## SMALL INTESTINE

# Gut mucosal granulocyte activation precedes nitric oxide production: studies in coeliac patients challenged with gluten and corn

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**Background and aims:** To elucidate the dynamics of nitric oxide (NO) production induced by rectal gluten challenge and the relation between NO production and mucosal granulocyte activation.
**Subjects and methods:** Release of rectal NO was measured in 13 patients with coeliac disease and in 18 controls before and after rectal wheat gluten challenge. Rectal gas was collected with a rectal balloon using a newly developed instrument/technique, the ''mucosal patch technique''. The instrument allows simultaneous measurements of concentrations of granulocyte mediators in the rectal mucosa. We measured myeloperoxidase (MPO), eosinophil cationic protein (ECP), and histamine. For comparison, we made similar measurements after corn (maize) gluten challenge.
**Results:** In all coeliac patients rectal NO concentration increased after gluten challenge and reached a peak after 15 hours (mean 9464 (SEM 2393) parts per billion (ppb); range 250–24982). The maximum MPO and ECP increase occurred five hours after challenge. A correlation was found between mucosal MPO and NO production at 15 hours. Six of the patients showed an increase in NO production 15 hours after rectal corn gluten challenge but this was much smaller than after gluten challenge. No increases were seen in the control group after either challenge.
**Conclusion:** Mucosal activation of neutrophils and eosinophils precedes pronounced enhancement of mucosal NO production after rectal wheat gluten challenge in patients with coeliac disease. Some of our coeliac patients displayed signs of an inflammatory reaction, as measured by NO and granulocyte markers, after rectal corn gluten challenge.

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N itric oxide (NO) production is part of the inflammatory process but its role is uncertain. Some studies indicate a protective role[1][2] and others a harmful role.[3] The dynamics of NO production during acute inflammation have not been elucidated previously. NO is synthesised from L-arginine by the enzyme NO synthase (NOS),[4] which exists in three isoforms: NOS I (also known as nNOS), NOS II (also known as iNOS), and NOS III (also known as eNOS).[5] NOS II is the major inducible isoform and may become activated as part of immune and inflammatory responses and produces great amounts of NO as long as the enzyme is activated. In order to elucidate NO production after antigen stimulation, we have studied the gut mucosal reaction to gluten in patients with coeliac disease (CD). Increased luminal NO concentrations in the small intestine have previously been reported in patients with untreated CD.[6] Although this disease affects predominately the proximal intestine, immune cells reactive to gluten antigens appear to be distributed along the mucosa of the entire gastrointestinal tract.[7–9] CD may be considered a prototype T cell mediated disease, being strongly associated with HLA class II and a triggering antigen (wheat gluten and related cereals).[10] Small intestinal inflammation usually remits on a strict gluten free diet. A novel aspect of the pathogenesis of CD has recently been described by Maiuri and colleagues.[11] Their data suggest that the adaptive immune reaction induced by gluten can be further driven by gliadin peptides by the less specific innate immune system.

Rectal challenge with gluten induces in coeliac patients a delayed rectal inflammatory reaction, which we studied using a newly developed technique (mucosal patch technique) designed to measure inflammatory substances in the gut mucosa.[12] This technique also allows simultaneous

collection of rectal luminal gas for NO measurements. In the present study, we attempted to elucidate the dynamics of NO production after rectal challenge with gluten as well as the kinetic relationship to mucosal granulocyte activation, defined by luminal concentrations of myeloperoxidase (MPO) and eosinophil cationic protein (ECP).

## PATIENTS AND METHODS
### Study subjects
The study comprised 13 patients (six males, seven females), mean age 48 years (range 25–68), and 18 healthy control subjects (13 males, five females), mean age 32 years (range 19–58). Criteria for a diagnosis of CD were: (a) a small intestinal biopsy specimen showing subtotal or partial villous atrophy at the time of the diagnosis and (b) recovery of the histopathology after a gluten free diet. All patients had been on a gluten free diet for an average of 12 years (range 6–22). After a gluten free diet, small intestinal biopsy results were normal (Marsh 0) in 9/13 patients; 4/13 had partial remission (Marsh 1). At the time of the present investigation, all patients had IgA tissue transglutaminase and IgG/IgA gliadin antibodies within the normal range and no detectable IgA endomysial antibodies. All patients with CD had normal blood test results at the time of investigation. Healthy controls had normal blood test values (no signs of inflammation, malabsorption, or disease) and no gastrointestinal symptoms. They had no history of illness during the past year. None of the controls had a rise in IgA antibodies to gliadin or endomysium. One had a border value for IgA

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**Abbreviations:** CD, coeliac disease; NO, nitric oxide; NOS, NO synthase; MPO, myeloperoxidase; ECP, eosinophil cationic protein; ppb, parts per billion; CTAB, N-Cetyl-N,N,N-trimethyl ammonium bromide

antibodies to tissue transglutaminase but no other signs of CD and normal duodenal biopsy results.

All subjects underwent rectal provocation with gluten, and mucosal and blood measurements were performed before and after the challenge. Initially, in 10 of the CD patients, measurements were made before and 5, 24, and 48 hours after rectal gluten challenge. These results indicated the need for a 15 hour measurement, and a further gluten challenge was therefore performed in all patients and measurements were carried out at 15 hours in addition to the other time points. Two patients also underwent a 10 hour test but as the NO peak occurred at 15 hours we decided not to continue with tests at 10 hours. Controls were tested before and 15 hours after the challenge. Ten of the patients and seven of the controls underwent a second test with corn gluten for evaluation of the specificity of the reaction.

The ethics committee of the Medical Faculty, Uppsala University, approved the study. All participants gave informed consent to participate in the study.

### Rectal challenge

Participants were challenged with wheat gluten 6.2–6.5 g (crude wheat gluten; Sigma Chemical Co., St Louis, Missouri, USA) and corn gluten 6.2-6-5 g (corn gluten meal; Sigma Chemical Co.) suspended in 25 ml of 0.9% NaCl solution. The suspension was instilled into the rectum with a syringe with the subject lying in the left lateral position. Subjects were then allowed to move about as they wished and were instructed to retain the enema for at least 60 minutes. Rectal challenge was performed between 4 and 6 pm and measurements 15 hours later, between 7 and 9 am. Subjects were told to fast for one hour before the challenge and one hour after the challenge and also from midnight before the measurements.

### Preparation

All patients and controls were given a rectal enema (Klyx 120 ml; Ferring, Copenhagen, Denmark) within one hour before introduction of the instrument.

### Mucosal evaluation

To elucidate inflammation after the rectal challenge with gluten, we measured inflammatory mediators of neutrophil activity (MPO), eosinophil activity (ECP), and histamine using a new instrument, a polyethylene catheter with a silicon balloon at its end, and with three patches attached to the balloon.[12] The instrument for the mucosal patch technique has been described previously and has been found to be rapid, simple, safe, and highly sensitive, and to produce reliable and reproducible data concerning mucosal inflammatory markers. The patches were made of highly absorptive cellulose material (Pharmacia Diagnostics AB, Uppsala, Sweden). When the instrument was positioned in the rectum, the balloon was inflated with air (60–80 ml), allowing the patches to come into contact with the mucosa. Two latex shields protected the patches during introduction of the catheter into the rectum and when it was retracted. If the patient had a strong feeling of distension or pain that did not disappear after 2–3 minutes, the volume of air was reduced by 5 ml at a time until the catheter and balloon were accepted. A minimum of 50 ml of air were left in the balloon to ensure that the patches adhered to the mucosa. The balloon was kept inflated for 20 minutes in all subjects, after which time it was deflated and the catheter removed. The patches were cut off and immediately put into 2 ml of 0.3% CTAB (N-Cetyl-N,N,N-trimethyl ammonium bromide; E Merck, Darmstadt, Germany) solution to extract the contents. Any discomfort, reactions, or other symptoms during the test procedure were recorded, as was the presence of any blood or faeces on the patches.

Each patch was collected and analysed separately. Patches were kept in the 0.3% CTAB solution for extraction for one hour. After completion of this step, the extraction solution was squeezed out of the patches, centrifuged, and frozen at −70°C until analysed.

Duplicate samples were analysed, using radioimmunoassays to measure concentrations of MPO, ECP, and histamine, according to the manufacture's instructions (Pharmacia Diagnostics AB, Uppsala, Sweden).

### NO measurements

NO was measured with a chemiluminescence NO analyser (model Sievers NOA 280; Ionics Instrument Business Group, Boulder, Colorado, USA). The system was calibrated with a mixture of NO in $N_2$ (AGA Gas AB, Lidingö, Sweden) with an NO concentration of 500 parts per billion (ppb). The calibration was tested every morning and zero was set before each measurement. Air samples were collected with three glass syringes from the cuff of the rectal catheter described above. Each sample was measured separately. Air was injected into the NO analyser and the peak level was monitored. The single measure for each subject represents the highest value obtained in the collected air samples to avoid false low NO measurement due to syringe leakage. An extraction test was performed to see how much NO could be recovered with our instrument and it was found that 80% was recovered (data not shown), a result in accordance with earlier reports.[13]

### Statistics and calculations

Results are presented as mean (SEM) (range). The Mann Whitney U test, Friedman ANOVA, sign test, and Spearman's sign rank test were used for statistical calculations.

## RESULTS
### Gluten challenge and NO production

The prechallenge rectal luminal NO value in patients with CD was 19 (4) ppb (range 6–41) and in controls 24 (6) ppb (6–90). No relation to age or sex was seen in patients or controls. The results of gluten challenge in patients with CD are illustrated in fig 1. No NO production was detected five hours after the challenge. The maximum NO concentration (9464 (2393) ppb; range 250–24982) was noted after 15 hours. NO values then gradually declined but had not returned to normal in all patients by 48 hours. Mean NO production in controls 15 hours after rectal gluten challenge
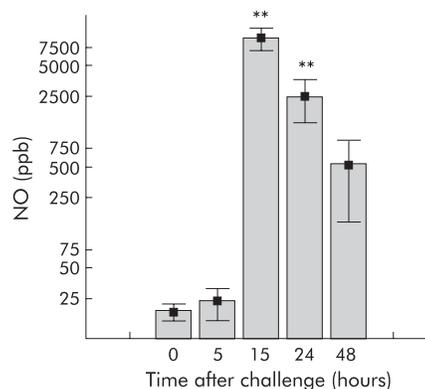


**Figure 1** Rectal luminal nitric oxide (NO) before and 5, 15, 24, and 48 hours after rectal gluten challenge in 10 patients with coeliac disease (mean (SEM)). Friedman ANOVA was significant (p<0.001); **p<0.01 compared with prechallenge value (sign test). ppb, parts per billion.
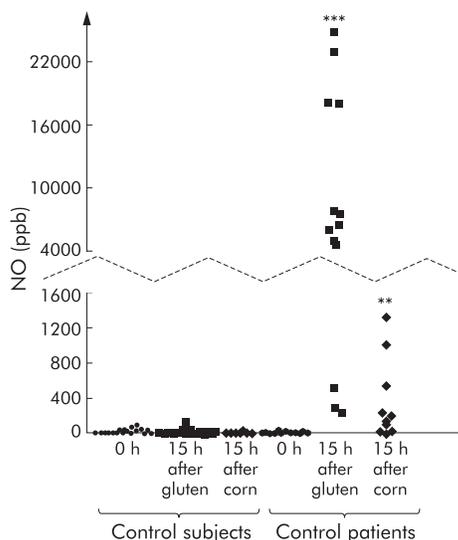
**Figure 2** Rectal concentrations of nitric oxide (NO) in 18 controls and 13 coeliac patients before challenge (0 h), 15 hours after rectal wheat gluten challenge (15 h after gluten), and 15 hours after rectal corn gluten challenge (15 h after corn). Note that the Y scale is broken, highlighting the differences between the two sections, and better illustrating the lower values. Significant increase was found when the patient and control groups were compared using the Mann-Whitney U test: **p<0.01, ***p<0.001.

was 22 (7) ppb (range 4–126). Rectal NO concentration in two patients with CD who were tested 10 hours after the challenge was almost half of that seen at 15 hours in the same patients.

## Corn challenge and NO production

In CD patients, NO production 15 hours after corn challenge was significantly increased by a mean of 368 (147) ppb (range 13–1348) compared with the prechallenge value

(p<0.05). However, as seen in fig 2, only 6/10 patients showed a postchallenge increase in NO. The relative increase was considerably greater after gluten challenge (on average 500-fold) than after corn challenge (on average 15-fold). In controls, no increase in NO was seen after corn challenge (fig 2.)

## Relation of NO production to signs of granulocyte and mast cell/basophil activation
### Gluten challenge

The kinetic responses of neutrophil activation in CD patients, assessed as release of MPO, are illustrated in fig 3B. In contrast with NO production, strong signs of neutrophil activation were already seen five hours after gluten challenge. After the initial increase, the concentration of MPO remained elevated throughout the observation period of 48 hours. There was a significant correlation ($r = 0.64$, $p<0.05$) between the increases in MPO ($\Delta$MPO) and the increase in NO ($\Delta$NO) 15 hours after the challenge (fig 3A). The kinetics of eosinophil activation, as represented by ECP values, is also illustrated (fig 3C). No correlation was found between $\Delta$ECP and $\Delta$NO, five and 15 hours after the challenge, with r values of 0.02 and 0.04, respectively. In controls, gluten induced no increase in MPO or ECP values; prechallenge values were 24 (6) µg/l (range 6–90) and 33 (7) µg/l (2–100) and post-challenge values 22 (7) µg/l (range 4–126) and 42 (12) µg/l (range 2–168), respectively.

No increase was found in methyl histamine at five or 15 hours after rectal gluten challenge in patients or controls. Prechallenge values in coeliac patients were 0.4 (0.03) µg/l (range 0.3–0.7) and in controls 0.5 (0.1) µg/l (0.2–1.1); five hours after the gluten challenge values were 1.3 (0.2) µg/l (range 0.7–2.6) and 1.2 (0.1) µg/l (1.0–1.3), respectively. At 15 hours values were 1.0 (0.2) µg/l (range 0.3–2.1) in coeliac patients and 1.0 (0.1) µg/l (0.3–1.8) in controls.

### Corn challenge

Corn challenge also induced an increase in MPO in some patients but the increase was not significant in the coeliac
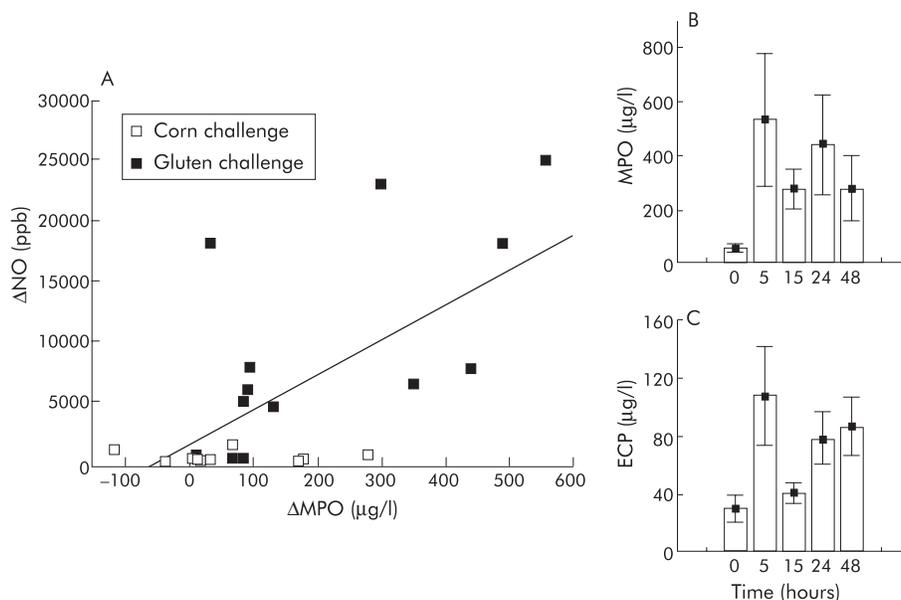


**Figure 3** Rectal mucosal nitric oxide (NO) production in patients with coeliac disease. (A) Relation between the increase in NO ($\Delta$NO) and the increase in mucosal myeloperoxidase ($\Delta$MPO) 15 hours after gluten and corn challenge. The correlation was significant (Spearman rank order correlation, $r=0.58$, $p<0.05$). Mean (SEM) rectal mucosal MPO (B) and eosinophil cationic protein (ECP) (C) concentrations before and 5, 15, 24, and 48 hours after gluten challenge in 10 coeliac patients for comparison. Friedman ANOVA was significant for both ECP and MPO ($p<0.05$). p<0.05 and p<0.01 compared with prechallenge values (0 hours) (sign test).

patients (p>0.05); mean baseline MPO value was 64 (17) μg/l (range 6–188) and 136 (42) μg/l (18–411) after 15 hours. Baseline ECP values were 29 (7) μg/l (range 1–102) and postchallenge ECP values were 36 (6) μg/l (5–74) (p>0.05). Control subjects showed no increase in MPO or ECP after the challenge.

## DISCUSSION

In this study we performed a kinetic analysis of gut mucosal NO production in relation to the local inflammatory response induced by instillation of wheat gluten into the rectum of patients with CD. This was made possible by the use of a newly developed technique (mucosal patch technique) that allows simultaneous measurements of NO and granule proteins released from inflammatory cells into the gut lumen. The obvious advantage of this approach is the gentle handling of the mucosa and the reduced distress for the patient compared with a study with repeated rectal biopsies, which also in fact, might influence NO synthesis.[14] Our main finding was that signs of granulocyte activation clearly preceded the increased synthesis of NO after gluten challenge. Increased NO synthesis was not apparent five hours after gluten instillation, a time point with maximum release of MPO, a granule constituent of neutrophils, and of ECP, a granule constituent of eosinophils. Luminal NO values peaked 15 hours after gluten challenge and then gradually declined, but were still increased after 48 hours. The granulocyte activation pattern described a biphasic pattern compatible with previous histological findings reporting a biphasic mucosal influx of neutrophils after rectal gluten challenge in coeliac patients.[7] This pattern might possibly reflect the fact that the gluten induced reaction involves both the adaptive as well as the innate system.[11]

NO is generated via NOS which has constitutive and inducible isoforms. The constitutive isoform of NOS (cNOS) is calcium dependent and involved in various physiological conditions. In response to physiological stimulation, for example of endothelial cells and neurones, NO is generated rapidly and transiently at low concentrations for purposes such as relaxation of vascular smooth muscle cells leading to vasodilatation and inhibition of leucocyte and platelet adhesion to vascular endothelium. The major inducible isoform of NOS (NOS II) produces NO in high concentrations for as long as the enzyme is activated as part of immune and inflammatory responses. This isoform is constitutively present in the epithelium of some tissues but is mainly expressed by inflammatory cells (macrophages, eosinophils, mast cells, and possibly also neutrophils and T lymphocytes[15–19]) on activation by proinflammatory cytokines and other inflammatory mediators. The role of NOS II in the inflammatory response to gluten instillation in the rectal mucosa has previously been elucidated by measurement of rectal mucosal biopsy samples obtained 4, 8, 24, and 48 hours after rectal gluten challenge.[20] A significant (approximately 50%) increase in NOS II was seen eight hours after gluten challenge but there was no change in cNOS. Increased NO production in the gut lumen has been observed not only in ulcerative colitis[21] but also 24 hours after gluten challenge in patients with CD.[22] Our finding of high production of NO starting 5–10 hours after gluten challenge is in accordance with the above mentioned biopsy results concerning NOS II expression,[20] as this expression has been found to precede the production of NO by several hours; the delay in NO production reflecting the time taken for mRNA and protein synthesis.[23 24] NOS II expression in rectal biopsy specimens gradually decreased and was lost 24–48 hours after challenge. Our NO measurements showed a return to prechallenge NO values in 30% of our patients but elevated NO values still remained in a few patients even 48 hours after the

challenge. In bronchial asthma an increase in luminal airway NO is also seen and it has been suggested that this may be useful for non-invasive determination of airway inflammation.[25] After allergen challenge in asthmatic patients, no increase in NO was observed in those who responded only with an acute reaction, while those who also had a late allergic response had a peak increase in NO at 10 hours and still showed elevated NO levels 21 hours after the challenge. The similarity to our NO reaction strengthens the idea that gut and airway luminal NO production might be a non-specific inflammatory response.

The cellular source of induced luminal NO synthesis has not been identified in asthmatic patients. There is evidence of increased expression of NOS II in asthmatic airways, especially in macrophages and epithelial cells, but activated neutrophils, eosinophils, and mast cells have also been proposed as major contributors to enhanced NO synthesis.[17 26 27] The early inflammatory reaction induced by gluten in patients with CD is characterised by expression of E-selectin, a specific adhesion molecule that is the main mediator involved in neutrophil recruitment in the first four hours after gluten exposure.[28] This is in concordance with our observations that a strong neutrophil activation, as assessed by luminal release of MPO, and less pronounced eosinophil activation, as measured by release of ECP, was already present five hours after the gluten instillation challenge and clearly preceded the NO response.

Gluten induced small intestinal mucosal inflammation is known to be patchy. Our technique is designed to reduce the influence of an uneven distribution of inflammatory signals by allowing measurements at different mucosal sites. Nevertheless, the observed interindividual variability in NO and MPO responses may partly be due to non-homogenous inflammation. Another, perhaps more likely, explanation is individual sensitivity.[29] The finding of a significant correlation between neutrophil and NO responses after challenge demonstrates that NO synthesis is linked to the intensity of the induced inflammatory reaction, but only suggests that neutrophils are the cellular NO source.

NO in exhaled air (eNO) has been proposed as a marker of bronchial inflammation. Some studies reported a significant association in patients with bronchial asthma between eNO and airway eosinophilia, as reflected by sputum eosinophilia[30 31] or density of eosinophilic granule constituents in airway mucosa. Because eosinophils can express NOS II,[32] these cell have been attributed a role in the synthesis of NO after allergen challenge. However, others have been unable to confirm a relationship between eNO and airway eosinophilia in asthma.[33 34] In the present study, signs of eosinophil activation, as reflected by ECP, were present after gluten challenge in patients with CD, with a similar time course of activation as neutrophils, but the eosinophil response was relatively weaker than the neutrophil response defined by MPO. The lack of relationship between the degree of eosinophil activation and NO production may suggest that NO reflects other aspects of gut mucosal inflammation, including neutrophil and possibly also T cell and monocyte activation.

The site of NOS II expression in the epithelium of gluten damaged rectal mucosa is controversial but immunohistochemical studies of such mucosa showed that NOS II was mostly localised in the lamina propria just beneath the surface epithelium and around the crypts.[20] The suggested protective effect of NO in inflammation has been partly attributed to a role in reducing granulocyte infiltration and consuming oxygen species.[3] Thus the observed relationship between signs of neutrophil activation and later NO synthesis may well reflect a counteracting system controlling the potentially tissue damaging principles delivered by activated

neutrophils. However, reports suggesting a protective role of NO in mucosal inflammatory damage are as numerous as those supporting a toxic role. It has been proposed, for example, that NO may promote inflammation by enhancing vascular permeability and by promoting chemotaxis of granulocytes and the production of proinflammatory cytokines.[35][36] Others have suggested that epithelial cells are the cellular source of luminal NO seen in bronchial asthma and inflammatory bowel diseases.[37][38] Our kinetic study is compatible with this hypothesis as activated granulocytes may induce enterocytes to increase NO synthesis. The results of our study give no answers concerning the possible toxic or protective role of NO in gluten induced damage. The possibility also remains that the association between granulocyte activation and NO production merely reflects the metabolic expression of neutrophils/eosinophils and other inflammatory cells activated by gluten challenge.

The lack of luminal histamine release after gluten challenge indicates that mast cells/basophils are not involved in induced NO synthesis. Niveloni et al observed in biopsy samples that the number of NOS II positive cells after gluten exposure increased in parallel with the CD3 cell infiltration observed around the subepithelial areas.[20] T cells have been attributed a central role in the pathogenesis of gluten induced mucosal damage but the NO producing capacity of T cells in inflammation remains unsolved.

The observation that corn gluten challenge induced an abnormal NO reaction in some of our patients with CD is intriguing as maize is considered safe and is recommended as the substitute cereal in a gluten free diet. However, a high incidence of serum antibodies against maize[39] has been reported in CD, and failure to normalise the mucosa in a fraction of adult patients with CD and on a strict wheat gluten free diet remains to be explained.[40] The manufacturer claimed that their corn product was free from wheat or other cereals. We tested the product at the Swedish National Food Administration (Livsmedelsverket) and it was found to be contaminated with 82 μg/g (ppm), which is less than the usual allowed amount in a gluten free diet (<200 ppm) according to the Codex Alimentarius Standard for gluten free foods, and far less than what has been found to be a safe amount of gluten contamination when correlated with histology in oral challenge studies.[41] It cannot be excluded that the small amounts of gluten present in the corn preparation induced an inflammatory reaction as the mucosal patch technique is very sensitive.

The major finding in the present study was that mucosal activation of neutrophils and eosinophils precedes pronounced enhancement of mucosal NO production after rectal wheat gluten challenge in CD. The observations were made possible by the use of a simple and safe procedure, which may also be of value for elucidating the possible role of other food antigens, such as milk and egg proteins, that may cause symptoms and mucosal damage in some patients with CD.

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Conflict of interest: declared (the declaration can be viewed on the Gut website at http://www.gut.com/supplemental).

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# EDITOR'S QUIZ: GI SNAPSHOT ...................................................................

**Robin Spiller,** *Editor*

## An unusual cause of pyrexia

### Clinical presentation

A 58 year old male patient presented with a 24 hour history of nausea, vomiting, and right shoulder tip pain with associated rigors. On examination he was tachycardic, 100 beats per minute, and pyrexial (38°C) but clinical examination was otherwise unremarkable. He was taking no medications and the only previous medical history of note related to a large birthmark on his leg which had been investigated when he was a child but had not required intervention. Initial laboratory investigations were also within normal limits apart from an elevated white cell count of $15.5\times10^9$/l and an elevated serum alkaline phosphatase of 256 U/l (normal range 44–132).

A chest radiograph revealed no focal lung lesion. Blood cultures were obtained prior to commencement of antibiotic therapy and *Streptococcus pneumoniae* was isolated, the source of which was unclear. In view of the abnormal liver function, ultrasound of the liver was undertaken and this demonstrated a 9 cm hyperechoic mass in the right lobe atypical for an abscess and suggestive of a tumour. The patient proceeded to computed tomography of the upper abdomen for further assessment and an unenhanced section through the right lobe of the liver and spleen is shown in fig 1.

### Question

What abnormalities are present? What is the explanation for the bacteraemia? What is the likely diagnosis?

*See page 802 for answer*



**Figure 1** Computed tomography of an unenhanced section through the right lobe of the liver and spleen.

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