



High levels of immunoglobulin E and a continuous increase in immunoglobulin G and immunoglobulin M by age in children with newly diagnosed type 1 diabetes

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ABSTRACT

The incidence of type 1 diabetes (T1D) is increasing, either because of environmental factors accelerating onset of the disease or because of inducement of autoimmune diabetes in children who previously were at lower risk. High levels of immunoglobulin (Ig), specifically, IgM and IgA, and a low level of IgG were reported in adult patients; however no studies have analyzed the increasing incidence in relation to Ig levels. Our aim was to describe Ig in children newly diagnosed with diabetes and in their healthy siblings. Children with T1D expressed significantly lower IgG ($p < 0.01$) and higher IgA levels ($p = 0.045$), whereas no differences in IgE or IgM ($p > 0.5$) levels were found. Age-specific levels were unchanged over a 9-year period. In patients and siblings IgG, IgA and IgE increased by age ($p < 0.001$); which was in contrast to IgM ($p > 0.05$). The continued increase in IgG levels by age indicates that adult levels are reached later than in previously studied cohorts, thereby indicating a slower maturation of the immune system.

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1. Introduction

Although several studies have shown an inverse relationship between atopic diseases, allergic diseases or both and type 1 diabetes (T1D) [1–6], there has been comparable increase in both diseases. In Denmark, the incidence of T1D has increased by more than 3% annually over the last 10 years [7]. Both T1D and atopic diseases, allergic diseases or both are associated with Western lifestyles, i.e., there are higher incidences in developed countries compared with developing countries [8–11]. Both diseases are the result of a complex gene–environment interaction leading to an imbalance in the immune system, but the triggers or accelerators of autoimmune disease and atopy are largely unknown. The hygiene hypothesis has been proposed as an explanation for the increased incidences of both disease types [12–15]. This hypothesis asserts that different exposures (e.g., to helminths, bacteria and viruses) in early life educate the immune system, causing it to acquire a more robust anti-inflammatory regulatory network that provides pro-

tection against autoimmune diseases and allergies. Better hygiene standards, extended maternity leave, and less crowded housing in developed countries mean that the immune defense is not challenged sufficiently, which results in the adaptive immunity developing more slowly and subsequently leaving the immune system at a more vulnerable level related to age. This hypothesis is supported by animal studies in which the frequency of diabetes has been shown to increase in rodents raised in a pathogen-free environment [16,17]. However infections may act to trigger autoimmunity processes and to provide protection by maturation of the immune system [18,19].

Immunoglobulins are important to the humoral adaptive immune system and in the response against infections. Previously, it has been demonstrated that adult patients with T1D diagnosed before the age of 40 years had higher plasma levels of immunoglobulin (Ig), specifically IgM and IgA, and a lower level of IgG compared with siblings and healthy controls [20]. However no Ig studies have been carried out based on children and adolescents aged 0 to 18 years or that examine changes over time following the clinical diagnosis of T1D.

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The population-based Danish National Childhood Diabetes Register and biobank (DIA-REG B&U) provides us with a unique opportunity to follow Ig levels in newly diagnosed patients over a 9-year period and to test the hypothesis that the increase in T1D is related to the immune system being triggered less early in life and subsequently leading to slower maturation of the immune system, as assessed by a change in age-specific levels of the different classes of Ig over time.

2. Subjects and methods

Data for the study were derived from a large population-based register of diabetic children and adolescents with an associated biobank. Established in 1996, the DIA-REG B&U currently contains data for more than 2200 children and adolescents diagnosed with diabetes between 0 and 18 years of age. The classification (T1D) is based on clinical phenotype; very few cases have C-peptide measurements. The biobank contains blood and serum samples from approximately 75% of all patients and their first-degree relatives. For this study, a random sample of 482 patients representing the entire period of the register was selected for which blood sampling had been performed less than 3 months after diagnosis. For comparison, 478 siblings less than 18 years of age at sampling were included. In all, 90% of the blood samples from the same family were taken less than 1 month apart. Serum samples were stored at -80°C .

2.1. Study population

Data were obtained from 482 patients, of whom 255 (53%) were male and 227 (47%) were female. Among the 479 siblings included in this study, 266 (56%) were male and 212 (44%) female. The age range was from 0 to 18 years. Table 1 shows the case-sib pairs. Of the 482 patients, 18 were immigrants, 451 were of Danish origin, and 13 had unreported ethnicity. The ethnicity of siblings was not reported, but because only siblings with the same parents as the patient were included, the distribution of ethnicity was comparable among patients and siblings.

2.2. Biomarkers

The Ig were measured using rate nephelometry on IMMAGE (Beckman Coulter, Fullerton, CA). The normal ranges of the classes were defined by Cejka et al. [21]. The upper normal range for IgE was 165.3 IU/ml (95% CI) for all ages according to the supplier. The sensitivity of levels above 165.3 U/ml is approximately 30% and the specificity approximately 90% for capturing an individual with atopy [22]. The detection limit of the IgE assay was 5 IU/ml; the detection limit for IgA measurements was 0.07 g/l, for IgG 0.3 g/l, and for IgM 0.04 g/l. The performance limits of the assays were 5.5% for IgA, 6.5% for IgG, and 8.7% for IgM. There were nine individuals with IgA and 80 (8.3%) with IgE below the detection limits; none of them had IgG or IgM below the detection limits.

Table 1
Patients and siblings in the study

	Siblings		Patients		Patients and siblings	
	P-S	N	P-S	n	P-S	n
	0-1	150	1-0	276	1-1	178
	0-2	38	2-0	1	1-2	22
	0-3	6			1-3	3
	0-4	1				
Families		195		277		203
Children		248		278		434

The children in the study come from families with at least one child diagnosed with type 1 diabetes before the age of 15 years. Only those patients with blood samples taken less than 3 months after diagnosis are included. The third column shows the families in which cases and siblings come from the same family.

Table 2
Immunoglobulin (Ig) levels and normal ranges

Immunoglobulin	Subject status	Below 95% range n (%)	Normal n (%)	Above 95% range n (%)
IgE	Patient	NA	386 (80.1)	96 (19.9)
	Sibling	NA	396 (83.0)	81 (17.0)
IgG	Patient	9 (1.9)	452 (93.8)	21 (4.4)
	Sibling	2 (0.4)	448 (93.9)	27 (5.7)
IgM	Patient	31 (6.4)	448 (93.0)	3 (0.6)
	Sibling	23 (4.8)	451 (94.6)	3 (0.6)
IgA	Patient	16 (3.3)	390 (80.9)	76 (15.8)
	Sibling	12 (2.5)	417 (87.4)	48 (10.1)

The table shows the number and percentage of patients and siblings with values inside and outside the age-specific normal range as stated by Cejka et al. [21]. Levels of IgE below limits are not applicable because the detection limits for IgE were above the lower limit.

Autoantibodies against glutamic acid decarboxylase (GAD65A) and insulinoma-associated antigen-2A (IA-2A) were measured in standard radioligand binding assays [23]. Antibody-bound was separated from free labeled antigen by Protein A Sepharose and the unbound antigen was removed by extensive washing. The 97.5% cut-off limits for GAD65A and IA2A were 31 and 5 U/ml, respectively.

2.3. Statistical methods

Measurements of Ig were log transformed before analysis to meet the assumption of the statistical model (normally distributed residuals); therefore relative changes, instead of absolute differences, are reported. Hence overall means are reported as geometric means, and regression results are reported as relative differences. IgE had several values and IgA had few values below the detection limit, for which a value corresponding to 50% of the detection limit was chosen. An alternative way of handling values below the detection limit is to select a certain value as the separator (e.g., the median) and to handle the biomarker as a dichotome variable. In this type of modeling the assumption is that the values above the cut-point are equal. Since the distribution of the biomarkers in this study is very wide, values 10 to 1,000 times higher would have had to be classified as the same. As a result, we chose to use log transformation and fixed values for the biomarkers below the detection limit, because we believe that the validity is higher and that the use of the data is better.

Log(Ig) levels were modeled based on a linear mixed model, including a random effect and correlation within families. We adjusted for the effects of gender, age at sampling, autoantibody status, season, and date of sampling (approximately the date of diagnosis for patients). We used two models. Model 1 analyzes the effect of the different variables on log(Ig), including patients status and correlation within families. Model 2 analyzes the effects of the different variables in separate models for patients and siblings, respectively.

Model 1: (families)

$\log(\text{Ig}) = \text{patients status} + \text{dates of sample} + \text{gender} + \text{auto} + \text{age}$
 $+ \text{age} * \text{age} + \text{season} + \text{fam-id}$

Model 2: (cases and sibs in separate models)

$\log(\text{Ig}) = \text{dates of sample} + \text{gender} + \text{auto} + \text{age} + \text{age} * \text{age}$
 $+ \text{season} \log(\text{Ig}) = \text{dates of sample} + \text{gender} + \text{auto} + \text{age}$
 $+ \text{age} * \text{age} + \text{season}$

Where the "date of sample" is the same as the blood sample date, auto is positive or negative for the autoantibodies GAD or IA2A. Age and sampling date are in the model as continuous variables, whereas gender and cases status are character variables. There was a nonlinear association with age, therefore age is reported as the estimated change by age and age*age. The ratios displayed in each

figure represent the adjusted ratio, *e.g.*, estimates from the above models. A given estimate for, *e.g.*, sample date is the difference between children with the same age and gender.

Results with *p* values <0.05 were considered statistically significant. No adjustments for multiple testing were applied but all analyses are reported to allow the reader to make any desired formal adjustments.

3. Results

3.1. Distribution of Ig levels

The IgE and IgA levels were above the normal range in more than 10% of this young population, and the IgG levels were above the normal range in at least 4%, whereas the IgM levels were below normal range in at least 5% of both patients and siblings (Table 2). The percentage of children with high levels of IgE varied from 12% in 2002 to 32% in 2001, with no clear trend indicating whether the levels were increasing or decreasing over the years.

3.2. Ig concentrations

For patients and siblings, the median IgE levels were, respectively 36.0 and 39.4 IU/ml and the IgM levels were 0.97 g/l and 0.94 g/l (Fig. 1). The patients had lower levels of IgG than the siblings (median 10.2 g/l vs 10.9 g/l), and the estimated ratio (siblings vs patients) from the adjusted model for cases was 1.09 (95% confidence interval [CI] = 1.04, 1.14; *p* < 0.001). The patients had higher levels of IgA (median levels 1.45 g/l vs 1.26 g/l; *p* < 0.01), when adjusted for age, gender, sample date and autoantibodies; the adjusted ratio (siblings vs patients) was 0.88 (95% CI = 0.77; 0.99; *p* = 0.045) (Fig. 2).

3.3. Changes in Ig concentrations with age

The four Ig classes increased significantly with age in both T1D and in the siblings except for the IgE in siblings, which showed a large variation (Fig. 3). The increase was tested to be nonlinear for IgA, IgE, and IgG and tended to continue after the age of 10 as illustrated in Fig 2 where the estimated increase in Ig by age and by age*age are reported. There was a nonsignificant increase for IgM after the age of 10

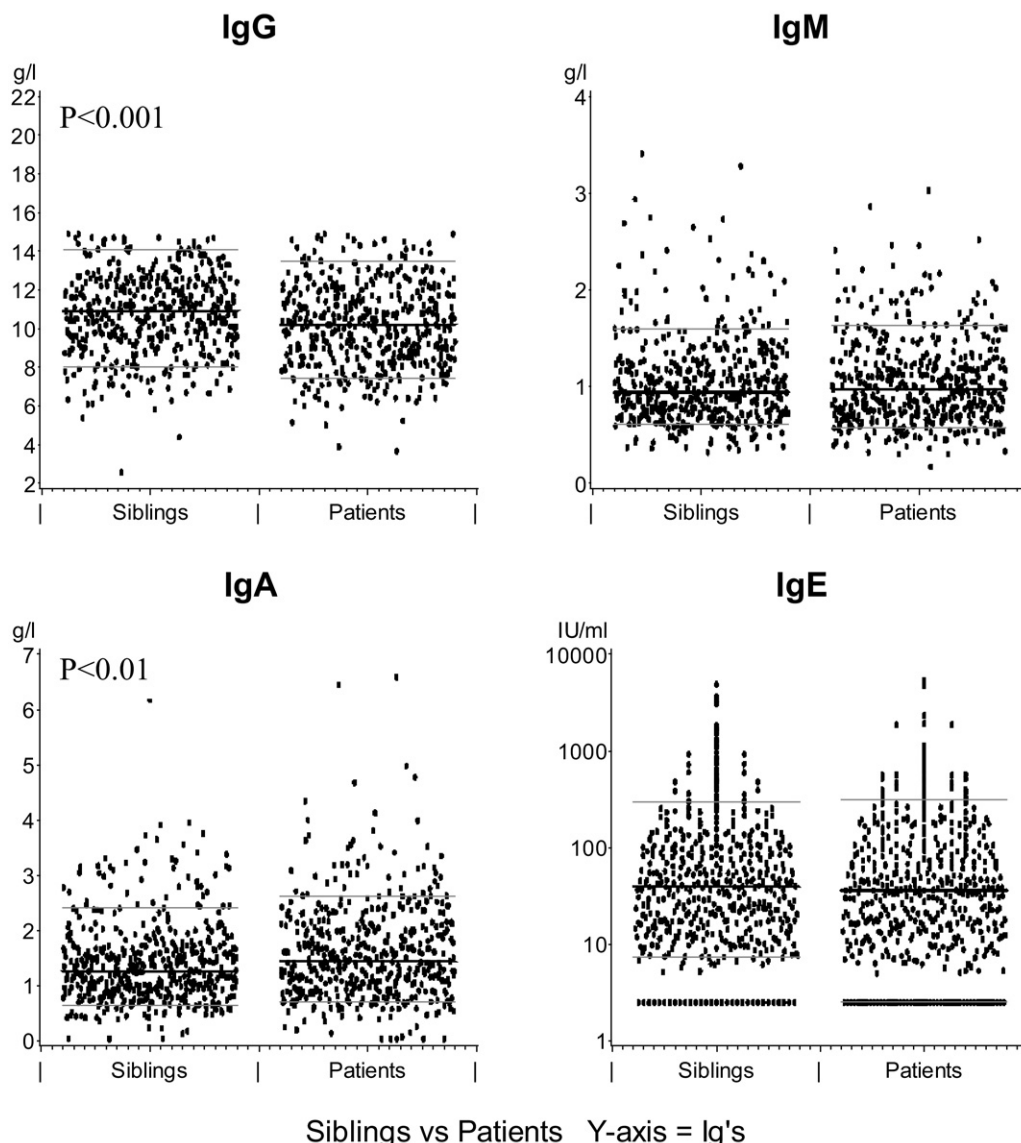


Fig. 1. The different immunoglobulin levels in siblings and patients. The thick line represents the median, the two thin lines are the 10th and 90th percentiles. The *p* value is from the univariate model without adjustment for age, gender, season, auto antibody status and period. The plot for IgE uses a log-scale because of the distribution; the scale used for the remaining Igs is an ordinary scale.

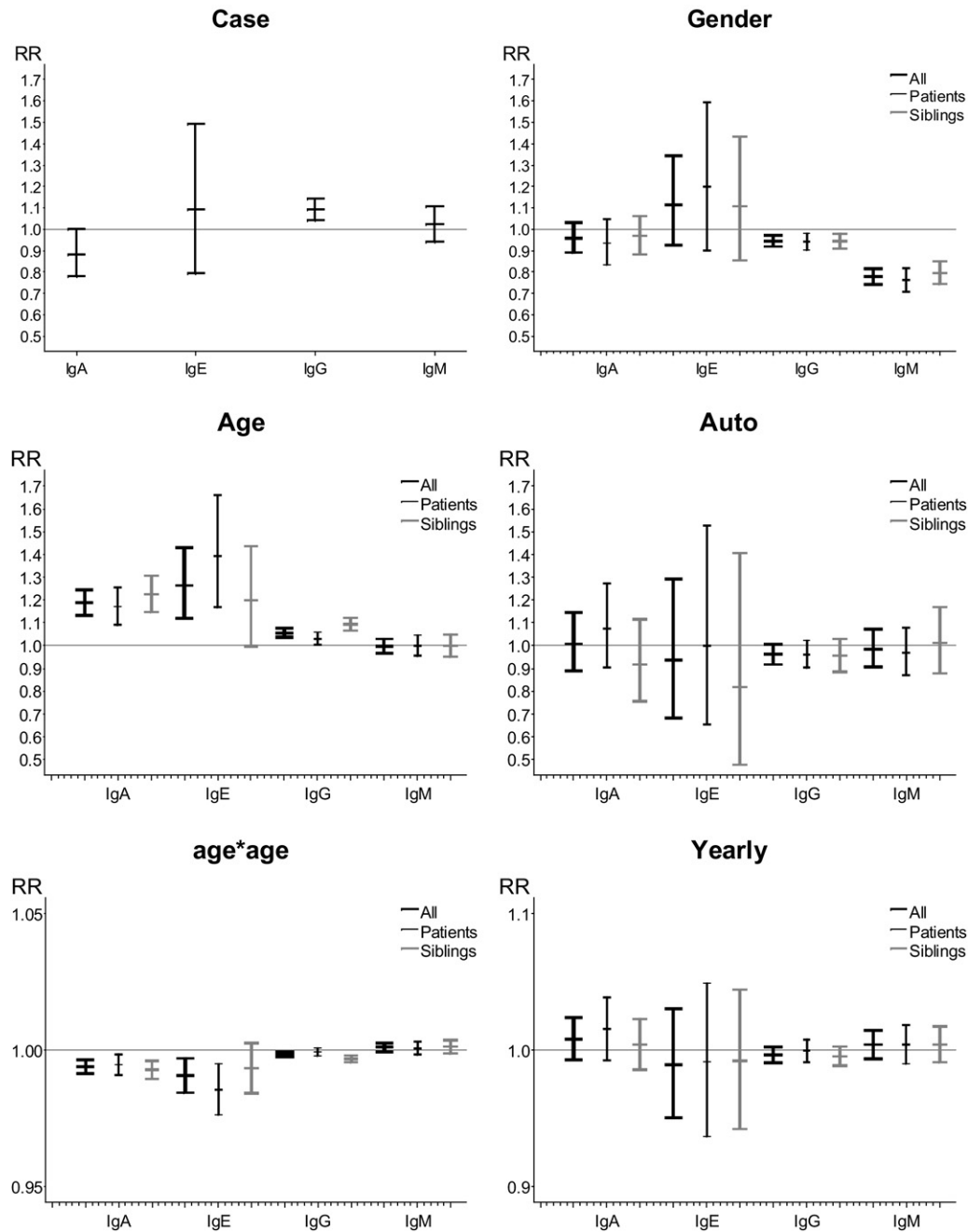


Fig. 2. Estimates of effects on the four different immunoglobulins. Thin lines refer to estimates and to 95% confidence intervals; bold black lines refer to a model with correlation within families; thick gray lines are for sibs and thin black lines for patients. Data were log transformed before analysis so the effects are relative effects. For example, boys have about 20% lower IgM levels than girls (the relative effect is 0.78 (CI95% 0.74; 0.82)). The age effect is non-linear so the figure shows the $\exp(\beta\text{-estimate})$ for age and age*age separately. The scale for the age*age and the yearly change is different from those listed above.

for patients and siblings, whereas the increase seemed to level off for IgA and IgE in patients and siblings and for IgG in siblings.

3.4. Changes in Ig with calendar time and onset of treatment

None of the Ig showed a significant change from 1997 to 2005 when age, patient status, and gender were taken into account (Fig. 4). Only the IgA levels decreased significantly within the first 3 months after initiation of insulin ($p = 0.015$).

3.5. Gender differences

Females showed significantly higher levels of IgG and IgM compared with males in patients and siblings (Fig. 5). None of the Ig

classes differed between T1D for children of Danish origin and children of foreign origin (data not shown).

3.6. Autoantibodies

None of the Ig differed significantly between those with positive autoantibodies (glutamic acid decarboxylase [GAD65] and insulinoma associated antigen-2A [IA-2A]) compared with those negative when age, gender and patient status were taken into account (Fig. 3).

3.7. Correlation between the Ig

The different classes of Ig were almost all significantly pairwise correlated, but the correlations were all weak and less than 0.4

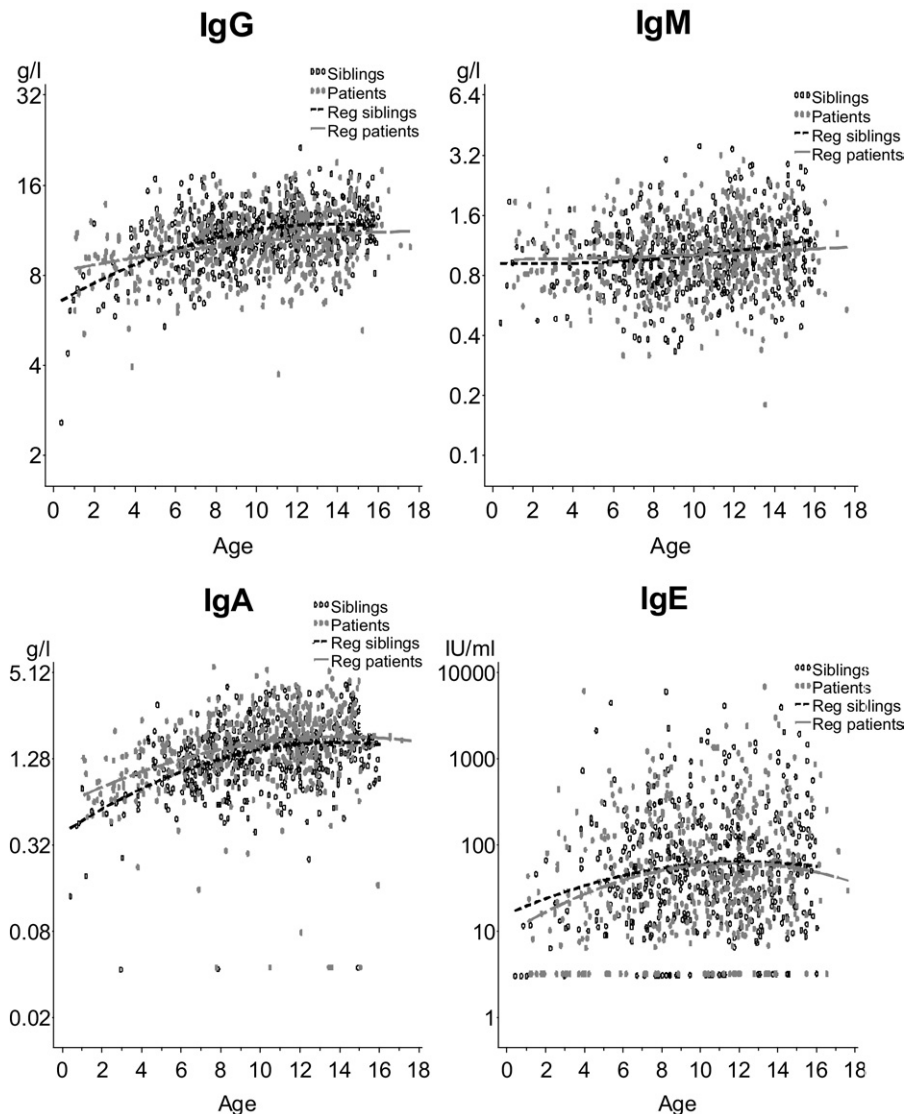


Fig. 3. This figure shows the change by age for the four different immunoglobulins. The y-axis for all four immunoglobulins is logarithmic. The regression line represents the quadratic curve for the increase by age. The β -coefficient for age*age is negative for IgG, IgA and IgE indicating a continuous but less steep increase after the age of ten except for IgM in patients, where there is no levelling off.

regardless of scale chosen for the calculation (Fig. 6). The correlation within family from the multiregression model was IgG = 0.48, IgM = 0.44, IgA = 0.31, and IgE = 0.38.

4. Discussion

This is the first study that examines Ig and their correlation to autoantibodies over a 9-year period that demonstrates a trend showing an increase in diabetes. The main findings were the absence of change in age-specific Ig levels over time for a cohort of patients and siblings comprised of children aged 0–18 years, but a significant increase in Ig levels with age in patients even after the age of 10, indicating that their adult Ig levels are reached later in life and may be interpreted as a slower maturation of the immune system.

4.1. IgE

We found more children (both patients and siblings) with IgE levels above the normal range, contrary to what would be expected if an inverse relationship existed between diabetes and allergic diseases. The relatively high levels of IgE may play a role in the autoimmune process, as IgE might affect mast cell survival and

activation [24]. Mast cells may play a role in the autoimmune process [25].

High IgE levels in a diabetic risk population have been found by other researchers. In children up to the age of 5 years, Ziegler et al. [26] found no inverse relationship between total IgE and islet autoimmunity. In 13% of islet autoantibody positive children IgE levels were above 150 kIU/l, compared with only 4% in the autoantibody negative group. A German study of latex allergy in T1D individuals concluded that there was no sign of an inverse relationship between diabetes and atopy, with a relatively high proportion of children with atopy in this diabetic population. Total IgE ranged from 2 to 1000 kIU/l, with the highest levels found in children with atopy [27]. In a study examining healthy adults total IgE levels were associated with the IL-13 gene variant (IL-13 plays a crucial role in the Th2 cytokine mediated immune response), but failed to find any association with T1D genes, such as CTLA4, PTPN22, and IL2RA [28]. Maier et al. make an apparently unsubstantiated assumption that there is an inverse relationship between IgE levels and the risk of diabetes.

Contrary to these studies Hopppu et al. found higher titers of IgE-IA-2 (a protein-tyrosine-phosphatase like protein) in the sib-

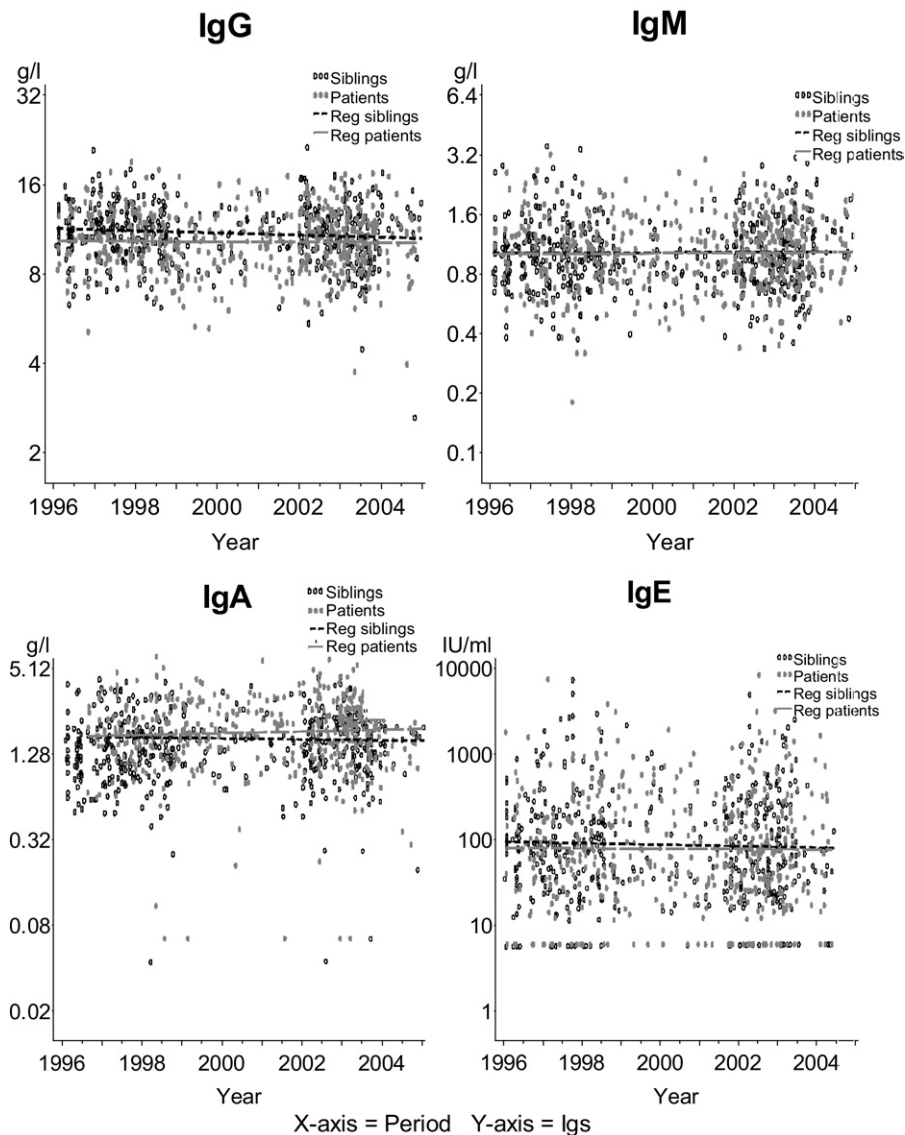


Fig. 4. The immunoglobulin levels in newly diagnosed patients and healthy siblings over nine years. The immunoglobulins did not show a tendency to increase or decrease over time or variation by seasonality.

lings who did not progress to diabetes, but they could not demonstrate differences in IgE-GAD65 (65 kDa isoform of glutamic acid decarboxylase) [29–31].

The high IgE levels in patients and siblings do not support the hygiene hypothesis. Studies in rodents have shown higher levels of IgE under unhygienic conditions [32]. Studies in humans comparing the developing area of Karelian with the rest of Finland found a higher total IgE in the Karelian population compared with the Finnish population [33]. In the Finnish population 26% had IgE levels above 100 IU/ml against 40% of the Karelians, indicating that the 17 to 20% of the population with IgE levels above 165.3 IU/l in our study may not be an uncommon finding.

The IgE level tended to increase with age, but only in patients. Other studies have observed an increase with age, but a levelling off at the age of 10 years when it reaches adult levels [34]. The fact that patients, contrary to their siblings, show an increase by age may indicate a slower maturation of the immune system in patients, but overall we could not demonstrate different levels of IgE in patients and siblings, and no signs of a decrease in IgE over the 9 years that would support the hypothesis. The lack of differences between patients and siblings may indicate that IgE levels probably are more

dependent on genetic background [35]. To explore the role of IgE further, documentation on levels in the background population is needed.

4.2. IgA

IgA is the main isotype found in sero mucous secretions. We found higher levels of IgA in patients compared with siblings, which is similar to the findings of other researchers [20]. Other studies of IgA in T1D have shown higher levels of bovine specific IgA, but not ovalbumin in children newly diagnosed with T1D compared with healthy controls, as well as higher levels of IgG against ovalbumin in controls, which indicates that the relationship is not straightforward. The switch to IgA production by plasma cells is driven by transforming growth factor (TGF)- β [36]. The higher IgA levels in patients probably reflects the stimulation by TGF- β , in line with our previous finding of higher levels of TGF- β in patients compared with siblings (unpublished results). The decrease in IgA levels with diabetes duration and the positive correlation with IgG and IgM in patients and siblings indicate that IgA levels are related to the inflammatory status of the immune system.

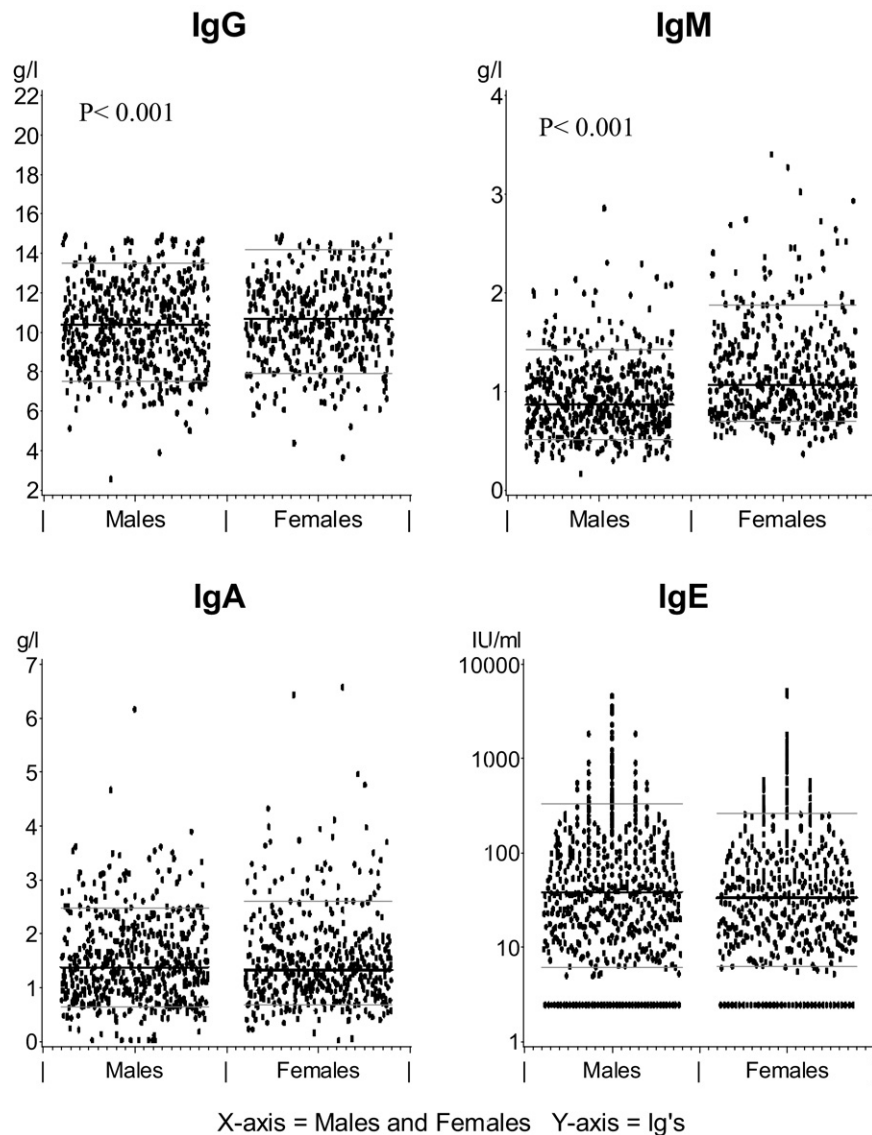


Fig. 5. The different immunoglobulins in males and females. The thick line represents the median; the two thin lines are the 10th and 90th percentiles. An ordinary scale is used for IgG, IgM and IgA, while a logarithmic one is used for IgE. The *p*-value is from the multiple regression model, with adjustment for patient status, age, period, season and autoantibody status.

The increase of IgA with age is in accordance with previous studies [21,37] showing that IgA levels increase with maturation of the immune system; as we could not confirm a decline over time in IgA levels, we were not able to support this hypothesis.

4.3. IgM

We found that more patients and siblings than expected had total IgM levels below normal range when compared with the reference values (0.6% above upper limit and more than 5% below). IgM is the first Ig produced by B cells in an immune response before switching to other isotypes. T-independent responses where IgM remains predominating do not show this switch [36].

IgM did not differ between patients and siblings, even though the patients were diagnosed with diabetes 3 months before the sampling. In addition the IgM levels increased insignificantly with diabetes duration.

A higher risk of diabetes in individuals with low IgM is supported by the Childhood Diabetes in Finland study (DiMe), which is based on only seven children who progressed to diabetes and six low risk siblings. The study found low IgM-GAD65 levels in pro-

gressors and T1D compared with high levels in low risk individuals [38]. Our results are in opposition to Gorus' [20], where IgM levels were higher in patients compared with siblings and controls, with siblings showing levels in-between; however, this was only the case in individuals over 20 years of age. These differences can be explained by differences in age groups and the period of the study. We found a nonsignificant tendency towards an increase after the age of 10, whereas in older studies IgM seems to level off at the age of 10 years [37,39]. The question is whether or not our patients actually expressed lower levels of IgM before the inflammatory response, but due to the activation of the immune system, reached the same levels of IgM as their healthy siblings.

The higher levels of IgM in females found in our study are in accordance with other research [39], thus highlighting the importance of taking gender into consideration when studying the immune system [40].

4.4. IgG

The IgG level was 9% lower in patients compared with siblings. The switch in B cells from IgM to IgG production is the second

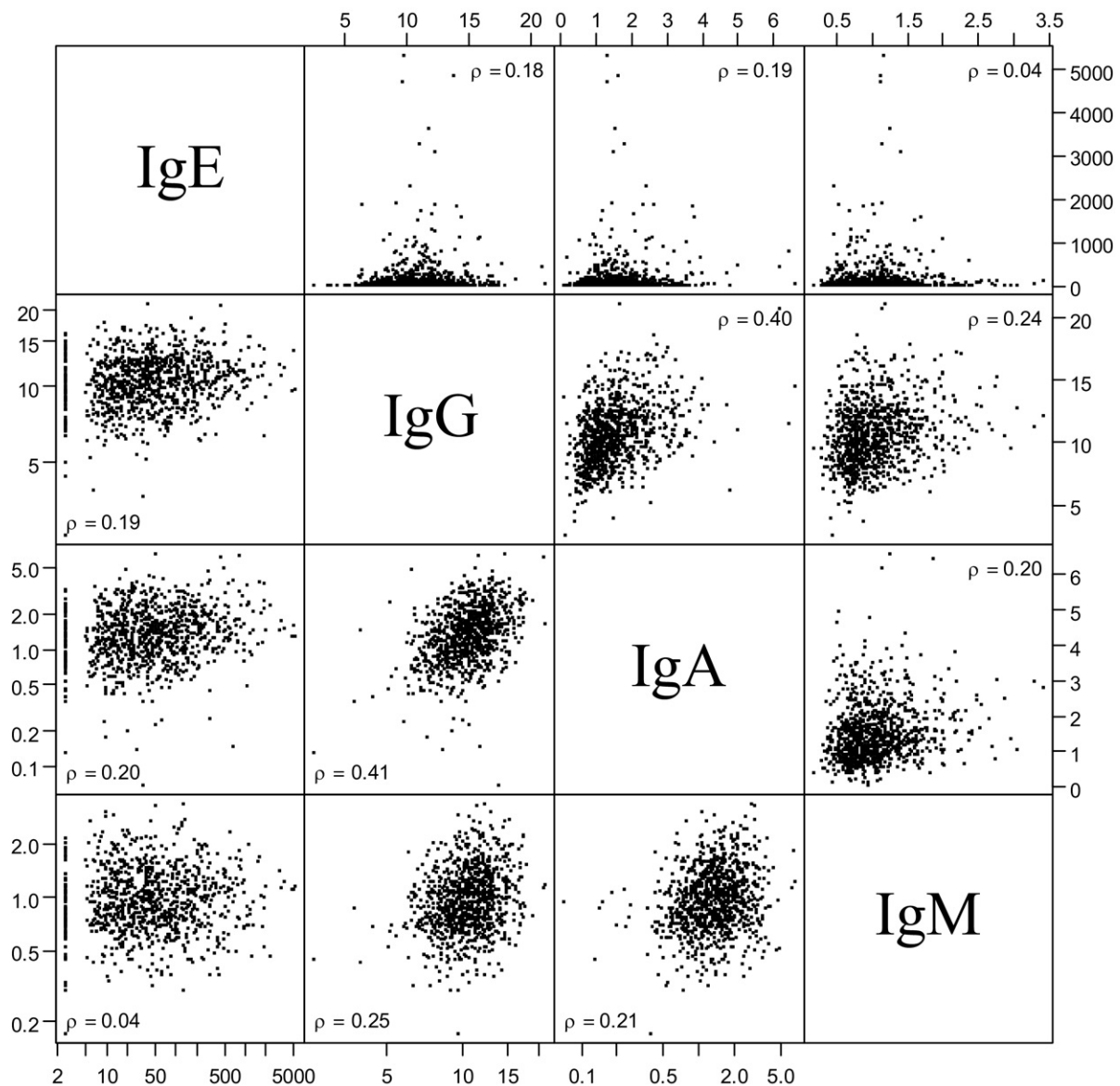


Fig. 6. The correlation between the four classes of immunoglobulins. The "upper-right triangle" shows the correlation between the absolute measures, whereas the "lower-left triangle" shows the correlation between the logarithm transformed values. The correlation, ρ , is shown in each plot for the untransformed data it is the Spearman correlation; for the transformed data it is the Pearson correlation. There was a significant positive correlation between the immunoglobulins ($p < 0.001$) with the exception of IgM: IgE ($p = 0.18$).

response of the adaptive immune system. The low level of IgG found in patients in this study is in accordance with Gorus et al. [20]. These results are in line with the hygiene hypothesis since high levels of IgG are found in animals living in their natural environment (unhygienic conditions) compared with laboratory-bred rodents [32].

We found that females have higher levels of IgG than males amongst both patients and siblings. IgG is associated with Th1-type immunity and this observation supports previous findings that females tend to be more Th-1 oriented than males [40]. Our findings are in contrast to Ritchie et al. [39], who found that the IgG levels of males and females were comparable. This discrepancy can perhaps be explained by the fact that our study population was a selected group of T1D related genotypes.

As shown in other studies, we corroborate that IgG increased significantly with age [21,37,39]. The increase with age was most prominent in patients with no leveling off as seen in siblings, supporting a slower maturation in patients; however, the role of

IgG levels in the rising epidemic could not be confirmed by a decrease in levels over time.

In this study the total Ig levels are unspecific. Consequently, one option for following up on this study would be to measure virus-specific antibodies.

In conclusion, this study provides some support for a delayed maturation of the immune system by demonstrating low levels of IgG in patients, a delayed increase in IgG, with age, and a lower level than expected of IgM. However we cannot confirm a decrease in immunoglobulin levels over time, to support that Ig levels play a role in the increase in T1D in children. The high level of IgE also conflicts with the hygiene hypothesis.

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