Universidade de Lisboa

Faculdade de Medicina

Instituto Politécnico de Lisboa - Escola Superior de Tecnologia da Saúde de Lisboa

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Dissertação especialmente elaborada para obtenção do grau de Mestre em Nutrição Clínica

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“A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de

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Abstract

Non Celiac Gluten Sensitivity (NCGS) is a syndrome characterized by intestinal and extra-intestinal symptoms, which appear with gluten consumption. It could be designated as "Non Celiac Wheat Sensitivity" (NCWS), as it is possible that the symptoms aren't related to gluten itself. Symptoms could be influenced by a lot of factors, like α-amylase/trypsin inhibitors (ATIs), which can modify intestinal barrier parameters and induce barrier dysfunction and immune activation. There's not enough evidence on wheat germ agglutinin (WGA), but it could lead to the activation of the immune system, increasing intestinal permeability and microbial translocation, and gliadin activates zonulin signaling regardless of the genetic expression of autoimmunity. Fermentable oligo-di-monosaccharides and polyols (FODMAPs), rather than being triggers, could exacerbate symptoms in some patients.

The Human gut microbiome is an important environmental factor that is modulated by diet composition, pre and probiotics, alcohol consumption, and also pharmacotherapy. Dietary changes affect gut microbiota, leading to changes in bacterial metabolites, such as short-chain fatty acids production.

There are very few studies investigating microbiota in NCGS patients. In the Mexican population, a Gluten-free diet (GFD) results in a higher abundance of duodenal *Pseudomonas*, without certainty of any beneficial effect or not to the integrity of the duodenal mucosa. A GFD leads to an increase in *Bacterioidetes* and a drop in *Firmicutes*, compared to the low FODMAP diet. Before the intervention diets, NCGS patients had more *Firmicutes* and less *Bacteroidetes* than healthy individuals. Also, consumption of a low-gliadin bread could be a positive component of the GFD, with the increase in *Faecalibacterium* and decrease in *Bacteroides*, suggesting a less inflammatory phenotype.

This review examines the impact on gut microbiota after gluten exclusion, particularly in NCGS patients, as well as being a summary of all evidence regarding NCGS. With a fuller understanding of the host-microbe interaction in the gut, perhaps the pathogenic side of this relationship can be suppressed, maintaining and/or restoring the beneficial effects of the microbiota. Efforts should be made in the future to increase research regarding the gut microbiota in these patients, and the changes that come from the inclusion and exclusion of gluten.

Keywords: Non Celiac Gluten Sensitivity; Gluten-free diet; Microbiota.

Resumo

A Sensibilidade Não Celíaca ao Glúten (SNCG) pode ser considerada como uma síndrome, caracterizada pela presença de sintomas gastrointestinais e extra-intestinais, que surgem com o consumo de glúten. Alguns estudos recentes têm designado esta condição clínica de Sensibilidade Não Celíaca ao Trigo, pois existe a possibilidade de que os sintomas experienciados por estes indivíduos não se devam apenas ao glúten. Os sintomas podem ser influenciados por vários fatores, como os Inibidores da α-amilase/tripsina, que poderão induzir uma disfunção na barreira intestinal, e desencadear a ativação do sistema imune. A evidência sugere que estas proteínas presentes no trigo ativam uma resposta da imunidade inata, induzida também por componentes do metabolismo microbiano, em particular Lipopolissacarídeos (LPS), através da ativação do recetor TLR4. Não existe evidência suficiente também relativamente ao papel das aglutininas do gérmen de trigo, mas estas parecem, da mesma forma, desencadear uma resposta da imunidade inata, aumentando a permeabilidade intestinal e translocação bacteriana. A gliadina desencadeia a libertação de zonulina, aumentando a permeabilidade intestinal, mesmo em indivíduos sem Doença Celíaca (DC) ou sensibilidade ao glúten, embora desencadeie uma resposta mais exacerbada na função da barreira intestinal em indivíduos com DC.

Os FODMAPs (grupo heterogéneo de hidratos de carbono de cadeia curta e poliálcoois, altamente fermentescíveis e de baixa absorção) podem ser considerados como exacerbadores de sintomas em alguns indivíduos. Estes não podem ser inteiramente responsáveis pelos sintomas percecionados por indivíduos com SNCG, visto que se observa uma resolução dos sintomas através de uma dieta isenta de glúten, continuando a ingerir alimentos pertencentes ao grupo dos FODMAPs, como por exemplo as leguminosas. Além disso, a dieta isenta de glúten parece ter resultados superiores na melhoria dos parâmetros psicológicos e consistência das fezes, comparativamente com a dieta com baixo teor de FODMAPs. Esta dieta pode ter potencial como intervenção dietética para um subgrupo de indivíduos com Síndrome do Intestino Irritável (SII), sensíveis ao glúten.

Como não existem métodos validados para avaliar a prevalência de SNCG, tem sido estimada por alguns estudos sem muito rigor, em indivíduos que reportam ter uma sensibilidade ao glúten ou que quando excluem o glúten por conta própria reportam melhoria clínica. O diagnóstico de SNCG deverá estará correto apenas numa minoria dos indivíduos que auto-excluem o glúten da sua dieta, ou que têm sintomas equivalentes aos reportados por indivíduos com SNCG. Isto pode ser parcialmente explicado por uma sobreposição de sintomas entre SNCG e SII. O diagnóstico mais específico de momento será uma monitorização dos indivíduos durante a eliminação e reintrodução de glúten, por desafio duplo cego controlado por placebo, após exclusão de DC e alergia ao trigo, como preconizado pelos Critérios de Salerno, guidelines criadas em 2015. Foi também sugerida uma dieta com baixo teor de FODMAPs/dieta isenta de

glúten seguida da introdução de glúten/placebo, com o intuito de identificar SNCG em indivíduos com SII, pois ambas as condições clínicas podem coexistir.

Os sintomas nestes indivíduos iniciam-se após ingestão de cereais contendo glúten, e resolvem-se após alguns dias, ou noutros casos até um mês, com a implementação da dieta isenta de glúten. Os sintomas são semelhantes aos associados à DC, mas parece existir um agravamento nos sintomas extra-intestinais. Os sintomas gastrointestinais mais prevalentes parecem ser dor e distensão abdominal. Outros sintomas gastrointestinais reportados são desconforto abdominal, obstipação, diarreia, dor epigástrica e flatulência. Relativamente aos sintomas extra-intestinais, estes indivíduos reportam fadiga crónica, dor de cabeça, ansiedade, confusão mental, prurido, depressão, mialgia, artralgia, dermatite, pior qualidade de vida, entre outros. Como não foram reportadas complicações a longo prazo na SNCG, pode ser recomendada a reintrodução de alimentos contendo glúten 1 a 2 anos depois do início da dieta. A SNCG começa a ser associada com doenças autoimunes, sendo principalmente associada à tiroidite autoimune.

Existe evidência de um comprometimento da integridade da barreira intestinal nestes indivíduos, promovendo uma translocação bacteriana aumentada. Uma proposta recente é que a diminuição de espécies produtoras de butirato, espécies pertencentes ao grupo dos *Firmicutes*, ou de *Bifidobacteria*, estão na base do aumento da permeabilidade intestinal em SNCG, resultando numa maior translocação de LPS e aumento da inflamação. Parece existir um envolvimento da imunidade inata, descrito por alguns autores, mas são necessários mais estudos para determinar se a imunidade adquirida contribui para a patogénese de indivíduos com SNCG.

O tratamento atual da SNCG é a dieta isenta de glúten, com vista à melhoria dos sintomas e qualidade de vida dos indivíduos. Têm surgido alguns complementos e alternativas à dieta isenta de glúten, como por exemplo o consumo de pão com menor teor de gliadina, que parece induzir mudanças positivas na composição da microbiota intestinal, como um aumento de *Faecalibacterium* (género anti-inflamatório), e uma diminuição em *Bacteroides* (género pro-inflamatório). Os sintomas são equivalentes aos obtidos com o consumo de pão sem glúten, e os resultados semelhantes aos obtidos através da variedade de farinha Senatore Cappelli. Esta última variedade de farinha gerou melhores resultados que a farinha de trigo comercial, reduzindo sintomas gastrointestinais e extra-intestinais nestes indivíduos. Variedades de trigo ancestral também podem ser consideradas como uma possível alternativa ao tratamento em alguns indivíduos, por terem menor concentração de inibidores da α-amilase/tripsina e péptidos tóxicos de glúten. Como grande parte dos alimentos sem glúten no mercado são constituídos por arroz, alimentos sem glúten beneficiariam da inclusão de grãos alternativos, como teff ou pseudocereais, além da inclusão de leguminosas e uso de pão de fermentação natural.

O microbioma intestinal humano pode ser modificado através da composição da dieta, pré e probióticos, consumo de álcool, e através de farmacoterapia. Alterações

alimentares afetam a microbiota intestinal, levando a uma alteração nos metabolitos produzidos por bactérias, nomeadamente na produção de ácidos gordos de cadeia curta.

A dieta parece induzir alterações rápidas na microbiota em alguns casos, nomeadamente entre dietas à base de carne e vegetarianas, ou alternando entre uma dieta com elevado teor lipídico e pouca fibra, e uma dieta de elevado teor de fibra e baixo teor lipídico.

Existem poucos estudos relativos à microbiota em indivíduos com SNCG. Foi observada uma maior abundância de Pseudomonas no duodeno, resultado de 4 semanas de dieta isenta de glúten, sem certezas do efeito exercido na integridade da mucosa duodenal. A maioria dos indivíduos com SNCG e SII apresenta um rácio *Firmicutes:Bacteroidetes* aumentado, que é parcialmente revertido através da dieta isenta de glúten. Através desta dieta, observou-se um aumento do nível de *Bacterioidetes* e uma diminuição de *Firmicutes*, em comparação com a dieta com baixo teor de FODMAPs. A microbiota de indivíduos com SNCG pode ser mais suscetível a alterações nutricionais, comparando com indivíduos saudáveis, e é sugerida a presença de um desequilíbrio na microbiota destes indivíduos.

Em indivíduos adultos e saudáveis, a dieta isenta de glúten parece reduzir populações de bactérias vistas como benéficas para a saúde humana, como por exemplo *Bifidobacterium* e *Lactobacillus*, e um aumento de bactérias oportunistas, como a *Escherichia coli* e *Enterobacteriaceae*. Uma possível explicação passa pela redução do teor de fibra através da dieta isenta de glúten, não se devendo à redução da ingestão de glúten. Na DC está demonstrado um estado de disbiose, e a microbiota pode ser apenas parcialmente restaurada através da dieta isenta de glúten, visto que esta pode se associar também a uma redução na diversidade de *Lactobacillus* e *Bifidobacterium*, e uma abundância superior de *Proteobacteria*.

Esta revisão da literatura analisa o impacto da exclusão do glúten na microbiota intestinal, particularmente em indivíduos com SNCG, além de sumarizar a evidência atual relativa à SNCG. Através de um melhor conhecimento das interações bactéria-hospedeiro no intestino, existe a possibilidade de ser controlado o aspeto patogénico desta relação, mantendo e/ou restaurando os benefícios associados à microbiota.

Devem ser feitos esforços no futuro para desenvolver biomarcadores sensíveis e específicos para auxiliar no diagnóstico da SNCG, além de serem necessários estudos relativamente à influência dos Inibidores da α-amilase/tripsina e aglutininas do gérmen de trigo nos sintomas e patogénese destes indivíduos. De momento, existe pouca evidência relativamente à microbiota destes indivíduos, sendo de extrema importância realizar estudos nesta temática, estudando as alterações causadas pela inclusão e exclusão de glúten nos mesmos, além de avaliar o impacto que uma dieta isenta de glúten com elevado ou reduzido teor de fibra terá na microbiota.

Palavras-chave: Sensibilidade Não Celíaca ao Glúten; Dieta isenta de glúten; Microbiota.

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List of abbreviations

ATI(s) - α-Amylase/trypsin inhibitor(s)
CD - Celiac Disease
FODMAPs - Fermentable oligo-, di-, and mono-saccharides and polyols
GFD - Gluten-free diet
IBS - Irritable Bowel Syndrome
LPS - Lipopolysaccharide
NCGS – Non-Celiac Gluten Sensitivity
SR-NCGS - Self-Reported Non Celiac Gluten Sensitivity
WA - Wheat Allergy
WGA - Wheat germ agglutinin

This dissertation, made in order to obtain a Master's degree in Clinical Nutrition, was divided in 2 parts.

The first part was the writing of the literature review, regarding all NCGS particularities (prevalence, diagnosis, symptoms and overlap with other diseases, pathology, possible causes for this syndrome, treatment with a GFD and microbiota). An extensive research regarding Non-Celiac Gluten Sensitivity was made on the PubMed platform, as well as Microbiota and GFD. The most relevant studies were selected for this review.

The second part was the development of a protocol for the implementation of the study, to evaluate symptoms and microbiota in Non-celiac gluten sensitivity patients, with and without gluten in their diets.

Introduction

Gluten is the main structural protein in wheat and other cereals, such as barley and rye, and is one of the main dietary components for most of the global population, particularly in the Mediterranean area were the mean daily gluten ingestion is around 10–20g[1].
There are some human diseases related to gluten exposure, like Wheat Allergy (WA) and Celiac Disease (CD). In both, the reaction to gluten is mediated by T-cell activation in the gastrointestinal mucosa.[2]

The number of individuals in a Gluten-free diet (GFD) is even higher than those estimated to be CD patients[3] or with WA diagnosis. Many of them claim a medical requirement to undertake a GFD. We can argue it could be a trend, but it can also be explained by cases of gluten reactions, generally defined as Gluten Sensitivity, in which neither allergic nor autoimmune mechanisms seem to be involved.[3]

Gluten Sensitivity patients are unable to tolerate gluten, develop an adverse reaction when eating gluten, with gastrointestinal symptoms that may resemble those associated with CD. The overall clinical picture associated with this reaction is not accompanied by the concurrence of tissue transglutaminase autoantibodies (tTG) or other specific Celiac-related antibodies and does not lead to classical CD small intestine damage.[2,4]

Various designations have been suggested and used to describe this new disorder, such as Gluten Hypersensitivity, Gluten Sensitivity and Non-Celiac Gluten Intolerance.[5] According to the 2012 consensus on new nomenclature and classification of gluten-related disorders, the designation of this disorder as a Non-Celiac Gluten Sensitivity (NCGS) is more adequate since it allows the differentiation from Celiac Disease.[3]
NCGS patients can present symptoms other than gastrointestinal impairment like fatigue, indigestion, skin rashes, joint pain, anemia, obstructive sleep apnea, cystitis, headaches. A myriad of neurological dysfunctions can also be triggered by the immune reaction to gluten. [2]

The diagnosis is based on a negative serology for Celiac Disease and the presence of: either symptoms improvement or disappearance after gluten is withdrawn from the diet and their recurrence when gluten is reintroduced.[3] A GFD should be implemented in a blinded fashion to prevent bias, mainly during the reintroduction of gluten-containing foods to test reappearance of symptoms.[3] NCGS patients have higher presence of HLA-DQ2[1] when compared to the general population, but unlike CD, not all present this haplotype. Thus, clinical and pathological features, possible serological biomarker - HLA-DQ2 expression - and increased intestinal CD-associated antibodies (but not villous atrophy) may help recognize the condition of wheat sensitivity.[6]

[1] HLA DQ - human leukocyte antigen

The Human gut microbiome is an important environmental factor that affects its host metabolism. Is dynamic and plays pivotal nutritional, physiological, immunological and pathological roles in the host. Various factors modulate microbiota, among these: diet composition including fatty acids, protein, pre and probiotics, and alcohol consumption, but also pharmacotherapy like non-steroidal anti-inflammatories and antibiotics.[7]

About 1.000 different prokaryotic bacterial species reside in the human gut with 99% belonging to about 40 species. The human normal flora, or microbiota, is vast, both in quantitative and in diversity. Recent technology applying high-throughput sequencing and molecular taxonomic methodologies have greatly increased our understanding of the population composition, dynamics, and ecology of the gut microbiota.[7]

The composition of the intestinal microbiome is known to play a critical role in shaping the immune system. It's vital to characterize the impact of gluten exclusion on the microbiome of NCGS patients. The knowledge of these changes could enhance the benefits of the GFD in these patients.

The main objective is to review the available evidence about microbiota, symptoms, and other particularities of NCGS patients.

Chapter 1 - Literature Review

1.1 Gluten

Gluten is one of the earliest protein fractions described by chemists, being first described by Beccari in 1728.[8]
Approximately 80% proteins of the total grain are formed by gluten proteins. Its main protein constituents are monomeric gliadins and polymeric glutenin subunits, in approximately equal amounts.[9–11]
Gluten proteins play a key role in determining the unique rheological dough properties and baking quality of wheat,[11] conferring the cohesiveness and viscoelasticity that allows dough to be processed into bread, noodles and other foods.[10,12] Hydrated gliadins contribute to the viscosity and extensibility of the dough, while hydrated glutenins are both cohesive and elastic, being responsible for dough strength and elasticity. A proper mixture of both fractions is essential to the quality of the end product.[11] These properties are particularly important in making leavened bread, as they allow the entrapment of carbon dioxide released during leavening. They are also exploited in the food industry where gluten proteins may be used as a binder in processed foods.[8]

Gluten proteins are responsible for intolerance in a large number of individuals. To date, the only therapy available for people with CD or sensitivity to gluten is the long-life avoidance of gluten from the diet.[13]

CD is an autoimmune disease of the small intestine caused by the exposure to gluten, inducing an inflammatory response and destruction of the villous structure of the intestine,[14] in genetically predisposed individuals. CD diagnosis comprises of positive serology, and an intestinal biopsy to confirm. Intestinal damage and systemic inflammation might explain the wide range of symptoms which accompany CD.[15] It commonly appears in early childhood, but in many patients, symptoms like fatigue, diarrhea, weight loss due to malabsorption, anemia, and neurological symptoms, may not develop until later in life.[16]

Human leucocyte antigen DQ2 and DQ8 (HLA- DQ2/ HLA-DQ8) determination is useful when there is discrepancy between serologic studies and histology.[17] The additional factors that influence CD development are unknown, but might include alterations in the intestinal microbiota.[18]

In WA, like in CD, the reaction to gluten is mediated by T-cell activation in the gastrointestinal mucosa.[2] WA is defined as an adverse immunologic reaction to wheat proteins, in which immunoglobulin E and release of chemical mediators such as histamine play a fundamental role. Depending on the route of allergen exposure and immunologic mechanisms, WA can be classified into classic food allergy affecting the skin, gastrointestinal tract or respiratory tract, wheat-dependent exercise-induced anaphylaxis, occupational asthma and rhinitis, and contact urticaria.[3]

1.2 Non-Celiac Gluten Sensitivity

NCGS 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 CD or WA.[19,20]

According to the 2012 consensus on new nomenclature and classification of gluten-related disorders, this disorder is designated as NCGS, to allow the differentiation from CD.[3]

NCGS was described as the no man's land because of the overlapping with CD and Irritable Bowel Syndrome (IBS). Patients without villous atrophy and tissue transglutaminase (tTG) serological responses that characterize CD, but with a symptomatic response to a GFD, were diagnosed as “IBS gluten sensitive,” particularly in the presence of genetic markers for CD.[21]

Some authors consider the terminology “Non Celiac Wheat Sensitivity” (NCWS) more appropriate than the current one,[22,23] because it can more accurately describe patients with different clinical presentations and different pathogenesis.[24] This terminology might be more appropriate if the non-gluten proteins of wheat grain are proved to play a role in the pathogenesis of the disease,[25] as it is possible that the symptoms improvement aren’t related to gluten itself. Other components in wheat may trigger the reported symptoms in these patients.[26] As a result of the broad symptom spectrum, NCGS might be regarded as a syndrome, rather than a gastrointestinal disease.[4]

The first time NCGS is described is in a case report published in 1978 in the Lancet, of a patient with diarrhea and intermittent abdominal pain, without abnormalities on biopsy and with improvement on a GFD,[27] and in 1980, describing very clearly patients with gluten-sensitive diarrhea without evidence of CD.[28]

1.3 Prevalence

Because there aren’t validated biomarkers for the diagnosis of NCGS, the diagnostic protocol remains difficult and not suitable for large epidemiological studies intended to establish the prevalence of this condition.[20] For this reason, it has been estimated by only a few studies, in Self-Reported Non Celiac Gluten Sensitive (SR-NCGS) patients.[29,30]

The prevalence of self-reported or suspected NCGS ranges from 0,97% to 14%.[29,31] In Italy, there are 3,19% patients suspected of having NCGS.[32] In the Netherlands, Argentina and Columbia, there are respectively 6,2%, 5,78% and 4,5% patients who report gluten sensitivity or gluten related symptoms.[33–35] In Australia, the prevalence of SR-NCGS in 2015 and 2018 was approximately 14%, and almost one-quarter of respondents were avoiding wheat or gluten.[31] This was similar to the UK prevalence, as

there were 13% SR-NCGS individuals.[30] There is also a prevalence of 5% Inflammatory Bowel Disease (IBD) patients that experience symptoms improvement with a GFD.[36]

The prevalence of individuals without CD adhering to a GFD is 0,548% in the USA,[37] and 3,7% and 5,9% adults, respectively, avoid gluten in Mexico and Colombia.[29,33]

Gluten challenge leads to a recurrence of symptoms in only a third of patients fulfilling the current diagnostic criteria for NCGS. Thus, NCGS is likely to be the correct diagnosis in only a minority of individuals who follow a GFD without CD. There's a clear need for a more strict way to correctly classify a patient as having NCGS, and one of the proposed criteria for this diagnosis was made by the Salerno experts.[38,39]

NCGS has a strong correlation with female gender,[30,34,37,40–42] with a F/M ratio of approximately 5:1,[40] and adult age.[32] Regarding family history, 12,8% to 18% of patients with suspected NCGS have a first or second degree relative affected by CD.[32,43]

Recent evidence reveals a presence of NCGS in 6,5% patients with Refractory functional dyspepsia. A reasonable approach could be a gluten challenge, or the evaluation of symptomatic responsiveness to GFD, in these patients.[44]

1.4 Diagnosis

The diagnosis of NCGS is established by exclusion criteria, based on a negative serology and biopsy for CD, and negative immuno-allergy tests to wheat for WA, besides symptoms improvement or disappearance after gluten withdrawal, and their recurrence with gluten reintroduction.[3]

In 2015, the Salerno experts made guidelines to help the clinician to reach a more solid diagnosis of NCGS, which included assessing the clinical response to a GFD and measuring the effect of reintroducing gluten, in a gluten controlled challenge after a period of treatment with the GFD, using a modified version of the Gastrointestinal Symptom Rating Scale (GSRS) to assess the response to a GFD and gluten containing diet. A symptomatic response is considered a decrease of at least 30% of the baseline score, and responders are defined as patients who fulfill the response criteria for at least 50% of the observation time. The gluten challenge should be a Double-Blind Placebo-Controlled Challenge, and the vehicle a muesli bar, muffin or bread, containing 8 g of gluten, with at least 0,3g of α-amylase/trypsin inhibitors (ATIs), and at the same time, FODMAP free. Summing up, in the absence of sensitive and specific biomarkers, a close and standardized monitoring of the patient during elimination and re-introduction of gluten is the most specific diagnostic approach and could be used as the diagnostic hallmark of NCGS.[20]

There isn't yet a specific biomarker identified for NCGS diagnostic.[45,46] However, in studies with suspected NCGS patients, the serological presence of immunoglobulin G (IgG) anti-gliadin antibodies (AGA) has been described in more than half of the

investigated patients, without the presence of anti-endomysium autoantibodies (EMA), tissue transglutaminase antibody, and Deamidated Gliadin Peptide anti-gliadin antibodies,[46,2,4] with a few exceptions.[46,32,24]

In gluten sensitive patients, immunoglobulin G anti-gliadin antibodies (IgG-AGA) are the most frequent antibody found, ranging from 10% to 68%.[24,32,46–51]

The prevalence of IgA anti-gliadin antibodies (IgA-AGA) is lower, ranging from 6% to 53%, lower than in CD patients.[24,32,46,48,51]

There are divergent opinions on this matter, as later IgA anti-gliadin antibodies (IgA-AGA) don't seem to be an adequately strong marker for its low statistical significance, and for its lacking diagnostic accuracy, reaching the undeniable conclusion that there isn't a serological marker useful to identify gluten sensitive patients, although it can partly help the NCGS diagnosis,[52] until a reliable biomarker is found.[53]

Elevated levels of immunoglobulin M (IgM) antibodies to flagellin are also found in NCGS patients.[54] IgM anti-gliadin antibodies are usually undetectable in healthy individuals, thus could be further investigated as a potential NCGS biomarker.[55]

A low clusters of differentiation 4 count could also be used as a biomarker, with a sensitivity of 100% and specificity of 90% against CD. A combination of clusters of differentiation 4 with the characterization of intraepithelial lymphocytes (IELs) could support a clinical diagnosis of NCGS.[56] The elevation in serum levels of interleukine-8 (IL-8) in CD subjects has potential for differentiating NCGS from CD, and the evaluation of interleukine-1 is also proposed as a distinguishing marker between NCGS patients and controls.[57]

There are some divergences about the presence of HLA-DQ2 in NCGS patients. When compared to the general population, the majority of the evidence points to NCGS patients having a higher presence of HLADQ2 and/or HLA-DQ8, but unlike CD, not all have this haplotype.[6]
The prevalence of gluten sensitive patients who carry HLADQ2 and/or HLA-DQ8 is around 50%,[18,36,39,40,42,47] in some cases higher (62%),[51] a percentage higher than that in the general population.[3] But in other cases is lower, similar to the general population, ranging from 26% to 41%.[54,56,58]

There are more gluten sensitive patients with the HLA-DQ2 allele than the HLA-DQ8,[50,51] and there is an association between Antinuclear Antibodies (ANA) positivity and HLA status.[59]

In NCGS patients, initiation of a GFD without adequate exclusion of CD is common,[60] yet, problematic, because of the immunoglobulin G anti-gliadin antibodies disappearance, that can also be seen as a sign of strict compliance to the GFD and an expression of a good clinical response to gluten withdrawal.[61] Only a third of the patients following a GFD without CD have indeed a NCGS diagnosis, after a gluten challenge.[38] This means that the NCGS diagnosis is only correct in a minority of

patients excluding gluten from their diet, and with gluten-related symptoms, showing a low predictivity of self-reported perceptions.[38,40]

1.4.1 Overlap with Irritable bowel syndrome

A significant number of NCGS patients fulfill the Rome III IBS criteria with a strong overlap between NCGS and IBS symptoms,[34] showing that gluten sensitivity is strongly associated with IBS.[35] About 25–30% of IBS patients, especially those with diarrhea-type IBS carrying the HLA haplo-types DQ2/DQ8, could be in fact NCGS patients.[45] Both IBS and NCGS may be common in the general population and can coexist independently.[62]

The most frequently associated disorder in patients with suspected NCGS is IBS, detected in 47% of the cases.[32] The high number of patients reporting symptoms with relation to FODMAPs suggests that they could be in fact responsible for part of the symptoms. SR-NCGS individuals frequently report symptoms upon consumption of products high in FODMAPs,[34] but in a lot of cases SR-NCGS is more likely IBS.[30,40] Approximately 80% of the SR-NCGS individuals can't be diagnosed as having NCGS after a double-blind, placebo-controlled, cross-over gluten challenge, as gluten is not the cause of their symptoms. This majority of individuals have, in fact, other causes for the symptoms, in particular IBS, as only 20% can correctly identify the two periods receiving muffins, thus leading to the NCGS diagnostic.[63,64]

NCGS is a well-defined clinical entity differing from IBS, as a limited role of the adaptive immune system has also been proposed in NCGS patients in response to wheat challenge, when compared with IBS patients.[65] Higher proportions of patients with NCGS develop autoimmune disorders, are positive for Antinuclear Antibodies (ANA), and show DQ2/DQ8 haplotypes compared to patients with IBS.[59,66] NCGS patients have also higher frequencies of weight loss, anemia, coexistent atopic diseases and family history of CD than IBS patients, as well as a significantly lower bone mineral density, more osteopenia and osteoporosis.[58,66]

Some studies argue that FODMAPs are the symptom inducers of NCGS. The results of a SR-NCGS patients study weakened the role of gluten as a symptom inducer, as most patients found no effect of gluten on a group level, indicating fructans are more likely to induce symptoms in those reporting sensitivity to wheat, rye, and barley, although some patients had their highest symptom score after gluten challenge.[67] But the dose of gluten used was 5,7g, far less than the consumed daily in the general population,[68] and a solid diagnostic of NCGS couldn't be established, like the one preconized by Salerno's criteria,[20] remaining only SR-NCGS individuals. It's been proposed that symptoms may depend on combined exposure to gluten and fructans with synergistic actions.[67] Other SR-NCGS individuals show no evidence of the specific effects of gluten, when placed on a low FODMAP diet, suggesting a confounding with FODMAP restriction, assuming

that gluten may not be a specific trigger of functional gut symptoms once dietary FODMAPs are reduced.[69]

Others downplay the role of FODMAPs in NCGS, raising the question whether FODMAPs exert any role in the clinical profile, as they provide the same symptoms improvement as the traditional diet advice in IBS patients.[70] FODMAPs cannot be entirely responsible for the symptoms experienced by NCGS patients, since these patients experience a resolution of symptoms while on a GFD, despite continuing to ingest FODMAPs from other sources like legumes, a richer source of FODMPs than wheat.[71] Also, the fructan content of gluten-free products depends on the product, and can't be assumed that gluten-free products are always lower in FODMAPs, because for e.g., gluten-free muesli has 0.96g fructan, more than the actual muesli with gluten,[72] and overall gluten-free bread contains similar quantities of fructan (1.00 g/100 g) as wheat bread.[73] FODMAPs do not trigger the extra-intestinal manifestations commonly observed in NCGS, likely elicited by wheat proteins (i.e., gluten, ATIs and possibly Wheat germ agglutinin),[46,74] as the well-described extra-intestinal symptoms in gluten-related disorders are usually ignored by IBS patients, implying that using Salerno criteria would help to differentiate between NCGS and IBS.[75] It's not that IBS patients don't have extra-intestinal symptoms,[76] but they do differ from NCGS in the fact that these symptoms are prominent and patients respond to dietary modification in NCGS, as in IBS, the symptom severity plays a minor role in determining health appraisals.[77] It has been suggested that NCGS may rather be characterized by a worsening of pre-existent chronic intestinal inflammation or extraintestinal inflammatory disease, difficult to explain based on the FODMAP hypothesis.[78]

Comparing both diets, an improvement in clinical symptoms is seen with a low FODMAP diet, but there's further improvement with a GFD regarding stool consistency, psychological parameters, a further decrease on IEL numbers and goblet cells on a GFD, increase in *Bacterioidetes* and a drop in *Firmicutes,* compared to the low FODMAP diet.[79]

Gluten-sensitive individuals, with IBS symptoms and immunoglobulin G antibodies to gluten were considered "orphans, living in no man's land, acknowledged neither by functional disease specialists nor by celiac disease specialists".[21] Some IBS patients without CD have reached satisfactory levels of symptom control with a GFD, showing gluten as a trigger of gut symptoms and tiredness.[19] They could be in fact gluten-sensitive, and their symptoms could be adequately controlled with a GFD only, including extra-gastrointestinal symptoms like anxiety, depression and fatigue.[80,81] There's a need to identify gluten sensitivity in this group of patients,[62,75] and a GFD appears to be a potential dietary intervention for this subgroup of IBS patients, denominated gluten sensitive IBS, not impairing quality of life as much as the Low-FODMAP diet.[74] Some diarrhea-predominant and mixed type IBS patients are responders to a GFD, thus classify as having wheat sensitivity. Long-term GFD seems necessary to identify all responders, justifying the recommendation of a GFD in non-constipated IBS patients as a therapeutic option.[82] In diarrhea-predominant IBS patients,

gastrointestinal symptom score decreased after a GFD. Decreased concentrations of immunoglobulin G after GFD were only seen in HLA-DQ2–positive diarrhea predominant-IBS patients, suggesting that serum immunoglobulin G antibodies against gliadin or tissue-transglutaminase in combination with HLA-DQ2 expression are useful markers to identify a subgroup of patients with diarrhea-predominant IBS who are likely responders to a GFD.[83] Zonulin serum levels could also be considered as a biomarker, combined with clinical data that differentiates NCGS from diarrhea-predominant IBS, as 6 months of wheat avoidance significantly reduced zonulin levels only in HLA-DQ2/8-positive patients with NCGS.[84]

Recent evidence suggests that the overlap of symptoms between these two pathologies has led to an overestimation of self-reported NCGS. Both clinical conditions can coexist, as almost half of the IBS patients were diagnosed with NCGS according to Salerno Criteria, and 19,2% according to the Di Sabatino criteria. FODMAP intolerance could hide the response to a challenge test with gluten for the identification of NCGS in IBS patients, and a low FODMAP-GFD followed by gluten/placebo challenge could better identify patients with NCGS.[85]

In conclusion, NCGS is likely a multi-factor-onset disorder, and rather than triggers, FODMAPs should be considered as additional elements of disturbance that could exacerbate symptoms in some patients.[86] Since there is an overlap between NCGS and other forms of wheat-exclusion responsive conditions, periodical patient reassessment is recommended.[87]

1.4.2 Autoimmune diseases

Gluten sensitivity was previously seen as a condition not accompanied by autoimmune comorbidities.[3,47] NCGS is now starting to be associated with autoimmune disorders, but to a lesser degree, compared to CD.[22]

There is evidence of a tendency towards autoimmunity in NCGS patients, characterized by the association of autoimmune diseases with the presence of serum Antinuclear Antibodies (ANA) positivity and HLA DQ2/DQ8 haplotypes. It seems that there are nearly a third of NCGS patients with autoimmune diseases,[40,88] slightly higher than in CD patients, contradicting the previous mentioned information, and significantly higher than in IBS patients, in particular autoimmune thyroiditis.[89] The prevalence of autoimmune thyroiditis is also higher among NCGS patients than in patients without a gluten-related disorder, being the most common autoimmune disease in these patients.[49,90] There's some evidence of type 1 diabetes, sarcoidisis and psoriasis, in a minor percentage of patients.[40]

In SR-NCGS patients, autoimmune diseases are present in 9,7%[30] to 14% of patients, also mainly represented by autoimmune thyroiditis.[32]

This association started when NCGS wasn't a defined entity. These patients couldn't be defined as having CD, due to the absence of significant mucosal abnormalities and of typical serology, but presented some markers of CD like an infiltrative pattern characterized by increased density of clusters of differentiation 3+ intra-epithelial cells in 50%, that could be associated with NCGS. In some of these patients, a presence Hashimoto's Thyroiditis,[91] atopic symptoms (asthma, allergic rhinitis, atopic dermatitis), vitiligo, alopecia areata and ulcerative colitis can be observed.[92]

1.4.3 Neurologic diseases

Some neurological symptoms improve or disappear upon gluten withdrawal, and recur when gluten-containing foods are reintroduced in the patient's diet.[4]

Approximately half of these patients could have neurological problems.[57] NCGS patients most common neurological manifestation is peripheral neuropathy (54%), followed by cerebellar ataxia (46%) and encephalopathy (10%). There isn't a clear distinguishing neurological feature between patients with CD and NCGS, as NCGS can present neurological dysfunction similar to CD, which is supported by the similar prevalence of Transglutaminase 6 antibodies in both.[51] Comorbidities associated with NCGS also include neuropsychiatric disorders, such as schizophrenia and autism,[93] and it could be argued that small fiber neuropathy is the first and only manifestation of CD and gluten sensitive patients.[94]

The gluten-mediated immune response is frequently associated with neurological and psychiatric manifestations in CD and gluten sensitivity.[95] As gluten ataxia is the most common cause of sporadic idiopathic ataxia, antigliadin antibody testing is essential to rule out other ataxias, before initiating a GFD. Diarrhea-predominant IBS-NCGS may trigger pathophysiological mechanisms that may involve alterations in the brain-gut axis, and these patients could have, for example, myopathy, arthritis, hypotonia, learning disorders, depression, migraine, and headache.[96]

But there is contradictory evidence, as it is also thought that non-celiac patients with normal duodenal mucosa, despite the presence of antigliadin, HLA genotype, and no other explanation for the cerebellar dysfunction, can be classified as having a form of latent CD, leaving ataxia to be the only clinical manifestation of gluten sensitive patients.[97] Other findings suggest that gluten sensitivity is common in patients with neurological disease of unknown cause, mostly ataxia and peripheral neuropathy, and may have etiological significance.[98]

It has been hypothesized that the reason why patients might feel better on a GFD is that gluten has a detrimental effect on their mental state, as a short-term exposure to gluten appears to induce feelings of depression.[99]

Some case reports studying this relationship have demonstrated the correlation of psychotic symptoms with gluten ingestion, and a diagnosis of NGCS followed by a GFD, that dramatically improved gastro-intestinal and psychiatric symptoms.[100,101]

1.5 Clinical presentation

NCGS clinical symptoms begin after the ingestion of gluten-containing grains. Symptoms improve or disappear with withdrawal of these grains from the diet, and reappear after gluten challenge, usually within hours or days.[17] NCGS symptoms may resemble those associated with CD, but with a bigger prevalence of extraintestinal symptoms.[3]

Some gastrointestinal symptoms mentioned are bloating, abdominal pain/discomfort, alternating bowel function, abdominal heaviness,[102] diarrhea, constipation,[50] epigastric pain,[40] flatulence,[103] and wind,[104] with the most prevalent symptoms being abdominal pain and bloating.[26,75,104,105]

Extra-intestinal reported symptoms are chronic fatigue,[50,75,104] headache, anxiety, 'confused/foggy mind', itching, arthralgia,[50] malaise, depression, joint/muscle pain resembling fibromyalgia, weight loss, anemia,[40,58] dermatitis, rash,[40] and history of food allergy in infancy, superior to both CD and IBS patients.[24,58] Other symptoms include bone disease and neurological problems,[57] contact dermatitis and nickel allergy, with high frequency of cutaneous manifestations after wheat ingestion,[106] asthenia of unknown etiology, oral aphthae,[107] multiple food sensitivity, osteopenia and osteoporosis, and elevated frequency of bone mass loss.[58]

NCGS patients have also lower overall well-being and quality of life,[103,108] lower physical health and health perception, more pain, and reduced social function, compared to healthy controls, reporting similar or even more complaints than CD patients. This loss of well-being can't be explained from the examination of personality and somatization.[109] There's a hypothesis that the reason why SR-NCGS patients might feel better on a GFD, is that gluten leads to a detrimental effect on their mental state and the cessation of gluten improves their well-being, rather than the gastrointestinal symptoms per se, as mentioned.[99]

An association with a wide range of rheumatic manifestations, including fibromyalgia,[110] spondyloarthritis and systemic autoimmune diseases can also be hypothesized, and gluten sensitivity may play an etiological and pathogenic role that acts as a trigger in some patients with systemic autoimmune diseases.[107] Recent evidence in patients with chronic lowback pain and spondyloarthritis shows that an improvement can be observed in approximately 60% of patients, and that approximately 50% are classifiable as NCGS, as they report a worsening of symptoms after gluten reintroduction. This supports the hypothesis that NCGS is associated with low back pain

with spondyloarthitis features, and that a GFD could be useful for treating these patients.[111]

In SR-NCGS patients, without the classification of NCGS, the most frequent symptoms are bloating and abdominal pain, diarrhea with 3 to 10 evacuations per day, alternating bowel habits and constipation. After bloating and abdominal pain, epigastric pain is the most frequent symptom, followed by nausea, aerophagia, gastroesophageal reflux disease, and aphthous stomatitis. The most frequent extraintestinal manifestations are tiredness and lack of well-being, headache, anxiety, 'foggy mind', arm/leg numbness, joint/muscle pain resembling fibromyalgia, weight loss, anemia, depression, dermatitis and skin rash. Less than 10% of these SR-NCGS patients show a clinical picture of allergic manifestations such as asthma or rhinitis, and symptoms occur mainly within 6 to 24 hours after gluten ingestion.[32,40]

In NCGS patients, symptoms induced by gluten appear in the first 48 hours or in the first week,[19,50] resolving within a few days after the implementation of a GFD,[47] and in other cases, within a month.[50] As no long-term complications in NCGS have been reported,[93] the reintroduction of gluten after 1–2 years on a GFD could be recommended. NCGS patients should not fear gluten contamination due to traces of gluten, but the level of tolerance varies, and there are patients who do not tolerate small amounts of gluten.[112] Dissimilar responses from patients with NCGS can be observed after the re-introduction of low, medium or high doses of gluten. The group of patients doing a low gluten dosage was more affected, in terms of quality of life and general well-being, than the other groups ingesting higher doses of dietary gluten, indicating that these latter groups can ingest some gluten without adverse health effects. This suggests that a greater gluten intake by patients with NCGS would not necessarily affect the quality of life of every patient, reinforcing the idea of inter-individual variability against gluten.[26]

1.6 Pathology

1.6.1 Permeability

It is currently thought that, when exposed to gluten, NCGS patients experience increased gut permeability, even if not as profoundly as CD patients,[25,113] although it still remains uncertain.

There is less recent evidence suggesting gluten sensitivity is not associated with increased intestinal permeability, as it is in fact reduced when tested with a lactulose/mannitol double sugar probe, and is significantly lower than in CD or dyspeptic control patients, showing that a normal to mild histology in GS is paralleled by a conserved barrier function.[47] This evidence was later criticized for the indirect methods used and the overlap of intestinal disorders in NCGS and IBS patients.[114]

There's also evidence of a higher intestinal barrier permeability in NCGS patients,[54] compared to patients with CD in remission and healthy individuals.[115] An elevated expression of fatty acid-binding protein 2 is found in NCGS patients, correlating with

the systemic immune responses to bacterial products, suggesting compromised intestinal epithelial barrier integrity and increased microbial translocation.[54] Also, gliadin exposure induces an increased intestinal permeability in all individuals, with a greater increase in celiac patients with active disease and gluten sensitive patients.[115] During the acute phase of CD, there's a significantly lower expression of both occludin and zonula occludens-1 genes compared to non-CD intestinal samples, but in remission, they show similar levels, suggesting that gliadin activates zonulin signaling irrespective of the genetic expression of autoimmunity, but with an increased amplitude and duration of induced zonulin release by gluten in CD intestinal samples, even when treated with a GFD.[116]

In diarrhea-predominant–IBS patients receiving a gluten-containing diet, there is an increased small-bowel permeability, demonstrated by increased mannitol excretion and increased lactulose to mannitol ratio, with a greater effect in the HLA-DQ2 or HLA-DQ8 positive patients.[80] An increased permeability in these patients is clinically relevant as serum zonulin levels correlate with diarrhea severity.[117] Zonulin is a novel human protein analogue to the Vibrio cholerae derived Zonula occludens toxin, which induces tight junction disassembly and a subsequent increase in intestinal permeability, which expression is raised in intestinal tissues during the acute phase of CD. This dysregulation might contribute to disrupted intestinal barrier functions, allowing the passage of environmental antigens involved in the pathogenesis of CD and related autoimmune disorders.[118] Zonulin serum levels are increased in patients with confirmed NCGS, as well as SR-NCGS, compared to controls and patients with diarrhea-predominant IBS, and Zonulin levels decrease with the elimination of wheat from the diet of individuals with a genetic predisposition to CD. This suggests that zonulin could be considered a useful biomarker for NCGS, with high accuracy, differentiating NCGS from healthy individuals.[84] Zonulin is indeed a mediator of gluten-induced changes, possibly paving the way for treatment alternatives to a GFD.[116]
In mice that are sensitized with gluten to develop an immune response to gliadin, gluten sensitization and long-term gluten challenge alters intestinal permeability. An environmental alteration of the intestinal barrier plays a critical role in determining host immune responses to gluten and intestinal microbiota antigens.[119]

A recent proposal is that the decrease of butyrate-producing *Firmicutes* or *Bifidobacteria* are at the basis of increased intestinal permeability of NCGS, via insufficient butyrate levels, and could result in a greater translocation of lipopolysaccharide (LPS) and inflammation, leading to a higher intestinal permeability.[86]

1.6.2 Innate and adaptive immunity

There's some evidence that NCGS' inflammatory response is triggered by an innate immune response.[22,62,93,120] The role of adaptive immunity is not as clear, as it's mostly rejected[4,121,122] or poorly supported.[54,55,65] Production of the anti-inflammatory cytokine

interleukine-10 is significantly lower in NCGS patients than in non-celiac controls, which could indicate an increased intestinal permeability and a less competent innate immune response.[115]

There's also contrary evidence, suggesting it is still impossible to establish whether immune responses are involved in the pathogenesis of NCGS, as there isn't any difference between the Toll-like receptors 2 (TLR2+) cell count in these patients and controls.[50] Patients with SR-NCGS show no abnormalities of the mucosal immune response, as the percentage of intraepithelial lymphocytes, production of pro-inflammatory cytokines and T helper cells do not significantly differ from treated CD patients and controls.[123]

Currently, most evidence points to NCGS showing signs of an activated innate immune response but without the enteropathy, typical of CD.[124] This is demonstrated by a significant infiltration of Innate lymphoid cell 1 in the rectal mucosa of NCGS patients upon wheat challenge, with a spontaneous interferon-γ production by these cells, supporting a role for a lymphoid cell subset in the pathogenesis of NCGS.[125] A 14-day GFD seems to significantly reduce numbers of intraepithelial lymphocytes in NCGS patients, underlining the involvement of the innate immune system.[79]

Although there seems to be more evidence supporting innate rather than adaptive immunity, differences concerning the immune response can be observed among patients with NCGS that may justify the division in subgroups that react differently, each characterized by a different pathogenesis and clinical course.[25] Increased small intestine expression of TLR2 and TLR1, but not TLR4, in gluten sensitive patients, suggests a prevalent role of the innate immune system, without markers of adaptive immunity. A reduced expression of regulatory T-cell markers in gluten sensitive patients, compared to controls, might be interpreted in the context of a reduced level of involvement of adaptive immunity, relative to CD, also shown by the approximately 50% of patients with gluten sensitivity that carry HLA-DQ2 and/or HLA-DQ8, slightly higher than that in the general population. A reduced expression of interferon-γ in gluten sensitivity relative to CD can also support the notion of a lower-level involvement of the adaptive immune system.[47] There's also evidence of an increased density of clusters of differentiation 3+ (CD3+) intraepithelial lymphocytes in the mucosa of NCGS patients following a GFD, and a significant increase in interferon-γ mRNA signal after gluten challenge, suggesting that the interferon-γ pathway is further activated by gluten challenge. A translocation of microbial products from the gastrointestinal tract has been suggested in these patients, contributing to the innate and adaptive immune activation.[126] Further studies are needed to determine whether adaptive immunity may contribute to NCGS pathogenesis.[127]

There's evidence that supports an alternative pathogenetic pathway, in which gliadin triggers a direct action through Toll-like receptors, without the involvement of the classical epitope presentation mediated by antigen-presenting cells and T helper lymphocytes. Clusters of differentiation 117 can be also expressed with a highest density for patients with NCGS, compared to controls and CD patients, supporting a hypothesis that mast cells could be both the pathogenetic and histopathological keystone

of NCGS.[56] There have been recent observations on neuro-immune (mast cells) interactions in the duodenal submucosa of NCGS patients, showing that the number of mast cells infiltrating the submucosa in these patients is similar to controls, being slightly increased compared to CD and significantly decreased compared to patients with functional dyspepsia. Results show different subsets in these patients, corroborating the NCGS' heterogeneity, with either increased infiltration of mast cells, or infiltration of eosinophils. Mast cells, the local innate immunity activation may play a role in the mechanisms leading to gastrointestinal symptoms in these patients.[128]

Recent evidence suggests that NCGS can be genetically defined, and gene expression profiling could be a suitable tool to support this diagnosis. The functional role of the dysregulated genes suggests that NCGS may especially result from a pathological innate immune response, and, besides immunity, ingenuity pathway analysis (IPA) shows that the hedgehog signaling pathway might have a role in NCGS.[129]

1.7 Possible causes

There is a consensus that NCGS symptoms are influenced by a lot of factors, like gluten-derived peptides, ATIs and Wheat germ agglutinin (WGA), which can trigger innate immune pathways, and also FODMAPs. The interplay between this various components of wheat may elicit both intestinal and extraintestinal symptoms in a patients sensitive to gluten, by modulation of intestinal permeability, microbiota composition, immune activation, which also affects the gut-brain axis activity, leading to symptom perception.[74]

1.7.1 α-Amylase/trypsin inhibitors

ATIs are nutritional proteins from wheat and related cereals.[130] They serve as adjuvants of an already ongoing immune reaction, which is not limited to the gut.[131]

There is preliminary evidence that ATIs can trigger innate immunity in both CD and NCGS patients,[3,25] and a possible dietary approach could be the use of ancient wheats, such as kamut, Einkorn or Triticum monococcum, as they have reduced amounts of gluten immune toxic peptides and low concentrations of ATIs.[25,78]

The combination of ATIs with gluten, in mice with a permissive celiac genetic background, results in an exacerbation of gluten immunopathology.[132] The pro-inflammatory effect of ATIs is confirmed in mice, with the activation of innate immune cells in pre-existing low-level small intestinal and colonic inflammation. ATIs trigger significant transcription of pro-inflammatory cytokines within 30 minutes after incubation. Their bioactivity varies comparing different levels of processing, with a modest increase in all immune cell subsets within the group of mice ingesting gluten

and high ATIs versus low ATIs, reducing pure dietary gluten responsibility on inflammation, as it had no relevant immune stimulatory activity itself.[78]

It has been hypothesized that NCGS triggers are ATIs plus microbial components, LPS in particular, and TLR4 activation by ATIs is similar to the activation induced by LPS, the strongest TLR4 agonist. NCGS could be considered an ATIs/low butyrate-producing *Firmicutes*/low *Bifidobacteria*-dysbiosis-induced disorder, more appropriately defined as "dysbiosis-induced ATIs sensitivity".[86] Addition of gliadin, the 33-mer peptide, or ATI alone to biopsies from patients with CD in remission, induces an increase in interleukine-8 mRNA expression, demonstrating that ATIs' structure is necessary to activate TLR4, and that they are indeed the main stimulants of wheat-induced TLR4-dependent innate immune reactions,[130] with a dose-dependent effect,[120] as the addition of ATI to the 33-mer further elevates interleukine-8 expression.[130]

There's probably an increased sensitivity towards ATIs in patients with CD or gluten sensitivity compared with healthy controls.[130] A significant reduction of daily ATI intake by a >90% avoidance of gluten could be enough to decrease ATI's costimulatory effect. Perhaps NCGS should be redefined as a syndrome with mainly extraintestinal symptoms and/or exacerbation of pre-existing disease upon consumption of gluten or ATI containing foods, allowing patients to adapt a liberal GFD.[120]

In mice, ATI supplementation with or without a large surplus of gluten significantly affects major taxa, with the suppression of *Lactobacillus* and the *Firmicutes/Bacteroidetes* ratio, suggesting a unique relationship between dietary ATIs and microbiota profile. Independently of genetic status and despite the absence of mucosal damage, ATIs can modify intestinal barrier parameters and play a role in wheat-related disorders in general, as they induce barrier dysfunction and immune activation.[132]

1.7.2 Fermentable oligo-, di-, and mono-saccharides and polyols

The presence of patients responsive to a GFD but negative to a gluten challenge is intriguing. A relevant part of the symptomatic response to GFD may be justified by a placebo effect, but some of those patients might be sensitive to other unspecified wheat components, additives or fermentable oligo-, di-, and mono-saccharides and polyols (FODMAPs).[108]

Past evidence suggested that NCGS might not be a discrete entity or that this entity might be confounded by FODMAP restriction, and that gluten might be not be a specific trigger of functional gut symptoms once dietary FODMAPs are reduced, because of the high nocebo response to gluten.[69] But the importance of FODMAPs is downplayed in eliciting IBS symptoms, as the low FODMAP diet seems equal to a traditional IBS dietary advice in terms of adherence and symptoms, raising the question whether they have a role in NCGS clinical manifestations.[70] Also, FODMAPs cannot be entirely responsible for the symptoms reported by NCGS patients, since they experience

a resolution of symptoms on a GFD, despite continued ingestion of FODMAPs from legumes and other sources, as mentioned.[133] When compared to the low FODMAP diet, a GFD further improves clinical symptoms regarding stool consistency and psychological parameters on these patients.[79]

FODMAPs cannot induce inflammatory responses in NCGS patients, but could contribute to "mechanically" worsen the symptoms.[86] In SR-NCGS, the high number of patients reporting symptoms resulting from FODMAPs suggests they are responsible for part of their symptoms. Abdominal discomfort relates to at least one high FODMAP-containing product, reported by 74% SR-NCGS patients, compared to 22% healthy individuals.[34]

1.7.3 Wheat germ agglutinin

Lectins are present in a variety of plants, especially in seeds, where they serve as defense mechanisms against other plants and fungi. There is a possibility that, together with gliadin, WGA increases intestinal permeability, resulting in an increased microbial translocation and dietary antigens interaction with cells of the immune system, leading to its activation.[134]

Previous work in mice demonstrates that WGA is an anti-nutritional factor when administered at high doses, damaging the proper functioning of the immune system. The nutritional evaluation of the effects of 7 g/kg of pure WGA shows that the lectin reduces the utilization of dietary proteins, induces wasteful growth of both the small intestine and the pancreas, causes thymus atrophy and depresses the growth of rats.[135] Also in rats, evidence suggests that cell surface-bound lectins potently inhibit plasma membrane repair, and the exocytosis of mucus that normally accompanies the repair response is toxic to wounded cells.[136]
At nanomolar concentrations, WGA can initiate cross-talk between immune and epithelial cells, involving the mobilization of pro-inflammatory cytokines. Lower WGA concentrations do not stimulate a significant increase of cytokine secretion, and evidence suggests that WGA could trigger the complete pathway of interleukine 1β (known to be synthesized as an inactive procytokine) biosynthesis and secretion, varying among different donors, but appeared to correlate with LPS' variation. As 1 ng/ml of LPS induces a significantly lower effect than WGA, the observed effects are not due to the contaminating endotoxin. This suggests that WGA can interact with immune cells at the gastrointestinal interface in vivo and tune their functions.[137]

1.7.4 Gliadin

The possibility of gliadin mediating a motility disorder of the gastrointestinal tract in NCGS patients is significant,[5] and the gliadin peptide 13-mer, in addition to the 33-mer peptide, can also be responsible for innate immunity.[45]

Comparing control peptides with 33-mer, the later demonstrates its stability by remaining largely intact for at least 15 hours, being highly resistant to digestion, as for the controls, they are nearly completely proteolyzed within 1 to 5 hours.[16]

Gliadin activates zonulin signaling, irrespective of the genetic expression of autoimmunity, leading to an altered barrier function in both active CD and NCGS patients and to increased intestinal permeability to macromolecules, but this threshold of intestinal permeability is higher in CD and in gluten sensitive patients than in the non-CD intestinal mucosa.[115,116]

There's also evidence contradicting gliadin effects, suggesting that mucosa from patients with NCGS does not express markers of inflammation, and their basophils are not activated by gliadin. Also, an early marker (PY-99) typical of the initiation of innate immunity, does not occur when gliadin is in contact with the intestinal mucosa of NCGS patients.[138]

1.8 Treatment

A GFD is the current NCGS treatment to improve patient symptoms,[17,19] dramatically improving their quality of life.[71] A GFD is based on the elimination of the alcohol-soluble protein fractions of gliadin in wheat, secalin in rye and hordein in barley, that are all toxic in CD and in gluten sensitivity.[127] It should be guided according to the symptomatology, and does not require strict adherence unlike CD, because no long-term complications have been described yet.[139] There is a rapid clinical and immunopathological resolution of symptoms after a month of GFD.[48] It seems that these patients maintain symptom remission while eating gluten in traces. The majority of patients can tolerate low gluten amounts (one biscuit daily) and show a relapse of symptoms within 2 days after the reintroduction of moderate amounts of gluten from modern wheat varieties (30–40 g of bread or pasta/day). Symptoms relapse after 2 weeks from the beginning of the diet containing a moderate amount of gluten, with symptoms appearing slowly and gradually with the use of ancient grain products, and to a lesser extent compared to the modern wheat. When most patients adopt a GFD, intestinal and extra-intestinal symptoms resolve after 24 hours.[140]

According to Codex Alimentarius, "gluten-free foods are dietary foods consisting of or made only from one or more ingredients which do not contain wheat (i.e. all *Triticum* species, such as durum wheat, spelt, and khorasan wheat, which is also marketed under different trademarks such as KAMUT), rye, barley, oats or their crossbred varieties, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer, and/or consisting of one or more ingredients from wheat (i.e. all *Triticum* species, such as durum wheat, spelt, and khorasan wheat, which is also marketed under different trademarks such as KAMUT), rye, barley, oats or their crossbred varieties, which have been specially processed to remove gluten, and the

gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer."[141]

Malt is toxic for CD patients, because it is a partial hydrolysate of barley prolamins. Thus, barley malt and all products based on malt (malt syrup, malt extract, malt flavorings) aren't allowed in a GFD. Millet, maize, sorghum and rice can be safely consumed.[142]

There's some evidence about the effectiveness of symptoms reduction by altered breads in NCGS patients, like the consumption of a low-gliadin E82 bread, which has great acceptability by these patients, and clinical symptoms are the same as those seen with gluten-free bread, possibly because of the long fermentation processes and baking reducing gluten content. The consumption of this low-gliadin bread could be a positive component of the GFD, since it induces positive changes in the gut microbiota composition, increasing the butyrate-producing bacteria and favoring the bacterial profile.[113] NCGS patients also experience lower gastrointestinal and extra-intestinal symptom scores with a Senatore Cappelli wheat variety flour, compared to standard commercial wheat.[143]

Buckwheat, teff, millet, and soy, reach up to 20% of activity of modern wheat, regarding ATIs. Technological food processing affects ATI bioactivity when it is completely extracted from cooked and baked products after pepsin and trypsin digestion, from a modest reduction (7%) in raw pasta to intermediate (30%–50%) reduction in bread, pizza, biscuits, and couscous, to a high reduction (70%) after boiling pasta with salt water, likely because of ATI extraction.[78] Ancient wheats, both for their reduced amount of gluten immune toxic peptides and low concentrations of ATIs, could be considered a new dietary approach for the management of NCGS patients.[25]

The monetary cost for patients following a GFD is much higher and it's a burden for people with a lower income to purchase these products without government support.[144] Gluten-free products are significantly more expensive than their wheat-based counterparts, although the cost declined from 2006 to 2016.[145]

Patients on a GFD should be closely supervised by dietitians and physicians to confirm adequate macronutrient, micronutrient, and dietary fiber intake, and monitor for signs and symptoms of deficiencies,[146] as wheat flour provides important nutritional components. Replacing these products represents a major task for the industry as well.[13] There are some marked differences between gluten-free and gluten-containing foods, and nutrient composition of gluten-free foods is not aligned with particular health benefits.[147] The preference of celiac patients for a high fat diet, high intake of sweets and soft drinks, and a low intake of vegetables, iron, calcium and folate raises concerns regarding the potential impact on chronic conditions such as type 2 diabetes and cardiovascular diseases,[148] as well as a higher risk of metabolic syndrome and hepatic steatosis.[149]

Comparing gluten-free products with their counterparts, it seems that there's great nutritional content variability between them, as gluten-free breads and pasta have almost

a third less protein and twice as much fat (mainly saturated), and gluten-free pasta and cereal bars or flour have more sodium and less fiber. This suggests that following a diet based on gluten-free products could lead to nutritional imbalances for celiac and non-celiac patients.[150]
Female patients adhering to a GFD have lower intakes of magnesium, iron, zinc, manganese, selenium and folate, and male patients have lower intakes of magnesium and selenium, emphasizing the importance of an experienced dietitian for advice on how to achieve a balanced diet or appropriate dietary supplementation, regarding low intakes of fiber and certain microminerals, and with attention to maintaining available carbohydrate intakes without resorting to sugary foods.[151]

Gluten-free foods would benefit from the inclusion of alternative grains with higher nutritional value, like teff or pseudocereals such as quinoa, amaranth and buckwheat, as rice flower is the most commonly used ingredient in the market of gluten-free foodstuff and has inferior nutritional value compared to wheat flour.[152]
There's more evidence supporting this idea, reinforcing pseudocereals as nutritious ingredients in gluten-free formulations because of their high quality protein and the presence of abundant quantities of fiber and minerals, such as calcium and iron.[152–155]
More than half of the patients consuming buckwheat products report significantly improved abdominal pain and bloating, compared with those who maintain their normal eating habits. This provides evidence for the positive effects of buckwheat in NCGS, showing that this alternative pseudocereal represents a possible dietary option for this patients.[102]

Besides pseudocereals, other alternatives to improve the nutritional value of gluten-free foods are legumes, and the use of sourdough, which seems to be a powerful tool to intercept various technological and nutritional aspects.[155,156]
There's evidence that a spaghetti made with chickpea, unripe plantain and maize flour shows higher protein and fat contents, but lower carbohydrates and total starch than spaghetti elaborated with semolina, suggesting a potential for the development of gluten-free spaghetti with reduced amount of glycemic carbohydrates.[157]

Other alternative to a GFD could be a two-step transamidation reaction of wheat flour with lysine ethyl ester, which largely produces fully transamidated water-soluble gliadins that lost their bio-immune activity without influencing main technological properties.[158] Also, a specific enzyme mixture, composed of peptidases and protease derived from microorganisms and papaya, could improve NCGS symptoms by digesting the 33-mer peptide generated from gliadin, and could be a useful dietary supplement to decrease NCGS symptoms.[159]

The use of a probiotic strain, Bifidobacterium longum ES1, as an adjuvant therapy to the GFD has led to a clinical response of greater magnitude than with a GFD alone, improving gastrointestinal and extra-gastrointestinal symptoms, and also improving results regarding the Bristol Stool Scale, with a slight loss of effectiveness in the following months.[160]

In mice, there's evidence suggesting that *lactobacilli* that more efficiently metabolize ATIs can prevent immune ATI-induced activation, ameliorating many aspects of gut dysfunction induced by wheat immunogenic proteins. Microbiome-modulating strategies based on the use of strains with specific ATI-degrading capacity could be developed and tested in clinical trials to support or replace dietary restrictions, in patients with wheat-sensitive disorders.[132]

Expert nutritionist assessment remains the gold standard for the standardized evaluation of GFD adherence in patients with CD, while clinical, demographic, and laboratory findings are imprecise and unsatisfactory indicators.[161,162]

Adherence to a GFD is frequently good. Most patients have excellent or good GFD adherence, measured by a standardized dietitian evaluation score.[163] Most patients have also a score of III or IV in a questionnaire developed to assess GFD compliance, confirming that most patients adhere to a GFD.[164,165] Interestingly, some of the patients who claim to follow a GFD 'very well' or 'well' have a score of II in the questionnaire, which is considered unsatisfactory.[164]

Most people who follow a GFD are doing it for reasons other than health-related benefits, and without health professional advice.[35,60] It is likely that the number of people worldwide following a GFD is higher than that of patients with true NCGS,[32] due to a low predictivity of self-reported perceptions.[40]

A previous review concluded that a GFD does not present side effects and is usually balanced and safe, and that supplementation therapy is not routinely needed, contradicting some previously mentioned studies.[45] Changes in cardiovascular risk factors with a GFD include beneficial effects (increases in HDL levels), detrimental effects (increases in total cholesterol or fasting glycaemia), and also neutral effects (small increases in BMI within the normal weight range). GFD's clinical relevance and overall effect on cardiovascular risk remains unclear.[166]

1.9 Microbiota in Non-Celiac Gluten Sensitivity

As mentioned, the human gut microbiome is an important environmental factor that affects its host metabolism. Various factors modulate microbiota, including diet composition, and the composition of the intestinal microbiome is known to play a critical role in shaping the immune system.[7] Dietary changes affect gut microbiota, leading to changes in bacterial metabolites, such as short-chain fatty acids production. The role of gut microbiota and strategies for modification of gut microbiota in food allergy require assessment.[167] With a fuller understanding of the host-microbe interaction in the gut, perhaps the pathogenic side of this relationship can be suppressed, whereas the beneficial effects of the gastrointestinal microbiota can be maintained and restored.[7]

Diet can induce rapid microbiota changes.[168,169,170] Switch between herbivorous and carnivorous functional profiles promotes changes in a single day after the diet reached the distal gut microbiota, and reverts to the original structure 2 days after the animal-based diet ends, demonstrating a rapid adaptation of human gut microbiome.[168] Microbiome composition can change detectably within 24 hours of initiating a high-fat/low-fiber or low-fat/high-fiber diet, but enterotype identity remains stable during ten days, thus only long-term diet correlates with enterotype clustering.[169] Diet-driven changes seem to appear rapidly, being detectable within 3–4 days, and are reversed equally rapidly. The colonic microbial community must typically be in a state of continuous change over time, although these changes can be expressed more by individual than by diet.[171] In mice, switching from a low-fat, plant polysaccharide-rich diet to a high-fat/high-sugar "Western" diet for 4 weeks, results in a shift in gut community structure that is evident after a single day, and stabilizes by day 7.[170] Although there's some evidence of a rather fast change in the microbiota population, it is unknown whether the changes that occur in the microbiota are acute (occurring very rapidly) or chronic (taking 4 weeks).[172]

Comparing the low FODMAP diet with the typical Australian diet in IBS and healthy patients, the low FODMAP diet lowers total bacteria by 47%, associated with lower absolute abundance of total bacteria, butyrate-producing bacteria, prebiotic bacteria, *A. muciniphila* and *R. gnavus*, lower relative abundances of *Clostridium* cluster XIVa, and a significantly higher abundance of *R. torques*.[173]

Comparing women and men, two different effects can be found. Bacterial taxa abundance changes differentially between genders, as consequence of the dietary intervention, and also regardless of the diet. This suggests that women and men could differentially benefit from the consumption of a specific diet, depending on their gender and disease, by diet-induced changes of gut microbiota.[174]

There are very few studies investigating microbiota in NCGS patients. There's recent evidence in the Mexican population that implementing a GFD for 4 weeks in NCGS, CD patients and healthy controls, has no significant difference in the abundance of any taxa between the two periods of time, despite the clear distinctive abundance and distribution of phyla in duodenum, likely due to the high inter-individual variability. Although most NCGS patients displayed little change after the GFD period, a higher abundance of duodenal *Pseudomonas* was clearly seen. LEfSe analysis of the taxa at the genus level showed that other *Proteobacteria* (e.g., *Stenophomonas* and *Novosphingobium*) were significantly more abundant on the GFD, while *Actinomycetaceae* was lower before the GFD, regarding the duodenal microbiota. At baseline, a higher abundance of *Actinobacillus*, *Finegoldia* and the phylum TM7 can be observed in the duodenal microbiota of NCGS patients, while *Sphingobacterium* (*Bacteroidetes*) is higher in healthy individuals. There's evidence of a high abundance of *Firmicutes* (~85%), a low abundance of *Bacteroidetes* (~1%), in all groups, and a significantly higher abundance of fecal *Ruminococcaceae* in the fecal microbiota of NCGS patients. The main finding is the increased abundance of *Pseudomonas* in

duodenal biopsies of NCGS patients, without certainty of any beneficial effect or not to the integrity of the duodenal mucosa.[175] This higher number of *P. aeruginosa* compared to healthy controls is also seen in IBS patients, suggesting that it may play a role in pathophysiology of IBS, combined with a lower number of *Bifidobacterium* species.[176] Similar to what is observed in NCGS patients, most IBS patients have an increased *Firmicutes:Bacteroidetes* ratio.[177,178]

Comparing the low FODMAP diet with a GFD, the later causes a significant increase in *Bacterioidetes* and a drop in *Firmicutes*, compared to the first. Before the intervention diets, NCGS patients have more *Firmicutes* and less *Bacteroidetes* than healthy controls, probably suggesting a microbiota disbalance in these patients. A 2 week GFD also results in a reduction of the families *Porphyromonadaceae* and *Acidaminococcaceae*, and increased *Eubacteriaceae* in NCGS patients, compared to controls. There's also a reduction of *Bifidobacteriaceae* with the GFD. Even with the elimination diet differences, the microbiota differences still persist, suggesting that these microorganisms per se may not be responsible for the clinical symptoms, which improve. There's evidence of a greater metabolomics variability in NCGS patients, suggesting that the microbiota in these patients may be more susceptible to nutrient changes, compared to healthy controls.[79]

The consumption of a low-gliadin wheat bread by NCGS patients for one week seems to have a positive impact on gut permeability. At the phylum level, only a lower abundance of the *Actinobacteria* can be observed with the low-gliadin wheat bread, compared to the gluten-free bread. At the family level, there is a significantly higher abundance of *Veillonellaceae* and *Ruminococcaceae* with the low-gliadin wheat bread, and a lower abundance of *Lachnospiraceae*, *Bacteroidaceae*, and *Coriobacteriaceae* families. At the genera level, a lower abundance of *Oscillospira*, *Dorea*, *Blautia*, *Bacteroides*, *Coprococcus*, and *Collinsella* can be observed, as well as a higher abundance of *Roseburia* and *Faecalibacterium*. At the species level, a lower abundance in *Collinsella aerofaciens* and a higher abundance in *Faecalibacterium prausnitzii* is observed. The increase in *Faecalibacterium* (anti-inflammatory genus) and decrease in *Bacteroides* (proinflammatory genus) suggests a less inflammatory phenotype, concluding that consumption of a low-gliadin bread could be a positive component of the GFD for NCGS patients, as it produces no adverse clinical symptoms and induces positive changes in the gut microbiota composition, increasing the butyrate-producing bacteria.[113] Indeed, a higher abundance of *F. prausnitzii* together with a lower abundance of Bacteroides is related to a low concentration of zonulin, and may play a role in intestinal epithelial integrity.[179]

Microbiota profiles of mice fed gluten-containing diets are more similar to each other compared to other diets, suggesting that gluten plays an important role in shaping microbiota composition. A substantial proportion of gluten-degrading bacteria, like strains belonging to *Enterococcus*, *Bifidobacterium*, *Clostridium*, *Bacillus*, and *Lactobacillus* genera, also have the capacity to efficiently degrade ATIs. This suggests an unique relationship between dietary ATIs and microbiota profiles, leading to the

possibility that *Lactobacillus* strains with high ATI-metabolizing capacity can ameliorate many adverse effects induced by wheat immunogenic proteins.[132]

Preliminary evidence in healthy adults after introducing a GFD for 1 month resulted in a reduction of bacterial populations generally regarded as beneficial for human health, such as *Bifidobacterium* and *Lactobacillus*, and an increase in opportunistic pathogens such as *Escherichia coli* and *Enterobacteriaceae* family in general, parallel with reductions in polysaccharide intake.[180] Similarly, a 8-week low-gluten diet intervention in healthy middle-aged adults induces changes in the intestinal microbiome and fermentation of complex carbohydrates, with a diminished abundance of *Bifidobacterium* species, *Dorea*, *Blautia wexlerae*, *Anaeostipes hadrus* and *Eubacterium hallii (*butyrate-producing bacteria), in comparison with the high-gluten diet, but with an increase of an unclassified species of *Clostridiales* and *Lachnospiraceae*. This change could be due to qualitative changes in dietary fiber upon reduction of gluten-rich food, rather than by the reduction of gluten intake.[181] Although the GFD has a significant effect on bacteria diversity, the inter-individual effect on the variation of the microbiome is stronger than the effect of the diet. In healthy subjects on a GFD, abundance of *Ruminococcus bromii*, *Roseburia faecis*, and mostly *Veillonellaceae* family, drop significantly in the gut, while families *Victivallaceae*, *Clostridiaceae*, *Coriobacteriaceae*, ML615J-28 order (*Tenericutes*), and genus *Slackia* increase. The lower abundance of *Veillonellaceae*, a pro-inflammatory bacterium, may lead to a reduction in gut inflammatory state, which may be linked with a beneficial effect of GFD for patients with gut disorders such as gluten-related disorders. Although with a lesser effect than what is seen in the shift from a meat-based diet to a vegetarian diet, gluten intake clearly influences the abundance of several species, in particular those involved in carbohydrate and starch metabolism.[182]

In vitro, a gluten-friendly bread (flour treated with microwaves) appears to exert a positive effect on the *Bifidobacteria* numbers of celiac donors, after 48 hours. In a complex ecosystem, such as the gut microbiota, this bread could induce a beneficial modulation in terms of bifidogenic effects, and on the growth of *lactobacilli.* This supports the utilization of a gluten-friendly bread to modulate the composition and metabolic profile of the intestinal microbiota of celiac and gluten sensitive individuals.[183] The same bread significantly increases *Bifidobacterial* numbers in the fecal microbiota of healthy and celiac donors, and *Clostridium cluster XIVa* in celiac samples, cluster which contains short-chain fatty acids producing species. This suggests a positive modulation of short-chain fatty acids by a gluten-friendly bread, with an increase of butyrate, acetate, and propionate.[184]

Bacteria such as *Lactobacillus mucosae*, *L. rhamnosus* and *Clostridium botulinum* and *sporogenes*, could have an important role in gluten metabolism, as they appear to have a higher activity with regard to the 33-mer peptide than other bacteria, like *Enterococcus faecalis* or *Bacillus licheniformis*, which exhibit activity but without complete hydrolysis of the peptide. These seem to be the same bacteria altered in CD patients, with an undefined effect in the gut, being a protecting or a pathogenic role.[185]

CD patients with persistent symptoms on a GFD seem to have an altered duodenal microbiota composition, such as lower abundance of *Bacteroidetes* and *Firmicutes,* and a higher abundance of *Proteobacteria,* compared with patients with no symptoms on a GFD. The difference in microbiota composition is also detected in the genus *Prevotella*. From this reduced richness of bacteria could be inferred a state of intestinal dysbiosis, associated with persistent symptoms in celiac patients with strict GFD and small bowel mucosal recovery.[186] More evidence points to a dysbiosis in the duodenal microbiome of active CD patients, showing an abundance of a *Neisseria flavescens* strain and other *Proteobacteria,* and less abundance of *Firmicutes* and *Actinobacteria*. *Neisseria flavescens* probably contributes to promoting inflammation in the small intestine of these patients. *Lactobacillus* and *Bacillus cereus,* which are involved in gluten metabolism, were not found in the active CD microbiome.[187]

Both active and non-active CD patients seem to have a lower number of total *Bifidobacterium* levels, compared to healthy individuals. *B.longum* levels are markedly lower in active CD patients, and to a lesser extent in non-active CD patients, than in healthy individuals, in both fecal and biopsy samples, and a GFD seems to partially restore duodenal *B. longum* levels.[188]

In another paper, untreated CD patients have more *B. bifidum*, *Bifidobacterium catenulatum,* and *Lactobacillus sakei,* similarly to healthy adults with a normal gluten diet. Treated CD patients have a reduction in the diversity of both *Lactobacillus* and *Bifidobacterium* species,[189] corroborating that the differences in fecal bacteria may be only partially restored by a GFD.[189,190]

Although studies in patients with CD clearly demonstrate an association between altered microbial composition and disease, as well as a state of dysbiosis,[191] there isn't a distinct celiac microbiota signature, making it difficult to determine the true pathogenic role of the gut microbiota in these patients. A modulation of the gut microbiota might improve ongoing symptoms, despite adherence to a GFD, however, the overall contribution of factors that disrupt homeostasis and disease development is poorly understood.[18] There's a hypothesis that microbiota alterations could also have a primary role by contributing to disease onset, instead of just aggravating CD pathogenesis.[191]

It is important to notice that the effect of a GFD on health and gut microbiota cannot be extrapolated from one population to others.[192]

Sequencing a well-chosen region of the 16S gene, 75–100 bases may suffice for drawing reliable conclusions from the data. The increased sample analysis capacity makes possible to explore in detail whether changes in rare or abundant species are primarily responsible for differences in microbial communities associated with health and disease.[193] Recently, there's evidence suggesting that 16S sequencing does not inform about the microbe-immune system interaction at the cell level.[175]

The human mycobiome, regarding fungi and their genome, is a relatively new advance in characterizing the residents of healthy individual's gastrointestinal tracts. A recent study reported an increased prevalence of *Candida albicans* and *Saccharomyces*

species, in CD patients with persistent symptoms. Their findings warrant larger scale studies to explore the significance of these organisms as both a cause of persistent symptoms in people adhering to a GFD, and also, the etiology of the disease.[194]

Chapter 2 – Development of a protocol for the implementation of the study: "Symptoms and microbiota in Non-celiac gluten sensitivity patients"

2.1 Objective

- Elaborate a protocol to evaluate diet's impact, regarding symptoms and microbiota change, after exclusion of gluten in a group of participants with NCGS.

2.2 Type of study

This study is a longitudinal dietetic intervention trial.

2.3 Materials and Methods

Within this aim, the following documents were elaborated: the Informed Consent; a symptom questionnaire for the selection of the participants; a muffin datasheet with recipe and needed ingredients; the study protocol in the format of a flowchart, including the exclusion criteria, delivered to the doctor and to be delivered to the participants; an explicative document for the GFD intervention, prepared to allow the participants to easily distinguish between foods with, without, or possibly having gluten, besides advices to apply on a daily basis.

2.3.1 Ethical considerations

The study protocol was submitted to and approved by the Ethics Committee from School of Health Technology of Lisbon (ESTeSL, Escola Superior de Tecnologia da Saúde de Lisboa). Informed consent will be requested, and oral & written information provided in advance. At any time, patients can decide to leave the study without any kind of penalty. All information on the outcome of the patient's evaluations will be available for the attending physician. Patients enrollment is completely voluntary, and won't entail costs, benefits, or any loss. Participants can withdraw authorization anytime, and their personal data will be destroyed. The confidentiality of the data will be preserved, and the purpose of the data will be explained to participants. All the information is available for patients, and results of the study can be accessed if they wish to. The participant will have time to decide whether he's willing to participate or not. The Informed Consent is available in Appendix I.

2.3.2 Recruitment of participants

Patients presenting symptoms included in NCGS diagnosis criteria will be recruited in the Clinical Institute of Allergology. Patients with CD, WA diagnosis, or taking antibiotic therapy will be excluded, after blood analyses.

2.3.2.1 Exclusion of Celiac Disease: for CD screening, laboratory screenings will be performed comprising IgA anti-tissue transglutaminase antibody, IgA and IgG anti-endomysial antibodies, total IgA and HLA DQ2 / DQ8 determinations.

2.3.2.2 Exclusion of Wheat Allergy: for WA screening, the following tests will be performed: gluten specific immunoglobulins E (IgE) (f79), wheat specific IgE, Triticum aestivum (sativum) (f4) and gliadin ω5 specific IgE.

2.4 Procedures

The study protocol will be presented and explained to participants. Patients who accept and sign the informed consent, will be asked to complete a symptom questionnaire (Appendix II).[34] This questionnaire records the past symptoms associated with NCGS and self-reported NCGS.

After the exclusion of CD and WA, if the patients are already doing a GFD, to confirm diagnosis, they will go through a single-blind experiment, reintroducing gluten for 1 week, with a week of washout, and another week of placebo, and the clinical evaluation performed using a self-administered instrument incorporating a modified version of the Gastrointestinal Symptom Rating Scale,[20] before and after the gluten challenge (Annex I). The nutritionist will phone call participants from 2 to 2 days in this part of the investigation. Gluten reintroduction is made through chocolate muffins, made in the university's laboratory. The muffins have the addition of 8 grams of gluten, or 8 grams of rice flower as the placebo, and should be identical to one another, and without FODMAPs, as Salerno experts recommend.[20] One muffin will be provided for each day of the first week, in the first meeting, and after the washout, in an arranged meeting, participants will receive the other 7 muffins.

The muffins were already made once, to test their acceptability, and the needed materials to make the muffins are at the university's laboratory. The ingredients are the following: lactose-free butter and milk, sugar, eggs, rice and gluten-free oat flour, gluten-free baking powder, and cocoa powder. The full recipe is in the muffin's datasheet (Appendix III).

Patients will then be nutritionally assessed, fulfill a food frequency questionnaire, and receive dietary advice from a nutritionist to implement a GFD for a period of 2 weeks, which will take place after the last week with the placebo muffins. During the period of GFD, nutritional monitoring will be done weekly for compliance, in order to clarify all doubts about the diet, thus promote its adherence. Patients should complete a 3-day food record, detailing nutritional intake.

At the end of each week, the modified version of the Gastrointestinal Symptom Rating Scale will be reapplied, and at the second and last meeting with the nutritionist, anthropometry will be assessed, and a new fecal crop done to evaluate the fecal

microbiota. Dietary adherence to the GFD will be assessed in all patients through a structured questionnaire, conducted by the nutritionist.[164]

The flowchart with the protocol delivered to the physician and to be delivered to participants is available in Appendix IV.

In Appendix V, there is an explicative document for the GFD intervention, prepared to allow the participants to easily distinguish between foods with, without, or possibly having gluten, besides advices to apply on a daily basis.

2.4.1 Microbiota Analysis

Fecal collection will be done in feces of one issue, and an aliquot of the size of a walnut transferred to a sterile container. Stool samples will be taken after the gluten challenge (day 7), and at the end of the intervention (day 35), after 2 weeks on a GFD, and stored and transported to the laboratory under refrigeration, being stable in these conditions for one week. This sample will be used to obtain the total genomic DNA samples for further PCR and sequencing of the 16S ribosomal RNA gene (16S rDNA), and test results will be delivered to patients and shown to the assistant doctor Pedro Da Mata.

Final Considerations

I really enjoyed writing this review. It was very interesting and fulfilling to study this area, regarding the NCGS. There's still a lot to uncover about this disorder, and I would like to continue my investigation in this area of great interest.

Although this disorder is still underestimated, any intervention in this area is of great interest for these patients. These patients feel groundless because of their recurrent symptoms allied to the lack of clear information regarding their disorder. With a fuller understanding of their microbiota, with and without the presence of gluten in the diet, perhaps the pathogenic side of this relationship can be suppressed, maintaining and restoring the beneficial effects of the microbiota. Studying gut microbiota is imperative, as it affects its host metabolism, playing a nutritional, physiological, immunological and pathological role.

There are very few studies investigating microbiota in NCGS, but those that exist suggest a microbiota disbalance in these patients. Efforts should be made in the future to increase research regarding the gut microbiota in these patients, and the changes that come from the inclusion and exclusion of gluten.

I hope to implement this protocol, that due to the pandemic situation of the unpredictable COVID-19, forced me to change the thesis' original objectives, and adapt them to what was possible, considering the situation.

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Appendix I- Informed Consent

CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM INVESTIGAÇÃO de acordo com a Declaração de Helsínquia [1] e a Convenção de Oviedo [2]

MICROBIOTA NOS DOENTES DE SENSIBILIDADE AO GLUTEN NÃO CELIACA

A autorização de participação neste estudo é completamente voluntária, não acarretará quaisquer custos, nem benefícios ou prejuízo aos seus participantes.
Os participantes no estudo têm o direito de solicitar o acesso aos seus dados pessoais, bem como à sua retificação ou a sua eliminação. Têm igualmente o direito de limitar ou opor-se ao tratamento destes dados e têm direito à portabilidade dos mesmos, e em qualquer altura podem retirar consentimento sem comprometer a licitude do tratamento efetuado com base no consentimento previamente dado. Os participantes têm o direito de apresentar reclamação junto da Comissão Nacional de Proteção de Dados caso seja necessário.

Procedimento: Ser-lhe-á pedido que preencha um questionário onde assinala os sintomas nos últimos meses, e fará análises com colheita de sangue para eliminação de diagnósticos confundidores. Para testar a sua tolerância ao glúten fará um teste de sobrecarga durante 2 semanas, onde deverá ingerir um queque e serão monitorizados os seus sintomas. O queque será fornecido pela Clínica e numa semana terá glúten, e na outra não terá glúten. Não saberá antecipadamente qual a semana em que o queque contém glúten, de forma a efetuar o diagnóstico da sua possível sensibilidade. Um nutricionista irá registar semanalmente os seus sintomas e estabelecer o disgnóstico de acordo com as referências atuais. Ao fim deste período ser-lhe-á pedida a recolha de fezes para análise da microbiota. Depois será aconselhado pelo nutricionista a fazer uma dieta com exclusão de glúten, e durante um período de 2 a 4 semanas irá ser monitorizada a adesão a essa dieta. Após esse período, preencherá novamente um questionário relativo aos sintomas. No final, ser-lhe-á pedida nova recolha de fezes. As análises fecais serão comparticipadas pela Clínica, não acarretará despesas aos participantes.

Compreendo que os dados recolhidos durante o estudo possam ser do conhecimento dos membros da equipa de investigação, sempre que necessário para o estudo e autorizo que os membros da equipa tenham acesso a esses dados. Estes não são objecto de tratamento nem decisões automatizadas, sendo exclusivamente tratados pelos membros da equipa de investigação. Os dados e as amostras biológicas colhidas no âmbito do estudo serão destruídos 1 ano após o seu término. No caso de publicação desta investigação, todos os dados serão mantidos anónimos e nenhuma informação será identificável.
Manifesto interesse em receber os resultados finais do estudo, que devem ser enviados para a morada a seguir indicada:
……
……

Foi-me dada a oportunidade de ler e considerar a informação apresentada, e fazer perguntas, as quais foram respondidas de forma satisfatória. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Foi-me igualmente garantida a possibilidade de solicitar o acesso aos meus dados pessoais, bem como à sua retificação ou a sua eliminação. Foi-me garantido o direito de limitar ou opor-me ao tratamento destes dados e o direito à portabilidade dos mesmos, e em qualquer altura posso retirar consentimento sem comprometer a licitude do tratamento efetuado com base no consentimento previamente dado. Caso seja necessário, poderei apresentar reclamação junto da Comissão Nacional de Proteção de Dados.

Desta forma, declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela pessoa que abaixo assina. Aceito participar neste

estudo, e tomo a minha decisão de forma inteiramente livre, e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pelos investigadores.

Eu, abaixo assinado ……., nascido(a) a ___/___/_____, tomei conhecimento dos objetivos do estudo "Microbiota nos doentes com sensibilidade ao glúten não celíaca" e ¨aceito / ¨não aceito (colocar um "X" na opção pretendida) participar.
Email (caso possua) ……
Assinatura: ……Data: …… /…… /……..

Qualquer questão sobre os seus direitos e deveres como participante, no contexto deste estudo, podem ser endereçadas à seguinte entidade independente, que apreciou e emitiu parecer favorável para este estudo clínico:
Email - Conselho de ética: conselhodeetica@estesl.ipl.pt

Confirmo que expliquei à pessoa abaixo indicada, de forma adequada e inteligível, os procedimentos necessários ao ato referido neste documento. Respondi a todas as questões que me foram colocadas e assegurei-me de que houve um período de reflexão suficiente para a tomada da decisão. Também garanti que, em caso de recusa, não haverá quaisquer consequências.

Nome legível do investigador: Ana Catarina Moreira
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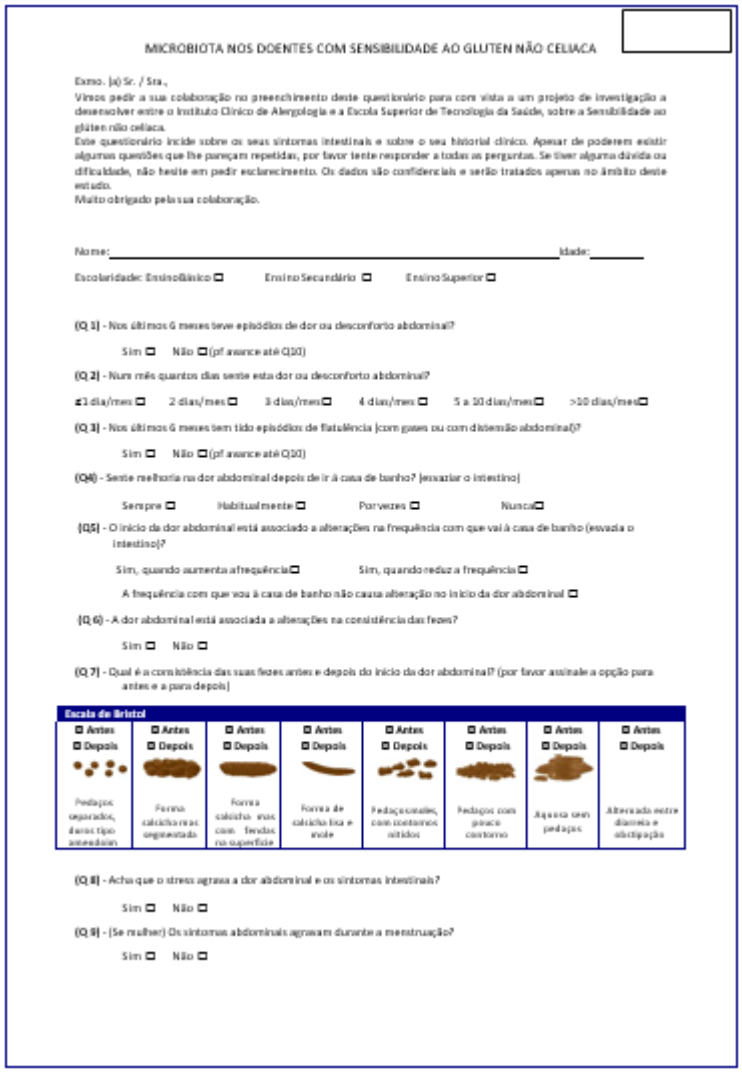
Telf. + 351 21 046 47 00 | + 351 21 046 47 08
Email: npires@net.ipl.pt

Assinatura: ……Data: …… /…… /……..

ESTE DOCUMENTO É COMPOSTO DE 2 PÁGINAS E É FEITO EM DUPLICADO: UMA VIA PARA O INVESTIGADOR E OUTRA PARA A PESSOA QUE CONSENTE.

Appendix II- Symptom Questionnaire



MICROBIOTA NOS DOENTES COM SENSIBILIDADE AO GLUTEN NÃO CELIACA

Exmo. (a) Sr. / Sra.,
Vimos pedir a sua colaboração no preenchimento deste questionário para com vista a um projeto de investigação a desenvolver entre o Instituto Clínico de Alergologia e a Escola Superior de Tecnologia da Saúde, sobre a Sensibilidade ao glúten não celíaca.
Este questionário incide sobre os seus sintomas intestinais e sobre o seu historial clínico. Apesar de poderem existir algumas questões que lhe pareçam repetidas, por favor tente responder a todas as perguntas. Se tiver alguma dúvida ou dificuldade, não hesite em pedir esclarecimento. Os dados são confidenciais e serão tratados apenas no âmbito deste estudo.
Muito obrigado pela sua colaboração.

Nome:_____ Idade:_____

Escolaridade: Ensino Básico ☐ Ensino Secundário ☐ Ensino Superior ☐

(Q 1) - Nos últimos 6 meses teve episódios de dor ou desconforto abdominal?

Sim ☐ Não ☐ (pf avance até Q10)

(Q 2) - Num mês quantos dias sente esta dor ou desconforto abdominal?

≤1 dia/mes ☐ 2 dias/mes ☐ 3 dias/mes ☐ 4 dias/mes ☐ 5 a 10 dias/mes ☐ >10 dias/mes ☐

(Q 3) - Nos últimos 6 meses tem tido episódios de flatulência (com gases ou com distensão abdominal)?

Sim ☐ Não ☐ (pf avance até Q10)

(Q4) - Sente melhoria na dor abdominal depois de ir à casa de banho? (esvaziar o intestino)

Sempre ☐ Habitualmente ☐ Por vezes ☐ Nunca ☐

(Q5) - O início da dor abdominal está associado a alterações na frequência com que vai à casa de banho (esvazia o intestino)?

Sim, quando aumenta a frequência ☐ Sim, quando reduz a frequência ☐

A frequência com que vou à casa de banho não causa alteração no início da dor abdominal ☐

(Q 6) - A dor abdominal está associada a alterações na consistência das fezes?

Sim ☐ Não ☐

(Q 7) - Qual é a consistência das suas fezes antes e depois do início da dor abdominal? (por favor assinale a opção para antes e a para depois)

Escala de Bristol

☐ Antes ☐ Depois	☐ Antes ☐ Depois	☐ Antes ☐ Depois	☐ Antes ☐ Depois	☐ Antes ☐ Depois	☐ Antes ☐ Depois	☐ Antes ☐ Depois	☐ Antes ☐ Depois
Pedaços separados, duros tipo amendoim	Forma salsicha mas segmentada	Forma salsicha mas com fendas na superfície	Forma de salsicha lisa e macia	Pedaços moles, com contornos nítidos	Pedaços com pouco contorno	Aquosa sem pedaços	Alternado entre diarreia e obstipação

(Q 8) - Acha que o stress agrava a dor abdominal e os sintomas intestinais?

Sim ☐ Não ☐

(Q 9) - (Se mulher) Os sintomas abdominais agravam durante a menstruação?

Sim ☐ Não ☐

(Q 10) - Conhece se sofre alguma destas doenças? (assinale todas as aplicáveis)

Ansiedade ☐ Dor de cabeça crónica ☐ Depressão ☐ Desordem bipolar ☐ Esquizofrenia ☐
Doença da tiróide ☐ Anemia ☐ Fadiga crónica ☐ Fibromialgia ☐ Artrite reumatóide ☐
Refluxo gastro-esofágico ☐ Intolerância à lactose ☐ Doença celíaca ☐ Diabetes ☐
Síndrome do intestino irritável ☐ Doença de Crohn ☐ Colite ulcerosa ☐ Cancro do colon ☐
Psoríase ☐ Alergia a alimentos, pf especifique a quais _____

Parte 2 - Sintomas relacionados com o glúten

(Q 11) - Antes de começar, sabe o que é o glúten?

Sim ☐ Não ☐

(Q 12) - Antes de começar, sabe o que é a doença celíaca?

Sim ☐ Não ☐

(Q 13) - Há alguma pessoa da sua família ou amigos que tenha sido diagnosticado com doença celíaca?

Sim ☐ Não ☐ (pf avance até Q15)

(Q 14) - A quem foi diagnosticada doença celíaca? (assinale todas as aplicáveis)

Pai/mãe ☐ Irmão/irmã ☐ Filho/filha ☐ Avô/avó ☐ Tio/tia/primo (de sangue) ☐

Outro ☐, pf especifique _____

(Q 15) - Algum dos seus familiares foi diagnosticado com alguma destas doenças? (assinale todas as aplicáveis)

Artrite reumatóide ☐ Diabetes juvenil ☐ Doença da tiróide ☐ Psoríase ☐
Doença de Crohn ☐ Colite ulcerosa ☐ Síndrome do intestino irritável ☐ Nenhum ☐

(Q 16a) – Há alguns sintomas que associa à ingestão de produtos com glúten?

Sim ☐ Não ☐ (pf avance até Q23)

(Q 16b) - Quais sintomas que relaciona com a ingestão de glúten? (assinale todas as aplicáveis)

Distensão abdominal ☐ Dor abdominal ☐ Desconforto abdominal ☐ Diarreia ☐
Obstipação ☐ Aerofagia (arrotar) ☐ Flatulência ☐ Náusea ☐ Dor de cabeça ☐ Fadiga ☐
Confusão mental (estar aéreo) ☐ Falta de coordenação ☐ Formigueiros ☐ Erupção cutânea ☐
Dores articulares ☐ Outras ☐, pf especifique _____

(Q 17) - Qual a frequência que estes sintomas se manifestam depois de consumir produtos com glúten?

Sempre que ingiro alimentos com glúten ☐ A maioria dos dias ☐ Alguns dias /semana ☐
Alguns dias/mês ☐ Algumas vezes/ano ☐

(Q 18) - Qual é a velocidade com que aparecem os sintomas?

Imediatamente |< hora após ingestão| ☐ 1 a 6 horas após ☐ 6 a 24h após ☐ No dia seguinte ☐

(Q 19) - Habitualmente qual é a duração dos sintomas?

Minutos ☐ Horas ☐ Dias ☐ Semanas ☐ Meses ☐

(Q 20) - Quais são os produtos com glúten que costumam de provocar estes problemas? (assinale todas as aplicáveis)

Pão ☐ Bolos ☐ Bolachas ☐ Cereais ☐ Massas ☐ Pizza ☐

Outros, pf especifique _____

(Q 21) - Há quanto tempo sente que tem problemas com a ingestão do glúten? (pode ser um valor aproximado)

_____meses OU_____anos

(Q 22a) - Alguma vez alterou a sua alimentação por causa dos sintomas sem a recomendação dum nutricionista ou de um médico?

Sim ☐ Não ☐, pf especifique porquê_____

(Q 22b) – Se sim, que tipo de alteração fez?

Dieta sem glúten ☐ Dieta com restrição de glúten ☐ Outra dieta, pf especifique qual _____

(Q 23) - Alguma vez teve consulta com um profissional da saúde por causa dos problemas relacionados com o glúten?

Sim ☐ Não ☐ (pf avance até Q28)

(Q 24) - Se a respondeu sim, especifique qual profissional é que visitou (assinale todas as aplicáveis)

Médico de clínica geral ☐ Médico de especialidade (pf especifique qual) ☐_____

Nutricionista/dietista ☐ Profissional de medicina alternativa (pf especifique qual) ☐_____

Outro (especifique) _____

(Q 25) - Quanto tempo teve os sintomas antes de visitar o profissional de saúde?

_____meses OU_____anos

(Q 26) - Sabe se foi submetido algum destes testes para pesquisar a causa dos sintomas?

Teste ao sangue para doença celíaca	Sim ☐	Não ☐	Não tenho a certeza ☐
Endoscopia	Sim ☐	Não ☐	Não tenho a certeza ☐
Não foi submetido a nenhum teste	Sim ☐		

Foi submetido a outro/s teste/s (pf especifique) ☐_____

(Q 27) - Se foi submetido algum dos testes mencionados na Q26, qual foi o resultado?

Diagnostico de doença celíaca ☐ Doença celíaca excluída ☐ Outro _____

(Q 28) - Alguma vez experimentou uma dieta sem glúten?

Sim ☐ Não ☐ (pf avance até Q31)

(Q 29) - Sentiu melhoria dos sintomas com a dieta sem glúten?

Sim ☐ Não ☐ Não tenho certeza ☐

(Q 30) - Ainda continua a fazer dieta sem glúten?

Sim ☐ Não ☐ Faço, mas raramente ☐

(Q 31) - Há algum tipo de pão ou alimento com cereais que provoque melhoria no aparecimento ou desenvolvimento dos sintomas? (assinale todas as aplicáveis)

Pão feito com espelta ☐ Pão de farinhas que não o trigo (tipo quinoa, cevada ou aveia) ☐

Não conheço nenhum pão que cause alívio dos meus sintomas ☐

(Q 32) - O consumo de algum destes alimentos provocou ou provoca-lhe desconforto abdominal?

Leguminosas (feijão, grão, favas, ervilhas) ☐	Couves ☐	Cebola ☐	Alho ☐	Gomas s/ açúcar ☐		
Couve-flor ☐	Cogumelos ☐	Tomate ou tomate cherry ☐	Maça ☐	Ameixa ☐	Pera ☐	
Manga ☐	Melancia ☐	Leite ☐	Iogarte ☐	Natas ☐	Outros ☐ _____	

Não experimentei desconforto depois de tomar algum destes produtos ☐

O questionário foi finalizado. Muito obrigado pela sua participação!

(adaptado de: Nutrients 2016,8:714-51.)

Appendix III- Muffin datasheet

FICHA TÉCNICA DO PRODUTO

PRODUTO				
Muffins de chocolate				
(L ou Kg)		(Doses)	6	

MODO DE PREPARAÇÃO:
1- Pré-aquecer o forno a 180ºC.
2- Juntar manteiga e açúcar e bater numa liquidificadora. (1 minuto na velocidade 3)
3- Numa taça à parte, bater os ovos juntamente com o leite.
4- Deitar a última mistura no liquidificador e bater 30 segundos na velocidade 4.
5- Acrescentar a farinha, o fermento e o cacau em pó. (1 minuto na velocidade 4)
6- Untar formas de queque com manteiga e encher com a massa obtida. Levar ao forno durante 15 a 20 minutos.

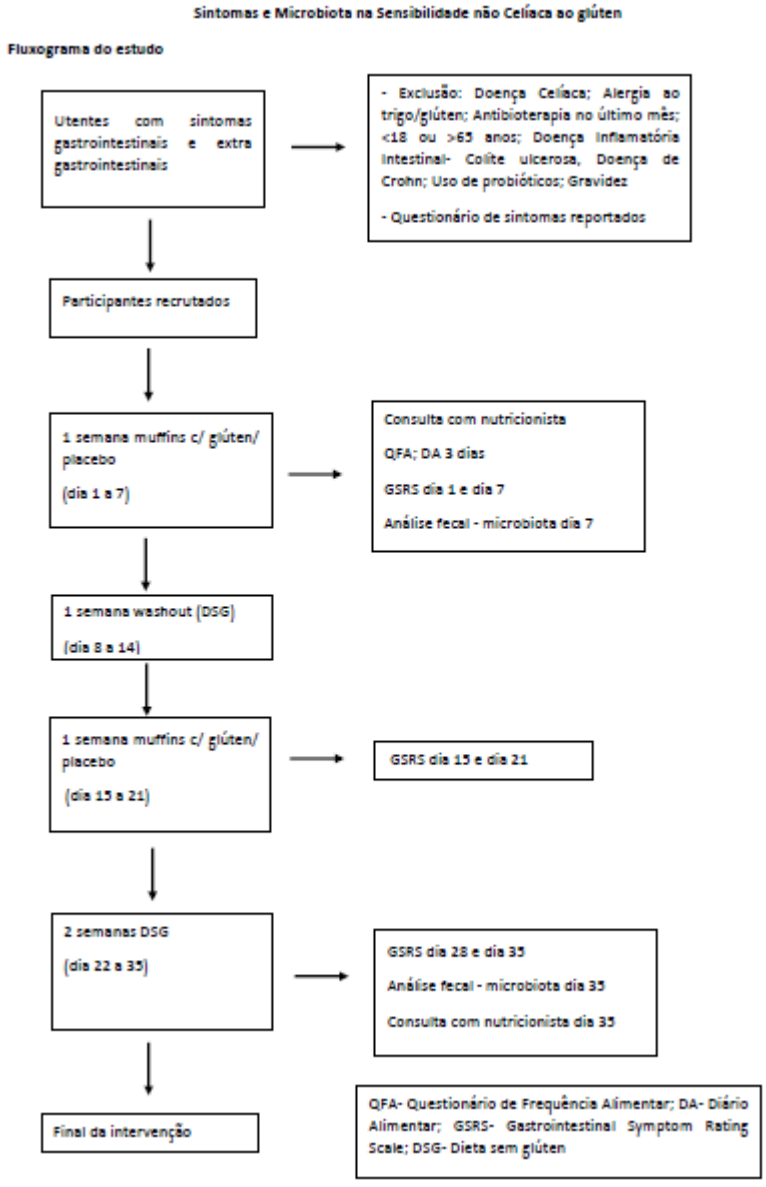
TEMPO DE PREPARAÇÃO	35 minutos

CÓDIGO	INGREDIENTES	QUANT. BRUTA (Kg ou L)	UNIDADES		PREÇO (€)	CUSTO (€)
			Nº	Kg ou L		
	manteiga sem lactose	0,075				0,000
	açúcar	0,110				0,000
	ovos		2,000			0,000
	leite sem lactose	0,060				0,000
	farinha de arroz	0,120				0,000
	farinha de aveia sem glúten	0,080				0,000
	fermento sem glúten	0,005	1 colher de	chá		0,000
	cacau em pó	0,030				0,000
	formas de queques		6,000			0,000
					CUSTO TOTAL (€)	0,000
					PVP sem IVA (€)	7,542
					% CUSTO	0%
					% MARGEM CONTRIBUIÇÃO	100%
					PVP com IVA (€)	8,900

CONSERVAÇÃO	congelação

VALIDADE: 4 dias temperatura ambiente

Appendix IV- Protocol Flowchart



Appendix V- Adjuvant document for the Gluten-free diet

Alimentos permitidos (naturalmente isentos de glúten)	**Alimentos perigosos** (leitura do rótulo para confirmar a lista de ingredientes, podem conter glúten)	**Alimentos a eliminar**
Arroz, pseudo-cereais (amaranto, quinoa, trigo sarraceno), milho, mandioca, tapioca, alfarroba, araruta, sorgo, fonio, teff, millet, aveia sem glúten; Batata, fécula e amido de batata; Açúcar, mel, melaço, compotas e marmeladas caseiras; Carne, pescado, ovos; Hortofrutícolas; Leguminosas (grão de bico, feijão, favas, lentilhas, soja, etc.), frutos oleaginosos (noz, pinhão, amêndoa, avelã, etc.) e sementes (sésamo, girassol, linhaça, abóbora, etc.); Leite simples, iogurtes (naturais e aromas), queijo fresco, requeijão; Azeite e óleos vegetais; Sal, especiarias (noz moscada, pimenta em grão, cravinho, etc.) e ervas aromáticas (salsa, coentros, orégãos, estragão, etc.); Água, chá, infusões, café puro, café descafeinado em grão; Vinho, vinho do Porto, espumante, champanhe; Fermento biológico fresco e seco; Néctares e sumos de fruta naturais e gaseificados.	Broa de milho; Queijos industriais e iogurtes de pedaços e cremosos; Leite achocolatado, maltado e aromatizado; Carnes processadas: carne picada, salsichas, hambúrgueres e almôndegas; Produtos pré-cozinhados, congelados, ultracongelados, enlatados, patés, produtos de charcutaria (fiambre, chouriço, etc.) e de soja; Caldos de cozinha, caril, vinagre, polpa de tomate e molhos industriais (ketchup, maionese, mostarda, chutney, etc.); Natas, manteigas, margarinas e banhas industriais; Batatas fritas de pacote com sabor e outros snacks salgados; Gelados, frutas em calda ou cristalizadas; Compotas de fruta comerciais e figos secos; Sumos concentrados e instantâneos (em pó); refrigerantes; Sobremesas instantâneas, gomas, caramelos, gelatinas e chocolates; Açúcar em pó (glacé) e fermento químico; whisky	Farinhas e amidos de trigo (triticale, espelta, trigo khorasan /kamut, Einkorn /Triticum monococcum/durum, graham), centeio, cevada e aveia, cuscuz, seitan, sêmola/semolina de trigo; massas alimentícias; Pão, produtos de pastelaria e confeitaria, bolachas e biscoitos; Iogurtes com cereais; Queijo creme e queijos comerciais de composição desconhecida; Farinheira, alheira e outros enchidos; Pizzas, lasanha, canelones e raviolis; Salgados (rissóis, croquetes, etc.) e panados; Delícias do mar e variantes; Cerveja, Malte e extrato de malte; Compotas ou sumos industriais com espessantes desconhecidos; Molho bechamel, molho de soja, sopa em cubo ou de pacote; Sobremesas instantâneas, gelados comerciais de composição desconhecida; Cereais de pequeno-almoço; hóstias.
Alimentos perigosos- analisar rótulo:		
Ingredientes a não incluir: Amido, amido modificado (de trigo ou de mistura, ou sem indicar a origem): aditivos E-14XX (E-1404, E-1412, E-1414, E-1422, E-1442, E-1410, E-1413, E-1420, E-1440, E-1450); Malte/ extracto ou xarope de malte; aromatizantes; dextrina; espessantes: farinha de vários cereais; extractos de levedura; amiláceos; fécula (excepto de batata); cereais (não especificados); proteína vegetal ou hidrolisada do trigo (sem indicar a origem); sêmola de trigo; farinha ou derivados de trigo, centeio, cevada, aveia; fibras alimentares (sem indicar a origem).		
Ingredientes que pode incluir: glicose, xarope de glicose, dextrina, dextrose, maltodextrina, dextrina de malte, sorbitol, maltitol, lecitina de soja, goma xantana, goma guar, inulina, levedura; restantes aditivos (que não E-14XX)		

No restaurante:

- Em caso de dúvida, informar sobre a sua sensibilidade ao glúten e fazer perguntas sobre as refeições;
- Preferir cozidos e grelhados, em detrimento de refeições com confeção mais complexa;
- Evitar o consumo de molhos e recheios, pois podem ser engrossadas com farinhas de cereais proibidos;
- Confirmar se o óleo utilizado é apenas para fritar produtos sem glúten e ter em atenção que, normalmente, os fritos são panados;
- Ter especial cuidado com confeções como empadões, sopas, almôndegas, pois podem conter farinhas de cereais proibidos.

Em viagem:

- Ter os contactos da Associação de Celíacos local (ver no site da APC, ligações externas);
- Levar alguns produtos sem glúten consigo;
- Informar-se previamente das marcas comercializadas e dos pontos de venda habituais;
- Ter atenção que as marcas dos produtos que habitualmente consome em Portugal, podem conter diferentes ingredientes no país onde se encontra.

No dia-a-dia:

- Planear o dia de forma a ter sempre consigo produtos sem glúten;
- Guardar os produtos sem glúten em local específico e bem identificado;
- Utilizar uma torradeira/tostadeira e uma manteigueira exclusivamente para os produtos sem glúten;
- Nas festas, informar previamente que tem sensibilidade ao glúten, levar produtos sem glúten e no próprio dia verificar que outras confeções disponíveis pode consumir;
- Manter o ritmo de vida social, planeando antecipadamente as refeições;
- Em caso de dúvida, NÃO CONSUMIR!

Na cozinha:

- Fazer refeições sem glúten para toda a família, sempre que possível, por facilidade e segurança. No entanto, quando não for possível: Preparar primeiro os alimentos sem glúten;

- Todos os utensílios utilizados para a confeção ou manipulação dos produtos sem glúten devem estar destinados apenas a esse fim ou, em alternativa, serem muito bem higienizados entre a utilização de produtos com e sem glúten (ex: panelas, travessas, talheres, material de corte, etc.);
- Para fritar, utilizar um óleo apenas destinado aos produtos sem glúten e nunca um óleo em que já foram fritos alimentos com glúten;
- Não confecionar alimentos sem glúten com alimentos com glúten e retirá-los antes de servir (ex.: farinheira no Cozido à Portuguesa, massa com glúten na sopa, etc.);
- Para polvilhar a forma dos bolos, utilizar farinha de arroz ou fécula de batata;
- Para engrossar molhos ou sopas, utilizar preferencialmente amido de milho e para panar, use farinha de arroz, de milho ou de mandioca;
- Para levar ao forno a torrar, utilizar pão ralado sem glúten ou farinha de mandioca;
- Se possível, optar por fazer o seu próprio pão em casa;
- Para o fabrico de produtos de pastelaria utilizar receitas «normais», substituindo a farinha de trigo por uma mistura de farinha de arroz, fécula de batata, farinha de mandioca e amido de milho. (ex. de mistura ideal: 45% farinha de arroz; 35% farinha de milho; 20% farinha de mandioca)

Nas compras:

- Verificar sempre se as embalagens dos alimentos sem glúten estão intactas;
- Verificar sempre a lista de ingredientes dos produtos, mesmo daqueles que está habituado a comprar/consumir. Não deve ficar preso a marcas, visto que a composição dos produtos pode mudar sem aviso prévio;
- Por segurança, evitar os alimentos a granel e todos aqueles em que não conseguir verificar a lista de ingredientes;
- Consultar a lista atualizada de produtos da APC. Um mesmo produto que é comercializado em vários países e com a mesma marca comercial, pode ter ingredientes diferentes e, como tal, a lista das outras Associações não é válida em Portugal;
- Procurar o segmento do supermercado destinado a alimentos sem glúten.

Annex I- Modified version of the Gastrointestinal Symptom Rating Scale

Sintomas	Início	1ª Semana	2ª Semana	3ª Semana	4ª Semana	5ª Semana
Intestinais						
Dor ou desconforto abdominal						
Azia						
Regurgitação ácida						
Inchaço						
Náuseas e vómitos						
Borborigmo						
Distensão abdominal						
Eructação						
Aumento da flatulência						
Diminuição da passagem de fezes						
Aumento da passagem de fezes						
Fezes moles						
Fezes duras						
Necessidade urgente de evacuação						
Sensação de evacuação incompleta						
Extra-intestinais						
Dermatite						
Dor de cabeça						
"Foggy mind"						
Fadiga						
Dormência nos membros						
Dor articular/muscular						
Desmaios						
Lesões orais/língua						
Outros (especifique)						

Durante a semana passada, qual foi a severidade do sintoma?