Basic Study

May the assessment of baseline mucosal molecular pattern predict the development of gluten related disorders among microscopic enteritis?

Giuseppe Losurdo, Floriana Giorgio, Domenico Piscitelli, Lucia Montenegro, Claudia Covelli, Maria Grazia Fiore, Antonio Giangaspero, Andrea Iannone, Mariabeatrice Principi, Annacinzia Amoruso, Michele Barone, Alfredo Di Leo, Enzo Ierardi

Giuseppe Losurdo, Floriana Giorgio, Lucia Montenegro, Antonio Giangaspero, Andrea Iannone, Mariabeatrice Principi, Annacinzia Amoruso, Michele Barone, Alfredo Di Leo, Enzo Ierardi, Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, 70124 Bari, Italy

Domenico Piscitelli, Claudia Covelli, Maria Grazia Fiore, Section of Pathology, Department of Emergency and Organ Transplantation, University of Bari, 70124 Bari, Italy

Author contributions: Principi M, Barone M, Di Leo A and Ierardi E conceived the study; Losurdo G, Montenegro L, Giangaspero A, Iannone A and Amoruso A collected the data; Piscitelli D, Covelli C and Fiore MG performed immunohistochemistry and pathological evaluations; Giorgio F performed molecular analysis; Losurdo G and Giorgio F performed statistical analysis; Principi M performed endoscopy; Losurdo G and Ierardi E wrote the manuscript; all authors read and approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved after two meetings of all the authors (all affiliated to the same Department) before and after immune-histochemical and molecular analysis.

Institutional animal care and use committee statement: This study was not performed on experimental animals.

Informed consent statement: All study participants provided informed written consent prior to endoscopic investigation. Additional oral consent to perform immunohistochemistry and molecular analysis was obtained. No ethical committee approval was required because all invasive procedures had been performed according to the current clinical patient management.

Conflict-of-interest statement: No conflict of interest is declared by authors.

Data sharing statement: No additional data are available.

Moreover, the presented data are anonymized and risk of identification is low.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Manuscript source: Invited manuscript

Correspondence to: Enzo Ierardi, Professor, Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Piazza Giulio Cesare 11, 1, 70124 Bari, Italy. ierardi.enzo@gmail.com
Telephone: +39-805-594034
Fax: +39-805-593088

Received: May 5, 2016
Peer-review started: May 6, 2016
First decision: June 20, 2016
Revised: June 30, 2016
Accepted: July 21, 2016
Article in press: July 21, 2016
Published online: September 21, 2016

Abstract

AIM
To evaluate mucosal baseline mRNA expression of tissue transglutaminase 2 (tTG2), interferon gamma (IFNγ), toll-like receptor 2 (TLR2) and Myeloid Differentiation factor 88 (MyD88) in patients with microscopic enteritis (ME).
METHODOLOGY

We retrospectively enrolled 89 patients with ME of different etiology, which was defined within a 2-year mean period of follow-up. Baseline histological examination was performed on Hematoxylin-Eosin stained sections and CD3 lymphocyte immunohistochemistry was used for intraepithelial lymphocyte count (IELs). ME was defined according to the criteria of Bucharest Consensus Conference. For each patient, formalin-embedded biopsy samples of the duodenum referred to the period of ME diagnosis were retrieved. Real-time polymerase chain reaction (RT-PCR) was used to detect the amount of mRNA coding for tTG2, IFNγ, TLR2 and MyD88, and the expression was counted as fold change compared to controls. Control group was represented by duodenal normal specimens from 15 healthy subjects undergoing endoscopy for functional symptoms. Comparisons among continuous variables were performed by One way analysis of variance (ANOVA) and Bonferroni’s test. The χ2 test was used for categorical variables. Pearson’s test was used to evaluate correlations. Receiver operating curves were drawn for all four markers to estimate sensitivity and specificity in discriminating the development of CD and GS.

RESULTS

After a period of follow up of 21.7 ± 11.7 mo, the following diagnoses were achieved: gluten related disorders in 48 subjects (31 CD; 17 GS) and non-gluten related ones in 41 (29 Irritable Bowel Syndrome - IBS; 12 Others). CD patients had the highest tTG2 levels (8.3 ± 4.5). The ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative controls. A cut off value of 2.258 was able to discriminate between CD and GS with a sensitivity of 52.94% and a specificity of 87.1%. Additionally, CD patients had the highest IFNγ levels (8.5 ± 4.1). ANOVA plus Bonferroni demonstrated CD > Other ME > GS = IBS > negative controls. A cut off of 1.853 was able to differentiate CD and GS with a sensitivity of 47.06% and a specificity of 96.77%. Patients with non-gluten-related causes of ME exhibited the highest TLR2 levels (6.1 ± 1.9) as follows: Other ME > CD = GS = IBS > negative controls. TLR2 was unable to discriminate CD from GS. Patients with CD overexpressed MyD88 levels similarly to non-gluten-related causes of DL (7.8 ± 4.9 and 6.7 ± 2.9), thus CD = Other ME > GS = IBS > negative controls. A cut off of 3.722 was able to differentiate CD from GS with a sensitivity of 52.94% and a specificity of 74.19%. IELs count (15-25 and more than 25/100 enterocytes) strongly correlated with mRNA levels of all tested molecules (P < 0.0001).

CONCLUSION

Our results confirm that a single marker is unable to predict a discrimination among ME underlying conditions as well as between CD and GS. Mucosal high levels of tTG and IFNγ mRNA may predict the development of CD more than GS with high specificity, despite an expected low sensitivity. TLR2 does not discriminate the development of CD from GS. MyD88 levels indicate that intestinal permeability is more increased when a severe intestinal damage underlies ME in both gluten related and unrelated conditions. Therefore, the results of the present paper do not seem to show a clear translational value.

Key words: Celiac disease; MyD88; Microscopic enteritis; Gluten sensitivity; Tissue transglutaminase; Interferon gamma; Toll-like receptor 2

© The Author(s) 2016. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Microscopic enteritis (ME) is an inflammatory condition, which is characterized by increased intraepithelial CD3 lymphocytes in the duodenum and can be due to both gluten and non-gluten related diseases. It is often difficult to achieve a final diagnosis in cases of ME, therefore the assessment of baseline mucosal molecular pattern may be helpful. In this study, we demonstrated that tissue transglutaminase and interferon gamma may predict the development of Celiac Disease more than Gluten Sensitivity with high specificity, despite an expected low sensitivity.

Losurdo G, Giorgio F, Piscitelli D, Montenegro L, Covelli C, Fiore MG, Giangaspero A, Iannone A, Principi M, Amoruso A, Barone M, Di Leo A, Ierardi E. May the assessment of baseline molecular pattern predict the development of gluten related disorders among microscopic enteritis? World J Gastroenterol 2016; 22(35): 8017-8025 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i35/8017.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i35.8017

INTRODUCTION

Duodenal lymphocytosis (DL) is a condition characterized by pathologic infiltration of lymphocytes in the epithelium (IELs) of duodenal mucosa[1]. It is not a single entity, since several conditions may underlie this picture. The main associated disorders may be gluten related (Celiac disease - CD; Non celiac gluten sensitivity - NCGS; and Wheat allergy - WA) and non-gluten related (Irritable bowel syndrome - IBS; infectious or parasitic diseases; autoimmune disorders: vasculitides, connective tissue diseases and inflammatory bowel disease; immunoglobulin deficiencies; drug damage)[9,10]. Therefore, DL may be considered as an "umbrella term" rather than a single entity. Recently, the Bucharest Consensus has proposed “Microscopic enteritis (ME)” as an alternative term to DL, and has reviewed and standardized an algorithm for its diagnosis and treatment[9,10].

In the ME scenario, it is well known that CD is an autoimmune enteropathy triggered by the ingestion of gluten and represents the most common cause of...
intestinal malabsorption and villous atrophy\textsuperscript{[11]}, NCGS [or simply gluten sensitivity (GS)] is a disorder showing intestinal and extra-intestinal symptoms related to the ingestion of gluten containing food, in subjects not suffering from either CD or WA\textsuperscript{[12]}. It is an emerging gluten related cause of ME and intestinal symptoms, with an increasing rate of diagnosis\textsuperscript{[13,14]}. However, the spectrum of ME encompasses even non gluten-related disorders. For instance, IBS may show increased IELs as well as organic diseases\textsuperscript{[11,15]}. For this reason, it is often difficult to formulate a differential diagnosis in ME, and the diagnostic \textit{iter} may be long and time consuming. This aspect is of relevance especially for GS, whose diagnosis is essentially clinical. Moreover, the clinical manifestations of GS often overlap with IBS, and this is a diagnostic challenge\textsuperscript{[16,17]}. A previous experience of our group found that IELs count of 15-25 IELs/100 enterocytes, autoimmune thyroiditis, folate deficiency and diarrhea may be predictive factors for GS\textsuperscript{[18]}, but a reliable marker has not been discovered yet despite the report that GS is characterized by the upregulation of toll-like receptor 2 (TLR2)\textsuperscript{[19]}. In our experience, a IELs infiltrate > 15 per 100 enterocytes paralleled an enhanced expression of pro-inflammatory cytokines, in particular interferon gamma (IFN$\gamma$), in subjects with suspected seronegative CD\textsuperscript{[20]}. Therefore, a pro-inflammatory status may underlie CD-related ME and the intestinal assessment of baseline mucosal molecular pattern could likely give useful information about ME underlying conditions. In detail, tissue Transglutaminase 2 (tTG2), IFN$\gamma$, TLR2 and Myeloid Differentiation factor 88 (MyD88) have been suggested as potential targets in this field\textsuperscript{[19]}. tTG2 is the main autoantigen involved in the pathogenesis of CD, and it has been demonstrated that it is over-expressed in the mucosa of patients with CD\textsuperscript{[21,22]}. IFN$\gamma$ is a pro-inflammatory cytokine that is essential for innate and adaptive immunity against infections\textsuperscript{[23]}. Aberrant IFN$\gamma$ expression is associated with a number of autoinflammatory and autoimmune diseases, including CD\textsuperscript{[24]}. TLR2 is a type of cellular receptor mainly involved in innate immunity, which has been proposed as a mediator of a potential inborn response to gliadin\textsuperscript{[25]}. MyD88 is an adapter protein mediating intracellular pathways triggered by TLRs to activate the transcription factor NF-$\kappa$B\textsuperscript{[26]}. Indeed, some gliadin peptides may bind TLR2 and drive the production of interleukin 1, a proinflammatory cytokine, through the mediation of MyD88\textsuperscript{[27]}. Moreover, the MyD88 was found to be a key protein mediating the release of zonulin in response to gliadin, thus leading to an increase of mucosal permeability in CD\textsuperscript{[28]}. Therefore, an increase of MyD88 may be considered as a marker of an alteration of intestinal barrier.

The aim of the present study was to investigate the duodenal mucosal transcriptomic expression of these four molecules in the prediction of ME underlying disorders at baseline, before a 2-year follow-up mean period, and to assess their potential accuracy in discriminating the development of CD and GS.

**MATERIALS AND METHODS**

**Patients**

We retrospectively enrolled 89 consecutive patients with ME followed up for a mean period of two years until a diagnosis was reached. ME was defined according to the criteria of the Bucharest Consensus Conference\textsuperscript{[9]}. Follow-up strategies which allowed achieving final diagnosis have been described elsewhere\textsuperscript{[18]}. In detail, CD was diagnosed if duodenal biopsy showed a microscopic picture of Marsh 1 or higher, along with the positivity of IgA anti tissue transglutaminase 2 (anti-tTG2) antibodies, according to current guidelines\textsuperscript{[29]}. The diagnosis of GS was made according to the Salerno criteria\textsuperscript{[30]}. Patients with IBS fulfilled the Rome III criteria and underwent a series of investigations (serology for CD, full blood count, folate, vitamin B$_{12}$, serum protein electrophoresis with immunoglobulin subclasses, stool investigations, fecal occult blood test, calprotectin, urea/lactose/glucose breath test and, if necessary, colonoscopy with random biopsy samples) in order to rule out organic diseases\textsuperscript{[31]}. Finally, patients with established non-gluten related cause of ME (\textit{Helicobacter pylori} infection, autoimmune disorders) were included.

We excluded subjects with immunoglobulin deficiencies, which may show possible molecular deregulation of duodenal mucosa. A group of 15 dyspeptic patients, undergoing upper endoscopy and duodenal biopsy without ME, represented the negative control group.

**Histology and immunohistochemistry**

For each patient, formalin embedded biopsy samples of the duodenum performed at baseline were retrieved. Histological examination had been carried out on Hematoxylin-Eosin stained sections. Immunohistochemistry of CD3 lymphocytes had been performed using monoclonal murine antibody (Novocastra Leica Biosystems Ltd, Newcastle, United Kingdom), according to the manufacturer’s instructions. In all subjects, IELs were counted in a field containing at least 1000 enterocytes and expressed as number per 100 enterocytes. We selected biopsy specimens with at least 15 IELs/100 enterocytes to define ME, as established in previous reports\textsuperscript{[17,19]}. The count was executed in the epithelial layer by two observers (DP and MGF) in a blinded fashion. Collection and processing was managed according to BRISQ recommendations\textsuperscript{[32]}. The diagnosis of GS was made according to the Salerno criteria\textsuperscript{[30]}. Patients with IBS fulfilled the Rome III criteria and underwent a series of investigations (serology for CD, full blood count, folate, vitamin B$_{12}$, serum protein electrophoresis with immunoglobulin subclasses, stool investigations, fecal occult blood test, calprotectin, urea/lactose/glucose breath test and, if necessary, colonoscopy with random biopsy samples) in order to rule out organic diseases\textsuperscript{[31]}. Finally, patients with established non-gluten related cause of ME (\textit{Helicobacter pylori} infection, autoimmune disorders) were included.

We excluded subjects with immunoglobulin deficiencies, which may show possible molecular deregulation of duodenal mucosa. A group of 15 dyspeptic patients, undergoing upper endoscopy and duodenal biopsy without ME, represented the negative control group.

**Molecular analysis**

Real time polymerase chain reaction (RT-PCR) was used to detect the amount of mRNA coding for tTG2, IFN$\gamma$, TLR2 and MyD88 in duodenal mucosa. As well-stated, mRNA levels were expressed as fold-change compared
Table 1 Primers and probes

| Tissue transglutaminase 2 | Primer Forward:ATAAGTTAGCGCCGCTCTCC | Primer Reverse:CCTGCCTCTCCTCAGTG |
|---------------------------|-------------------------------------|-----------------------------------|
| Interferon gamma           | Primer Forward:CCGGTTACTTATAGAAACCTTGA | Primer Reverse:TCAATGAGAAGAATCTTGCACTTC |
| Toll-like receptor 2       | Primer Forward:CTGGTGAATCTCAA        | Primer Reverse:TGCCCCTACTCAA      |
| MyD88                      | Primer Forward:CAAGCTTCTGCCCGTGC    | Primer Reverse:TCTGCCCTGCTCCTC   |
| Tissue transglutaminase 2  | Probe: GCCACGCGCCAGTG                | Probe: AGGGCCCTGGGTGTGTGT        |

of paraffin sections of paraffin block using the RNaseasy FFPE Kit (Qiagen, GmbH, Heidelberg, Germany), specifically designed for the purification of total RNA from formalin-fixed paraffin-embedded (FFPE) tissue sections, according to a validated protocol[33]. Five hundred microliters (µL) of xylene were added to the sections to yield a solution that was vortexed for 10s and then incubated for 10 min at room temperature (25 ℃). This step was repeated twice. Subsequently, 500 µL of absolute ethanol was added and the novel solution was again vortexed vigorously for 10 s and centrifuged for 2 min at 11000 rpm in order to remove residual xylene. The supernatant was carefully removed by pipetting without disturbing the pellet. Finally, the mRNA concentrations were estimated by ultraviolet absorbance at 260/280 nm. We performed the agarose formaldehyde gel run to confirm the RNA integrity. Imaging analysis after this procedure was performed with the Bio-Rad Chemidoch Analyzer (Bio-Rad Laboratories S. r.l., Milan, Italy). Aliquots of total mRNA (1 mg) were reverse-transcribed using random hexamers and TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, United States) in a final volume of 50 µL. Two step reverse transcription PCR was performed using the first-strand cDNA with a final concentration of 1 × TaqMan gene expression assay, i.e., the analyzed molecules and glyceraldehyde 3 phosphate dehydrogenase as reference gene (Applied Biosystems, Foster City, CA). The final reaction volume was 25 µL and analyzed in triplicate (all experiments were repeated twice). A non template control (Rnase free water) was included on every plate. Our method was further validated by including in each assay fresh samples from three normal patients, frozen at -90 ℃ until the analysis. These samples were treated with the same technique as the paraffin embedded samples, except for the paraffin removal and rehydration procedures. Specific thermal cycler conditions were employed using a real time PCR System (Applied Biosystems). A standard curve plus validation experiment was performed for each primer/probe set. The reference gene was represented by glyceraldehydes3phosphate dehydrogenase. Primers and probes are reported in Table 1.

Statistical analysis
Comparisons among continuous data obtained in our groups of patients were performed by one way analysis of variance (ANOVA) and Bonferroni’s test as post-hoc analysis to compare head-to-head each group. The χ2 test was used for categorical variables. Values of P < 0.05 were considered significant. Receiver operating curves (ROC) were drawn to estimate sensitivity and specificity of tTG2, IFNγ, TLR2 and MyD88. Correlations between IELs count, tTG2, IFNγ, TLR2 and MyD88 were assessed by Pearson’s test. Diagnostic agreement for the IEL count was tested by calculating the weighted Cohen’s k coefficient interpreted in accordance with the Landis and Koch benchmarks, whereby a value of more than 0.8 indicated excellent agreement. Statistical analyses were performed using the statistical software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, United States).

RESULTS

Patients baseline features
We enrolled 89 patients with ME. After the follow up the following diagnoses were observed: 31 CD, 17 GS, 29 IBS and 12 other non gluten-related ME. Among the 12 non gluten-related ME, 4 had small bowel Crohn’s disease, 6 H. pylori infection, 1 scleroderma and 1 lymphocytic colitis. The most relevant clinical, demographic and histopathological characteristics are summarized in Table 2. A good agreement among pathologists was achieved (κ = 0.86, 95%CI: 0.75-0.91). The IELs count, DQ 2 or 8 positivity and the presence of weight loss, abdominal pain and diarrhea were the most important discriminating factors between CD, GS and non-gluten-related diseases (Table 2). Villous atrophy (Marsh 3 stage) was found in 14 out of 31 CD patients, Marsh 2 in 3 and Marsh 1 in 14 patients.

tTG2
The mucosal expression of mRNA-tTG2 is represented in Figure 1A. In detail, CD patients had the highest levels (8.3 ± 4.5) compared to GS (3.6 ± 2.7), IBS (3.5 ± 1.8), other ME (5.3 ± 2.3) and negative controls (1.001 ± 0.089). The ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative controls.

The ROC curve analysis, displayed in Figure 1B showed that a cut off of 2.258 was able to discriminate between CD and GS with a low sensitivity (52.94%) and a good specificity (87.1%; AUC = 0.804).
The mucosal expression of mRNA-IFNγ is displayed in Figure 2A. We observed that CD patients had the highest levels (8.5 ± 4.1) compared to GS (3.3 ± 2.8), IBS (3.3 ± 2.6), other ME (4.6 ± 2.1) and negative controls (1.001 ± 0.15). The ANOVA plus Bonferroni analysis showed that CD > Other ME > GS = IBS > negative controls.

The analysis of ROC curve (Figure 2B) showed that a cut off of 1.853 was able to differentiate CD and GS with a sensitivity of 47.06% and a specificity of 96.77%, with an AUC of 0.816.

The mucosal expression of mRNA-TLR2 is represented in Figure 3. Patients with non gluten-related causes of ME were characterized by the highest levels (6.1 ± 1.9), greater than GS (3.1 ± 1.8), IBS (3.5 ± 2.0), CD (4.1 ± 2.4) and negative controls (1.006 ± 0.18). The ANOVA plus Bonferroni analysis showed that Other ME > CD = GS = IBS > negative controls.

The mucosal expression of mRNA-MyD88 is represented in Figure 4A. Patients with CD expressed levels similar as non gluten-related causes of ME (7.8 ± 4.9 and 6.7 ± 2.9), higher than GS (4.2 ± 2.3), IBS (4.3 ± 2.4), and negative controls (0.99 ± 0.17). The ANOVA plus Bonferroni analysis demonstrated that CD = Other ME > GS = IBS > negative controls.

The analysis of ROC curve (Figure 4B) showed that a cut off of 3.722 was able to differentiate CD and GS with a sensitivity of 52.94% and a specificity of 74.19%, with an AUC of 0.712.

Correlation between IELs count and transcriptome analysis
In all cases, the IELs count correlated with mRNA levels with a strong significance (P < 0.0001). The expression of tTG2 directly correlated to IELs (r = 0.66, 95%CI: 0.53-0.76). IFNγ showed a similar pattern, with an r = 0.56, 95%CI: 0.42-0.68. A less relevant, despite significant correlation, was found for TLR2 (r...
DISCUSSION

ME often represents a diagnostic dilemma, therefore a careful algorithm should be applied in such cases to achieve the final diagnosis\[^{9,18}\]. IELs infiltrate represents the only common denominator; therefore, in our series we selected only subjects showing IELs levels higher than 15/100 enterocytes, independently from ME etiology. This cut-off value was associated with molecular mucosal changes suggestive of inflammatory damage/repair in our experience\[^{20}\]. IELs infiltrate is a well known feature of GS and CD\[^{34,35}\], but it has been described even in patients with duodenal involvement in systemic disorders\[^{36}\]. IBS may show an increased IELs infiltrate, thus confirming that it cannot be considered only as a functional entity\[^{37}\]. In a previous experience of our group, we demonstrated a positive correlation between the amount of IELs infiltrate and the mucosal expression of tTG2 and IFN\(_{\gamma}\) in patients with seronegative suspected CD\[^{20,38}\]. In the present study, moreover, we confirmed that IELs infiltrate parallels mucosal molecular expression, more markedly for tTG2 and IFN\(_{\gamma}\), but also for TLR2 and MyD88. Therefore, our results demonstrate that ME clearly underlies a local inflammatory status.

tTG2 is the main autoantigen involved in CD pathogenesis\[^{21}\], however it is an enzyme essential for the process of wound healing and tissue repair, since it is able to create crosslinks between peptides\[^{39}\]. For this reason, tTG2 is over-expressed not only in gluten-related disorders, but even in all pathological conditions that induce mucosal injury, as confirmed in the present study. In our analysis, we reported that tTG2 has a good performance in the comparison between CD and GS or IBS. This detail could be useful especially in the subgroup of subjects with IBS who show positivity of anti-tTG antibodies, without histological evidence of CD\[^{40}\]. Moreover, our result may explain the reason of a four-fold increased risk of CD in subjects with IBS\[^{41}\]. A pattern similar to tTG was found for IFN\(_{\gamma}\). Indeed, patients with CD show the upregulation of IELs-secreted IFN\(_{\gamma}\). This ability has been demonstrated even in peripheral T lymphocytes of CD patients, which are able to produce high IFN\(_{\gamma}\) levels when stimulated by gliadin peptides\[^{42,43}\]. For these reasons, tTG2 and IFN\(_{\gamma}\) could represent a good diagnostic tool in gluten related-disorders. In summary, we found a high specificity of tTG2 and IFN\(_{\gamma}\),
despite an expected low sensitivity, in discriminating CD from GS development. The expression of these molecules in GS has been poorly explored until now and available data are controversial. Some reports have shown an IFN\(_\gamma\) overexpression in GS similar to CD\([44]\), while others described a marked increase only in CD\([19]\). In the present study, we have found that high levels of IFN\(_\gamma\) are more predictive of CD than GS.

TLR2 is a receptor involved in the innate immune response against non-self antigens. It has been demonstrated that some gliadin peptides may bind such receptor and address the production of interleukin 1, a proinflammatory cytokine, trough the mediation of MyD88\[27\]. Moreover, MyD88 was found to be a key protein mediating the release of zonulin in response to gliadin, thus leading to an increase of mucosal permeability in CD\[28\]. In a previous report\[19\], patients with GS expressed higher levels of TLR2 than subjects with CD. However, we found that ME had similar baseline mRNA levels encoding for TLR2, independently from the successive development of CD or GS within a two-year period. Surprisingly, in our series, non gluten related ME had the highest levels of TLR2. This finding could be related to the deep deregulation of TLRs, which has been described in IBD (a possible cause of non gluten related ME). Indeed, such receptors mediate the immune response against the microbiota, a phenomenon that has been claimed as a trigger in IBD pathogenesis\[45-47\].

In regard to the molecular pattern of MyD88, similarly to TLR2, we found that patients with non gluten related causes of ME showed higher levels than IBS and GS, but comparable with CD. This finding suggests that a potential increase of intestinal permeability may be more marked when a severe intestinal damage underlies ME. On the other hand, MyD88 has been poorly investigated in CD. Eiró \textit{et al}\[48\] demonstrated that its increased expression paralleled mucosal TLR4 in CD. Therefore, MyD88 overexpression overtaking TLR2 may be explained by a MyD88-independent pathway for TLR2 in CD, as described by Junker \textit{et al}\[25\].

In conclusion, our results suggest that a single marker is unable to discriminate the development of different ME underlying conditions as well as between CD and GS. High mucosal levels of tTG and IFN\(_\gamma\) mRNA may predict the development of CD more than GS with high specificity. TLR2 does not discriminate the development of CD from GS. High MyD88 levels may indicate that intestinal permeability is more increased when a severe intestinal damage underlies ME (CD as well as Crohn’s disease). Finally, a reliable marker for GS diagnosis has not yet been found; however, further studies need to be addressed to evaluate whether the combination of different mucosal markers could help the differential diagnosis with CD and support the identification of doubtful cases of GS\[49,50\]. Therefore, the results of the present paper do not seem to show a clear translational value.

**COMMENTS**

**Background**

Microscopic enteritis is an inflammatory condition, which is characterized by increased intraepithelial CD3 lymphocytes in the duodenum and can be due to both gluten and non-gluten related diseases.

**Research frontiers**

The MyD88 was found to be a key protein mediating the release of zonulin in response to gliadin, thus leading to an increase of mucosal permeability in CD. Therefore, an increase of MyD88 may be considered as a marker of an alteration of intestinal barrier.

**Innovations and breakthroughs**

The authors demonstrated that tissue transglutaminase and interferon gamma...
may predict the development of Celiac Disease more than Gluten Sensitivity with high specificity, despite an expected low sensitivity.

**Peer-review**

This report seeks to distinguish among 4 causes of duodenal lymphocytosis (celiac disease, non-celiac gluten sensitivity, wheat allergy and irritable bowel syndrome) by retrospectively comparing the mRNA expression of tissue transglutaminase 2, interferon gamma, toll-like receptor 2 and myeloid differentiation factor 88 in duodenal biopsies from 89 patients obtained up to two years previously.

**REFERENCES**

1. Lauwers GY, Fasano A, Brown IS. Duodenal lymphocytosis with no or minimal enteropathy: much ado about nothing? *Mod Pathol* 2015; 28 Suppl 1: S22-S29 [PMID: 25560597 DOI: 10.1038/modpathol.2014.135]

2. Aziz I, Key T, Goodwin JG, Sanders DS. Predictors for Celiac Disease in Adult Cases of Duodenal Intraepithelial Lymphocytosis. *J Clin Gastroenterol* 2015; 49: 477-482 [PMID: 25014240 DOI: 10.1097/MCG.0000000000000184]

3. Simondi D, Ribaldone DG, Bonagara GA, Foi S, Sapone N, Garavagno M, Villanacci V, Bernardi D, Pellicano R, Rizzetto M, Asteigiano M. Helicobacter pylori in celiac disease and in duodenal intraepithelial lymphocytosis: Active protagonist or innocent bystander? *Clin Res Hepatol Gastroenterol* 2015; 39: 740-745 [PMID: 25956489 DOI: 10.1016/j.clinres.2015.03.005]

4. Losurdo G, Principi M, Di Leo A, Ierardi E. Letter: Helicobacter-negative gastritis--a distinct condition? *Aliment Pharmacol Ther* 2015; 41: 597-598 [PMID: 25692171 DOI: 10.1111/apt.13080]

5. Patterson ER, Shmidt E, Oxentenko AS, Enders FT, Smyrk TC. Normal villous architecture with increased intraepithelial lymphocytes: a duodenal manifestation of Crohn disease. *Am J Clin Pathol* 2015; 143: 445-450 [PMID: 25968040 DOI: 10.1309/AJCPBKQNQD45HVFVXQ]

6. Malamut G, Verkarre V, Suarez F, Viallaf JD, Lascaux AS, Cosnes J, Bouhnik Y, Lambotte O, Béchade D, Ziol M, Lavergne A, Hermine O, Cerf-Bensussan N, Cellier C. The enteropathy associated with common variable immunodeficiency: the delineated frontiers with celiac disease. *Am J Gastroenterol* 2010; 105: 2262-2275 [PMID: 20551941 DOI: 10.1038/jaig.2010.214]

7. Giorgio F, Principi M, Losurdo G, Piscitelli D, Iannone A, Barone M, Amoruso A, Ierardi E, Di Leo A. Evolution of nonspecific duodenal lymphocytosis over 2 years of follow-up. *World J Gastroenterol* 2015; 21: 7545-7552 [PMID: 26140001 DOI: 10.3748/wjg.v21.i24.7545]

8. Sapone A, Lammers KM, Casolaro V, Cammarota M, Giuliano MT, De Rosa M, Stefanelle R, Mazzarella G, Tolone C, Russo MI, Esposito F, Herrera C, Carteri M, Riegel G, de Magistris L, Fasano A. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. *BMJ Med* 2011; 9: 23 [PMID: 21392369 DOI: 10.1136/bmj.343.dj4169]

9. Ierardi E, Amoruso A, Giorgio F, Principi M, Losurdo G, Piscitelli D, Buffelli F, Fiore MG, Mongelli A, Castellaneta NM, Giangaspero A, De Francesco V, Montenegro L, Di Leo A. Mucosal molecular pattern of tissue transglutaminase and interferon gamma in suspected seronegative celiac disease at marsh 1 and 0 stages. *Saudi J Gastroenterol* 2015; 21: 379-385 [PMID: 26655133 DOI: 10.4103/1319-3767.167189]

10. Dieterich EW, Ethin N, Baurer M, Donner P, Volta U, Riecken EO, Schuppan D. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 1997; 3: 797-801 [PMID: 9212111]

11. Esposito C, Paparo F, Caputo I, Porta R, Salvati VM, Mazzarella G, Auricchio S, Troncone R. Expression and enzymatic activity of small intestinal tissue transglutaminase in celiac disease. *Am J Gastroenterol* 2003; 98: 1813-1820 [PMID: 12907337]

12. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol* 2007; 96: 41-101 [PMID: 17981204]

13. Lademperer AI, Fält-Magnusson K, Högborg L, Ludvigsson J, Varrala O. Expression pattern of T-helper cell signaling pathway and mucosal inflammation in celiac disease. *Scand J Gastroenterol* 2014; 49: 145-156 [PMID: 24325470 DOI: 10.1080/00365521.2013.836966]

14. Junker V, Zeissig S, Kim SJ, Barisani D, Wieser H, Leffler DA, Zevulov V, Libermann TA, Dillon S, Freitag TL, Kelly CP, Schuppan D. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. *J Exp Med* 2012; 209: 2395-2408 [PMID: 23209313 DOI: 10.1084/jem.20120660]

15. Arancibia SA, Beltrán CJ, Aguirre IM, Silva P, Peralta AL, Malinách F, Hermoso MA. Toll-like receptors are key participants in innate immune responses. *Biol Res* 2007; 40: 97-112 [PMID: 18064347]

16. Paloviš-Jelínková L, Daňová K, Drasárová H, Dvořák M, Fonda...
Principi M, Montenegro L, Amoruso A, Di Leo A. Seronegative celiac disease: where is the specific setting? Gastroenterol Hepatol Bed Bench 2015; 8: 110-116 [PMID: 25926935]

Nonminskaya MV, Belkin AM. Cellulose functions of tissue transglutaminase. Int Rev Cell Mol Biol 2012; 294: 1-97 [PMID: 22364871 DOI: 10.1016/B978-0-12-394305-7.00001-X]

Locke GR, Murray JA, Zinsmeister AR, Melton LJ, Talley NJ. Celiac disease serology in irritable bowel syndrome and dyspepsia: a population-based case-control study. Mayo Clin Proc 2004; 79: 476-482 [PMID: 15065612]

Ford AC, Chey WD, Talley NJ, Malhotra A, Spiegel BM, Moayyedi P. Yield of diagnostic tests for celiac disease in individuals with symptoms suggestive of irritable bowel syndrome: systematic review and meta-analysis. Arch Intern Med 2009; 169: 651-658 [PMID: 19364994 DOI: 10.1016/archinternmed.2009.22]

van Leeuwen MA, Lindenbergh-Kortleve DJ, Raatgeep HC, de Ruiter LF, de Krüger RR, Groeneweg M, Escher JC, Samsom JN. Increased production of interleukin-21, but not interleukin-17A, in the small intestine characterizes pediatric celiac disease. Mucosal Immunol 2013; 6: 1202-1213 [PMID: 23571506 DOI: 10.1038/mi.2013.19]

Ontiveros N, Tye-Din JA, Hardy MY, Anderson RP. Ex-vivo whole blood secretion of interferon-γ and IFN-γ-inducible protein-10 measured by enzyme-linked immunosorbent assay are as sensitive as IFN-γ enzyme-linked immunospot for the detection of gluten-reactive T cells in human leucocyte antigen (HLA)-DQ2:5(+) associated celiac disease. Clin Exp Immunol 2014; 175: 305-315 [PMID: 24192268 DOI: 10.1111/cei.12232]

Brotveit M, Beintes AC, Tollefsen S, Bratlie JE, Jahnsten FL, Johansen FE, Sollid LM, Lundin KE. Mucosal cytokine response after short-term gluten challenge in celiac disease and non-celiac gluten sensitivity. Am J Gastroenterol 2013; 108: 842-850 [PMID: 23588237 DOI: 10.1038/ajg.2013.91]

Bran P, Giron MC, Qureshi M, Porziano A, Caputi V, Zoppellaro G, Banzanto S, Grillo AR, Spagnoli L, De Caro R, Pizzuti D, Barbieri V, Rosato A, Starnolo GC, Martines D, Zaninotto G, Palù G, Castagliuolo I. Toll-like receptor 2 regulates intestinal barrier integrity in CD: a population-based case-control study. Gut 2013; 62: 2143-2148 [PMID: 23548250 DOI: 10.1136/gutjnl-2013-306578]

Moore HM, Kelly AB, Jewell SD, McShane LM, Clark DP, Tovoli F, Cicola R, Parisi C, Fabbri A, Piscaglia M, Discepolo V, Jabri B. Intraepithelial lymphocytes in celiac disease: role of the innate immune response in Celiac disease. J Immunol 2006; 176: 2512-2521 [PMID: 16456012]

Ludvigsson JF, Bai JC, Biagi F, Card TR, Ciacci C, Ciccittà PJ, Cellier C, Cristofori F, de Magistris L, Dolinsek J, Dieterich W, Francavilla R, Hadjivassiliou M, Holtmeier W, Körner U, Leffler DA, Lundin KE, Mazzarella G, Mulier CJ, Nastasi K, Sanders D, Skodje GI, Schepman D, Ullrich R, Volta U, Williams M, Zevallas VF, Zopf Y, Fasano A. Diagnosis of Non-Celiac Gluten Sensitivity (NCGS): The Salerno Experts’ Criteria. Nutrients 2013; 5: 3497-3511 [PMID: 23838686 DOI: 10.3390/nu5093497]

Catassi C, Eilli L, Bonaz B, Bouna G, Carroccio A, Castilletto G, Cellier C, Cristofori F, de Magistris L, Dolinsek J, Dieterich W, Francavilla R, Hadjivassiliou M, Holtmeier W, Körner U, Leffler DA, Lundin KE, Mazzarella G, Mulier CJ, Pelegrini N, Rostami K, Sanders D, Skodje GI, Schepman D, Ullrich R, Volta U, Williams M, Zevallas VF, Zopf Y, Fasano A. Diagnosis of Non-Celiac Gluten Sensitivity (NGCS): The Salerno Experts’ Criteria. Nutrients 2015; 7: 4966-4977 [PMID: 26096570 DOI: 10.3390/nu7040696]

Ford AC, Moayyedi P, Lacey B, Lembo AJ, Saito Y, Schiller LR, Soffer EE, Spiegel BM, Quigley EM. American College of Gastroenterology monograph on the management of irritable bowel syndrome and chronic idiopathic constipation. Am J Gastroenterol 2014; 109 Suppl 1: S2-26; quiz S27 [PMID: 25091148 DOI: 10.1038/ajg.2014.187]

Moore HM, Kelly AB, Jewell SD, McShane LM, Clark DP, Greenspan R, Hayes DF, Hainaut P, Kim P, Mansfield EA, Potapova VM, Moore HM, Pirisi M, Sanders DS, Skodje GI, Schuppan D, Ullrich R, Volta U, Williams M, Milla PJ. Celiac disease serology in irritable bowel syndrome and chronic idiopathic constipation. J Pediatr Gastroenterol Nutr 2013; 56: 51-55 [PMID: 22785416 DOI: 10.1097/MPG.0b013e3182766ef]

Aladie V, Discepolo V, Jahri I. Intraepithelial lymphocytes in celiac disease immunopathology. Semin Immunopathol 2012; 34: 551-566 [PMID: 22660791 DOI: 10.1007/s00281-012-0316-x]

Vulva N, Calabro A, Randone A, Corazza GR. An Italian prospective multicenter survey on patients suspected of having non-celiac gluten sensitivity. BMC Med 2014; 12: 85 [PMID: 24885375 DOI: 10.1186/1741-7015-12-85]

Shmiedt E, Smyrk TC, Fuchbon JA, Oxensten AK, Duodenal intraepithelial lymphocytes with normal villous architecture in healthy population from the western region of Saudi Arabia. Saudi J Gastroenterol 2014; 20: 23-25 [PMID: 24290098 DOI: 10.4291/wjg.v5.i3.441]

Eiró N, González-Reyes S, González L, González LO, Altadill A, Andicociea A, Fresno-Forracellado MF, Rodrigo-Sáez L, Vizoso FJ. Duodenal expression of Toll-like receptors and interleukins are increased in both children and adult celiac patients. Dig Dis Sci 2012; 57: 2278-2285 [PMID: 22562536 DOI: 10.1007/s10620-012-2184-6]

Khayyat YM. Serologic markers of gluten sensitivity in a healthy population from the western region of Saudi Arabia. Saudi J Gastroenterol 2012; 18: 19-25 [PMID: 222409088 DOI: 10.4103/1397-7857.100001]

Vulva N, Tovoli F, Cicola R, Parisi C, Fabbri A, Piscaglia M, Forni E, Caio G. Serological tests in gluten sensitivity (nonceliac gluten intolerance). J Clin Gastroenterol 2012; 46: 680-685 [PMID: 22138844 DOI: 10.1097/MCG.0b013e3182372541]

P- Reviewer: Freeman HJ, Mishra PK, Rhoads JM S- Editor: Qi Y L- Editor: A E- Editor: Ma S
