**Supplementary material**

**Randomized Double-Blind Placebo-Controlled Crossover Trial for the Diagnosis of Non-Celiac Gluten Sensitivity: First Pediatric Experience**

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**Methods:**

**Serological tests**

Quantitative detection of AGA of -IgA and –IgG class in human serum or plasma were assessed by an indirect solid phase enzyme immunoassay (ELISA) test. The cut-off value was set for values greater than 10 arbitrary units (AU) for both (ORGENTEC Diagnostika; Mainz, Deutschland).

Quantitative detection of TTG-IgA and -IgG class in human serum or plasma were assessed by an indirect solid phase enzyme immunoassay (ELISA) test (ORGENTEC Diagnostika; Mainz, Deutschland). The cut-off value was set for values greater than 10 AU for both.

EMA-IgA was determined by indirect immunofluorescence using monkeys esophagus sections as substrate (Euroimmun Italia Diagnostica Medica SRL; Padova, Italia). Dilutions greater that 1:10 were considered positive and then titrated.

To exclude the presence of selective IgA deficiency (serum IgA concentrations <0.05 g/L), serum IgA levels were assayed by nephelometry in all patients.

**Allergical assessment**

Skin prick tests (SPT), prick by prick and atopy patch test (APT) were performed as follows, in order to exclude WA. For SPT wheat extract (Alyostal, Stallergenes, France) and fresh food (1 g of wheat powder dissolved in 10 ml of isotonic saline solution) were applied to the patient volar forearm. Skin prick tests were performed using a 1-mm single peak lancet (Allergy Therapeutics, UK), with histamine hydrochloride (10 mg/ml) as positive control and isotonic saline solution (NaCl 0.9%) as negative control. Reactions were recorded on the basis of the largest diameter (mm) of the wheal and flare at 15 minutes compared to histamine. The SPT result was considered positive if the wheal was 3 mm or larger, without reaction of the negative control.

Serum samples from patients were analyzed for specific IgE antibody titers against wheat and gluten using a commercially available kit (Immuno CAP Phadia 250, Sweden). The detection limit of the system was 0.35 kU/l IgE.

APT was performed in all children using fresh food (1 g of wheat powder dissolved in 10 ml of isotonic saline solution) put on filter paper and applied with adhesive tape to the unaffected skin of the child’s back, using 12-mm aluminium cups (Finn Chambers on Scanpor, Allergopharma, Germany). Isotonic saline solution was used as negative control. The occlusion time was 48 hours and results were read 20 min and 24 h after removal of the cups. Seventy-two hours after the start of the test, reactions were classified as follows: (-) negative; (+/-) doubtful: erythema only; (+) weak positive: erythema and slight infiltration; (++) strong positive: erythema, infiltration, papules; (+++) very strong positive: erythema, infiltration, papules, vesicles.

**HLA Typing**

Class II antigens HLA typing (DQA1\*02:01, DQA1\*03, DQA1\*05, DQB1\*02, DQB1\*03:01/03:04, DQB1\*03:02/03:05, DRB1\*03, DRB1\*04, DRB1\*07, DRB1\*11, DRB1\*12) was performed by polymerase chain reaction sequence specific oligonucleotide using DQ-CD Typing Plus; (DiaGene, Palermo, Italy).

**Supplementary table: Symptoms of children according to the result of the challenge in the different phases of the trial**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Symptoms (%)** | **phase 2**  **GFD open** | | **phase 3**  **Gluten challenge gluten** | | **phase 3**  **Gluten challenge placebo** | |
| **Gluten challenge positive** | **Gluten challenge negative** | **Gluten challenge positive** | **Gluten challenge negative** | **Gluten challenge positive** | **Gluten challenge negative** |
| **Gastrointestinal**  Abdominal Pain  Diarrhea  Dyspepsia  Bloating  Mixed bowel habits  Nausea  Constipation  Regurgitation | 36%  0%  0%  0%  0%  0%  9%  0% | 17%  0%  0%  5%  0%  5%  5%  0% | 81%  18%  27%  18%  9%  0%  9%  0% | 47%  23%  23%  5%  23%  0%  11%  0% | 18%  0%  0%  0%  0%  0%  0%  0% | 52%  11%  17%  23%  17%  5%  17%  5% |
| **Extra-intestinal**  Fatigue  Headache  Joint/muscle pains  Skin Rash  Irritability  Inappetence  Oral ulceration  Paresthesia | 72%  54%  54%  9%  0%  9%  0%  0% | 52%  35%  41%  17%  0%  0%  0%  0% | 100%^  81%  54%  9%  0%  9%  0%  0% | 52%^  47%  35%  11%  9%  0%  17%  5% | 54%  45%  18%  9%  0%  0%  0%  0% | 70%  64%  52%  23%  0%  0%  0%  0% |

^ p=<0,05