The PreventCD Study design: towards new strategies for the prevention of coeliac disease

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Background PreventCD (*www.preventcd.com*) is a European multicentre study, which studies the influence of infant nutrition, and that of genetic, immunologic and environmental factors, on the risk of developing coeliac disease (CD). The hypothesis is that it is possible to induce tolerance to gluten by introducing small quantities of gluten to infants, preferably while they are still being breast-fed, and that this might also reduce the risk for related autoimmune disorders.

Aim To describe the design of this ongoing European CD research project.

Methods PreventCD encompasses two study designs and two study populations: (i) a European multicentre study: a prospective, double-blind, randomized dietary-intervention study among infants from families with high risk of CD, and (ii) a Swedish population-based CD screening study among 12-year-olds from the general population, divided into two birth cohorts that differ with respect to infant feeding practices.

Discussion PreventCD is expected to elucidate some of the genetic and immunological mechanisms involved in the process of immune intolerance. *Eur J Gastroenterol Hepatol* 22:1424–1430 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Coeliac disease (CD) is an autoimmune enteropathy. The aetiology of CD is complex and not fully understood yet. In genetically susceptible individuals, ingestion of the gluten peptides present in wheat, rye and barley triggers an aberrant inflammatory T-cell response, subsequently resulting in histological alterations of the small intestinal mucosa, which may cause the malabsorption of nutrients [1–4]. The only treatment is adherence to a gluten-free diet, which may considerably affect the patient's overall quality of life [5–7].

The prevalence of CD among the European population has been estimated at 0.5–1%, which means that approximately 2.5 million Europeans are affected [4,8,9]. The health burden of CD is considerable, as it increases the overall mortality risk [10,11], reduces quality of life [12] and, at a societal level, has extensive negative economic consequences [13]. For these reasons, the prevention of the disease is strongly warranted [14]. To develop strategies for primary prevention, the underlying genetic and immuno-logical mechanisms of CD should be further explored. CD has a strong genetic component, and is one of the hereditary complex disorders, with a prevalence of 5–15% in first-degree relatives of a CD patient and a strikingly high-concordance rate among monozygotic twins as compared with dizygotic twins [15]. Ninety-eight percent of CD patients possess the human leucocyte antigen (HLA)-DQ2/DQ8 molecules [3], and about 30% of the general population. Hence, HLA is a necessary, though not sufficient, factor in the development of CD [16].

Genome-wide association studies have recently identified 26 non-HLA loci that might be involved in the development of CD; 22 of these contain genes controlling immunological responses [17–20]. CD patients have an increased risk of other autoimmune diseases, for example, diabetes mellitus type I (20%) [21,22] and some 5% of patients with other autoimmune diseases suffer from CD [4]. Notably, a gluten-dependent occurrence of diabetes mellitus type I and thyroid-related antibodies has been shown [23]. Although such comorbidity can partly be explained by shared genetic factors [24], it is

*See appendix.

nevertheless suggested that environmental and lifestyle factors may also contribute to the development of CD.

Importantly, many studies have focused on the effect of gluten intake by infants on the development of CD [25–31]; however early exposure to gluten has never been identified as an independent risk factor in the development of CD. The study of Norris *et al.* [32] suggested a 'window of opportunity' for lowering the risk of CD by introducing gluten into the child's diet at the age of 4–6 months. This period in infancy is thought to be important for the development of the immune system and also for the discrimination between tolerance and intolerance to specific nutritional antigens [32,33]. Data from the Swedish CD epidemic, which arose in the mid-1980s, suggest that CD may be prevented by adjusting infant nutrition [30,31]. In the case of this epidemic, the incidence rate of symptomatic CD among children below 2 years of age increased 4-fold, and declined equally abruptly approximately a decade later. Potential contributing factors were investigated, with the main cause subsequently established as changes in infant feeding practices [30,31]. Prior to the epidemic, it was recommended nationally that the introduction of gluten into children's diet should be postponed from the age of 4–6 months, the interval during which breast-feeding is commonly discontinued. At the same time, although notably unrelated to this, the gluten content of commercially available cereal milk drinks and porridges was increased. The decline of the epidemic was preceded by a reinstatement of the earlier recommendation, and to reduce the gluten content in commercial infant foods.

The Swedish experience suggests that a gradual introduction of gluten, if possible while breast-feeding, will reduce the risk of CD [30,31,34,35]. Moreover, a meta-analysis, which includes the Swedish findings, confirms the protective effects of breast-feeding at the time of gluten introduction [36]. Additional studies also suggest that infant nutrition may have a significant impact on the subsequent risk of developing CD, and other autoimmune disorders [32,34,35,37]. These findings corroborate the need to develop prevention strategies, particularly as the current guideline recommends the introduction of gluten only after 6 months [38].

This study describes the design of an ongoing European CD research project, PreventCD, which aims to develop strategies for the prevention of CD and other autoimmune diseases by optimizing infant feeding practices.

Objectives

The objective of PreventCD is to reduce the number of people suffering from CD by developing primary prevention strategies. The hypothesis is that it is possible to induce tolerance to gluten by exposing infants to small quantities of gluten, preferably while they are still being breast-fed, and that this might similarly reduce the risk

for related autoimmune disorders. To achieve this objective, PreventCD studies the influence of infant nutrition on the development of CD and related autoimmune phenomena, and how genetic, immunological and environmental factors relate to this development.

More specifically, PreventCD studies:

(1) The role of HLA and non-HLA risk alleles;
(2) The early immunological response to gluten introduction; and
(3) The role of infant nutrition, with respect to gluten introduction and breast-feeding.

PreventCD also makes a special effort to disseminate its findings and raise awareness of CD.

Participants and funding

PreventCD (*www.preventcd.com*) is a European multicentre study comprising 17 partners in 11 countries: Belgium, Croatia, Germany, Hungary, Italy, Israel, The Netherlands, Norway, Poland, Spain and Sweden. PreventCD involves 13 clinical centres and/or universities, three industrial companies and the Association of European CD Societies. It is supervised by the Leiden University Medical Center (LUMC) in Leiden, The Netherlands. PreventCD has been funded by a grant from the European Commission (FP6-2005-FOOD-4B-36383 – PREVENTCD) from 2007 to 2010.

The study entitled ETICS (Exploring the Iceberg of CD patients in Sweden) has been partly incorporated into PreventCD. ETICS is a Swedish multicentre study initiated in 2005, overseen by the Epidemiology and Global Health division of the Department of Public Health and Clinical Medicine at Umeå University (*www.etics.se*). ETICS also receives support from the Center for Global Health at Umeå University (*www.globalhealthresearch.net*) and several Swedish research councils.
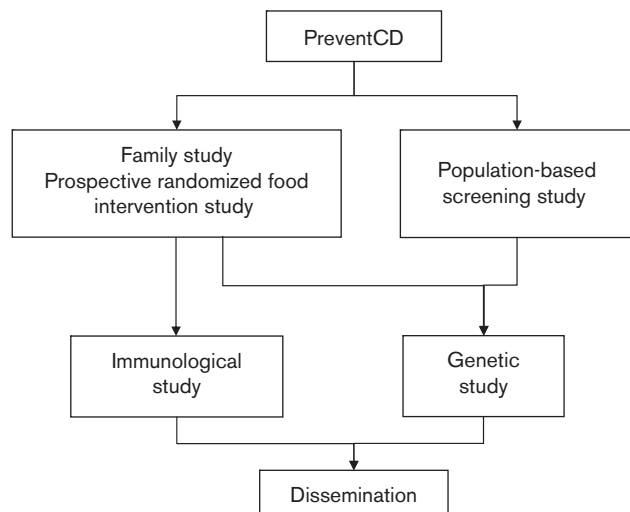
Design

PreventCD encompasses two distinct study populations (Fig. 1). The family study is a multicentre study among infants from families with high risk of CD, whose parents have consented to prospective double-blind randomized dietary intervention and repeated CD screening up to the age of 3 years. The Swedish study is based on the screening for CD carried out among the general population after the epidemic [30,35]. The immunological study analyzes only the material from the family study, whereas the genetic study used material from both the populations.

Family study
Participants

A cohort of at least 1000 newborns with an increased genetic risk of CD is enrolled for eligibility (Fig. 2). Included are:

Fig. 1

Flow chart of the European multicentre study PreventCD.

Fig. 2

Flow chart of the PreventCD Family study structure. CD, coeliac disease; HLA, human leucocyte antigen.

(1) Children, 0–3 months of age at high risk of developing CD, that is, those who: (i) have at least one first-degree family member with CD confirmed by small-bowel biopsy, and (ii) is HLA-DQ2 or HLA-DQ8 positive, or otherwise carrying the allele DQB1*02; and

(2) Children whose parents or legal guardians consented to their participation in the trial.

Excluded are:

(1) Children born prematurely or small for gestational age; and/or

(2) Children diagnosed with syndromes associated with an increased risk of CD, for example, trisomy 21 and Turner's syndrome.
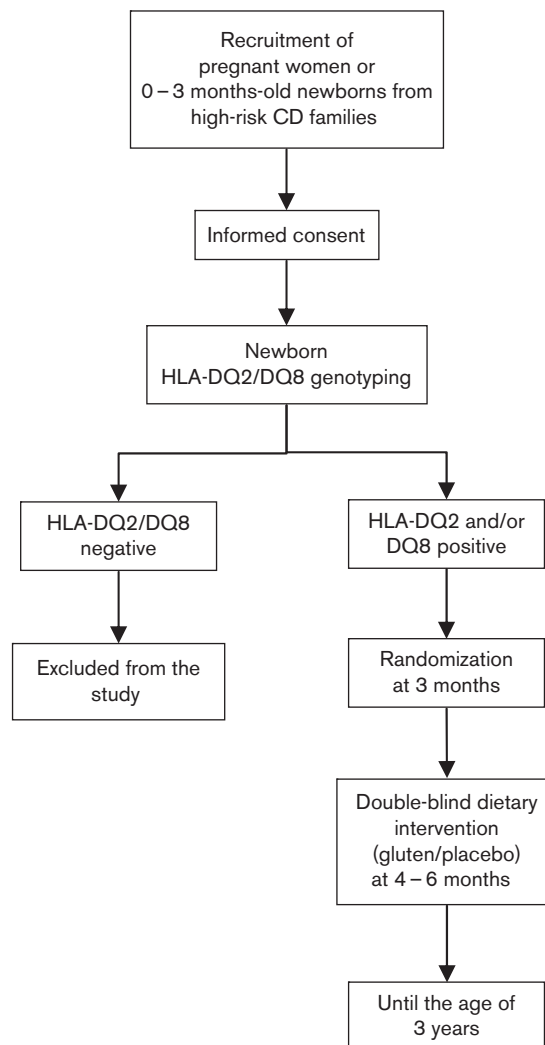
Power of the study

The study aims to achieve a 50% reduction of CD development at the age of 3 years among the gluten intervention group. This amounts to a 5% risk of developing CD in the gluten group versus a 10% risk in the placebo group [39]. With a two-sided significance level of 5 and 80% power to detect a difference of 10% versus 5%, 474 children are required in both the groups. Allowing for dropouts and crossovers, this study aims to include a total of 1000 genetically susceptible children.

Intervention

At the age of 3 months, the HLA-DQ2 and/or HLA-DQ8 positive children are blindly randomized to either placebo or gluten intervention (Table 1). The randomization codes are generated at the Department of Medical Statistics, LUMC, Leiden, The Netherlands. The gluten intervention product contains 1.9 g of lactose mixed with 100 mg gluten and the placebo product contains 2 g of lactose. Both the products are supplied in identical packages by Danone

Research BV (Wageningen, The Netherlands), in collaboration with NIZO Food research, Ede, The Netherlands. The dose of 100 mg – which is less than 3% of the amount of gluten introduced in the diet at weaning time [40] – is the lowest that may be expected to have an effect on the immune system [41,42]. Moreover, this amount is a requirement for immunomodulatory effects and for the possible induction of tolerance to prevent the development of CD. Lactose was selected as a placebo to avoid introducing any new nutrients during the intervention period, as this carbohydrate is present in breast-feeding and in formula feeding. Starting from the age of 4 months, children are given the gluten or placebo product on a daily basis for a period of 8 weeks. The period of 8 weeks was selected in congruence with Norris *et al.* [32] 'window of opportunity' for lowering the risk of CD by introducing gluten into the child's diet at the age of 4–6 months. In our

Table 1 Family study – timetable of intervention and follow-up activities

Age in months	1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24	28	30	34	36
Randomization			x																			
Intervention				x	x																	
Anthropometric measurements	x	x	x	x	x	x			x			x			x			x				x
Health check	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Food questionnaire	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Breast milk sampling	x	x	x	x	x	x	x	x	x													
Blood sampling	x[a]			x[b]		x[b]			x[b]			x[b]			x[b]			x[b]				x[b]

[a]HLA genotyping performed.
[b]Presence of serological markers tested and immunological studies performed.

study, all participating families are strongly encouraged to breast-feed their child during the first 6 months of life. Neither the participating family nor the researchers know whether the child is receiving gluten or placebo. Monthly visits or interviews assess the parent's compliance to the procedure (Table 1). In case of health complaints, extra check-ups take place and, if required, the randomized intervention is discontinued. After the 8-week intervention period, all families are advised to gradually introduce gluten into the diet of their child until the age of 10 months.

Follow-up
A timetable of all follow-up activities is presented in Table 1. Health status, anthropometrics and nutritional habits of the infants are monitored regularly, and the presence of immunological markers of gluten intolerance. Information on feeding habits, for example, breast-feeding and/or formula feeding and quantitative gluten consumption, is collected using validated food frequency questionnaires [40], translated and adapted for use in the participating countries. All data generated from the family study are entered into a central SQL server database, using the web-based data management application ProMISe at the Department of Medical Statistics and BioInformatics, LUMC (*www.msbi.nl*/promise).

Serological markers
The children's blood is collected at least seven times during the study (Table 1) and analyzed for gliadin IgA and antihuman tissue transglutaminase IgA (tTG-IgA), respectively (expressed as arbitrary units per millilitre, U/ml), using Varelisa Gliadin IgA and Celikey (Phadia GmbH, Freiburg, Germany; cut-offs 17 and 6 U/ml, respectively). Total serum IgA is measured locally in all children using the local standard cutoff point. Samples from children with IgA deficiencies are subjected to additional analyses for gliadin IgG and anti-human tTG-IgG using Varelisa Gliadin IgG and Celikey (cutoffs 17 and 10 U/ml, respectively). These serological measurements are performed at Phadia GmbH.

Breast milk
Breast milk samples are collected monthly after birth for quantitative and qualitative analysis of gluten content. For this purpose, a monoclonal antibody-based competi-

tion assay is used to monitor the presence of CD-toxic gluten peptides [43]. These analyses are performed at the Department of Immunohaematology and Blood Transfusion, LUMC, Leiden, The Netherlands.

Diagnosis of coeliac disease
Children with elevated levels of antibodies indicating CD and/or with clinical suspicion of CD are offered to undergo a small-bowel biopsy to diagnose the disorder. The small-bowel specimens are assessed for morphology, both by a local pathologist and also by an independent pathologist (Professor V. Villanacci, Spedali Civili, Brescia, Italy). The findings of all mucosal specimens are graded according to the revised Marsh–Oberhuber classification [44,45].

Study endpoint
The primary endpoint of the family study is to establish whether or not there is a significant difference in the frequency of CD between the children with gluten intervention and the children with placebo at 3 years of age. Any difference will be tested using the Cochran–Mantel–Haenszel test, stratified by the different HLA genotypic subgroups [46]. The analysis will be based on the principle of intention-to-treat.

Population study
Participants
In 2005–2006 and in 2009–2010, all 12-year-old children attending school in one of the five study areas across Sweden (Lund, Norrköping, Norrtälje, Umeå or Växjö) were invited to participate in a CD screening study ($n = 18\,000$). The majority of these children were born either in 1993 or in 1997 – respectively during and after the epidemic – and thus belong to birth cohorts, which differ with respect to dietary exposure during infancy, and with respect to the prevalence of clinically diagnosed CD at comparable ages [30,31,34,35].

Power of the study
In determining the sample size, the priority was to detect any considerable difference in CD prevalence at the age of 12 years – including both previously diagnosed cases and those detected by screening – between the birth cohorts of 1993 and 1997. With a statistical power of 90%, the necessary sample size was 10 000 children, with 5000 in each cohort.

Methods

Field work

The study is supervised from the ETICS project office in Umeå; the field work at each study site is overseen by a local pediatrician. The screening of the first cohort and that of the second cohort cover the same geographical areas, and are also comparable with respect to the methods used for data collection and analyses [47]. All children in grade 6 are invited to participate in the screening, after obtaining their parents' written consent. Research nurses visit each school and, in cooperation with school nurses, measure the children's weight and height and take venous blood samples. The children fill out a questionnaire at school, and take home another questionnaire for their parents to fill out.

Parental and child questionnaires

The parental questionnaire contains questions regarding the child's nutrition during infancy with respect to breast-feeding and gluten exposure. In addition, both the parents' and the child's questionnaires cover aspects, such as heredity with respect to CD and other autoimmune diseases, socioeconomic situation, nutritional habits, symptoms that might indicate untreated CD and health-related quality of life.

Coeliac disease serological markers

tTG-IgA and tTG-IgG are analyzed with Celikey (Phadia GmbH). Endomysial antibodies of isotype IgA and IgG are analyzed using indirect immunofluorescence technique using monkey oesophagus (Euroimmun, Luebeck, Germany). Total serum IgA is analyzed using a routine nephelometric method (BN Pro Spec System, Dade Behring, Marburg GmbH, Germany) at Clinical Immunology, Umeå University, Sweden.

Autoimmunity other than coeliac disease

Blood samples from all identified CD cases – plus samples from four controls per case – are analyzed for markers associated with insulin-dependent diabetes, such as islet-cell antibodies (IA2), insulin autoantibodies and glutamyldecarboxylase antibodies. The samples are also tested for thyroid peroxidase antibodies, as a marker for autoimmune thyroid disease. These serological measurements are performed at Phadia GmbH.

Diagnosis of coeliac disease

After giving their informed consent, parents are asked to report whether or not their child has been diagnosed previously with CD and/or has been prescribed a gluten-free diet. If so, the National Swedish Childhood Celiac Disease Register [35,48] or the child's medical record is consulted to check whether or not the diagnosis was based on evaluation of the small intestinal mucosa. Children fulfilling the criteria for a small intestinal biopsy are subsequently referred to the nearest paediatric department. Biopsies are taken either by endoscope or by capsule, and mucosal specimens are evaluated according to local clinical routines. Finally, all mucosal specimens are re-evaluated by an expert pathologist and graded according to the revised Marsh–Oberhuber classification [44,45].

Study endpoints

The primary endpoint of the population study is to compare the two birth cohorts exposed to different infant feeding practices with respect to the prevalence of CD at age 12 years. The prevalence will be determined by including both previously diagnosed cases and those detected by screening during the study. The two cohorts will also be compared with respect to the prevalence of autoimmunity other than CD, as will be done for the following groups within each cohort: (i) previously diagnosed cases, (ii) cases detected by screening and (iii) children without CD.

Immunological study

To identify the immunological mechanisms, which could be potentially involved in initiating an aberrant response to gluten in genetically predisposed infants, all blood samples and small-bowel biopsy specimens obtained during the family study (Table 1) are subjected to the following investigations:

(1) Detection of alterations in gene expression profiles, which are known to correlate with gluten introduction and/or disease development;
(2) Development of the T-regulatory compartment, in particular the induction and/or presence of (gluten specific) IL-10 and/or TGF-β-producing cells;
(3) Determination of the gluten-specific T-cell response. For this purpose, polyclonal gluten-specific T-cell lines are generated from the biopsies for subsequent testing against a panel of gluten peptides, which spans all the currently known T-cell-stimulatory gluten peptides; and
(4) Immunohistochemistry analysis, including detection of tTG-specific immunoglobulin bound to the small intestinal tissue.

The first three analyses are performed at the Department of Immunohaematology and Blood Transfusion, LUMC, The Netherlands and at the Institute of Immunology, University of Oslo, Norway. The final immunological assessment is performed at the Coeliac Disease Center of Heim Pál Children's Hospital, Budapest, Hungary.

Genetic study

In the family study, DNA samples are obtained from all participating newborns and their first-degree family members, and in the population study in all cases of CD and four controls per case. These samples are genotyped for HLA-DQ-alleles, DQA1*05, DQA1*0201,

DQB1*02 and DQB1*0302, encoding HLA-DQ2 and DQ8, performed by oligonucleotide probe hybridization (Eu-DQ test, Eurospital SpA, Trieste, Italy). Moreover, the Genetics Department of the University Medical Center Groningen and University of Groningen in The Netherlands also genotypes these samples for HLA and the 26 currently known non-HLA loci identified by the genome-wide association studies and their follow-ups [17–20]. Genotyping for both HLA and non-HLA single-nucleotide polymorphisms (SNPs) will permit the validation of a genetic risk score as recently described [49]. To predict homozygosity or heterozygosity for the CD-risk haplotypes (DQ2.5, DQ2.2, DQ7, DQ8), DNA is also genotyped for six HLA SNPs based on the tag-SNP approach [50].

Dissemination

PreventCD aims to enhance public participation in, and general awareness of, its studies by widely disseminating its activities and accomplishments to interested parties, such as the food industry, CD patients and patient associations. PreventCD runs a website (*www.preventcd. com*) and publishes a newsletter every 6 months with the objective to facilitate internal communication and to make the process and results easily accessible externally. Irrespective of whether or not the hypothesis of induction of tolerance to gluten in susceptible infants proves to be correct, recommendations addressing the issues relating to gluten introduction will be made in collaboration with the Committee of Nutrition of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition.

Ethical considerations

The family study has been approved by all medical ethics committees of the participating centres. The population study was approved by the Regional Ethical Review Board at Umeå University in Umeå, Sweden. For the entire PreventCD research project, an independent ethical advisor, Professor Moshe Berant (Helsinki Committee, Rambam Health Care Campus, Haifa, Israel), has been appointed to provide guidance on ethical issues and decisions throughout the research period. He also acts to ensure compliance with the International Conference on Harmonisation and Good Clinical Practices regulations.

Potential impact of PreventCD

CD was previously considered to be an unavoidable phenomenon that would set in if a genetically susceptible person would consume gluten-containing food. However, this view has been challenged by studies, which suggest that infant nutrition may have a significant effect on the risk of developing CD and other autoimmune disorders [23,32,35,51]. The PreventCD project carries out innovative research regarding the primary prevention of CD, by studying the effects of infant nutrition on the development of early and late (in)tolerance to gluten and other autoimmune phenomena. Taking full advantage of genomics techniques, PreventCD is expected to eluci-

date various genetic and immunological mechanisms involved in this process, both in the case of high-risk children and in children from the general population. Thus, PreventCD represents a significant step forward in the application of knowledge from basic science in the prevention and treatment of a highly prevalent food-related disease, CD.

Acknowledgements

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Appendix

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