PROTOCOL

(Version April 2015)

TRIGR

Trial to Reduce IDDM in the Genetically at Risk

RESEARCH DESIGN AND METHODS

This is a randomized double blinded intervention study using an intention to treat statistical analysis to compare the incidence of predictive autoantibodies and clinical diabetes in the two treatment groups.

A. STUDY POPULATION

Newborn infants who have a first degree relative (FDR) with type 1 diabetes (i.e. a mother, father or full sibling), and who meet the inclusion but not the exclusion criteria are recruited:

Inclusion criteria:
1. The biological parent and/or full (not half) sibling of the newborn infant has type 1 diabetes as defined by the World Health Organization
2. The infant's parent or legal guardians give signed consent to participate

Exclusion criteria:
1. An older sibling of the newborn infant has been included in the TRIGR intervention
2. Multiple gestation
3. The parents are unwilling or unable to feed the infant cow's milk (CM) based products for any reason (e.g., religious, cultural).
4. The newborn infant has a recognizable severe illness such as those due to chromosomal abnormality, congenital malformation, respiratory failure needing assisted ventilation, enzyme deficiencies, etc.
5. The gestational age of the newborn infant is less than 35 weeks
6. Inability of the family to take part in the study (e.g. the family has no access to any of the Study Centers, the family has no telephone)
7. The infant has received any infant formula other than Nutramigen prior to randomization
8. The infant is older than 7 days at randomization
9. No HLA sample drawn before the age of 8 days

B. SAMPLE SIZE

The sample size estimation presented in Table 1 is based on experiences from family studies analyzing the occurrence of autoantibodies in siblings of children with type 1 diabetes or in offspring of affected parents and progression to clinical disease in such young first-degree relatives. The data on the frequency of multiple ($\geq$ 2) autoantibodies by the age of 6 years and the cumulative incidence of type 1 diabetes by the age of 10 years are based on 82 young siblings from the DiMe Study carrying increased genetic risk for type 1 diabetes as defined according to the criteria in the Pilot-2 trial (106).

Nineteen out of 325 offspring (5.9%) with increased genetic risk according to the criteria used in the Pilot-2 trial tested positive for at least one autoantibody by the age of 2.4 years in the German BABYDIAB study (107). Seventeen of these 19 subjects (89%) developed multiple autoantibodies during prospective observation. Assuming a constant increase in autoantibody frequency from age 2 to

age 6 years, the expected cumulative incidence of at least one antibody would be 14.8% and that of at least two antibodies 13.0% by the age of 6, which is well within the 95% CI of the observed frequency of 20.7% of multiple autoantibodies in the young DiMe siblings.

The cumulative incidence of at least one autoantibody by the age of 4 years was 10.6% in siblings and offspring of affected subjects in the DAISY study from Denver (108). The frequency reached 37.9% in those carrying the DR3/4, DQB1*0302 combination. Published data are not available allowing an estimation of the frequency of at least one or at least two autoantibodies in subjects with increased genetic risk as defined in the Pilot-2 trial. Nevertheless a constant increase in the frequency of at least one autoantibody from age 4 to age 6 years, would result in an autoantibody prevalence of 15.9% by the age of 6 years in siblings and offspring irrespective of HLA-defined genetic risk. The DiMe study was used for the power calculation as it includes the largest long term follow-up of young relatives.

The estimation that 2032 infants should be randomized for the trial (Table 1) is based on the following assumptions:

1. a confidence level of 95%;
2. a statistical power of 80%;
3. a reduction of 40% in the hazard rate of type 1 diabetes in the intervention group;
4. a drop-out rate of 20%; and
5. a frequency of 10% of exclusive breast feeding (BF) up to the age of 6 months

These figures represent a conservative estimate, since they are based on the lower 95% CI (7.6%) of the observed cumulative incidence of type 1 diabetes by the age of 10 years (15.4%) in young siblings with moderate (DQB1*0302/x) and slightly increased genetic risk (DQB1*02/y), and since the high risk (DQB1*02/0302) group was not considered in the calculations. In the Pilot-2 trial the prevalence of the high risk genotype (DQB1*02/0302) was about two times higher (absolute frequency 20%) among siblings of affected children than among offspring of affected parents. If also the young siblings carrying the high risk genotype in the DiMe Study should be included in the group at risk, the lower 95% CI would be 10.6%, which should result in a recommendation of 1920 infants to be randomized for the trial. However, fewer data from non-Finnish populations are available, explaining the caution of this sample size estimate.

Table 1. Estimation of the sample size for the trial

Assumptions:

- Confidence level: 95%
- Power: 80%
- Reduction in the annual frequency of multiple auto-antibodies (≥ 2 AB) by the age of 6 years 30, 40 or 50%
- Reduction in the annual frequency of type 1 diabetes by the age of 10 years 30, 40 or 50%

Frequency of ≥ 2 AB by the age of 6	**SAMPLE SIZE**								
	30% effect			40% effect			50% effect		
	I	II	III	I	II	III	I	II	III
20.7% (*02 and/or *0302)	1048	1132	1165	558	602	626	338	364	382
18.5% (*0302/x, *02/y	1196	1294	1330	636	688	716	386	418	438
9.9% (lower 95% CI)	2252	2438	2511	1200	1300	1334	732	792	832

Frequency of type 1 diabetes by the age of 10	**SAMPLE SIZE**								
	30% effect			40% effect			50% effect		
	I	II	III	I	II	III	I	II	III
18.3% (*02 and/or *0302)	1330	1492	1536	706	794	824	428	482	502
15.4% (*0302/x, *02/y)	1548	1782	1834	844	948	986	512	576	606
7.6% (lower 95% CI)	3250	3658	3768	1735	1952	2032	1056	1190	1250

Column I: Mathematical sample size

Column II: Sample size corrected for a drop-out rate of 20%

Column III: Sample size corrected in addition for a 10% frequency of exclusive BF up to the age of 6 months.

RECOMMENDATION: 2032 infants and families

SCREENING TARGET: 4516 infants

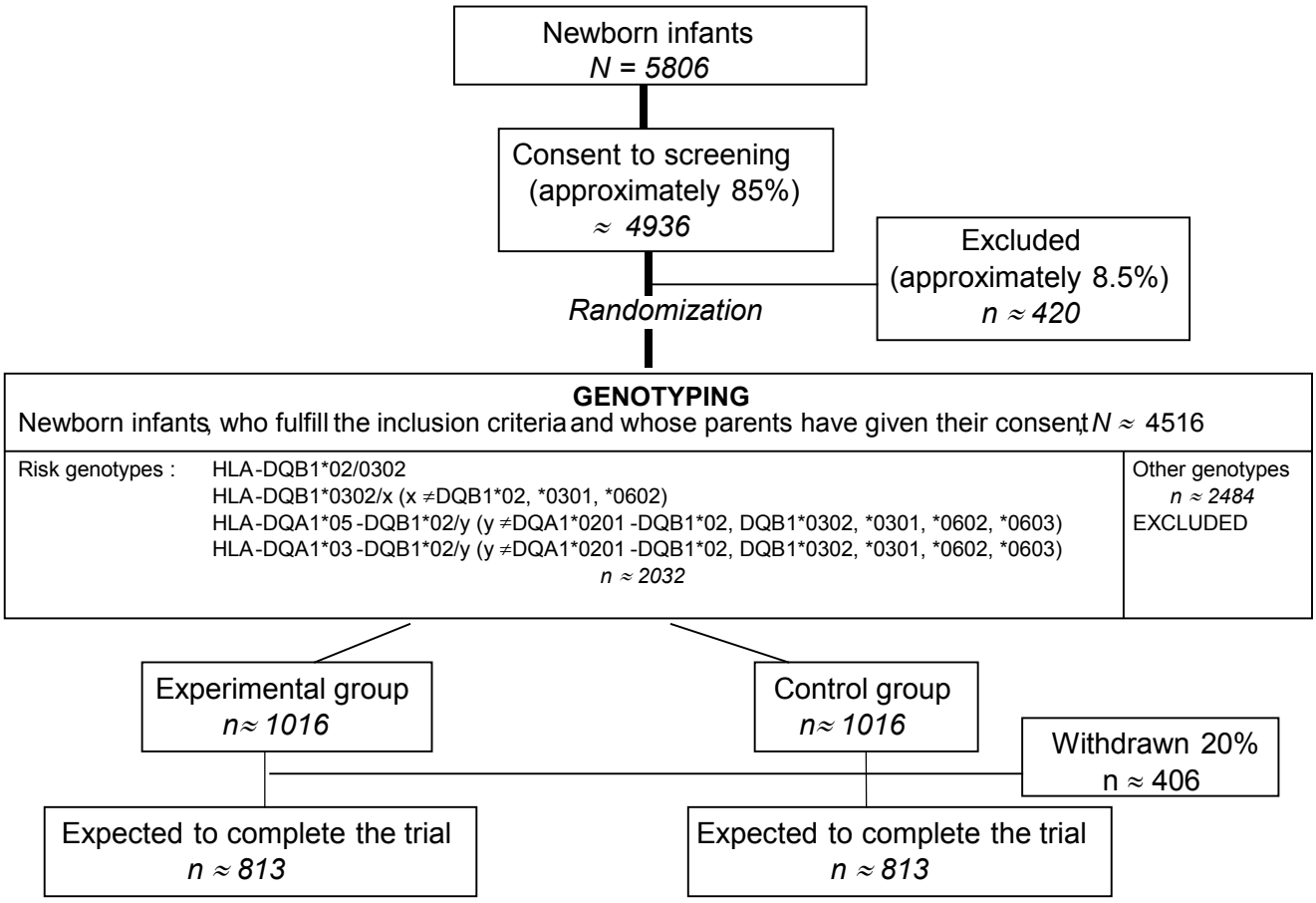
All statistical analyses will be based upon the total cohort of subjects randomized into the trial. Although data on some subjects may be missing at points in time, all relevant data available from each subject will be employed in the analyses. In all principal analyses, subjects will be included in the group

to which they were initially assigned and this assignment will not be altered based on a subject's adherence to the assigned treatment program. Analyses of each outcome will include an assessment of possible differences among clinical centers. Baseline variables that will be used in stratified analyses include gender, HLA risk category and other prognostic characteristics. If differences in baseline characteristics are observed, analyses will be conducted of the effects of the treatments on outcomes adjusting for the potential confounding effects of these baseline characteristics. If only a few such baseline characteristics are identified, analyses will be conducted stratifying for those characteristics. If any more than a few baseline characteristics are identified, because of small sample size, it will be necessary that regression models be employed to adjust the treatment comparison for the confounding effects of those characteristics. A number of subgroup analyses are planned to help identify individuals more likely to benefit from, or to be harmed by, the treatment. In regard to treatment effects, definition of such subgroups will rely on baseline data, not data measured after randomization. Such subgroups might include: gender, gender of the relative with type 1 diabetes, HLA risk group and other factors suspected to be associated with the event. Exploratory data derived through subgroup analyses will serve primarily to generate new hypotheses for subsequent testing, and conclusions drawn from subgroup hypotheses not explicitly stated before data analysis will have less credibility than those from hypotheses stated in the protocol. For safety monitoring, the Data Management Unit also will perform any and all analyses that are appropriate to identify subgroups that may be at significantly increased safety risk. The Kaplan-Meier method will be used to construct survival curves and the logrank statistic used to compare treatment arms with respect to time until the development of autoimmunity or type 1 diabetes.

We therefore aim to randomize 2032 infants with increased genetic risk as defined in the genetic screening section (E). To achieve that number 4516 infants must be screened assuming a frequency of 45% of the genotypes conferring increased risk. The observed prevalence of risk genotypes was 50% among the 471 infants screened for the Pilot-2 trial in Finland. An enrollment scheme for the trial proper is presented in Figure 1. This flow sheet indicates that the trial requires initial access to 5806 pregnancies. This should provide at least 4936 families with consent to participate in the trial before the birth of the child provided that the consent rate is 85%. With an exclusion rate of 8.5% after birth based on the exclusion criteria approximately 4516 infants will be available for genetic screening. The observed exclusion rate in the Pilot-2 trial was 8.6%.

Infants will be recruited over a four-year period and the planned follow-up will be 6 years after the last infant has been accrued for the antibody endpoint and 10 years after the last infant is accrued for the type 1 diabetes end point. Thus, all subjects will have at least 10 years of follow-up.

Figure 1. Enrollment scheme for TRIGR



C. RECRUITMENT

Recruitment is carried out in six major centers in USA, in 18 centers in Canada, in 12 European countries, and three centers in N.S.W., Australia. A list of participating countries and recruitment estimates are presented in Appendix 1. The figures represent an estimate of eligible pregnant women available in each country. The background of these estimates is that the appraisal of the acceptance rate (best scenario 70-80%, average 60%) may vary from country to country, and accordingly the total recruitment figures are a rough estimate. According to the figures given in Appendix 1 about 65% of the participants will be recruited in North America and 35% in Europe + Australia. The recruitment will be continued past the initial 4-year period, if we have not obtained 2,032 newborn infants with increased genetic risk to the series by that time.

To facilitate recruitment and to minimize any possibility of unintentional exposure to CM protein, every attempt is made to identify eligible families before the child is born. Written consent is obtained at this time; the child will participate after birth if he/she meets the inclusion but not the exclusion criteria. Families not identified until just prior to the onset of maternal labor are approached after birth and written consent is obtained at that time.

Mothers with type 1 diabetes are identified during pregnancy via hospitals monitoring pregnant women with type 1 diabetes. Fathers with type 1 diabetes are identified by (i) available history or data already in the medical records of pregnant women, (ii) interviewing women at prenatal maternity clinic visits, and (iii) existing registries of type 1 diabetes in some centers. Pregnant women already having one or more children affected with diabetes are approached through various diabetes clinics. Children with type 1 diabetes are usually followed by pediatricians or pediatric endocrinologists, who are aware of a pregnant mother. The identification of such mothers is arranged through these physicians.

Experience gained in the Pilot-2 study of this project is utilized to optimize the efficacy of the recruitment in the study proper (use of diabetes societies, information in mass media etc.). In that pilot study we also found that weekly staff meetings and continuing recruitment during weekends and vacation periods substantially raises recruitment efficiency. Leaflets, distributed to "candidate" mothers, describing the project can be very helpful.

D. SUBJECT ALLOCATION

Randomization takes place before birth or immediately after the birth of the child. The research assistant or investigator obtains the formula allocation code from the Data Management Unit (DMU) by completing the Randomization Form electronically. Subjects who meet the inclusion and do not meet exclusion criteria are randomized as follows: randomization in each strata will be within four blocks. Subjects will receive either the test formula, casein hydrolysate (Nutramigen™, Mead Johnson Nutritionals), not containing antigenic CM protein, or a CM protein containing control formula which has an addition (20 %) of Nutramigen, whenever breast milk is not available. Any subject requiring supplemental feeding prior to randomization (e.g. infants born at night or on weekends) is given banked breast milk or Nutramigen. The randomization code will be kept by the manufacturer and it will be opened when TRIGR study is completed.

Cord blood is obtained from newborn infants whenever possible or alternatively a heel prick is performed to obtain capillary blood as soon as possible after birth, at the latest at the age of 7 days. The blood sample is forwarded to the continental genotyping center and screening performed for the presence of the genotypes listed below indicating an increased genetic risk. Results are entered into the Central Data Base immediately when available (within 2 weeks after the sampling) and are sent by e-mail from DMU to the national coordinating centers and/or to the local study center in question and to the Study Monitors. Only subjects with these genotypes are included in the nutritional prevention trial; all other subjects are withdrawn from the study at that time; their parents are told that genetic screening suggests that their child does not fall into the increased genetic risk group for type 1 diabetes and they are appropriately thanked for their participation. It is emphasized that their child might still develop diabetes despite not meeting the eligibility criteria.

E. GENETIC SCREENING

The options for genetic screening vary between the extremes of a very high risk population requiring a small sample size (e.g. DQA1*0301-DQB1*0302/DQA1*0501-DQB1*02 heterozygotes), and a lower average risk population with larger sample size (e.g. newborn infants with a FDR but with no additional genetic selection applied). Based on anticipated rates of identification of eligible newborn infants and a feasible enrollment period, we propose to base the selection on the presence of the following genotypes, representing different risk categories:

1. HLA-DQB1*0302/DQB1*02
2. HLA-DQB1*0302/x (x not DQB1*02, DQB1*0301 or DQB1*0602)
3. HLA-DQA1*05-DQB1*02/y (y not DQA1*0201-DQB1*02, DQB1*0301, DQB1*0302, DQB1*0602 or DQB1*0603)
4. HLA-DQA1*03-DQB1*02/y (y not DQA1*0201-DQB1*02, DQB1*0301, DQB1*0302, DQB1*0602 or DQB1*0603)

The HLA-DQB1 genotyping used in pilot studies has been completed by additional DQA1 typing in samples positive for DQB1*02 allele but without protective DQB1 alleles. These samples are analyzed for the presence of DQA1 alleles DQA1*0201, DQA1*03 and DQA1*05. This procedure allows the distinction between the diabetes risk associated (DQA1*05-DQB1*02 and DQA1*03-DQB1*02) and non-associated haplotypes (DQA1*0201-DQB1*02). The effect of this further step is relatively small in the populations of Northern European descent, but more important in some Mediterranean and Black populations.

The efficiency of this new screening procedure was tested in an enlarged series of 289 familial cases of type 1 diabetes in Finland as well as in the newborn infants recruited for the Pilot-2 study. 85.8% of familial cases were positive for the selected genotypes which on the other hand were present in 50% of the 471 newborn infants with a FDR affected by type 1 diabetes. If an approximate figure of 10% is used for the risk of a FDR to develop multiple autoantibodies before the age of 6 yrs, positive predictive values delineate a risk of 17.1% for those with risk genotypes compared to a risk of 2.8% of those without a risk genotype. This figure is close to the 20.7% proportion given in Table 1 for the positivity of at least two autoantibodies by the age of 6 years.

The selected risk genotypes are present in 81.0% of Finnish children with type 1 diabetes and in 22.5% of the background population, the higher frequency in familial cases confirms our earlier observations (4). We do not have available data on familial cases and newborn infants with a FDR with type 1 diabetes in other populations, but comparative analyses of genotype frequencies in patients with type 1 diabetes and healthy controls indicate that the genotyping protocol would function with quite similar efficiency in various populations to enrich high risk subjects for the study (Table 2). Several published data also support this view, although exact proportions cannot be deduced because of the lack of detailed genotype frequencies (5-7).

Table 2. The HLA risk genotypes in patients and healthy controls from various populations

Population	Type 1 Diabetes		Healthy Controls		Reference
	%	N studied	%	N studied	
Estonia	78.4	97	14.1	269	Nejentsev et al.:Tissue Antigens 1998:52:473
Finland	81.0	316	22.5	1000	Ilonen et al.: Eur. J. Immunogenet. 2000 27:225
Hungary	87.5	112	21.0	210	Hermann et al., personal communication
Italy (Sardinia)	90.0	100	40.0	100	Songini & Ilonen, personal communication
The Netherlands	77.6	205	26.9	840	Schipper et al., personal communication
USA (Puerto Rico)	78.9	114	26.9	108	Santiago, Trucco & Frazer, personal communication

F. STUDY FORMULA COMPOSITION, LABELLING AND DISTRIBUTION

Each study formula is either a nutritionally complete infant formula, "Nutramigen" powder, manufactured by one company (Mead-Johnson Nutritionals, Evansville, IN, USA) containing extensively hydrolyzed casein as the protein source, vegetable oils as the fat source, and glucose polymers and modified starch as the carbohydrate source, or a control formula which is a mixture of commercial routine CM-based formula powder made by the same company plus casein hydrolysate powder in a 4:1 ratio designed to mask the flavor and smell distinctions between the two study formulas. The casein hydrolysate has been shown to reduce diabetes frequency in the NOD mouse (8) and BB (9) rat models. The Study Formula contains all the nutrients infants need. Their nutritive value is analogous to the regular infant formulas used in the TRIGR countries.

The formula has four different code numbers and has been packed in four different colours - two colors for test formula and two colors for control formula. The 4-color coding scheme has been extensively tested in formula studies, it aids the blinding process, provides a hard control for randomization during data analysis, and avoids accidental mis-shipments as the families recognize “their” color. Only Mead Johnson Nutritionals knows which colors correspond to test and control formulas. Subjects are allocated to receive one color formula, which is maintained throughout the study. Each center maintains a reserve supply of each "color" formula and is responsible for ensuring that the home supplies of each participant are maintained. The company provides the coded formulas free of charge, whereas the shipping costs are paid by the study grant.

G. IMPLEMENTATION OF INTERVENTION

All recruited mothers are encouraged to breast-feed; the newborn infants are randomized before birth or as soon as possible after birth so that any elective formula supplementation or weaning by the mother will be done with the appropriate study formula. The duration of the intervention will be until at least 6 months of age. If the mother chooses to exclusively breastfeed up to the age of 6 months she is advised,

thereafter, if milk supplementation is needed, to give the study formula until the age of 8 months. Similarly, if exclusive BF lasts for 5 months, the infant would receive study formula for 2 months until the age of 7 months. Infant feeding practices are altered as little as possible by the trial. In particular, BF practice(s) is entirely at the discretion of participating mothers.

The rationale for the 6-8 month intervention period is based on the following considerations: in early infancy the child receives the major part of energy in liquid form, either as human milk or CM-based formula. The period chosen provides a reasonable and practical safety margin over the 2-3 months "vulnerable" time when the gut is permeable to proteins, as suggested in several epidemiological studies (reviewed in ref. 10).

Dietary information

Dietary advice is given by a member of the study team at the first contact with the family after randomization, at the 2 week, 1, 2, 4 and 5 month telephone calls from the center to the mother, and at 7 and 8 months for those infants who continue in the intervention beyond the age of 6 months, and at the 3 and 6 month visits. The families receive both written and oral instructions about infant feeding during the intervention period. Parents will be given a pamphlet which describes the sequence and amounts of food recommended at specific ages, according to local guidelines. It is important to avoid any contamination of the dietary intervention with sources of CM protein (including milk products, beef, and veal) contained in foods ingested by the infant. Thus, parents are provided with a list of all solid foods giving choices of brand names which can be given to the infant and which do not contain CM protein; they are also provided with a list of foods to be avoided, as they contain CM protein. Dietary advice leaflets, dietary forms and questionnaires are translated into relevant languages and adapted to national practices. The TRIGR Manual of Operations contains detailed instructions about the dietary interviews and forms. Post-intervention diets follow generally accepted practices. Designated personnel will be available during working hours to deal with urgent nutritional questions by telephone (e.g. feeding intolerance).

Monitoring of compliance and retention strategy

The diet of the infant and the compliance with the avoidance of CM proteins are assessed at the delivery hospital by interview, by a telephone interview when the infant 2 week-old, 1, 2, 4, and 5 month-old, and by an interview during the visits at the age of 3, 6 and 9 months. Those families, whose infants continue in the intervention beyond the age of 6 months, are in addition interviewed by phone, when the infant is 7 and 8-month-old. The dietary interviews are similar at all stages. We ask the families about the age at the end of exclusive and total breastfeeding. We have several questions on the use of Study Formula: Age when the feeding was started and age when regular, daily use started, whether the infant receives Study Formula at the time of the interview, how much Study Formula the infant is receiving per feeding, how much Study Formula the family has available at home at the time of the interview, and whether they need more or not. In the interview form we also have questions on the frequency of use of breast milk, Study Formula and other foods. The frequency of use of foods to be avoided during the intervention period is also asked. The Study Centers record the amount of Study Formula given to the family, and all the unused formula must be returned to the local study center the clinical visit closest to the end of the intervention period (at the 6 or 9 month visit). Compliance with the avoidance of CM proteins is also assessed by measuring antibody levels to CM proteins from sera at 3 and 6 months, and the results are analyzed by the Data Safety and Monitoring Board. We do not intend to employ serum titers of antibodies to CM proteins as a surrogate of islet cell damage.

The Nutrition Epidemiology Unit (Dr. Suvi M. Virtanen, National Institute for Health and Welfare, Helsinki, Finland) of the International Coordinating Center is responsible for the education and supervision concerning the assessment and maintenance of the dietary compliance and the dietary advice given by the centers involved. A continuous quality control system is implemented in each center for the data collection and content, especially to ensure that the delivery of dietary advice and the measurement of dietary compliance function properly. A specially trained Study Monitor supervises the implementation of these measures. A Nutrition Fellow at the Nutrition Epidemiology Unit oversees the dietary intervention closely supervised by Dr. Virtanen, by means of data collected, reports from the monitors, and continuous contacts with the Study Centers.

H. STUDY ASSESSMENTS

Baseline

After the delivery medical and perinatal history of the infant and mother (including birth weight and gestational age) and the results of the newborn physical examination are recorded on the case report forms.

Follow-up Period during and after Intervention

The subjects visit the research center or have a home visit at the age of 3, 6, 9, 12, 18 and 24 months, and at 3, 4, 5, 6, 7, 8, 9 and 10 years of age, or when clinical diabetes develops.

Protocol change in 2011: The follow-up of the subjects will be continued until the youngest participant reaches the age of 10 years. The oldest participants will then be 14-year old. During the prolonged follow-up period the follow-visits will take place annually at the ages of 11, 12, 13 and 14 years. The presence or absence of diabetes is determined according to the criteria outlined below (see Outcome Assessment, below). Clinical findings at each visit (e.g. weight, height) are recorded on the case report forms.

Protocol Change in 2015: Puberty will be assessed based on hormonal analyses. Serum estradiol will be analyzed in girls and serum testosterone in boys starting from the age of 8 years. In addition girls will be asked about their age at menarche and the use of medication containing estrogens and/or androgens starting from the age of 10 years.

Blood for serology (diabetes-associated autoantibodies and CM antibodies) is drawn after the application of an analgesic ointment on the venipuncture site at the above mentioned visits and serum samples are stored centrally. In addition, the 3 and 6 month serum samples are available for use in the assessment of dietary compliance. All serum samples are aliquoted, and the samples are stored at -70 C° in the TRIGR Core Laboratory in Helsinki. Local measurement of plasma glucose and glycated hemoglobin takes place at 12, 18, 24 months, and 3, 4, 5, 6, 7, 8, 9 and 10 years. During the prolonged follow-up period these measurements will be performed at the age of 11, 12, 13 and 14 years. The specimens for plasma glucose are preferentially taken 1-2 hours postprandially. If the glycated hemoglobin is higher than the reference range, an OGTT may be required to confirm the diagnosis as below. An OGTT will be performed at the age of 6 and 10 years in all study subjects. In addition, an

OGTT will be performed in all study subjects at the last follow-up visit in 2016-2017. A heparin blood sample (3-5 ml blood depending on age) is obtained at each sampling time for the isolation of mononuclear cells. These cells are sent fresh to central labs (Professors Outi Vaarala, Helsinki, Finland and Hans-Michael Dosch, Toronto, ON, Canada) and used for mechanistic studies. An EDTA blood sample, 4 ml, for DNA extraction will be collected from all study participants. Samples will be obtained in association of collection of blood samples for autoantibody analyses once at the age of 5 years or later. In the case of children who already have developed type 1 diabetes or have dropped-out from the follow-up a separate sample will be collected if possible.

Protocol Change in 2015: After TRIGR, any remaining samples will be placed into the National Institutes of Health (NIH) repository for future studies related to type 1 diabetes and its complications. They will be stored there indefinitely without names or any other identifying information on them. Once the samples are in the repository, subjects will not be able to have them removed. Researchers must first get permission from the NIH to use samples from the repository.

A summary and timetable of the various events are given in Table 3.

Table 3. Study schedule

	0 mo*	½, 1, 2 mo Call	3 mo Visit	4, 5 mo Call	6 mo Visit	7 mo Call	8 mo Call	9 mo Visit	12 mo Visit	18 mo Visit	2 yr Visit	3 yr Visit	4 yr Visit	5 yr Visit	6 yr Visit	7 yr Visit	8 yr Visit	9 yr Visit	10 yr Visit	11 - 14yr Visit
Contact number	1	2-4	5	6-7	8	8B	8C	9	10	11	12	13	14	15	16	17	18	19	20	21-24
Weight and height	X		X		X			X	X	X	X	X	X	X	X	X	X	X	X	X
Blood Specimens																				
- Cord blood	X																			
- Venous blood			X		X			X	X	X	X	X	X	X	X	X	X	X	X	X
- Heparin blood			X		X			X	X	X	X	X	X	X	X	X	X	X	X	X
- Blood glucose									X	X	X	X	X	X	X	X	X	X	X	X
- HbA1c									X	X	X	X	X	X	X	X	X	X	X	X
- EDTA blood														X***	X***	X***	X***	X***	X***	
Dietary Interview	X	X	X	X	X	X	X	X												
Dietary counselling	X	X	X	X	X	X	X													
Delivery of Study Formula	X	+ When	needed																	
Examination by Study Doctor	X		X		X				X		X				X				X	X**
OGTT															X				X	X**

* Contact number 1 takes place in the delivery hospital but in case of home deliveries it is arranged at home

** OGTT and examination by the Study Doctor will be performed in all study subjects at the last follow-up visit in 2016-2017.

*** An EDTA blood sample, 4 ml, for DNA extraction will be collected from all study participants once at the age of 5 years or later

Outcome Assessment

The major outcome for the first phase will be the frequency of type 1 diabetes-associated autoantibodies and/or the development of clinical diabetes by the age of 6 years. The outcome of the second phase will be the manifestation of diabetes by the age of 10 years. The manifest diabetes outcome is assessed as the proportion of subjects in each group who develop type 1 diabetes, as well as age at diagnosis. These subjects will be classified as having type 1 diabetes if they fulfill one of the following criteria:

1) Symptoms + a single random plasma glucose ≥ 11.1 mmol/l = 200 mg/dl (venous blood glucose > 10.0 mmol /l = 180 mg/dl)
2) If the child has no symptoms the diagnosis requires

a) A raised random plasma glucose reading ≥ 11.1 mmol/l = 200 mg/dl (venous blood glucose ≥ 10.0 mmol/l = 180 mg/dl) on two occasions or

b) A raised fasting plasma glucose readings ≥ 7.0 mmol/l = 126 mg/dl (venous blood glucose ≥ 6.1 mmol/l = 110 mg/dl) on two occasions or

c) A diabetic oral glucose tolerance test by WHO criteria

- fasting venous plasma glucose ≥ 7.0 mmol/l = 126 mg/dl (fasting venous blood glucose ≥ 6.1 mmol/l =110 mg/dl) on two occasions
  or
- 2 hour venous plasma glucose ≥ 11.1 mmol/l = 200 mg/dl (2 hour venous blood glucose ≥ 10.0 mmol/l = 180 mg/dl)] on two occasions.

  Accordingly a second OGTT should be performed, if the first one is diabetic. There should be an interval of at least one week between these two OGTTs.

The diabetes-associated autoantibody data will be disclosed when the autoantibodies have been assayed after the 6-year visit, and the family will be informed by the Study Center as soon as the autoantibody results have become available.

I. METHODOLOGY TO BE APPLIED

Methodology for Genetic Screening

The procedures used for HLA-DQ typing were specifically developed for screening relevant DQB1 and DQA1 alleles (11, 12). EDTA treated cord blood is collected. Alternatively capillary blood is taken postnatally if cord blood is not available. The blood samples are sent by air mail or special delivery from the U.S. and Canadian centers to the tissue typing laboratory of Professor M. Trucco, University of Pittsburgh, PA, USA, and from the various European and other centers to the tissue typing laboratory of Dr. J. Ilonen, University of Turku, Finland. According to experiences from the Pilot-2 study of the project (Finland, Sweden, Estonia and Hungary), the result of the genetic screening is available within 2 weeks to assess the eligibility of the infants. The results are entered into the Central Data Base immediately when available, and the Study Center is alerted by e-mail.

The polymorphic gene region is amplified in a microtiter format using biotinylated primers. The amplification product is subsequently transferred to streptavidin coated microtiter plates where it is bound by solid phase streptavidin. After denaturation the hybridization reaction is performed with a mixture of lanthanide (Europium, Samarium or Terbium) labeled sequence specific oligonucleotide

probes and specific hybridization signals detected by time-resolved fluorometry after repeated washes and the addition of enhancement solution.

Methodology for Autoantibodies

We will use islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA) and antibodies to the protein tyrosine phosphatase related IA-2 molecule (IA-2A) as humoral markers of β-cell autoimmunity. All autoantibody analyses will be performed in the laboratory of Professor M. Knip, University of Helsinki. His laboratory has a long experience and acknowledged expertise in the analysis of diabetes-associated autoantibodies. The laboratory will pay particular attention to quality control procedures of the assays for autoantibodies. All samples are bar-coded, and the analyses will be performed on a blinded basis. Samples from the same individual will, however, be stored in such a way that they can be run in the same assay. Samples will be analyzed in the following assay rounds: the first including samples from the baseline visit (0 month visit) up to the 2-year visit, the second from the 3-year visit to the 6-year visit, and after the 6-year visit annually visit by visit. Possible assay drift over time will be monitored by analyzing blindly with each assay three standards (low, medium and high antibody levels) once a month. An assay drift exceeding 10% is considered unacceptable. The laboratory has completed a process of collecting optimal standards with sufficient volume for a period of 15 years.

ICA are analyzed with a standard immunofluorescence assay performed on sections of frozen human pancreas from a blood group O donor (13). Fluorescein-conjugated anti-human IgG (Sigma, St Louis, MO, USA) is used to detect ICA. All initially ICA-positive samples are re-tested to confirm antibody positivity. End-point dilution titers are identified and the results are expressed in Juvenile Diabetes Foundation (JDF) units relative to an international reference standard (14). The detection and cut-off limit is 2.5 JDF units. The sensitivity of this ICA assay was 100%, the specificity 98% the validity 98%, and the consistency 98% in the most relevant international standardization round.

IAA are measured with a micro-assay, modified from Williams et al. (15). According to the assay protocol endogenous insulin is not removed before the assay. Immune complexes are precipitated with Protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) after incubation for 72 h of the serum sample (5 µl/well) with mono ^{125}I-(Tyr^{A14})-labeled human insulin (Amersham, Little Chalfont, Bucks, UK) in the presence or absence of an excess of unlabeled insulin. The volume of the incubation reaction is doubled by adding the reaction buffer (TBT; 50 mM Tris, pH 8.0, 1 % (v/v) Tween 20). After thorough washing with the reaction buffer the samples are transferred from the deep well plates to microtiter plates, scintillation liquid is added and the bound activity measured with a liquid scintillation counter (1450 MicroBeta Trilux; Perkin Elmer Life Sciences Wallac, Turku, Finland). The specific binding is expressed in relative units (RU) based on a standard curve run on each plate using the MultiCalc™ software program (Perkin Elmer Life Sciences Wallac). The standard curve is constructed from nine serial dilutions of a serum from a patient with a high IAA titer and a serum of an IAA-negative subject. The negative human serum is considered as the lowest point of the standard curve. The cut-off limit for IAA positivity is set at the 99th centile in 371 non-diabetic Finnish subjects. Samples with an initial IAA level exceeding the 95th centile are reanalyzed to verify the antibody status. The disease sensitivity of this assay was 35 % and the disease specificity 100 % based on 140 samples included in the Multiple Autoantibody Workshop (16).

GADA are detected in an immunoprecipitation radioligand assay (17, 18). The recombinant plasmid pGEM3 encoding the whole 65 kD form of the GAD protein (585 amino acids) is propagated in *E. coli* JM 109 by standard techniques. The GAD65 protein is produced by *in vitro* transcription and translation of the purified plasmid using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) in the presence of ^{35}S-methionine (Amersham). Unincorporated ^{35}S-methionine is removed by gel chromatography on NAP-5 columns (Pharmacia Biotech). Sera (2 µl) are incubated overnight at + 4 °C with approximately 20,000 cpm human GAD65 in a total volume of 50 µl TBST. To isolate the immune complexes, 10 µl Protein A-Sepharose® CL-4B (Pharmacia Biotech) is added the following day. A scintillation counter is used to count the amount of precipitated immune complexes. The results are expressed in relative units (RU) based on a standard curve run on each plate using a commercial software program (MultiCalc™, Perkin Elmer Life Sciences Wallac). The cut-off limit for antibody positivity is set at the 99th percentile in 373 non-diabetic children and adolescents. All samples with an initial GADA level between the 97.5th and 99.5th percentiles are reanalyzed to verify the antibody status. This assay had a disease sensitivity of 69% and a specificity of 100% based on 140 samples included in the 1995 Multiple Autoantibody Workshop (16).

IA-2A are detected in a similar radiobinding assay (19). The recombinant plasmid pSP64poly(A) encoding the intracellular portion of the full length IA-2 protein, including amino acids 605-979 is propagated in *E. coli* JM 109 cells. The radioactive IA-2 protein is produced with the TNT Coupled Reticulocyte Lysate System (Promega) by *in vitro* transcription and translation of the purified plasmid in the presence of ^{35}S-methionine. Sera are incubated overnight at +4 °C with 10,000 cpm of labeled IA-2 protein. Protein-A Sepharose® (Pharmacia Biotech) is used to isolate the immune complexes on the following day. After thorough washing the radioactivity of the samples is measured by a liquid scintillation counter (1450 Microbeta® Trilux, Perkin Elmer Life Sciences Wallac). The results are expressed in RU based on a standard curve constructed from the dilution of a pool of strongly positive samples and a pool of negative samples. The standard curve is run on each plate. A subject is considered IA-2A positive, if the serum antibody levels are equal to or exceed the 99th percentile in 374 non-diabetic Finnish children and adolescents. Samples with an initial IA-2A level between the 97.5th and 99.5th percentiles are reanalyzed to verify the antibody status. The disease sensitivity of this assay was 62% and the specificity 97% based on 140 samples included in the 1995 Multiple Autoantibody Workshop (16).

Methodology for Antibodies to Cow's Milk Proteins

CM antibodies (IgG, IgA and IgM), β-lactoglobulin antibodies (IgG and IgA), α-casein antibodies (IgG and IgA) and BSA antibodies (IgG and IgA) will be measured with a modification of the original ELISA technique by Dr. E. Savilahti, Helsinki, Finland.

Microtitre plates (MaxiSorp®, Nunc A/S, Roskilde, Denmark) are coated with one of the following antigens: 1. adapted liquid CM formula (Tutteli®, Valio Ltd, Helsinki, Finland) defatted, diluted (1:500 in carbonate buffer, pH 9.6) bovernight, 2. bovine β-lactoglobulin (Sigma Pharmaceuticals, St. Louis, MO, USA), at a concentration of 1 mg/ml in carbonate buffer, pH 9.6, overnight, 3. BSA (2 mg/ml, grade V, Sigma), 4. α-casein (Sigma) 2 mg/ml in phosphate buffered saline (PBS), pH 7.4. Thereafter they are incubated overnight. Wells are blocked either with 0.5% sheep serum (for anti-CM and β-lactoglobulin assays) or with 1% gelatin in PBS pH 7.4 (for BSA and α-casein assays). Serum samples are diluted in the blocking buffers. Triplicate dilutions for assays are 1:20 for β-lactoglobulin, 1:40 for CM and BSA and 1:100 for α-casein. In assays for CM and β-lactoglobulin antibodies, plates are

incubated with serum dilutions overnight at room temperature, in assays for BSA and α-casein at 37 °C for 1 hour. After washing, 100 µl alkaline-phosphatase-conjugated affinity purified rabbit $F(ab')_2$ anti-human IgG, IgA or IgM antisera (dilutions between 1:600 to 1:1200) (Dako A/S, Glostrup, Denmark) are added for 60 minutes at 37 °C. After washing, 100 µl of p-nitrophenyl-phosphate substrate, 2 mg/ml in diethanolamine buffer, pH 10.0, (Medix Biochemica, Helsinki, Finland) is added. The reaction is stopped after 30 minutes with 100 µl 1 M NaOH. The end point measure of OD405 nm is obtained in a semi-automatic multiwell photometer (Titertek Multiscan®, Elflab Inc., Helsinki, Finland). The mean value of two absorbances for wells coated with blocking solution is subtracted from the mean value for the three absorbances in antigen-coated wells. Results are subjected to point-to-point analysis in a computerized photometer using 2-fold serial dilutions of a high titer standard serum as reference. Sample dilutions must fall within the linear part of the standard curve, and antibody levels are expressed as percentages of the standard.

J. CONTAMINATION

It is critical to ensure that subjects who are randomized to receive the intervention formula, are not exposed to CM protein during the intervention period. The risk is especially high in the immediate postpartum period when a newborn infant may be given the wrong formula for supplementation in the hospital nursery. To prevent such contamination, infants who are in the study are clearly identified to all nursery personnel. Any formula supplementation for these infants is only with Nutramigen if formula is required prior to randomization (interim formula).

K. DATA BASE

The database is held at the Pediatrics Epidemiology Center at the University of South Florida, Tampa, FL, headed by Professor Jeffrey Krischer. The web sites were built by the DMU in Tampa with the assistance of the TRIGR International Coordination Center in Helsinki. The DMU has established the data transfer system between the laboratories and the database using File Transfer Protocol (FTP) technique. The system has recently been tested. The DMU created a password-based security management system for all the users who are authorized in the system and the user information was collected and saved in the system.

L. STATISTICAL METHODS

A two part hypotheses will be tested. The first hypothesis is that the children in the group fed casein hydrolysate will have a decreased occurrence of diabetes-associated antibodies in comparison with the control group receiving conventional CM-based formula. The second hypothesis is that the group weaned to a casein hydrolysate formula has a reduced incidence of type 1 diabetes.

The first hypothesis will be studied based on a longitudinal data set consisting of repeated measurements of several variables at standard time points. The variables measured at these time points are potential confounding factors (i.e., factors the effects of which have not fully been eliminated by the randomization), effect modifying factors (such as possible occurrence of differences in weight and height development, which may have the ability to modify the effect of the CM exposure considered)

and the outcome variables (e.g., the status of the diabetes-associated antibodies). At every time point the single antibody variable will be dichotomized as negative or positive, and a summary variable will be produced (negative if less than two variables are antibody positive and positive if two or more variables are antibody positive). The exposure to formula vs. placebo will be included in the model as an indicator variable. Age at initiation, duration of breast feeding and/or formula use and cumulative formula dose will be included in the model as potential effect modifying and confounding factors. The presence of interactions will be tested and the possible effect of the components considered will be estimated.

Two different statistical methods will be used to analyze the hypothesis that weaning to a casein hydrolysate decreases the occurrence of diabetes-associated antibodies. First, generalized linear models will be fitted to the data (20). Also random effect models will be used (21). These models which represent a general approach to the problem of modeling repeated measurements with fairly general error structures, can allow for missing observations, serial correlations, time-varying covariates, and irregular measurement occasions. Second, for the description of the data, the association of covariates with the risk of an elevated antibody at a given point of time will be assessed using logistic regression models (22). A rather similar statistical approach was used in the DCCT Study (23).

The analyses of the second hypothesis will use the time of diagnosis of manifest type 1 diabetes as the only outcome measure. The dataset will thus be similar to that of a cohort study, and the statistical analyses will be carried out using the proportional hazards regression model (24) including the milk exposure as the risk factor and potential confounding factors a covariates in the model. Also time-dependent covariates can be included to assess the effects of potential modifying factors. The adjusted relative risk of type 1 diabetes between the two groups of milk exposure will be estimated. Also the adjusted incidence of the disease in the two groups can be assessed.

M. INTERIM ANALYSIS

An interim analysis of autoantibodies and clinical diabetes will be performed repeatedly by the Data Safety and Monitoring Board, beginning 2 years after the last subject is recruited, in order to ensure that an unexpectedly large protective effect of the intervention will be detected early. Although the follow-up will be continued, the code will be opened and the results published if either of these analyses reach sufficient statistical significance, as defined by the Data Safety and Monitoring Board.

N. ETHICAL ISSUES

Ethical approval has been obtained at each study center. Written informed consent is requested from the parents and if needed also from the child. If, during the course of this study, an alternative strategy for diabetes prevention is proven to be effective, then we will inform the families of this possibility and discuss, and likely offer its use in a rational fashion.

Detailed HLA information will be released by qualified personnel only on the request by the parents.

The diabetes associated autoantibody results will be disclosed to the families after each annual visit starting from the 6-year visit. The family will be informed by the Study Center as soon as the autoantibody results have become available.

O. TRAINING, PROJECT PLANNING AND TIME TABLE

To achieve standardization in the implementation of the study protocol, a detailed training program for the study personnel is essential. The national coordinators, nurse and nutrition coordinators participate at vital training sessions in their respective regions. We will utilize the experience gained during the second pilot study in training and informing the national investigators and their staff, when we expanded the study to Sweden, Estonia and Hungary (investigators' meetings, site visits by project staff etc.). Additional training is provided by the Monitors and other clinical team mentors to their counterparts during their visits to the centers.

1. year
- Investigator meeting at the beginning of this period to review the recruitment strategies and protocol
- Training sessions are organized for study personnel
- Recruitment and genetic screening are started
- Clinical follow-up of the children and collection of the follow-up samples are initiated
- Dietary advice, assessment and maintenance of dietary compliance of families start
- Monitoring of the implementation of the dietary intervention and the clinical follow-up
- Investigators meeting in the middle of this period to review the start of the project, genetic screening, recruitment and participation rate, subject allocation (randomization), study formula distribution, implementation of intervention, study baseline assessments (e.g. case report forms), start of the blood specimen collection and laboratory functions, avoidance of contamination, protocol adherence (compliance), ethical aspects, collaboration in the network
- Investigators meeting at the end of this period to evaluate the same items as above, and follow-up assessments of case report forms and the suitability of dietary advisory material

2. year
- Recruitment and genetic screening are continued
- Clinical follow-up of the children and collection of the follow-up samples are continued
- Dietary advice and assessment continue
- Monitoring of the implementation of the dietary intervention and the clinical follow-up continues
- Investigators meeting in the middle of this period, review of items as listed above for the first year
- Investigators meeting at the end of this period to evaluate the progress of the project

3. year
- Recruitment and genetic screening are continued
- Clinical follow-up of the children and collection of the follow-up samples are continued

- Dietary advice and assessment continue
- Monitoring of the implementation of the dietary intervention and the clinical follow-up continues
- Analysis of the dietary data from the intervention period
- Investigators meeting at the end of this period to evaluate the progress of the project

4. year
- Recruitment and genetic screening are continued
- Clinical follow-up of the children and collection of the follow-up samples are continued

- Start of the assays of immunological markers
- Dietary advice and assessment continue
- Monitoring of the implementation of the dietary intervention and the clinical follow-up continues
- Analysis of the dietary data are continued
- Investigators meeting at the end of this period to evaluate the progress of the study

5. year
- Clinical follow-up of the children and collection of the follow-up samples are continued
- Continuation of the assays of immunological markers
- Dietary advice and assessment continue
- Monitoring of the implementation of the dietary intervention and the clinical follow-up continues
- Analysis of the dietary data are continued
- Investigators meeting at the end of the 5. year to evaluate the progress of the study

6. year
- Recruitment is completed
- Clinical follow-up of the children and collection of the follow-up samples are continued
- Continuation of the assays of immunological markers
- Dietary advice and assessment, and monitoring of the implementation of the dietary intervention are completed
- Monitoring of the implementation of the clinical follow-up continues
- Analysis of the dietary data are continued
- Investigators meeting to evaluate the progress of the study

7. year
- Clinical follow-up of the children and collection of the follow-up samples are continued
- Monitoring of the implementation of the clinical follow-up continues
- Continuation of the assays of immunological markers
- Analysis of the dietary data are continued
- Investigators meeting to evaluate the progress of the study

8.- 11. year
- Clinical follow-up of the children and collection of the follow-up samples are continued
- Monitoring of the implementation of the clinical follow-up continues
- Continuation of the assays of immunological markers
- Investigators meeting to evaluate the progress of the study

12. year
- The last recruited child reaches the age of 6 years
- Interim analysis of autoantibodies and clinical diabetes is performed
- Clinical follow-up of the children and collection of the follow-up samples are continued
- Monitoring of the implementation of the clinical follow-up continues
- Continuation of the assays of immunological markers
- Investigators meeting to evaluate the progress of the study

13.- 15. year
- Clinical follow-up of the children and collection of the follow-up samples are continued
- Monitoring of the implementation of the clinical follow-up continues
- Continuation of the assays of immunological markers
- Investigators meeting to evaluate the progress of the study

16. year
- The last recruited child reaches the age of 10 years
- Clinical follow-up of the children and collection of the follow-up samples are completed

- Monitoring of the implementation of the clinical follow-up is completed
- The randomization code is opened

17. year
- The assays of immunological markers are completed
- Final analysis of autoantibodies and clinical diabetes is performed
- Investigators meeting at the end of the study

P. PUBLICATION POLICY

The publication policy follows the guidelines published in New England Journal of Medicine (25) and the TRIGR Publication and Presentation document.

Q. ORGANIZATION AND ADMINISTRATION OF TRIGR

The trial will be executed by a multinational consortium of clinical research groups, in two large regional organizations. The scale of the study is dictated by the logistics of recruitment, the necessity of completion within a reasonable period of time, and the need for ethnic diversity in the study populations. Based on the successful development and execution of TRIGR pilot studies, the central coordinating center with the study PI will continue to be located in Helsinki (Dr. M. Knip and his deputy, Dr. H.K. Åkerblom (PI until July 1, 2008). The two major regional groups will be: 1. Europe, with a coordinating center in Helsinki; 2. North America with a coordinating center in Pittsburgh, PA (Dr. D. Becker) and a co-coordinating center in London, Ont. (Dr. J. Dupré). Each of these regional groups is multicentered, as described in Appendix 1. The trial is nationwide in Finland, Germany, the Netherlands and Canada. Satellite centers with investigators who have participated in the development of TRIGR, in New South Wales, Australia, will be 'attached' to the European group. The central major organization functions of the study will be carried out in Helsinki and Tampa. These functions include data acquisition, processing and storage, randomization and protocol administration. Monitoring and budgetary administration will be carried out by the three major regional coordinating centers**.**

The essential laboratory functions required for TRIGR will be conducted in three central internationally recognized study laboratories 1. for determination of diabetes-related autoantibodies (Dr. M. Knip, Helsinki), and 2. for determination of compliance through measurements of CM antibodies (Dr. E. Savilahti, Helsinki) 3. MHC typing with optimized turnaround time for Europe will involve the laboratory in Turku, Finland (Dr. J. Ilonen). These laboratories served the same functions in the human pilot studies. An experienced MHC laboratory in Pittsburgh, PA (Dr. M. Trucco) has been added to serve the North American centers with rapid turnaround time. Pilot experiments have been performed to optimize tissue typing for the satellites. Dr. S.M. Virtanen will be responsible for the analysis of dietary data.

Three regional fiscal units in Helsinki (Europe), London (Canada), and Pittsburgh (USA) will be responsible for operations in these respective regions, and for acquisition and transfer of data to the DMU in Tampa, Florida. However, for governance purposes North America and Europe constitute two governance regions. Communication among participants in each region will be largely by electronic means, but group meetings will be essential during the initiation of the study, and throughout the recruitment and treatment phase. Special provision will be required for communication among, and support of, the trial coordinators modeled after the DCCT and its epidemiological follow-up (EDIC).

Committees

The two Regional Executive Committees will maintain effective electronic communication among the regions, with personal meetings of representatives as needed, through the TRIGR International Executive Committee (IEC). This coordinating committee will be under the Chairmanship of the lead principal investigator or his delegate, with the co-chairmanship of the national coordinators in Canada and United States or their delegates, and with representation of the several constituencies in the study groups.

The Data Safety and Monitoring Board supervises the safety issues in the study, and performs the interim analyses, described above. It also supervises compliance issues, like the interpretation of CM antibody assays.

Communication

During randomization and recruitment, rapid communication between each center, the genetics laboratories and the central randomization center is essential. This will be achieved by the development of an internet website with Email and fax backup. Once established, this communication format will be used throughout the study.

Participating centers

The study group members represent a versatile and high-standard expertise in the fields of pediatrics, pediatric diabetology, infant nutrition, pediatric gastroenterology, neonatology, obstetrics, nutrition science, genetics, immunology and epidemiology. Many members are internationally recognized experts in their fields related to research on type 1 diabetes, particularly on the etiology, pathogenesis and prevention of the disease, and they have published extensively on the prediction, prevention and etiology of type 1 diabetes. In addition, most centers in all regions have had substantial clinical trial experience in diabetes.

R. ANCILLARY STUDIES OUTSIDE THE CORE PROTOCOL

The present core protocol deals with type 1 diabetes associated autoantibodies and/or manifest type 1 diabetes. In addition we intend, with support from other sources (e.g. JDRF) carry out in certain centers mechanistic and other ancillary studies approved by the TRIGR Ancillary Studies Committee.

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APPENDIX 1

TRIGR:
Participating Countries and Annual Recruitment Prospects

Country	Number of centers	Registered/year
Australia	3	95
Canada	18	850
The Czech Republic	7	40
Estonia	2	30
Finland	16	300
Germany	1	80
Hungary	1	50
Italy	2	90
Luxembourg	1	10
The Netherlands	1	60
Poland	5	120
Spain	3	75
Sweden	10	100
Switzerland	1	25
USA	6	1,050
TOTAL	**77**	**2,975**