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The clinical and phenotypic assessment of seronegative villous atrophy; a prospective UK centre experience evaluating 200 adult cases over a 15 year period (2000-2015)

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Ethics

The initial ethics was from the South Sheffield Research and Ethics Committee, then the Humber Research and Ethics Committee 09/H1304/69. The study was also registered under Sheffield Teaching Hospitals audit number 2954.

Abbreviations:

A2RB, Angiotensin-2-receptor-blockers; CD, Coeliac disease; GFD, Gluten-free diet; HIV, Human immunodeficiency virus; HLA, Human Leukocyte antigen; NSAIDS, Non-steroidal anti-inflammatory drugs; SNVA, Seronegative villous atrophy; SNCD, Seronegative coeliac disease; SN-non-CD, Seronegative non-CD; SPCD: Seropositive coeliac disease; UK, United Kingdom; US, United States

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ABSTRACT

Background: Seronegative villous atrophy (SNVA) is commonly attributed to coeliac disease. However, there are other causes of SNVA. More recently angiotensin-2-receptor-blockers have been reported as an association but data on SNVA has been limited to centres evaluating complex case referrals and not SNVA in general.

Objectives: To provide clinical outcomes and associations in a large prospective study overseeing all newcomers with SNVA.

Design: Over a 15-year period (2000-2015) we evaluated 200 adult patients with SNVA at a UK centre. A diagnosis of either seronegative coeliac disease (SNCD) or SN-non-CD was reached. Baseline comparisons were made between the groups, with 343 seropositive CD subjects serving as controls.

Results: Of the 200 SNVA cases, SNCD represented 31% (n=62) and SN-non-CD 69% (n=138). The HLA-DQ2 and/or DQ8 genotype was present in 61%, with a 51% positive predictive value for SNCD. The breakdown of identifiable causes in the SN-non-CD group comprised infections (27%, n=54), inflammatory/immune-mediated disorders (17.5%, n=35) and drugs (6.5%, n=13; two cases related to angiotensin-2-receptor-blockers). However, no cause was found in 18% (n=36) and of these 72% (n=26/36) spontaneously normalised duodenal histology whilst consuming a gluten-enriched diet.

Following multivariable logistic regression analysis an independent factor associated with SN-non-CD was non-white ethnicity (odds ratio 10.8, 95% confidence interval 2.2-52.8); in fact, 66% of non-whites had gastrointestinal infections. On immunohistochemistry all groups stained positive for CD8-T-cytotoxic intraepithelial lymphocytes. However, additional CD4-T-helper intraepithelial lymphocytes were occasionally seen in SN-non-CD mimicking the changes associated with refractory CD.

Conclusions: Most patients with SNVA do not have coeliac disease, in particular those who are not white. Furthermore, a subgroup with no obvious aetiology will show spontaneous histological resolution whilst consuming gluten. These findings suggest caution in empirically prescribing a gluten-free diet without investigation.

Significance of this study

What is already known on this subject?

Seronegative villous atrophy (SNVA) is a diagnostic and therapeutic dilemma.

The causes of SNVA are vast but can be broadly grouped into seronegative CD (SNCD) and SN-non-CD.

To date no study has systematically evaluated all newcomers with SNVA.

What are the new findings?

SNCD accounts for 31% of SNVA cases, with the remaining 69% due to SN-non-CD.

A positive HLA-DQ2 and/or DQ8 status is seen in 61% of SNVA cases; its positive predictive value for SNCD is roughly 51%.

An independent risk factor associated with SN-non-CD is non-white ethnicity, suggestive of infective aetiology.

Overall, almost one-in-five SNVA patients will have no identifiable cause; reassuringly, the majority of these will spontaneously normalise duodenal histology despite undertaking a gluten challenge.

How might it impact on clinical practice in the foreseeable future?

Individuals with SNVA should not be prescribed a gluten-free diet prior to further investigations. This is because of the wide differential diagnoses and that a subgroup with no obvious aetiology spontaneously normalise their duodenal histology whilst maintaining gluten intake.

Introduction

Coeliac disease (CD) affects 0.7-1% of the population and can be defined as a state of heightened immune response to ingested gluten in genetically susceptible individuals.[1,2] All patients with CD carry the HLA-DQ2 and/or DQ8 genotypes, although these alleles are also present in approximately 40% of the general population.[3] A cast-iron diagnosis of CD can be made on the basis of demonstrating duodenal villous atrophy in the presence of serum IgA endomysial and/or tissue transglutaminase antibodies.[4-6] This mode of presentation may be termed seropositive CD (SPCD) and following a systematic review accounts for approximately 93% of cases with villous atrophy,[7] although some international groups have reported a lower prevalence (Table 1).[8-15]

With this in regard, diagnostic and therapeutic dilemmas occur when villous atrophy is found in the context of negative coeliac serology.[8-15] This clinical entity is termed seronegative villous atrophy (SNVA), of which the causes can be broadly grouped into CD or non-CD related.[16,17] The reasons for seronegative CD (SNCD) include patients who have reduced gluten intake prior to investigations,[18] lesser degrees of villous atrophy,[13] selective IgA deficiency,[19] immunosuppressive therapy, or those with long-standing advanced CD within the spectrum of ulcerative jejunitis/enteropathy associated T-cell lymphoma.[15] The causes of seronegative non-CD (SN-non-CD) are vast ranging from infective, inflammatory, immune-mediated and drug-related.[16,17] Such examples include autoimmune enteropathy,[20] bacterial overgrowth,[16] common variable immunodeficiency,[21] crohn's disease,[22] gastroenteritis,[23] giardiasis,[24,25] graft vs. host disease,[26] HIV enteropathy,[27], mycobacterium tuberculosis,[25,28] peptic duodentitis +/- helicobacter pylori,[17,29-32] radiation enteritis,[33] tropical sprue,[25,34] and Whipple's disease.[35] Medications include non-steroidal anti-inflammatory drugs,[36-38] azathioprine,[39] methotrexate,[40] mycophenolate mofetil,[41] and most recently angiotensin-2-receptor-blockers (A2RB), in particular olmesartan.[16,42-45] Finally, in some instances no unifying cause can be found and such patients are classified as idiopathic/unclassified sprue, the natural history of which is unknown.[16,17]

Studies attempting to evaluate diagnostic outcomes in SNVA have thus far been limited to a US centre overseeing complex case referrals from a wide catchment area.[16] In such circumstances a high prevalence of SNCD and olmesartan-related enteropathy has been reported, the latter accounting for a striking 22% of SNVA cases.[16] However, we hypothesise that this may not be reflective of SNVA as seen in routine gastrointestinal practice. Furthermore, the clinical and histological phenotype of SNCD and SN-non-CD has not been established, nor how these entities contrast to the more conventionally seen SPCD. Such an evaluation may prove useful in

understanding the spectrum of villous atrophy whilst also aiding clinicians towards the correct diagnosis when posed with the challenges of SNVA.

In light of this, the aim of our study was to provide a large comprehensive overview of all SNVA patients seen at a UK centre over a 15 year period. Furthermore, we sought to identify differences between SNCD and SN-non-CD, using SPCD as controls.

Material and Methods

Setting

This study was carried out between the time-periods of 2000 to 2015 at the Royal Hallamshire Hospital, Sheffield, South Yorkshire, UK. The hospital is located in Northern England and provides a secondary/tertiary-care service to a population of 500 000 people. The unit undertakes approximately 6000 oesophagogastroduodenoscopies per year.

Participants

Over the 15 year period we prospectively recruited 200 consecutive adult patients presenting with SNVA. The identification of SNVA was based upon duodenal biopsies showing villous atrophy yet with negative serum IgA endomysial and tissue transglutaminase antibodies from the outset.

As for our control group we recruited 343 SPCD patients diagnosed within the same department between the years 2005 to 2011.

Histology

Throughout the study period the gastroenterology department had a policy of taking 4 duodenal biopsy specimens from the second part of the duodenum in those with suspected malabsorption. All duodenal biopsy specimens were fixed in buffered formalin and embedded in paraffin wax. Standard 3µm thick sections at 3 levels were stained with haematoxylin and eosin. The duodenal biopsies were routinely reported by one of a team of seven gastrointestinal histopathologists. Agreement was then performed by one of two expert GI histopathologists reviewing SNVA biopsy samples (co-authors SSC and PV). Intraepithelial lymphocytosis was defined as >25 per 100 enterocytes. Villous atrophy was identified according to the Marsh-Oberhuber criteria, using the most severe lesion present: Marsh 3a (partial villous atrophy, PVA); Marsh 3b (subtotal villous atrophy, SVA); or Marsh 3c (total villous atrophy, TVA).[46,47]

The groups were also assessed for differences in immunohistochemistry based on CD3 pan-lymphocyte marker and specific CD8-T-cytotoxic and CD4-T-helper intraepithelial lymphocyte expression.

Coeliac serology

The initial panel of coeliac serology testing was IgA based, with endomysial antibodies detected by immunofluoresence on primate oesophagus sections from The Binding Site (Birmingham, UK). IgA

tissue transglutaminase antibodies were assayed by using enzyme-linked immunosorbent assay kits (Aesku Diagnostics, Wendelsheim, Germany), with titres less than or equal to 15 U/ml taken as negative. Of note, our immunology department does not automatically test for immunoglobulin or total IgA levels when processing coeliac serology. Rather, these have to be specifically requested as does IgG coeliac serology.

Baseline characteristics

We collected baseline characteristic data on the SNVA and SPCD cohort. Taking into consideration the potential aetiologies and clinical manifestations this included age, gender, ethnicity, city residence, clinical symptoms, past medical history, current medication, grading of villous atrophy, HLA-DQ2/8 status, as well as laboratory parameters in the form of haemoglobin, ferritin, folate, vitamin B12, albumin, calcium, erythrocyte sedimentation rate and/or C-reactive protein.

All the data (other than age) was inputted as categorical. This included converting numerical laboratory values into either within the normal or abnormal range, thereby overcoming the difficulties that arise over a 15 year period with departmental changes in testing kits and reference values.

Diagnostic work-up for SNVA

All patients with SNVA were investigated in line with a systematic protocol, similar to that proposed by other expert groups, aiming to diagnose either SNCD or SN-non-CD (Figure 1).[16,17] It is important to bear in mind that, despite several international guidelines on CD, there is no consensus on how to approach subjects with SNVA.[4-6] Some physicians may suggest a trial of a gluten-free diet (GFD) followed by clinical and histological reassessment.[4-6] However, this can be fraught with uncertainty given that up to 32% of patients with SN-non-CD report favourable clinical response to a GFD.[17] Furthermore, mucosal recovery in adult CD is slow with histological abnormalities often persisting beyond 2-5 years and in some cases never normalising.[48-50] Therefore, adopting such an approach in SNVA could potentially lead to unnecessary delays given the wide differential diagnoses. Hence, patients with SNVA in our study were asked to continue a gluten-containing diet until investigations were complete and a firm diagnosis reached. This approach is also useful in that it allows progression of villous atrophy and detectable serum antibodies in some cases of SNCD.[51]

Mortality

At the end of December 2015 mortality rates were calculated. Overall survival was calculated in years and defined as the time from diagnosis to death. Surviving patients were censored at the time of last follow-up.

Statistics

Statistical analysis was carried out using SPSS version 21.0 software (SPSS Inc. Chicago, USA), with significance set at a p-value of <0.05. A complete-case analysis approach was adopted to address the limited data which was missing completely at random. Categorical variables were summarized by descriptive statistics, including total numbers and percentages, with comparisons between groups performed using the chi-squared or Fisher's exact test. Normally-distributed continuous variables were summarized by mean and standard deviation with comparisons between groups performed using the unpaired student's t-test. We performed dichotomous logistic regression between the SNCD and SN-non-CD groups using a forward stepwise method with p-value of <0.1 for entry into the analysis with all variables available for inclusion into the model. Finally, overall survival was analysed using Kaplan–Meier curves and significance compared using the Log-rank test.

Results

Characteristics of SNVA

The baseline characteristics of the 200 SNVA patients are provided in Table 2. The patient cohort comprised 83% (n=166) who were residents of Sheffield and thus classed as secondary-care referrals. There were 17% (n=34) who were referred from another city for a tertiary-care opinion. The mean-age was 51.2 years, with 63.5% (n=127) female, and 82.5% (n=165) of white ethnicity.

The most frequently reported clinical symptoms were diarrhoea (60%, n=120), abdominal pain (49%, n=98), weight loss (35.5%, n=71), and bloating (31%, n=62). Autoimmunity was present in 18.5% (n=37) of cases, with 3.5% (n=7) also having a family history of CD. A recent history suggestive of gastroenteritis was elicited in 11.5% (n=23) of cases. The use of A2RB was seen in 4% (n=8), of which 7 were on candesartan and 1 was on irbesartan; no patients were taking olmesartan.

Blood tests revealed anaemia in 30.8%, with associated haematinic deficiencies ranging from 16.1% to 39.5%. A raised erythrocyte sedimentation rate and/or C-reactive protein was present in 26.6% of patients. The presence of positive HLA DQ2 and/or DQ8 was seen in 61.1% (n=118/193).

Finally, histological grading of duodenal biopsies showed intraepithelial lymphocytosis in 88.5% (n=177), with the majority of patients found to have partial villous atrophy at 79.5% (n=159). In contrast, subtotal villous atrophy was seen in 12.5% (n=25) and total villous atrophy in 8% (n=16).

Aetiology of SNVA

Following systematic evaluation of 200 SNVA cases, we diagnosed SNCD in 31% (n=62) of cases with the remaining 69% (n=138) due to SN-non-CD. The breakdown of all causes is shown in Figure 2.

In the 62 cases identified as having SNCD, 14 were diagnosed with relative ease based on i) selective IgA deficiency but with raised IgG coeliac serology (n=9, three also had associated first degree family history of CD), ii) first degree family history of CD alone with subsequent response to a GFD (n=4), and iii) dermatitis herpetiformis (n=1). The other 48 patients were diagnosed with SNCD on the basis of having positive HLA-DQ2 and/or DQ8 status, no alternate cause found, persisting villous atrophy following a gluten re-challenge, with subsequent clinical +/- histological response to a GFD.

A wide range of aetiologies were established in the 138 SN-non-CD cases, commonly infective, medication-induced, and inflammatory in nature. In total, there were 54 cases attributed to an infection. This included H.pylori induced duodenitis alone (n=21) or in conjunction with

Mycobacterium tuberculosis (n=2), Mycobacterium avium intracellulare (n=1) and HIV (n=1). Other causes included viral gastroenteritis based upon clinical history (n=7), giardiasis (n=6), small bowel bacterial overgrowth (n=4), HIV enteropathy (n=2), Ascariasis (n=2), Mycobacterium tuberculosis (n=2), tropical sprue (n=2), Campylobacter (n=1), Candidiasis (n=1), Whipple's disease (n=1) and Mycobacterium avium intracellulare (n=1). There were 13 cases which occurred as a result of medication; 9 due to NSAID-related duodenitis, and the others were a case each related to methotrexate, mycophenolate mofetil, irbesartan and a possible association with candesartan. There were 23 cases of non-specific peptic duodenitis, 6 cases of Crohn's disease, and 4 cases due to systemic immune-mediated disorders which included a case each of sarcoidosis, graft vs. host disease, autoimmune enteropathy and common variable immunodeficiency. There was a case each of radiation enteritis and eosinophillic enteritis. Following appropriate treatment these showed clinical and histological improvement.

Finally, in 36 cases of SN-non-CD, despite extensive investigations, we were unable to elicit any cause and these patients were labelled as idiopathic/unclassified sprue. Interestingly, 72% (n=26/36, eleven of whom were HLA-DQ2/8 positive) had spontaneously normalised their duodenal biopsies when re-challenged with gluten, suggesting transient villous atrophy. This was seen on average nine months after the index biopsy had shown villous atrophy. Of the remaining 10 cases, all HLA-DQ2/8 negative, four required immunosuppressive therapy for persisting unexplained villous atrophy with the other 6 either lost to follow-up or refusing further endoscopic investigations given their clinical stability.

Risk factors for diagnostic outcomes

Univariate analysis comparing the SNVA subgroups and SPCD controls are shown in table 2. In summary, the SNVA cohort were older at the time of presentation and more likely to present with symptoms of diarrhoea, abdominal pain, nausea and weight loss.

In contrast, subjects with SPCD or SNCD were more likely than SN-non-CD to have autoimmunity, family history, and HLA-DQ positivity; however, the positive predictive value of HLA-DQ2/8 for SNCD in the context of SNVA was only 51% (n=60/118). There was also a significant trend towards lesser degrees of villous atrophy from SPCD towards SNCD and then SN-non-CD.

Factors significantly associated with SN-non-CD included non-white ethnicity, dyspepsia, negative HLA-DQ status, lack of intraepithelial lymphocytosis/crypt hyperplasia, and hypoalbuminemia. Multivariable logistic regression analysis of the SNVA cohort showed that an independent factor

associated with a diagnosis of SN-non-CD was non-white ethnicity (odds ratio 10.8, 95% confidence interval 2.2-52.8, p=0.003). Indeed, 23 of the 35 (66%) non-white subjects presenting with SNVA had a gastrointestinal infection, commonly Helicobacter pylori induced duodenitis; Table 3. Only 2 of 35 (5.7%) non-whites with SNVA had SNCD compared to 60 of 165 (36%) whites.

Immunophenotyping of intraepithelial lymphocytes

Immunohistochemistry was performed in 19 SNVA cases of which 14 were SN-non-CD and 5 SNCD. Both groups showed CD8 positive T-cytotoxic intraepithelial lymphocytes, similar to that seen in SPCD controls. However, 4 cases of SN-non-CD also contained CD4 positive T-helper cells amongst the intra-epithelial lymphocytes; these cells are associated with refractory CD within the context of CD but in other contexts, such as gastrointestinal infection, they are a normal component of the immune response (Figure 3 and Supplementary Figure 1).

Survival Analysis

There have been 19 deaths within the 200 SNVA cohort, of which 7/60 (11.2%) were in the SNCD group and 12/138 (8.7%) in the SN-non-CD group. In comparison there have been 11/343 (3.2%) deaths in the SPCD controls. On Kaplan-Meier there were no statistical differences in estimated survival between the SNVA groups although this was less favourable compared to SPCD (Figure 4: log rank p=0.002).

Discussion

Main findings

We believe that our findings represent a major conceptual change in the understanding and management of SNVA. Having used a systematic clinical algorithm we have shown that SNCD accounts for 31% of SNVA cases, with the remaining 69% due to SN-non-CD related causes. Furthermore, HLA-DQ2 and/or DQ8 genotype was present in 61% of SNVA cases, with a positive predictive value of only 51% for a diagnosis of SNCD. This is not surprising given that these alleles are common as seen in approximately 40% of the general population.[3]

Importantly, we have identified that non-white ethnicity is a risk factor to alert clinicians to the possibility of SN-non-CD, in particular with regards to an infective aetiology. These findings are the first to be reported outside of the Tropics and in a Western society.[25] The clinical relevance of this also expands to the US where results from a national pathology database have identified that among patients undergoing duodenal biopsies it is those from the Punjab region of India that constitute the ethnic group with the highest prevalence of villous atrophy.[52] It remains to be determined whether such patients had SNVA given that the US National Health and Nutrition Examination survey has found positive coeliac serology to be rare amongst non-whites.[1]

In addition, in almost one-in-five cases of SNVA no identifiable cause was found although, reassuringly, the majority spontaneously normalised duodenal histology whilst being investigated on a gluten-enriched diet; had these patients been commenced on a GFD at the outset instead then they would have erroneously been diagnosed with SNCD and wrongfully subjected to a lifelong, restrictive diet. This, along with previous studies showing that an empirical trial of a GFD to be a poor predictor of CD,[17] further supports the notion that clinicians must not start a GFD in SNVA until investigations are complete and a firm diagnosis of SNCD has been established.

Finally, differences in survival outcomes between SNVA and SPCD controls were noted. A recent English study involving more than 10 000 CD patients found no major excess risk of cancer, digestive disease or respiratory disease related or cardiovascular mortality compared with the general population.[53] However, it is recognised that those with SNCD tend to be older and run a more advanced disease course than SPCD.[15] With regards to SN-non-CD this entity has a number of heterogeneous disease associations (i.e. HIV, tuberculosis, common variable immunodeficiency) which are associated with poorer outcomes.

Strengths and limitations

The main strength of this study is that it is the largest and most comprehensive to date, having prospectively evaluated 200 consecutive adult patients with SNVA at a UK secondary/tertiary-care centre over a 15-year period. The cohort studied included both inner and outer city referrals. Moreover, systematic and rigorous investigations were performed using testing modalities available amongst most gastroenterology departments. We therefore feel that our findings can be used as a benchmark and generalized to other physicians seeing similar patients.

However, our study does have several limitations. Firstly, we do not perform serum deamidated gliadin peptide antibodies or intestinal coeliac antibody deposits, both of which are relatively novel markers and can aid towards the diagnosis of CD.[54-56] Secondly, it may also be perceived that by identifying and including IgA deficient patients who were subsequently found to be IgG coeliac serology positive (n=9) is a weakness in that this should be common knowledge. However, our findings are those of real life practise and would be supported by other groups who have shown that inadequate evaluation of IgA deficiency occurs frequently when testing for CD.[57] Nevertheless, had we excluded such patients from our analysis then the prevalence of SNCD would have been 27.7% (n=53/191) instead of the 31% (n=62/200) stated. Thirdly, we have unanswered questions in those in whom no cause was found (so called idiopathic/unclassified sprue) but spontaneously normalised duodenal biopsies whilst consuming high-dose gluten. A recent case series has highlighted that self-limiting enteropathies can occur in the context of gastrointestinal infections,[23] which raises the possibility that our patients may have experienced a similar insult although this was not recalled from their clinical history nor isolated from stool cultures. Furthermore, these individuals had their repeat biopsy performed on average nine months after the index case which had shown villous atrophy. We do not know when their histology started improving and at what exact time-point it had normalised. Had the biopsies been performed earlier then these patients may still have had persisting villous atrophy and, in those with the correct HLA-DQ genotype, subsequently categorised as having CD. However, our study was performed pragmatically and is a reflection of routine out-patient clinical practise. Nevertheless, future research studies should aim to perform biopsies at sequential time-points. Finally, of those carrying the HLA-DQ genotype it could be hypothesised that these individuals may still belong to the spectrum of CD and have simply experienced an unexplained gastrointestinal insult transiently manifesting as SNVA but having not yet reached the cumulative threshold required for CD to become apparent.[58] Hence, longitudinal follow-up data is now required in this particular group.

Other studies

To our knowledge only one other study has evaluated diagnostic outcomes in SNVA.[16] This was performed by the New York group who evaluated 72 complex case referrals of SNVA over a 10-year period. The investigators found that 22% (n=16/72) of their SNVA cases were due to olmesartan-related enteropathy.[16] This novel association has generated substantial interest and is of importance given its presentation may be that of a severe form of enteropathy necessitating hospitalization for the management of intractable diarrhoea, weight loss, dehydration, hypotension, acute renal failure and metabolic acidosis.[16,42-45] Yet, these findings are in contrast to ours where the use of A2RB was seen in 8 of 200 SNVA cases, with A2RB a responsible cause for enteropathy in 2 patients; overall prevalence of A2RB-enteropathy being 1% (n=2/200). In the other 6 patients we found an alternate aetiology for SNVA with patients well maintained on their A2RB; these include CD (n=2), giardiasis (n=1), eosinophillic enteritis (n=1), small bowel bacterial overgrowth (n=1), and one lost to follow-up. Given that A2RB including olmesartan are dispensed in the UK then the discrepancy in results raises two main points. Firstly, the high prevalence of olmesartan-related enteropathy reported elsewhere may not be reflecting SNVA in general but rather groups overseeing and presenting the outcomes of cases referred from wide-catchment areas with presumed "poorly responsive/refractory CD".[16] In fact, the initial case series highlighting this association came from the Mayo clinic where 22 patients with olmesartan-related enteropathy were reported following referrals from 16 US states over a 3-year period.[42] Following on, a nationwide multicentre French survey identified 36 patients with olmesartan-related enteropathy.[44] Most recently, the crude incidence rates of olmesartan and other A2RB-enteropathy in France has been calculated at 5.6 and 1.8 per 100 000 patient years, respectively.[45] These findings suggest that olmesartan- and in particular other A2RB-related enteropathies are rare adverse events. Secondly, despite the growing awareness of A2RB-related enteropathy clinicians must still remain vigilant that on occasions A2RB will merely be innocent bystanders and an alternate aetiology for SNVA will be found.

Conclusion

This large UK centre study provides a prospective, systematic, and clinically pragmatic evaluation of SNVA. We have shown that most patients with SNVA do not have CD or angiotensin-2-receptor-blocker enteropathy. Further, a subgroup in whom no cause is found will show spontaneous histological resolution whilst still consuming gluten and this phenomenon requires further evaluation. The presence of non-white ethnicity was found to be a factor predicting a non-coeliac cause, in particular infective aetiology.

Table 1: Studies where coeliac serology have shown low sensitivities in villous atrophy

First author	Year	Country	No of villous atrophy cases	Positive coeliac serology, n (%)	Negative coeliac serology, n (%)
Rostami[8]	1999	Netherlands	69	42 (61%)	27 (39%)
Dickey[9]	2000	Northern Ireland	89	69 (78%)	20 (22%)
Dahele[10]	2001	Scotland	53	42 (79%)	11 (21%)
Dahele[11]	2001	Scotland	114	92 (81%) - 99 (87%)	15 (11%)-22(19%)
Clemente[12]	2002	Italy	111	95 (86%)	16 (14%)
Abrams[13]	2004	United States	115	74 (64%)	41 (36%)
Collin[14]	2005	European Multicentre	126	112 (89%) - 118 (94%)	8 (6%)-14 (11%)
Salmi[15]	2006	Finland	177	151 (85%)	26 (15%)

Coeliac serology as defined by endomysial and/or tissue transglutaminase antibodies

Figure 1: Step-wise proposed algorithm used to investigate and diagnose causes of SNVA

Step-wise algorithm to investigate seronegative villous atrophy (SNVA)

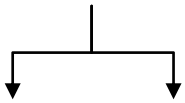
1. Exclude immunoglobulin/selective IgA deficiency. Check for raised IgG coeliac serology if needed.
2. Review clinical history and medication.
3. Seek opinion from expert GI histopathologist i.e. neutrophils, granulomas, crypt abscesses, duodenitis
4. a) Check HLA-DQ2 and DQ8 status.

   b) Check HIV status, Tuberculosis quantiferon, anti-enterocyte and anti-goblet cell antibodies.

   c) Check stool for giardia antigen, bacterial and viral enteritis.

   d) Repeat upper GI endoscopy following a gluten-challenge of 10g/day for six weeks. Obtain further duodenal biopsies, H.pylori rapid urease test, small bowel aspirate, and Whipples PCR.

   e) Consider ileo-colonoscopy with biopsies if lower gastro-intestinal symptoms.
5. Further investigations i.e. small bowel video capsule endoscopy at the discretion of clinician and depending on local availability.



Diagnosis of SNCD (any of the following criteria)	**Diagnosis of SN-non-CD (any of the following criteria)**
1. IgA deficiency with raised IgG coeliac serology 2. Co-existing dermatitis herpetiformis 3. First degree family history of coeliac disease with subsequent response to a GFD. 4. HLA DQ2 and/or DQ8 positive, no alternate cause found, persisting villous atrophy on gluten-challenge. Subsequent clinical +/- histological response to a GFD.	1. HLA DQ2 and DQ8 negative. 2. HLA DQ2 and/or DQ8 positive with confident alternate cause identified. Subsequent treatment leads to clinical + histological improvement. 3. HLA DQ2 and/or DQ8 positive with no alternate cause identified. Spontaneous histological resolution whilst still on a gluten-containing diet.

Table 2: Baseline characteristics of SNVA subjects and SPCD controls

	Total SNVA (n=200)	SN-non-CD (n=138; 69%)	SNCD (n=62; 31%)	P-value (between SNCD and SN-non-CD)	SPCD controls (n=343)	P-value (SPCD controls vs. SN-non-CD)	P-value (SPCD controls vs. SNCD)
Demographics							
Mean-age ± SD	51.2 ± 17.6	51.4 ± 17.3	50.9 ± 18.2	0.89	43.5 ± 17.3	**<0.001**	**0.002**
Female, n	127 (63.5%)	84 (60.9%)	43 (69.4%)	0.25	242 (70.6%)	**0.04**	0.85
White ethnicity, n	165 (82.5%)	105 (76.1%)	60 (96.8%)	**<0.001**	307 (89.5%)	**<0.001**	0.1
Sheffield city resident, n	166 (83%)	116 (84%)	50 (80.6%)	0.55	343 (100%)	**<0.001**	**<0.001**
Clinical symptoms							
Diarrhoea, n	120 (60%)	84 (61%)	36 (58.1%)	0.71	153 (44.6%)	**0.001**	**0.05**
Weight Loss, n	71 (35.5%)	52 (37.7%)	19 (30.6%)	0.34	40 (11.7%)	**<0.001**	**<0.001**
Abdominal pain, n	98 (49%)	69 (50%)	29 (46.8%)	0.67	121 (35.3%)	**0.003**	0.08
Bloating, n	62 (31%)	43 (31.2%)	19 (30.6%)	0.94	102 (29.7%)	0.76	0.89
Dyspepsia, n	28 (14%)	24 (17.4%)	4 (6.5%)	**0.04**	19 (5.5%)	**<0.001**	0.79
Reflux, n	32 (16%)	24 (17.4%)	8 (12.9%)	0.42	27 (7.9%)	**0.002**	0.19
Nausea, n	39 (19.5%)	27 (19.6%)	12 (19.4%)	0.97	22 (6.4%)	**<0.001**	**0.001**
Constipation, n	32 (16%)	24 (17.4%)	8 (12.9%)	0.42	52 (15.2%)	0.54	0.65
Fatigue, n	32 (16%)	19 (13.8%)	13 (21%)	0.2	91 (26.5%)	**0.003**	0.36
Past medical history							
Autoimmunity, n	37 (18.5%)	18 (13%)	19 (30.6%)	**0.003**	72 (21%)	**0.04**	0.09
Family history of CD, n	7 (3.5%)	0 (0%)	7 (11.2%)	**<0.001**	55 (16%)	**<0.001**	0.34
Recent gastroenteritis-type history, n	23 (11.5%)	18 (13%)	5 (8.1%)	0.31	8 (2.3%)	**<0.001**	**0.03**
Crohn's disease, n	1 (0.5%)	1 (0.7%)	0 (0%)	0.5	1 (0.3%)	0.49	1.0
Lymphoproliferative disorders, n	4 (2%)	3 (2.2%)	1 (1.6%)	1.0	2 (0.6%)	0.15	0.39
HIV, n	2 (1%)	2 (1.4%)	0 (0%)	1.0	1 (0.3%)	0.2	1.0
Tuberculosis, n	2 (1%)	2 (1.4%)	0 (0%)	1.0	1 (0.3%)	0.2	1.0
Medication							
A2RB, n	8 (4%)	6 (4.3%)	2 (3.2%)	1.0	8 (2.3%)	0.23	0.66
Aspirin, n	29 (14.5%)	21 (15.2%)	8 (12.9%)	0.67	29 (8.5%)	**0.03**	0.26
NSAIDs, n	19 (9.5%)	16 (11.6%)	3 (4.8%)	0.13	11 (3.2%)	**<0.001**	0.46
Methotrexate, n	2 (1%)	1 (0.7%)	1 (1.6%)	0.5	2 (0.6%)	1.0	0.39

Mycophenolate, n	1 (0.5%)	1 (0.7%)	0 (0%)	1.0	1 (0.3%)	0.49	1.0
Azathioprine, n	0 (0%)	0 (0%)	0 (0%)	-	3 (0.9%)	0.56	1.0
Bloods							
Anaemia, n	61/198(30.8%)	43/137 (31.4%)	18/61 (29.5%)	0.79	154/343 (44.9%)	**0.007**	**0.03**
Low ferritin, n	75/190 (39.5%)	51/130 (39.2%)	24/60 (40%)	0.92	207/333 (62.2%)	**<0.001**	**0.001**
Low folate, n	35/192 (18.3%)	28/131 (21.4%)	7/61 (11.5%)	0.1	100/333 (30%)	0.06	**0.003**
Low vitamin B12, n	31/193 (16.1%)	21/131 (16%)	10/62 (16.1%)	0.99	61/331 (18.4%)	0.54	0.67
Low calcium, n	22/186 (11.8%)	17/126 (13.5%)	5/60 (8.3%)	0.31	28/332 (8.4%)	0.1	0.98
Low albumin, n	21/198 (10.6%)	19/137 (13.9%)	2/61 (3.3%)	**0.03**	16/336 (4.8)	**0.001**	1.0
Raised ESR and/or CRP, n	51/192 (26.6%)	40/132 (30.3%)	11/60 (18.3%)	**0.08**	82/324 (25.3%)	0.28	0.25
HLA-DQ2 and/or DQ8 positive, n	118/193 (61%)	58/133(43.6%)	60/60(100%)	**<0.001**	112/112 (100%)	**<0.001**	1.0
Duodenal histology							
Intraepithelial lymphocytosis, n	177 (88.5%)	116 (84%)	61 (98.4%)	**0.003**	343 (100%)	**<0.001**	0.15
Crypt hyperplasia, n	177 (89%)	117 (84.8%)	61 (98.4%)	**0.003**	343 (100%)	**<0.001**	0.15
Partial villous atrophy , n	159 (79.5%)	120 (87%)	39(62.9%)		82 (23.9%)		
Subtotal villous atrophy, n	25 (12.5%)	10 (7.2%)	15 (24.2%)	**<0.001**	144 (42%)	**<0.001**	**<0.001**
Total villous atrophy, n	16 (8%)	8 (5.8%)	8 (12.9%)		117 (34.1%)		

A2RB, Angiotensin-2-receptor-blockers; CD, Coeliac disease; CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; HIV, Human immunodeficiency virus; HLA, Human Leukocyte antigen; NSAIDS, Non-steroidal anti-inflammatory drugs; SNVA, Seronegative villous atrophy; SNCD, Seronegative coeliac disease; SN-non-CD, Seronegative non-coeliac disease; SPCD: Seropositive coeliac disease

Figure 2: Causes of SNVA at a UK centre (n=200)

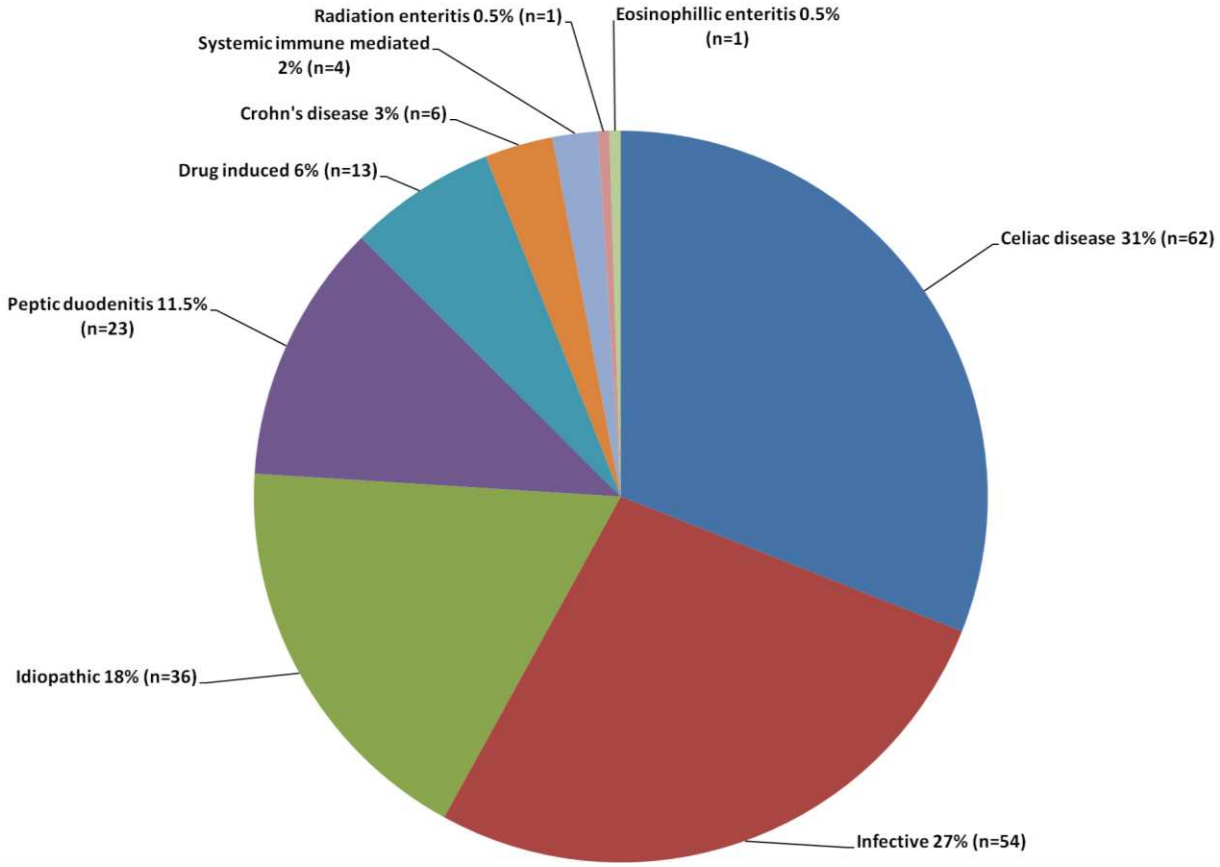
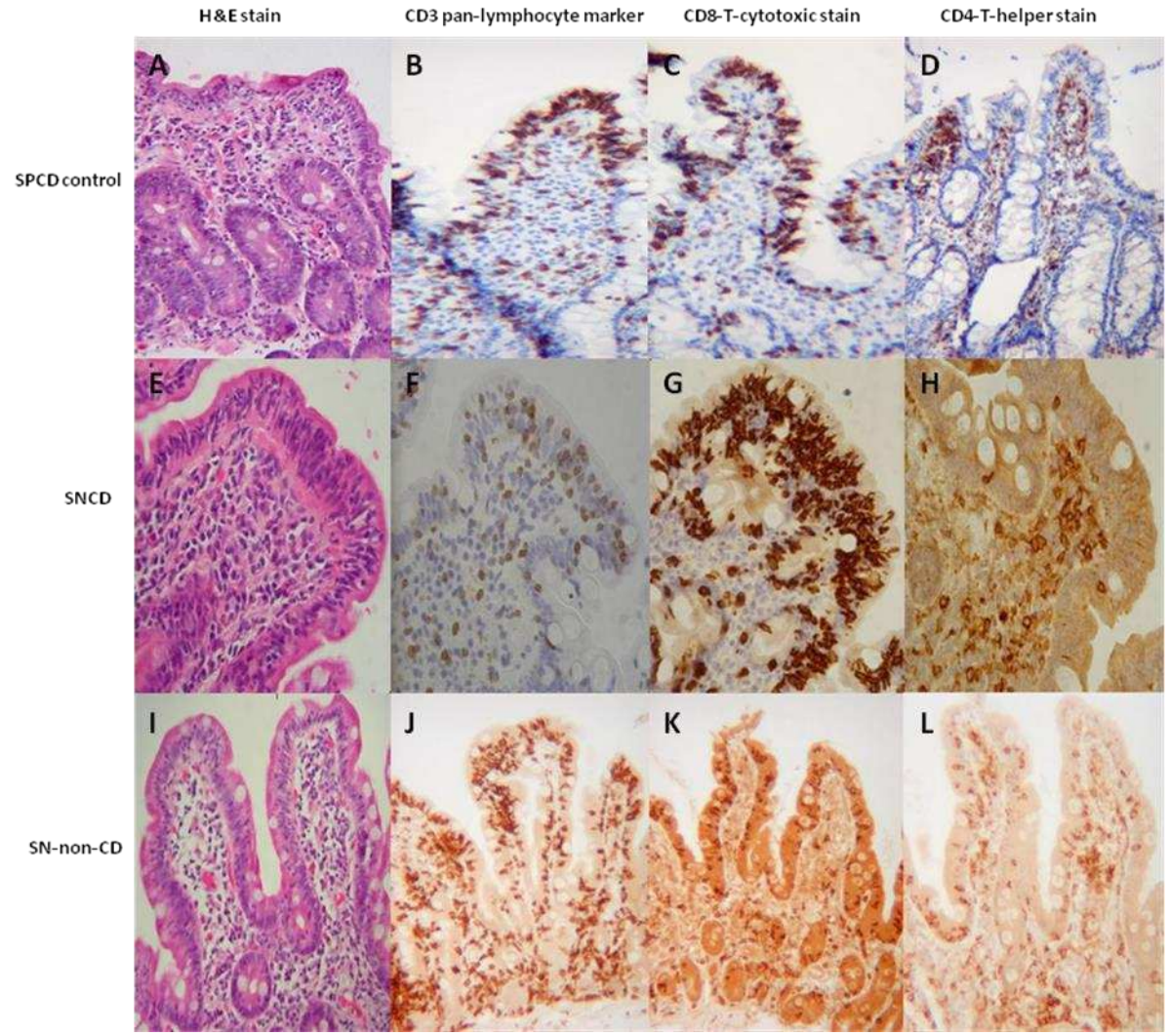


Table 3: Characteristics and diagnostic outcomes in non-whites with SNVA seen at a UK centre

Case	Age/Sex	Ethnicity	Symptoms	Marsh Grading	HLA-DQ2/8 status	Aetiology of SNVA
1	28/female	Pakistan	Abdominal pain, reflux, weight loss	PVA	+	Helicobacter pylori induced duodenitis
2	29/male	Oman	Diarrhoea, nausea, bloating, dyspepsia	PVA	+	No cause found – SNVA resolved
3	30/female	Tunisia	Diarrhoea, anaemia, bloating	PVA	+	Giardiasis
4	33/male	Ghana	Diarrhoea, weight loss, abdominal pain, cough	PVA	+	Sarcoidosis
5	39/male	Somalia	Diarrhoea, bloating, abdominal pain	PVA	+	Small intestinal bacterial overgrowth
6	43/male	Iran	Nausea, dyspepsia, cough, weight loss	PVA	+	Tuberculosis
7	49/female	Pakistan	Anaemia, abdominal pain, bloating, reflux, fatigue, constipation	PVA	+	Small intestinal bacterial overgrowth
8	49/female	India	Anaemia	PVA	+	Helicobacter pylori induced duodenitis
9	49/female	Somalia	Diarrhoea, bloating, abdominal pain, anaemia	PVA	+	No cause found – SNVA resolved
10	58/female	India	Abdominal pain, dyspepsia, bloating	PVA	+	No cause found – SNVA resolved
11	65/female	Iraq	Abdominal pain, bloating, dyspepsia, anaemia	PVA	+	Whipple's disease
12	82/female	Yemen	Abdominal pain, bloating, reflux	PVA	+	Peptic duodenitis
13	18/male	Pakistan	Anaemia	SVA	+	Coeliac disease. Associated Sjogren's and IgA deficiency
14	31/female	Pakistan	Diarrhoea, anaemia, nausea, bloating	SVA	+	Mycobacterium avium, Helicobacter pylori induced duodenitis
15	53/female	India	Anaemia	SVA	+	NSAIDs
16	71/male	Bangladesh	Anaemia, dyspepsia, bloating	SVA	+	Helicobacter pylori induced duodenitis
17	30/male	Iran	Dyspepsia, reflux, weight loss	SVA	+	Coeliac disease
18	22/female	Pakistan	Abdominal pain, nausea, fatigue	PVA	-	Helicobacter pylori induced duodenitis
19	26/female	Yemen	Anaemia, weight loss, abdominal pain. nausea, bloating, fatigue	PVA	-	Helicobacter pylori induced duodenitis
20	35/female	Caribbean	Diarrhoea, bloating, abdominal pain	PVA	-	Helicobacter pylori induced duodenitis
21	36/male	Iraq	Diarrhoea, abdominal pain, bloating	PVA	-	NSAIDs
22	38/male	Zambia	Anaemia, abdominal pain	PVA	-	Tuberculosis, Helicobacter pylori induced duodenitis
23	38/female	Bangladesh	Diarrhoea, anaemia, abdominal pain, fatigue	PVA	-	Helicobacter pylori induced duodenitis
24	45/female	Pakistan	Anaemia	PVA	-	Helicobacter pylori induced duodenitis
25	47/female	Vietnam	Dyspepsia	PVA	-	No cause found – lost to follow-up
26	47/male	Pakistan	Abdominal pain, weight loss, bloating, reflux	PVA	-	Peptic duodenitis
27	47/female	Pakistan	Diarrhoea, bloating	PVA	-	Ascariasis
28	49/female	Bangladesh	Diarrhoea, anaemia, fatigue, fevers, night sweats	PVA	-	Tuberculosis
29	50/male	Caribbean	Diarrhoea, abdominal pain, reflux	PVA	-	Helicobacter pylori induced duodenitis
30	51/female	Yemen	Abdominal pain, weight loss	PVA	-	Helicobacter pylori induced duodenitis
31	54/female	Bangladesh	Diarrhoea, weight loss, abdominal pain, dyspepsia, fatigue, constipation	PVA	-	No cause found – SNVA resolved
32	75/female	Hong Kong	Diarrhoea, weight loss, anaemia	PVA	-	Small intestinal bacterial overgrowth
33	35/male	Bangladesh	Reflux, dyspepsia, weight loss	SVA	-	Helicobacter pylori induced duodenitis
34	57/male	Yemen	Diarrhoea, weight loss	PVA	not stated	Helicobacter pylori induced duodenitis
35	25/male	Caribbean	Diarrhoea	SVA	not stated	HIV enteropathy, Helicobacter pylori induced duodenitis

Figure 3: Duodenal histology of SNVA and SPCD control

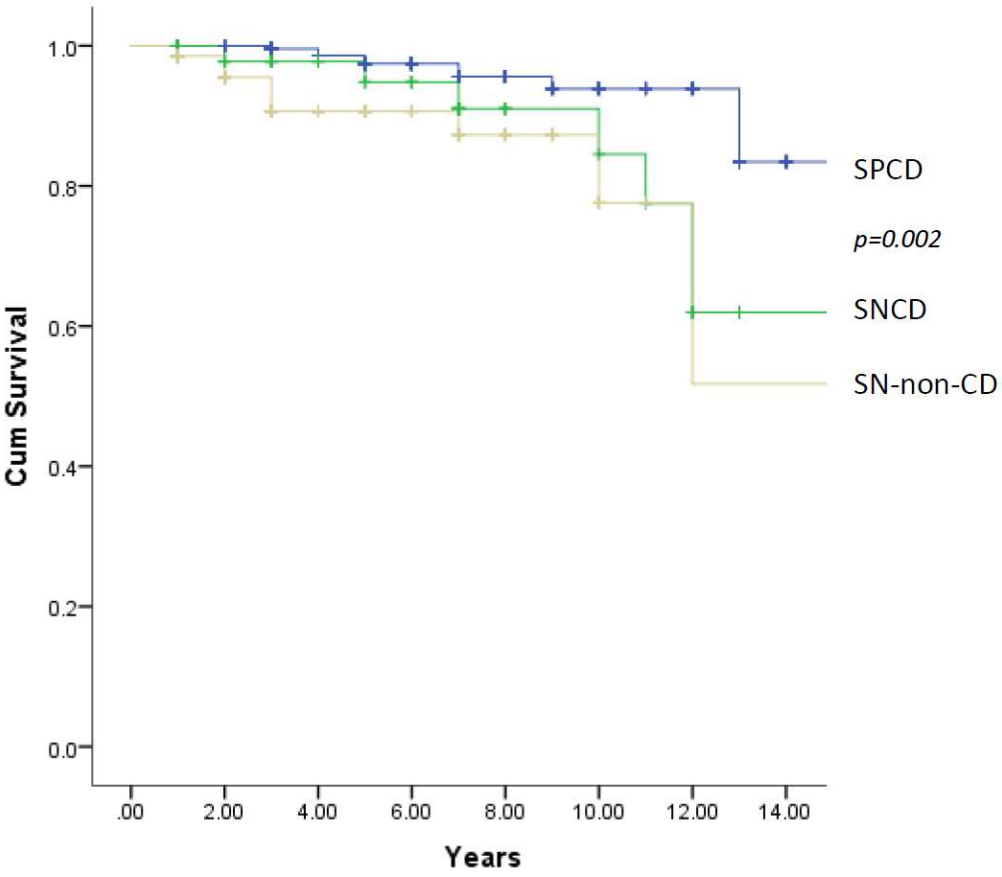


Plates A to D: SPCD control. A white woman presenting with anaemia and positive serum IgA endomysial antibody. Duodenal biopsy demonstrated subtotal villous atrophy when stained with haemotoxylin and eosin (H&E). It can also be seen that there is an increased number of intra-epithelial lymphocytes stained by the pan-lymphocyte marker CD3. Furthermore, staining of antibodies against different intraepithelial lymphocyte phenotype revealed that they are all of the CD8-T-cytotoxic stain and not CD4-T-helper cells. This is the classical pattern of coeliac disease.

Plates E to H: SNCD patient. A white woman presenting with diarrhoea. Serum IgA endomysial antibodies were negative but duodenal biopsy showed subtotal villous atrophy. There were increased intraepithelial lymphocytes noted following CD3 pan-lymphocyte stain, which on immunophenotypic differentiation revealed CD8-T-cytotoxic cells but not CD4-T-helper cells. Her HLA-DQ2 was positive, no alternate cause was found, and she responded to a gluten-free diet.

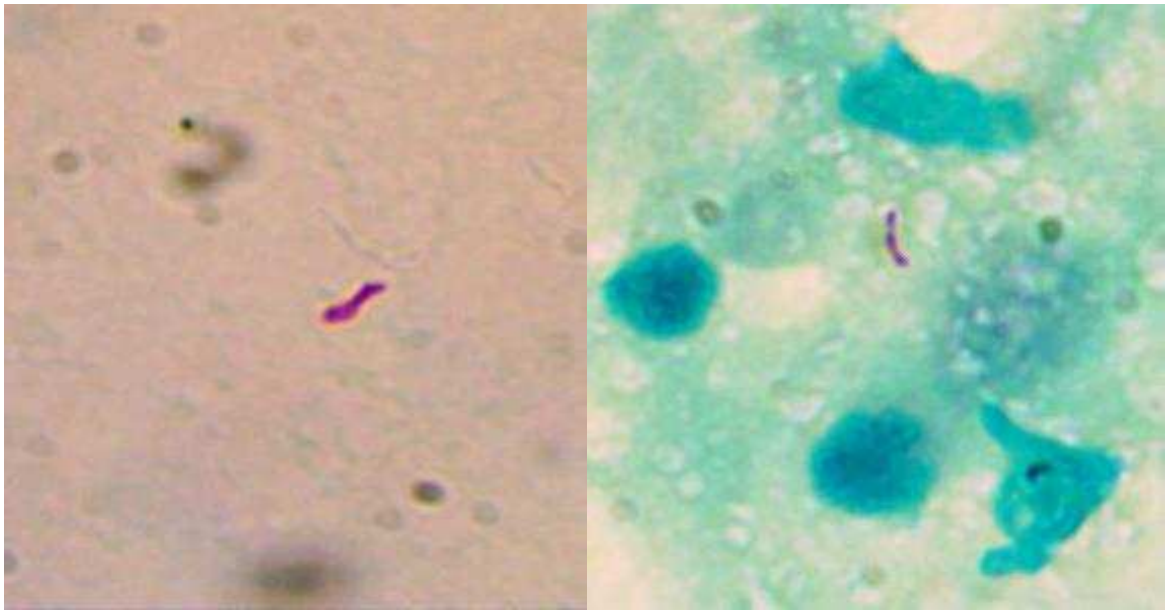
Plates I to L: SN-non-CD patient. Bengali woman presenting with diarrhoea, anaemia, night sweats and fevers. Her serum IgA endomysial antibody was negative but duodenal biopsy showed partial villous atrophy with raised intraepithelial lymphocytes. She stained positive for CD8-T-cytotoxic cells but also for CD4-T-helper cells. This could have been mistaken for refractory coeliac disease. However, her HLA-DQ2/8 genotype was negative and on microbiology assessment her duodenal sample revealed mycobacteria (supplementary picture). She was commenced on anti-tuberculosis therapy.

Figure 4: Kaplan-Meier estimated survival curves for SNVA and SPCD controls



Supplementary picture

A **B**



Mycobacterium demonstrating acid-alcohol fast staining using Ziehl-Neelsen's method following culture in liquid medium (A) and on direct staining of tissue sample (B). The lipid rich mycolic acids in the organism's cell wall prevent decolorisation with acid-alcohol after initial staining with carbol fuchsin (red)

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