Statistical Analysis Plan

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1   Abbreviations and Definitions

AE   Adverse event

AGA   Antigliadin antibodies

CD   Coeliac disease

ESPGHAN   European Society of Paediatric Gastroenterology, Hepatology and
                Nutrition

EC   European Commission

HLA   Human Leukocyte Antigen

IgA   Immunoglobulin A

IgG   Immunoglobulin G

LUMC   LeidenUniversityMedicalCenter

Numico   Royal Numico N.V

PREVENTCD   Multicenter European study funded by the European Commission

                FP-6-2005-FOOD-4B; Proposal/Contract no 036383: Influence of the
                dietary history in the prevention of coeliac disease: possibilities of
                induction of tolerance for gluten in genetic predisposed children

SAP   Statistical Analysis Plan

tTGA   Anti-tissuetransglutaminase antibodies

AGA   Anti-gliadin antibodies

UIN   Unique Identification number

2 Introduction

2.1 Preface

Coeliac disease (CD) is a chronic disorder caused by hypersensitivity to some of the most common proteins (gluten) in the diet of the European population. Gluten is a common name used for proteins (prolamins and glutenins) of wheat, barley, and rye. CD affects as many as 1% of the Europeans (2.5 million people) and is the most common food intolerance in Europe. If recognised, CD patients have only limited access to safe foods and there is not causal therapy available. This study is part of the multicenter European project PREVENTCD, funded from 2008–2012 by the European Commission FP-6-2005-FOOD-4B; Proposal/Contract no.: 036383. The general objective of PREVENTCD is to significantly reduce the number of people suffering from CD in Europe, by developing primary prevention strategies for CD[1].

2.2 Purpose of the analyses

This analysis will assess the efficacy of introduction of small quantities of gluten during the period of breast-feeding in preventing the development of CD at the age of 3 yearsin genetically predisposed individuals from CD families.

3 Study Objectives and Endpoints

3.1 Study Objectives

The hypothesis is that early dietary history, i.e. the introduction of small quantities of gluten during the period of breast-feeding, may prevent coeliac disease (CD) in genetically predisposed individuals by induction of tolerance for gluten and for other related auto-antigens.

The primary objective is to test this hypothesisin a prospective early dietary intervention study in about 1000 young HLA-DQ2 and/or -DQ8 positive children from high-risk families for CD with at least one case of CD among their siblings and/or the parents.

3.2 Endpoints

The primary endpoint is the cumulative incidence of CD, including the cumulative incidence at 3 years of age.

The time scale is age of the subject (child). Time of development of CD for a subject in the study is defined as the age at diagnosis of CD (see below for precise definition). Subjects without CD are censored at the time of last CD antibody determination, defined as either

- Immunoglobulin A (IgA) anti-tissuetransglutaminase antibodies (tTGA)

- IgA antigliadin antibodies (AGA)

- In case of IgA deficiency, tTGA and AGA of the IgG class

Diagnosis of CD: Small bowel biopsies for the diagnosis of CD are offered to parents of participating children if the following criteria are met:

a) In asymptomatic children biopsies will be performed if they have either positive tTGA on two occasions in a 3 month interval or high positive AGA on three occasions in a 3 month interval and/or clearly increasing positive AGA in two tests performed with 3 month interval.

Positive anti-tTGA means a result of > 6 U/ml

High positive AGA means a result of > 50 U/ml

Increasing positive AGA means a result of > 17 and < 50 U/ml for the first positive sample and an increase of at least 20 U/ml in a 3 month interval for the second positive sample.

b) In symptomatic children biopsies areoffered

– in subjects with mild clinical symptoms (i.e. loose stools, anorexia, constipation) if AGAand/or anti-tTG are positive (see above for the criteria).
– in subjects with severe clinical symptoms (i.e. chronic diarrhoea, abdominal distension, failure to thrive) if these persist for more than 1 month, independently of the presence of AGA or anti-tTG.

Biopsies areonly performed when medically indicated, that is: only in these children highly suspected for active CD, and not just for purpose of the study. Such children would undergobiopsies also in non-study circumstances. The diagnosis of all children who undergo small bowel biopsies are centrally reviewed by a diagnostic committee, blinded for theinterventionassignment.All the biopsies taken during the study are centrally assessed by one pathologist (Professor V. Villanacci,SpedaliCivili,

Brescia, Italy).Time of diagnosis of CD is defined as the date of biopsies sampling, in case of biopsies with histology results highly suggestive for CD. There are a small number of children with high suspicion of CD whose parents refused biopsies, but who could be diagnosed according to the new ESPGHAN criteria. In these children the date of the diagnosis is defined as the date, before the meeting of the diagnostic committee, at which theCD antibodies were highest.

4 Study Methods

4.1 General Study Design and Plan

PREVENTCD is adouble blind prospective randomized food intervention study comparing early gluten introduction with placebo.The study has been performed in collaboration between partners in eight different European countries (codes in parenthesis): The Netherlands (101); Italy (102); Poland (103); Spain (Madrid: 104, Valencia: 105, Reus: 109); Israel (106); Croatia (107); Hungary (108); Germany (110). The data from the participant children (Table 1) are recorded by every partner in identical, standardized, anonymous (UIN) forms and sent to the project office in Leiden. A secure website as well as monthly overviews of the results and several working meetings, including telephonic ones, guarantees good communication between the partners.

The (blind) intervention starts at the age of 4 months and continues for a period of 8 weeks. This time frame is chosen because it is known to represent a "window of opportunity" to introduce gluten into the diet, with respect to development of autoimmune phenomena. The present evidence over this "window of opportunity" is based on publications covering, among others, two studies in Germany and the USA and a recent population study from Sweden, also part of the PreventCD project[2].

Shortly before the dietary intervention, subjects are randomized to the group for "early gluten introduction" or to the "control" group.

*Tolerance induction*is attempted in the randomised group of children by blind daily intake of 100 mg glutenduring 8 weeks. The gluten and the placebo (100mg lactose) intervention products in identically measured packages are supplied by the industry partner Danone (before Numico). Compliance is assessed by frequent visits or interviews (Table 1).

4.2 Inclusion-Exclusion Criteria and General Study Population

Inclusion criteria:

1. Children, 0-3 months of age at high risk for developing CD, that is those who:

> (i) have at least one first degree familymember with CD confirmed by small-bowel histology;

> (ii) areHLA-DQ2 and/or HLA-DQ8 positive, or otherwise carrying the allele DQB1*02;

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

Exclusion criteria:

1. Children born prematurely (≤36 weeks gestational age). Exceptions are made for healthy children born between 34-36 weeks with a birth weight above 2 kg, and for healthy multiple births born between 34-36 weeks regardless of birth weight.

2. Children with an increased risk for CD because they are diagnosed with trisomy 21 and Turner's syndrome.

4.3 Randomisation and Blinding

Subjects are randomized before 4 months of age, after checking of inclusion criteria. Randomization was performed by the LUMC using SPSS 18, stratified by participating country and using variable block sizes ranging from 4 to 8. Results were sent to industry partner Danone (formerlyNumico), who created randomization stickers for the gluten products / placebo.

4.4 Study Variables

Table 1. Time scheme of the assessments in the enrolled children until the age of 3 years

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.

The early immune response measurements at months 4 (baseline), 6, 9, 12, 18, 24, and 36, include:

- IgA CD serum antibodies:tTGA and antigliadin antibodies (AGA)

- IgGtTGAand AGA in case of IgA deficiency

5   Sample Size

The sample size calculation was based on a significance level of 5% (two-sided) and a power of 80%. Assuming a frequency of 10% CD among the enrolled infants, 474 children need to be randomized to early gluten introduction and 474 to control intervention to be able to detect a 50% reduction of CD development following the early dietary intervention.

6   General Considerations

6.1 Timing of Analyses

The final analysis will be performed after the last child entered into the study has reached the age of 3 years. The randomization code will not be broken before the finalization, approval and publication of this SAP document.

6.2 Analysis Populations

[Please include some text announcing the pilot study, if they are to be included in any of the secondary analyses.]

6.2.1 Full Analysis Population

All randomized subjects who are eligible (i.e., who fulfil all inclusion criteria and fail all exclusion criteria).

6.2.2 Per Protocol Population

According to the study protocol, compliance with the food intervention was assessed by the number of sachets with intervention material remaining after finishing the intervention. The intervention period was 56 days, and could occur at 4, 5 or 6 months of age, (1 sachet per day, with a maximum of 7 skipped sachets). The families received 62 sachets (6 extra) before the start of the intervention period. The child was considered compliant with the food intervention if the family had 13 or less sachets left.

If information about the number of sachets left after finishing the intervention is missing, but the self-reported information about the percentage of the sachets that was ingested is known in month 4, 5 and 6, then the child is considered compliant with the food intervention if either:

- at each of these months everything was ingested.

- in two of these months everything was ingested and in one of them there was some ingestion.

- in one of these months, everything was ingested and in two months, more than half was ingested.

Compliance is only relevant for the gluten induction group; for the placebo group no compliance restriction is applied.

Before breaking the codes, the compliance of every child with the food intervention and the decision whether or not the child is included and in which of the blinded intervention groups in the per protocol analyses will be documented. The primary analysis of the primary endpoint will be on intention-to-treat, based on the full analysis population as specified above.

After breaking the codes, compliant randomized eligible subjects (according to the above definition) of the gluten intervention group are part of the per protocol population and will be analysed in the gluten intervention group. In addition, all randomized eligible subjects in the placebo group, irrespective of their compliance status, are included in the per protocol population and will be analysed in the placebo group in all per protocol analyses. Also, randomized eligible subjects of the gluten intervention group that never started intervention and/or never ingested any sachets are included in the per protocol population and will be analysed in the placebo group in all per protocol analyses.

6.3 Covariates and Subgroups

The risk of developing CD is expected to differ between:

- participating countries,

- gender,

- genetic risk for CD in five classes according to HLA-DQ genotype (hereafter referred to as HLA risk group in five)[3]:

Group 1= DR3-DQ2/DR3-DQ2; DR3-DQ2/DR7-DQ2;

Group 2 = DR7-DQ2/DR5-DQ7;

Group 3 = DR3-DQ2/DR5-DQ7; DR3-DQ2/DR4-DQ8; DR3-DQ2/other;

Group 4 = DR7-DQ2/DR7-DQ2; DR7-DQ2/DR4-DQ8; DR4-DQ8/DR4-DQ8;

Group 5 = DR7-DQ2/other; DR4-DQ8/DR5-DQ7; DR4-DQ8/other

- family history (number of affected first degree relatives) at time of inclusion (1, 2, 3 or more)

Exploratory subgroup analyses will be performed for the above mentioned variables. These will be presented as forest plots. Tests for interaction between intervention and covariate will be used to test for differential effects of the intervention across subgroups.

6.4 Missing Data

For the primary endpoint, intermittent missing visits will have no consequence. If later visits are recorded, then it is assumed that in between these visits no CD has occurred. Rationale for this decision is that in case of clinical problems leading to a diagnosis of CD, these children would most probably present themselves. In case the missing visits are not intermittent, but the child is lost to follow-up, then the child will be treated as censored at the date of last visit.

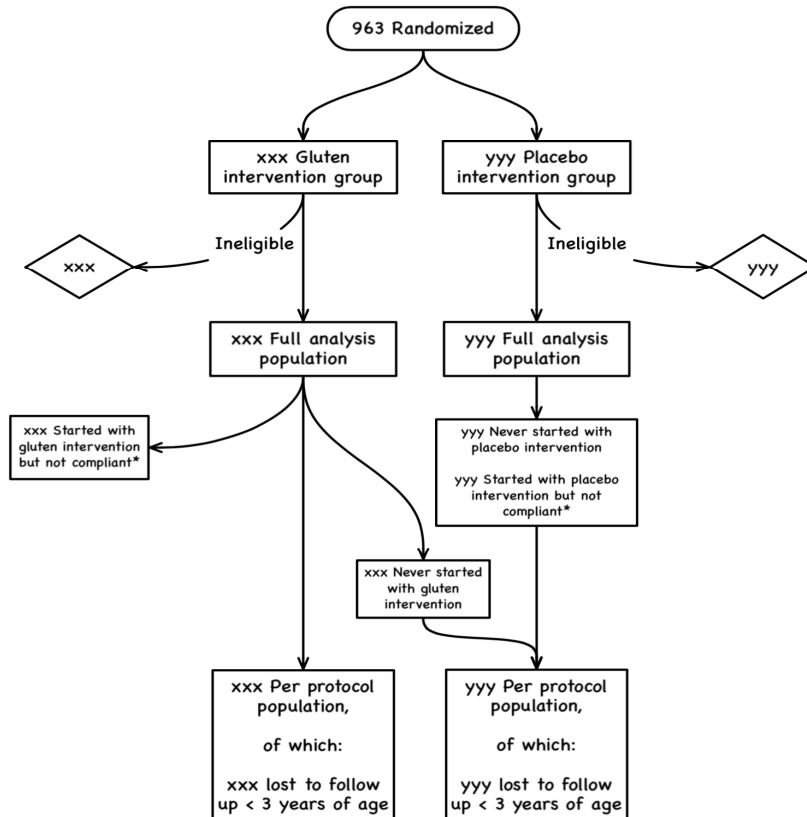## 6.5 Interim Analyses and Data Monitoring

No interim analyses or data monitoring are performed.

7   Summary of Study Data

Continuous variables will be summarized as n(non-missing sample size), plus mean(standard deviation) or median (minimum-maximum), when non-normally distributed. Categorical variables will be summarized as frequencies and percentages of observed levels (based on non-missing sample size).

7.1 Subject Disposition

The flow of subject disposition is presented in the chart below.

* See section 6.2.2. for the definition of compliance with the food intervention.

7.2 Protocol Deviations

Protocol deviations that could impact the analysis and specification of methods used to accommodate them:

- Not fulfilling inclusion criteria

    o These subjects will not be included in full analysis population nor in the per protocol population, when detected before deblinding (otherwise they will be included in the full analysis population but not in the per protocol population)

- Withdrawing consent to be included in the study

    o These subjects will not be included in full analysis population nor in the per protocol population

- Withdrawing consent for further follow-up in the study

- These subjects will be included in both full analysis and per protocol populations (but only information until consent withdrawal is used for the per protocol analyses)

- Drop out for other reasons

  - These subjects will be included in both full analysis and per protocol populations (but only information until drop out is used for the per protocol analyses)

- Not starting study food intervention

  - These subjects will be included in full analysis population, but for gluten induction not in the per protocol population

- Stopping study food intervention or not complying to study food intervention

  - These subjects will be included in full analysis population; they will be included in the per protocol population, provided compliance according to protocol as described in Section 6.2.2

- Intermittent missing visits

  - These subjects will be included in both full analysis and per protocol populations

7.3 Demographic and Baseline Variables

Baseline variables, recorded at, or shortly, before randomisation are

- Country

- HLA risk group in five, see Section 6.3

- Family history

  - Number of affected 1st degree relatives

  - Father affected

  - Mother affected

  - Sibling affected

- Gender

Summary statistics for these variables will be produced in accordance with Section 7.

7.4   Intervention Compliance

See section 6.2.2.

8   Efficacy Analyses

8.1 Primary Efficacy Analysis

The primary efficacy analysis is different from what was specified in the protocol, see Section 10 for motivation. For estimation of the cumulative incidence of CD, Kaplan–Meier curves are calculated and plotted as cumulative incidence curves (one minus survival) for each of the intervention arms. The cumulative incidence at 3 years of age, obtained from the Kaplan–Meier estimates, will be reported for each of the intervention arms, with 95% confidence intervals. For comparison of the cumulative incidence of CD between arms, a stratified log–rank test (two-sided) will be used, stratified for country(as used in the randomization procedure) and HLA risk group (in five groups). To quantify treatment effect, the hazard ratio of CD for gluten induction with respect to control (with 95% confidence interval) will be provided, based on a Cox proportional hazards regression, stratified for the same factors as the stratified log–rank test.

8.2 Secondary Efficacy Analyses

A secondary efficacy analysis of the primary endpoint will consist of the analyses specified in Section 8.1, applied to the per protocol population, using the grouping defined in Subsection 6.2.2.

For the longitudinal analysis of immune response, linear mixed models will be used, with random subject intercepts and treatment effects (unstructured covariance), and with time (categorical) and treatment and their interaction, as well as the variables named in section 6.3, as fixed effects. Immune response outcomes will be log-transformed, if appropriate, before longitudinal analysis.

In all longitudinal analyses, the following limits of tolerable deviations for the time of assessment will be used: in the first year of life, the assessment has to be

performed within 1 month of the scheduled moment, in the second year within 3 months and from the third year within 4 months.

In addition to the above mentioned rules for tolerable deviations for the time of assessment, the first of the seven scheduled blood samples has to be obtained before start of the intervention.

If start date of intervention is missing, then the child will be excluded for this analysis. If date of blood withdrawal is missing, the measurement will not be used, but other data of the same child may still be included. The analysis (linear mixed models, see Section 8.2) assumes (dates of) measurements are missing at random.

Secondary endpoints are the occurrence of clinical events related to gluten intolerance, and early immune response to gluten, defined as high CD antibodies in serum.

Frequencies of clinical events (anorexia, diarrhoea, failure to thrive, abdominal distension, constipation, vomiting, abdominal pain, non-gastroenterology symptoms, (viral) respiratory tract infection, and gastrointestinal tract infection) will be tabulated (in accordance with Section 7) by treatment and time. This will be done separately by type of clinical event and aggregated over types of event ("any clinical event").

8.3 Exploratory Efficacy Analyses

There is a possibility that censoring due to loss to follow-up is informative (caused by children without any clinical complaints being more inclined to leave the study). A sensitivity analysis will be performed where these children are censored at three years, rather than at the time of last study visit.

In a few cases, two children from the same family have been included in the study. Because of the small number of these occasions, the resulting possible correlation of event times is ignored in the primary efficacy analysis. As sensitivity analysis a Cox regression is performed with family as cluster, using sandwich estimators of the variance of the regression parameters[4].

Differences in cumulative incidence of CD will be assessed according to the baseline variables in Section 6.3, country, HLAriskgroup, family history, and gender, as well as according to variables which are not yet known at baseline such as duration of breast feeding, formula feeding, daily gluten intake and infections. For the former

variables (multivariate) Cox regression will be used. For the latter type of variables, landmark analysis will be used, with 6 months (12 months as sensitivity analysis) as landmark time point.

9 Safety Analyses

The product under investigation in the protocol is regularly used. Therefore no formal safety analyses will be performed. Tabulation of clinical events are mentioned in Section 8.2.

10 Summary of Changes to the Protocol

In the protocol, a Mantel-Haenszel test comparing frequency of CD within three years of age has been specified for the primary efficacy analysis. However, it was felt that a method based on survival analysis techniques was more appropriate, because of the possibility to use (partial) information of subjects leaving the study before three years and to use information on CD events beyond three years of age. Furthermore, even with comparable frequency of CD at three years, differences in timing of occurrence were felt to be clinically important. The Mantel-Haenszel test has therefore been replaced by a stratified log-rank test and stratified Cox regression.

11 Reference List

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2. Ivarsson A, Myleus A, Norstrom F, van der Pals M, Rosen A, Hogberg L, et al. Prevalence of childhood celiac disease and changes in infant feeding. Pediatrics. 2013;131:e687-e694.

3. Bourgey M, Calcagno G, Tinto N, Gennarelli D, Margaritte-Jeannin P, Greco L, et al. HLA related genetic risk for coeliac disease. Gut. 2007;56:1054-1059.

4. Lin DY, Wei LJ. The robust inference for the Cox proportional hazards model. J Amer Statist Assoc. 1989;84:1074-1078.

12 Listing of Tables, Listings and Figures

Fig   Flow of subject disposition