 1.48 |
| CXCL9 | Chemokine (C-X-C motif) ligand 9 | 1.59 |
| CSF3 | Colony-stimulating factor 3 (granulocyte) | 1.46 |
| IL6 | Interleukin 6 (interferon, beta 2) | 1.51 |
| CD84 | CD84 molecule | 3.40 |
| FCGR2B | Fc fragment of IgG, low affinity IIb, receptor (CD32) | 1.77 |
| LAT2 | Linker for activation of T cells family, member 2 | 1.85 |
| C7 | Complement component 7 | 3.11 |
| CCR1 | Chemokine (C-C motif) receptor 1 | 3.27 |
| CCR3 | Chemokine (C-C motif) receptor 3 | 2.80 |
| CFP | Complement factor properdin | 2.92 |
| IL24 | Interleukin 24 | 2.19 |
| IL8 | Interleukin 8 | 1.86 |
| CXCL10 | Chemokine (C-X-C motif) ligand 10 | 1.82 |
| IL1F7 | Interleukin 1 family, member 7 (zeta) | −2.26 |
| IKBKB | Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta | −2.25 |
| CCL11 | Chemokine (C-C motif) ligand 11 | 1.96 |
| **Type I interferon signaling** | | |
| FCAR | Fc fragment of IgA, receptor for | 2.15 |
| **Type I interferon signaling** | | |
| IFNA16 | Interferon, alpha 16 | 1.58 |
| STAT1 | Signal transducer and activator of transcription 1, 91 kDa | −1.46 |
| IFNA17 | Interferon, alpha 17 | 1.56 |
| YY1 | YY1 transcription factor | −2.24 |
| IFNA4 | Interferon, alpha 4 | 1.73 |
| IRF8 | Interferon regulatory factor 8 | 1.68 |
| IFNA5 | Interferon, alpha 5 | −2.85 |
| IRF2 | Interferon regulatory factor 2 | 1.58 |
| IFNA8 | Interferon, alpha 8 | 2.23 |
| IRF5 | Interferon regulatory factor 5 | 1.52 |
| IFI6 | Interferon, alpha-inducible protein 6 | 1.56 |
| IFNA6 | Interferon, alpha 6 | 2.08 |
| **Positive regulation of interferon alpha production** | | |
| IRF5 | Interferon regulatory factor 5 | 1.52 |
| **Positive regulation of interferon beta production** | | |
| DDX3X | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked | −1.49 |
| IRF5 | Interferon regulatory factor 5 | 1.52 |
| **Negative regulation of interferon beta production** | | |
| LILRB1 | Leukocyte immunoglobulin-like receptor, subfamily B, member 1 | −1.60 |
| Gene symbol | Gene title                                                                 | FC  |
|-------------|-----------------------------------------------------------------------------|-----|
| **Positive regulation of Type I interferon production** |                                                                             |     |
| IFI16       | Interferon, gamma-inducible protein 16                                      | −1.68|
| CREBBP      | CREB-binding protein (Rubinstein-Taybi syndrome)                             | 1.51 |
| **Negative regulation of Type I interferon production** |                                                                             |     |
| CYLD        | Cylindromatosis (turban tumor syndrome)                                     | −3.04|
| **Gamma interferon signaling** |                                                                             |     |
| **Cellular response to Interferon Gamma signaling** |                                                                             |     |
| FCAR        | Fc fragment of IgA, receptor for                                             | 2.15 |
| MRC1        | Mannose receptor, C type 1                                                  | 2.52 |
| SYNCRIP     | Synaptotagmin-binding, cytoplasmic RNA-interacting protein                  | −1.69|
| CCL8        | Chemokine (C-C motif) ligand 8                                              | 1.63 |
| **Interferon gamma signaling** |                                                                             |     |
| STAT1       | Signal transducer and activator of transcription 1, 91 kDa                 | −1.46|
| MID1        | Midline 1 (Opitz/BBB syndrome)                                              | −1.99|
| HLA-DRB4    | Major histocompatibility complex class II, DR beta 4                        | 2.39 |
| SDK1        | Sidekick homolog 1 (chicken)                                                | 1.61 |
| IFNGR1      | Interferon gamma receptor 1 interferon gamma receptor 1                    | 1.67 |
| **Negative regulation of gamma interferon production** |                                                                             |     |
| LILRB1      | Leukocyte immunoglobulin-like receptor, subfamily B, member 1              | −1.60|
| CD244       | CD244 molecule, natural killer cell receptor 2B4                             | −1.69|
| IL10        | Interleukin 10                                                              | −3.56|
| **Positive regulation of gamma interferon production** |                                                                             |     |
| FOXP3       | Forkhead box P3                                                             | 1.49 |
| IL1B        | Interleukin 1, beta                                                         | 1.80 |
| **Toll-like receptor signaling** |                                                                             |     |
| TANK        | TRAF family member-associated NFKB activator                                | −1.91|
| CHUK        | Conserved helix-loop-helix ubiquituous kinase                               | −1.72|
| ELK1        | ELK1, member of ETS oncogene family                                        | 3.70 |
| MAP3K8      | Mitogen-activated protein kinase kinase kinase 8                            | −2.16|
| TLR6        | Toll-like receptor 6                                                         | 2.43 |
| TLR1        | Toll-like receptor 1                                                         | 1.57 |
| TLR7        | Toll-like receptor 7                                                         | −1.64|
| MAP3K7      | Mitogen-activated protein kinase kinase kinase 7                            | −1.89|
| LY96        | Lymphocyte antigen 96                                                       | −1.81|
| NFKB2       | Nuclear factor of kappa light polypeptide gene enhancer in B cells 2 (p49/p100) | 1.54 |
| REL         | v-rel reticuloendotheliosis viral oncogene homolog (avian)                  | −1.82|
| PTGS2       | Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | 1.76 |
| TNFAIP3     | Tumor necrosis factor, alpha-induced protein 3                              | 1.73 |
| MAP2K3      | Mitogen-activated protein kinase kinase 3                                   | 1.59 |
| IKBKB       | Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta  | −2.25|
| TLR3        | Toll-like receptor 3                                                         | −2.03|
| IFNB1       | Interferon, beta 1, fibroblast                                               | −1.84|
| IRAK3       | Interleukin-1 receptor-associated kinase 3                                   | 1.70 |
| TLR4        | Toll-like receptor 4                                                         | 1.46 |
| IKBKE       | Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase epsilon | 2.06 |
| MAP2K2      | Mitogen-activated protein kinase kinase 2                                   | 1.98 |
| TLR2        | Toll-like receptor 2                                                         | −2.30|
Table 7: Selection of genes modulated in PBCs in course of the acute phase of rotavirus infection, involved in immune response and in molecular signaling related to the viral infection process.

| Gene symbol | Gene title                                                                 | FC   |
|-------------|-----------------------------------------------------------------------------|------|
| **Immune response** |                                |      |
| ADGRE3      | Adhesion G protein-coupled receptor E3; ADGRE3 ortholog                     | −3.35|
| ADIPOQ      | Adiponectin, C1Q and collagen domain containing                            | −1.60|
| BLK         | BLK proto-oncogene, Src family tyrosine kinase                              | 1.72 |
| BRAF        | B-Raf proto-oncogene, serine/threonine kinase                              | 1.57 |
| BTK         | Bruton agammaglobulinemia tyrosine kinase                                   | −1.59|
| C1QTNF9     | C1q and tumor necrosis factor related protein 9                             | −1.69|
| CD109       | CD109 molecule                                                              | −1.84|
| CD79B       | CD79b molecule, immunoglobulin-associated beta                             | −1.67|
| CLEC7A      | C-type lectin domain family 7, member A                                     | −1.62|
| CMIP         | c-Maf inducing protein                                                      | −3.50|
| CSF2RA      | Colony-stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophase) | −2.30|
| CXCL2       | Chemokine (C-X-C motif) ligand 2                                            | −3.76|
| CXCL8       | Chemokine (C-X-C motif) ligand 8                                            | −8.65|
| FCER1A      | Fc fragment of IgE, high affinity I, receptor for, alpha polypeptide        | −5.64|
| HIST         | Hematopoietic cell signal transducer                                        | 1.85 |
| IL18BP      | Interleukin 18 binding protein                                              | 1.56 |
| JAG1        | Jagged 1                                                                   | −2.06|
| KLRB1       | Killer cell lectin-like receptor subfamily B, member 1                      | −5.99|
| MAP3K11     | Mitogen-activated protein kinase kinase kinase 11                           | 1.55 |
| MAP1        | Mannan-binding lectin serine peptidase 1                                   | −1.50|
| MIR1        | Major histocompatibility complex, class I-related                          | 8.43 |
| PLEKHN1     | Pleckstrin homology domain containing, family N member 1                    | −1.99|
| PPP2R2C     | Protein phosphatase 2, regulatory subunit B, gamma                          | −1.84|
| PPP3CA      | Protein phosphatase 3, catalytic subunit, alpha isozyme                    | −1.89|
| PSME3       | Proteasome activator subunit 3                                             | 1.91 |
| PVR         | Poliovirus receptor                                                         | −1.59|
| STAT5B      | Signal transducer and activator of transcription 5B                        | −2.06|
| TNFRSF10C   | Tumor necrosis factor receptor superfamily, member 10c decay without an intracellular domain | −1.75|
| TNFRSF4     | Tumor necrosis factor receptor superfamily, member 4                        | 1.94 |
| **Type I interferon signaling** |                                |      |
| Positive regulation of Type I interferon production |                                 |      |
| EP300       | E1A-binding protein p300                                                     | −1.56|
| POLR3G      | Polymerase (RNA) III (DNA directed) polypeptide G (32kD)                    | −1.98|
| CREBBP      | CREB-binding protein                                                        | −1.83|
| LRRFIP1     | Leucine rich repeat (in FLII) interacting protein 1                         | −2.14|
| SOCS1       | Suppressor of cytokine signaling 1                                          | 2.32 |
| Negative regulation of Type I interferon production |                                |      |
| UBB         | Ubiquitin B                                                                 | 1.75 |
| ITCH        | Itchy E3 ubiquitin protein ligase                                            | 2.26 |
| TAX1BP1     | Tax1 (human T cell leukemia virus type I) binding protein 1                 | −4.01|
| Negative regulation of Type I interferon pathway |                                |      |
| PTPN2       | Protein tyrosine phosphatase, nonreceptor type 2                            | 2.10 |
| Positive regulation of interferon beta production |                                |      |
| ZBTB20      | Zinc finger and BTB domain containing 20                                    | 4.27 |
| Negative regulation of interferon Beta production |                                |      |
| PTPRS       | Protein tyrosine phosphatase, receptor type, S                              | −2.00|
| CACTIN      | Cactin, spliceosome C complex subunit                                       | −2.53|
Table 5 shows selected genes modulated in the three datasets that are ascribed to the most representative GO terms, including viral transcription, viral gene expression, response to virus, viral genome replication, and viral life cycle. Moreover, the GO analysis of the abovementioned datasets was complemented by searching for transcripts involved in immune response.

In the “T84 dataset,” we found upregulation for the T cell costimulatory molecule ICOSLG, the transcriptional...
regulator that is crucial for the development and inhibitory function of regulatory T cells, [38] interleukin-6 that is pivotal for the development of Th-17 cells [39], and FCGR2B that is involved in the phagocytosis of immune complexes and in modulation of antibody production by B cells [40] (Table 6).

In the “Rotavirus infection” dataset, we found upregulation for molecules that are crucial in the immune response including the BLK gene, involved in transmitting signals through surface immunoglobulins, supporting the pro-B to pre-B transition, [41] MR1/MAIT playing a role in the development of the mucosal-associated invariant T cells (MAIT), [42] TNFRSF4 involved in T cell proliferation [43], and HCST/DAP10 playing a role in triggering cytotoxicity against both stressed and infected by virus target cells [44] (Table 7).

Interestingly, in all the datasets, we found the presence of modulated genes involved in the type I interferon signaling, that is central in autoimmunity, and in molecular pathways involved in the immune response to viral infection, including the Toll-like receptors, and the type I and gamma interferon pathways (see Tables 2, 6, and 7).

Taken together, our data seem to indicate that NCGS has features of autoimmunity and that an immune response to rotavirus may play a role in some cases of NCGS.

4. Conclusions

NCGS is an emerging new clinical entity lacking specific diagnostic biomarkers which has been reported to occur in 6–10% of the population. Interestingly, up to 50% of these patients carry HLA-DQ2 and/or HLA-DQ8 genes. NCGS patients may complain gastrointestinal (e.g., diarrhea/constipation, abdominal pain, bloating) and/or extraintestinal symptoms (“foggy mind,” headache, dermatitis, etc.) which recede with GFD. The pathogenesis of NCGS is still unclear and the data, so far obtained, suggest a predominant activation of the innate immune responses.

Our data indicate a concomitant involvement of the adaptive immune system and suggest that NCGS may have an autoimmune origin. This is based both on gene expression data (i.e., TH17-IFNA I signatures) and on the presence of TH17 cells and of serological markers of autoimmunity in NCGS. Our results also indicate a possible involvement of rotavirus infection in the pathogenesis of NCGS, similarly to what we have previously shown in CD.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors’ Contributions

Antonio Puccetti, Daniele Saverino, Roberta Opri, Claudio Lunardi, and Marzia Dolcino equally contributed to this paper.

Supplementary Materials

Supplementary 1. Additional Table 1: genes modulated in NGCS samples versus healthy controls.

Supplementary 2. Additional Table 2: genes modulated in the “Rotavirus infection” dataset.

Supplementary 3. Additional Table 3: GO terms containing the words “virus” and “viral” to which are associated genes modulated in the three datasets.

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