PRELIMINARY COMMUNICATION

Omega-3 Polyunsaturated Fatty Acid Intake and Islet Autoimmunity in Children at Increased Risk for Type 1 Diabetes

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Context Cod liver oil supplements in infancy have been associated with a decreased risk of type 1 diabetes mellitus in a retrospective study.

Objective To examine whether intakes of omega-3 and omega-6 fatty acids are associated with the development of islet autoimmunity (IA) in children.

Design, Setting, and Participants A longitudinal, observational study, the Diabetes Autoimmunity Study in the Young (DAISY), conducted in Denver, Colorado, between January 1994 and November 2006, of 1770 children at increased risk for type 1 diabetes, defined as either possession of a high diabetes risk HLA genotype or having a sibling or parent with type 1 diabetes. The mean age at follow-up was 6.2 years. Islet autoimmunity was assessed in association with reported dietary intake of polyunsaturated fatty acids starting at age 1 year. A case-cohort study (N=244) was also conducted in which risk of IA by polyunsaturated fatty acid content of erythrocyte membranes (as a percentage of total lipids) was examined.

Main Outcome Measure Risk of IA, defined as being positive for insulin, glutamic acid decarboxylase, or insulinoma-associated antigen-2 autoantibodies on 2 consecutive visits and still autoantibody positive or having diabetes at last follow-up visit.

Results Fifty-eight children developed IA. Adjusting for HLA genotype, family history of type 1 diabetes, caloric intake, and omega-6 fatty acid intake, omega-3 fatty acid intake was inversely associated with risk of IA (hazard ratio [HR], 0.45; 95% confidence interval [CI], 0.21-0.96; $P=.04$). The association was strengthened when the definition of the outcome was limited to those positive for 2 or more autoantibodies (HR, 0.23; 95% CI, 0.09-0.58; $P=.002$). In the case-cohort study, omega-3 fatty acid content of erythrocyte membranes was also inversely associated with IA risk (HR, 0.63; 95% CI, 0.41-0.96; $P=.03$).

Conclusion Dietary intake of omega-3 fatty acids is associated with reduced risk of IA in children at increased genetic risk for type 1 diabetes.

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TYPE 1 DIABETES MELLITUS IS AN autoimmune disease that is characterized by the destruction of insulin-producing beta cells in the pancreatic islets. Although it is not yet known what initiates the autoimmune process, it is likely that both genetic background and environmental factors contribute to the disease process. Dietary factors have been implicated in the etiology of type 1 diabetes as well as in initiating the autoimmune process that leads to clinical disease. A case-control study from Norway[1] reported that children with diabetes were less likely to have been given cod liver oil during infancy than children without diabetes. Given that cod liver oil contains both vitamin D and the marine omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), it was not clear whether the protective factor in cod liver oil was the vitamin D, the marine fatty acids, or both. Although 2 studies reported that children with diabetes were less likely to have taken vitamin D supplements in infancy than children without diabetes,[2,3] similar investigations focusing on the intake of marine omega-3 fatty acids have not

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been conducted to resolve this important question.

The clinical phase of type 1 diabetes, where hyperglycemia manifests, is preceded by an asymptomatic period that varies in duration, ranging from a few months to several years, in which autoantibodies to the beta cells and their antigens are detectable in the blood. Persistent positivity of these autoantibodies confers a high risk of subsequent development of type 1 diabetes in relatives of those individuals with diabetes[4] and in the general population.[5] Because autoantibodies appear before clinical diabetes, examination of risk factors for the appearance of these autoantibodies would yield important clues regarding the early pathogenic events leading to autoimmunity, and perhaps the pathogenesis of type 1 diabetes itself.

Studies suggest that macrophage infiltration and inflammatory cytokine production are early events in the pathogenesis of type 1 diabetes.[6-9] Therefore, identifying factors that either promote or block the impact of these early pathogenic inflammatory events may be key to promoting or inhibiting the development of type 1 diabetes. Several studies have demonstrated a strong effect of omega-3 fatty acids on inflammatory responses in animals and humans.[10-13] A relative deficiency of omega-3 fatty acids, a characteristic of many Western diets, may predispose to heightened inflammatory reactions and thus increase the risk for autoimmune diseases, such as type 1 diabetes.

Alpha-linolenic acid (ALA) is the principal omega-3 fatty acid in Western diets and is found in the green leaves of plants, and also in selected seeds, nuts, and legumes (eg, flax, canola, walnuts, and soy). Alpha-linolenic acid may serve in a limited capacity as a precursor for EPA and DHA, 2 omega-3 fatty acids that are primarily obtained from fish. Linoleic acid is the most abundant omega-6 fatty acid in the diet and is found primarily in nut, seed, and vegetable oils. Arachidonic acid is an omega-6 fatty acid that can be derived from linoleic acid and is also found in meat and poultry. Because ALA and linoleic acid compete for key enzymes involved in fatty acid metabolism and conversion to either pro-inflammatory or anti-inflammatory eicosanoids, it is important to examine the effects of omega-3 and omega-6 fatty acid intakes together.

To examine the role of polyunsaturated fatty acids (PUFAs) in the etiology of diabetes, we conducted 2 separate yet related studies in the Diabetes Autoimmunity Study in the Young (DAISY), which followed a cohort of children at risk for diabetes for the appearance of islet autoantibodies. First, we examined the association between reported dietary intake of omega-3 and omega-6 fatty acids and the appearance of islet autoantibodies in the entire DAISY population. Second, a case-cohort study within DAISY was conducted to examine the association between fatty acid content of the erythrocyte membranes, a biomarker of PUFA status, and the appearance of islet autoantibodies.

METHODS

Dietary Intake and Risk of Islet Autoantibodies in the Entire DAISY Population (Study 1)

Study Population. DAISY is a prospective study of 2 groups of young children at increased risk for developing type 1 diabetes.[14] One group consists of unaffected first-degree relatives of patients with type 1A diabetes, identified and recruited between birth and 8 years through the Barbara Davis Center for Childhood Diabetes in Denver, Colorado, other diabetes care clinics, and the Colorado Insulin-Dependent Diabetes Mellitus Registry.[15] The second group consists of babies born at St Josephs Hospital in Denver, Colorado, and screened by umbilical cord blood samples for diabetes-susceptibility alleles in the HLA region. The St Josephs Hospital newborn population is representative of the general population of the Denver metropolitan area. This longitudinal observational study was conducted between January 1994 and November 2006. Cord blood was sent to Roche Molecular Systems (Alameda, California) for polymerase chain reaction–based HLA class II typing. The details of the newborn screening[14] and follow-up[16] have been published elsewhere. Written informed consent was obtained from the parents of each study participant. The Colorado Multiple Institutional Review Board approved all study protocols.

Collection and Analysis of Dietary Intake. Early childhood diet was measured prospectively using a 111-item semiquantitative food frequency questionnaire (FFQ) that has been altered and validated for use in preschool children.[17] Starting at the age of 2 years, or at enrollment if after the age of 2 years, the FFQ was administered annually and asked the mothers to recall the diets of their children in the previous year. Thus, the dietary intake data available to this study began from the age of 1 year (ie, the second year of life). A comparable quantitative dietary assessment was not available for the first year of life. This was an observational study; no dietary advice was given to the families.

To calculate intakes of omega-3 and omega-6 fatty acids and other nutrients, a commonly used unit or portion size for each food (eg, 1 egg or 3-4 oz of fish) was specified on the FFQ and the parents were asked how often, on average, during the previous year their child had consumed that amount. Nine responses were possible, ranging from "never" to "≥6 times per day." Specifically, the questionnaire asked about the frequency of intake of canned tuna, dark-meat fish (mackerel, salmon, sardines, bluefish, and swordfish), other fish (not specified), and shrimp, lobster, and scallops. The questionnaire also inquired about the kind of fat usually used for frying, sautéing, and baking (vegetable oil, solid vegetable oil shortening, butter, margarine, lard, or none). The intake of nutrients was computed for each child by multiplying the frequency of consumption of each unit of food by the nutrient content of the specified portions. Composition values for fatty acids and other nutrients were obtained from the Harvard University Food Composition

Database, which was derived from US Department of Agriculture sources[18] and supplemented by manufacturer information.[19]

The total omega-3 fatty acid intake variable was calculated by summing the intakes of the following fatty acids available in the FFQ data: ALA, EPA, DHA, and docosapentaenoic acid. The calculation of EPA and DHA intake from this FFQ and database is described in detail elsewhere.[20] The total omega-6 fatty acid intake variable was calculated as the sum of linoleic acid, arachidonic acid, and gamma-linolenic acid intake.

The validity of the nutrient intakes as assessed by the FFQ in children was evaluated by comparing these with nutrient intakes from four 24-hour recalls collected from the parent throughout the year in 68 DAISY children aged 1 to 3 years. The correlation between energy-adjusted intake of fat measured by the recalls and by the FFQ was 0.39 ($P < .05$).[21] We also compared the intake of omega-3 and omega-6 PUFAs, which were assessed by our FFQ to erythrocyte membrane composition of the same fatty acids in 404 DAISY children over time, for a total of 917 visits.[22] Longitudinal analysis showed that estimates of energy-adjusted intakes of marine PUFAs ($r = 0.38$, $P < .001$), total omega-3 fatty acids ($r = 0.25$, $P = .001$), and total omega-6 fatty acids ($r = 0.16$, $P < .001$) were associated with the sums of EPA and DHA, of all omega-3 fatty acids, and of all omega-6 fatty acids (as a percentage of total lipids) in the erythrocyte membrane, respectively.

Because fish, which is the primary source of marine PUFA, is also a good source of vitamin D and because vitamin D intake has been implicated as a protective factor in type 1 diabetes,[2,3] we investigated vitamin D intake as a potential confounder of our analyses. Intake of vitamin D was calculated from the sum of the frequency of consumption of specified portion sizes of those foods containing vitamin D naturally and after fortification, and the consumption of multivitamins and specific supplements that contain vitamin D.

Measurement of Islet Autoantibodies. In the DAISY follow-up, all children who were recruited at birth were tested at 9 months, 15 months, 24 months, and annually thereafter for antibodies to pancreatic islet antigens. Children who were recruited after birth had their blood first drawn at enrollment and then annually thereafter. Children who tested positive for any of the 3 autoantibodies were placed on an accelerated schedule on which they returned for a blood draw every 3 to 6 months for the duration of the study. Individuals who were negative for the autoantibodies remained on the aforementioned clinic visit schedule.

Glutamic acid decarboxylase 65 (GAD) autoantibodies and insulinoma-associated antigen-2 autoantibodies were measured with a combined radiobinding assay as previously described.[23] In brief, the sera were incubated with 3-H labeled GAD65 and 35-S label ICA512 and then precipitated with protein A Sepharose (Amersham, Little Chalfont, England). The assay was performed on a 96-well filtration plate (Fisher Scientific, Loughborough, England) and radioactivity was counted on a Topcount 96-well plate beta counter (PerkinElmer Life Sciences, Wilmington, Delaware). The antibody levels were expressed as an index. The interassay coefficients of variation (n = 50) are 10% and 5% for GAD and insulinoma-associated antigen-2 autoantibodies, respectively. The upper limits of normal controls (0.032 for GAD and 0.049 for insulinoma-associated antigen-2 autoantibodies) were established as the 99th percentile in 198 healthy controls. In the most recent Diabetes Autoantibody Standardization Program workshop (2005), the sensitivity and specificity were 76% and 99%, respectively, for GAD, and 66% and 100%, respectively, for insulinoma-associated antigen-2 autoantibodies.

Insulin autoantibody was measured by a micro–insulin autoantibody assay as described previously.[24] Briefly, iodine 125I–labeled human insulin (Amersham) was incubated with patient serum with and without cold human insulin, and immune complex was precipitated with protein A and G Sepharose. The assay was performed on a 96-well filtration plate and radioactivity was counted on a Topcount 96-well plate beta counter. An index was calculated on delta counts per minute between wells without and with cold human insulin, with a positivity criterion of 0.010, which was the 99th percentile of 106 normal controls. The interassay coefficient of variation is 20% (n = 100) at low-positive levels. In the most recent Diabetes Autoantibody Standardization Program workshop (2005), the sensitivity and specificity for insulin autoantibody were 58% and 99%, respectively.

Random blood glucose and glycated hemoglobin A_{1c} measures were obtained at each clinic visit on all children positive for an autoantibody. Children with a random blood glucose level of more than 200 mg/dL (to convert glucose to mmol/L, multiply by 0.0555) or a glycated hemoglobin A_{1c} level of 6.3% or more were referred to a physician for clinical evaluation and diagnosis of type 1 diabetes.

Statistical Analysis. SAS version 9.1 (SAS Institute Inc, Cary, North Carolina) statistical software package was used for all statistical analyses. Because recruitment into the DAISY cohort could occur anytime between 1994 and 2004, there are varying lengths of follow-up on the children, producing right-censored data. Cox proportional hazards regression model, which allows for right censoring, was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for time to appearance of islet autoimmunity (IA). Survival analysis methods adjusting for tied event times was used to accommodate the situation of having fixed intervals of blood draws that determine the outcome of IA.[25,26] Calculation of follow-up time began at birth.

We conducted our analyses using 2 different, yet nested definitions of an event. The primary case definition was primary cases having persistent IA, which was defined as testing positive

for at least 1 of 3 autoantibodies (ie, insulin autoantibody, GAD, or insulinoma-associated antigen-2 autoantibodies above the 99th percentile) on at least 2 consecutive visits 3 to 6 months apart, and either still positive for autoantibodies or having diabetes on the most recent follow-up visit, and was meant to examine predictors of autoimmunity. The secondary case definition was a subgroup of the primary cases who were positive for at least 2 of the aforementioned autoantibodies or who had converted to type 1 diabetes, and was meant to examine the children at highest risk of converting to type 1 diabetes.

Two children who were left-censored (ie, positive for autoantibodies at their first DAISY blood draw) were eliminated from the data set because Cox proportional hazards regression analyses cannot accommodate both right-censored and left-censored data in the same model. Therefore, there were 58 children with the primary event of persistent IA, out of a total 1770 DAISY children with childhood dietary data. Forty-five of the 58 cases met the secondary definition of multiple autoantibodies or type 1 diabetes. For both case definitions, time to event was calculated as time from birth to the first autoantibody positive visit (for cases), and the analyses were conducted in the entire cohort of 1770 DAISY children with childhood dietary data.

We ran 2 separate models (1 model with the total omega-3 fatty acids and 1 model with the marine PUFA variables as the dietary variables of interest). Given the multiple measures of intake over time before the outcome, the dietary variables were analyzed as time-varying covariates. This meant that intake information was updated dynamically each time an IA event occurred. In this way, the most recent available value of intake was used for children who were still at risk of IA at a given event time. Children were included in the risk set at each event time only if they had dietary intake data regarding that time period. The HR reflects the average effect of intake over time.

Covariates were retained in the model if they were statistically significant or if their exclusion resulted in a more than 10% change in the HR of the omega-3 fatty acid intake variable. Other dietary intake variables that were explored were total omega-6 fatty acids, arachidonic acid, total calories, and vitamin D. Sociodemographic factors (sex, maternal education [≤ high school vs at least some college], maternal age at birth [in years], and ethnicity [non-Hispanic white vs other {composed of Hispanic American, African American, biracial, Asian, and American Indian}]) were reported by the parent of the child via the questionnaire and were examined as potential confounders in the association with IA. We also considered timing of cereal introduction during infancy as a covariate, as this was found to be significantly associated with IA in a previous analysis of this population.[16] Finally, we adjusted for genetic susceptibility for type 1 diabetes, which was defined by the participant's HLA-DR genotype (HLA-DR3/4,DQB1*0302 vs other genotypes) and whether the child had a first-degree relative with type 1 diabetes.

We calculated adjusted HRs and 95% CIs based on a standard deviation difference in the fatty acid intake. This allowed us to ask the question, "What was the decrease in risk associated with an increase in fatty acid intake equal to the standard deviation of that intake variable?"

Case-Cohort Study of Erythrocyte Membrane Fatty Acid Content and Risk of IA (Study 2)

Study Population. In 2000, DAISY began collecting and storing erythrocytes from enrolled children with the intent of analyzing the erythrocyte membranes for fatty acid content as a biomarker of omega-3 and omega-6 fatty acids status. To conduct the case-cohort study, a sample of the DAISY children on whom we had erythrocyte samples was assembled as a reference group (subcohort, n=214). This representative subcohort was obtained using stratified random sampling of the DAISY population based on HLA-DR genotype and family history of type 1 diabetes. Five cases of IA developed within this subcohort during follow-up. Thirty cases of IA that developed in DAISY outside of this subcohort were later added to complete our case-cohort study population. The number of cases in this case-cohort analysis (n=35) is less than the number of cases in the dietary intake analysis (study 1, n=58), because erythrocyte samples were not available until 2000 and some of the DAISY cases developed before that date.

Measurement of Membrane Fatty Acids. On collection at each clinic visit, erythrocytes from the blood sample were separated within 30 minutes of blood draw, flash frozen in liquid nitrogen, and stored at –70°C. Samples from all visits of children in the case-cohort study were shipped to the University of Florida laboratories of 2 investigators (M.C.-S. and N.J.S.). Samples of erythrocytes were extracted for lipids following the method developed by Bligh and Dyer[27] and stored at –20°C in sealed cryotubes following flushing with nitrogen gas. The fatty acids present in the lipid isolates were subsequently methylated using the base-catalyzed procedures by Maxwell and Marmer[28] in preparation for analysis by gas chromatography (Hewlett-Packard 6890, Wilmington, Delaware) with mass spectral detection (Hewlett-Packard 5973). The samples, separated across a CP-WAX column (Varian [Palo Alto, California], 25 m × 0.25 mm, 0.2-μm film), were identified by comparing the retention times and mass-to-charge ratios (m/z) of selected ions from analytes in the samples to those of authentic standards (NuCheckPrep, Elysian, Minnesota; and Supelco, Bellefonte, Pennsylvania). Quantitation was determined against 5-point standard curves and reported as a gram of fatty acid per 100 g of red blood cell lipid.

We measured the following fatty acids in the membranes: 18:2n-6 (linoleic acid), 20:4n-6 (arachidonic acid), 18:3n-6 (gamma-linolenic acid), 18:3n-3 (ALA), 20:5n-3 (EPA), 22:6n-3 (DHA), and 22:5n-3 (docosapentaenoic acid). Eicosapentaenoic acid and

DHA were combined to estimate total marine PUFAs; ALA, DHA, EPA, and docosapentaenoic acid were combined to estimate total omega-3 fatty acid intake; and linoleic acid, arachidonic acid, and gamma-linolenic acid were combined to estimate total omega-6 fatty acid intake. Measures of erythrocyte membrane fatty acids were expressed as the percentage of total lipids (gram of fatty acid per 100 g of red blood cell lipid).

Statistical Analysis. The HRs and 95% CIs for the development of IA in relation to erythrocyte membrane fatty acid content were calculated by weighted Cox proportional hazards regression models, using the Barlow method[29] and a SAS macro program developed by Ichikawa and Barlow[30] (http://lib.stat.cmu.edu/general/robphreg) to account for the sampling and case-cohort design. The erythrocyte fatty acid content variables were analyzed as time-varying covariates. This means that the fatty acid content data were updated dynamically each time an IA event occurred. In this way, the most recent available value was used for children who were still at risk of IA at a given event time. Children were included in the risk set at each event time only if they had fatty acid content data at that time. The HR reflects the average effect of fatty acid content of the erythrocyte membranes over time. We calculated adjusted HRs based on a standard deviation difference in the fatty acid level (percentage of total lipids). We adjusted for genetic susceptibility for type 1 diabetes, which was defined by the participant's HLA-DR genotype (HLA-DR3/4,DQB1*0302 vs other genotypes), and whether the child had a first-degree relative with type 1 diabetes.

RESULTS

Dietary Intake and Risk of Islet Autoantibodies in the Entire DAISY Population (Study 1)

Dietary data were available for 1770 DAISY children aged 1 year or older, although due to staggered enrollment, the amount of data differed by child. We collected 1 annual FFQ for 396 children, 2 FFQs for 310 children, 3 FFQs for 224 children, 4 FFQs for 211 children, 5 FFQs for 160 children, 6 FFQs for 158 children, 7 FFQs for 167 children, and at least 8 FFQs for 144 children. To provide a simple description of the dietary intake data by age, we selected the 3-year-old, 5-year-old, 7-year-old, and 9-year-old children in our study population and calculated mean nutrient intakes of the variables of interest (TABLE 1). However, for our analysis of the predictors of IA, we used dietary data from the FFQs collected at every age in our cohort. These dietary intake variables were analyzed as time-varying covariates, which allowed us to examine the association between IA positivity and the dietary intake directly preceding it, and to account for changes in diet over time.

Fifty-eight children became positive for IA during follow-up for a rate of 8.6 per 1000 person-years of follow-up. The mean (SD) age at first-positive visit for children with IA was 4.8 (2.6) years and the mean (SD) age at the last follow-up for children without IA was 6.2 (3.2) years (TABLE 2). HLA-DR3/4,DQB1*0302 status was significantly associated with an increased risk of IA in univariate analyses.

Adjusting for HLA-DR3/4,DQB1*0302 status, family history of type 1 diabetes, caloric intake, and total omega-6 fatty acid intake, total omega-3 fatty acid intake was inversely associated with IA risk (HR, 0.45; 95% CI, 0.21-0.96; $P = .04$) (TABLE 3, model 1). Although total omega-6 fatty acid intake was not associated with risk of IA, it was retained in the model because (1) its inclusion strengthened the omega-3 fatty acid association and (2) studies have suggested that levels of omega-3 and

Table 1. Mean Dietary Intakes of Children in DAISY Cohort by Age (Study 1)[a]

	Group of Children, Mean (SD)			
Dietary Intake Variable	3-Year-Olds (n = 979)	5-Year-Olds (n = 762)	7-Year-Olds (n = 587)	9-Year-Olds (n = 411)
Calorie intake, kcal/d	2158.89 (735.16)	2114.82 (719.59)	2114.49 (735.27)	2079.93 (654.09)
Total omega-3 fatty acids, g/d[b]	1.17 (0.57)	1.19 (0.53)	1.21 (0.54)	1.29 (0.56)
Alpha-linolenic acid	1.02 (0.52)	1.02 (0.48)	1.03 (0.46)	1.14 (0.51)
Eicosapentaenoic acid	0.037 (0.048)	0.045 (0.058)	0.049 (0.057)	0.041 (0.048)
Docosahexaenoic acid	0.093 (0.087)	0.106 (0.094)	0.109 (0.103)	0.094 (0.090)
Docosapentaenoic acid	0.018 (0.015)	0.019 (0.016)	0.019 (0.017)	0.017 (0.014)
Marine PUFAs intake, g/d[c]	0.131 (0.133)	0.151 (0.148)	0.158 (0.159)	0.134 (0.137)
Total omega-6 fatty acids, g/d[d]	10.53 (4.67)	10.80 (4.54)	11.09 (4.61)	11.33 (4.30)
Linoleic acid	10.38 (4.62)	10.65 (4.50)	10.94 (4.56)	11.19 (4.26)
Arachidonic acid	0.131 (0.070)	0.133 (0.075)	0.133 (0.076)	0.124 (0.063)
Gamma-linolenic acid	0.013 (0.007)	0.013 (0.007)	0.012 (0.007)	0.011 (0.007)
Vitamin D intake, IU/d	431.62 (225.91)	409.72 (208.11)	372.21 (181.38)	372.68 (189.02)

Abbreviations: DAISY, Diabetes Autoimmunity Study in the Young; PUFAs, polyunsaturated fatty acids.
[a] These are cross-sectional slices of the DAISY cohort. The same child may be in one or all of these age group samples depending on whether dietary data were available at these ages.
[b] Total omega-3 fatty acids consisted of alpha-linolenic acid (18:3n3), eicosapentaenoic acid (20:5n3), docosahexaenoic acid (22:6n3), and docosapentaenoic acid (22:5n3).
[c] Marine PUFAs consisted of eicosapentaenoic acid (20:5n3) and docosahexaenoic acid (22:6n3).
[d] Total omega-6 fatty acids consisted of linoleic acid (18:2n6), arachidonic acid (20:4n6), and gamma-linolenic acid (18:3n6).

omega-6 fatty acids exhibit a competitive interrelationship in the body. In a separate model (Table 3, model 2), we examined marine PUFA intake and found no significant association with IA. Although arachidonic acid intake itself was also not associated with risk of IA, it was retained in the model because its inclusion strengthened the marine PUFA association. Given that fish are a source of both marine PUFAs and vitamin D, we initially included vitamin D intake in both of the above models and found that this was not significant and did not alter the HR of either the total omega-3 fatty acid intake variable or the marine PUFA intake variable, suggesting that vitamin D was neither a covariate nor a confounder in the association between PUFA intake and IA.

In the analysis of the secondary outcome (multiple autoantibodies or type 1 diabetes), we limited our cases to those 45 children who had developed 2 or more autoantibodies or who had developed type 1 diabetes, and then we examined predictors of time to positivity of the first autoantibody. Adjusting for HLA-DR3/4,DQB1*0302 status, family history of type 1 diabetes, caloric intake, and intake of omega-6 fatty acids, omega-3 fatty acid intake was significantly associated with a decreased risk of multiple autoantibodies or type 1 diabetes (HR, 0.23; 95% CI, 0.09-0.58; $P=.002$) (TABLE 4). There were nonsignificant associations between marine PUFA intake and arachidonic acid intake, with decreased and increased risk of multiple autoantibodies or type 1 diabetes, respectively. We did not find an association between timing of cereal introduction and risk of IA, as we had in a previous analysis,[16] which may be explained by our recent findings that this exposure may have an age-dependent effect, whereby timing of cereal introduction is associated with early onset but not with later onset IA.[31] Therefore, the longer follow-up time (ie, older age) of the current cohort compared with the previous analysis cohort may explain the lack of an association with timing of cereal introduction.

Case-Cohort Study of Erythrocyte Membrane Fatty Acid Content (Study 2)

Membrane fatty acid data were available for an average of 4 visits (time points) per child (25 had 1 time point, 20 had 2 time points, 26 had 3 time points, 39 had 4 time points, 58 had 5 time points, and 46 had ≥6 time points) in the 214 subcohort population. TABLE 5 describes the case-cohort study population. Adjusting for HLA-DR3/4,DQB1*0302 status and family history of type 1 diabetes, increased level of omega-3 fatty acids in the erythrocyte membranes (as a percentage of total lipids) was associated with decreased risk of IA (HR, 0.63; 95% CI, 0.41-0.96; $P=.03$) (TABLE 6). Marine fatty acids, a subset of total omega-3 fatty acids, showed a weaker and nonsignificant association with risk of IA.

COMMENT

Our study suggests that higher consumption of total omega-3 fatty acids, which was reported on the FFQ, is associated with a lower risk of IA in children at increased genetic risk of type 1 diabetes. This association is further substantiated by the observation that a higher proportion of omega-3 fatty acids in the erythrocyte membranes is as-

Table 2. Descriptive Characteristics and Unadjusted Risk Estimates for 1770 Children at Increased Genetic Risk for Type 1 Diabetes (Study 1)[a]

Characteristic	Children Positive for IA (n = 58)	Children Negative for IA (n = 1712)	Unadjusted HR (95% CI)	*P* Value
Age, mean (SD), y[b]	4.8 (2.6)	6.2 (3.2)	NA	NA
HLA-DR3/4,DQB1*0302 genotype	25 (43)	348 (20)	3.13 (1.85-5.28)	<.001
Family history of type 1 diabetes	38 (66)	849 (50)	1.50 (0.87-2.60)	.15
Female sex	32 (55)	815 (48)	1.35 (0.81-2.27)	.25
Non-Hispanic white ethnicity[c]	48 (83)	1294 (76)	1.24 (0.63-2.46)	.54
Maternal education >high school[c]	52 (90)	1341 (79)	2.18 (0.94-5.08)	.07
Maternal age at birth, mean (SD), y[c]	30.9 (5.0)	30.2 (5.5)	1.03 (0.98-1.08)	.29
Month of cereal introduction in the infant diet[c]				
0-3	8 (14)	316 (19)	0.69 (0.33-1.48)	.34
4-6	42 (72)	1219 (72)	1 [Reference]	
≥7	8 (14)	147 (9)	1.42 (0.67-3.02)	.36

Abbreviations: CI, confidence interval; HR, hazard ratio; IA, islet autoimmunity; NA, not applicable.
[a] Data are presented as No. (%) unless otherwise specified.
[b] For children positive for IA, age represents the age at first-positive autoantibody visit. For children negative for IA, age represents the age at last follow-up.
[c] Ethnicity data were missing for 10 children; maternal education data were missing for 15 children; maternal age data were missing for 23 children; and infant cereal data were missing for 30 children.

Table 3. Risk of Developing the Outcome of Islet Autoimmunity by Dietary Intake of PUFAs (Study 1)[a]

	Adjusted HR (95% CI)[b]	*P* Value
Model 1		
Total omega-3 fatty acid intake[c]	0.45 (0.21-0.96)	.04
Total omega-6 fatty acid intake[d]	1.68 (0.83-3.39)	.15
Model 2		
Marine PUFAs intake[e]	0.81 (0.46-1.42)	.47
Arachidonic acid intake	1.27 (0.78-2.09)	.33

Abbreviations: CI, confidence interval; HR, hazard ratio; PUFAs, polyunsaturated fatty acids.
[a] Fifty-eight children developed islet autoimmunity for a rate of 8.6 per 1000 person-years of follow-up.
[b] Adjusting for total caloric intake, HLA-DR3/4,DQB1*0302 status, and family history of type 1 diabetes. Fatty acid intakes were modeled as continuous variables. The adjusted HRs (95% CIs) reflect the risk associated with a standard deviation difference in intake. The standard deviations for total omega-3 fatty acid, total omega-6 fatty acid, marine PUFAs, and arachidonic acid intakes are 0.778, 6.252, 0.245, and 0.107, respectively.
[c] Total omega-3 fatty acids consisted of alpha-linolenic acid (18:3n3), eicosapentaenoic acid (20:5n3), docosahexaenoic acid (22:6n3), and docosapentaenoic acid (22:5n3).
[d] Total omega-6 fatty acids consisted of linoleic acid (18:2n6), arachidonic acid (20:4n6), and gamma-linolenic acid (18:3n6).
[e] Marine PUFAs consisted of eicosapentaenoic acid (20:5n3) and docosahexaenoic acid (22:6n3).

sociated with a decreased risk of IA in a subset of this same population.

Several animal studies have suggested that omega-3 fatty acids may be involved in the etiology of type 1 diabetes and autoimmunity. Long-chain fatty acids have been shown to reduce the risk of chemically induced diabetes in animal models.[32] Kleemann et al[33] investigated the impact of fish oil feeding in BB (BioBreeding) rats and found that although a specific anti-inflammatory effect of fish oil was not observed in the pancreas, a shift from "beta cell destructive" to "benign" (from Th1 to Th2 cytokine mRNA ratio) was observed in the gut-associated immune system in the BB rats fed a diet supplemented with fish oil. Interestingly, another animal study[34] suggested that essential fatty acid deficiency, including both omega-3 and omega-6 fatty acids, was associated with decreased diabetes risk.

Table 4. Risk of Developing the Outcome of Multiple Autoantibodies or Type 1 Diabetes by Dietary Intake of PUFAs (Study 1)[a]

	Adjusted HR (95% CI)[b]	*P* Value
Model 1		
Total omega-3 fatty acid intake[c]	0.23 (0.09-0.58)	.002
Total omega-6 fatty acid intake[d]	1.50 (0.67-3.35)	.32
Model 2		
Marine PUFAs intake[e]	0.48 (0.21-1.09)	.08
Arachidonic acid intake	1.48 (0.88-2.49)	.14

Abbreviations: CI, confidence interval; HR, hazard ratio; PUFAs, polyunsaturated fatty acids.
[a] Forty-five children developed multiple autoantibodies or type 1 diabetes for a rate of 6.7 per 1000 person-years of follow-up.
[b] Adjusting for total caloric intake, HLA-DR3/4,DQB1*0302 status, and family history of type 1 diabetes. Fatty acid intakes were modeled as continuous variables. The adjusted HRs (95% CIs) reflect the risk associated with a standard deviation difference in intake. The standard deviations for total omega-3 fatty acid, total omega-6 fatty acid, marine PUFAs, and arachidonic acid intakes are 0.778, 6.252, 0.245, and 0.107, respectively.
[c] Total omega-3 fatty acids consisted of alpha-linolenic acid (18:3n3), eicosapentaenoic acid (20:5n3), docosahexaenoic acid (22:6n3), and docosapentaenoic acid (22:5n3).
[d] Total omega-6 fatty acids consisted of linoleic acid (18:2n6), arachidonic acid (20:4n6), and gamma-linolenic acid (18:3n6).
[e] Marine PUFAs consisted of eicosapentaenoic acid (20:5n3) and docosahexaenoic acid (22:6n3).

Table 5. Descriptive Characteristics of Children in the DAISY Case-Cohort Study (Study 2)[a]

Characteristic	Children Positive for IA (n = 35)	Children Negative for IA (n = 209)
Age, mean (SD), y[b]	5.3 (3.3)	8.2 (3.1)
HLA-DR3/4,DQB1*0302 genotype	15 (43)	82 (39)
Family history of type 1 diabetes	16 (46)	21 (10)
Female sex	22 (63)	99 (47)
Non-Hispanic white ethnicity	28 (80)	148 (71)

Abbreviations: DAISY, Diabetes Autoimmunity Study in the Young; IA, islet autoimmunity.
[a] Data are presented as No. (%) unless otherwise specified. The entire case-cohort (N = 244) consisted of a subcohort of 214 children selected from DAISY (within which 5 cases of IA developed) and 30 cases that developed in DAISY outside of the subcohort that were added to the subcohort, for a total of 35 children positive for IA and 209 children negative for IA.
[b] For children positive for IA, age represents the age at first-positive autoantibody visit. For children negative for IA, age represents the age at last follow-up.

Table 6. Association Between Omega-3 and Omega-6 Fatty Acids in Erythrocyte Membranes and Risk of IA (Study 2)[a]

Fatty Acids (as Percentage of Total Lipids)	Adjusted HR (95% CI)[b]	*P* Value
Total omega-3 fatty acids[c]	0.63 (0.41-0.96)	.03
Marine PUFAs[d]	0.87 (0.53-1.43)	.59
Total omega-6 fatty acids[e]	1.02 (0.68-1.53)	.92
Arachidonic acid	0.79 (0.52-1.21)	.28

Abbreviations: CI, confidence interval; DAISY, Diabetes Autoimmunity Study in the Young; HR, hazard ratio; IA, islet autoimmunity; PUFAs, polyunsaturated fatty acids.
[a] The entire case-cohort study (N = 244) consisted of a subcohort of 214 children selected from DAISY (within which 5 cases of IA developed) and 30 cases that developed in DAISY outside of the subcohort that were added to the subcohort, for a total of 35 children positive for IA and 209 children negative for IA.
[b] Separate models were run for each fatty acid. Models were adjusted for HLA-DR3/4,DQB1*0302 status and family history of type 1 diabetes. Fatty acid levels were modeled as continuous variables. The adjusted HRs (95% CIs) reflect the risk associated with a standard deviation difference as percentage of total fatty acids. The standard deviations for total omega-3 fatty acid, total omega-6 fatty acid, marine PUFAs, and arachidonic acid (as percentage of total lipids) are 1.328, 3.952, 0.831, and 2.003, respectively.
[c] Total omega-3 fatty acids consisted of alpha-linolenic acid (18:3n3), eicosapentaenoic acid (20:5n3), docosahexaenoic acid (22:6n3), and docosapentaenoic acid (22:5n3).
[d] Marine PUFAs consisted of eicosapentaenoic acid (20:5n3) and docosahexaenoic acid (22:6n3).
[e] Total omega-6 fatty acids consisted of linoleic acid (18:2n6), arachidonic acid (20:4n6), and gamma-linolenic acid (18:3n6).

The only published human study examining the contribution of omega-3 fatty acid intake on type 1 diabetes was a case-control study from Norway showing that children with diabetes were less likely to have been given cod liver oil during infancy than children without diabetes.[1] We could not examine an association between cod liver oil and IA in DAISY because fish oil supplements are not commonly given during infancy in the United States. Unfortunately, we were also unable to quantify dietary intake of omega-3 and omega-6 fatty acids during infancy in the DAISY children due to limitations in the infant diet data collection instrument for quantifying fatty acids. Because of this, the 12 children who developed IA during infancy had to be excluded from study 1, because we did not have PUFA intake data for them before autoantibody conversion. Therefore, our study 1 findings may not be representative of the very earliest-onset IA. However, the findings of study 2 (the case-cohort study) would reflect children of all ages, including infants, because erythrocyte membrane fatty acids were measured at all ages, and 28 of 913 total erythrocyte samples in study 2 were collected before 1 year of age.

Cell membranes require unsaturated fatty acids to maintain their structure, fluidity, and function. Long-chain omega-3 fatty acids are incorporated into cell membranes, usually in the *sn*-2 position of membrane phospholipids, where they serve as substrate reservoirs for several enzymes in-

volved in the production of a class of anti-inflammatory eicosanoids, known as resolvins and protectins.[35] These potent anti-inflammatory lipid molecules are produced by the 5 and 12/15 lipoxygenase enzyme systems, and through cyclooxygenase 2 [COX-2], particularly in the presence of aspirin. Resolvins and protectins exert a panopoly of anti-inflammatory effects, including suppression of inflammatory cytokines (eg, interleukin [IL]-1β, tumor necrosis factor α, IL-12), reduction of Th1 responses, and suppression of antigen presenting cell maturation (M.C.-S., unpublished data, 2007[10,36]), all relevant for the prevention of type 1 diabetes. The long-chain omega-3 fatty acids also play an important role in decreasing proinflammatory eicosanoid production by functioning as substrate competitors with arachidonic acid and through their role as substrates for protectin and resolvin production. Finally, omega-3 fatty acids have also been shown to reduce levels of oxidative stress; wherein, the addition of fish meals reduced in vivo lipid peroxidation, measured by F_2-isoprostanes, in patients with dyslipidemic type 2 diabetes.[37]

The omega-3 fatty acid intake variable becomes more significantly associated with IA when it is included in the model together with omega-6 fatty acid intake compared with when it is tested alone. This suggests a complex interrelationship that could be related to their competition for enzymes involved in fatty acid metabolism and conversion to either proinflammatory or anti-inflammatory eicosanoids. Increased consumption of omega-3 fatty acids, especially with low omega-6 fatty acid intake, results in increased content of omega-3 fatty acids in the cell membranes in contrast with diets where omega-6 intake is higher.[38,39] At low omega-3 to omega-6 membrane fatty acid ratios, the 2 will compete to be transformed to eicosanoids with a resultant increased production of proinflammatory eicosanoids with a relative deficiency in production of lipid molecules directed toward resolving inflammation. We suggest that increased intake of omega-3 fatty acids will lead to increased membrane concentration of these fatty acids, resulting in increased levels of anti-inflammatory resolvins and protectins, to bring chronic inflammation to a homeostatic end point.

Heightened production of proinflammatory prostaglandins by macrophages may contribute to non–major histocompatibility complex–encoded antigen-presenting cell dysfunction[40,41] and contribute to type 1 diabetes pathogenesis. Interestingly, reducing macrophage prostaglandin production in vivo by dietary fatty acid manipulation reduces diabetes incidence in nonobese diabetic mice by 70%.[40] Prostaglandins are produced by cyclooxygenases, of which there are 2 forms: COX-1 and COX-2, a form that is expressed under conditions of inflammation. On activation, monocytes and macrophages express COX-2 and markedly increase proinflammatory prostaglandin output from arachidonic acid.[42] Ingestion of fish oils that contain omega-3 PUFAs results in a decrease in membrane arachidonic acid levels, and a concomitant decrease in the capacity to synthesize proinflammatory prostaglandins from arachidonic acid.[11] In humans, constitutive COX-2 expression is significantly greater in monocytes of patients with type 1 diabetes, those at risk for the disease, and their relatives, than monocytes of healthy controls.[43] Therefore, we hypothesize that under conditions of relative abundance of membrane omega-6 fatty acids, production of the COX-2–mediated proinflammatory prostaglandins may predominate and contribute to the etiology of type 1 diabetes; whereas, increased levels of omega-3 fatty acids may limit production of prostaglandins and promote the generation of anti-inflammatory resolvins and protectins.

A major strength of our study is the use of 2 different exposure assessment methods, the parent-reported FFQ and the biomarker of erythrocyte membrane fatty acid content. Intake of PUFAs can be measured through diet surveys, such as FFQs and diet records; however, the ability of these self-reported data to adequately measure PUFA intake has been questioned. Both observational studies[44-46] and clinical trials[47,48] have shown that fatty acid levels in the body are known to change as a result of changes in dietary intake of fatty acids. Erythrocyte cell membrane fatty acid status has been shown to be a good indicator of medium-term (4-6 weeks) intake of omega-3 and omega-6 PUFAs in children younger than 2 years.[49] The semiquantitative FFQ used in our study has shown good correlation between reported EPA intake and percentage of EPA in adipose tissue in adults ($r=0.49$, $P<.001$).[50]

Overall, our data suggest that ingestion of omega-3 fatty acids throughout childhood may decrease the risk of IA. Recently, a TrialNet-based clinical trial, called "The Nutritional Intervention for the Prevention of Type 1 Diabetes," was established and will address the hypothesis that dietary supplementation with anti-inflammatory doses of DHA in utero and in infancy will block early islet inflammatory events key to the pathogenesis of type 1 diabetes and thus prevent the development of early IA in infants with a high genetic risk for this disease. If this trial confirms this hypothesis, dietary supplementation with omega-3 fatty acids could become a mainstay for early intervention to safely prevent the development of type 1 diabetes.

Author Contributions: Drs Norris and Rewers had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Norris, Clare-Salzler, Rewers.
Acquisition of data: Norris, Lamb, Barriga, Seifert, Hoffman, Clare-Salzler, Szabo, Erlich, Eisenbarth.
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