Enterovirus as trigger of coeliac disease: nested case-control study within prospective birth cohort

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ABSTRACT

OBJECTIVE
To determine whether infection with human enterovirus or adenovirus, both common intestinal viruses, predicts development of coeliac disease.

DESIGN
Case-control study nested within Norwegian birth cohort recruited between 2001 and 2007 and followed to September 2016.

SETTING
Norwegian population.

PARTICIPANTS
Children carrying the HLA genotype DR4-DQ8/DR3-DQ2 conferring increased risk of coeliac disease.

EXPOSURES
Enterovirus and adenovirus detected using real time polymerase chain reaction in monthly stool samples from age 3 to 36 months.

MAIN OUTCOME MEASURE
Coeliac disease diagnosed according to standard criteria. Coeliac disease antibodies were tested in blood samples taken at age 3, 6, 9, and 12 months and then annually. Adjusted odds ratios from mixed effects logistic regression model were used to assess the relation between viral infections before development of coeliac disease antibodies and coeliac disease.

RESULTS
Among 220 children, and after a mean of 9.9 (SD 1.6) years, 25 children were diagnosed as having coeliac disease after screening and were matched to two controls each. Enterovirus was found in 370 (17%) of 2135 samples and was significantly more frequent in samples collected before development of coeliac disease antibodies in cases than in controls (adjusted odds ratio 1.49, 95% confidence interval 1.07 to 2.06; P=0.02). The association was restricted to infections after introduction of gluten. High quantity samples (>100 000 copies/μL) (adjusted odds ratio 2.11, 1.24 to 3.60; P=0.01) and long lasting infections (>2 months) (2.16, 1.16 to 4.04; P=0.02) gave higher risk estimates. Both the commonly detected enterovirus species *Enterovirus A* and *Enterovirus B* were significantly associated with coeliac disease. The association was not found for infections during or after development of coeliac disease antibodies. Adenovirus was not associated with coeliac disease.

CONCLUSIONS
In this longitudinal study, a higher frequency of enterovirus, but not adenovirus, during early childhood was associated with later coeliac disease. The finding adds new information on the role of viral infections in the aetiology of coeliac disease.

WHAT IS ALREADY KNOWN ON THIS TOPIC

Recent evidence suggests that viral infections are involved in an animal model of coeliac disease

Prospective studies have shown a higher prevalence of infections in children before diagnosis of coeliac disease

Previous studies of adenoviruses and enteroviruses have been limited to cross sectional designs, and reverse causality is a possible explanation for these observations

WHAT THIS STUDY ADDS

Children with increased genetic risk of coeliac disease had a higher frequency of enteroviruses in stool samples before development of coeliac disease than did healthy controls

Adenovirus was not associated with coeliac disease

Identification of specific viruses as triggers of coeliac disease may have implications for preventive strategies and justify future studies to clarify mechanisms

Introduction

Coeliac disease is an immune mediated disease believed to result from gluten intake and unknown environmental trigger factors in genetically susceptible individuals.[1] Coeliac disease develops almost exclusively in people with the HLA-DQ2 (*DQA1*05:01-DQB1*02:01*) or HLA-DQ8 (*DQA1*03-DQB1*03:02*) haplotype. The HLA-DQ2 and HLA-DQ8 haplotypes also occur in about 40% of the general population. Although non-HLA genetic variants have also been identified, the predictive value of these variants, even in combination, is limited.[2 3] As most children are exposed to gluten, this suggests that additional genetic and environmental triggers are involved in development of coeliac disease.[4] The autoimmune process may start months or years before clinical manifestations,[5] and the identification and confirmation of environmental triggers remain a major challenge.[6]

Both experimental studies and epidemiological studies based on parental reporting of illness suggest a role for infections in the development of coeliac disease, particularly gastrointestinal infections.[6-9] Gastrointestinal infections are common in childhood and may impair the mucosal barrier for transfer of dietary proteins as gluten regardless of the presence of clinical symptoms.[10 11] The only prospective study on coeliac disease and viral infection suggested that frequent rotavirus infections might increase the risk of development of coeliac disease antibodies in a cohort at high risk.[12] Other studies with retrospective

designs have studied adenovirus, enterovirus, and orthoreovirus as potential triggers of coeliac disease with conflicting or inconclusive results.[9] [13-15] Study designs including patients diagnosed as having coeliac disease and controls may be subject to bias due to reverse causality. Therefore, long term follow-up of longitudinal birth cohorts is needed.

In this study, we approached the question of potential gastrointestinal triggers by using a longitudinal birth cohort analysis of the most frequently occurring viruses: enterovirus and adenovirus. We chose these two viruses on the basis of a pilot screening of the stool virome in the cohort members. We aimed to test whether the presence of human enterovirus and adenovirus in monthly faecal samples was more common before development of coeliac disease antibodies in cases subsequently diagnosed as having coeliac disease compared with children not developing the disease.

Methods

Participants and study design
We designed a nested case-control study of coeliac disease within a birth cohort of children with the HLA-DQ2/DQ8 genotype, with monthly stool samples tested for nucleic acid of enterovirus and adenovirus. Figure 1 outlines the enrolment of the study sample, and figure 2 gives an overview of the study design. The MIDIA study, which was originally designed to study type 1 diabetes, is described in detail by Stene et al.[16]

Briefly, during 2001-07, 46 939 newborns throughout Norway were screened for the HLA-DQ2/DQ8 genotype conferring an increased risk of both coeliac disease and type 1 diabetes (HLA-*DRB1*04:01-DQA1*03-DQB1*03:02/DRB1*03-DQA1*05:01-DQB1*02:01*). This risk genotype was identified in 912 (1.9%) children, who were followed with repeated blood and faecal samples from the age of 3 months. We collected plasma samples at age 3, 6, 9, and 12 months and annually thereafter. Monthly stool samples collected at age 3-36 months were diluted in preservation buffer, centrifuged, and supernatant separated. All samples were stored at −80°C until testing.[17]

Children who still actively contributed with blood samples during 2014-16 (n=501) were invited to coeliac disease screening, of whom 220 consented to participate. These participants tended to have a higher prevalence of type 1 diabetes, fewer siblings, and slightly higher prevalence of family history of coeliac disease at initial recruitment around 3 months of age, than the whole cohort (supplementary table A).

Case definition and selection of matched controls
From the 220 consenting participants, we first identified those who had coeliac disease by measuring serological markers of coeliac disease in their most recent sample and by review of clinical documentation, followed by an invitation for a confirmatory blood sample and for standard paediatric diagnostic investigation for coeliac disease. We identified previously diagnosed cases by using a parental questionnaire followed by a review of medical files. For an overview of the coeliac screening process, see supplementary figure A. We diagnosed coeliac disease according to the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) 2012 criteria.[18] By this procedure, we identified 27 cases of coeliac disease by February 2016 and confirmed that the remaining 193 participants were free from coeliac disease. Mean age at end of follow-up was 9.9 (SD 1.6) years. After evaluation for eligibility, we matched 25 cases (16 girls and 9 boys; 11% of participants) with coeliac disease to two controls each. Matching was done for duration of follow-up, date of birth, and county of residence. We excluded one matched control from analysis owing to missing stool samples. See table 1 for characteristics of the nested cases and controls. For more details about case definition, see supplementary methods.

We then determined the time interval when cases seroconverted for coeliac disease markers by retrospectively analysing biobanked plasma samples that had been collected longitudinally since age 3 months, searching for the last sample that was negative for the serological markers and the first sample indicative of coeliac disease. Of the 25 case-control groups, 15 had this seroconversion period covered by monthly stool sampling, whereas the remaining 10 seroconverted after the collection of stools was terminated (after 36 months of age). For characteristics of the children with coeliac disease, see supplementary table B.

Serological testing for coeliac disease
IgA anti-tissue transglutaminase and IgG anti-deamidated gliadin peptide (EliA Celikey IgA/EliA GliadinDP IgG, Thermo Fischer Scientific, Phadia AB; Uppsala, Sweden) were measured in the most recent blood sample from each participant at the laboratory of Akershus University Hospital, Lørenskog, Norway. Mean age at screening was 8.6 (SD 2.0) years. Screening with IgA anti-tissue transglutaminase in combination with IgG anti-deamidated gliadin peptide should better capture people with IgA deficiency and samples in which tissue transglutaminase concentrations may have been influenced by haemolysis.[19-21] In accordance with a previous screening study and to increase sensitivity,[22] we defined IgA anti-tissue transglutaminase values of 3 U/mL or higher as positive. For IgG anti-deamidated gliadin peptide, we used 7 U/mL as the cut-off value in accordance with the manufacturer's instruction. We contacted children with positive serological results (n=20) for a second blood sample (Varelisa tissue transglutaminase IgA, Phadia, cut-off <3 U/mL (<5 U/mL until 2015); IgG anti-deamidated gliadin peptide, QUANTA Lite Gliadin IgG IIInova, cut-off <20 U/mL) which was analysed at Oslo University Hospital Ullevål, Oslo, Norway.

Detection of enterovirus and adenovirus in stool samples
All available faecal samples from cases and controls were subjected to RNA and DNA extraction using
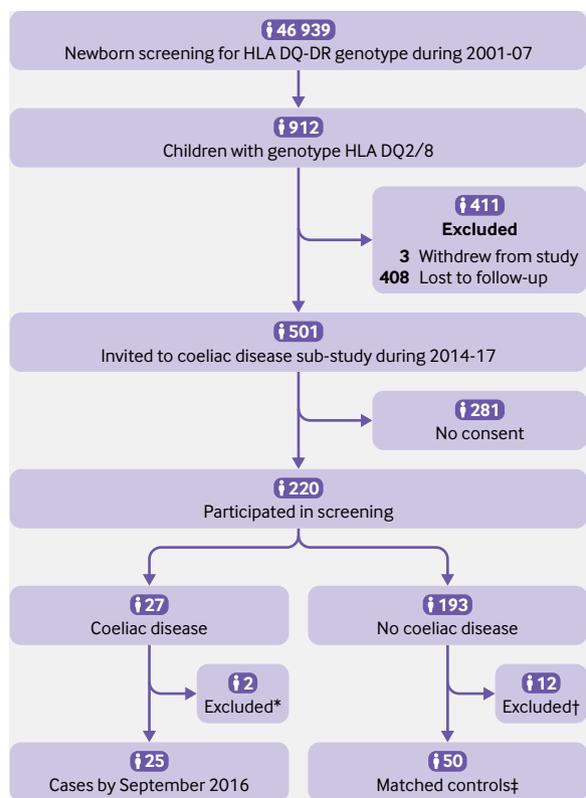
Fig 1 | Enrolment of study sample. "Invited" children are those who were still actively participating (delivering samples, questionnaires) at start of coeliac disease sub-study and were invited to participate; "participated" are those who consented to participate and were screened for coeliac disease antibodies. *Two cases were excluded from analyses, one owing to missing stool samples and one owing to incorrect use of diagnostic criteria. †Children with positive coeliac disease antibodies at first screening test but for whom follow-up did not confirm coeliac disease diagnosis (n=10) were excluded from further analyses; control children with high antibody titres (>10 times cut-off) (n=2) in old samples but with normal values at screening were excluded and replaced by new controls selected using same matching criteria. ‡One matched control was excluded from analysis owing to missing stool samples

Qiagen chemistry (Qiagen, Hilden, Germany). We tested enterovirus by quantitative real time polymerase chain reaction (PCR).[23] The primers detected with an equal sensitivity the *Enterovirus A-D* species (that is, members of species *Enterovirus A*, *Enterovirus B*, *Enterovirus C*, and *Enterovirus D* of the genus *Enterovirus*, family *Picornaviridae*, order *Picornavirales*; the former nomenclature of these species was *Human enterovirus A-D*) but did not react with human rhinoviruses (members of species *Rhinovirus A-C*). Adenovirus was tested using a previously published real time PCR assay.[24] The threshold of enterovirus and adenovirus positivity was set to 10 copies/µL nucleic acid. We tested 2161 stool samples, of which 2135 and 2006 provided data on quantity of enterovirus and adenovirus, respectively. All samples were tested blinded as to the case-control status.

All samples with more than 100 copies/µL of enterovirus were subjected to genotyping with Sanger or next generation amplicon sequencing of PCR amplicons of the VP1 gene segment informative for the virus type. Samples with more than 10 copies/µL of adenovirus were genotyped by a similar protocol amplifying the seventh hypervariable region of its hexon gene. Primers, probes, and a detailed protocol have been described in Cinek et al.[25] See also supplementary methods for more details.

Statistical analysis

We analysed the association of virus with coeliac disease primarily by using a mixed effects logistic regression model with random intercepts for each matched set and for each individual child, thus accounting for the matched design. We used faecal sample virus positivity as the dependent variable (separate models for enterovirus and adenovirus) and case-control status as an independent variable. The odds ratio for coeliac disease status is then interpreted as the odds of a faecal sample being positive for virus given that it came from a child who later developed coeliac disease, compared with the same odds for virus positivity for samples from matched controls. We included the following potential predictors of viral infection and coeliac disease as covariates in the main regression model: sex, age, age squared, season of sample collection, number of siblings (categorised as none, 1, or ≥2), and family history of coeliac disease. In the primary analysis, we included only stool samples from the matched sets collected before the case child developed coeliac disease antibodies to avoid reverse causality (fig 3).

In pre-planned sub-analyses, we explored the association between enterovirus and adenovirus and subsequent coeliac disease for stool samples collected during and after the development of coeliac disease
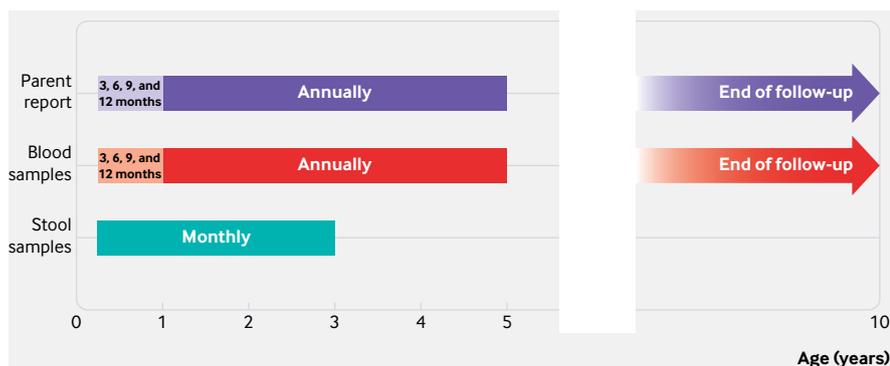

Fig 2 | Timeline of study

Table 1 | Characteristics of nested cases and controls. Values are numbers (percentages) unless stated otherwise

Characteristics	Cases (n=25)	Controls (n=49)
Female sex	16 (64)	26 (53)
Other children in household:		
None	7 (28)	15 (31)
1	10 (40)	28 (57)
≥2	8 (32)	6 (12)
Type 1 diabetes	4 (16)	1 (2)
Family history of coeliac disease*	7 (28)	4 (8)
Mean (SD) age (months) at antibody screening test†	99 (30)	106 (21)
Mean (SD) age (months) at end of follow-up‡	119 (19)	119 (19)
Mean (SD) age (months) at last stool sample	32 (7)	34 (3)
Stool samples§	703	1458
Enterovirus:		
Stool samples providing enterovirus data	690 (98)	1445 (99)
Enterovirus positive stool samples	135 (20)	235 (16)
Median (range) count of positive samples per child	6 (0-11)	4 (0-9)
Adenovirus:		
Stool samples providing adenovirus data	649 (92)	1357 (93)
Adenovirus positive samples	85 (13)	173 (13)
Median (range) count of positive samples per child	4 (0-9)	3 (0-11)
Blood samples§:		
Total	326	593
Samples providing coeliac disease antibody data¶	259 (79)	374 (63)
Coeliac disease antibody positive samples¶	131 (51)	7 (2)

*Known coeliac disease in first degree relative or half-sibling ascertained at coeliac disease screening 2014-16.
†Antibody screening test performed on both cases and controls at time of inclusion in coeliac disease sub-study of MIDIA.
‡At time when all 25 cases were ascertained by end of February 2016.
§Discrepancy between number of stool samples and samples providing virus data and number of blood samples and samples providing data on coeliac disease antibodies was due to missing samples or failed laboratory test.
¶Antibodies to tissue transglutaminase.

as long infectious episodes (at least two positive consecutive monthly samples) and per infection with high quantity (≥100 000 copies per µL nucleic acid). We investigated specific virus types, using an a priori determined threshold of at least 20 positive samples to be analysed. We grouped enteroviruses into *Enterovirus A-D* species as detailed above. We grouped adenovirus types only into specific types (for example, human adenovirus C2), as the species *Adenovirus C* dominated in our samples.

Additionally, we adjusted the primary analysis for the timing of introduction of gluten and breast feeding. As exploratory analyses, we investigated time periods less than six months, six to 12 months, more than 12 months, after the end of breast feeding, and after the introduction of gluten. We also investigated whether infectious symptoms, as reported by parents in longitudinal questionnaires in early life, were associated with coeliac disease antibodies and whether specific symptoms were linked to infections. In sensitivity analyses, we also adjusted for type 1 diabetes.

Patient and public involvement

Patients were not involved in setting the research question or the outcome measures, nor were they involved in developing plans for recruitment, design, or implementation of the study. No patients were asked to advise on interpretation or writing up of results. We will disseminate the results of the research to study participants and the general public.

Results

Enterovirus for whole observational period

We detected enterovirus in 370 (17%) of 2135 stool samples (table 1), with 73 children having at least one positive sample. The distribution of enterovirus in cases and controls of the 25 matching groups is shown in supplementary figure B. Enterovirus showed variation with age and season, with a peak in autumn (supplementary figures C and D). Several different

antibodies (fig 3). We analysed the samples before development of coeliac disease antibodies, defining the exposure as number of infectious episodes, counting a sequence of consecutively virus positive faecal samples as a single episode (a negative stool sample is required before a new episode is defined). We speculated that a higher quantity of enterovirus, longer duration of viral shedding, or symptomatic infections would have a greater effect on development of coeliac disease. Therefore, we analysed infections with reported symptoms and defining exposures
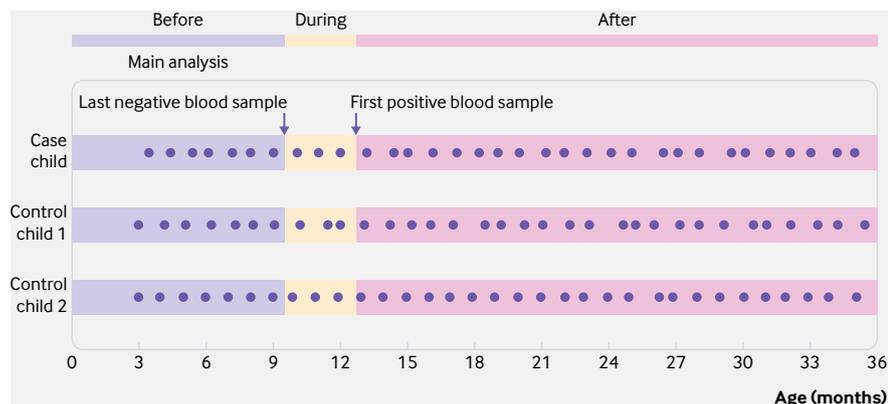

Fig 3 | Development of coeliac disease antibodies. Figure illustrating definition of before, during, and after development of coeliac disease antibodies. Dots represent faecal samples. Primary analysis included only stool samples collected up to age at last antibody negative blood sample for cases and corresponding age for matched controls. Pre-planned sub-analyses explored same association for stool samples collected during development of coeliac disease antibodies (between last negative and first antibody positive blood sample) and samples collected after first antibody positive blood sample

types were detected, occasionally as co-infections, with coxsackievirus A2 and A4 being most common (supplementary table C).

Enterovirus and coeliac disease

The frequency of enterovirus positive stool samples before development of coeliac disease antibodies was 84/429 (20%) in cases and 129/855 (15%) in matched controls (adjusted odds ratio 1.49, 95% confidence interval 1.07 to 2.06; P=0.02) (table 2). The adjusted odds ratio was 2.11 (1.24 to 3.60; P=0.01) for high quantity samples (>100 000 copies/µL), 2.16 (1.16 to 4.04; P=0.02) for long lasting infections (more than two months), and 1.27 (0.87 to 1.86; P=0.21) for infectious episodes (consecutive positive samples counted as a single episode) (supplementary table D). The frequency of enterovirus in stool samples during or after development of coeliac disease antibodies was not associated with coeliac disease (table 2).

Exploratory analyses of enterovirus and coeliac disease

Both the commonly identified enterovirus species *Enterovirus A* (adjusted odds ratio 1.62, 1.04 to 2.53; P=0.03) and *Enterovirus B* (2.27, 1.33 to 3.88; P=0.003) were significantly associated with later coeliac disease. *Enterovirus C* and *Enterovirus D* were detected in few or no samples (table 2).

Enterovirus infections after the first year of life showed increased estimates, whereas infections from age 3 to 6 months or from 6 to 12 months did not. Enterovirus infections after introduction of gluten were associated with coeliac disease, whereas infections before or at the time of gluten introduction were not (supplementary table E). Similarly, infections after the end of breast feeding were associated with coeliac disease, but enterovirus infections during breast feeding were not. Enterovirus infections in shorter time windows before the last sample negative for coeliac disease antibody showed borderline increased

estimates (supplementary table E). We found no association between reported infectious symptoms and coeliac disease or between infectious symptoms and enterovirus positivity (supplementary tables F and G). Enterovirus positivity at the same time of reported fever or diarrhoea was associated with development of coeliac disease, but other symptoms were not (supplementary table F). Adjustment for type 1 diabetes only marginally changed the estimates (adjusted odds ratio 1.43, 1.02 to 2.02).

Adenovirus and coeliac disease

We detected adenovirus in 258 (13%) of 2006 faecal samples (table 1), with 61 children having at least one positive sample. Adenovirus showed variation with age but did not follow a seasonal pattern (supplementary figures C and D). Several different types were detected, occasionally as co-infections, with human adenovirus C2 and C1 being most common (supplementary table H).

We found no significant difference between cases and controls in adenovirus positive stool samples before (adjusted odds ratio 0.82, 0.49 to 1.38; P=0.46), during, or after development of coeliac disease antibodies. No adenovirus types were associated with development of coeliac disease (table 3). Adenovirus was associated with fever, but adenovirus positive samples with reported symptoms were not associated with development of coeliac disease (supplementary table F).

Discussion

We found a significant association between exposure to enterovirus and subsequent risk of coeliac disease. Adenovirus was not associated with coeliac disease.

Strengths and limitations of study

This is the first population based study on viruses in stool samples collected longitudinally before development of coeliac disease antibody markers in children later diagnosed as having coeliac disease. We

Table 2 | Enterovirus positive stool samples and subsequent coeliac disease*

	Positive samples/total samples (%)			Adjusted†	
	Cases (n=25)	Controls (n=49)	Unadjusted odds ratio (95% CI)	Odds ratio (95% CI)†	P value
Before development of coeliac disease antibodies‡					
All enteroviruses	84/429 (20)	129/855 (15)	1.37 (1.01 to 1.87)	1.49 (1.07 to 2.06)	0.02
Enterovirus A‡	42/387 (11)	60/787 (8)	1.47 (0.97 to 2.23)	1.62 (1.04 to 2.53)	0.03
CV-A2	10/355 (3)	10/737 (1)	2.11 (0.87 to 5.11)	2.29 (0.89 to 5.87)	0.09
CV-A4	5/350 (1)	11/738 (1)	0.96 (0.33 to 2.78)	0.93 (0.30 to 2.86)	0.90
CV-A6	7/352 (2)	9/736 (1)	1.64 (0.60 to 4.44)	1.59 (0.56 to 4.57)	0.39
CV-A10	4/349 (1)	10/737 (1)	0.84 (0.25 to 2.76)	1.03 (0.30 to 3.53)	0.96
CV-A16	4/349 (1)	7/734 (1)	1.20 (0.35 to 4.14)	1.39 (0.36 to 5.30)	0.63
Enterovirus B‡	34/379 (9)	43/770 (6)	1.74 (1.08 to 2.81)	2.27 (1.33 to 3.88)	0.003
During/after development of coeliac disease antibodies§					
All enteroviruses during	30/140 (21)	58/332 (17)	1.24 (0.74 to 2.10)	1.26 (0.66 to 2.39)	0.48
All enteroviruses after	21/121 (17)	48/258 (19)	0.92 (0.52 to 1.62)	0.82 (0.44 to 1.53)	0.54

*Number of positive/total stool samples and results from mixed effects logistic regression are given for cases and controls at different time periods. In analysis for specific genotypes, samples positive for other enteroviruses have been set to missing. *Enterovirus C* and *Enterovirus D* were too few for a meaningful analysis and are not presented.
†Adjusted for sex, age, age squared, season of sample collection, number of siblings, and family history of coeliac disease.
‡Only enterovirus types found in more than 10 samples before development of coeliac disease antibodies are presented.
§Before: before last coeliac disease antibody negative blood sample. During: between last coeliac disease antibody negative and first coeliac disease antibody positive blood sample. After: after first coeliac disease antibody positive blood sample.

Table 3 | Adenovirus positive stool samples and subsequent coeliac disease*

	Positive samples/total samples (%)			Adjusted†		
	Cases (n=25)	Controls (n=49)	Unadjusted odds ratio (95% CI)	Odds ratio (95% CI)	P value
Before development of coeliac disease antibodies‡					
All adenoviruses	47/390 (12)	111/775 (14)	0.82 (0.50 to 1.35)	0.82 (0.49 to 1.38)	0.46
HAdV-C1	17/360 (5)	60/724 (8)	0.65 (0.19 to 2.19)	0.66 (0.19 to 2.29)	0.51
HAdV-C2	20/363 (6)	43/707 (6)	0.88 (0.43 to 1.80)	0.84 (0.38 to 1.87)	0.67
HAdV-C5	5/348 (1)	7/671 (1)	1.42 (0.29 to 6.96)	2.22 (0.52 to 9.57)	0.28
During/after development of coeliac disease antibodies‡					
All adenoviruses during	26/139 (19)	36/325 (11)	1.84 (0.89 to 3.79)	1.76 (0.87 to 3.56)	0.12
All adenoviruses after	12/120 (10)	26/257 (10)	0.99 (0.41 to 2.39)	1.29 (0.47 to 3.52)	0.62

HAdV=human adenovirus.
*Number of positive/total stool samples and results from mixed effects logistic regression are given for cases and controls at different time periods. In analysis for specific genotypes, samples positive for other adenoviruses have been set to missing. Adenovirus types were grouped into only specific genotypes (eg, HAdV-C2), as species *Adenovirus C* dominated in samples.
†Adjusted for sex, age, age squared, season of sample collection, number of siblings, and family history of coeliac disease.
‡Before: before last coeliac disease antibody negative sample. During: between last coeliac disease antibody negative and first coeliac disease antibody positive blood sample. After: after first coeliac disease antibody positive blood sample.

used accepted diagnostic criteria for coeliac disease. The development of coeliac disease antibodies, which is believed to be the first sign of disease, may start months or even years before clinical manifestations and diagnosis. Importantly, we analysed exposure before development of coeliac disease antibodies and thus avoided the potential reverse causation that may bias studies of infections at or after clinical diagnosis of coeliac disease. The long time period from development of coeliac disease antibodies to diagnosis of coeliac disease makes it highly unlikely that infections before development of coeliac disease antibodies prompted symptoms and diagnostic investigation for coeliac disease. Enterovirus positivity and type distribution in our study was in line with previous studies.[26] We used a PCR technique, which is a highly sensitive method for detection of virus locally in the gut but does not measure the immune response as serological testing does.[27] We believe that PCR was most appropriate in our study, as most infections were asymptomatic and current enterovirus serology not is designed for this kind of setting. We used a quantitative assay that enabled us to follow the dynamics of the viral load. Regular monthly stool sampling and high completeness were important strengths, because duration of viral shedding is expected to be around three to four weeks.[28 29] Administration of oral poliovirus vaccine may increase the detection of enterovirus in stool samples, but this cohort received inactivated poliovirus vaccine injections.[30 31]

Our study also had some limitations. The participants were followed up for about 10 years, and some children (particularly among those with the shortest follow-up) would be likely to be diagnosed as having coeliac disease after that age. Even though we analysed a large number of samples, the number of children with coeliac disease was limited. Furthermore, the limited sample size for some of the sub-analyses could have led to spurious associations. Loss to follow-up and modest response rate is inevitable in most cohort studies and also raises the possibility of selection bias. The nested cases and controls tended to have a higher prevalence of type 1 diabetes, fewer siblings, and a slightly higher frequency of family history of coeliac disease than the whole cohort. This could have led to non-representativeness, but it does not necessarily bias exposure-outcome associations.[32] The study was originally designed to study type 1 diabetes, with inclusion of only children with the HLA-DQ2/DQ8 genotype. Although we cannot generalise our findings beyond this genotype, we note that this genotype accommodates the two well established susceptibility haplotypes (DQ2 and DQ8) present in nearly all patients. We therefore believe that our findings are likely to apply to a sizeable proportion of patients with coeliac disease. Furthermore, the frequency of faecal enterovirus shedding did not differ significantly between people with the HLA-DQ2/DQ8 genotype and other genotypes in a previous study.[33] We did our best to adjust for potential confounding factors, and we are not aware of any obvious confounding factors that could plausibly explain our findings, but unmeasured confounding factors or residual confounding can never be entirely ruled out in non-randomised studies. Further studies in other settings would therefore be necessary to corroborate our findings.

Comparison with other studies
Coeliac disease and type 1 diabetes have several common features, and viral infections are candidate triggers in both diseases. In type 1 diabetes, enterovirus infections before islet autoimmunity, as well as persistent enterovirus infections in the gut mucosa or pancreatic tissue, have been described.[34-36] The single study on enterovirus and coeliac disease was cross sectional but could not find persistent viral infection in intestinal biopsies from untreated coeliac disease patients.[15] The study did not investigate signs of previous exposure to enterovirus. Finally, adjusting for type 1 diabetes in our analyses yielded similar results. This is consistent with the lack of association between enterovirus and diabetes autoimmunity in the same cohort.[37]

Adenoviruses have been proposed to precipitate coeliac disease via molecular mimicry.[13 38 39] However, later studies did not confirm the association.[14 40] Our negative findings are in line with the latter studies and

suggest that adenovirus in general does not influence risk of coeliac disease. An important strength of our study was the prospective design, whereas the studies mentioned above were cross sectional. Although we cannot rule out moderate effects from our data, the 95% confidence interval around the odds ratios estimated from our data suggest that strong associations with adenovirus infections are unlikely.

Interpretation

Although the effect sizes are relatively small, this study suggests that infections with enterovirus in early life could be one among several key risk factors for development of a disease with lifelong consequences. Our observations suggest that, rather than a specific enterovirus species or type driving the association, several enterovirus types, high titre, and long duration infections in the period after introduction of gluten were involved. Collectively, our results are compatible with a mechanism whereby viral infections may disrupt the mucosal barrier with increased translocation of gluten peptides into the mucosa as the initial event in the loss of tolerance.[41] We speculate that enteroviruses may provide a danger signal that activates dendritic cells acting as antigen presenting cells for CD4 positive gluten reactive T cells in the presence of transglutaminase modified gluten peptides.[9 42 43]

Patients with coeliac disease may have enteric barrier disruption before the development of autoantibodies and thus a susceptibility to enterovirus. However, we believe a more plausible explanation is that enterovirus causes impaired barrier function, which in turn increases the risk of coeliac disease. A challenge in studying the temporal association is the potential separation in time between the triggering event and disease onset as marked by appearance in the blood of coeliac disease antibodies. The results from our study suggest a certain time window between these events. As stool sampling was restricted to the period between birth and 3 years of age, we are lacking data just before the development of coeliac disease antibodies in a few cases diagnosed after 4 years of age.

Our study design is less sensitive to detection of infections occurring with lower frequency and shorter duration of viral shedding (rotavirus, orthoreovirus), so a non-specific response to several virus infections should be further investigated. If enterovirus is confirmed as a trigger factor, vaccination could reduce the risk of development of coeliac disease. Currently, except for poliovirus, non-polio enterovirus vaccines are not commercially available, but efforts are ongoing to develop such vaccines.[27 44] Further research in larger sample sets are needed to confirm our results.

Conclusion

In this longitudinal study, we found that a higher frequency of enterovirus infections was associated with increased risk of coeliac disease. Given the limited number of cases, we call for corroboration in similar studies and preferably interventional studies to reach conclusions about causality.

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Supplementary materials