Increased inhibitor incidence in severe haemophilia A since 1990 attributable to more low titre inhibitors

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Summary

Many studies have reported an increased incidence of inhibitors in previously untreated patients (PUPs) with severe haemophilia A after the introduction of recombinant products. It was the objective of this study to investigate whether the inhibitor incidence has increased between 1990 and 2009 in an unselected cohort of PUPs with severe haemophilia A (FVIII< 1 %). Patients were consecutively recruited from 31 haemophilia treatment centres in 16 countries and followed until 50 exposure days or until inhibitor development. Inhibitor development was studied in five-year birth cohorts comparing cumulative incidences. Furthermore the risk for inhibitor development per five-year birth cohort was studied using multivariable Cox regression, adjusting for potential genetic and treatment-related confounders. A total of 926 PUPs were included with a total cumulative inhibitor incidence of 27.5 %. The inhibitor incidence increased from 19.5 % in 1990–1994 (lowest) to 30.9 % in 2000–2004 (highest; p-value 0.011). Low titre inhibitor incidence increased from 3.1 % in 1990–1994 to 10.5 % in 2005–2009 (p-value 0.009). High titre inhibitor incidences remained stable over time. After 2000, risk of all inhibitor development was increased with adjusted hazard ratios 1.96 (95 % CI 1.06–2.83) in 2000–2004 and 2.34 (1.42–4.92) in 2005–2009. Screening for inhibitors was intensified over this 20-year study period from a median of 1.9 to 2.9 tests/year before 2000 to 2.7 to 4.3 tests/year after 2000. In conclusion, the cumulative inhibitor incidence has significantly increased between 1990 and 2009. The high titre inhibitor incidence has remained stable.

Keywords

Risk factors, haemophilia A / B, factor VIII inhibitors, epidemiological studies

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Introduction

Haemophilia is a rare coagulation disorder, which occurs in 1:10.000 newborns. Without treatment, patients suffer from frequent bleeds, principally in muscles and joints (1). Primary prophylaxis is the preferred treatment and should be started early in patients with severe haemophilia in order to prevent joint bleeding and joint disease (2). Presently, inhibitor development is the most serious side effect of treatment, occurring in 25–32 % of all patients with severe haemophilia A (3–6). Because inhibitors develop mostly within the first 50 exposure days, inhibitors are diagnosed at an early age.

While patients on prophylaxis who are adherent to the treatment have very few bleeds and an almost normal life expectancy, inhibitor patients often have large bleeds which are difficult to treat with very costly bypassing agents (7). The development of an inhibitor has a large impact on the patient and his family. This provides strong motivation for studies on risk factors for inhibitor development and strategies to potentially reduce the risk of inhibitor development (6–8).

In particular single centre studies from the 1980s reported very conflicting results, which were often attributed to the specific concentrate involved (9–13). From the 1990s recombinant concentrates introduced and it was reported that they cause more in-

hibitors than plasma-derived factor concentrates (14–16). In the same period treatment practices has changed. Before the adoption of primary prophylaxis it could take many years until patients reached 50 exposure days (EDs) and the diagnosis of an inhibitor was primarily a clinical diagnosis based on the observation of increased bleeding and reduced responsiveness to the treatment. Nowadays frequent testing in the first 50 EDs is common practice and has had potentially an effect on the overall detection of inhibitors.

A significant limitation in comparing inhibitor incidences between studies is the limited number of study subjects, which hampered the ability to adjust for confounding factors (6, 18).

The aim of the present study is to report the cumulative incidence of low and high titre inhibitors adjusted for genetic and non-genetic risk factors over a 20-year period in a large, well defined cohort of children with severe haemophilia A.

Methods

Patients

Previously untreated patients (PUPs) were consecutively recruited from 14 haemophilia treatment centres (HTCs) in the period between 1990 and 2000 (CANAL Study) and from 29 HTCs in the period between 2000 and 2009 (PedNet Registry) (5, 19). The PedNet study group has collected data from a total of 31 haemophilia treatment centres (HTCs) from 16 countries, of which 12 centres participated in both studies (see Appendix) (19).

For this study only patients with FVIII activity < 0.01 IU/ml were included and follow-up data until 50 exposure days (EDs) were used. Patients who were referred to the participating centres because of the presence of an inhibitor were excluded to avoid selection bias.

Approval was obtained from each centre's institutional review board. Written informed consent was obtained from the parents or guardians of all participants.

Data collection

For all PUPs, detailed data on disease and treatment characteristics were collected from the medical files through similar case report forms (CRFs), and included reason for treatment, types of bleeding, surgical episodes and administrations of FVIII including doses and product brands.

For patients who ever had a positive inhibitor titre, details on all inhibitor tests and recovery measurements (in case of borderline positive inhibitor tests) were collected. All laboratory testing was done in the local laboratory of each participating centre and then the results were sent to the central study staff for classification according to the definition of a clinically relevant inhibitor, performed by two independent investigators.

Outcomes

Patients were followed until the development of a clinically relevant inhibitor or a cumulative number of 50 EDs to FVIII. Clinically relevant inhibitor development was determined as at least two positive inhibitor titres and a decreased FVIII recovery (< 66 %) (19). Positive inhibitor titres were defined according to the cut-off levels of local laboratories. Almost all laboratories used the Nijmegen modification of the Bethesda assay after 2000 with cut-off values between 0.3 and 0.6 BU. High titre inhibitor development was defined as a peak inhibitor titre of ≥ 5 BU.

Inhibitor-testing

Inhibitor-testing rates were defined as the number of inhibitor tests performed in 50 EDs in non-inhibitor patients, starting from ED1. To evaluate across birth cohorts the number of tests per year was calculated.

Data on inhibitor-testing rates were collected from 319 non-inhibitor patients: In birth cohort 1990–1999 we had data on 181 non-inhibitors, distributed across all centres (73.8 % of all non-inhibitors). In birth cohort 2000–2009 we randomly selected 20 % of non-inhibitors (n=65) from the nine largest HTCs of the PedNet Registry. Furthermore a random selection of eight of the 19 smaller HTCs (42 %) provided data on the number of tests for all their non-inhibitor patients (n=73). Since the data for the largest HTCs were randomly collected to represent all non-inhibitors from these centres, we calculated a weighted average for inhibitor-testing rate.

Potential confounding factors

- *Ethnicity* was categorised into Caucasian or non-Caucasian ethnicities. This was self-reported by the parents or guardians of the children.
- *Family history* for inhibitors was defined as positive if present in any first, second or third degree relatives as assessed at time of diagnosis or initial treatment.
- *F8 gene mutation type* was defined as either large mutations (large deletions of > 200 base pairs missing, nonsense mutations and intron 1 and/or 22 inversions) or small mutations (missense mutations, small deletions of < 200 base pairs, insertions and splice-site defects). We opted for this general categorisation, because detailed information on the specific small deletions/insertions and splice-site defects (conserved and unconserved) was not available for the 1990–1999 cohort. Patients in which no genetic analysis was performed or no mutation was found were categorized as unknown. Genotyping results were provided by the centres and assessed centrally (20, 21).
- *Peak treatment at first exposure* was defined as receiving FVIII for at least five consecutive days from the first exposure day onwards.
- *Dose during first 5 exposure days* was defined as the mean dose of FVIII in IU/kg received during the first five exposure days.

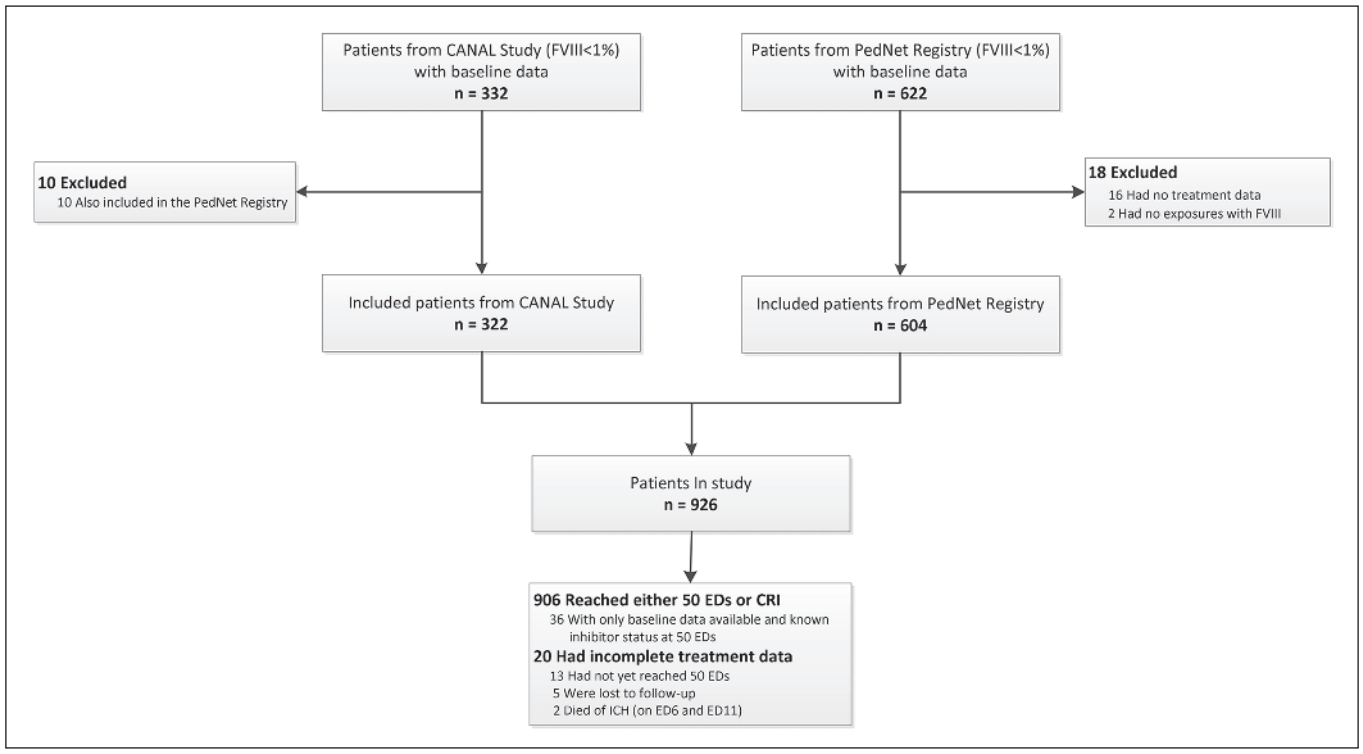

Figure 1: Enrollment of patients from two separate cohorts. ED, exposure days; CRI, clinically relevant inhibitor; ICH, intracranial haemorrhage.

- *Prophylaxis* was defined as regular administration of FVIII with the aim to prevent bleeding on at least three exposure days within 14 calendar days, excluding follow-up treatment for bleeds or surgeries, started before the 50th exposure day (3).

Statistical analyses

To investigate a trend over time in the incidence of clinically relevant inhibitor development, four five-year birth cohorts were created: patients born between 1990–1994 (Period 1), patients born between 1995–1999 (Period 2), patients born between 2000–2004 (Period 3) and patients born between 2005–2009 (Period 4). Cumulative inhibitor incidences were calculated for these four five-year birth cohorts taking into account the time to inhibitor development. Comparisons in the cumulative incidences of all, high titre and low titre inhibitor development between the birth cohorts were performed using time-stratified Cochran-Mantel-Haenszel tests (log-rank tests) with p-values <0.05 considered significant (22). Patients who had not yet reached their 50th ED were included in the analysis and censored at the time of their last exposure day.

Multivariable Cox regression was used to assess the risk of inhibitor development per birth cohort with Period 1 (1990–1994) as the reference birth cohort and reaching 50 EDs or inhibitor development as the time variable. Hazard ratios were adjusted for inhibitor testing rate (mean testing rate per period), non-Caucasian ethnicity, positive family history of inhibitors, large *F8* gene mutation type, ≥5 EDs peak treatment at first exposure, mean dose (IU/kg) during first five EDs, and start and exposure day of regular prophylaxis (before the 50th ED).

Missing values were imputed using multiple imputation (23). Family history of inhibitors: n=74 (8.0%), F8 gene mutation type: n=93 (10.0%), Peak treatment at first exposure: n=2 (0.2%), Dose during first exposure: n=40 (4.3%), and Prophylaxis before 50th ED: n=48 (5.2%). Statistical analyses were performed using IMB SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

Results

A flow chart for enrollment and exclusion of patients from the two cohorts for the current study is shown in ▶Figure 1.

In total 960 patients with severe haemophilia A were eligible for inclusion from both cohorts (CANAL; n=332 and PedNet; n=628). Of these 28 patients (3.5%) were excluded: 10 because they were included in both cohorts, 22 were excluded because they did not have any treatment data available and two patients were excluded because they had not been exposed to FVIII. Finally, 926 patients with severe haemophilia A (FVIII activity <0.01 IU/ml) were included in this study. From these patients, 906 (98%) reached 50 exposure days or developed a clinically relevant inhibitor.

A total of 255 patients (cumulative incidence, 27.5%; 95% confidence interval [CI] 25.0–30.8) developed a clinically relevant inhibitor: high titre inhibitors in 188 (21.4%; 18.7–24.1) patients and low titre inhibitors in 67 (8.2%; 6.2–10.2). Period 1 (1990–1994) consisted of 144 patients of whom 28 (19.5%; 13.0–26.0) devel-

Table 1: Inhibitor characteristics of patients in the entire cohort and in four 5-year birth cohorts.

	Birth cohort 1990–1994 N = 144	Birth cohort 1995–1999 N = 178	Birth cohort 2000–2004 N = 299	Birth cohort 2005–2009 N = 305	Entire cohort 1990–2009 N = 926
Clinically relevant inhibitors					
Number of patients	28	49	92	86	255
% of patients in birth cohort	19.4	27.5	30.8	28.2	27.5
cumulative incidence (95% CI)	19.5 (13.0– 26.0)	27.6 (20.9 – 34.3)	30.9 (25.6 – 36.2)*	29.0 (23.9 – 34.1)*	27.9 (25.0 – 30.8)
ED at inhibitor development					
Median (IQR)	15 (10 – 25)	12 (8 – 20)	14 (9 – 22)	14 (9 – 17)	14 (9 – 19)
High titre inhibitors					
Number of patients	24	39	67	58	188
% of patients in birth cohort	16.7	21.9	22.4	19.0	20.3
cumulative incidence (95% CI)	16.9 (10.6 – 23.2)	22.7 (16.4 – 29.0)	23.5 (18.6 – 28.4)	20.5 (15.8 – 25.2)	21.4 (18.7 – 24.1)
Peak inhibitor titre, BU	34.5 (12.3 – 100.3)	30.5 (10.8 – 139.5)	84.5 (18.0 – 380.0)	52.5 (16 – 367.6)	52.0 (15.4 – 260.0)
Low titre inhibitors					
Number of patients	4	10	25	28	67
% of patients in birth cohort	2.8	5.6	8.4	9.2	7.2
cumulative incidence (95% CI)	3.1 (0.2 – 6.0)	6.3 (2.6 – 10.0)	9.6 (6.1 – 13.1)#	10.5 (6.8 – 14.2)#	8.2 (6.2 – 10.2)
Peak inhibitor titre, BU	2.6 (1.0 – 3.0)	2.3 (1.2 – 3.3)	2.2 (1.3 – 3.5)	2.0 (1.2 – 3.4)	2.2 (1.2 – 3.3)
Inhibitor testing rate$					
Tests/year, median (IQR)	1.9 (1.3 – 3.2)	2.9 (1.7 – 4.6)$	2.7 (1.7 – 5.6)$	4.3 (2.5 – 8.9)$	3.1 (1.9 – 5.9)
Tests/50 EDs, median (IQR)	3 (2 – 6)	5 (3 – 7) ¥	4 (2 – 7) ¥	5 (3 – 8) ¥	5 (3 – 7)

Chi-square test performed for categorical variables and independent samples t-tests performed for continuous variables. In all tests the birth cohort with the lowest value for the specific variable is the reference birth cohort. * Birth cohort 1990–1994 vs birth cohort 2000–2004: p-value 0.011, Birth cohort 1990–1994 vs birth cohort 2005–2009: p-value 0.026. # Birth cohort 1990–1994 vs birth cohort 2000–2004: p-value 0.020, Birth cohort 1990–1994 vs birth cohort 2005–2009: p-value 0.009. $ Birth cohort 1990–1994 vs birth cohort 1995–1999: p-value 0.001, Birth cohort 1990–1994 vs birth cohort 2000–2004: p-value 0.012, Birth cohort 1990–1994 vs birth cohort 2005–2009: p-value <0.001. ¥ Birth cohort 1990–1994 vs birth cohort 1995–1999: p-value 0.001, Birth cohort 1990–1994 vs birth cohort 2000–2004: p-value 0.004, Birth cohort 1990–1994 vs birth cohort 2005–2009: p-value <0.001.

oped an inhibitor; Period 2 (1995–1999) consisted of 178 patients of whom 49 (27.6 %; 20.9–34.3) developed an inhibitor; Period 3 (2000–2004) included 299 patients of whom 92 (30.9 %; 25.6–36.2) developed an inhibitor and Period 4 (2005–2009) included 305 patients of whom 86 (29.0 %; 23.9–34.1) developed an inhibitor. The difference in cumulative inhibitor incidence was the largest between Period 1 and Period 3 (P-value 0.011). An overview of patient characteristics and cumulative incidences of inhibitor development in the entire cohort and for each of the four five-year periods is shown in ▶Table 1.

Incidence of high and low titre inhibitors and inhibitor-testing rate

The cumulative incidence of high titre inhibitor development during the four time periods was: 16.7 %; 95 % CI 10.6–23.2 (Period 1); 22.7 % CI 16.4–29.0 (Period 2); 23.5 %; CI 18.6–28.4 (Period 3) and 20.5 %; CI 15.8–25.2 (Period 4: ▶Table 1). The cumulative high titre incidences in all the time periods were stable (all p-values > 0.05). High titre inhibitors accounted for the following proportion of all inhibitors diagnosed during the time periods: 85.7 % (Period 1); 79.6 % (Period 2); 72.8 % (Period 3) and 67.4 % (Period 4). The cumulative incidences of patients detected with a low titre inhibitor increased over time from 3.1 %; 0.2–6.0 (Period 1) to 10.5 %; 6.8–14.2 (Period 4; p-value 0.009: ▶Table 1).

The inhibitor-testing rate in the first 50 exposure days increased from a median 3 tests/50 EDs in Period 1 to 5 tests/50 EDs in Period 2, 4 tests/50 EDs in Period 3 and finally 5 tests/50 EDs in Period 4. When calculated as tests/year the inhibitor-testing rate increased from a median 1.9 tests/year in Period 1 to 2.9 tests/year (Period 2), 2.7 tests/year (Period 3) and finally 4.3 tests/year in Period 4 (▶Table 1). To demonstrate the impact of difference in treatment intensity over the four periods, the analysis was repeated in three groups; patients that reached 50 EDs in < 1 year (group 1; N=103), between 1–2 years (group 2; N=100) and > 3 years (group 3; N=106: ▶Figure 2). The median number of tests varied from 2-6 for all patients through all periods. No clear association of treatment intensity with inhibitor testing over time was observed, except for a trend towards more frequent testing in patients reaching 50 EDs in > 3 years (▶Figure 2).

The distribution of genetic and treatment-related possible confounding factors is shown in ▶Table 2. *F8* gene mutations were available for 90 % of the whole study population (833 patients). Large mutations (large deletions, inversions and nonsense mutations) were present in 60 % of the patients, and proportions were

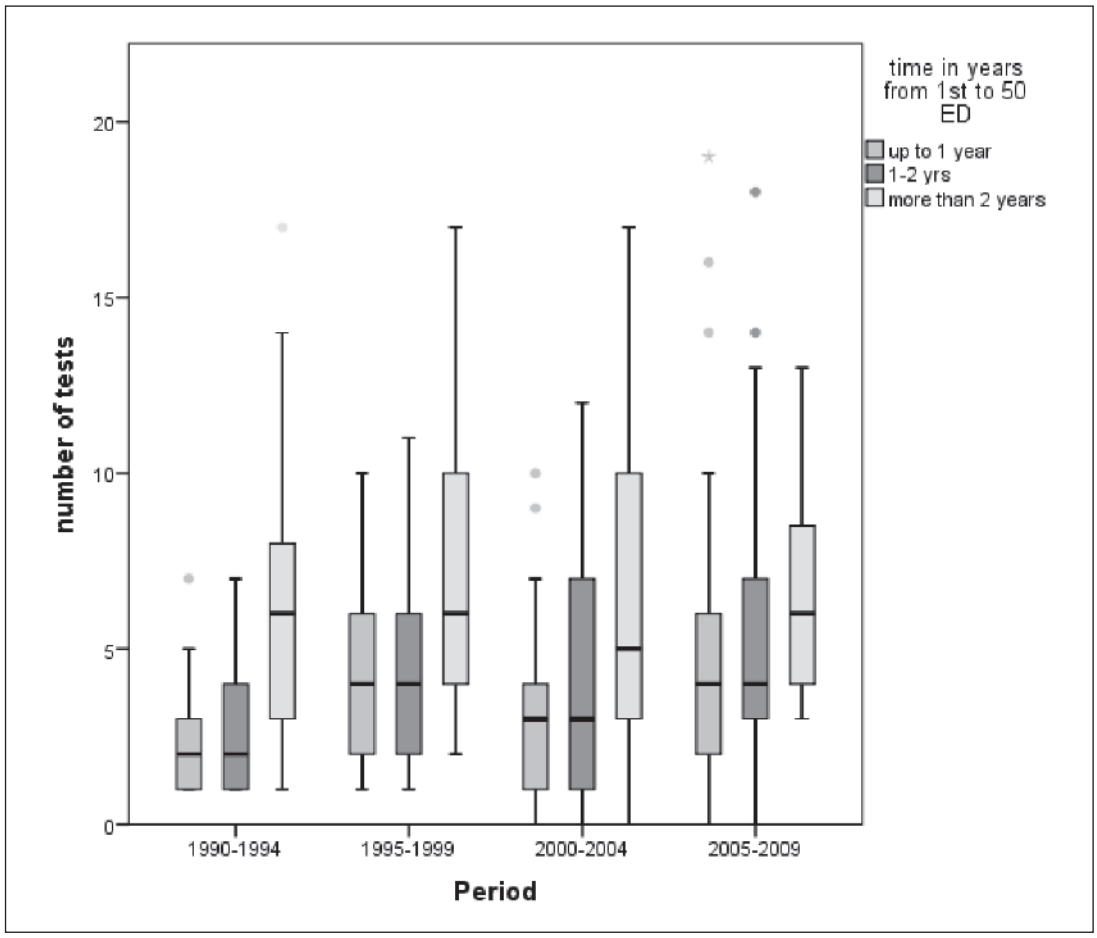

Figure 2: Comparison of number of tests in patients that reached 50 exposure days in 1 year, 1–2 years or more than 2 years. No significant difference found (p-values>0.05) between the three groups in all periods.

similar across the five-year periods (lowest; 59.0 %, highest; 64.9 %: ►Table 2).

In 887 patients (95.8 %) the FVIII product at first exposure was known. In total 25.6 % used a plasma-derived FVIII product as a first exposure product and 74.4 % used a recombinant FVIII product. Because there was such large variety in the different FVIII products used during the 20-year study period, we chose not to adjust for this parameter in the multivariable analysis. We did however perform univariable analyses on product type at first exposure and inhibitor development: All inhibitors hazard ratio (HR) 0.9 (95 % CI 0.7–1.2), high titre inhibitors HR 1.0 (0.7–1.4) and low titre inhibitors HR 0.6 (0.4–1.1), concluding that this risk factor was not associated with inhibitor development. Furthermore we repeated the analyses without the patients using the FVIII product Kogenate FS which yielded the same results.

Adjustment for genetic and treatment related risk factors

Period 1 (1990–1994) was chosen as the reference period to compare the risk of inhibitor development. Because the number of low titre inhibitors in Period 1 and 2 (4 and 10, respectively, ►Table 1) was considered too small to perform meaningful adjustments, it was chosen only to adjust for all and high titre inhibitors.

HRs were adjusted for inhibitor testing rate (mean testing rate per period), non-Caucasian ethnicity, positive family history of inhibitors, large *F8* gene mutation type, ≥ 5 EDs peak treatment at first exposure, mean dose (IU/kg) during first 5 EDs, and start and exposure day of regular prophylaxis (before the 50th ED).

After adjustment the hazard ratios (aHR) were significantly increased for all inhibitors in Period 3 (aHR 1.96; CI 1.06–2.83) and Period 4 (aHR 2.34; 1.42–4.92). Risk for high titre inhibitor development was not significantly different for any of the four periods (►Table 3).

Discussion

In this analysis involving 926 previously untreated children with severe haemophilia A the inhibitor incidence was investigated over a 20-year period. During the whole study period the same definition for an inhibitor was used; every positive blood sample was confirmed by a consecutive positive sample and preferably accompanied by a reduced recovery. The cumulative inhibitor incidence of all inhibitors increased significantly between 1990–1994 and

Table 2: Distribution of potential confounding genetic and treatment-related factors per 5-year birth cohort.

	Birth cohort 1990–1994 N = 144	Birth cohort 1995–1999 N = 178	Birth cohort 2000–2004 N = 299	Birth cohort 2005–2009 N = 305	P-value
Caucasian ethnicity Number of patients (%)	129 (89.6)	160 (89.9)	259 (86.6)	257 (84.3)	0.241
Family history of inhibitors % Positive	9 (6.3)	13 (7.3)	27 (9.0)	30 (9.8)	0.409
F8 gene mutation type, % Large mutations	85 (59.0)	105 (59.0)	194 (64.9)	172 (56.4)	0.338
Peak treatment at first exposure ≥5 EDs, n (%)	17 (11.8)	34 (19.1)	55 (18.4)	38 (12.5)	0.059
Dose during first 5 EDs (IU/kg) All patients, median (IQR) No peak treatment, median (IQR) Peak of ≥5 EDs, median (IQR)	 43 (31 – 50) 42 (30 – 50) 56 (37 – 91)	 47 (37 – 66) 43 (34 – 53) 88 (54 – 106)	 48 (38 – 67) 45 (35 – 59) 72 (50 – 110)	 44 (33 – 58) 42 (33 – 54) 78 (46 – 120)	 0.057 0.650 0.048
Started prophylaxis before 50th ED Yes (%)	70 (48.6)	90 (50.6)	200 (66.9)	228 (74.8)	<0.001
ED at start prophylaxis Median (IQR)	17 (9 – 29)	17 (8 – 28)	13 (6 – 22)	11 (4 – 19)	0.023

Chi-square test performed for categorical variables and independent samples t-tests performed for continuous variables. In all tests the birth cohort with the lowest value for the specific variable is the reference birth cohort.

2000–2004 and 2005–2009, from 19.5% to 30.9% and 29.0%, respectively. This was mainly due to the diagnosis of more low titre inhibitors, the percentage of low titre inhibitor patients increased significantly from 3.1% to 9.6% (2000–2004) and 10.5% (2005–2009). Interestingly, the incidence of high titre inhibitors was quite stable over birth cohorts ranging from 16.9% to 23.5% (p-values > 0.05).

To address the differences in genetic and treatment related factors, aHRs were calculated to compare the inhibitor risk over the four time periods. Because the total inhibitor incidence is much influenced by the increase in detection of low titre inhibitors, we chose to adjust only for all and high titre inhibitor risk (►Table 3). The aHRs for high titre inhibitor development reflected the same results as shown with the cumulative incidences, i.e. the risk of inhibitor development was not significantly increased in the periods in reference to Period 1 (►Table 1 and ►Table 3). The increased detection of low titre inhibitors seems likely to be referable in part to more frequent testing, in part to more sensitive laboratory assays and in part to the changes in treatment modalities (►Table 1 and ►Table 2).

Changes in treatment modalities

From 1990 onwards, treatment regimens and dosing were intensified and regular prophylaxis was more widely adopted and started earlier (►Table 2). Data on changing practice in haemophilia in our group have recently been published (24).

Furthermore, ►Tables 1 and 2 show that the only factors that are significantly different between the periods are regular prophylaxis and inhibitor-testing rate. Periods 2 and 3 had more peak moments at first exposure with 19.1% and 18.4%, respectively. In the CANAL study the impact of intensive treatment as an impor-

Table 3: Risk of inhibitor development.

Birth cohorts	All inhibitors		High titre inhibitors		Low titre inhibitors[$]
	Unadjusted Hazard ratio	Adjusted Hazard ratio#	Unadjusted Hazard ratio	Adjusted Hazard ratio#	Unadjusted Hazard ratio
Period 1990–1994	Reference	Reference	Reference	Reference	Reference
Period 1995–1999	1.52 (0.95 – 2.41)	1.53 (0.94 – 2.50)	1.41 (0.85 – 2.34)	1.43 (0.83 – 2.44)	2.17 (0.68 – 6.91)
Period 2000–2004	1.70 (1.11 – 2.60)*	1.96 (1.06 – 2.83)*	1.45 (0.91 – 2.31)	1.35 (0.74 – 2.13)	3.24 (1.13 – 9.30)*
Period 2005–2009	1.61 (1.05 – 2.47)*	2.34 (1.42 – 4.92) *	1.27 (0.79 – 2.04)	1.71 (1.00 – 3.13)	3.68 (1.29 – 10.49)*

* p-value of the Wald test <0.05. # Hazard ratios were adjusted for inhibitor testing rate (mean testing rate per period), non-Caucasian ethnicity, positive family history of inhibitors, large *F8* gene mutation type, ≥ 5 EDs peak treatment at first exposure, mean dose (IU/kg) during first 5 EDs, and start and exposure day of regular prophylaxis (before the 50th ED). [$]Hazard ratios not adjusted because of very low number of low titer inhibitors in Periods 1 and 2.

tant risk factor for inhibitor development was clearly established (5). These results were available in 2006 and participants of the PedNet Study group might have changed their practice afterwards, which led to a lower number of peak moments at first exposure in period 4 (12.5 %).

Changes in inhibitor-testing

To address whether the frequency of inhibitor-testing was changed, data of non-inhibitor patients were collected until 50 EDs. The inhibitor-testing rate in the first 50 exposure days for non-inhibitor patients was relatively stable, fluctuating between median 3–5 tests/50 EDs over the four periods. When calculated as the number of tests per year the rates showed a trend towards increased frequency (►Table 1). This was also demonstrated when we calculated the median tests per 50 EDs per period, stratified for patients who reached their 50 EDs within one year, between 1–2 years and >3 years, as a proxy for treatment intensity (►Figure 2). Median tests per 50 EDs fluctuated between 2–6 tests. However a distinct trend was observed in increased tests per 50 EDs in patients who reached their 50 EDs in >3 years versus the other two groups.

The impact of more frequent testing on the increased diagnosis of low titre inhibitors was already recognised by several studies on the association of the type of FVIII concentrate and inhibitor development (12, 25, 26).

Another factor that could have influenced the results is the change of practice in the laboratory assay for inhibitors. In the European PedNet study group, 26 out of 29 centres changed to the Nijmegen modification of the Bethesda assay after it was introduced. It is well described that the Nijmegen modification allows for lower cut-off values for positivity (27). It seems possible that this had also an effect on the increased detection of low titre inhibitors.

Quality of test results

In this study inhibitor test results from the local laboratory of participating centres were used. Additional samples for central confirmation are often very difficult to be obtained in young children. Mandatory central confirmation could result in missing samples at crucial time points and subsequent selection bias of cases for the main outcome parameter. Stringent follow up of all patients makes it unlikely that high titre inhibitors were left undiagnosed. In addition, cost of central testing was not covered and would have increased the logistical complexity of the study.

To improve quality 80 % of the participating centres are involved in external validation studies on the Bethesda assay, such as UKQHAS, NEQAS and ECAT (17). From the early nineties the Bethesda assay as the standard assay for inhibitor detection has received much attention (28). By improved standardisation of the Bethesda assay such as through the Nijmegen modification the cut-off value for what constitutes an inhibitor has been reduced (29, 30). The effect of the increased assay sensitivity on the detection of the total number of inhibitors is unknown (27).

What is known about this topic?

- Since the introduction of recombinant FVIII concentrates an increased incidence of inhibitors has been reported
- Comparison between studies on the incidence and risk factors of inhibitor development in previously untreated patients is limited due to the small number of study subjects
- Frequent testing common practice and has had potentially an effect on the overall detection of inhibitors

What does this paper add?

- This study is performed in the largest multi-center birth cohort (N=926) of previously untreated patients, spanning 20 years
- The cumulative inhibitor incidence has indeed significantly increased between 1990 and 2009, because of the enhanced diagnosis of low titre inhibitors.

Further follow-up data from patients who developed a low titre inhibitor is currently ongoing and will establish whether these low titre inhibitors have the tendency to cause bleeding and need immune tolerance induction or disappeared spontaneously and should be redefined as transient inhibitors.

In conclusion, the cumulative inhibitor incidence has significantly increased mainly due to the enhanced diagnosis of low titre inhibitors. This increase seems to be attributable to a combination of more frequent testing together with more sensitive laboratory assays along with changes in treatment modalities. Future studies on inhibitor development should preferably use the development of high titre inhibitors as the primary study outcome instead of any clinically relevant inhibitor development.

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Author contributions

Contributions: H. M. v. d. B., S. M. H. and E. S. equally contributed, designed the research, analysed and interpreted the data, and wrote the first draft of the manuscript; K. F., P. P., R. L., A. R., M.C, G. A., K. K., G. K., N. C., E. A. C., and A. T. collected and interpreted the data and co-authored the manuscript.

Conflicts of interest

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