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NON-CELIAC GLUTEN SENSITIVITY IN CHILDREN WITH HEADACHE: A FOCUS ON CLINICAL AND IMMUNOLOGICAL ASPECTS

S.S.D. MED/04 – General Pathology, Immunology

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Non-celiac Gluten Sensitivity in children with headache: a focus on clinical and immunological aspects.
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SOMMARIO

INTRODUZIONE

Le cefalee sono patologie frequenti in età pediatrica, tendono ad avere decorso cronico e spesso comportano un notevole peggioramento della qualità di vita. Identificare eventuali cause o fattori scatenanti potenzialmente trattabili è un obiettivo primario nella gestione del paziente cefalalgico. Recenti evidenze indicano come la cefalea rappresenti uno dei sintomi maggiormente prevalenti nel contesto di patologie glutine-correlate, in particolare nella Sensibilità al glutine non celiaca (NCGS). Tuttavia la prevalenza di NCGS nei pazienti cefalalgici in età pediatrica, così come i meccanismi etio-patogenetici della cefalea nell'ambito di NCGS, sono per lo più sconosciuti.

SCOPI DELLO STUDIO

Gli obiettivi dello studio sono: 1) indagare la prevalenza ed il quadro clinico di NCGS in una coorte di pazienti in età pediatrica che presentano markers sierologici di possibile sensibilità al glutine (anticorpi anti-gliadina nativa – AGA); 2) valutare il profilo di espressione genica in cellule mononucleate del sangue periferico (PBMCs) raccolte in pazienti con NCGS e cefalea; 3) misurare nel siero dei pazienti cefalalgici alcuni mediatori solubili identificati mediante l'analisi del profilo di espressione genica di PBMCs e valutare l'eventuale positività per anticorpi anti - Tranglutaminasi 6 (TG6), markers di patologie neurologiche glutine-dipendenti.

PAZIENTI E METODI

Sono stati considerati per il reclutamento i soggetti di età inferiore ai 18 anni valutati per cefalea nel periodo 1/03/13 – 31/07/15 presso l'Unità di Neuropsichiatria Infantile dell'Azienda Ospedaliera Universitaria Integrata di Verona. I criteri di inclusione erano i seguenti: 1) diagnosi di cefalea primaria posta secondo i criteri ICHD III-beta; 2) accettare indagini diagnostiche comprendenti dosaggio di IgA totali, AGA IgG ed IgA, anticorpi anti-tranglutaminasi 2 IgA (TGA), IgE specifiche per grano, glutine, gliadina. Una volta acquisito il consenso informato, i pazienti sono stati sottoposti a valutazione di esame emocromocitometrico, profilo biochimico, indici di flogosi, studio della funzionalità epatica, renale e tiroidea, dosaggio di IgA totali, TGA, AGA IgA e IgG. Sono stati esclusi i pazienti che presentavano: cefalea secondaria o nevralgia cranica; trattamenti introdotti o modificati negli ultimi 2 mesi; deficit di IgA parziale o totale, secondo i riferimenti per età; soggetti positivi per TGA (sospetta celiachia) o IgE specifiche per grano, glutine, gliadina (sospetta allergia al grano); riscontro bioptico duodenale di enteropatia (indagine a completamento dell'iter diagnostico effettuata nei soggetti con riscontro di AGA-positività). Come da attuale protocollo diagnostico per sospetta Sensibilità al glutine non celiaca, i soggetti AGA-positivi

sono quindi stati sottoposti ad una dieta strettamente priva di glutine (DPG) per 3 mesi, con successiva reintroduzione di glutine nella dieta per ulteriori 3 mesi (gluten challenge). I soggetti AGA-negativi hanno proseguito una dieta a normale contenuto di glutine. L'andamento della cefalea è stato valutato al reclutamento e nel corso del follow-up mediante somministrazione di scale standardizzate e questionari specifici per rilevazione di disturbi emotivi e comportamentali in età pediatrica.

Per i pazienti con conferma diagnostica di NCGS è stata effettuata l'analisi dell'espressione genica in PBMCs raccolte in fase attiva di malattia. In relazione ai dati di espressione genica, sono stati scelti CTLA-4 solubile (sCTLA-4) e gp130 solubile (sgp130) come mediatori da dosare nel siero dei pazienti cefalalgici. E' stato inoltre effettuato il dosaggio sierico del mediatore CD25 solubile (sCD25), selezionato come marker di attivazione T-linfocitaria, e degli anticorpi TG6 IgA ed IgG.

RISULTATI

Sono stati reclutati 17 pazienti con emicrania o cefalea muscolo-tensiva: 11 soggetti AGA-positivi (4 maschi, 7 femmine; età media 10,6 anni; range d'età 7,9-12,5 anni), 6 soggetti AGA-negativi (2 maschi, 4 femmine; età media 11,6 anni; range d'età 9,1-13,8 anni). Tutti i soggetti AGA-positivi sono stati sottoposti a biopsia duodenale in corso di dieta a normale contenuto di glutine e nessuno ha avuto riscontro istologico di enteropatia. Successivamente i soggetti AGA-positivi hanno effettuato dieta aglutinata a scopo diagnostico seguita da challenge con glutine. Sei pazienti AGA-positivi su 11 (55%) hanno quindi ricevuto la diagnosi definitiva di NCGS, in relazione al miglioramento clinico in corso di DPG e ricaduta in fase di challenge.

Non sono emerse peculiarità cliniche in merito alle caratteristiche della cefalea nei pazienti con NCGS. Sintomi gastrointestinali sono risultati più frequenti in questi pazienti (50% dei soggetti) rispetto ai soggetti AGA-positivi non-NCGS o ai soggetti AGA-negativi. Sintomi extra-intestinali (astenia, tono dell'umore depresso, anemia, febbricola persistente, dermatite, scarso accrescimento) sono risultati più rappresentati nei soggetti AGA-positivi, ma non utili nel discriminare tra questi i soggetti con NCGS.

Tre soggetti AGA-positivi su 11 (27%), ovvero 1/6 (17%) AGA-positivi con NCGS e 2/5 (40%) AGA-positivi non-NCGS, presentavano anche anticorpi TG6 IgA. Nessuno dei soggetti AGA-negativi ha avuto riscontro di TG6 IgA. Nessun soggetto nei 3 gruppi è risultato positivo per anticorpi TG6 IgG. I soggetti con titoli elevati di anticorpi TG6 IgA non hanno mostrato miglioramento clinico nel corso dei 3 mesi in dieta aglutinata.

Il profilo di espressione genica in PBMCs di pazienti con NCGS ha documentato up-regolazione, in fase attiva di malattia, di numerosi geni correlati ad attivazione del

compartimento T- e B-linfocitario, nonché up-regolazione di geni correlati al compatimento Th17 e geni con signature di Interferone tipo I.

Al reclutamento, in dieta a normale contenuto di glutine, i livelli sierici di sCTLA-4 sono risultati più elevati nei soggetti AGA-positivi rispetto agli AGA-negativi, con differenza statisticamente significativa (livello medio 14.46±3.74 ng/ml vs 0.53±0.67 ng/ml; p=0.001). In corso di dieta aglutinata si è osservata una riduzione dei livelli di sCTLA4 in tutti i soggetti AGA-positivi, anche se la diagnosi di NCGS non è stata confermata in alcuni di essi. La differenza nei livelli di sCTLA-4 al reclutamento e dopo DPG è risultata statisticamente significativa nei pazienti con NCGS confermata (livello medio 14.46±3.74 ng/ml vs 4.28±3.46 ng/ml; p=0.036).

I livelli sierici di sgp130 non sono risultati significativamente differenti al momento del reclutamento nei soggetti con NCGS, quando confrontati con i soggetti AGA-positivi non-NCGS o AGA-negativi, né si è potuto osservare un significativo effetto della dieta aglutinata sul livello sierico di questo mediatore. Analogamente, i livelli sierici di sCD25 sono risultati simili nei 3 gruppi di pazienti.

CONCLUSIONI

Un sottogruppo di pazienti in età pediatrica con cefalea primaria soddisfa i criteri clinici per NCGS.

I dati sperimentali di analisi dell'espressione genica sono suggestivi di una condizione immuno-mediata con signature "Interferone di tipo I", tipicamente riscontrata in malattie autoimmuni. Elevati livelli sierici di sCTLA-4 in soggetti AGA-positivi potrebbero costituire un ulteriore indizio di sensibilizzazione al glutine e coinvolgimento della risposta immunitaria adattativa.

Il riscontro di anticorpi anti-TG6 nel siero di pazienti AGA-positivi con cefalea potrebbe essere predittivo di un'insoddisfacente risposta clinica alla dieta aglutinata perseguita per soli 3 mesi, giustificando pertanto un tentativo di trattamento dietetico più prolungato, verosimilmente di 6-12 mesi, come attualmente raccomandato per i soggetti con sospetta atassia da glutine positivi per anticorpi anti-TG6.

ABSTRACT

BACKGROUND

Headaches frequently occur in childhood, often with a relevant burden on quality of life. The identification of any underlying treatable causes or precipitating factors should be a primary endpoint in headache management. Headache is one of the most frequently reported complaints in patients with gluten-related disorders, particularly Non-celiac Gluten Sensitivity (NCGS), although prevalence of NCGS in headache patients in childhood is unknown. Moreover, etio-pathogenetic bases for headache in NCGS are poorly understood.

AIMS OF THE STUDY

The study aimed: 1) to explore the prevalence and the clinical picture of NCGS in a cohort of pediatric headache patients with serological markers of gluten sensitivity (native anti-gliadin antibodies - AGA); 2) to analyze gene expression profiles in peripheral blood mononuclear cells (PBMCs) from pediatric headache patients with NCGS; 3) to validate some of gene array results by the measurement of soluble mediators in sera of patients, and to determine serum levels of Tranglutaminase 6 (TG6) antibodies, markers of neurological gluten-related disorders.

PATIENTS AND METHODS

Patients aged <18 years and evaluated for headache at the Child Neuropsychiatry Unit, University Hospital of Verona, Italy, in the period 1/03/13 – 31/07/15 were considered for recruitment in the study. Inclusion criteria were: 1) to be diagnosed with a type of primary headaches according to the ICHD III-beta criteria; 2) to accept determination of total IgA, AGA IgG and IgA, anti-tranglutaminase 2 IgA antibodies (TGA), and specific IgE to wheat, gluten, gliadin on serum samples. After informed consent, patients were investigated with a complete blood count, biochemical analyses and evaluation of liver, renal and thyroid function and dosage of total IgA and TGA. Patients were excluded if one of the following conditions occurred: fulfilled criteria for secondary headaches or cranial nerve neuralgias; ongoing treatments for headache introduced or modified less than 2 months before; total or partial IgA deficiency, according to reference values for age; to have tested positive for TGA (suspected diagnosis of celiac disease) or IgE to wheat, gluten, gliadin (suspected diagnosis for wheat allergy); presence of enteropathy at duodenal biopsy (performed only in AGA-positive patients). Determination of AGA was performed. According to the diagnostic algorithm for NCGS, AGA-positive patients underwent a 3 months – period on gluten-free diet (GFD) followed by reintroduction of dietary gluten for 3 months (gluten challenge). AGA-negative patients continued on their normal, gluten-containing diet. The headache clinical course was evaluated by means of standardized scales and questionnaires.

PBMCs were collected from NCGS patients in active phase of the disease for gene expression profiling.

Based on results of gene expression profiling, soluble CTLA-4 (sCTLA-4) and soluble gp130 (sgp130) were chosen to be determined in sera of headache patients. Soluble CD25 (sCD25), chosen as a biomarker for T lymphocyte activation, and TG6 IgA and IgG were also measured in sera.

RESULTS

Seventeen patients were recruited with migraine or tension-type headache: 11 subjects (4 males, 7 females; mean age, 10.6 years; age range, 7.9-12.5 years) tested positive for AGA, 6 children (2 males, 4 females; mean age, 11.6 years; age range, 9.1-13.8 years) were AGA-negative. All AGA-positive patients had duodenal biopsy on gluten-containing diet: None of the subjects displayed histological signs of enteropathy. AGA-positive patients underwent GFD followed by gluten challenge. Six out 11 AGA-positive patients (55%) received the diagnosis of NCGS, due to clinical improvement on GFD and relapse on gluten challenge.

As for clinical picture, no headache features or migraine-associated syndromes resulted to be typical of NCGS patients. Gastrointestinal symptoms occurred more frequently in NCGS patients (50%) than in other patients. Extra-intestinal symptoms (fatigue, depression, anemia, long-lasting low-grade fever, dermatitis, failure to thrive) were more frequent in AGA-positive patients and very similar to those reported in NCGS cohorts, although they could not distinguish NCGS patients among other AGA-positive patients.

Three AGA-positive patients out 11 (27%), i.e. 1/6 (17%) AGA-positive NCGS patients and 2/5 (40%) AGA-positive no-NCGS patients tested positive for TG6 IgA. None of the subjects tested positive for TG6 IgA in the AGA-negative group. No one tested positive for TG6 IgG either. Patients with high levels of TG6 antibodies did not show clinical amelioration on a 3 months - period on gluten-free diet.

The gene expression profiling of PBMCs from NCGS patients documented up-regulation, in the active phase of the disease, of a high number of genes related to T- and B-lymphocyte activation and up-regulation of genes related to Th17 cell subset and type I Interferon response.

Levels of sCTLA-4 were significantly higher in headache AGA-positive patients than in AGA-negative subjects (mean levels 14.46±3.74 ng/ml vs 0.53±0.67 ng/ml; p=0.001), and sCTLA-4 levels markedly reduced on gluten-free diet in all subjects, despite NCGS diagnosis was not confirmed in some of them. The difference in mean levels at recruitment and on GFD was statistically significant in NCGS patients (mean levels 14.46±3.74 ng/ml vs 4.28±3.46 ng/ml; p=0.036).

Serum levels of sgp130 were not significantly different at recruitment in NCGS patient, as compared to other AGA-positive subjects or to AGA-negative headache patients, neither levels of this soluble mediator significantly changed on gluten-free diet. Serum levels of sCD25 were similar in NCGS patients, no-NCGS patients and AGA-negative subjects.

CONCLUSIONS

A subgroup of children or adolescents fully satisfying criteria for primary headaches has Non-celiac Gluten Sensitivity.

Experimental data from NCGS patients suggest the existence of an activated immune response with a type-1 Interferon signature. High levels of serum sCTLA-4 in AGA-positive patients may be a further clue for gluten sensitization and involvement of adaptive immunity.

The presence of TG6 antibodies in sera may predict a poor clinical response to gluten-free diet upon a 3 months – period, thus justifying a dietary treatment for 6-12 months at least, as recommended for patients with gluten ataxia who tested positive for TG6 antibodies.

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INTRODUCTION

1. Headache in the pediatric age

Headache is one of the most common health complaints in children and adolescents, often with a relevant burden on quality of life. Primary headaches, mainly migraine and tension-type headache, account for the great majority of cases in this age group. Nevertheless, secondary headaches should always be considered, and an accurate diagnostic work-up focused on early recognition of any underlying treatable causes should represent a primary endpoint for physicians [Bigal 2010, Ozge 2011].
Recently, several areas of research have contributed to better understand headache features in childhood, although significant gaps remain in our understanding of pathophysiology of this condition [Hershey 2012].

1.1 Epidemiology

Applying the International Classification of Headache Disorders second edition (ICHD II) criteria, Abu-Arafeh et al. [2010] found that the prevalence of headache was at approximately 58% in subjects up to 20 years of age (female to male ratio 1.5:1) and that it increases with age, ranging from 20% in children younger than 5 years to 75% in children aged 15 years [Bille 1962, Silanpoo 1991]. Migraines in childhood range 3.9% to 7%, whereas 4% to 20% of adolescent boys and 10% to 27% of adolescent girls will develop migraines [Carlsson 1996]. Boys suffer from migraines more than girls in early childhood, but this figure reverses at puberty [Bille 1962].

1.2 Disease characterization

In recent decades several definitions of pediatric migraine have been proposed, until the currently accepted classification system for headaches, which was published by the International Headache Society in 2013, the International Classification of Headache Disorders third edition beta version (ICHD-III beta) [[Bigal 2010, Headache Classification Committee 2013].

Primary headaches, secondary headaches, and painful cranial neuropathies are separately classified in ICHD-III beta. Migraine (with or without aura), and tension-type headache belong to the primary headache group and account for the vast majority of primary headaches in children. Secondary headaches represent a widely heterogeneous group of head pains due to traumas, intracranial or extracranial disorders (e.g., vascular diseases, masses, infections), assumption or withdrawal of substances, or disorders of homeostasis.

Requirements for correct and timely diagnoses are demographic data of patients, inter-ictal symptoms, and profiles of headache attacks, as well as personal and family history, and a complete physical examination [Hershey 2010, Karli 2006]. Moreover, the unique features in childhood headaches should be taken into account for disease characterization in this age group.

1.2.1 Migraine

Migraine attacks are often shorter in the pediatric population than in adults, lasting from 1 hour (in younger patients) to 48-72 hours [Slover 2015, Tarasco 2015].

The most frequent symptoms, for children, in migraine attacks are nausea and vomiting, photophobia and phonophobia, as expected for adult migraineurs, but also dizziness and vertigo, which are more likely in children than in adults [Tarasco 2015].

Dizziness and vertigo, as well as vomiting or abdominal pain, but also limb and body pain without traumatic or rheumatologic findings, occur frequently in children with migraine also as isolated symptoms in the inter-ictal period, and all together account for the so-called "migraine variants" and "migraine-associated syndromes".

Some of these migraine-associated syndromes (Cyclical vomiting syndrome, Abdominal migraine, Benign paroxysmal vertigo, Vestibular migraine, Benign paroxysmal torticollis), previously known as "Periodic syndromes of childhood", have been defined by the ICHD-III beta [Headache Classification Committee 2013].

Other common episodic syndromes in pediatric migraineurs (e.g. limb pain, paroxysmal dizziness, motion sickness) have not been included in ICHD-III beta classification yet, even though currently recognized by experts in childhood headaches as belonging to the spectrum of migraine variants and migraine-related conditions [Gupta 2016].

Notably, younger patients may have recurrent vomiting or abdominal pain even before developing recurrent head pain [Carson 2011]. Since these disorders typically start in young childhood prior to the ability to express headache, they may be underdiagnosed. Indeed, many patients receive in-deep evaluation for gastrointestinal disorders without clinically relevant findings, or even *ex-adjuvantibus* treatments for gastritis or gastroesophageal reflux, whereas they lack information and treatment for their migraine variants, which would be likely to respond to specific interventions for migraines.

1.2.2 Tension-type headache

Tension-type headaches (TTHs) occur in approximately 10% to 15% of youth, and they has been reported to be equal or more common than migraine in childhood, although studied only in a limited degree [Seshia 2009, Matthew 2011]. TTHs are usually less painful than migraines, shorter in duration, bilateral, posterior in location, and do not meet criteria for migraines, even though they can co-occur with migraine headaches and lead to chronic daily headaches [Slover 2015].

1.2.3 Secondary Headaches

Although primary headaches are the most common headaches, "red flags" for underlying causes should be systematically searched: an onset in the preschool age, a short history of headache or an accelerated course, elements suggesting a raised intracranial pressure, systemic illnesses or inter-ictal symptoms, such as fever, weakness, or personality changes [Celle 2010, John 2014].

In a small minority of patients, headaches are secondary to serious life-threatening acute or subacute disorders (e.g., subarachnoid or cerebral parenchymal hemorrhage, carotid or vertebral dissection, brain mass lesions) [Conicella 2008, Scagni 2008]. However, the most frequently secondary headaches in various studies of pediatric emergency setting are those related to viral infections (29–39%) or minor head traumas [Lewis 2000, Conicella 2008, Abend 2010]. Nevertheless, symptoms and signs for other systemic disorders should be considered, e.g. causes for hypoxia, hypercapnia, hypoglycemia, but also iron deficiency, obstructive sleep apnea syndrome, or autoimmune disorders such as thyroiditis or celiac disease [Abend

2010]. As for the latter, physicians should remind that celiac disease often occurs without gastrointestinal symptoms, and it seems to be 4- to 10-fold more prevalent in headache patients than in the healthy general population [Gabrielli 2003, Dimitrova 2013].

1.3 Long-term prognosis

Prognosis of headache primarily depends on headache phenotype. If the priority for secondary headaches is the identification and the proper management of underlying disorders, the main endpoint for primary headaches should be the reduction of interference of headache on quality of life through developmental ages.
It is well-established that primary headache often have their onset in childhood and become progressively more prevalent. It was estimated that 25% of pediatric patients had recurrent headaches along a 3 years-period, and that the prevalence of high-frequency episodic headaches or chronic daily headaches was about 1.7-2% in children and adolescents from the general population [Arruda 2010, Dunn 2011, Lipton 2011].

1.4 Pathophysiology of headache

1.4.1 Migraine

The etio-pathogenetic features of migraine have been extensively studied, but many aspects are still unclear. Burstein et al. [2015] provided an extensive review on etio-pathogenesis of migraine.
Migraine is a multifactorial condition. A family history for migraine is reported in 60% to 88% of patients, and some susceptibility genes have been identified, dealing with regulation of neurotransmitter release, glial ability to reuptake glutamate from the synapse, and generation of action potentials, but also regulation of synaptic development and plasticity [Ophoff 1996, Hernandez-Latorre 2011, Tarasco 2015, Burstein 2015]. However, environmental factors are necessary for the onset of attacks.

Currently, the neurovascular theory is the most accredited to explain onset of pain in migraine attacks. According to this theory, the initial phenomenon in migraine is cortical spreading depression, i.e. a slowly propagating wave of depolarization/excitation in cortical neurons and glia, followed by hyperpolarization/inhibition, possibly due to mechanisms that invoke inflammatory molecules as a result of physiological or emotional stress [Cui 2014, Ferrari 2015]. The initial membrane depolarization of cells in the cortex is associated with efflux of potassium, influx of sodium and calcium, release of glutamate, ATP, and hydrogen ions, with neuronal swelling, upregulation of genes involved in inflammatory processing, and activation of enzymes, such as caspase-1. This event can initiate inflammation by releasing interleukin-1 into the cerebro-spinal fluid, which in turns activates nuclear factor-kB in astrocytes, and release of cyclooxygenase-2 and inducible nitric oxide - synthase into the subarachnoid space. The introduction of pro-inflammatory molecules into the meninges and a high level of hydrogen ions alters the molecular environment in which meningeal nociceptors exist, and activates pain fibers which densely innervate meninges. When activated in an altered molecular environment, peripheral trigemino-vascular neurons become sensitized and begin to respond to dura stimuli and innocuous mechanical and thermal stimulation of cephalic and extracephalic skin areas as if it were noxious. The stimulation of meningeal nociceptors leads to the activation of the trigemino-vascular pathway. This pathway originates in trigeminal ganglion neurons whose peripheral axons reach the pia, dura, and large cerebral arteries and whose central axons reach the spinal trigeminal nucleus and then brainstem nuclei, hypothalamic nuclei, and basal ganglia nuclei. These projections may be critical for the initiation of nausea, vomiting, yawning, lacrimation, urination, loss of appetite, fatigue, anxiety, irritability, and depression by the headache itself. Additional projections of spinal trigeminal nucleus neurons are found in the thalamic nuclei, where relay trigemino-vascular thalamic neurons project to the somatosensory, insular, motor, parietal association, auditory, visual, and olfactory cortices, which are finally altered in their functioning, providing an explanation for the cortically-mediated symptoms in migraine (e.g., motor clumsiness, difficulty focusing, amnesia, allodynia, phonophobia, photophobia, and osmophobia).

The clinical practice with migraineurs suggests that migraine brains are extremely sensitive to deviations from homeostasis. Therefore, it has been hypothesized that hypothalamic neurons regulating homeostasis might be at the origin of some of the migraine prodromes (e.g., fatigue, depression, irritability, food cravings), by altering the balance between parasympathetic and sympathetic tone in the meninges toward the predominance of parasympathetic tone, thus activating meningeal nociceptors.

1.4.2 Tension-type headache

Information about pathophysiology of tension-type headache is less complete, but some mechanisms leading to this prevalent primary headache disease have been clarified and extensively reviewed by Bendtsen et al. [2011].

The processing of pain from myofascial tissues is finely regulated. Under some conditions, the painful stimulus from the pericranial myofascial tissues may be more prolonged or more intense than normal. The underlying mechanisms may include an increased muscle activity or the release of various chemical mediators secondary to local pathological conditions. An increased muscle activity secondary to psychogenic stress is likely to be of relevance in this respect. The reason may be because the psychogenic stress condition may cause a prolonged increase of muscle tone via the limbic system and potentiate pain facilitation from the brainstem to the spinal dorsal horn.

In predisposed individuals, the prolonged nociceptive input from the pericranial myofascial tissues may lead to sensitization of nociceptive second-order neurons at the level of the spinal dorsal/trigeminal nucleus. It is possible that low-grade tension, that normally does not result in pain, does so in the presence of central sensitization. It also has been demonstrated that emotional factors like stress and anxiety may contribute to the chronicization of TTH.

1.4.3 Headache and the sickness behavior

It is well known that animals dealing with infections and inflammation show various behavioral symptoms of sickness, including lethargy, anorexia and reduced social activities. A body of experimental data supports the hypothesis that these behavioral changes are not merely the result of physical debilitation due to the infection, but

they represent a set of coordinated responses to the presence of infection and inflammation [Dantzer 1998]. Similar behavioral changes occur when humans experience some types of pain, particularly "inescapable" pain, i.e. the pain which comes from the activation of C-fibers localized in the deep viscera, meninges and cerebral vascular tree included, and is implied in the activation and transmission of visceral homeostatic pain [Bonavita 2011]. Inescapable pain leads to passive coping strategies characterized by a disengagement from the external environment, and includes motor quiescence, hyporeactivity, decreased vigilance, reduced responsiveness to tactile or visual stimuli, sympatho-inhibition and vasodepression. This complex defensive reaction has been termed as sickness behavior [Hart 1988]. According to this observation, it seems arguable that pain may promote the complex and coordinated sensorial-emotive behavioral experience underlying the allostatic processes, i.e. all mechanisms finalized in restoring homeostasis, including anticipatory behavioral measures to prevent whatever impending derangement of the homeostatic balance, or to maintain or restore internal homeostasis of the body [Cortelli 2010, Bonavita 2011].

As other experiences of pain, headache and all multifaceted symptoms and signs associated to headache attacks may be read as a complex and integrated allostatic defensive reaction to an inescapable pain, that allows the restoration of biologic homeostasis through a temporary disengagement from active interaction with environment. The neurochemical basis of such reaction might involve the cytokines system, mainly TNFα, IL-1β, and IL-6, implied in hyperalgesia and allodynia as well as in visceral homeostasis signaling and in the so-called immune-to-brain communication [Maier 1998, Watkins 2000, Quan 2008, Montagna 2010, Benson 2012].

Even more convincingly, it is well-established that system inflammation due to infections or injuries is able to induce brain inflammation [Poon 2015]. Peripheral cytokines can communicate with the brain via the humoral routes and the neural routes. In respect to the humoral routes, plasma cytokines may enter the brain at the choroid plexus and at the circumventricular organs which lack a complete blood-brain barrier, thus activating local macrophage-like cells to produce cytokines into the brain parenchyma, or acting on the brain endothelium to stimulate the secretion of cytokines and prostaglandins. Furthermore, there is evidence to indicate that IL-1

and TNF can be actively and selectively transported into the brain via saturable transporter mechanisms. In terms of the neural routes, systemic cytokines can take effect on the primary afferent nerves. For example, the vagus nerve expresses IL-1 receptors and prostaglandin-E receptors, and its electrical firing can be triggered by ligand-receptor interaction, thus conveying electrical signals to the brain, where other cytokines are produced.

2. Gluten-related disorders

Gluten is the main structural protein complex of wheat; equivalent proteins are present in other cereals, including rye and barley. In genetically predisposed individuals, gluten or some protein fractions, such as gliadins and glutenins, can be toxic [Sapone 2012].

The Figure 1 summarizes the recently accepted classification of gluten-related disorders, based on their pathogenesis.

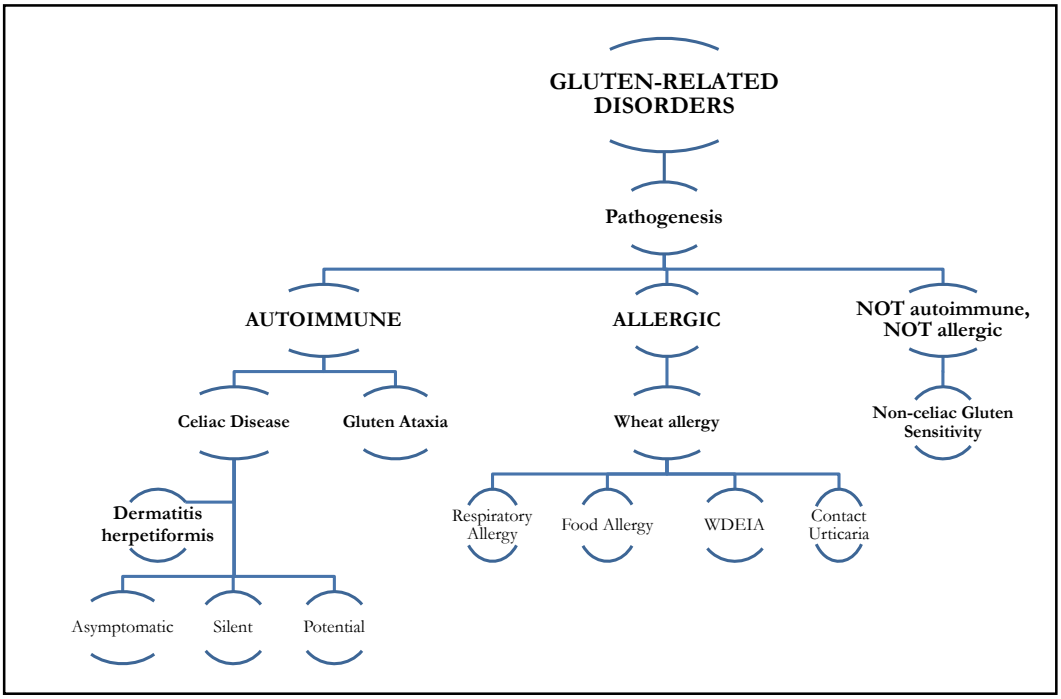

Figure 1. Classification of gluten-related disorders. WDEIA: wheat-dependent, exercise-induced allergy. Modified from Sapone *et al.,* BMC Med. 2012 Feb 7;10:13.

The best known human diseases related to gluten exposure, i.e. wheat allergy and celiac disease, are mediated by the adaptive immune system. In celiac disease (CD), the reaction to gluten is mediated by T-cell activation in the gastrointestinal mucosa. In wheat allergy the cross-linking of specific IgE by repeat sequences in gluten peptides triggers the release of histamine from basophils and mast cells [Tanabe 2008].

CD is an autoimmune disorder, as demonstrated by specific serologic autoantibodies, most notably serum anti-tissue transglutaminase 2 (TGA) and anti-endomysial antibodies (EMA). CD is responsible for enteropathy in genetically susceptible individuals, who carry HLA-DQ2 or HLA-DQ8 haplotypes. Genetic predisposition plays a key role in CD because the presence of HLA-DQ2 and/or HLA-DQ8 is necessary for disease development, although it is not sufficient on its own. The clinical spectrum of CD includes symptomatic cases with either classical intestinal (e.g., chronic diarrhea, weight loss) or non-classical extra-intestinal features (e.g., anemia, osteoporosis, neurological symptoms), and silent forms that are occasionally discovered because of serological screening. Complications associated with untreated celiac disease include osteoporosis, impaired splenic function, neurologic disorders, infertility or recurrent abortion, ulcerative jejuno-ileitis, and cancer. Enteropathy-associated T-cell lymphoma and adenocarcinoma of the jejunum are rare complications of celiac disease [Fasano 2012]. Enteropathy in CD is characterized by histological changes in duodenal mucosa, including an increased number of intraepithelial lymphocytes (IELs) (>25 lymphocytes per 100 enterocytes), elongation of the crypts, partial to total villous atrophy and a decreased villous/crypt ratio. The etio-pathogenesis of CD has been extensively investigated. Gluten, the environmental trigger, crosses the intestinal epithelium through different routes, as gut permeability is increased in CD. Gliadin peptides bind to the chemokine receptor CXCR3, which is over-expressed at the apical side of enterocytes in CD patients, leading to an increased release of zonulin, which enables paracellular translocation of gliadin peptides to the lamina propria by disassembling intercellular tight junctions. Once gluten reaches the lamina propria, it is deamidated by tissue tranglutaminases (TG), so that it becomes rich in negatively charged glutamate residues with increased affinity for the HLA-DQ2/-DQ8 molecules. Deamidated gluten is then presented by antigen presenting cells to HLA-DQ-restricted gluten-reactive CD4+ T cells. Upon

their activation, gluten-specific CD4+ T cells produce pro-inflammatory cytokines, dominated by IFNγ, thus inducing a Th1 response. In addition to Th1 cells, a pathogenic role in CD is also played by IL-17A-producing Th17 cells. Both Th1 and Th17 immune responses are enhanced by IL-21 in active CD. On the other hand, Th2 cytokines drive the activation and clonal expansion of B cells with subsequent production of anti-tranglutaminase 2 antibodies (TGA), the serological hallmark of CD [Sapone 2012, Di Sabatino 2015].

Dermatitis herpetiformis and gluten ataxia are autoimmune disorders too.

Dermatitis herpetiformis (DH) is a skin manifestation of CD: EMA and TGA occur in the serum, and the rash is gluten-sensitive; recently, antibodies directed at epidermal transglutaminase (TG3) have been identified in patients with DH and this may be the dominant autoantigen in the disorder [Sapone 2012].

Gluten ataxia (GA) was originally defined as an otherwise idiopathic, sporadic ataxia characterized by autoimmune processes resulting in damage of the cerebellum and positive serological markers for gluten sensitization. GA is usually of insidious onset with a mean age at onset of 50 years. Pediatric cases, however, have also been described. GA usually presents with pure cerebellar ataxia primarily affecting gait and lower limbs; rarely, it can present with ataxia in combination with focal myoclonus, palatal tremor, or opsoclonus. Specific autoantibodies in GA have not been definitely established: TGA are present in about 40% of patients, some patients have AGA. One out 3 patients has bioptic evidence of enteropathy, but less than 10% of patients with GA has any gastrointestinal symptoms. Recently, antibodies directed at brain transglutaminase (TG6) have been identified. TG6 antibodies react with Purkinje cells and cells within the granular layer in the cerebellum. Moreover, accumulation of IgA deposits in the cerebellum and brainstem were observed in post-mortem brain studies, most prominently within the muscular layer surrounding vessels, with perivascular inflammation and changes in vessel permeability. Thus, the blood-brain barrier function is altered, and gluten and antibodies are allowed to enter into the Central Nervous System [Mitoma 2015]. It has been estimated that antibodies against TG2 and antibodies against TG6 can be found up to 85% of patients with ataxia who are positive for AGA. Thus, neurologists expert in gluten-related disorders recommend screening of patients with progressive cerebellar ataxia using AGA, TGA and anti-TG6 antibodies. Subjects positive for any of these antibodies with no

alternative cause for their ataxia should be offered a strict GFD: stabilization of neurological symptoms after one year on GFD would be a strong indicator that the patient suffers from GA, even though response to treatment with a gluten-free diet depends on the duration of the ataxia prior to the diagnosis. Autoantibodies should disappear within 6-12 months on GFD [Sapone 2012].

A third clinical entity, for neither which allergic, nor autoimmune mechanisms have been identified, has been introduced among gluten-related disorders and generically named Non-Celiac Gluten Sensitivity (NCGS) [Sapone 2012, Catassi 2013].

2.1 Non-Celiac Gluten Sensitivity

Non-Celiac Gluten Sensitivity is a syndrome characterized by intestinal and extra-intestinal symptoms related to the ingestion of gluten-containing food, in subjects that are not affected by either celiac disease or wheat allergy. The terminology "Non-Celiac Gluten Sensitivity" is still a matter of debate, because NCGS is triggered by gluten-containing cereals, but the offending dietary protein has not been identified yet, and could include components other than gluten itself (e.g., the cereal protein amylase-trypsin inhibitors) [Junker 2012, Catassi 2015].

2.1.1 Epidemiology

Epidemiological data on NCGS are scant [Volta 2015]. According to data from US, the prevalence of NCGS was 0.6% at a primary care level, and about 6% in a tertiary care center for celiac disease [Sapone 2012, Di Giacomo 2013]. An Italian multicenter prospective survey compared the prevalence of NCGS and CD diagnoses in referral pediatric and adult centers for gluten-related disorders, being NCGS and CD diagnosed in 3.2% and 2.8%, respectively, with an estimated NCGS/CD ratio about 1/1 in Italy [Volta 2014].

NCGS appears to occur more frequently in adults (mean age at onset 40 years) and among females (female/male ratio 5:1) [Volta 2014]. The prevalence of markers of gluten-related disorders or ascertained celiac disease and NCGS are reported as 4- to 10-fold higher in headache patients, both adults and children, than in general population [Gabrielli 2003, Lionetti 2009, Lionetti 2010, Dimitrova 2013].

2.1.2 Clinical Picture

The clinical involvement in NCGS appears to be multi-systemic.
Gastrointestinal symptoms overlap to those reported by patients with irritable bowel syndrome and include abdominal pain/discomfort, bloating and bowel habit changes (diarrhea, alternating bowel, constipation) [Catassi 2013, Volta 2014, Volta 2015]. Upper gastrointestinal manifestations include epigastric pain, nausea, aerophagia, gastroesophageal reflux disease, and aphthous stomatitis. Extra-intestinal features include fatigue, headache, "foggy mind", arm/leg numbness and anxiety/depression. Other systemic signs are joint/muscle pain resembling fibromyalgia, skin rash/dermatitis, weight loss, iron-deficiency and/or folic acid anemia and, although rarely, allergic manifestations such as asthma and rhinitis [Volta 2014].
Gastrointestinal symptoms are supposed to be more frequent in young patients than extra-intestinal manifestations (fatigue, headache, limb pain) [Francavilla 2014].
More than 20% of NCGS patients show symptoms of allergy, autoimmune disorders seem to be rare [Sapone 2012, Volta 2012, Catassi 2013, Volta BMC Med 2014]. Further data are needed to establish whether complications might occur, as in CD [Sapone 2012, Volta 2013].

2.1.3 Diagnostic Criteria

NCGS should be suspected in patients with persistent intestinal and/or extra-intestinal complaints which worsen after eating gluten-rich food but do not display the CD and wheat allergy serologic markers.
Although the most specific CD serologic markers, such as TGA and EMA, are negative in NCGS patients by definition, IgG-class AGA directed against native gliadin are found more frequently in these cases (about 50%) than in the general population, when eating a gluten-containing diet. Therefore, the finding of isolated IgG-AGA positivity may be a clue to the diagnosis of NCGS. When initially positive, some authors observed that IgG AGA normalize more quickly in NCGS than CD patients after starting treatment with the GFD [Caio 2014]. However, the determination of IgG AGA cannot be recommended for clinical use due to the poor

specificity of this test, being found in CD (80–90% of patients), autoimmune liver disorders (21,5%), connective tissue disease (9%) and Irritable Bowel Syndrome (20%), as well as in healthy controls (2-8%) [Volta 2008, 2012 Catassi 2015].

Any correlation with HLA-DQ2 and/or -DQ8 has been identified [Sapone 2012, Volta 2012].

Since no biomarker is sensitive and specific enough for diagnostic purposes, the aim of the confirmation of the diagnosis of NCGS should include two subsequent stages in the patient who is on a normal, gluten-containing diet: (1) assessing the clinical response to the gluten-free diet; (2) measuring the effect of reintroducing gluten for one week followed by a one-week washout of strict GFD and by the crossover to the second one-week challenge, possibly in a blinded manner. The standardization of the diagnostic procedure has been published last year. The threshold of a 30% increment in symptoms in the gluten challenge stage has been proposed as a positive challenge, but this threshold is arbitrary and needs validation [Catassi 2015].

Performing a duodenal biopsy on a gluten-containing diet is another important step in the diagnostic approach to NCGS, particularly to rule out an underlying seronegative CD [Volta 2015]. Although small bowel villous architecture is normal in NCGS, the number of intraepithelial lymphocytes (IELs), defined normal if ≤25% [], is increased in a high proportion of patients, thus suggesting the existence of a mucosal low-grade inflammation [Hayat 2002, Sapone 2011, Volta 2014]. This mild lymphocytic intestinal infiltration can result in a specific NCGS histological pattern, characterized by a linear distribution of CD3+ T lymphocytes in the deeper part of the mucosa, together with clusters of the same cells in the superficial epithelium [Villanacci 2013]. Unlike CD, in NCGS there is no increase of T-cell receptor gamma/delta IELs [Sapone 2011]. Although small intestinal mucosa in NCGS shows a normal (i.e. >3:1) villous/crypt ratio, it is acknowledged that a mild inflammation may contribute to impair intestinal absorption [Volta 2012].

The Figure 2 summarizes the clinical and diagnostic features of NCGS, compared to celiac disease and wheat allergy.

	CD	NCGS	WA
Time interval between gluten exposure and onset of symptoms	Weeks–years	Hours–days	Minutes–hours
Pathogenesis	Autoimmunity (innate and adaptive immunity)	Immunity? (innate immunity?)	Allergic immune response
HLA	HLA-DQ2/8 restricted (~97% positive cases)	Not HLA-DQ2/8 restricted (50% DQ2/8-positive cases)	Not HLA-DQ2/8 restricted (35–40% positive cases as in the general population)
Autoantibodies	Almost always present	Always absent	Always absent
Enteropathy	Almost always present	Always absent (slight increase in IEL)	Always absent (eosinophils in the lamina propria)
Symptoms	Both intestinal and extra-intestinal (not distinguishable from GS and WA with GI symptoms)	Both intestinal and extraintestinal (not distinguishable from CD and WA with GI symptoms)	Both intestinal and extra-intestinal (not distinguishable from CD and GS with GI symptoms)
Complications	Co-morbidities, long-term complications	Absence of co-morbidities and long-term complications (long follow-up studies needed to confirm it)	Absence of co-morbidities, short-term complications (including anaphylaxis)

GI = Gastrointestinal; GS = gluten sensitivity; IEL = intraepithelial lymphocytes.

Figure 2. Clinical and diagnostic features of Non-celiac Gluten Sensitivity (NCGS), compared to Celiac Disease (CD) and Wheat Allergy (WA). From Catassi C. Gluten Sensitivity. Ann Nutr Metab 2015; 67 Suppl 2:16-26.

2.1.4 Pathophysiology

The etio-pathogenetic features of NCGS are still unclear [Elli 2015].

In a study conducted by Sapone et al. [2011], NCGS subjects did not show increased intestinal permeability. Coherently with this observation, authors documented that the claudin-1 and zonulin-1 expression in the intestinal epithelium was similar in NCGS, celiac patients and dyspeptic patients. Conversely, in NCGS patients a significantly higher expression of claudin-4, i.e. a claudin isoform postulated to decrease tight junction-dependent permeability in the intestinal epithelium, was observed [Sapone 2011]. As for the immune system activation, an increased expression of toll-like receptor (TLR)-2, TLR-1 and TLR-4, and a significant reduction of T-regulatory cell marker FoxP3 relative to controls and CD patients was observed, without any increase in adaptive immunity-related gut mucosal gene expression, including interleukin (IL)-6, IL-21 and interferon-gamma. These changes in NCGS led to hypothesize a primary role of the innate immune system in the pathogenesis of the disease, without any relevant involvement of the adaptive immune response [Sapone 2011].

By exploring the initial mucosal immunologic events in NCGS patients before and after a gluten challenge, Brottveit et al. [2013] observed a significant increase in expression of IFNγ after challenge with gluten, thus supporting a possible pathogenic role for adaptive immunity in this syndrome. Changes neither in density of CD3+ IELs, nor in expression of IL-8, TNFα, MCP-1, Hsp27, or Hsp70 were found in response to challenge.

Bucci and colleagues [2013] evaluated the expression of markers of activated innate and adaptive immunity after stimulation with gliadin in duodenal biopsies of NCGS patients, but they failed to identify any increase in the expression of these markers. Shuppan and colleagues [2015] suggested that molecules other than gluten, i.e. wheat amylase-trypsin inhibitors (ATIs), might play a major role as triggers of the innate immune response in NCGS. ATIs are water-soluble, wheat globulins, which are resistant to proteolytic digestion by the gastrointestinal proteases trypsin and pepsin. In *in vitro* studies, ATIs engage the TLR4-MD2-CD14 complex, leading to an up-regulation of dendritic cell maturation markers and to the release of pro-inflammatory cytokines in myeloid cells. Data from healthy controls are comparable to those from celiac and NCGS patients, in line with innate immune activation. Valerii and collaborators [2015] studied how a mix of wheat proteins (gliadin, glutenin, lectins, wheat germ agglutinin and ATIs) activated cultured peripheral blood mononuclear cells from NCGS patients. Authors documented over-expression of CXCL10, a pro-inflammatory chemokine produced mainly by neutrophils and macrophages to recruit leukocytes which is able to decrease the intestinal epithelial barrier function in vitro.

In a different line of research, Biesiekierski et al. [2013] indicated wheat fermentable oligosaccharides, disaccharides, monosaccharides and polyoils (FODMAPs) as poorly absorbed short-chain carbohydrates, which could be responsible for intestinal distension after fermentation and thus for gastrointestinal complaints, in a cohort of NCGS patients with predominant irritable-bowel syndrome symptoms.

CHAPTER I: AIMS OF THE STUDY

The study design accounted for three subsequent stages.

The first stage aimed to explore:

- the prevalence of IgA and/or IgG native anti-gliadin antibodies (AGA) and IgA tissue transglutaminase-2 antibodies (TGA) in sera from children and adolescents evaluated for headache in a tertiary referral hospital for pediatric neurological diseases;
- the prevalence of Non-celiac Gluten Sensitivity in pediatric headache patients with AGA;
- the clinical picture of pediatric headache patients with NCGS.

The second stage aimed to analyze gene expression profiles in peripheral blood mononuclear cells from pediatric headache patients with NCGS.

The third stage aimed to validate some of gene array results by the measurement of soluble mediators in sera of AGA-positive headache patients with NCGS, compared to AGA-positive, no-NCGS, headache patients, or to AGA-negative headache patients, and to determine serum levels of TG6 antibodies, as markers of neurological gluten-related disorders.

CHAPTER II: PATIENTS AND METHODS

2.1 Stage 1: Observational, prospective cohort study exploring NCGS in pediatric headache patients

2.1.1 Inclusion and exclusion criteria

All consecutive patients aged less than 18 years and evaluated for headache at the Child Neuropsychiatry Unit, University Hospital of Verona, Italy, in the period 1/03/13 – 31/07/15 were considered for recruitment in the study.

Inclusion criteria were the following: 1) to be diagnosed with a kind of primary headaches according to the ICHD III-beta criteria; 2) to accept dosage of total IgA, AGA IgG and IgA, TGA IgA, and specific IgE to wheat, gluten, gliadin on serum samples.

A written informed consent was obtained from legal representatives of all participants. The study was approved by the local Ethical Committee.

Data on personal and family history of headache, allergy, autoimmune, and gastrointestinal disorders were collected. Patients' age, sex, headache phenotypes, as well as intestinal and extra-intestinal complaints, were registered at the recruitment.

All patients were investigated with a complete blood count, biochemical analyses and evaluation of liver, renal and thyroid function and dosage of total IgA and TGA IgA (Eu-tTG® IgA kit, Eurospital, Trieste, Italy; cut-off levels: negative <9 U/ml, borderline 9-16 U/ml, positive >16 U/ml).

Patients were excluded if one of the following conditions occurred, at least: fulfilled criteria for secondary headaches or cranial nerve neuralgias; ongoing treatments for headache introduced or modified less than 2 months before; partial or total IgA deficiency, according to reference values for age; to have tested positive for TGA IgA (suspected diagnosis of celiac disease) or IgE to wheat, gluten, gliadin (suspected diagnosis for wheat allergy); presence of enteropathy as in celiac disease at duodenal biopsy.

Determination of AGA IgA was performed with the commercial ELISA kit α-Gliatest® S IgA kit, Eurospital, Trieste, Italy (cut-off levels: negative ≤8 U/ml, borderline 8.01-15 U/ml, positive >15 U/ml), determination of AGA IgG with α-

Gliatest® S IgG kit, Eurospital, Trieste, Italy (cut-off levels: negative <50 U/ml, positive ≥50 U/ml). Although, according to unpublished preliminary data and taken into account the sensitivity and specificity of AGA test, and of the adopted commercial method in particular, we considered the cut-off levels of 8 U/ml for AGA IgA and 25 U/ml for AGA IgG. Based on the determination of AGA, patients were assigned to the "AGA-positive" or the "AGA-negative" group.

2.1.2 AGA-positive patients

At the recruitment, AGA-positive patients also underwent a duodenal biopsy to exclude CD. Biopsy specimens were histologically evaluated according to the Marsh classification scheme [Marsh 1992]. Immunostaining to identify intraepithelial lymphocytes (IELs) was performed; intraepithelial lymphocytosis was defined as the presence of >25 IELs/100 epithelial cells. Immunohistochemistry with CD3, CD4 and CD8 markers was performed to better evaluate the lymphocyte phenotype. If patients were diagnosed with enteropathy, they would have to exit the study.

Clinical evaluation and standardized, self-administered questionnaires dealing with emotional/behavioral problems of patients (Child Behavior Checklist – CBCL) and disability due to headache (Migraine Disability Score - MIDAS) were obtained at every follow-up visit, or twice at least, i.e. at recruitment and after a 3 months - period on gluten-free diet (GFD). Patients and their parents were informed about the management of a strict gluten-free diet (i.e., no cross contamination, no gluten traces) by a dietitian with expertise in celiac disease and nutritional advices in the pediatric age.

If clinical improvement was observed on GFD, patients have been requested to reintroduce gluten in their diet (i.e., an average daily intake of gluten about 10–15 g) for the subsequent 3 months; then, they were newly evaluated clinically and by means of questionnaires (follow-up visit 1). Patients experiencing headache amelioration on GFD and relapsing on gluten challenge received the diagnosis of "AGA-positive NCGS" headache patients.

MIDAS questionnaires were completed by parents of participants after instructions provided by child neurologists attending the visit; in order to minimize biases, physicians had to verify correspondence in information between questionnaires and anamnestic data obtained by anamnestic interviews and patients' headache attacks diaries. MIDAS

questionnaires were scored as indicated by the questionnaire protocol. In order to evaluate amelioration, the MIDAS score at recruitment was compared to that at follow-up visit 1; similarly, the MIDAS score at post-GFD visit was compared to the score at follow-up visit 2, in order to evaluate relapse. Amelioration and relapse of symptoms were defined as a difference ≥50% between MIDAS total scores in 2 subsequent visits. Pain severity on a 0 (no pain) to 10 (maximum pain) - scale was also registered in MIDAS questionnaires at every follow-up visit.

CBCL questionnaires, completed by parents of participants as regards the last 3 months-period after instructions provided by child neurologists attending the visit, were scored as indicated by the questionnaire protocol. The broad-band scores "Internalizing problems" and "Externalizing problems" were registered, as well as the narrow-band sub-score "Somatic complaints". Scores were expressed as *t*-scores, in order to consent comparison among heterogeneous groups for age and sex.

Whenever possible, blood samples were collected from AGA-positive patients at every follow-up visit. Data from patients, whose samples were collected at recruitment and follow-up visit 1 at least, were considered suitable for the analysis. Additionally, blood samples for collection of peripheral blood mononuclear cells were obtained from patients diagnosed with NCGS at follow-up visit 2.

2.1.3 AGA-negative patients

Clinical evaluation and standardized, self-administered questionnaires dealing with emotional/behavioral problems of patients (Child Behavior Checklist – CBCL) and disability due to headache (Migraine Disability Score - MIDAS) were obtained from AGA-negative patients at the recruitment and two subsequent follow-up visits, after 3 months apart, on normal, gluten-containing diet (i.e., an average daily intake of gluten about 10–15 g).

MIDAS and CBCL questionnaires were scored as indicated by the questionnaire protocol, and scores managed as in AGA-positive patients.

Whenever possible, serum samples were collected at every follow-up visit; although, data from patients, whose samples were collected at recruitment and follow-up visit 1 at least, were considered suitable for the analysis.

Serum samples separated from blood collected from patients and controls were stored

frozen at -20°C until assayed.

2.1.4 Statistical Analyses

For continuous variables, the Wilcoxon test was used for comparison of the mean values among groups. All statistical tests were 2-tailed and performed at a 5% level of significance.

2.2 Stage 2: Analysis of gene expression profiles in peripheral blood mononuclear cells from patients with headache associated to NCGS.

2.2.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood through density gradient centrifugation using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, NO) according to manufacturer's instruction. Briefly, 10mL peripheral blood was diluted with 10mL PBS and then layered over 10mL Lymphoprep in a 50mL centrifuge tube. Samples were centrifuged at 800×g for 20 minutes and cells were collected using a Pasteur pipette. Cells were washed twice with PBS at 1200 rpm for 10 minutes and then counted in a Burker chamber using acridine orange staining.

2.2.2 Preparation of cRNA and array analysis

Total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions.
Preparation of cRNA hybridization and scanning of probe arrays for each sample were performed according to the protocols of the manufacturer (Affymetrix, Santa Clara, CA, USA) by Cogentech Affymetrix microarray unit (Campus IFOM IEO, Milan, Italy) using the Human Genome U133A 2.0 Gene Chip (Affymetrix). The Human Genome U133A Gene Chip is a single array representing 14,500 well-characterized human genes and including more than 22,000 probe sets and 500,000 distinct oligonucleotide features. The different gene expression patterns were analyzed using the Gene Spring software, version 12.1 (Agilent Technologies, Santa Clara, CA, USA) that calculated a robust

multi-array average of background-adjusted, normalized, and log-transformed intensity values applying the Robust Multi-Array Average algorithm (RMA). With this software the mean optical background level for each array was subtracted from the signal intensity for each probe. The normalized background-corrected data were transformed to the log2 scale. Signal log2 ratio of 1.0 indicates an increase in the transcript level by twofold change (2 F.C.) and -1.0 indicates a decrease by twofold (-2 F.C.). A signal log2 ratio of zero would indicate no change. The unpaired t-test was performed to determine which genes were modulated at a significant level ($p<0.01$) and *p*-values were corrected for multiple testing by using Bonferroni correction. Finally, statistically significant genes were selected for final consideration when their expression was at least 1.5-fold different in the test sample versus control sample. Genes that passed both the *p*-value and the FC restriction were submitted to a functional classification according to the Gene Ontology (GO) annotations (http://www.geneontology.org/).

2.3 Stage 3: Detection of soluble mediators and Transglutaminase-6 antibodies in serum samples from patients with headache associated to NCGS

The analysis of gene expression profiles was paralleled by the detection of some soluble mediators in serum samples of patients, by means of commercially available ELISA kits. We decided to analyze levels of soluble CTLA-4 (sCTLA-4) and soluble gp130 (sgp130) in serum samples collected at recruitment and after the 3 months - period on gluten-free diet, from AGA-positive NCGS headache patients. Findings in these patients were compared to those from AGA-positive, no-NCGS, headache patients, and from AGA-negative headache patients. Commercial ELISA kits were used for the determination of serum levels of sCTLA-4 (sCD152/CTLA-4 Human ELISA®, BioVendor Laboratory Medicine, Inc., Bratislava, Slovakia) and sgp130 (Quantikinine ELISA Human soluble gp130®, R&D Systems, Minneapolis, USA).

Additionally, we decided to analyze levels of soluble IL-2 receptor alfa (sCD25), a well-established biomarker of activated peripheral blood T-lymphocytes. The commercially available ELISA kit purchased from Novus Biologicals (Littleton CO, USA) was used according to the manufacturer's instructions.

Further, being TG6 antibodies described as serological markers of gluten-related disorders with a major neurological involvement, the determination of IgA and IgG

Tranglutaminase-6 antibodies was performed in sera of headache patients. The commercial ELISA kits from Zedira, Ebringen, Germany (cut-off levels for Tg6 IgA: negative <26 U/ml, borderline 26-41 U/ml, positive >41 U/ml; cut-off levels for Tg6 IgG: negative <28 U/ml, borderline 28-44 U/ml, positive >44 U/ml) were used.

Figures 3-5 show calibration curves obtained for ELISA assays and applied to calculate analyte concentrations in serum samples.

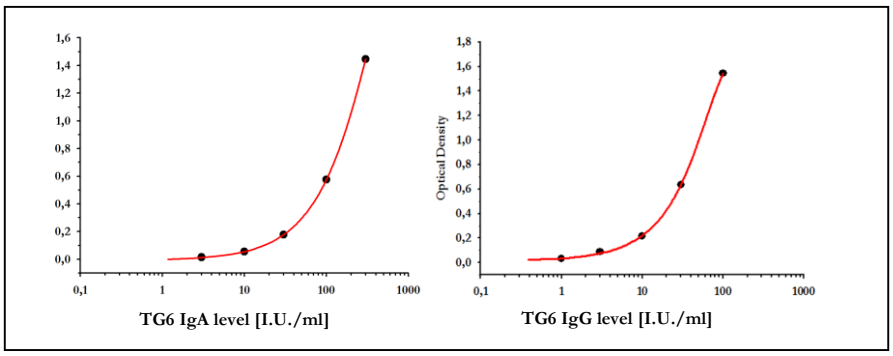

Figure 3. Calibration curves for TG6 IgA ELISA assay and TG6 IgG ELISA assay.

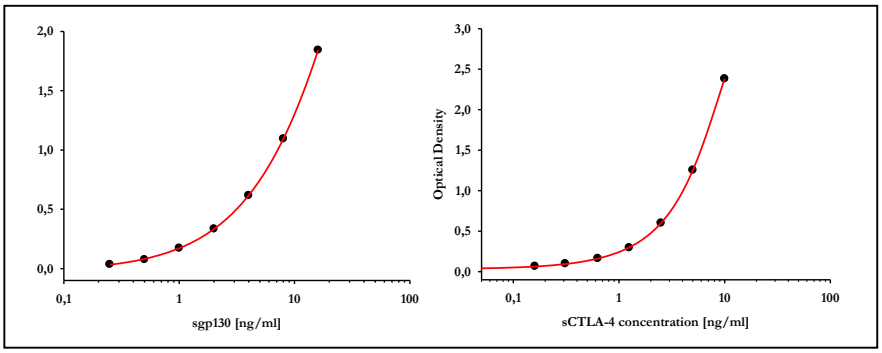

Figure 4. Calibration curves for sgp130 ELISA assay and sCTLA-4 ELISA assay.

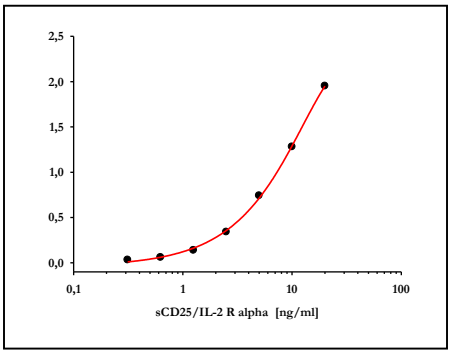

Figure 5. The calibration curve for sCD25 ELISA assay.

CHAPTER III: RESULTS

3.1 Stage 1: Observational, prospective cohort study exploring NCGS in pediatric headache patients

Among 60 patients aged less than 18 years with primary headache examined for eligibility, 40 subjects accepted serologic examination for gluten-related disorders. None of the subjects tested positive for TGA IgA, IgE to wheat, gluten, gliadin, or AGA IgA; no one had IgA deficiency. Twenty patients (50%) tested positive for AGA IgG. Consequently, all were confirmed eligible for the study.

Seventeen patients out 40 who had serologic tests (43%) accepted to enter the study: 11 subjects (4 males, 7 females; mean age, 10.6 years; age range, 7.9-12.5 years) belonged to the AGA-positive group, 6 children (2 males, 4 females; mean age, 11.6 years; age range, 9.1-13.8 years) to the AGA-negative group. Thus, 55% of patients who tested positive for AGA and 30% of patients who tested negative for AGA participated in the study.

3.1.1 Headache clinical course during the diagnostic phase

AGA-positive patients underwent the 3 months – period on GFD and then the gluten challenge was performed for patients who experienced clinical amelioration. Thus, 6 out 11 AGA-positive patients (55%) received the diagnosis of NCGS, confirmed by clinical improvement on GFD and relapse on gluten challenge. NCGS was not confirmed in 5 AGA-positive patients due to lack of clinical benefit on GFD (one case), or lack of symptom relapse with the gluten challenge (4 patients). The flow-chart in Figure 6 shows the study design.

Figures 7-9 and Tables 1-2 summarize data from MIDAS questionnaires, completed at recruitment and within the follow-up period, in AGA-positive and AGA-negative patients; AGA-positive patients were further stratified, on the basis of their response to GFD and gluten challenge.

Tables 3-5 summarize data from CBCL questionnaires, completed at recruitment and within the follow-up period, in AGA-positive and AGA-negative patients. Headache appeared to have a similar burden on AGA-positive and AGA-negative patients at recruitment. However, a subgroup of AGA-positive patients experienced clinical benefit

on GFD and relapse on gluten challenge, and consensually CBCL "Internalizing problem" score and "Somatic complaints" score decreased at the first follow-up visit and returned at the recruitment level after challenge. In other AGA-positive patients, and in AGA-negative subjects, CBCL scores were nearly unchanged within the follow-up period.

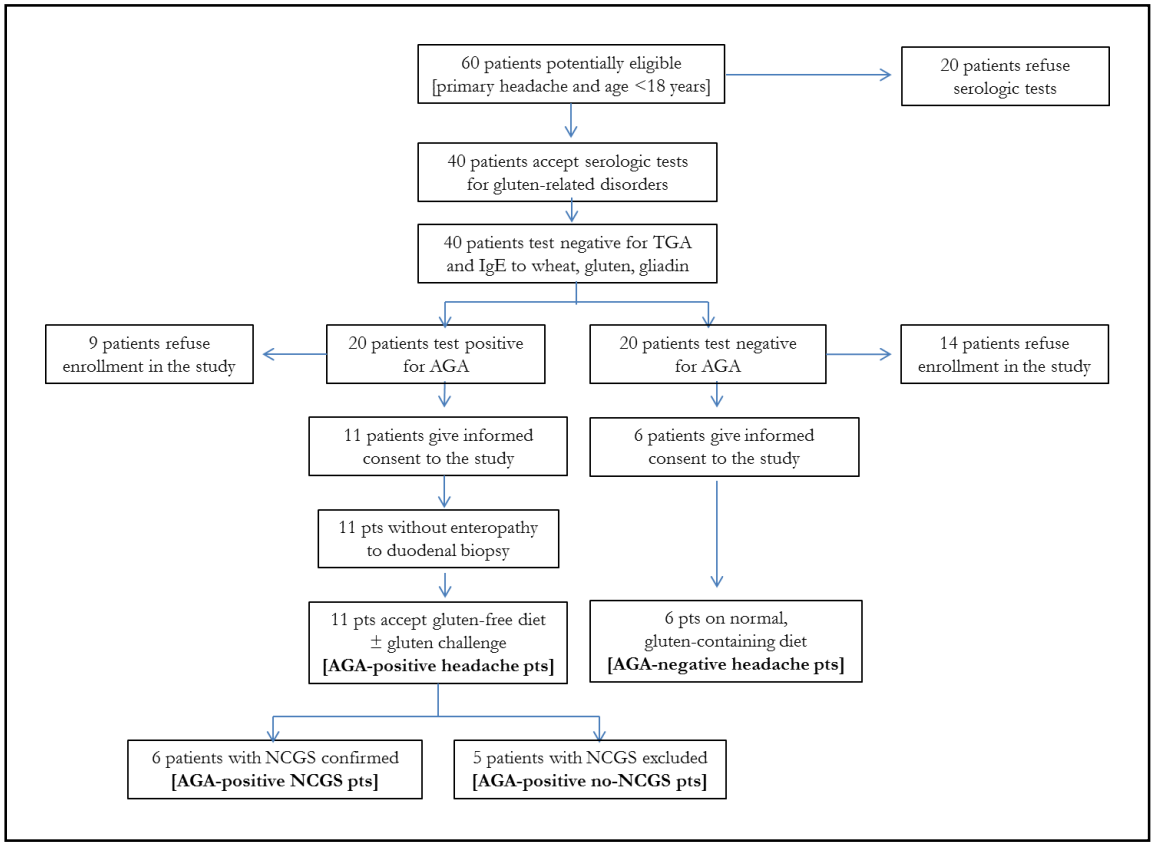

Figure 6. The study design.

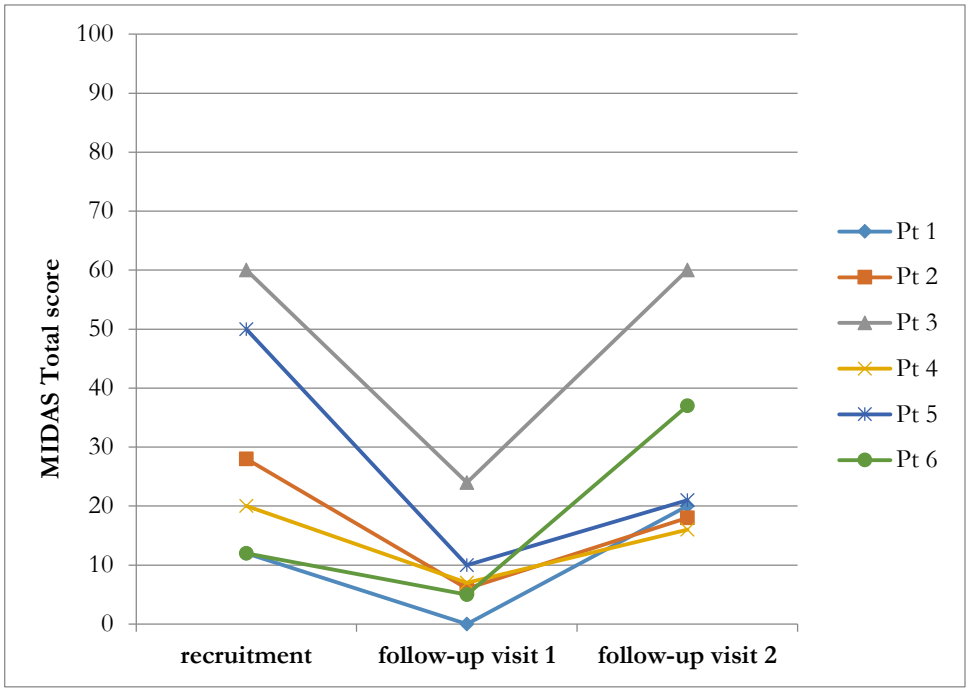

Figure 7. MIDAS Total score at recruitment and within the follow-up period in AGA-positive, NCGS patients. Patients displayed clinical improvement on GFD (follow-up visit 1) and relapse on gluten challenge (follow-up visit 2).

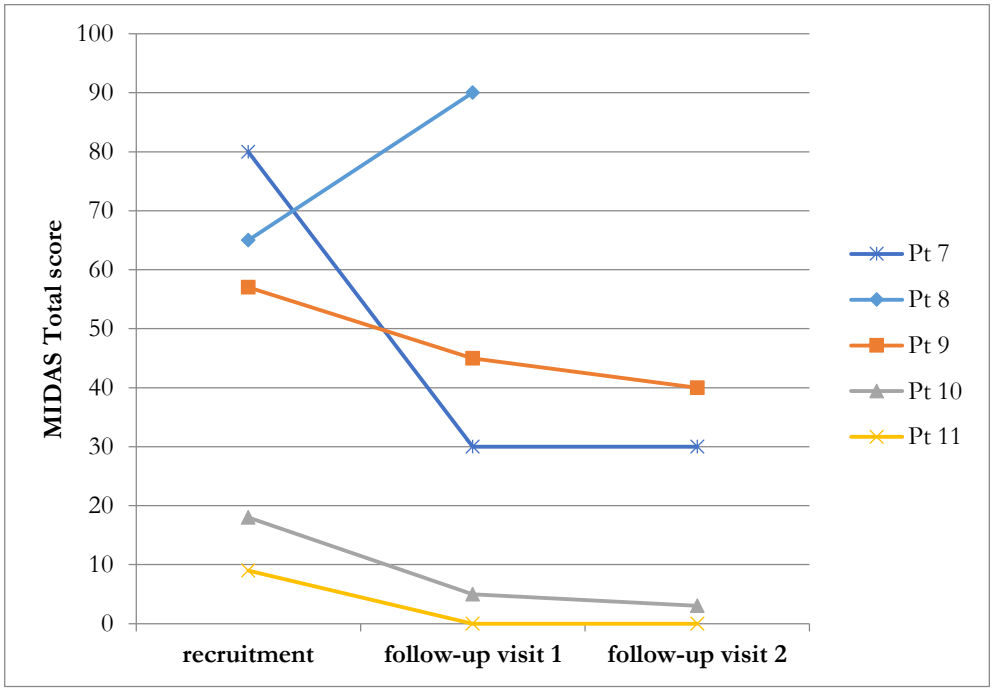

Figure 8. MIDAS Total score at recruitment and within the follow-up period in AGA-positive, no-NCGS patients. Patients were on GFD at F.U. visit 1 and on gluten-containing diet on F.U. visit 2. A patient lacked clinical benefit on GFD, 4 patients lacked symptom relapse with the gluten challenge (follow-up visit 2).

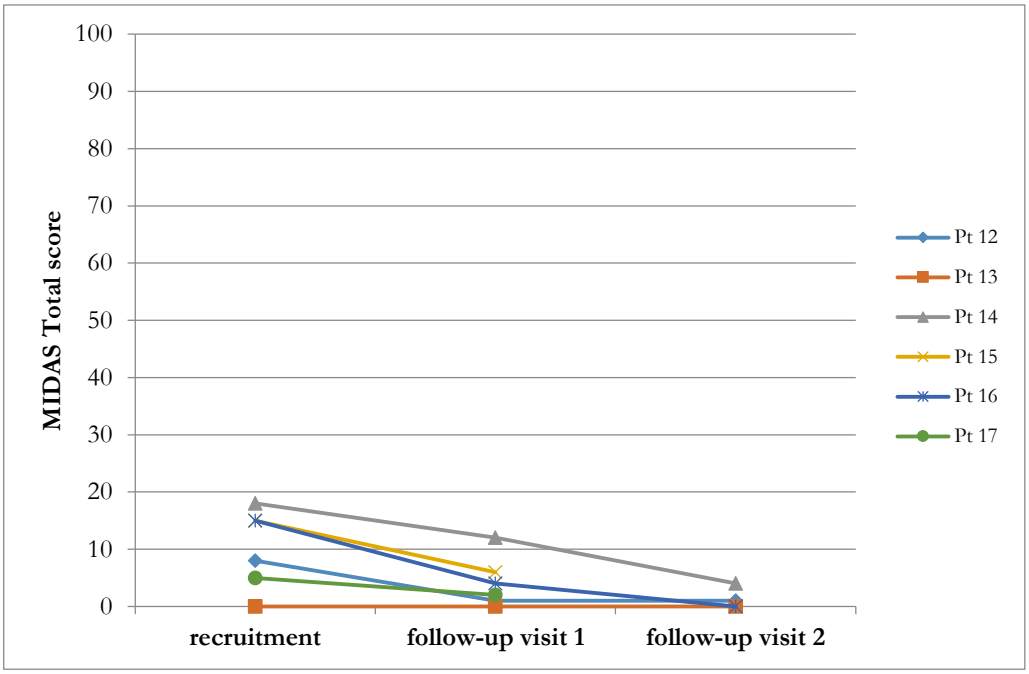

Figure 9. MIDAS Total score at recruitment and within the follow-up period in AGA-negative patients. Patients were on normal, gluten-containing diet within the follow-up period.

Table 1. MIDAS scores in AGA-positive and AGA-negative patients, at the recruitment and within the follow-up period.

	MIDAS score recruitment (mean ± SD)	MIDAS score F.U. visit 1 (mean ± SD)	MIDAS score F.U. visit 2 (mean ± SD)
AGA-positive pts (all)	37 ± 25	20 ± 27	25 ± 18
AGA-positive NCGS	30 ± 20	9 ± 8	29 ± 17
AGA-positive no-NCGS	46 ± 31	34 ± 36	18 ± 20
AGA-negative pts	10 ± 7	4 ± 4	1 ± 2

Table 2. Pain intensity scores on a 0-to-10 scale in AGA-positive and AGA-negative patients, at the recruitment and within the follow-up period.

	Pain intensity recruitment (mean ± SD)	Pain intensity F.U. visit 1 (mean ± SD)	Pain intensity F.U. visit 2 (mean ± SD)
AGA-positive pts (all)	8 ± 2	6 ± 3	7 ± 2
AGA-positive NCGS	8 ± 2	6 ± 2	8 ± 2
AGA-positive no-NCGS	7 ± 1	6 ± 3	6 ± 1
AGA-negative pts	8 ± 1	6 ± 1	4 ± 3

Table 3. CBCL "Internalizing problems" (INT) t-scores in AGA-positive and AGA-negative patients, at the recruitment and within the follow-up period. A t-score ≥67 corresponds to a clinically significant condition.

	INT t-score recruitment (mean ± SD)	INT t-score F.U. visit 1 (mean ± SD)	INT t-score F.U. visit 2 (mean ± SD)
AGA-positive pts (all)	56 ± 7	52 ± 6	53 ± 5
AGA-positive NCGS	59 ± 7	52 ± 5	55 ± 5
AGA-positive no-NCGS	51 ± 5	52 ± 7	52 ± 6
AGA-negative pts	60 ± 6	62 ± 8	56 ± 5

Table 4. CBCL "Somatic complaints" (SC) subscale t-scores in AGA-positive and AGA-negative patients, at the recruitment and within the follow-up (F.U.) period. A t-score ≥67 corresponds to a clinically significant condition.

	SC t-score recruitment (mean ± SD)	SC t-score F.U. visit 1 (mean ± SD)	SC t-score F.U. visit 2 (mean ± SD)
All AGA-positive pts (all)	65 ± 7	58 ± 7	64 ± 8
AGA-positive NCGS	69 ± 6	56 ± 7	68 ± 7
AGA-positive no-NCGS	60 ± 5	61 ± 8	58 ± 7
AGA-negative pts	62 ± 7	61 ± 7	59 ± 12

Table 5. CBCL "Externalizing problems" (EXT) t-scores in AGA-positive and AGA-negative patients, at the recruitment and within the follow-up (F.U.) period. A t-score ≥67 corresponds to a clinically significant condition.

	EXT t-score recruitment (mean ± SD)	EXT t-score F.U. visit 1 (mean ± SD)	EXT t-score F.U. visit 2 (mean ± SD)
AGA-positive pts (all)	46 ± 6	46 ± 7	43 ± 8
AGA-positive NCGS	46 ± 5	44 ± 7	43 ± 7
AGA-positive no-NCGS	45 ± 8	47 ± 7	43 ± 11
AGA-negative pts	53 ± 5	51 ± 15	45 ± 9

3.1.2 Headache phenotype and migraine-related conditions

Primary headache phenotypes (migraine, or migraine associated to tension-type headache, and tension-type headache) were similarly distributed among AGA-positive NCGS patients, AGA-positive no-NCGS patients, and AGA-negative patients (Table 6). Syndromes belonging to the spectrum of migraine variants and migraine-related conditions (i.e., abdominal migraine, acetonemic crises, cyclic vomiting, paroxysmal vertigo, paroxysmal torticollis, motion sickness, limb pain) occurred more frequently in AGA-positive patients, even though they were similarly distributed among AGA-positive NCGS patients, and AGA-positive no-NCGS patients (Table 6).

Table 6. Headache phenotypes in AGA-positive and AGA-negative patients.

	AGA-positive pts			AGA-negative pts
	NCGS	no-NCGS	All	All
	N. pts (%)	N. pts (%)	N. pts (%)	N. pts (%)
Migraine or migraine+TTH *	5/6 (83%)	5/5 (100%)	10/11 (91%)	5/6 (83%)
Pure TTH *	1/6 (17%)	0/5 (0%)	1/11 (9%)	1/6 (17%)
Migraine-associated syndromes	6/6 (100%)	5/5 (100%)	11/11 (100%)	4/6 (67%)

* TTH: tension-type headache.

3.1.3 Gastrointestinal and extra-intestinal complaints

Gastrointestinal symptoms (i.e., bloating, diarrhea) occurred more frequently in AGA-positive NCGS patients than in other groups (Table 7).

Extra-intestinal symptoms (fatigue, depression, anemia, long-lasting low-grade fever, dermatitis, failure to thrive) were similarly distributed in AGA-positive NCGS patients and AGA-positive no-NCGS patients, although they occurred more frequently in AGA-positive patients than in AGA-negative subjects (Table 7).

Table 7. Clinical features other than headache in AGA-positive and AGA-negative patients.

	AGA-positive pts			AGA-negative pts
	NCGS N. pts (%)	no-NCGS N. pts (%)	All N. pts (%)	All N. pts (%)
Gastrointestinal symptoms	3/6 (50%)	1/5 (20%)	4/11 (36%)	1/6 (17%)
Extra-intestinal symptoms	6/6 (100%)	4/5 (80%)	10/11 (91%)	2/6 (33%)

3.1.4 Associated disorders

Symptoms of inhalant and food allergy were similarly distributed in AGA-positive NCGS patients, and AGA-positive no-NCGS patients. Although, they occurred more frequently in AGA-positive patients than in AGA-negative subjects (Table 8).
None of the subjects had autoimmune disorders; oral lichen planus occurred in one case in the AGA-positive, no-NCGS group (Table 8).

Table 8. Comorbidities in AGA-positive and AGA-negative patients.

	AGA-positive pts			AGA-negative pts
	NCGS N. pts (%)	no-NCGS N. pts (%)	All N. pts (%)	All N. pts (%)
Allergy	2/6 (33%)	2/5 (40%)	4/11 (36%)	0/6 (0%)
Other immune-mediated diseases	0/6 (0%)	1/5 (20%)	1/11 (9%)	0/6 (0%)

3.1.5 Family antecedents

As shown in Table 9, family antecedents for primary headaches were similarly distributed in AGA-positive NCGS patients, AGA-positive no-NCGS patients, and AGA-negative patients.
A family history of allergy occurred similarly in AGA-positive NCGS patients, and AGA-positive no-NCGS patients. Although, it was more frequent in AGA-positive patients than in AGA-negative subjects.

A family history of autoimmune diseases was more frequent in AGA-positive no-NCGS patients than in other groups, whereas it was similarly distributed in AGA-positive NCGS patients, and AGA-negative patients.

Table 9. Family antecedents in AGA-positive and AGA-negative patients.

	AGA-positive pts			AGA-negative pts
	NCGS	no-NCGS	All	All
	N. pts (%)	N. pts (%)	N. pts (%)	N. pts (%)
Headache	6/6 (100%)	5/5 (100%)	11/11 (100%)	5/6 (83%)
Allergy	4/6 (66%)	4/5 (80%)	8/11 (73%)	2/6 (33%)
Autoimmune diseases	3/6 (50%)	4/5 (80%)	7/11 (64%)	3/6 (50%)

3.1.6 Duodenal biopsy

All AGA-positive patients had duodenal biopsy on gluten-containing diet.
None of the subjects displayed histological signs of enteropathy or an increased number of IELs in duodenal mucosa, as confirmed by immunohistochemistry. In a single patient the mucosal eosinophilic count appeared to be mildly increased, without any other pathological findings.

3.2 Stage 2: Analysis of gene expression profiles in peripheral blood mononuclear cells from patients with headache associated to NCGS

In order to identify specific gene signatures possibly associated to NCGS, we compared gene expression profiles of peripheral blood mononuclear cells obtained from NCGS patients with samples obtained from 5 healthy, age- and sex-matched donors. We observed that 1585 modulated genes complied with the Bonferroni–corrected *p*-value criterion and the fold change criterion, showing robust and statistically significant variation between healthy controls and NCGS samples. In particular, 860 transcripts resulted to be up-regulated and 725 transcripts were down-regulated. According to the Gene Ontology analysis, modulated genes were then classified in different functional classes, including immune response, inflammation, signal transduction, apoptosis and cell proliferation (Figures 10-11). Interestingly, 80 modulated genes belong to the functional category "Immune Response". Therefore, in the attempt to dissect the role of immune system in this clinical syndrome, we focused our attention on genes related to immune system functions. Modulated genes were classified on the basis of the role played in the immune response and listed in Tables 10A-C, which also includes GeneBank accession numbers and fold changes.

We observed that genes involved in the innate immune response, as well as those related to the adaptive immune response, were represented. Twenty genes prevalently involved in T-cell activation (e.g., CD28, CD3E, CD3G, CTLA-4, IL24, IL32, TNFRSF25) were up-regulated. Interestingly, 9 Th17 lymphocyte-related genes (e.g. IL4R, IL2RG, gp130, IL1B, IL7R, STAT6, STAT5B, SOCS3 and CXCL2) were up-regulated. Thirty-eight transcripts prevalently involved in B-cell activity (e.g., gp130, CLEC2D, IL2RG, IL6R, KLF12 and CD27) were modulated, thus suggesting a concomitant activation of this cell subset. Also 14 genes involved in NK activity were modulated: CLEC2D was up-regulated, whereas LILRA1, LILRA2, LILRA2, LILRA5, LILRB2 and LILRB4 were down-regulated. Finally, 6 type I Interferon inducible genes were up-regulated (IFNA17, IRF3, IRF5, STAT2, STAT1, LY9), thus indicating a type I IFN signature. Moreover, we noticed that a number of genes related to macrophage activation (e.g. MARCO) were down-regulated.

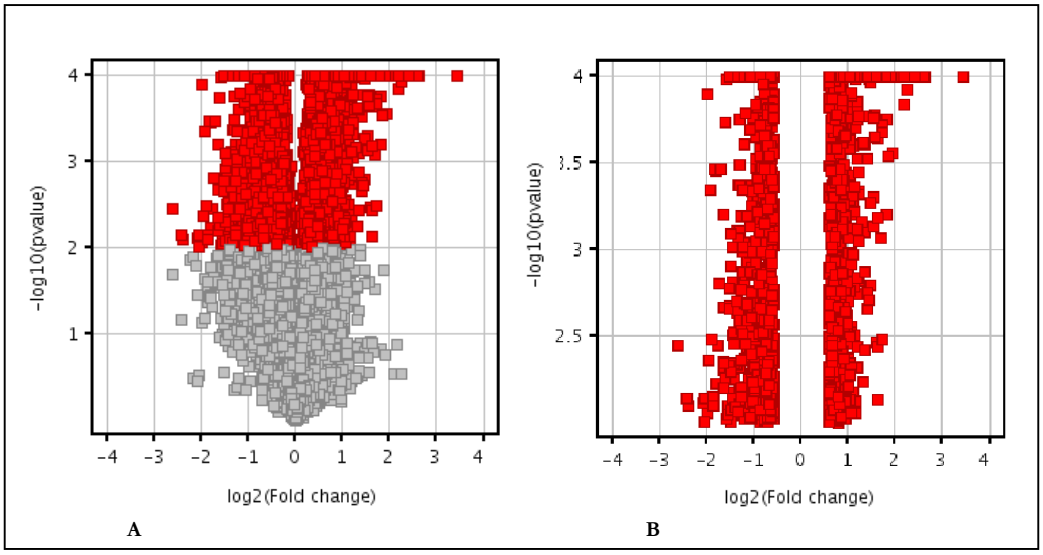

Figure 10. A. Scatter plot of normalized relative signal intensity (log10) of genes satisfying the p-value cut-off ($p<0.01$). **B.** Scatter plot of normalized relative signal intensity (log10) of genes differently expressed with 1.5-fold change.

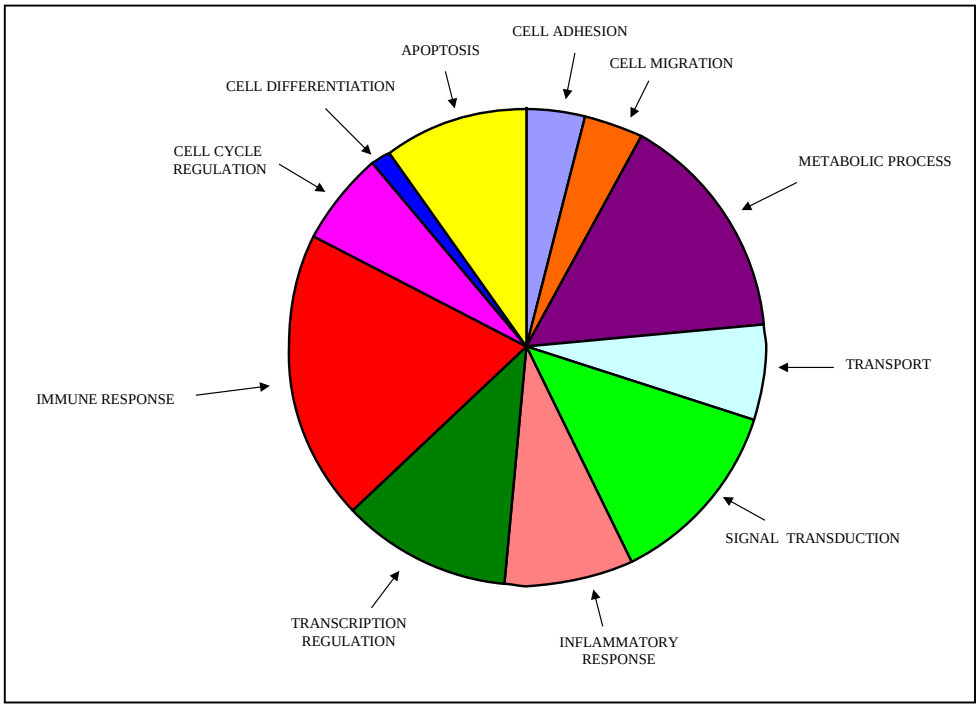

Figure 11. Distribution of genes which were modulated in the analysis of gene expression profiles in peripheral blood mononuclear cells from AGA-positive NCGS patients.

Table 10A. The most relevant genes related to innate and adaptive immune response and differently expressed.

Public ID	Gene Title	Symbol	FC
T cell activation			
NM_001626	v-akt murine thymoma viral oncogene homolog 2	AKT2	2,36
AF222343	CD28 molecule	CD28	2,75
NM_000733	CD3e molecule, epsilon (CD3-TCR complex)	CD3E	2,67
NM_000073	CD3g molecule, gamma (CD3-TCR complex)	CD3G	2,13
NM_001190720	inhibitor of K light polypeptide gene enhancer in B-cells, kinase beta	IKBKB	2,69
NM_002228.3	jun proto-oncogene	JUN	3,07
U07236	lymphocyte-specific protein tyrosine kinase	LCK	2,05
NM_030662	mitogen-activated protein kinase kinase 2	MAP2K2	1,83
NM_005921	mitogen-activated protein kinase kinase kinase 1	MAP3K1	1,52
NM_001278548	mitogen-activated protein kinase 8	MAPK8	2,33
U57843	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta	PIK3CD	1,88
NM_181523	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	PIK3R1	2,76
NM_002660	phospholipase C, gamma 1	PLCG1	1,53
NM_006908	rho family, small GTP binding protein Rac1	RAC1	-1,92
NM_002872	rho family, small GTP binding protein Rac2	RAC2	2,31
NM_006939	son of sevenless homolog 2	SOS2	1,90
NM_148965	tumor necrosis factor receptor superfamily, member 25	TNFRSF25	2,40
NM_005214	cytotoxic T-lymphocyte-associated protein 4	CTLA4	2,26
NM_006850	interleukin 24	IL24	2,84
NM_004221	interleukin 32	IL32	2,10
TH17 related genes			
NM_000418	interleukin 4 receptor	IL4R	1,50
NM_000206	interleukin 2 receptor, gamma	IL2RG	1,84
NM_002184	interleukin 6 signal transducer (gp130, oncostatin M receptor)	IL6ST	4,52
NM_000576	interleukin 1, beta	IL1B	1,52
NM_002185	interleukin 7 receptor	IL7R	1,55
AH006951	signal transducer and activator of transcription 6	STAT6	1,54
NM_012448	signal transducer and activator of transcription 5B	STAT5B	1,60
NM_003955	suppressor of cytokine signaling 3	SOCS3	1,83
M57731	chemokine (C-X-C motif) ligand 2	CXCL2	1,53
Type I interferon signaling			
NM_021268	interferon, alpha 17	IFNA17	1,59
NM_032643	interferon regulatory factor 5	IRF5	1,52
NM_001571	interferon regulatory factor 3	IRF3	1,67
S81491	signal transducer and activator of transcription 2, 113kDa	STAT2	1,59
NM_007315	signal transducer and activator of transcription 1, 91kDa	STAT1	2,73
NM_002348	lymphocyte antigen 9	LY9	2,05

Table 10B. Continues.

Public ID	Gene Title	Symbol	FC
B cell mediated immune response			
NM_001190720	inhibitor of k light polypeptide gene enhancer in Bcells, kinase beta	IKBKB	2,69
NM_002228.3	jun proto-oncogene	JUN	3,07
AI356412	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	LYN	-2,22
NM_030662	mitogen-activated protein kinase kinase 2	MAP2K2	1,83
NM_001278548	mitogen-activated protein kinase 8	MAPK8	2,33
U57843	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta	PIK3CD	1,88
NM_001243975	protein phosphatase 3, catalytic subunit, gamma isozyme	PPP3CC	2,00
NM_006908	rho family, small GTP binding protein Rac1	RAC1	-1,92
NM_002872	rho family, small GTP binding protein Rac2	RAC2	2,31
NM_006939	son of sevenless homolog 2	SOS2	1,90
NM_003177	spleen tyrosine kinase	SYK	-1,92
NM_016543	sialic acid binding Ig-like lectin 7	SIGLEC7	-2,21
NM_001242	CD27 molecule	CD27	1,96
NM_005238	v-ets erythroblastosis virus E26 oncogene homolog 1	ETS1	2,25
NM_005239	v-ets erythroblastosis virus E26 oncogene homolog 2	ETS2	-1,83
KJ896761	E74-like factor 1 (ets domain transcription factor)	ELF1	1,94
AF327066	Friend leukemia virus integration 1	FLI1	2,28
NM_002348	lymphocyte antigen 9	LY9	2,08
U96845	killer cell lectin-like receptor subfamily C, member 4	KLRC4	2,23
NM_000206	interleukin 2 receptor, gamma	IL2RG	1,84
S72848	interleukin 6 receptor	IL6R	1,79
NM_002184	interleukin 6 signal transducer (gp130, oncostatin M receptor)	IL6ST	4,52
AH010423	Kruppel-like factor 12	KLF12	1,83
NM_015995	Kruppel-like factor 13	KLF13	1,89
NM_001767	CD2 molecule	CD2	2,00
NM_020125	SLAM family member 8	SLAMF8	-2,27
NM_003842	tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	1,50
NM_001066	tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	-2,06
NM_148965	tumor necrosis factor receptor superfamily, member 25	TNFRSF25	2,40
NM_014452	tumor necrosis factor receptor superfamily, member 21	TNFRSF21	-1,68
NM_003037	signaling lymphocytic activation molecule family member 1	SLAMF1	1,65
D86359	sialic acid binding Ig-like lectin 6	SIGLEC6	1,58
AF054818	CD84 molecule	CD84	2,55
NM_013269	C-type lectin domain family 2, member D	CLEC2D	3,18
AY532110	interleukin 11 receptor, alpha	IL11RA	1,56
AF000672	ELK1, member of ETS oncogene family	ELK1	1,60
U50748	leptin receptor	LEPR	-2,10
NM_002342	lymphotoxin beta receptor (TNFR superfamily, member 3)	LTBR	-1,92

Table 10C. Continues.

Public ID	Gene Title	Symbol	FC
Response to gamma interferon			
NM_001767	CD2 molecule	CD2	2,00
NM_032643	interferon regulatory factor 5	IRF5	1,52
NM_020125	SLAM family member 8	SLAMF8	-2,27
AF054818	CD84 molecule	CD84	2,55
NM_002053	guanylate binding protein 1, interferon-inducible	GBP1	-1,77
NM_001571	interferon regulatory factor 3	IRF3	1,67
NM_016107	zinc finger RNA binding protein	ZFR	2,19
NM_003037	signaling lymphocytic activation molecule family member 1	SLAMF1	1,65
NM_002348	lymphocyte antigen 9	LY9	2,08
NK cell activation			
NM_013269	C-type lectin domain family 2, member D	CLEC2D	3,18
NM_000418	interleukin 4 receptor	IL4R	1,50
NM_001278426	leukocyte immunoglobulin-like receptor, subfamily B, member 4	LILRB4	-1,83
NM_024318	leukocyte immunoglobulin-like receptor, subfamily A, member 6	LILRA6	-1,74
NM_021268	interferon, alpha 17	IFNA17	1,59
NM_001278319	leukocyte immunoglobulin-like receptor, subfamily A, member 1	LILRA1	-2,81
NM_006866	leukocyte immunoglobulin-like receptor, subfamily A, member 2	LILRA2	-2,35
U96845	killer cell lectin-like receptor subfamily C, member 4	KLRC4	2,23
NM_006865	leukocyte immunoglobulin-like receptor, subfamily A, member 3	LILRA3	-2,96
NM_012276	leukocyte immunoglobulin-like receptor, subfamily A, member 4	LILRA4	-1,83
NM_181985	leukocyte immunoglobulin-like receptor, subfamily A, member 5	LILRA5	-3,15
AF004231	leukocyte immunoglobulin-like receptor, subfamily B, member 2	LILRB2	-3,41
M37780	platelet/endothelial cell adhesion molecule 1	PECAM1	-1,73
NM_004221	interleukin 32	IL32	2,10
Macrophage activation			
NM_003842	tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	1,50
NM_005202	collagen, type VIII, alpha 2	COL8A2	-1,62
NM_006770	macrophage receptor with collagenous structure	MARCO	-3,01
NM_006725	CD6 molecule	CD6	3,67
NM_003177	spleen tyrosine kinase	SYK	-1,92
NM_001066	tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	-2,06
NM_002438	mannose receptor, C type 1	MRC1	-2,04
NM_002053	guanylate binding protein 1, interferon-inducible	GBP1	-1,77
M37780	platelet/endothelial cell adhesion molecule 1	PECAM1	-1,73
Complement activation			
NM_001735	complement component 5	C5	-1,60
NM_000573	complement component (3b/4b) receptor 1 (Knops blood group)	CR1	-1,79
Antigen processing and presentation			
NM_002939	ribonuclease/angiogenin inhibitor 1	RNH1	-1,53
NM_000544	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	TAP2	1,86

3.3 Stage 3: Detection of soluble mediators and Tranglutaminase-6 antibodies in serum samples from patients with headache associated to NCGS

The analysis of gene expression profiles was complemented by detection of some corresponding soluble mediators in sera of patients.

We decided to analyze levels of soluble CTLA-4 (sCTLA-4) and soluble gp130 (sgp130) due to relevant changes in their transcript levels in the analysis of gene profiles, and literature data suggesting a role of these molecules in celiac disease and other autoimmune disorders. Soluble forms of the gp130 receptor protein, which are mainly generated by alternatively splicing of the gp130 mRNA, have been identified as able to selectively block IL-6 trans-signaling, thus protecting hosts from unwanted stimulation via IL-6 trans-signaling, i.e. the pro-inflammatory signaling pathway mediated by IL-6 [Rose-John 2015]. Soluble forms of CTLA-4 derive from CTLA-4, a type I glycoprotein on the surface of activated T-cells, whose function is to attenuate the ongoing immune response [Brunet 1987, Linsley 1991A, Linsley 1991B].

The determination of sCD25 concentration and TG6 IgA and IgG levels were also performed at this stage.

Figures 12-13 represent the concentration of sCTLA-4 and sgp130 in sera collected at recruitment and the first follow-up visit from AGA-positive NCGS patients, as compared to those of AGA-positive, no-NCGS patients and AGA-negative patients. All patients were on normal, gluten-containing diet at recruitment; at follow-up visit 1, all AGA-positive patients have been on gluten-free diet for 3 months, AGA-negative patients on normal, gluten-containing diet.

Soluble CTLA-4 levels at recruitment on gluten-containing diet were significantly higher in AGA-positive patients, as compared to AGA-negative subjects (mean 14.46±3.74 ng/ml vs mean 0.53±0.67 ng/ml, p=0.001; AGA-positive NCGS - mean 14.90±3.97 ng/ml vs AGA-negative subjects - mean 0.53±0.67 ng/ml, p=0.005; AGA-positive no-NCGS - mean 13.93±3.82 ng/ml vs AGA-negative subjects - mean 0.53±0.67 ng/ml, p=0.008), and they were similar among AGA-positive NCGS and no-NCGS patients. Soluble CTLA-4 levels decreased on GFD, both in AGA-positive NCGS patients (mean 4.28±3.46 ng/ml; p=0.036) and in no-NCGS subjects (mean 2.84±1.96 ng/ml; p=0.059), although remaining significantly higher in AGA-positive NCGS, as compared to AGA-negative patients (mean 0.46±0.57 ng/ml; p=0,013). Levels of sCTLA-4 in

AGA-positive no-NCGS were higher than in AGA-negative patients, without reaching a statistical significance (mean 0.46±0.57 ng/ml; p=0,055).

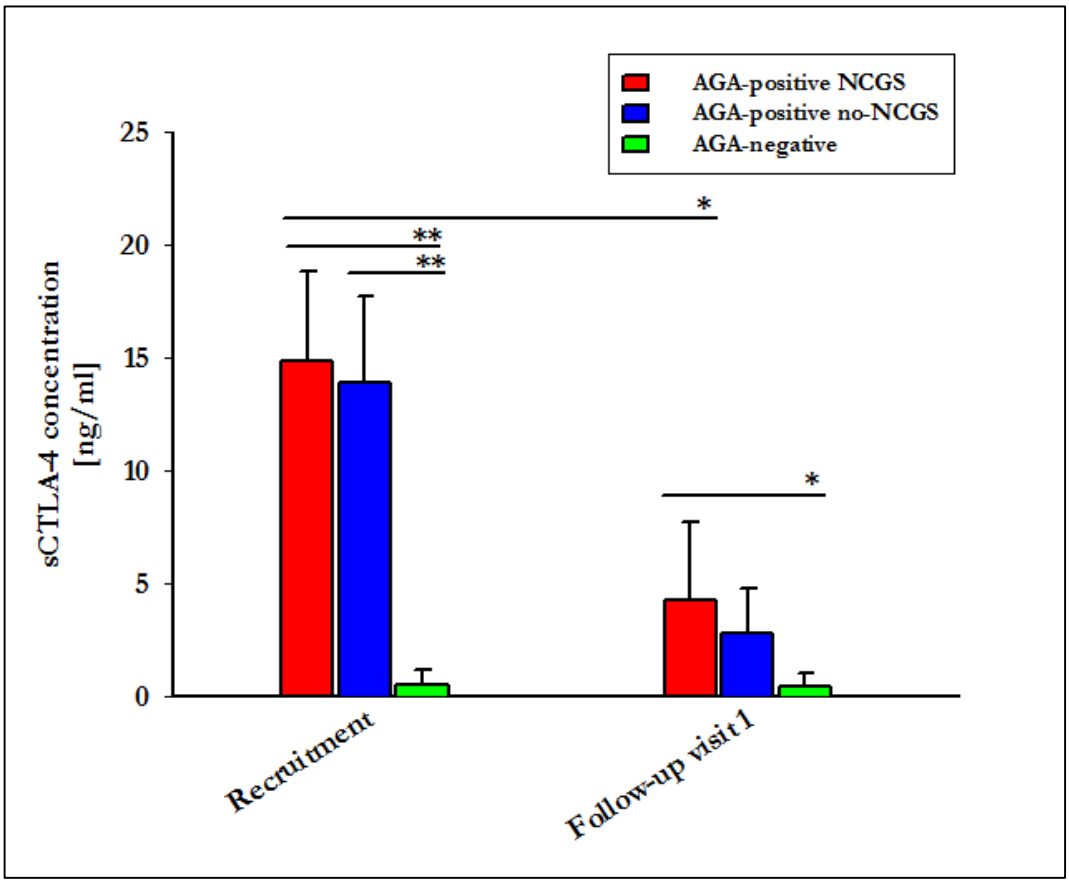

Figure 12. Soluble CTLA-4 levels at recruitment and at the first follow-up visit. All patients were on normal, gluten-containing diet at recruitment. At follow-up visit 1, all AGA-positive patients have been on gluten-free diet for 3 months, AGA-negative patients on normal, gluten-containing diet. Legend: (*) p<0.05; (**) p<0.01.

Levels of soluble gp130 were similar at recruitment in AGA-positive NCGS patients, AGA-positive no-NCGS patients and AGA-negative patients (mean concentration 316.83±37.85, 306.00±20.78, 312.80±39.92, respectively). Moreover, mean sgp130 concentrations were not modified significantly on GFD, neither in AGA-positive NCGS patients (316.83±37.85 vs 307.50±23.89), nor in AGA-positive no-NCGS patients (306.00±20.78 vs 318.25±66.78); similarly, after the gluten challenge only a slight change in sgp130 concentration, both in AGA-positive NCGS patients (307.50±23.89 vs

316.83±45.89), and in AGA-positive no-NCGS patients (318.25±66.78 vs 337.33±57.83) was observed.

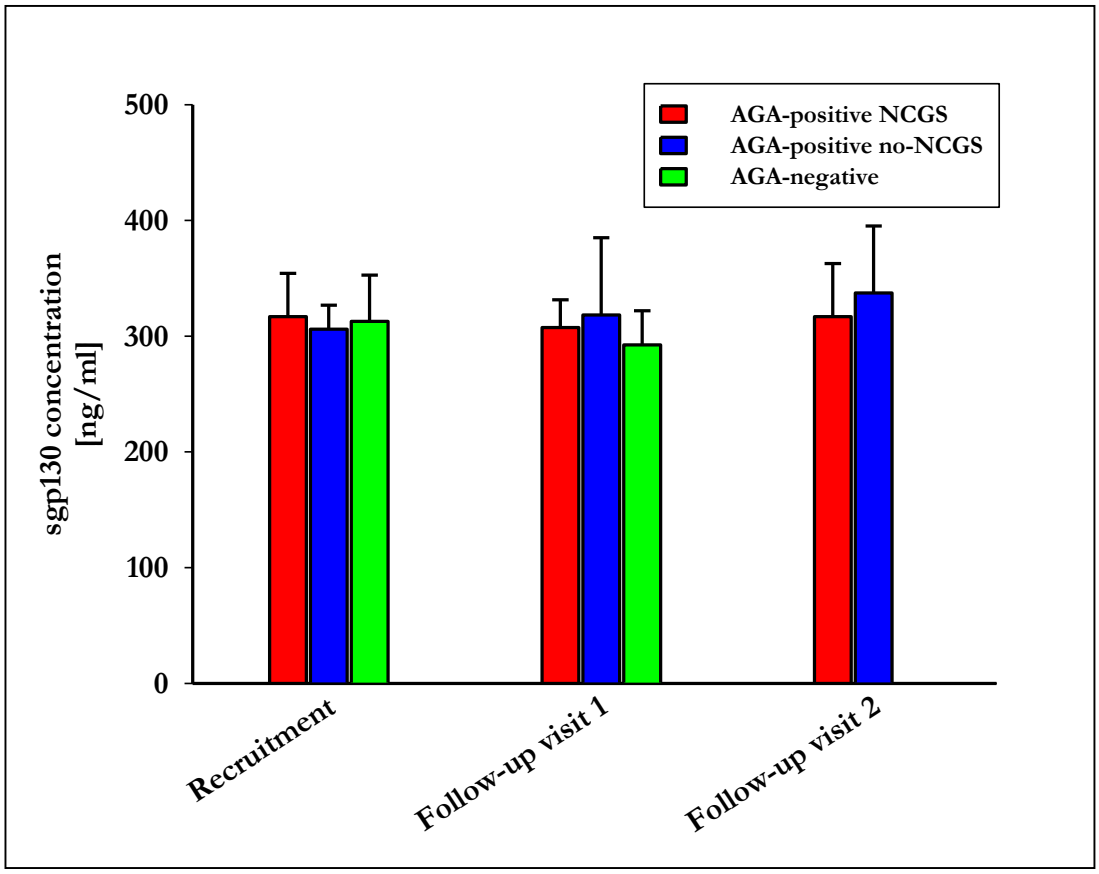

Figure 13. Soluble gp130 levels at recruitment and within the follow-up period. All patients were on normal, gluten-containing diet at recruitment. At follow-up visit 1, AGA-positive patients were on gluten-free diet, AGA-negative patients on normal, gluten-containing diet. At follow-up visit 2, AGA-positive patients were on gluten-containing diet (gluten challenge).

The determination of sCD25 concentration was performed for samples collected at recruitment and first follow-up visit (Figure 14). Soluble CD25 concentrations were similar at recruitment among AGA-positive NCGS patients, AGA-positive no-NCGS patients and AGA-negative patients (mean concentration 5.12±2.67, 3.81±1.16, 4.01±0.12, respectively). Levels of sCD25 were slightly reduced on GFD both in AGA-positive NCGS patients (5.12±2.67 vs 4.34±2.18) and in AGA-positive no-NCGS patients (3.81±1.16 vs 3.27±0.51), even though differences in mean concentrations was not statistically significant. Soluble CD25 concentrations on samples collected on GFD were similar both in AGA-positive NCGS patients and AGA-positive no-NCGS

patients to those in samples of AGA-negative patients (mean sCD25 concentration 4.06±1.98), who were on gluten-containing diet at the follow-up visit.

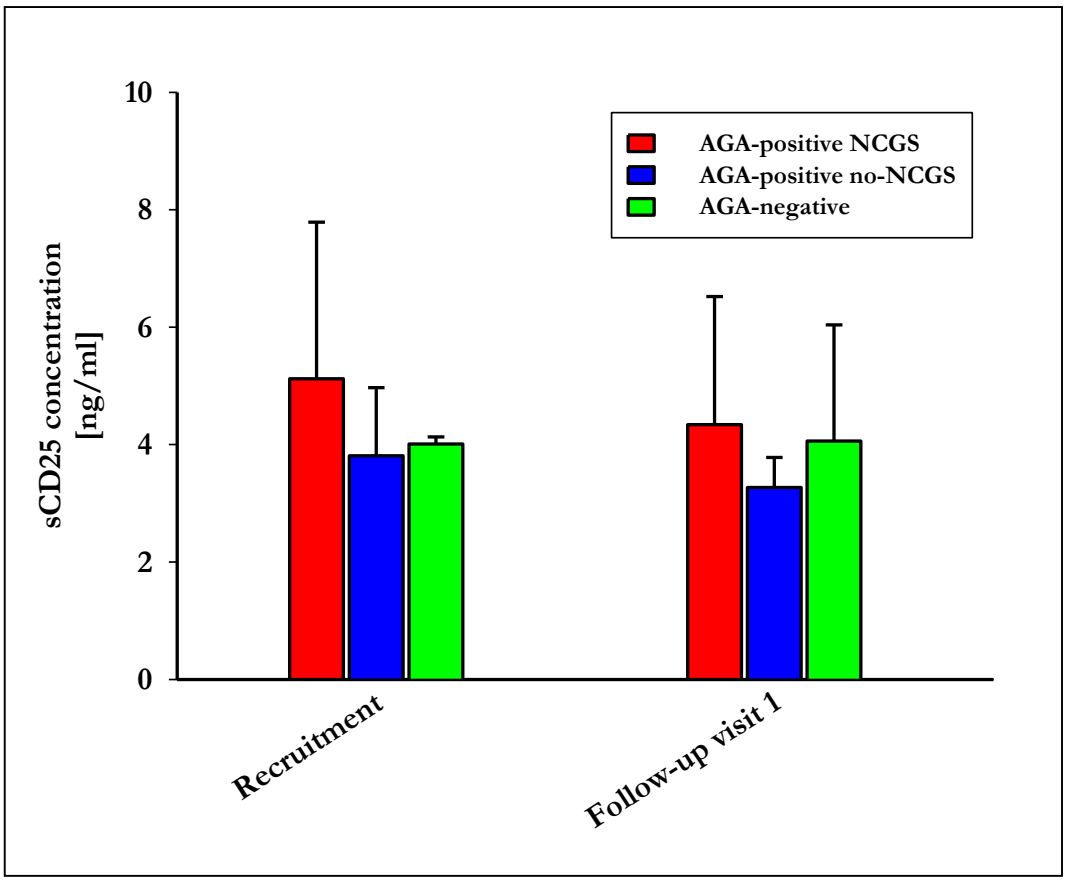

Figure 14. Soluble CD25 levels at recruitment and at follow-up visit. All patients were on normal, gluten-containing diet at recruitment. At follow-up visit 1, AGA-positive patients were on gluten-free diet, AGA-negative patients on gluten-containing diet.

As for the determination of TG6 IgA antibodies (Figure 15), differences in mean TG6 IgA levels among 3 patient groups were not statistically significant, neither at recruitment (mean concentration 11.10±15.92 I.U./ml, 49.46±71.87 I.U./ml, 4.17±3.67 I.U./ml, in AGA-positive NCGS patients, AGA-positive no-NCGS and AGA-negative patients, respectively), nor within the follow-up period (mean concentration at the first follow-up visit: 9.84±18.29 I.U./ml, 40.43±51.21 I.U./ml, 7.92±9.88 I.U./ml; mean concentration at the second follow-up visit: 14.58±12.68 I.U./ml, 40.18±61.00 I.U./ml, 11.73±3.77 I.U./ml).

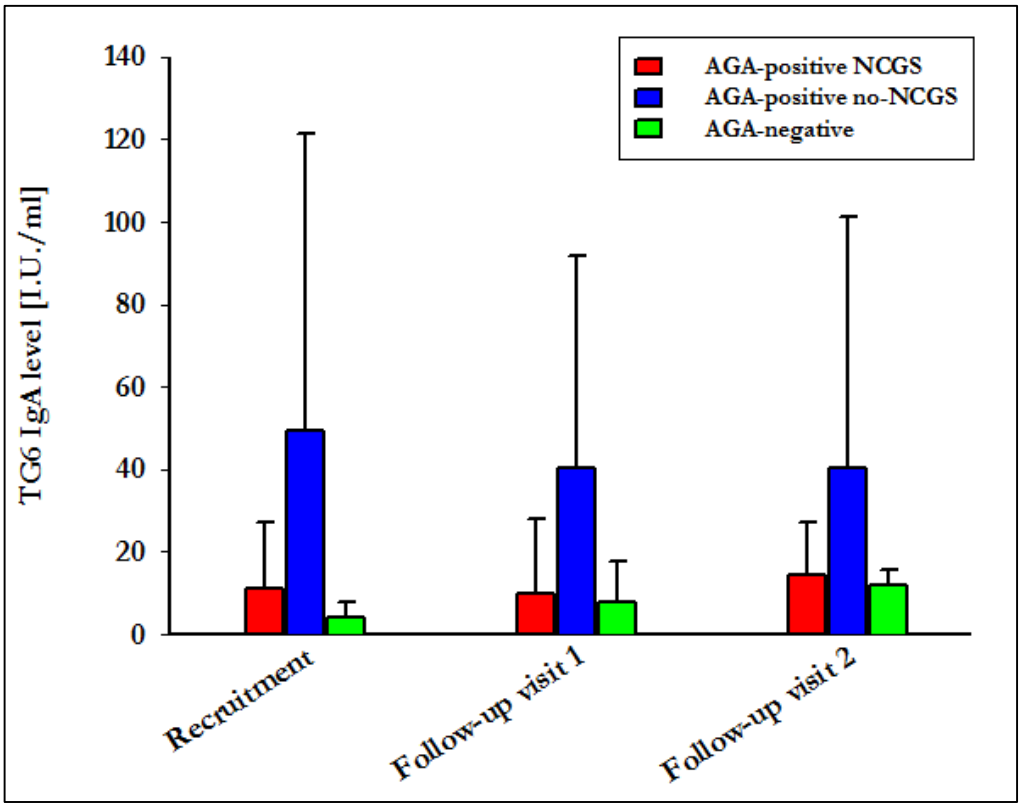

Figure 15. TG6 IgA levels at recruitment and within the follow-up period. All patients were on normal, gluten-containing diet at recruitment. At follow-up visit 1, AGA-positive patients were on gluten-free diet, AGA-negative patients on gluten-containing diet. At follow-up visit 2, all AGA-positive patients were on gluten-containing diet (gluten challenge), whereas AGA-negative patients continued on gluten-containing diet.

According to the reference values indicated by the manufacturers of the commercial ELISA test, none of the subjects among AGA-negative patients was positive for TG6 IgA, neither at recruitment, nor within the follow-up (Table 11, p. 52). Three AGA-positive patients out 11 (27%) tested positive for TG6 IgA (Table 11). In particular, one out 6 (17%) AGA-positive NCGS patients and 2 out 5 (40%) AGA-positive no-NCGS patients had positivity to TG6 IgA. The antibody level was 43.36 I.U./ml in the AGA-positive NCGS patient, 166 I.U./ml and 72.6 I.U./ml in the AGA-positive no-NCGS patients, respectively. Levels of TG6 IgA resulted to be nearly unchanged in the AGA-positive NCGS patient (43.36 vs 42.4 U/ml – 2% reduction) after 3 months on GFD, or mildly reduced in AGA-positive no-NCGS patients (166 vs 120 I.U./ml – 28% reduction; 72.6 vs 64 I.U./ml – 12% reduction). At follow-up visit 2 (i.e., on gluten-containing diet for 3 months), TG6 IgA antibody level was further reduced in the AGA-positive NCGS patient (42.4 vs 38 U/ml – 10% reduction), whereas the antibody level

was mildly increased in one of AGA-positive no-NCGS patients (120 vs 131,5 U/ml – 10% increase). Subsequent evaluation was not available for the other AGA-positive no-NCGS patient who tested positive for TG6 IgA, because he decided to exit the study after the first follow-up visit.

As for the determination of TG6 IgG antibodies (Figure 16), differences in mean TG6 IgG levels were not statistically significant in 3 different groups, neither at recruitment (mean concentration 10.68±6.17 I.U./ml, 10.98±7.87 I.U./ml, 6.83±2.09 I.U./ml, in AGA-positive NCGS patients, AGA-positive no-NCGS and AGA-negative patients, respectively), nor within the follow-up period (mean concentration at the first follow-up visit: 12.78±5.86 I.U./ml, 10.71±10.02 I.U./ml, 5.20±3.06 I.U./ml; mean concentration at the second follow-up visit: 10.43±7.32 I.U./ml, 10.03±8.89 I.U./ml, 5.77±4.64 I.U./ml).

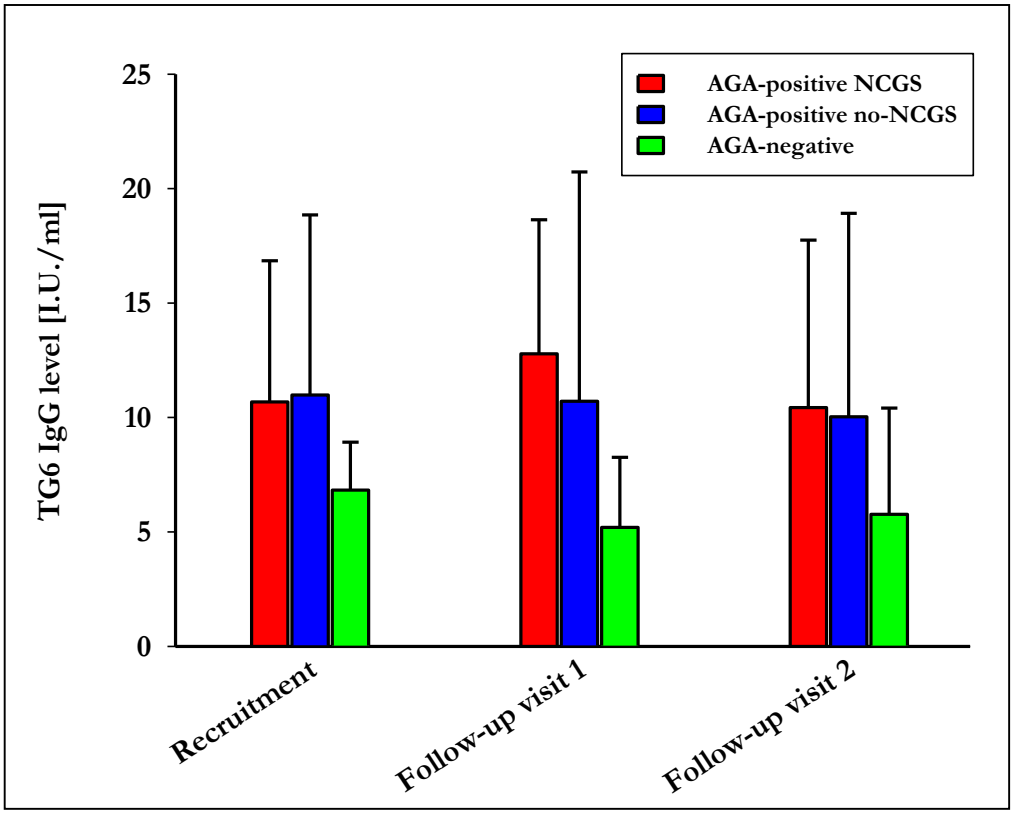

Figure 16. TG6 IgG levels at recruitment and within the follow-up period. All patients were on normal, gluten-containing diet at recruitment. At follow-up visit 1, AGA-positive patients were on gluten-free diet, AGA-negative patients on gluten-containing diet. At follow-up visit 2, all AGA-positive patients were on gluten-containing diet (gluten challenge), whereas AGA-negative patients continued on gluten-containing diet.

According to the reference values indicated by the manufacturers of the commercial ELISA test, all patients tested negative for TG6 IgG antibodies, both at recruitment and within the follow-up period (Table 11).

Table 11. Patients who tested positive to TG6 antibodies.

	AGA-positive pts			AGA-negative pts
	NCGS N. pts (%)	no-NCGS N. pts (%)	All N. pts (%)	All N. pts (%)
Positive to TG6 IgA	1/6 (17%)	2/5 (40%)	3/11 (27%)	0/6 (0%)
Positive to TG6 IgG	0/6 (0%)	0/5 (0%)	0/11 (0%)	0/6 (0%)

CHAPTER IV: DISCUSSION

4.1 Non-celiac Gluten Sensitivity in children with headache

This study suggests that it is worth exploring the presence of gluten-related diseases in subjects with headache.

We found that about 50% children and adolescents, who were evaluated for primary headache in a specialized referral center, tested positive for native anti-gliadin antibodies. Thus, the estimated prevalence of AGA-positivity resulted about 5-fold higher in this cohort than in the general population, according to published data [Volta 2008, Volta 2012, Caio 2014, Catassi 2015]. The prevalence of AGA-positivity was about 2-fold higher in children with headache than in general population even when considering cut-off values of AGA tests for celiac disease, which were fixed at 50 I.U./ml, instead of 25 I.U./ml, for the commercial ELISA test we used for AGA IgG determination. Conversely, none of the subjects in this cohort had serological markers for celiac disease.

Our findings are consistent with previous data reporting a 4- to 10-fold higher prevalence of markers of gluten-related disorders or ascertained celiac disease and NCGS, in headache patients than in general population, even though celiac disease appeared to be less prevalent in our cohort than in other headache pediatric cohorts, where it was estimated about 5% [Gabrielli 2003, Lionetti 2009, Lionetti 2010, Dimitrova 2013].

When the diagnostic algorithm for NCGS was applied, the prevalence of the disease in our cohort of AGA-positive pediatric headache patients was 55%. This is a quite elevated figure, possibly influenced by lack of blindness for patients and physicians in gluten withdrawal or gluten challenge. However, in the study design we considered that our increased duration of both phases, 2-fold longer than in the most recently proposed diagnostic algorithm, could consent a better evaluation of the clinical course for fluctuating symptoms such as headache, and reasonably reduce the impact of placebo and nocebo effects. Indeed, response to placebo is high in acute headache attacks, whereas the placebo effect in chronic headache remission is smaller and reported to be approximately one patient in four [Gabrielli 2003].

As for the clinical picture, we were not able to identify any headache features or

migraine-associated syndromes which might be typical for AGA-positive, NCGS patients. Conversely, we noted that gastrointestinal symptoms occurred more frequently in these patients (50%) than in AGA-positive patients without NCGS or in AGA-negative subjects, being gastrointestinal complaints similarly prevalent in the latter 2 groups (20% and 17%, respectively). Extra-intestinal symptoms other than headache were more frequent in AGA-positive patients, and very similar to those reported in almost all published NCGS cohorts, but they could not identify NCGS patients in the AGA-positive group [Catassi 2013, Volta 2014, Volta 2015]. Personal and family history for allergy was reported in about one patient out 3 AGA-positive NCGS subjects, and this finding is consistent with literature data [Volta 2014]; nevertheless, a similar figure was found in AGA-positive no-NCGS in this cohort, being not a key factor for NCGS again. No one had autoimmune diseases in NCGS patients in this headache cohort. In fact, autoimmune disorders seem to occur rarely in NCGS patients [Volta 2014].

4.2 Gene expression profiles in peripheral blood mononuclear cells from headache, NCGS patients

Previous studies from collaborators to this research project provided important information on the pathogenetic mechanisms involved in celiac disease [Zanoni 2006, Dolcino 2013]. Data on etio-pathogenesis of NCGS are preliminary and conflicting. Some research groups hypothesized a primary role of the innate immune system, without any relevant involvement of the adaptive immune response [Sapone 2011, Shuppan 2015]; other authors observed that expression of IFNγ increased in duodenal mucosa after challenge with gluten, thus supporting a possible pathogenic role for adaptive immunity in this syndrome [Brottveit 2013]. A third line of research considered gastrointestinal symptoms as independent from immunologic mechanisms, and possibly due to fermentation of poorly absorbable molecules in foods [Biesiekierski 2013].
From the present analysis, genes belonging to functional classes of immune response, both innate and adaptive, resulted modulated in the active phase of NCGS. The gene expression profiling documented up-regulation of a high number of genes related to T- and B-lymphocyte activation and, even more interestingly, up-regulation of genes related to Th17 cell subset and type I Interferon response. These findings are in line with experimental data by Brottveit and colleagues, and provide further evidence for a major

role of adaptive immune response in NCGS. Notably, both type I IFN-driven inflammation and activation of Th17 pathway are primarily involved in chronic inflammatory disorders of the gut, such as Crohn disease, and in systemic autoimmune diseases, e.g. systemic lupus erythematosus, Sjogren's Syndrome, and rheumatoid arthritis [Eken 2014, De Jong 2015, Ghodke-Puranik 2015, Nezos 2015]. Moreover, type I Interferon is the proximate driver of the tissue damage resulting in villous atrophy in celiac disease, and a role of Th17 cell subset has been hypothesized for this disorder [Mazzarella 2015].

In our cohort of NCGS patients IL24, IL32, TNFRSF25 and CTLA-4 genes of the "T-lymphocyte activation" cluster were up-regulated. Notably, many of these gene products are shown to be associated with chronic inflammatory and autoimmune disorders.

IL-24 is upregulated in T cells, when c-Jun is overexpressed after an activating stimulus, and induces secretion of IL-6, TNF-α and INF-γ at high levels. A body of evidence accounts for a role of IL-24 in autoimmune diseases such as psoriasis, rheumatoid arthritis and spondylo-arthropathies [Menezes 2014].

IL-32 is expressed in NK cells and T lymphocytes after stimulation with TNFα and IL-23, and induces the differentiation of monocytes into macrophage-like cells which secrete pro-inflammatory cytokines such as IL-6 and TNFα. IL-32 also induces activation of dendritic cells leading to an increased production of IL-12 and IL-6, which are Th1- and Th17-polarizing cytokines, and the production of this cytokine is associated with several inflammatory diseases, including rheumatoid arthritis and intestinal chronic inflammation [Xu 2013, Joosten 2013].

TNFRSF25, also known as DR3, is expressed in NK cells and T cells, particularly on Th17 cells, and it is enhanced upon cell activation. Since the ligand of DR3 is induced by inflammatory cytokines or TLR ligands, its interaction with DR3 represents a key event connecting innate immune responses to adaptive immune responses, and it is critically involved in the induction of autoimmune and inflammatory diseases, such as rheumatoid arthritis and Crohn disease [Aiba 2013].

CTLA-4 is a type I glycoprotein on the surface of activated T-cells, which is a B7-binding protein. The function of CTLA-4 is attenuation of ongoing immune responses, both for cellular and humoral immune responses. The CTLA-4 gene is able to generate messenger RNA for a full-length isoform (flCTLA-4) and a soluble form (sCTLA-4), which originates from alternative splicing and is found in the serum. The blockade of

B7/CTLA-4 interaction exacerbates autoimmune diseases in animal models. The soluble CTLA-4 is able to decrease *in vitro* proliferation of T-cells and production of IFNγ, whereas it increases anti-inflammatory cytokines, such as TGF-β and IL-10. Immuno-regulatory functions of sCTLA-4 appear to be dependent on the activation state of cells. Since resting T-cells express sCTLA-4 but not flCTLA4, sCTLA4 inhibits B7–CD28 interaction and sustains T-cell resting state; when flCTLA4 is overexpressed after T-cell activation, sCTLA4 interferes with B7–flCTLA4 interaction, blocks the flCTLA4 inhibitory pathway, and finally results in a sustained T-cell activation [Saverino 2007, Simone 2014]. This regulatory pathway may be dependent on the duration of the activating stimulus, as documented by *in vitro* experimental data: sCTLA-4 arrests lymphocyte proliferation when it is added within 24 hours, but it leads to a sustained lymphocyte proliferation if added after 24 hours [Saverino 2007]. Such an effect, we argue, may be primarily directed to control acute inflammatory states, e.g. infectious diseases, and provide an explanation for high levels of sCTLA-4 in chronic inflammatory disorders. In fact, increased concentrations of sCTLA-4 were observed in patients with celiac disease, thyroid autoimmune diseases, type 1 diabetes, systemic sclerosis, and systemic lupus erythematosus, but also in patients with allergy or neoplastic disorders [Saverino 2007, Erfani 2010, Simone 2014]. The highest sCTLA-4 levels were measured in the active phase of the disease in the majority of published case series, but a positive correlation with symptom severity was not confirmed. Interestingly, sCTLA-4 levels markedly decreased on gluten-free diet in patients with celiac disease [Simone 2009] and also in our headache AGA-positive cohort, providing evidence for sCTLA-4 as a biomarker of gluten sensitization which is modulated by trigger withdrawal.

As for the Th17 compartment, we observed that 9 genes, including IL6ST, STAT6, STAT5B and SOCS3, were up-regulated. The IL6ST protein, also known as gp130, is the beta-receptor for cytokines of the IL-6 family which binds to the complex IL-6R/IL-6 and starts the signaling cascade, which involves the Jak/STAT pathway; at the same time, the negative feedback regulator SOCS3 is transcriptionally up-regulated and drives to degradation of gp130 [Yao 2014, Garbers 2015]. T lymphocytes stimulated by IL-6 differentiate into Th17 cells, and this process is sustained in time [Calabrese 2014]. A body of evidence shows that a deregulated cytokine signaling via gp130 is associated with chronic inflammation and autoimmune diseases (e.g.,

rheumatoid arthritis and inflammatory bowel diseases) [Gabers 2015]. Therefore, the evidence for up-regulation of genes primarily involved in IL-6 signaling pathways, at different levels, in our cohort of headache patients, may be in favor of a pathogenic role of Th17 cells in NCGS.

Even more interestingly, NCGS patients also showed up-regulation of type I Interferon inducible genes (e.g. IRF5, IRF3, Ly-9), thus displaying a type I IFN signature. IRFs are transcriptional regulators of the production of type I Interferons (INFs). Type I IFNs drive the immune response polarizing T cells to a Th1 phenotype, in order to control viral infections. Interferon Regulatory Factor 5 (IRF5) is extremely important for increased and prolonged TNF production, and "gain of function" IRF5 gene mutations have been associated with susceptibility to autoimmune diseases, such as systemic lupus erythematosus and inflammatory bowel diseases [Eames 2016]. Interferon Regulatory Factor 3 (IRF3) plays a role in Th17 cell development and function, as suggested by *in vitro* impaired IL-17 production in IRF3-deficient cells [Fitzgerald 2014]. Ly9 is expressed on T and B lymphocytes and regulates lymphocyte development and survival, cytotoxicity and humoral immunity, thus exerting crucial immunomodulatory functions. Moreover, it acts as an inhibitory receptor of IFNγ-producing CD4+ T cells. A role for Ly9 in the maintenance of immune cell tolerance is confirmed by experimental data in Ly9-deficient mice, which spontaneously develop features of systemic autoimmunity. Alike for CTLA-4 gene, up-regulation of LY-9 gene in NCGS patients would be consistent with activated immunomodulatory mechanisms meant to attenuate the ongoing immune response [De Salort 2013].

The transcriptional modulation of genes related to the "Response to INFγ" cluster, namely up-regulation of CD84 and down-regulation of SLAMF8 genes, seems in line with observations above. Up-regulated CD84 in T-, B-, and dendritic cells increases activation of these cells [Veillette 2010]. Instead, SLAMF8 is a surface receptor which modulates macrophage functions when it is induced by cell exposure to INFγ and dampens an ongoing innate immune response [Wang 2012]. The down-regulation of this receptor, as we observed in NCGS patients, would presumably lead to a prolonged activation of innate immune responses. Coherently, the MARCO gene, which codes for a macrophage scavenger receptor increasing phagocytosis after repetitive stimulation in order to protect hosts from excessive inflammation as documented by Jing et al. [2013], resulted down-regulated in our cohort of NCGS patients.

A number of transcripts involved in B cell and NK cell activity were modulated in this cohort of NCGS patients, thus suggesting a concomitant activation of this cell subset. Among them CLEC2D, also named LLT1, and the Leucocyte Immunoglobulin-like Receptors (LILRs) deserve further discussion.

LLT1 is expressed on Toll-like receptor-activated plasmacytoid dendritic cells, but also on activated B-, T- and NK cells when they are stimulated with IFNγ in a Th1-polarizing environment. LLT1 in turn increases IFNγ secretion by T cells, and IL-17 secretion by Th17 cells. Thus, the interaction of LLT1 with its receptor may participate in the switch from innate to adaptive immunity and regulate both innate and adaptive immune responses [Germain 2011]. The LLT gene was up-regulated in NCGS headache patients, thus providing evidence for a key role of Th1 and Th17 cell activation in this disorder.

LILRs are predominantly expressed on myelo-monocytic cells and B cells, interact with self-proteins and modulate dendritic cell activation status, antigen-presenting functions and their capacity to elicit T-cell responses. High levels of LILRB2 and LILRB4 decrease the expression of costimulatory proteins, hindering dendritic cells' ability to activate Th cells. LILRA2 engagement inhibits dendritic cell differentiation and antigen presentation, LILRA4 negatively regulates the secretion of type I Interferons by plasmacytoid dendritic cells. Notably, all these genes were down-regulated in NCGS patients, providing further evidence for an ongoing, predominantly type I INF-driven, immune response.

In a physiopathological perspective, the gene expression profile in headache NCGS patients is suggestive for a type I Interferon response - related syndrome. In this context, symptoms such as headache, fatigue, "foggy mind", and "lack of well-being", so frequently reported by these patients, might be attributable to a "sickness behavior" phenomenon, alike it is well-known to occur in systemic inflammatory conditions [Maier 1998, Watkins 2000, Quan 2008, Benson 2012, Poon 2015].

However, since the great majority of patients in this cohort also had family antecedents for headache, we can assume that this systemic inflammatory environment may contribute to potentiate, in genetically predisposed subjects, those neuro-inflammatory mechanisms responsible for the sensitization of nociceptive second-order neurons, a phenomenon which is well-known to occur in patients suffering from recurrent headaches and leads to chronicization of headache [Bendtsen 2011, Burstein 2015].

4.3 Soluble mediators in sera and TG6 antibodies in headache patients

We found that serum levels of soluble CTLA-4 were significantly higher in headache AGA-positive patients than in AGA-negative subjects, and that sCTLA-4 levels markedly decreased on gluten-free diet in all subjects, despite NCGS diagnosis was not confirmed in a subgroup of them. AGA-negative patients had very low levels of sCTLA-4, which were nearly unchanged on a normal, gluten-containing diet. Similar effects of GFD on serum sCTLA4 levels were reported by Simone and colleagues [2009] on a cohort of patients with celiac disease, although these authors found that levels of sCTLA-4 were positively correlated with the severity of enteropathy. Conversely, our findings are consistent with data from patients with autoimmune thyroid diseases or breast cancer, where no correlation between sCTLA-4 levels and disease stage or severity was found [Saverino 2007, Erfani 2010].

Therefore, although sCTLA-4 cannot be considered a predictive biomarker for confirmed NCGS, high levels of this soluble mediator might represent a clue for an ongoing inflammatory response related to gluten ingestion and adaptive immune response activation. Unfortunately, neither sCD25 nor sgp130 levels in sera could add any useful information, at least in this cohort of patients.

Finally, we observed that both patients who had high levels of TG6 antibodies in sera did not display clinical amelioration on a 3 months - period on gluten-free diet. Therefore, we speculate that a longer duration of the dietary treatment, maybe 6-12 months as recommended for gluten ataxia, might be requested for headache patients who test positive for TG6 antibodies, in order to better evaluate a gluten-related disorder.

CONCLUSIONS

The present work provides evidence for considering the diagnostic hypothesis of Non-celiac Gluten Sensitivity in children and adolescents with recurrent headaches fully satisfying criteria for primary headaches.

Laboratory data from NCGS patients in this cohort suggest the existence of an activated immune response with a type I Interferon signature. High levels of soluble CTLA-4 in serum in headache AGA-positive patients could be a further clue for gluten sensitization and adaptive immune response activation.

The presence of TG6 antibodies in sera may predict a poor clinical response to gluten-free diet upon a 3 months – period, thus justifying a gluten-free dietary treatment for 6-12 months at least.

Further investigation in larger series of patients is needed to validate these preliminary results. Histo-pathological studies on duodenal specimens might be useful to evaluate local immune response, and to explore the role of mucosal innate lymphoid cell compartment and inter-relationships between these innate immunity cells and other actors of the immune response.

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LIST OF ABBREVIATIONS FREQUENTLY USED IN THE TEXT

AGA: native anti-gliadin antibodies

AGA-positive NCGS patient: patient who tested positive for AGA and had the diagnosis of Non-celiac Gluten Sensitivity confirmed

AGA-positive no-NCGS patient: patient who tested positive for AGA and had the diagnosis of Non-celiac Gluten Sensitivity excluded

AGA-negative patient: patient who tested negative for AGA

CD: Celiac disease

CBCL: Child Behavior Checklist

DH: Dermatitis Herpetiformis

ELISA: enzyme-linked immunosorbent assay

EMA: anti-endomysial antibodies

GA: Gluten Ataxia

GFD: gluten-free diet

ICHD: International Classification of Headache Disorders

IELs: intraepithelial lymphocytes

IFN: Interferon

IL-6: Interleukin-6

IL-17: Interleukin-17

MIDAS: Migraine Disability Score

NCGS: Non-celiac Gluten Sensitivity

PBMCs: Peripheral blood mononuclear cells

sCD25: soluble form of IL-2 Receptor α-Chain

sCTLA-4: soluble form of Cytotoxic T-Lymphocyte Antigen 4

sgp130: soluble form of Glycoprotein 130 (also known as IL6ST)

TGA: anti-tissue transglutaminase 2 (intestinal transglutaminase) antibodies

TG3: tranglutaminase 3 (epidermal transglutaminase)

TG6: tranglutaminase 6 (brain transglutaminase)

Th1: T helper 1 lymphocyte

Th17: T helper 17 lymphocyte

TNF: Tumor necrosis factor

TTH: Tension-type headache

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