Joana Clímaco Henggeler Antunes

NON-COELIAC GLUTEN SENSIVITY AND COELIAC DISASE: ANALYSIS OF A PORTUGUESE POPULATION

Dissertation of Food Safety Master Degree under orientation of Professor Ph.D. Fernando Jorge Ramos and Professor Ph.D. Manuel Teixeira Veríssimo, presented to the Faculty of Pharmacy of University of Coimbra

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UNIVERSIDADE DE COIMBRA

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Dissertation of Food Safety Master Degree presented to the Faculty of Pharmacy, University of Coimbra to obtain the degree of Master

Oriented by: Professor Ph.D. Fernando Jorge Ramos and Professor Ph.D. Manuel Teixeira Veríssimo

Coimbra, 2017

DECLARAÇÃO

Eu, Joana Clímaco Henggeler Antunes, estudante do Mestrado em Segurança Alimentar, com o nº 2008110921, declaro assumir toda a responsabilidade pelo conteúdo da Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, no âmbito da unidade curricular de Dissertação/Projecto.

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Coimbra, 31 de Agosto de 2017

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RESUMO

Durante a última década, a sensibilidade ao glúten não celíaca (SGNC) tem vindo a crescer em popularidade e em controvérsia tanto entre a população em geral como na literatura. A SGNC é considerada parte do espectro dos distúrbios relacionados com a ingestão de glúten, mas ao contrário, por exemplo, da doença celíaca (DC), os seus mecanismos não são inteiramente conhecidos.

Esta dissertação teve como objectivo descrever adequadamente a SGNC e as suas características, para depois determinar se esta doença estaria presente numa amostra populacional de pacientes portugueses com doença celíaca. Tal foi conseguido através de uma revisão da literatura, que foi por sua vez seguida por uma análise dos dados recolhidos dos processos médicos de 47 pacientes com DC.

Depois de uma análise completa destes dados, foi concluído que 4 pacientes sofriam potencialmente de SGNC. É possível que uma quantidade significativa da população, incluindo alguns diagnosticados com DC, possa também sofrer deste distúrbio. Por conseguinte, os profissionais de saúde deveriam ter esta possibilidade em mente durante o processo de diagnóstico, especialmente no caso de pacientes com lesões histológicas pouco severas e serologia específica para DC negativa.

Palavras-chave: distúrbios relacionados com a ingestão de glúten, doença celíaca, glúten, sensibilidade ao glúten não celíaca

ABSTRACT

Over the past decade, non-coeliac gluten sensitivity (NCGS) has increasingly grown in popularity and controversy both among the general population and in literature. NCGS is considered part of the spectrum of gluten-related disorders, but unlike, for instance, coeliac disease (CD), its mechanisms are not fully known.

This dissertation was aimed at adequately describing NCGS and its features, to then determine whether this disorder was present in a population sample of Portuguese CD patients. This was achieved through a review of the literature, which was then followed by an analysis of data from medical files of 47 CD patients.

After a full analysis of these data, it was concluded that 4 patients potentially suffered from NCGS. It is possible that a significant amount of the population, including some of those diagnosed as having CD, could also suffer from this disorder. Therefore, clinicians should keep this possibility in mind during the diagnostic process, especially when considering patients with minor histological damage and negative CD-specific serology.

Keywords: coeliac disease, gluten, gluten-related disorders, non-coeliac gluten sensitivity

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LIST OF ABBREVIATIONS

AGA - anti-gliadin antibodies

ASD - autism spectrum disorders

ATI(s) - amylase trypsin inhibitor(s)

CCL2 - chemokine (C-C motif) ligand 2

CD - coeliac disease

CD3, CD4, CD8, CD14 - clusters of differentiation 3, 4, 8 and 14

CLDN1, CLDN4 - claudins 1 and 4

COPD - chronic obstructive pulmonary disease

DBPC - double-blind placebo-controlled

DBPCFC - double-blind placebo-controlled food challenge

DGP - deamidated gliadin peptide(s)

DQ2, DQ8 - human major histocompatibility class II alleles

EATL - enteropathy-associated T-cell lymphoma

EI - extra-intestinal

EMA - anti-endomysium autoantibodies

FABP2 - fatty acid-binding protein 2

FODMAPs - fermentable oligo-, di-, and mono-saccharides and polyols

FOXP3 - forkhead box P3

GFD - gluten-free diet

GI - gastrointestinal

GS - gluten sensitivity/gluten sensitive

HLA - human leukocyte antigen

HUC – Hospitais da Universidade de Coimbra

IBS - inflammatory/irritable bowel syndrome

IBS-D - diarrhoea-predominant inflammatory/irritable bowel syndrome

IFN-γ - interferon-gamma

IgA, IgE, IgG - immunoglobulins A, E and G

IL-8 - interleukin-8

LBP - lipopolysaccharide-binding protein

LPS - lipopolysaccharide

MD2 - lymphocyte antigen 96

NCGI - non-coeliac gluten intolerance

NCGS - non-coeliac gluten sensitivity

NCWS - non-coeliac wheat sensitivity

RCD - refractory coeliac disease

sCD14 - soluble CD14

T1DM - type 1 diabetes mellitus

T2DM - type 2 diabetes mellitus

TGF - transforming growth factor

TLR-1, TLR-2, TLR-4 - Toll-like receptors -1, -2 and -4

$\mathbf{T_{REG}}$ - regulatory T-cell

tTG - tissue transglutaminase

WA - wheat allergy

WIS - wheat intolerance syndrome

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1. INTRODUCTION

The spectrum of gluten-related disorders has been the target of numerous studies in the past few years. While the underlying mechanisms behind coeliac disease (CD) have been well understood for the past few decades, non-coeliac gluten sensitivity (NCGS) has been the subject of much controversy since it was first documented (Di Sabatino & Corazza, 2009; Sapone *et al.*, 2012).

The main objective of this dissertation was to contribute to understand how exactly this disorder fits in the spectrum of gluten-related disorders, and eventually attempt to discover if it was present among Portuguese patients.

The initial step in this process was a review of the literature, mainly based on articles published between 2011 and 2017, focusing on NCGS as a clinical entity and its symptoms, diagnosis, treatment, history, prevalence and potential mechanisms.

The second step was to analyse the medical files of a sample of coeliac patients, since it is known that a reasonable number of NCGS patients is misdiagnosed as having CD. Data pertaining to age at diagnosis, family history, symptoms and associated pathologies, serological, genetic and histological testing and treatment (among other parameters) were evaluated for this analysis.

2. STATE OF THE ART[1]

2.1. Gluten and the spectrum of gluten-related disorders

Gluten is the main storage protein complex in cereals such as wheat, barley, rye and spelt. Its main protein constituents are gliadin and glutenin (Dupont *et al.*, 2011). These proteins are rich in glutamines and prolamines, which causes them to be incompletely digested by gastric, pancreatic, and brush border peptidases, leading to the formation of large peptides (Shan *et al.*, 2002). These pass through the intestinal epithelial barrier and enter the lamina propria by way of a transcellular or paracellular route (Matysiak-Budnik *et al.*, 2008; Visser *et al.*, 2009).

When flours which contain gluten are kneaded with water, the dough acquires viscosity and elasticity due to the action of gliadins and glutenins (Blomfeldt *et al.*, 2011; Turabi *et al.*, 2008). This happens by way of the formation of an elastic network which retains the gases resulting from fermentation, which allows the expansion and rise of the dough during the baking process (Gallagher *et al.*, 2004; Moore *et al.*, 2004).

The proteins of gluten possess resistance to gastric and intestinal digestion and cause an increase in intestinal permeability through cytoskeletal rearrangement, zonulin (protein which modulates intestinal permeability) overexpression and dysfunction of tight junctions (Drago *et al.*, 2006). Intestinal homeostasis is altered via the inhibition of epithelial cell growth and the induction of apoptosis (Dolfini *et al.*, 2005a).

The ingestion of gluten can trigger an array of conditions; these are designated by the broader term "gluten-related disorders". They are divided into: disorders with autoimmune pathogenesis, including coeliac disease (CD); disorders characterized by allergic mechanisms, which include wheat allergy (WA); and the controversial non-coeliac gluten sensitivity (NCGS), whose causes are neither autoimmune nor allergic in nature (Di Sabatino & Corazza, 2009; Sapone *et al.*, 2012).

[1] The entirety of this chapter was adapted from: ANTUNES, J.C.; VERÍSSIMO, M.; RAMOS, F. - Non-coeliac gluten sensitivity: A review of the literature. **Trends Food Sci Technol**. 66 (2017) 84-92.

2.1.1. Coeliac disease

Coeliac disease (CD) is a systemic immune-mediated disorder caused by gluten and analogous prolamines (found in rye and barley) in individuals with a genetic susceptibility (Di Sabatino & Corazza, 2009; Sapone *et al.*, 2012). It is considered to affect between 1 and 2% of the general population (DiGiacomo *et al.*, 2013; Kang *et al.*, 2013; Sapone *et al.*, 2012).

CD possesses well-documented genetic makeup and environmental triggers (such as gliadin peptides) (Sapone *et al.*, 2010). Markers used in the confirmation of a diagnosis are IgA anti-tissue transglutaminase (tTG) and anti-endomysial (EMA) antibodies (Sapone *et al.*, 2010; Sapone *et al.*, 2011; Sollid & Jabri, 2011).

The common feature for all patients suffering from CD is the presence of a variable combination of gluten-dependent clinical manifestations, specific antibodies (anti-tTG, anti-EMA), human leukocyte antigen (HLA)-DQ2 and/or -DQ8 haplotypes (present in 90-95% of CD patients) and degrees of enteropathy varying from lymphocytic infiltration of the epithelium to complete villous atrophy (Husby *et al.*, 2012; Sollid & Jabri, 2011; van der Windt *et al.*, 2010).

The immune response to gluten in this disorder involves both the adaptive and innate immune systems (though the former is more heavily implicated in this mechanism) (Nilsen *et al.*, 1998; Sollid, 2002). An adaptive immune reaction takes place within the lamina propria. Gliadin is deamidated by tTG, and the latter increases its immunogenicity by altering the charge of the gliadin fragments that aid in the binding between antigen presenting cells and the HLA-DQ2 or -DQ8 molecule (Sollid, 2002). This reaction leads to inflammatory responses in the small intestine that are mediated by CD4+ T cells (Palová-Jelinková *et al.*, 2005; Sollid, 2002). Said cells recognize gliadin peptides, leading to the subsequent production of pro-inflammatory cytokines, specifically interferon gamma (IFN-γ) (Nilsen *et al.*, 1998; Sollid, 2002). The release of metalloproteinases and other tissue damaging mediators concurrent with the inflammatory cascade induce tissue injury (Mohamed *et al.*, 2006).

Diagnosis is based on clinical symptoms (mainly chronic diarrhoea and weight loss), histopathological evaluation of biopsies of the small intestine with varying degrees of villous atrophy, as well as serological detection of tTG and/or EMA IgA antibodies (Guandalini & Assiri, 2014; Sollid & Jabri, 2011).

Until recently, CD was thought to be uncommon during infancy or childhood, while also being detected more frequently in individuals of that age (Matthias *et al.*, 2011). However, it is now recognized that the majority of CD cases occur in adults in the 40-60 years age range (Matthias *et al.*, 2011). This disorder is also more common in females than in males (Volta *et al.*, 2013). First-degree family members of CD patients have been found to possess an increased risk for CD, which can vary from 2 to 20% depending on gender and HLA-haplotype (Mearin *et al.*, 1985; Vriezinga *et al.*, 2014).

The foundation of treatment for CD is the permanent adherence to a strict gluten-free diet (GFD), which will usually lead to a rapid improvement, both clinical and histological (Green & Cellier, 2007). This treatment calls for the exclusion of wheat and other cereal grains from the diet (Green & Cellier, 2007). Patients on a GFD will have a quick response in most cases, leading to a resolution of clinical symptoms. Nonetheless, the resolution of histological changes is a longer process (Sestak & Fortgang, 2013).

Some patients will, however, develop refractory forms of CD (RCD), being unable to respond to the GFD and maintaining an inflammation. Repeated dietary transgressions and continuing gluten consumption (e.g., in non-diagnosed individuals) are thought to be contributing factors to the development of RCD (Hadithi & Peña, 2010).

Finally, regarding the association of CD and the risk of malignant lymphomas, a GFD does not appear to alter the risk of lymphoma. However, it is not possible to eliminate a cause-effect relationship (Olén *et al.*, 2011). The risk of occurrence of malignant lymphoma in CD seems to be related with small intestinal histopathology, without increasing the risk of latent CD (Elfström *et al.*, 2011).

More recently, Lebwohl and colleagues found that the risk is mainly related to the development of T-cell lymphoma rather than B-cell lymphoma, and concluded that follow-up biopsies may be a means to effectively stratify CD patients regarding subsequent malignant lymphoma risks (Lebwohl *et al.*, 2013).

2.1.2. Wheat allergy

Wheat allergy (WA) is an adverse immunological reaction to gluten and other proteins found in wheat (National Institute of Allergy and Infectious Diseases, 2010; Sapone

et al., 2012). As it is mediated by IgE, the pathogenesis of CD and WA are believed to be unrelated, in spite of their optimal treatments being the same (Sestak & Fortgang, 2013).

In WA, the ingestion of wheat causes typical IgE-mediated reactions of immediate onset, whose symptoms include: swelling and itching of the lips or mouth, urticaria, angioedema, bronchial obstruction, nausea and abdominal pain, or, in severe cases, systemic anaphylaxis (Domínguez-Ortega *et al.*, 2014; National Institute of Allergy and Infectious Diseases, 2010). Late-onset manifestations appear around 24 hours after the ingestion of wheat, including gastrointestinal (GI) symptoms (such as bloating, diarrhoea, vomiting and constipation) and aggravation of atopic dermatitis (Domínguez-Ortega *et al.*, 2014; National Institute of Allergy and Infectious Diseases, 2010). Additional manifestations include fatigue, weight loss, joint pains and headaches, and, in rarer cases, eosinophilic esophagitis (Domínguez-Ortega *et al.*, 2014). Sensitization to wheat proteins can be proved via the measurement of circulating IgE-specific antibodies against the suspected allergen and by skin sensitivity testing (which can be done either by prick or patch technique or by intradermal injection) (Mowszet *et al.*, 2014; Soares-Weiser *et al.*, 2014).

2.2. Non-coeliac gluten sensitivity

Non-coeliac gluten sensitivity (NCGS), also known as gluten sensitivity (GS), non-coeliac gluten intolerance (NCGI), non-coeliac wheat sensitivity (NCWS) and wheat intolerance syndrome (WIS) has been an intriguing and poorly defined entity for decades (Di Sabatino & Corazza, 2012; Jericho *et al.*, 2017; Sapone *et al.*, 2012; Valenti *et al.*, 2017).

There is no officially established definition of NCGS (DiGiacomo *et al.*, 2013). Some studies have defined it as a reaction to gluten in which allergic and autoimmune mechanisms have been excluded, but ingestion of foods containing wheat, rye and barley still lead to one or more of a wide range of immunological, morphological and symptomatic manifestations (Di Sabatino & Corazza, 2013; Ludvigsson *et al.*, 2013a; Sapone *et al.*, 2012). However, it must be said is not certain that this condition is triggered by gluten at all, with other components of wheat and other gluten-containing grains being possibly responsible, as detailed in later sections (Biesiekierski *et al.*, 2013; Catassi *et al.*, 2013; Fasano *et al.*, 2015; Gibson *et al.*, 2015).

NCGS patients are traditionally defined as individuals who develop symptoms (which can be GI and similar to those of CD or irritable/inflammatory bowel syndrome (IBS), or

extra-intestinal (EI)) after the ingestion of gluten-containing food but do not have definite evidence of CD or WA (Biesiekierski *et al.*, 2014; Catassi *et al.*, 2013; Fasano *et al.*, 2015; Volta *et al.*, 2013).

Said symptoms seem to develop after the ingestion of gluten-containing food, improve (or disappear altogether) when it is withdrawn from the diet and reappear when it is reintroduced (Catassi *et al.*, 2013; Sapone *et al.*, 2012).

2.2.1. Symptoms

The time interval between the ingestion of gluten and the advent of symptoms in NCGS can vary from several hours to several days (Catassi *et al.*, 2013; Sapone *et al.*, 2012; Volta *et al.*, 2013). The majority of patients with NCGS will already have been suffering from these symptoms for months or even years by the time a diagnosis is reached (Volta *et al.*, 2012).

NCGS incorporates a wide array of GI symptoms, such as abdominal discomfort and pain, bloating, aerophagia, flatulence, bowel habit abnormalities (either constipation or diarrhoea), dissatisfaction with stool consistency and gastroesophageal reflux (Di Sabatino & Corazza, 2012; National Institute of Allergy and Infectious Diseases, 2010; National Institute of Diabetes and Digestive and Kidney Diseases, 2008; Sapone *et al.*, 2012; Volta *et al.*, 2013).

Its EI symptoms include headaches, nausea, "foggy mind"/"brain fog", anxiety, depression, blurry vision, fatigue, lethargy, musculoskeletal pains, dermatitis (eczema or skin rash), paraesthesia of the legs, arms, hands and feet, anaemia and attention-deficit/hyperactivity disorder (Carroccio *et al.*, 2012; Catassi *et al.*, 2013; Di Sabatino & Corazza, 2012; DiGiacomo *et al.*, 2013; Sapone *et al.*, 2012).

More severe neurologic and psychiatric conditions including schizophrenia, "idiopathic" cerebellar ataxia, neuropathies, epilepsy, mood swings, and autism spectrum disorders (ASD) have also been claimed to be associated with NCGS (Canales *et al.*, 2006; Dickerson *et al.*, 2010; Hadjivassiliou *et al.*, 2010; Ludvigsson *et al.*, 2013b).

2.2.2. Diagnosis

The diagnosis is mostly based on an association between the ingestion of gluten-containing grains and the development of adverse symptoms, once CD and WA have been ruled out and the patient has started a GFD (Carroccio *et al.*, 2012; Fasano *et al.*, 2015; Sapone *et al.*, 2012; Volta & De Giorgio, 2012).

NCGS patients do not suffer from the lesions in the duodenal mucosa that are characteristic of CD, but can in some cases present lymphocytic enteritis (Rosinach *et al.*, 2016; Rostami *et al.*, 2015; Volta *et al.*, 2013). The recurrence of symptoms after the reintroduction of gluten to the diet has been proposed as a final step in the diagnostic process of NCGS (Volta & De Giorgio, 2012).

Currently, there are no specific biomarkers for this condition, neither in the form of antibodies or proteins or peptides in serum, nor of histological changes in the small intestine or elsewhere (Catassi *et al.*, 2013; Sapone *et al.*, 2012; Volta et al., 2012).

In the diagnostic process, CD should be eliminated by means of negative serology (IgA anti-tTG and anti-EMA) (Sapone *et al.*, 2012; Volta *et al.*, 2013). A duodenal biopsy is also strongly recommended due to the possibility of seronegative or "silent" CD (Volta & Villanacci, 2011). Patients presenting borderline serology should undergo HLA typing to determine the need for biopsy (Kabbani *et al.*, 2014).

IgA-anti-gliadin antibodies (AGA), found in the vast majority of CD patients, are present in about half of NCGS patients (Carroccio *et al.*, 2012; Volta & De Giorgio, 2012; Volta *et al.*, 2012). Even taking this into account, AGA might not be sufficiently adequate to be used as markers for a diagnosis of NCGS (Infantino *et al.*, 2015).

WA should be ruled out through negative testing for specific IgE and/or prick tests (Mowszet *et al.*, 2014; Sapone *et al.*, 2012).

At present, NCGS is seen as a diagnosis of exclusion (Catassi *et al.*, 2015; Kabbani *et al.*, 2014; Sapone *et al.*, 2012). The suspected overlap between IBS and GS raises the need for even more stringent diagnostic criteria (Catassi *et al.*, 2013; Verdu *et al.*, 2009; Volta *et al.*, 2013).

Excluding gluten from the diet causes significant improvement, which includes the disappearance of both GI and EI symptoms; reintroducing gluten causes the recurrence of symptoms (Catassi *et al.*, 2013; Sapone *et al.*, 2012; Volta & De Giorgio, 2012). The

improvement or cessation of symptoms, along with their reappearance, attributable to the absence or presence of dietary gluten, suggests the presence of NCGS (Volta *et al.*, 2013).

Nonetheless, double-blind, placebo-controlled (DBPC) challenge trials are actively recommended for the confirmation of the NCGS diagnosis, since a placebo effect induced by gluten withdrawal cannot be excluded (Lillie *et al.*, 2011). A significant number of patients often refuse to return to a diet containing gluten if they have already independently started a GFD and have experienced symptom relief (which could lead to missed or at least delayed diagnoses) (Leffler, 2010). It is also necessary to consider that self-reporting of symptoms can be inaccurate, and as such, there will likely be a considerable difference in perceived versus actual NCGS (Biesiekierski *et al.*, 2014). The constitution of a proper gluten challenge is also an area where there is little agreement, since modalities and amounts of gluten used in clinical trials have been varied (Biesiekierski *et al.*, 2011a; Carroccio *et al.*, 2012; Francavilla *et al.*, 2014). In addition, there is a risk of positive results based only on chance, with some studies therefore defending that the double-blind placebo-controlled food challenge (DBPCFC) should be repeated at least twice to minimize this risk (Husby & Murray, 2015).

The DBPCFC is not without limitations, however, as it has been linked to false-positive results and to a nocebo effect by reason of the issues associated with the measurement of subjective symptoms (Godlee, 2012). More recently, a review article has raised concerns about the adequacy of the DBPC gluten challenge as the gold standard for the confirmation of a NCGS diagnosis (Molina-Infante & Carroccio, 2017).

2.2.3. Gluten-free diet and its issues

The recommended treatment after a diagnosis of NCGS, as in the case of CD and WA, is a GFD (Pietzak, 2012). Patients must make the necessary alterations to their dietary habits and consume foods with minimal gluten content (less than 10 mg/day) (Volta *et al.*, 2013). Cereals such as rice, corn, buckwheat, millet and teff and leguminosae such as quinoa, amaranth and soybean are recommended as substitutes for gluten-containing products (Foschia *et al.*, 2016; Volta *et al.*, 2013). Many naturally gluten-free starches that can complement the GFD with important nutrients also exist; these include sweet potatoes, butternut or acorn squashes, beans, and lentils (Theethira & Dennis, 2015).

Gluten-free products which are commercially available are useful in helping NCGS patients achieve a fully gluten-free diet; however, naturally gluten-free foods (such as meat,

fish, eggs, fruit and vegetables) should still be integrated into their diets to provide proper nutrition (Volta *et al.*, 2013). The overall use of commercially available gluten-free products ought to remain low to limit the introduction of chemical additives and preservatives abundant in these products, which are a potential cause of functional GI symptoms (Volta *et al.*, 2013).

It must be said that the tolerance level to gluten varies across individuals, with some NCGS patients not being able to tolerate even minimal amounts of gluten (Volta *et al.*, 2013). Most NCGS patients will experience the complete disappearance of symptoms on a GFD, while in some cases this improvement will only be partial (Volta *et al.*, 2013).

Moreover, it should be considered that in the absence of a confirmed diagnosis of CD or NCGS, a GFD is no healthier than an analogous gluten-containing diet (Kulai & Rashid, 2014). Actually, the implementation of a GFD in the full absence of symptoms might only result in an increased cost with no benefit (Burden *et al.*, 2015). In the absence of the adequate knowledge or supervision by a dietician and the necessary nutritional substitutions, the levels of fibre, vitamins (specifically B vitamins and folic acid) and minerals (such as iron, zinc, phosphorus, magnesium and calcium) can be too low and those of saturated fat and calories too high, originating nutritional deficiencies (Miranda *et al.*, 2014; Theethira & Dennis, 2015).

Long-term adherence to a GFD with a lack of nutritional supervision can even cause the development of obesity, new-onset insulin resistance and metabolic syndrome as the worst scenario (Kabbani *et al.*, 2012; Tortora *et al.*, 2015). There is also emerging evidence that a GFD which is not sufficiently diverse may cause a greater risk of exposure to certain toxins (such as mycotoxins, heavy metals and pesticides) when compared to a diet containing gluten (Clarke *et al.*, 2015; Lai *et al.*, 2015; Pellegrini & Agostoni, 2015).

Additionally, there is the issue of the increased costs of gluten-free food products, leading to many patients finding a GFD unsatisfactory (Burden *et al.*, 2015). Social isolation is also a concern with this diet, particularly in settings such as communal celebrations, religious rituals, and dining out (Jordan *et al.*, 2013; Simsek *et al.*, 2015). Furthermore, much like in the case of coeliac patients, rigorous avoidance of gluten can prove challenging due to its many hidden sources (Thompson & Simpson, 2015).

Future progress in the proper characterization of the factors related to CD and non-CD pathogenesis might aid in the development of new treatment strategies alternative to the GFD (Plugis & Khosla, 2015).

2.2.4. History

The existence of NCGS was first hypothesised in 1978 in a case report of a patient suffering from diarrhoea and intermittent abdominal pain, lacking abnormalities on a biopsy and experiencing improvement on a GFD (Ellis & Linaker, 1978).

This was followed by a double-blind crossover trial in 8 adult female patients who suffered from abdominal pain and chronic diarrhoea (in the absence of CD and WA) and had startling relief on a GFD, with the symptoms reappearing after a gluten challenge. All patients subsequently decided to remain on a GFD, having been on it for 4-6 years by the time of the report (Cooper *et al.*, 1980).

Possibly due to a lack of a proper definition and specific tests for this condition and the fact its symptoms had an overlap with those of IBS, interest ceased (Holmes, 2013).

After more than two decades with no mention of NCGS, in 2000, Kaukinen and colleagues reported that 63% of 94 adults who complained of abdominal symptoms after gluten ingestion did not comply to the diagnostic criteria for CD and WA; as they also benefited from a GFD, they were considered to suffer from NCGS (Kaukinen *et al.*, 2000).

A subsequent scarcity of data followed until 2007, when Wahnschaffe and co-workers described a group of patients with diarrhoea-predominant IBS (IBS-D) who showed clinical improvement on a GFD in the absence of CD. This response, however, was only significant in HLA-DQ2 positive patients who possessed IgG antibodies against tTG and gliadin (Wahnschaffe *et al.*, 2007).

The modern age of NCGS truly began in 2011, when Biesiekierski *et al.* performed the first randomized, DBPC, clinical trial in IBS patients with a history of GI symptoms kept in control by a GFD. This study decisively established the double-blind randomized controlled trial as feasible, which in turn led to its adoption as the optimal design of NCGS clinical studies. The success and influence garnered by this study stemmed from its appropriately simple primary aim ("to determine whether gluten ingestion can induce symptoms in non-coeliac individuals") and design. Even though it was unable to reach a

conclusion about the underlying mechanism, the study suggested gluten as a dietary trigger for symptoms (Biesiekierski *et al.*, 2011a).

More recently, in 2012, a revision of the nomenclature for gluten-related disorders was the first to officially include NCGS (Sapone *et al.*, 2012).

2.2.5. Prevalence

The estimated prevalence of NCGS is widely variable in the literature, with cited figures ranging between 0.6 and 6% of the general population (this meaning it could be as high as six times that of CD) (Choung *et al.*, 2015; DiGiacomo *et al.*, 2013; Sapone *et al.*, 2012).

In addition, it might be as high as 25-30% among IBS patients (Carroccio *et al.*, 2012).

The clinical features of NCGS as determined from existing studies show that this disorder is rare, or at least uncommon, in infancy and childhood (or possibly underdiagnosed in said age groups), being more frequent in adolescents and young to middle-aged adults, with a significant number of cases also diagnosed in the elderly (Catassi *et al.*, 2013; Volta *et al.*, 2012; Volta *et al.*, 2013).

Similarly to CD, NCGS seems to be more common in females than in males (DiGiacomo *et al.*, 2013; Volta & De Giorgio, 2012; Volta *et al.*, 2012). It also seems to be more frequent in first-degree relatives of CD patients (Volta *et al.*, 2012).

Importantly, it should be noted that the CD-predisposing HLA-DQ2 and -DQ8 genotypes are found in 50% of NCGS patients, a prevalence lower than in CD (95%) and only slightly higher than in the general population (30%) (Catassi *et al.*, 2013; Sapone *et al.*, 2012).

2.3. Possible mechanisms of non-coeliac gluten sensitivity

2.3.1. Innate or adaptive immune response

Many studies consider that NCGS has an enhanced innate immune response as its underlying mechanism, in contrast to the adaptive immunity seen in CD (Junker *et al.*, 2012; Sapone *et al.*, 2010; Sapone *et al.*, 2011).

Innate immunity relies on a receptor-mediated and immediate response to molecular patterns that are conserved and common in bacterial and viral antigens, and to some molecules that act as "danger" signals (Beutler, 2009).

Higher organisms are endowed with a diversity of innate, pathogen and danger signal recognizing receptors; these include the Toll-like receptors (TLRs), primarily expressed on myeloid cells (monocytes, macrophages and dendritic cells) (Beutler, 2009). It has been hypothesised that gluten and its related peptides may be the triggers, inducing the innate immune response and stimulating dendritic cells, which results in the infiltration of leukocytes and inflammation of the gut mucosa (Sapone *et al.*, 2011; Sollid & Jabri, 2013).

It should be pointed out that the innate immune response is a first line of defence that is dependent on cytokines and does not provide long-lasting immunity, in contrast to the adaptive response seen in CD, which relies on antibody production and confers immunologic memory for future antigenic exposure (Sapone *et al.*, 2011). The behaviour of the aforementioned TLRs (that play a key role in the innate immune system as well as the digestive system) gives support to the hypothesis of a stimulation of the innate immune system in NCGS that does not involve the IFN-γ pathway (Sapone *et al.*, 2011; Vazquez-Roque *et al.*, 2013).

TLRs are single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells (such as macrophages and dendritic cells). They recognize structurally conserved molecules derived from microbes (Sapone *et al.*, 2011). When physical barriers, such as the skin or intestinal mucosa, are breached by said microbes, the latter are recognized by TLRs, which activate immune cell responses (Sapone *et al.*, 2011).

The expression of TLR-2 (and, to a lesser extent, of TLR-1 and TLR-4) in the small intestinal is greater in NCGS patients than in coeliacs (Sapone *et al.*, 2011; Vazquez-Roque *et al.*, 2013). The two disorders also differ in the small intestinal expression of the T-regulatory (T_{REG}) marker forkhead box P3 (FOXP3), considerably weaker in gluten-sensitive patients than in coeliacs (Sapone *et al.*, 2010; Sapone *et al.*, 2011).

In vivo short-term gluten challenge of NCGS patients' mucosa has been observed not to cause alterations in the transcript levels of interleukin-8 (IL-8) and chemokine (C-C motif) ligand 2 (CCL2), two chemokines involved in the recruitment of immune cells in the inflamed gut (Brottveit *et al.*, 2013).

An additional involvement of an adaptive immune response in NCGS has also been hypothesised (Brottveit *et al.*, 2013). Said hypothesis is supported by observations of increased levels of IFN-γ and density of intraepithelial CD3+ T-cells and decreased levels of transforming growth factor β1 (TGF-β1) and FOXP3 markers after a gluten challenge. These data indicate that there is a lower likelihood of recruitment of regulatory T lymphocytes to the small intestine in NCGS patients than in healthy individuals (Brottveit *et al.*, 2013).

2.3.1.1. Amylase trypsin inhibitors

Amylase trypsin inhibitors (ATIs) are a family of homologous proteins of low molecular weight (Junker *et al.*, 2012; Mäkelä *et al.*, 2014). They can be found in a variety of plants, where they inhibit enzymes of common parasites (such as mealworms and bugs) and are involved in regulating processes such as grain maturation and storage of carbohydrates (Dupont *et al.*, 2011; Junker *et al.*, 2012).

ATIs represent 2-4% of total wheat protein (in contrast to 80-90% for gluten) (Dupont *et al.*, 2011). They are approximately 120-150 amino acids in length and display a compact secondary structure with 5 intramolecular disulphide bonds, which confer them a degree of resistance to proteolytic digestion by the GI proteases trypsin and pepsin (Junker *et al.*, 2012; Schuppan & Zevallos, 2015).

These proteins have been shown to engage the TLR4–MD2–CD14 complex and lead to the upregulation of maturation markers, eliciting release of pro-inflammatory cytokines in cells from patients with and without CD and in coeliac patients' biopsies (Schuppan *et al.*, 2015; Schuppan & Zevallos, 2015). Consequently, after ingestion and during their passage through the intestine, ATIs preserve their biological activity of TLR-4 activation (Junker *et al.*, 2012; Schuppan & Zevallos, 2015).

Members of the ATI family are strong activators of the innate immune responses of monocytes, macrophages and dendritic cells (Beutler, 2009; Junker *et al.*, 2012; Schuppan & Zevallos, 2015). ATIs have also been previously characterized as allergens in baker's asthma and GI hypersensitivity to wheat (Junker *et al.*, 2012; Tatham & Shewry, 2008). These observations have led to the hypothesis that ATIs might fuel inflammation and immune responses in other intestinal and non-intestinal immune disorders, such as CD and NCGS (Junker *et al.*, 2012).

Of note, ATIs are present and even enriched in commercial gluten (Makharia *et al.*, 2015). Although they are not classified as gluten proteins, they are found in grain endosperm along with these, usually co-fractionating with the gliadin fraction (Makharia *et al.*, 2015).

TLR-4 stimulating activity is mainly restricted to ATIs of gluten-containing grains (Junker *et al.*, 2012; Schuppan *et al.*, 2015; Schuppan & Zevallos, 2015). Therefore, plants other than wheat, rye, barley or their related ancestors show only minimal or absent TLR-4-activating activity, even though they contain (structurally different) inhibitors of amylase and trypsin-like activities (Junker *et al.*, 2012; Schuppan *et al.*, 2015; Schuppan & Zevallos, 2015). A GFD is, thus, also essentially ATI-free (Makharia *et al.*, 2015; Schuppan *et al.*, 2015). These findings prompted the suggestion that the term "non-coeliac wheat sensitivity" (NCWS) might be more appropriate than the previously established "NCGS" (Makharia *et al.*, 2015; Schuppan *et al.*, 2015).

The effect of ATIs is dose-dependent, and a significant reduction of their daily intake by a >90% avoidance of gluten-containing foods could be enough to decrease their effect of co-stimulation to an irrelevant level (Schuppan *et al.*, 2015).

2.3.2. Gut dysbiosis

The microbiota, an integral part of both animal and human organisms, has a close inter-relationship with its host; it is an essential inherent factor that impacts human health (Daulatzai, 2015). Recent research has shown how changes in the composition of the gut microbiome, as well as microbial translocation, can influence animal and human physiology, contributing to a vast array of diseases (Bäckhed, 2012; Cryan & Dinan, 2012; Foster & McVey Neufeld, 2013).

An alteration in microbiome (dysbiosis) has thus been identified as the driving force behind development of diseases and dyshomeostasis (Daulatzai, 2015). Compromised integrity of the intestinal epithelium has been linked to extensive systemic immune responses, both innate and adaptive, resulting from translocation of microbes from the lumen into circulation (Estes *et al.*, 2010).

Several studies point to the possibility of NCGS being a subset of IBS, rather than a gluten-related disorder (Fritscher-Ravens *et al.*, 2014; Vazquez-Roque *et al.*, 2013). This is mainly based on the overlap between the GI symptoms characteristic of both conditions,

which could have a common mechanism (Di Sabatino & Corazza, 2012). Alterations in the gut microbiome have been speculated to be able to lead to an abnormal immune function in the gut, translating clinically into the classic IBS (and NCGS) symptoms (Wahnschaffe *et al.*, 2001). It has also been hypothesised that the changes in gut microbiota possibly observed in IBS and NCGS might be caused by certain non-gluten components of wheat (Simrén *et al.*, 2013). However, said alterations could also originate from a radical change in dietary habits (Simrén *et al.*, 2013).

Dysbiosis may also be responsible for several of the extra-intestinal symptoms present in NCGS through the enhancement of gut inflammation, dysfunction of the intestinal barrier, and promotion of systemic- to neuro-inflammation (Collins *et al.*, 2009; Collins *et al.*, 2012). It has also been linked to an accelerated development of CD through certain species of rod-shaped bacteria with the ability to colonise the human small intestine (Sjöberg *et al.,* 2013; Xu *et al.*, 2013). The regulation of microflora composition (e.g. by probiotics and prebiotics) offers the possibility to restore GI homeostasis and decrease neuro-inflammation by influencing the mucosal and systemic immunity dysfunction (Daulatzai, 2015).

Some alterations in gut microbiota were observed to have been restored to normal levels after adherence to a GFD (Sanz *et al.*, 2011). This suggests that these alterations are secondary consequences of the disease and possibly directly related to gluten consumption (Sanz *et al.*, 2011; Tillisch *et al.*, 2013).

2.3.3. Epithelial cell damage and systemic immune activation

It has been suggested that NCGS symptoms may result from an impaired barrier function of the intestinal mucosa (Ciccocioppo *et al.*, 2006; Sander *et al.*, 2005; Vogelsang *et al.*, 2001). It is thought that gluten induces malabsorption of various nutrients, leading to systemic deficiencies (Morant, 2011).

Various *in vitro* studies on cell cultures demonstrated that treatment with gluten can cause alterations in cellular morphology and motility, as well as in cytoskeleton organisation and intercellular contact through tight junction proteins (Dolfini *et al.*, 2005a; Roncoroni et al., 2009; Roncoroni *et al.*, 2013). Treated cells show a reduction in viability due to the stimulation of apoptosis and reduction of the synthesis of nucleic acids and proteins (Caputo *et al.*, 2012; Dolfini *et al.*, 2005b).

CD patients have already been well-documented as having impaired intestinal permeability as a result of abnormal tight junction morphology (Vogelsang *et al.*, 2001). Tight junctions are known to open in the presence of gluten, allowing exposure of luminal antigens within the lamina propria (Ciccocioppo *et al.*, 2006; Sander *et al.*, 2005).

Claudins (CLDN) are protein components essential to the function of tight junctions (Sapone *et al.*, 2011). When compared to CD patients, patients with NCGS have showed increased expression of CLDN4, which, along with CLDN1, is associated with normal tight junction function and intestinal permeability (Sapone *et al.*, 2011).

Gluten having a role in mediating cholinergic activation is also a possibility (Biesiekierski *et al.*, 2011a; Verdu *et al.*, 2008). This could lead to increased smooth muscle contractility and have indirect effects on luminal water content (Biesiekierski *et al.*, 2011a; Verdu *et al.*, 2008).

The hypothesis that the enhanced antibody response to native gliadin in NCGS/NCWS patients could be a consequence of ongoing defects in the intestinal epithelial barrier was advanced in a more recent study (Uhde *et al.*, 2016). These defects would lead to an inadequate regulation of the interaction between the gut microbiota and systemic circulation, with peripheral immune activation as the result (Uhde *et al.*, 2016). Translocated circulating lipopolysaccharides (LPS) can lead to the rapid secretion of lipopolysaccharide-binding protein (LBP) by GI and hepatic epithelial cells, as well as of soluble CD14 (sCD14) by CD14+ monocytes/macrophages (Brenchley & Douek, 2012). The latter binds LPS in the presence of LBP to activate TLR-4 (Miller *et al.*, 2005). The observed increased serum levels of sCD14 and LBP, elevated expression of fatty-acid binding protein 2 (FABP2) and antibody reactivity to microbial antigens indicated systemic immune activation and compromised integrity of the intestinal epithelial barrier (Uhde *et al.*, 2016). This study also puts forward the possibility that these markers of systemic immune activation and epithelial cell damage might be of future use as potential biomarkers for the diagnosis of NCGS/NCWS, given that they are present in high concentrations in individuals suffering from this condition and decrease to normal levels after implementation of a diet excluding wheat and related cereals (Uhde *et al.*, 2016).

2.3.4. Effect of non-protein components

Fermentable oligo-, di-, and mono-saccharides and polyols (FODMAPs) are poorly absorbed short-chain carbohydrates that cause distension of the intestinal lumen due to their small molecular size and rapid fermentability, originating functional GI symptoms (Eswaran *et al.*, 2013; Gibson & Shepherd, 2012).

FODMAPs are found in a variety of foods, including those which contain lactose, fructose, fructans, galactans, and polyols (sorbitol, mannitol, and xylitol) (Gibson & Shepherd, 2012; Yao *et al.*, 2013). Some common foods which are high in FODMAPs include wheat, rye and barley, as well as milk, honey, some fruits (e.g. apples, watermelons, cherries, mangos and pears) and vegetables (e.g. chicory, fennel, beetroot, leek, lentils and chickpeas) (Biesiekierski *et al.*, 2011b; Volta *et al.*, 2013).

These carbohydrates can trigger functional GI symptoms by inducing luminal distension through a combination of osmotic effects and production of gas, since they provide a substrate for rapid bacterial fermentation in the small and large intestine (Fasano *et al.*, 2015; Gibson & Shepherd, 2012; Gibson *et al.*, 2015).

Many cereals which contain gluten are high in fructans, thus posing a problem for IBS patients, as their reduction accompanied by the introduction of a GFD may lead to an improvement in symptoms, wrongly perceived to result from a reduction in gluten intake (Biesiekierski *et al.*, 2011b; Gibson & Shepherd, 2012).

A diet low in FODMAPs (containing, for instance, almond and rice milk, rice, oats and quinoa) has been observed to improve GI symptoms (such as abdominal pain, bloating, gas and diarrhoea) in NCGS patients (Biesiekierski et al., 2013; Halmos *et al.*, 2014). Despite this, a low-FODMAP diet is strict to follow and not intended to be a prolonged diet regimen (Öhman & Simrén, 2013; Staudacher *et al.*, 2012).

In contrast, the role of FODMAPs in NCGS has been dismissed by some studies, which state that, although FODMAPs can cause certain GI symptoms, they inhibit, rather than cause, intestinal inflammation (Gibson *et al.*, 2015). Moreover, they have also been shown to generate short-chain fatty acids and induce beneficial alterations in the intestinal microbiota (Makharia *et al.*, 2015).

2.3.5. Placebo or nocebo effect

The profound effect of placebo has been well documented for a variety of functional GI disorders (Shah & Pimentel, 2014). The existence of a relevant placebo/nocebo phenomenon has actually been reported in DBPC trials in adult patients with self-reported food intolerances, and the possibility of gluten withdrawal resulting in a placebo effect caused has been pointed out (Godlee, 2012). Therefore, it is likely that a portion of patients with NCGS, and arguably a considerable one, fall in this category (Carroccio et al., 2010; Godlee, 2012).

In spite of all the recent studies, it is clear that NCGS is still a controversial issue (Di Sabatino & Corazza, 2012; Godlee, 2012). There is the definite probability that many patients present an imaginary syndrome with a subjective sensation of improvement resulting from a placebo effect or the withdrawal of gluten (Godlee, 2012). Many patients might be strongly influenced by the general popularity of the GFD as a healthy diet option (Brown, 2012; Di Sabatino & Corazza, 2012).

There is also the possibility that a nocebo effect could explain the current prevalence of NCGS to an extent, as patients believing themselves to be food sensitive would be preconditioned to avoidance (Biesiekierski *et al.*, 2013). The results of a DBPC trial of gluten vs placebo demonstrate that gluten is the trigger of symptoms in a subgroup of patients fulfilling the diagnostic criteria for NCGS (Rosinach *et al.*, 2016).

2.3.6. Other proposed mechanisms

2.3.6.1. Opioid activity of gluten

Recently, NCGS has been associated with the development of neuro-psychiatric disorders such as ASDs, schizophrenia and depression (Cascella *et al.*, 2013; Dickerson *et al.*, 2010; Ludvigsson *et al.*, 2013b; Marcason, 2009).

The mechanism proposed as being responsible is a CD-unrelated, primary alteration of the small intestinal barrier ("leaky gut") that leads to an abnormal absorption of gluten peptides (resulting from incomplete breakdown of gluten) (Catassi *et al.*, 2013; Marcason, 2009). Said peptides are able to eventually cross the intestinal membrane, enter the bloodstream, and cross the blood-brain barrier, affecting the endogenous opiate system and neurotransmission within the nervous system (Catassi *et al.*, 2013; Millward *et al.*, 2008).

Gluten proteins can in fact mimic some of the effects of opiates by causing alterations in the intestinal transit of healthy volunteers in a naloxone-reversible manner (Corazza *et al.*, 1984).

2.3.6.2. Food additives

Chemical additives such as glutamates, benzoates, sulphites and nitrates, added to many commercial products for a variety of reasons (for improvement of flavour, colour and preservative function), may have a role in eliciting the functional GI symptoms of NCGS and other conditions with characteristic intestinal inflammation, such as IBS (Gibson & Shepherd, 2012). Supporting this hypothesis is the fact that a subgroup of NCGS patients does not improve by eating commercially gluten-free products (rich in additives and preservatives), and only experience symptom resolution through implementation of a diet based on naturally gluten-free foods (Volta *et al.*, 2013).

2.3.6.3. Early-stage coeliac disease

Even though NCGS and CD are in all likelihood two separate entities with distinct primary pathogenic pathways represented by innate and adaptive immunity, respectively, the probability that NCGS might evolve into CD cannot be discarded (Not *et al.*, 2011; Volta *et al.*, 2013).

The diagnosis of CD is clearly defined and follows a well-standardized process, but instances where the disease cannot be recognized remain, as the process has yet to reach the point of being detectable (either by serum antibody testing or by pathology) (Borghini *et al.*, 2014; Picarelli *et al.*, 2013). Therefore, it can be speculated that a fraction of patients labelled with NCGS may in fact suffer from seronegative CD and still lack overt intestinal damage (Borghini *et al.*, 2014; Picarelli *et al.*, 2013).

The detection of deposits of IgA through small intestine biopsies may help in the identification of NCGS patients at risk of developing CD (Not *et al.*, 2011; Volta & Villanacci, 2011). NCGS patients possessing HLA-DQ2/-DQ8 haplotypes actually suffering from CD is also a possibility (Mooney *et al.*, 2013).

3. MATERIALS AND METHODS

3.1. Data collection

From March to May of 2017, data were collected from files of patients that had at some point been admitted to the Hospitals of the University of Coimbra (Hospitais da Universidade de Coimbra, HUC). These patients had been admitted to the HUC either for regular consultations or surgery, and had a prior diagnosis of coeliac disease.

Data were collected from a total of 47 individual medical files. The selected information included: general statistics (gender, date of birth, date of death when applicable, place of residence), age at diagnosis, family history, symptoms (GI, EI and malabsorption-associated conditions), associated pathologies, serology (IgA levels, testing for CD-specific antibodies and HLA testing), histology (prior biopsies and Marsh-Oberhuber classifications), adherence to a GFD and presence of RCD (when applicable).

3.2. Data analysis

All data were organised and analysed with SPSS version 24. All tables were likewise generated with SPSS.

4. RESULTS AND DISCUSSION

4.1. General statistics

Data were collected from a total of 47 patients, of whom 8 (17.02%) were male and 39 (82.98%) were female. 5 (10.64%) of the patients (all female) were deceased (**Table 1**).

The fact that most of the patients were female is in accordance to the literature, as in adults, CD is more commonly diagnosed in females than in males (Ciclitira *et al.*, 2001; Thomas *et al.*, 2009).

Table 1 – Gender and status of patients.

		N	%
Gender	Female	39	82,98%
	Male	8	17,02%
	Total	47	
Individual deceased?	No	42	89,36%
	Yes	5	10,64%
	Total	47	

The mean age of the (living) patients (computed as their age on September 1st, 2017) was 43.19±16.02 years (range 10-77 years) (**Table 2**).

Table 2 – Current age of patients.

N	42
Mean	43,19
Median	42,00
Standard deviation	16,02
Minimum	10
Maximum	77

Most of the patients (24, 51.06%) resided in the Coimbra district. A considerable percentage resided in the Aveiro district (9, 19.15%). The remainder resided in the Viseu (4, 8.51%), Castelo Branco (3, 6.38%), Guarda (2, 4.26%), Leiria (2, 4.26%) and Santarém (2, 4.26%) districts and in the Autonomous Region of the Azores (1, 2.13%) (**Table 3**).

Table 3 – Places of residence of patients.

Place of residence	N	%
Azores	1	2,13%
Aveiro	9	19,15%
Castelo Branco	3	6,38%
Coimbra	24	51,06%
Guarda	2	4,26%
Leiria	2	4,26%
Santarém	2	4,26%
Viseu	4	8,51%
Total	47	

The general prevalence of CD in Western populations is around 1% (though it is increasing worldwide over time), and it might be higher in Northern European countries (Catassi *et al.*, 2010; Dydensborg *et al.*, 2012; Mustalahti *et al.*, 2010). There has only been one study to date that determined the prevalence of CD in a Portuguese population, which was found to be 1/134, though it is important to note that the results of this study were only based on serological testing in adolescents (Antunes *et al.*, 2006).

4.2. Age at diagnosis and family history

4.2.1. Age at diagnosis

Thirty-one (65.96%) of the patients had a stated age at which they were diagnosed with coeliac disease. The mean age at diagnosis was 34.48±17.40 years (range 9 months-69 years) (**Table 4**).

Table 4 – Patients' age at diagnosis.

N	31
Mean	34,48
Median	30,00
Standard deviation	17,40
Minimum	,75
Maximum	69,00

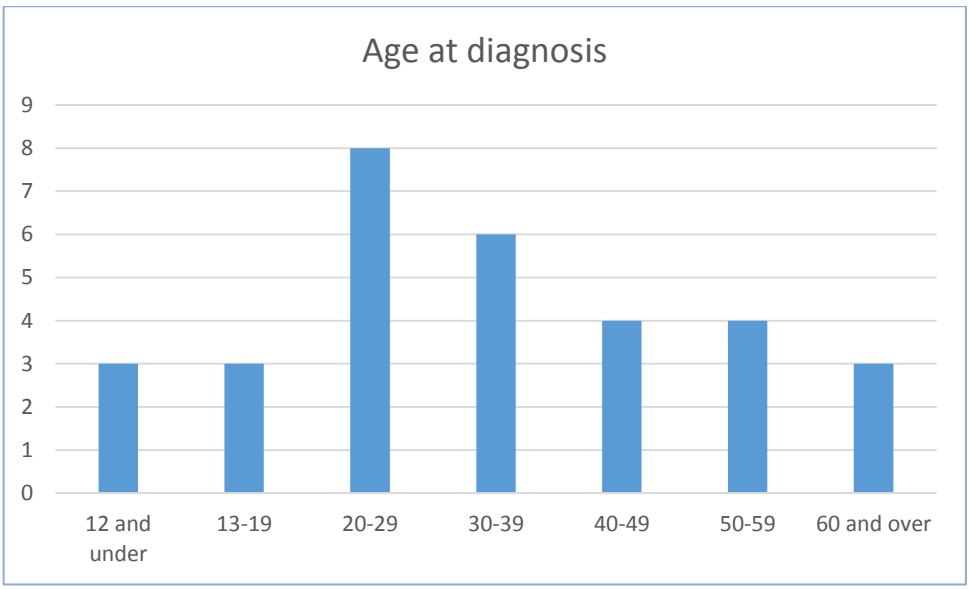

Figure 1 – Patients' age at diagnosis per age groups.

These findings are largely consistent with data found in literature. While CD used to be mostly diagnosed in children, it is currently known to manifest in any age group, with a large percentage of coeliacs diagnosed only later in life (Ciclitira *et al.*, 2001; Fasano & Catassi, 2001). A significant amount is over 60 at the time of diagnosis (Freeman, 1995; Gasbarrini *et al.*, 2001).

As it can be seen in this sample of patients, only a minority (3 patients, 6.38%) were diagnosed as children under the age of 13, while a reasonable percentage (11 patients, 23.40%) were diagnosed at age 40 and over (**Figure 1**).

CD was most commonly diagnosed in the 20 to 29 age range (8 patients, 25.81%), however, which is something not frequently reported. It must be noted, though, that this perceived difference may result from the fact that these data only pertain to a total of 31 people, and are not representative of the general population.

4.2.2. Family history

A family history of CD was present in 3 of the patients (6.38%). In all these cases, the mother also suffered from the disease (all other mentioned relatives being healthy).

CD has indeed been documented as having a higher prevalence in first-degree relatives of coeliacs, such as parents, siblings and children (around 10%), than in the general population (about 1%) (Bardella *et al.*, 2007; Rubio-Tapia *et al.*, 2008).

4.3. Symptoms and other manifestations

4.3.1. Gastrointestinal symptoms

Several of the patients presented with classic GI manifestations of CD such as diarrhoea (16), constipation (3) and abdominal distension (2) (**Figure 2**).

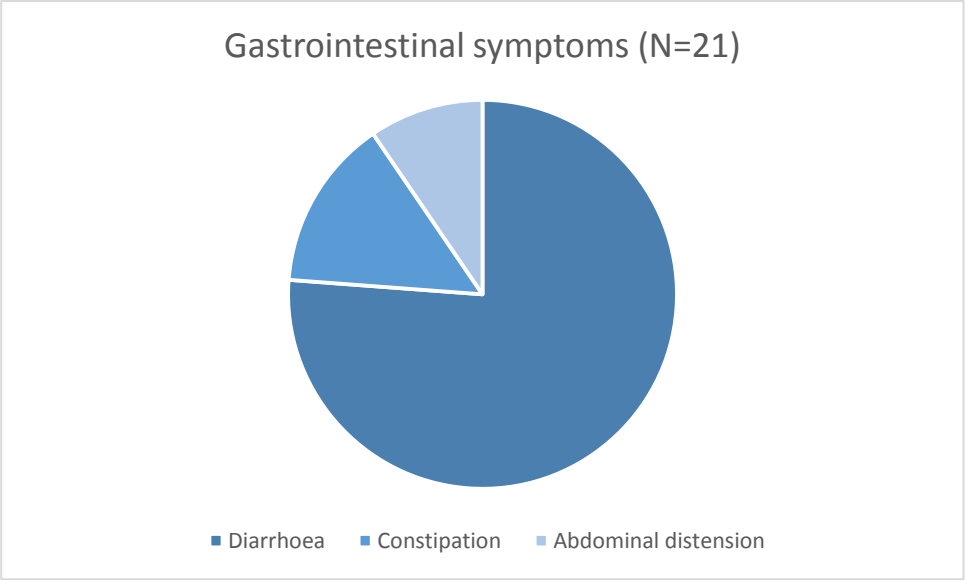

Figure 2 – Gastrointestinal symptoms observed.

While GI symptoms are more common in children than in adults (Fasano & Catassi, 2001; Hill *et al.*, 2016), the latter can still present with a variety of GI symptoms, which include chronic diarrhoea, (Emami *et al.*, 2008; Fasano & Catassi, 2012; Rodrigo-Sáez *et al.*, 2011), chronic constipation (Rashid *et al.*, 2005; Rodrigo-Sáez *et al.*, 2011; Sadik *et al.*, 2004) and abdominal distension (Emami *et al.*, 2008; Fasano & Catassi, 2012; Garampazzi *et al.*, 2007).

4.3.2. Extra-intestinal symptoms

The EI symptoms observed in these patients that are known to be associated with CD were anxiety (10), depression (10) and ataxia (3) (**Figure 3**).

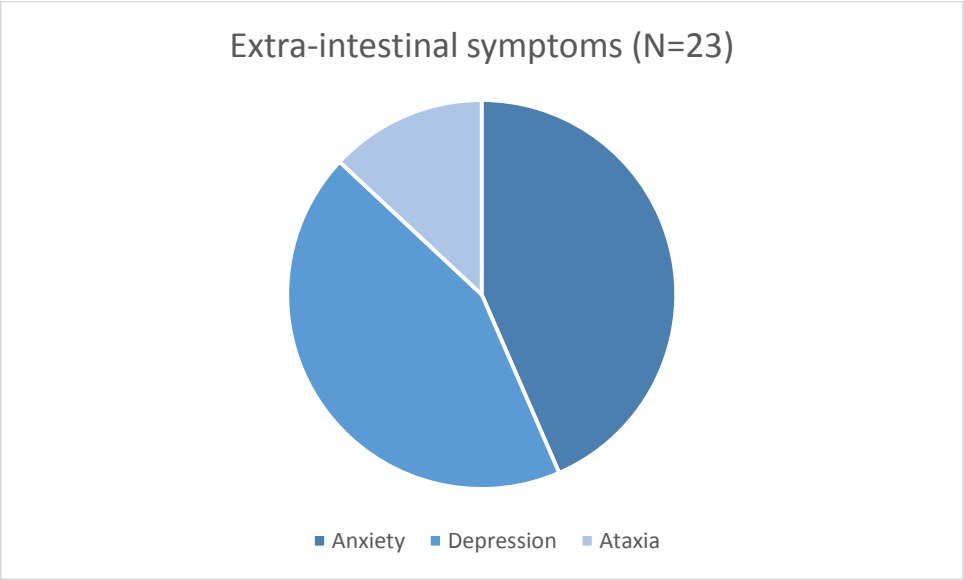

Figure 3 – Extra-intestinal symptoms observed.

EI manifestations of CD can be neuropsychiatric, and include depression (Ludvigsson *et al.*, 2007a; Smith & Gerdes, 2012; van Hees *et al.*, 2013), anxiety (Cannings-John *et al.*, 2007; Häuser *et al.*, 2010; Smith & Gerdes, 2012) and ataxia (Hadjivassiliou *et al.*, 2003; Hadjivassiliou *et al.*, 2016; Sarrigiannis *et al.*, 2014).

It is important to note that anxiety and depression were by far the most common manifestations of CD observed among this sample of patients, with each one present in 10 out of 47 patients (21.28%). Despite the small size of the sample, this is a significant percentage and most likely representative of an actual association.

4.3.3. Malabsorption-associated conditions

Conditions often observed in CD as the result of malabsorption were also present: osteoporosis (7), iron deficiency anaemia (6), other types of anaemia (4), folic acid deficiency (2), vitamin D deficiency (2), aphthous stomatitis (1) and growth failure (1) (**Figure 4**).

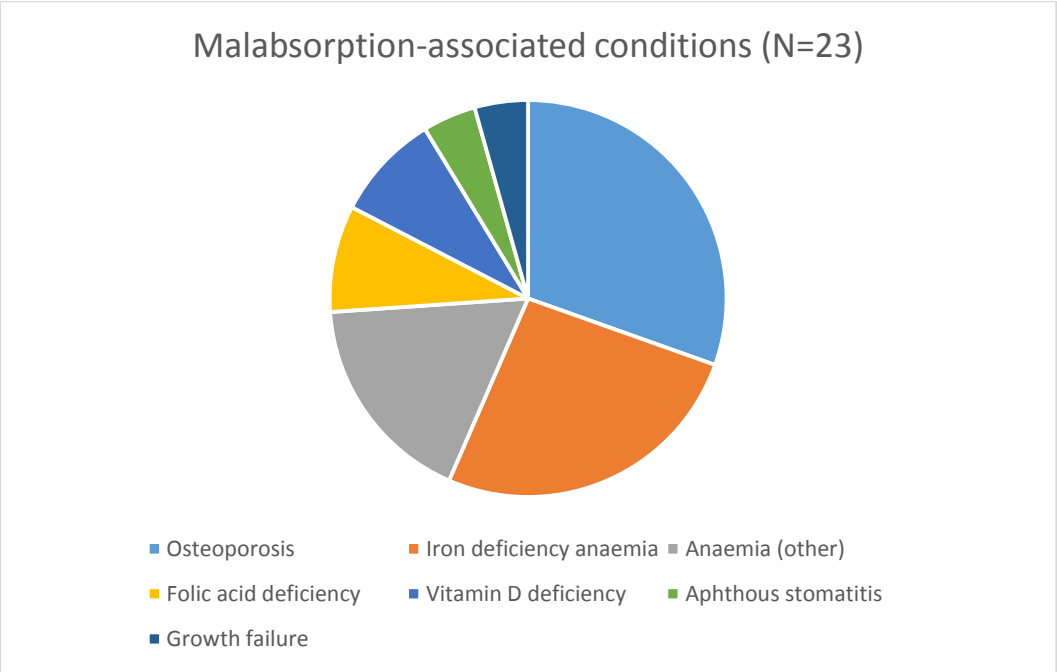

Figure 4 – Malabsorption-associated conditions observed.

A variety of CD manifestations ultimately resulting from malabsorption have all been documented in the literature: they indeed include iron-deficiency anaemia (Bergamaschi *et al.*, 2008; Elli & Bardella, 2005; Rostom *et al.*, 2006), anaemia resulting from other causes (such as malabsorption of folate and vitamin B12) (Bergamaschi *et al.*, 2008; Harper *et al.*, 2007; Vilppula *et al.*, 2008), impaired absorption of vitamins A, B12, D, E and K and of folic acid (Halfdanarson *et al.*, 2007; Reilly *et al.*, 2012; Tikkakoski *et al.*, 2007), growth failure (in children) (Fasano & Catassi, 2012; Troncone & Kosova, 2010; van Rijn *et al.*, 2004), osteoporosis and osteopenia (resulting from vitamin D malabsorption) (Krupa-Kozak, 2014; Ludvigsson *et al.*, 2007b; Olmos *et al.*, 2008) and aphthous stomatitis (Fasano & Catassi, 2012; Volta *et al.*, 2014).

These manifestations were considered separately from the associated conditions in the following section, because even though they are not symptoms of CD per se, their origin can be traced to the malabsorption resulting from CD.

4.4. Associated pathologies

Several pathologies associated with CD were observed in these patients. These included conditions with a well-documented association with CD: lactose intolerance (4), type I diabetes mellitus (T1DM) (4), Down syndrome (3), hypothyroidism (3), goitre (2), hyperparathyroidism (2), IgA deficiency (2), non-IgA immunodeficiency (2), Sjögren

syndrome (2), autoimmune hepatitis (1), primary biliary cirrhosis (PBC) (1) and vitiligo (1) (**Figure 5**).

Other conditions observed were: sarcoidosis (2), type 2 diabetes mellitus (T2DM) (2), anorexia nervosa (1), bulimia nervosa (1), chronic gastritis (1), chronic obstructive pulmonary disease (COPD) (1), Gilbert's syndrome (1), Graves' disease (1), hyperhidrosis (1), hypoparathyroidism (1), protein S deficiency (1), Raynaud syndrome (1), retinopathy (1) and spongiotic dermatitis (1).

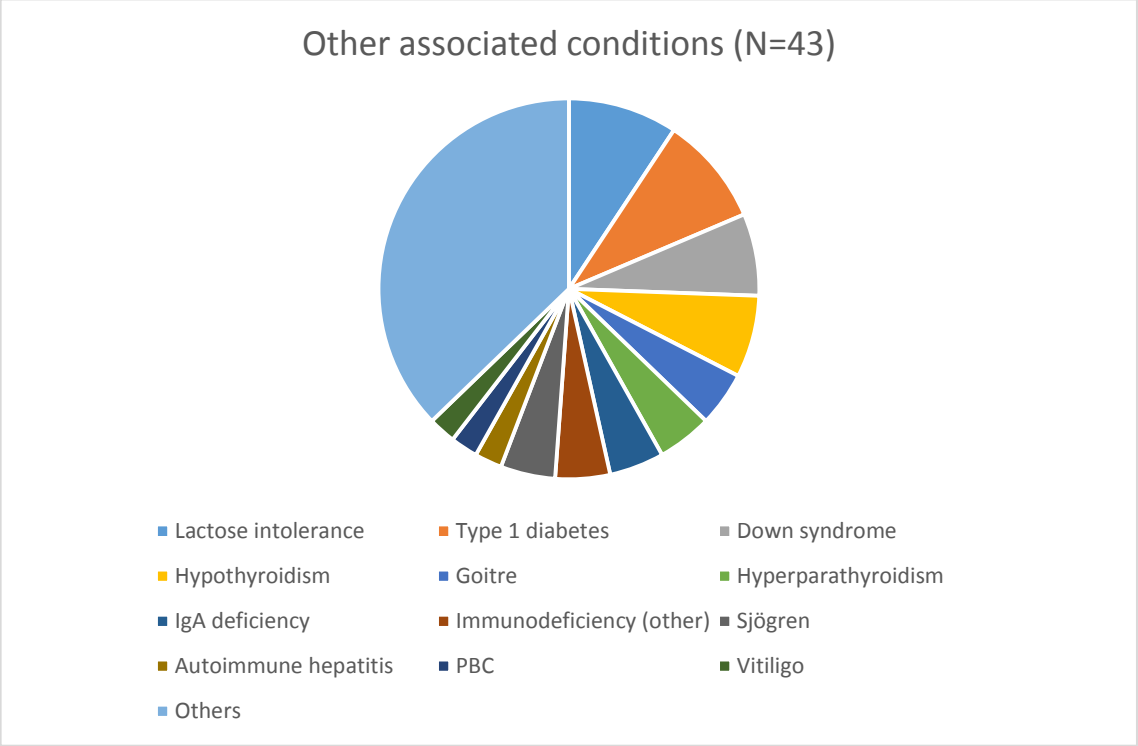

Figure 5 – Other associated conditions observed.

Some disorders, mostly but not limited to those with an autoimmune pathogenesis, have a known association with CD, being found in coeliacs at a higher frequency than in the general population (Hill *et al.*, 2016; Lebwohl *et al.*, 2015; Sapone *et al.*, 2012). These include T1DM (Garud *et al.*, 2009; Gutierrez-Achury *et al.*, 2011; Smyth *et al.*, 2008), various autoimmune thyroid diseases (Berti *et al.*, 2000; Elfström *et al.*, 2008), Sjögren's syndrome (Iltanen *et al.*, 1999; Teppo & Maury, 1984), autoimmune hepatitis (Caprai *et al.*, 2008; Vajro *et al.*, 2013), PBC (Freeman, 2010; Rubio-Tapia & Murray, 2007), selective IgA deficiency (Conrad *et al.*, 2012; McGowan *et al.*, 2008), Down's syndrome (Goldacre *et al.*, 2004; Mårild *et al.*, 2013; Rumbo *et al.*, 2002) and vitiligo (Rodríguez-García *et al.*, 2011; Volta *et al.*, 1997). Individuals presenting with one or more of these conditions, along with first-degree relatives of coeliac patients, are considered as belonging to high-risk groups for the development of

CD, and it is recommended that they be screened for the disease (Gujral *et al.*, 2012; Lebwohl *et al.*, 2015; Sapone *et al.*, 2012).

The other pathologies that were observed in these patients are not commonly associated with CD, but an association can be found in the literature for most of them. Regarding thyroid-related conditions, patients presented with goitre (Cuoco *et al.*, 1999), Graves' disease (Samasca *et al.*, 2014; Teixeira *et al.*, 2014), hyperparathyroidism (Abboud *et al.*, 2011; Deressa *et al.*, 2006), hypoparathyroidism (Abboud *et al.*, 2011; Freeman, 2016) and hypothyroidism (Canova *et al.*, 2016; Roy *et al.*, 2016). Associations have also been proposed for anorexia nervosa (Golden & Park, 2017; Mårild *et al.*, 2017), bulimia nervosa (Karwautz et al., 2008; Leffler *et al.*, 2007), COPD (de Menthon *et al.*, 2010; Ludvigsson *et al.*, 2012), Gilbert's syndrome (de Freitas *et al.*, 2002), non-IgA immunodeficiencies (Jørgensen *et al.*, 2016; Pituch-Noworolska *et al.*, 2013), lactose intolerance (Chiu *et al.*, 2016; Volta *et al.*, 2014), protein S deficiency (Bahloul *et al.*, 2005; Kallel *et al.*, 2009), Raynaud syndrome (de Almeida Menezes *et al.*, 2016; Gabrielli *et al.*, 2003), retinopathy (Leeds *et al.*, 2014; Mollazadegan *et al.*, 2013), sarcoidosis (Hwang *et al.*, 2008; Rutherford *et al.*, 2004), spongiosis (Campisi *et al.*, 2009) and T2DM (Mostowy *et al.*, 2016). Interestingly, retinopathy (Bakker *et al.*, 2013) and T2DM (Kabbani *et al.*, 2013) have been documented as having a lower prevalence in CD patients in some studies.

4.5. Serology

4.5.1. IgA levels

IgA levels (given in g/L) were noted as the earliest documented levels available (before the patient had been started on a GFD or even diagnosed, whenever possible).

Thirty-four patients (72.34%) had IgA levels noted in their medical files. These varied between 0.07 g/L and 9.35 g/L (mean 2.46±1.90 g/L) (**Table 5**).

Table 5 – IgA levels of patients.

N	34
Mean	2,46
Median	2,18
Standard deviation	1,90

The normal range for IgA levels is 0.7-4.0 g/L: taking this into account, there were 4 patients (8.51%) with IgA levels below normal range (which included the 2 patients with diagnosed IgA deficiency) and 6 patients (12.77%) with IgA levels above normal range (**Figure 6**).

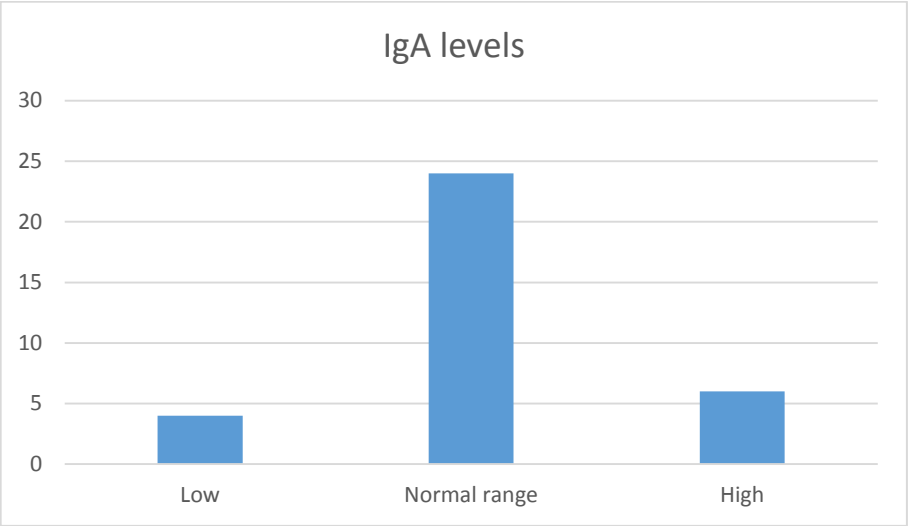

Figure 6 – Patients' IgA levels per categories.

It is recommended for total serum IgA to be measured if unknown in the diagnostic process for CD (Chow *et al.*, 2012; Husby *et al.*, 2012).

Since IgA deficiency is much more common in CD patients than in the general population, this should be taken into account when undergoing serological testing (Chow *et al.*, 2012; Korponay-Szabó *et al.*, 2003; Rashid & Lee, 2016). If the patient is found to have IgA deficiency following measurement of total serum IgA (usually defined as the serum IgA level being less than 0.2 g/L) (Srinivas *et al.*, 2014), IgG anti-EMA and IgG anti-tTG should be used as markers rather than their IgA counterparts (Dahlbom *et al.*, 2005; Korponay-Szabó *et al.*, 2003; Villalta *et al.*, 2010).

Indeed, as previously mentioned, 2 of the patients did suffer from IgA deficiency, and serological testing revealed 2 others to have low serum IgA levels (though the latter were both greater than 0.2 g/L, and thus did not indicate a deficiency).

4.5.2. CD-specific antibodies

As for the specific antibodies used in the detection of CD, a total of 29 patients (61.70%) had been tested for IgA AGA, 28 (59.57%) had been tested for IgG AGA, 27 (57.45%) for IgA anti-tTG and 27 (57.45%) for IgA anti-EMA (**Table 6**).

Table 6 – Results of patients' serological tests.

		N	%
Anti-gliadin antibodies (IgA)	Negative	14	48,28%
	Positive	15	51,72%
	Total	29	
Anti-gliadin antibodies (IgG)	Negative	15	53,57%
	Positive	13	46,43%
	Total	28	
Anti-tissue transglutaminase antibodies (IgA)	Negative	14	51,85%
	Positive	13	48,15%
	Total	27	
Anti-endomysial antibodies (IgA)	Negative	10	37,04%
	Positive	17	62,96%
	Total	27	

Out of the 47 patients, 13 (27.66%) did not have any serological testing done concerning these antibodies. Seventeen (36.17%) had been tested for all four antibodies. One (12.77%) had been tested for all but IgA anti-tTG. Five (10.64%) had been tested for all but IgA anti-EMA. Two (4.26%) had been tested for IgA anti-tTG and IgA anti-EMA. Two (4.26%) had only been tested for IgA anti-tTG. One (2.13%) had only been tested for IgA anti-EMA. One (2.13%) had been tested for all but IgG AGA.

Testing for IgA anti-tTG is used as an initial step in diagnosis, as it is the most sensitive test for CD (up to 97%) (Lewis & Scott, 2010; van der Windt *et al.*, 2010). Measurement of IgA anti-EMA should follow, being generally used as a confirmatory test if the anti-tTG test is positive (Di Sabatino & Corazza, 2009; Liu *et al.*, 2007), due to these antibodies' higher specificity (around 100% compared to 91% for IgA anti-tTG) (Giersiepen *et al.*, 2012; Husby *et al.*, 2012; Rubio-Tapia *et al.*, 2013).

IgA and IgG AGA should no longer be used when testing for CD, as they are now considered obsolete due to their low sensitivity and specificity when compared to IgA anti-tTG and IgA anti-EMA (Leffler & Schuppan, 2010; Rubio-Tapia *et al.*, 2013).

As mentioned in the previous section, for patients with IgA deficiency, IgG anti-EMA and IgG anti-tTG should be used as diagnostic markers (Dahlbom *et al.*, 2005; Korponay-Szabó *et al.*, 2003; Villalta *et al.*, 2010).

The above results show that, while serological testing is considered an important step in the diagnostic process for CD, a significant percentage (27.66%) did not have any testing done, and yet a diagnosis of CD was put forward. Furthermore, 14 of the 34 patients who did undergo serological testing (41.18%) were not tested for either IgA anti-tTG or IgA anti-EMA, both of which are strongly recommended as part of the diagnostic process.

IgA and IgG AGA were also commonly used as markers for CD (in 29 of the 34 patients who underwent serological testing, 85.29%) despite their well-known inadequacy. This happened even in the cases of patients whose diagnosis was fairly recent. These tests should have either been dispensed with or replaced by testing for the far more reliable IgA and IgG anti-deamidated gliadin peptides (DGP) (Giersiepen *et al.*, 2012; Husby *et al.*, 2012; Villalta *et al.*, 2010).

Concerning the 2 patients suffering from selective IgA deficiency, one of them was tested for IgG anti-tTG and IgG anti-EMA as recommended (she was also tested for IgG AGA and IgA AGA, anti-tTG and anti-EMA, the latter three predictably yielding negative results), being positive for both. The other was not tested for IgG anti-tTG or IgG anti-EMA, being only tested for their IgA counterparts and for IgA and IgG AGA (all four tests were negative), which could have been prejudicial for the diagnostic process.

4.5.3. HLA testing

Five (10.64%) of the patients had been tested for the presence of HLA-DQ2.2, HLA-DQ2.5 and HLA-DQ8. Out of these, 2 (40%) were positive for HLA-DQ2.2, 2 (40%) were positive for HLA-DQ2.5 and 1 (20%) was positive for both HLA-DQ2.2 and HLA-DQ2.5 (**Table 7**).

Table 7 – Results of patients' HLA testing.

		N	%
HLA-DQ2.2	Negative	2	40%
	Positive	3	60%
	Total	5	
HLA-DQ2.5	Negative	2	40%
	Positive	3	60%
	Total	5	
HLA-DQ8	Negative	5	100%
	Positive	0	0%
	Total	5	

The negative predictive value of this genetic test is very high, meaning that the vast majority of HLA-DQ2 and HLA-DQ8 patients will never develop CD (Rashtak & Murray, 2007; Rubio-Tapia *et al.*, 2013; Wolters & Wijmenga, 2008).

HLA-DQ typing is not enough for a diagnosis, but is useful as a means of ruling it out as a possibility. (Greco *et al.*, 1998; Husby *et al.*, 2012; Rashtak & Murray, 2007). This has particular importance in the cases of high-risk patients (especially asymptomatic ones), such as first-degree relatives of coeliacs and patients with T1DM or other disorders known to be associated with CD (Hill *et al.*, 2005; Husby *et al.*, 2012; Rashtak & Murray, 2007). It is also recommended in cases where a discrepancy between histology and serology results exists, or when a GFD has been started before any testing was performed (Kochhar *et al.*, 2016; Rubio-Tapia *et al.*, 2013).

In this case, while 5 patients underwent HLA testing, none of them belonged to high-risk groups (family history of CD or associated T1DM, Down syndrome and/or selective IgA deficiency). However, as mentioned in an earlier section, there were several high-risk patients present in this sample, to whom genetic testing should have been recommended as part of a proper diagnostic process.

4.6. Histology

Thirty (63.83%) patients had had at least one prior duodenal biopsy.

Nineteen (63.33%) of these biopsies had resulted in a definite Marsh-Oberhuber classification. Two patients (10.53%) had a Marsh-Oberhuber degree of 1, four (21.05%) a

degree of 2, four (21.05%) a degree of 3a, six (31.58%) a degree of 3b and three (15.79%) a degree of 3c (**Table 8**).

Table 8 – Patients' Marsh-Oberhuber classifications.

		N	%
Marsh-Oberhuber classification	1	2	10,53%
	2	4	21,05%
	3a	4	21,05%
	3b	6	31,58%
	3c	3	15,79%
	Total	19	

When applying the alternate classification proposed by Corazza and Villanacci, these values would be equivalent to 6 patients (31.58%) with a "A" degree, 10 (52.63%) with a "B1" degree and 3 (15.79%) with a "B2" degree (**Table 9**).

Table 9 – Patients' Corazza-Villanacci classifications.

		N	%
Corazza-Villanacci classification	A	6	31,58%
	B1	10	52,63%
	B2	3	15,79%
	Total	19	

The diagnosis of CD is based on characteristic lesions found in duodenal biopsies following (in most but not all cases) positive or genetic serological tests (Lebwohl *et al.*, 2011; Ludvigsson *et al.*, 2014). The distinctive histological abnormalities present in CD include partial to total villous atrophy, elongated crypts, decreased villous:crypt ratio, increased mitotic index in the crypts, increased IEL density, increased IEL mitotic index and infiltration of plasma cells, lymphocytes, mast cells, and eosinophils and basophils into the lamina propria (Corazza & Villanacci, 2005; Ludvigsson *et al.*, 2014).

The Marsh classification, later modified by Oberhuber and colleagues, was defined to grade the severity of these lesions (Marsh, 1992; Oberhuber *et al.*, 1999). Initially, normal mucosa was classified as Type 0 (preinfiltrative stage), intraepithelial lymphocytosis as Type 1 (infiltrative lesion), intraepithelial lymphocytosis and crypt hyperplasia as Type 2 (hyperplastic

lesion), intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy as Type 3 (destructive lesion), and total villous atrophy with crypt hypoplasia as Type 4 (hypoplastic lesion) (Marsh, 1992). Type 3 was later modified and divided into Type 3a (partial villous atrophy), Type 3b (subtotal villous atrophy) and Type 3c (total villous atrophy) (Oberhuber *et al.*, 1999).

As the Marsh-Oberhuber classification proved unreliable at times due to the high number of categories often resulting in low inter-observer and intra-observer agreement, a new simplified classification was eventually developed by Corazza and colleagues (Corazza & Villanacci, 2005; Corazza *et al.*, 2007). This new grading system classified the lesions characteristic of CD into non-atrophic (Grade A) and atrophic (Grade B). Grade A was characterised by the isolated increase of IELs (>25/100 enterocytes). Grade B was split into B1, in which the villous:crypt ratio is less than 3:1, with still detectable villi, and B2, in which the villi are no longer detectable (Corazza & Villanacci, 2005; Corazza *et al.*, 2007). Grade A includes Type 1 and Type 2 of the Marsh-Oberhuber classification, Grade B1 includes Type 3a and Type 3b, and Grade B2 is equivalent to Type 3c (Corazza *et al.*, 2007). This system's inter-observer reproducibility proved to be significantly higher than that of the Marsh-Oberhuber classification (Corazza *et al.*, 2007).

The guidelines for the diagnosis of CD currently recommend that a minimum of four endoscopic biopsies, two from the duodenal bulb and two from (preferably two different locations of) the second third of the duodenum, be performed (as lesions may be patchy and irregular) (Lebwohl *et al.*, 2011; Rubio-Tapia *et al.*, 2013; Villanacci, 2009).

The presence of lesions corresponding to Type 2 or Type 3a, 3b or 3c of the Marsh-Oberhuber classification tends to support a diagnosis of CD (Hill *et al.*, 2005; Vriezinga *et al.*, 2015), but it is important to note that there are other disorders that might be responsible for a non-gluten-dependent villous atrophy (Oberhuber *et al.*, 1999; Villanacci *et al.*, 2010).

While the majority of patients (63.83%) had duodenal biopsies performed as is recommended for an adequate diagnosis of CD to be made, it was not possible to determine the actual number of samples collected in any of these cases. It was observed, however, that several patients (12 out of 30, 40%) had multiple prior biopsies performed, though not all were related to a diagnosis of confirmation of CD.

There was also a significant percentage of patients (36.67%) who underwent one or more duodenal biopsies but did not have a Marsh-Oberhuber degree determined. This is not

a mandatory requirement for a diagnosis of CD to be made, but it is recommended, as it provides better documentation on the severity of histological lesions present.

As noted before, 2 patients had a Marsh-Oberhuber degree of 1, which is not usually indicative of CD. In one of these cases, additional information raised the possibility that the patient actually suffered from NCGS rather than CD, as will be discussed in a later section.

4.7. Treatment (GFD)

Twenty-six patients (55.32%) were mentioned to be on a GFD.

A lifelong GFD, to which patients must strictly, adhere, is the current gold standard of treatment for CD (Bascuñán *et al.*, 2017; Ludvigsson *et al.*, 2014; Sapone *et al.*, 2012). Symptoms usually improve after the GFD has been in effect for 4 weeks, and normalization of serological markers occurs approximately 1 year after the diet has been initiated (Bao *et al.*, 2012; Bascuñán *et al.*, 2017; Nasr *et al.*, 2012).

Patients will benefit from dietary counselling provided by a dietitian, as there might be hidden sources of gluten they have not considered, and some patients will have a very high sensitivity to even trace amounts (Hill *et al.*, 2016; Rubio-Tapia *et al.*, 2013; Sapone *et al.*, 2012). For instance, some products presumed to be gluten-free might be in fact contaminated, and gluten might be an inactive ingredient in medications and vitamin and mineral supplements (Fasano & Catassi, 2001). Adequate nutritional supplementation may also be needed, as a GFD has been observed to be high in saturated fats, sugar and sodium and low in fibre and some vitamins and minerals (Martin *et al.*, 2013; Pellegrini & Agostoni, 2015; Welstead, 2015), which can lead to excessive weigh gain in patients after starting the diet (Ferrara *et al.*, 2009; Valletta *et al.*, 2010).

The compliance to the GFD tends to be imperfect, mostly due to the decrease in quality of life which comes with its implementation (Lee & Newman, 2003; O'Leary *et al.*, 2004). Untreated CD, however, is known to be associated with an increase in morbidity and mortality, which might be reduced by the prolonged adherence to a GFD (Kochhar *et al.*, 2016; Lebwohl *et al.*, 2013). Thus, it is desirable to diagnose CD and implement a GFD as soon as possible (Hill *et al.*, 2005; Kochhar *et al.*, 2016). A full assessment of dietary adherence should have four steps: clinical assessment of symptoms, dietetic review, serum antibodies and follow-up biopsy (Ludvigsson *et al.*, 2014).

Despite the small size of this sample, the percentage of patients on a GFD (55.32%) was not as high as it could be expected, especially when considering the risks present when CD is left untreated. It was also not possible to determine how many of the patients on a GFD had adequate dietary counselling or regular assessment of their adherence to the diet.

4.8. Refractory coeliac disease

Two (4.26%) of the patients were diagnosed with refractory coeliac disease (one of these patients was diagnosed with type I and the other with type II of this form of the disease). The patient suffering from RCD type II was undergoing treatment with cladribine.

Refractory coeliac disease (RCD) is defined by as the persistence and recurrence of symptoms and signs of malabsorption and villous atrophy in spite of adherence to a strict GFD for over 12 months and in the absence of other disorders (Di Sabatino & Corazza, 2009; Kochhar *et al.*, 2016; Malamut & Cellier, 2014). It is an uncommon complication of CD, affecting 1-2% of patients with the disease (Malamut *et al.*, 2009; Roshan *et al.*, 2011; Rubio-Tapia *et al.*, 2009), though a prevalence as high as 5% has been reported (Al-Toma *et al.*, 2007; Rubio-Tapia *et al.*, 2009).

RCD can be divided into two categories – type I and type II (Kochhar *et al.*, 2016; Malamut *et al.*, 2009; Rubio-Tapia & Murray, 2010). Type I presents with intraepithelial lymphocytosis similar to that found in untreated CD (Kochhar *et al.*, 2016; Malamut *et al.*, 2009; Verbeek *et al.*, 2008). Type II presents with CD3-positive intraepithelial T-cells possessing an abnormal immunophenotype, lacking expression of normal cell surface differentiation markers such as CD8 (Kochhar *et al.*, 2016; Verbeek *et al.*, 2008). The T-cell abnormalities seen in type II RCD are associated with a much less favourable prognosis due to the higher risk of development of enteropathy-associated T-cell lymphoma (EATL) within 4-6 years after the diagnosis of RCD (Malamut *et al.*, 2009; Malamut & Cellier, 2014; Rubio-Tapia & Murray, 2010).

There is no standard treatment for RCD. Patients have been known to undergo elemental diets (Goerres *et al.*, 2003; Mauriño *et al.*, 2002) or treatment with steroids (Stuart & Gent, 1998) or immunosuppressants such as azathioprine (Vaidya *et al.*, 1999). These are known to sometimes be beneficial in the case of RCD type I, but have limited benefits in type II (Goerres *et al.*, 2003; Mauriño *et al.*, 2002). Treatment for RCD type II has been reported to include cladribine (a chemotherapeutic agent used to treat hairy cell leukemia)

(Tack *et al.*, 2011), cyclosporine (Rolny *et al.*, 1999) and high-dose chemotherapy with autologous stem cell support, though it seems to be preferable that therapy be individualized (Rubio-Tapia & Murray, 2010).

The number of patients affected by RCD was perhaps slightly higher than it was to be expected, but this is likely due to the small size of the sample. While it was not mentioned what treatment, if any, the patient with type I was undergoing, the patient with type II RCD (who was deceased – it was not possible to determine if the cause of death was related to RCD) had been treated with cladribine, one of the therapeutic agents documented in literature.

4.9. Non-coeliac gluten sensitivity

Despite the initial criterion of a diagnosis of CD used in the selection of the 47 patients, more in-depth analysis of their medical files showed that 4 of them (8.51%) did not have a definite CD diagnosis, and could possibly be considered to have NCGS. All were still living. Three were female and aged 55, 36 and 28 respectively. The fourth was male and aged 26.

The 55-year-old presented with diarrhoea as a GI symptom, and also suffered from common variable immunodeficiency and iron deficiency anaemia. As for serological testing, she had only been tested for IgA anti-EMA (which had yielded a negative result). She had undergone 6 different duodenal biopsies in the past, and was on a GFD. Reports from duodenal biopsies and endoscopies concluded with the following diagnoses (in chronological order): "chronic atrophic gastritis", "villous atrophy associated with common variable immunodeficiency", "chronic duodenitis of mild intensity", "'coeliac-like' villous atrophy" and "villous atrophy without features of CD".

The 36-year-old had a family history of CD (coeliac mother) and presented with anxiety as an EI symptom and hypothyroidism as an associated condition. Regarding serology, she had been tested for IgA AGA, IgG AGA and IgA anti-EMA (the second of these tests being positive and the others negative). She had only had 1 prior duodenal biopsy performed. The biopsy report diagnosed her with "unspecific mild duodenitis".

The 28-year-old presented with diarrhoea as a GI symptom and depression as an EI symptom. Multiple other associated conditions (some resulting from malabsorption) were

present, these being: iron deficiency anaemia, osteoporosis, hyperparathyroidism, IgA deficiency and sarcoidosis. Concerning serology, she had been tested for IgA AGA, IgG AGA, IgA anti-tTG and IgA anti-EMA (all tests had been negative). No IgG anti-tTG or IgG anti-EMA tests had been performed, despite her IgA deficiency. She had had 3 prior duodenal biopsies and was on a GFD. The reports of duodenal biopsies and endoscopies diagnosed her with "congenital immune deficit", "nodular lymphoid hyperplasia of the duodenum" and "atrophic chronic gastritis".

The 26-year-old did not present with any GI symptoms. He did present with an EI symptom, ataxia, and a malabsorption-associated condition, folic acid deficiency. Tests for IgA AGA, IgG AGA and IgA anti-tTG (the first being positive and the other two negative) had been performed. Genetic testing had also been performed (and had yielded a positive result for HLA-DQ2.2). He had undergone 3 prior duodenal biopsies and was on a GFD. The biopsy reports did not mention have any alternative diagnoses, but an official diagnosis of CD was not reached either. He was also noted as having a Marsh-Oberhuber degree of 1.

It seems probable that these patients might in fact suffer from NCGS rather than CD. The GI and EI symptoms and other associated conditions which may also be observed in CD observed in these particular cases can all be present in NCGS as well. Serology (which predated the implementation of a GFD for the 3 patients on this diet) was mostly negative for these patients, and only AGA tests, which are currently considered unreliable and insufficient for a CD diagnosis, had yielded positive results.

The histological lesions observed in duodenal biopsies could be attributed to common variable immunodeficiency in the case of the first patient. The second and fourth patients had mild histological lesions, which, while possible for coeliacs, tend to be more indicative of NCGS. The third patient suffered from many other conditions, and could have a yet undiagnosed underlying immune disorder as the cause of intestinal damage.

Finally, while the fourth patient did have a positive HLA-DQ2.2 result, this by itself is not enough for a diagnosis of CD (with HLA-DQ2 being found in about 30% of the population), especially when considering the lack of GI symptoms, minor histological damage (CD is only present in about 10% of cases with non-atrophic lesions) (Kakar *et al.*, 2003) and mostly negative serology.

5. CONCLUSION

After a review of the literature and a full analysis of the data collected from the medical files of 47 Portuguese CD patients, it can be concluded that there are in fact potential NCGS patients among those diagnosed as coeliac patients. Given that this was a relatively small sample, it is likely that a significant percentage of the population could suffer from NCGS (since the prevalence of this disorder has been estimated to be higher than that of CD).

It would be advisable that, in the future, the possibility of this diagnosis be considered for "coeliac" patients with negative serology and minor histological damage. Likewise, NCGS should be considered when there is a clear reaction to gluten but there is not enough evidence for a diagnosis of CD to be made.

Following the analysis of the data collected from these patients' files, there are several other topics (not necessarily related to NCGS) to consider:

Firstly, clinicians should take into account that CD (or NCGS) patients with associated selective IgA deficiency should be tested using IgG CD-specific antibodies (which was not always the case among this particular sample of patients).

Secondly, IgA and IgG AGA antibodies should not be used to diagnose CD (these tests should be replaced by IgA and IgG anti-DGP or simply not performed at all).

Thirdly, HLA testing should always be performed on high-risk patients (such as first-degree relatives of CD patients and patients with T1DM, Down syndrome and/or selective IgA deficiency). Again, while this is strongly recommended in literature, it was not the case for patients belonging to these groups in this sample.

Furthermore, a Marsh-Oberhuber classification (or alternatively, a Corazza-Villanacci classification) should be determined when performing duodenal biopsies. It might not be a strictly necessary step for the diagnosis of CD (or NCGS) but it could yield more specific information and help with the diagnostic process overall.

Finally, patients diagnosed with CD (or NCGS) should be strongly encouraged to start a GFD. It is currently the gold standard of treatment for both these disorders, yet only slightly more than half of the patients in this sample were following this dietary regimen.

Patients should also be offered nutritional counselling to be able to avoid hidden sources of gluten and supplement their diet as necessary (as a GFD is not usually balanced from a nutritional standpoint).

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