Mast cell-nerve interactions correlate with bloating and abdominal pain severity in patients with non-celiac gluten / wheat sensitivity

Title: Mast cell-nerve interactions correlate with bloating and abdominal pain severity in patients with non-celiac gluten / wheat sensitivity

Running Title: Neuro-immune cross-talk in gluten sensitivity

Fiorella Giancola, PhD[1], Umberto Volta, MD[1], Roberta Repossi, MSc[1], Rocco Latorre, PhD[2], Dorien Beeckmans, PhD[3], Florencia Carbone, PhD[3], Karen Van den Houte, MSc[3], Francesca Bianco, PhD[1], Elena Bonora, PhD[1], Alessandra Gori, MSc[1], Anna Costanzini, PhD[4], Elisa Boschetti, PhD[1], Giacomo Caio, PhD[1], Tim Vanuytsel, PhD[3], Vincenzo Stanghellini, MD[1], Jan Tack, PhD[3*] and Roberto De Giorgio, PhD[4*]

*These authors share co-last authorship

1 Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy;

2 Department Basic Science & Craniofacial Biology, New York University, New York, USA;

3 Translational Research Center for Gastrointestinal Disorders (TARGID), KU Leuven, Leuven, Belgium;

4 Department of Medical Sciences, University of Ferrara, Ferrara, Italy

Word count: 3139

Corresponding author:
Roberto De Giorgio
Internal Medicine Unit - Department of Medical Sciences
University of Ferrara, Italy
Arcispedale St. Anna, Via A. Moro, 8 - 44124 Cona, Ferrara, Italy
E-mail: roberto.degiorgio@unife.it; dgrrrt@unife.it

Abstract

Background: Gastrointestinal (GI) and extra-GI symptoms/manifestations represent key clinical features of patients with non-celiac gluten/wheat sensitivity (NCG/WS). This study aimed to investigate neuro-immune (focusing on mast cells, MCs) interactions in the duodenal submucosa of patients with NCG/WS.

Methods: Submucosal whole mounts from duodenal biopsies of 34 patients with self-reported NCG/WS, 28 with celiac disease (CD), 13 with functional dyspepsia (FD) and 24 healthy controls (HC) were analyzed by immunohistochemistry. Quantitative data on neuronal and MCs density and the percentage of MCs in close vicinity to nerves were obtained and correlations among neurons, MC density and MC-nerve distance (D) and symptoms were assessed in the three groups.

Key results: The number of submucosal neurons was not different among groups. In NCG/WS, MC density was not different from HC, while it was slightly increased *vs.* CD (P=0.07) and significantly decreased *vs.* FD (P<0.05). The percentage of MCs close to nerves (D<15 μm) was similarly increased in all three pathological groups *vs.* HC (P<0.001). In NCG/WS, MC infiltration correlated to bloating (P=0.001) and abdominal pain severity (P=0.03) and the percentage of MCs in proximity to neurons correlated with the number of GI symptoms (D<5 μm; P=0.05), bloating and abdominal pain severity (D<15um; P=0.01).

Conclusions & Inferences: Submucosal MC infiltration and the close (within 15 μm) MC-to-nerve proximity in the duodenum of NCG/WS patients are features providing a histopathological basis to better understand GI symptoms in this condition.

Keywords: Food sensitivity; Functional bloating; Functional abdominal pain; Functional dyspepsia; Gluten sensitive enteropathy.

Key Points

- Non-celiac gluten/wheat sensitivity (NCG/WS) is a condition characterized by gastrointestinal (GI) and extra-intestinal symptoms evoked by gluten/wheat-containing food consumption.
- Although innate and adaptive immunity may contribute to NCG/WS pathophysiology, the mechanisms underlying symptom generation remain unsolved.
- This study provided new morphological findings indicating that submucosal mast cell (MC) infiltration and their interaction with nerves in the upper GI tract correlate to symptom profile.
- A close MC-to-nerve proximity in the duodenum of patients with NCG/WS is a feature underlying severity of abdominal pain and bloating.
- Our histopathological data may help detecting patients with NCG/WS and provide cellular targets for the development of new therapeutic approaches in this condition.

1. **Introduction**

Non-celiac gluten / wheat sensitivity (NCG/WS) is a clinical condition presenting with either gastrointestinal (GI) and /or extra-GI manifestations which typically occur in strict relation to gluten and other wheat proteins ingestion in patients in whom celiac disease (CD) and wheat allergy have been ruled out.[1-6]

The overlap of clinical signs with functional GI disorders such as IBS and / or functional FD[4,7-10] and the lack of biomarkers make the diagnosis of NCG/WS challenging. Furthermore, the actual dietary triggers and the putative mechanisms underlying GI symptoms and extra-GI manifestations in NCG/WS patients remain still poorly understood. The role for gluten in GI and extra-GI symptom generation is still controversial[11-15] since non-gluten proteins and fermentable short-chain carbohydrates have also shown similar effects although mainly in the GI spectrum.[16-18] Gut microbiota changes along with a compromised intestinal epithelial barrier appear to play a prominent role in the clinical expression of NCG/WS,[19,20] plausibly leading to the activation of the adaptive, and even more of the innate ,immune response.[21]

Immune activation, mainly based on the identification of mucosal mast cells (MCs) in close vicinity to the nerves supplying the gut, appears to play a role in sensory-motor dysfunction and symptom generation in patients with IBS and FD.[22,23] Based on the similarity of intestinal symptoms in NCG/WS, CD, IBS and FD, it is conceivable that neuro-immune interactions between MCs and nerves in the mucosa or submucosa of the upper gut can contribute to symptoms reported by NCG/WS patients. In order to establish whether MC-nerve interactions occur in NCG/SW patients, this study was designed to investigate the neuro-immune profile in the duodenal submucosa by exploiting a novel technical approach through which routine biopsies can

be processed to separate the mucosa from submucosa.[24] We focused on the duodenal submucosa for the following reasons: 1) the diagnostic work-up of patients with NCG/WS may include an upper GI endoscopy in order to show possible changes of duodenal mucosa,[2] where from 26% to 96% of NCG/WS patients show a Marsh 1 degree of lesions at duodenal biopsy histology; [21] 2) duodenal submucosa whole mounts, derived from mucosal separation, show a denser innervation than the few nerve endings detectable in a single biopsy. Neuronal density, MC infiltration, MC-nerve interactions in the duodenal mucosa and their relationship with GI symptoms were assessed comparatively in NCG/WS, FD, CD patients, and in healthy asymptomatic controls.

2. Patients and Methods

2.1 Study Protocol and patient recruitment

The Ethical Committee of the St. Orsola Hospital in Bologna (N° 119/2012/U/Tess) and of the University Hospital in Leuven (S60477) approved the study protocol.

Adult subjects (n=62,18-68 year range, 50 females) referred for GI and extraintestinal symptoms related to gluten / wheat ingestion were prospectively recruited at St. Orsola Hospital, by obtaining their informed content. They were then stratified in CD or NCG/WS according to the diagnostic work-up, including serological and genetic tests and histopathological evaluation.[25-28]

All the diagnoses of CD patients were characterized by villous atrophy and positive serology. NCG/WS patients had a non-atrophic duodenal mucosa and tested negative for CD serology. Their diagnosis was confirmed by a trial of 6-month gluten free diet (GFD) showing a significant symptom improvement followed by 1-month gluten challenge with symptom exacerbation.[6] Thirteen patients (19-60 years, 9 females) meeting the Rome III criteria for FD [29]

referred and submitted to upper gastroscopy with duodenal biopsies, were prospectively recruited at Translational Research Center for Gastrointestinal Disorders (TARGID) in Leuven.

Twenty-four healthy volunteers (19-29 years, 9 females), belonging to an existing mailing list, were recruited at Leuven Center following screening questionnaires to evaluate the general health and to exclude the presence of GI symptoms or a history of GI disease.

Excluding criteria applied to the four study cohorts included: no restrictive diet over the past 6 months; absence of wheat allergy (assessed by specific IgE or skin prick tests); no previous history of liver disease and/or abnormal liver function tests.

2.2 Diagnosis of CD and Characterization of NCG/WS

Serology. In patients suspected for NCG/WS and CD established serological markers of coeliac disease, including IgA antibodies to transglutaminase-2 (TG2), IgG antibodies to deamidated gliadin (DGP - IgG) and IgG and IgA antibodies to native gliadin (AGA - IgG and AGA- IgA, respectively), were measured as previously described[25,26] at the laboratory of Immunogenetics of the St. Orsola-Malpighi Hospital, Bologna.

HLA Typing. In the patients suspected for NCG/WS and CD, HLA typing was performed at the laboratory of Immunogenetics of the St. Orsola-Malpighi Hospital, Bologna. The patients were genotyped for HLA DQA1 and DQB1 alleles.[27] HLA-DQ2 and HLA-DQ8 positivities were based on DQB1*02 and DQA1*05 and DQB1*0302 findings, respectively.

Duodenal Biopsy. In the patients suspected for NCG/WS or CD, the diagnostic work-up included six well-oriented duodenal biopsies (2 from the duodenal bulb and 4 from the distal duodenum) taken during upper gastroduodenoscopy. Biopsies were evaluated by 2 pathologists who were blinded to the clinical history of the patients and graded according to Marsh-Oberhüber.[28]

2.3 Symptom evaluation

Intestinal symptoms (bloating, abdominal pain, diarrhoea, epigastric pain and nausea) and extra-GI symptoms (fatigue, headache, anxiety, memory and cognitive disturbances, and numbness of arms or legs),[3] were examined. All recruited subjects completed a modified version of the Gastrointestinal Symptom Rating Scale[30-32] designed to rate (0 to 10) severity of symptoms commonly associated with NCG/WS.

2.4 Tissue collection and processing

According to the diagnostic work-up for NCG/WS and CD patients and specifically for the study protocol for FD and HC, all participants underwent an upper gastroduodenoscopy.

During this procedure, n= 4 mucosal biopsies (including the submucosa) were taken from the second portion of the duodenum and immediately collected in ice-cold Krebs buffer. Specimens were then oriented with the mucosal side face-down and dissected under a stereomicroscope (Leica S6E, Leica Microsystems, Italy) in order to obtain submucosal whole-mounts. These specimens were pinned flat and fixed in 4% paraformaldehyde buffered solution for 2 hours at room temperature. After three washes in phosphate-buffered saline (pH 7.2) solution, submucosal whole mounts were processed for immunohistochemistry (Figure 1A).

2.5 Immunohistochemistry

Submucosal whole mounts were analysed using a previously validated immunohistochemical protocol.[24] The antibody against neurofilament 220KDa (NF220KDa, rabbit polyclonal; 1:500, N4142, Sigma, USA) was used to identify perikarya and nerve fibers (Figure 1B),[33-35] and a mouse monoclonal antibody (working dilution 1:1000; MAB1222, Millipore, Germany) against the specific human tryptase was used to identify mast cells (Figure 1B).[36] Specificity for immunostaining was evaluated by a number of experiments including

omission of the primary or the secondary antibody, substitution of the primary antibody with a preimmune (generic) serum and, for the anti-tryptase antibody, with specific preabsorption tests yielding always negative immunolabeling in line with previous demonstration.[36]

2.6 Image acquisition and analysis

Submucosal whole mount preparations were examined by three different blinded observers on a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes and a motorized XYZ stage with auto-focus capability. The images were recorded with a DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe, Amsterdam, The Netherlands). Large images of the entire submucosal specimens (Figure 1A) were obtained by combining single field acquisitions (magnification 4x), which were automatically scanned and measured (mm^2) by the software. In each specimen, four three-dimensional (3-D) images were obtained by acquiring 4 randomly selected fields (magnification 20x, XY) scanned automatically by using a motorized XYZ stage with a step of 1 mm along the Z axis for the whole thickness of the sample (Figure 1C). For each 3-D image acquired, the total volume scanned was calculated.

In each 3-D image, the total number of neurons identified by the ant-NF220KDa antibody and the total number of MCs identified by the anti-tryptase antibody were counted (Figures 1 D-E). The density of neurons in each specimen was expressed as total number of neurons / volume (mm^2 * um) and as number of neurons / ganglion (means ± SD). The density of MCs infiltrating the submucosa was counted and expressed as number of cells / volume (mm^2 * um). The spatial relation (distance, D) between a MC and the closest nerve fiber was measured in these four 3-D spots (XYZ) / subject (Figure 1F).

Specifically, the planar D between one MC on focus and the closest nerve fiber and / or neuronal cell body at the same focus was manually measured in planar field (XY). These fields

were selected each 10 mm along the Z axis (i.e. the average diameter of a MC) in order to avoid re-count a given MC twice. We evaluated 4 fields (20x each of magnification) / sample because of the size of the specimens and considering that some fields close to blood vessels were excluded to avoid MC extravasation, which would have biased our measurements.[37] Then, the percentage of MCs localized at D< 5 μm and D < 15 μm was calculated according to the total number of tryptase-immunopositive MCs.

2.7 Statistical analysis

Statistical analysis was performed according to the appropriate tests for each considered variable. A D'Agostino & Pearson normality test was applied to verify the normality of the distributions. Continuous data were reported as mean ± SD, and categorical data were described as frequencies. Mann Whitney test, One-way analysis of variance (Kruskal-Wallis with Dunn's Multiple comparison test), X^2 and Fisher's exact test were applied. Correlation analysis was performed by Spearman's test. Two-tailed *P* values less than .05 were considered significant. Graphical representations of data were obtained using GraphPad software (GraphPad Prism version 5.00 for Windows, GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1 Clinical features of NCG/WS patients

Thirty-four adult patients (22-50 years, 27 females) with negative CD-specific serology and self-reported NCG/WS reported GI and/or extra-GI symptoms after ingestion of gluten-containing foods. In all subjects symptoms improved or disappeared when those foods were withdrawn for a period of 6 months, and recurred when re-introduced for a period of up to 1 month.

3.2 Symptom differences among NCG/WS, CD and FD patients

GI symptoms differed significantly between the NCG/WS, CD and FD group ($P<0.0001$). At diagnosis, 9.4% of NCG/WS patients and 43.5% of CD reported one GI symptom; 21.9% of NCG/WS and 30.4% of CD complained two GI symptoms; 68.8% of NCG/WS *vs.* 4.3% of CD reported three or more GI symptoms in contrast to the 100% of FD patients (Figure 2A).

There were no significant differences in bowel habit among NCG/WS, CD and FD groups (Figure 2B) ($P= 0.08$).

Notably, bloating and abdominal pain differed significantly among the three groups (Figures 2C-F). All NCG/WS patients complained of bloating *vs.* 43% of CD and 87.5% of FD ($P< 0.0001$), whereas 84.4% of NCG/WS patients *vs.* 17.4% of CD and 75% of FD ($P< 0.0001$) reported abdominal pain (Figures 2C and 2E). Bloating and abdominal pain severity scores differed significantly among the three groups ($P= 0.0008$ and $P< 0.0001$, respectively). Specifically, 6% of NCG/WS patients reported mild (score <5) bloating *vs.* 43.3% of CD and 37.5% of FD. The remaining 94% of NCG/WS complained of intense bloating (between 6 and 9) with two peaks corresponding to score 8 (30.3%) and 9 (21.2%) *vs.* 56.7% of CD and 62.5% of FD (Figure 2D). Abdominal pain was reported with a mild symptom score by 24% of NCG/WS patients *vs.* 78.3% of CD and 25% of FD patients. The remaining 76% of NCG/WS patients reported intense abdominal pain (score 6 to 9) with the highest peak (45.5%) at 8 *vs.* 21% of CD and 75% of FD (Figure 2F).

3.3 Submucosal neuronal density

The quantitative assessment on duodenal submucosal whole mount preparations revealed no significant differences ($P= 0.2996$) of neuronal density in the three patient groups (Figure 3A) and of the mean number of cell bodies / ganglia ($P= 0.3669$) (not shown).

3.4 Mast cell density

MC density was higher in FD *vs*. NCGS/WS ($P< 0.05$), CD ($P< 0.001$) and HC ($P< 0.001$). There were no differences comparing NCGS/WS and CD *vs*. HC. Notably, MC density was increased, although not significantly, in NCGS/WS *vs*. CD ($P= 0.07$) (Figure 3B).

3.5 Interspatial relation between mast cells and nerves

The percentage of MCs localized at D< 5 μm and D< 15 μm from the closest nerves was calculated on the total number of MCs. The percentage of MCs at D< 15 was significantly higher in NCGS/WS (61.7±26.23%), CD (60.2±19.36%) and FD (64.3±24.84%) *vs*. HC (27.8±11.22%) ($P< 0.0001$, $P< 0.001$ and $P< 0.001$, respectively), but not different among the three groups of patients (Figure 3C). The percentage of MCs at D< 5 μm was significantly higher in NCGS/WS (49.5±24.17%), CD (50±19.81%) and FD (47.6±22.5%) *vs.* HC (18.9±10.39%) ($P<0.0001$, $P<0.001$ and $P<0.001$, respectively), but not different among the three groups of patients (Figure 3D).

3.6 Clinical-pathological correlations

In NCG/WS patients, MC density was not correlated to the number of GI symptoms (data not shown), while it correlated with bloating ($P= 0.001$; R= 0.64) and abdominal pain severity ($P= 0.03$; R= 0.46) (Figures 4A-B). The percentage of MCs close to nerves (D< 5 μm) correlated with the number of GI symptoms (not shown) ($P= 0.05$; R= 0.48). Notably, the percentage of MCs in the range of D< 15 μm form nerves correlated with bloating ($P= 0.01$; R= 0.61) and abdominal pain severity ($P= 0.01$; R= 0.40) (Figures 4C-D).

In CD, no correlation resulted between MC density / proximity to nerves and GI symptoms, while in FD, MC density was highly correlated to the number of GI symptoms ($P= 0.03$; R= 0.88) and the presence of abdominal pain ($P= 0.05$; R= 0.83) (not shown).

In all three patient groups, MC density or MC-nerve spatial relation did not correlate to bowel habit, while the severity of bloating and abdominal pain were significantly correlated to each other (NCGS/WS: *P*= 0.0006; R= 0.57; CD: *P*= 0.0001; R= 0.83; FD: *P*= 0.05; R= 0.70) (not shown).

4. Discussion

Despite expanding research on gluten-related disorders, NCG/WS remains challenging for physicians for the lack of diagnostic biomarkers and the little knowledge of underlying pathophysiology of symptoms.

Considering the existing link between the neuro-immune activation and GI symptoms in functional disorders, such as IBS and FD, [9] we explored whether MC-nerve interactions in the submucosa of the upper gut could contribute to symptoms / manifestations of NCG/WS patients. Thus, we assessed comparatively MC infiltration and MC-nerve spatial relationship in whole mount preparations of the submucosal layer from routine duodenal biopsies of NCG/WS, CD and FD patients and healthy / asymptomatic controls. Consistently, the absence of neuronal abnormalities, as indicated by an unchanged number of neuronal density, was shown in the three patients groups. However, the number of MCs infiltrating the submucosa in NCG/WS patients, although not different form HC, was slightly increased compared to CD and significantly decreased compared to FD. Consequently, FD patients showed the highest number of MCs, while CD patients and HC had similar numbers. Taken together, in NCG/WS, as well as in FD, MCs and the local innate immunity activation may play a role in the mechanisms leading to GI symptoms in NCG/WS and FD.

Notably, the proportion of MCs in proximity to nerve fibers (within <15 μm) was a common feature to all three groups of patients *vs.* HC ($P< 0.001$). Specifically, about 60% and 45% of the total MCs infiltrating the submucosal was within< 15 μm and <5 μm , respectively, from the closest nerve fiber in NCG/WS, CD, and FD patients, which contrasts to the 20% MC density in HCs. In the NCGS/WS group MC density and the proportion of MCs proximal to nerves significantly correlated to the severity of bloating and abdominal pain, an association reported in patients with functional GI disorders such as FD and IBS.[38] Previous studies showed MC infiltration in colonic mucosa of IBS or post-infectious IBS patients[39] and MC vicinity to nerve endings contribute to severity and frequency of abdominal pain through the release of inflammatory/pro-nociceptive mediators.[40,41] Conceivably abdominal pain and bloating in NCG/WS patients may similarly arise, as in IBS, by MC-mediated release of messengers/bioactive substances activating upper gut afferent sensory nerves. In the duodenal submucosa of FD patients, Cirillo et al.[42] using immunohistochemical and calcium imaging techniques revealed that neuronal and glial cell morpho-functional abnormalities correlated with a significant MC and eosinophil infiltration, but the distance between MCs and nerve fibers was not assessed and abdominal (epigastric) pain was not associated with the number of MCs. Furthermore, an increased number of degranulated eosinophils in the duodenum has been identified as hallmark of FD.[43-45] Moreover, Carroccio et al.[46] investigated in detail the histologic characteristics of duodenal mucosa in 78 NCG/WS patients, 39 non-NCG/WS patients and 16 CD enrolled as positive control group. Interestingly, the duodenal mucosal biopsies of NCG/WS had a significantly higher number of intraepithelial CD3+ T cells, lamina propria CD45+ immunocytes, and eosinophils compared to non-NCG/WS controls. A significantly higher number of eosinophils was found in the duodenal lamina propria of dyspeptic NCG/WS patients compared to NCG/WS

patients without upper GI symptoms. In addition the rectal mucosa of the NCG/WS patients had more enlarged lymphoid follicles, intraepithelial CD3+ T cells, lamina propria CD45+ cells, and eosinophils *vs.* CD and non-NCG/WS controls. Carroccio et al. did not detect a significant increase of MCs in any examined gut segments, but, differently from their study performed in mucosal sections from paraffin-embedded biopsies, we analyzed submucosal whole mount preparations to better define the innervation and immune/inflammatory infiltrate throughout the submucosal layer. Clearly, distinct technical approaches may yield different results. Also, the heterogeneity of NCG/WS patients may account for different subsets with either MC or eosinophil infiltration. It would thus appear that submucosal duodenal MCs in close vicinity to nerves may involve MC-induced nerve sensitization, leading to symptoms including bloating and abdominal pain.[47] Furthermore, eosinophil infiltration may play a role for predominant dyspeptic symptoms. Clearly, further studies are necessary to understand whether MCs or eosinophils (or both) can be responsible for symptom-predominant subsets of NCG/WS patients.

The present study showed some limitations. First, we investigated only the second portion of the duodenum, and therefore we cannot exclude that MC infiltrate occurs also in the colon or other intestinal segments. Secondly, we did not enroll IBS patients. The lack of this subset prevented us to establish whether MCs could be detectable throughout the duodenal submucosal layer and their relationship with nerves in the upper GI tract. Thirdly, we cannot categorically exclude that some of NCG/WS patients overlapped with IBS, improving their symptoms after gluten withdrawal; however, the presence of skin and neurological manifestations in our series of patients points more towards a diagnosis of NCG/WS rather than IBS.[2, 6] Finally, although we provided new data on

the presence of MCs, neuro-MC interaction and associated GI clinical features in NCG/WS, the mechanisms underlying these relationships remain to be clarified.

In conclusion, our data demonstrated that, duodenal submucosal MC infiltration and the close proximity of MCs to nerves may be histopathological features underlying GI symptoms, i.e. abdominal pain and bloating severity, in NCG/WS. We support the idea that unraveling neuro-immune interactions may help the identification of reliable bio-markers and novel therapeutic approaches in patients with NCG/WS.

Funding information: This study was supported by grant RF 2016-02364814 from the Italian Ministry of Health and by University of Bologna and Ferrara (RFO and FAR funds, respectively) to RDG. RDG received research grants from Fondazione Del Monte of Bologna and Ravenna.

JT is supported by a Methusalem grant from Leuven University. TV is a clinical researcher supported by the FWO Flanders.

Conflict of interest: VS has participated as a consultant for Alfasigma, Admirall, Angelini, Bayer, Malesci, Takeda, and receives scientific support from AlfaSigma and Allergan unrelated to the present work. These consultancies did not influence the content of this article.

RDG has participated as a consultant for Shire, Sucampo, Coloplast, Kyowa Kirin International and Takeda and received grant support from Shire and Takeda unrelated to the present work. These consultancies did not influence the content of this article.

JT has given Scientific advice to AlfaWassermann, Allergan, Christian Hansen, Danone, Grünenthal, Ironwood, Janssen, Kiowa Kirin, Menarini, Mylan, Neutec, Novartis, Noventure, Nutricia, Shionogi, Shire, Takeda, Theravance, Tramedico, Truvion, Tsumura, Zealand and Zeria pharmaceuticals and has served on the Speaker bureau for Abbott, Allergan, AstraZeneca, Janssen, Kyowa Kirin, Menarini, Mylan, Novartis, Shire, Takeda, Truvion and Zeria. These interactions did not influence the content of this article.

Author's contributions: FG was involved in the study concept and design, data acquisition, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content; UV was involved in the study concept and design, data acquisition, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content; RR was involved in data acquisition, critical revision of the manuscript for important intellectual content; RL was involved in data acquisition, critical revision of the manuscript for important intellectual content; DB was involved in data acquisition, interpretation of data, critical revision of the manuscript for important intellectual content; FC was involved in data acquisition, interpretation of data, critical revision of the manuscript for important intellectual content; KVdH was involved in data acquisition, interpretation of data, critical revision of the manuscript for important intellectual content;

FB was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content; EB was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content; AG was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content; AC was involved in analysis and interpretation of data, critical revision of the manuscript for important

intellectual content; EB was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content; GC was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content; TV was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content; VS was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content; JT was involved in analysis and interpretation of data, critical revision of the manuscript for important intellectual content, study supervision. RDG was involved in the study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, study supervision.

Abbreviations: CD, celiac disease; FD, functional dyspepsia; GFD, gluten /wheat free diet; GI, gastrointestinal; IBS, irritable bowel syndrome; MC, mast cells; NCG/WS, non -celiac gluten / wheat sensitivity.

References

1. Volta U, De Giorgio R. New understanding of gluten sensitivity. Nat Rev Gastroenterol Hepatol 2012;9:295-9.
2. Catassi C, Alaedini A, Bojarski C, et al. The Overlapping Area of Non-Celiac Gluten Sensitivity (NCGS) and Wheat-Sensitive Irritable Bowel Syndrome (IBS): An Update. Nutrients 2017;9.
3. Volta U, Bardella MT, Calabro A, et al. An Italian prospective multicenter survey on patients suspected of having non-celiac gluten sensitivity. BMC Med 2014;12:85.
4. Volta U, De Giorgio R, Caio G, et al. Nonceliac Wheat Sensitivity: An Immune-Mediated Condition with Systemic Manifestations. Gastroenterol Clin North Am 2019;48:165-82.
5. Potter MDE, Walker MM, Jones MP, et al. Wheat Intolerance and Chronic Gastrointestinal Symptoms in an Australian Population-based Study: Association Between Wheat Sensitivity, Celiac Disease and Functional Gastrointestinal Disorders. Am J Gastroenterol 2018;113:1036-44.
6. Volta U, Tovoli F, Cicola R, et al. Serological tests in gluten sensitivity (nonceliac gluten intolerance). J Clin Gastroenterol 2012;46:680-5.
7. Barbaro MR, Cremon C, Stanghellini V, Barbara G. Recent advances in understanding non-celiac gluten sensitivity. F1000Res 2018;7.
8. Elli L, Tomba C, Branchi F, et al. Evidence for the Presence of Non-Celiac Gluten Sensitivity in Patients with Functional Gastrointestinal Symptoms: Results from a Multicenter Randomized Double-Blind Placebo-Controlled Gluten Challenge. Nutrients 2016;8:84.
9. Potter MDE, Walker MM, Keely S, Talley NJ. What's in a name? 'Non-coeliac gluten or wheat sensitivity': controversies and mechanisms related to wheat and gluten causing gastrointestinal symptoms or disease. Gut 2018;67:2073-7.
10. Jarbrink-Sehgal ME, Talley NJ. Duodenal and Rectal Eosinophilia Are New Biomarkers of Nonceliac Gluten Sensitivity. Clin Gastroenterol Hepatol 2019;17:613-5.
11. Biesiekierski JR, Newnham ED, Irving PM, et al. Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial. Am J Gastroenterol 2011;106:508-14; quiz 15.
12. Di Sabatino A, Volta U, Salvatore C, et al. Small Amounts of Gluten in Subjects With Suspected Nonceliac Gluten Sensitivity: A Randomized, Double-Blind, Placebo-Controlled, Cross-Over Trial. Clin Gastroenterol Hepatol 2015;13:1604-12 e3.
13. Volta U, Pinto-Sanchez MI, Boschetti E, et al. Dietary Triggers in Irritable Bowel Syndrome: Is There a Role for Gluten? J Neurogastroenterol Motil 2016;22:547-57.
14. Dale HF, Hatlebakk JG, Hovdenak N, Ystad SO, Lied GA. The effect of a controlled gluten challenge in a group of patients with suspected non-coeliac gluten sensitivity: A randomized, double-blind placebo-controlled challenge. Neurogastroenterol Motil 2018.
15. Dale HF, Biesiekierski JR, Lied GA. Non-coeliac gluten sensitivity and the spectrum of gluten-related disorders: an updated overview. Nutr Res Rev 2019;32:28-37.
16. Biesiekierski JR, Peters SL, Newnham ED, et al. No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates. Gastroenterology 2013;145:320-8 e1-3.
17. Skodje GI, Sarna VK, Minelle IH, et al. Fructan, Rather Than Gluten, Induces Symptoms in Patients With Self-Reported Non-Celiac Gluten Sensitivity. Gastroenterology 2018;154:529-39 e2.

417 18. Junker Y, Zeissig S, Kim SJ, et al. Wheat amylase trypsin inhibitors drive intestinal
418 inflammation via activation of toll-like receptor 4. J Exp Med 2012;209:2395-408.
419 19. Hollon J, Puppa EL, Greenwald B, et al. Effect of gliadin on permeability of intestinal
420 biopsy explants from celiac disease patients and patients with non-celiac gluten sensitivity.
421 Nutrients 2015;7:1565-76.
422 20. Uhde M, Ajamian M, Caio G, et al. Intestinal cell damage and systemic immune activation
423 in individuals reporting sensitivity to wheat in the absence of coeliac disease. Gut 2016;65:1930-7.
424 21. Volta U, Caio G, Karunaratne TB, Alaedini A, De Giorgio R. Non-coeliac gluten/wheat
425 sensitivity: advances in knowledge and relevant questions. Expert Rev Gastroenterol Hepatol
426 2017;11:9-18.
427 22. Barbara G, Cremon C, Carini G, et al. The immune system in irritable bowel syndrome. J
428 Neurogastroenterol Motil 2011;17:349-59.
429 23. Burns G, Carroll G, Mathe A, et al. Evidence for Local and Systemic Immune Activation
430 in Functional Dyspepsia and the Irritable Bowel Syndrome: A Systematic Review. Am J
431 Gastroenterol 2018.
432 24. Giancola F, Torresan F, Repossi R, et al. Downregulation of neuronal vasoactive intestinal
433 polypeptide in Parkinson's disease and chronic constipation. Neurogastroenterol Motil 2017;29.
434 25. Moeller S, Canetta PA, Taylor AK, et al. Lack of serologic evidence to link IgA
435 nephropathy with celiac disease or immune reactivity to gluten. PLoS One 2014;9:e94677.
436 26. Lau NM, Green PH, Taylor AK, et al. Markers of Celiac Disease and Gluten Sensitivity in
437 Children with Autism. PLoS One 2013;8:e66155.
438 27. Karell K, Louka AS, Moodie SJ, et al. HLA types in celiac disease patients not carrying
439 the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European Genetics Cluster on
440 Celiac Disease. Hum Immunol 2003;64:469-77.
441 28. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for
442 a standardized report scheme for pathologists. Eur J Gastroenterol Hepatol 1999;11:1185-94.
443 29. Tack J, Talley NJ, Camilleri M, et al. Functional gastroduodenal disorders.
444 Gastroenterology 2006;130:1466-79.
445 30. Catassi C, Elli L, Bonaz B, et al. Diagnosis of Non-Celiac Gluten Sensitivity (NCGS): The
446 Salerno Experts' Criteria. Nutrients 2015;7:4966-77.
447 31. Caio G, Volta U, Tovoli F, De Giorgio R. Effect of gluten free diet on immune response to
448 gliadin in patients with non-celiac gluten sensitivity. BMC Gastroenterol 2014;14:26.
449 32. Volta U, Caio G, De Giorgio R, et al. Non-celiac gluten sensitivity: a work-in-progress
450 entity in the spectrum of wheat-related disorders. Best Pract Res Clin Gastroenterol 2015;29:477-
451 91.
452 33. Lebouvier T, Neunlist M, Bruley des Varannes S, et al. Colonic biopsies to assess the
453 neuropathology of Parkinson's disease and its relationship with symptoms. PLoS One
454 2010;5:e12728.
455 34. Brehmer A, Croner R, Dimmler A, et al. Immunohistochemical characterization of putative
456 primary afferent (sensory) myenteric neurons in human small intestine. Auton Neurosci
457 2004;112:49-59.
458 35. Ganns D, Schrodl F, Neuhuber W, Brehmer A. Investigation of general and cytoskeletal
459 markers to estimate numbers and proportions of neurons in the human intestine. Histol Histopathol
460 2006;21:41-51.

36. Wang GD, Wang XY, Liu S, et al. Innervation of enteric mast cells by primary spinal afferents in guinea pig and human small intestine. Am J Physiol Gastrointest Liver Physiol 2014;307:G719-31.
37. Schemann M, Camilleri M. Functions and imaging of mast cell and neural axis of the gut. Gastroenterology 2013;144:698-704 e4.
38. GB BB, Carroll G, Mathe A, et al. Evidence for Local and Systemic Immune Activation in Functional Dyspepsia and the Irritable Bowel Syndrome: A Systematic Review. Am J Gastroenterol 2019;114:429-36.
39. Bashashati M, Moossavi S, Cremon C, et al. Colonic immune cells in irritable bowel syndrome: A systematic review and meta-analysis. Neurogastroenterol Motil 2018;30.
40. Barbara G, Stanghellini V, De Giorgio R, et al. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology 2004;126:693-702.
41. Barbara G, Wang B, Stanghellini V, et al. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 2007;132:26-37.
42. Cirillo C, Bessissow T, Desmet AS, et al. Evidence for neuronal and structural changes in submucous ganglia of patients with functional dyspepsia. Am J Gastroenterol 2015;110:1205-15.
43. Walker MM, Talley NJ, Prabhakar M, et al. Duodenal mastocytosis, eosinophilia and intraepithelial lymphocytosis as possible disease markers in the irritable bowel syndrome and functional dyspepsia. Aliment Pharmacol Ther 2009;29:765-73.
44. Du L, Shen J, Kim JJ, et al. Corrigendum: Increased Duodenal Eosinophil Degranulation in Patients with Functional Dyspepsia: A Prospective Study. Sci Rep 2017;7:46121.
45. Vanheel H, Vicario M, Boesmans W, et al. Activation of Eosinophils and Mast Cells in Functional Dyspepsia: an Ultrastructural Evaluation. Sci Rep 2018;8:5383.
46. Carroccio A, Giannone G, Mansueto P, et al. Duodenal and Rectal Mucosa Inflammation in Patients With Non-celiac Wheat Sensitivity. Clin Gastroenterol Hepatol 2019;17:682-90 e3.
47. Wouters MM, Vicario M, Santos J. The role of mast cells in functional GI disorders. Gut 2016;65:155-68.

498

499 **Figure Legends**

500 **Figure 1. Photomicrographs illustrating the quantitative immunohistochemical**
501 **analysis performed in this study.** Figure A illustrates a low-magnification picture of a duodenal
502 submucosal whole-mount preparation (the contour is highlighted in yellow line) from a HC;
503 figures B-F are representative examples of NCG/WS patients. Figure B shows the neurofilament
504 (NF)-immunoreactive (green fluorescence) neuronal network and the tryptase immunolabeled (red
505 fluorescence) mast cells along with the 3-D profile on the Z-axis for the two markers. The NF-
506 tryptase overlap is readily detectable in C. Figure C indicates three representative fields capturing
507 close neuro-mast cell contacts (in the inset). Pictures D and E illustrate nerve-mast cell distance
508 providing the basis for the quantitative analysis performed in this study. Figure F illustrates a
509 high-magnification of the insert in figure E. Scale bar: 100 μm in D; 150 μm in E.

510

511 **Figure 2. Gastrointestinal symptoms in the three pathological groups: NCG/WS**
512 **(orange), CD (red), FD (purple).** Percentages of patients showing: (A) N=0, N=1, N=2 or N≥3
513 gastrointestinal symptoms; a specific bowel habit phenotype (B); abdominal bloating (C) and
514 bloating according to the severity score (D); abdominal pain (E) and abdominal pain according to
515 the severity score for each group (F). (A) Fisher's exact test of contingency, ****P<0.0001; (B-F)
516 Chi-square test, ***P<0.001, ****P<0.0001.

517

518 **Figure 3. Submucosal neuron and mast cell densities in NCG/WS (orange), CD (red),**
519 **FD (purple) and HC (light blue).** Data are presented as means ± SD. (A) Neuronal density
520 expressed as number of neurons / volume (1 mm^2 of submucosa / each mm of thickness). (B) Mast

cell density expressed as number of mast cells (MCs) / volume; (C) Percentages of mast cells localized at less than < 15 μm from nerves in each group. (D) Percentages of mast cells localized at less than < 5 μm from nerves in each group. Kruskal-Wallis test with Dunn's Multiple Comparison test; +P=0.07; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 4. Clinical-pathological correlations in NCG/WS patients. (A) Correlation between mast cell density and bloating severity score; (B) Correlation between mast cell density and abdominal pain severity score; (C) Correlation between the percentage of mast cells localized at less than 15 μm form nerves and bloating severity score; (D) Correlation between the percentage of mast cells localized at less than 15 μm form nerves and abdominal pain severity score. Spearman Correlation test; *P<0.05; **P<0.01; ***P<0.001.