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

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

3  
4 **Running Title:** Neuro-immune cross-talk in gluten sensitivity

5  
6 Fiorella Giancola, PhD<sup>1</sup>, Umberto Volta, MD<sup>1</sup>, Roberta Repossi, MSc<sup>1</sup>,

7 Rocco Latorre, PhD<sup>2</sup>, Dorien Beeckmans, PhD<sup>3</sup>, Florencia Carbone, PhD<sup>3</sup>,

8 Karen Van den Houte, MSc<sup>3</sup>, Francesca Bianco, PhD<sup>1</sup>, Elena Bonora, PhD<sup>1</sup>,

9 Alessandra Gori, MSc<sup>1</sup>, Anna Costanzini, PhD<sup>4</sup>, Elisa Boschetti, PhD<sup>1</sup>,

10 Giacomo Caio, PhD<sup>1</sup>, Tim Vanuytsel, PhD<sup>3</sup>, Vincenzo Stanghellini, MD<sup>1</sup>,

11 Jan Tack, PhD<sup>3\*</sup> and Roberto De Giorgio, PhD<sup>4\*</sup>

12 \*These authors share co-last authorship

13  
14 *1 Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy;*

15 *2 Department Basic Science & Craniofacial Biology, New York University, New York, USA;*

16 *3 Translational Research Center for Gastrointestinal Disorders (TARGID), KU Leuven,*  
17 *Leuven, Belgium;*

18 *4 Department of Medical Sciences, University of Ferrara, Ferrara, Italy*

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20 **Corresponding author:**

21 Roberto De Giorgio

22 Internal Medicine Unit - Department of Medical Sciences

23 University of Ferrara, Italy

24 Arcispedale St. Anna, Via A. Moro, 8 - 44124 Cona, Ferrara, Italy

25 E-mail: [roberto.degiorgio@unife.it](mailto:roberto.degiorgio@unife.it); [dgrrrt@unife.it](mailto:dgrrrt@unife.it)

26 **Abstract**

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

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

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

44           **Conclusions & Inferences:** Submucosal MC infiltration and the close (within  $15\ \mu\text{m}$ ) MC-to-  
45 nerve proximity in the duodenum of NCG/WS patients are features providing a histopathological  
46 basis to better understand GI symptoms in this condition.

47

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

50

51        **Key Points**

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

64

65

66 **1. Introduction**

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

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

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

88 be processed to separate the mucosa from submucosa.<sup>24</sup> We focused on the duodenal submucosa  
89 for the following reasons: 1) the diagnostic work-up of patients with NCG/WS may include an  
90 upper GI endoscopy in order to show possible changes of duodenal mucosa,<sup>2</sup> where from 26% to  
91 96% of NCG/WS patients show a Marsh 1 degree of lesions at duodenal biopsy histology;<sup>21</sup> 2)  
92 duodenal submucosa whole mounts, derived from mucosal separation, show a denser innervation  
93 than the few nerve endings detectable in a single biopsy. Neuronal density, MC infiltration, MC-  
94 nerve interactions in the duodenal mucosa and their relationship with GI symptoms were assessed  
95 comparatively in NCG/WS, FD, CD patients, and in healthy asymptomatic controls.

96

## 97 **2. Patients and Methods**

### 98 ***2.1 Study Protocol and patient recruitment***

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

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

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

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

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

115 Excluding criteria applied to the four study cohorts included: no restrictive diet over the past  
116 6 months; absence of wheat allergy (assessed by specific IgE or skin prick tests); no previous  
117 history of liver disease and/or abnormal liver function tests.

## 118 ***2.2 Diagnosis of CD and Characterization of NCG/WS***

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

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

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

### 133 **2.3 Symptom evaluation**

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

### 139 **2.4 Tissue collection and processing**

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

142 During this procedure, n= 4 mucosal biopsies (including the submucosa) were taken from  
143 the second portion of the duodenum and immediately collected in ice-cold Krebs buffer.

144 Specimens were then oriented with the mucosal side face-down and dissected under a  
145 stereomicroscope (Leica S6E, Leica Microsystems, Italy) in order to obtain submucosal whole-  
146 mounts. These specimens were pinned flat and fixed in 4% paraformaldehyde buffered solution  
147 for 2 hours at room temperature. After three washes in phosphate-buffered saline (pH 7.2)  
148 solution, submucosal whole mounts were processed for immunohistochemistry (Figure 1A).

### 149 **2.5 Immunohistochemistry**

150 Submucosal whole mounts were analysed using a previously validated  
151 immunohistochemical protocol.<sup>24</sup> The antibody against neurofilament 220KDa (NF220KDa,  
152 rabbit polyclonal; 1:500, N4142, Sigma, USA) was used to identify perikarya and nerve fibers  
153 (Figure 1B),<sup>33-35</sup> and a mouse monoclonal antibody (working dilution 1:1000; MAB1222,  
154 Millipore, Germany) against the specific human tryptase was used to identify mast cells (Figure  
155 1B).<sup>36</sup> Specificity for immunostaining was evaluated by a number of experiments including



156 omission of the primary or the secondary antibody, substitution of the primary antibody with a  
157 preimmune (generic) serum and, for the anti-tryptase antibody, with specific preabsorption tests  
158 yielding always negative immunolabeling in line with previous demonstration.<sup>36</sup>

## 159 ***2.6 Image acquisition and analysis***

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

170 In each 3-D image, the total number of neurons identified by the ant-NF220KDa antibody  
171 and the total number of MCs identified by the anti-tryptase antibody were counted (Figures 1 D-  
172 E). The density of neurons in each specimen was expressed as total number of neurons / volume  
173 ( $\text{mm}^2 * \mu\text{m}$ ) and as number of neurons / ganglion (means  $\pm$  SD). The density of MCs infiltrating  
174 the submucosa was counted and expressed as number of cells / volume ( $\text{mm}^2 * \mu\text{m}$ ). The spatial  
175 relation (distance, D) between a MC and the closest nerve fiber was measured in these four 3-D  
176 spots (XYZ) / subject (Figure 1F).

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

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

### 185 ***2.7 Statistical analysis***

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

194

## 195 **3. Results**

### 196 ***3.1 Clinical features of NCG/WS patients***

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

### 201 ***3.2 Symptom differences among NCG/WS, CD and FD patients***

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

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

208 Notably, bloating and abdominal pain differed significantly among the three groups  
209 (Figures 2C-F). All NCG/WS patients complained of bloating *vs.* 43% of CD and 87.5% of FD  
210 ( $P < 0.0001$ ), whereas 84.4% of NCG/WS patients *vs.* 17.4% of CD and 75% of FD ( $P < 0.0001$ )  
211 reported abdominal pain (Figures 2C and 2E). Bloating and abdominal pain severity scores  
212 differed significantly among the three groups ( $P = 0.0008$  and  $P < 0.0001$ , respectively).  
213 Specifically, 6% of NCG/WS patients reported mild (score  $< 5$ ) bloating *vs.* 43.3% of CD and  
214 37.5% of FD. The remaining 94% of NCG/WS complained of intense bloating (between 6 and 9)  
215 with two peaks corresponding to score 8 (30.3%) and 9 (21.2%) *vs.* 56.7% of CD and 62.5% of  
216 FD (Figure 2D). Abdominal pain was reported with a mild symptom score by 24% of NCG/WS  
217 patients *vs.* 78.3% of CD and 25% of FD patients. The remaining 76% of NCG/WS patients  
218 reported intense abdominal pain (score 6 to 9) with the highest peak (45.5%) at 8 *vs.* 21% of CD  
219 and 75% of FD (Figure 2F).

### 220 **3.3 Submucosal neuronal density**

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

### 224 **3.4 Mast cell density**

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

### 228 ***3.5 Interspatial relation between mast cells and nerves***

229 The percentage of MCs localized at  $D < 5 \mu\text{m}$  and  $D < 15 \mu\text{m}$  from the closest nerves was  
230 calculated on the total number of MCs. The percentage of MCs at  $D < 15 \mu\text{m}$  was significantly higher  
231 in NCGS/WS ( $61.7 \pm 26.23\%$ ), CD ( $60.2 \pm 19.36\%$ ) and FD ( $64.3 \pm 24.84\%$ ) *vs.* HC ( $27.8 \pm 11.22\%$ )  
232 ( $P < 0.0001$ ,  $P < 0.001$  and  $P < 0.001$ , respectively), but not different among the three groups of  
233 patients (Figure 3C). The percentage of MCs at  $D < 5 \mu\text{m}$  was significantly higher in NCGS/WS  
234 ( $49.5 \pm 24.17\%$ ), CD ( $50 \pm 19.81\%$ ) and FD ( $47.6 \pm 22.5\%$ ) *vs.* HC ( $18.9 \pm 10.39\%$ ) ( $P < 0.0001$ ,  
235  $P < 0.001$  and  $P < 0.001$ , respectively), but not different among the three groups of patients (Figure  
236 3D).

### 237 ***3.6 Clinical-pathological correlations***

238 In NCG/WS patients, MC density was not correlated to the number of GI symptoms (data  
239 not shown), while it correlated with bloating ( $P = 0.001$ ;  $R = 0.64$ ) and abdominal pain severity ( $P =$   
240  $0.03$ ;  $R = 0.46$ ) (Figures 4A-B). The percentage of MCs close to nerves ( $D < 5 \mu\text{m}$ ) correlated with  
241 the number of GI symptoms (not shown) ( $P = 0.05$ ;  $R = 0.48$ ). Notably, the percentage of MCs in  
242 the range of  $D < 15 \mu\text{m}$  from nerves correlated with bloating ( $P = 0.01$ ;  $R = 0.61$ ) and abdominal  
243 pain severity ( $P = 0.01$ ;  $R = 0.40$ ) (Figures 4C-D).

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

247 In all three patient groups, MC density or MC-nerve spatial relation did not correlate to  
248 bowel habit, while the severity of bloating and abdominal pain were significantly correlated to  
249 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  
250 shown).

251

#### 252 **4. Discussion**

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

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

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

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

305  
306 The present study showed some limitations. First, we investigated only the second portion of the  
307 duodenum, and therefore we cannot exclude that MC infiltrate occurs also in the colon or other  
308 intestinal segments. Secondly, we did not enroll IBS patients. The lack of this subset prevented us  
309 to establish whether MCs could be detectable throughout the duodenal submucosal layer and their  
310 relationship with nerves in the upper GI tract. Thirdly, we cannot categorically exclude that some  
311 of NCG/WS patients overlapped with IBS, improving their symptoms after gluten withdrawal;  
312 however, the presence of skin and neurological manifestations in our series of patients points more  
313 towards a diagnosis of NCG/WS rather than IBS.<sup>2, 6</sup> Finally, although we provided new data on

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

316  
317 In conclusion, our data demonstrated that, duodenal submucosal MC infiltration and the close  
318 proximity of MCs to nerves may be histopathological features underlying GI symptoms, i.e.  
319 abdominal pain and bloating severity, in NCG/WS. We support the idea that unraveling neuro-  
320 immune interactions may help the identification of reliable bio-markers and novel therapeutic  
321 approaches in patients with NCG/WS.

322  
323  
324  
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369 supervision.

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

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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  $\mu\text{m}$  in D; 150  $\mu\text{m}$  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 $\geq$ 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  $\pm$  SD. (A) Neuronal density  
 520 expressed as number of neurons / volume (1  $\text{mm}^2$  of submucosa / each mm of thickness). (B) Mast

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

525  
526 **Figure 4. Clinical-pathological correlations in NCG/WS patients.** (A) Correlation  
527 between mast cell density and bloating severity score; (B) Correlation between mast cell density  
528 and abdominal pain severity score; (C) Correlation between the percentage of mast cells localized  
529 at less than  $15 \mu\text{m}$  from nerves and bloating severity score; (D) Correlation between the  
530 percentage of mast cells localized at less than  $15 \mu\text{m}$  from nerves and abdominal pain severity  
531 score. Spearman Correlation test;  $*P<0.05$ ;  $**P<0.01$ ;  $***P<0.001$ .

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