

## Short duration of breast-feeding as a risk-factor for $\beta$ -cell autoantibodies in 5-year-old children from the general population

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Breast-feeding has been suggested to have a protective effect against the development of type 1 diabetes. In the present study, we investigated the relation between duration of breast-feeding and  $\beta$ -cell autoantibodies in 5-year-old non-diabetic children who participated in a prospective population-based follow-up study (the All Babies in Southeast Sweden study). Autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA) and the protein tyrosine phosphatase-like IA-2 (IA-2A) were measured by radiobinding assays. A short duration of total breast-feeding was associated with an increased risk of GADA and/or IAA above the ninety-fifth percentile at 5 years of age (OR 2.09, 95 % CI 1.45, 3.02;  $P < 0.000$ ) as well as with an increased risk of IAA above the ninety-fifth percentile at this age (OR 2.89, 95 % CI 1.81, 4.62;  $P < 0.000$ ). A short duration of exclusive breast-feeding was associated with an increased risk of GADA, IAA and/or IA-2A above the ninety-ninth percentile (OR 2.01, 95 % CI 1.08, 3.73;  $P = 0.028$ ) as well as with an increased risk of IA-2A above the ninety-ninth percentile (OR 3.50, 95 % CI 1.38, 8.92;  $P = 0.009$ ) at 5 years of age. An early introduction of formula was associated with an increased risk of GADA, IAA and/or IA-2A above the ninety-ninth percentile (OR 1.84, 95 % CI 1.01, 3.37;  $P = 0.047$ ) at 5 years of age. The positive association between a short duration of both total and exclusive breast-feeding, as well as an early introduction of formula, and positivity for  $\beta$ -cell autoantibodies in children from the general population suggests that breast-feeding modifies the risk of  $\beta$ -cell autoimmunity, even years after finishing breast-feeding.

### Breast-feeding: $\beta$ -cell autoantibodies: Children: General population

Type 1 diabetes (T1D) is considered to be an autoimmune disease in which the insulin-producing  $\beta$ -cells are destroyed in genetically predisposed individuals (Castano & Eisenbarth, 1990). Environmental factors are suggested to trigger the autoimmune response, as reviewed in Åkerblom *et al.* (2002). In a number of studies, breast-feeding has been proposed to have a protective effect: a high frequency of breast-feeding has been reported to be associated with a low incidence of T1D (Borch-Johnsen *et al.* 1984; Dahl-Jorgensen *et al.* 1991). A duration of breast-feeding of less than 3–4 months has been shown to be associated with development of T1D in a meta-analysis by Gerstein (1994). The possible protective effect of breast-feeding may be due to a delayed introduction of cows milk, and several studies have reported an earlier exposure to cows milk or solid foods in children with T1D compared with healthy children (Kostraba *et al.* 1993; Verge *et al.* 1994).

Most studies on the association between breast-feeding and T1D are retrospective, and the results may be compromised by recall bias. Thus, prospective studies are needed to investigate the possible association between  $\beta$ -cell autoimmunity, infant feeding in general and breast-feeding in particular.

In a Finnish study, short-term exclusive breast-feeding and an early introduction of a cows milk-based formula were associated with an increased risk of  $\beta$ -cell autoimmunity in genetically predisposed children (Kimpimäki *et al.* 2001), but the duration of breast-feeding has not been associated with an increased risk of  $\beta$ -cell autoimmunity in children with a first-degree relative with T1D in Germany, Australia or the USA (Couper *et al.* 1999; Hummel *et al.* 2000; Norris *et al.* 2003; Ziegler *et al.* 2003).

The protective effect of breast-feeding may also be due to the protective agents in the breast milk, which may affect the child's immature immune system, as reviewed in Newburg (2005). In epidemiological studies, an increased risk of T1D has been associated with an early introduction of cows milk formula in infancy, indicating that triggering of the gut immune system in early infancy may contribute to the later development of  $\beta$ -cell autoimmunity (Vaarala, 1999).

In predicting T1D, the presence of circulating autoantibodies to insulin (IAA) glutamic acid decarboxylase (GADA) and the protein tyrosine phosphatase-like IA-2 (IA-2A) are important, and multiple autoantibodies confer a

**Abbreviations:** ABIS, All Babies in Southeast Sweden; CD, coeliac disease; GADA, glutamic acid decarboxylase autoantibodies; IAA, insulin autoantibodies; IA-2A, protein tyrosine phosphatase-like (IA-2) autoantibodies; T1D, type 1 diabetes.

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higher risk of developing T1D (Bingley *et al.* 1994; Verge *et al.* 1998). We investigated the relation between duration of breast-feeding and  $\beta$ -cell autoantibodies in 5-year-old Swedish children from the general population who participated in a prospective population-based follow-up study (the All Babies in Southeast Sweden (ABIS) study).

## Subjects and methods

### Subjects

The current study was part of a prospective population-based follow-up study of all infants born between 1 October 1997 and 1 October 1999 in Southeast Sweden (the ABIS study; Ludvigsson *et al.* 2001). Data on total and exclusive breast-feeding, dietary factors, hereditary factors, delivery, infections and parents' age, education and ethnicity were obtained through questionnaires filled in at birth and 1, 2–3 and 5–6 years of age.

The duration of total breast-feeding was defined as the period when any breast milk was given regardless of other food supplements, and exclusive breast-feeding as the period when only breast milk was given. The duration of total breast-feeding was categorised into 0–3 months and 4 months or longer. The duration of exclusive breast-feeding was categorised 1–3 months and 4 months or longer.

The external factors of maternal age, maternal education, infections during pregnancy, mode of delivery, low birth weight (<2500 g), early age of gestation ( $\leq 37$  weeks), a first-degree relative with T1D, coeliac disease (CD) or type 2 diabetes, or gastroenteritis in the child during the first year of life or recurrently until 5 years of age were considered as potential confounder variables and tested by the  $\chi^2$  test.

Data regarding breast-feeding, the introduction of formula, external factors and additional background factors were available from questionnaires filled in at birth and 1-year, 2–3-year and 5–6-year follow-up. At the 5–6-year follow-up, data from questionnaires were available from 7443 families and a venous blood sample from 3788 children. The sera were stored at  $-20^\circ\text{C}$  until analysis of autoantibodies. The group of children with available questionnaire data at 5–6-year follow-up ( $n$  7443) did not differ significantly from the group of children with available venous blood samples ( $n$  3788) with regard to the additional background factors (mother's and father's age, educational level and ethnicity as well as T1D, type 2 diabetes and CD among first-degree relatives, or premature birth, low birth weight and delivery by caesarean section). Parents/guardians gave their informed consent, and the protocol of the ABIS study was approved by the Research Ethics Committees of the Medical Faculty of Linköping University, Linköping, and of the Medical Faculty of Lund University, Lund, Sweden.

### Methods

Measurements of GADA and IA-2A by radiobinding assays were performed as previously described (Holmberg *et al.* 2006). The cut-off for positivity at the ninety-ninth percentile of 5–6-year-old Swedish children was 160.9 relative units (RU)/ml, corresponding to 61.4 WHO units, for GADA ( $n$  3251) and 5.6 RU/ml, corresponding to 4.0 WHO units,

for IA-2A ( $n$  3459). The cut-off for positivity at the ninety-fifth percentile for GADA was 62.3 RU/ml, corresponding to 20.9 WHO units. The cut-off for positivity was determined as the ninety-ninth percentile for IA-2A as the detection level of the IA-2A assay is 5.5 RU/ml. In the Diabetes Auto-antibody Standardization Program for the year 2005, at the ninety-ninth percentile the specificity was 99% for GADA and 100% for IA-2A, whereas the sensitivity was 74% for GADA and 72% for IA-2A. At the ninety-fifth percentile, the specificity for GADA was 94% and the sensitivity 80%. Interassay variation for negative and positive controls was 10% and 8% for GADA and 11% and 12% for IA-2A, respectively.

The measurement of IAA by radiobinding assays was performed according to the method of Williams *et al.* (1997), with some modifications (Holmberg *et al.* 2006). The cut-off for positivity at the ninety-ninth percentile of 2201 Swedish children (age 5–6 years) was 6.3 units/ml, and the cut-off for positivity at the ninety-fifth percentile was 2.6 units/ml. In the 2005 Diabetes Auto-antibody Standardization Program, at the ninety-ninth percentile, the specificity for IAA was 100%, whereas the sensitivity was 24%; the corresponding figures at the ninety-fifth percentile were 97% and 34%, respectively. Interassay variation for negative and positive controls was 11% and 8%, respectively. Standard curves and interpolated values of samples were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA).

In the present study, IAA levels above the ninety-fifth percentile or IA-2A above the ninety-ninth percentile was used as an outcome variable and marker of  $\beta$ -cell autoimmunity. In addition, GADA and/or IAA values above the ninety-fifth percentile or any one of GADA, IAA or IA-2A above the ninety-ninth percentile were used as outcome variables.

### Statistics

The data from all the questionnaires were optically scanned. The data on breast-feeding in relation to autoantibody status have been analysed statistically using  $\chi^2$  tests after classifying the children into autoantibody positive or negative. The duration of breast-feeding was divided into two categories.  $\chi^2$  tests were also used to test the external factors and the additional background factors.

OR with 95% CI were estimated using logistic regression with autoantibody positivity as the dependent variable and entering the significant variables. Autoantibodies as the dependent variable, and duration of either total or exclusive breast-feeding and the external factors as covariates, were analysed simultaneously and included in the multivariate model by stepwise forward selection for the significance of the explanatory variable. A  $P$  value below 0.05 and a 95% CI not overlapping the null value 1.00 for the OR was regarded as statistically significant. Intercorrelations between variables were investigated using Spearman's rank correlation coefficients. A two-tailed  $P$  value of 0.05 or less was considered statistically significant. Calculations were performed with the statistical package SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Descriptive

Among the 3788 children studied with autoantibody analysis, 12 (0.3%) developed T1D before the age of 5–6 years and were therefore excluded from further analysis. Among the remaining 3776 non-diabetic children, the median duration of total breast-feeding was 8 months (data available on 2916 children) and that of exclusive breast-feeding was 4 months (data available on 2867 children). At 3 months of age, 2724/2916 (93.4%) of the children were breast-fed and 2491/2867 (86.9%) were exclusively breast-fed. Sixty-four of 3776 children (1.7%) were positive for at least one of GADA, IA-2A and IAA above the ninety-ninth percentile, and 266/3776 children (7.0%) were positive for GADA and/or IAA above the ninety-fifth percentile.

### Breast-feeding and autoantibodies

The number of children positive for GADA and/or IAA above the ninety-fifth percentile was increased in children with a duration of total breast-feeding of less than 4 months compared with those breast-fed for longer (14.0% and 7.2%, respectively;  $P < 0.000$ ). Similarly, the prevalence of IAA above the ninety-fifth percentile was higher in children with a duration of total breast-feeding less than 4 months compared with those breast-fed for longer (12.3% and 4.6%, respectively;  $P < 0.000$ ). The number of children with GADA, IAA and/or IA-2A above the ninety-ninth percentile was non-significantly increased in children with a duration of total breast-feeding less than 4 months (3.0% and 1.6% for 0–3 months *v.* 4 months or longer;  $P = 0.090$ ; Table 1). The number of children with IA-2A above the ninety-ninth percentile or IAA above the ninety-ninth percentile was not associated with the duration of total breast-feeding (data not shown). Similarly, the number of children with GADA above the ninety-fifth or ninety-ninth percentile was not associated with the duration of total breast-feeding (data not shown).

A short duration of total breast-feeding was associated with an increased risk of GADA and/or IAA above the ninety-fifth percentile at 5 years of age (OR 2.09, 95% CI 1.45, 3.02;  $P < 0.000$ ) as well as with an increased risk of IAA above

the ninety-fifth percentile at this age (OR 2.89, 95% CI 1.81, 4.62;  $P < 0.000$ ; Table 1).

The number of children positive for GADA and/or IAA above the ninety-fifth percentile was non-significantly increased in children with a duration of exclusive breast-feeding of less than 4 months (8.9% and 6.9% for 1–3 months *v.* 4 months or longer;  $P = 0.121$ ). The number of children positive for IAA above the ninety-fifth percentile was also non-significantly increased in children with a duration of exclusive breast-feeding of under 4 months (6.5% and 4.2% for 1–3 months *v.* 4 months or longer;  $P = 0.070$ ). The number of children with GADA, IAA and/or IA-2A above the ninety-ninth percentile was increased in children with a duration of exclusive breast-feeding of less than 4 months (2.7% and 1.4%, respectively;  $P = 0.025$ ). The number of children with IA-2A above the ninety-ninth percentile was also increased in children with a duration of exclusive breast-feeding of less than 4 months (1.6% and 0.5%, respectively;  $P = 0.013$ ; Table 1). The number of children with GADA above the ninety-fifth or ninety-ninth percentile, or IAA above the ninety-ninth percentile, was not associated with the duration of exclusive breast-feeding (data not shown).

A short duration of exclusive breast-feeding was associated with an increased risk of GADA, IAA and/or IA-2A above the ninety-ninth percentile (OR 2.01, 95% CI 1.08, 3.73;  $P = 0.028$ ), as well as with an increased risk of IA-2A above the ninety-ninth percentile (OR 3.50, 95% CI 1.38, 8.92;  $P = 0.009$ ) at 5 years of age (Table 1).

### Introduction of cows milk proteins and autoantibodies

We also investigated whether the early introduction of cows milk protein in formula was associated with the development of  $\beta$ -cell autoantibodies. The duration of exclusive breast-feeding correlated to the age of exposure to formula ( $\rho = 0.591$ ,  $P < 0.000$ ). The majority of the non-diabetic children (1619/2675; 60.5%) received formula for the first time at 5–9 months of age. In children who received formula early, at 1–3 months of age, the prevalence of GADA, IAA and/or IA-2A above the ninety-ninth percentile was increased compared with children who received formula later (2.4% and 1.3%, respectively;  $P = 0.043$ ) and was associated with an

**Table 1.** Duration of total and exclusive breast-feeding in non-diabetic children with glutamic acid decarboxylase autoantibodies (GADA) and/or insulin autoantibodies (IAA) above the ninety-fifth percentile, IAA above the ninety-fifth percentile, GADA, IAA and/or protein tyrosine phosphatase-like (IA-2) autoantibodies (IA-2A) above the ninety-ninth percentile or IA-2A above the ninety-ninth percentile

Breast-feeding		GADA, IAA 95th percentile		IAA 95th percentile		GADA, IAA, IA-2A 99th percentile		IA-2A 99th percentile	
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Total	0–3 months	40	14.0	27	12.3	9	3.0		
	≥4 months	179	7.2	70	4.6	42	1.6		
	$\chi^2$ P value	<0.000		<0.000		0.090			
	OR	2.09		2.89		Not done			
	(95% CI) P value	1.45, 3.02 <0.000		1.81, 4.62 <0.000					
Exclusive	1–3 months	46	8.9	22	6.5	15	2.7	8	1.6
	≥4 months	151	6.9	56	4.2	32	1.4	10	0.5
	$\chi^2$ P value	0.121		0.070		0.025		0.013	
	OR	Not done		Not done		2.01		3.50	
	95% CI P value					1.08, 3.73 0.028		1.38, 8.92 0.009	

increased risk of GADA, IAA and/or IA-2A above the ninety-ninth percentile (OR 1.84, 95 % CI 1.01, 3.37;  $P=0.047$ ). In children who received formula early, at 1–3 months of age, the prevalence of IAA above the ninety-fifth percentile was not significantly different from that of children who received formula later (6.1 % and 4.3 %, respectively;  $P=0.1$ ).

#### External factors and autoantibodies

The prevalence of the external factors (possible confounder variables) among the 3776 non-diabetic children is given in Table 2. The prevalence of IAA above the ninety-fifth percentile was higher in children with a first-degree relative with CD compared with those with no CD in the family (12.9 % and 4.9 %; respectively,  $P=0.044$ ). In a forward stepwise logistic regression with IAA as the dependent variable and duration of total breast-feeding and CD in the family as covariates, CD in the family did not affect the risk mediated by breast-feeding.

The prevalence of IA-2A above the ninety-ninth percentile was higher in children when the mother reported having an infection during pregnancy compared with those with no infection (1.1 % and 0.5 %, respectively;  $P=0.051$ ) and when the child was delivered via caesarean section compared with vaginal delivery (1.6 % and 0.5 %, respectively;  $P=0.027$ ). In a forward stepwise logistic regression with IA-2A as the dependent variable and the duration of exclusive breast-feeding and either infection during pregnancy or mode of delivery as covariates, neither of these affected the risk mediated by breast-feeding.

The prevalence of GADA and/or IAA above the ninety-fifth percentile tended to increase in children when the mother reported not having an infection during pregnancy compared with those with an infection (8.0 % and 6.0 %, respectively;  $P=0.051$ ). In a forward stepwise logistic regression with GADA and/or IAA as the dependent variable and duration of exclusive breast-feeding and infection during pregnancy as covariates, infection during pregnancy did not affect the risk mediated by breast-feeding.

All the other external variables mentioned earlier were found to be non-significant in  $\chi^2$  tests with either IAA

above the ninety-fifth percentile, IA-2A above the ninety-ninth percentile, GADA and/or IAA above the ninety-fifth percentile or GADA, IAA and/or IA-2A above the ninety-ninth percentile; they were therefore not included in any logistic regression analysis.

#### Discussion

We found a positive association between a short duration of total breast-feeding and positivity for GADA and/or IAA at the age of 5 years in non-diabetic children from the general population, which suggests that breast-feeding has a long-term effect on the risk of  $\beta$ -cell autoimmunity several years after completing breast-feeding. We also found a positive association between a short duration of exclusive breast-feeding, as well as the early introduction of formula, and positivity for GADA, IAA and/or IA-2A at the age of 5 years in non-diabetic children.

The risk of T1D associated with short breast-feeding (less than 4 months) may be mediated by a diabetogenic effect of cows milk formula, which is usually used for weaning, or by a protective effect of breast milk itself. As breast milk contains growth factors and cytokines, it promotes the maturation of the intestinal mucosa, and the protective effect of breast-feeding might be explained by an improved function of the gut immune system, as reviewed in Harrison & Honeyman (1999). Immunisation to bovine insulin in cows milk has been suggested to be the link explaining the cows milk-mediated risk of T1D in children with aberrant function of the gut immune system (Vaarala, 1999; Knip *et al.* 2005).

Insulin is an important autoantigen, especially in young children who develop T1D, as autoantibodies to insulin (IAA) are often the first autoantibody detected in the young children (Ziegler *et al.* 1999) and the prevalence of IAA is highest in the youngest children (Arslanian *et al.* 1985; Karjalainen *et al.* 1986; Vardi *et al.* 1988). Although a short duration of total breast-feeding was associated specifically with IAA, suggesting a link with insulin, we observed only a weak positive association between IAA and the duration of exclusive breast-feeding and

**Table 2.** Prevalence of external factors (possible confounder variables) among 3776 non-diabetic Swedish children

External factors	Prevalence		
	Mean	<i>n</i>	%
Mother's age (years)	30.0	3337	
Mother's education			
< 10 years	259	3646	7.1
11–14 years	2124	3646	58.3
> 15 years	1263	3646	34.6
Infections during pregnancy	916	3512	26.1
Vaginal delivery	3031	3424	88.5
Caesarean delivery	393	3424	11.5
Low birth weight (<2500 g)	98	3658	2.7
Early gestation ( $\leq 37$ weeks)	295	3598	9.2
First-degree family member with:			
Type 1 diabetes	85	3776	2.3
Type 2 diabetes	40	3776	1.1
Coeliac disease	54	3776	1.4
Gastroenteritis			
In first year of life	765	2673	28.6
Until 5 years	2907	3259	89.2

early exposure to cows milk formula in the Swedish population. Instead, the combination of GADA, IAA and/or IA-2A was associated with both a short duration of exclusive breast-feeding and an early introduction of formula.

Exposure to dietary factors introduced at the weaning, such as cows milk formula, is lower if breast-feeding is still continued. Infants with a short duration of total breast-feeding (i.e. those who are breast-fed for less than 4 months) have therefore been exposed to higher doses of cows milk formula at the age of 3 months than those infants who have been reported to have a duration of exclusive breast-feeding of less than 4 months. This kind of dose-effect was seen in our previous study (Vaarala *et al.* 2002). In addition, in studies on the risk of CD, children with CD were exposed to a larger amount of gluten at first exposure than children without CD (Ivarsson *et al.* 2002).

In previous studies of children with an increased genetic risk of T1D (Norris *et al.* 2003) or a first-degree relative with T1D (Couper *et al.* 1999; Ziegler *et al.* 2003), no association was found between duration of either total or exclusive breast-feeding and  $\beta$ -cell autoimmunity. In addition, the early introduction of food supplements containing cows milk did not increase the risk of  $\beta$ -cell autoimmunity (Norris *et al.* 2003; Ziegler *et al.* 2003). Prospective studies in Germany (Ziegler *et al.* 2003) and the USA (Norris *et al.* 2003) did not reveal any association between duration of breast-feeding or early introduction of cows milk and  $\beta$ -cell autoimmunity, as in the Finnish study (Kimpimäki *et al.* 2001) as well as the present one.

The different findings in these studies may be due to differences in the populations or may reflect different infant-feeding practices in different countries. When weaning to formula in Germany and the USA, a hydrolysed formula is often used (Åkerblom *et al.* 2005), which is less immunogenic (Vaarala *et al.* 1995). This kind of formula is less often used in Sweden (according to the recommendations of the National Board of Health and Welfare) and Finland (Åkerblom *et al.* 2005). The high frequency of children receiving breast milk at 3 months of age in our study cohort correlates with numbers from the whole ABIS study population (Brekke *et al.* 2005), as well as the National Board of Health and Welfare (Official Statistics of Sweden, 2000). It is extremely difficult to dissect the effect of different mediators in the pathogenic process leading to the manifestation of disease or the appearance of surrogate markers such as  $\beta$ -cell autoimmunity for T1D.

We do not expect a large number of children with T1D in our study population at this age so the end-point is  $\beta$ -cell autoimmunity. One may speculate whether this  $\beta$ -cell autoimmunity is transient or whether the aetiology differs from that of T1D. It is possible that environmental factors triggering the initial  $\beta$ -cell autoimmunity are different from those which later cause the ensuing T1D, as recently suggested (Knip *et al.* 2005). Despite this, the environmental factors that trigger the  $\beta$ -cell autoimmunity determine the population at risk of T1D. Additional studies may shed light on the importance of these factors, such as a short duration of breast-feeding, in the progression from  $\beta$ -cell autoimmunity to clinical disease.

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