Gut Mucosal Reactivity to Gluten and Cow’s Milk Protein in Rheumatic Diseases

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**Abstract**


This thesis comprised patients with chronic rheumatic diseases. The studies aimed to elucidate food sensitivity by measuring mucosal inflammatory reactivity and thereby a possible link between the gut and joints. In all the studies, the mucosal path technique was used to evaluate the rectal mucosal response to rectal challenge with gluten and/or cow’s milk protein (CM).

In some patients with primary Sjögren’s syndrome (pSS) and the genetic susceptibility genes HLA DQ2, mucosal reactivity measured with nitric oxide (NO) was found after rectal gluten challenge without detectable serum antibodies to gluten or transglutaminase. This gluten sensitivity was not linked to coeliac disease.

After rectal CM challenge, a rectal mucosal inflammatory response measured with NO and myeloperoxidase (MPO) was detected in 38% of pSS patients, all of whom fulfilled the criteria for irritable bowel syndrome.

In a questionnaire study of self-experienced adverse reactions to food, 27% of patients with rheumatoid arthritis (RA) reported intolerance to various foods and CM in particular. After rectal CM challenge performed in RA patients (n=27), strong mucosal reactivity to CM was observed in a few patients and a moderate increase in 23%. After gluten challenge, a moderate increase in mucosal reactivity was found in 35% of patients. No correlation to self-perceived intolerance and mucosal reactivity measured with NO and MPO was seen.

Inflammation of the gut is a prominent feature of spondyloarthropathies (SpA). After rectal challenges with CM protein and gluten, an increase in rectal NO production was seen in 26% and 19% respectively (p<0.001). An increase in the mucosal release of MPO as a sign of neutrophil activation was seen in the CM- and gluten-sensitive patients. NO production in SpA patients was more enhanced compared with RA and pSS patients and could contribute to the increased barrier permeability described in SpA patients.

**Keywords:** Primary Sjögren’s syndrome, rheumatoid arthritis, spondyloarthopathies, rectal challenge, food sensitivity, myeloperoxidase, nitric oxide and barrier permeability

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Dedication
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

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<th>Description</th>
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<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
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<tr>
<td>AS</td>
<td>Ankylosing spondylitis</td>
</tr>
<tr>
<td>EmA</td>
<td>Endomysial antibodies</td>
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<tr>
<td>Anti-CCP</td>
<td>Anti-cyclic citrullinated peptide</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<td>CD</td>
<td>Coeliac disease</td>
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<tr>
<td>CM</td>
<td>Cow’s milk protein</td>
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<tr>
<td>DBPCFC</td>
<td>Double-blinded, placebo-controlled food challenge</td>
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<tr>
<td>FA</td>
<td>Food allergy</td>
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<tr>
<td>FI</td>
<td>Food intolerance</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>IBS</td>
<td>Irritable bowel disease</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroid anti-inflammatory drug</td>
</tr>
<tr>
<td>Ppb</td>
<td>Part per billion</td>
</tr>
<tr>
<td>PsA</td>
<td>Psoriatic arthritis</td>
</tr>
<tr>
<td>pSS</td>
<td>Primary Sjögren’s syndrome</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>ReA</td>
<td>Reactive arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>SpA</td>
<td>Spondyloarthropathies</td>
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<tr>
<td>tTG</td>
<td>Tissue transglutaminase</td>
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Introduction

This thesis includes studies of rheumatological disorders and their possible relationship to food sensitivity. In all the studies, the mucosal patch technique was used.

Food and the relationship of food to illness and well-being have occupied the minds of people for many years. We are constantly receiving information in magazines and newspapers about different strategies relating to lifestyle changes and different diet regimens and the urge to influence the course of disease for which there is no cure has created various strategies among our patients. For instance, in patients with rheumatoid arthritis, between 33% and 75% regard diet as a factor contributing to their disease and 20-50% have tried dietary manipulation. (1, 2)

Knowledge of how to answer and advise patients is sparse and, in an attempt possibly to shed some light on the role of food protein in rheumatic diseases, we performed these studies. In all four papers, rectal food protein challenges with gluten and/or cow’s milk (CM) protein, followed by rectal measurements of inflammatory mediators, have been performed.

The studies were performed in patients with rheumatoid arthritis, primary Sjögren’s syndrome and spondyloarthropathies, and, together with celiac disease, a shortly presentation will be given in the following section.
Background

Food allergy

Adverse food reactions include any abnormal reaction from the ingestion of a food and could be the result of food intolerance (non-immune mediated) or food allergy (immune mediated), according to the nomenclature for allergy from the European Academy of Allergy and Clinical Immunology (EAACI). Food intolerance (FI) is defined as an adverse response caused by some unique physiological characteristics of the host and the best known is lactase deficiency. Food allergies (FA) are adverse immunological reactions that might be mediated by IgE or non-IgE immune mechanisms. Moreover, psychological mechanisms could be involved with the pure avoidance of certain food. (3, 4)

There is a huge difference in beliefs and objective assessments regarding the prevalence of food allergy. Studies of the adult general population in different European countries state that up to 20-30% report self-experienced adverse food reactions. (5-7) However, using objective measures like dietary exclusion and controlled food antigen challenge, the prevalence of food allergy and food intolerance is estimated to be much less frequent. (3, 7)

Food allergy is more common in children than in adults, with an estimated prevalence of 6-9% in children and 1-2% in adults. The best studied is the immediate IgE-mediated hypersensitivity, often called type I allergy. (3) The non-IgE allergy is a cell-mediated reaction and is thought to be involved in milk and soy protein enteropathy and in gluten enteropathy (coeliac disease). (8) For the diagnosis of IgE-mediated allergy, the skin-prick test together with measurements of specific IgE is mostly used, but they have their limitations. For example, the skin-prick test has a poor positive predicted value. (8) No single laboratory test can offer an effective tool for the diagnosis of non-IgE-mediated food allergy. The double-blinded, placebo-controlled food challenge (DBPCFC) has been accepted as the gold standard for diagnosing food hypersensitivity, but the DBPCFC does not discriminate between allergy and non-immune-mediated intolerance and has limitations with regard to food allergy manifesting primarily in the gastrointestinal (GI) tract. (3, 8)

The pathophysiology behind food allergy is not known, but disturbed gut barrier function with the increased uptake of food antigens and immunological alterations with the loss of oral tolerance to dietary proteins have been proposed. (9)
Gluten and coeliac disease

Coeliac disease (CD) is the most common, with an estimated frequency of 1%, (10) and best studied, of the non-IgE-mediated food allergies. It is a food allergy induced by the ingestion of dietary gluten and is genetically linked to the histo-compatibility leukocyte antigen (HLA) DQ2 and/or DQ8 haplotypes. The vast majority (90-95%) of patients carry a variant of DQ2 (alleles DQA1*05/DQB1*02), while the remainder carry a variant of DQ8 (alleles DQA1*03/DQB1*0302). (11, 12) Classical gluten sensitivity is histologically characterised by the inflammatorily induced villous atrophy of the small intestine and the therapy is a strict gluten-free diet resulting in the recovery of the mucosal lesions. (13) Today, gluten sensitivity is viewed in a broader sense, with different clinical manifestations, as illustrated by the coeliac “iceberg”. Fig. 1

![Figure 1](image_url)

*Figure 1.* An illustration of the different clinical manifestations of coeliac disease and its genetic predisposition, the “iceberg” model

Individuals with the classical CD disease manifestations, with symptoms such as diarrhoea, weight loss and malabsorption, together with the typical histological signs of crypt hyperplasia and villous atrophy that lead to diagnosis, can all be found. Beneath the surface, there are those diagnosed in population screenings with mild and atypical symptoms or asymptomatic individuals. The mucosal lesions could be absent or include villous atrophy. (14) Small mucosal damage develops gradually and, in the early lesions, lymphocyte infiltration in the epithelium and the lamina propria is present, with a predominant high density of $\gamma$- T-cell receptors bearing intra epithelial lymphocytes. (15) Since about 25% of the population in Northern
Europe carry the susceptibility genes and the majority do not develop CD, (12) other unknown triggers/factors have to be involved. Inflammation, as induced by an infection, might breach the epithelial barrier and lead to a further influx of gluten peptides. There are reports of increased intestinal permeability in CD (16, 17) which is normalised on a gluten-free diet. (17, 18) Furthermore, a high percentage of first-degree relatives of coeliac patients have increased intestinal permeability (19) and, in an animal model, abnormal paracellular permeability precedes the development of gluten-sensitive enteropathy (Irish setter dogs), (20) thus suggesting that a change in permeability precedes the gliadin-induced intestinal damage. Gluten has been reported to be a trigger for the release of zonulin, a protein regulating intracellular tight junctions. (21)

One possible important factor is the introduction of dietary gluten in the infant diet. However, the results relating to the timing and the relationship to breastfeeding are conflicting. (22) One study from Sweden revealed that the introduction of dietary gluten while infants were still being breastfed and the introduction of small amounts rather than large amounts irrespective of time only are protective factors. (23) Others suggested an increased risk when introducing gluten during the first three months and did not observe the protective effect of breastfeeding. (24)

The pathophysiological mechanism in CD suggested to date is an adaptive gluten-specific T-cell response. From the gut lumen, gliadin (derived from gluten) reaches the lamina propria and binds to DQ2- or DQ8- positive antigen presenting cells (APC), which are then presented to CD4+ T-lymphocytes and these activated T cells drive a T-helper-cell type 1 response to inflammation. The enzyme tissue transglutaminase (tTG), which is released and activated in the presence of gliadin and strengthens the binding of gliadin to APC, is central in this cascade. The modification of tTG to the gliadin peptide takes place via deamidation; from neutral glutamines to glutamic acid given negatively charges which are preferred in the antigen-binding groove of HLA-DQ 2 positive APC. As a consequence of the gluten-specific T-cell response, an auto-antibody to the enzyme tTG is produced. (13, 16, 25) This antibody is used as a serological marker for CD, together with anti-gliadin antibodies (AGA). AGA have low sensitivity and specificity compared with anti-tTG, and they become undetectable on a gluten-free diet. (13) Apart from the adaptive immune system, the innate immune system could also contribute to the pathogenesis via a fragment of gliadin for example, which appears to dictate the type and intensity of the adaptive response controlled by pathogenic CD4+ lamina propria cells. (26)

A number of associated auto-immune diseases, in particular insulin-dependent diabetes mellitus, autoimmune thyroiditis and primary Sjögren’s syndrome, are associated with CD. (16, 27-29) Symptoms attributable to gluten may also appear outside the gut, in cerebellar ataxia and dermatitis herpetiformes, for example. (30-32)
Gluten consists of storage proteins from wheat, rye and barley that remain after starch is removed from the grain. Gluten can be separated into two fractions, the ethanol-soluble prolaminues and the ethanol-insoluble glutenins. Gliadines are the prolaminues in wheat, secalines in rye and hordeins in barley. The prolaminues have the toxic properties and are rich in glutamine and proline. Proline strengthens the binding with HLA-DQ2 and DQ8 molecules on APC and glutamine residues are the preferred substrate for tTG mediated deamidation which enhances the T-cell response. (25)

Cow’s milk protein
Adverse reactions to cow’s milk (CM), related to both lactase deficiency and allergy to CM proteins, are frequently reported. (33) The most common manifestation is IgE-mediated, especially in childhood, (3) but a non-IgE-mediated enteropathy may also be seen, especially as a transient condition in children. The histological inflammation in CM protein enteropathy is more discrete compared with CD, without the classical villous atrophy but instead with a nodular lymphoid hyperplasia, suggesting a delayed immune response. (34, 35) In eosinophilic reflux disease in children, one third of cases are attributed to CM allergy and they improve on a CM free-diet. (8) In adults, very little is known about adverse allergic reactions to CM protein.

The diagnosis of IgE-mediated allergy to CM is based on a positive skin-prick test and/or an increase in IgE antibody levels to milk protein, together with a typical clinical picture. (36) The non-IgE food allergies, on the other hand, are more difficult to diagnose and require procedures with food elimination and food challenges. (3, 8) In diabetes mellitus type 1, the incidence has risen during the last decade and the role of environmental factors including diet and early exposure to CM protein has been debated. (37)

CM protein consists of several fractions; the most abundant is casein (76-86%), followed by β-lactoglobulin, α-lactalbumin, serum albumin and serum immunoglobulins. The two most allergenic are thought to be casein and β-lactoglobulin. (33)

Gut immune system and barrier function
The gastrointestinal tract is the largest immunological organ in the body and, as a result of its large area in relation to the environment, it is in contact with a variety of antigens. Its dual function of protection people from unwanted antigens and, at the same time, allowing nutrients to gain access requires special properties in the intestinal barrier. Perhaps the most important issue is the role the gut immune system plays in preserving oral tolerance – in other words, “educating” the immune system not to react inappropriately to gut luminal antigen.
The intestinal barrier is composed of an outer mucus layer and a layer of epithelial cells. Beneath is the lamina propria and organised lymphoid tissue called Peyer’s patches in the small bowel. The composition of the intestinal barrier varies in different regions of the intestine and the barrier is a highly dynamic structure allowing permeability for nutrients and macromolecules and, at the same time, providing an effective barrier to foreign antigens, micro-organisms and their toxins. Firstly, there is the thick mucosal layer consisting of a variety of peptides (defensins, lysozymes and lectines) with direct antimicrobial activity and immunoglobulins (sIgA, IgG, IgM) which are responsible for the opsonisation of microbes. Secondly, the gut epithelium acts as a physical barrier separating luminal antigens from the underlying immune cells. The sealing of the epithelial lining includes desmosomes, adherent junctions and tight junctions. However, epithelial crosstalk with underlying immune cells in order to regulate the immune response exists in specific areas. Luminal antigens can cross the epithelium through breaks in the tight junctions (paracellular pathway); through M cells in the epithelium that overlies the organised lymph tissue; or via the antigen-presenting dendritic cells which reach into the gut lumen and sample gut bacteria, for example. Through pattern recognition receptors, which recognise the conservative structures of bacteria and viruses, the gut epithelium itself can also directly sense commensal bacteria and pathogens. For this reason, the mucosal immune system of the gut has the capacity to identify potentially harmful antigens and prevent excessive immune responses to foods and gut bacteria in an extremely dynamic way. (38-40)

The Payer’s patches and the isolated lymphoid follicles, together with the mesenteric lymph nodes, work as an inductive site for mucosal immunity upholding oral tolerance. In the lamina propria, on the other hand, the antibody-producing plasma cells, CD4+ T cells, macrophages, dendritic cells, mast cells, eosinophils and intra-epithelial lymphocytes work as the effector site, responsible for the elimination of unwanted antigens. (41)

The innate immune response is our most ancient response primarily to infections and generally involves immediate, non-specific responses mainly to foreign infectious agents. These actions involve the direct killing and/or further presentation of the antigen to the adaptive system. The cells involved in the innate immune response include granulocytes, natural killer cells, macrophages and dendritic cells. The adaptive immune system, on the other hand, involves the engagement of receptors that are selected for reactivity with specific antigens, making it a flexible and specific immune response with a memory. The B and T-lymphocytes recognise antigens presented by APC and start either antibody production or direct cell-mediated actions. This involves the HLA antigens for recognition. These two, the innate and the adaptive immune mechanisms, interact to form our immune system. The disruption of this complex immune system and the immunological equilibrium of the gut can lead to auto-immune diseases, (42-44) and the gastro
intestinal disorders that are regarded as being auto-immune are CD and Crohn’s disease. In Crohn’s disease, a chronic inflammation of the bowel, the lesions are characterised by patchy mucosal ulcerations, strictures and granuloma formations. The etiology remains incompletely understood, but a disturbed barrier function has been proposed as a causal factor behind the development of inflammatory bowel disease (IBD), supported by gene discoveries with mutations in genes that control epithelial permeability. (45-47) Among healthy first-degree relatives to patients with Crohn’s disease, increased intestinal permeability is also demonstrated. (48, 49) Increased intestinal permeability precedes clinical relapses in clinically asymptomatic Crohn’s disease patients, thus indicating an early event. (50) This theory is supported by animal experimental studies showing that alterations in the intestinal barrier alone, in the absence of immune dysfunction, lead to colitis. (51)

Upholding intestinal permeability is essential and the structure of tight junctions is highly complex and involves a number of proteins. Several of these proteins are supposed to regulate permeability, including the zonulin/zonulin receptor pathway. The stimulation of the receptor by a luminal trigger leads ultimately to the release of zonulin and eventually to the opening of the paracellular space. Infection with cholera, which leads to excessive diarrhoea, illustrates this pathway and the importance of upholding intestinal permeability. Vibrio cholera toxins alter the intestinal barrier by disrupting the tight junctions acting via the zonulin pathway. (40) In coeliac disease, gliadin has been shown directly to stimulate zonulin production, thereby increasing intestinal permeability. (21) It has also been suggested that barrier dysfunction plays a role in the pathogenesis of other auto-immune diseases such as CD and diabetes mellitus type 1. (40)

Our normal commensal gut bacterial flora is of great importance, mainly in the interaction with the immune system, when it comes to upholding oral tolerance. (44) The balance between enteric infections and the normal flora may be partly responsible for the increase in food allergies and IBD. Infectious diseases of the gut have decreased in western countries and gastrointestinal food allergies have increased, thereby raising the question of whether the absence of overt gut infections has altered the balance between the normal bacterial flora in the healthy gut and the mucosal immune system. (8) The incidence of IBD also appears also to be increasing in countries “beyond the west” where is has traditionally been considered rare. This indicates that the reduction in enteric infections and in particularly helmet infections lies behind this increase in IBD. (52) The alteration and involvement of the commensal gut flora in IBD has attracted a great deal of attention in recent years; this includes probiotics to normalise an alteration. (53)
Primary Sjögren’s syndrome

Primary Sjögren’s syndrome (pSS) is a chronic auto-immune disease characterised by dryness of the mouth and eyes caused by lymphocytic infiltrations of the lacrimal and salivary glands, resulting in keratoconjunctivitis sicca and xerostomia. (54) Sicca symptoms may also appear in other exocrine glands, resulting in tracheitis sicca, vaginitis sicca and dry skin. General accompanying symptoms are fatigue, myalgia and arthralgia. In addition, systemic manifestations of pSS may involve the lungs, liver, kidneys, vasculature and blood. The most common manifestations are Raynaud’s phenomenon and/or a non-erosive arthritis. (55)

The disease primarily affects women and the prevalence is estimated to be between 0.5-3%, depending on the patient selection and classification. (56-58) The recently modified criteria from the European-American Consensus Group require symptoms and signs of dry eyes and dry mouth, as well as characteristic histopathological findings in minor salivary glands or the presence of auto-antibodies to Ro/SS-A or La/SS-B. (54) A high prevalence (65%) of various allergic manifestations and, in particular allergic drug reactions (46%) has previously been reported in pSS patients. (59, 60)

The etiology of pSS is largely unknown and a combination of genetic susceptibility, infectious agents and hormonal effect has been proposed. (61, 62) Apart from studies regarding infectious agents and other environmental factors, for example, the literature is sparse. Treatment with gammalinolenic acid has been studied, with conflicting results. In a randomized, controlled trial, the treatment showed no effect on fatigue or sicca signs. (63)

Associations between gut involvement and pSS

Gastrointestinal symptoms are frequent and include dysphagia, nausea and epigastric pain. The dysphagia could be due to either sicca manifestations or abnormal esophageal motility, while the epigastric symptoms could be due to chronic atrophic gastritis. Impaired pancreatic function, most commonly subclinical, and auto-immune liver disease are also found. Reports on small bowel and colonic manifestations are rare but include nutritional deficiencies due to malabsorption, possibly associated with CD. (64-66) An increased incidence of CD is seen in pSS. The frequency has been suggested to be as high as 15%, with predominantly latent or silent manifestation. In some of these studies, the classification criteria did not require an auto-immune component, thereby raising some suspicion regarding the high frequencies. (67-70) HLA haplotypes DRB1*0301, DQA1*0501 and DQB1*0201 are associated with pSS and, in particular with the existence of anti-SS-A and/or anti-SS-B antibodies. Susceptibility to CD is strongly associated with the same HLA haplotypes. (71-73) Interestingly, histological examinations have also
demonstrated increased amounts of intra-epithelial lymphocytes, the hallmark of CD, in the gut mucosa of pSS patients without CD. (70)

**Rheumatoid arthritis**

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease and is characterised by symmetrical polyarthritis in peripheral joints. The clinical manifestations range from mild to rapidly progressive multisystem inflammation and joint destruction that can develop early. To stop the destructive process, early treatment with disease-modifying drugs (DMARDs) is required. In recent years, new therapeutic drugs, the biological-response modifiers, have provided better tools for suppressing the inflammatory process. (74) Despite the decreasing rates of life-threatening extra-articular manifestations such as amyloidosis and the need for total hip replacement and other surgical procedures, other co-morbidities still exist. Increased mortality, mainly due to an increased risk of developing cardiovascular disease, (75) where the inflammation per se is suggested as the risk factor and not the traditional cardiovascular risk factors, is also seen. Moreover, an over-representation of lymphoma is known and the increased risk is mainly due to long-term disease activity. (76)

The diagnosis is based on a number of criteria for classification. These criteria, the American College of Rheumatology (ACR) 1987 revised criteria, are the most recently revised and have been used in this thesis. (Table 1) (77) Today, when early treatment can prevent disability in many patients, it is desirable to start before rheumatic nodules and radiographic changes are seen and, as a result, these criteria are the subject of debate. Antibodies to proteins that are post-translationally modified by the enzymatic conversion of arginine to citrulline have recently been recognized. They are present in approximately 70% of RA patients and can be detected before disease onset. (78, 79) These antibodies include anti-cyclic citrullinated peptide antibody (ACPA or anti-CCP), which predicts a more severe and erosive disease, (80, 81) and are present in the synovial tissue (82), implying a possible pathogenic role.
Table 1. The 1987 revised ACR criteria for the classification of RA.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Definition</th>
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<tbody>
<tr>
<td>1. Morning stiffness</td>
<td>Morning stiffness in and around the joints, lasting at least one hour before maximum improvement</td>
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<tr>
<td>2. Arthritis in three or more joint areas</td>
<td>At least three joint areas simultaneously observed by a physician</td>
</tr>
<tr>
<td>3. Arthritis in hand joints</td>
<td>At least one area</td>
</tr>
<tr>
<td>4. Symmetrical arthritis</td>
<td>On both sides of the body</td>
</tr>
<tr>
<td>5. Rheumatic nodules</td>
<td>Subcutaneous nodules observed by a physician</td>
</tr>
<tr>
<td>6. Serum rheumatoid factor</td>
<td></td>
</tr>
<tr>
<td>7. Radiographic changes</td>
<td>Erosions or unequivocal or decalcification adjacent to the involved joints</td>
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Patients fulfilling four or more criteria for at least six weeks are classified as having RA.

The estimated prevalence in Europe is between 0.5-1%, with a 2.5 times higher prevalence in females than in males. (83, 84) The etiology of RA is unknown, but it is thought to be interaction between both genetic and environmental factors. (42, 85) The most important genes are located within the human major histocompatibility complex (MHC) or HLA region and the overall genetic risk may account for a risk of developing RA of up to 60%. (86) RA is associated with several HLA DRB1* alleles. They all encode similar amino acids in the DRβ chain and these shared sequences are referred to as a shared epitope. One of the recently established environmental risk factors is smoking which, together with HLA-DR shared epitope genes, increases the risk of developing RA in anti-CCP positive patients. (87)

Associations between gut and food involvement and RA

The incidence has been progressively declining over a period of 40 years in both women and men, although a cyclic variation may exist, indicating the possibility of changes in exposure to environmental factors, such as infectious and hormonal factors. (88, 89) The evidence regarding the importance of nutritional factors is sparse (90, 91), although a possible protective role for fish high rich in omega-3 fatty acids and olive oil has been suggested.
This is supported by epidemiological studies in the Faroe Islands, where the course of RA had a milder form and the diet is high in fish and whale meat, and in north-western Greece, where the consumption of olive oil is high and the prevalence of RA is low. This contrasts with a prospective study from the UK, where a low intake of fruit and vitamin C and a high intake of red meat was associated with an increased risk of RA.

A fast of seven to 10 days has been shown to reduce the symptoms and the acute phase reaction in patients with established RA, thereby suggesting that food intake may play a role in the pathogenesis of the disease through a putative immunological link between gut immunity and RA. A vegan diet free from gluten and a Mediterranean diet may alleviate arthritic symptoms in a subset of patients. The gluten-free vegan diet has also demonstrated a decrease in LDL and oxLDL levels and raised antibodies to phosphorylcholine, changes that are potentially atheroprotective and anti-inflammatory. Pattison et al. systematically review studies with comparison groups that examine dietary intake and suggest that evidence regarding dietary factors may play a role in the etiology of RA, but the small number of studies and the variation in study design make this inconclusive.

**Spondyloarthropathy**

Spondyloarthropathy (SpA) is a heterogeneous group of inflammatory disorders including ankylosing spondylitis (AS), reactive arthritis (ReA), psoriatic arthritis (PsA), arthritis associated with inflammatory bowel disease (IBD) and undifferentiated SpA. The existence of both axial and/or peripheral joint involvement and entheses (inflammatory lesions at ligamentous attachments to the bone) are the most important clinical hallmarks. A number of extra-articular manifestations, such as mucocutaneous, ocular, heart and the aortic valve, and inflammatory gut lesions may also be seen in SpA.

The estimated prevalence of AS among white populations in western countries is estimated at about 0.5-1.4%, and in the whole group of SpA patients, at about 1-2%. Exercise and NSAIDs have been the main cornerstone of symptom control for decades, but the recently developed biological agents have shown an improvement in disease control, especially in patients with short disease duration. However, whether anti-TNF treatment can stop radiographic progression (new bone formation) has not yet been proven. The most used diagnostic criteria are the European Spondyloarthropathy Study Group criteria and the Modified New York criteria for AS. However, in AS, the Modified New York criteria perform well in patients with established disease, but they have limitations in
early disease and a call for new diagnostic and classification criteria has therefore emerged. (112)

A remarkably high association between AS and ReA and the expression of MHC class I molecule HLA-B27 has long been known; 90-95% of AS are HLA-B27 positive. The prevalence of HLA B27 in the general population shows considerable variation from 0%-50% and, in northern Scandinavia, the prevalence is estimated at 16%. (113) The etiology is still unknown, and the way HLA B27 is involved in the pathogenesis has still not been entirely elucidated.

However, due to the strong association with HLA-B27, some people argue that SpA are autoimmune diseases that can be attributed to cross-reactivity between bacteria and HLA-B27. (114) However, the pathogenic significance of molecular mimicry between various bacteria including yersinia, shigella, salmonella, campylobacter and clamydia in ReA and klebsiella pneumonia in AS has been questioned. (115)

Associations between gut involvement, barrier dysfunction and SpA

It has been clearly established that a close relationship exists between gut inflammation and the development of arthritis. In patients with IBD, up to 30% present muscular skeletal manifestations including arthritis and the most common arthritis is peripheral and it comes and goes with the bowel flares. An SpA indistinguishable from AS and an isolated sacroiliitis are also seen. (116-118) It is also known that inflammatory bowel disease is overrepresented in patients with SpA. In endoscopic studies 5-10% have been diagnosed with IBD. Microscopic signs of gut inflammation without clinical symptoms, especially in the ileum, have been demonstrated in more than 50% of SpA patients and, with time, 6-13% develop overt IBD, especially Crohn’s disease. (119-125) In SpA patients, those with peripheral arthritis are more prone to develop intestinal inflammation. (126, 127) Subclinical intestinal inflammation in first-degree relatives of patients with AS has also been demonstrated. (128)

The relationship between the genetic background, gut inflammation and arthritis is illustrated in HLA-B27 transgenic rats, which spontaneously develop an SpA-like disease and inflammatory lesions in the gut. However, they develop neither joint inflammation nor gut lesions in germ-free conditions, thereby also illustrating the association with the environment, such as bacteria. (129) In humans, joint symptoms and ReA are known to be induced after certain bacterial infections, such as salmonella typhimurium, yersinia enterocolitica, shigella, campylobacter jejuni and chlamydia. In patients with a certain genetic background, HLA B27, the symptoms persist for longer and can even develop into a persistent disease. (130, 131)
Shared disease-susceptible genes and shared environmental triggers to generate immune activation could explain the close association between SpA and IBD, (132) supported by a genealogic study in Iceland. (133) In fact, the same altered gene expression in non-inflamed colon in SpA and Crohn’s patients is reported by Laukens et al. (134) Further support for the link between the diseases is provided by the increased intestinal permeability that has been observed in AS, ReA, PsA, CD and IBD. (119, 135-138) Furthermore, increased intestinal permeability is also seen in healthy first-degree relatives of AS and IBD patients. (135, 139)

Unlike RA, there are just a few studies of SpA elucidating the possible effect of diet regimens on disease activity. It has been suggested that treatment with seal oil, fish oil and omega-3 fatty acids could be beneficial in patients with PsA and AS. (140, 141) Arguing the cross-reactivity theory, Ebringer recommends a low-starch diet in AS to impair the intestinal growth of klebsiella. (142) In an open study, a diet without CM has been reported to result in the subjective improvement of symptoms in SpA. (143) In PsA patients a proposed role for gluten has been implicated, since increased lymphocyte infiltration in the duodenal mucosa and raised serum levels of antibodies to gliadin has been found. (144, 145)

**Irritable bowel syndrome**

Irritable bowel syndrome (IBS) is regarded as a functional bowel disorder with abdominal pain or discomfort and is associated with defecation or changes in bowel habits and with features of disordered defecation with prevalence estimates of up to 20%. Irritable bowel syndrome is defined according to the most recent ROME III criteria by the presence of these symptoms for at least three days a month in the last three months and can be divided into subgroups according to whether diarrhoea or constipation or both are the most predominant symptom (see appendix). (146, 147) IBS symptoms are often chronic and the patient’s score is lower on measures of quality of life than that of the general population, as well as those with other chronic disorders. The etiology is largely unknown, although different mechanisms for the symptoms have been proposed, including an imbalance in serotonin signalling, which can affect GI motility, secretion and visceral sensitivity. (148, 149)

In some IBS patients, a non-specific mild mucosal inflammation has been seen, with an increase in the number of neutrophils and mast cells. Why this inflammation occurs is unknown, but the suggested factors include infection and food, (150) including food allergy. (151, 152)
The rectal mucosal patch technique

One approach to detecting food sensitivity is based on the idea that rectal challenges with food antigens may induce a local mucosal inflammatory reaction in sensitive patients. Previous biopsy studies have supported this idea and also show that immune cells reacting to gluten are found in the entire gastrointestinal tract. (153, 154) After rectal challenge with gluten in CD patients on a gluten-free diet, the rectal mucosa is inflamed with an increased number of intraepithelial lymphocytes and neutrophils, (153, 155) as well as an increase in iNOS synthase. (156)

The mucosal patch technique has been evaluated in a doctor’s thesis by G. Kristjánsson on patients with IBD, IBS and CD. The idea is based on previous perfusion studies evaluating inflammation in the gut mucosa with measurements of released substances from activated neutrophils, eosinophils and mast cells. (157-159) The rectal mucosal technique provides an opportunity to measure inflammatory activity in the rectal mucosa by simultaneous measurements of nitric oxide (NO) and soluble released mediators like myeloperoxidase (MPO) from activated neutrophils and eosinophil cationic protein (ECP) from eosinophils. (160) The kinetics of the local inflammatory reaction induced after rectal challenge with gluten have been evaluated in patients with CD. The mucosal activation of neutrophils and eosinophils starts to appear five hours after challenge and precedes the mucosal production of NO, which peaks after 15 hours. (Fig 2) (161, 162)
Figure 2. Rectal luminal NO before and five, 15, 24 and 48 hours after rectal gluten challenge in 10 patients with coeliac disease. Friedman ANOVA was significant (p< 0.001), ** p< 0.01 compared with prechallenge values (Sign test). The open squares illustrate the rectal mucosal MPO concentrations at each time and the relationship to NO production.

Markers of inflammation

In inflammatory processes in the gut, as well as in inflammatory disease in the gut, granulocyte mucosal infiltration is present, followed by the release of humoral mediators. In our studies, we have used two of these humoral mediators, myeloperoxidase (MPO) and eosinophil cationic protein (ECP), which have previously been found to increase in inflammatory processes in the gut. (163-165)

Markers of neutrophils

The neutrophil granulocytes are our first line of defence against invading micro-organisms. If they are not activated, they are found in the blood circulation and arrested in narrow capillaries (the marginating pool). They have a high turnover rate and die within a few hours unless activated. When reacting to chemoattractants, released by activated cells such as macrophages epithelial cells and bacteria or dying cells, they migrate immediately to the inflamed tissue and phagocytosis and degranulation take place. This involves
the production of toxic oxygen radicals, known as the respiratory burst, and the release of preformed proteins stored in granules. If the neutrophils do not encounter the micro-organism or if the target is too large to engulf, they release their granule constituents extracellularly with potential damage to adjacent tissue. The neutrophils also play an important role in tissue healing and, by producing chemotactic signals, they can influence the recruitment, activation and programming of APC, as well as affecting both B and T lymphocytes. (166, 167)

There are two major types of granule in neutrophils, the azurophil (or primary) and the specific (secondary). The azurophil granules contain MPO and proteinases, lysozymes and other factors directed at microbial killing and digestion. The specific granules contain lysozyme, membrane proteins, metalloprotease, lactoferrin and transcobalamine. (168) In gut inflammation, granulocyte mucosal infiltration is prominent and the release of MPO is used as a marker of inflammation. (163, 165)

**Markers of eosinophils**

The migrated mature eosinophil granulocytes are predominantly found in epithelial surfaces that interact with the external environment; skin; lung and mainly in the lamina propria of the gastrointestinal tract. Intestinal eosinophils protect us from parasitic infections and in particular from helmets. (169) In addition, they can act as antigen-presentation cells and stimulate T-cells to activate and proliferate. The eosinophils respond to for example, to non-specific tissue injury, allergens and infections. Once activated, they can phagocytose, upregulate their cytokine production, produce IgE and release their toxic granule proteins and produce oxygen-free radicals. Activated eosinophils release four cationic proteins; ECP, eosinophil-derived neurotoxin, eosinophil peroxidase and major basic protein. (169)

The accumulation of eosinophils is seen in several gastrointestinal diseases; IgE-mediated food allergy, eosinophilic gastroenteritis and esophagitis and in IBD causing tissue destruction and inflammation. (170) However, in inactive ulcerative colitis, activated eosinophils are also found, suggesting a possible role in tissue repair. (171) The involvement of the eosinophils in the pathogenesis of asthma bronchiale and atopic dermatitis is also implicated. (172) Eosinophil-derived neurotoxin, eosinophil peroxidase and ECP are thought to be specific for eosinophils (173) and are used as markers of eosinophil activation.

**Nitric oxide as a marker of inflammation**

Since the discovery of NO as a biological agent, the recognition of its importance in many physiological and pathophysiological processes has grown tremendously. Researchers in this field were in fact presented with the Nobel Prize in 1998. NO is a short-lived reactive small molecule that can freely
diffuse due to its uncharged properties. The production of NO is under the control of three distinct synthase (NOS) genes. NO is the product of NO synthase (NOS). There are three isoforms of NOS and two of them are expressed constitutively at low levels (cNOS). The cNOS produces small amounts of NO in response to increases in intracellular calcium levels. The third isoform, inducible NOS (iNOS), is not expressed under normal conditions but is induced at high levels during inflammation. Once iNOS is expressed, it produces NO in high concentrations for prolonged periods of time. (174) NO is regulated at transcription level and is inducible in virtually every cell after the appropriate stimuli. The NOS catalyses the conversion of arginine and oxygen into equimolar amounts of citrulline and NO. (175)

NO has been proposed as a marker of airway inflammation and exhaled NO is elevated in patients with asthma bronchiale and correlates with the eosinophil count in sputum. (176) The regular monitoring of NO is suggested as a tool to identify whether a corticosteroid responsibility exists and thereby monitor treatment with inhaled corticosteroids. (177) When it comes to the role of NO in IBD, there are conflicting reports about whether NO is beneficial or devastating and the likely explanation is that, in low doses produced by cNOS, NO is beneficial compared with the large amounts produced by iNOS under inflammatory conditions. Moreover, direct measurements of rectal NO have been proposed as an objective method for monitoring disease activity in IBD. (178) In the jejunum of patients with untreated coeliac disease, the increased production of NO has also been reported directly and indirectly. (179) The idea that rectal challenge with food proteins may produce NO as a marker of inflammation has been reported by others. (180) Kristjánsson et al. have reported the fairly strong synthesis of NO in the rectal lumen after local gluten challenge, as well as after CM challenge, in CD patients. (162, 181) The cellular sources of NO after food protein challenge have not been identified, but they are probably the same as those seen in the inflamed mucosa of patients with ulcerative colitis, i.e. enterocytes, macrophages and other inflammatory cells. (179)
Overall aim

To broaden our understanding of the immunological connection between the gut and joints in patients with rheumatic diseases by using the mucosal patch technique, a technique used to evaluate gut mucosal inflammatory reactivity before and after food challenge. It is hoped that these studies will make it possible to identify subgroups of patients with rheumatic diseases who might benefit from diet.

The specific aims of the studies are:

- To evaluate the rectal mucosal response to gluten in patients with pSS, as a sign of gluten sensitivity

- To evaluate the rectal mucosal response to rectal challenge with CM protein in patients with pSS and relate possible CM protein reactivity to their intestinal symptoms

- To evaluate the subjective conception of food intolerance in patients with RA and relate this to the rectal mucosal reactivity to gluten and CM protein

- To evaluate the rectal mucosal activity in patients with SpA and the reactivity to rectal challenge with gluten and CM protein
Subjects and Methods

Food protein challenge for all patient groups

The patients and control subjects were challenged with 6.5g of dried milk powder (Semper AB, Stockholm, Sweden) and/or wheat gluten (crude wheat gluten; Sigma Chemical Co.), suspended in 25 ml of 0.9% NaCl solution. The solution was instilled into the rectum with a syringe with the participant lying in the left lateral supine position. The subjects were instructed to retain the enema for at least 60 minutes. Rectal challenge was performed between 4 pm and 6 pm and samplings were made 15 hours later, between 7 am and 9 am. The challenge with CM and gluten was performed at different times and with a minimum of two weeks between the examinations. The subjects were told to fast from midnight until the samplings were made. The patients were not on any specific diet and did not change any ongoing medication during the challenge studies. The mucosal reactivity after challenge was studied by using the mucosal patch technique.

All patients and controls were given a rectal laxative enema (Klyx 120 ml; Ferring, Copenhagen, Denmark) within one hour before being tested with the rectal mucosal patch technique.

Analytical measurements

The device used is a plastic catheter with a silicon balloon at the end of the catheter. Three patches made of highly absorbent cellulose material (Phadia AB) are attached to the balloon. After the instrument is positioned in the rectum with the subject lying in the left lateral supine position with sub maximum flexed hips, the balloon is inflated with air, bringing the patches in contact with the mucosa. The catheter is left in place for 20 minutes. The balloon is then deflated and the catheter removed. The patches are cut off and immediately put into 2 ml of 0.3% CTAB solution (N-Cetyl-N,N,N-trimethyl ammonium bromide; Merck, Darmstadt, Germany) to extract the absorbed material. The extraction solution is squeezed out of the patches, pooled, centrifuged, and frozen at -70°C.

The extraction solutions were analysed for the concentrations of granule constituents from neutrophils (myeloperoxidase; MPO) and eosinophils (eosinophil cationic protein; ECP). MPO was analysed by an ELISA assay (Diagnostics Development, Uppsala, Sweden) and ECP by the ImmunoCap
assay (Phadia AB, Uppsala, Sweden) according to the instructions of the manufacturers.

Air samples were collected with glass syringes during deflation of the balloons and analysed for nitric oxide (NO) with a chemiluminescence NO analyser (Sievers NOA 280; Ionic Instrument Business Group, Boulder, Colorado, USA), as described previously. (162)

![Figure 3. Schematic drawing of the procedures in the mucosal patch technique. A. The instrument with a non-inflated balloon and with the patches covered. B. The instrument with the inflated balloon in the rectal ampulla and in contact with the mucosa. After 20 minutes, the balloon is deflated and the air samples are collected in a glass syringe for the analysis of NO. C. The patches are cut off after removal from the rectum into the extraction solution and frozen for subsequent analysis.](image)

**Questionnaires**

**Questionnaires about food intolerance in Paper III**

Questions were asked about food ingestion and various symptoms; intestinal symptoms, urticaria, itching, eczema, dyspnoea, angio-oedema, rhinitis, anaphylaxis, joint and muscle symptoms and fatigue. Further, questions about other allergic manifestations, first-degree family history of allergy, food allergy as a child, smoking habits, previous vegetarian diets and whether food has some relevance for their disease activity were asked. The questions
were based on a questionnaire used in a population study of food intolerance in the UK (7) see appendix.

Questionnaires about irritable bowel syndrome (IBS) in Paper II
Questions about gastrointestinal symptoms were asked according to the Roma III criteria and subgroup according to the manual (146) see appendix.

Serum antibodies
Serum IgA and IgG antibodies to casein, β-lactoglobulin and α-lactalbumin were measured according to the manufacturer’s instructions (Phadia AB, Uppsala, Sweden).

IgG and IgA antibodies to gliadin (IgG-AGA and IgA-AGA) were measured with ELISA. IgA-endomyosium antibodies were measured using an indirect fluorescence technique (Kallestad, Diagnostics, Chaska, Minnesota, USA). IgA-antibodies to tissue-transglutaminase were measured with ELISA and the antigen from guinea pig was obtained from Sigma Chemical Co, St.Louis, Missouri, USA. All measurements were performed at the Department of Clinical Immunology at Uppsala University Hospital, Uppsala, Sweden.

HLA typing
The HLA-DQB1 typing was performed with PCR-SSOP using the Luminex flow bead platform (One Lambda Inc, Canoga Park, CA, USA) at the Department of Clinical Immunology at Uppsala University Hospital, Uppsala, Sweden.

Paper I – Gluten sensitivity in patients with primary Sjögren’s syndrome
Twenty patients (two males) with pSS according to the revised American Consensus Criteria (54) (Table 2) were included consecutively from the outpatient clinic at the Department of Rheumatology, Uppsala University Hospital. Patients with secondary pSS or previously diagnosed CD were excluded. The mean age of the patients was 56 years (range 34-74, median duration of sicca symptom nine years (rang 1-37). The results of laboratory tests did not reveal any signs of inflammatory activity. Eight of the patients had extra-glandular manifestations, the most common of which was arthritis,
and seven had other auto-immune diseases, the most common of which was thyroiditis. Three patients were being treated with corticosteroids in low doses and four with chloroquinephosphate.

All the participants were asked about gastrointestinal symptoms, the existence of eczema, allergic rhinitis, asthma or anaphylaxis and self-experienced adverse food or drug reactions before challenge studies. All the subjects were challenged with gluten and mucosal NO measurements were performed 15 h after the challenge. HLA typing was performed and antibodies to gliadin, endomysium and tissue transglutaminase were measured.

Paper II – Cow’s milk protein sensitivity assessed by the mucosal patch technique is related to irritable bowel syndrome in patients with primary Sjögren’s syndrome

Twenty-one patients (two males) with pSS according to the revised American Consensus Criteria (54) (Table 2) were included consecutively from the outpatient clinic at the Department of Rheumatology, Uppsala University Hospital. Patients with secondary pSS and IgE type of reactions to CM were excluded. Using partly the same cohort as in Paper I, the patients were challenged with CM protein and rectal mucosal reactivity was studied by measuring NO, ECP and MPO. The mean age of the patients was 56 (range 34-73) and the mean duration of sicca symptoms was nine years (range 1-37). Eight of the patients had extra-glandular manifestations. Three patients were being treated with corticosteroids in low dosages and four with chloroquine phosphate.

All the participants were asked about gastrointestinal symptoms, the existence of eczema, allergic rhinitis, asthma or anaphylaxis and self-experienced adverse food or drug reactions before challenge studies. Based on the high frequency of reported gastrointestinal symptoms, a questionnaire to support the diagnosis of IBS according to the Rome III criteria (146) was used in order to evaluate gastrointestinal symptoms. Serum IgA and IgG antibodies to casein, β-lactoglobulin and α-lactalbumin were also measured.
Table 2. Revised international classification criteria for Sjögren’s syndrome

I. Ocular symptoms: a positive response to at least one of the following questions:
1. Have you had daily, persistent, troublesome dry eyes for more than three months?
2. Do you have a recurring sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than three times a day?

II. Oral symptoms: a positive response to at least one of the following questions:
1. Have you had a daily feeling of dry mouth for more than three months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular signs - that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests:
1. Schirmer’s I test, performed without anaesthesia (< 5 mm in 5 minutes)
2. Rose bengal score or other ocular dye score (> 4 according to van Bijsterveld’s scoring system)

IV. Histopathology: In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score of >1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm2 of glandular tissue

V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result in at least one of the following diagnostic tests:
1. Unstimulated whole salivary flow (<1.5 ml in 15 minutes)
2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer

VI. Auto-antibodies: presence in serum of the following auto-antibodies:
1. Antibodies to Ro(SSA) or La(SSB) antigens, or both

Table 2 Revised rules for classification

For primary SS
In patients without any potentially associated disease, primary SS may be defined as follows:
a. The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive
b. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)
c. The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in a clinical-epidemiological survey

Exclusion criteria:
Past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency disease (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease, use of anticholinergic drugs (since a time shorter than 4-fold the half life of the drug)
Paper III – Self reported food intolerance and mucosal reactivity after rectal food protein challenge in patients with rheumatoid arthritis

A questionnaire (see appendix) about allergy and food intolerance and perceived connection between food ingestion and symptoms was sent by mail to patients (n=347) born in 1942-1983 and suffering from RA according to ACR criteria. (77) (Table 1) The patients were selected from the out-patient register at the Department of Rheumatology, University Hospital of Uppsala, Sweden.

A total of 27 subjects (5 males) underwent rectal challenges. The mean age in the challenge group was 50 years (range 28-60), with a mean disease duration of 14 years (range 1-46) and 14/27 had one or more swollen joints. Twenty-one of 27 were being treated with DMARDs, and 16/27 with corticosteroids. The mucosal synthesis of NO and the mucosal release of ECP and MPO were measured in order to study the mucosal reactivity. Serum IgA and IgG antibodies to casein, β-lactoglobulin, α-lactalbumin, gliadin, endomysium and tissue transglutaminase were analysed together with CRP, ESR and hemoglobin.

Paper IV – Cow’s milk protein and gluten – environmental factors to be considered in the pathogenesis of spondyloarthropathies

Fifty-eight adult patients (28 females) with SpA according to the European Spondylarthropathy Study Group (110) and modified New York criteria(111) (see page 35) for AS were investigated. Thirteen had AS, two had ReA, four had arthritis associated with IBD, three UspA, ten had sacroiliitis with our without peripheral arthritis, five had HLA B27-associated peripheral arthritis and twenty-one patients had PsA. The patients were included consecutively from the outpatient clinic at the Department of Rheumatology, Uppsala University Hospital. All the participants were asked about gastrointestinal symptoms, the existence of eczema, allergic rhinitis, asthma or anaphylaxis and self-experienced adverse food or drug reactions before challenge studies.

The mean age was forty-four years (range 19-71) and the median disease duration was eleven years (range 1-39). Twenty-three patients were being treated with corticosteroids, nineteen with DMARDs, three with biologics and thirty-three with NSAIDs on a regular basis. All the participants underwent rectal challenges with CM protein and gluten, while NO and MPO were measured to study the mucosal reactivity. Serum IgA and IgG antibodies to casein, β-lactoglobulin and α-lactalbumin were measured. In addition,
serum antibodies to gliadin, endomysium and tissue transglutaminase were analysed, together with CRP, ESR and hemoglobin. HLA typing was also performed.

**European spondyloarthropathy study group classification criteria** from Dougados et al. (110)

*Criteria*

Inflammatory spinal pain

_or_

Synovitis (asymmetric, predominantly in lower limbs)

_and any of the following:*

- Positive family history
- Psoriasis
- Inflammatory bowel disease
- Alternating buttock pain
- Enthesopathy
- Urethritis or cervicitis or acute diarrhoea occurring within one month before arthritis

*Sensitivity, 77%, specificity, 89% adding:*

- Sacroiliitis

*Sensitivity, 87%, specificity, 87%*

**Modified New York criteria, 1984** from van der Linden et al. (111)

*Criteria*

1. Low back pain with at least three months’ duration improved by exercise and not relieved by rest
2. Limitation of lumbar spine motion in sagittal and frontal planes
3. Chest expansion decreased relative to normal values for age and sex
4a. Unilateral sacroiliitis grade 3-4
4b. Bilateral sacroiliitis grade 2-4

*Definite ankylosing spondylitis if*

(4a OR 4b) AND any clinical criterion (1-3)

**Controls**

Eighteen adult healthy control subjects (13 males, 5 females) were recruited for the rectal challenge studies from students, nurses and their relatives. The mean age of the control subjects was 34 years (range 19-58). In Paper IV, sixteen additional control subjects were recruited, making a total of thirty-four (16 males, 18 females). They were recruited from nurses and their relatives. The mean age of the total control group was 35.5 years (range 19-64).
None of the included controls had a history of gastrointestinal complaints or symptoms of any kind of illness during the two months preceding the rectal challenge. All the control subjects had normal results following a wide range of blood screening tests at the time of the study and no rise in IgA antibodies to gliadin or endomysium.

Statistical methods

The mean and standard error of the mean (SEM) of patients and control groups are presented in figures, tables and text, unless otherwise stated. Non-parametric tests and comparisons between groups were performed using the Mann-Whitney U test. For comparisons within groups, the chi-square test was used. Comparisons of frequencies were made by Fisher’s test and correlations were sought using Spearman’s rank correlation. The statistical calculations were performed using the Statistica statistical package (Statsoft Inc., Tulsa, Oklahoma, USA).
Results

Paper I: Gluten sensitivity in patients with primary Sjögren’s syndrome

Five of twenty patients (25%) with pSS had a significant increase in the luminal release of NO after rectal gluten challenge compared with the controls and were classified as gluten reactive. Two patients had increases in NO above the +2 SD level of the controls but at the same level as one of the healthy controls and were classified as possibly gluten reactive. (Fig. 4) Seven of our patients had HLA type DQ2 and three had DQ8. All five patients with gluten reactivity were DQ2 positive. The two patients with possible gluten reactivity were DQ2 and DQ8 negative. We did not find any differences regarding auto-antibody patterns between the DQ2/DQ8-positive and DQ2/DQ8-negative patients.

Figure 4. Increase in rectal luminal nitric oxide (ΔNO) in patients with primary Sjögren’s syndrome after rectal gluten challenge. The level of two standard deviations (SD) above the mean of the control subjects (n=18) is marked with a line (81.1 ppb). HLA-DQ and/or DQ8 are marked with + if this/these genes/s is/are present and with – if they are absent.
Two of the patients with increased NO had antibodies to transglutaminase, with increased levels of rectal mucosal NO after gluten. One of these patients also had high IgA antibody levels against gliadin and endomysium and a duodenal biopsy showed a totally flat mucosa consistent with CD. The other patient with significant antibody titres to transglutaminase refused an endoscopic biopsy since she was already on a low-gluten diet. (Table 3)

Table 3. Serological findings in patients with pSS and gluten reactivity demonstrated by an increase in luminal NO after rectal gluten challenge. Abbreviations: pSS=primary Sjögren’s syndrome; NO=nitric oxide; EMA=endomysium antibodies; IgA-AGA= immunoglobulin A-antibodies to gliadin; IgG-AGA=immunoglobulin G-antibodies to gliadin; tTGA= tissue-transglutaminase antibodies

<table>
<thead>
<tr>
<th>Patient</th>
<th>ΔNO</th>
<th>IgA-AGA kU/L</th>
<th>IgG-AGA kU/L</th>
<th>tTGA U/L</th>
<th>EMA</th>
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<tr>
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<td>240</td>
<td>6</td>
<td>310</td>
<td>&gt;1/160</td>
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<td>&lt;72</td>
<td>&lt;21</td>
<td>&lt;6</td>
<td>0</td>
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</tbody>
</table>

Before gluten challenge, fifteen of the pSS patients reported gastrointestinal symptoms and eight reported intolerance to various food products. No correlation was found between gluten sensitivity and self-reported food intolerance or GI symptoms.
Paper II: Cow’s milk protein sensitivity assessed by the mucosal patch technique is related to irritable bowel syndrome in patients with primary Sjögren’s syndrome.

Eight of 21 (38%) patients with pSS had a mucosal reaction to CM defined at a level of two SD above the mean of the control subjects. The individual results for the increase in rectal mucosal NO and MPO concentrations in patients and controls are illustrated in the figure below. (Fig 5) No significant increase in ECP was seen in either patients or controls after challenge.

![Figure 5](image)

*Figure 5. Increase in luminal nitric oxide (ΔNO) and myeloperoxidase (ΔMPO) in patients with primary Sjögren’s syndrome (pSS) after rectal cow’s milk (CM) challenge. The level of two standard deviations above the mean of the control subjects (n=18) is marked by a line (ΔNO=123ppb and ΔMPO=49μg/L)*

The serum levels of IgA and IgG antibodies to casein, β-lactoglobulin and α-lactalbumin were similar in the patients with pSS compared with the controls. Nor was the sign of CM sensitivity linked to IgG/IgA antibodies to milk proteins. Allergic drug reactions were frequently reported (n=9, 43%). Gastrointestinal symptoms were reported by 16 patients (76%) and ten patients (48%) attributed these symptoms to an adverse food reaction to CM products and wheat gluten in particular. The majority of our patients who suspected CM intolerance were also reactive to rectal CM challenge. CM reactivity also appeared in patients who had no suspicion of food intolerance. Sixty-two per cent fulfilled the criteria for IBS according to the Rome III criteria. (146) All the patients with CM reactivity fulfilled the criteria for IBS. Those patients who were CM reactive were offered help by a dietician
to identify foods containing CM, seven patients excluded CM products for at least six months and six reported improved well-being and less abdominal problems assessed by symptoms reported according to the IBS protocol. One patient no longer fulfilled the criteria for IBS.

Table 4. Patients with pSS and mucosal sensitivity to CM, both CM and gluten and their relationship to IBS subgroups

<table>
<thead>
<tr>
<th>Sensitivity to;</th>
<th>IDS-D</th>
<th>IBS-M</th>
<th>IBS-C</th>
<th>IBS-U</th>
<th>CD</th>
</tr>
</thead>
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<td>2</td>
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<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CM+gluten</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Gluten</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
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Abbreviations: IBS-D=diarrhoea predominant subgroup, IBS-M=alternating diarrhoea-constipation subgroup, IBS-C=constipated predominant subgroup, IBS-U=undifferentiated subgroup, CD=coeliac disease, pSS=primary Sjögren’s syndrome, CM=cows’s milk protein

Paper III: Self reported food intolerance and mucosal reactivity after rectal food protein challenge in patients with rheumatoid arthritis

The questionnaire about allergy and food intolerance and the perceived connection between food ingestion and symptoms was sent to 347 RA patients and 241 patients replied, giving a response rate of 69%. Twenty-seven per cent of the RA patients reported food intolerance to various foods and, in particular, to CM and meat. Adverse reactions to wheat were also frequently reported. Forty-four per cent of the patients believed that food was relevant to their disease. The most common symptoms related to food were gastrointestinal complaints, followed by atopic symptoms and joint and muscle pain.

Twenty-seven RA patients, eight in the non-FI group and nineteen in the FI group, agreed to undergo a rectal challenge with CM and gluten. The baseline mean value for rectal luminal MPO in RA patients was significantly higher than in the controls, whereas the baseline mean value for rectal luminal NO was significantly lower. (Table 5)

Table 5. Baseline mean values with actual range in parentheses of rectal luminal MPO and NO in RA patients versus the control group. * p<0.05, ** p<0.01

<table>
<thead>
<tr>
<th></th>
<th>MPO μg/L</th>
<th>NO ppb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA patients</td>
<td>22(4-79)*</td>
<td>8(3-20)**</td>
</tr>
<tr>
<td>Controls</td>
<td>12(3-43)</td>
<td>24(6-90)</td>
</tr>
</tbody>
</table>
Compared with the controls, rectal challenges induced no significant increase in MPO, ECP and NO in the RA patient group, or in subgroups of patients with or without FI. However, nine of the RA patients (35%) had MPO values above the highest MPO value in the control group after challenge with gluten. After challenge with CM apparent increases in Δ-MPO were found in only three patients (12%). These patients had a self-reported FI. Marginally increased values above the highest MPO level in the controls were seen in six patients (23%) (Fig. 6). An increase in Δ–NO values after gluten challenge was seen in one patient and after CM challenge in three. One had a significant increase in ECP after CM challenge and had a reported allergy, as well as self-reported FI to CM.

**Figure 6.** The individual results for Δ MPO after challenge with cow’s milk protein and gluten in patients with rheumatoid arthritis and controls.

Abbreviations: MPO = myeloperoxidase, CM = cow’s milk protein

The serum levels of IgA and IgG antibodies to casein, β-lactoglobulin, α-lactalbumin and gliadin were similar in RA patients and controls and were not related to self-experienced adverse food reactions. IgA antibodies to endomysium and tTG were not found in any patients or controls. No relationship was seen between self-experienced adverse reactions to CM and gluten and mucosal reactivity to these proteins.
At baseline, the rectal mean NO values were significantly lower in the patient group (18 ±3 ppb p<0.05) compared with the control group (30±4 ppb). In the SpA patient group, the average increase in rectal NO (Δ NO) was after rectal challenge with CM and gluten significantly higher (p<0.001) compared with the Δ NO values obtained in the control group. Table 6

### Table 6. Δ NO values after challenge with cow’s milk protein (CM) and gluten in patients with spondyloarthropathies (SpA), SpA separated into SpA without psoriasis (non-PsA) and with psoriasis (PsA) and controls. Mean ±SEM *p<0.05, **p<0.01, ***p<0.001 (Mann-Whitney U-test)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>CM Δ NO ppb</th>
<th>Gluten Δ NO ppb</th>
</tr>
</thead>
<tbody>
<tr>
<td>All SpA</td>
<td>58</td>
<td>267±85***</td>
<td>38 ±9***</td>
</tr>
<tr>
<td>Non PsA</td>
<td>37</td>
<td>279±112***</td>
<td>36 ±10**</td>
</tr>
<tr>
<td>PsA</td>
<td>21</td>
<td>247±130***</td>
<td>42 ±18*</td>
</tr>
<tr>
<td>Control</td>
<td>34</td>
<td>-3±8</td>
<td>2 ±6</td>
</tr>
</tbody>
</table>

The individual values for Δ NO in the patients are shown in Fig 7. The patients were divided into PsA and non-PsA patients. They had similar increased Δ NO values for CM and gluten. (Table 6) Nor was any difference in Δ NO values seen between the subgroups of non-PsA patients (data not shown). The Δ MPO values after CM challenge were significantly (p<0.05) higher (89 ± 40 μg/L) in the NO reactive group compared with the Δ MPO value seen in the non-NO reactive group (50 ±21 μg/L).

No correlation was seen between the HLA genotype and CM and gluten reactivity. The number of patients treated with corticosteroids, DMARDs and NSAIDs did not differ in the food-reactive and non-reactive groups (Yates correlated chi-square test). No relation between food antigen reactivity and self-reported gastrointestinal symptoms, allergy and food intolerance was seen.
Figure 7. The individual results for \( \Delta \text{NO} \) after challenge with cow’s milk protein \((p<0.001)\) and gluten \((p<0.001)\) in patients with spondyloarthropathies and controls. The level of two SD above the mean for the control subjects \((n=34)\) is marked by a horizontal line.

Abbreviations: NO = nitric oxide, ppb = parts per billion, CM = cow’s milk protein
Food hypersensitivity in primary Sjögren’s syndrome

The findings in these two studies of food hypersensitivity to gluten and/or CM in patients with primary Sjögren’s syndrome are discussed in the context of a close association between coeliac disease and Sjögren’s syndrome and the patients’ experience of IBS-like gastrointestinal symptoms.

Gluten

The results in this study show that gluten induces an inflammatory response measured by the local production of NO after rectal provocation in a subset of pSS patients with the genetic susceptibility genes HLA DQ 2 and without antibodies to tTG, as seen in CD.

As illustrated by the “coeliac iceberg” in genetic susceptibility individuals, the mucosal damage develops gradually from CD latency to silent or early CD to villous atrophy. The cause of the development from having the genetic susceptibility to silent or active disease is not known. (14) Perhaps separate genetic factors may regulate the capacity for gluten sensitisation and the susceptibility to develop enteropathy, (182, 183) or perhaps gluten sensitivity constitutes a separate entity. Our findings of mucosal reactivity after rectal challenge with gluten might reflect gluten sensitivity, defined by Marsh as “a state of heightened immunological responsiveness to ingested gluten in genetically susceptible individuals”. (15)

This immunological responsiveness to dietary gluten is illustrated in healthy first-degree relatives of coeliac patients, where about 30% had a high density of gut mucosal intraepithelial γ δ T-cell receptors bearing lymphocytes, despite normal villous architecture. (184) These intraepithelial γ δ lymphocytes are regarded as a marker of potential CD and remain elevated, even if they are lower, on a gluten-free diet (185) and, in pSS patients, these lymphocytes are also found. (70) Gluten sensitivity has also been shown in almost half of first-degree relatives to coeliac patients or non-atrophic siblings of coeliac children after rectal gluten challenge. (186)

Data regarding the concept of gluten sensitivity with or without enteropathy have been presented in several auto-immune diseases. After rectal gluten challenge and subsequent rectal biopsies, a mucosal response was observed in a subset of HLA DQ-2 positive children with diabetes mellitus without
entheropathy. (187) The same pattern has also been found in patients with primary biliary cirrhosis. (188) Hadjivassiliou suggested that a mechanism of this kind lay behind cerebellar ataxia, where anti-gliadin antibodies cross-react with epitopes on Purkinje cells. The majority of these patients share the same genetic susceptibility genes for coeliac disease; HLA DQ2 and DQ8. (30) The same group has also detected the presence of intestinal IgA deposits targeted against type 2 tissue transglutaminase (TG2) in the gut and brain of patients with gluten ataxia in a similar fashion to that in patients with CD (189) and dermatitis herpetiformes (190). They suggest that this auto-antibody could trigger the disruption of the blood-brain barrier, thereby enabling the anti-gliadin antibodies to react with Purkinje cells. (31) Hadjivassiliou also suggests that gastrointestinal symptoms are absent in most patients with CD, that the definition of gluten sensitivity cannot be based on the presence of only an enteropathy and that genetic susceptibility may be an important additional marker of gluten sensitivity. Recently, in a review article on gluten sensitivity, Verdu et al hypothesise that gluten may be one of the underlying trigger mechanisms for gastroenterological symptoms in some patients with IBS who would otherwise be considered for a diagnosis of IBS. Moreover, this gluten sensitivity could explain a part of the spectrum in IBS. (191)

HLA DQ2 and DQ8 are strongly correlated to the development of CD (13) and, in our group of pSS patients, 60% were either DQ2 or DQ8 positive compared with 25-30% of the population in the western hemisphere. (11, 12) The finding that all our pSS patients that we defined as definitely gluten reactive were HLADQ2 strengthens our hypothesis that the gluten reactivity found after challenge represents gluten sensitivity. This suggests that the same abnormal mucosal inflammatory response to rectal gluten as seen in CD is also present in a subset of pSS patients with HLA DQ2/DQ8 haplotypes. We are unable to define the risk that this group of patients will develop CD, but, expressed in guarded terms, a follow-up is recommended. There is one follow-up study of first-degree relatives of patients with CD, previously characterised as gluten sensitive. The authors were able to diagnose an additional 5% of new CD patients, all DQ2 positive, and recommended follow-up in the subgroup of relatives with gluten sensitivity. (192) Among our pSS patients, we found one patient with villous atrophy and antibodies to tTG, in accordance with CD. This patient had no anaemia and no signs of diarrhoea and malabsorption. Another gluten-reactive patient with elevated tTG levels could possibly also have CD. The finding of one definite and one possible CD in this cohort of twenty patients illustrates that we should be alert and consider the possibility of CD in pSS patients and this corresponds with previously noted associations between pSS and CD.

In the other patients, no elevated antibodies to tTG were seen. One reasonable explanation could be that the observed mucosal reactivity could reflect an innate immune response. This is supported by observations that
certain gluten peptides have the ability to induce the direct activation of innate systems through the activation of macrophages (193) and dendritic cells, (194) and may further be involved in epithelial cell destruction through intra-epithelial lymphocytes. (195) Mauri et al. have shown that cells in the innate system recognise gluten peptides directly via pattern recognition receptors and release cytokines that drive adaptive responses but only in HLA DQ2-positive individuals. (26, 196) An unspecific response to gluten may also contribute to reported signs of small bowel mucosal inflammation in patients with pSS without CD. (70) Another possible link between pSS and CD is the observation that patients with pSS and DQ2 have alterations in circulating T-cell subsets similar to those found in coeliac patients, suggesting that they are immunologically more active. (197)

The question whether gluten could be directly involved in the pathogenesis of various autoimmune diseases in genetically susceptible individuals such as pSS has been discussed. Moreover, it has been argued that the prevalence of associated auto-immunity in CD is related to the duration of exposure. (198) This has however, been contradicted of others, arguing that it instead increases with age. (199) The possibility that the introduction of an early gluten-free diet could influence the development of autoimmune disease, such as type 1 diabetes mellitus and CD, is interesting, but the question is still unanswered. (22, 24, 187, 200, 201)

The value of population screening for undetected CD is the subject of debate. Currently, the approach is to focus on case-finding by measurement of antibodies to tTG, especially by screening in high-risk groups including relatives of CD patients with pSS, IBS and diabetes mellitus. In pSS patients, this was recently stressed by Moutsopoulos et al. in a review article. (16, 202-204) However, antibodies are undetectable in gluten-sensitive patients or patients with latent CD and a gluten endoscopic biopsy and/or DQ 2 typing are then suggested as tools to find these individuals. (205) Perhaps the mucosal path technique could be of value in this respect, as indicated in our study. There is no consensus on the remaining question of whether patients who do not fully meet the criteria for CD should be recommended a gluten free diet.

Cow’s milk protein

The findings in this study of a high prevalence of reported various allergic manifestations (62%) and allergic drug reactions (43%), in particular to antimicrobials and salicylates, is in keeping with previous reports on pSS patients, especially anti-Ro-positive patients. (59, 60) More than half our pSS patients with reported allergic drug reactions were anti-Ro positive. This tendency towards drug reactions, particularly to antimicrobials, is also recognised from clinical practice. The prevalence of all kinds of adverse reaction to food reported by our pSS patients was high (48%) compared with one
population study from the UK in which 20% of the population attributed atopic, intestinal and joint symptoms and migraine to certain foods, including cow’s milk products and wheat. (7) A slightly higher number was reported in a representative cross-sectional study in Germany where the prevalence of adverse food reactions was estimated to be 35% of the population. (6)

Another, perhaps unrecognised finding among our patients was the high incidence of reported intestinal symptoms (76%) and, in particular, IBS-associated symptoms (62%). The literature on intestinal involvement in pSS is sparse, but IBS has been reported to be associated with sicca complex; sicca syndrome without an auto-immune component, a central disturbance of the neuroendocrine system, was suggested as the mechanism. (206) In this study, we found eight patients with a mucosal reaction to CM after provocation and they all fulfilled the criteria for IBS according to ROME III, with diarrhoea or alternating diarrhoea-constipation as the predominant IBS subgroups.

There are reports of food-induced aggravation of IBS and improvement after dietary exclusion and one of the offenders is CM products. (151, 207-210) In a questionnaire study about perceived symptoms related to food, 30% reported moderate to very severe symptoms after milk intake and 23% avoided milk completely. (211) In particular, some patients with diarrhoea-predominant or alternating bowel habits responded to an exclusion diet. (151) In our pSS patients, the foods reported to cause adverse reactions were CM and gluten in particular and the majority who suspected CM intolerance were actually CM reactive. An improvement with fewer abdominal problems assessed according to the IBS protocol was noted in six of seven of our pSS patients and one no longer fulfilled the criteria for IBS. It therefore appears that a subgroup of IBS and pSS patients share some features regarding intolerance of CM.

We do not know the mechanism behind this food reactivity, but it is most probably related to the innate immune system, as there is no increase in serum IgG or IgA antibodies to CM and no association with HLA alleles DQ2/DQ8. This suggests that other additional genes may be found in CM sensitivity or that there is a general sensitivity to food and not food intolerance confirmed by DBPCFC, as has been proposed by Simrén, M et al. This explanation is supported by reported symptoms after the majority of the foods they included in the questionnaire. (211)

As noted from the gluten provocation study, the subgroup with gluten sensitivity share the CD susceptibility genes and three CM-reactive patients from this study were also gluten sensitive, resembling the mucosal inflammatory reaction after rectal CM and gluten challenge in CD patients. Recently, Kristánsson et al. reported an induced inflammatory reaction after rectal CM challenge in 40% of adult CD patients. These patients were on a gluten-free diet and had no increase in serum antibodies to t-TG and gliadin
and CM proteins. The inflammatory reaction was similar to that produced by gluten. (181) These findings have resulted in the hypothesis that CM sensitivity may contribute to persistent intestinal symptoms in some coeliac patients, despite a gluten-free diet, as some adult patients with CD do not restore their gut lesions and the contribution of other food antigens has been discussed. (212) Among other food components, it has been suggested that CM protein may induce food reactivity in some CD patients. (213)

The fact that all eight pSS patients with mucosal sensitivity after CM challenge fulfilled the ROME III criteria for IBS patients may suggest that food allergy to CM could play a role in their intestinal symptoms. The existence of a low-grade mucosal inflammation with the activation of the immune system in some patients with IBS and pSS, (70, 151) thereby creating a state of hypersensitivity, suggests that food allergy could be one of the contributory factors to persistent inflammation. A knowledge of the tendency towards various allergic manifestations in pSS patients lends further support to our suggestion that gluten and CM protein could reflect an allergic hypersensitivity and contribute to their gastrointestinal symptoms.

Food hypersensitivity in patients with RA

The prevalence of self-reported food intolerance in our RA patients corresponds with the prevalence of self-reported adverse reactions found in Europe. (5-7, 214) Compared with the population in the UK, (7) adverse reactions to CM protein and wheat were more frequently reported, but it is worth issuing a few words of warning that this might be a biased, as it has previously been suggested that dairy protein and gluten have a negative influence on arthritic symptoms. (101) In our study, gastrointestinal symptoms were the most commonly reported, followed by atopic symptoms and joint and muscle pain. In the UK study, the most frequently reported symptom was food allergy symptoms. Nearly half our patients believed that food affected their disease and symptoms. Knowing this, it is clear that the patients test dietary manipulations. Our aim was to identify the individuals that might benefit from diet, with the idea that sensitivity to the persistent intake of food antigens could induce a gut mucosal inflammation.

We identified a small fraction with an apparent release of MPO or NO after challenge with CM protein and another fraction with moderately increased values after challenge with CM and gluten. Since the majority of our patients are treated with corticosteroids and DMARDs, this may have reduced the rectal mucosal reactivity. Studies of patients with asthma bronchiale reveal that corticosteroids are potent inhibitors of NO synthesis; this could have reduced the production of NO. (215) Furthermore, the RA patients had significantly lower basal levels of NO compared with the controls, even considering the possibility of medication influence. The ideal situation
for studying the influence of food proteins would be before the administra-

tion of any DMARDs or corticosteroids, but, as it takes several weeks to

perform these provocation studies, this would be difficult to accomplish. The

perceived self-reported adverse reactions to CM and gluten was not related

to the challenge-induced sensitivity. In population studies, there is a large
discrepancy between self-reported and objective assessments of food allergy

and our data agree with these findings. One patient had an increase in ECP

after CM challenge and had a self-reported allergy and food intolerance to

CM, most probably reflecting eosinophil activation and perhaps an IgE-

mediated allergy.

Various diets have been tested and reported in clinical studies with a

beneficial effect in some patients, but dietary advice has not been extended
to RA patients as a group. One of the problems is identifying the individual

patients who could benefit from a change of diet. Previous attempt to iden-
tify food-sensitive RA patients by measuring food-specific IgG and IgA
antibodies in circulation have been disappointing and the results conflict.

(214) However, by measuring IgG, IgA and IgM antibodies to food antigens

in perfusion fluid from the jejunum in patients with RA, increased food-
specific activity and the cross-reactivity of IgM and IgA antibodies have

been found. (216) Immune activation in the gut intestinal immune system is

probably not reflected in blood. In accordance with previous attempts, we

found no elevated serum antibodies to cow’s milk and gluten in our patients.

The increase in baseline MPO mucosal release that we observed in our

RA patients could reflect a subclinical inflammation in the rectum, or the

fact that, in some patients, gluten and CM protein contribute to inflamma-
tion. Inflammation of the small intestine, independent of NSAIDs has been

reported in RA (217-219) and there are some studies indicating immune

activation in the gut, (220, 221) as well as suggestions of an accumulation of
gut-derived lymphocytes in the inflamed joints of RA patients. (222, 223)
However, drug treatment and especially NSAIDs could also contribute to
inflammation.

It is estimated that the overall genetic influence behind the development

of RA contributes to 60% (86), leaving a large percentage of as yet unknown

factors. The environmental factors that has been suggested as initiating im-
mune reactions and contributing to the development of RA inculde hor-
mones, such as estrogen, smoking, occupational exposure, primarily silica,
infections, such as Epstein-Barr virus, and diet. (224) Even events during
fetal life are discussed and it appears that greater growth and less infectious
exposure increases the risk of developing RA. (225) There are difficulties in
identifying the environmental trigger, as the importance of the environ-
mental trigger is probably seen before the disease onset. These problems
exist in diet studies, where there is a lack of prospective studies that can
answer the question of whether variations in diet precede the disease, and
because of the fact that there are many confounding problems in diet analy-
ses that could mask real associations. (224) However, one established risk factor is smoking. (226) In a subset of rheumatoid-factor positive and/or anti-CCP-positive patients with HLA-DR SE genes, the risk of developing RA is higher and it is even higher if it is combined with another genetic risk allele of PTNPN22. This indicates that a different subset could have different susceptibility genes and different environmental triggers. (227) From clinical practice, the manifestations of the RA disease show variability and the possibility that there are different subsets of patients is a reality. Food antigens could perhaps interact with additional genes and induce immune reactions in certain subsets of RA patients. This is supported by our reports that mucosal reactions, albeit low, are found. Perhaps other foods in addition to CM protein and gluten, to which gut a luminal response has been observed, are important.

Another hypothesis relating to the aetio-pathogenesis of RA and the role of the normal intestinal bacteria has been put forward by Toivanen. He suggests that the normal microbiota degradation products from bacteria can induce arthritis in interaction with yet undiscovered genes, probably encoding proteins in the intestinal epithelium. This could result in intestinal colonisation by arthritogenic bacteria and eventually lead to synovial inflammation. The fact that the intestinal microbiota differs between subjects and that changes in diet induce alterations in the microflora leads to another possible explanation of the involvement of food in RA. (228)

Food hypersensitivity in patients with SpA

In patients with SpA, including AS, ReA and PsA, we found a pronounced enhanced rectal NO production after challenge with gluten (19%) and, in particular after CM challenges (26%). Compared with the pSS and RA patients, the reactions were far more enhanced, raising questions about the mechanism that is involved. In this respect, the reports of barrier dysfunction in patients with SpA, IBD, CD and diabetes mellitus, as well as in their healthy first-degree relatives, are interesting. The observation that increased permeability precedes the development of the disease, as has been shown in IBD and CD is even more intriguing. Moreover, mutations in genes that appear to be involved in barrier function have been reported in association with Crohn’s disease and CD. (229) In CD, this could be one explanation of the way the relatively large peptide, gliadin, is able to cross the epithelium, (139) and, in IBD, the translocation of bacteria.

We suggest that the increased high NO levels after challenge with CM or gluten in our SpA patients are mediated through inflammation. In this way, the increased mucosal release of MPO, would be derived from the neutrophil granules. (168) We are unable to say which cells are responsible for the NO production, but it is reasonable to suppose that the cellular source is the en-
dothelium, supported by observations in active ulcerative colitis where high levels of iNOS are present, in particular in the colonic epithelium. (179) The inducible NOS, expressed in almost all cells, is induced during inflammation and produces NO in large amounts for as long as five days. (230, 231) Furthermore, experiments with isolated enterocytes can induce them to express iNOS and produce NO after endotoxin or cytokine challenge. (232)

The dual role of NO in the gut is a matter of controversy. (231) On the one hand, NO performs several protective functions in the gastrointestinal tract; the stimulation of mucus secretion, the resistance of epithelial cells to injury, the inhibition of adherence of leukocytes to the mucosa and the down-regulation of the release of inflammatory mediators. (233) In addition, experiments with NOS inhibitors lead to increased permeability. The significantly lower values of NO at baseline in our study could thus be attributed to the impairment of cNOS activity maintaining mucosal integrity. On the other hand, NO could also contribute to the inflammation seen in intestinal diseases such as IBD and coeliac disease. (231, 234) The protective role of NO in the gut is believed to be regulated by the cNOS and the deleterious role of iNOS, depending on the amount, duration and where iNOS is persistently up-regulated. (175) When NO is overproduced, it may cause damage to the gut wall. Endotoxin can induce exaggerated NO production and impaired gut barrier function. The mechanisms involved have indicated that NO is able directly to increase enterocyte permeability by reducing cellular ATP levels, deranging the cytoskeleton and affecting tight junctions. (232, 235-237) A far more toxic oxidant that promotes the loss of barrier function is peroxynitrite (ONOO\(^-\)), which is formed when NO reacts with superoxide (\(^{O_2-}\)), produced by neutrophils and macrophages in inflamed conditions. Peroxynitrite can cause the apoptosis of the epithelial cells and direct administration is reported to result in colitis, while treatment with peroxynitrite scavengers reduces colon damage in experimental colitis. (175, 231, 238-240) We suggest that the large amounts of NO produced after CM and gluten challenge affect the barrier function either directly or via peroxynitrite. The mucosal release of MPO lends support to the belief that the superoxide is derived from activated neutrophils.

Another aspect is the concept of molecular mimicry. Although it is known that certain bacterial species induce arthritis, researchers have been unable to find bacteria in the joint cavity, but DNA fragments have been found. (241, 242) The concept of “molecular mimicry”, whereby microbial antigens or epitopes are postulated to resemble self-antigens, has been the subject of ongoing debate. In AS, Ebringer argues that molecular mimicry between klebsiella and the HLA B27 molecule, as well as the spinal collagen types I,II and IV, indicates a pathological mechanism involving auto-immunity. He also recommends a low-starch diet to prevent the intestinal growth of bowel bacterial flora, such as klebsiella.(114) In this respect, a recent observation indicates that human chondrocytes in AS can act as non-professional
APCs and stimulate peptide-specific cytotoxic CD8+ T cells to destroy tissue. (243) However, others claim there is no clear proof of molecular mimicry in human diseases, (115, 244) while still others suggest an impact on an already existing auto-immune process, rather than breaking of tolerance from the beginning. (245)

Moreover, it has been suggested that food proteins and molecular mimicry are involved in some auto-immune diseases. Hadjivassiliou has suggested that gluten plays a role in cerebellar ataxia. (31) In diabetes mellitus type 1, CM has been proposed as a trigger for beta-cell auto-immunity, (246) while experimental auto-immune uveitis in rats has been induced by the administration of casein with molecular mimicry between casein and retinal antigens. (247)

As suggested by Medding, barrier dysfunction and, as a result, the translocation of luminal antigens to abrogate oral tolerance leading to inflammation could match the interpretation of our data most effectively. In some SpA patients, primarily with an affected gut barrier, CM and gluten induce NO production that increases barrier permeability and opens the way for the translocation of luminal antigens leading to inflammation. However, we can only speculate about whether the “breach” of the intestinal barrier by non-self-antigens could lead to an immune response targeting extra-intestinal organs. This has, however, been suggested in diabetes mellitus type 1, (139, 248) supported by experiments in BB rats which spontaneously develop diabetes. They develop increased permeability before pancreas islet destruction and manifest disease and (249) zonulin, a protein regulating intracellular tight junctions, has been implicated in causing abnormality in antigen delivery. (250) A potential antigen trigger could be gluten, as has been identified in a NOD mouse model (251) and suggested in children with DM and HLA DQ2, (252) and a recent review has addressed this question. (253)

NSAIDs, a drug commonly used in SpA patients, can also contribute to intestinal inflammation, as well as to increased intestinal permeability. (254) However, we found no correlation between CM and gluten reaction and the use of NSAIDs and, as increased intestinal permeability has also been seen in first-degree relatives of patients with SpA, as well as in patients not taking NSAIDs, this suggests that a defective barrier function is not only a consequence of NSAIDs. (135, 137)

The elevated serum levels of IgG antibodies to β-lactoglobulin and α-lactalbumin found in our group of patients with SpA could be related to the affected intestinal mucosa, causing an increased influx of undegraded proteins and not reflecting an adaptive immune response. This has been observed in CD (255) and IBD. The mucosal inflammatory reaction found after CM and gluten challenge is most probably mediated through the innate immune system, due in part to the rapid onset of the inflammatory response and to the fact that peptide fragments from bovine casein are chemotactic for
neutrophils and macrophages (256) and that gluten can cause the maturation of APC and the attraction of leukocytes. (194)

In our patients, we did not find any elevated levels of IgA anti-tTG antibodies and no correlation of HLA DR2 or DR8 and we conclude that the elevated levels of NO after challenge with gluten were not consistent with CD, as IgA anti-tTG antibodies are very sensitive markers, (257) together with HLA DR2 or DR8, for the diagnosis of coeliac disease.

Our data open the possibility that, by inducing a strong mucosal NO synthesis, dietary antigens contribute to barrier dysfunction in this group of patients and this suggests that increased intestinal permeability facilitates enhanced luminal antigen uptake and facilitates interactions between luminal antigen and the immune system. The luminal antigen could be a bacterium or perhaps gliadin or casein, which are known to activate antigen presenting cells.

Clinical implications

Some patients were willing to test whether the withdrawal of the offending food could have a positive impact on their disease. They were offered help by a dietician and a few patients responded dramatically. There are only anecdotal reports to suggest that, in some patients, food protein could possibly contribute to persistent chronic inflammation

For example, one of the PsA patients with a strong mucosal reaction to CM protein showed both a clinical and an objective improvement after CM withdrawal.

He presented at the age of 14 with psoriasis and, for the last eight years, also with peripheral arthritis. During the last year, both joint and skin manifestations worsened, with extensive skin lesions and arthritis in several joints, and he also experienced periods of gastrointestinal involvement with diarrhoea. He had no antibodies to tTG and gliadin and no antibodies to CM proteins.

Three months after CM withdrawal, his ESR had dropped from 48 to 13, his haemoglobin had increased from 128 to 164 and his albumin from 32 to 39. The diarrhoea had also improved, together with the arthritis, but the skin showed a marginal improvement. Before and six months after a CM exclusion, endoscopic biopsies were taken. A moderate infiltration of lymphocytes and eosinophil granulocytes was seen in lamina propria and the epithelium, as well as broadened villi, but after six months, after the inflammatory cell infiltration was less prominent and the villi were more gracile.
Figure 8. Biopsy from the duodenum before CM withdrawal, showing variable villus abnormalities with shortened and broadened villi and, in the interstitium, mild chronic inflammatory changes. There was no increase in intra-epithelial lymphocytes.

Attempts to identify a possible role for dietary factors in the development of auto-immune diseases have not succeeded. The same thing applies to the selection of individuals that could benefit from diet regimens. (200) Based on our present results, we suggest that a demonstration of biological sensitivity to gluten or other food antigens could be of potential value when it comes to identifying patients at risk of developing not only CD but also other auto-immune disorders possibly also induced by dietary exposure. Our expectation is that the rectal challenge procedure could be of clinical assistance in identifying foods causing non-IgE-mediated adverse reactions. In the future, more systematic diet studies, together with challenge provocations, could prove useful.
Conclusion

Based on the findings in Papers I-V, the following conclusions have been drawn.

In some patients with primary Sjögren’s syndrome, a mucosal reactivity is observed after rectal challenge with gluten. These patients have no detectable serum antibodies to gluten or transglutaminase, as might be expected in coeliac disease. The gluten-reactive patients share the genetic susceptibility haplotypes HLADQ2 or DQ8 with coeliac patients, thereby indicating gluten sensitivity in these patients.

A high prevalence of gastrointestinal symptoms was observed in patients with pSS and more than half the patients fulfilled the criteria for IBS. After challenge with cow’s milk, we found mucosal reactivity and no elevated serum antibodies to CM protein in 38% of the patients with pSS. All the patients with CM reactivity fulfilled the IBS criteria.

In patients with RA, we found that 27% reported adverse food reactions, which is similar to the prevalence in population studies. In the subsequent rectal provocation study, we found a group of patients with mucosal reactivity to CM and gluten which did not correlate to self reported adverse reactions and to elevated serum antibodies to cow’s milk protein.

CM sensitivity was seen in 26% of patients with SpA after rectal challenge with CM. After gluten challenge, sensitivity was seen in 19%. The NO responses were stronger after CM than after gluten and much stronger than the responses in the group of pSS and RA patients. We suggest that, by inducing a strong mucosal NO synthesis, gluten and CM protein contribute to increased intestinal permeability and open the way to the translocation of gut constituents.
Acknowledgements

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Frågeformulär angående födoämnesöverkänslighet

Kön :
- Man
- Kvinna

Vilken reumatisk sjukdom lider Du av ?
- Reumatoid artrit
- Bechterews sjukdom
- Reaktiv artrit
- Psoriasis artrit
- Sjögrens syndrom
- SLE
- Sklerodermi
- Annan ledsjukdom ................

Tål du all mat?
- Ja
- Nej

Vad tål du inte?
- Mjölk/Mejeripro dukter
- Ägg
- Vete, råg, korn, havre
- Fisk
- Jordnötter
- Soja
- Skaldjur
- Choklad
- Ost
- Nötkött
- Griskött
- Apelsin
- Tomat
- Annat: ......................

Vilka symtom?
- Magbesvär
- Diarré
- Utslag
- Klåda
Andfäddhet/Astma
Klåda i halsen
Hösnuva
Allergichock
Ledbesvär
Muskelvärk
Trötthet
Annan:……………………
Hur fort får Du besvär efter matintag?
Inom 1 timme
2-4 timmar
4-24 timmar
Dagar
Har Du provat vegetarisk kost?
Nej
Ja
Blev du bättre?
Nej
Ja
Tror Du att kosten har betydelse för Din sjukdom?
Nej
Ja
Har Du allergiska besvär (astma, hösnuva, rinnande ögon, eksem)?
Nej
Ja
Om ja, mot vad?
Pollen
Damm
Pålsdjur
Nickel
Läkemedel, vilka? ..............................
Nötter
Skaldjur
Annan:.................................
Har Du haft eksem som barn?
Nej
Ja
Har Du eksem nu?
Nej
Ja
Finns astma, hösnuva eller annan allergi hos nära släktingar (föräldrar, sys-
kon, barn)?
Nej
Ja
Röker Du?
- Nej
- Ja
- Har slutat
Tålde Du som barn all mat?
- Nej
- Ja
Om nej, jag reagerade som barn mot:
- Mjölkprodukter
- Komjölk
- Annat:..................  

Tack för Din medverkan! Var god returnera enkäten i bifogad kuvert inom 14 dagar
**Figure 9. Protocol for diagnosis of IBS**

<table>
<thead>
<tr>
<th>IBS Module</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. In the last 3 months, how often did you have discomfort or pain anywhere in your abdomen?</td>
<td>☐ Never  ➔  ☐ Less than one day a month  ☐ One day a month  ☐ Two to three days a month  ☐ One day a week  ☐ More than one day a week  ☐ Every day</td>
</tr>
<tr>
<td>2. For women: Did this discomfort or pain occur only during your menstrual bleeding and not at other times?</td>
<td>☐ No  ☐ Yes  ☐ Does not apply because I have had the change in life (menopause) or I am a male</td>
</tr>
<tr>
<td>3. Have you had this discomfort or pain 6 months or longer?</td>
<td>☐ No  ☐ Yes</td>
</tr>
<tr>
<td>4. How often did this discomfort or pain get better or stop after you had a bowel movement?</td>
<td>☐ Never or rarely  ☐ Sometimes  ☐ Often  ☐ Most of the time  ☐ Always</td>
</tr>
<tr>
<td>5. When this discomfort or pain started, did you have more frequent bowel movements?</td>
<td>☐ Never or rarely  ☐ Sometimes  ☐ Often  ☐ Most of the time  ☐ Always</td>
</tr>
<tr>
<td>6. When this discomfort or pain started, did you have less frequent bowel movements?</td>
<td>☐ Never or rarely  ☐ Sometimes  ☐ Often  ☐ Most of the time  ☐ Always</td>
</tr>
<tr>
<td>7. When this discomfort or pain started, were your stools (bowel movements) looser?</td>
<td>☐ Never or rarely  ☐ Sometimes  ☐ Often  ☐ Most of the time  ☐ Always</td>
</tr>
<tr>
<td>8. When this discomfort or pain started, how often did you have harder stools?</td>
<td>☐ Never or rarely  ☐ Sometimes  ☐ Often  ☐ Most of the time  ☐ Always</td>
</tr>
<tr>
<td>9. In the last 3 months, how often did you have hard or lumpy stools?</td>
<td>☐ Never or rarely  ☐ Sometimes  ☐ Often  ☐ Most of the time  ☐ Always</td>
</tr>
<tr>
<td>10. In the last 3 months, how often did you have loose, mushy or watery stools?</td>
<td>☐ Never or rarely  ☐ Sometimes  ☐ Often  ☐ Most of the time  ☐ Always</td>
</tr>
</tbody>
</table>

**Alternative scale:**

- ☐ Never or rarely
- ☐ About 25% of the time
- ☐ About 50% of the time
- ☐ About 75% of the time
- ☐ Always, 100% of the time
C1. Irritable Bowel Syndrome

Diagnostic Criteria*

Recurrent abdominal pain or discomfort** at least 3 days/month in last 3 months associated with two or more of criteria #1 - #3 below:

1. Pain or discomfort at least 2-3 days/month (question 1>2)
   For women, does pain occur only during menstrual bleeding? (question 2=0 or 2)
2. Improvement with defecation
   Pain or discomfort gets better after BM at least sometimes (question 4=0)
3. Onset associated with a change in frequency of stool
   Onset of pain or discomfort associated with more stools at least sometimes (question 5=0), OR
   Onset of pain or discomfort associated with fewer stools at least sometimes (question 6=0)
4. Onset associated with a change in form (appearance) of stool
   Onset of pain or discomfort associated with looser stools at least sometimes (question 7=0), OR
   Onset of pain or discomfort associated with harder stools at least sometimes (question 8=0)

* Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis

**“Discomfort” means an uncomfortable sensation not described as pain.

In pathophysiology research and clinical trials, a pain/discomfort frequency of at least two days a week is recommended for subject eligibility.

Pain or discomfort more than one day per week (question 1>4)

Criteria for IBS-C

(question 9=0) and (question 10=0)

Criteria for IBS-D

(question 9=0) and (question 10=0)

Criteria for IBS-M

(question 9=0) and (question 10=0)

Criteria for IBS-U

(question 9=0) and (question 10=0)

Figure 10. Protocol for interpretation of IBS symptoms
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