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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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(Article begins on next page)

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	
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26	Abstract

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 selfreported 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).

44 Conclusions & Inferences: Submucosal MC infiltration and the close (within 15 μm) MC-to45 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

27

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

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-} 71 ¹⁰ and the lack of biomarkers make the diagnosis of NCG/WS challenging. Furthermore, the actual 72 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 extra-GI symptom generation is still controversial¹¹⁻¹⁵ since non-gluten proteins and fermentable 75 short-chain carbohydrates have also shown similar effects although mainly in the GI spectrum.¹⁶⁻¹⁸ 76 77 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 78 adaptive, and even more of the innate ,immune response.²¹ 79

80 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 81 symptom generation in patients with IBS and FD.^{22,23} Based on the similarity of intestinal 82 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 duodenal submucosa by exploiting a novel technical approach through which routine biopsies can 87

be processed to separate the mucosa from submucosa.²⁴ We focused on the duodenal submucosa 88 89 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 90 96% of NCG/WS patients show a Marsh 1 degree of lesions at duodenal biopsy histology; ²¹ 2) 91 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 The Ethical Committee of the St. Orsola Hospital in Bologna (N° 119/2012/U/Tess) and of 99 100 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 content. They were then stratified in CD or NCG/WS according to the diagnostic work-up, including serological and genetic tests and histopathological evaluation.²⁵⁻²⁸ 104 105 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.⁶ Thirteen patients (19-60 years, 9 females) meeting the Rome III criteria for FD²⁹

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 ^{25,26} 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. ²⁷ 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. ²⁸

133 **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 Scale³⁰⁻³² designed to rate (0 to 10) severity of symptoms
 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 immunohistochemical protocol.²⁴ The antibody against neurofilament 220KDa (NF220KDa, 151 152 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, 153 154 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 155

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

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 (mm²) 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 $(mm^2 * um)$ and as number of neurons / ganglion (means \pm SD). The density of MCs infiltrating 173 174 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 175 176 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

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. ³⁷ Then, the percentage of
183	MCs localized at D< 5 μm and D < 15 μm 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), X^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

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).

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 μ m and D< 15 μ m from the closest nerves was 230 calculated on the total number of MCs. The percentage of MCs at D<15 was significantly higher 231 in NCGS/WS (61.7±26.23%), CD (60.2±19.36%) and FD (64.3±24.84%) vs. HC (27.8±11.22%) 232 (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 233 234 (49.5±24.17%), CD (50±19.81%) and FD (47.6±22.5%) vs. HC (18.9±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

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).

251

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.

256 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 257 258 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 patients groups. However, the number of MCs infiltrating the submucosa in NCG/WS patients. 263 264 although not different form 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 $<15 \mu$ m) was a common feature to all three groups of patients vs. HC (P < 0.001). Specifically, about 60% and 45% of the 270 271 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 patients with functional GI disorders such as FD and IBS.³⁸ Previous studies showed MC 275 276 infiltration in colonic mucosa of IBS or post-infectious IBS patients³⁹ and MC vicinity to nerve 277 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 278 279 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 submucosa of FD patients, Cirillo et al.⁴² using immunohistochemical and calcium imaging 281 282 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 identified as hallmark of FD.⁴³⁻⁴⁵ Moreover, Carroccio et al.⁴⁶ investigated in detail the histologic 286 287 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 was found in the duodenal lamina propria of dyspeptic NCG/WS patients compared to NCG/WS 291

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 vield 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.⁴⁷ 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.^{2, 6} 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.
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323	
324	
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343

344 Author's contributions: FG was involved in the study concept and design, data acquisition, 345 analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript 346 for important intellectual content; UV was involved in the study concept and design, data 347 acquisition, analysis and interpretation of data, drafting of the manuscript, critical revision of the 348 manuscript for important intellectual content; RR was involved in data acquisition, critical 349 revision of the manuscript for important intellectual content; RL was involved in data acquisition, 350 critical revision of the manuscript for important intellectual content; DB was involved in data 351 acquisition, interpretation of data, critical revision of the manuscript for important intellectual 352 content; FC was involved in data acquisition, interpretation of data, critical revision of the 353 manuscript for important intellectual content; KVdH was involved in data acquisition, 354 interpretation of data, critical revision of the manuscript for important intellectual content; 355 FB was involved in analysis and interpretation of data, critical revision of the manuscript for 356 important intellectual content; EB was involved in analysis and interpretation of data, critical 357 revision of the manuscript for important intellectual content; AG was involved in analysis and 358 interpretation of data, critical revision of the manuscript for important intellectual content; AC was 359 involved in analysis and interpretation of data, critical revision of the manuscript for important

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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.

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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 vellow 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.

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511 Figure 2. Gastrointestinal symptoms in the three pathological groups: NCG/WS

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

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Figure 3. Submucosal neuron and mast cell densities in NCG/WS (orange), CD (red),
FD (purple) and HC (light blue). Data are presented as means ± SD. (A) Neuronal density
expressed as number of neurons / volume (1 mm² 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 $< 15 \ \mu m$ from nerves in each group. (D) Percentages of mast cells localized
523	at less than $< 5 \ \mu m$ from nerves in each group. Kruskal-Wallis test with Dunn's Multiple
524	Comparison test; + <i>P</i> =0.07; * <i>P</i> <0.05; ** <i>P</i> <0.01; *** <i>P</i> <0.001; **** <i>P</i> <0.0001.
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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 μ m form nerves and bloating severity score; (D) Correlation between the
530	percentage of mast cells localized at less than 15 μ m form nerves and abdominal pain severity
531	score. Spearman Correlation test; * <i>P</i> <0.05; ** <i>P</i> <0.01; *** <i>P</i> <0.001.
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