# 34th Hemophilia Symposium Hamburg 2003

Editors: I. Scharrer, W. Schramm

HIV Infection and Epidemiology

Management of Bleedings in Hemophiliacs with Inhibitors

Orthopedic Problems and Therapy in Hemophiliacs

Therapy with Protein C

Pediatric Hemostaseology

Free Lectures





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# HIV Infection and Causes of Death in Patients with Hemophilia in Germany (Year 2002/2003 Survey)

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## Introduction

The annually survey »HIV Infection and Causes of Death in Patients with Hemophilia in Germany« already goes along with a fine tradition. Already in the late 1970s Professor Landbeck began to survey annually hemophiliacs living at that time in West Germany for causes of death and the prevalence of diseases. This was carried on till today, so that our actual insights rest upon a broad database. However data quality could be much more improved in future.

## Patients and Methods

Questionnaires called »Todesursachenstatistik 2002/2003« were sent to all established hemophilia centers in Germany. Prompted was information about patients with hemophilia A, B and von Willebrand disease. In particular, anonymous data concerning the last 12 months about the number of treated patients, type and severity of illness, HIV-status and causes of death was inquired. This data was merged with existing data returning to 1982 and analyzed statistically. In the 2001/2002 survey, a total number of 8070 patients (including possible double registrations) have been reported from the participating centers.

## Results

### Participating Centers

Since the first survey the number of participating centers has increased every year with a particular rise in 1991 when the hemophilia treatment centers of the former East Germany joined in. Today these centers contribute a significant portion of the overall data (Fig. 1). In this year's survey the number of reporting hemophilia centers slightly decreases from 75 centers last year to 71 centers this year (Table 1). Thereby the total number of patients (including patients with von Willebrand disease) reported from all centers remained relatively constant and added up to 8070 patients compared to 7759 patients in last year's survey (Table 2).

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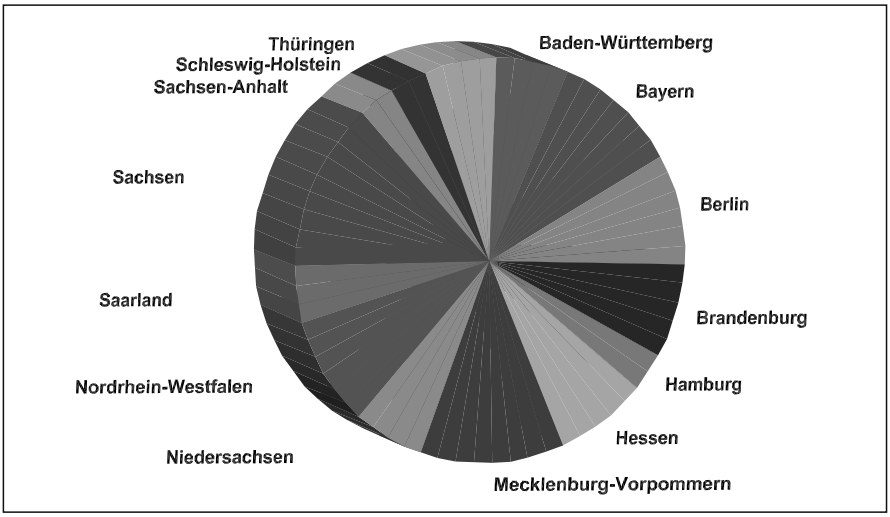


**Fig. 1a, b.** Participating hemophilia centers

**Table 1.** Numbers of participating hemophilia centers

| | 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| East | 47 | 62 | 79 | | | | | | | | | | |
| West | 18 | 18 | 24 | | | | | | | | | | |
| Totals | 65 | 80 | 103 | 111 | 119 | 119 | 71 | 75 | 93 | 87 | 72 | 75 | 71 |

## Patients

The distribution of patients with hemophilia A (48.31%), B (8.41%) and patients with von Willebrand disease (43.27%) is given in Table 2. Compared to the data of the previous surveys these are relative consistent findings. When severity of disease is analyzed with a cut-off of 2% factor activity, the distribution between the two subgroups, i.e. below 2% and above 2%, is almost similar in patients with hemophilia A and B as shown in Table 2. 18.36% of patients with von Willebrand disease showed ristocetin co-factor levels below 30%.

## Inhibitors

In 4.46% (174) of the patients with hemophilia A and in 2.21% [15] of the patients with hemophilia B an inhibitor was found (see Fig. 2 and Table 2). These findings correspond to international large-scale prevalence studies and registry data indicating that the prevalence of inhibitors in the hemophilia A population overall is between 5% and 7% [10].

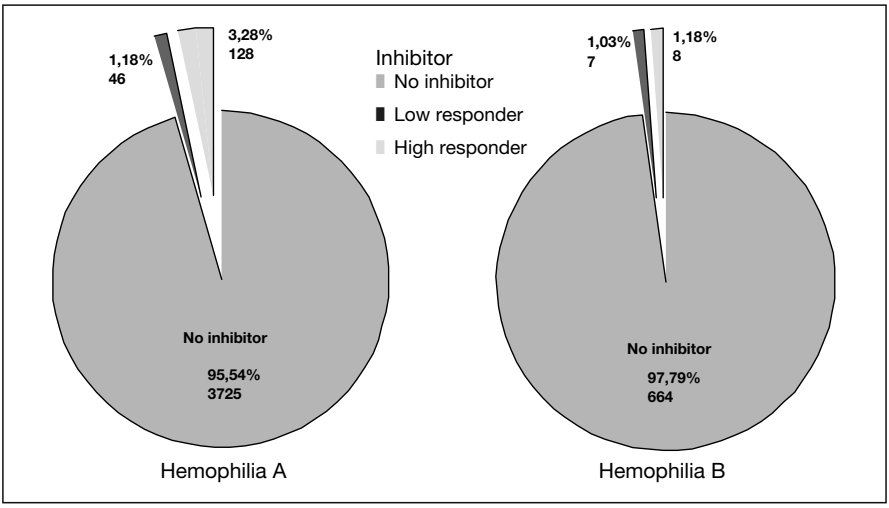


**Fig. 2.** Distribution of inhibitors in patients with hemophilia A and hemophilia B

**Table 2.** Cumulative data from 71 centers as of 2002/2003

| | Hemophilia A | | Hemophilia B | | von Willebrand disease | | Total |
|---|---|---|---|---|---|---|---|
| | N | % | N | % | N | % | N |
| Total | 3899 | 48.3% | 679 | 8.41% | 3492 | 43.27% | 8070 |
| Factor activity = 2% | 1978 | 50.73% | 326 | 48.01% | — | — | 2304 |
| Factor activity > 2% | 1921 | 49.27% | 353 | 51.99% | — | — | 2274 |
| Ristocetin Cofactor = 30% | — | — | — | — | 641 | 18.36% | 641 |
| Ristocetin Cofactor > 30% | — | — | — | — | 2851 | 81.64% | 2851 |
| Inhibitor (low responders) | 46 | 1.18% | 7 | 1.03% | — | — | 53 |
| Inhibitor (high responders) | 128 | 3.28% | 8 | 1.18% | — | — | 136 |
| Total HIV negative | 3238 | — | 571 | — | 2951 | — | 6760 |
| Total HIV positive | 583 | — | 89 | — | 7 | — | 679 |
| HIV positive, no AIDS | 273 | — | 47 | — | 5 | — | 325 |
| HIV positive, CD4<200 cells/µl | 59 | — | 13 | — | 1 | — | 73 |
| HIV positive, full blown AIDS | 35 | — | 3 | — | 0 | — | 38 |
| HIV positive, no comment | 216 | — | 26 | — | 1 | — | 243 |

## HIV Status

Of all reported patients a total of 679 were infected with HIV. Analyzed for HIV distribution in subgroups nearly 15% of all patients with hemophilia A, 13% of all patients with hemophilia B, and 0.2% of all patients with von Willebrand disease were HIV-infected (Fig. 3). A total of 38 patients (5.6% of all HIV positive patients) has reached the stage of full-blown AIDS, compared to 325 patients (47.9% of all

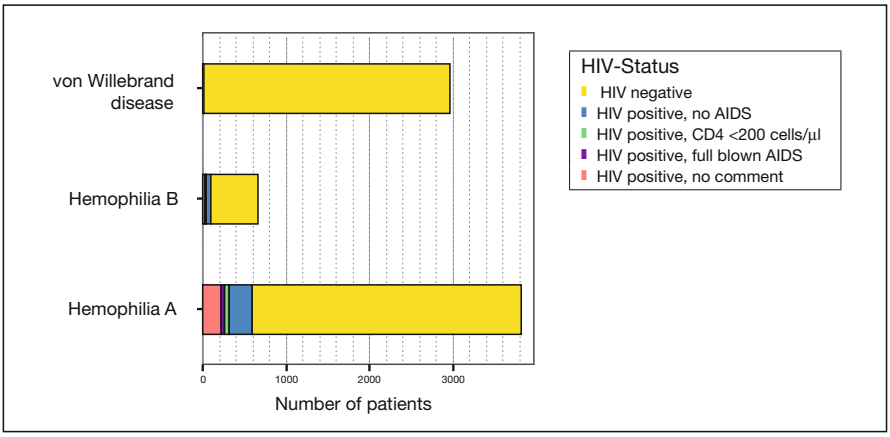


**Fig. 3.** Distribution of HIV-infected patients

HIV positive patients) that have up to now not shown severe symptoms of the immune disease (Tab. 3). Unfortunately 243 HIV positive patients with no further details concerning stadium were reported. As this bates data quality considerable further investigation is needed to fill in the missing information.

**Tab. 3.** HIV status

| HIV status | Hem. A | Hem. B | von Willebrand disease | Total |
|---|---|---|---|---|
| HIV negative | 3238 | 571 | 2951 | 6760 |
| HIV positive, no AIDS | 273 | 47 | 5 | 325 |
| HIV positive, CD4+ < 200 cell/µ | 59 | 13 | 1 | 73 |
| HIV positive, full-blown AIDS | 35 | 3 | 0 | 38 |
| HIV positive, no comment | 216 | 26 | 1 | 243 |
| Total HIV positive | 583 | 89 | 7 | 679 |

## Mortality from all Cases

In the 2002/2003 period a total of 16 patients were reported dead with the distribution of causes of death given in Table 4. Since the beginning of the survey in 1982 a total of nearly 800 patients have been reported dead. The development of mortality and causes of death since 82/83 are depicted in Fig. 6 to Fig. 8. In this year's survey liver disease (38%) and cancer (19%) have been the main causes of death while AIDS (6%) loses ground anymore (see Fig. 7a). Up to 1995 the number of AIDS-related deaths increased continuously with decline taking place since then. As at present more than half of the primary HIV-infected patients with hemophilia are still alive (679), the main reason for this development can probably be attributed to improved antiretroviral therapies as described by many authors [1, 3, 6]. In contrast liver

disease showed a sharp increase from 14% last year except for 38% this year. No patient died of a bleeding. Cancer as a cause of death remained relatively constant still staying on an alarming high level (19%). Overall annual mortality in patients with bleeding disorders in the 2002/2003 survey adds up to 0.2% per year. No indications for Creutzfeld-Jakob disease in our patient collective has been reported since 1978. Once again mentionable is the low portion of reported deaths with no comment, improving data quality clearly.

**Table 4.** Distribution of death causes

| Patients | N | % |
|---|---|---|
| AIDS | 1 | 6 |
| Liver disease | 6 | 38 |
| Bleeding | 0 | 0 |
| Cancer | 3 | 19 |
| Other diseases | 3 | 19 |
| No comment | 3 | 19 |
| Total | 16 | 100 |

Arranging data for greater periods of time one can see these changes obviously (see Fig. 4 b, d, f). Clustered data for the years 1982 to 1994 and 1994 to 2003 gives us a statistically significant difference between these periods concerning all important causes of death as HIV ($p < 0.022$), liver disease ($p < 0.023$) and cancer ($p < 0.001$). The same numerical picture shows the HIV/liver disease/cancer deaths expressed as percentage of all deaths per year (see Fig. 5 a–c).

## Mortality from Liver Disease

Therewith in this year's survey the increase of liver disease as a cause of death has reached statistical significance the first time, suggesting a further increase in future (see Fig. 4 d). The obvious reason for this probably can be attributed to the increasing number of deaths induced by liver cirrhosis and hepatocellular carcinoma due to chronic HCV [7]. As we did not discriminate type of cancer in our surveys up to now there might be a relevant portion of patients in this group having died of primary hepatocellular carcinoma induced by chronic HCV intensifying the impact of liver disease on causes of death in patients with hemophilia even more.

Clustered data analyzed for HIV negative and positive patients for the period 1999 to 2003 only indicates a slight difference in mortality (45 vs. 53 deceased patients), not reaching statistical significance. However the same data separated for causes of death (liver disease and cancer) shows a clear difference in the percentage of total number of deaths between the two subgroups (see Fig. 9). These findings harden the suspicion that the combination of HIV/HCV coinfection accelerate progression of liver disease [4, 9, 11].

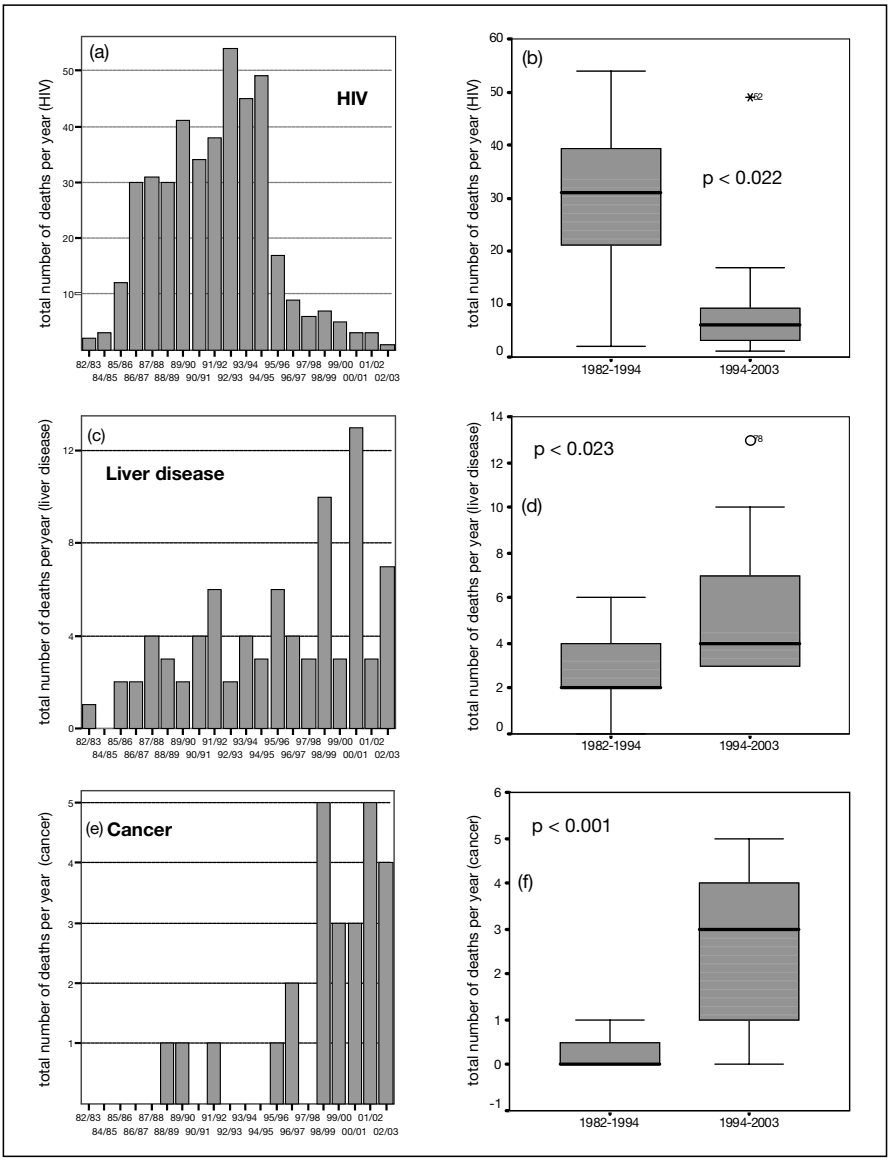


**Fig. 4a–f.** Comparison total number of deaths of HIV, liver disease and cancer (a – f)

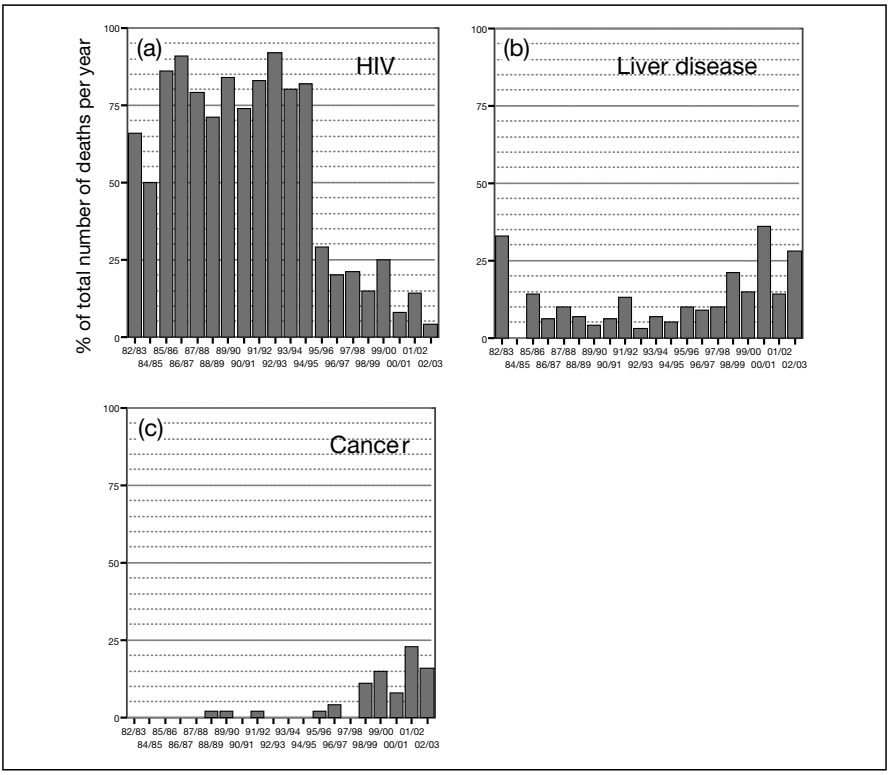


**Fig. 5.** Comparison % of total number of deaths of HIV, liver disease and cancer (a–c)

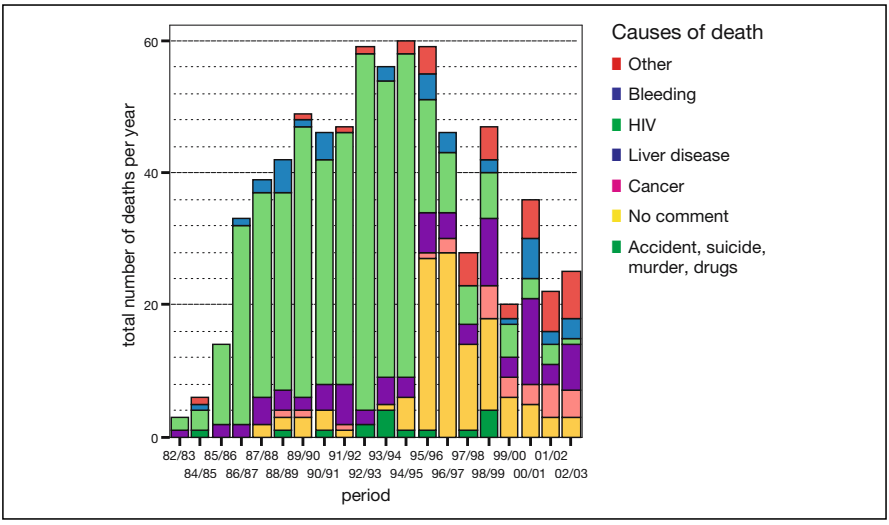


**Fig 6.** Causes of death since the beginning of the survey

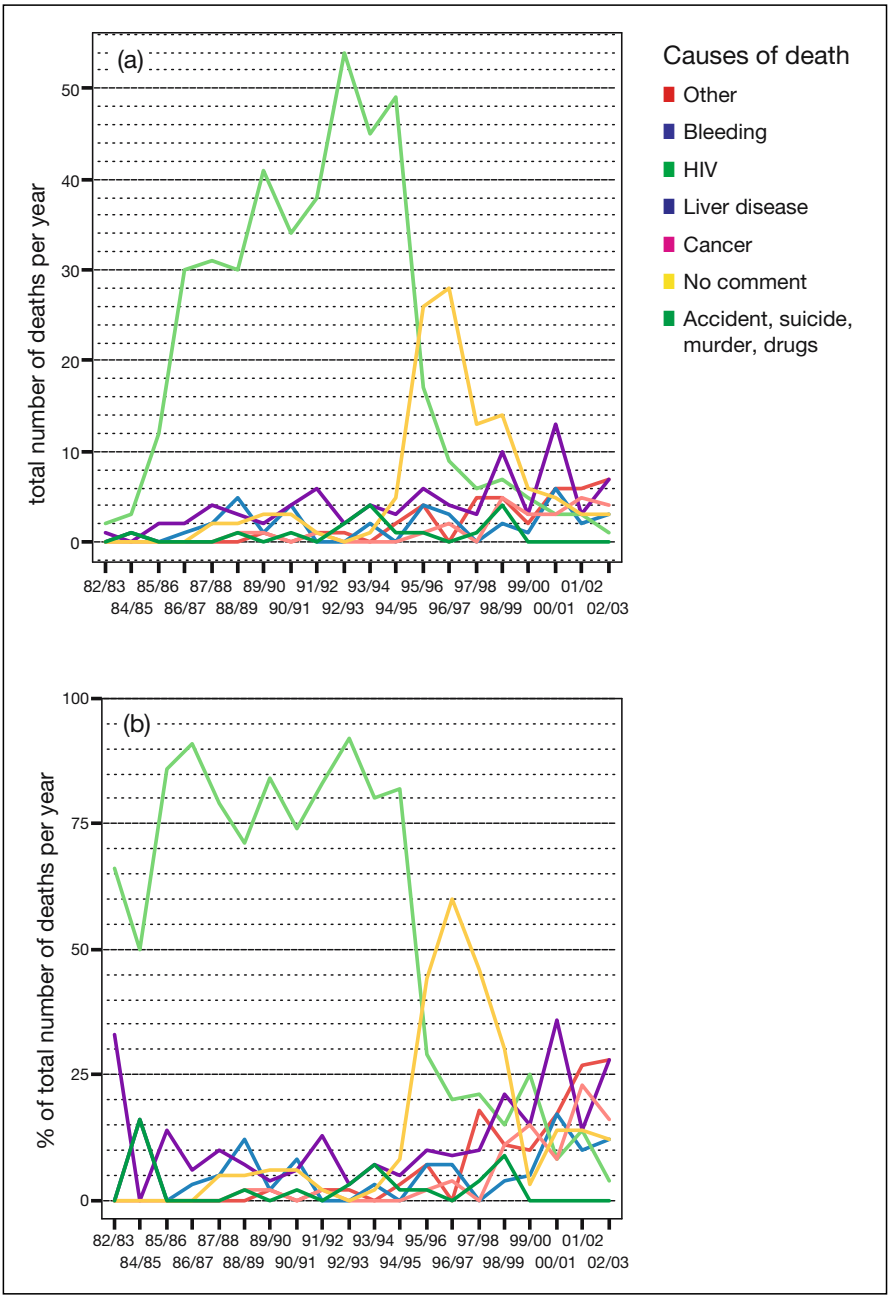


**Fig. 7.** Chart of deceased patients per year, separated for causes of death

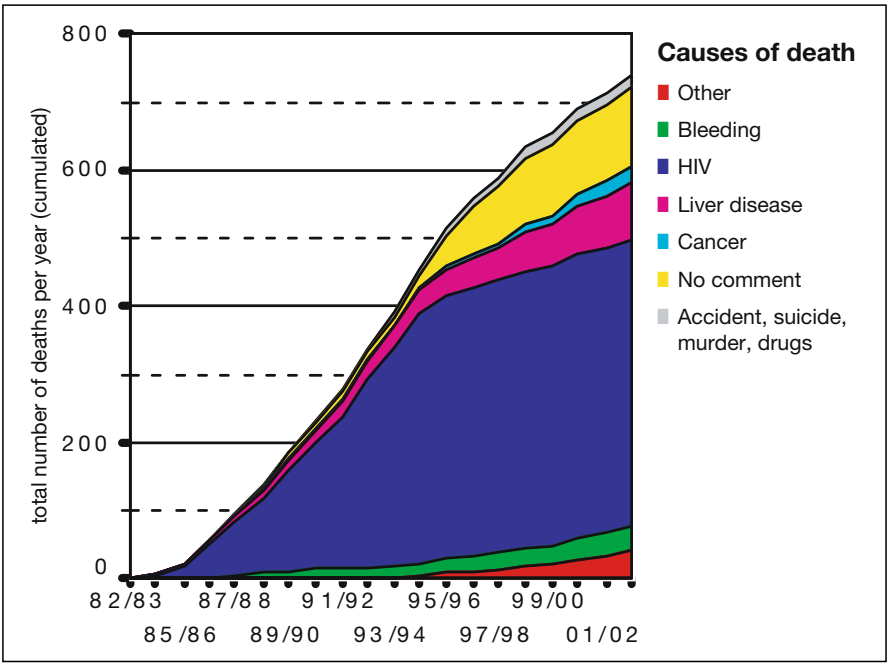


**Fig. 8.** Cumulative chart of deceased patients, separated for causes of death

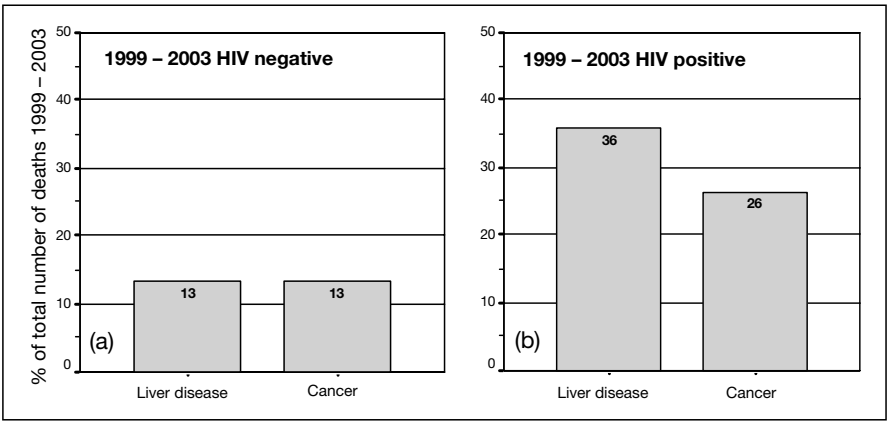


**Fig. 9a, b.** Comparison % of total number of deaths of HIV negative (a) vs. HIV positive (b) patients separated for liver disease and cancer, period 1999 – 2003.

## Conclusion

Comparing actual data to those of the previous surveys we got very consistent findings indicating good data quality. In addition our data is comparable to that of international large-scale prevalence studies and registry data. Despite mortality from HIV in patients with hemophilia is keeping on decreasing, HIV still remains an important factor as an HIV/HCV coinfection seems to increase the risk of progression of liver disease to cirrhosis and hepatocellular carcinoma [8, 11]. A relevant portion of patients reported dead of cancer might have died of primary hepatocellular carcinoma induced by chronic HCV. This hypothesis has to be proved in future surveys by discriminating type of cancer. Moreover we will have to investigate the cohort of chronic HCV-infected patients in order to be able to calculate cumulative risks of death from liver disease in patients with hemophilia infected between 1969 and 1985 with HCV-contaminated blood products. Therefore there might be evident arguments especially in HIV-coinfected patients for an early onset of an HCV-therapy in spite of a good liver capacity and plain immunological conditions [2, 5].

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# Hemophilia in Austria – The Annual Survey of the Austrian Hemophilia Centers

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## Patients and Methods

The Institute of Social Medicine of Vienna organizes the annual survey of the Hemophilia Treatment Centers (HTC) in Austria. Anonymous questionnaires are collected by the heads, or staff, of the HTC, on the basis of the data they provided the year before.

The analysis 2003 shows the distribution of the patients within the Austrian counties in respect to the place where they live, the number of patients according to the severity of the disease and the distribution according to age. Further the type of the disease and the number of HIV-infections among Austria's hemophilia patients are analyzed.

## Results

We received evaluable data from 359 patients with a mean age of 29 (+/– 20) in a range between 1 and 91 years (Table 1).

23.4% of the PwH are integrated in the working process, 35.9% are children and 9.5% are retired (Table 2).

265 (73.8%) patients suffer from hemophilia A, 48 (13.4 %) from hemophilia B, and 18 (5.8%) are reported inhibitor-patients (Table 3).
30 have a diagnosis of von-Willebrand-Syndrome (8.4%) and 16 (4.5%) other bleeding disorders (Table 4). 260 (72.4) are in HTC-care and 92 (25.6%) patients are lost to follow up.

**Table 1.** Age

| | | frequency | % | valid % | cumulated % |
|---|---|---|---|---|---|
| valid | 0 - 14 yrs | 102 | 28.4 | 28.7 | 28.7 |
| | 15 - 29 yrs | 102 | 28.4 | 28.7 | 57.3 |
| | 30 - 54 yrs | 107 | 29.8 | 30.1 | 87.4 |
| | 55 yrs + | 45 | 12.5 | 12.6 | 100.0 |
| | total | 356 | 99.2 | 100.0 | |
| missing system | | 3 | 0.8 | | |
| total | | 359 | 100.0 | | |

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**Table 2.** Profession

| | | frequency | % | valid % | cumulated % |
|---|---|---|---|---|---|
| valid | blue collar worker | 9 | 2.5 | 3.0 | 3.0 |
| | white collar worker | 58 | 16.2 | 19.1 | 22.1 |
| | self-employee | 8 | 2.2 | 2.6 | 24.8 |
| | official | 9 | 2.5 | 3.0 | 27.7 |
| | pensioner | 34 | 9.5 | 11.2 | 38.9 |
| | others | 56 | 15.6 | 18.5 | 57.4 |
| | (school-) children | 129 | 35.9 | 42.6 | 100.0 |
| | total | 303 | 84.4 | 100.0 | |
| missing | system | 56 | 15.6 | | |
| total | | 359 | 100.0 | | |

**Table 3.** Inhibitor

| | | | age group | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0-14 years | 15-29 years | 30-54 years | 55 years + | total |
| inhibitor | yes | number | 5 | 5 | 5 | 3 | 18 |
| | | % | 5.7 | 5.4 | 5.2 | 8.3 | 5.8 |
| | no | number | 82 | 87 | 91 | 33 | 293 |
| | | % | 94.3 | 94.6 | 94.8 | 91.7 | 94.2 |
| total | | number | 87 | 92 | 96 | 36 | 31 |
| | | % | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

**Table 4.** Hemophilia Type

| | | frequency | % | valid % | cumulated % |
|---|---|---|---|---|---|
| valid | hemophilia A | 265 | 73.8 | 73.8 | 73.8 |
| | hemophilia B | 48 | 13.4 | 13.4 | 87.2 |
| | von-Willebrand-Syndrome | 30 | 8.4 | 8.4 | 95.5 |
| | other bleeding orders | 16 | 4.5 | 4.5 | 100.0 |
| | total | 359 | 100.0 | 100.0 | |

The relation of patients with mild hemophilia to patients with the severe form of hemophilia is similar (Table 5), the relation of hemophilia A to hemophilia B is according to the literature.

The distribution of the patients according to age and severity of the disease is also balanced, as expected, but there is a significantly fewer number of patients in the age cohort of older than 55 years (Table 6), which is explained by the HIV-infection.

**Table 5.** Severity

| | | frequency | % | valid % | cumulated % |
|---|---|---|---|---|---|
| valid | mild | 150 | 41.8 | 46.9 | 46.9 |
| | moderate | 31 | 8.6 | 9.7 | 56.6 |
| | severe | 139 | 38.7 | 43.4 | 100.0 |
| | total | 320 | 89.1 | 100.0 | |
| missing | system | 39 | 10.9 | | |
| total | | 359 | 100.0 | | |

**Table 6.** Severity / Age

| | | | age group | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0-14 years | 15-29 years | 30-54 years | 55 years + | total |
| severity | mild | number | 37 | 40 | 44 | 27 | 148 |
| | | % | 38.9 | 43.5 | 46.3 | 77.1 | 46.7 |
| | moderate | number | 23 | 3 | 4 | 1 | 31 |
| | | % | 24.2 | 3.3 | 4.2 | 2.9 | 9.8 |
| | severe | number | 35 | 49 | 47 | 7 | 138 |
| | | % | 36.8 | 53.3 | 49.5 | 20.0 | 43.5 |
| total | | number | 95 | 92 | 95 | 35 | 317 |
| | | % | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

**Table 7.** Hepatitis C

| | | | age group | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0-14 years | 15-29 years | 30-54 years | 55 years + | total |
| Hepatitis C | yes | number | 2 | 22 | 61 | 19 | 104 |
| | | % | 2.2 | 30.1 | 73.5 | 70.4 | 38.0 |
| | no | number | 89 | 51 | 22 | 8 | 170 |
| | | % | 97.8 | 69.9 | 26.5 | 29.6 | 62.0 |
| total | | number | 91 | 73 | 83 | 27 | 274 |
| | | % | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

104 out of 274 patients are diagnosed HCV-antibody positive. The majority is found in the age cohort of 30 to 54 years (Table 7). 31 patients out of 275 are diagnosed HIV-antibody positive, 6 out of them developed AIDS (Table 8), the majority again found in the group of 30 to 54 years old.

But the data of the Austrian HIV-/AIDS-Fund (Fig. 1) are very different, in 2003 there were 58 PwH recognized recipients of benefit.

**Table 8.** HIV / AIDS

| | | | age group | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0-14 years | 15-29 years | 30-54 years | 55 years + | total |
| **HIV** pos. | yes | number | | 9 | 20 | 2 | 31 |
| | | % | | 11.5 | 23.0 | 7.7 | 11.3 |
| | no | number | 84 | 69 | 67 | 24 | 244 |
| | | % | 100.0 | 88.5 | 77.0 | 92.3 | 88.7 |
| total | | number | 84 | 78 | 87 | 26 | 275 |
| | | % | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

| | | | age group | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0-14 years | 15-29 years | 30-54 years | 55 years + | total |
| **AIDS** | yes | number | | 2 | 3 | 1 | 6 |
| | | % | | 2.6 | 3.4 | 3.7 | 2.1 |
| | no | number | 92 | 74 | 84 | 26 | 276 |
| | | % | 100.0 | 97.4 | 96.6 | 96.3 | 97.9 |
| total | | number | 92 | 76 | 87 | 27 | 282 |
| | | % | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

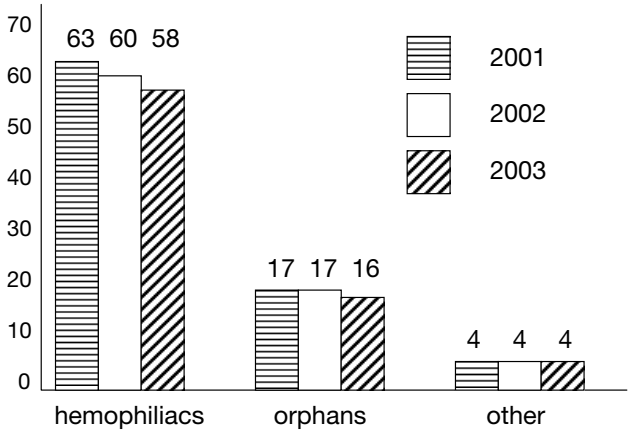


**Fig. 1.** HIV-Fund

## Discussion

The annual survey on Hemophilia in Austria tries to give a representative overview. There is no significant change within the Austrian hemophilia population over the last years detectable. Over the last years we found a slight increase in average age and a reduction of the percentage of iatrogenic virus-infected patients due to safe clotting factors. HAART reduced the mortality rates of HIV-infected patients and the HCV-antibody positive patients benefit from new antiviral therapies.

For the year 2004 we have created an online questionnaire and included questions on bleeding frequencies, treatment regimen and target joints, as well as other bleeding disorders as von-Willebrand-disease.

We know that the data presented is partly biased, but surveys like these are depending on the collaboration of HTCs and the engagement of the caregivers. We hope to encourage our contributors in simplifying the participation by technical means even more, especially as we have to face the difficulties of financing health care and need to antagonize and to arrive with valuable conclusions to perpetuate the state-of-the-art-care for our patients.

# Lithuanian Hemophilia Register: Update 2003

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## Background

Lithuanian Hemophilia Register (LHR) was constructed in 1994 under the supervision of Dr. R. Jurgutis in Klaipeda Seamen's Hospital (Klaipeda, Lithuania). The aims of the register were:

1. to gather data from lithuanian patients suffering from inherited bleeding disorders including hemophilia A (HA), hemophilia B (HB), von Willebrand disease (VWD), rare coagulation factor deficiencies, e.g. FVII deficiency, FX deficiency
2. controlling of infectious diseases (HBV, HCV and HIV) in hemophiliacs,
3. controlling of bleeding and concentrates consumption,
4. identification of molecular defects in corresponding genes,
5. comparison of phenotype-genotype correlation,
6. providing carrier and prenatal diagnosis in affected families [1, 2].

## Results and Discussion

To date, 229 patients were reported in LHR, comprising 118 patients with HA, 17 patients with HB, 75 patients with VWD, 9 patients with FVII deficiency, 4 patients with FXII deficiency, 2 patients with FX deficiency, 2 patients with FXIII deficiency, single patients with FXI deficiency and Bernard-Soulier syndrom, respectively.

The concentrate consumption (FVIII/FIX) was 0.7U / inhibitant in year 2001, and 0.9U / inhibitant in year 2002. Seventy-five percent of hemophiliacs are infected by HCV, 25% by HBV, none of the patients were HIV positive.

So far mutation screening was done in 104 individuals (89 patients with HA, 10 with HB, 1 with FXIII deficiency, 3 with FVII deficiency, and 1 with factor X deficiency). The phenotype correlates with genotype in many but not in all cases. Carrier status was elucidated in 51 of 92 females. One female has been shown to have somatic mosaicism. Prenatal diagnosis has been performed in 2 cases, revealing two healthy girls.

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## Conclusions

Such a register is of essential importance for Lithuania. To the physicians, the register allows the prediction of the clinical course of a patient with respect to risk of inhibitor development or the identification of mitigated severe hemophilia A phenotypes. To the health care system such a registry provides an important tool for the provision of health services to the hemophilia community.

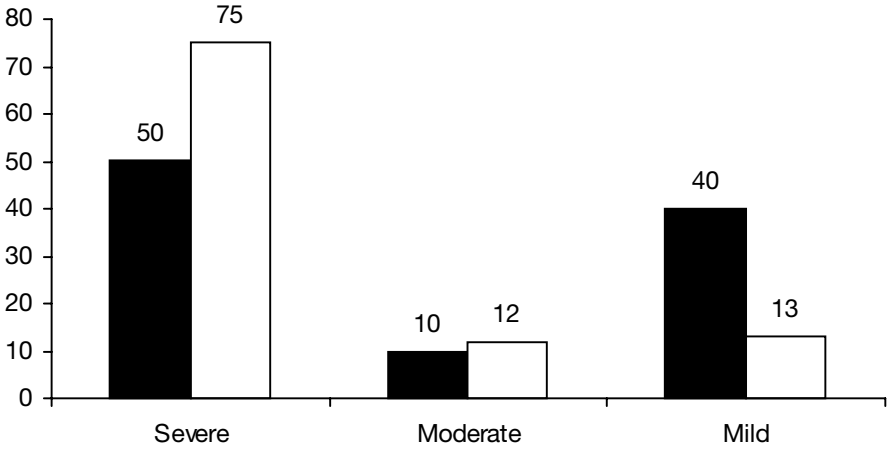


**Fig. 1.** The percentage of severe, moderate and mild hemophiliacs (HA) are shown. Black columns according to Antonarakis et al. [3], white columns in Lithuanian patients. The majority (75%) of Lithuanian patients are suffering from severe HA, while only 13% from mild HA.

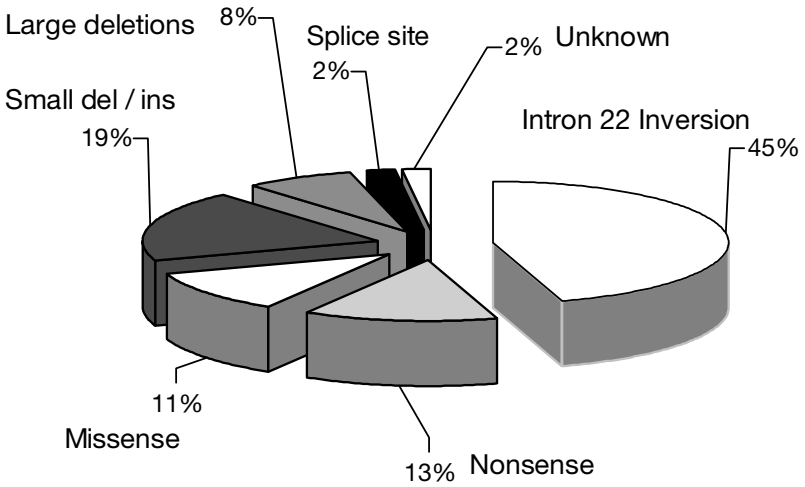


**Fig. 2.** Mutation profile in 53 unrelated lithuanian hemophilia A patients.

**Table 1.** Estimated Prevalence of coagulation disorders in Lithuania

| Disorder | Number of Patients | Estimated Prevalence |
|---|---|---|
| HA | 118 | 1: 15 000* |
| HB | 17 | 1: 100 000* |
| VWD | 75 | 1: 50 000 |
| FVII | 9 | 1: 400 000 |
| FXII | 4 | 1: 875 000 |
| FXIII | 2 | 1: 1 750 000 |
| FX | 2 | 1: 1 750 000 |
| FXI | 1 | 1: 3 500 000 |
| BSS | 1 | 1: 3 500 000 |

* Frequency in males, BSS- Bernard-Soulier Syndrom. The prevalence of HA and HB is lower when compared to the literature. This can be explained by the lack of diagnosis in patients with mild deficiencies.

**Table 2.** Analysis of genetic defects in patients with coagulation disorders

| Disease | Number of analyzed patients | Number of identified different mutations | Number of detected carriers |
|---|---|---|---|
| HA | 89 | 34 | 43 |
| HB | 10 | 8 | 5 |
| FVII | 3 | 3 | 1 |
| FX | 1 | 2 | 0 |
| FXIII | 1 | 1 | 2 |

HA= hemophilia A, HB= hemophilia B, FVII= factor VII deficiency, FX= factor X deficiency, FXIII= factor XIII deficiency.

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# *II. Management of Bleedings in Hemophiliacs with Inhibitors*

Chairmen:

H.-H. Brackmann (Bonn)
W. Schramm (Munich)

# Guidelines for Treatment of Patients with Hemophilia and Inhibitors

A. Gringeri for the Italian Association of Hemophilia Centres

## Introduction

The treatment of patients with hemophilia who develop clotting factor-inhibiting antibodies is the most challenging and difficult for the hemophilia physicians. This issue is made even more difficult by the absence of evidence-based guidelines. We report the guidelines for treatment of patients with hemophilia and inhibitors approved by the Italian Association of Hemophilia Centres (AICE). In particular these guidelines aim to address many specific open issues in the treatment of these patients. How should immune tolerance be induced? How should bleeding events be managed? Is thrombogenicity an issue in the use of bypassing agents? Which are possible guidelines for switching bypassing products? What is the therapeutic role of antifibrinolytic agents? Consensus statements are presented.

## Guidelines for Immunotolerance Induction

Eradication of the inhibitor in patients with haemophilia represents the main goal of the treatment since it allows substitution therapy with specific clotting factor concentrates, being this therapy with the lowest cost-efficacy ratio. Inhibitor eradication can be achieved in about three fourth of patients by induction of factor immune tolerance [1–11]. This is usually accomplished by repeated injections of high clotting factor doses, even though lower doses have been shown to be effective [12, 13]. Some authors have suggested the concomitant use of immunosuppressive agents and/or high dose intravenous immunoglobulins with or without extra corporeal immunoadsorbtion of inhibitory antibodies [13, 14]. Open issues of immune tolerance induction (ITI) are the dose regimen, the product type, the time to start and the role of immunomodulating treatments.

AICE recommends that an effort to induce immune tolerance should be done in all children with hemophilia A who develop inhibitors. Children with hemophilia B and inhibitor can develop inhibitory antibodies which bind the complement: a further exposure to factor IX can cause anaphylactoid reactions and/or nephrotic syndrome [15–18]. Therefore particular precautions should be adopted in these patients.

ITI should start as soon as possible, however spontaneous remissions in patients with lower inhibitor titres are frequent [19]. In any case, ITI should not be delayed beyond one year from inhibitor detection.

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Because of the urgent need of controlled clinical trials in order to gather evidences of regimen and product type to use first, AICE recommends that all children eligible should be enrolled in the international study coordinated by D. Di Michele and C. Hay, designed to compare high daily doses (200 IU/kg) with lower doses (50 IU/kg) three times a week. This study does not indicate which product should be used, with the aim to carry on a *post hoc* analysis on the type of concentrate.

Common practice is to start with the same product that induced the inhibitory response: usually previously untreated patients (PUPs) are treated with recombinant FVIII and FIX products and consequently tolerized with recombinant products, while plasma-derived products are used in case of shortage or as rescue treatment after a previous ITI failure. In fact, some experiences seem to indicate that plasma-derived products with an higher content of von Willebrand factor can play a role in ITI-resistant patients [19–22]. More evidences should be gathered in order to know the real impact of these observations, the right time to judge as failed an on-going ITI and consequently the time to switch to a different product.

Too poor is the experience about the use of immunosuppressive agents with or without extra corporeal immune absorption [13, 14]: concerns were raised about the potential risks of immunosuppressive therapy in children not counterbalanced by a significant efficacy. On the other hand extracorporeal immune absorption can be problematic particularly in children because of poor venous access and high exchange volumes.

## Guidelines for Management of Bleeding Events

Therapeutic decisions should be based on previous exposure to plasma-derived factors, inhibitor titre, anamnestic inhibitor response, site and severity of the bleed [23–26].

At diagnosis of inhibitor development, usually done in children previously exposed to a recombinant product, recombinant activated factor VII (rFVIIa) is generally recommended in order to avoid to expos the child to blood products and to elicit an anamnestic response with high doses of FVIII/FIX concentrates or with activated prothrombin complex concentrate (APCC) [27–29]. Moreover, occurrence of severe adverse reactions are reported in some hemophilia B patients who develop inhibitor when re-exposed to factor IX [15–18].

During ITI, patients can experience breakthrough bleedings, that can be treated with higher doses of FVIII/FIX concentrates or by-passing agents such as rFVIIa and APCC [23–27, 29–38].

In ITI-resistant patients, rFVIIa or APCC are equally recommended as first line treatment: both are effective, both can fail [23, 25–27, 29, 31, 33–38]. No comparative study is available in order to compare efficacy. An international, randomized, cross-over, clinical trial is on-going in Europe and the U.S.A., called FENOC and coordinated by Eric Berntorp. This study will compare efficacy of NovoSeven and FEIBA in controlling hemostasis in patients experiencing joint bleedings at knees, elbows or ankles. Although this study cannot be logically blind, it will offer the chance to evaluate the equivalence of these two approaches.

FVIII/FIX substitution therapy is recommended always when inhibitor anamnestic response is low or when inhibitor titre is low in high responders with acute life-threatening bleeding episodes [30–32].

Major surgery is the most difficult situation in high-responders with high inhibitor titres. RFVIIa is usually used as first choice, even though successful surgical procedures with APCC have been reported [23, 25, 29, 34–40]. This attitude seems based on a more largely reported experience with rFVIIa in the literature particularly for major surgery.

Doses, number of and intervals between injections of rFVIIa represent still quite open issues after 10 years of experience, not sufficiently addressed by clinical trials yet [23]. Doses ranges from 90 to 120 μg/kg, even though lower and higher doses have been used and reported as efficacious. In fact, hemostatic effects of by-passing agents is unpredictable on the basis of laboratory assays [37]. No precise guidelines are available for the use of rFVIIa by continuous infusion [41–43], which actually is not licensed.

By contrast, large uniformity of approach exists for treatment with APCC, that is recommended at doses of 50-100 IU/kg every 8-24 hours, never exceeding 200 IU/kg per day, in order to prevent adverse events. APCC was also suggested for secondary prophylaxis in patients with recurrent bleedings [44], but a risk/efficacy ratio and a cost/benefit ratio evaluation are still missing as well as the appropriate dose and administration schedule. An international study is ongoing to address these issues.

Porcine FVIII concentrate might be a valid option in haemophilia A high responding patients with low cross-reaction for treatment of severe bleedings and for surgical procedures [45], but it has become no longer available.

Thrombogenicity is a rare adverse event during the use of APCCs and rFVIIa [46-51]. Transient hypofibrinogenemia, thrombotic stroke, DIC, and acute myocardial infarction have been reported with APCCs as well as with rFVIIa. These serious complications have been always reported in adults with other risk factors (age, obesity, major surgical procedures, DIC, diabetes, cirrhosis, atherosclerotic heart disease, presence of concomitant thrombophilia, immobilization) or associated with misuse of these products (doses of APCC exceeding 120 IU/kg per infusion or 200 IU/kg a day, concomitant use of APCC and rFVIIa and/or antifibrinolytics). Case-control studies are warranted in order to define risk factors and consequently to prevent these rare but life-threatening events. In any case the switching between the 2 bypassing agents and the concomitant use of antifibrinolytics should follow precise guidelines.

### Guidelines for Switching Bypassing Agents

No guidelines or clinical data are available about when it is safe to substitute one type of bypassing products with another, but some thrombogenic events have been probably correlated with short intervals between them [51]. To switch to another bypassing factor concentrate, when the previous one has failed, that is in critical situations, it is per se at risk of adverse events. An interval of at least 3–6 hours

should be respected after a rFVIIa injection to use APCCs, while a longer interval of about 8–12 hours should follow an infusion of APCCs. These empirical intervals are based on product half-lifes (about 3 hours for rFVIIa and 6-24 hours for APCCs). An international survey is warranted in order to better identify minimal safety intervals. Monitoring of systemic activation of hemostasis should be possibly carried out before and after this product switch.

## Guidelines for the Use of Antifibrinolytic Agents

Antifibrinolytic agents are frequently used in association with FVIII/FIX substitution therapy and with bypassing products. Their use seems generally safe, with the exception of the association with APCC, linked with higher risk of thrombogenicity, and in case of hematuria, in which formation of clots in the urinary tract has been frequently observed [52–54]. Despite of their safety, their efficacy is poorly demonstrated [55]. In particular, antifibrinolytics are frequently use together with rFVIIa, many publications suggesting an additional efficacy [56–58].

Mouth washes with antifibrinolytics have been shown to be safe and effective for mouth bleeding, also in association with APCCs [59-62]. The use of antifibrinolytic agents as mouth washes is therefore particularly recommended for oral cavity bleedings.

## Conclusions

Many issues remain open in the treatment of children with hemophilia and inhibitors. AICE has attempted to address some of them. Consensus statements can be summarized as follows:

*ITI*

1. The main goal of treatment of patients with hemophilia and inhibitors is to eradicate the inhibitor by inducing immune tolerance.
2. In all patients immune tolerance induction should be tried.
3. The use of immunosuppressive agents is not justified by the risk/benefit ratio.

*Bleeding events*

4. Either rFVIIa or APCCs for patients on ITI can be used to treat breakthrough bleeding events.
5. Caution for anaphylactoid reactions must be used when FIX-containing concentrates are infused in hemophilia B patients.
6. High dose FVIII/FIX substitution therapy is recommended in low inhibitor responders.
7. All the currently available bypassing agents used in inhibitor patients have limitations and are associated with potential/although rare/problems.
8. The major drawbacks of the bypassing agents are their unpredictable hemostatic effect, lack of laboratory assays to determine hemostatic dose and the risk of thrombosis.

*Switching from one type of bypassing agent to another*

9. When it is allowed by the clinical situation, the recommended intervals are 3-6 hours for switching from rFVIIa to APCCs and 8-12 hours for switching from APCCs to rFVIIa.
10. Monitoring of coagulation assay before and after switching bypassing products is recommended.

*Use of antifibrinolytics*

11. Avoid the use of antifibrinolytic agents when APCCs are infused
12. Avoid the use of antifibrinolytic agents when hematuria is present.
13. Mouth washes for mouth bleedings are recommended, being safe and cost-effective.

Many dilemmas are still to be resolved: it can be only accomplished by well-designed clinical trials, which would take also in account costs and health-related quality of life. Accurate national and international registries of adverse events occurring in occasion of treatments of hemophilic children with inhibitors can weight the real risk of adverse events of the different therapeutic options.

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# Bolus Injection of Recombinant Factor VIIa (NovoSeven) can be More Effective than Continuous Infusion in Inhibitor Patients with Severe Hemophilia A

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## Background

Although in most inhibitor patients with hemophilia A, immune tolerance therapy (ITT) according to the Bonn protocol [1] is successful, a small number of patients does not reach a remission. These patients suffer from relapsing severe bleedings and are at high risk to develop hemophilic arthropathy. Bleedings have to be treated either by an activated prothrombin complex (FEIBA) [2, 3] or by recombinant activated factor VII (rFVIIa, NovoSeven), given as bolus injections (BI) [4–8] or continuous infusion (CI). Because of its short half-life, rFVIIa requires infusions every 2 hours in the immediate post-operative period [9, 10]. Then, time intervals can be increased but infusions often have to be continued for days. Treatment schedules are shown in Table 1 and 2. The administration of rFVIIa by continuous infusion is a wide-spread practice since several authors have shown that it is effective, less expensive [11–15], and more convenient than bolus injections.

**Table 1.** Treatment schedule for recombinant FVIIa – bolus injections.

| NovoSeven - Bolus-Injection | |
|---|---|
| Initial dose until bleeding stops: | 4.5 kIU (90 µg)/kg bw, every 2h |
| in case of failure: | 6.0 kIU (120 µg)/kg bw , every 2h |
| 24h after beginning of the treatment: | 4.5 kIU (90 µg)/kg bw, every 3h |
| 48h after beginning of the treatment: | 4.5 kIU (90 µg)/kg bw, every 3–4h |

**Table 2.** Treatment schedule for recombinant FVIIa – continuous infusion.

| NovoSeven - Continuous Infusion (not licensed) | |
|---|---|
| Initial bolus: | 4.5 kIU (90 µg)/kg bw |
| Continuous infusion: | 1.0–1.5 kIU (20–30µg)/kg bw |
| *24 h stable at 25°C after reconstitution* | |

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## Case-Report

### History

The 12-year-old boy with severe hemophilia A (factor VIII <1%) developed a high-titer factor VIII inhibitor in early infancy. ITT with the Bonn protocol was without success. Recurrent bleedings were treated initially with FEIBA. Because of allergic reactions the "on demand treatment" was continued with NovoSeven in case of bleedings. Recurrent joint bleedings had led to the development of hemophilic arthropathy of several joints at stage II° – III° according to Arnold and Hilgartner [16]. For the clinical status of the joints see Table 3.

Long-time ITT had led to irreversible damage of his peripheral veins. In the past, several central venous lines (two Broviak-catheters and 4 port-a-cath systems) had been implanted. The actual port-a-cath was occluded by a thrombus and treatment of bleedings became impossible because of lack of venous access. Once more, a port-a-cath implantation was indicated.

**Table 3.** Clinical status of the patient's joints using the Neutral-0-Method

| Hemophilic arthropathy | | |
|---|---|---|
| Right knee | E/F | 0 - 5 - 140° |
| Left knee | E/F | 0 - 10 - 140° |
| Right ankle | E/F | 0 - 5 - 40°, reduced movement |
| Left ankle | E/F | 0 - 5 - 30°, reduced movement |
| Right elbow | E/F | 0 – 20 - 130° |
| Left elbow | E/F | 0 – 0 - 135° |
| Pronation/Supination normal | | |

(E=extension, F=flexion).

### Treatment Course

In Figures 1 and 2, rFVIIa bolus doses are shown as daily doses per kilogram (kg) body weight (bw) per hour (h). For example, on day 9 (Fig. 2) he received 12 boli of rFVIIa with 6 kIU/kg each, which amounted to 3 kIU/kg/h. In this way, doses of bolus treatment and continuous infusion can be compared easily.

The patient was prepared for surgery by starting a CI. He received a bolus of 180 kIU rFVIIa (4.5 kIU/kg bw), followed by CI with 60 kIU per hour (1.5 kIU/kg/h) as shown in Figure 1. At the beginning of the operation the boy showed respiratory symptoms that were suspected to be an allergic reaction to NovoSeven. Operation and the treatment with NovoSeven were stopped.

Two days later the boy developed an hemarthros of his right knee. The circumference of the knee increased 3.5 to 4 centimeters. Again CI with NovoSeven (1.0 kIU/kg/hour) was started, giving first a test dose with steroid and antihistamine premedication (Fig. 2). The treatment with NovoSeven was well tolerated but was without clinical effect concerning the bleeding of his knee. The swelling persisted.

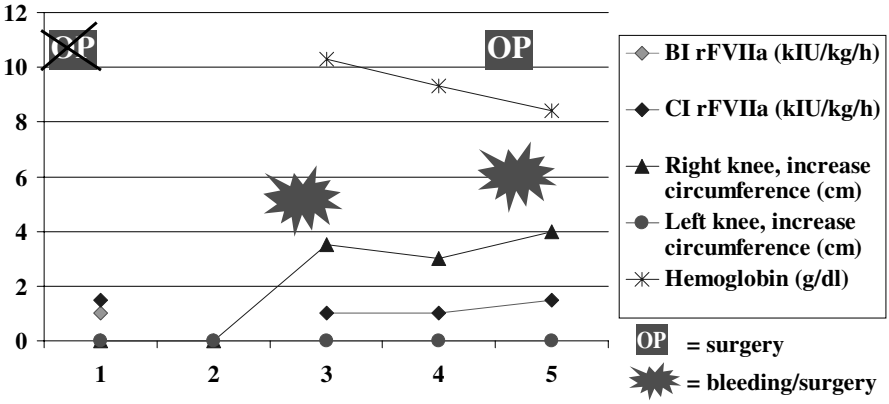


**Fig. 1.** Treatment course with rFVIIa (continuous infusion) before the first surgery (interrupted) and during a joint bleeding.

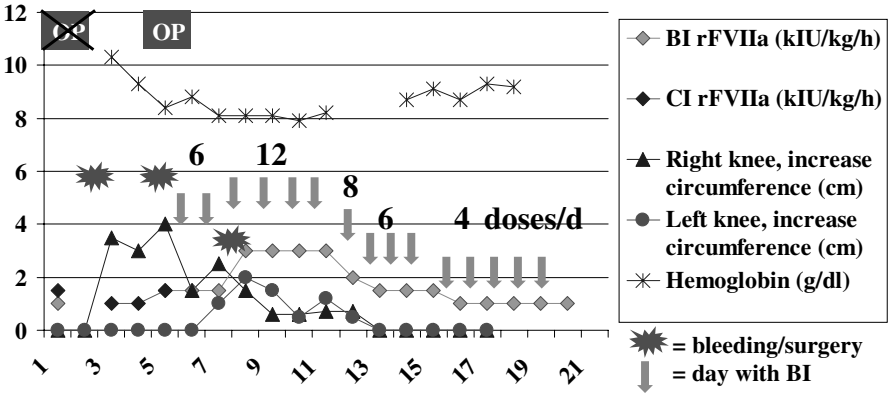


**Fig. 2.** The whole treatment course with rFVIIa during a port-a-cath implantation and for two joint bleedings. Comparison of two different treatment schedules: continuous infusion versus bolus injections.

The port-a-cath implantation took place two days later (day 5) under CI with NovoSeven (1.0 kIU/kg/hour) as shown in figure 2. Because of severe post-operative bleedings, with life-threatening swelling of the neck and the throat and a decrease in hemoglobin level (>2.0g/dl), the NovoSeven dose was increased up to 1.5 kIU/kg/hour without success.

After changing the treatment regime to bolus injections (240 kIU every 4 hours = 6 kIU/kg) instead of CI, the bleeding in the operative site stopped. Also, the bleeding in the right knee could be treated successfully although the daily dose had not been changed in comparison to CI.

Three days later (day 8) the boy suffered from a bleeding in the other knee under the treatment with rFVIIa. The circumference of the knee increased 2.0 cm. For the therapy of this bleeding, a higher dose of NovoSeven (240 kIU (6 kIU/kg) every 2

hours) was needed (Fig. 2). Therefore the frequency of the injections was increased using constant single doses. Also the higher daily dose was well tolerated without any side-effects and could be reduced after 5 days (day 13). At this time both knees had reached the original circumferences and no more signs of acute bleedings were seen. For the port-a-cath implantation the patient was treated for a total of 12 days with rFVIIa without side-effects. The initial suspicion of an allergic reaction to NovoSeven was not confirmed.

## Discussion

In this patient, a joint-bleeding of his right knee could not be treated effectively by CI. In addition, after port-a-cath implantation the boy suffered from life-threatening postoperative bleedings during a regular treatment with CI. Only when the treatment regime was changed and bolus injections were given, the bleedings could be treated successfully. So, in this patient, bolus injections of rFVIIa were better in controlling his bleedings than CI, although CI has been described to be effective and safe by several authors [9-15].

Recombinant FVIIa is approved as a "bypassing" agent to promote hemostasis in hemophilia with inhibitors. High doses of FVIIa (peaks) can optimize the thrombin formation via tissue factor. In addition, several studies suggest that high-dose FVIIa acts independently of its usual cofactor, tissue factor, to enhance platelet surface thrombin generation [17-19]. A platelet-dependent mechanism of action probably explains why factor VIIa does not cause systemic activation of coagulation, since the activated platelets on which FVIIa acts localize it to sites of injury.

In conclusion, the management of bolus injections of rFVIIa in the postoperative period seems to be more effective than CI in some patients and should be tried when continuous infusion failed. The daily dose does not necessarily have to be increased.

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# The Role of Therapy Regimen and Age at First Exposure on Inhibitor Development in Patients with Severe Hemophilia A

C. Escuriola Ettingshausen, R. Linde, I. Martinez Saguer, A. Zyschka, C. Kessel, H. Stoll and W. Kreuz

## Introduction

Today the development of neutralizing antibodies is one of the most serious complications in the replacement therapy with Factor (F) VIII and IX concentrates. Prospective studies with previously untreated patients (PUPs) demonstrated that severe hemophilia A patients are mostly affected (21-52%) within the first 50 exposure days (ED). Incidence is lower in patients with moderate or mild hemophilia A and hemophilia B [1].

Genetic risk factors as the patients´ ethnicity/race, the underlying gene mutation and to a lesser extent the HLA-type have an impact on the risk to develop inhibitors [2]. Recent publications indicate that early age at first exposure leads to an increased risk of inhibitor development in severe hemophilia A patients [3, 4]. In order to assess whether the risk of inhibitor development is associated with the age at first exposure and the treatment strategy during the initial phase of treatment with FVIII we are conducting a prospective PUP-study.

## Patients and Methods

Over a 24-years-study period (start 1979) a total of 74 severely affected hemophilia A-PUPs have been consecutively recruited at the outpatient clinic of the Children's University Hospital Frankfurt, Dept. of Hematology, Oncology and Hemostaseology. They have been treated regularly with FVIII concentrates. The following parameters which may have impact on inhibitor development were prospectively investigated: Type and severity of hemophilia, reason for first ED (bleeds, surgery etc), age at first exposure and at inhibitor development, inhibitor titer (at start of ITI, peak titer), number of exposure days (ED) until inhibitor development, therapy regime (prophylaxis vs on demand), type and dosage of factor concentrate administered, mutation type and HLA-type.

From 1976-1992 inhibitor testing according to the Bethesda method was performed before the first exposure to FVIII and thereafter every 20th ED or in any situation of suspected inhibitor formation. Since 1993 inhibitor testing was done more frequently: Before the first ED, every 3rd-4th exposure day (ED) for the first 20 EDs, thereafter until the 200th ED every 10th ED, after the 200th ED every 3 months.

Additional testing is recommended in any suspicion of inhibitor development.

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Rarely exposed patients should be followed up every 3 months.

Definitions of inhibitors: Positive inhibitor = 0.6 Bethesda Units (BU). Low titer inhibitor (0.6–5 BU), high titer inhibitor >5BU.

### Therapy Regimens

Primary prophylaxis: Regular prophylactic FVIII administration in order to prevent bleeds (25-40 IU FVIII/kg bw 3 times per week or every other day) started before or immediately after the first relevant bleeding episode.

A prophylactic treatment is defined as a secondary prophylaxis when started after 2 or more bleeds.

## Results

Until January 2003, 23 out of 74 severe hemophiliacs developed an inhibitor (31%).

Preliminary evaluations over the years of this still ongoing study showed decreasing inhibitor incidences for patients with severe hemophilia A (Ehrenforth et al, Lancet 1992: 52% [5]; Kreuz et al, Sem Thromb Haemost 1999: 43% [6]; 2003: 31%). More than half of those patients developed high titer inhibitors (>5 Bethesda Units).

### Age at First Exposure to FVIII and Inhibitor Development

No linear correlation was found between the age at first exposure to F VIII and inhibitor formation. However, in the group of very early exposed severe hemophiliacs (age at first ED <0.5 years) inhibitor incidence was remarkably high (62%, 5/8 patients) (Table 1).

**Table 1.** Inhibitor incidence dependent on age at first exposure to FVIII (n.a.-data not available)

| Age at 1st ED [years] | Inhibitor/total patients |
|---|---|
| < 0.5 | 5/8 (62.5%) |
| 0.5–1 | 7/32 (22%) |
| 1–1.5 | 4/16 (25%) |
| > 1.5 | 4/13 (31%) |
| n.a. | 3/5 |
| total | 23/74 (31%) |

### Therapy Regimen and Inhibitor Development

Inhibitor incidence was 0% (1 transient inhibitor out of 23 patients) in those patients who received prophylaxis before or immediately after the first relevant bleed and 42 % (18/43 patients) in case of delayed prophylaxis or on-demand treatment. A significant difference in inhibitor incidence was found between the two groups (p=0.002) (Table 2).

**Table 2.** Inhibitor incidence dependent on the therapy regimen

| Therapy regimen | total (n=74)<br>Inh/total |
|---|---|
| Primary prophylaxis (start 0.8-2 yr) | 0/23 (0%)*<br>(1 trans./23) |
| Secondary prophylaxis or on-demand treatment (age >0.5 yr) | 18/43 (42%)* |
| Inhibitor development during treatment of 1st bleed (age<0.5 yr) | 5/8 (62.5%) |
| total | 23/74 (31%) |

*p=0.002 (Fisher's exact test); trans (transient inhibitor); yr (year)

## Discussion

Evaluations on inhibitor incidence over the years revealed a continuously decreasing incidence in our patient cohort. The decreasing inhibitor incidence might be explained by the changing therapeutic strategies over the years: In the 80ies patients were usually treated on-demand and long-term prophylaxis was started after several bleeds. Since the early 90ies long-term prophylaxis was started earlier in severe hemophilia A patients. Accordingly the majority of those patients started prophylaxis at the age of 1 year (before or immediately after the first relevant bleeding event).

Patients starting prophylaxis before or immediately after the first relevant bleed showed a significant lower inhibitor incidence than those treated on-demand in the initial phase of FVIII replacement therapy. In a group of Swedish patients who started prophylaxis at early age, inhibitor incidence was also low for severe hemophilia A patients [7]. According to these observations a protective character of early prophylaxis can be discussed and should be investigated in a larger number of patients. This evaluation should include the mutation type as well as the type of concentrate used.

Age at first exposure did not correlate with the occurrence of inhibitors. However, we found a high rate of inhibitors in patients who were treated at very early age (<0.5 years; 62.5%). All those patients received clotting factor concentrate for treatment of severe bleeding or for surgical interventions. This observation confirms the hypothesis that early age at first exposure is associated with a high risk to

develop inhibitors. On the other hand we could not confirm, that severe hemophiliacs, who get their first exposure at the age >1.5 years show a low inhibitor incidence as postulated by the Spanish and the Dutch group [3, 4].

In order to confirm the influence of age at first exposure and therapy regimen in the initial phase of treatment on inhibitor development in severe hemophiliacs a larger number of patients should be investigated.

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# Presentation of the Inhibitor-Immunology-Study

I. Wieland, C. Wermes, K. Welte and K.-W. Sykora

**Abstract**

We are presenting a new study in which risk factors for the development of inhibitors in patients with hemophilia are to be explored. The ultimate goal is to find out why some children suffering from severe or moderate hemophilia develop inhibitory antibodies during replacement therapy and others do not. Genetic and immunological risk factors defining the individual tendency to develop inhibitors against coagulation factors VIII or IX are to be explored.

About 31% of the patients with a factor VIII replacement therapy develop a factor VIII inhibitor. Well-known predisposing factors for inhibitor formation are genetic features of factor VIII, which include large deletions, nonsense mutations or intrachromosomal recombinations. Also, ethnic groups other than Caucasians (e.g. Africans) have a higher risk of inhibitor development.

Other risk factors are presumably derived from the immune system.

Within the scope of our study we want to compare the following factors between patients with and without inhibitors and between patients and the normal population:

1. T-cell and B-cell activation by factors VIII or IX, studied by the synthesis and secretion of cytokines. We want to show differences between hemophilic patients with and without inhibitors and a normal population as well as between high and low responders. The influence of the HLA-antigens is also to be examined. Also, allergic reactions, infections and vaccinations could influence inhibitor development and should be documented.

2. Genetic polymorphisms in immune-response associated genes. The characterization of genetic polymorphisms including CTLA-4, IL-4, IL-5, IL-10 and IL-6 are emphasized. The correlation between some genotypes or polymorphism in cytokines and the development of inhibitory antigens is to be studied.

## Introduction

The development of inhibitors is one of the most important complications of the replacement therapy in hemophilia affecting mortality and morbidity. The development of inhibitory antigens is based on complex immunological factors, and to date, too little is known about the basic mechanisms of inhibitor development.

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About 31% of the patients with a factor VIII replacement therapy develop a factor VIII inhibitor. From these are 23% low-responder (<5BE) and 77% high-responder (>5BE) [8]. In the case of severe hemophilia B, about 10.5% of the patients develop inhibitory antibodies [9]. Anti-factor VIII-antibodies are also seen in 15-78% healthy people without hemophilia [7, 18, 20]. Lacroix-Desmazes et al. [10, 11] showed anti-idiotypic antibodies neutralizing the inhibitory activity of the anti-factor VIII antibodies in healthy people.

Well-known predisposing factors for inhibitor formation are genetic features of factor VIII, which include large deletions, nonsense mutations or intrachromosomal recombinations [5, 24]. Also, ethnic groups other than Caucasians (e.g. Africans) have a higher risk of developing inhibitors. Other risk factors are presumably derived from the immune system. For instance, a reduction of the inhibitor was seen with lower CD4+ T helper cell counts in HIV positive hemophilic patients [3, 4]. The development of inhibitors is very likely to be a Th-2 mediated event where cytokines and their receptors, T-cell receptors and the major histocompatibility complex may also play an important role.

## Theoretical Background

The substituted factor is an unknown protein for patients with a severe hemophilia. Unknown proteins are taken up in macrophages by pinocytosis, split into peptides and bound to MHC class II molecules. This complex is transported to the cell membrane and is presented to T cells on the cell surface. The peptide MHC class II complex is recognized by the T cell receptor. The T cell is activated by binding of the complex to the T-cell receptor. Costimulatory molecules, including the B7 - CD28 interacting molecules, provide additional signals and start the differentiation into the Th-1 or Th-2 direction. Th-2 specific T cells produce IL-4, IL-5, IL-6, IL-10 and TGF-β, leading to the switch to IgG4 and IgE. Th-1 specific T cells cause the switch to IgG1 and IgG3 by production of IL-2, IFN-γ and TNF (Fig. 1).

Three epitopes in the structure of the factor VIII molecule were identified against which most antibodies are directed. These are regions in the A2, C2 and A3 domain [13]. In healthy people and in patients with hemophilia without inhibitory antibodies the most frequently recognized epitopes are located in the A3 and A1 domain [7]. Lacroix-Desmazes et al. [10] proposed that, in patients with hemophilia and inhibitory antibodies, affinity maturation and hypermutation of the V-region of the antibodies take place.

In previous studies, no significant association between HLA class and inhibitor development was found [1, 12, 14, 16]. Later, different studies could show a higher risk of developing inhibitors when special factor VIII mutations were combined with special HLA class II molecules. For example, higher inhibitor development was associated with HLA-DRB1*1501/ DRB1*0602/DQA1*0102 in patients with the Intron 22 inversion and with HLA-DRB1*01/DQA1*0101/ DRB1*0501 in patients without the intron 22 inversion [6, 15].

Factor VIII and IX inhibitory antibodies are mostly IgG-type immunoglobulins, especially subclass 4, rarely subclass 1 [4]. Therefore, inhibitor development was

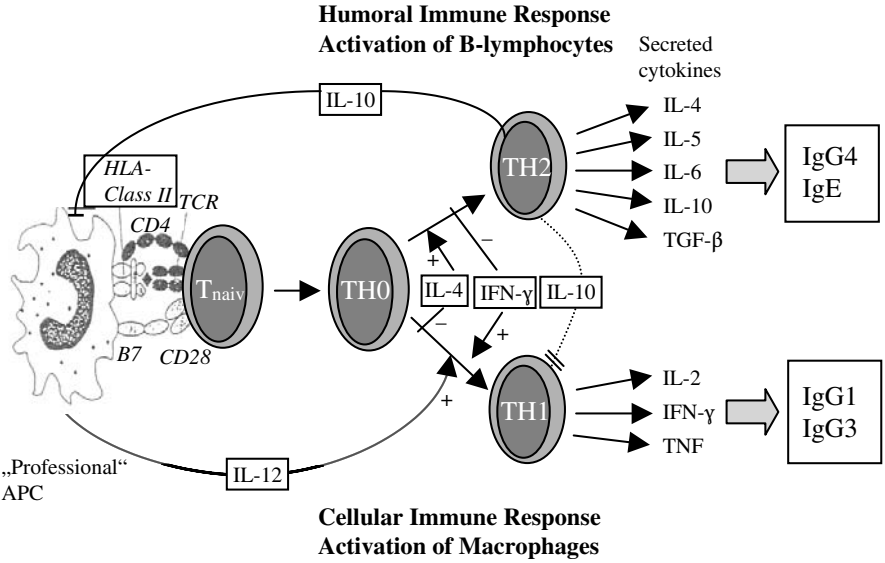


**Fig. 1.** The normal immunoresponse (according to Baenkler et al. [2])
Abbreviations: TCR - T cell –receptor; APC – antigen presenting cell

considered to be a Th-2 mediated event. However, Reding at al. [19] could show that Th-1 specific immunoglobulins play a more important role than expected before. If the patient has a high inhibitor titer, IgG4 antibodies were more commonly found. In the case of low anti-factor VIII antibody titers, IgG1 and IgG2 immunoglobulins were dominant. This was also true when the low inhibitor titer was achieved by immune tolerance therapy. During this therapy, a switch occurred from the Th-2 to the Th-1 direction.

**Table 1.** Different dominance of cytokine expression in different groups

| Dominance | Wu et al. (2001) | Sasgary et al. (2002) | Reipert et al. (2001) |
|---|---|---|---|
| 1 | IL-10 | *INF-γ* | *IL-2* |
| 2 | *INF-γ* (some) | IL-10 | *INF-γ* |
| 3 | IL-4 (one) | *IL-2* | IL-4 not always |
| 4 | IL-4 + *INF-γ* never *IL-2* | IL-10 + *INF-γ* rather not IL-4 | |

Th-2 specific cytokines: IL-4, IL-10.
Th-1 specific cytokines: *INF-γ, IL-2.*

In order to have better conditions studying the development of inhibitory antibodies, mouse models were established. Partly conflicting results were reported about the dominance of either Th-2 [25] or Th-1 [21, 23] specific cytokines. All authors agree that both Th-2 and Th-1 specific T cells are involved in the development of anti factor VIII antibodies (Table 1).

Mouse models also showed that the development of anti-factor VIII-antibodies was inhibited by the blockade of the costimulatory interactions of B7/CD28 [17, 18] and CD40-CD40L interaction [21, 22].

## The Inhibitor-Immunology-Study

The ultimate goal of this study is to find out why some children suffering from severe or moderate hemophilia develop inhibitory antibodies during replacement therapy and others do not.

Within the scope of our study we want to compare the following factors between patients with and without inhibitors and between patients and the normal population:

1. T-cell and B-cell activation by factors VIII or IX, studied by the synthesis and secretion of cytokines.
2. Genetic polymorphisms in immune-response associated genes.

Goals for question 1 are:
- the determination of the differentiation tendency in Th-1 or Th-2 direction,
- the quantification of the factor VIII or IX reacting CD 4-T-cell count,
- the determination of the pattern of the synthesis, expression and secretion of Th-1 or Th-2 specific cytokines in CD 4-T-cells after the stimulation with the coagulation factor
- the determination of factor VIII or IX binding B-cells
- the concentration of the cytokines in the blood of the patient with the Hemophilia in the initial time of treatment.

Goals for question 2 are:
- the characterization of genetic polymorphisms of genes that are associated with the immune response (for instance cytokines)
- the determination of the distribution of the alleles and genotypes.

The characterization of genetic polymorphisms including CTLA-4, IL-4, IL-5, IL-10 or IL-6 are emphasized. The correlation between some genotypes or polymorphism in cytokines and the development of inhibitory antigens is to be studied.

In order to elucidate these goals we want to answer the following questions:

Are there differences in the activation of CD 4-cells defined by HLA-Cass II antigens by factor VIII or IX?

Are there differences in the production of cytokines in the Th-1 or Th-2 direction by CD 4- T-cells?

How is the expression and secretion of cytokines changed during different states of treatment or during the development of inhibitors?

Are there differences in the distribution of genetic polymorphisms in immune response associated genes?

The following methods are planned: All patients and controls are HLA-class II-typed. Then, the CD 4– T-cells are isolated and stimulated with coagulation factors VIII or IX as well as tetanus toxoid and intracellular cytokine synthesis is measured by FACS-analysis. Cytokine secretion is measured by Elispot. Genetic polymorphisms are characterized by PCR and sequence analysis.

This study is an open, longitudinal case control study. The patients are not randomized. The patients are divided into four groups. These groups are:

I. No inhibitor development,
II. Low responder (< 5BE),
III. High responder (> 5BE),
IV. Patients in different phases of immune tolerance therapy.

Participants can be all patients with a hemophilia needing regular factor replacement therapy.

In part 1 of the study, a minimum of 100 patients should be included.

Part 2 will start with a pilot phase in order to test how many patients would be necessary to show a significantly different distribution of genotypes between patients with or without inhibitors. This pilot phase will be the basis for the decision whether sufficient statistical power can be achieved with the available patient population to see a difference between inhibitor- and non-inhibitor-patients for a specific genetic variant. If no significant differences can be expected for a variant, examination of this variant will not be pursued anymore.

In addition, a control group of healthy blood donors and children corresponding in gender and age will be recruited.

The only exclusion criterion for part one is a known HIV-infection. There are not exclusion criteria for part two.

Since a single centre will not be able to recruit a sufficient patient number it is planned to establish this study as a multicentric study.

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# Current Clinical Investigations Involving FEIBA

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The management of hemophilia A and B is complicated by the appearance of neutralizing antibodies to factor VIII or factor IX in roughly one third of hemophilia A patients, and in about 3% of hemophilia B patients. This condition leads to a reduction in the FVIII or FIX pharmacokinetics, which, depending on the inhibitor status and the hemorrhagic situation, may or may not be managed with increased factor dosage, or may necessitate the use of bypassing agents. FEIBA VH (FEIBA S-TIM4) has for a number of years been a well established safe and efficacious therapeutic for hemophilia patients with an inhibitor. Over the years, clinicians have developed innovative strategies to manage these clinically challenging patients through various hemorrhagic circumstances. In order to better understand the effectiveness of these approaches, a number of clinical research projects have been started in order to study prophylactic use, comparisons with other therapeutic options regarding efficacy and pharmacoeconomics, but also surgical applications, the latter having become a focus of attention in the advent of newly adapted therapy monitoring technologies

There are three projects that I will focus on: The FENOC project, the Prophylaxis FEIBA Study and the monitoring of FEIBA VH therapy by the thrombin generation assay.

## The FENOC Study

In the FENOC study, Feiba is compared with NovoSeven in a comparative efficacy analysis. This is a multicenter study with the principal investigator being Jan Astermark, but with leadership also being provided by Drs. Berntorp, Gringeri and Di Michele. There are collaborating centers in Europe and also in North America providing study subjects for the project. In addition there is a study data and safety monitoring committee with leadership provided by Dr. Lou Aledort. This is an investigator-driven study sponsored by the University of Lund with funding provided to the University of Lund by Baxter. The objectives are to assess and compare the hemostatic effect of a single dose of FEIBA VH with that of two doses of NovoSeven on joint bleeds in hemophilia A patients with inhibitors. Secondary outcomes include the evaluation of the average number of doses required for each concentrate to achieve hemostasis, and the cost-efficacy of each treatment will also be assessed.

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The study is a prospective, open-labeled, randomized, cross-over study. We are targeting 60 patients for accrual into the study. Clinical evaluations will extend over a period of time and the key inclusion criteria are patients who are at least 2 years of age with hemophilia A and have an inhibitor. Each study subject with a joint bleed will be randomized to receive either product A or product B. The site of hemorrhage may be either an ankle, knee or elbow, with product A being used to treat the first joint bleed, or it could be product B. The alternate therapeutic agent will be used for the subsequent joint bleed in cross-over fashion. This clinical trial is designed as an equivalence study with the goal of demonstrating that the two drugs differ by less than 15% in efficacy with a power of 95%. The study drugs are FEIBA at a dose targeting 85 U/kg, ranging between 70-100 U/kg as one dose. NovoSeven is being used with a target dose of 105 μg/kg with a range of 90-120 μg/kg as two doses given two hours apart. The infusion to manage the joint bleed for the study could be carried out either at home or in hospital, but therapy should be initiated within 4 hours of the start of the hemorrhage in the specific joint for the study. The evaluation of the joint bleed from the point of view of efficacy, will take place at 6 hours post infusion. The treatment as to the dosage as well as any subsequent infusions – either FEIBA or NovoSeven – will be at the discretion of each investigator. It is expected that the achievement of efficacy at 6 hours will result in the study subject exiting that particular arm of the study. However, if there is continuing bleeding, then again at the discretion of the investigator, there will be the recommendation to continue with the same therapy. Thus repeat infusions with NovoSeven at 2 hour intervals, or at 12 hours intervals with FEIBA will be given until the bleeding ceases. Immunosuppression and anti-fibrinolytics will be permitted after the key efficacy analysis at 6 hours. The primary endpoint will be hemostatic effect at 6 hours post infusion. The secondary endpoints will be

a) Hemostatic effect at the end of 2 hours (in the case of NovoSeven related to the first infusion) and also 12, 24 and 48 hours post infusion.
b) The average number of infusions required for hemostasis.
c) The pain score.
d) The use of analgesics.
e) Sub-analysis of target joints.
f) Rebleeds and
g) Cost-efficacy assessment both at 6 and at 48 hours post infusion.

This study has been ongoing for a couple of years, with 28 patients in Europe and United States being entered to date. Further eligible patients have been identified and we are hoping to close the enrollment in late spring of next year.

### The Prophylaxis-Feiba (Pro-FEIBA) Study

The Pro-Feiba study will evaluate the use of FEIBA VH in a prophylaxis situation. This is a prospective, randomized, multicenter, international cross-over study focused on patients with inhibitors who require bypassing agents in the management of their bleeding episodes. This is an investigator driven study with Dr. Cindy

Lessinger as the principal investigator and Professor Piero Mannucci as co-principal investigator. Acute hemorrhagic episodes in patients with persistent inhibitors, particularly of high titer, pose a substantial therapeutic challenge. The prevention of bleeding episodes is a well-established avenue of management for patients who do not have inhibitors. However, from an anecdotal point of view, prophylaxis is now standard of care in a few centers for patients with inhibitors. Consequently this approach forms the basis of the study rationale. In addition, there are also some reports using FEIBA and also NovoSeven in the prevention of bleeding. These agents are also used to control bleeding at the time of surgery, but at this point in time, there are no prospective studies or clinical trials to evaluate the efficacy and safety of FEIBA for regular prophylaxis of hemorrhages in patients with inhibitors. The study design is focused on a treatment period of 6 months at approximately 85 U/kg + 15% three times weekly with FEIBA. Once this is completed, there will be a washout period of approximately 3 months with the patient's physician determining the therapeutic agent and dose to manage acute bleeds. Then the patient will enter a period of ›demand‹ therapy also for 6 months. Patients will be randomized at entry to either the ›prophylaxis‹ or the ›demand‹ arm, so some patients will initially go onto ›demand‹ therapy, then to washout and then ›prophylaxis‹, whereas the other half will be the opposite way around.

The primary endpoint of the Pro-Feiba study will be the number of relevant bleeds on both the prophylaxis and demand arms of the study. The secondary outcomes will be change in inhibitor titers, laboratory indications of thrombin generation and thrombogenicity, treatment-related adverse events, differences related to the sequence of study arms and, of course, pharmacoeconomic parameters. The study statistics is based on an estimated reduction in the number of bleeds on prophylaxis at about 50% and the power is 80%. Therefore we expect to target 42 patients for entry with the number of patients needed to meet these numbers being 36. This study is in the process of final development phase, centers are being recruited, and the study protocol is in the process of going through IRBs with some centers having approval and study accrual being initiated at a small number of centers at this time. So this study has started.

### The Thrombin Generation Assay

The thrombin generation assay is conceived as a procedure to monitor the management of bleeding episodes in patients with inhibitors undergoing FVIII-by-passing agent treatments while undergoing surgery. Ideally this procedure would be employed as a bedside assay to control efficacy and safety for these inhibitor bypassing therapies. This technology, focused on the measurement of thrombin that is generated, has been developed by a number of laboratories. The technology that is currently being evaluated under clinical circumstances, has been developed by my colleagues Drs. Turecek and Varadi who are located in Baxter's Vienna research laboratories. This is being currently employed in laboratories and under clinical research circumstances in Germany, but also in other countries as well, including data that is being generated by Professor Negrier in Lyon where he has studied this

specific thrombin generation assay in 2 patients to evaluate the »half-life« in patients A and B. With »half-life« we define the time to reduction to 50% of thrombin generation starting from maximum values. He has observed that the half-life was much longer in patient B than in patient A with 250 versus 300 min. This technology is also being evaluated in other laboratories from the point of view of a meaningful application of the use of FEIBA VH. We hope it would be a sensitive routine assay for perioperative monitoring and also as pilot investigations to understand the link between thrombin generation and the clinical response.

## *III. Orthopedic Problems and Therapy in Hemophiliacs*

Chairmen:

I. Scharrer (Frankfurt/Main)
A. Kurth (Frankfurt/Main)

# Major Orthopedic Reconstructions in an Inhibitor Patient – A Case Report

U. Stumpf, C. Eberhardt, M. Krause, W. Miesbach, I. Scharrer and A. A. Kurth

## Abstract

Inhibitors against FVIII or FIX in patients with hemophilia are a common and serious complication. Until recently elective surgery was associated with major bleeding despite of a substitution therapy. Only emergency medical care was done if necessary. We report about one patient with hemophilia A and inhibitors who underwent 3 major reconstructions of the right knee. Because of severe joint destruction due to hemophilic arthropathy a total knee replacement was necessary. After half a year a periprosthetic fracture of the femur occurred. An open reduction and an internal fixation was performed. In the rehabilitation phase after the second operation a second fracture of both the hardware and the bone occurred. We were faced with the problem, that we had to remove the distal part of the femur and the joint replacement and reconstruct it with a mega-tumor prosthesis. This is to our knowledge the first patient with inhibitors undergoing such a complicated reconstruction of a limb. All operations were done under factor VIIa substitution. Even in this high risk patient the operations were done without any bleeding complications. After a follow up of nine months after the last operation no complications like infections, recurrent bleedings, or limited range of motion were seen.

## Introduction

Patients with hemophilia A (FVIII deficiency) or hemophilia B (FXI deficiency) who receive factor concentrates may develop antibodies (IgG) to factors and prevent their effectiveness. This development of FVIII inhibitors remains one of the most serious complications in the treatment of patients with hemophilia. The incidence of inhibitors is about 5–32 % [1]. New prospective studies confirmed that young children with severe hemophilia A are at highest risk of inhibitor development [1]. Until recently elective surgery was mostly associated with major bleeding despite a substitution therapy. Only emergency care was done if necessary. Recombinant FVIIa (rFVIIa, NovoSeven) is a potentially effective hemostatic drug. Its beneficial effect was demonstrated in hemophilia patients with inhibitors to FVIII or FIX in several trials [2, 3] and the use of rFVIIa in these patients is generally perceived [4]. NovoSeven (rFVIIa) is a recombinant product, which contains no human derivates and no traces of FXIII or FIX. So no rise of inhibitor titres

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under this treatment must be expected. Recurrent spontaneous hemarthros results in significant joint damage and require surgical intervention. Contrary to some predictions that the use of prophylaxis would eliminate orthopedic complications of hemophilia there will be a need for orthopedic surgery because of the more active living of the patients, which may result in more sports related injuries, and an aging population with an increase of degenerative arthritis.

## Case Report

A 43-year-old male patient suffers from a severe hemophilia A with an inhibitor. He is HIV and HCV, HBV and HAV positive. He has a very active daily living with sports and motor-biking. He had to undergo three major reconstructions of the right knee within 8 months. As a result of recurrent hemarthros he developed a severe hemophilic arthropathy over years involving several joints. He already got an arthrodesis of the right elbow in 1998 and of the left knee in 1999. His right knee showed progressive loss of function with maximal limitation of range of motion and difficulties to walk. Based on this the indication for elective joint replacement was given. All following substitutions of rFVIIa (NovoSeven)/ tranexamic acid (Anvitoff)/ Feiba were done in close interdisciplinary cooperation with the hemophilia ambulance of the University Hospital Frankfurt/Main (Prof. Scharrer). The first operation, the implantation of a cemented constrained hinge knee replacement, was done under substitution in combination with Feiba (prothrombin complexes), NovoSeven (rVIIa) and Anvitoff (tranexamic acid). Postoperative course showed no further bleedings, no infections, and under physiotherapy full weight-baring after 6 weeks. A sufficient function of the knee with a flexion of 90° could be achieved. Only six months later a traumatic periprosthetic fracture of the right femur occurred. Under immediately started substitution with NovoSeven (rFVIIa) and Anvitoff (tranexamic acid) an osteosynthesis with LCDC-plate could be done. Five days after surgery the substitution could be changed from Novo Seven/Anvitoff to Feiba. In the postoperative course the patient was restricted to no weight bearing for eight weeks. The further mobilization was good, and no further complications like bleeding episodes or infections occurred. Two months after this second operation we were faced with another fracture of the right femur (secondary dislocated spiral femur fracture with loosening of the implanted osteosynthesis material of the femur fracture), which took place within the first weight-baring test of physiotherapy without trauma.

Because of the large bone defect in the right distal femur we decided to implant a modular tumor prosthesis Type MUTARS (ImplantCast, Buxtehude, Germany). For this application parts of the prosthesis were custom made. This prosthesis should replace the total right knee, bridge the bone defect of the distal femur (20 cm) and was anchored in the proximal 1/3 of the femur. During the time to manufacture and deliver the implant the fracture was stabilized by an extension bandage with 5 kg and strict bed rest. The posttraumatic hematoma was controlled with a substitution of FEIBA (6000 IU 12h). Two days prior the third and largest operation, substitution was changed from Feiba to NovoSeven / Anvitoff to start surgery under optimal conditions. In this reconstruction of the right limb a bone defect of

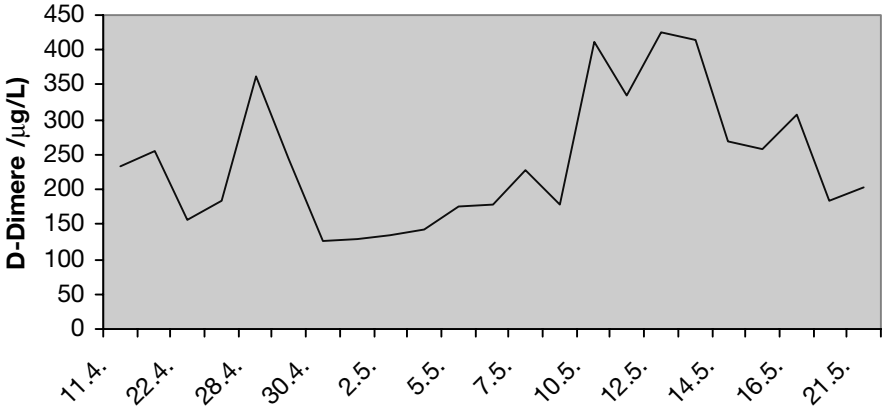


**Fig. 1.** Course of d-dimers Feiba/NovoSeven/Feiba

the distal femur of 24 cm had to be bridged. The proximal anchorage was achieved by outstanding of a part of the prosthesis between trochanter major and femur neck. During the implantation of the tibial part a fissure of the distal tibia occurred. This was stabilized by cerclage-bandage. The resting holes of the trephine after the plate osteosynthesis in the femur were stabilized with cerclages, too. Intraoperative and postoperative hemostasis was secured with NovoSeven. Postoperative x-rays showed the properly positioned prosthesis and no further fracture of the remaining bone. Within the follow-up of the last nine months neither further bleeding episodes, nor infections were seen. With regard to function a full range of motion was achieved. The rehabilitation started carefully: for the first eight weeks no weight-baring of the left leg was allowed, mobilization started with a wheel-chair. Weight-baring was slowly increased with scrutches, up to full weight-baring of the right limb was achieved. Flexion in the right knee within the first two weeks after surgery was only up to 60° allowed. Then flexion was carefully raised up to actually 90°. Further x-ray controls showed no signs of loosening or fractures.

It is mandatory to reflect about the costs of such an outstanding procedure in an inhibitor patient. The substitutive therapy rFVIIa (NovoSeven) and Feiba: Knee prosthesis (Type Blauth): NovoSeven was € 1,015,004.52; LCDCP osteosynthesis: € 592,028.46 NovoSeven 444,847.66 €/Feiba 147,180.90 €). Implantation of MUTARS prosthesis right limb: 1,026,096.34 € (NovoSeven 879,292.02 €/Feiba 326,804.34 €). The total reconstruction of the right limb with the necessary factor substitution was 2,813,129.32 €. Even in emergency situation we try to keep a close contact and give early information to the patients health insurance. In this special case all costs were reimbursed by the health insurance company.

## Discussion

We conclude that it is possible to perform major reconstruction of a limb even in patients with hemophilia A and inhibitors. Under therapy with NovoSeven the

bleedings are intraoperative under control even in operations with large wounds. This is to our knowledge the first patient with inhibitors undergoing such a complicated reconstruction of a limb.

The incidence of infection after surgery is higher in patients with hemophilia, HIV and hepatitis C infection than in the general population. We are aware of this risk and avoided infection by combined antibiotic prophylaxis intra- and postoperative. However, the benefits of this procedure are substantial and must be weighed against the risks.

Substitutive treatment is always a combination; successful hemostasis is not possible without adverse events: one of the adverse events of Feiba (prothrombin complexes) is the increase of d-dimers under treatment; tranexamic acid (Anvitoff) is used for inhibition of fibrinolysis under NovoSeven. The increasing of d-dimers level was in the expected range after such large operations, if it is getting too high due to the experience of our hemophilia center this was indication for us to reduce the units of Feiba per day.

After stabilizing the patient postoperatively we changed the therapy from NovoSeven to Feiba. The costs of such an procedure can not be judged disregarding the benefits of prevention of invalidity, the ability to return to work and the improvement of life quality.

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# Total Hip Replacement in Patients with Severe Bleeding Disorders
# A 30 Years Single Center Experience

A.A. Kurth, C. Eberhardt, U. Stumpf, I. Scharrer and L. Hovy

## Introduction

Hemophilic arthropathy of the hip is less common in patients with bleeding disorders.

If the destruction of the hip joint is severe enough replacement of the joint is indicated.

## Material and Methods

We performed a retrospective analysis of the cases done at our center to determine the outcome of hip replacements in this specific group of patients.

In July 1972 the first elective total hip replacement (THR) in a hemophilic patient was performed at the University Hospital in Frankfurt. Between then and 2002 in 13 patients 15 hips were found to be necessary to replace. In 10 hemophilic patients and 3 VWD patients a hip replacement was performed. The mean age of the patients was at the time of surgery 42 years. During that time we changed the implants used for the replacement. At the beginning we used only cemented cups and stems. Later hybrid THR was use with a cemented stem and a cementless cup. Recently we use only cementless implants even in those high risk patients.

The charts of the patients were evaluated for complications during surgery, early complications after surgery, survival of the implants, cases of revision surgery, and infections. To determine the functional outcome the widely accepted Harris Hip Score was calculated for the time before surgery and at the time of follow up. The earliest follow-up was 12 months after surgery.

## Results

Good long term results after a follow up time of 132 months (12–363) were found. No perioperative complications like bleeding or infection were found. Only one aseptic loosening of a cemented cup occurred after 14 years as well as one septic loosening 14 months postoperatively in a HIV positive patient. Another HIV positive patient developed a hematogenic abscess on both operated hips without loosening.

The calculated survival time after a mean follow up of 11 years was 86.7%.

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The Harris Hip Score increased remarkably from 48 points (range 32 – 66) preoperatively to 89 points (range 76 – 100) at the time of follow up. We found excellent and good results in 72%, fair results in 19%, and bad results in 9% of the cases.

## Discussion

End-stage hemophilic arthropathy is further complicated by arthrofibrosis and loss of motion as the hypertrophic synovium is replaced by dense fibrous scar tissue. Arthropathy of the hip is moderate in frequency but is less common than ankle, knee or elbow arthropathy. It has two modes of onset. In childhood, rapidly progressive severe arthropathy may result from a single hemarthrosis because of increased intracapsular pressure leading to osteonecrosis of the capital femoral epiphysis [1]. More often, hip arthropathy is the result of chronic synovitis similar to that which occurs in other joints. Between the second and fourth decades many hemophilic patients develop severe articular destruction. At this stage, possible treatments include arthrodesis and arthroplasty. For the hip, total hip arthroplasty remains the best solution [2].

Teitelbaum [3] reviewed pelvic radiographs of 34 patients complaining of pain in the hip (64 hips) from a population of 175 patients seen at one hemophilia centre. Sixteen (80%) of 20 hips that had an open proximal femoralepiohysis had a valgus deformity, but none had osteoarthritic changes. Fifteen (31%) of 48 skeletally mature hips had degenerative changes, including protusion acetabuli in eight hips. Thus, end-stage hemophilic arthropathy necessitating arthroplasty is infrequent in the hip.

There are few reports of the results of total hip arthroplasty in patients with hemophilia. The indication for a total hip arthroplasty in young patients who have hemophilic arthropathy of the hip should be severe disabling pain occurring both during activity and at rest that is unresponsive to non-operative treatment.

The first important series was published by Luck and Kasper of the Orthopedic Hospital, Los Angeles, USA in 1989 [4], announcing that the first prosthetic arthroplasty of the hip in a hemophiliac in the United States was a cup arthroplasty performed by J. Vernon Luck in 1968. Luck and Kasper reported that between 1968 and 1982, three more cup arthroplasties and ten primary, cemented hip arthroplasties were performed at the Orthopedic Hospital by six different surgeons. Prostheses varied with surgeon and include Müller (five cases), Ausfranc-Turner (two cases), Charnley (one case), Harris (one case) and Wagner resurfacing (one case). Since 1982, two cementless primary hip arthroplasties had been performed.

Eight patients had required revision, and one required an excision arthroplasty. The revisions included three cup arthroplasties because of pain and bleeding, three cemented total hips for aseptic loosening, two cemented total hips for infection, and one Wagner resurfacing for a fracture of the femoral head sustained in a fall 5 years postoperatively. One patient had a Girdlestone resection arthroplasty for a pseudomonas aeruginosa infection. Thus, five of nine conventional, cemented total hip arthroplasties had failed over the last 16 years. Including the three cup arthroplasties, eight revisions had been performed. Seven of these patients were symptom-free. One patient developed Serratia marcescens infection 1 year after revision. In

another of the revision case, there was radiographic evidence of component loosening. This cup arthroplasty was revised to a cemented Charnley low-friction prosthesis in 1972. The femoral component loosened at 3 years and subsided, but it had stabilized and remained asymptomatic over the last 12 years.

The long-term experience of Luck and Kasper with various types of hip prostheses can be rated as only fair, with a revision rate of about 60% over 20 years. However, the clinical status of all patients was quite satisfactory in that all were free from hip pain and were capable of unlimited, unassisted, community ambulation. They were substantially better than they had been prior to their initial hip surgery. End-stage hip disease in hemophilic patients poses an unsolved problem. Primary cemented prostheses have a 33% aseptic failure rate 5-14 years after operation, which is higher than would be expected in a comparable group of patients with another form of polyarthritis. One reason for the loosening of cemented hip prostheses in hemophilic patients may be the increased stresses of a stiff-legged gait.

In 1992, Nelson et al. [5] reported the experience of the Nuffield Orthopedic Centre, Oxford, UK. From 1969 until 1985, 39 total hip arthroplasties were performed in 38 patients for hemophilic arthropathy. The median age of the patients at operation was 48 years. In 21 patients, 22 hip replacements were reviewed clinically and radiographically, with a median follow-up of 7.6 years. Five of the 22 hips had been revised and three were likely to require revision in the near future. The incidence of revision was compared to other studies of total hip arthroplasty in young patients and the influence of HIV infection was examined. Total hip arthroplasty was believed to be an appropriate operation for disabling hemophilic arthropathy.

As hemophilic arthropathy infrequently affects the hip joint, Kelley et al. [6] in 1995 reported a multicenter retrospective study to determine the results of hip arthroplasty in hemophilic patients. Thirty-four hip arthroplasties were performed in 27 male patients at four major hemophilia centres between October 1972 and September 1990 in the United States.

The mean age of patients at the time of operation was 38 years (range 15-73 years). Four patients were seropositive for HIV at the time of the operation, and 16 patients were seropositive at the latest follow-up examination. Nine patients (33%) died before the latest review, seven of whom had been seropositive for HIV. The mean duration of follow-up was 8 years, with a minimum of 2 years for all patients who were still alive at the latest review.

Surgery was carried out as follows: 26 total hip arthroplasties performed with cement, six total hip arthroplasties performed without cement, one total hip arthroplasty in which the femoral component was inserted with cement and the acetabular component was inserted without it (so-called hybrid arthroplasty), and one bipolar arthroplasty performed with cement. There were no early infections after these 34 primary arthroplasties. Three late infections occurred around prostheses inserted with cement, all of which led to a resection arthroplasty. Six (21%) of the 28 cemented femoral components and six (23%) of the 26 cemented acetabular components were revised because of aseptic loosening.

Of the 24 cemented femoral components for which radiographs were available and which were still in place at the time of latest review or at the time of death, ten were definitely loose, two were probably loose, five were possibly loose and seven had

no evidence of loosening. Of the 23 cemented acetabular components for which radiographs were available and which were still in place at the time of review, ten were definitely loose, seven were probably loose, three were possibly loose, and three were not loose. None of the cementless prostheses was loose. There was a high rate of loosening of the cemented hip prosthesis in this series. There was also a high rate of mortality overall, and a high rate of late deep infection in the patients who were seropositive for HIV. Kelley et al. advised caution when a total hip arthroplasty is considered for a patient with hemophilia. Despite the aforementioned complications, Kelly et al. stated that total hip arthroplasty has a continuing role in the treatment of hemophilic arthropathy in patients who have severe pain and disability.

During 1973-88, at the University Hospital, Malmö, Sweden, 13 total hip replacements were performed in 11 hemophilic patients with a mean age of 46 years. According to Löfqvist et al. [7], the indication for surgery was disabling pain due to advanced hemophilic arthropathy. The surgical technique was the same as for other patients: cemented Charnley prostheses were inserted, using the transtrochanteric approach. The mean duration of follow-up was 7 years. Five hips became loose within 6 years, and a further one after 13 years. Four hips were revised, two of them due to infection in patients who were also seropositive for HIV. At the latest follow-up, ten patients were alive, six had no hip pain, and seven could walk a distance of at least 1000 meters. Although these results were inferior to those obtained in arthrosis, total hip replacement should be considered in patients with hemophilia. Löfqvist et al. concluded that this group of patients can expect a fairly high frequency of aseptic loosening after total hip replacement. HIV-positive patients also seem to have an increased infection rate. However, according to their findings, they concluded that total hip replacement is of value in some hemophilic individuals.

In 1998 Heeg et al. [8] evaluated the long-term results of three total hip arthroplasties. One hip was revised after 9 years for aseptic loosening.

During 1976-99, six total hip arthroplasties were performed in six hemophilic patients at La Paz University Hospital, Madrid, Spain. The indication for total hip arthroplasty in people with hemophilia was severe disabling pain, both during activity and at rest, that was unresponsive to non-operative treatment. The mean age of the patients was 42 years (range 35-47). Four Harris-Galante hybrid prostheses (acetabular component without cement and a precoated femoral component with cement), one uncemented isoelastic prosthesis and one cemented Charnley arthroplasty (for an ankylosed hip; see next section in this chapter) were used in these procedures. At the time of the index hip arthroplasty, no patient was known to be seropositive for HIV. The mean duration of follow-up for all the patients was 7 years (range 1-15 years). One patient (the one with the isoelastic prosthesis) died before the time of this review, and five were alive. So far, both clinical and radiographic results are satisfactory.

Studies from several hemophilia centres suggest that between 33% and 92% of patients with hemophilia B, carry the HIV antibody [9]. In two studies of hip arthroplasty for hemophilic arthropathy with more than 20 patients and more than 5 years follow-up, approximately 50% of the patients were known to be seropositive for HIV, contributing to an overall mortality rate at median 7-year follow-up of 20% to 33% [5,6]. Patients with CD4 levels of greater than 500 cells/$mm^3$, a positive re-

action with energy testing to intradermal skin antigens, platelet count greater than 60 000, total leukocyte count greater than 1000, serum albumin greater than 25g/l, and no history of opportunistic infections of neoplasm have a postoperative complication risk similar to the general population.

In addition to thorough preoperative medical preparation of the patient, considerable surgical preparation is also required. Depending on the age at which significant bleeding began, the proximal anatomy of the femur can be distorted and, in the most severe cases, there can be an extremely small femoral medullary canal, valgus and excessive anteversion of the head and neck, and protrusion acetabuli [10].

## Conclusion

Despite FVIII prophylaxis and better joints in hemophilic patients this group of patients will get older and arthritis of the hip is an increasing problem in the older generation. Regardless hemophilic arthropathy hemophilic patients will develop hip and knee arthritis and the need for surgery will increase.

We conclude after more than 30 years of experience with THR in hemophilic patients and based on our positive results that for advanced severe hemophilic hip arthropathy, THR results in pain-relief and an improvement in quality of life. Thus, total hip replacement should be considered regardless the age of the patient and the surgical intervention should be performed in specialized centers with experience in the treatment of bleeding disorders.

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# MRI Staging of Hemophilic Joints in Comparison with the Radiological Score and the Clinical Dates of Children and Young Adults

R. Jentzsch, H. Lenk, D. Weber and F. Schmidt

## Introduction

The magnetic resonance imaging (MRI) is currently establishing itself as a significant procedure in the diagnostics of hemophilic arthropathy.

Various MRI scores (Nuss, Funk) have been designed on the basis of MRI findings to demonstrate quantitative and qualitative pathologic changes in joints. In a lot of cases, it is difficult for pediatricians [6] to evaluate the radiologic results and to use them as a parameter of progression. Often, it is not easy to find a connection between the clinical dates of a patient and his partially extensive MRI findings.

The aim of this study was to investigate the value of the MRI diagnostics in assessing the hemophilic arthropathy of children and young adults in comparison with their radiological and clinical dates.

## Patients and Methods

31 MRI examinations (T1 and T2 weighting, with and without contrast medium) of 23 hemophiliacs at the age of 8 to 29 have been performed. The target joints were defined as joints with the highest bleeding rate per lifetime of the patient. 21 patients suffered from a hemophilia A and two young adults suffered from a hemophilia B. According to their age, patients were divided into three groups. The average age was 17.4 years.

19 out of 23 patients suffered from a severe hemophilia (4/19 with an inhibitor), 3 out of 23 suffered from a moderate hemophilia and 1 out of 23 (1/1 with an inhibitor) patients suffered from a mild hemophilia. 2 boys had an active inhibitor. In the case of two patients the inhibitor was eradicated and one patient has had a transient inhibitor.

**Table 1.** Age of the patients

| age in years | number of patients | group |
|---|---|---|
| 8–14 | 6 | 1 |
| 15–18 | 9 | 2 |
| 19–29 | 8 | 3 |

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**Table 2.** Overview - bleeding episodes

| bleedings / joint per lifetime | 1-3 | 4-6 | 7-10 | 11-20 | >20 | Total |
|---|---|---|---|---|---|---|
| number of joints | 10 | 6 | 6 | 6 | 3 | 31 |

18 out of 23 patients received prophylactic treatment since their childhood and 5 out of 23 were treated on demand. All together, 31 joints (13 ankles, 10 knees and 8 elbows) were examined. The most affected joint regarding bleedings per lifetime was the right ankle (8/31). Only joints with at least one reported bleeding were investigated. The mean of bleedings per joint per lifetime amounted to 8.9 (median 6.5), and the average yearly bleeding frequency came to 0.59 bleedings per year. The bleeding episodes of our patients are separately listed in Table 2.

A partly retrospective and partly prospective study on only hemophilic patients, which showed no other joint associated diseases, was accomplished. From each target joint we performed a MRI examination, a conventional x-ray and a clinical investigation. Only joints without bleeding symptoms between the examinations and without bleedings at the time of investigation were included. Those inspections were carried out in a short period of time. Through anamnestic research we were able to determine quite precisely the total number of bleedings which occurred in each investigated joint. The radiological joint score [1,4,7] was evaluated by two radiologists according to the Petterson score (12 p.max.), and the orthopedic joint score (12 p.max.) was calculated through a pediatrician as recommended by WFH. The MR images were evaluated according to a score that we set up by ourselves (Table 3). This score is a modified score of Funk et al [2,5]. In addition, we rated the

**Table 3.** modified MRI

| criterion of value | dimension | points |
|---|---|---|
| effusion/ bleeding | small | 1 |
| | large | 2 |
| hemosiderin present | small | 1 |
| | large | 2 |
| synovial hyperplasia | small | 1 |
| | large | 2 |
| subchondral cyst | 1 cyst | 1 |
| | >1 cyst | 2 |
| erosion/destruction of the bony joint surface | single erosion | 1 |
| | < 50% | 2 |
| | > 50% | 3 |
| cartilage loss | surface lesion | 1 |
| | < 50% | 2 |
| | > 50% | 3 |
| bone marrow edema | present | 1 |
| maximum score | | 15 |

»bone marrow edema« (which is close to joint) with 1 point and graded the »presence of hemosiderin« into 2 severity degrees. MRI was performed with a field strength of 1.5 Tesla (Magnetom VISION-Siemens Erlangen) with the following method: T1w, T2w, DESS 3D and in the most cases with contrast medium (0.2 ml/kg Gd-DTPA).

## Results

17 out of 31 joints (54%) showed radiological perceptible alterations. Regarding to the clinical score (max. 12 points) of the investigated joints, 42% (13/31) had 0 points, 32% (10/31) had 1 point, 17% (5/31) had 2 points and 9% (3/31) had 3 points.

Regarding to the Petterson score of all examined joints, 45% (14/31) had 0 points, 29% (9/31) had 1-4 points and 26% (8/31) had 5-8 points. The MRI score of each investigated joint was higher than 0. Our patients showed the following results in the MRI score: 32 % (10/31) had 1-2 points, 29% (9/31) had 3-8 points and 39% (12/31) had 9-14 points.

11 joints had neither in the clinical score nor in the Petterson score any joint alterations respectively positive score values (11/31=0 points). Those 11 joints had 3.27 bleedings on average and a medial MRI score of 2 points (6/11 had 1 point, 3/11 had 2-3 points and 2/11 had 4-5 points in the MRI score).

By means of this selection one can see the significant predominance of the MRI diagnostics compared to the conventional radiological diagnostics.

We found a rather weak correlation ($r=0.63$, $p<0.01$ Spearman) between the age of the patients and the MRI score. A significant connection could be found between the MRI score and the Petterson score ($r=0.84$, $p<0.01$ and $r=0.88$, $p<0.01$ Spearman).

As far as the number of bleedings and the Petterson score are concerned, we could only find a weak relationship between them, but between the number of bleedings and the MRI score we determined a significant correlation ($r=0.69$, $p<0.01$ and $r=0.76$, $p<0.01$ Spearman).

Figures 2 and 3 show a right knee with 3 bleedings, which appeared until to the day of the examinations, of an 18 years old patient, who was given prophylactic treatment since 1991.

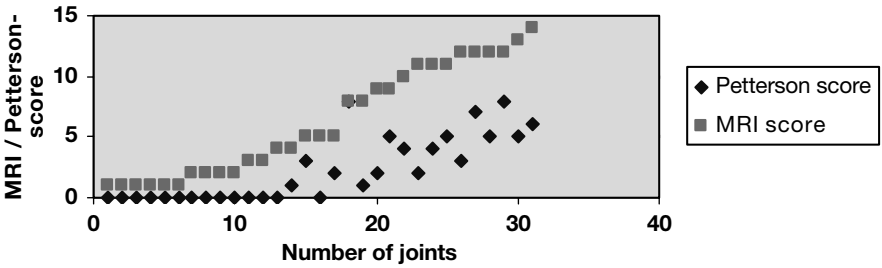


**Fig. 1.** Correlation between MRI and Petterson-score

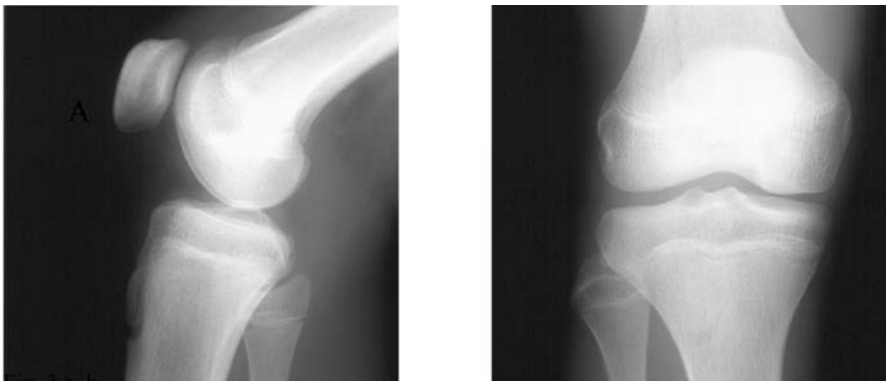

**Fig. 2a, b.** x-ray of a right knee (anterior-posterior and lateral)

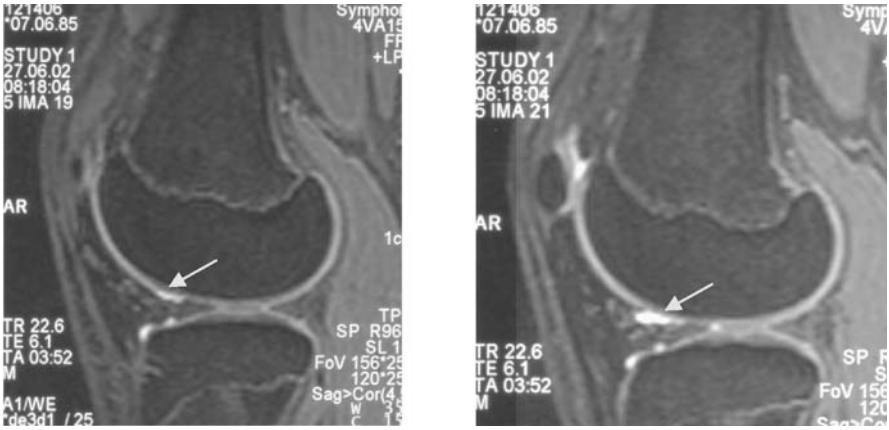


**Fig. 3a, b.** MRI, DESS 3D right knee

His factor VIII residual activity is on average between 4-5 %. He has a MRI score of 4 and a Petterson score of 0. As a band of high strength of intensity one can see in Figure 3a and 3b the hyaline articular cartilage of the right distal femur. The arrow (in the Figs. 3a and 3b) is pointing out the clearly visible superficial damage of cartilage on the femoral surface of joint with effusion. On the other hand one can not see any pathological findings in analyzing the conventional radiographies.

Table 4 shows a comparison of the established scores (Petterson and clinical score) with our modified MRI score and the number of bleedings of patients suffering from only a few joint bleedings.

In joints with less than 5 bleedings, one can see, with few exceptions, no pathological findings in the Petterson score as well as in the clinical score. However, in the MRI score, those joints show distinct findings (MRI score from 1-5 points).

**Table 4.** Representation of number of bleedings, Petterson score and MRI score of patients with few bleedings.

| joint | age | Petterson score | MRI score | bleedings/ lifetime | grade of hemophilia* | clinical score |
|---|---|---|---|---|---|---|
| 1 | 8 | 0 | 1 | 1 | 1 | 0 |
| 2 | 8 | 0 | 1 | 1 | 1 | 0 |
| 3 | 15 | 0 | 1 | 1 | 1 | 0 |
| 4 | 11 | 0 | 2 | 2 | 3 | 1 |
| 5 | 12 | 0 | 1 | 2 | 1 | 0 |
| 6 | 17 | 0 | 2 | 2 | 2 | 1 |
| 7 | 18 | 1 | 4 | 2 | 3 | 1 |
| 8 | 10 | 0 | 1 | 3 | 1 | 0 |
| 9 | 18 | 0 | 4 | 3 | 2 | 0 |
| 10 | 13 | 3 | 5 | 4 | 1 | 1 |
| 11 | 14 | 0 | 5 | 4 | 1 | 0 |
| 12 | 15 | 0 | 2 | 4 | 1 | 0 |
| 13 | 18 | 0 | 1 | 4 | 1 | 0 |
| 14 | 20 | 2 | 5 | 4 | 1 | 2 |
| 15 | 23 | 0 | 2 | 6 | 2 | 0 |
| 16 | 29 | 5 | 12 | 7 | 1 | 1 |
| 17 | 18 | 0 | 3 | 7 | 1 | 1 |
| 18 | 22 | 8 | 8 | 7 | 1 | 3 |

(* 1=severe hemophilia, 2=moderate hemophilia, 3=mild hemophilia)

The percentage distribution of the specific MRI findings [9] is shown in Table 5. Joints where we were not able to value the specific alterations like synovial hyperplasia or hemosiderin were mainly joints, where we could not perform a MRI examination with contrast medium for technical and other individual reasons.

The alteration to be found most frequently emerged to be effusion – intraarticular fluid. The assessment of the quantity of the effusion is difficult. Every person has some fluid in his or her joints. Due to this fact, the pathogenicity of the findings has to be evaluated only with reservations. A real pathological finding exists, if the score at the MRI score in the rubric »effusion/bleeding« amounts to more than one.

**Table 5.** Distribution of specific MRI findings

| modified MRI-score | MRI with findings | impossible to grade | total no. of MRI | in % |
|---|---|---|---|---|
| effusion/bleeding | 25 | 0 | 31 | 80.65 |
| cartilage loss /damage | 21 | 0 | 31 | 67.74 |
| subchondral cysts | 18 | 0 | 31 | 58.06 |
| hemosiderin | 16 | 2 | 31 | 51.61 |
| synovial hyperplasia | 15 | 4 | 31 | 48.39 |
| erosion/ destruction of the surface of the joint | 18 | 0 | 31 | 58.06 |
| bone marrow edema | 7 | 0 | 31 | 22.58 |

The synovial hyperplasia could only be valued sufficiently in the cases of patients which we had been given contrast medium [3]. 9 out of 31 joints were investigated without contrast medium. In 5 of those joints, it was not possible to evaluate the synovial hyperplasia sufficiently. The remaining 4 joints without contrast medium could only be graded partially: in the single categories of the MRI score.

In 22 of the 31 joints we had administered contrast medium; and we diagnosed a synovial hyperplasia in 50% of them. It turned out that there is a significant difference between joints with and without synovial hyperplasia regarding to the number of bleeding per joint per lifetime.

In our patients on average, 13.2 bleedings occurred in joints with synovial hyperplasia and only 3.2 bleeding in joints without synovial hyperplasia.

The independent-samples T-Test of Student confirms this predication ($p=0.02$).

## Discussion and Summary

There are 2 reasons why we have less patients of younger age; first because of the drop of number of birth during the last 10 years in our country and secondly due to the fact that we determined a minimum age of 6 years to avoid investigating children with an anesthesia.

The partly low correlation between the age and the MRI score of the patients has to be attributed partially to the fact that several patients with an inhibitor are still in the younger age group, but have already suffered a lot of bleedings in their joints. As a result, they had a higher MRI score.

The reason for the more exact graduation of the presence of hemosiderin is based on the prognostic relevance of the product of catabolism of hemoglobin regarding the maturation and the worsening of the synovial hyperplasia [10], and therefore for the development of an arthropathy.

We added the bone marrow edema (which is close to the joint) to our MRI score because this finding was manifest in 7 of 31 patients. In one example, a bone marrow edema at the superior margin of the talus in the right ankle of a young man turned into a considerable osteonecrosis within the period of only 4 years. This example shows the prognostic meaning of this finding.

In cases of patients with 1 to 4 bleedings per joint per lifetime we found, with only a few exceptions, no radiological changes, but a MRI score of 1-5 points.

This shows the higher sensitivity of the MRI regarding to the representation of joint specific pathological changes in comparison with the conventional radiography.

It was not until frequent bleeding occurred in a joint, that alterations in the radiological picture were demonstrable [8]. The MRI as a diagnostic tool is prepotent in comparison with the radiological x-ray diagnostics, especially in joints with just few bleedings. Due to this fact, and in agreement with other authors [2], it should be used preferably in these cases.

It would be very helpful to perform more MRI examinations of joints which are not affected by bleedings to determine the objectivity of MRI-findings in hemophilic joints.

During the MRI examination the synovial hyperplasia as a prognostic parameter can only be evaluated sufficiently, if contrast medium will be given.

Even for patients who already have radiological perceptible alterations, the MRI leads to a more differentiated predication of the status of the joint, regarding to the valuation of hemosiderin, the synovia and of the dimension of the damage of the articular cartilage.

Investigations have shown that manifest pathological alterations do already appear after a small number of joint bleedings. These changes can be demonstrated with the MRI very well. This predication supports the recommendation for an early start of prophylactic treatment.

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# Orthopedic Knee Replacement in Hemophilic Patients

H.H. Wolf, H. Reichel, O. Dorligshaw and H.J. Schmoll

Hemophilic arthropathy is a major complication in hemophilia and may become cause of immobilization and social disability.

After multiple spontaneous or traumatic bleedings acute synovitis may occur.

Deposits of hemosiderin induce chronic synovitis resembling hypertrophy, hyperplasia and hypervascularisation of the synovial membrane followed by intra-articular fibrosis and extra-articular muscle contracture.

Cartilage and bone destruction lead to severe malformation and axial deviation with contractures, chondral atrophy, erosive destruction, and subchondral cysts. Figure 2 shows radiographs of an hemophilia B patient born in October 1959, who presented at the age of 7 years with pain, swelling, and axial deviation due to hemorrhages into the left knee.

In 1998, the patient has been admitted to our hospital for knee replacement surgery.

Radiological examination showed complete destruction of the knee, immobilization, and loss of knee function. Furthermore, we found axial deviation as well as shortening of the femur after fracture and inadequate reposition.

Usually, replacement surgery is performed in the elderly. In hemophilic patients, however, severe arthropathy impairs patient's quality of life in an early age [1].

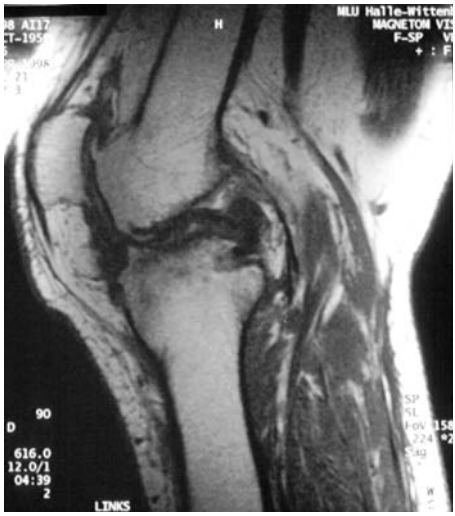


**Fig. 1** Magnetic resonance imaging of the knee: destruction of cartilage and bone structure; hemophilia B patient, 38 years old

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34th Hemophilia Symposium Hamburg 2003

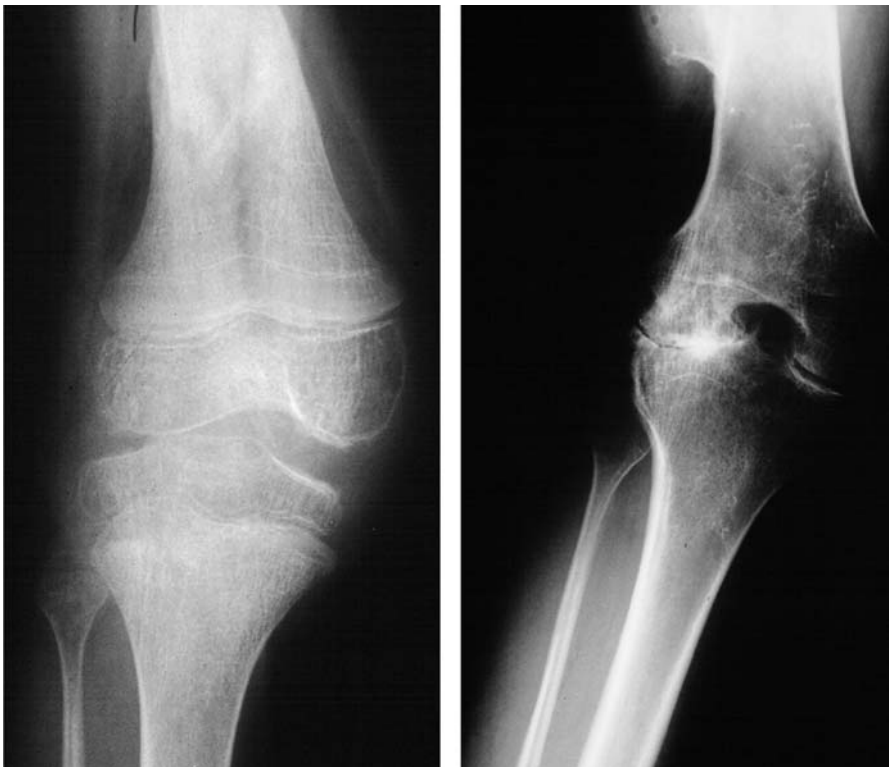

**Fig. 2.** Radiographs of the right knee, hemophilia B patient, 7 and 38 years old
Hemarthros with axial deviation in the boy, ossification of the joint in the adult patient

As tendinal and muscular contractures may complicate further orthopedic replacement, surgery should be done in an earlier course of disease compared to arthropathy patients.

Different protocols exist in hemophilic centres concerning time to undergo knee replacement and perioperative substitution therapy.

## Patients` Characteristics

We report the clinical course of 7 hemophilic patients (hemophilia A 6 patients, hemophilia B 1 patient) who underwent knee replacement surgery.

Plasma activity of coagulation factor VIII or IX was < 1 %.

Patients` age was 27 to 62 years, median age 47.3 years.

Hemophilic arthropathy stage IV was found in 1 patient, stage V in 6 patients according to the Arnold and Hilgartner classification.

All patients suffered from severe pain and impairment of knee function.

Stage II or III hemophilic arthropathy of the upper ankle was found.

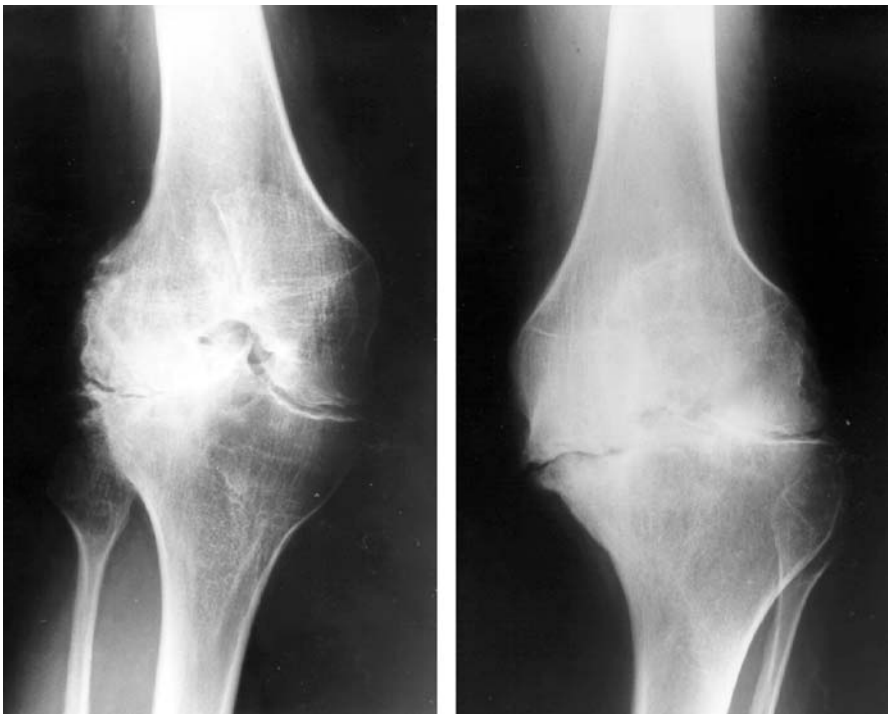

**Fig. 3.** Severe hemophilic arthropathy, hemophilia A patient

### Surgical Therapy

Implantation of the prostheses was performed after arterial closure mostly via medial dissection. However, in 2 patients osteotomy of tuberositas tibiae became necessary.

Endoprostheses of the whole knee with cemented shafts have been performed in 4 joints; prostheses with cementless shafts with minimal fixation in 10 joints.

Intraoperative extension was complete and flexion was at least 80 degrees. Postoperatively, flexion was found to be at least 60 degrees up to day +5, and 90 degrees up to day +14, respectively.

Active training had to be performed using CPM extension.

Only in 1 patient replacement of the second knee was performed consecutively within a 5 month period.

Surgery was performed within 98 to 295 minutes time, depending whether there were one or two surgeons to perform arthroplasty simultaneously.

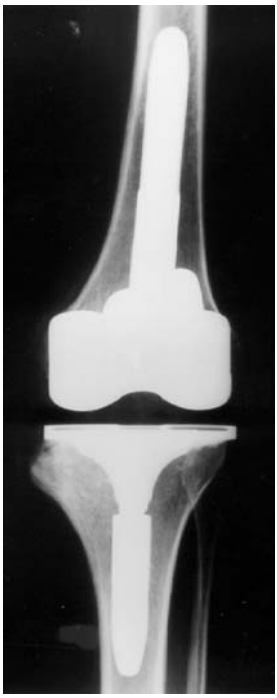

**Fig. 4.** Postoperative radiographs of the left knee, hemophilia A patient.

### Hemostaseologic Therapy

Substitution therapy with coagulation factor concentrates was performed as bolus injection in 3 patients, as continuous infusion in the following patients. All hemophilia A patients were treated with recombinant FVIII products.

Continuous infusion therapy and reduction of bleeding were correlated positively.

Postoperative blood loss was 75–940 ml, depending on the modus of administration.

Substitution therapy started the day before surgery by bolus injection of 40 IU/kg body weight. The same dosage was administered immediately before surgery as well as postoperatively.

Continuous infusion was performed in 3 patients with hemophilia A with recombinant FVIII products, in 1 patient with hemophilia B with plasmatic derivative.

In order to maintain median plasma concentration of 70% substitution of FVIII/FIX concentrates was done:

Median dosage administered was 50 IU/kg bw until day + 8, 30 IU/kg bw day + 9 to +15, respectively.

The median total dosage of coagulation factor concentrates was 92,000 IU (range 80,000 to 152,000 IU) depending on modus of administration. The dosage

was lower in patients treated with continuous infusion than with bolus injection regimen.

Thromboembolic prophylaxis was performed with low-molecular heparin subcutaneously day 1–15. On day 15, patients started out-patient training.

## Results

HSS-Score improved significantly (34.5 to 77.9).

Functional results were found to be good (excellent n= 4, well n=7, sufficient n=2 joints, respectively).

Function of only 1 prosthesis was found to be not sufficient.

Median time of follow-up was 3.7 years (1 up to 7 years). There was no radiological evidence of instability.

## Complications

No postoperative hemorrhages have been found. No development of inhibitors has been seen. 6 years after implantation, late infection of bilateral endoprostheses by staphylococcus species was seen in one patient.

**Table 1.** Functional results after bilateral arthroplasty of the knee

| patient | age (years) | preoperative function (degree) | postoperative function (degree) | difference (degree) | follow-up (years) |
|---|---|---|---|---|---|
| A | 49 | left 0-30-45<br>right 0-30-50 | left 0-30-45<br>right 0-5-90 | + 45<br>+ 65 | 7.7<br>7.2 |
| B | 27 | left 0-25-100<br>right 0-10-90 | left 0-15-80<br>right 0-0-90 | – 10<br>+ 10 | 5.1 |
| G | 49 | left 0-20-85<br>right 0-15-90 | left 0-0-110<br>right 0-0-110 | + 65<br>+ 35 | 4.1 |
| D | 46 | left 0-30-100<br>right 0-35-100 | left 0-5-110<br>right 0-5-100 | + 35<br>+ 30 | 3.2 |
| E | 38 | left 0-20-40<br>right 0-5-50 | left 0-10-100<br>right 0-0-110 | + 70<br>+ 65 | 1.5 |
| Z | 62 | left 0-30-40<br>right 0-20-60 | left 0-5-60<br>right 0-0-60 | + 45<br>+ 20 | 1.3 |
| I | 50 | left 0-40-70<br>right 0-15-50 | left 0-0-90<br>right 0-5-50 | + 60<br>+ 10 | 1.1 |

## Conclusions

Arthroplasty of the whole knee improved motility and function in younger hemophilic patients [2].

Simultaneous bilateral arthroplasty was performed without intraoperative bleeding complications in patients with continuous infusion therapy of coagulation factor concentrates [3, 4].

We prefer continuous infusion compared to bolus injection in order to proof stable and sufficient plasma concentrations of coagulation factors [5].

Prospective studies are required to define state of arthropathy for the patients to undergo surgery, modus of surgery (unilateral or simultaneous bilateral arthroplasty), as well as material of endoprosthesis which has to be used.

Bilateral arthroplasty may be useful concerning postoperative functional training and cost reduction, but intraoperative hemorrhage could become a complication in case of insufficient substitution.

Data are requested whether continuous infusion or bolus injection provide more safety and comfort to patients and physicians.

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## *IV. Therapy with Protein C*

Chairmen:

A. Sutor (Freiburg)
P. Knöbl (Vienna)

# Treatment of Adults with Sepsis-Induced Coagulopathy and Purpura Fulminans with a Plasma-Derived Protein C Concentrate (Ceprotin)

P. Schellongowski, U. Eidher, E. Bauer, P. Schenk and P. Knöbl

## Introduction

One of the most important pathophysiological effects of sepsis and septic shock is a disturbance of the hemostatic equilibrium. A pronounced activation of the procoagulatory mechanisms, together with a shut-down of fibrinolysis, leads to the formation of fibrin microthrombi in the microvasculature. The consumption of coagulation factors and platelets enhances the bleeding tendency – the result is the clinical picture of disseminated intravascular coagulation (DIC). The resulting hypoperfusion of tissue causes damage of organs: septic patients are considerable sick, need intensive treatment and have, in part, severe compromised organ dysfunction (MODS), associated with a high mortality [1].

A distinct manifestation of sepsis-associated DIC is purpura fulminans with its typical cutaneous morphology (Fig. 1). The coagulopathic processes in the microcir-

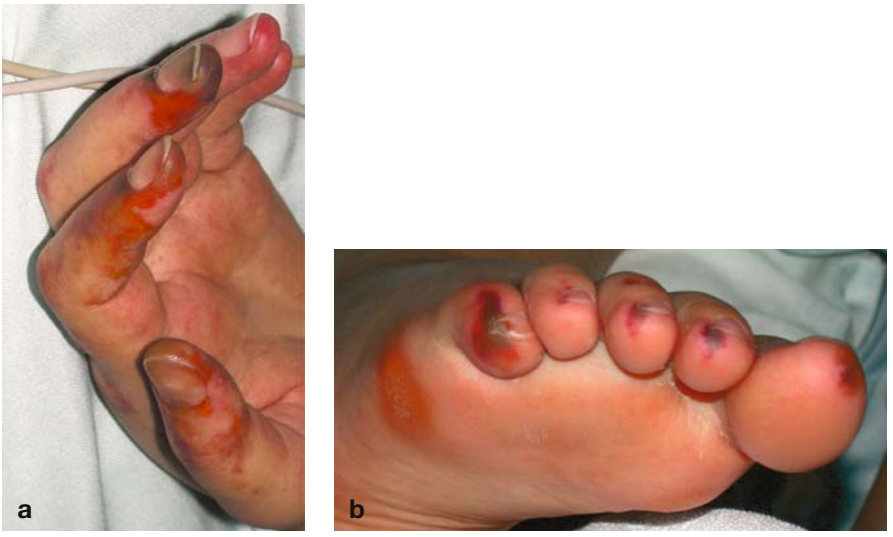


**Fig. 1.** Example on skin lesions of purpura fulminans. **a** peripheral microembolization with inflammatory reaction; **b** peripheral microembolization with inflammatory reaction

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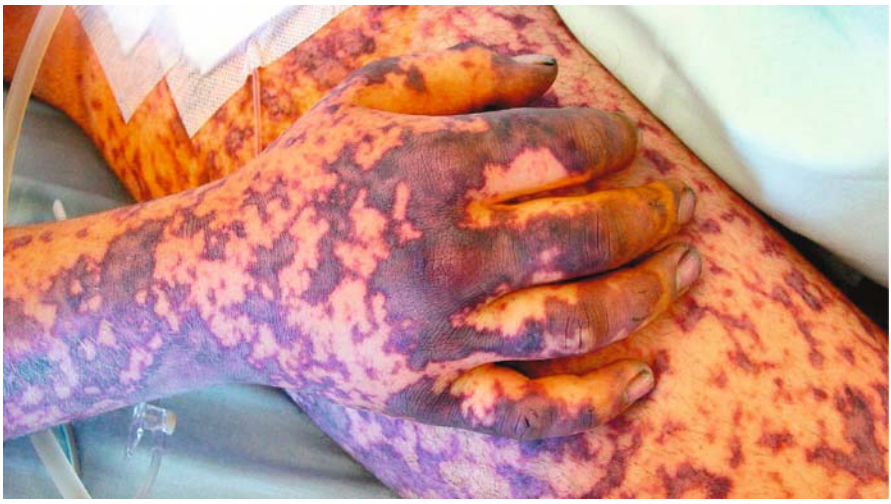

**Fig. 1c.** generalized occurrence of purpura fulminans at the skin

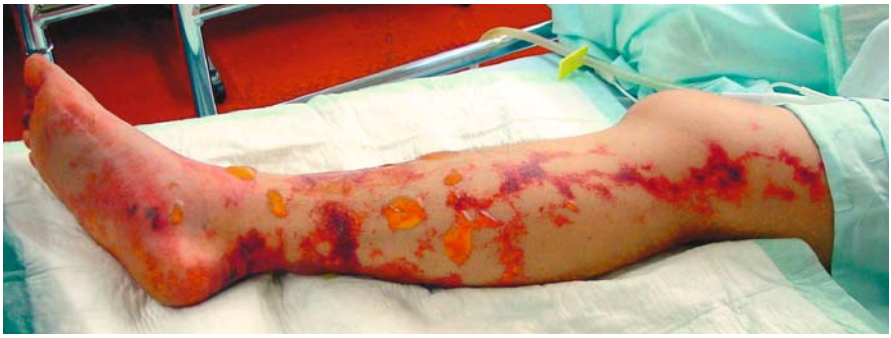

**Fig. 1d.** lower limb with skin lesions of purpura fulminans, edema, bullae, and beginning compartment syndrome

culation manifest on the one hand as microthrombosis and necrosis, and on the other hand as petecchiae, ecchymosis and sometimes hemorrhagic bullae. Often, even acral gangrene develops. Furthermore, there is an accompanying enhanced inflammatory reaction. The trigger of sepsis induced purpura fulminans is often an infection with some kinds of pathogens, for example neisseria meningitidis, streptococcus pneumoniae, or others. A dysfunction of the protein C pathway is always present in purpura fulminans and a reason for coagulopathy and necrosis. Besides low levels of fibrinogen and antithrombin, a pronounced decrease of the protein C activity is obvious. This is caused either by consumption during systemic activation of blood coagulation and by reduced hepatic synthesis due to septic liver dysfunction, but also by degradation of protein C by proteolytic enzymes released by white blood cells, as elastase. This results in a markedly shortened half life time of protein C compared with healthy subjects (6 hours). Several studies demonstrated that low levels of

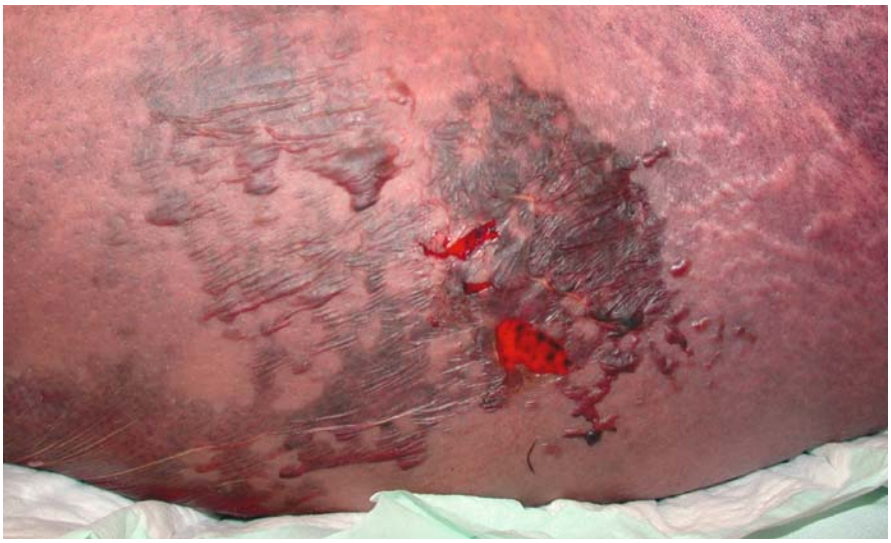

**Fig. 1e.** progressed consumption coagulopathy with skin bleeding

protein C in patients with sepsis and/or DIC are associated with a bad prognosis [1–3]. Protein C is a potent regulator of the coagulation cascade and possesses anti-coagulatory, profibrinolytic and anti-inflammatory properties. Data of some case reports, as well as of an open label study, lead to the hypothesis that an early substitution with protein C could have a positive influence on the progress of DIC in sepsis induced purpura fulminans, and so may improve its prognosis [4–11]. These series are mainly concerning children and teenagers, reports on adults are rare.

We report our experience with substitution of protein C in adult patients with sepsis induced purpura fulminans.

## Patients

Seven adult patients (6 female, 1 male; median age 35 years, [range 19-48]) were treated with a plasma-derived protein C concentrate (Ceprotin, Baxter, Vienna, Austria). The patient's clinical data are shown in table 1, examples of the cutaneous manifestations are presented in Figure 1.

At the time of admission all patients presented with skin lesions appearing as characteristic purpura fulminans (Fig. 1), as well as with signs of severe infection. Four patients fulfilled the ASCCM criteria of septical shock, five patients had acute renal dysfunction or neurological alteration, six patients suffered from acral necrosis, and five had laboratory signs of rhabdomyolysis. The proven causative organisms were neisseria menigitidis in three cases, streptococcus pneumoniae in two cases, pseudomonas aeruginosa and cytomegaly virus in one case each. As a focus meningitis could be discovered in three patients, pneumonia, tonsillitis and cutaneous infections in the others.

**Table 1.** Demographic data

| | |
|---|---|
| Number of patients | 7 |
| Age (years); median (range) | 34 (19–48) |
| Sex | 1 male, 6 female |
| **Accompanying illness** | |
| Myasthenia gravis | 1 |
| Post splenectomy | 1 |
| Unknown | 5 |
| **Focus:** | |
| Meningitis | 3 |
| Pneumonia | 1 |
| Skin lesion | 1 |
| Tonsillitis | 1 |
| Not identified | 1 |
| **Identified pathogens** | |
| Neisseria meningitidis | 3 |
| Streptococcus pneumoniae | 2 |
| Unspecified streptococci | 1 |
| Pseudomonas aeruginosa | 1 |
| Cytomegaly virus | 1 |
| **Clinical symptoms** | |
| APACHE II | 20 (9–29) |
| SAPS II | 40 (14–64) |
| Purpura fulminans | 7 |
| Consumption coagulopathy | 5 |
| Rhabdomyolysis | 5 |
| Necrosis | 6 |
| Renal failure | 5 |
| Septic shock | 4 |

Coagulation analysis showed DIC [12] in 5 patients (Fig. 2): median platelet count was 19 G/l (range 17–23 G/l), fibrinogen concentration 60 mg/dl (44–103 mg/dl), antithrombin activity 0.47 U/ml (0.25–0.76 U/ml), prothrombin time 32 % (14–39%), APTT 88 sec (42–160 sec), D-dimer 66 ng/ml (3.3–140 ng/ml). The remaining 2 patients were treated with the protein C concentrate because of the typical skin lesions and proven meningococcal infection only. Nevertheless, in all reported patients the plasma protein C activity was significantly decreased (median 0.34 U/ml (range 0.19–0.56 U/ml)).

## Methods

Five patients were treated with Ceprotin according to the protocol by White and al. [4]. Following a testing dose of 10 U/kg body weight, patients received a single shot bolus of 100 U/kg followed by a level-controlled continuous infusion starting with 10 U/kg/h. Determinations of protein C activity were performed at least daily, and the infusion rate was adjusted to obtain a target protein C activity of 1.0 U/ml.

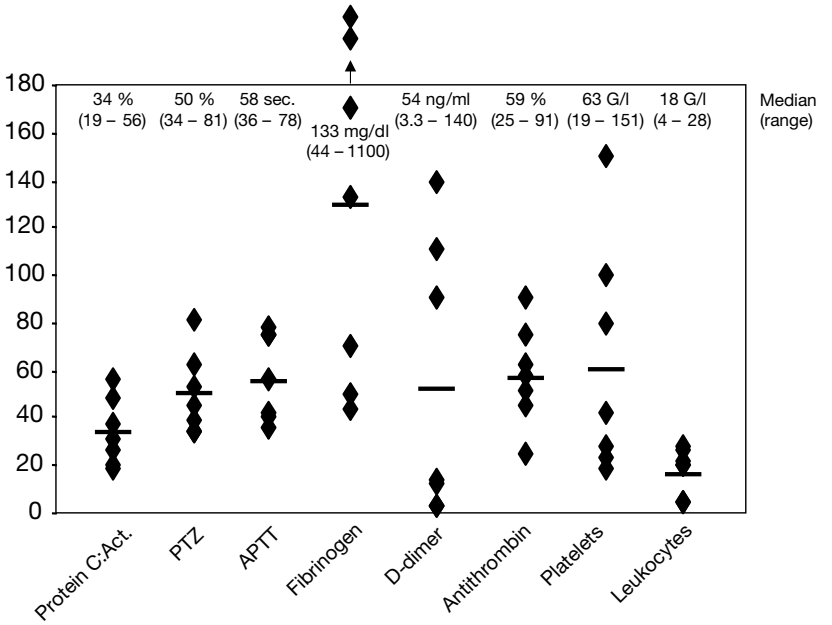


**Fig. 2.** Coagulation values at admission

**Table 2.** Therapy protocol for Ceprotin treatment

| | Level-adjusted continuos infusion (according to White et al.)<br><br>Test dose 10 U/kg b.w.<br>Bolus 100 U/kg b.w.<br>continuous infusion 10 U/kg/h, adjusted to Protein C-activity (goal >1.00 U/ml) | Bolus treatment (according to Rintaala et al.)<br><br>100 U/kg every 6 hours | Total |
|---|---|---|---|
| N | 5 | 2 | 7 |
| Total Ceprotin dose<br>Median (units)<br>Range | 17000<br>(7800–40800) | 62900<br>(48000–77700) | 40000<br>(7800–77700) |
| Duration of Ceprotin therapy<br>Median (hours)<br>Range | 24<br>(8–168) | 99<br>(61–136) | 61<br>(8– 168) |
| Max. Protein C activity<br>Median (U/ml)<br>Range | 1.83<br>(1.34-2.0) | 1.97<br>(1.93-2.0) | 1.85<br>(1.34 – 2.0) |

**Table 3.** Additional therapy of patients with DIC and purpura fulminans

| | |
|---|---|
| **Standard intensive treatment** | |
| Antibiotics | 7 / 7 |
| Vasopressors | 4 / 7 |
| Hemofiltration | 3 / 7 |
| Mechanical ventilation | 4 / 7 |
| Hydrocortisone | 4 / 7 |
| **Coagulation therapy** | |
| Unfractionated heparin | 7 |
| Low molecular weight heparin | 0 |
| Fresh frozen plasma | 4 |
| Antithrombin concentrates | 2 |
| Fibrinogen concentrates | 4 |
| Prothrombin-complex concentrate | 0 |
| Fibrinolytic therapy (rh-tPA) | 2 |
| Platelet concentrates | 5 |
| Prostacyclin | 3 |
| Others | 1 |

Two patients were treated with Ceprotin as reported by Rintala et al [6] (100 U/kg bolus every 6 h) (Table 2).

Additional coagulation-active therapy was given (Table 3): unfractionated heparin as an APTT-controlled continuous infusion (7 patients), fresh-frozen plasma (5 patients), antithrombin-concentrates if levels were below 0.5 U/ml (3 patients), fibrinogen concentrates if levels were lower than 80 mg/dl (2 patients), platelet concentrates if counts were lower than 30 G/l (4 patients) and prostacyclin when continuos veno-venous hemofiltration was necessary (3 patients). In 2 patients a fibrinolytic therapy with low doses of rtPA (5 and 20 mg i.v.) was performed due to expansive ischemia with threat of compartment syndrome and myolysis.

The further therapy consisted of antibiotic treatment as well as fluid, vasopressors and hydrocortison [13, 14] according to the guidelines of early goal-directed therapy (4 patients with septic shock). Mechanical ventilation due to respiratory failure was necessary in 4 patients.

## Results

### Protein C Substitution

Patients were treated in median with 40000 units of Ceprotin (range: 7800–77700 U) for a median period of 61 (8-168) hours, corresponding to 2.5 (0.3–7.0) days (Table 2). Patients with continuous infusion received in median 17000 units, patients on bolus treatment received 62900 units (mean). Protein C activity increased in all patients to supranormal values (maximal protein C activity 1.85 (1.34–2.0) U/ml).

## Influence on Coagulopathy

Coagulopathy could be controlled in six of the reported cases; normal coagulation parameters were observed after 1 to 6 days after treatment start (Fig. 3). We could monitor the response by the fast spontaneous rise of fibrinogen and antithrombin values. Platelet counts increased after a delay of several days.

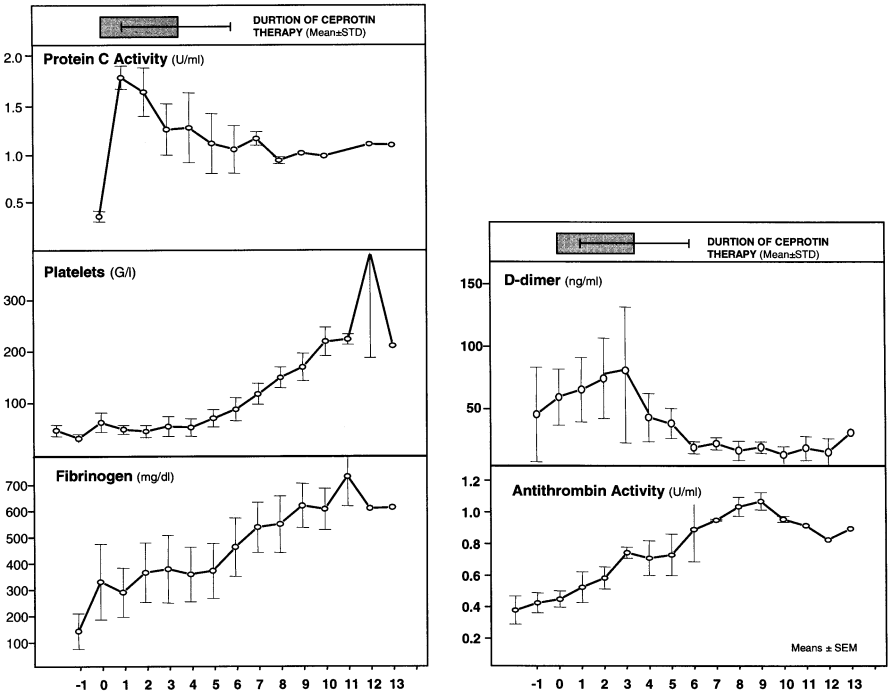


**Fig. 3.** Course of coagulation parameters during therapy

## Outcome

One patient died within the first 24 hours after admission due to fulminant septic multiorgan failure, another patient 14 days after start of therapy because of a new caught sepsis caused by systemic candidiasis. The other five patients survived (Table 4). The median stay at the intensive care unit was 11 days (range 2–41 days). In 2 patients, one or more phalanges had to be amputated, while another patient had to receive a meshcraft following a surface necrosectomy. One patient suffered from a large liver necrosis, which recovered spontaneously without surgical treatment within several weeks after demission. No one of the surviving patients suffered from any persistent neurological or other organ deficit.

**Table 4.** Outcome

| | |
|---|---|
| Resolve of coagulopathy | 6 / 7 |
| Survival | 5 / 7 |
| Death (n / day) | 2 (d1, d14) |
| Days at ICU | 11 (2–41) |
| Total – TISS-28 | 295 (49–1444) |
| TISS-28 per day | 92 (45–299) |
| **Side effects / events:** | |
| Mild bleeding | 0 |
| Severe bleeding | 1 (after rtPA) |
| Thrombosis | 0 |
| **Late sequelae:** | |
| Amputations | 3 / 5 |
| Neurological disturbance | 0 / 5 |

### Side-effects

After starting of Ceprotin therapy no hemorrhage or thrombosis was observed despite abnormal coagulation values due to DIC. One of the patients who was treated with rtPA because of a compartment syndrome of the right lower extremity suffered from severe bleeding from an arterial puncture site directly after lysis, but this event was treatable with conservative measures and was not related with the Ceprotin treatment.

## Discussion

Ceprotin seems to be an effective and save therapeutic option in patients with DIC and purpura fulminans, also in adult patients. Especially in some patients with an otherwise extremely bad prognosis, we found a very good response to this therapy. However, it must be considered that our patients cohort was heterogeneous and received different other additional coagulation active therapy as well as different doses of Ceprotin.

Our results and the data from the other uncontrolled trials and case reports urge the need for a standardized protocol in further studies on the treatment with Ceprotin in sepsis induced coagulopathy. Several unresolved questions should be answered in appropriately designed studies: what criteria should the patients fulfill before they can receive treatment with Ceprotin? Should only the existence of typical skin lesions lead to the initiation of protein C substitution or is the presence of coagulopathy a necessary condition? Is it necessary to measure protein C levels prior to therapy, and which value is the cut-off to decide whether treatment is necessary? Which are the target levels of protein C under therapy? What kind of administration is better: bolus or continuous infusion? What is the optimal duration of therapy? Until abnormal coagulation values have normalized or until skin lesions have resolved. What additional therapy (heparin, fibrinolytics, antithrombin, etc.) is of benefit?

It is absolutely necessary to answer these questions, since protein C substitution is still an experimental therapy in DIC and purpura fulminans, and Ceprotin has not been approved for this indication. Moreover, it is extremely expensive and regimen should be used which are effective, but as cheap as possible. Our data and the results of others suggest that during a level-adjusted continuous infusion lower daily doses of Ceprotin are necessary than with a fixed-dose bolus regimen. On the contrary, the opportunity to measure protein C activity throughout the whole day is necessary for such strategies.

However, it is always necessary to keep in mind that coagulopathy is a secondary phenomenon in sepsis. The early therapy of the cause is still the strategy with the greatest impact on survival. Surgical therapy of a focus, early and specific antibiotic treatment, and goal-directed intensive care medicine are the main columns of sepsis treatment. The new forms of sepsis therapy, activated protein C, Ceprotin, hydrocortisone, insulin treatment, etc. can improve survival, but are indicated only as additional measures to conventional therapy.

*Acknowledgement.* We gratefully acknowledge the competent assistance of the nursery team of the intensive care units 13i2 and 13h1 of the University Hospital of Vienna. We would like to give our special thanks to Dr. Christian Sillaber for kindly providing some of the pictures of the patients.

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# Comparison of the Anticoagulant Action of Recombinant Human Activated Protein C in Cord with that in Adult Plasma

G. Cvirn, S. Gallistl, M. Köstenberger, K. Baier, P. Fritsch, G. Jürgens and W. Muntean

## Background

Recently, recombinant human activated protein C (rhAPC) has been shown to be a good candidate for the treatment of microvascular thrombosis, disseminated intravascular coagulation, and septicemia in both adults and neonates [1]. In the present study we focus our attention on the influence of tissue factor pathway inhibitor (TFPI) and antithrombin (AT) on the anticoagulant action of rhAPC. TFPI and AT have been shown to amplify the anticoagulant action of APC in a synergistic manner in plasma activated by addition of lipidated tissue factor (TF) [2, 3]. TFPI and AT levels in neonatal plasma are reduced to approximately 50% of adult value [4]. Thus, different anticoagulation action has to be expected between rhAPC administered to neonates compared with rhAPC administered to adults.

Therefore, we comparatively evaluate in the present study the anticoagulant action of rhAPC in terms of prolongation of clotting time, suppression of thrombin potential (TP), and suppression of prothrombin fragment 1+2 (F1+2) generation in cord plasma containing TFPI and AT at neonatal levels, in cord plasma adjusted to contain TFPI and AT at adult levels, and in adult plasma. Clot formation is induced not only by addition of high (30 μM final concentration) amounts of lipidated tissue factor (TF), as usual in standard assay systems (activated partial thromboplastin time, prothrombin time) [5], but also by addition of low (20 pM final concentration) amounts of TF.

## Methods

### Collection and Preparation of Plasma

Cord blood was obtained immediately following the delivery of 21 full term infants (38-42 weeks gestational age). Newborns with Apgar scores of 9 or less five minutes after delivery were excluded from the study. Blood (2.7 ml) was collected into precitrated S-Monovette premarked tubes from Sarstedt (Nümbrecht, Germany), containing 300 μl 0.106 M citrate, centrifuged at room temperature for 15 min at 2800 x g, pooled and stored at -70°C in propylene tubes until assayed. The hematocrit of cord blood was similar to that of adult blood. FV and FVIII activities were elevated over the respective adult values, other pro- and anticoagulant factors were in the normal range for neonates.

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**Preparation of cord Plasma Containing TFPI at Adult Level**

The TFPI level of the pooled cord plasma was raised up to adult value by addition of 10.5 µL of TFPI stock solution to 989.5 µL of cord plasma.

**Determination of the Plasma TFPI Level**

TFPI activity in cord and adult plasma was determined by means of the chromogenic assay ActichromeTM TFPI activity assay.

**Preparation of cord Plasma Containing AT at Adult Level**

AT level of pooled cord plasma was raised up to the respective adult value by addition of 12.5 µl of the AT stock solution to 987.5 µl of cord plasma.

**Determination of the AT Content**

Determination of the AT content of cord and adult plasma was performed by means of a standard chromogenic method on a BM/Hitachi 917 from Boehringer Mannheim (Vienna, Austria).

**Activation of Plasma**

Three hundred eighty µl of plasma were incubated with 20 µl of PBS containing different amounts of rhAPC (0.1–16 µg mL-1 final concentration) for 1 min at 37°C. Subsequently, 20 µl of PBS containing H-Gly-Pro-Arg-Pro-OH (Pefabloc FG, 1.0 mg ml-1 final concentration) to inhibit fibrin polymerization [6] were added. Subsequently, 40 µl of PBS were added containing TF (20 pM final concentration) or Thromborel S (30 µM final concentration of TF) for 1 minute at 37°C. Finally, 20 µl of 0.5 M CaCl 2 were added to trigger clot formation.

**Determination of Clotting Time**

Clotting times were determined by means of the optomechanical coagulation analyzer Behring Fibrintimer II from Behring Diagnostics GmbH (Marburg, Germany).

**Determination of Thrombin Generation**

We used a subsampling method derived from a recently described technique [7]. Plasmas were prepared and activated as described above. At timed intervals 10 µl

aliquots were withdrawn from the activated plasma and subsampled into 490 µl PBS containing 255 µM pNAPEP 0238. Amidolysis of pNAPEP 0238 was stopped after 6 min by addition of 250 µl 50% acetic acid.

## Results

### Effect of Increasing Amounts of rhAPC on Thrombin Potential

Suppression of thrombin generation dose-dependently increased in the presence of increasing amounts of rhAPC in both cord and adult plasma (Fig. 1). Whereby, the anticoagulant action of rhAPC was significantly more pronounced in cord plasma under high coagulant challenge compared with that in adult plasma (P of differences <0.01, panel A) and, in contrast, significantly more pronounced in adult plasma under low coagulant challenge compared with that in cord plasma (P of differences <0.01, panel B).

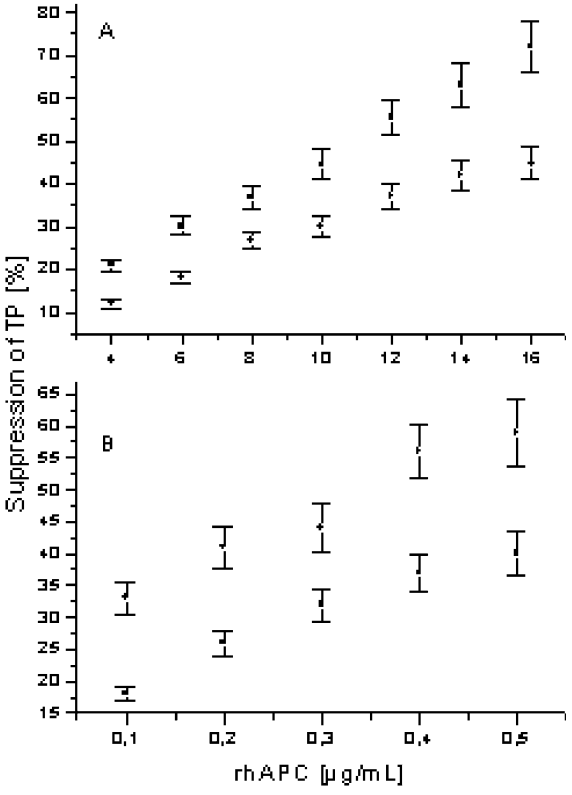


**Fig. 1.** Effect of addition of rhAPC on suppression of thrombin potential in cord (■) and adult (●) plasma in the presence of 30 µM (A) or 20 pM (B) tissue factor to induce clot formation.

**Anticoagulant action of rhAPC in cord plasma containing neonatal levels of TFPI and AT compared with that in cord plasma containing both inhibitors at adult levels**

Raising TFPI and AT in cord plasma up to adult values resulted in significantly prolonged clotting time (348 ± 16 vs. 282 ± 11 s, P <0.01), suppressed TP (234 ± 18 vs. 309 ± 23 nM min, P <0.01), and suppressed F1+2 generation (0.402 ± 0.03 vs. 0.512 ± 0.04 μmol L-1, P <0.01) in the presence of 20 pM TF to initiate clot formation (Fig. 2). Significantly enhanced anticoagulant action of 0.2 μg $mL^{-1}$ rhAPC was observed in cord plasma containing adult levels of TFPI and AT compared with cord plasma containing both inhibitors at neonatal levels: addition of 0.2 μg $ml^{-1}$ rhAPC significantly prolonged clotting time by 35.1 % in cord plasma containing neonatal levels of TFPI and AT, and by 49.7% in cord plasma containing both inhibitors at adult levels (P of differences < 0.01). TP and F1+2 generation were suppressed by 26.2 and 15.2%, respectively, in cord plasma containing neonatal levels of TFPI and AT, and by 47.8 and 28.1%, respectively, in cord plasma containing both inhibitors at adult levels (P of differences < 0.01).

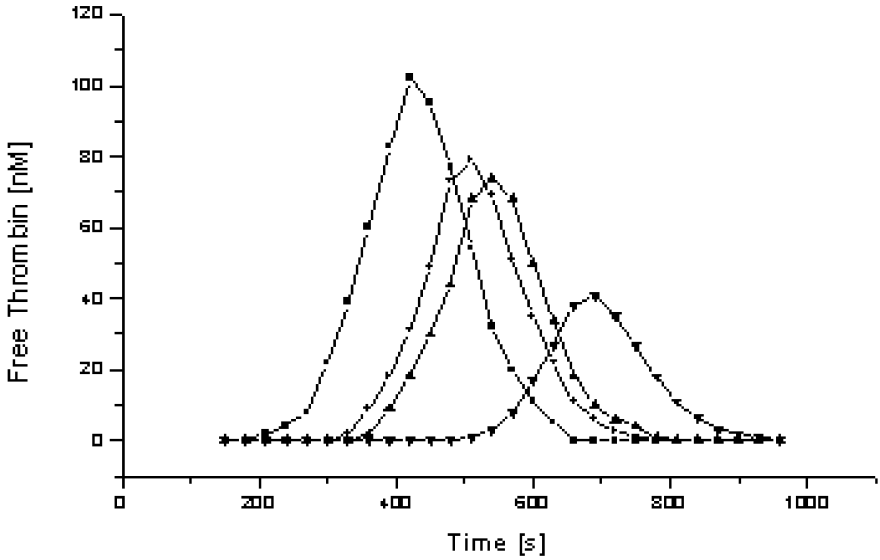


**Fig. 2:** Effect of TFPI and AT on the anticoagulant action of rhAPC in cord plasma. Free thrombin generation over time is displayed in reactions initiated by 20 pM TF in cord plasma containing physiological levels of TFPI and AT in the absence of rhAPC (■), in the presence of 0.2 μg $ml^{-1}$ rhAPC (●), and in cord plasma containing TFPI and AT at adult levels in the absence of rhAPC (▲), and in the presence of 0.2 μg $ml^{-1}$ rhAPC (+). Results are expressed as means (n = 3). SDs were not shown for clarity of graph reading but represented less than 10 % of the mean.

## Conclusions

We found that under both high and low coagulant challenge clotting time was dose-dependently prolonged, thrombin potential and prothrombin fragment 1+2 generation were dose-dependently suppressed in the presence of increasing amounts of rhAPC in both cord and adult plasma. Whereby, cord plasma was significantly more susceptible to addition of rhAPC in the presence of high amounts of TF as trigger for clot formation and adult plasma was significantly more susceptible to addition of rhAPC in the presence of low amounts of TF. Whereas the elevated anticoagulant action of rhAPC in cord plasma under high coagulant challenge might be caused by the low neonatal levels of prothrombin, we demonstrate that the increased anticoagulant efficacy of rhAPC in adult plasma under low coagulant challenge is attributable to the physiologically high levels of TFPI and AT present in adults.

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# The Relationship between Protein C Activity and the Endogenous Thrombin Potential in Controls and Septic Patients

A. Siegemund, T. Siegemund, U. Scholz, S. Petros and L. Engelmann

## Background

The physiologic effects of activated protein C (APC) are manifold. It inactivates the clotting factors Va and VIIIa, thereby inhibiting the prothrombinase and tenase activities. It also inhibits the proinflammatory consequences of local thrombin generation (platelet activation and secretion of vasoactive and proinflammatory substances) and activates endogenous fibrinolysis by binding to PAI-1.

In a randomized double-blind placebo-controlled study [1], administration of the activated protein C concentrate drotrecogin alfa (Xigris) has been found to result in an absolute mortality reduction of 6.1%. On the contrary, the positive effects of human inactive protein C concentrate have been described in small studies [2] and case reports [3] only. These are particularly patients with a hereditary protein C deficiency and with meningococcal sepsis, but also others with severe sepsis and consumption coagulopathy.

Considering these facts and the positive effects of the (inactive) human protein C concentrate (although not proven in a randomized clinical study), the present ex-vivo study was aimed to discuss the following questions:

1. Can the thrombin generation curve and the variables computed thereof be suitable to depict the complex processes involving protein C?
2. Are there differences between administration of activated and inactive human protein C regarding the thrombin generation curve?
3. Are there differences between healthy controls and septic patients in their reaction to the administration of protein C?

## Patients and Methods

The study was conducted using blood samples from 10 healthy controls without thrombotic risk factors and patients with severe sepsis under treatment at the Medical ICU of the University of Leipzig. Sepsis was defined according to the ACP/SCCM criteria [4]. Blood was drawn via an antecubital venipuncture in controls and through an indwelling central vein catheter in patients after an informed consent. Patients did not receive any anticoagulant during the previous 12 hours before the blood draw.

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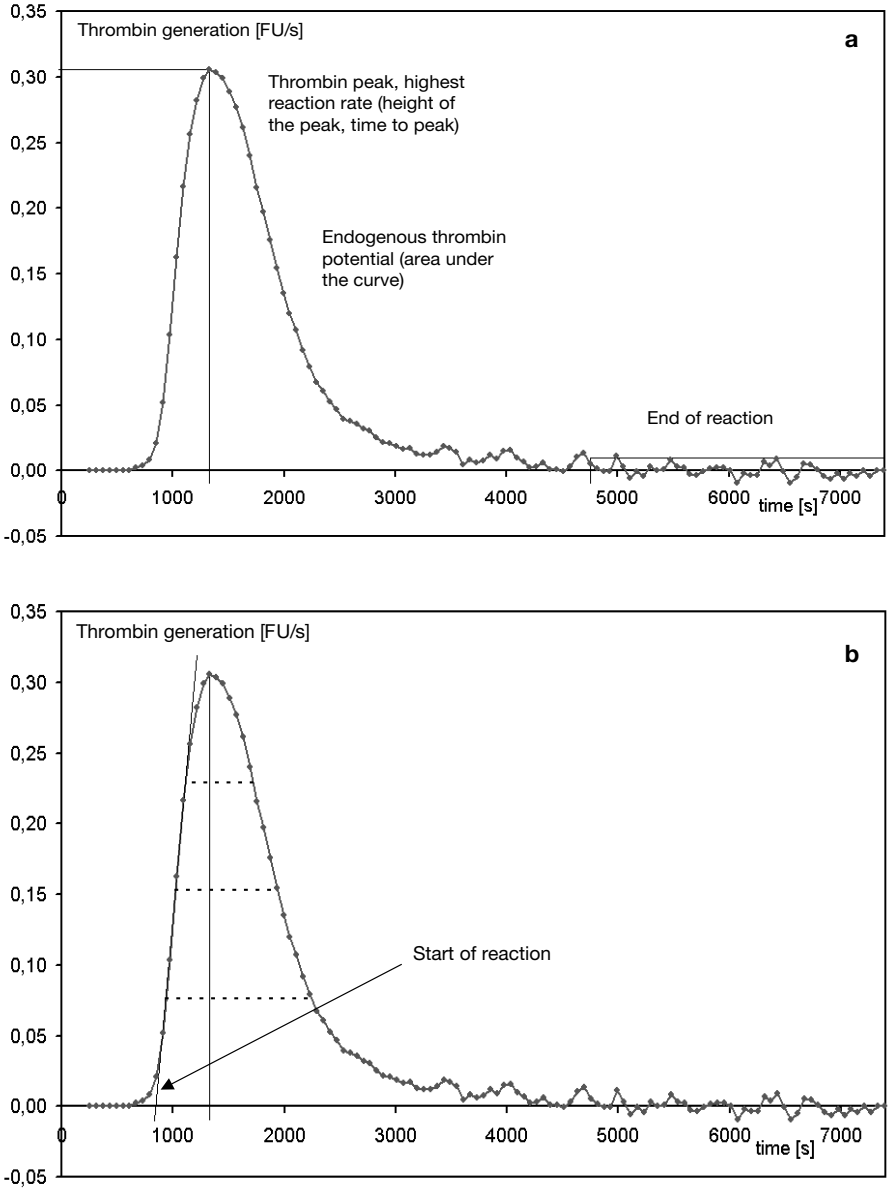


**Fig. 1a–c.** Parameters of thrombin genertion curve

Thrombin generation was measured in platelet-rich plasma (PRP) as previously described [5]. The endogenous thrombin potential (ETP), thrombin peak (peak_h), time to peak (peak_t), start vs. end of the reaction as well as thrombin inhibition were computed. Protein C activity was measured with the Behring Coagulation

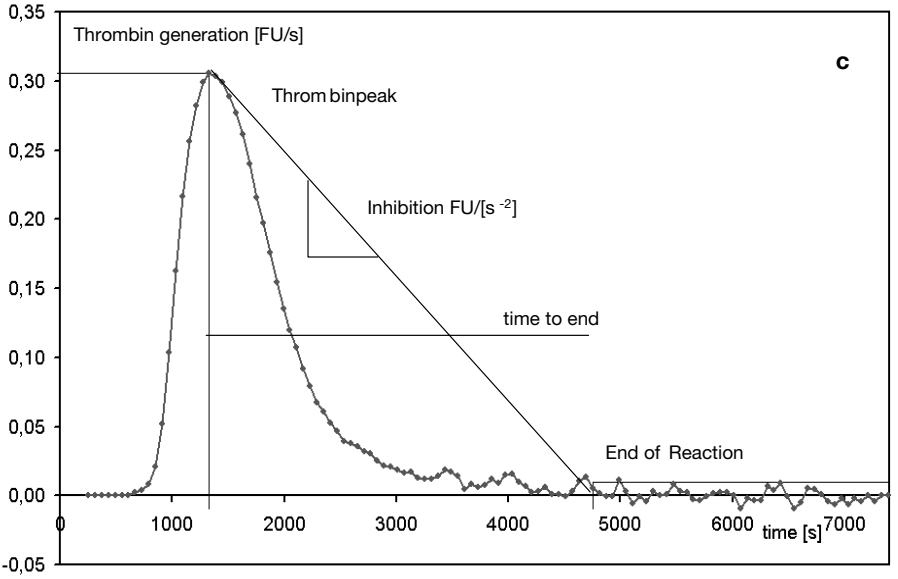


**Fig. 1c**

System (Dade Behring) applying the chromogenic method (Berichrom Protein C, Dade Behring Marburg GmbH). Measurements were carried out before and after addition of APC (drotrecogin-α, Xigris, Lilly Deutschland GmbH) and inactive protein C (Ceprotin, Baxter Deutschland GmbH) ex vivo.

Data analysis was conducted using SPSS for windows version 10.0 (Chicago, Illinois). The Student t-test was applied for data comparison. Parametric data are given as mean ± SD, and a $p < 0.05$ was considered statistically significant.

## Results

Thrombin generation is dependent on platelet count in healthy controls as well as in patients. Therefore, inter-individual comparison is only possible with a defined platelet count (Fig. 2).

Drotrecogin-a reduced ETP and thrombin peak significantly ($p<0.001$); it also markedly delayed the start of thrombin formation (i.e. it prolonged the lag phase) ($p<0.001$). These reactions were concentration-dependent (Fig. 3). The inactive human protein C concentrate Ceprotin reduced the thrombin peak and prolonged the lag phase, but these effects were not statistically significant.

Another important physiological aspect is the thrombin inhibition, which is defined as the mean negative reaction velocity between maximum thrombin peak and the end of the reaction. It could be demonstrated that thrombin inhibition is also dependent on the platelet count and protein C activity (Fig. 4). At low platelet counts, small amount of APC is enough to prevent thrombin formation, while high-

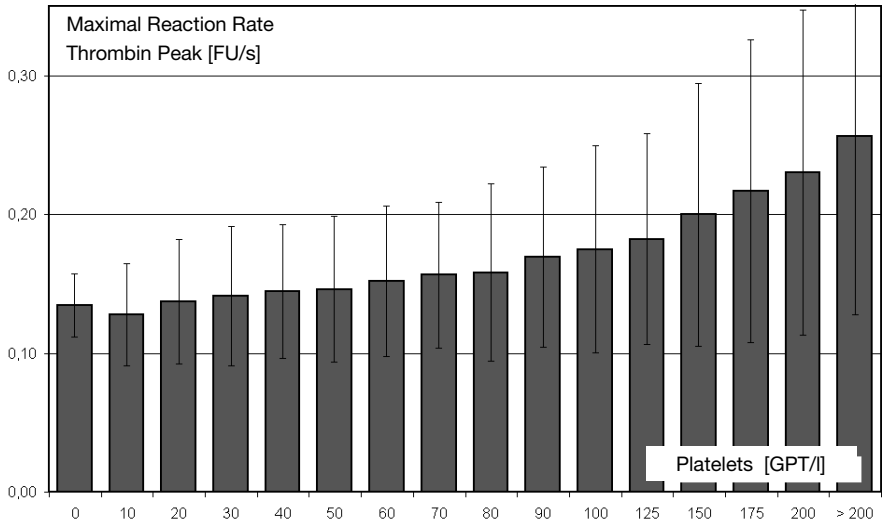


**Fig. 2.** Thrombin peak as function of platelets

er APC concentrations are needed with increasing platelet counts. The study on our healthy controls have shown that at a platelet count of 20 x $10^9$/l only a quarter of the APC required at a platelet count of 200 x $10^9$/l was enough for a comparable degree of thrombin inhibition.

In septic patients, it could be demonstrated that thrombin generation decreases with increasing APC concentrations in a similar manner to that observed in healthy controls (Fig. 5).

## Discussion

Protein C plays a significant role as a physiological anticoagulant in the hemostatic system. After being activated via the thrombin-thrombomodulin mechanism, it inactivates the coagulation factors Va and VIIIa, thereby slowing the activities of the prothrombinase and tenase complexes, respectively. This process contributes to a marked reduction in thrombin generation. Protein C also activates endogenous fibrinolysis by binding to plasminogen activator inhibitor 1 (PAI-1). It also inhibits the proinflammatory consequences of local thrombin formation.

Platelets play a significant role in the process of thrombin generation, a role that has been underestimated in the past. Our data demonstrate that the amount of protein C to be administered in a given subject should be dependent on the platelet count.

The endogenous thrombin potential represents all the factors involved in thrombin formation. Indirect measurements of thrombin generation, such as thrombin-antithrombin complex or the prothrombin fragments have also shown to be significantly reduced after administration of the activated protein C concentrate drotrecogin-α [1] or purified inactive human protein C [6]. In our study, the effect

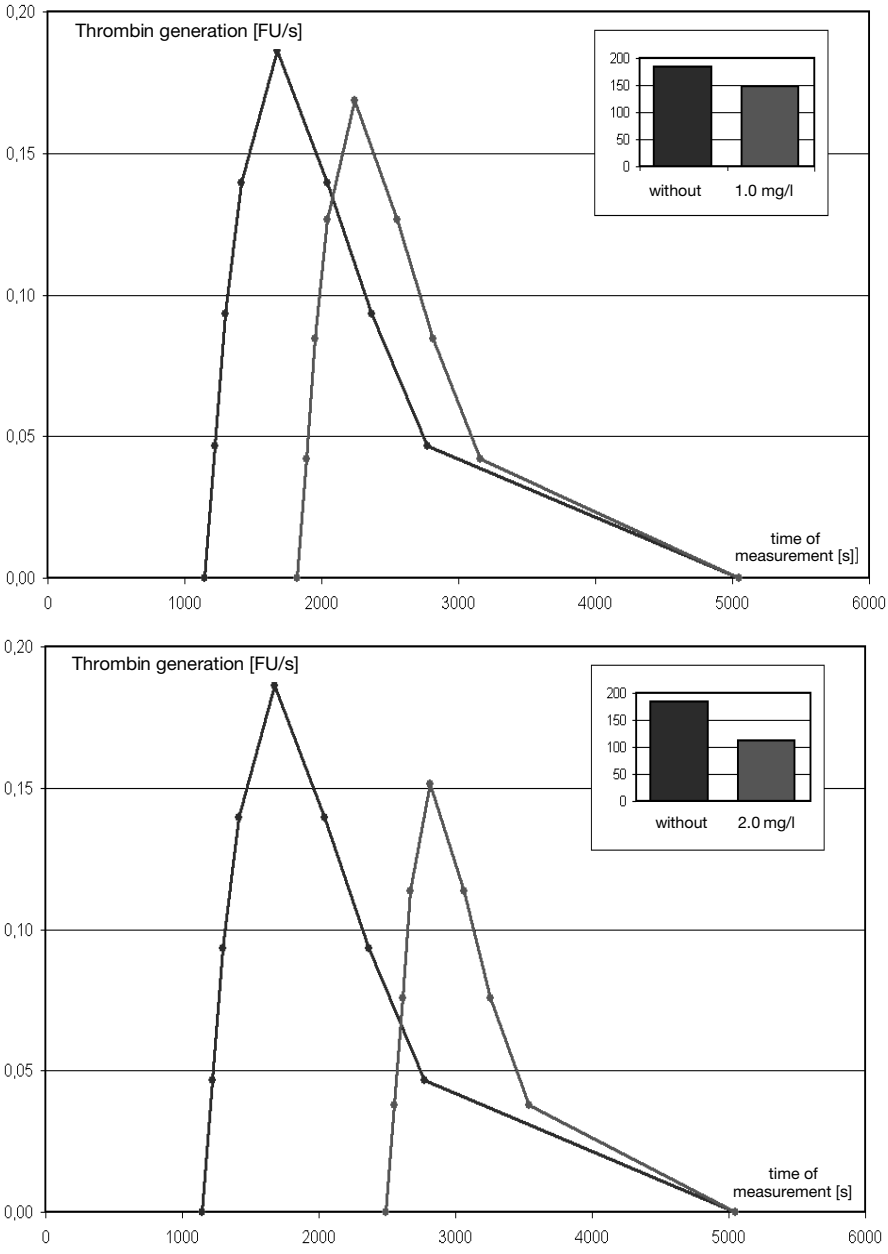


**Fig. 3.** Influence of activated Protein C on Thrombin Generation (Platelets 100 Gpt/I)

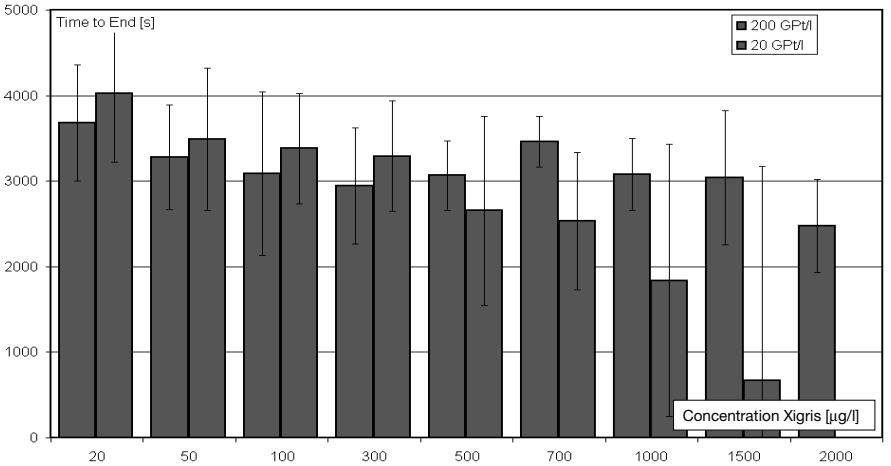


**Fig. 4.** Inhibition of thrombin generation – Influence of activeted protein C and platelets

of inactive human protein C on the thrombin generation curve was not significant. The reason for this may be the lack of thrombomodulin or the endothelial thrombin receptor in the test system, so that activation of protein C would be very slow. The transition of protein C from its inactive zymogen to the active form is slowed in septic patients (7), so that administration of inactive protein C would not result in an optimal influence on the hemostatic system.

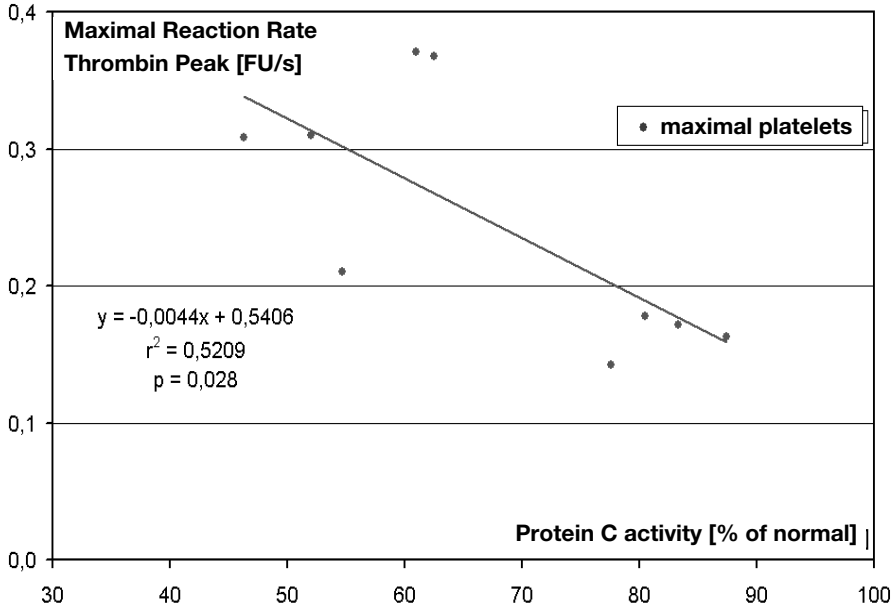


**Fig. 5a.** Influence of protein C activity in Sepsis – ETP (maximal reaction rate)

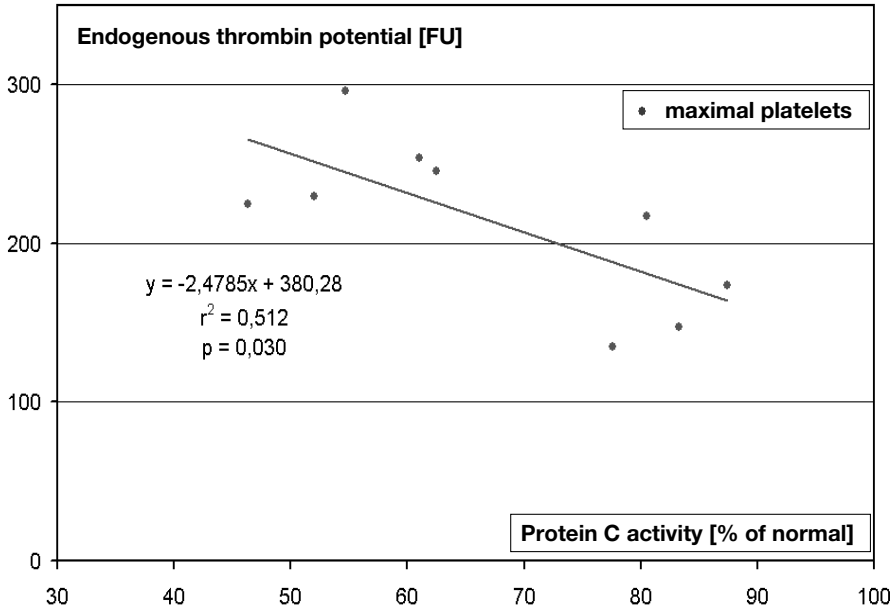


**Fig. 5b.** Influence of protein C activity in Sepsis – ETP (area under the curve)

This is a preliminary study demonstrating that measurement of thrombin generation in platelet-rich plasma may be a valuable tool to describe the complex processes of coagulation in sepsis. Further investigations are necessary to elaborate on the role of active and inactive protein C.

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## *V. Pediatric Hemostaseology*

Chairmen:

G. Auerswald (Bremen)
H. Lenk (Leipzig)

# Antibodies Against Annexin V, Cardiolipin and $\beta_2$-Glycoprotein 1 or APC-Resistance in Patients with Recurrent Miscarriage or In-Vitro-Fertilization-Failures

W. M. Halbmayer, W. Feichtinger, C. Kindermann, B. Prendinger and M. Böhm

## Background

Antibodies against annexin-V, a potent anticoagulant abundant in placental tissues, were recently controversially reported to be associated with recurrent abortions or failures of in-vitro-fertilization (IVF) attempts [1, 2]. Recognition by anti-annexin V antibody of adhered annexin V on trophoblast cell structures might represent a potential pathologic mechanism by which these antibodies can cause defective placentation [3].

## Objective

To evaluate the prevalence of annexin V antibodies in women with early recurrent pregnancy losses and/or recurrent IVF-failures.

## Material and Methods

We screened 56 women (34.7 +/- 4.3 years of age: mean +/- 1 SD) with recurrent IVF failures and/or early pregnancy losses for:

- **APC-resistance:** original clotting assay with factor V deficient plasma predilution.
- **Lupus anticoagulant**: dilute Russells Viper Venom Time screening and confirmatory test and Kaolin Clotting Time.
- **Antibodies against annexin V** IgG/IgM isotypes: ELISA-research kit using micro ELISA plates coated with highly purified human recombinant Annexin V and affinity purified goat antibodies specific fur human IgG/IgM Fcγ coupled to HRPO.
- **Antibodies against cardiolipin** IgG/IgM and **$\beta_2$-glycoprotein 1** IgG/IgM: commercially available ELISA test kits using micro ELISA plates coated with cardiolipin and human $\beta_2$gp-1 and purified antibodies specific fur human IgG/IgM Fcγ coupled to HRPO.

All together the 56 women have had 67 pregnancies (1.2 +/- 1.6 mean +/- 1 SD), 9 ectopic pregnancies (0.2 +/- 0.5), 4 deliveries (0.07 +/- 0.3), 43 early miscarriages

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(0.8 +/- 1.3) and 180 IVF attempts (3.2 +/-2.2) when they entered the hemostaseological evaluation.

Fourty-two (75%) of these women had another IVF-attempt after the hemostaseological evaluation and received low molecular weight heparin (40 mg Enoxaparin »Lovenox«) subcutaneously once daily and/or low-dose (100 mg o.d.) aspirin in the case of positivity for APC-resistance, lupus anticoagulant or antibodies against annexin V, cardiolipin or $\beta_2$-glycoprotein-1.

## Results

In 21 women out of 56 (37.5%) »defects« could be detected.

**Table 1.** Prevalence of »defects«

| | n | % |
|---|---|---|
| Annexin V Ab. | 1/56 | 2 |
| Cardiolipin Ab. | 7/56 | 12 |
| ß$_2$-gp1 Ab. | 3/56 | 5 |
| LA | 8/56 | 14 |
| APCR | 8/56 | 14 |

No statistically significant difference between women with- and without hemostaseological »defect« was identified concerning the patient's age or the number of previous pregnancies, ectopic pregnancies, deliveries, miscarriages or IVF-attempts (Table 2).

**Table 2.** Data and patient's history of women with and without »defect« (mean+/-1 SD)

| | n | age | pregn. | ectop. p. | delivery | miscarriage | IVF |
|---|---|---|---|---|---|---|---|
| no defect | 35 | 34.7+/-4.3 | 1.1+/-1.7 | 0.1+/-0.3 | 0.09+/-0.4 | 0.7+/-1.4 | 3.3+/-2.1 |
| with defect | 21 | 35.5+/-4.2 | 1.3+/-1.2 | 0.2+/-0.7 | 0.05+/-0.2 | 0.9+/-1.0 | 3.2+/-2.0 |
| Fisher's Exact Test | | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

Interestingly, the next IVF-attempt after hemostaseological evaluation showed a trend of more pregnancies within the group of women with »defect« (Table 3).

**Table 3.** Outcome of next IVF attempt in women with and without »defect«

| | n | another IVF | pos.pregn.test | normal preg. | early miscarriage |
|---|---|---|---|---|---|
| no defect | 35 | 26 (74%) | 10/26 (38%) | 9/10 (90%) | 1/10 (10%) |
| with defect | 21 | 17 (81%) | 9/17 (53%) | 6/9 (67%) | 3/9 (33%) |
| Fisher's Exact Test | | | n.s. | n.s. | n.s. |

The reason for this trend might be the therapy (aspirin and/or low molecular heparin), which has been given to women with detected »defects«. We found a better IVF-outcome (more positive pregnancy-tests) among women receiving low molecular heparin as a »mono-prophylaxis« of venous thromboembolism (Table 4, Fig. 1).

**Table 4.** Effect of Enoxaparin or Enoxaparin and Aspirin in women with »defect« and another IVF attempt.

| | n | pos.pregn.test | early miscarriage |
|---|---|---|---|
| Enoxaparin | 9/17 (53%) | 8/9 (89%) | 3/9 (33%) |
| Enoxaparin + ASS | 5/17 (29%) | 1/5 (20%) | 0/5 (0%) |
| Fisher's Exact Test | $p < 0.02$ | n.s. | |

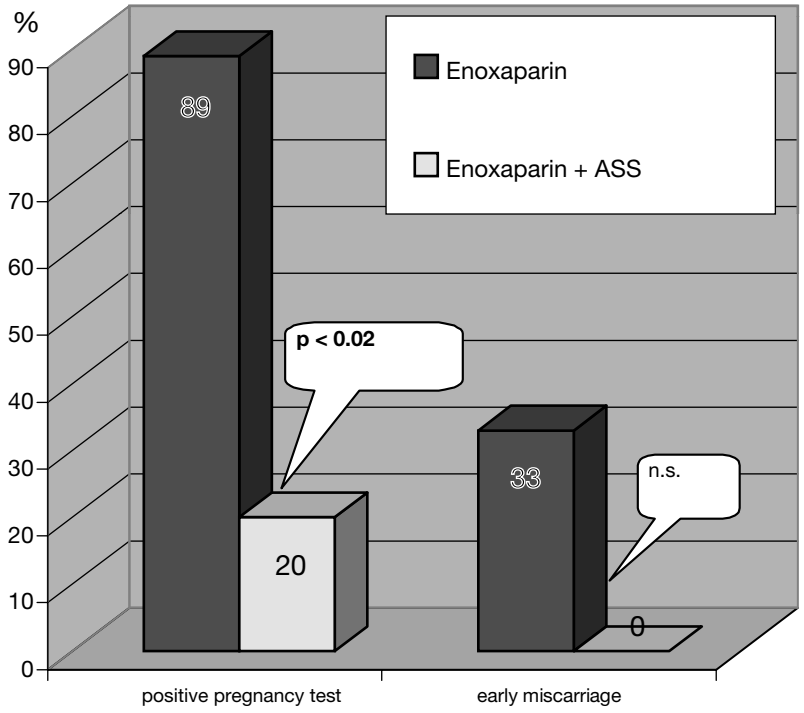


**Fig. 1.** Effect of Enoxaparin or Enoxaparin and Aspirin in women with »defect«

The only woman with an elevated anti-Annexin V (IgG) level has had 7 IVF attempts previously and received 40 mg Enoxaparin (Lovenox) subcutaneously once daily during the 8th IVF, which resulted in a healthy pregnancy.

## Conclusion

Our findings suggest that among women with recurrent miscarriages and recurrent IVF failures anti-annexin V antibody positivity is less prevalent than APC-resistance, lupus anticoagulant or elevated levels of antibodies against cardiolipin or ß$_2$-glycoprotein-1 and that the IVF-result in women with APC-resistance, lupus anticoagulants or elevated levels of antibodies against annexin V, cardiolipin or $\beta_2$-glycoprotein-1 might be positively influenced by low molecular weight heparin.

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# Diagnostics of Platelet Function Disorders by Lumi-Aggregometry – Results and Comparison of Methods

R. Knoefler, G. Siegert, E. Kuhlisch and G. Weissbach

## Introduction

Agonist-induced platelet aggregation and ATP release from platelets can be measured simultaneously in a small amount of 450 µl citrated whole blood per test by lumi-aggregometry. Despite the fact that this method might be suitable especially for pediatric patients due to the small blood volume requirement only few studies have been published so far [7, 15, 25, 26]. The method was standardized on normal controls and reference values for different age groups were determined [12]. The aim of study was to evaluate lumi-aggregometry for detection of platelet function disorders in children and adults and to compare the results with those obtained from established platelet function tests such as the aggregometry in platelet rich plasma, the in vivo bleeding time and the PFA 100 test.

## Materials and Methods

### Blood Sampling

Blood was collected from peripheral veins into plastic tubes containing 3.8% trisodium citrate (Sarstedt, Germany). For the determination of closure times by the Platelet Function Analyzer (PFA 100, Dade Behring, Germany), tubes with buffered trisodium citrate (pH 5.5) purchased from Sarstedt were used. Nine volumes of blood were anticoagulated with one volume of the citrate solution. The samples were stored for at least 10 minutes at room temperature. Hematocrit, leukocyte and thrombocyte counts were determined by an electronic counter in an additional ethylenediamintetraacetic acid (EDTA)-anticoagulated blood sample.

### Patients

Samples from 32 patients with the suspicion of disturbed primary hemostasis were investigated. Among them were 22 children (mean age: 10.5 years, range: 1.25 to 17 years) and 10 adults (mean age: 38.5 years, range: 20 to 64 years). The different reasons for platelet function diagnostics in the 32 patients are given in Table 1. The majority of patients (n= 22) had bleeding symptoms such as skin and mucosal

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**Table 1.** Reasons for platelet function diagnostics in 32 patients

| Reasons for Diagnostics | Number of Patients |
|---|---|
| recurrent epistaxis | 8 |
| bleedings related to surgical procedures and dental extractions | 8 |
| petechial skin and mucosal bleedings | 6 |
| congenital thrombocytopenia | 5 |
| prolonged closure times in the PFA 100® test | 5 |

bleedings as well as bleedings related to surgical procedures or dental extractions. Ten patients were examined because of pathological prefindings. Five of them suffered from hereditary thrombocytopenia and five had pathological results in PFA 100 tests performed in preparation for surgical procedures. For comparison some patients were investigated by additional platelet function tests such as the aggregometry in platelet rich plasma (n= 26), the PFA 100 test (n= 27) and the in vivo bleeding time (n= 24).

**Test Procedures**

Lumi-aggregometry in whole blood (WB)

Samples were tested using the whole blood lumi-aggregometer type 550 VS (Chronolog Corporation, Havertown, PA, USA). The aggregation was determined by the impedance technique [5], and the adenosine triphosphate (ATP) release reaction was measured using the luciferin-luciferase system [8].

The final concentrations of agonists were as follows: adenosine diphosphate (ADP) 20.0 µM; arachidonic acid, 1.0 mM; collagen, 1.0 µg/ml; thrombin, 0.5 U/ml; and ristocetin, 1.25 mg/ml. In the case of thrombin only the ATP release was measured, and in case of ristocetin only the aggregation was determined. The reagents were purchased from Chronolog Corporation.

The calibration of the system and the measurement of aggregation and ATP release were performed according to the producer's instructions. For the test procedure 450 µl whole blood were mixed with 450 µl isotonic saline and 100 µl luciferin-luciferase reagent in plastic cuvettes and stirred at 1000 rpm. In the case of ristocetin, 500 µl of blood and 500 µl of saline without the luciferin-luciferase reagent were used. The aggregation curves were evaluated by measuring the maximal aggregation within 6 minutes. The ATP release curves were recorded until the peak was reached. The free ATP content in the sample was subtracted from the peak height which was transformed into the ATP amount by the ATP standard. The values measured were compared with the reference values which were already obtained on normal controls [12]. The agonist-specific reference range was determined by the mean ± 1 standard deviation (Table 2). The experiments were carried out within 120 minutes after blood sampling.

**Table 2.** Reference ranges for maximal aggregation and peak of ATP release in whole blood samples from children (mean age: 9.3 years, age range: 1–17 years). Data are given as means ± 1 standard deviation (SD).

| Agonists | Parameters | Reference Range |
|---|---|---|
| ADP (20 μM) | maximal aggregation (ohms) | 23.5 ± 9.1 (n=17) |
| ADP (20 μM) | peak of ATP release (nmol) | 1.64 ± 1.1 (n=17) |
| arachidonic acid (1.0 mM) | maximal aggregation (ohms) | 21.7 ± 8.5 (n=16) |
| arachidonic acid (1.0 mM) | peak of ATP release (nmol) | 0.79 ± 0.47 (n=16) |
| collagen (1.0 μg/ml) | maximal aggregation (ohms) | 33.4 ± 7.5 (n=18) |
| collagen (1.0 μg/ml) | peak of ATP release (nmol) | 0.91 ± 0.46 (n=18) |
| thrombin (0.5 U/ml) | peak of ATP release (nmol) | 1.24 ± 0.64 (n=19) |
| ristocetin (1.25 mg/ml) | maximal aggregation (ohms) | 19.6 ± 9.1 (n=19) |

### Aggregometry in platelet rich plasma (PRP)

This method was used only in patients with platelet counts of more than 100 G/l. Platelet aggregation was measured according to the turbidimetric method of Born [3] using a PAP-4 four channel aggregometer (BioData Corp., Hatboro, PA, USA).

The final concentrations of agonists were as follows: adenosine diphosphate (ADP) 4.0 μM; arachidonic acid, 500 μg/ml; collagen, 10 μg/ml; and ristocetin, 1.5 mg/ml. All reagents were purchased from Helena Corporation (Sunderland, Great Britain).

PRP was obtained by centrifugation of blood at 150 x g for 15 minutes. Then, PRP was centrifuged further for 15 min at 1500 x g to yield platelet poor plasma (PPP). Because the platelet count in PRP ranged from 200 to 300 G/l, the platelet counts in the samples were not modified by dilution with autologous PPP.

The calibration of the system and the measurement of aggregation were performed according to the producer's instructions. Aggregation was measured in samples containing 450 μl PRP stirred at 1000 rpm. The aggregation curves were recorded for 10 minutes and evaluated by measuring the maximal aggregation and the desaggregation within this time. Independent on the agonist used, the values were considered to be normal if the maximal aggregation ranged between 60 to 90% and a desaggregation of maximal 10% occurred. This corresponds to the established reference range in our clinical coagulation laboratory. The experiments were carried out within 120 minutes after blood sampling.

### In vivo bleeding time

The bleeding time was measured using the Precisette (Knoll Feinmechanik, Umkirch, Germany). This instrument contains a disposable, automatically retractable lancet and was developed by Sutor and coworkers [23]. On the scale of instrument the puncture depth was adjusted to 3.5 mm leading to an incision depth and length in the skin of about 1.5 mm. A blood pressure cuff was placed on the upper arm of the lying patient and inflated to 40 mm Hg. The puncture was done at the

volar side of the forearm at a 90-degree angle to the long axis of the arm. The blood was absorbed by a filter paper at 30-second intervals avoiding the direct contact to the wound. Because this test needs the cooperation of the patient, it was performed only in children older than 5 years of age. According to the data published from Sutor et al. [24] bleeding times of less than 5 minutes were considered to be normal.

PFA 100 test

The principle of the PFA 100 test was described by Kundu and coworkers [14]. Briefly, an anticoagulated whole blood sample is aspirated under constant vacuum into a collagen/ADP- or collagen/epinephrine-coated membrane, leading to the formation of a platelet plug that obstructs the flow through the aperture. The test result is reported as closure time. The reference range for the closure times in children is given in Table 3 and has been published previously [12]. The measurements were carried out within 4 hours after blood sampling.

**Table 3.** Reference range of closure times in the PFA 100 test. Data are given as means ± 2 standard deviations

| | collagen/ADP-cartridge | collagen/epinephrine-cartridge |
|---|---|---|
| Infants and schoolchildren (>1 to < 18 years, n= 59) | 86 ± 27 s | 107 ± 46 s |
| Adults (> 17 years, n = 46) | 74 ± 26 s | 106 ± 33 s |

### Statistical Analysis

Results of different platelet function tests were compared by determination of the degree of correspondence »kappa«. Statistical analysis was performed using SPSS software for Windows.

## Results

### Aggregation and ATP release by lumi-aggregometry

Platelet function disorders were detected in 20 out of 32 patients by lumi-aggregometry (Table 4). Normal test results were obtained in 10 patients. Fifteen out of 17 hereditary platelet function disorders were clearly classified by this method. In the majority of patients (n = 12) the aspirin like defect characterized by a decreased synthesis of prostaglandins due to an absent or diminished platelet cyclooxygenase activity was detected. In figure 1 a typical disease-related finding is presented – the absence of arachidonic acid-induced aggregation and ATP release.

In 2 patients the platelet function disorder was not classified by the test results of lumi-aggregometry. Later on in one of these patients, a 32 years old woman,

**Table 4.** Findings by lumi-aggregometry in 32 patients

| platelet function disorders | | n = 20 |
|---|---|---|
| aspirin like defect | n = 12 | |
| drug-induced (ASS, valproic acid) | n = 3 | |
| storage pool defect | n = 2 | |
| thrombasthenia Glanzmann | n = 1 | |
| unclassified platelet defect | n = 2 | |
| nonpathological findings | | n = 12 |

the Fechtner syndrome caused by a defect of the nonmuscle myosin heavy chain type IIA was found by immunohistochemical and molecular genetic investigations at the University Greifswald, department of transfusion medicine (head: Prof. Greinacher). In the other case, a 3-years-old girl, the electrone microscopic investigation of platelets at the University Homburg, department of biology (head: Prof. Morgenstern) revealed a complex disturbance of platelet adhesion, aggregation and release reaction without a definite classification of the underlying defect.

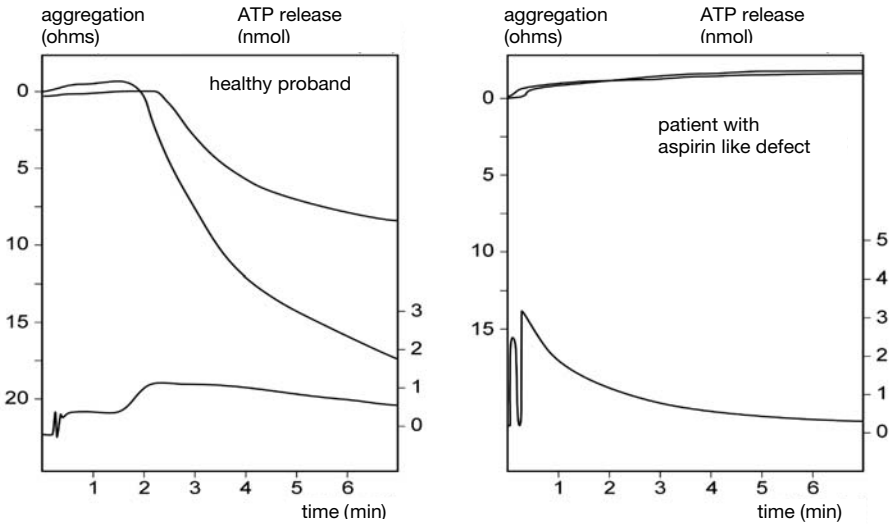


**Fig. 1.** Aggregation and ATP release reaction in whole blood after addition of arachidonic acid at a final concentration of 1 mM. In contrast to the healthy proband, in the patient with aspirin like defect neither an aggregation (upper curve) nor an ATP release reaction (lower curve) were detected.

### Comparison of Methods

The findings obtained from lumi-aggregometry were compared with those from the other 3 methods (Table 5). The highest percentage of similar results was detected for the aggregometry in PRP (81%). Different results were found only in 5 cases where the ADP-induced aggregation was normal in whole blood but diminished in PRP. The results from lumi-aggregometry corresponded to 63% with those from the in vivo bleeding time and to 70% with those from the PFA 100 test. Contrastingly, the results from aggregometry in PRP were in agreement with the results from the in vivo bleeding time only to 48% and from the PFA 100 to 57% (Table 6).

**Table 5.** Comparison of findings by lumi-aggregometry in whole blood with the other methods concerning similar and different findings.

| Methods | Lumi-aggregometry with similar findings | Lumi-aggregometry with different findings | Total number | Degree of correspondence (kappa) |
|---|---|---|---|---|
| aggregometry in PRP | n = 21 (81%) | n = 5 (19%) | n = 26 | 0.58 |
| bleeding time | n = 15 (63%) | n = 9 (37%) | n = 24 | 0.26 |
| PFA 100 | n = 19 (70%) | n = 8 (30%) | n = 27 | 0.40 |

**Table 6.** Comparison of findings by aggregometry in platelet rich plasma (PRP) with the other methods concerning similar and different findings.

| Methods | Aggregometry in PRP with similar findings | Aggregometry in PRP with different findings | Total number | Degree of correspondence (kappa) |
|---|---|---|---|---|
| lumi-aggregometry | n = 21 (81%) | n = 5 (19%) | n = 26 | 0.58 |
| bleeding time | n = 10 (48%) | n = 11 (52%) | n = 21 | 0.05 |
| PFA 100 | n = 12 (57%) | n = 9 (43%) | n = 21 | 0.16 |

## Discussion

The successful use of the lumi-aggregometry for the diagnostics of hereditary or acquired platelet function disorders in adults was firstly described in 1984 [10], and later on confirmed by others [1, 2, 6, 18]. Using the same method in the study presented here platelet function disorders were detected in 20 out of 32 patients and 15 out of 17 hereditary platelet defects and could be clearly classified. Among the 32 patients were 22 children of whom the youngest was 15 months old. Therefore, it has been proven that lumi-aggregometry is suitable also for diagnostics of platelet function disorders in children.

Aggregometry with platelet activating substances (agonists) in platelet rich plasma by the turbidimetric method has been used successfully in many clinical coagulation laboratories for years (reviews to platelet function tests in 21 and 27). However, for a test with four different agonists at least 20 ml of anticoagulated whole blood are needed to obtain sufficient amounts of platelet rich and platelet poor plasma. Contrastingly, for the lumi-aggregometry with four agonists and the ATP standard only 3 ml of whole blood are needed. It has already been demonstrated that both methods provide comparable results concerning the diagnostics of platelet function defects [9]. This is confirmed by our results. Comparing the results from lumi-aggregometry with those from the aggregometry in PRP, the in vivo bleeding time and the PFA 100 test the highest percentage of similar results was found for the aggregometry in PRP (81% compared to 63% and 70%).

The PFA 100 test and the in vivo bleeding time are influenced by different cell components and represent established tests for the detection of disturbed primary hemostasis in children [11, 17, 22, 24]. Therefore, they were chosen for the comparative investigations. The results of both tests corresponded only in part with those obtained from lumi-aggregometry and from aggregometry in PRP. However, the bleeding time as in vivo test detects the bleeding tendency in patients with disturbed primary hemostasis [29]. By this test the clinical relevance of pathological findings of in vitro tests such as the aggregometry in PRP or in whole blood may be determined. Therefore, in patients with suspected disturbance of primary hemostasis in vitro tests should be used to clarify the underlying defect, and a standardized in vivo bleeding time technique is needed to determine the clinical relevance of the in vitro findings. The experiences with the PFA 100 as a screening test for the detection of disturbances of primary hemostasis in children are limited and the published data are partially contradictory [4, 20, 28]. In adults the findings from the PFA 100 test correspond to more than 90% with those obtained from aggregometry in PRP [13]. A close relation between the closure times of the PFA 100 and the results of ADP- and collagen-induced aggregation in platelet rich plasma from adults has been described [16, 19]. These observations could not be confirmed in our study where only 57% of the results from the PFA 100 corresponded to those from the aggregometry in PRP. From this data it may be hypothesized that the PFA 100 is not a reliable test for the diagnostics of platelet function disorders in children.

## Conclusions

The lumi-aggregometry is a useful method for detection of platelet function disorders. The results correspond well with those obtained from aggregometry in PRP which is one the most established platelet function test in the clinical laboratory. However, lumi-aggregometry is superior because of the smaller blood volume requirement which is important especially in pediatrics, the measurement in the more physiologic whole blood environment and the minimal sample manipulation. The bleeding time as in-vivo test is needed to clarify the bleeding tendency in patients with disturbed primary hemostasis. The PFA 100 test seems to be only partially suitable for the detection of platelet function disorders in children.

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# Management of a Premature Infant below 1500 g with Hemophilia A

C. Bidlingmaier, A. Garhammer, S. Jenkins, M. Deml and K. Kurnik

## Introduction

The incidence of prematurity in Germany is approximately 10%. Obtainable literature regarding the management of hemophilic newborns is nearly exclusively targeting at mature babies. The recommendations of the World Federation of Hemophilia for those children are shown in the table (Table). General factor substitution is not recommended [1]. The risk of intraventricular hemorrhage (IVH) is approximately 10% in mature hemophiliacs [2] but already 30% in non-hemophilic premature infants, weighing below 1500 g. Therefore, the prophylaxis and treatment of IVH in hemophilic premature newborns forms a major goal for the pediatrician in care. Information on half-time and recovery of factor VIII concentrate in hemophilic newborns is only rarely available. To our knowledge, the case report of Gale et al. forms the only explicit report on the management of a premature boy with moderate hemophilia weighing 1590 g [3]. We report the case of an infant weighing only 1200 g with moderate to mild hemophilia A.

**Table 1.** Recommendations of the World Federation of Hemophilia for birth-management of possible hemophiliacs [see Ref. 1]

- if wished by the parents: prenatal diagnostics
- gender determination by sonography
- if possible vaginal delivery (not if problems are to be expected)
- no vacuum extraction
- no fetal-scalp-pH-measurements
- early postpartum determination of the factor VIII level
- factor substitution only on demand
- early sonography of the brain
- no intramuscularly application of drugs
- CAVE: bleeding risk of the mother

## Case Report

A known carrier of hemophilia A (reported factor VIII activity in the family: 0.16 IU $ml^{-1}$) was admitted at 26 weeks of gestation after spontaneous rupture of membranes. No prenatal diagnostics were performed apart from gender determination by sonography. The male patient was born via sectio in the 28+3 gestational week

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weighing 1200g. Factor VIII measured directly after birth was surprisingly low (0.03 IU $ml^{-1}$). Sonography of the brain showed intraventricular hemorrhage °I. Therefore the patient was substituted 1 hour after birth with 50 U plasma derived factor VIII concentrate/kg body weight. Due to respiratory problems, the patient had to be ventilated by CPAP. He was consecutively substituted every 12 hours for the first 11 days. The IVH did not worsen and no other bleedings were observed with factor VIII levels of 60-180% 30 minutes after substitution and 22-60% before substitution. Factor VIII replacement therapy was then continued with 30-50 U/kg once daily until week 5. No operations were necessary and any invasive procedure (i.e. correction of feeding tube, blood drawing) was done after substitution. Apart from the mild respiratory problems the only other complication was hemorrhagic enterocolitis, which did improve spontaneously after discontinuation of oral feeding. After the stop of factor substitutions, factor VIII levels dropped to 0.03 IU $ml^{-1}$ again (Fig. 1). No factor VIII inhibitor was observed after 35 exposure days. Factor VIII levels were controlled every day for the first week, then approximately every two to three days. At day 27 recovery (96%) and half-life (6 hrs) were measured (Fig. 2). Due to low plasma volume more repeated measurements were considered unethical, but the data collected showed longer half life during the first days of life. Re-exposure to factor VIII in the sixth month of life after a minor trauma did not lead to the development of an inhibitor. 12 month after birth the factor VIII level increased to 0.09 IU $ml^{-1}$, probably due to maturation of the liver. Genetics of the grandfather and the patient are still unknown, but the rise of factor VIII levels indicates that the boy might only suffer from mild hemophilia, despite the lower factor VIII levels during the first 9 months of life.

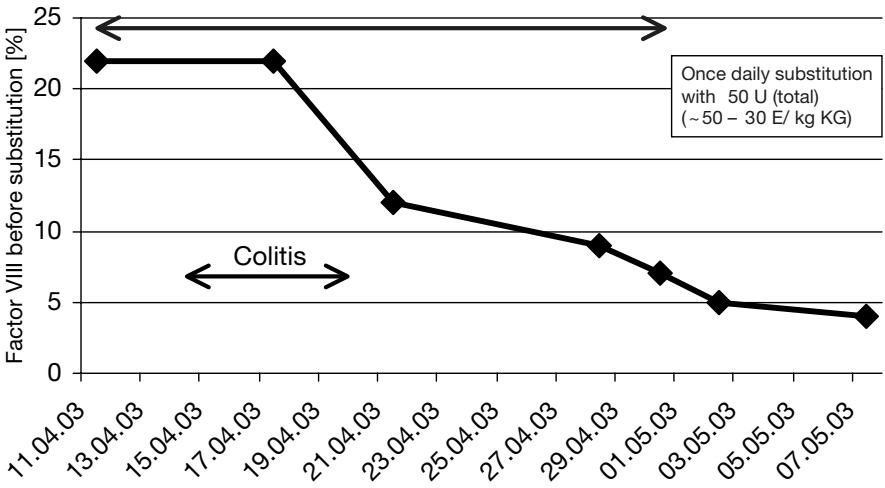


**Fig. 1.** Course of factor VIII levels after day 11. The boy was substituted once daily with 50 U factor VIII concentrate (~ 50-30 U/kg body weight) for the time indicated, the decrease of pre-substitution factor levels after the colitis is caused by the increase in body weight.

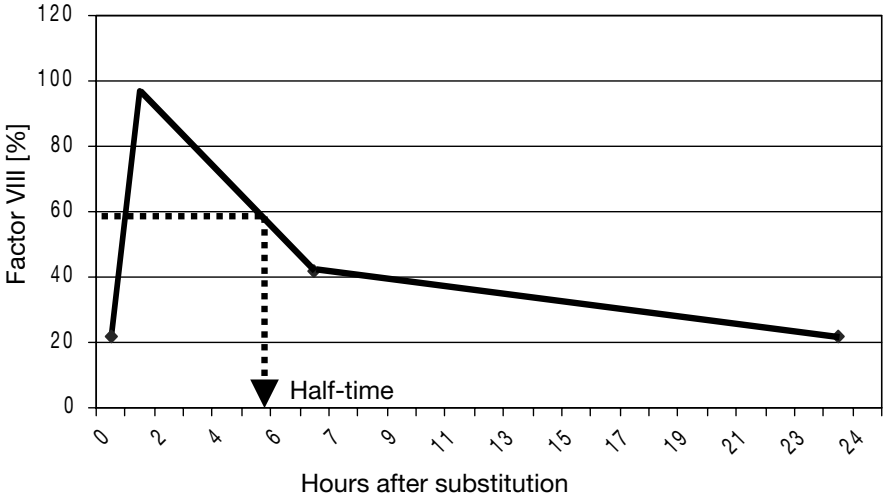


**Fig. 2.** Half-life of factor VIII after substitution of 40 U/kg at day 27 (~ 6 hrs)

## Conclusion

The very benign course of our case report shows that early substitution in a premature infant with hemophilia A can prevent bleeding complications despite low post partum factor levels. Obviously this observation is limited by the fortunate lack of common complications in premature infants below 1500 g, such as the often necessary operations. In concordance with the publication by Gale et al. we report good recovery (96%) but shortened half-life (6 hrs) of the factor VIII concentrate, but both swayed considerably particularly at the beginning of the therapy. The low post partum factor VIII levels increased during the first year of life. Close cooperation between obstetricians, neonatologists and the pediatric hemophilia centre was essential for the successful treatment of our patient.

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# Elevated Factor VIIIC:Ag in Children with Venous Thrombosis and Stroke – Preliminary Results of a Case-Control Study

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## Summary

Elevated factor (F) VIIIC: concentrations are associated with an increased risk of venous thrombosis in adults. To evaluate the role of persistent elevated FVIII:C-Ag in children with venous thrombosis (VT) or ischemic stroke (IS) the present study was performed. FVIII:C-Ag concentrations (Asserachrom VIIIC:Ag, Diagnostica Stago) were measured along with established prothrombotic risk factors six to twelve months after the acute thrombotic onset in 171 white children aged neonate to 18 years suffering a first VT (n = 69) or IS (n =102) and in 255 healthy controls. The cut-off values defined as age-dependent 90$^{th}$ percentiles were obtained from the 255 healthy children. The hereditary nature of elevated FVIII:C-Ag was confirmed in family studies. Median (range) values of FVIII:C-Ag were significantly elevated in children with VT compared with controls [117(25-374)IU/dl vs. 96(21-192)IU/dl; Mann-Whitney tied p-value < 0.000], but not in children with IS [95(49-192)IU/dl vs. 96(21-192)IU/dl; p = 0.7]. In addition, when comparing FVIII:C-Ag in subjects above the 90$^{th}$ age-dependent percentiles with children below the cut-off logistic regression adjusted for age and the presence of established prothrombotic risk factors (factor V G1691A, factor II G20210A, elevated lipoprotein (a), deficiency states of protein C, S and antithrombin, elevated antiphospholipid antibodies) showed a significantly increased odds ratio (OR) and 95% confidence interval (CI) in children with VT [OR/CI: 2.9/1.4-6.1; p = 0.004] but not in patients with IS [1.8/0.9-3.6; p = 0.11]. Data shown here give evidence that familiarly elevated FVIII:C-Ag concentrations independently increase the risk of VT in children 2.9-fold. In contrast, elevated FVIII:C-Ag levels did not play a significant role in pediatric IS so far.


## Introduction

Venous and arterial thrombosis are rare diseases that are being increasingly diagnosed and recognized also in infancy and childhood. Due to the special properties of the hemostatic system during infancy and childhood, symptomatic thrombotic manifestation occurs in 0.07/10,000 children, 5.3/10,000 admissions of children, and 2.4/1000 admissions of newborns to intensive care units [1-4]. Within the entire childhood population, possibly due to the lower concentrations of antithrombin, heparin cofactor II and protein C along with a reduced fibrinolytic capacity, neona-

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tes are at a greater risk of thromboembolic complications than older children. The incidence of vascular accidents decreases significantly after the first year of life, with a second peak during puberty and adolescence again associated with reduced fibrinolytic activity [1, 2]. Numerous clinical and environmental conditions, such as peripartal asphyxia, neonatal infections, fetal diabetes, the use of central lines, trauma or surgery, dehydration, malignant diseases, renal diseases, autoimmune diseases, or the intake of oral contraceptives by adolescent girls resulted in elevated thrombin generation with subsequent thrombus formation in infancy and childhood [2-7]. In addition to the stated underlying clinical conditions, various genetic prothrombotic defects, particularly those affecting the physiological anticoagulant systems, i.e. antithrombin, protein C and protein S deficiency, the mutation of coagulation factor V (G1691A), and the prothrombin gene variant (G20210A), have been well established as risk factors for thrombotic events [8]. In addition, metabolic diseases such as homozygous homocysteinuria and moderate hyperhomocysteinemia as well as increased concentrations of lipoprotein (a), have recently been shown to significantly enhance the risk of thromboembolic arterial and venous thrombosis in pediatric and adult patients [9-13]. The association of multiple hemostatic prothrombotic defects or the combination of established prothrombotic risk factors with acquired environmental or clinical conditions greatly increases the risk of thrombosis, not only in adults but also in infants and children [11].

Several studies have been demonstrated that elevated factor VIIIC: concentrations are associated with an increased risk of venous thrombosis in young and elderly adults [14-20]. Thus, to evaluate the role of persistent elevated FVIII:C-Ag in children with venous thrombosis (VT) or ischemic stroke (IS) the present study was performed.

## Materials and Methods

### Ethics

The present study was performed in accordance with the ethical standards laid down in the updated relevant version of the Declaration of Helsinki and approved by the medical ethics committee at the Westfälische Wilhelms-University, Münster, Germany.

### Study Period

1018 pediatric patients aged neonate to 18 years with a first symptomatic thromboembolic event were consecutively recruited and screened for hereditary prothrombotic risk factors between January 1995 and June 2003 by the following participating centres in the catchment areas of Hamburg, Kiel, Lübeck, Münster, Bielefeld, Düsseldorf, Berlin, Magdeburg, Halle and Munich [9, 11, 12].

**Inclusion Criteria**

Inclusion criteria were a thromboembolic event confirmed objectively by standard imaging methods, i.e., duplex sonography, venography, CT or MR imaging for the diagnosis of venous thromboembolism, and cerebral CT scanning, MR imaging, MR angiography, or transcranial Doppler ultrasonography for the diagnosis of thromboembolic ischemic stroke.

**Exclusion Criteria**

Patients older than 18 years at onset, children not of Caucasian origin, patients with incomplete clinical or laboratory work-up (established prothrombotic risk factors), and subjects lost for follow-up or without parental consent were not enrolled in the present study.

In addition, children during the acute thromboembolic phase, patients with renal insufficiency, liver disease or malignancies, subjects on heparin therapy, and adolescent girls taking oral contraceptives were excluded.

**Final Study Population**

From the ongoing multicenter study, a subgroup of 171 white patients with a median (range) age of 6.4 (0.6 to 18) years was analyzed. Thromboembolic manifestation included VT (n=69) and thromboembolic IS (n=102).

**Control Group**

Patients were compared with 255 white infants from different geographic areas of Germany recruited between January 1996 and June 2003. These control infants, who had no history of chronic disease or of thromboembolic events and were not on medication at the time of recruitment, had presented as outpatients for evaluation before minor surgery (planned circumcisions and hernias) or bone marrow donation [9, 11, 12].

**Laboratory Tests**

With informed parental consent, the factor V G1691A (FV) and factor II G20210A (FII) mutations, concentration of lipoprotein (Lp) (a), protein C (PC), protein S (PS) and antithrombin (AT) levels were investigated in patients and controls 6-12 months after the acute event [16], using standard laboratory techniques [9, 11, 12]. Factor VIIIC:Ag was investigated with a commercially available ELISA (Asserachrom VIIIC:Ag, Diagnostica Stago). Since data on normal values for factor VIIIC:Ag in children are sparse, FVIII:C-Ag concentrations > age-dependent 90$^{th}$

percentiles derived from the healthy control children were used as cut-off values. A type I deficiency (antithrombin, protein C) was diagnosed when functional plasma activity and immunological antigen concentrations of a protein were repeatedly shown to be below 50% of the normal age-related limit. A type II deficiency (antithrombin, protein C) was diagnosed in patients with repeatedly low functional activity along with normal antigen concentrations. The diagnosis of protein S deficiency was based on reduced free protein S antigen levels combined with decreased or normal total protein S antigen concentrations respectively. Criteria for the hereditary nature of a hemostatic defect were its presence in at least one first degree family member, or the identification of a causative gene mutation, or both.

### Statistical analysis

All statistical analyses were performed with the StatView 5 software package (SAS Institute) and the MedCalc software package (MedCalc, Mariakerke, Belgium). Prevalence rates of prothrombotic risk factors in patients and control subjects were calculated by $\chi^2$-analysis or by Fisher's exact test. To compare the overall rate of prothrombotic risk factors between patients and controls and to evaluate the interaction between decreased FVIII:C-Ag concentrations and established prothrombotic risk factors, odds ratios (ORs) together with 95% confidence intervals (CIs) were estimated from a multivariate analysis using a logistic regression model. The significance level was set at 0.05. For variables with a non-Gaussian frequency distribution, data were presented as medians and ranges. All evaluations and comparisons between patients and controls were conducted using the Mann-Whitney test (p-values $< 0.05$ were considered significant).

## Results

### Age-dependent Distribution of FVIII:C-Ag

The upper age-specific $90^{th}$ percentile was 113 IU/dl in children aged 3-6 months, 142 IU/dl in children from 6.1 to 12 months, 138 IU/dl in subjects from 1-9 years, and 148 IU/dl in subjects aged 9.1 to 18 years.

### Median/Range FVIII:C-Ag Concentration in Patients and Controls

The median (range) level of total FVIII:C-Ag was 117 IU/dl (25-374) in the children with VT compared with 96 IU/dl (21-192: $p < 0.0001$) in the controls. Children with IS showed no elevated FVIII:C-Ag values 95 IU/dl (49-192; $p= 0.7$).

### Logistic Regression Analysis

In the multivariate analysis, including the overall rate of established prothrombotic risk factors known to be significantly associated with symptomatic thromboembolism in pediatric patients in Germany, e.g. the FV G1691A mutation, the FII G20210A variant, deficiency states of protein C, S or antithrombin, or elevated Lp(a) concentrations, elevated levels of FVIII:C-Ag > the age-specific 90$^{th}$ percentiles retained their statistically significant and independent association in children with venous thrombosis (OR/CI 2.9/1.4-6.1; p=0.004). No such association was found for the stroke children tested (OR/CI 1.8/0.9-3.6; p=0.11). In children with persistent elevated FVIII:C-Ag levels the inheritance of low concentrations was confirmed in at least one family member [18].

## Discussion

The importance of various hereditary hemostatic abnormalities in contributing to the risk of pediatric thromboembolism is well established [8]. To date, the rate of single or combined prothrombotic risk factors detected in Caucasian children is approximately 50%, while no thrombophilia has been found in the remaining subjects [9-12]. Confirming the findings of adult patients with venous thrombosis [14-20], data presented here give evidence that elevated FVIII:C-Ag concentrations > age dependent 90$^{th}$ percentiles further contribute as a significant and independent risk factor to symptomatic venous thrombosis in Caucasian children [21]. The OR found was 2.9 for children with venous thrombosis, i.e. clearly within the range reported for further inherited thrombophilic risk factors in white children, e.g. the heterozygous FV G1691A mutation, the FII G20210A variant and elevated lipoprotein (a) levels.

In summary, besides the established risk factors, a FVIII:C-Ag concentration > 90$^{th}$ age-dependent percentiles is another risk factor for venous thrombosis in Caucasian children. However, the findings of the present study are restricted to white German patients, and further studies are recommended to clarify the role of elevated FVIII:C-Ag in pediatric populations not of Caucasian origin.

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# Functional Disorders and Treatment Modalities in Hemophilic Children

A. Seuser, G. Schumpe, H.H. Brackmann and T. Wallny

## Introduction

Functional defects are identified by motion analysis today. Motion analysis is done in three spatial dimensions.

Functional analysis is the only way to detect subclinical functional defects of the knee joint. Independently of bleeds, x-rays, MRI imaging, and clinical and orthopedic procedures, motion analysis is a suitable means of identifying functional defects of coordination or strength in the muscles supporting a joint.

Why is it important to investigate functional defects of this kind?

An excursion into physics is necessary at this point. Knee joint motion is crucially determined by what is called the center of motion or center of rotation.

The way we move our knee joint determines the position of the respective center of rotation, and the position of the center of rotation determines the length of the lever, which, by definition, is at right angles to the forces acting upon the center of rotation.

Displacements of the center of rotation may therefore cause very major strains on the joint in a worst-case scenario. The increased strain goes unnoticed but may cause microtrauma in the joints and significantly impair joint integrity in the long term.

## Material and Methods

Functional analysis has been conducted to date in 98 children (196 knees) by treadmill and knee bend tests. The children's ages ranged from 3 to 16 years. Analysis was conducted using an ultrasound-based motion analysis system (original ultrasound topometry). Ultrasound transmitters attached above and below the knee joint send ultrasound impulses to ultrasound receivers positioned at fixed points in space. Run time is taken and, in the presence of a known ultrasound velocity and known distance between the receivers, the three-dimensional coordinates of the individual transmitter can be determined accurate to less than 1 mm. A software algorithm calculates the angle, angular velocity, angular acceleration and roll and glide motion of the knee joint under strain.

The studies were conducted in collaboration with hemophilia treatment centers in Berlin, Bonn, Bremen, Brunswick, Dresden, Erlangen, Erfurt, Frankfurt, Munich, Halle, Hanover, Potsdam and Würzburg.

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Joint angle, angular velocity and angular acceleration curves during locomotion and during knee bends, and the roll and glide pattern of both knee joints during weight-bearing were assessed by qualitative criteria.

## Results

Age-dependent typical deviations from healthy adult locomotion were identified. Four main problems were pinpointed.

Three examples from different age groups are shown.

Figure 1 above shows a normal gait with shaded areas highlighting regular stance phase changes of 12° to 14° in a 16-year-old hemophiliac. The stance phase comprises the following cycles:

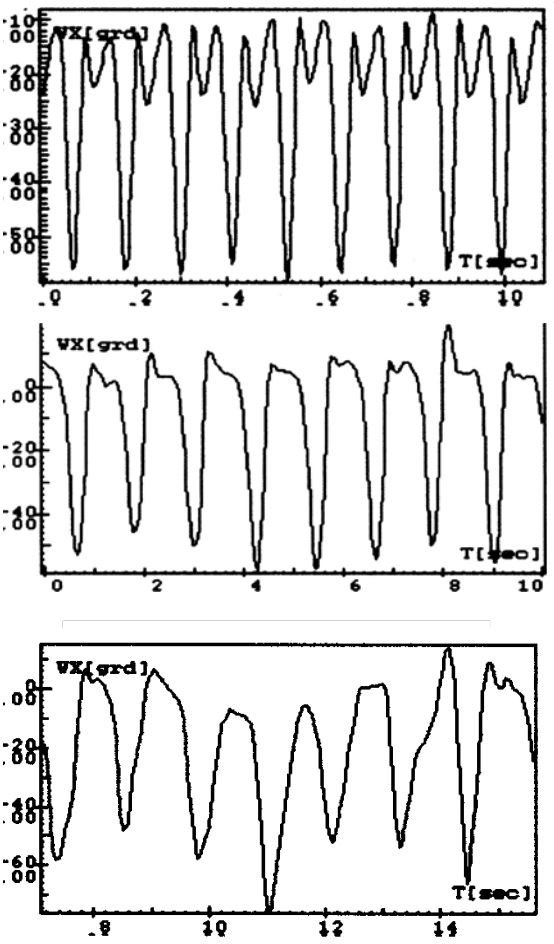


**Fig. 1.** Absent, inadequate or poorly controlled stance phase activity of the knee joint during locomotion (shadowed: stance phase of knee jointg).

1. Heel strike: the knee is almost fully extended as it touches down
2. Load transfer: the knee is first flexed, then extended
3. Toe off: the knee enters the swing phase from an almost fully extended position

The larger downward excursions designate the swing phase.

Figure 1 in the middle shows a significant difference in a child aged 10.

The stance phase is indicated by a small tick only. Although the knee joint extends almost fully at heel strike, it is only flexed initially and transfers to the swing phase during flexion.

Figure 1 below shows a typical gait of a boy aged 3 at the time of investigation. Gait analysis detects almost no real knee joint activity during the stance phase.

Full stance phase activity of the knee joint is very important because load surface transfer takes place and distributes body weight to the largest possible knee joint surface area. If there is no transfer of load-bearing surfaces, i.e. there is no change of angle during weight-bearing, the cartilage comes under strain at that point and overstrain may result in the long-term, with the possibility of microtraumata.

This is a brief illustration of the possible differences in leg axis compliance during gait. Figure 2 above shows fairly regular, rhythmic, sinusoidal lateral deviation in a subject walking on a treadmill.

The lateral deflections range from 10° to 25° and are well above normal. However, because the deviation is regular, rhythmic and sinusoidal, it does not qualify as detrimental. However, it significantly increases the energy requirement and results in more rapid exhaustion.

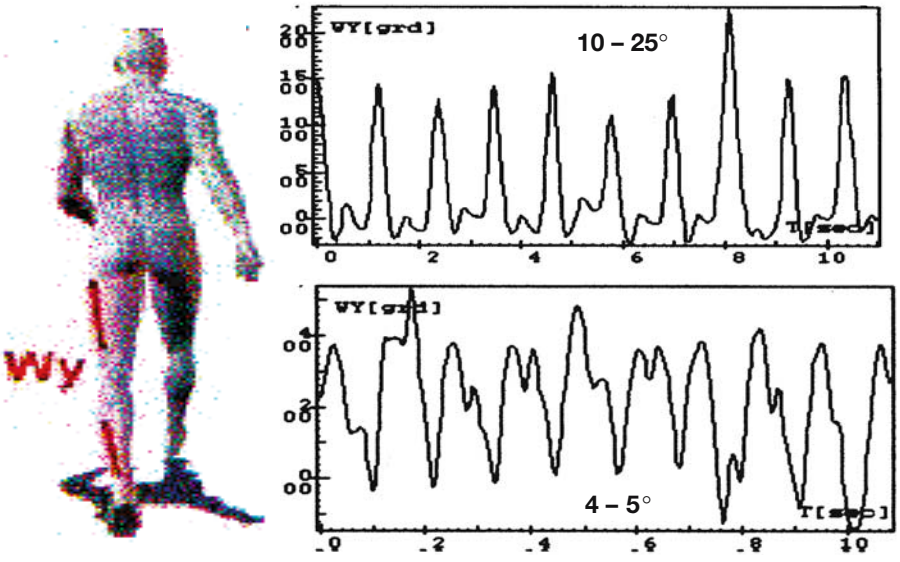


**Fig. 2.** Leg axis deviation during gait

Figure 2 below: Another patient displays a significantly smaller degree of lateral deviation (4°-5°) but a more arrhythmic overall pattern with loss of sinusoidal curvature. Energy consumption is much lower but muscular control is more difficult as smaller amplitudes need to be controlled. However, the peak velocities and accelerations are smaller, with the result that, on the whole, the smaller lateral deviations make for better kinematics despite the increased dysrhythmia.

### Acceleration Peaks in Motion Transition Phases

This is mainly seen during knee bends (Fig. 3) but is also evident during weight-bearing phases of locomotion.

Figure 3 shows on top the knee bend angle of a 16-year-old male patient performing knee bends from 0° to 100°. The corresponding acceleration curve shows the abnormality that is not represented in the angle curvature.

Interim acceleration occurs near full extension in the acceleration curves between ± 20°. This happens during each repetition only when nearing full extension and does not occur in flexion phases. Motion is otherwise regular and rhythmic.

These interim accelerations significantly increase the load on the joint during a very sensitive phase of motion, where forces are in transition from one direction to the other (similar to the baton exchange zone in a 4x100 m relay race, where difficulties are most likely to occur).

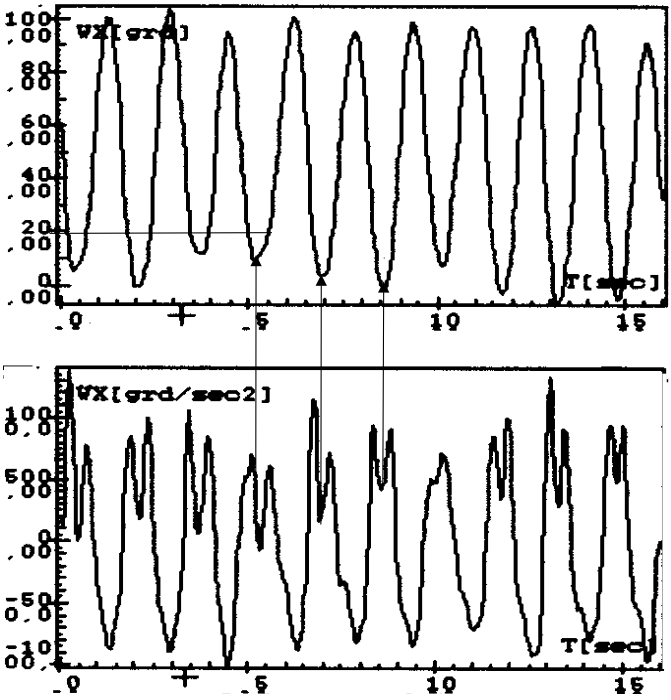


**Fig. 3.** Acceleration peaks in motion transition phases (arrows)

This redirection of forces and the associated interim acceleration as shown in our example impose a shock and heavy load on the joint in a closely circumscribed area of cartilage. The cartilage can be assumed to come under heavy strain.

**Loss of the Roll Element in the Roll and Glide Pattern during Weight-Bearing** (Fig. 4)

Figure 4 shows an ideal roll and glide pattern of a weight-bearing knee joint. This 16-year-old boy has a roll element of 25° when performing knee bends in the 20° to 90° range. The curve rises in an almost linear pattern with a high degree of repeatability and congruence from the lower left to the upper right. (Fig. 4 top)

In contrast, the curvatures observed in a 10-year-old subject are very different. He produces the same linear rise when flexing his knees from 20° to 90° consistent with rolling (R). When straightening up from a hunkering position, gliding on its own is seen from 60° onward (G). (Fig. 4 middle)

Our 3-year-old subject produced the bottommost curve (Fig. 4). A roll and glide pattern is barely discernible apart from a negative roll (nR) with linear progression of the angle presented from the upper left to the lower right.

A perfect roll and glide motion is the ideal way of distributing load on the knee joint. Pronounced gliding elements in themselves are associated with oblique forces

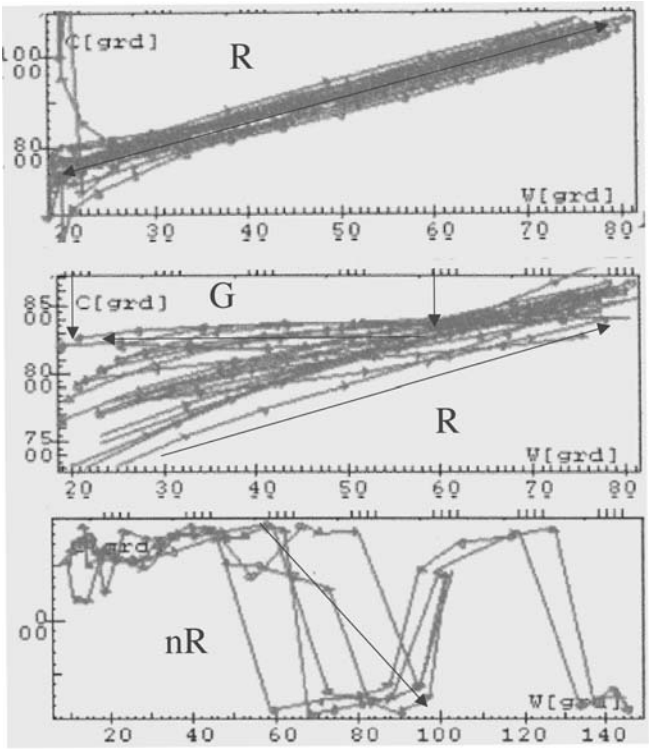


**Fig. 4.** Loss of the roll element in the roll and glide pattern during weight-bearing (R = rolling, G = gliding, nR = negative rolling)

and increased strain on joint elements. Negative roll can be described by the analogy of a car that rolls backwards when attempting to ascend a steep hill.

On the basis of these main functional defects, all of which increase the physical forces acting on the joints and thus may promote bleeding due to the higher load on the joints, an easily implemented physiotherapeutic activity program was developed with the aim of counteracting the functional deficits with just a few exercises. Care was taken to ensure that all the exercises included in the routine are easy to perform and feasible in the home situation under parental guidance.

1. Stance phase exercises for the knee joint (Fig. 5):
The figure above shows a fully normal process from right to left. Heel strike with the knee joint almost fully extended, transfer of body weight, knee bend, and raising of the leg almost fully extended for the swing phase.

This process should be performed carefully and deliberately in slow motion. The speed of the exercise can be increased once the subject has mastered the technique.

This exercise can be varied through the use of different surfaces (solid to more unstable surfaces) and by the application of resistance over the upper legs as shown in Figure 5 below from left to right.

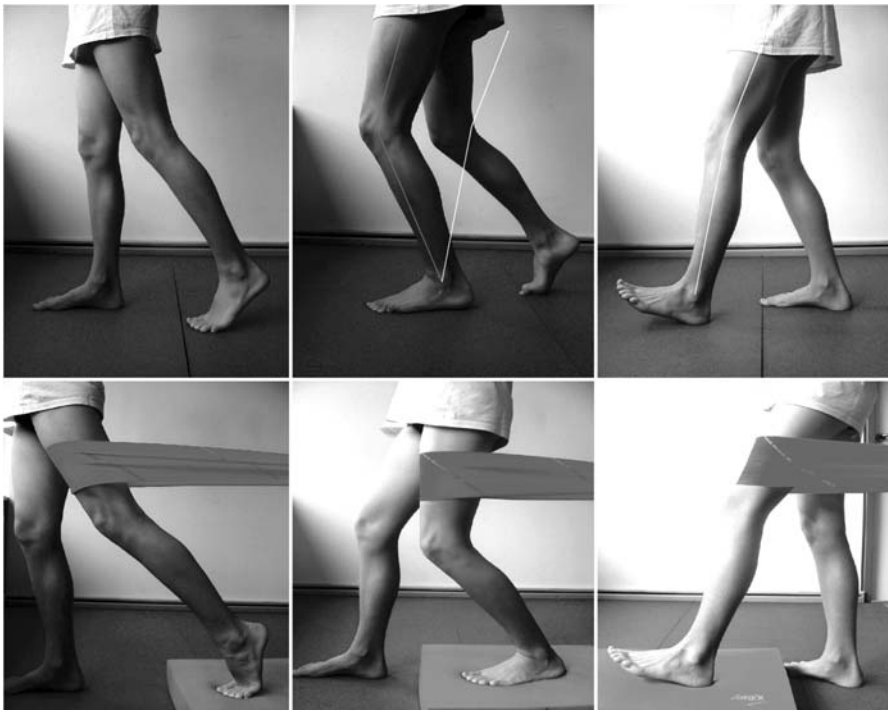

**Fig. 5.** Stance phase exercises for the knee joint

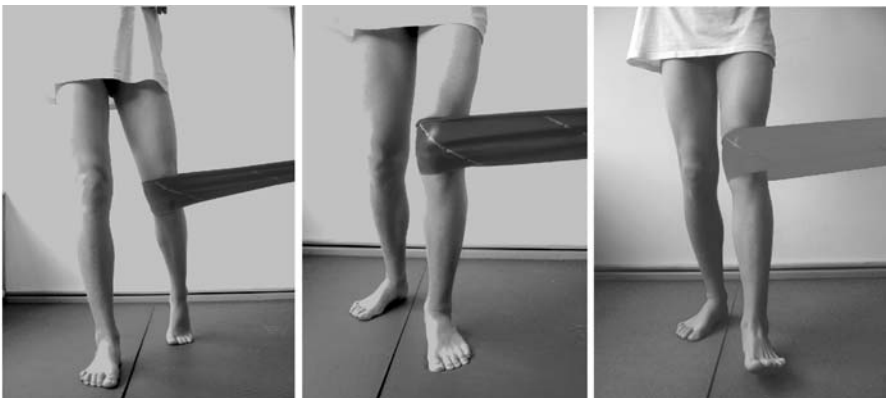

**Fig. 6.** Transition phase training

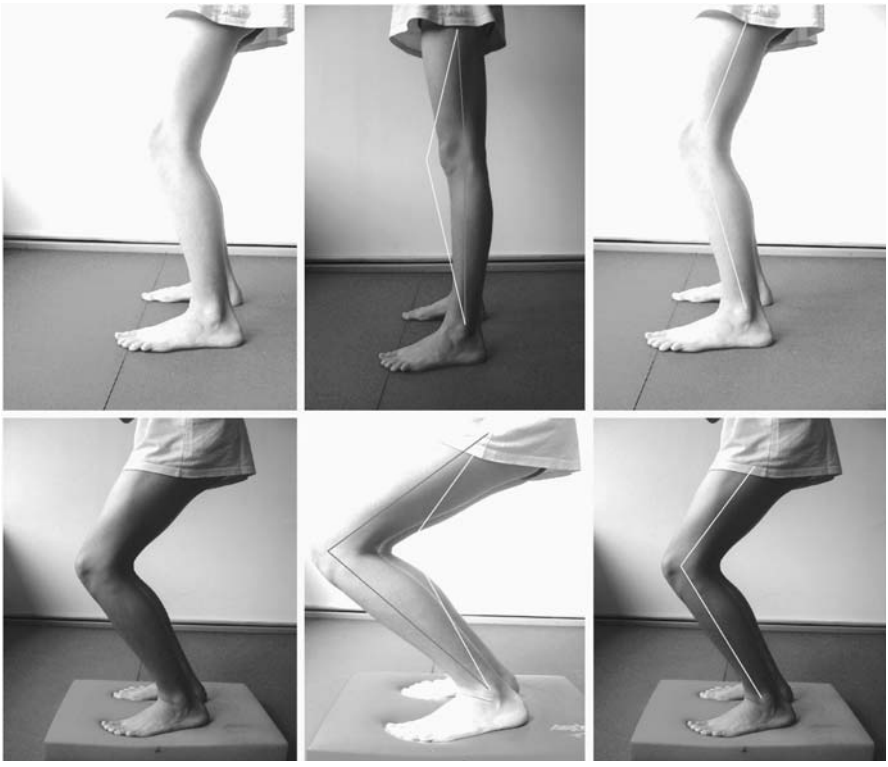

**Fig. 7.** Leg axis exercises

2. Leg axis exercises
Lateral deflection of the leg during gait or knee bends can be counteracted by leg axis exercises. The training program should be started in slow motion, the child's job being to keep his leg compliant to the load axis. An additional force may be applied from outside or inside to strengthen the axis muscles (Fig. 6).

3. Transition phase training (Fig. 7)
Acceleration peaks in the motion transition phase can be counteracted by transition phase exercises. The movement should be repeated in slow motion depending on where the acceleration peaks were identified, for example between 20° flexion and 20° extension.

If the motion transition defects were localized in squatting position, the motion transition exercises should mainly take place during knee bends. The exercise can be varied by starting on stable surfaces and progressing to unstable surfaces, by progressing from slow to rapid motion, and by applying resistance (standing on a Theraband).

4. Training with weights
Loss of the rolling element in the roll and glide pattern during weight-bearing should be treated with suitable weight-lifting exercises (Fig. 8). The program should

**Fig. 8.** Training with weights and coordination

start in glide-only areas, with small excursion movements (escalation through coordination-enhanced effects). Weight training should be initiated precisely in the area where gliding takes place, starting with an endurance training. The size of the motions and also the weights should be increased gradually until a submaximal weight has been reached.

Negative roll phases should be treated by physiotherapy to start with. The primary aim is to improve internal knee joint kinematics through manual therapy, including rotation in the knee bend situation.

## Conclusion

As presented above, greater or lesser deviations from normal adult motion can be seen in the various age groups. Children with hemophilia should be encouraged at the earliest possible age to promote optimum inner knee kinematics in order to minimize external bleeding risks through overstrain and false motion as well as the internal bleeding risks. The largest existing study on this subject has clearly defined these abnormal movements and established an exercise program for prevention and control of these kinematic abnormalities. Follow-up studies after implementation of the exercise program can help show whether the small effort involved managed to improve the patient's situation.

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## *VI. Free Lectures*

Chairmen:

R. Schneppenheim (Hamburg)
M. von Depka Prondzinski (Hanover)

# HLA Profile in Acquired Hemophilia

J. Oldenburg, A. Pavlova, J. Schröder, H.-H. Brackmann, W. Effenberger, R. Zimmermann, A. Huth-Kühne, I. Scharrer, R. Grossmann, E. Seifried and C. Seidl

## Introduction

Acquired hemophilia A is a rare but serious coagulopathy affecting 1 in about 1,000,000 inhabitants/year. Mainly the elderly, persons with autoimmune disorders and infrequently, women in the immediate postpartum period are exhibiting this condition, that results in bleeding into the skin, muscles, gastrointestinal and genitourinary tracts. It is due to autoantibodies directed against specific domains of the factor VIII molecule [1]. The pathogenesis if this disorder is only partly understood. Only in 50% of the patients an underlying disease can be diagnosed, the other 50% of the patients are idiopathic.

## Results and Discussion

In order to investigate whether specific HLA alleles increase the risk of developing acquired haemophilia A we determined the HLA profile in 45 patients with acquired hemophilia A that were diagnosed during the last 6 years in Bonn, Frankfurt, Heidelberg and Würzburg. In the HLA-A, -B and -C systems small relative risk figures were found for A2 (1.6), A3 (0.4), B7 (0.5), respectively (Fig 1). However,

**Table 1.** Evaluation of the relative risk in different HLA alleles with respect to inhibitor formation in acquired hemophilia, normal Caucasians and hemophilia patients with and without inhibitors

| Ac HA vs N | | Inh HA vs Non- Inh. HA | |
|---|---|---|---|
| Higher Risk | RR | Lower Risk | RR |
| A2 | 1.5 | A2 | 0.5 |
| DQB 0502 | 9.9 | DQB 0502 | 1.1 |
| DR13 | 1.9 | DR13 | 0.1 |
| DR16 | 9.9 | DR16 | 1.5 |
| | | | |
| Lower Risk | | Higher Risk | |
| A3 | 0.4 | A3 | 2.2 |
| B7 | 0.5 | B7 | 4.0 |
| DQB0602 | 0.3 | DQB0602 | 3.7 |
| Dr15 | 0.6 | DR | 2.2 |

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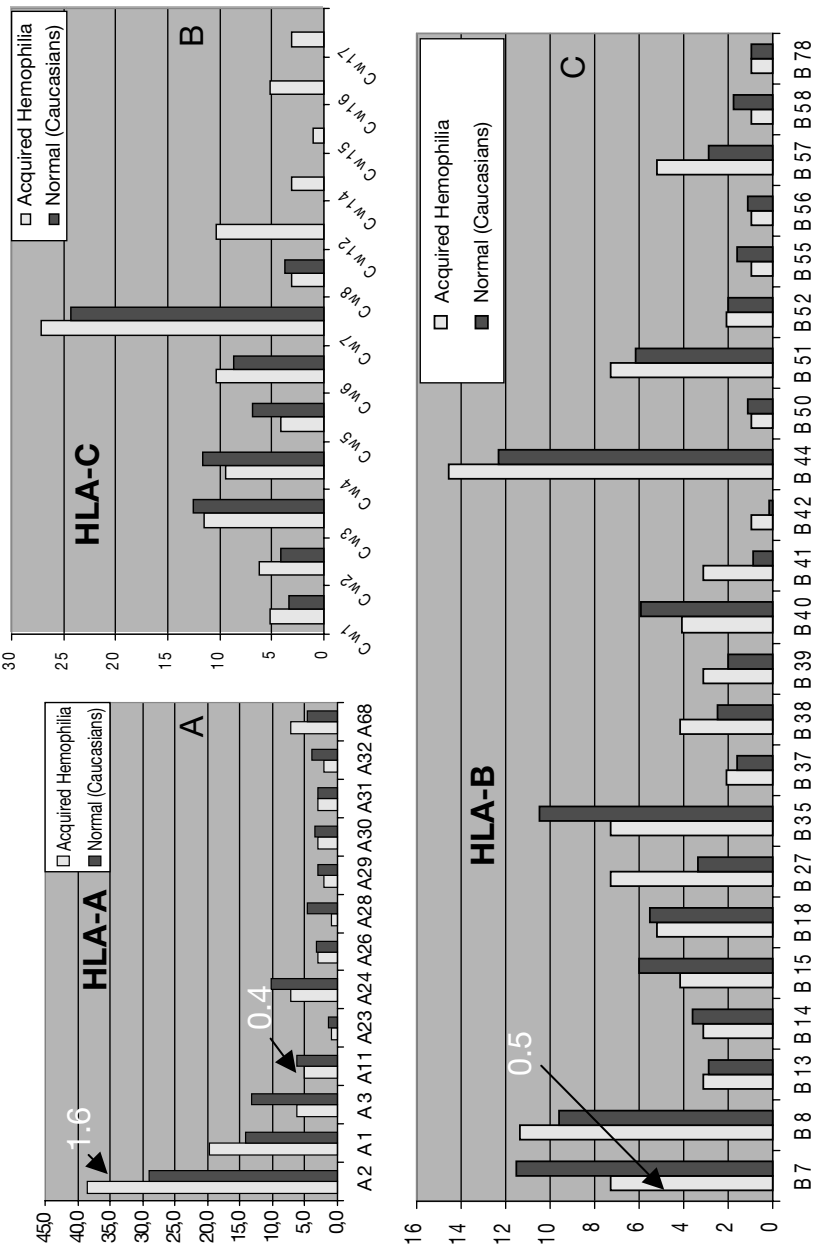


**Fig. 1.** Comparison of HLA class I antigen distribution in patients with acquired hemophilia and Caucasian population. (A: HLA -locus A; B: HLA -locus B; C: HLA -locus C)

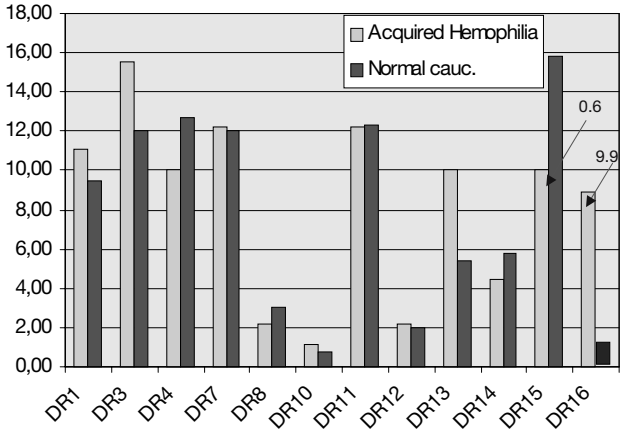


**Fig. 2.** Comparison of HLA class II - DR antigen distribution in patients with acquired hemophilia and Caucasian population

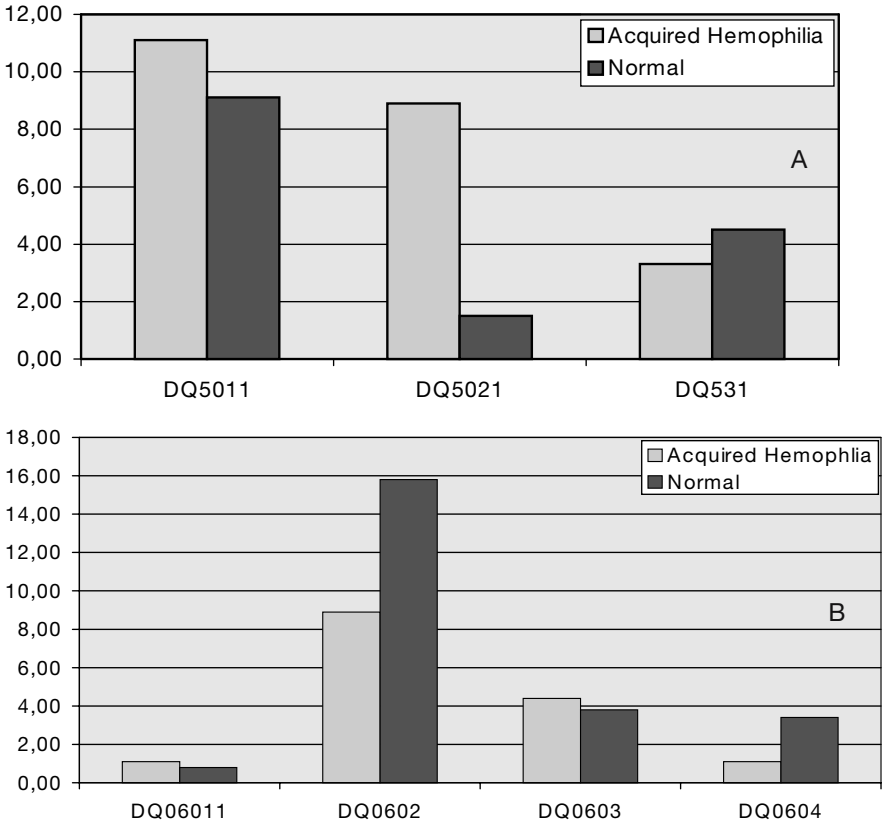


**Fig. 3.** Comparison of HLA class II - DQ5 (A) and DQ6 (B) subclass distribution in patients with acquired hemophilia and Caucasian population

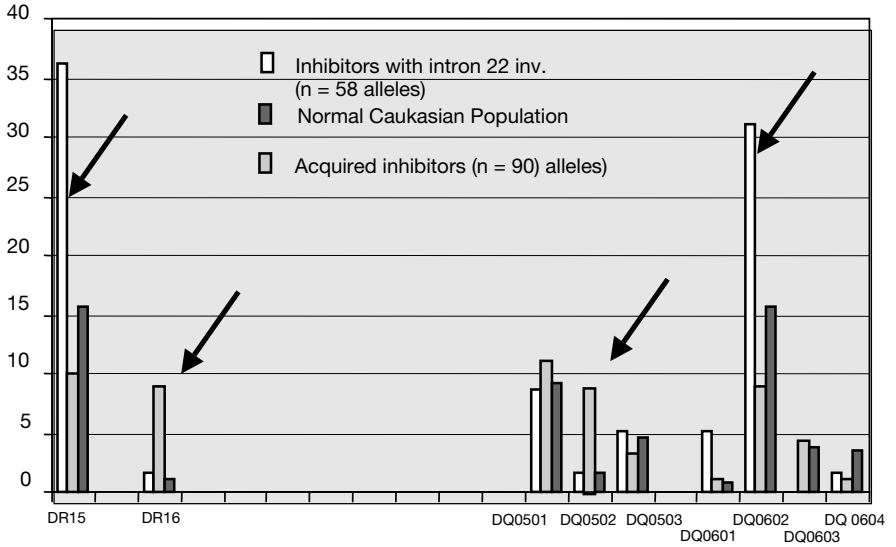


**Fig. 4.** MHC ClassII profile in inherited hemophilia with inhibitors, acquired hemophilia and normal Caucasians

DR16 and DQB0502 showed a substantial increase of frequency in acquired inhibitor patients compared to normal population (10% vs 1%), resulting in a relative risk of 9.9, while DR15 and DQB0602 showed decreased relative risks of 0.6 and 0.3 (Fig 2, 3 and 4) [2, 3]. This finding may be of special interest because HLA alleles with higher risk figures in acquired HA have lower risk figures in inhibitors with inherited HA and vice versa (Table 1). Thus in the presence of endogenous FVIII other HLA alleles may promote immune response than in the absence of endogenous FVIII [4, 5]. Moreover, it has been reported by Chicz et al. (1993) that a FVIII peptide comprising amino acids 1705 to 1721 has been eluted from a DR15 cell line and therefore may be of significance for the immune response towards FVIII [6].

## Conclusions

The DR16 and DQB0502 alleles were more frequent in acquired hemophilia A than in the normal population. Although not statistically significant, the HLA profile in acquired hemophilia A gains importance because of its antipodal expression compared to inhibitors in inherited hemophilia A.

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# Characterization of Polyclonal Factor VIII-Inhibitory Antibodies

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## Introduction

Clotting factor VIII (FVIII)-inhibitory antibodies develop in approximately 30 % of patients with severe hemophilia A and render further classical substitution therapy ineffective [1-4]. Although certain variables are known to play crucial roles in the development of FVIII-inhibitory antibodies [5], the pathogenesis of these »inhibitors« is still not fully understood. The FVIII molecule is a glycoprotein consisting of a series of repeated, homologous domains A1-A2-B-A3-C1-C2 [6]. The heavily glycosylated B domain with no known function [7] is least immunogenic [8]. In contrast, a large number of inhibitory antibodies have been found to bind to the FVIII domains A2 and C2 [9–11], but until now only few epitopes have been clearly defined and characterized [12]. However, to understand the inactivation mechanisms and to pave the way towards modifications of recombinant clotting factors that reduce their immunogenicity, the exact localization of immunodominant epitopes is required. Here, immunoprecipitation of radioactively labelled FVIII fragments by the patient's antibodies was used to assess the distribution of epitopes with respect to FVIII domains in twelve hemophilia A patients. In addition, a strategy for precise epitope mapping in the polyclonal FVIII antibody population of one individual is presented. This strategy employs a random peptide phage display library to identify epitopes of antibodies isolated from the patient's plasma by affinity chromatography.

Epitope mapping can be performed by different methods including Western blotting or immunoprecipitation of candidate regions [9, 13], site-directed mutagenesis [14], deletion analysis of recombinant FVIII fragments [15], homologue scanning mutagenesis using recombinant hybrid human/porcine FVIII [11] or screening of libraries of overlapping target sequence-specific peptides [16]. However, the use of random peptide phage display libraries may offer advantages over all these approaches, because the precise localization of the antibody binding site could be achieved very rapidly by screening a large number of short random peptides (more than $10^9$ different sequences), allowing even for identification of discontinuous epitopes [17]. The method is based on the *in vitro*-selection of peptides genetically fused to a coat protein of a bacteriophage resulting in their display on the surface of the virion. This selection (biopanning) is carried out by incubating the pool of phage-displayed peptides with the target molecule immobilized on a plate or beads, washing away unbound phages and eluting bound phages either spe-

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cifically with the target itself, its ligand or by non-specific disruption of binding interactions, e.g. with an acidic buffer. Selection and amplification of the phage eluate are repeated several times to enrich the pool iteratively in favour of the best binding partners.

## Material and Methods

### Plasma Samples

Twelve plasma samples from hemophilia A patients aged between 21 months and 45 years, who had developed inhibitors in response to treatment with purified human or recombinant FVIII, were investigated. The inhibitory activity of each plasma sample as quantified by the Nijmegen modification of the Bethesda assay [18] is given in Table 1. For patient I, HLA analysis and DNA sequencing to identify the mutation in the factor VIII gene were performed.

### Radio-Immunoprecipitation

Immunoprecipitation of radioactively labelled FVIII fragments was performed as described previously [8–10]. Briefly, serial dilutions of the plasma samples (1:10, 1:50, 1:250, 1:500, 1:1000 and 1:3000) were incubated with 10 µl $^{125}$I-labelled recombinant FVIII at a concentration of 0.75 nM, which equals the normal concentration in human plasma, for 20 hours at 4°C with agitation. Immune complexes consisting of $^{125}$I-labelled FVIII and the patient's FVIII antibodies were captured by Protein G-sepharose beads (Pharmacia). After washing the beads three times with TBS containing 0.05 % Tween 20 (Sigma), the radioactivity of bound FVIII was quantified with a γ-counter (Perkin-Elmer). Immunoprecipitation units (IPU) were calculated using the formula (cpm precipitated – cpm precipitated by control resin without plasma)/(total cpm added to the assay) taking into account the plasma dilution (cpm = counts per minute). To localize the epitopes on the FVIII domains, the individual domains A1, A2, A3, C2 and the entire light chain were labelled with $^{125}$I and used in the same manner for immunoprecipitation.

### Antibody Purification

The antibodies were isolated from the patient's plasma sample by affinity chromatography in two steps. After buffer exchange, first the total immunoglobulin content was collected from the flow-through of a DEAE Affi-Gel Blue Gel column (BioRad). In the second step, FVIII-specific antibodies were isolated from the total IgG pool by chromatography on a FVIII column produced by coupling 4000 IU of recombinant human fVIII (ReFacto, Wyeth) to the matrix using either the UltraLink kit (PIERCE) or CNBr-activated sepharose (Amersham) according to the manufacturer's instructions. The eluate was dialyzed and concentrated, and total amount and

purity of FVIII-specific antibodies were determined by ELISA using human IgG (Jackson ImmunoResearch Laboratories) and a monoclonal human FVIII antibody (Bo2C11, a gift of Prof. Saint-Remy, University of Leuven, Belgium) as standards. Plates were coated either with goat $F(ab')_2$ fragment-specific anti-human IgG (Jackson ImmunoResearch Laboratories, dilution 1:1000) or recombinant human FVIII (0.5 µg/ml) and incubated with the samples. Donkey anti-human IgG (H+L) coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, dilution 1:2000) was used for the detection of bound patient's antibodies in a color reaction with *o*-phenylenediamine.

**Selection of random Peptides Displayed by Phages**

In this study the Ph.D.-7 Phage Display Peptide Library kit (New England Biolabs), a combinatorial library of random heptamers, was used for biopanning according to the manufacturer's instructions. For each round of selection $2 \times 10^{11}$ phages were applied to wells of Maxisorp microtiter plates (Nunc) coated with the purified antibody preparation in 0.1 M $NaHCO_3$ buffer, pH 8.6, (0.1 µg/ml FVIII-specific IgG) overnight in a humidified box. Unbound phages were then removed by repeated washing with Tris-buffered saline (50 mM Tri-HCl, 150 mM NaCl, pH 7.5), bound phages were specifically eluted with recombinant FVIII (ReFacto, Wyeth) at a concentration of 700 nM. The eluted phage pool was amplified in *E. coli*, purified and used for three additional binding and amplification cycles, in which the stringency of the selection was increased by adding the detergent Tween-20 (up to 0.5 % final concentration) and stepwise reducing the phage binding time from 60 to 10 minutes. Finally, 24 to 48 individual clones were isolated and characterized by DNA sequencing. The corresponding peptides were aligned with the human FVIII amino acid sequence to identify regions of homology. The human FVIII antibody F14A12 (a gift of Sylvie Villard, University of Montpellier, France) served as control for the biopanning experiment.

**Phage Binding Assays**

The binding of the isolated amplified phages to the purified patient's antibodies was investigated by ELISA as described previously [19]. Briefly, serial dilutions of the phages were applied to the wells of microtiter plates coated with the purified antibody preparation (0.1 µg/ml FVIII-specific IgG) and blocked with 2 % bovine serum albumin in phosphate-buffered saline. For the competitive ELISA, recombinant human FVIII (ReFacto, Wyeth) at a concentration of 1 µg/ml was applied simultaneously with the phages. Unbound phages were removed by repeated washing, and the bound phages were detected using an anti-M13 antibody-horseradish peroxidase conjugate (Pharmacia) diluted 1:2000 in blocking buffer. The color reaction with *o*-phenylenediamine substrate was quantified by measuring the absorbency at 490 nm. All values were corrected for phage binding to blocked uncoated wells.

### Competitive ELISA with Peptides

Diluted patient's plasma containing FVIII-specific antibodies at a concentration of approximately 1 nM was pre-treated overnight at 4°C with serial dilutions (1 μM to 1 mM) of synthetic peptides (ISIS, Markredewitz) corresponding to the putative epitopes on the FVIII molecule, irrelevant control peptides or with phosphate-buffered saline (PBS) as a control. The pre-treated plasma samples were then applied to microtiter plates coated overnight with recombinant FVIII (5 nM ReFacto, Wyeth). After repeated washing bound FVIII antibodies were detected using a horse radish peroxidase-coupled donkey anti-human IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, dilution 1:2000) and *o*-phenylenediamine as substrate for the color reaction.

### Bethesda Assay

The effects of synthetic peptides on the inhibitory activity of the respective plasma samples were studied by the Nijmegen modification of the Bethesda assay [18]. Plasma dilutions giving approximately 50 % residual FVIII activity were pre-treated overnight at 4°C with synthetic peptides (ISIS, Marktredewitz) corresponding to the putative epitopes on the FVIII molecule or with PBS as a control, then mixed with equal volumes of pooled normal human plasma (PreciClot Plus I, Stago/Roche) in parallel to FVIII-depleted human plasma controls and incubated at 37°C for 2 hours. FVIII residual activity was measured in a one stage FVIII assay, and the inhibitory activity in Bethesda units/ml was calculated as described previously [18]. The data were analyzed statistically by calculating the p-value using the two-tailed student's t-test.

**Table 1.** Immunoprecipitation of radioactively labelled FVIII fragments by IgG in the plasma of hemophilia A patients (IPU/ml with standard deviation, n = 3; n.d., not determined)

| Patients | A1 | A2 | A3 | B | C2 | light chain |
|---|---|---|---|---|---|---|
| I | 112 ± 9 | 98 ± 11 | 0 | 0 | 0 | 228 ± 12 |
| II | 68 ± 3 | 94 ± 4 | 3.1 ± 0.4 | 0 | 3.8 ± 0.5 | 484 ± 18 |
| III | 0 | 8.6 ± 0.7 | 1.2 ± 0.6 | 0 | 7.1 ± 0.4 | 480 ± 18 |
| IV | 81 ± 3 | 171 ± 6 | 5.2 ± 0.2 | 0 | 8.2 ± 0.9 | 16.4 ± 1.4 |
| V | 0 | 0 | 4.6 ± 0.6 | 0 | 14.3 ± 0.9 | 15.0 ± 0.7 |
| VI | 15 ± 3 | 160 ± 14 | n. d. | n. d. | 37 ± 3 | 157 ± 16 |
| VII | 78 ± 2 | 272 ± 22 | n. d. | n. d. | 1117 ± 60 | 500 ± 15 |
| VIII | 2 ± 2 | 6 ± 1 | n. d. | n. d. | 0 | 159 ± 17 |
| IX | 85 ± 12 | 112 ± 16 | n. d. | n. d. | 665 ± 50 | 444 ± 27 |
| X | 2 ± 1 | 100 ± 12 | n. d. | n. d. | 0 | 15 ± 4 |
| XI | 1 ± 3 | 3 ± 2 | n. d. | n. d. | 0 | 2 ± 3 |
| XII | 90 ± 3 | 184 ± 15 | n. d. | n. d. | 446 ± 20 | 341 ± 24 |

## Results and Discussion

The FVIII antibodies of twelve hemophilia A patients were characterized by immunoprecipitation with radioactively labelled FVIII fragments (Table 1). The data obtained illustrate the heterogeneity of the antibody epitopes with respect to number and specifity and emphasize the variety of factors such as type of mutation in the FVIII gene, purity of the FVIII product administered and MHC and other immune response genes which influence the risk and extent of inhibitor formation. Except for the B-domain, all FVIII domains seem to be able to elicit an immune response as shown by the high diversity of antigenic domains in our patients which is in agreement with the distribution of already identified epitopes in the A2, A3, C1, C2 domains of the FVIII molecule [20].

For patient I this method revealed antibody binding to the A1 domain, the A2 domain and the entire light chain, whereas no signal was detected for the domains A3, B and C2 of FVIII. Although there was no C1 domain available for direct testing, the binding to the light chain consisting of A3, C1 and C2, together with the lack of binding to the individual domains A3 and C2 clearly indicated an epitope in the C1 domain. This patient, who carries an intron 22 inversion on both alleles of the factor VIII gene, has an increased risk for the development of inhibitory antibodies, because the inhibitor prevalence in patients with such severe molecular defects (e.g. large deletions, nonsense mutations, intron 22 inversion) and complete absence of endogenous FVIII is 7-10 times higher than in patients with milder molecular gene defects (e.g. missense mutations, small deletions, splice site mutations) [20]. HLA-analysis revealed A2, A30(19), B40, DRB104, DRB109, DQB10302 and DQB10303 alleles, but not A3, B7, C7, DQA0102, DQB0602 or DR15 which have been reported to occur more often in inhibitor patients with an intron 22 inversion [21]. However, a strong correlation of any HLA-allele to inhibitor or non-inhibitor status has not yet been established.

FVIII-specific antibodies of patient I were analyzed in detail by epitope mapping using a random peptide phage display library. The antibodies were purified from the plasma in two steps of affinity chromatography. First, total IgGs were isolated by collecting the flow-through of a DEAE Affi-Gel Blue Gel column (BioRad) and their purity was checked by protein gel electrophoresis (Fig. 1). In a second purification step, FVIII-specific antibodies were bound to a FVIII-column, eluted and quantified by ELISA. Typical yields after purification of 1 ml plasma were 0.5–5 μg of FVIII-specific antibodies with a purity of approximately 40 % of the total IgG as determined by ELISA. The FVIII inhibitory activity of the purified antibody samples was quantified in Bethesda assays and ranged from 10 to 140 Bethesda Units/μg total IgG.

For each purified sample at least three phage binding and amplification cycles were performed, in which the stringency of the selection was increased gradually. Control biopanning experiments were carried out on plates coated with the monoclonal human FVIII antibody F14A12 specific for the known epitope EMDVVRF in the A1 domain (Sylvie Villard, personal communication). In these controls the phage concentrations in the eluates increased from the first to the third round by almost 5 orders of magnitude despite constant input of $2 \times 10^{11}$ phages, indicating a

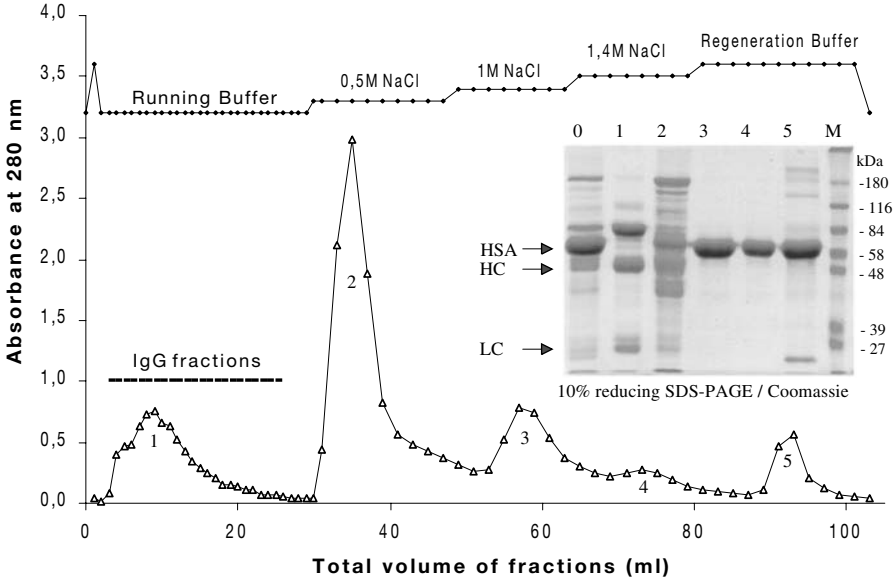


**Fig. 1.** Chromatogram of the purification of total immunoglobulins and protein gel of fractions

Patient's plasma (1 ml) was loaded on a DEAE Affi-Gel Blue Gel column (5 ml, BioRad) followed by running buffer as described in the manufacturer's manual. Total IgGs were collected from the flow-through while other plasma plasma proteins including the predominant albumin fraction remained bound to the column material and were only eluted at increasing concentrations of salt during the regeneration steps.

Insert: Proteins of denatured samples of original plasma (0) and of peak fractions (1-5) were separated on a reducing 10 % SDS-polyacrylamide gel and stained with Coomassie-Blue. HSA, human serum albumin; HC, IgG heavy chain; LC, IgG light chain; M, protein molecular weight marker.

selective enrichment of antibody-binding phages. After three rounds of panning more than 75 % of the isolated phages showed the known or an equivalent motif as well as strong binding to human FVIII in ELISA binding studies. This control experiment proved the concentration of the recombinant hFVIII and the strength of its interaction with the antibodies to be sufficient for the elution of phages bound to FVIII-specific antibodies.

In the panning rounds with purified patient's antibodies, stepwise increasing phage concentrations in the eluate also indicated a selective enrichment of antibody-binding phages in all samples analyzed. However, among the phages isolated after the first panning on the antibody preparation of patient I only few consensus motifs were found. Phage I-A1 containing the peptide NPVERSL with the highest similarity to the human FVIII amino acid sequence (Fig. 2a) was the only one from this panning which bound clearly to the corresponding antibody preparation (Fig. 2b). These data suggested an epitope of 5 amino acids in the A1 domain of FVIII. However, repetition of the panning resulted also in selective enrichment of the phage I-A2 displaying the peptide MLPSVLD and the phage I-C1 displaying the

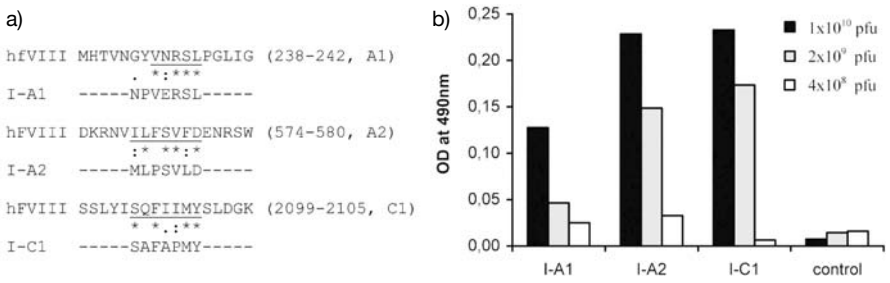


**Fig. 2.** Characterization of phages selected on purified FVIII antibodies of patient I.
a) Alignment of the phage-displayed peptides with the amino acid sequence of human FVIII (* = fully conserved residue; : = strong conservation, . = weak conservation)
b) Analysis of phage binding to the purified FVIII antibodies by ELISA. Serial dilutions of amplified phages (1 x $10^{10}$ to 4 x $10^8$ plaque forming units, irrelevant phage as control) were added to microtiter plates coated with the antibody preparation. Bound phages were detected using an anti-M13 antibody-horseradish peroxidase conjugate. All values were corrected for phage binding to blocked uncoated wells.

peptide SAFAPMY. These peptides showed significant similarity to amino acid sequences in the A2 and C1 domain of human FVIII, respectively (Fig. 2a), and both phages bound strongly to the purified antibody preparation (Fig. 2b). The specificity of this interaction was demonstrated by competitive ELISA in which the binding of all three phages was reduced significantly in the presence of recombinant human FVIII (Fig. 3a).

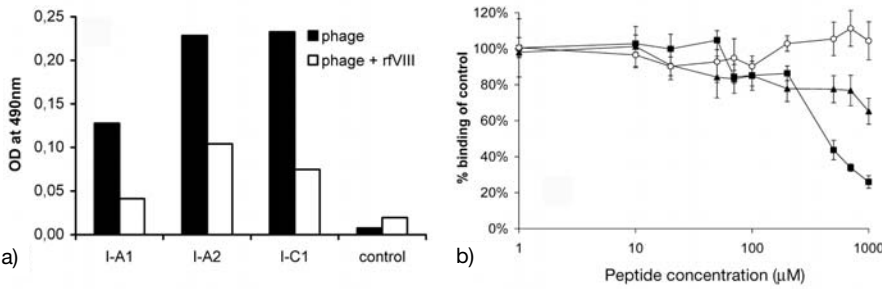


**Fig. 3.** Competitive ELISAs for antibodies of patient I
**a)** Inhibition of phage binding to the FVIII antibodies of patient I by recombinant FVIII. Recombinant human FVIII (0.5 µg/ml) was applied simultaneously with the respective phages (1 x $10^{10}$ pfu each, irrelevant phage as control) to wells coated with the purified FVIII antibodies of patient I and detected by ELISA as described above. All values were corrected for phage binding to blocked uncoated wells.
**b)** Inhibition of the binding of FVIII antibodies to FVIII by the peptides sI-A2 and sI-C1
Plasma of patient I (1 : 500 dilution) was pre-treated overnight at 4°C with serial dilutions of the synthetic peptides sI-A2 (ILFSVFD, triangles), sI-C1 (SQFIIMY, squares), a control peptide (RSNAWRP, open circles) or PBS as control and then applied to microtiter plates coated with recombinant human FVIII (3 nM). Bound FVIII antibodies were detected using a horse-radish peroxidase-coupled donkey anti-human IgG antibody. Error bars represent the standard deviations (n = 3).

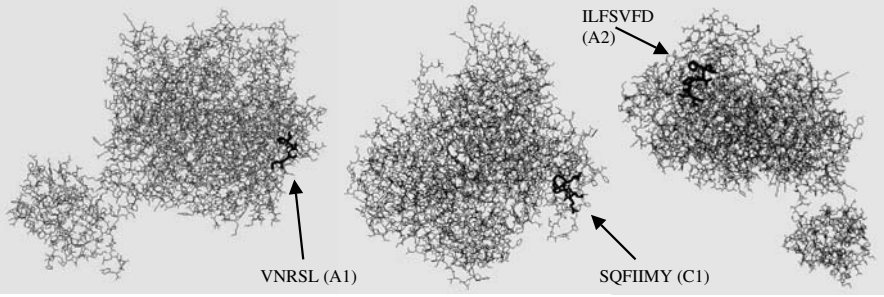


**Fig. 4.** Location of the putative epitopes in the three dimensional structural model of human FVIII
The localization of the putative epitopes inferred from selected phages was determined using the Swiss PdbViewer v3.7 (www.expasy.org/spdbv) and published structural data [22]

These findings were in agreement with the immunoprecipitation data, since all three putative epitopes were located in domains that had been shown to be antigenic (Table 1). However, the presence of further epitopes in each of these domains could not be excluded.

In the three-dimensional structural model of human FVIII [22] all three putative epitopes, valine-asparagine-arginine-serine-leucine (VNRSL), isoleucine-leucine-phenylalanine-serine-valine-phenylalanine-aspartic acid (ILFSVFD) and serine-glutamine-phenylalanine-isoleucine-isoleucine-methionine-tyrosine (SQFIIMY) appear to be located at the surface of the molecule and thus likely to be accessible to antibodies (Fig. 4).

To determine whether the respective antibodies only bound to the epitopes identified without inhibiting the activity of FVIII or whether they had indeed an inhibitory effect, synthetic peptides (sI-A1, sI-A2 and sI-C1) corresponding to the putative epitopes on the FVIII molecule were made and investigated in competitive Bethesda assays. Pre-incubation of the plasma from patient I with the peptide sI-A2 (ILFSVFD) at a concentration of 1 mM reduced its total inhibitory activity by 12.4 % from 304 to 266 BU/ml ($p < 0.01$), whereas pre-incubation with the peptide sI-C1 (SQFIIMY) at the same concentration resulted in a decrease by only 8.6 % to 277 BU/ml ($p < 0.1$). The combination of both peptides led to a reduction of the inhibitory activity by 17 % to 252 BU/ml. However, the peptide sI-A1 (GYVNRSL) did not have any significant neutralizing effect (Fig. 5). A control peptide (RSNA-WRP) did not reduce the inhibitory activity of the plasma. These data indicate that the peptides sI-A2 and sI-C1 mimic functional epitopes for FVIII-inhibitory antibodies and are able to neutralize the inhibitory activity of such antibodies at least partially. The lack of a neutralizing effect of the peptide sI-A1 is not contradictory to the observations described above, because the phage selection by biopanning, the ELISA binding studies and competitive ELISA as well as the radio-immunoprecipitation only investigated the binding of a particular amino acid sequence to the respective antibody sample but not the functional effect of this binding on the FVIII molecule.

A competitive ELISA, in which increasing concentrations of the peptides sI-A2

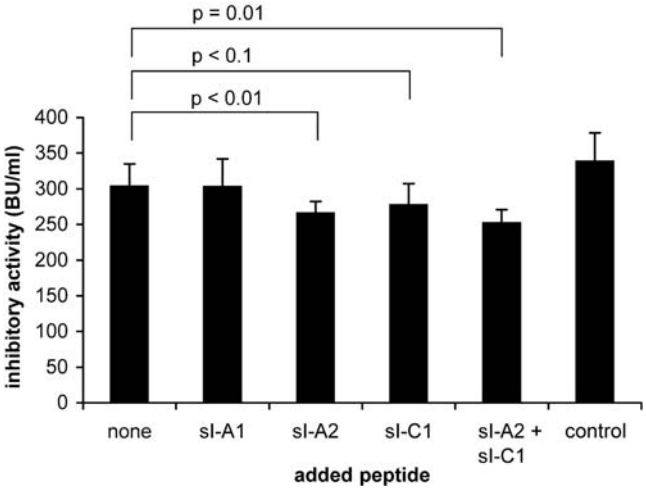


**Fig. 5.** Partial neutralization of the inhibitory activity of patient's plasma samples by synthetic peptides
Dilutions of patient's plasma (1 : 400) giving approximately 50 % residual FVIII activity were pre-treated overnight at 4°C with the synthetic peptides sI-A1 (GYVNRSL), sI-A2 (ILFSVFD), sI-C1 (SQFIIMY) or a control peptide (RSNAWRP), respectively, at a concentration of 1 mM or with PBS as a control, then mixed with equal volumes of pooled normal human plasma (PreciClot Plus I, Stago/Roche) and incubated for 2 hours at 37°C in parallel to FVIII-depleted human plasma controls. FVIII residual activity was measured in a one-stage FVIII assay, and the inhibitory activity was calculated in Bethesda units/ml. Error bars represent the standard deviations (n = 10, except for the combination of sI-A2 and sI-C1 with n = 4).

and sI-C1 competed with recombinant FVIII for binding to factor VIII-specific antibodies in the plasma of patient I, showed a dose-dependent inhibition of the antibody binding by these peptides (Fig. 3b). The effects of synthetic peptides with an amino acid sequence identical with that of a phage-displayed peptide were significantly smaller than those of peptides corresponding exactly to the amino acids of the putative epitope in the FVIII molecule (data not shown). A comparison of the two neutralizing peptides sI-A2 and sI-C1 suggests that a higher quantity of antibodies recognizing the sI-C1 sequence may be present in the patient's sample, accounting for the greater reduction of antibody binding by sI-C1. Taking this difference into consideration, the epitope corresponding to sI-C1 seems to play a minor role for the total inhibitory activity of the plasma than the epitope corresponding to sI-A2, because similar neutralizing effects had been found in the Bethesda assays. Since the patient's immune response to FVIII is typically polyclonal [5], there should be several inhibitor epitopes and even total blocking of the antibodies recognizing the sI-A2 and sI-C1 sequences would reduce the inhibitory effect of the plasma of patient I only partially. Thus, with these two synthetic peptides alone a complete neutralization as described for example in a recent study by Villard et al. [23] for peptides binding to the monoclonal FVIII-antibody ESH8 cannot be expected for this patient's plasma. The lack of a neutralizing effect of the

peptide sI-A1 indicates that more yet undetected epitopes of inhibitory antibodies in the A1, A2 or C1 domains exist, which are responsible for the residual inhibitory activity of patient's plasma. Furthermore, it cannot be excluded that a significant proportion of its FVIII-specific antibodies did not bind to the column during affinity chromatography and could thus not be isolated from the plasma sample.

Although these results provide the proof of principle that random phage display libraries can be used for the mapping of epitopes in polyclonal antibody samples such as inhibitors from the plasma of hemophilia patients, the sensitivity of this method needs to be investigated in further studies which are not limited by the amount of patient's material available for analysis. Alterations in the phage display protocol such as subtractive panning strategies on total IgG samples not purified by affinity chromatography may also be considered. However, future studies may allow to identify immunodominant epitopes and may thus be an important step towards understanding the inactivation mechanism and a precondition for the modification of recombinant coagulation factors to reduce their immunogenicity. An alternative therapeutic approach might be the clinical application of peptides inferred from the epitopes identifiable by this method to directly neutralize the inhibitory activity of the plasma as shown for the monoclonal antibodies ESH8 and Bo2C11 in a murine model of hemophilia [23, 24].

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# Frequency and Inhibitor Risk of the Intron-1-Inversion Mutation in the German Hemophilia Population

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## Background

The Intron-1-Inversion represents the most recently discovered mutation mechanism of the factor VIII (FVIII) gene (Bagnall et al. 2002). The mutation is caused by an intrachromosomal recombination of two repeats, located within intron 1 and the telomeric end of the FVIII gene. It can be easily analyzed by a PCR shown in Figure 1. Several groups reported an intron 1 inversion prevalence of 4-5% in severely affected hemophilia A patients. However, these studies were based on small patient numbers. Moreover, because of the low frequency of the intron 1 inversion, no data on inhibitor risk were available. The reported studies suggest a low inhibitor prevalence of 10-15%. Based on a large scale mutation profiling in the German hemophilia population this study aims to provide more exact information on frequency and inhibitor risk of this specific FVIII gene mutation.

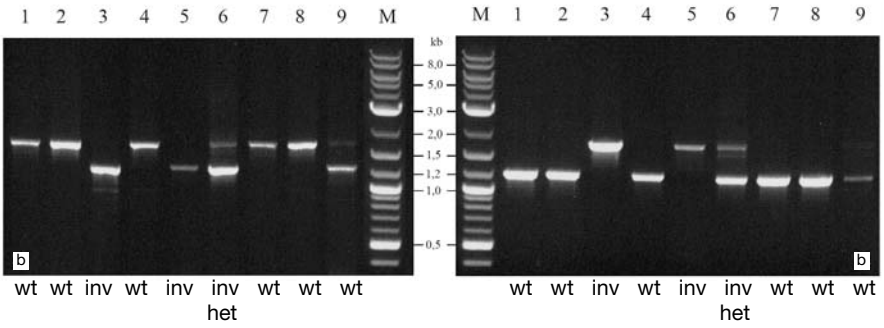


**Fig. 1a, b.** Fragment Inth1-1: Lanes 1,2,4,7,8,9 wildtype (wt); 3,5 Intron-1-Inversion (inv); 6: heterozygous Intron-1-Inversion (inv het); **b)** Fragment Inth1-2: Lanes 1,2,4,7,8,9 wildtype 3,5 Intron-1-Inversion; 6 heterozygous Intron-1-Inversion

## Results and Discussion

Analyzing 794 index patients from German families with severe hemophilia A we found 19 patients with an inversion resulting in a prevalence of 2.4%. The other mutation types distributed as follows: intron-22-inversion in 363 (45.7%) patients,

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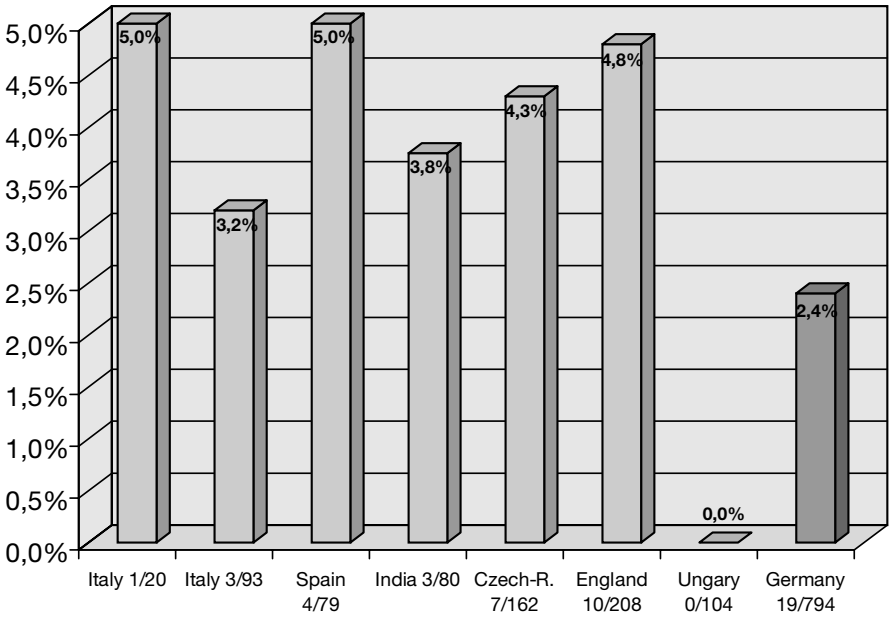


**Fig. 2.** Summarized data for prevalence of intron 1 inversion

big deletions/insertions in 31 (3.9%) patients, small deletions/insertions in 118 (14.9%) patients, missense- and stop mutations in 211 (26.6%) patients and splice site mutations in 20 (2.5%) patients. No mutation could be detected in 32 (4.0%) of the severely affected hemophilia A patients. 6 (31.6%) of the patients with an intron 1 inversion developed an inhibitor against factor-VIII-protein, including 3 patients with a low titer and 2 patients with a high titer inhibitor. In one patient the inhibitor titer was not known.

## Conclusion

Compared to published data, our study revealed a significantly lower frequency of the intron 1 inversion mutation (2.4% vs 4-5%). The inhibitor prevalence of 30% clearly indicates a high risk for inhibitor formation which agrees with other null mutations.

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# Effect of FVIII on Clotting Time and Thrombin Potential in Tissue Factor-Activated Hemophilia A Plasma

P. Fritsch, G.Cvirn, K. Baier, B. Leschnik and W. Muntean

## Introduction

FVIII-deficiency (hemophilia A) is known to be associated with severe bleeding. The quantity and intensity of bleeding in hemophiliacs can differ individually in patients despite similar factor VIII-concentrations. Also neonates have a lower bleeding tendency than older hemophiliacs. This could be the effect of factors like prothrombin, factor VII, but also of inhibitors like tissue factor pathway inhibitor (TFPI), antithrombin (AT) and protein C (PC) [1]. To measure the effect of these factors on hemostasis sensitive assays are necessary. We can not use standard assay systems via the intrinsic pathway (aPTT), because the plasma is too highly diluted and the activation is done with too high amounts of activator. However, it has been shown in recent years that plasma activation via the extrinsic pathway by addition of low amounts of lipidated tissue factor (TF) as activator is probably more compatible with the physiological milieu [2]. Butenas et al. [3] have shown, that low concentrations of lipidated TF as trigger are suitable for a sensitive detection of the effects of different levels of pro- and anticoagulants on thrombin-generation. Cvirn et al. [4, 5] showed in their studies the influences of the above mentioned factors after activation with low amounts of TF in cord and adult plasma. Therefore, our study was performed to investigate whether low concentrations of TF as activator can also be used to determine the influence of factor VIII on clotting time and thrombin generation.

## Materials and Methods

FVIII-deficient plasma (containing 0 % FVIII-clotting FVIII deficient plasma Dade Behring) was spiked with increasing amounts of purified FVIII-concentrate (HaemateP Aventis Behring) and the effect on clotting time (CT) and thrombin potential (TP) was evaluated. It was evaluated in the plasma of hemophilic patients.

For the activation we used different amounts of recombinant lipidated TF (Innovin Dade Behring).The concentration of TF was measured with TF-Elisa (American diagnostica).

380 micro liters of plasma with different FVIII-concentrations were incubated with 100 micro liters of buffer containing different amounts of TF for 2 min at 37°C. The activation of the plasma samples was done by addition of 20 microliters $CaCl_2$.

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The CT were determined by means of the optomechanical coagulation analyzer Behring Fibrintimer II from Dade Behring Marburg, Germany, which applies the turbodensitometric measuring principle. For the determination of the TP we used additionally H-Gly-Pro-Arg-Pro-OH (Pefabloc FG). Also the subsampling method was used derived from a recently described technique [6]. Plasmas were prepared and activated as described above. At timed intervals, 10 µL aliquots were withdrawn from the activated plasma and subsampled into 490 µl buffer B containing 255 µmol L S-2238. The extinction of the plasma was measured in the Anthos micro plate-reader 2001, Salzburg, Austria [7].

## Results

### Effect of TF and FVIII on Clotting Time (CT)

Activation of plasma with high concentration of TF showed no significant increase of the CT with decreasing amounts of F VIII. With small amounts of TF (2.5 pM $L^{-1}$) the CT in FVIII-deficient plasma was dose-dependently shortened when increasing amounts of FVIII-concentrate were added (Fig. 1).

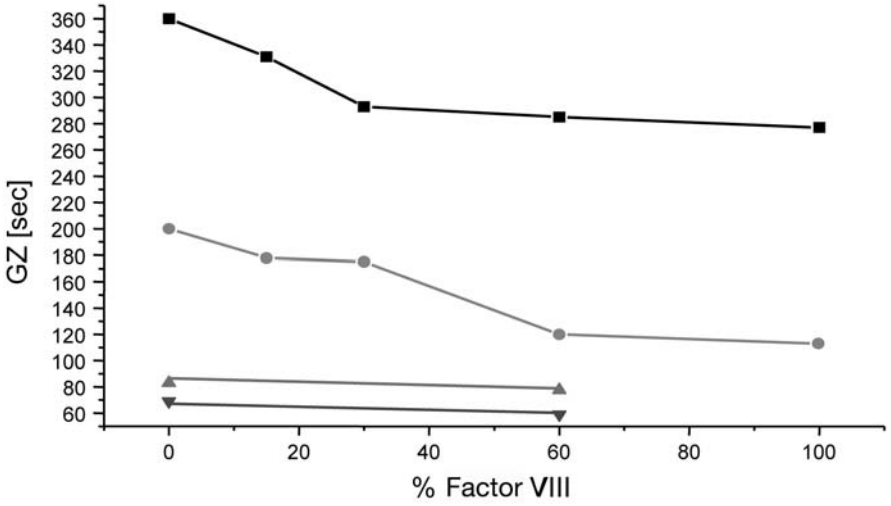


**Fig. 1.** Effect of activation with different TF-concentration in plasma with different FVIII-content on clotting time. 13 pM $L^{-1}$ TF (▼), 6 pM $L^{-1}$ TF (▲), 2.5 pM $L^{-1}$ TF (●), 0.25 pM $L^{-1}$ TF (■).

### Effect of FVIII on Thrombin Potential (TP)

There was no significant effect on the TP using high concentrations of TF in plasma with different F VIII-content. But using low concentration of TF (2,5 pM $L^{-1}$), the TP was increased when the FVIII-content was successively raised (Fig. 2).

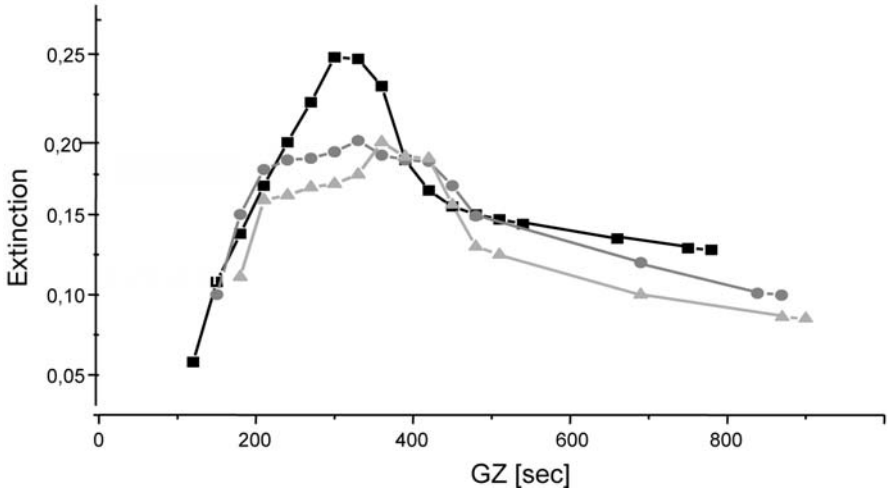


**Fig. 2.** Effect of different FVIII-concentration on the thrombin generation after an activation with 2.5 pM $L^{-1}$ TF. Plasma with 60 % F VIII (■), plasma with 15 % FVIII (●), plasma with 0 % FVIII (▲).

## Clotting Time in Plasma from Hemophilia A Patients

Plasma of hemophiliacs with different FVIII-content, showed an increase of the CT with decreasing FVIII-content (Fig. 3).

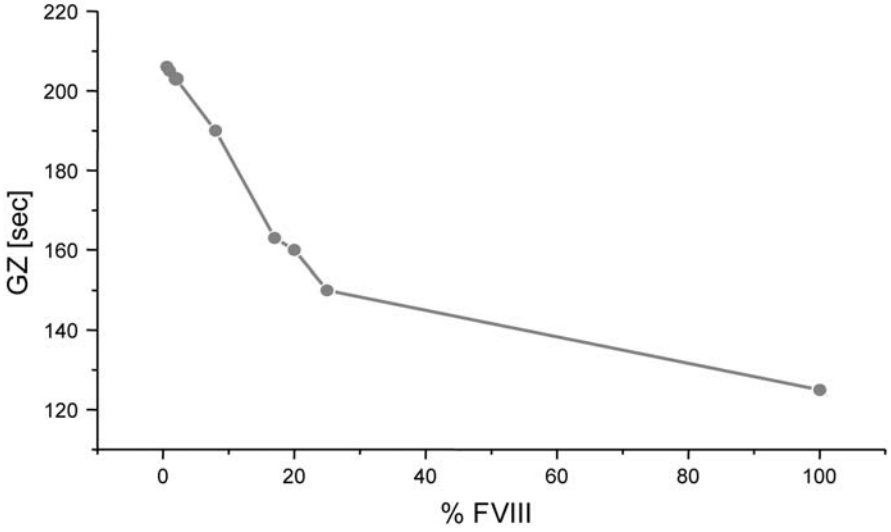


**Fig. 3.** Effects on CT in plasma of hemophiliacs with different FVIII-content after an activation with 1.25 pM $L^{-1}$ TF.

**Thrombin Generation in Plasma from Hemophilia A Patients**

In contrast to the CT, the height of the TP did not correlate that strictly as CT with the FVIII-content. While in general the TP of mild hemophiliacs were higher and the TP of the severe hemophiliacs were lower, in two patients with the same FVIII-content (both 20 %- ▲ and ▼ in Figure 4) had different high TP-levels (Fig. 4).

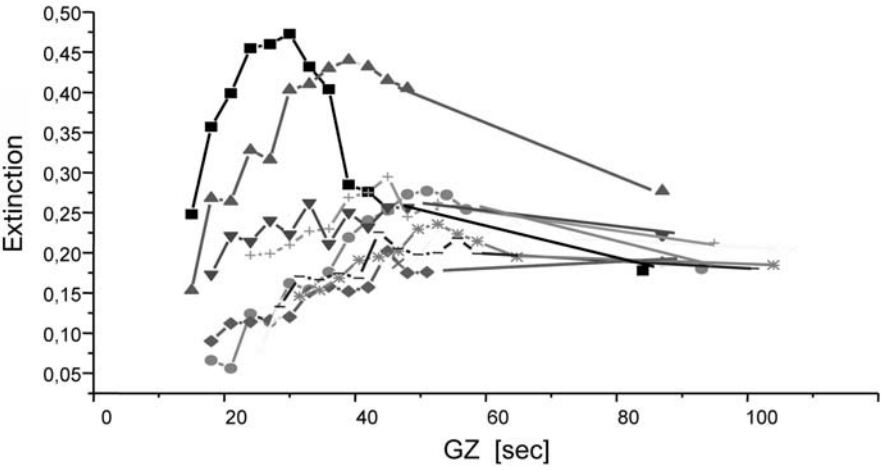


**Fig. 4.** Effects on TP in plasma of hemophiliacs with different FVIII-content after an activation with 1.25 pM $L^{-1}$ TF. Healthy proband (■), patients with 25 % FVIII (●), 20 % F VIII (▲), 20 % FVIII (▼), 17 % FVIII (◆), 8 % FVIII (+), 2 % FVIII (x), 1 % FVIII (*), 0.6 % FVIII (–).

## Discussion

Recent investigations showed that hemophiliacs with factor V Leiden disease [9, 10, 12] or protein C deficiency [13] had lower bleeding tendency than hemophiliacs with no prothrombotic risk factors. Some data even report of sporadic cases of symptomatic thromboembolism in patients with severe hemophilia and prothrombotic risk factors after factor replacement therapy [12, 16–18]. This is not confirmed by others [11,19–21]. They postulate that there must be other variables, that influence the bleeding tendency and the lower factor concentrate utilization in severe hemophiliacs. Van't Veer [1] could show the influence of factors like TFPI and AT on the TG in plasma of healthy adults.

It is known that excessive bleeding during delivery and in the neonatal period is a relatively rare event in hemophiliacs. In a French study of 754 neonates with hemophilia, only 8 % showed clinically overt bleeding , in 71,5 % of patients the diagnosis was made after the neonatal period. Intracranial bleeding is found in only 3.9 % and 3.6 % of neonates with hemophilia [14, 15].

In previous works we have focused on the problem why healthy neonates do not bleed easily in spite of low clotting factors: It was shown that after activation with low concentrations of TF physiological low levels of natural inhibitors, like TFPI, AT

and PC, compensate for low concentrations of clotting factors, like the prothrombin complex, and allow sufficient thrombin generation [4, 5].

Conventional clotting assays, that are used for factor VIII-measurement, are not sensitive enough for the detection of these factors. Davie et al. [2] showed in their work, that an activation via the extrinsic pathway, using small amounts of TF, is more physiological than the conventional clotting assays. The group of Mann [3] showed that this method is sensitive enough to detect the effects of different levels of pro- and anticoagulants on the thrombin generation.

In our study there was no significant difference in plasmas with different FVIII-content using high concentrations of TF for the activation. Using low concentrations of TF, the clotting time decreased dose-dependently and the TP increased dose-dependently when the FVIII-content was successively raised by addition of increasing amounts of purified FVIII-concentrate to FVIII-deficient plasma.

First measurement of the TP in plasma of hemophiliacs showed the expected data. There was a correlation measuring the CT in plasma of hemophilia A patients to FVIII-content. But in accordance to Ingerslev [8] and »the FVIII and FIX Subcommittee of the International Society on Thrombosis and Haemostasis«, the TP showed a heterogeneity in patients with the same phenotypes (FVIII:C 0.2 IU mL-1). An explanation might be that other compounds of the hemostatic system, e.g. FII, FVII, tissue factor pathway inhibitor (TFPI), or antithrombin (AT) effectively influence thrombin generation in TF-activated plasma. Another explanation could be the different age of our patients (11 months to 18 years). As mentioned before, there is a discrepancy between the good hemostasis and the prolonged values in neonates [4, 5].

Our investigation suggest that low concentrations of TF can be used for a sensitive detection of prothrombotic and inhibitory variables in plasma of hemophiliacs. Therefore, we intent to investigate the effects of these variables on the CT and TP in plasma of hemophilia A patients in the future, to possibly explain the difference in bleeding manifestation between patients, different age groups and especially between neonates and older children.

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## *VII. Poster*

## *VIIa. Hemophilia*

# Bleeding Tendency in Factor XI Deficiency: Report on two Families and the Detection of a Novel Mutation within the Factor XI Gene

B. Maak, L. Kochhan, J. Schröder, J. Oldenburg, P. Heuchel, B. Harwardt and U. Budde

The inherited Factor XI deficiency seems to be, with only few exceptions, a rare disorder worldwide. In patients with factor XI deficiency spontaneous bleedings are uncommon, bleeding events occur mostly after surgical procedures or injuries in persons with a markedly decreased residual factor XI-activity (homozygous or compound-heterozygous factor XI deficiency). However, there are patients with only mildly reduced factor XI activity (factor XI activity between 15 and approximately 60%) and significant bleeding problems. In addition, families with a quite varying bleeding behavior in the individual members exist. The reason for this difference is not fully understood as yet. Therefore, it may be speculated that in such patients an additional defect of the hemostatic system is present. In some studies the coexistence of factor XI deficiency and hemophilia A, von Willebrand's disease and also platelet function disorders has been described [1, 5, 7], furthermore, in two reports it could be demonstrated that patients with factor XI deficiency and bleeding symptoms tended to have lower values of von Willebrand factor in comparison with patients without bleeding symptoms [1, 3].

We describe here the results of our studies of two patients and their family members with mild factor XI deficiency. In both cases a second defect of the hemostatic system could be detected and in our opinion this defect was responsible for the different bleeding tendency of the patients in comparison with the other family members. The factor XI deficiency of the investigated individuals was confirmed by evidence of mutations within the factor XI gene. In one of the families we identified a novel mutation (Ala 464 (GCC) > Val (GTC), exon 12).

## Material and Methods

### Patients and Family Members

Case 1:

In the patient, a 4 month old boy, a premature synostosis of the skull was surgically treated. A massive bleeding occurred during the procedure and persisted for the next hours. After administration of fresh frozen plasma the bleeding ceased immediately. As the cause of the bleeding factor XI deficiency was presumed. Five months later the parents and the boy contacted our hospital. The boy was in good health and without any bleeding signs. The mother of the boy reported easy bruising and

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marked menstrual blood loss before oral contraceptive treatment. The maternal grand-grandmother also reported easy bruising, her menstrual blood loss was not remarkable and no bleeding problems occurred after two deliveries. Dental extractions were also uneventful. After a fracture of the right radius an unusually large hematoma was observed.

The father of the patient reported no bleedings after tonsillectomy and appendectomy.

Case 2:

The patient, a 17 year old girl reported a life-long tendency to easy bruising and delayed bleedings after incidental injuries. The menstrual blood loss was strong and decreased after introduction of oral contraceptive treatment.

The father of the girl is also factor XI deficient. He reported frequent epistaxis only during childhood and is currently symptom-free. After multiple dental extractions and two herniotomies no bleeding events occurred.

### Coagulation Tests

Blood was taken from an antecubital vein using a commercially available device (BD vacutainer). Nine parts of blood were carefully mixed with one part of 0.129 m trinatrium citrate solution. Thromboplastin time (PT, Quick value) and activated partial thromboplastin time (aPTT) were measured with an automated device. The fibrinogen content was determined as so-called derived fibrinogen during the PT assay. Coagulation factor assays were performed as clotting tests with specifically deficient plasmas according to the instructions given by the manufacturer. Antithrombin was estimated by a colorimetric assay. The APC-resistance was determined with an aPTT-based reagent kit. All reagents for the coagulation assays were purchased from IL. The concentration of von Willebrand factor (VWF:Ag) was assayed by an ELISA kit (Bio-Merieux). Protein Z and PAI-1 were measured also by ELISA techniques.

### Genomic Analysis

Total DNA of all patients was extracted from whole blood using micro spin columns (Quiagen, Hilden, Germany). Exons of the factor XI gene were amplified by polymerase chain reaction (PCR). Cycle sequencing was performed using the Big Dye Terminator Cycle Sequenzing kit (Perkin Elmer Applied Biosystems, Foster City, USA) and analyzed using an automated DNA sequencer (ABI PRISM/377 DNA Sequencer, Applied Biosystems, Weiterstadt, Germany). Primers used for sequencing were the same as those used for PCR amplification. Mutation analysis of the factor VIII gene was performed as previously described [8].

## Results

In our first case the in an other hospital presumed factor XI deficiency was confirmed (Table 1). We found on different occasions factor XI-activities between 32 and 37%. In his mother the factor XI-activities were in the same magnitude and also in his maternal grand-grandmother a decreased factor XI-activity has been found. The mutation Thr 475 (ACA) > Ile (ATA) in the exon 12 of the factor XI gene is responsible for the reduced factor XI-activities. As an additional defect we found in the patient reduced factor VIII-activities and consequently the mild form of hemophilia A. As the cause of the slight factor VIII deficiency the mutation Gly 22 (GGT) > Cys (TGT) within the exon 1 of the factor VIII gene was identified. The mutation was also present in the patients mother, her factor VIII values could be found within the normal range. In the grand-grandmother the factor VIII activity was also normal.

**Table 1**

| | | Patient | Mother | Father | Maternal grand-grandmother | Normal range |
|---|---|---|---|---|---|---|
| Quick | [%] | 94 | 101 | 117 | 121 | 70–120 |
| PTT | [sec] | 46 | 31 | 31 | 36 | 25–35 |
| Fibrinogen | [g/l] | 2.15 | 2.35 | 1.66 | 3.34 | 1.8–4.50 |
| VWF:Ag | [%] | 84/84 | 80/87/96 | >120 | >120 | 50–150 |
| VWF:RiCoF | [%] | 84/110 | 56/84/56 | 110 | 112 | 60–150 |
| VIII:C | [%] | 38/40/32 | 74/81/68 | 110 | 97 | 70–150 |
| IX :C | [%] | 67 | 92 | 110 | 79 | 50–150 |
| XI :C | [%] | 37/32/34 | 53/34/42 | 78 | 36 | 70–150 |
| XII:C | [%] | 80 | 126 | 101 | 82 | 70–150 |
| AT | [%] | n.d. | 93 | n.b. | 102 | 80–120 |
| APC-Ratio | | n.d. | 2.35 | 2.50 | 2.50 | 2.0–3.5 |
| Protein C | [%] | n.d. | 114 | n.d. | 125 | 70–120 |
| Protein S | [%] | n.d. | 75 | n.d. | 118 | 70–130 |
| Protein Z | [mg/l] | 1.26 | 1.48 | n.d. | 2.46 | 0.8–2.0 |
| PAI-1 | [µg/l] | 36 | 7 | 20 | 20 | 4–5 |
| Mutation XI-Gene | | Thr 475 (ACA), > Ile (ATA) Exon 12, heterozygous | Thr 475 (ACA), > Ile (ATA) Exon 12, heterozygous | n.d. | Thr 475 (ACA), > Ile (ATA) Exon 12, heterozygous | |
| Mutation VIII-Gene | | Gly 22 (GGT)> Cys (TGT) Exon 1 | Gly 22 (GGT)> Cys (TGT) Exon 1, heterozygous | n.d. | n.d. | |

n.d. = not determined

**Table 2**

| | | Patient | Mother | Father | Normal range |
|---|---|---|---|---|---|
| Quick | [%] | 117 | 117 | 107 | 70–120 |
| PTT | [sec] | 40/39 | 32 | 38 | 25-35 |
| Fibrinogen | [g/l] | 3.19 | 2.88 | 1.95 | 1.8–4.50 |
| VWF:Ag | [%] | 65 | 134 | 77 | 50–150 |
| VWF:RCoF | [%] | 56 | 225(CBA)* | 56 | 60–150 |
| VWF-Multimeric analysis | | normal | normal | n.d. | |
| VIII:C | [%] | 72 | 102 | 81 | 70–150 |
| IX:C | [%] | 95 | n.d. | 72 | 70–150 |
| XI:C | [%] | 34/26 | 85 | 35 | 70–150 |
| XII:C | [%] | 66/72 | 107 | 50 | 70–150 |
| AT | [%] | 98 | n.d. | 75 | 80–120 |
| APC-Ratio | | 2.35 | n.d. | 3.0 | 2.0–3.5 |
| Protein C | [%] | 99 | n.d. | 49 | 70 -120 |
| Protein S | [%] | 87 | n.d. | 75 | 70–130 |
| Protein Z | [mg/l] | 1.84 | n.d. | 0.91 | 0.8–2.0 |
| PAI–1 | [µg/l] | 6 | n.d. | 8 | 4–45 |
| Mutation XI-Gene | | Ala 464 (GCC) > Val (GTC) Exon 12, heterozygous | n.d. | Ala 464 (GCC) >Val (GTC) Exon 12 heterozygous | |

*CBA = Collagen binding activity
n.d. = not determined

In the second family investigated the factor XI-activities were found between 26–35%, father and daughter are hemizygous for the mutation Ala 464 (GCC) > Val (GTC) which is localized within the exon 12 of the factor XI gene.

In both persons the levels of the von Willebrand factor have been found near the lower limit of the normal range. In contrast to the young girl her father offers a distinctly-decreased protein C activity.

## Discussion and Conclusions

In the two families presented here the reduced factor XI activity is of hereditary origin. The analysis of the factor XI genes of the first patient and his family members revealed a base exchange (C > T) within the exon 12. This mutation was described for the first time in 1995 and is characterized by a reduced stability of the

factor XI-mRNA and therefore a reduced expression of factor XI [4]. The molecular mechanism of the novel mutation (case 2) in the factor XI gene (Ala 464 (GCC) > Val (GTC)) remains to be established.

In the first family there is a clear-cut difference between the aPTT-value of the patient to the corresponding values of his mother and his grand-grandmother. In our opinion, this difference cannot be explained by the age of the patient alone. Therefore a second defect was supposed, the results of further coagulation testing revealed a reduced factor VIII activity. The cause of this low factor VIII level is the mutation Gly 22 (GGT) > Cys (TGT) within the exon 1 of the factor VIII gene [2]. The same mutation was found in the patients mother in a heterozygous state. We believe that the massive peri- and postoperative bleeding in the patient could be attributed to the mild hemophilia rather than to the factor XI deficiency. Mother and grand-grandmother of the patient offer a bleeding tendency which may be the result of the mild factor XI deficiency.

In our second patient and also in the father there is a combination of mild factor XI deficiency and low normal values of von Willebrand factor. This may be the reason for the bleeding tendency in the girl. The father shows additionally a protein C deficiency which counteracts to the effects of factor XI deficiency and low von Willebrand factor. The cessation of the bleeding tendency after the childhood may support this assumption.

Finally we emphasize that the aPTT assay is not always suitable for the detection of mild factor XI deficiency [6]. In a great number of patients normal or only slightly prolonged aPTT values could be found. Therefore we recommend a specific factor XI assay in patients with bleeding symptoms which can not be explained by other diagnostic measures.

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# Multiple Distal Pseudotumours in a Patient with Severe Hemophilia A and High Titer Inhibitors

C.A. Petrescu, M. Şerban, P. Ţepeneu, W. Schramm, R. Bădeţi and B. Popescu

## Introduction

Hemophilic pseudotumours (PTs ), first described by Fernandez de Valderrama and Matthews [7], are rare complications of bleedings: 1-2% of patients in a large study over 25 years [1]; 1.89% in a study on 212 patients from Romania, aged between 0-25 years, followed for 11 years [15]. PTs develop after repeated, unresolved muscular hematomas, or subperiosteal hemorrhages [5, 16, 17]. The distinct clinical features of PTs are progressive enlargement, with destruction of the adjacent structures (bones, vessels, nerves) compressed by, or included within the PT. Pathologically, the PTs are blood collection in different states of organization, with a liquid or solid content, having one or more cavities; PTs are surrounded by a fibrous capsule with very rich blood supply, usually originating from more than one artery; calcifications, and ossification of the capsule, inflammatory cells, many histiocytes containing copious amounts of siderin may be seen [2, 4, 16].

## Objectives, Patients

The study aims to analyze the characteristics (favoring factors, locations, imagistic aspects, evolution) and to evaluate the results of the medical and surgical approaches in a rare case of multiple consecutive distal PTs appeared in a single patient. The patient (G.E.) with severe familial hemophilia A (factor VIII:C <0.2%) was diagnosed at 9 months of age (1990), and treated in the III$^{rd}$ Pediatric Clinic, and respectively, in the Pediatric Surgical and Orthopedic Clinic Timişoara. Very high titre inhibitors were first diagnosed in 1998, and persisted at high levels (>10 BU). No treatment for immune tolerance induction (ITI) was applied. The molecular defect is not known. Inhibitors were absent in a patient's affected cousin, with only a few exposures to FVIII.

The patient developed five consecutive distal PTs with different locations, between his ages of 8-13 years. The conservative measures were inefficient (progressive bone destruction, infection, hemorrhage), and radical surgical interventions were necessary in order to save the patient's life. Replacement therapy with rFVIIa, and aPCC was used. An antiangiogenic treatment with Interferon alpha (IFNα) was applied for eight months, apparently with good results.

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## Results and Discussions

There are two types of PTs described by Gilbert [6]. **Proximal PTs** occur more frequently in adults, are localized in the proximal axial skeleton, especially around the femur and pelvis, probably start in the soft tissue and than secondarily erode the bones, and have a slow evolution, presenting as painless, firm, expanding mass. **Distal PTs** affect usually young, skeletally immature patients (children, adolescents), being located especially in the small cancellous bones (calcaneus, talus and metatarsals of the feet, seldom the carpus or other locations). Distal PTs are generally the result of direct trauma, are most probably secondary to unresolved intraosseous hemorrhages, and are characterized by rapid and painful evolution [6]. The extension, dimensions, content, and the number of cavities may be evaluated by x-ray, ultrasounds, CT, and MRI investigations; the epiphyseal, or the epiphysometaphysary distal PTs usually have multiple cavities, while the diaphyseal, or diaphyso-methaphysary PTs have a single cavity [2, 16, 17].

Multiple PTs were extremely rare described [8]. The presented patient is the single one in our experience with this type of complication. The PTs appeared consecutively, between the patients' age of 8-13 years, being located at the both foots, the left knee, and the right shank.

The patient G.E. was diagnosed at the age of 9 months (1990) presenting a muscular hematoma after intramuscularly injection, and severe anemia. It's important to underline that the familial care was inadequate (low socio-economic level, alcoholic father with advanced tuberculosis, unemployed, two brothers at school ages, mother working all days long), the patient being admitted many times with multiple muscular hematomas, advanced hemarthroses of the knees, opened wounds, gingival, lingual, and pharyngeal hemorrhages, epistaxis, epicranial hematomas, frequently with severe anemia or hemorrhagic shock. Chronic arthropathy of the knees and ankles developed in time. The replacement treatment with plasma, cryoprecipitate, and erythrocyte concentrate in his first two years of life was complicated with HCV infection (donors screening for HCV was introduced in our transfusions center in 1992), but he remained HIV negative (donors screening for HIV was introduced in our transfusions center in 1990); between 1992-1997 he was treated only with factor VIII concentrates, with good clinical response, although one surgical intervention for an infected hematoma, performed in 1995 under FVIII concentrate coverage, was complicated by severe hemorrhage. The psychological disturbances, repeated urinary tract infections with *E.coli,* and *Proteus spp.*, pulmonary tuberculosis (contact with father), and multiple dental problems complicated the patient's state.

In July 1998, the patient (8 years old) was admitted presenting a tumoral mass of the left heel, intense pain, with obvious signs of infection, and the imminence of spontaneous rupture. The problem has been neglected, the patient being previously taken care in a related family (Moldavia) for two months. The x-ray showed destruction of the calcaneus (Fig. 1-A), and the presence of a distal PT was not recognized, the case being initially interpreted as osteomyelitis. The surgical intervention (evacuation) under FVIII concentrate coverage and antibiotics was complicated by severe hemorrhage; repeated evacuations, application of local hemostatics and anti-

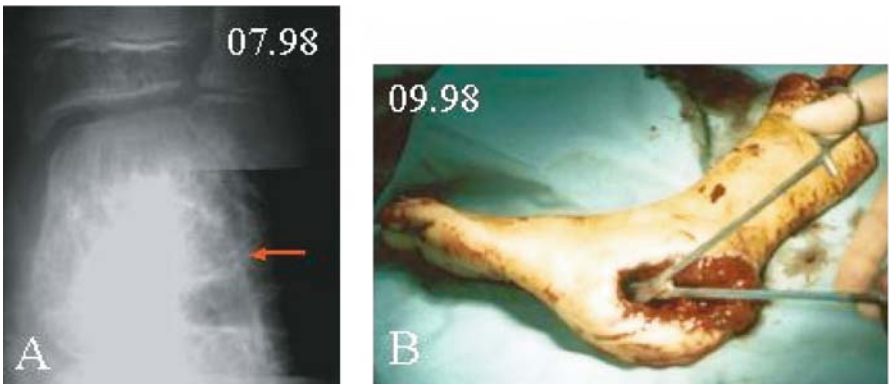


**Fig. 1. A.** Left heel PT destructing the calcaneus; **B.** Amputated fragment showing massive bone and tissues destruction

biotics, selective arterial ligature, were inefficient, with extensive foot bone destruction, extension of the infection, and repeated life-threatening hemorrhages. About 140,000 IU of FVIII concentrate were administered in that period. After the diagnosis of very high titer inhibitors, no FVIII concentrates were administered. Although the presence of a ***left heel PT*** was accepted, and amputation was considered necessary in order to save the patient's life, the shortage of bypassing agents imposed the temporization of the radical surgery. Amputation from the medium left shank level (Fig. 1-B) was performed in September 1998, under rFVIIa (2,940 kIU) and FEIBA (21,000 IU) coverage, in good conditions. However, post-surgical infection with extensive soft tissues destruction appeared (*S.aureus, P.aeruginosa)*, and remodelling of the amputated fragment was necessary (October 1998).

A ***second right heel PT*** destructing the calcaneus was diagnosed in May 1999 (Fig. 2-A). Local trauma (vicious sitting position after amputation, with persistent

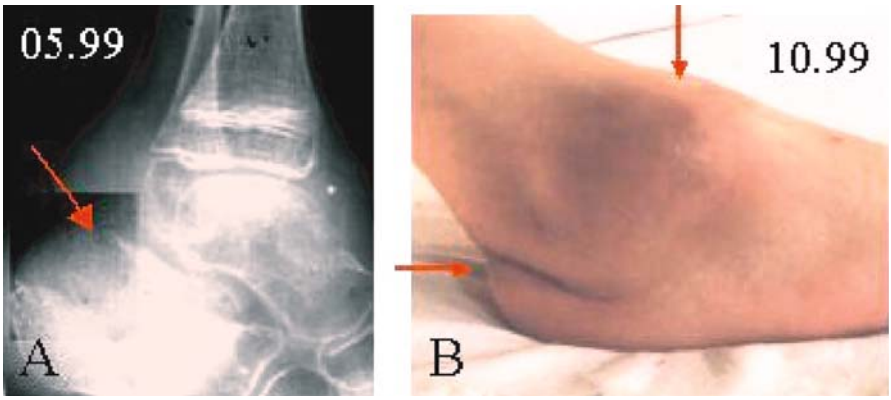


**Fig. 2. A.** Right heel PT with calcaneus destruction; **B.** Good result after complete excision; appearance of the third PT

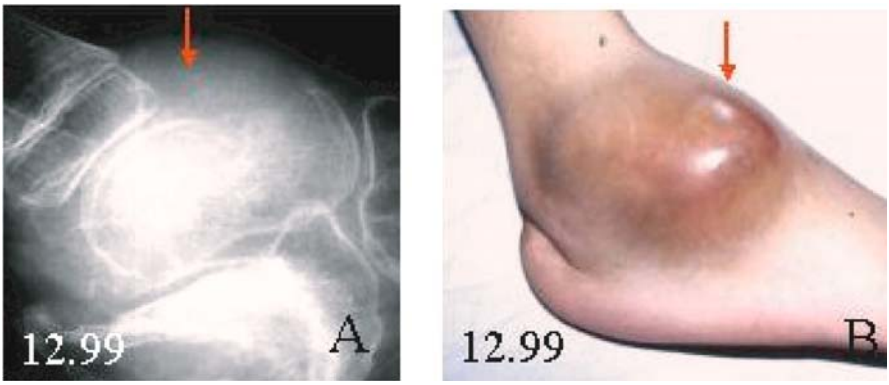


**Fig. 3. A.** Right foot PT destructing the astragalus; **B.** Infection and rebleeding of the PT

pressure on the right heel, insufficient home care, psychological disturbances with auto-aggression), and severe secondary osteoporosis were most probably favoring factors. Repeated episodes of infection and rebleeding, associated with intense pain, only responding to opioids, were treated with antibiotics and bypassing agents, but the medical treatment didn't stop the progression of the PT. After two months of evolution, a minimal surgical intervention was performed (percutaneous evacuation, drainage, local hemostatic agents) but rebleeding appeared in a short time. In September 1999, a radical surgical cure was performed, with complete excision after selective arterial ligature, curettage of the residual cavity, and application of local hemostatic agents, under rFVIIa coverage (8,280 kIU), with good results (Fig. 2-B).

A ***third PT at the dorsum of the right foot*** appeared in October 1999 (Fig. 1-B); the same pattern of undulant evolution, with periods of partial remissions, and periods of rapid enlargement with severe pain was noted. Progressive destruction the right astragalus (Fig. 3-A), and repeated episodes of infections appeared (Fig. 3-B). Because of the shortage of bypassing agents (inhibitors were still at very high levels), the surgery was postponed; medical conservative methods were applied for seven months, with no long-term result. The PT was evacuated in May 2000 with temporary results, with a consumption of 5,760 kIU rFVIIa. In February 2001 the evolution was complicated by a fulminating hemorrhage (vascular erosion determined by the progression of the PT). Repeated evacuations, drainage, local hemostatic agents were not efficient, and in May 2001 a second amputation from the inferior right shank level was performed. The by-passing agent consumption in this period was 9,700 KIU of rFVIIa, respectively 26,300 IU of FEIBA.

Beginning with September 2002, intense pain and progressive growth of the left knee, and the radiological aspect, raised the suspicion of another PT development (Fig. 4-A). The presence of the ***fourth PT of the left knee*** was confirmed by the MRI investigation showing a PT with multiple cavities, with femoral and tibial destruction (Fig. 4-B).

Taken into account the history of this patient, and the previous unsatisfactory results in treating his PTs, leading to amputation of both legs, we've decided to try an alternative therapeutic approach. The idea of an **antiangiogenic** treatment was

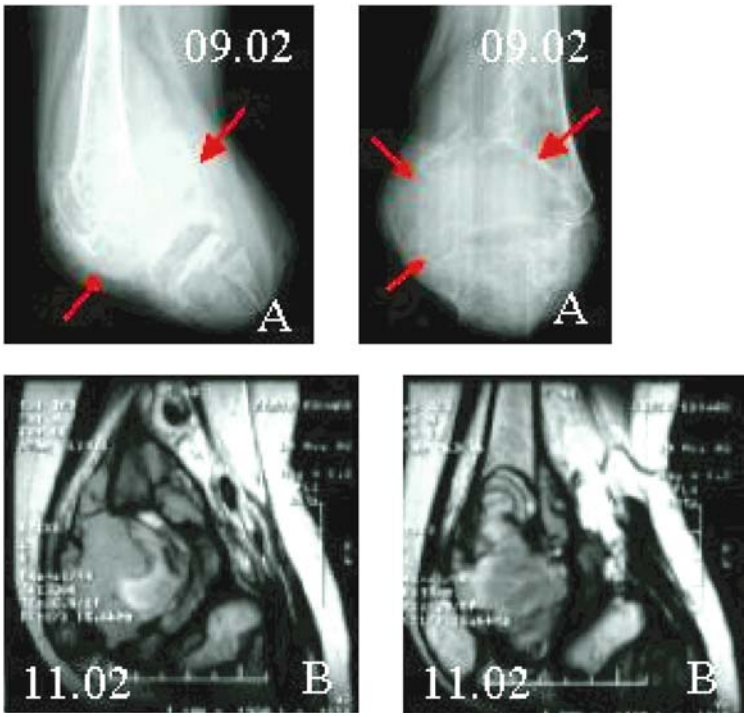


**Fig. 4.** A. Left knee PT with femoral and tibial destruction; B. PT with multiple cavities

based on the successful use of interferon alpha (IFNα) in children's angiomatous diseases (hemangiomas, angioblastomas) [7, 12, 13]. In fact, PTs can be considered vascular tumors. The very rich vascular supply of the capsule [23], usually originating from more than one artery, and the progressive fibrosis, characteristic features of PTs, suggests an endothelial and fibroblastic proliferation. This is most probably the result of the »toxic« effect of iron; iron deposits in the capsule of PTs, and of the presence of histiocytes with large amounts of hemosiderin were described [4, 10, 16]. It was clearly shown that iron has a proinflammatory role [3, 10, 20], through formation of radical oxygen species (ROS) that produce tissue damage [10] and induce endothelial and fibroblastic proliferation [14, 19].

The use of IFNα, in a daily regimen of 3 millions IU/m$^2$/day, seemed to have good effects: the rapid (in a few days) disappearance of the previously very intense pain (only responding to opioids), suggesting no bleeding, and stopping of the PT progression. We've continued the treatment for six months (November 2003-April 2004). After the IFNα treatment interruption (April-June 2004), an unexpectedly rapid »rebound effect« was noted (intense pain, rapid growing of the PT – 4 cm in diameter in one month); this »rebound effect« was also described in case of hemangioma treatment with IFNα [1].

In April 2004, a ***fifth PT of the right shank*** producing tibial diaphyseal destruction (Fig. 5-A), with a multilocular structure (Fig. 5-B) was diagnosed; this time the

local trauma produced by an inadequate splint was clearly involved. Intense pain only responding to opioids, associated to the pain from the left knee PT after IFNα interruption, almost transformed the patient's life in a calvary.

Based on our previous good experience with IFNα therapy, and the results with IFNα treatment in case of hemangiomas, showing permanent efficacy only after more than six months of treatment, daily IFNα was restarted (June 2004). Once again, we were surprise by the rapid disappearance of pain.

Unexpected events have unfortunately interrupted the evolution. A local trauma exerted in the time of the MRI investigation (July 2004) determined the rupture of the right shank PT, finally producing a life-threatening hemorrhage (August 2004). In the mean time, the externalization of amputated left tibia fragment, determined by the normal bone growing, appeared. Another amputation, with complete excision of the both PTs was considered to be the only right option (August 2004). A total

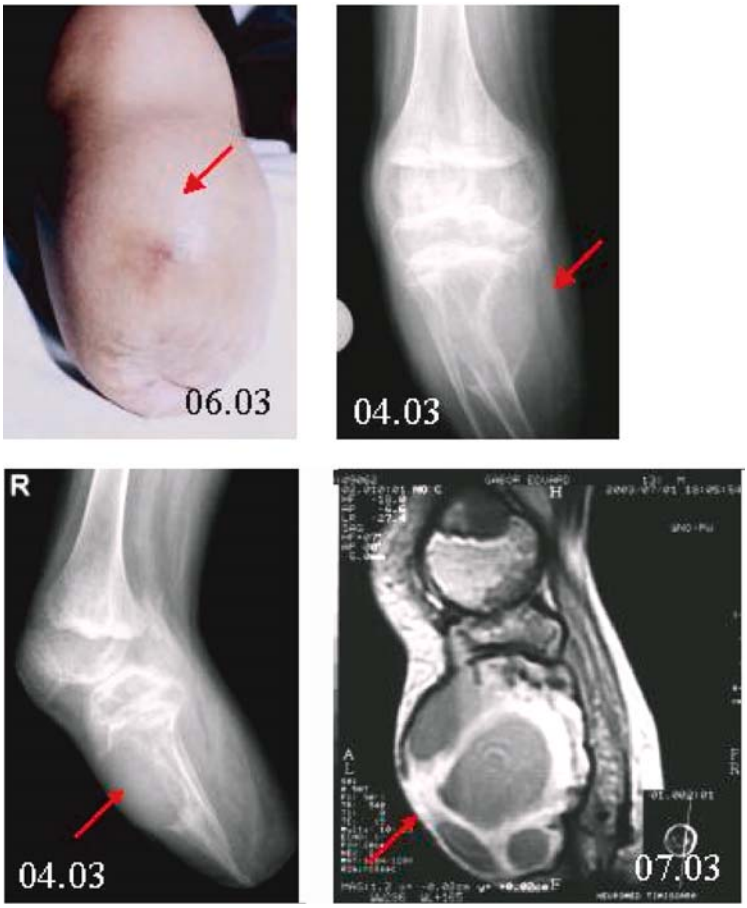


**Fig. 5. A.** Right shank PT; **B.** Tibial diaphyseal destruction; C: Multilocular PT

amount of 12,060 kIU of rFVIIa, and respectively 420,000 IU of FEIBA was consumed with these last two PTs.

Multiple PTs of the limbs were extremely rarely described [8]. The chronology of events in our patient could be probably explained by some particular **favoring factors**:

- the severe secondary osteoporosis (x-ray, osteodensitometry) induced by the long-term immobilization, favoring intraosseous hemorrhage, even after minor trauma;
- the vicious positions adopted by the patient, mainly in time of sitting; the secondary psychological disorders (dysphoria with auto-aggression, anxiety, conversion disorders) favoring multiple trauma;
- the persistent high titer inhibitors (ITI was considered to be most probably inefficient, tacking into account the inhibitors level and the late moment of intervention); because of the bypassing agents shortage, prophylaxis was not possible, and on demand treatment was assured only at minimal levels in case of bleeding episodes; except for the first PT, radical surgery (amputation) was performed in good conditions;
- the secondary immunodeficiency (previous treatment with low/intermediate purity products [15], infection with HCV [15]), favoring the infection of PTs, the infectious complications after surgery;
- the associated health problems (chronic hepatitis with HCV, pulmonary tuberculosis, repeated urinary tract infections);
- the insufficient home care (three brothers at school ages, unemployed father with advanced tuberculosis, and alcoholism).

## Conclusions

The described PTs had the characteristics of distal PTs, appeared most probably after intraosseous hemorrhage. The presented case of multiple distal PTs is extremely rare. The most important factors favoring the apparition of multiple PTs were: high inhibitors level, shortage of bypassing agents, and severe osteoporosis. The spontaneous evolution of PTs was characterized by repeated episodes of hemorrhage and infection, and progressive growth. The conservative surgical approach (evacuation, drainage) was almost always inefficient. PTs could be solved only by complete excision/amputation. Alternative approaches to radical surgery, in patients with distal PTs and inhibitors merit to be discussed (antiangiogenic therapy, long-term therapy with bypassing agents, early conservative surgery – percutaneous management).

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# HIV Resistance to Antiretroviral Therapy in Romania

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## Abstract

One of the major aspects of HIV dynamics is the appearance of resistant viral variants. An increasing number of studies is related to the occurrence of drug resistance in patients receiving different ARV (antiretroviral) therapy regimen.

In Romania the access to ARV's started in 1995, but HAART (highly active antiretroviral therapy) could be used only from 1998 on. Taking into account the particularities of AIDS epidemics in Romania (predominance in children, horizontal way of contamination, evolution of more than 13 years of infection and last but not least the subtype F of HIV-1 infection) we studied the profile of resistance in 20 children and 4 adults that received different ARV's regimens.

## Introduction

AIDS epidemics in Romania broke out in the late 80's, in more than 90% of the cases affecting children. The predominant way of transmission was horizontal, probably in a nosocomial way, but with a large number with unknown route of infection. Children were infected during the first 3 years of life. Since 1990 in Romania more than 10,000 children have been diagnosed with HIV infection, about 85% of them being born in 1988-1990. Studies performed in 1995-1997 in different regions of the country revealed that almost all cases were infected with subtype F of HIV-1 [1].

Antiretroviral therapy in Romania could be started in 1995 as monotherapy with AZT (Zidovudine). In 1996-1997 there were available other 2 NRTI's (nucleoside reversetranscriptase inhibitors): ddC (Zalcitabine) and 3TC (Lamivudine), so the patients received bitherapy (AZT+ddC or AZT+3TC). Protease inhibitors (PI) became available from 1998: first IDV (Indinavir) and in short time NFV (Nelfinavir). Patients receiving mono- or bitherapy switched to HAART and in the same time naïve patients could start ARV directly with HAART. Since 1999-2000 almost all ARV drugs are available in Romania: NRTI's [AZT, ddC, 3TC, ddI (Didanosine), d4T (Stavudine), ABC (Abacavir)], NNRTI's (non-nucleoside reverstranscriptase inhibitors) [EFV (Efavirenz) and NVP (Nevirapine)] and PI's [IDV, NFV, RTV (Ritonavir), AMP (Amprenavir), SAQ (Saquinavir) and Lop/r (Lopinavir/Ritonavir)]. In 2001 Romania declared HIV/AIDS as a top health priority and launched the Action Plan for Universal Access to HIV/AIDS treatment, becoming the first European country benefiting from the price reduction and facilities for ARV's. The number of patients under

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therapy increased yearly: from 3% in 1995 to more than 60% in 2002. The guidance for changing therapy was firstly clinical and immunological. Viral load could be performed only from the year 2000 and not in all cases.

An increasing number of studies are showing the presence of drug resistance in patients with and even without ARV therapy. Most of these studies have involved subtype B of HIV-1, but there are very few data about subtype F. In a study [2] it is revealed a natural reduction in susceptibility to nonnucleoside RT inhibitors in HIV-1 subtype F strains.

## Patients and Methods

Resistance tests were made in Germany (Max Pettenkofer Institute from Munich and Friedrich Loeffler Institut from Greifswald).

Genotyping tests were performed in 24 symptomatic patients (20 children and 4 adults) in stage B or C of HIV infection that received different regimen of ARV's. Resistance to nucleoside and nonnucleoside RT inhibitors (AZT, d4T, ddC, ddI, 3TC, ABC, NVP, DLV, EFV and TDF) and the P inhibitors (SAQ, IDV, RTV, NFV, APV and Lop/r) were tested.

## Results and Discussion

Eight patients from Timis county and 16 from Sibiu county were investigated (Fig. 1). There were 10 males and 14 females. All children were infected with HIV-1 subtype F; 2 adults were infected with HIV-1 subtype F and 2 with subtype B. They had a chronic infection with more than 10 years of evolution. All patients were under HAART for more than 3 years.

Patients were under different ARV's regimen, with 2 NRTI's + 1 PI or 2 NRTI's + 1 NNRTI. We noticed the appearance of resistance to all used drugs in 8 to 29% of the cases (Table 1).

In some cases we noticed the appearance of cross-resistance between NRTI's or PI's, or natural resistance to NNRTI's. We illustrate with B.A, female, 12 years old diagnosed with HIV infection stage C3 in 1995. She received in 97-98 AZT+ddC

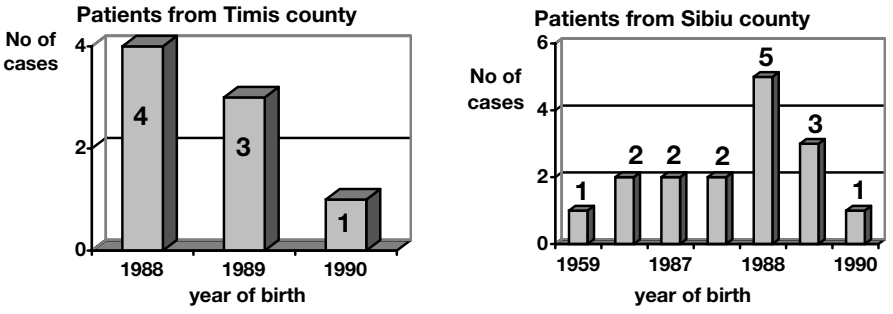


**Fig. 1.** Distribution of the cases by county and year of birth

**Table 1.**

| Antiretroviral drugs | Number of patients (24) | Percentage of cases with resistance to ARV's |
|---|---|---|
| NRTI's | | |
| AZT | 7 | 29.16% |
| ddI | 5 | 20.8% |
| ddC | 5 | 20.8% |
| d4T | 4 | 16.6% |
| 3TC | 6 | 25% |
| ABC | 5 | 20.8% |
| NNRTI's | | |
| EFV | 6 | 25% |
| NVP | 6 | 25% |
| PI's | | |
| IDV | 7 | 29.16% |
| NFV | 5 | 20.8% |
| RTV | 6 | 25% |
| SAQ | 2 | 8.33% |
| AMP | 6 | 25% |
| Lop/r | 6 | 25% |

and, from 1998 AZT+3TC+IDV. At the moment of sample taking she had interrupted ARV's because of hyperlactacidemia. The viral load was 350,000 copies/ml. She had the following mutations: D67N, T69N, K70R, M184I/M and T215Y for NRTI's, K101P/Q, K103N and G190A/G for NNRTI's and, L10I/V, K20R, M36I, G48V, I54V, L63T and V82A for PI's. Despite the fact that she didn't receive NNRTI's the patient showed resistance mutations to EFV and NVP.

In other cases we concluded that the patient was not compliant to the prescribed treatment, as after more than 2 years of ARV therapy he had no resistance mutations. For example M.N, male 14 years old, diagnosed with HIV infection stage B3 in 1995. He was prescribed AZT in 1997, AZT+3TC+IDV in 1998–1999 and EFV+IDV from 1999. The viral load was 660,000 copies/ml. He missed resistance mutations.

If the adherence to the treatment was good, the viral suppression would be assured. For example H.M, female, 11 years old, diagnosed with HIV infection stage C3 in 2000. Between November 2000 and June 2002 she received AZT+3TC+IDV. The viral load was 120 copies/ml. She had no resistance mutations.

## Conclusions

1. Combined antiretroviral therapy represents a remarkable success in controlling AIDS. The key of success is to adapt HAART to the spectrum of viral resistance. The conditions of pediatric HIV infection, encountered also in our country, claim optimal viral suppression and continuous refinements to insure the best chance of life for these patients.
2. The occurrence of natural resistance to NNRTI's in patients infected with HIV-1 subtype F should also be mentioned.

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# Ileopsoas Hemophilic Pseudotumour with Externalized Bowel Fistulation

M. Şerban, D. Bordoş, M. Siebeck, C. Petrescu, L. Petcana, M. Batăneănţ, A. Lăcătusu and W. Schramm

**Summary**

A case of ileopsoas hemophilic pseudotumour is presented. Its particularity is a fistula, developed between the pseudotumour and the large bowel, followed later by a second externalized fistula on the abdominal wall. A large abscess involving the pseudotumour and chronic sepsis developed as life-threatening complications. Diversion colostomy with anus praeter and drainage under FVIII substitution resolved the unusual, severe clinical condition after almost 6 years of evolution.

## Introduction

Hemophilic pseudotumour (HPT) is a well organized, encapsulated, progressive blood mass, expanding, compressing and frequently eroding local tissues.

Usually rare, but severe complication of hemophilia and related disorders, HPT is unfortunately quite frequent in developing countries.

Out of a total of 24 patients with HPT (17 proximal and 7 distal) we present a most impressive case. It illustrates the natural history of ileopsoas hematoma with all its burden of complications, focusing the attention on the unusual aspect of HPT.

## Case Presentation

M.A. is a 30-year-old man, with severe form of type A hemophilia (Factor VIII concentration $<0.5\%$; inhibitor status- 0 BU). He presents chronic hemophilic arthropathy in the following target joints: knees, right shoulder and left elbow and a total clinical joint score of 35.

He experienced a left ileopsoas hematoma (IPH) in July 1997 (Fig. 1): he developed pain and swelling in the left flank, loss of sensation in the femoral nerve distribution of the left leg, weakness of quadriceps and anemia. He was treated with regular cryoprecipitate and supplementation of FVIII concentrate for 14 days. Over the next 9 months he recovered partially, but in April 1998 his clinical status worsened suddenly: a huge abdominal mass appeared in the left flank and fossa (Fig. 2), followed by a posthemorrhagic shock. Surgical removal and drainage was attempted under a substitutive treatment with FVIII concentrate and cryoprecipitate.

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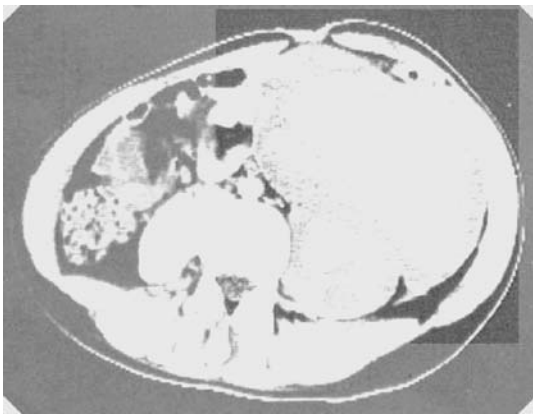

**Fig. 1.** Posthemorrhagic shock, 1st rupture of left ileopsoas hematoma – July 1997

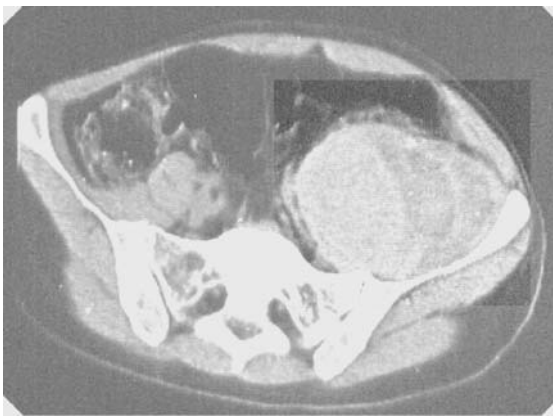

**Fig. 2.** 2nd rupture left ileopsoas hematoma, surgical removal and drainage – April 1998

Repositioning of drains was intermittently required. After 2 months he was discharged in stable condition.

March 2002 marked the start of serious worsening in the patient's evolution. He presented septic fever, with pain in the left iliac fossa, diarrhea and a nasal and malar cutaneous redish erruption. He lost 13 kg in weight. He was serologically negative for HIV, but positive for HCV; his urine and stool cultures remained negative. FVIII inhibitors and autoantibodies (anti-DNA, lupus cells, and rheumatoid factor) were negative. In blood cultures grew Klebsiella. The CT scan revealed that the HPT had doubled in size. Under continuos intermittently adapted antibiotic therapy (ampicillin/sulbactam, ceftriaxone, ceftazidime, gentamicin, amikacin sulphate, metronidazole, chloramphenicol, ciprofloxacin, meropenem, imipenem-cilastatin, vancomycin) he became afebrile with sterile blood culture; but the discontinuance of antibiotics was immediately followed by the reappearance of septic fever.

In the period of April-August 2002 the patient experienced 5 episodes of maelena, with negative gastroscopy. A repeat CT showed gas filled cavities (Fig. 3), suggesting a communication between the HPT and the bowel. The colonscopy and

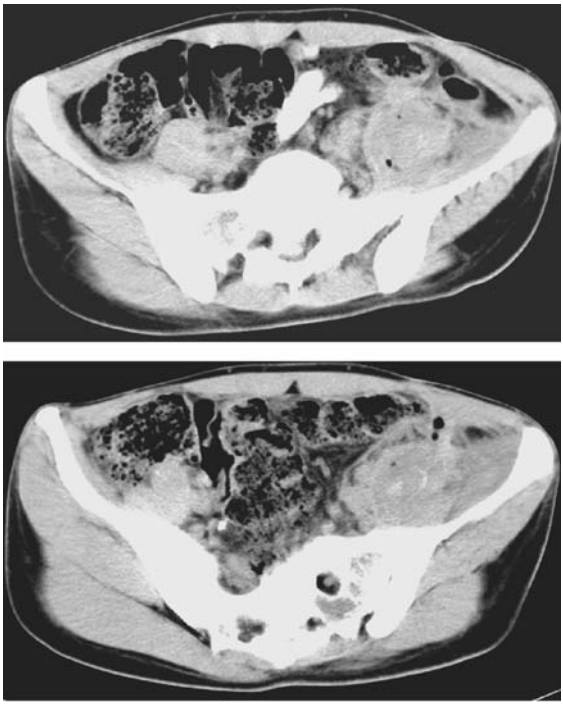

**Fig. 3.** Bowel adherence and air within the left pelvic pseudotumor, intestinal hemorrhage - November 2002

barium enema failed to asses the fistula. Surgical excision or percutaneous drainage of the HPT were considered but deemed much too risky in our situation of FVIII shortage. Antibiotic therapy was performed almost continuously.

In December 2002 macroscopic hematuria complicated the evolution. In February 2003 an inguino-crural abscess was assessed.

March 2003 marked the externalization of the retroperitoneal abscess on the abdominal wall, precipitating the surgical intervention (Fig. 4–6).

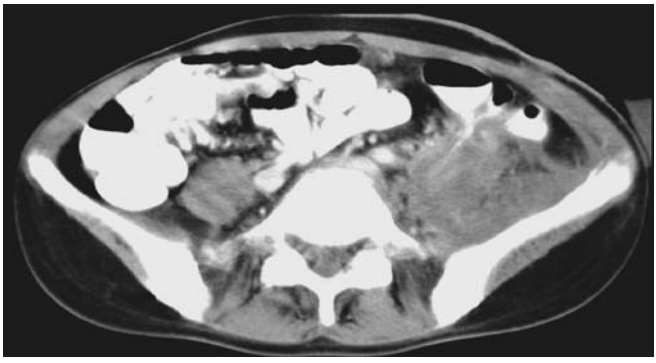

**Fig. 4.** Bowel fistula, contrast substance and air within the pseudotumor – March 2003

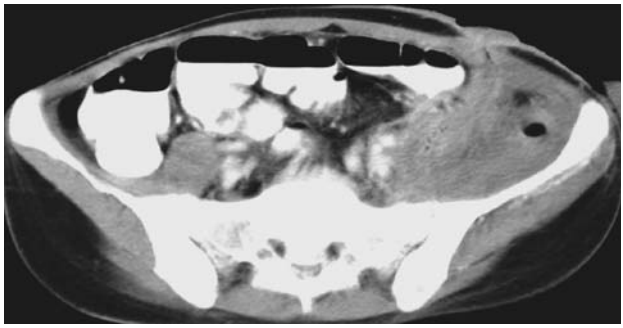

**Fig. 5.** Externalization of fistula on the abdominal wall - March 2003

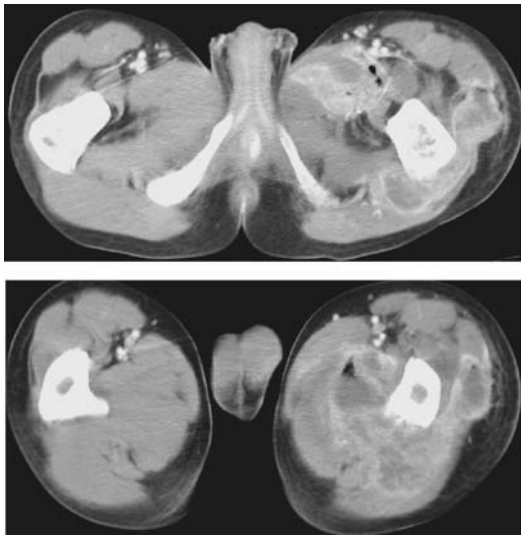

**Fig. 6.** Inguino-crural involvement, abscess in the left tigh - March 2003

On 22 March 2003 under FVIII substitution (80,000 IU) a diversion colostomy and anus praeter were performed; retroperitoneal and thigh drainage were inserted. An amount of more than 1000ml bloody pus was discharged, followed by immediate clinical improvement. Under continuous secondary prophylaxis (FVIII 50,000 IU) repositioning of drains was intermittently required. In June 2003 the drainage was discharged. In September 2003 closure of the externalized fistula on the abdominal wall was obtained.

The present status is satisfactory: the patient is nonfebrile since April 2003; he has a weight gain of 14 kg; the locomotor performance has improved; the total clinical joint score has dropped to 25; the dimension of the ileopsoas HPT has reduced, the present diameter of HPT being of 18 mm; the external segment of fistula has closed; the fistulation route of HPT is still present but there are no signs of communication with the bowel (Fig. 7).

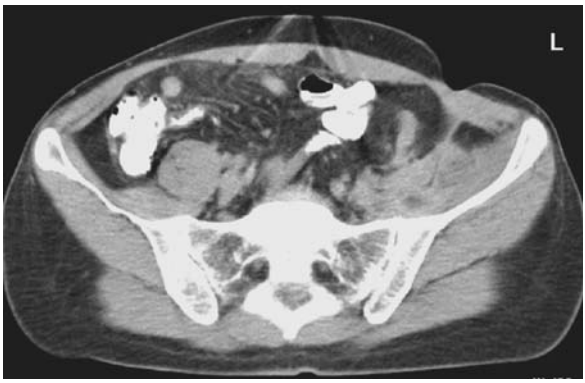


**Fig. 7.** Fistulation route present, closed external segment, no signs of communication with the bowel – November 2003

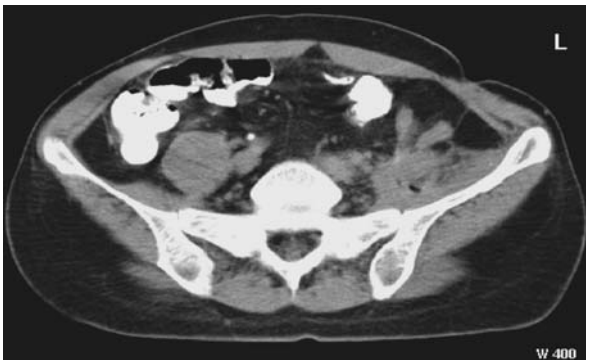


**Fig. 8.** Reduced HPT dimension, ø18 mm – November 2003

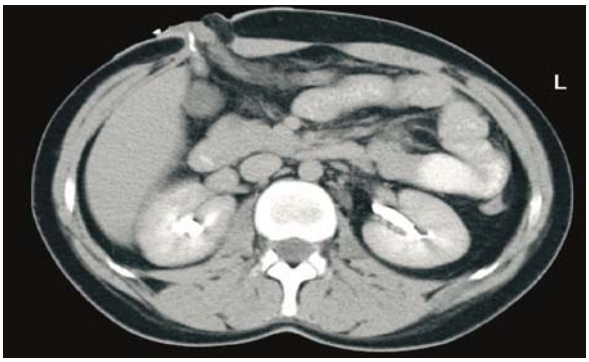


**Fig. 9.** Anus praeter – November 2003

## Discussion

In countries where adequate substitution is performed, the occurrence of HPT is rare (<1%); they usually complicate the evolution of patients with high level inhibitors. In countries where the accessibility to appropriate replacement therapy is limited, the prevalence of HPT is quite frequent (10% or even more) [1]. Fistula formation is a common complication of HPT, usually in the form of cutaneous fistulae;

but fistulae between pseudotumor and bowel seem to be particularly uncommon; externalization, like in our patient, is only exceptionally observed [2].

Pseudotumour management is complex and challenging and must be tailored to each patient's situation and to the quality of FVIII substitution. An initial trial of conservative management is the first choice. A complete surgical removal of the HPT and filling of the cavity with gluteus medius or rectus abdominis muscles would be the treatment of choice, but it is complicated by a high mortality rate (>20%). Embolization alone has only a temporary effect. Irradiation of unresectable lesions can further damage the walls of the bowel fistula. Often, more conservative approaches are preferred. However, residual HPT always remains a potential focus for recurrence with growth and infection [3, 4, 5]. Despite adequate clotting factor replacement bleeding may develop in the dead space remaining after the HPT is resected. Complete resection of the HPT should be the goal, but, like in our case, may not be possible. A new surgical intervention with the interposition of omentum between PT and the bowel fistula could represent a salvage solution for our patient [6].

## Conclusions

Because of many and diverse shortcomings, HPT remain a severe life threatening burden for our patients with hemophilia. A successful outcome was achieved in the presented patient due to a persistent, multidisciplinary approach; his long term prognosis is depending on the potential risks of bleeding and infection.

*Acknowledgement.* We thank Prof. Wolfgang Schramm for the generous support in FVIII concentrates, allowing the surgical intervention.

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# Reduction of Proteoglycan Synthesis in Chondrocytes Depending on Concentration and Duration of Iron Overload

U. Hartl, K. Hochmuth and A.A. Kurth

## Introduction

Typically, arthropathy in hemocromatosis or hemophilia is strongly associated with an iron overload in chondrocytes [1, 2]. So far, the effect of iron has been shown on myocardium cells, but has not been quantified on articular chondrocytes [3, 4]. In addition, change in the proteoglycan synthesis is an indication of cell damage to the chondrocytes, therefore it is still unknown whether changes of iron overload will lead to variation in the proteoglycan synthesis [5].

The aim of this study was to develop a new in vitro model for iron overload in articular chondrocytes and to show the influence on the proteoglycan synthesis. The proteoglycan synthesis was measured depending on the concentration and exposition time of iron. Results were compared with histopathologic specimens.

## Methods

Chondrocytes were cultured as monolayer after being isolated from slices of bovine articular cartilage. The cells were isolated by means of sequential digestion of the matrix using pronase and collagenase. They were then seeded in 24 well plates at a density of 1 x $10^5$ per ml media.

Chondrocytes were maintained by feeding daily with 0.5 ml medium per well (Ham's F-12 medium with 10 % FBS, 50µg/ml gentamicin and 25µg/ml ascorbic acid), incubated in a humidified atmosphere of 5 % $CO_2$ in air at 37 °C.

Together with media exchange, iron was introduced as $FeSO_4$ every 48 hours. 5 treatment groups were formed at different $FeSO_4$ concentrations (0.5 / 1.0 / 1.5 / 2.0 / 2.5 mMol). The medium was changed every 24 hours . The cells were cultured for 14 days. The residue was removed and the proteoglycan release was measured using a photometric assay (DMMB-assay, Hoechst-Marion-Roussel/Germany).

In addition, after the above treatment some of the cells were removed for histopathologic reappraisal by means of Pearls reaction. To demonstrate the level of iron, the culture was dyed using Pearls reaction. It was now possible to view the iron intake under the microscope. By means of regular observation of the degree of discoloration it was possible to determine the level of the proteoglycan synthesis. This semiquantitative analysis showed a direct relationship between iron overload and the proteoglycan synthesis.

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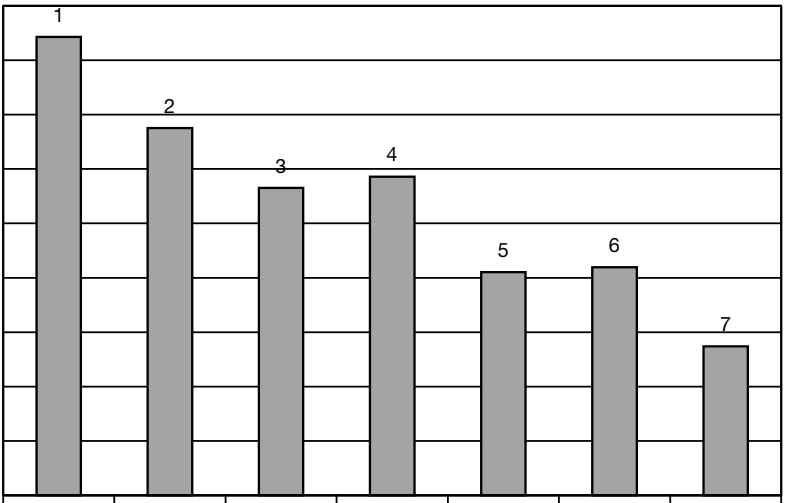
## Essential Results

The chondrocyte cell culture exhibited a high intake of stored iron. Over a period of 14 days the study showed an increase in the concentration of $FeSO_4$. Simultaneously, a reduction of the proteoglycan synthesis was found (see graph below.)

Figure 1 shows a typical example of the Pearls reaction corresponding to the eighth day of incubating the chondrocytes with $FeSO_4$ at a concentration of 1.5 mMol.




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| 1,5 mMol | 42,137 | 33,776 | 28,266 | 29,298 | 20,527 | 20,977 | 13,688 |

**Reduction of the proteoglycan synthesis in comparison to control group for 7 treatment terms**

**Fig. 1.** 1,5 mMol

Figure 2 shows for a sample of 1.5 mMol $FeSO_4$ the proteoglycan synthesis over the 14 day period.

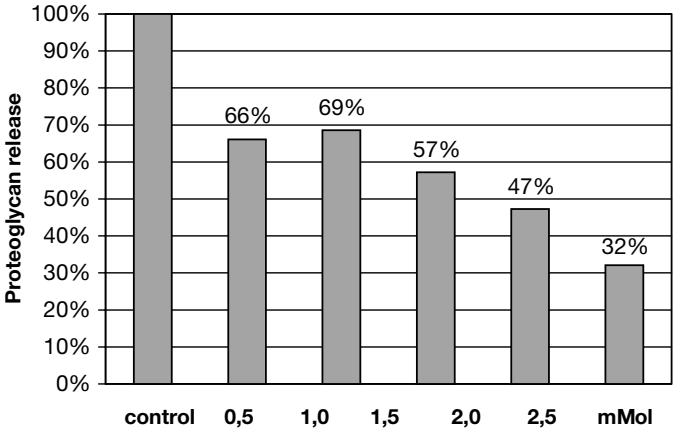



**Fig. 2.** Proteoglycan synthesis of culture with different Ferrumsulfat concentrations

## Discussion

In this study we have developed and applied a new in-vitro model which is able to prove the influence of iron on the proteoglycan synthesis.

The direct impact of iron on the articular cartilage could be measured. In addition the reduction of the proteoglycan synthesis is directly dependent on the concentration of iron. Furthermore we determined a semiquantitative histopathologic score for the correlation between iron overload and the proteoglycan synthesis. The results demonstrate that it is very important to prevent the joint and therefore the cartilage from iron overload. These findings support, amongst others, the current concept of blood–induced cartilage damage.

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# Bleeding Tendency of Carriers of Hemophilia A – Dependent on the Age of the Carriers?

W. Miesbach, J. Oldenburg, I. Stier-Brück, T. Vigh and I. Scharrer

Carriers of hemophilia A often present with normal clotting factor VIII levels but occasionally also report a considerable tendency to bleeding. Recently it was shown that reduced concentrations of factor VIII and IX in carriers of hemophilia A and B are associated with favorable effects on blood coagulation and hemostasis which contributes to cardiovascular protection and a decreased mortality of ischemic heart disease. [1]

Hemophilia A is an X-linked recessive bleeding disorder of variable severity that is caused by a deficiency of factor VIII (FVIII). The disease results from mutations in the FVIII gene which are heterogeneous both in type and position within the gene.

The frequency of hemophilia A is 1–2 in 10 000 male births in all ethnic groups.

Because carriers have usually one unaffected allele the FVIII concentration is often of about 50 % of normal. But symptomatic carriers of hemophilia A can present with bleeding symptoms like a male patient with mild hemophilia A. A small number of carriers may have very low levels of FVIII [2] due to co-inheritance of a variant von Willebrand factor allele (i.e. von Willebrand's disease Normandy), homozygosity for hemophilia gene [3] or extreme lyonization.

The daughters of men with hemophilia are obligate carriers and carriers have a 50:50 chance of passing on the condition to a son and a 50:50 chance that a daughter will be a carrier.

Members of the same family usually have the same FVIII level and the severity of hemophilia within a family remains constant.

Over the last 20 years the advances in molecular biology and prenatal diagnostic techniques have faciliated the diagnosis of hemophilia.

The gene for factor VIII was cloned in 1984 [4-6]. The FVIII gene is 186 kb in size and contains 26 exons.

The factor VIII mutations causing hemophilia A are heterogeneous. In hemophilia A patients many mutations in the FVIII gene have been identified including deletions, point mutations or mutations resulting in stop codons [7]. In about 5 % of the patients with hemophilia A there are large (more than 100 nucleotides) deletions in the FVIII gene [8]. Deletions almost always produce severe hemophilia A. Small deletions or insertions in the coding region of the factor VIII gene that result in frameshifts may cause severe hemophilia A, too. FVIII gene inversion mutations were identified in 45 % of severe hemophilia [9].

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It is not always possible to exclude carrier status by measuring a women's FVIII level. These levels fluctuate in normal people under stress, during pregnancy and for those under oral contraceptives. Thus, for this tests calculations are made after taking blood samples on several separate occasions. Carrier detection in hemophilia A can give definite answers in 80–90% of cases after DNA analysis. The tests sometimes require blood samples from other members of the family, particularly the person with hemophilia and an unaffected male.

The aim of this investigation was to document the occurrence of bleedings in carriers of hemophilia A and to study the genotype and phenotype correlation as well as the dependency of the bleeding events on the age of the carriers.

## Material and Methods

The subjects included in this study were carriers of hemophilia A who attended the Hemophilia Ambulance of the University Hospital Frankfurt/Main, Germany.

The fact that they were carriers was confirmed in all cases by analysis of the factor VIII gene mutation (done by PD Dr. J. Oldenburg,Würzburg/Frankfurt).

42 carriers were questioned about their tendency to bleedings.

The following symptoms were noted: easy bruising, nose bleeding, gum bleeding, bleeding after small or larger traumata, bleeding after surgical procedures, long or heavy menstrual bleedings.

Measurement of factor VIII was carried out by an one-stage clotting assay (Instrument ACL 300, normal range: 64 %–167 %) with FVIII-deficient plasma from Instrumentation Laboratory (IL).

To exclude the additional presence of a von Willebrand syndrome the von Willebrand antigen was measured in all carriers by an home-made ELISA.

## Results

The median age of the carriers was 43 years (16–75 years).

In all carriers the FVIII mutation could be detected. Nine carriers had an intron-22-inversion, one carrier a small deletion and the majority of the carriers (32 carriers) a point mutation.

The median FVIII concentration of all carriers was 54.5 % (4–136 %). Divided into two age-dependent groups, the younger patients (median age 32 years, range 16–43 years) had a lower FVIII concentration (median 53 %, range 4–120 %), the older patients (median age 57 years, range 43–75 years) had a higher FVIII concentration (median 62 %, range 16–136 %) (Table 1).

28/42 carriers (67 %) reported on the occurrence of bleeding events.

The following bleeding symptoms were noted (Fig. 1).

The most frequently mentioned events were bleedings after traumata or operations (24/28, 86 %). Tendency for bruising was reported by 11/28 carriers (39 %), strikingly long and heavy menstrual bleeding by 3/28 (11 %), and frequently gum bleeding by 2/28 (7 %) carriers.

**Table 1.** Characteristics of the carriers

| | |
|---|---|
| Median age | 43 years (16–75) |
| Factor VIII-activity, median | 54.5 % (4–136 %) |
| | |
| Factor VIII –gene mutation | |
| Intron-22-Inversion | 9 carriers |
| Point Mutation | 32 carriers |
| Small Deletion | 1 carrier |
| | |
| Factor VIII concentration – dependent on the age of the carriers? | |
| Median age | Factor VIII-activity |
| 32 years (16–43, n = 21) | 53 % (4–120 %) |
| 57 years (43–75, n = 21) | 62 % (16–136 %) |

14/42 (33 %) carriers reported that they did not remember any tendency to bleeding.

In the 28/42 carriers who presented with bleeding tendency the FVIII concentration was lower (median factor VIII 51 %, 4–136 %) than in 14/42 carriers without any bleeding tendency (median factor VIII 66 %, 30–120 %) (Table 2).

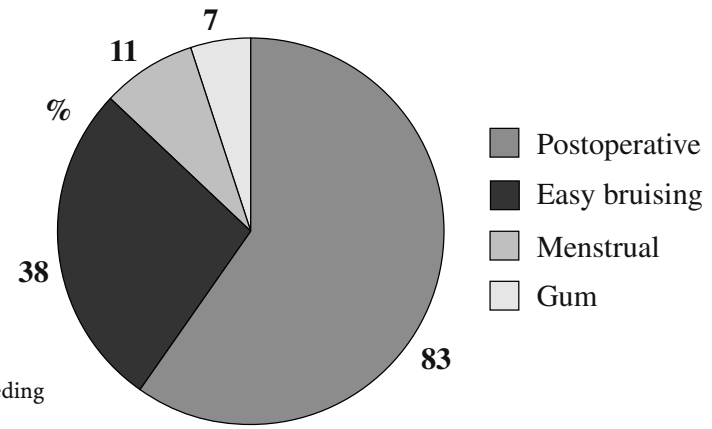


**Fig. 1.** Type of bleeding events

**Table 2.** Association of bleeding tendency to the FVIII concentration and the age of the carriers

| Bleeding tendency | yes | no |
|---|---|---|
| Carriers | 28 / 42 (67 %) | 14 / 42 (33 %) |
| Factor VIII-activity | 51 % (4 % - 136 %) | 66 % (30 % - 120 %) |
| Age | 47 years (21–73) | 32 years (16–75 J.) |

The age of the 28/42 carriers with bleeding tendency was median 47 years (21–73 years).

The age of the 14/42 carriers without any bleeding tendency was median 32 years (16–75 years).

By separation the carriers into two age-groups from 16 to 43 years (21 carriers, factor VIII: 53 %, 4–120 %) and from 43 to 75 years (21 carriers, factor VIII: 62 %, 16–136 %) it could be shown that in the group of younger carriers 50 % of them had so far no bleedings in comparison with only 20 % of the carriers with older age although in these carriers factor VIII levels were higher (Table 3).

**Table 3.** Bleeding tendency of two age-dependent groups of carriers

| Median age | Bleeding tendency |
|---|---|
| 32 years (16–43, n = 21) | 52 % (11 / 21) |
| 57 years (43–75, n = 21) | 81 % (17 / 21) |

## Discussion

Mostly not enough attention is paid to the bleeding history of carriers of hemophilia A.

Recurrent bleeding events however are a frequent symptom in carriers of hemophilia A. Bleedings after traumata or operations are the most frequent manifestations of this. Bruising or prolonged menstrual bleeding occur markedly less.

Recently we have demonstrated that the incidence of bleeding symptoms is correlated to the activity of FVIII and the underlying FVIII gene mutation. Carriers with low levels of FVIII are at risk of excessive bleeding from surgery or other invasive procedures. [10]

In this investigation we studied the dependency of the bleeding events on the age of the carriers.

It could be shown that in the group of younger carriers less carriers had a history of bleeding events in comparison to the older carriers. The younger age of the carriers who could not remember any tendency to bleeding is striking. This correlation was in inverse proportion to the FVIII concentration. Although we demonstrated a correlation between the age of the carriers and the level of FVIII before [10], in this case the bleeding tendency correlated to the age of the carriers and not to the FVIII level.

Although their factor VIII levels were lower, younger carriers were less likely to report a tendency to bleeding. Older carriers with higher concentrations of FVIII reported more common bleeding events.

This may be due to the fact that the diagnosis of a carrier was made earlier in younger carriers and that frequently postoperative bleeding was dealt by prophylactic treatment.

Finally it can be postulated that carriers were less likely to report a tendency for bleeding when timely diagnosis and treatment for carriers of hemophilia A can prevent the tendency to bleeding.

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# Clinical Investigation of Orthopedic Outcome in Patients with Severe Hemophilia Under Prophylactic Treatment. Disadvantage of a late Prophylactic Treatment?

S. Meister, T. Spranger, K. Jersch and G. Auerswald

## Introduction

One essential aim in the hemophilia treatment is to prevent hemorrhaging into joints by prophylactic treatment with factor concentrates. Hemophilic arthropathy is a possible consequence of recurrent joint bleeds. Efficacy of early prophylactic treatment has been shown within the last years.

A physical examination and a joint status was done of all regularly treated patients in our treatment centre born 1985–1996 (40 patients) with a FVIII/IX residual activity of <2%. Excluded was one patient with former cranial hemorrhage and spastic hemiplegia. All patients were on prophylactic FVIII or IX treatment at the time of investigation.

## Patients

Two groups were formed: the first group with patients born 1985-1990 (17 patients, average age 15.1 years), and the second group with patients born 1991–1996 (23 patients, average age 9.3 years). Prophylaxis was started at the average age of 6.1 years (median 4.9 years) in the first group. Prophylactic treatment was started earlier at the average age of 4.1 years (median 3.6 years) in the second group.

| | Group 1 | Group 2 |
|---|---|---|
| Year of birth | 1985–1990 | 1991–1996 |
| Number of patients | 17 | 23 |
| Hemophilia A/B patients | 14/3 | 21/2 |
| Average age | 15.1 | 9.3 |
| Median age | 15 | 9.3 |
| Start of prophylactic treatment (average) | 6.1 | 4.1 |
| Start of prophylactic treatment (median) | 4.9 | 3.6 |

## Anamnestic data

Most bleeds were reported in the ankle joints (33 patients, 82.5%). Other locations were the knees (25 patients, 62.5%) and elbows (20 patients, 50%). Bleeds were less

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**Fig. 1.** Locations of bleeds

often reported in hip, hand and feet. This distribution was found in both groups; patients of the second group were affected less often (Fig. 1).

## Physical Examination

3 patients had synovitis in the ankle joint, in two other patients synovitis was found in the left knee. Crepitations were found in 13 patients, mostly in the knees (10 patients, 25%), in 6 patients even both knees were affected. Also crepitations were found in elbow and ankle.

10 patients of the first group (59%) and 8 patients of the second group (35%) had limited range of motion. The decreased range of movement was in most patients mild. The ankle joint was affected most often (Fig. 2) (47% group 1, 35% group 2) followed by the elbow (29% group 1, 9% group 2). Discreet ankle joint disorders occurred especially more often in group 2 (Fig. 3). Surprisingly we found no decreased range of motion in the knees. A total of 5 patients (29%) in the first group and in contrast only one patient (4%) in the second group had more than one joint with limited range of motion (Fig. 4).

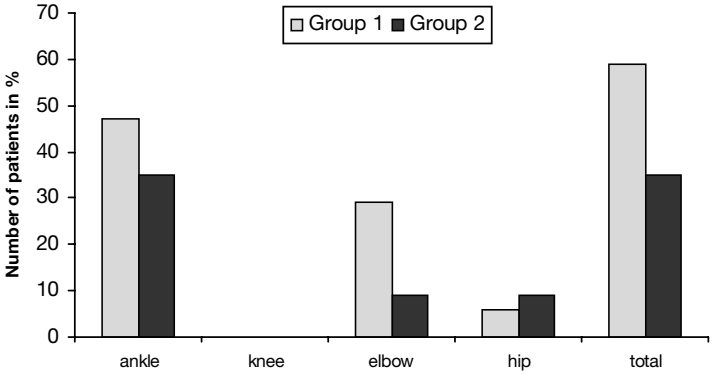


**Fig. 2.** Joints with limited ranges of motion

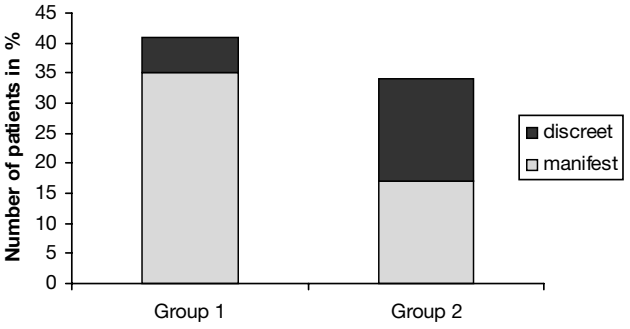


**Fig. 3.** Limited ranges of motion of the ankle joints

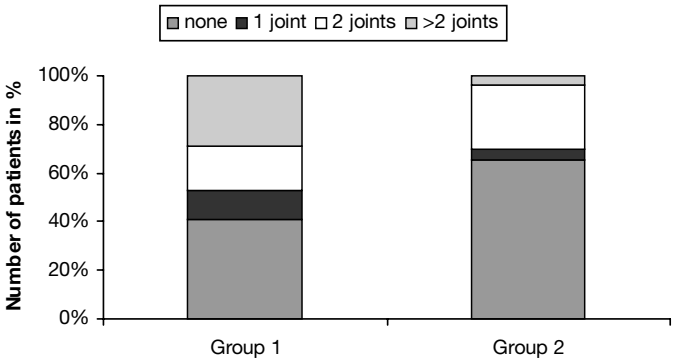


**Fig. 4.** Joints with limited ranges of motion in each patient

## Summary

This clinical investigation supports former data regarding the efficacy of early prophylactic treatment (Kreuz et al., Aledort et al.). It becomes most clear looking at the number of joint disorders in each group. Decreased ranges of movements in several joints were also found clearly more often in the first group. Possible causes are either the prophylactic treatment (which was started later in the first group) or the patient's age at the time of investigation. Patients who were older than 8 years when prophylactic treatment was started often had limitations in the range of movement in several joints. Further investigation in patients born 1997-2002 is planned. Also a follow up of the two groups will be done with a functional motion study (a three dimensional ultrasound study was done in some patients already). The prophylactic treatment with factor concentrates was in most cases started after occurrence of the first joint hemorrhage; in the rest of the cases it was started after the second joint bleeding.

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# Operative Treatment of Ankle Equinus Deformity in Hemophiliacs

T. A. Wallny, P. Pennekamp, H.-H. Brackmann, P. Hofmann, C. N. Kraft and A. Seuser

## Introduction

In the juvenile and adolescent hemophiliac with no or only inadequate substitution therapy, the calf muscles and the ankle joint remain one of the primary localizations of bleeding episodes [1, 2]. Subsequent fibrous atrophy after recurrent bleeding typically causes an ankle equinus deformity, which may functionally be complicated by an extension-deficit of the knee-joint [3].

In cases where non-surgical management options remain ineffective, moderately simple operative techniques such as Achilles tendon lengthening and simultaneous dorsal capsulotomy have been propagated [3-5]. The aim of this study is to assess long-term clinical and radiographic results after Achilles tendon lengthening for hemophilic equinus, in an effort to determine whether this procedure is a feasible management option.

## Materials and Methods

Between 1975 and 1986, 30 hemophilic patients with secondary equinus deformity of the ankle due to recurrent bleeding episodes underwent Achilles tendon lengthening. Of these, 23 patients, all with severe hemophilia A (factor VIII less than 1 %), could be followed up. 12 were operated on the right side, 11 on the left side. The average age at the time of operation was 28.7 years (range 14 to 46). Clinical results were assessed by using the section »clinical examination« and »pain« of the Advisory Committee of the World Federation of Hemophilia (WFH)-Score [6], while roentgenographic results were evaluated using the Petterson-Score [7]. The clinical score is still assessed prospectively at least twice a year, the radiographic score every two years.

The average preoperative WFH-score for the treated ankle was 4.2 points (min. 1, max. 8) and the average preoperative Petterson-score was 4.4 (range 1 to 10) points (see Table 1).

Preoperatively an equinus position averaging 21° (range 5-55°) was found.

The factors pain (0-3 points), bleeding (0-3 points) and physical examination (0-12 points) make up the clinical score (WFH), whereby a normal joint will have 0 points and a severely afflicted joint a maximum of 18 points. The radiographic score ranges from 0 points for a healthy joint and 13 points for apparent massive radiographic destruction. Average follow-up was 13 years *(range 1 to 24).*

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**Table 1.** Patients and Results

| | number of patients | average age | number of procedures | follow-up (average) | clinical score (points) | radiographic score (points) |
|---|---|---|---|---|---|---|
| | 23 | 28.7 years | 23 | 11 years | 4.2 | 4.4 |
| at follow-up | | | | | 2.7 | 6.9 |

## Operative Technique

The indication for surgical management was a persistent or a progressively deteriorating equinus position of the ankle. Lengthening was performed by an open Z-incision of the tendon and, if considered necessary, a simultaneous posterior capsule release of the ankle-joint. In case of a tendency to hindfoot valgus the distal lateral limb of the split tendon was detached and in patients with a hindfoot varus the distal medial limb of the incised tendon was detached. Postoperatively the treated lower leg and foot was fixed with a circular cast in a neutral position over a period of 6 weeks to maintain correction and allow tendon healing. After this, intensive physiotherapy was performed and patients were permitted to fully weight bear.

## Factor Replacement

Factor substitution to achieve an activity of 30-40% was performed preoperatively. Directly prior to surgery, factor activity was raised up to 80-100% by giving approximately 30-40 units per kg body-weight. A coagulation-test was performed postoperatively and, if required, factor was again given. Factor activity was kept at 60% up to the fourth postoperative day and subsequently maintained at 50% until day 14 after surgery. In the following period factor activity was held at 20%. After rehabilitation, a return to normal concentrations was allowed.

## Results

The average WFH-clinical score (clinical examination) improved after surgery to 2.7 points (range 1 to 4). 11 (47.8%) ankles showed clinical improvement, 1 (4.4%) ankle showed a postoperative deterioration while 11 (47.8%) further ankles remained unchanged. Taking the postoperative observation period into consideration, patients who were observed over a period of up to 10 years (n=6) showed an average improvement of the clinical score by 3 points. Those patients with an observation period of over 10 years after surgery improved clinically by an average 1 point.

The Petterson-Score increased to an average of 6.9 (range 3 to 12) points. Here the radiographs of 18 (78.2%) patients indicated postoperative deterioration, 4 (17.4%) remained unaltered and 1 (4.4%) patient showed improvement. Radiographic assessment of patients 10 years or less after surgery (n=12) showed an aver-

age drop of the Petterson-Score by 2 points, while those patients radiographically over a period of more than 10 years deteriorated radiographically by an average 4 points.

The average postoperative equinus position was 10° (range –4° to 20°). Of the 20/23 (87%) patients with a measurable improvement of the ankle equinus at follow-up. Only 7 patients still had complete persisting correction of the initial deformity.

The range of motion (ROM) of the ankle for dorsal extension and plantar flexion was improved in 8 patients (average 10°) and deteriorated in 15 patients (average 9°), suggesting that an improvement of the plantar flexion was frequently associated with a loss of dorsal extension.

6 patients had a persistent reduction of pain while 17 patients remained unchanged.

Achieved improvement of the postoperative equinus position did not significantly alter over the observation period: an average measured postoperative improvement of 16° of the equinus deformity was measured. Overall these remained unchanged over the subsequent observation period. Even after 15 and 20 years, the increase of ROM compared to preoperative values were much the same as after initial surgery (Fig. 1). In contrast the time-span between surgery and follow-up had an influence on the total ROM of the ankle. Scrutiny of plantar flexion and dorsiflexion showed values more or less unchanged 1, 5 and 10 years postoperatively (on

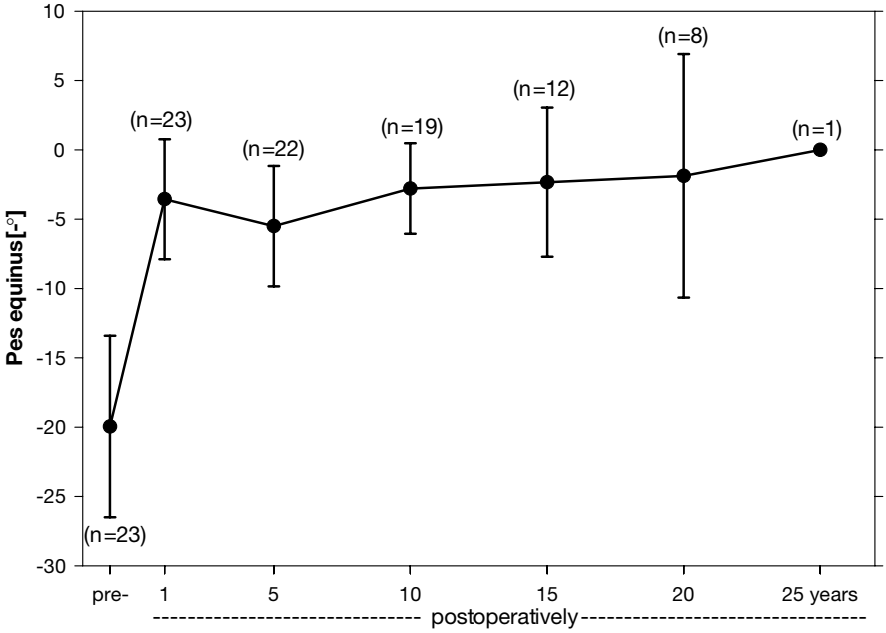


**Fig. 1.** Average change of the severity of equinus deformity pre- to postoperatively (95% confidence interval; negative values = equinus deformity, pos. values = dorsiflexion possible). Deformity showed an average improvement of 16° pre- to post-Achilles tendon lengthening, and remained almost unchanged over the subsequent years (n=number of patients)

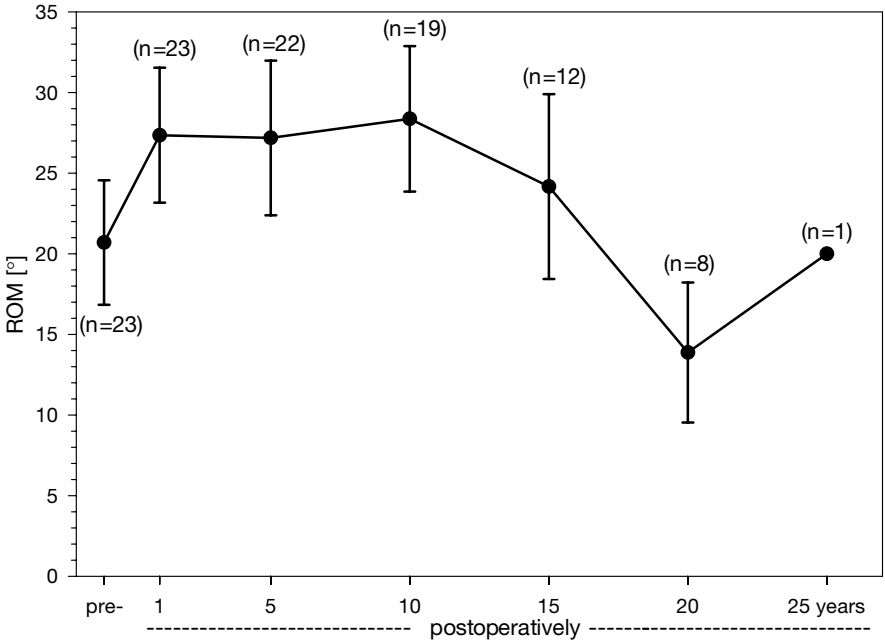


**Fig. 2.** Mean values of pre-and post-operative total range of motion in plantarflexion and dorsiflexion after Achilles tendon lengthening for hemophilic equinus deformity (95% confidence interval; n=number of patients).

average 6° higher than preoperatively). After this, average ROM of the ankle joint tended to deteriorate, and at 15 years postoperatively values equal to or worse than the preoperative situation were documented (Fig. 2). In this context it must be pointed out that for the last two observation periods relatively few patients were assessed. Therefore conclusions can only be drawn very tentatively.

Pearson's coefficient of correlation showed no association between the clinical score (discrepancy pre- to post-operative), equinus deformity and the improvement of ROM ($p>0.145$-1.0). Furthermore a correlation between the preoperative clinical and radiographic score and the equinus deformity, respectively the ROM was not found. The preoperative situation obviously hat no statistically verifiable influence on the postoperative ROM.

## Discussion

### Clinical Results

Achilles-tendon lengthening is a probate method for the treatment of hemophilic pes equinus. As from an equinus contracture larger than 30° tendinous correction is usually insufficient and additional corrective wedge-osteotomy should be con-

templated. Interestingly we found no suture-insufficiencies in our patients, despite the fact that in some cases a marked lengthening of the Achilles tendon was performed.

Though no surgery of the ankle joint as such was performed, tendon lengthening in general had a positive effect on the patients post-operative clinical score of the ankle. This substantiates the conclusion that by tendinous correction and the simultaneously achieved indirect slackening of the periarticular soft-tissue structures, the motion pattern of the ankle joint is brought closer to the physiological situation. Whether surgery had an influence on the intraarticular structures of the ankle cannot satisfactorily be answered by this study. The attempt to rectify the motion pattern of the ankle may have a positive effect on intraarticular cartilage.

Through Achilles tendon lengthening the ankle's overall range of motion is only slightly improved, as a gain of extension is typically associated with loss of flexion. Johnson and Babbitt (1985) [8] could show a correlation between the loss of ROM and the successive receding of articular cartilage in the hemophiliac. One can therefore hypothesize that the preoperative documented loss of ROM in our patients is not solely a result of the ankle equinus deformity but probably has multiple factors. Consequently Achilles tendon lengthening treats only part of the problem.

This study shows that Achilles-tendon lengthening is an acceptable method for treatment of hemophilic ankle equinus deformity. Nonetheless it must be pointed out that these results also suggest that in the long-run there is a tendency of overall loss of ankle ROM. This may be due to the almost always associated arthropathy of the ankle. Most of our patients had some degree of radiographically verified arthropathy of the treated ankle prior to surgery. Whether loss of motion is due to a progressive arthropathy or possibly based on an insufficient surgical management cannot conclusively be answered through this study.

**Radiographic Results**

The long-term postoperative radiographic assessment of our patients showed that, despite surgical management, joint damage in hemophiliacs with an ankle equinus deformity continually progresses. This finding is not new. In previous studies we could demonstrate that operative procedures in the vicinity of afflicted hemophilic joints do not necessarily prevent further destruction [9,10]. Yet untreated or only conservatively managed equinus deformities also show continuous arthropathy [11]. It therefore seems that with or without surgical management subsequent deterioration of the ankle joint is inevitable and most likely not a direct result of Achilles tendon lengthening.

**Conclusion**

The majority of patients treated for hemophilic pes equinus by Achilles tendon lengthening showed a temporary or permanent functional improvement. Long term improvement of the equinus deformity is achieved by the procedure, yet this is fre-

quently associated with loss of plantar flexion. Achilles tendon lengthening does not prevent progression of hemophilic ankle arthropathy. In view of the fact that in a significant number of our patients the clinical status was improved, the procedure seems a feasible management option for equinus deformity of the ankle, with acceptable long-term results.

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# Spontaneous Empyema of Joints and Staphylococcal Sepsis in a Patient with Severe Hemophilia A

W. Mondorf, L. Hovy, S. Habisreutinger and A. Hümmer

## Clinical Course

A 38-year-old man with severe hemophilia A reported about a sudden onset of painful swelling of his left knee during a vacation in Italy. Immediate transfusion of 4000 IU of factor VIII concentrate (Recombinate, Baxter) did not leed to any relieve. Due to further swelling and pain as well as general weakness the patient decided to return to Germany on the following day. At immediate hospital admission the patient presented in a moderately reduced general condition and slightly elevated body temperature. The knee was tender and warm. Knee motion was markedly reduced due to swelling and severe pain. As factor VIII treatment did not lead to any improvement a diagnostic tab of the knee was performed showing blood and pus (Staph. areus). In spite of surgical drainage the general condition of the patient deteriorated rapidly so that intubation and intensive care treatment was necessary. As swelling of other joints such as right knee, left elbow and right ankle was noted, further surgical drainages were performed and shock treatment on swing bed as well as intravenous antibiotic treatment with Imipenem was started. During the next days machine aided respiration improved slowly so that extubation was possible after 10 days. Factor VIII concentrate (Recombinate, Baxter) was transfused 3000 IU BID and reduced to 2000 IU BID after 6 days. No bleeding occurred during the time of hospitalization.

## History and Further Outcome

The patient suffered from severe hemophilia A leading to hemarthrosis of knees, elbows and ankles. Most bleedings occurred in childhood and very seldom during the recent years so that on demand treatment with factor VIII concentrate was performed. Hepatitis C infection was treated 2001 with a 12 month period of Interferon and Ribavirin leading to a sustained viral response. HIV was negative. History prior to admission as well as clinical examination did not show any source of Staph. aureus so that the reason leading to the hereby presented clinical course is still obscure. In spite of successful management of knee empyema, pain and functional deterioration was worse in comparison to prior status so that a total knee replacement was performed one year later without any problems.

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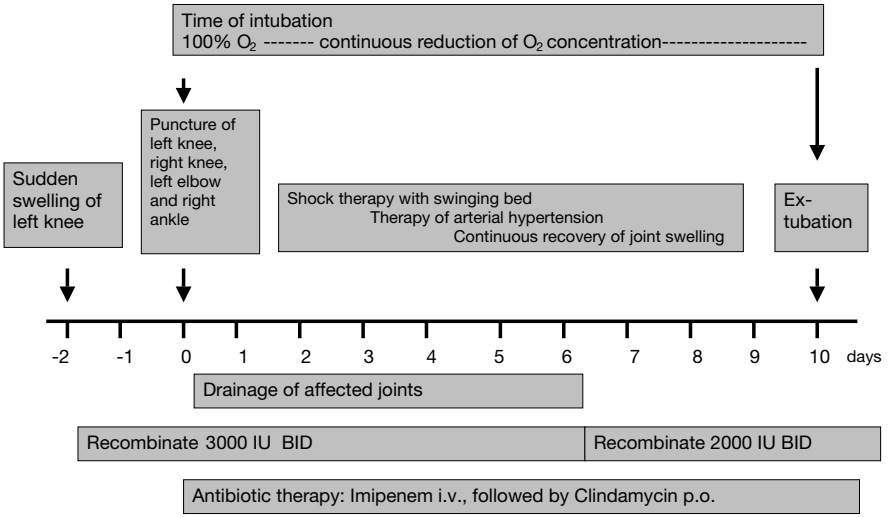


**Fig. 1.** Clinical course and therapy prior (–2 to –1) and during first 10 days of hospitalization

## Summary

Sudden swelling of joints in patients with hemophilia A may not necessarily be due to bleeding. Especially, when factor treatment does not lead to any improvement, empyema may be present. Diagnostic puncture may be necessary as well as rapid drainage. Life threatening sepsis may occur rapidly in these situations.

# The Role of VWF:Ag II in Patients with Acquired von Willebrand Disease

M. Krause, C. Caron, I. Stier-Brück, T. Vigh and I. Scharrer

## Introduction

Acquired von Willebrand syndrome (AVWS) is a rare disease, characterized by a late onset of bleeding diathesis, and absence of personal and family history of bleeding. The clinical symptoms are similar to those of congenital von Willebrand syndrome (VWS). The majority of cases of acquired von Willebrand disease are associated with myeloproliferative, lymphoproliferative or autoimmune disorders, neoplasia, drugs and agents, and other disorders. Laboratory features are associated with normal or prolonged bleeding time and/or activated partial thromboplastin time (APTT), a decreased ristocetin cofactor activity (VWF:RCo) or collagen binding activity (VWF:CBA), a reduced VWF:RCo/Ag or VWF:CBA/Ag ratio, and a reduced concentration of all the multimeric forms or selective loss of the largest multimeric form of von Willebrand factor simulating a type 1 or 2 von Willebrand disease. An additional test to characterize of acquired von Willebrand disease is the measurement of VWF:AgII.

Von Willebrand factor is a multimeric glycoprotein, that originates from the VWF precursor pro VWF and results in mature VWF and in the VWF propeptide, called VWF:AgII. Von Willebrand factor is synthesized by vascular endothelial cells and megakaryocytes. The biological functions of VWF and VWF:AgII are differing, VWF:AgII is contributed to intracellular, posttranslational multimerisation and targeting of VWF to storage vesicles. Systemic VWF propeptide levels can reflect more endothelial secretion processes than VWF levels do.

In patients with acquired von Willebrand disease, VWF is normally synthesized but rapidly removed from the circulation. Normal or increased levels of VWF:AgII compared to decreased levels of VWF:Ag show an accelerated removal of VWF:Ag from plasma. Decreased levels of VWF compared to low VWF:AgII levels might be a predictor of a decrease in VWF synthesis.

The aim of the present study was to assess the role of VWF:AgII in the diagnosis of acquired von Willebrand disease in our patients.

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## Methods

### Blood Sampling

With informed consent, blood samples were collected by peripheral vein puncture into 3.8% trisodium citrate (Sarstedt tubes, Germany). Platelet poor plasma was prepared by centrifugation at 3000 g for 40 minutes at 4°C, aliquoted in polystyrene tubes, stored at -70°C and thawed immediately before the assay.

### Assays of Coagulations Factors

**APTT** (normal range: 28–38 sec): conventional method; **FVIII:C** (normal range: 60-150%): one-stage clotting assay, ACL; **VWF:RCo** (normal range: 62-131%): turbidimetric method, BCT; **VWF:Ag** (normal range: 58-135%): in-house ELISA; **VWF:AgII** (normal values: 81+/-17%): in-home ELISA; **autoantibodies to VWF (AbVWF):** in-house ELISA [de Romeuf C, Mazurier C. Comparison between plasma von Willebrand factor (VWF) and VWF antigen II levels in normal individuals and patients with von Willebrand disease. Thromb Haemost 1998; 80:37-41]

### Other Methods

**Platelet count:** conventional methods; **Bleeding time** (normal range: <9.30 minutes): template method, Simplate; **Platelet function analyzer PFA-100,** DADE AG (normal range collagen/ADP: 71-118 seconds; collagen/epinephrine: 85-165 seconds); **Multimeric analysis of von Willebrand factor:** sodium dodecyl sulphate agarose gel electrophoresis

### Statistical Analysis

The Chi-squared test was used for group comparison and P-values. P-values less than 0.05 were considered significant. All statistical analyses were performed using the BIAS program (BIAS. Windows 7.04, Germany).

## Subjects

11 patients (female: 5/male: 6) with acquired von Willebrand syndrome, associated with Valproat replacement (n=3), essential thrombocythemia (ET; n=2), chronic lymphatic leukemia (CLL; n=1), chronic myeloid leukemia (CML; n=1), thrombotic thrombocytopenic purpura (TTP; n=1), Paget von Schroetter syndrome (n=1) and idiopathic form (n=2), were studied. At first onset, patients were aged 8-71 years. None of the patients enrolled had bleeding and family histories for the von Willebrand disease.

**Table 1.** Laboratory characteristics

| Pts. | FVIII:C (%) | vWF:Ag (%) | vWF:RCo (%) | vWF:RCo/Ag | multimeric form |
|---|---|---|---|---|---|
| 1# | 74 | 74 | 35 | 0.47 | type 1 |
| 2# | 47 | 13 | 26 | 2.00 | type 1 |
| 3# | 61 | 48 | 35 | 0.73 | type 1 |
| 4# | 21 | 20 | <5 | 0.25 | n.a. |
| 5# | 5 | <6.25 | <5 | 0.80 | type 1 |
| 6# | 61 | 51 | 64 | 1.25 | type 1 |
| 7# | 84 | 63 | 49 | 0.77 | type 1 |
| 8# | 46 | 69 | 19 | 0.27 | type 1 |
| 9# | 74 | 36 | 61 | 1.69 | n.a. |
| 10# | 95 | 61 | 43 | 0.70 | type 1 |
| 11# | 128 | 56 | 56 | 1.00 | type 1 |

Laboratory characteristics in our patients shows Table 1.

22 healthy subjects and 19 patients with inherited VWS type 1 and 13 patients with VWS type 2 constituted the control group.

## Results

The mean level of VWF:AgII (69%; range:131-47%) was increased as compared to the mean level of VWF:Ag (51%; range:74-6%) in the patients with AVWS. In contrast to the patients with inherited VWS (VWF:AgII – mean level: 64% in type 1; mean level: 81% in type 2) and the healthy subjects (VWF:AgII-level: 81%), the mean level of VWF:AgII in our study group was not different. Furthermore, in 10/11 patients with AVWS the levels of VWF:AgII were normal or increased. In only two of the patients VWF:AgII levels were decreased. The mean value of VWF:AgII/Ag ratio in the patients was 1.35 as compared to the ratio 1.63 in type 1 VWS, 2.53 in type 2 VWS and 0.76 in healthy controls. In 8/11 patients VWF:AgII/Ag ratio was about 1, and in 3 of these patients about 5 (range: >22-6.55).

We found in 3/11 patients VWF:AgII/Ag ratio lower than 1, associated with VWF:RCo levels <35% (range: 35-19%), compatible with an inherited VWS, but lack of bleeding history inspite of surgery and deliveries. None of the patients with Valproat treatment had ratio about 5 or positive autoantibodies to VWF.

In our study group we found positive autoantibodies to VWF in 3/11 patients (27%), only one of them had VWF:AgII/Ag ratio higher than 5, but in all of these patients the ratio was about 1. These patients were associated with CLL, CML or TTP.

The laboratory findings in our patients with AVWS are documented in Table 2.

**Table 2.** Laboratory results

| Pts. | vWF:AgII (%) | Ratio vWF:AgII/Ag | Anti vWF IgG/IgM |
|---|---|---|---|
| 1# | 69 | 0.93 | neg/neg |
| 2# | 113 | 8.69 | neg/pos+++ |
| 3# | 47 | 0.98 | neg/neg |
| 4# | 131 | 6.55 | neg/neg |
| 5# | 110 | >22 | neg/neg |
| 6# | 77 | 1.51 | neg/neg |
| 7# | 85 | 1.35 | neg/neg |
| 8# | 68 | 0.99 | neg/neg |
| 9# | 66 | 1.83 | neg/neg |
| 10# | 64 | 1.05 | pos+/neg |
| 11# | 61 | 1.09 | weak pos/neg |

## Discussion and Conclusions

The acquired von Willebrand disease was diagnosed in all of our patients based upon the data from an international registry of acquired von Willebrand syndrome, and the guidelines for the diagnosis of the acquired von Willebrand syndrome the Scientific and Standardization Committee Communication of the International Society on Thrombosis and Hemostasis (SSC-ISTH).

Von Willebrand factor is synthesized normally in the majority of patients with AVWS, however it is rapidly removed by pathogenic mechanisms, associated with presence of autoantibodies to VWF, adsorption of VWF and proteolytic degradation of VWF. Patients with inherited VWS shows low VWF:AgII levels, as an indicator of a decreased VWF synthesis, compared to normal or increased VWF:AgII levels in patients with AVWS. A number of studies have shown that elevated VWF:AgII levels may occur in acute phases of vascular disease, which more reflect the endothelial cell activation.

In our study group of the patients with AVWS we found normal or increased levels of VWF:AgII compared to decreased levels of VWF:Ag, which reflects an accelerated removal of VWF from the circulation. The ratio VWF:AgII/Ag of all patients was higher, but not significantly higher, as compared to the healthy controls (1.35 vs. 0.76; p=0.87). In 8/11 patients (73%) we found VWF:AgII/Ag ratio >1.0. These results are similar to the observations made by other investigators.

In the international registry of acquired von Willebrand syndrome, the prevalence of autoantibodies to VWF was 16% (27/170), that is similar to that reported in other studies. We found in our patients a strikingly high prevalence of autoantibodies (27%, 3/11). The presence of autoantibodies may be influence both the risk of bleeding and the response to treatment. In only one of our patients with antibodies bleeding episodes in case of surgery was documented. The bleeding tendency was not different in patients with autoantibodies compared to patients without auto-

antibodies, but the patients with VWF:AgII/Ag ratio upper 5 were characterized by severe bleeding events.

- In our present study of patients with acquired von Willebrand syndrome we could demonstrate, that VWF:Ag II levels, the VWF:AgII/VWF:Ag ratio and the presence of antibodies to VWF appears to be helpful in the characterization between inherited von Willebrand syndrome and acquired von Willebrand syndrome and should be performed in suspected acquired von Willebrand syndrome.
- This suggests an important role of VWF:AgII in pathogenesis, in the diagnosis and management of acquired von Willebrand syndrome.

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# Contamination of Coagulation Factor Concentrates with Human Parvovirus Genotype 2 DNA is Less Frequent than Contamination with Genotype 1 (B19) DNA

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## Introduction

Human parvovirus B19 (B19) DNA has been frequently detected in plasma-derived coagulation factor concentrates [5, 11, 14, 16]. Furthermore, transmission of B19 infection by derivatives produced from pooled plasma has been repeatedly reported thus indicating presence of infectious virus despite routine application of inactivation or virus removal procedures [1, 2, 9, 15]. However, the effectiveness against B19 of the current inactivation procedures is unclear due to physical robustness of the non-enveloped virus. Within the virus family *Parvoviridae*, parvovirus B19 belongs to the genus *Erythrovirus*.

Recently, it has been shown that the genetic diversity of the human parvoviruses is higher than previously expected [10, 12, 17; for review 7]. Therefore, human parvoviruses are now subdivided into three different genotypes, with parvovirus B19-like viruses belonging to human erythrovirus genotype 1. Up to now, human erythrovirus genotype 2 viruses have been detected in several European countries including Germany, and in the United States [10, 13, Eis-Hübinger et al., manuscript in preparation], whereas detection of genotype 3 viruses was limited to France [17]. According to current data, genotype 2 and genotype 3 virus infections cause the same spectrum of illnesses, including intrauterine infections, as »classical« B19 infections [17].

Since there is no information on the frequency of contamination with human erythrovirus genotype 2, we investigated by nested polymerase chain reaction 202 lots of coagulation factor concentrates representing 21 commercially available products for erythrovirus genotype 2 DNA and B19 (erythrovirus genotype 1) DNA. One hundred eighty-one lots (13 different products) were administered during the last three years, 21 lots (8 different products) were stored products applied until the beginning of the eighties. In case of a positive genotype 2 result, DNA sequencing analysis was performed. Viral load of genotype 1 (B19) virus was quantitatively measured by real-time polymerase chain reaction.

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## Materials and Methods

### Coagulation Factor Concentrates

In total, 202 lots of 21 commercially available plasma-derived coagulation factor products were investigated. One hundred eighty-one lots (13 different products) were currently available products, administered during the last three years (specimens collected between October 18, 2000 and February 28, 2003), 21 lots (8 different products) were stored products, administered until the beginning of the eighties, not treated by viral inactivation procedures. Details of the coagulation factor concentrates are given in Table 1 and Table 2.

### DNA Isolation and Polymerase Chain Reaction

DNA was prepared from 200 µL of reconstituted factor by spin column procedure (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Nested polymerase chain reaction (PCR) for detection of human erythrovirus genotype 1 (B19) DNA was performed as described previously [4, 6]. PCR was specific for B19-like DNA. Amplification of human erythrovirus genotype 2 DNA was performed using the primers described by Hokynar et al. (2002) [10]. PCR was carried out in volumes of 50 µl with the following concentration of reagents: 250 µM of each deoxynucleoside triphosphate (Ultrapure dNTPs, Amersham Biosciences, Freiburg, Germany), 25 pmol of each primer (Sigma-Genosys, Steinheim, Germany), 5 µl 10× PCR-buffer (Expand High Fidelity PCR System, Roche Diagnostics, Mannheim, Germany), 2 mM $MgCl_2$ (Roche Diagnostics) and 1.75 U DNA polymerase (Expand High Fidelity PCR System, Roche), and 5 µl of DNA preparation. Five microliters from the first round reaction mixture was transferred to second round reaction mixture containing the same constituents as the first round mix except for the nested primers. PCR was performed in a cycler (T3 Thermocycler, Biometra, Göttingen), for 5 min at 95 °C (initial denaturation) and for 35 cycles each cycle consisting of 10 s at 94 °C (denaturation), 10 s at 50 °C (hybridization of primers) and 20 s at 72 °C (elongation). A final elongation step followed for 3 min at 72 °C. Identical conditions were used for the first and second round amplification.

10 µl of the second-round PCR mixture were analyzed by electrophoresis on agarose composite minigels of 1.5%NuSieve GTG [FMC]/0.5%SeaKem LE [FMC](Cambrex, supplied by Biozym, Hessisch Oldendorf, Germany). Amplified products were visualized by ethidium bromide staining and UV illumination. Positive and negative controls were included in every run. For negative control, all PCR reagents and sterile bidistilled water instead of the sample was used. Strict precautions to avoid contaminations were taken.

**Table 1.** Detection of human erythrovirus genotype 1 (B19) DNA and genotype 2 DNA in presently used plasma-derived coagulation factor concentrates.

| Coagulation factor | Product (manufacturer) | Virus inactivation | I.U. | No. of lots tested | PCR results | |
|---|---|---|---|---|---|---|
| | | | | | Genotype 1 (B19) | Genotype 2 |
| | | | | | No. of positive lots (%) | |
| Factor VIII | A | S/D & dry heat 80 °C 72 h | 1000 | 35 | 7 | 0 |
| | | | 500 | 7 | 4 | 0 |
| | | | | Σ 42 | 11 | 0 |
| | B | Tween 80 & vapor heat 60 °C 10 h | 1000 | 16 | 12 | 0 |
| | | | 500 | 8 | 4 | 0 |
| | | | 250 | 4 | 0 | 0 |
| | | | | Σ 28 | 16 | 0 |
| | C | S/D & dry heat 100 °C 0.5 h | 1000 | 25 | 20 | 2 |
| | | | 500 | 2 | 0 | 0 |
| | | | | Σ 27 | 20 | 2 |
| | D | S/D | 1000 | 11 | 3 | 0 |
| | E | Pasteurization 60 °C 10 h | 1000 | 7 | 1 | 0 |
| | | | 500 | 2 | 0 | 0 |
| | | | | Σ 9 | 1 | 0 |
| | F | Pasteurization 60 °C 10 h | 1000 | 10 | 7 | 0 |
| | G | S/D & dry heat 80 °C 72 h | 1000 | 1 | 1 | 0 |
| | H | S/D & dry heat 100 °C 0.5 h | 250 | 1 | 1 | 0 |
| | | | Total | 129 | 60 (46.5%) | 2 (1.6%) |
| Factor IX | I | Tween 80 & vapor heat 60 °C 10 h, 80 °C 1 h | 1000 | 14 | 5 | 0 |
| | | | 600 | 4 | 2 | 0 |
| | | | | Σ 18 | 7 | 0 |
| | J | S/D & nanofiltration | 1000 | 10 | 4 | 0 |
| | | | 500 | 3 | 1 | 0 |
| | | | | Σ 13 | 5 | 0 |
| | K | S/D | 500 | 2 | 0 | 0 |
| | | | Total | 33 | 12 (36.4%) | 0 |
| Factor VII | L | Vapor heat 60 °C 10 h, 80 °C 1 h | 500 | 2 | 0 | 0 |
| Act. prothromb. complex conc. | M | Vapor heat 60 °C 10 h, 80 °C 1 h | 1000 | 8 | 3 | 0 |
| | | | 500 | 9 | 2 | 0 |
| | | | Total | 17 | 5 | 0 |
| All products | | | Total | 181 | 77 (42.5%) | 2 (1.1%) |

**Table 2.** Detection of human erythrovirus genotype 1 (B19) and genotype 2 DNA in coagulation factor concentrates administered until the beginning of the eighties.

| Coagulation factor | Product | I.U. | No. of lots tested | PCR results | |
|---|---|---|---|---|---|
| | | | | Genotype 1 (B19) | Genotype 2 |
| | | | | No. of positive lots (%) | |
| Factor VIII | a | 250-1100 | 8 | 6 | 0 |
| | b | 250 / 500 | 4 | 4 | 1 (in 500) |
| | c | 250 / 500 / 1000 | 4 | 2 | 0 |
| | d | 1000 | 1 | 1 | 1 |
| | e | 250 | 1 | 1 | 0 |
| | f | 1000 | 1 | 1 | 0 |
| | g | 500 | 1 | 1 | 1 |
| | h | 1000 | 1 | 1 | 0 |
| | | | Σ 21 | 17 (81%) | 3 (14%) |

**DNA Sequence Analysis**

For DNA sequence analysis, half of the genome region was amplified by nested PCR using the following genotype-specific oligonucleotide primers: For sequencing of the B19 DNA amplification regions NS1-C, ΔV, VP1/VP2 and VPC, the primers originally described by Hemauer et al. (1996) [8] were used. Additionally, primers for amplification of the genome region between VP1/VP2 and VPC were applied: outer forward 5′-ACAATGCCAGTGGAAAGGAG-3′ (nucleotide (nt) 3318-3337; all positions according to B19 prototype strain Au [18; GenBank accession no. M13178]; outer reverse 5′-CCCAGGGCGTAAGGATATT-3′ (nt 4117-4099); nested forward 5′-AAGGTTTGCACCATCAGTCC-3′ (nt 3341-3360); nested reverse 5′-TTAAGGCTTTTCCAGCTCCA-3′ (nt 4064-4045).

For amplification of human erythrovirus genotype 2 DNA the sequences of the oligonucleotide primers used for the amplification regions NS1-C, ΔV, VP1/VP2 and VPC were modified according to the sequence of the genotype 2 prototype strain LaLi [10; GenBank accession no. AY044266]. Primers for amplification of the genome region between amplification region VP1/VP2 and VPC were as follows: outer forward 5′-CAGTGGAAAAGAGGCAAAGG-3′ (nt 3174-3193; nucleotide positions according to genotype 2 prototype strain LaLi [10]); outer reverse 5′-CCAGTGATGGTATGGCTGTG-3′ (nt 3993-3974); nested forward 5′-CATAATGGGCTACTCAACACCA-3′ (nt 3210-3231); nested reverse 5′-GCGCCTGTATTGGAAGTGTC-3′ (nt 3899-3880).

The five overlapping amplicons generated by nested PCR represented homologous regions in the genomes of human erythrovirus genotype 1 and 2 (genotype 1, nt 1877-4726; genotype 2, nt 1726-4575). For DNA sequencing, nested PCR products were purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequencing reactions were carried out using approximately 5-20 ng of the purified PCR product. Amplicons from two independent PCR reactions were sequenced in forward and reverse directions using the nested primers. Sequencing was per-

formed with the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (ABI, Applied Biosystems, Weiterstadt, Germany), unincorporated dye terminators removed using SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma-Aldrich, Steinheim, Germany) and reactions were run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequencing data were manually reviewed. Alignments were generated by ClustalX 1.81. Editing was performed using BioEdit.

### Quantitative Polymerase Chain Reaction

For quantitative measurement of B19 DNA contamination a real-time PCR (LightCycler – Parvovirus B19 Quantification Kit, Roche Diagnostics, Mannheim, Germany) was carried out using a LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer´s instructions except that in addition to the Roche B19 DNA standards the International Standard for B19 DNA (1st World Health Organization International Standard 99/800 for Parvovirus B19 DNA [National Institute of Biological Standard and Control (NIBSC), London, UK]; 5 x $10^5$ IU per vial) was included in each run. The international standard was run undiluted and in 5 serial tenfold dilutions. The LightCycler Parvovirus B19 Quantification Kit (Roche) amplified B19-like (genotype 1) viruses.

### Statistical Analysis

The statistical analysis was performed using the $\chi^2$ test.

## Results

B19 (genotype 1) DNA was detected by genotype 1-specific PCR in 77 of 181 (42.5%) lots of coagulation factor concentrates used for therapy in the last three years (Table 1). Overall, the frequency of contamination was higher in factor VIII concentrates than in factor IX and activated prothrombin complex concentrates (47% vs. 36%, 29%, resp.). The highest contamination rate was found in product C (74%; factor VIII concentrate).

Analysis of the coagulation factor VIII products formerly used for therapy showed that the frequency of B19 DNA contamination was significantly higher (17/21 = 81%; $p < 0.01$) than in the currently used products (Table 2).

Quantitative real-time PCR that amplified B19 (genotype 1) DNA showed that contamination ranged from less than $10^2$ IU/ml to $10^7$ IU/ml. Sixteen percent of concentrates proved to be highly contaminated ($10^6$ to $10^7$ IU/ml). The level of B19 DNA contamination was similar in currently used and previously used factor concentrates (Fig. 1).

Genotype 2 DNA was detected by genotype 2-specific PCR in 2 of 181 (1.1%) lots of currently applied concentrates and in 3 of 21 (14%) lots of formerly applied products. Thus, the number of concentrates contaminated with genotype 2 DNA was

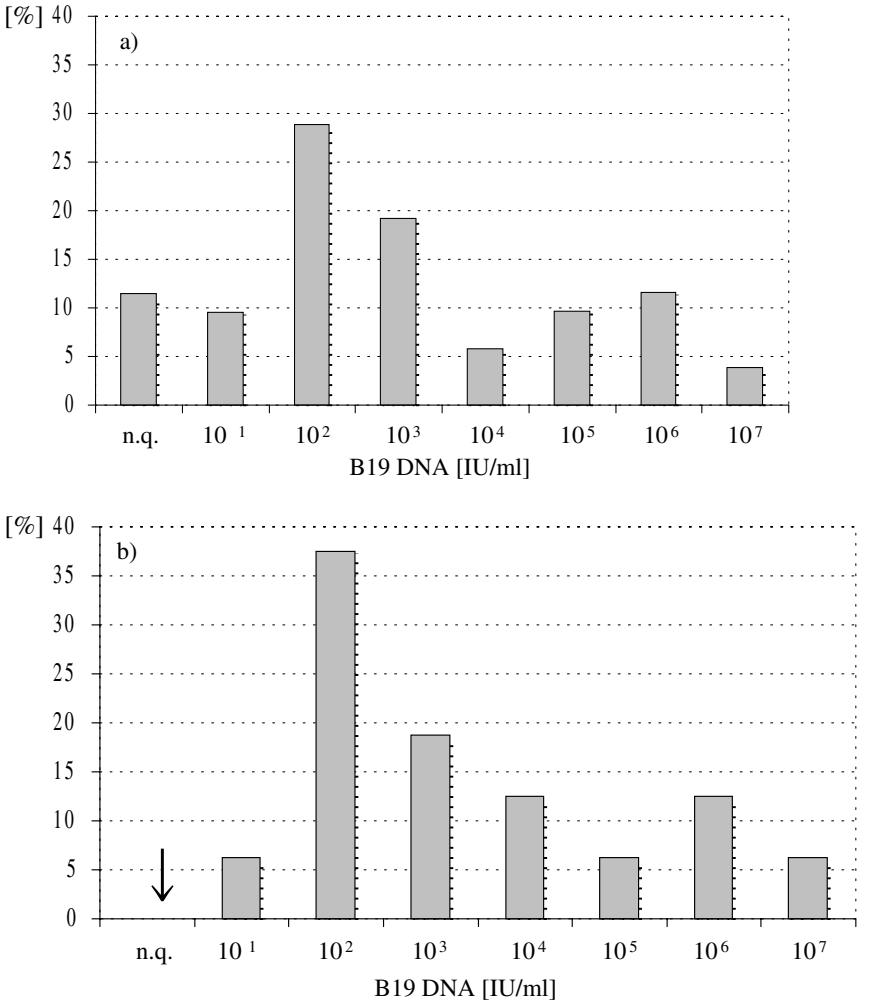


**Fig. 1.** Parvovirus B19 (genotype 1) DNA load in plasma-derived coagulation factor concentrates. Nested PCR-positive lots were quantified by real-time PCR and classified in $\log_{10}$ levels (x-fold value not considered). **a)** Results from 52 presently used lots (42 lots of factor VIII, 10 lots of factor IX). **b)** Results from 16 factor VIII lots administered until the beginning of the eighties.
n.q. = not quantifiable.

significantly less than the number of concentrates contaminated with B19 (genotype 1) DNA ($p < 0.001$). All genotype 2 DNA positive lots were co-contaminated with B19 DNA.

To verify the PCR results sequencing analysis of the five PCR-double positive concentrates was carried out. Due to the low genetic variability of parvovirus, half of the viral genome of each isolate was sequenced. Nucleotide sequence comparison


nt*
1901
Product g ·T····· ····· ····· ·····AC··· A·····A·· ·····A ····· ····· ····· ·····
Genotype 1 TTAATGCAGA TGCCCTCCAC CCAGACCTCC AAACCACCCC AATTGTCACA GACACCAGTA TCAGCAGCAG TGGTGGTGAA AGCTCTGAAG AACTCAGTGA
Genotype 2 TAAATGCAGA TGCCCTCCAC CCAGACCTCC AAACCGTCCC CATTGTCGCA GACACCAGTG TCAGCAGCAG TGGTGGTGAA AGCTCTGAAG AACTCAGTGA
Product g ····· ····· ·····GT····C·····G·· ·····G ····· ····· ····· ·····
2001
Product g ····· ··T····· ····C····· ·····C····· ····· ···G···A·· ····· ····· ·····
Genotype 1 AAGCAGCTTT TTTAACCTCA TCACCCCAGG CGCCTGGAAC ACTGAAACCC CGCGCTCTAG TACGCCCATC CCCGGGACCA GTTCAGGAGA ATCATTTGTC
Genotype 2 AAGCAGCTTT TTCAACCTCA TCACTCCAGG CGCCTGGAAC AGTGAAACCC CGCGCTCTAG TACACCCGTC CCCGGGACCA GTTCAGGAGA ATCATTTGTC
Product g ····· ··C····· ····T····· ·····G····· ····· ···A···G·· ····· ····· ·····
2101
Product g ·····**T**··· ····· ··**C**T·····T ··A····· ·A····· C·····A··T T·G····· ····· ····· ·····T·
Genotype 1 GGAAGCCCAG TTTCCTCCGA AGTTGTAGCT GCATCGTGGG AAGAAGCCTT CTACACACCT TTGGCAGACC AGTTTCGTGA ACTGTTAGTT GGGGTTGATT
Genotype 2 GGAAGCCCAG TTTCCTCCGA AGTGGTAGCC GCGTCGTGGG AGGAAGCCTT TTACACTCCA CTTGCAGACC AGTTTCGTGA ACTGTTAGTT GGGGTTGACT
Product g ····· ····· ···G···· C ··G····· ·G····· T·····T··A C·T····· ····· ····· ·····C·
2201
Product g ····· C·····A··· ··T··A···· ·G····· ···A····· ··C····· ·····**C**·· G··A····· ··C··**C**··C· ·····A··
Genotype 1 ATGTGTGGGA CGGTGTAAGG GGTTTACCTG TGTGTTGTGT GCAACATATT AACAATAGTG GGGGAGGGTT GGGACTTTGT CCCCATTGCA TTAATGTAGG
Genotype 2 ATGTGTGGGA TGGTGTGAGG GGATTGCCTG TTTGTTGTGT GCAGCATATT AATAATAGTG GGGGAGGGTT AGGCCTTTGT CCTCATTGTA TTAATGTGGG
Product g ····· T·····G··· ··A··G···· ·T····· ···G····· ··T····· ····· A··C····· ··T·····T· ·····G··
2301
Product g G····· ····· ·A····· ·····C··· ·····G···**G**····· C·····G··· ····· ····· ·C·····**T**
Genotype 1 GGCTTGGTAT AATGGATGGA AATTTCGAGA ATTTACCCCA GATTTGGTGC GATGTAGCTG CCATGTGGGA GCTTCTAATC CCTTTTCTGT GCTAACCTGC
Genotype 2 AGCTTGGTAT AATGGATGGA AGTTTCGAGA ATTTACTCCA GATTTGGTAC GGTGTAGCTG TCATGTAGGA GCTTCTAATC CCTTTTCTGT GTTAACCTGC
Product g A····· ····· ·G··**C**····· ·····T··· ·····A· ·G····· **C**·····A··· ····· ····· ·T·····
2401
Product g ····· ·**C**··**T**C···· ····· ··C·····A· ····· ··**G**····· C····· ····· ····· ···A·C···**C**
Genotype 1 AAAAAATGTG CTTACCTGTC TGGATTGCAA AGCTTTGTAG ATTATGAGTA AAAAAAGTGG CAAATGGTGG GAAAGTGATG ATAAATTTGC TAAAGCTGTG
Genotype 2 AAAAAATGTG CTTACTTGTC TGGATTGCAA AGTTTTGTGG ATTATGAGTA AAGAAAGTGG TAAATGGTGG GAAAGTGATG ATAAATTTGC TAAGGATGTG
Product g ····· ·····T···· ····· ··T·····**A**· ····· ··**A**····· **C**····· ····· ····· ···G·A**C**···
2501
Product g ···**G**····· ····**A**····· ····G····G ·····A· ····· ·····T··· ····· ·····T·· ····· ···**C**·····
Genotype 1 TATCAGCAAT TTGTGGAATT TTATGAAAAG GTTACTGGAA CAGACTTAGA GCTTATTCAA ATATTAAAAG ATCATTATAA TATTTCTTTA GATAATCCCC
Genotype 2 TATAAGCAAT TTGTAGAATT TTATAAAAAA GTTACTGGGA CAGACTTAGA GCTTATACAA ATATTAAAAG ATCATTACAA TATTTCTTTA GATAATCCCC
Product g ···A····· ····A····· ····**G**····A ·····G· ····· ·····**T**··· ····· ·**C**·····C·· ····· ·····
2601
Product g ····· ···C··T··· ····· ····· ·····A···C ·····A··· ·····T· ····· ····· ·····
Genotype 1 TAGAAAACCC ATCCTCTCTG TTTGACTTAG TTGCTCGTAT TAAAAATAAC CTTAAAAACT CTCCAGACTT ATATAGTCAT CATTTTCAAA GTCATGGACA
Genotype 2 TAGAAAACCC ATCTTCCCTG TTTGACTTAG TTGCTCGTAT TAAAAGTAAT CTTAAAGACT CTCCAGACCT ATATAGTCAT CATTTTCAAA GTCATGGACA
Product g ····· ···T··C··· ····· ····· ·····G···**C** ·····G··**A** ·····C· ····· ····· ·····

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2701|
Product g  ..........  ..........  ..........T·  ..........  ...G......  ..........  ..........  ..........  ..........  ..........
Genotype 1 GTTATCTGAC  CACCCCCATG  CCTTATCATC  CAGTAGCAGT  CATGCAGAAC  CTAGAGGAGA  AAATGCAGTA  TTATCTAGTG  AAGACTTACA  CAAGCCTGGG
Genotype 2 GTTATCTGAC  CACCCCCATG  CCTTATCACC  CAGTAGCAGT  CATACAGAAC  CTAGAGGAGA  AAATGCAGTA  TTATCTAGTG  AAGACTTACA  CAAGCCTGGG
Product g  ..........  ..........  ..........C·  ..........  ...A......  ..........  ..........  ..........  ..........  ..........

2801|
Product g  ..........G  ..........  ..........  ..........  ..........  ..........  ..........  ..........  T.........  ..........
Genotype 1 CAAGTTAGCG  TACAACTACC  CGGTACTAAC  TATGTTGGGC  CTGGCAATGA  GCTACAAGCT  GGGCCCCCGC  AAAGTGCTGT  TGACAGTGCT  GCAAGGATTC
Genotype 2 CAAGTTAGCA  TACAACTACC  CGGTACTAAC  TATGTTGGGC  CTGGCAATGA  GCTACAAGCT  GGGCCCCCGC  AAAGTGCTGT  GGACAGTGCT  GCAAGGATTC
Product g  ·····T·A··  ..........  ..........  ..........  ..........  ..........  ..........  ..........  G.........  ..........

2901|
Product g  ..........  ..........  C.........T  ..........  T.........  ..........  ..........  A··G··C···  ..........  ..........
Genotype 1 ATGACTTTAG  GTATAGCCAA  CTGGCTAAGT  TGGGAATAAA  TCCATATACT  CATTGGACTG  TAGCAGATGA  AGAGCTTTTA  AAAAATATAA  AAAATGAAAC
Genotype 2 ATGACTTTAG  GTATAGCCAA  TTGGCTAAGC  TGGGAATAAA  CCCATATACT  CATTGGACTG  TAGCAGATGA  GGAACTGTTA  AAAAATATAA  AAAATGAAAC
Product g  ·····T·A··  ..........  T.........C  ..........  T.........  ..........  ..........  A··A··G···  ..........  ..........

3001|
Product g  ..........  ........T··  ........C··  ..........  ..........  ..........  ..........  ..........  ..........  .......T···
Genotype 1 TGGGTTTCAA  GCACAAGTAG  TAAAAGACTA  CTTTACTTTA  AAAGGTGCAG  CTGCCCCTGT  GGCCCATTTT  CAAGGAAGTT  TGCCGGAAGT  TCCCGCTTAC
Genotype 2 TGGGTTTCAA  GCACAAGCAG  TAAAAGATTA  CTTTACTTTA  AAAGGTGCAG  CTGCCCCTGT  GGCCCATTTT  CAAGGAAGTT  TGCCGGAAGT  TCCCGCATAC
Product g  ·····T·A··  ........C··  ·····T····  ..........  ..........  ..........  ..........  ..........  ..........  .....A····

3101|
Product g  ..........  ....A.....  ..........  ..........  ..........  ..........  ..........  ·T·.......  TC···C····  ..........
Genotype 1 AACGCCTCAG  AAAAATACCC  AAGCATGACT  TCAGTTAATT  CTGCAGAAGC  CAGCACTGGT  GCAGGAGGGG  GGGGCAGTAA  TTCTGTCAAA  AGCATGTGGA
Genotype 2 AACGCCTCAG  AAAAGTACCC  AAGCATGACT  TCAGTTAATT  CTGCAGAAGC  CAGCACTGGT  GCAGGAGGGG  GAGGCAGTAA  CCCTGTCAAA  AGCATGTGGA
Product g  ..........  ....G.....  ..........  ..........  ..........  ..........  ..........  ·A·.......  TC·.......  ..........

3201|
Product g  ..........  ..........C·  ··C··C····  ....T.....  ..........  ..........  ..........  ..........  .....T··G·  ..........
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Genotype 2 GTGAGGGGGC  CACTTTTACT  GCCAATTCTG  TAACCTGTAC  ATTTTCCAGA  CAGTTTTTAA  TTCCATATGA  CCCAGAGCAC  CATTACAAAG  TGTTTTCTCC
Product g  ..........  ..........C·  ··C··C····  ....T.....  ......G···  ..........  ..........  ..........  .....T··A·  ..........

3301|
Product g  ......T···  ..........C·  ..........  G··G·····  ..........  ·C··T·····  ..........A  ..........C·  ..........  T·····T···
Genotype 1 CGCAGCGAGT  AGCTGCCACA  ATGCCAGTGG  AAAGGAGGCA  AAGGTTTGCA  CCATCAGTCC  CATTAATGGA  TACTCAACCC  CATGGAGATA  TTTTAGATTT
Genotype 2 CGCAGCTAGT  AGCTGCCATA  ATGCCAGTGG  AAAAGAGGCA  AAGGTTTGCA  CTATTAGTCC  CATTAATGGC  TACTCAACAC  CATGGAGATA  CTTAGACTTT
Product g  ·····T··C·  ..........T·  ..........  ···A·····  ··A·......  ·T··T·····  ..........C  ·........G·  ..........  C·····C···

3401|
Product g  ..........  .T·.......  ..........  ··G·····G·  ·C·.......  ..........  .........T·  ..........  ......A··T  .........A·
Genotype 1 AATGCTTTAA  ATTTATTTTT  TTCACCTTTA  GAGTTTCAGC  ACTTAATTGA  AAATTATGGA  AGTATAGCTC  CTGATGCTTT  AACTGTAACC  ATATCAGAAA
Genotype 2 AATGCTTTAA  ACTTATTTTT  TTCACCTTTA  GAATTTCAAC  ATTTAATTGA  AAATTATGGA  AGTATAGCCC  CTGATGCTTT  AACTGTTACC  ATATCAGAGA
Product g  ..........  ·C·.......  ..........  ··A·····G·  ·T·.......  ..........  .........C·  ..........  ......T···  ........A·
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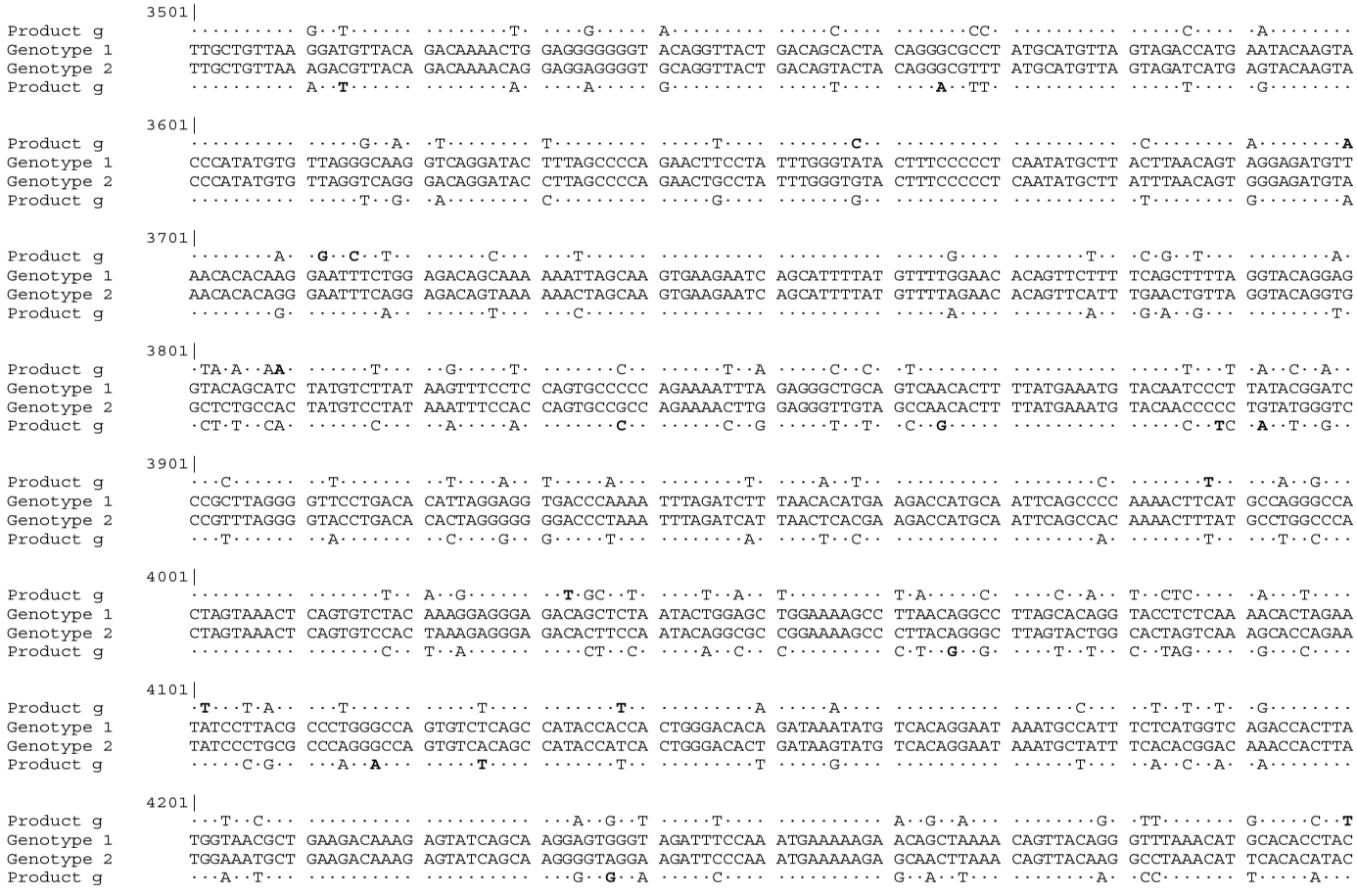
3501
Product g ·····
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Genotype 2 TTGCTGTTAA AGACGTTACA GACAAAACAG GAGGAGGGGT GCAGGTTACT GACAGTACTA CAGGGCGTTT ATGCATGTTA GTAGATCATG AGTACAAGTA
Product g ·····
3601
Product g ·····
Genotype 1 CCCATATGTG TTAGGGCAAG GTCAGGATAC TTTAGCCCCA GAACTTCCTA TTTGGGTATA CTTTCCCCCT CAATATGCTT ACTTAACAGT AGGAGATGTT
Genotype 2 CCCATATGTG TTAGGTCAGG GACAGGATAC CTTAGCCCCA GAACTGCCTA TTTGGGTGTA CTTTCCCCCT CAATATGCTT ATTTAACAGT GGGAGATGTA
Product g ·····
3701
Product g ·····
Genotype 1 AACACACAAG GAATTTCTGG AGACAGCAAA AAATTAGCAA GTGAAGAATC AGCATTTTAT GTTTTGGAAC ACAGTTCTTT TCAGCTTTTA GGTACAGGAG
Genotype 2 AACACACAGG GAATTTCAGG AGACAGTAAA AAACTAGCAA GTGAAGAATC AGCATTTTAT GTTTTAGAAC ACAGTTCATT TGAACTGTTA GGTACAGGTG
Product g ·····
3801
Product g ·····
Genotype 1 GTACAGCATC TATGTCTTAT AAGTTTCCTC CAGTGCCCCC AGAAAATTTA GAGGGCTGCA GTCAACACTT TTATGAAATG TACAATCCCT TATACGGATC
Genotype 2 GCTCTGCCAC TATGTCCTAT AAATTTCCAC CAGTGCCGCC AGAAAACTTG GAGGGTTGTA GCCAACACTT TTATGAAATG TACAACCCCC TGTATGGGTC
Product g ·····
3901
Product g ·····
Genotype 1 CCGCTTAGGG GTTCCTGACA CATTAGGAGG TGACCCAAAA TTTAGATCTT TAACACATGA AGACCATGCA ATTCAGCCCC AAAACTTCAT GCCAGGGCCA
Genotype 2 CCGTTTAGGG GTACCTGACA CACTAGGGGG GGACCCTAAA TTTAGATCAT TAACTCACGA AGACCATGCA ATTCAGCCAC AAAACTTTAT GCCTGGCCCA
Product g ·····
4001
Product g ·····
Genotype 1 CTAGTAAACT CAGTGTCTAC AAAGGAGGGA GACAGCTCTA ATACTGGAGC TGGAAAAGCC TTAACAGGCC TTAGCACAGG TACCTCTCAA AACACTAGAA
Genotype 2 CTAGTAAACT CAGTGTCCAC TAAAGAGGGA GACACTTCCA ATACAGGCGC CGGAAAAGCC CTTACAGGGC TTAGTACTGG CACTAGTCAA AGCACCAGAA
Product g ·····
4101
Product g ·····
Genotype 1 TATCCTTACG CCCTGGGCCA GTGTCTCAGC CATACCACCA CTGGGACACA GATAAATATG TCACAGGAAT AAATGCCATT TCTCATGGTC AGACCACTTA
Genotype 2 TATCCCTGCG CCCAGGGCCA GTGTCACAGC CATACCATCA CTGGGACACT GATAAGTATG TCACAGGAAT AAATGCTATT TCACACGGAC AAACCACTTA
Product g ·····
4201
Product g ·····
Genotype 1 TGGTAACGCT GAAGACAAAG AGTATCAGCA AGGAGTGGGT AGATTTCCAA ATGAAAAAGA ACAGCTAAAA CAGTTACAGG GTTTAAACAT GCACACCTAC
Genotype 2 TGGAAATGCT GAAGACAAAG AGTATCAGCA AGGGGTAGGA AGATTCCCAA ATGAAAAAGA GCAACTTAAA CAGTTACAAG GCCTAAACAT TCACACATAC
Product g ·····

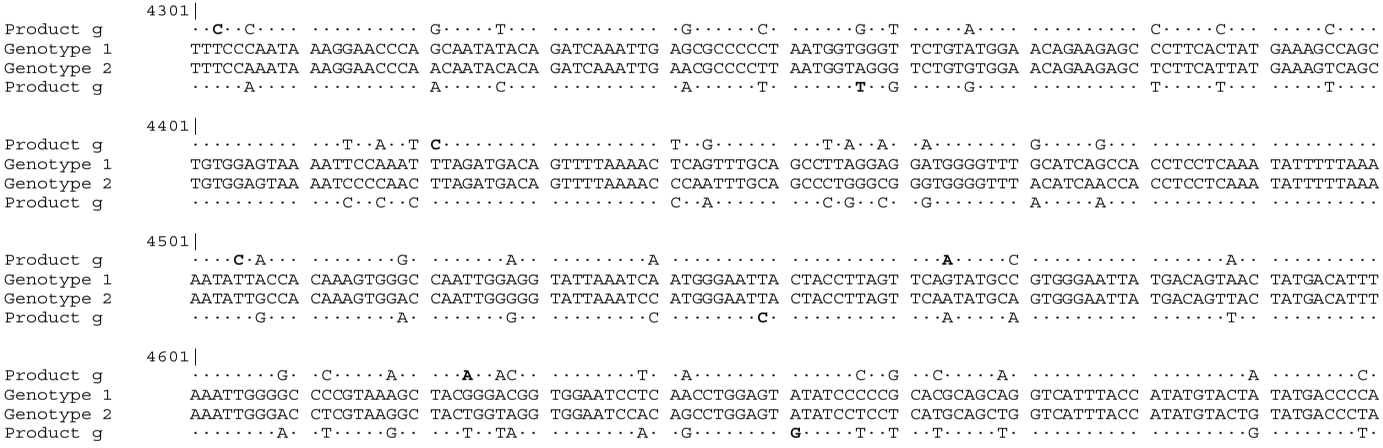


**Fig. 2.** Nucleotide sequence alignment of human erythrovirus genotype 1 (B19) DNA (strain Au; GenBank accession no. M13178), genotype 2 DNA (strain LaLi; GenBank accession no. AY044266) and 2 isolates from a coagulation factor VIII concentrate (PCR double-positive product g, Table 2). Dots indicate nucleotides that are identical to both reference sequences. Bold letters specify nucleotides different from the prototype nucleotides. * = Nucleotide (nt) numbering of the prototype sequences is based on Shade et al. [18].

was performed with B19 (genotype 1) prototype strain Au [18; GenBank accession no. M13178] and genotype 2 prototype strain LaLi [10; GenBank accession no. AY044266].

Sequencing analysis revealed that each of the five PCR-double positive concentrates contained both typical genotype 1 and genotype 2 DNA. Figure 2 shows, exemplarily, the alignment of the nucleotide sequences of the two parvovirus isolates detected in one lot (product g, Table 2).

## Discussion

The results show that human erythrovirus genotype 2 DNA is detected in coagulation factor concentrates much less frequent than genotype 1 DNA. In the presently used coagulation factors the detection rate for genotype 2 was 1.1% whereas the rate for genotype 1 was 42.5%. In old products used until the beginning of the eighties the frequency rate for genotype 2 was 14% and 81% for genotype 1, respectively. Although the number of old products investigated was rather small the differences in the contamination frequency between genotype 1 and 2 were highly significant ($p < 0.001$).

There is only one report in the literature describing the genotype 2 virus prevalence in human blood. Nguyen et al. [13] tested by PCR 62 plasma pools each derived from plasma from 2000 Danish voluntary blood donors. No genotype 2 viremic pool was detected in this study. In contrast, screening of the plasma pools for B19 identified 40 pools (65%) containing B19 DNA. Furthermore, among 207 serum samples submitted to the NIH specifically for testing for B19 between 1991 and 2001, only one sample collected from an Italian HIV-positive patient with chronic anemia tested positive for genotype 2 DNA. This low detection rate of human erythrovirus genotype 2 in blood donations would be in agreement with the low frequency of the viral DNA in factor concentrates prepared from pooled plasma measured in the present study.

On the other hand, we and others have shown that genotype 2 DNA is present in human tissue in a relatively high proportion [10, 20, Eis-Hübinger et al., manuscript in preparation]. Using PCR Hokynar et al. [10] detected genotype 2 DNA in 9 out of 19 (47%) human skin samples collected from B19 seropositive individuals. Furthermore, genotype 2 DNA was found in 5 of 83 (6%) livers from patients with fulminant hepatitis or hepatitis-associated aplastic anemia [20], and in 27 of 88 (31%) liver specimens collected from randomly selected adults undergoing liver transplantation or liver biopsy or from autopsied individuals [Eis-Hübinger et al., manuscript in preparation]. These findings indicate that genotype 2 virus can persist in human tissue similar to genotype 1 virus [3, 6, 19].

Thus, according to the present data is seems that long-term presence in specific tissues is a common phenomenon of both genotype 1 (B19) or genotype 2 virus but the characteristics of the viraemic phase of infection are highly different between the two genotypes. Further studies are needed to address whether the low detection rate of genotype 2 virus DNA in coagulation factor concentrates reflects differences in the duration and magnitude of genotype 2 viremia or other reason

have to be considered such as a different behavior during the viral inactivation procedures. The results of these studies can contribute to efforts to improve viral safety of blood-derived therapeutics.

## Conclusion

Human parvovirus genotype 1 (B19-like) DNA was frequently present in coagulation factor VIII concentrates used until the beginning of the eighties (contamination rate 81%). In concentrates presently used for replacement therapy, overall genotype 1 DNA contamination was about 42.5%. The level of viral load was similar in formerly used and presently used concentrates.

Genotype 2 DNA was detected much less frequently in coagulation factor concentrates than genotype 1 DNA. Overall, 2.5% of the lots proved to be contaminated. In the present study, all genotype 2 DNA-contaminated lots were also genotype 1-contaminated.

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# The Hemophilia Assistant in Germany

Hemophilia Assistant of the German Hemophilia Centers

## Introduction

At hemophilia treatment centers, the Hemophilia Assistant plays a central role as the most important non-medical team member. The Hemophilia Assistant supports the attending physicians in their work at the hemophilia treatment center and acts as a link between the physician and the patient and his family. For the patients, the Hemophilia Assistant is something of a confidante who helps them in their everyday life in case of any problems in connection with their hemophilia. One major goal is to give the patients as much support as possible.

## Required Qualifications of the Hemophilia Assistant

- Registered nurse/MTA/physician assistant or similar
- Interest in the tasks of a Hemophilia Assistant
- Commitment to the well-being of the hemophilia patients
- Flexibility
- Team player
- Willingness for further education

## Role and Tasks of the Hemophilia Assistant

The main role includes the follow-up discussion of the issues addresses in the first doctor-patient meeting and practical training of the patient and/or his family in physician-controlled self-treatment.

The following tasks may vary depending on the responsible physician and the availability of other team members at the hemophilia treatment center.

A. Data collection and collaboration in keeping the patient records
B. Participation or cooperation in studies and research projects
C. Interest in own further education
D. Cooperation in the certification of the hemophilia treatment center
E. Patient education on general hemophilia issues:
   1. Basic information
      - Description of hemophilia as a chronic hereditary disease – illustration of blood clotting and discussion of general aspects of hemophilia

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- Type of hemophilia, severity, (inhibitors)
- Basics of factor substitution including the preparations available
- Bleedings to be expected – so-called spontaneous bleeds, slight and severe injuries
- Bleedings into joints and muscles – the most serious injuries, with the possibility of permanent damage
- Importance of substitution as early as possible and at an adequate degree in the event of bleeding
- Possibility of side effects during or after administration of clotting factors
- Management of any pain that may occur
- Special aspects pertaining to hemophilia:
  - Medications that must be avoided
  - Safety precautions for children
  - Appropriate sporting activities in order to build up the muscular system as a means to prevent joint and muscle bleeds, which may result in lasting damage
  - Dental care
  - Control of vaccine titers (point out the importance of vaccine titers)

2. Information on bleeding sites and the possible consequences
   - Explanation of hemophilic arthropathies
   - Explanation of the different types of muscle bleeds (e.g. iliopsoas bleeds, pseudo-tumor etc.)
   - Signs and symptoms
   - Cerebral hemorrhage
   - Kidneys
   - Oropharynx
   - Gastrointestinal tract
   - Soft tissues
   - Other

F. Treatment related tasks

1. Focal points
   - Coordination and conduction of blood collection
   - Assistance during infusion of the factor concentrates
   - Explanation of the use of other medications
   - Explanation of measures in order to improve the quality of life
   - Support of the physician during treatment and documentation
   - Referral to other qualified team members
2. Physician-controlled home treatment
   - Training and further education of the patients
     - Detection of bleedings and their treatment by individual dosing
     - Technical aspects: sterility, reconstitution, injection technique, disposal, Storage
     - Documentation of bleeding events and their treatment including batch recording
   - Distribution of the clotting factors and monitoring their consumption

3. Follow-up treatments
   - Ensuring appropriate follow-up examinations and treatment at the center for all patients and – if required – their families
   - If required – arranging for treatment in local institutions
   - Maintenance of regular contact between patients and/or their families and the center
   - Encouraging patients to lead a normal life (school, professional training, leisure time, recreation, appropriate sporting activities)
   - Early notification of the attending physician about stress factors and any family issues in order to be able to offer appropriate support and solutions
   - Information about self-help groups
4. Genetics
   - Basics of heredity
   - Carrier status
   - Possibilities of genetic counseling

G. Function as link
   - Physicians at the hemophilia treatment center
   - Other team members such as orthopedist, psychologist, dentist, physiotherapist, etc.
   - Patient/family
   - Society
     - General practitioners/specialists
     - School/nursery school/employer
     - Local health authorities
     - Regular contact and exchange with other hemophilia treatment centers
     - Relationship with Hemophilia Associations (e.g. German Hemophilia Society [DHG], WFH)

# »Need« in Hemophilia A – a Qualitative Study

K. Hespe-Jungesblut, M. von Depka Prondzinski, T. Schöpke and A. Dörries

## Introduction

Although the German health care system is based on the idea that the patient ought to receive everything that is »needed«[1] in her or his situation (SGB V), this »medical need«[2] is not further defined anywhere. The question arises whether some medical services are »more needed« than others and how these different degrees of »need« can be described. It is also important to find out whether different groups (patients, doctors, nursing staff and the patients' relatives) differ in their evaluation of »medical need«. The following pilot study analyses the different perspectives of this questions for professional experts, patients, and their relatives in the case of the hemophilia A disease.

## Method

Qualitative interviews were carried out with patients of different degrees of quality of life (n=8), relatives of hemophiliacs (n=3), as well as doctors (n=3) and nursing staff (n=3) working with hemophilia A patients. The interviews were about »medical need« in the medical care of hemophilia A; the evaluation took place in 14 preposition listed categories.

## Results

### Judgement about Hemophilia A Disease

For all interview partners hemophilia A is a disease which can be treated quite well and therefore enables the patients to live a good life. Although hemophilia A is often associated with limitations in movement, pain and a permanent affinity to bleedings, the accompanying diseases (HIV/Aids, Hepatitis) are much more cumbersome to the individuals than Hemophilia A itself.

[1] In German: »notwendig«
[2] In German: »medizinische Notwendigkeit«

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Most patients state that they have come to terms with their disease and try to be as little limited as possible by it. It was often expressed that the patient lives a »normal life«. The patients do not avoid all risks, but most of them weight out the advantages and disadvantages for each single situation.

The patients' relatives sometimes feel even more burdened by the hemophilia A disease than the patients themselves.

**Treatment of Hemophilia A and Treatment Goals**

For all interview partners, the substitution of factor VIII is the most important medical service for hemophiliacs. Three persons named a service that they require »not necessary«: A physician considered cosmetic surgery for hemophiliacs »not necessary«, and one doctor and one nurse listed the extra supply of factor concentrate for exclusive hobbies like soccer or motor cross.

Most individuals demand all available medical services as »needed« that help them live a »normal life«. Most of the nursing staff, relatives and patients wish that anything that can be normalized should be treated until normality is reached. There is a tendency among physicians to accept a »sub-optimal« treatment at some point of the treatment. One physician for example accepts a good pain therapy instead of joint surgery in some cases.

**What is »Medically Needed«?**

Physicians assume that everything is needed which enables the patient to live a human life, a »normal« life and a life with no harm or pain but activity and autonomy.

Nursing staff claim that everything is needed which allows the patients to live a »normal« life, a life with a good quality of life, or »generally everything«.

The relatives of hemophilia A-patients and the patients themselves show great hesitation in answering the questions. While the relatives can not or do not want to say what is medically needed from their point of view, the patients' answers ranged from a life with dignity to everything that improves the patient's situation.

**Intensive Sports for Hemophiliacs**

Except for three persons, all interview partners refuse to bear the costs for extra factor concentrates via the solidarity-funded health insurance for those who practice sports associated with a high risk of injury to hemophilia A-patients (e.g. cross-biking, sky-diving). They expect some solidarity from hemophilia A-patients and think that a patient can be required to refrain from such dangerous sports. However, two relatives and one patient expect the costs to be borne by the health insurance. The argument is that hemophiliacs do not stand a chance of any other form of insurance cover for themselves in order to live their lives the way they want to; therefore the solidarity-funded health insurance should be required to pay.

### Who Should Define »Medical Need«?

With one exception, all interview partners prefer a decision-making between two or more expert groups to define what is »needed« in health care and therefore should be financed by the solidarity-funded health insurance. Physicians are the group mostly mentioned to participate in the decision-making process, and physicians together with patients are the most desirable combination of decision makers. All patients list the physicians' group in first place whereas all relatives of hemophiliacs name the patient group as first on the list. While some individuals would like some participation of members from the health insurance, politics, the churches, or ethics organizations to define »medical need«, others refuse any participation of such groups. Politicians are the group mentioned most as being inappropriate to define »medical need«.

## Discussion

Our question is how the criteria of »need« can be used to allocate medical resources and who should define the »medical need« paid for by the solidarity financed health insurance. In Germany the *Gemeinsame Bundesausschuss der Ärzte und Krankenkassen* is supposed to prove among other things the medical need of new methods of examination and treatment. This is done based on relevance to the medical problem, the prevalence of the disease, the natural course of the diseases and the diagnostic and therapeutic alternatives. In our study, we talked to representatives of physicians, nursing stuff, patients and relatives to find out how they judge »medical need« associated with the disease of hemophilia A.

We found out that the interview partners' expectations towards the medical treatment for hemophilia A-patients and the financing of this treatment is very high. The interview partners do not list any treatment that is not needed in the therapy of hemophilia A and only three persons characterize a situation in which a certain treatment seems exaggerated to them. This observation might be explained by the fact that thanks to the progress in treatment in the last decades hemophilia A has turned from a crippling disease granting patients only a shortened life expectancy to a disease allowing most patients to live long with a high quality of life.

The interviews show that the categorical term of »need«, which describes what is needed to reach a certain goal, can be insufficient to describe different levels of need or to describe whether one thing is more needed than others. Instead the term of »comparative need« might be an adequate criteria to describe medical services within the disease of hemophilia A. It needs to be proved if the term of »comparative need« is an adequate criterion to describe medical services within the hemophilia A disease and among different diseases. Furthermore the question could be asked if »medical need« is an adequate criteria to describe a medical service that has to be paid for by health insurance after all.

Concerning the question who should define »medical need« the interviews showed that almost all individuals want two or more expert groups to jointly define it. While there is major agreement about physicians participating in this process, the

participation of patient-groups, politicians and representatives of health insurances is discussed very controversially. Representatives of physicians and patient are the most preferred combination.

## Summary

Qualitative interviews about »medical need« were held with hemophilia A-patients, relatives of patients, doctors and nursing staff. In the interviews divergent answers in the interviews could be found to the question what the individuals claim to be »medically needed«, while approximate agreement could be reached for most other items of the interview:

- The hemophilia A disease is a disease that allows the patients to live a »good« life.
- Medical treatment is claimed to be »needed« to bring the patients' life to a normal standard. This implies that everything that can be done should be done.
- Extra factor concentrate for hemophiliacs to exercise intensive sports is refused by most interview partners.
- »Medical need« should be defined by two or more expert groups. The physicians should be involved in the decision.
- The interviews showed the lack of general agreement on a definition of »medical need« if the term is supposed to limit medical services.
- The high expectations towards medical treatment show the importance of an open public discussion about the medical budget itself and resource allocation within it and in general.

# Fit for Life – Fitness Levels of Young Hemophiliacs Today

A. Seuser, A. Kurme, E. Trunz, P. Böhm, S. Ochs, H.-H. Brackmann and T. Wallny

## Introduction

Hemophilic children nowadays lead a life largely unbothered by bleeding events. This multicenter study was conducted to investigate whether this correlates with a general fitness level corresponding to that of their age-matched non-hemophiliac peers.

## Material and Methods

101 youngsters aged 8 to 25 (mean 13.3 years) underwent a 5-stage fitness check at six different investigational sites.

The 5-stage fitness check comprises a coordination measurement element measuring the excursion of a two-dimensional mobile floor platform (Posturomed) in millimeters over a period of 30 seconds with the subject standing on one leg. Flexibility testing analyzes chest muscles and ischiocrural (hamstring) muscle using an electronic inclinometer. Muscle strength is measured by isometry of the abdominal and back muscles, for each set of muscles on its own and in relation to the other.

The endurance test is done on an exercise bike. Resting heart rate and frequency of training are entered as baseline parameters. This test is based on validated IPN endurance testing which produces an individual result in four to six minutes.

Test element 5 is infrared body fat analysis on the anterior biceps of the dominant hand. This is the site with the best correlation to hydrostatic weighing.

All tests are normalized on the basis of age, gender, height, weight, our own parallel measurements and height and weight percentiles taken from the literature. The results are rated by comparing the specific readings with the relevant age-matched means. Scores are given on a scale of 1 to 5.

## Results

The best result (average score of 3.1) was obtained for endurance (Fig. 1). The frequency of above-average results (33%) was highest in this category. However, the range of variation was very high, at 43%.

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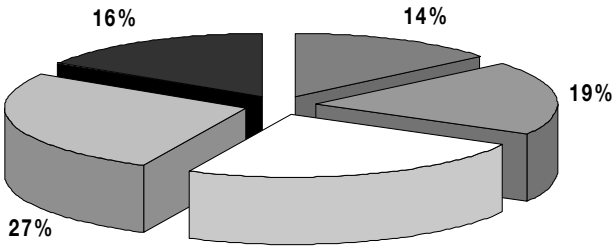


**Fig. 1.** Results endurance test

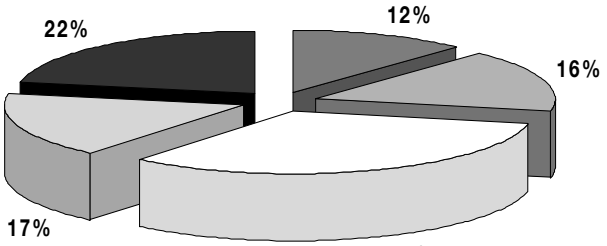


**Fig. 2.** Results body fat

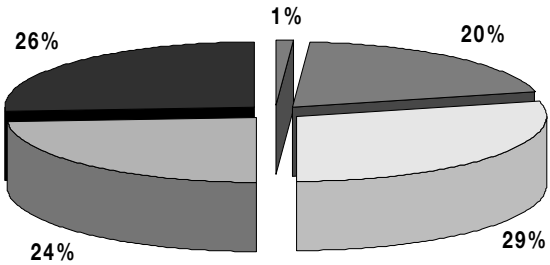


**Fig. 3.** Results coordination

In the body fat category (Fig. 2), young hemophiliacs match their peers with a tendency toward mild obesity.

Coordination is the most important fitness marker for hemophiliacs (Fig. 3). The overall score of 2.5 in this category is well below average. This category also contained the largest percentage of subjects (26%) scoring just one out of 5 points. Half of the total number of hemophilic subjects scored below the age-matched average and only 1% achieved full marks.

Upper body strength (Fig. 4, A-D) came in just behind coordination, occupying the second last place with a score of 2.4. Although there were fewer total failures, a larger percentage (68%) of subjects scored below average.

Detailed analysis of the extensor of the back and abdominal muscle shows an imbalance between underdeveloped abdominal muscle and stronger back muscle, resulting in below-average scores in terms of trunk strength ratio in two thirds of the youngsters. Depending on age, the ratio of abdominal to back muscle should

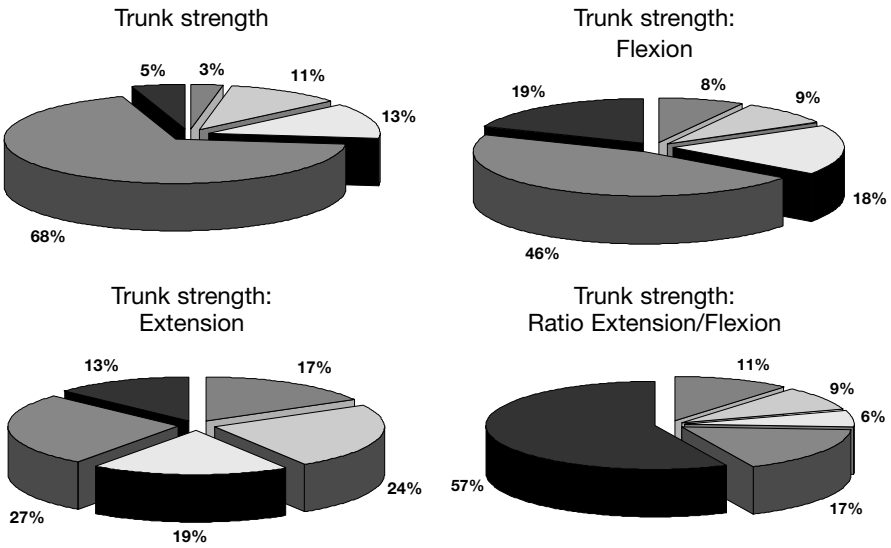


**Fig. 4.** Results trunk strength

range from approximately 100 to 130 for an 18-year-old to 100 to 150 for an 8-year-old in order to ensure optimum balance and stability of the lumbar spine.

The least developed athletic ability among young hemophiliacs is flexibility (Fig. 5, A-C). More than 2/3 of the youngsters tested scored below the age-matched average. Poor hamstring flexibility is to blame.

Only 13% of the young people tested scored as high as their non-hemophilic peers. The result is so striking as to be virtually pathognomonic.

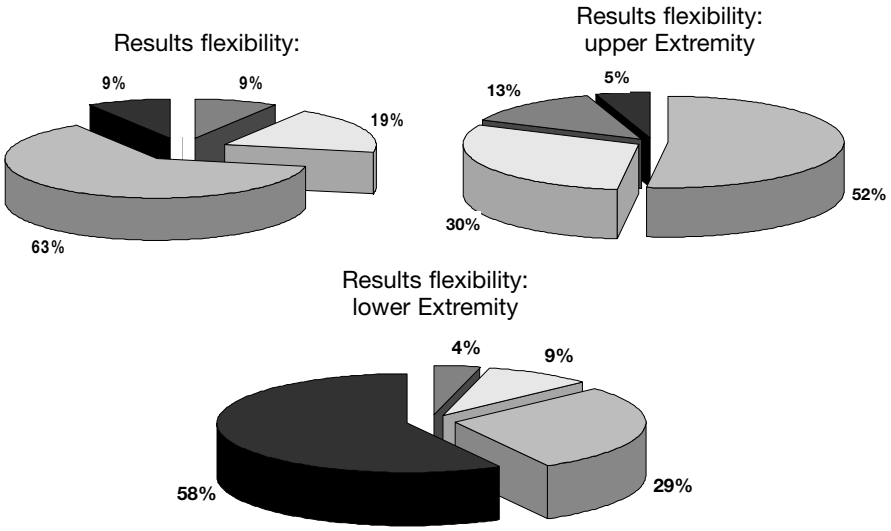


**Fig. 5.** Results flexibility

## Fitness Profile

The fitness profile or hemophilia-specific weighted average result from the five fitness tests (Figs. 6, 7) illustrates very clearly that half of the young hemophiliacs scored below the age-matched average. The mean score was 2.6.

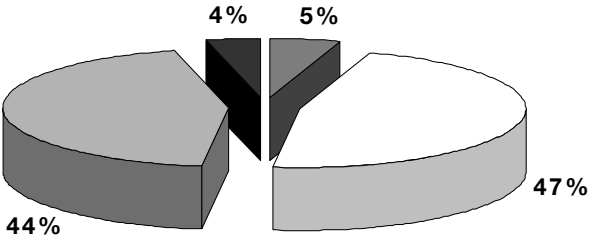


**Fig. 6.** Fitness profile

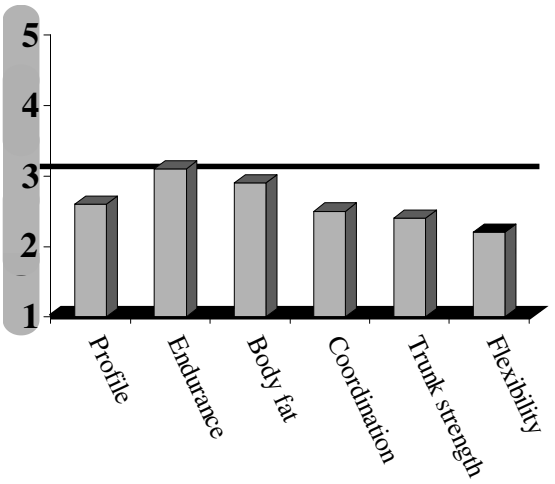


**Fig. 7.** Total results

## Discussion

This study provides entirely new facts which may help to normalize life for young hemophiliacs. Each individual subject is given an exercise program based on his or her individual fitness profile that is designed to improve individual weaknesses, build on existing strengths, and thus improve the overall profile. Many of these exercises are very simple and easily integrated into the subject's normal daily routine. Abdominal muscle exercises should be done to prevent back pain and offset the existing imbalance. Failure may result in more rapid degeneration of the spine and a higher energy requirement for activity of any kind, as well as a loss of coordination of the upper and lower extremities.

To address hemophilia as a disease, the main emphasis should be placed on training coordination and improving the mobility of the flexor muscles of the leg.

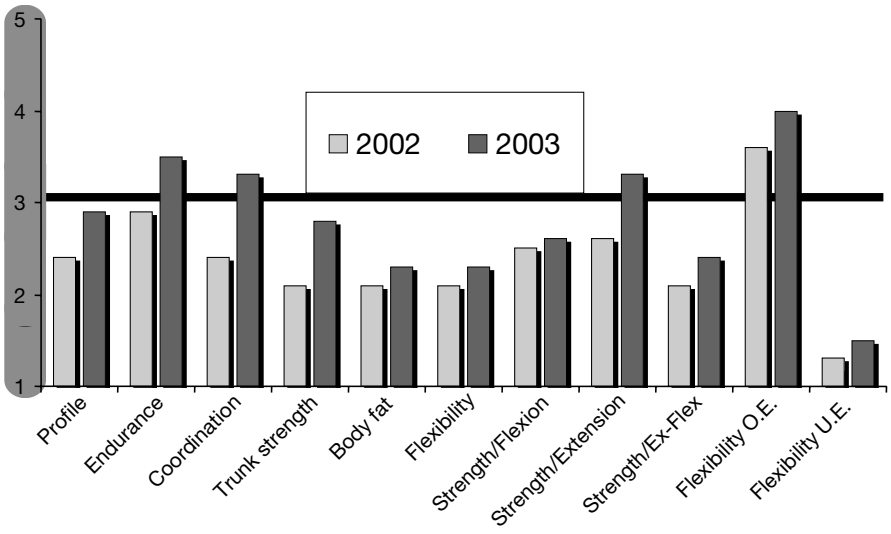


**Fig. 8.** Results retest

Shortening of the flexor muscles of the lower extremity is a stimulus-response driven process. Lesions in the knee joint (which may be subclinical) produce monotonous response mechanisms, resulting in weakening of the vastus medialis and shortening of the flexor muscles of the leg. There are two explanations for the average score of 1.6 for mobility of the lower extremity:

1. Modern prophylactic therapy is not enough to prevent such processes.
2. Young hemophiliacs get too little mobility training; any training they do receive is too unspecific.

The fact that individual fitness can improve if subjects are sufficiently motivated is shown by the retest done at one investigating site exactly one year on (Fig. 8), where the 8 retested subjects showed that they had improved their overall profile by 20%. Disproportionately high improvement was achieved in the categories coordination, endurance and upper body strength. Any improvement in mobility of the lower extremities was minor.

## Conclusion

Hemophilia is a condition with long-term sequelae. Not all of its subtle long-term effects on the musculoskeletal system are known. We need more data for greater statistical power. More specific treatment programs and more incentives are required in order to give young hemophiliacs more motivation to work on their fitness. A nationwide comparison of centers, like a fitness Olympics, might be a way of bringing dynamism to the fitness promotion campaign. Fit children are more confident in their movements, less likely to fall and may be less likely to have serious bleeds.

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# The Austrian »Haemofit-Program« – a two Years work-out Experience of People with Hemophilia (PwH)

H.K. Hartl, T. Kienbacher, H. Ramschak, U. Eidher, M. Fischill and P. Frigo

## Background

In early 2000 we developed a concept for a work-out program for adolescent and adult Persons with Hemophilia (PwH) at the »Arnold Schwarzenegger-Fitness Center«, a professional, public work-out studio in Graz, Styria. The model was based on the experiences of a pilot project »Haemofit« in Vienna (Fig. 1), where an excellent equiped out patient-center for physiotherapy and rehabilitation with experienced staff was chosen as location. As the interest decreased over a period of 18 months the project was stopped and, after a local group of members of the Austrian Haemophilia Society was interested to start the program, reorganized in the (smaller) city of Graz, but not in a medical-care-unit.

After a parallel survey in the patient organization »Österreichische Hämophilie Gesellschaft« it turned out very clear, that there is a very impressive wish to train

**Fig. 1**

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together with other PwH, 90% of the responses. Nevertheless most of the patients do not workout at all, but not because of lack of time or bleeding complications; 30% specify joint problems.

50% would like to be coached at exercising professionally and only 10% expect more joint bleedings.

## Patients and Methods

The mean age of the ten patients was 36.4 years, range 22 to 63. The mean bodyweight was 63.5 kg. 5 patients were diagnosed HIV-Ab positive, 7 patients suffer from severe hemophilia A, 2 from severe hemophilia B and all patients were on prophylactic therapy at the beginning, the therapy was again changed to treatment-on-demand, if the bleeding frequency did not increase or the patient wished to do so.

4 patients were married, 4 were single; 3 patients were students, 2 white collar worker and 2 retired.

After physical examination at the local hemophilia Center and QoL-survey (SF-36) the PwH was allowed to workout according to a individualized training program in the fitness center (Fig. 2) according to the special needs once or twice a week for free (Fig. 3). The training program of each participant was controlled, supported and evaluated by physiotherapists and a hemophilia specialist for a period of at least one year. One trainer and one dedicated, work-out experienced PwH accompanied the PwH during the whole period. The QoL-survey as well as the physical examination was repeated in 6–12 months intervals, to document the mobility ranges of the afflicted joints.

**Fig. 2**

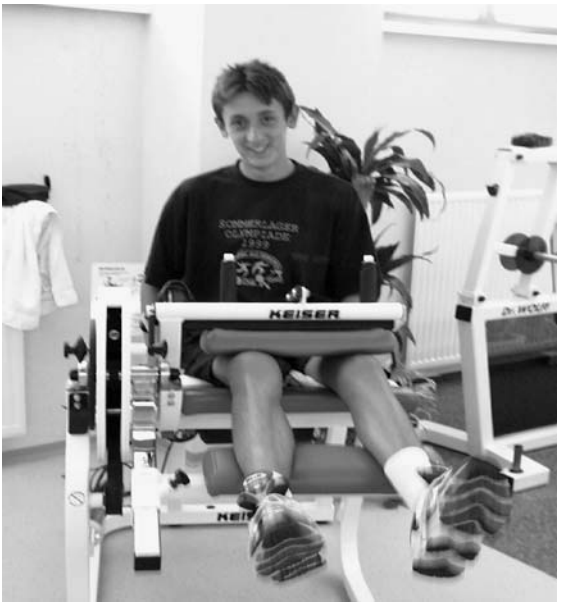


Fig. 3

## Results

Due to the small number of the evaluated group of ten PwH we concentrated only at range of motion, muscle strength, self perceived quality of life and clotting-factor consumption.

Only muscle strength was significantly improved (Fig. 4); motility was improved over the period (Fig. 5), quality of life increased and clotting factor consumption as

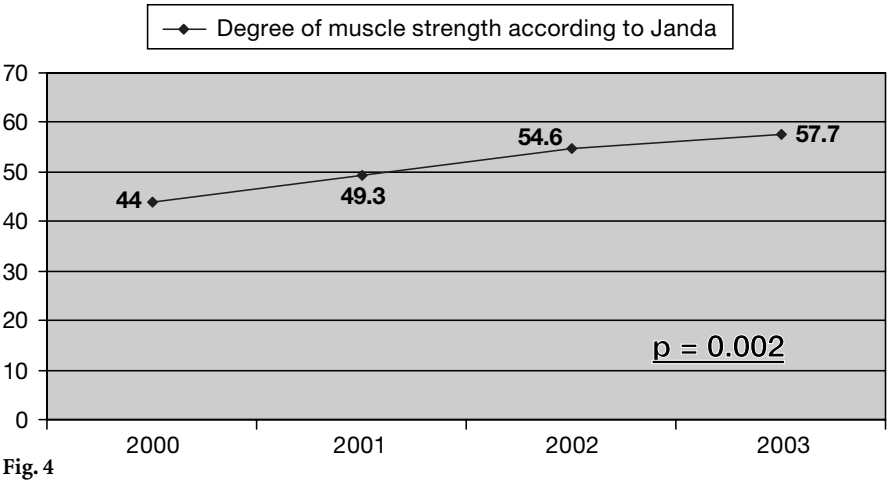


Fig. 4

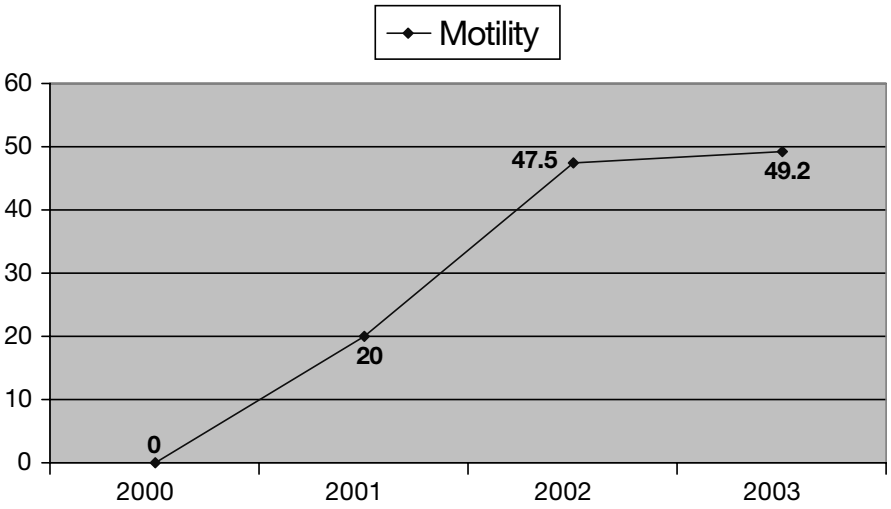


**Fig. 5**

well as bleeding episodes decreased, even after switch from prophylactic to treatment-on-demand.

## Discussion

These preliminary results encouraged us to enlarge the program and to offer it for free to all PwH in Austria, in a fitness center of personal choice, but with strict biannual physical examinations after composition of an individual training program and regular controls by a project officer.

Today there are some 30 PwH in the program and the first evaluation of all participants is scheduled after the first year of training.

## *VIIb. Hemophilia with Inhibitors*

# Methods for Testing Pharmacodynamic Variables of Hemophilia and Inhibitor Therapy: Thrombin Generation Assay and Other Tests

K. Váradi, H. P. Schwarz and P. L. Turecek

## Introduction

Prothrombin complex zymogens and enzymes are used as pharmaceutical agents for inhibitor-bypassing therapy. These agents include activated prothrombin complex concentrates, such as FEIBA (Baxter Healthcare, CA, USA), which are composed of vitamin-K-dependent coagulation factor zymogens and small amounts of their activated forms and preparations containing activated factor VII, like recombinant activated FVII (rFVIIa; NovoSeven; Novo Nordisk, Glostrup, Denmark). These products induce the activation of the clotting cascade by multiple reactions resulting in thrombin generation and thus achieving hemostasis independently from and bypassing factor VIII [1–3]. Because of the complex mechanism of action, no direct monitoring of the drug substance is available for either treatment regimes. The measurement of the individual factor activities or their derivatives does not reflect the effectivity of these products. To measure the effect of FVIII-bypassing agents new types of assay systems, assessing the time-dependent changes in the hemostatic system are required.

## Kinetics of Hemostasis. Mode of Action of FVIII-Bypassing Agents

Tissue injury triggers a series of reactions. Platelets adhere to damaged tissue at the site of injury providing an active surface for the hemostatic reactions. The complex formed by tissue factor (TF) and factor VII (FVII) activates factor X (FX) and factor IX (FIX). Enzyme-cofactor complexes assemble on the activated platelet surface, and small amounts of thrombin are generated resulting in fibrin formation. The various feedback effects of thrombin including the activation of factor V (FV), FVIII and factor XI (FXI) produce a burst of thrombin generation, mostly after the clot is formed. The fibrin clot is stabilized due to cross-linking of the fibrin chains by factor XIII (FXIII), activated by thrombin, and due to the retraction of activated platelets. The enhanced thrombin generation is controlled by the physiological inhibitor systems (TF pathway inhibitor, antithrombin, and the activated protein C-protein S system) slowing down thrombin activation by inactivating the active enzymes or degrading their cofactors. Circulating inhibitors, such as $\alpha_2$-macroglobulin inactivate thrombin directly and thrombin concentration gradually decreases. Tissue injury also induces the fibrinolytic system. Tissue plasminogen activator is liberated resulting in the generation of

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the active fibrinolytic enzyme, plasmin. To act effectively plasmin has to be bound to the fibrin clot and protected from inhibitors, some of which are activated by thrombin.

In the absence of FVIII the rate of reactions leading to thrombin generation and subsequent clot formation are very slow, which also delays all the thrombin-mediated feedback reactions. The FVIII-bypassing agents increase the concentration(s) of some active enzymes, but the complex preparations like activated prothrombin complex concentrates (APCCs) also increase the concentrations of the potential zymogene substrates, and thus raise the rate of reactions independent of FVIII [4, 5].

## General Assays to Assess the Effect of FVIII-Bypassing Therapy

To measure the effect of FVIII-bypassing agents an assay system is required that not only measures the activity of the single factors, but assesses the kinetics of those interactions described above. General screening assays just measuring the time of clotting cannot access the fine balance of these reactions. Therefore several attempts were made to modify the assays to increase their sensitivity and specificity for this purpose.

## Thrombelastography

The thrombelastography (TEG) was first described more than 50 years ago [6]. This method measures the viscoelastic properties of blood induced to clot under low shear conditions, corresponding to venous flow. It evaluates the kinetics of formation, stabilization and subsequent lysis of the clot. TEG analyses whole blood and thus measures the effect of both cellular and plasmatic components on clot formation. Inhibitor-bypassing agents both *in vitro* and *in vivo* reduce the clotting time of a FVIII-inhibitor plasma and thus also respond in thrombelastography. The main components of the TEG are a cylindrical cup and a pin hanging on a torsion wire. Depending on the equipment system used, either the pin or the cup rotates in an oscillatory way at a low angle. When blood is added torque is transmitted as the clot forms linking the cup and pin together. The torque increases as the clot strengthens and decreases as the clot lyses. The changes in the clot elasticity measured by the TEG can be drawn as the function of time, giving specific, characteristic curves (Fig. 1). These curves assess the clotting time by distinguishing the time to initial clot formation and the time to reach an arbitrary clot strength. Thus some kinetic information on the initial clot formation and various physicochemical properties of the clot are provided. The rate of the clot strengthening, the maximum clot strength and the time at maximum clot strength are indicative of the combined effect of the coagulation factors and platelet functions. The clot strength measured at a defined time (e.g. 60 minutes) after the maximum is reached and the calculated clot lysis index, i.e. the percentual ratio of the two measured amplitudes, inform about the fibrinolytic activity of the blood sample.

The critical step for monitoring the FVIII-bypassing therapy is the rate of thrombin generation, which also plays an important role even in the rate of clot

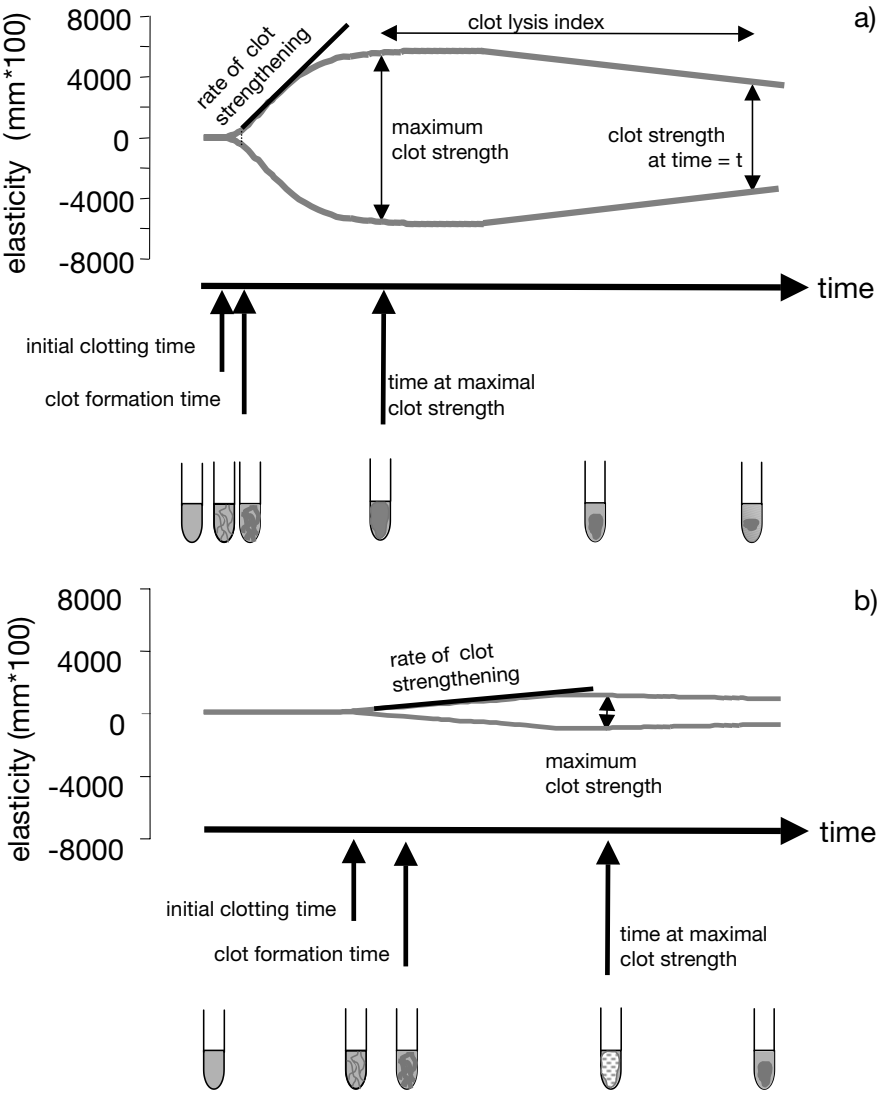


**Fig. 1a, b.** Characteristics of thrombelastogram. **a)** Schematic thrombelastogram of a normal blood sample. **b)** Schematic thrombelastogram of a severe hemophilic blood sample. The explanation of characteristic parameters is described in detail in the text. The tubes symbolize the clot formation and lysis in whole blood during the time course of TEG.

formation. To obtain further information on this step a new evaluation method, assessing the rate of clot formation in more detail was introduced recently by Sorensen et al. [7]. This method anticipates that the time-dependent changes in the clot strengthening indirectly reflect the course of thrombin generation. Even though the assay measures the thrombin activation, it only does so up to clot for-

mation and therefore cannot assess the whole kinetics of thrombin generation, which continues after the clot has been formed and leads to the main bulk of thrombin.

### Thrombin Generation Assays

The first assay that measured the rate of thrombin generation was called the »thrombin generation test« and was introduced by Macfarlane and Biggs in 1953 [8]. In this assay defibrinated plasma samples were activated, and the thrombin formed determined by subsampling to a fibrinogen source, the natural substrate of thrombin. Hemker et al. (1986) were the first to describe the detailed kinetic analysis of prothrombin activation in plasma [9]. In 1989, a platelet-based thrombin generation assay for measuring the bioavailability of FEIBA was presented [10]. In the following year Gill et al. [11] were able to show clinical correlations between thrombin generation and clinical outcome after patients with hemophilia and inhibitors were treated with APCCs. In 1993, Sultan and Loyer [12] used such a thrombin generation test for in vitro evaluation of the FVIII-bypassing activity of APCCs and FVIIa. Hemker et al. subsequently introduced a new form of thrombin generation tests, using small peptide substrates for thrombin instead of fibrinogen. Recent thrombin generation assays (TGAs) measure the time-dependent changes in thrombin concentrations in platelet-rich or in platelet-poor plasma samples [13, 14]. In these assays thrombin generation is triggered by a low concentration of tissue factor, and the thrombin activity is measured continuously using a specific fluorescence peptide substrate, which is cleaved by thrombin and liberates a fluorophore. The rate of development in the fluorescence intensity can be converted to thrombin-equivalent concentrations (nM) using a reference curve prepared by measuring the rate of substrate conversion by a thrombin calibrator.

The changes in the effective thrombin concentration can be drawn as the function of time giving specific, characteristic curves. Figure 2 shows the typical thrombin generation curves measured in normal human plasma and in a FVIII-deficient plasma with inhibitors.

The assay detects the time from trigger to starting and completing the initial clot formation, defined as the onset of thrombin generation and the kinetic lag phase, i.e. the time intercept of the maximum slope. The rate of thrombin generation, the highest thrombin concentration (peak thrombin) and the time required to reach it (peak time) is assessed. The assay informs about the rate of inactivation, and the amount of the residual thrombin concentration, reflecting the overall inhibitor capacity of the plasma. The thrombin potential, which is the area under the curve, defined first by Hemker at al. [15], is a measure of the total thrombin that could possibly have been formed after coagulation was triggered.

The absolute time parameters depend on the type and concentration of reagents used to trigger the clot formation.

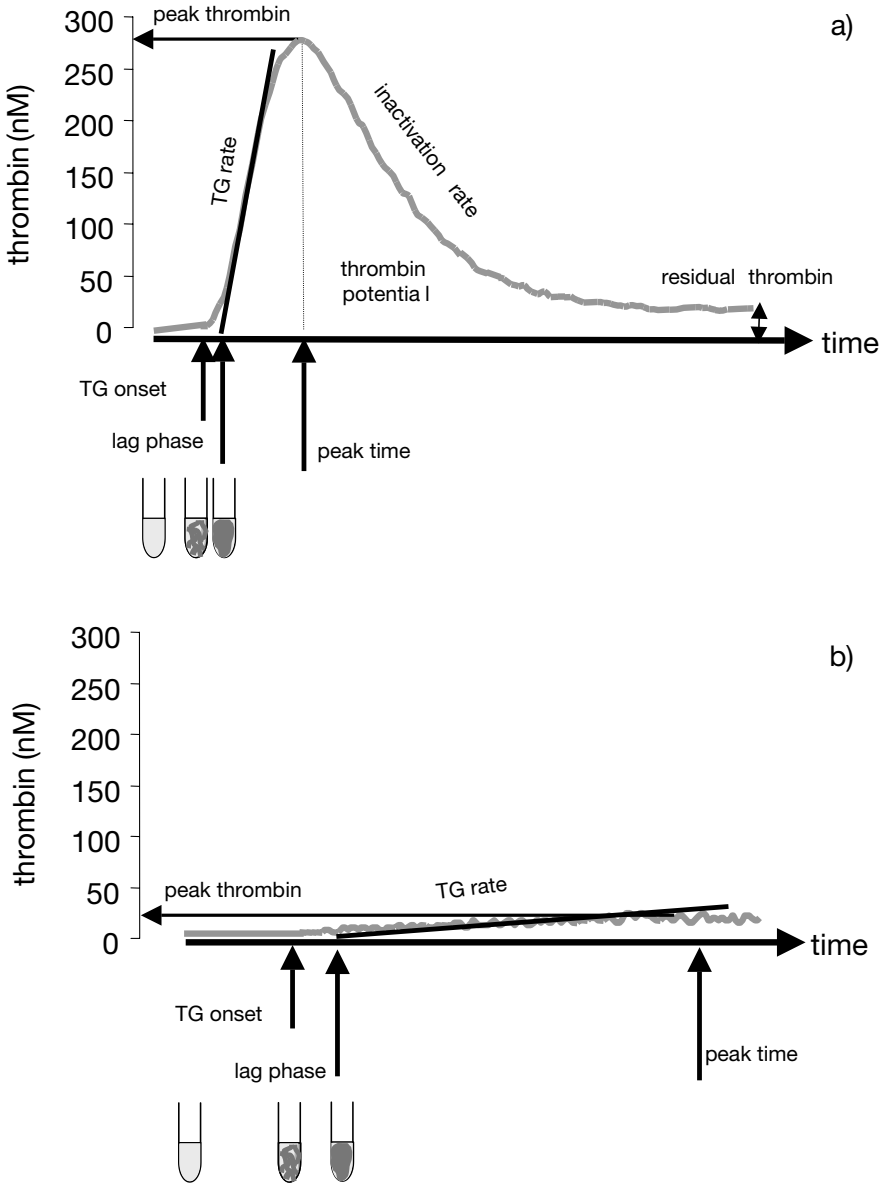


**Fig. 2a, b.** Characteristics of the thrombin generation curves triggered by a low concentration of tissue factor and phospholipid complex. **a)** Thrombin generation curve of a normal plasma sample. **b)** Thrombin generation curve of a severe hemophilic plasma sample. The explanation of characteristic parameters is described in detail in the text. The tubes symbolize the clot formation in the plasma samples during the time course of thrombin generation.

## Application of the Global Assays in the Monitoring of FVIII-Bypassing Therapy

In recent years there have been several new attempts to measure overall hemostasis, instead of measuring isolated factor activities, to assess the entire complex processes in which hemostatic components interact in various bleeding and thrombotic disorders and the results of their treatment.

Some »old assays« began their renaissance with modern computerized analytic techniques. TEG evaluates the kinetics of formation, stabilization and subsequent lysis of the clot. Yoshioka at al. [16] showed that after a single dose of rFVIIa the parameters reflecting the clotting times (time to initial clot formation and the time to reach an arbitrary clot strength) and also the maximum amplitude was corrected but the activated partial thromboplastin time (APTT) values did not normalize. The authors suggested that TEG was more suitable for monitoring treatment than APTT.

Clot waveform analysis is also a new technique for investigating the structural changes in clot formation during APTT or prothrombin time measurement [17]. The clot waveform analysis seems to be a very sensitive method for detecting minor changes in the low factor levels [18]. Clear changes were observed in the parameters of the APTT waveforms after a single infusion of rFVIIa, FEIBA or a FVIII concentrate in a hemophilia patient with low titer inhibitor (2.6 Bethesda U/mL) [16]. The FVIII concentrate had a greater improvement effect than rFVIIa or FEIBA. In contrast, when the results were compared using TEG analysis, the two bypassing agents had the more pronounced correction capacity. The differences were interpreted as arising from the different assay conditions used of whole blood or plasma.

New evaluation parameters, the maximum velocity and time of maximum velocity of the initial clot formation of the TEG were published recently [19]. The courses of whole blood clot formation seemed to be similar to the thrombin generation curves measured in plasma. Using this evaluation method the in vitro addition of recombinant FVIIa and APCC to the whole blood of hemophilia patients with inhibitor was shown to correct the diminished clot formation measured before [20]. Despite the apparent similarity of the transformed TEG curves to the thrombin generation ones, the assay measures the thrombin activation indirectly and only up to clot formation.

Thrombin is a multipotent enzyme that has many other roles apart from fibrin formation, all of which influence hemostasis. Physiologically the majority of thrombin generation occurs after the clot has been formed. Therefore the information obtained from the TEG or from the waveform analysis cannot assess the whole kinetics of thrombin generation.

In contrast, the thrombin generation assay measures the actual thrombin concentrations before and after the clot formation and is very sensitive to variations in individual factors or groups of coagulation factors.

Al Dieri at al. [21] showed a good correlation between thrombin potential and bleeding tendency in rare coagulation disorders, showing that patients with a bleeding tendency had a thrombin potential below 20% of normal. In vitro spiking of high-titer inhibitor plasma with increasing concentrations of FEIBA resulted in the dose-dependent restoration of the thrombin-generating capacity of the

FVIII-inhibitor plasma. Normal thrombin generation was obtained with concentrations corresponding to the expected concentrations that might have been achieved with the usual therapeutic doses of 50-100 U/kg FEIBA, calculated on the assumption of a 50 to 100% recovery [14]. The defective thrombin generation is also improved in patients with FVIII inhibitors within 30 minutes after a single injection of the therapeutical doses of FEIBA. The thrombin generation gradually decreased back to baseline values with a biological half-life of approximately 5 hours for FEIBA [22].

The changes in the kinetics of thrombin generation measured before, during and after a substitution or FVIII-bypassing therapy reflect the pharmacological effect of the drugs. Thus, this assay enables monitoring of the in vivo effect of the therapy, giving the possibility of further optimizing and individualizing the treatment.

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# Immune Tolerance in an Inhibitor Patient with Severe Hemophilia A – Comparison of two Different Treatment Schedules Including Rituximab

C. Wermes, I. Wieland, M. von Depka Prondzinski and K.W. Sykora

## Background

Up to 40 % of the patients with hemophilia A develop factor VIII inhibitors [1]. In more than 80 % of them, successful immune tolerance therapy (ITT) can be performed by the Bonn protocol [2] or the Malmoe treatment schedule [3]. Unfortunately, in a small amount of patients these treatment efforts are ineffective. Rituximab leads to B-cell depletion and has been demonstrated to be an effective tool in inhibitor patients with hemophilia A, but this effect seems to be only short-lived [4]. Based on the Malmoe treatment schedule and our experiences in the treatment of inhibitor patients with hemophilia B [5], we developed an immunosuppressive ITT regimen that includes Rituximab and mycophenolate-mofetil (MMF, Cellcept).

## Case Report

The 14-year-old boy with severe hemophilia A developed a high-titer factor VIII inhibitor in his early infancy. ITT with the Bonn protocol over 10 years including the use of different factor preparations (plasma products and recombinant factor VIII concentrates) was without success. Recurrent bleedings were treated with FEIBA.

## Treatment Course I

The current ITT was started one year ago. He received two different treatment schedules which are shown in Figure 1. For the first ITT (treatment I), the patient was started on MMF, 2 x 300 mg/m$^2$ po daily and after 7 days received his first dexamethasone (DEXA) pulse (2 x 12 mg/m$^2$/day po for 4 days) followed by Rituximab 375 mg/m$^2$ iv on day 5. Rituximab was repeated weekly over 4 weeks and the DEXA pulse was given again in week 3. In the fifth week the patient received again DEXA for 4 days followed by iv-immunoglobuline (IVIG, Octagam, 1 g/kg/day for 1 day) on day 5. The DEXA ivIg-pulses were repeated every 3 weeks. High dose factor VIII (Haemoctin SDH, Biotest; 1 x 100 units/kg bw) was continued the whole treatment period. MMF was stopped after 2.5 months.

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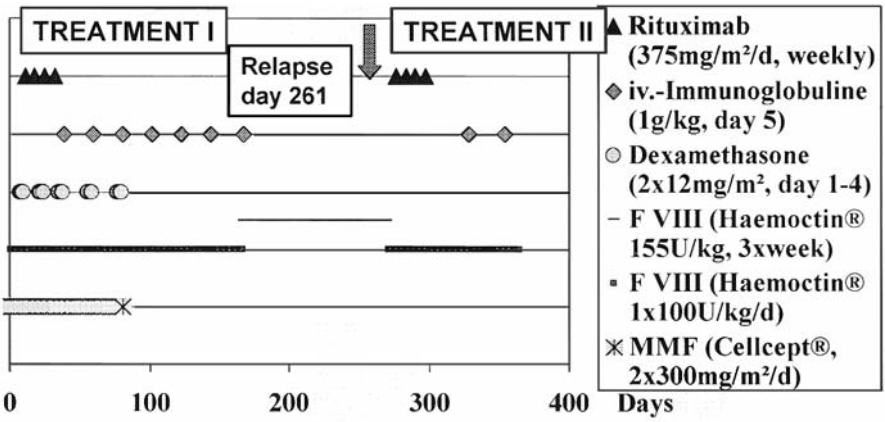


**Fig. 1.** Treatment schedules (ITT)

**Outcome**

After the third application of Rituximab the factor VIII inhibitor had disappeared and recovery and half-life of the substituted factor VIII had normalized rapidly (Fig. 2 and 3). No bleedings occurred and no treatment complications had been observed. ITT was stopped after a total period of 5 months, followed by prophylaxis with Haemoctin (155 units/kg) 3x per week. 8 months after the patient reached remission, the inhibitor reappeared.

### Treatment Course II

A different regimen of ITT (treatment II) including daily high dose FVIII administration (6000 Units = 95 units/kg) and Rituximab alone in the above mentioned

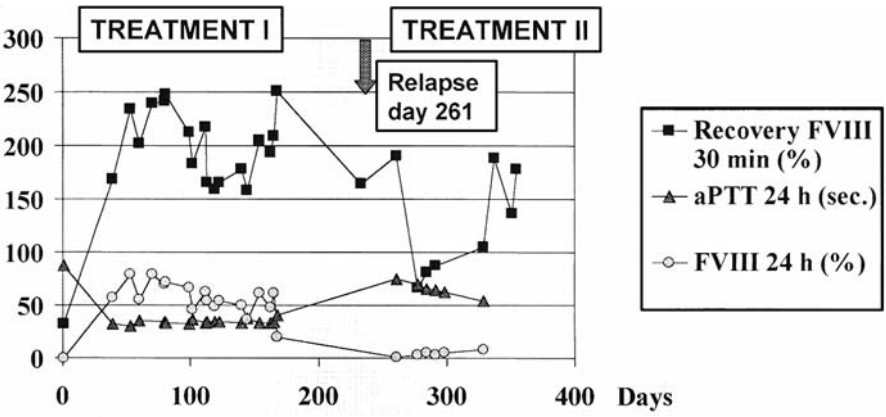


**Fig. 2.** Laboratory parameters (factor VIII, aPTT) during ITT

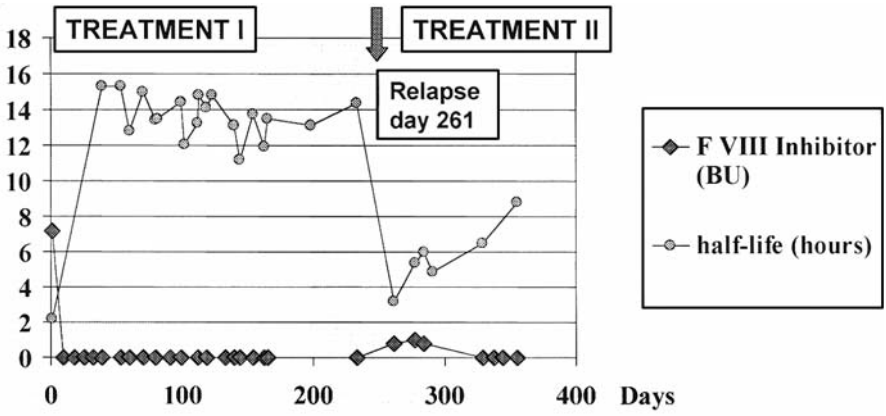


**Fig. 3.** Laboratory parameters (F VIII inhibitor and half-life) during ITT

dose over a period of 4 weeks was administered. The treatment has been intensified by regular application of IVIG in a dose of 0,95 g/kg bw which has been given every 3 weeks.

### Outcome

8 weeks after the start of another 4 courses of Rituximab the patient showed a normal recovery and the inhibitor titer was negative, but half-life was not completely normalized (Fig. 2 and 3). At this time the treatment was extended by the application of IVIG (Flebogamma). This was complicated by the development of aseptic meningitis. Treatment was continued with Octagam and again it was tolerated well.

### Conclusion

In this patient, the combination MMF, DEXA, Rituximab and IVIG (Octagam) was a well tolerated regimen leading to rapid inhibitor elimination without the development of complications or bleedings. The characteristics of MMF, being not only a T-cell, but also a B-cell immunosuppressant may be especially useful in ITT. It may be more successful than Rituximab alone, which leads to an isolated B-cell depletion. Combination treatment in course I resulted in faster clearance of the inhibitor than monotherapy with Rituximab. Because two courses of Rituximab were given we plan to give ivIg over a prolonged period of time of at least one year. All treatments were administered on an outpatient or singly day hospital basis, mainly on the weekends. The boy missed totally only 14 days of school during the whole year.

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# The Endogenous Thrombin Potential as a Monitoring Parameter in a Patient with an Acquired Hemophilia A

U. Scholz, A. Siegemund, T. Siegemund, S. Petros and L. Engelmann

## Introduction

Acquired hemophilia is a rare bleeding disorder of unknown etiology that mainly affects elderly patients. The bleeding diathesis mostly involves soft tissues and the severity is variable, from mild subcutaneous to life threatening internal bleeding episodes. The management of this bleeding disorder is not yet standardized. Laboratory diagnosis and monitoring is based on the finding of prolonged activated partial thromboplastin time (APTT), marked reduction of the coagulation factor involved (mostly factor VIII) and presence of an inhibitor on Bethesda assay [1–3]. However, these parameters do not correlate with the severely of bleeding [4].

Based on our current understanding of the coagulation system, measurement of thrombin generation (TG) in platelet-rich plasma may present a possibility to better define the hemostatic status of a given individual. A recent study by our group [5] has shown that TG is decreased in patients with hereditary hemophilia A and B, and this is the result of a reduced factor Xa-generation on the surface of activated platelets due to the deficiency of the factor VIIIa-IXa (tenase) complex.

Based on these findings, the TG assay was used in a patient with an acquired hemophilia A in order to define the interaction of coagulation factors and platelets and improve the coagulation monitoring.

## Patient

A 62-year-old woman was referred to our center due to a massive uncontrolled macrohematuria of a sudden onset. Other than a type 2 diabetes mellitus, no serious disease state was identified. She never had a bleeding diathesis prior to the present admission and family history was not remarkable.

On admission, factor VIII activity was 5.2% and factor VIII inhibitor was 7.6 Bethesda units. Bleeding was managed with activated prothrombin complex (FEIBA, Baxter) or recombinant activated factor VII (NovoSeven, NovoNordisk). Initial treatment for inhibitor eradication included immunoadsorption, followed by administration of the anti-CD20 monoclonal antibody MabThera (Rituximab) and finally immunosuppression with cyclophosphamide and prednisolone. After 6 months of immunosuppressive treatment, a complete elimination of the inhibitor could be achieved.

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### Method

Coagulation factor VIII activity was measured with the Behring Coagulation System (Dade Behring, Marburg) using a one-stage clotting assay and factor-deficient plasma. Factor VIII inhibitor was measured with the Bethesda assay.

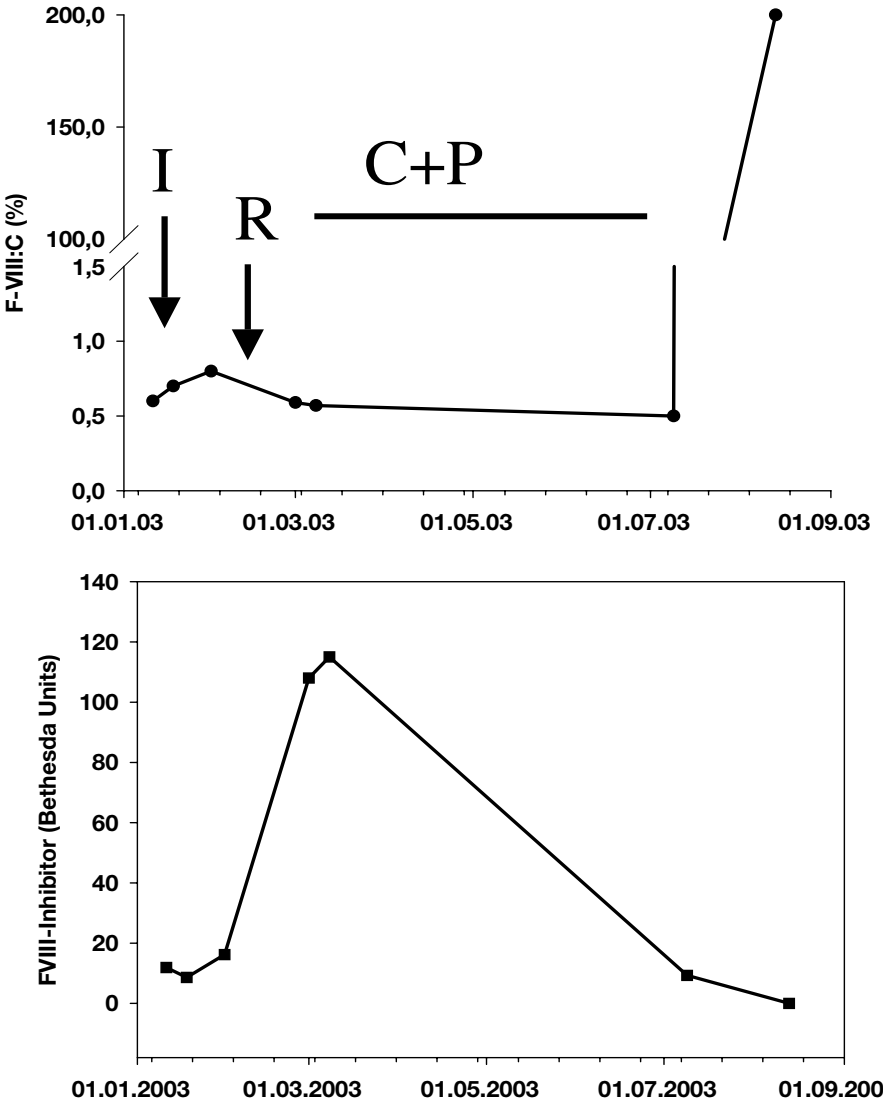


**Fig. 1.** Factor VIII activity and inhibitor concentration during the course of inhibitor treatment. (I = immunoadsorption; R = administration of Rituximab; C = cyclophosphamide; P = prednisolone)

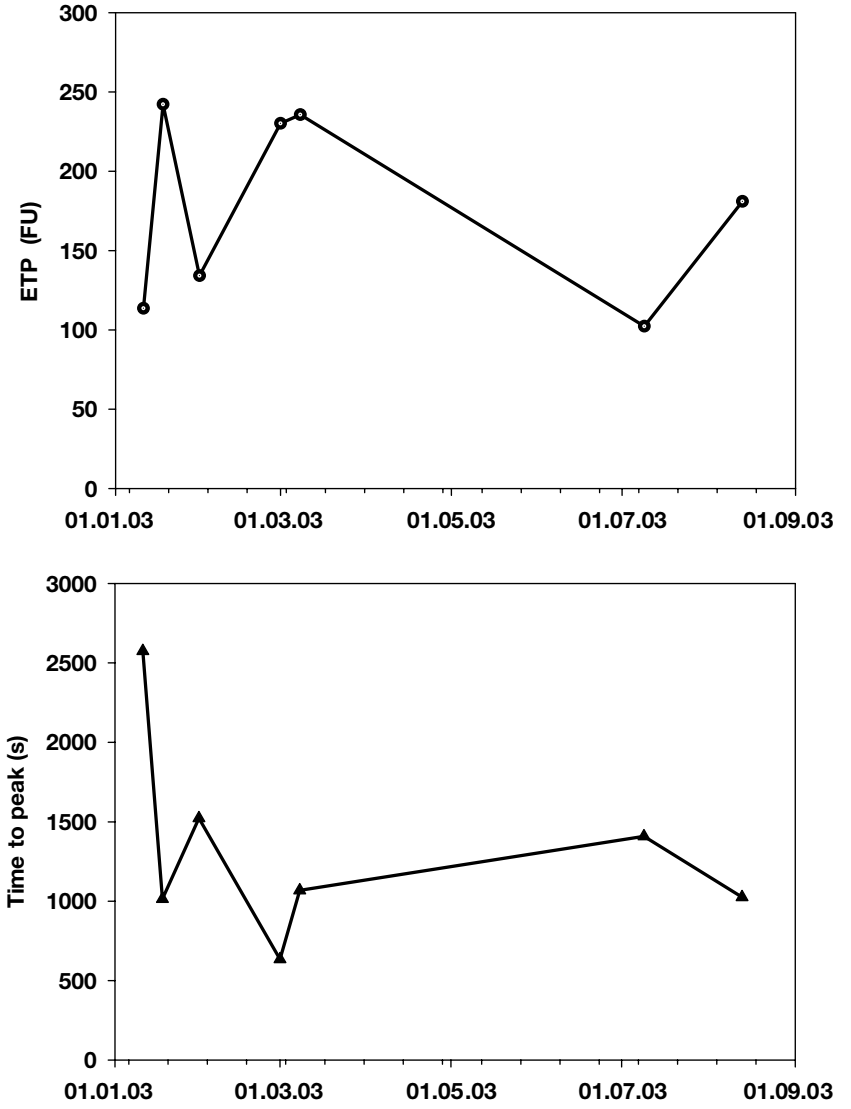


**Fig. 2.** ETP and time to peak measurements during the course of inhibitor treatment

TG was measured in a platelet-rich plasma (PRP) as previously described [5]. In short, freshly drawn citrated blood was centrifuged for 10 minutes at 170 g. 25 µl of the test reagent (fluorogenic substrate, dimethyl sulfoxide, isotonic saline, $CaCl_2$, tris puffer and exogenous and endogenous activator) was added to 100 µl of PRP. Fluorescence intensity was measured with the Fluorescan Ascent 2.2 (LabSystems, Helsinki, Finland) at wavelengths of 340 nm (excitation) and 440 nm (emission). The measurement lasted 120 minutes and data are given in arbitrary fluorescence units

(FU). Not only the area under the TG curve (i.e., the endogenous thrombin potential, ETP), but the time to peak is also presented to define the coagulation capacity [5].

## Results

Figures 1 shows the plasma activity of factor VIII and the inhibitor during the course of treatment. Simultaneous TG measurements are shown to be useful for treatment monitoring (Fig. 2). TG was increased immediately after immunoadsorption and administration of rituximab. This corresponded with the clinical observation of cessation of bleeding, although factor VIII activity was still low and inhibitor titer high. Further observation under immunosuppressive treatment demonstrated inhibitor eradication and normalization of factor VIII activity and TG.

## Discussion

The thrombin generation assay in PRP allows monitoring the contribution of the plasmatic and the cellular (platelets) system in the coagulation process. In contrast to the measurement of individual coagulation factors or PTT or prothrombin time (PT), TG represents the total coagulation capacity.

Acquired hemophilia A is a rare disorder that may result in serious bleeding episodes. The measurement of factor VIII activity or inhibitor level does not correlate well with the extent of bleeding, thus not sufficient enough for monitoring efficacy of acute care. In the present case, improvement in thrombin generation was shown after immunoadsorption and administration of rituximab, although no improvement in factor VIII activity or inhibitor level was observed. Particularly, inhibitor level markedly increased after application of rituximab (the mechanism is not known) without any deterioration in the hemostatic status of the patient. Therefore, the TG assay may be a useful tool to define the coagulation state of such patients and monitor treatment. Further investigation is necessary to verify these findings and establish the method in the clinical routine. The fact that PRP should be processed immediately and the long period of measurement required are problems yet to be solved.

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# Case report: 58-Year-Old Hemophilia A Patient with High-Titer Inhibitor Development and Introduction of a Multicenter PTP-Inhibitor Study

Ch. von Auer, M. Krause, W. Miesbach, G. Asmelash, I. Scharrer and PTP Study Group/Germany

## Background

Today the development of inhibitors to FVIII or FIX is the most serious complication in hemophilia treatment. The risk for inhibitor development is highest after the first exposure to factor concentrate and in young children (< 5 years). After 20 days of exposure and in children between 6–10 years of age the risk is levelling off [1]. Older patients with more than 50-100 exposure days (ED) are at low risk for inhibitor development. If such a patients develops an inhibitor, other influences such as immunogenicity or way of factor application should be considered [2].

In July 2003 a 58-year-old patient with hemophilia A developed a high-titer inhibitor against FVIII in our centre.

## Case history

58-year-old patient, normal body weight, severe hemophilia A, severe hemophilic arthropathy in all main joints with resulting immobility in a wheelchair, chronic hepatitis C-infection, tooth decay with bad dental status, cholecystectomy 9/01. Previous hemophilia treatment (not done in a hemophilia treatment centre): 6 ED with plasma or whole blood samples, 14 ED with Beriate for cholecystectomy. Gene mutation: Large deletion (Exon 23-24). Risk for inhibitor development: 35 %.

## Case report

The patient was admitted to a regional hospital with spontaneous hematoma of the right thorax. He was treated with two boli of FVIII concentrate (Beriate, 2 x 3500 IU) and transferred to our centre. A CT-scan revealed a soft tissue hemorrhage of the right thorax and back muscles. Hemoglobin was 13.7 g/dl, FVIII:C was 48 % before morning substitution, the inhibitor titer was 0 BU. We continued the FVIII substitution with Beriate (3000-0-2000 IU/d) and saw no progression of hematoma and no suspect laboratory findings. After three days of treatment we found a low FVIII:C level (5%) before morning substitution, hemoglobin was 8.4 g/dl even though the patient had received 4 red blood cell concentrates. A massive hematoma in the soft tissue of right thorax and back muscles had developed. FVIII inhibitor testing:12.8

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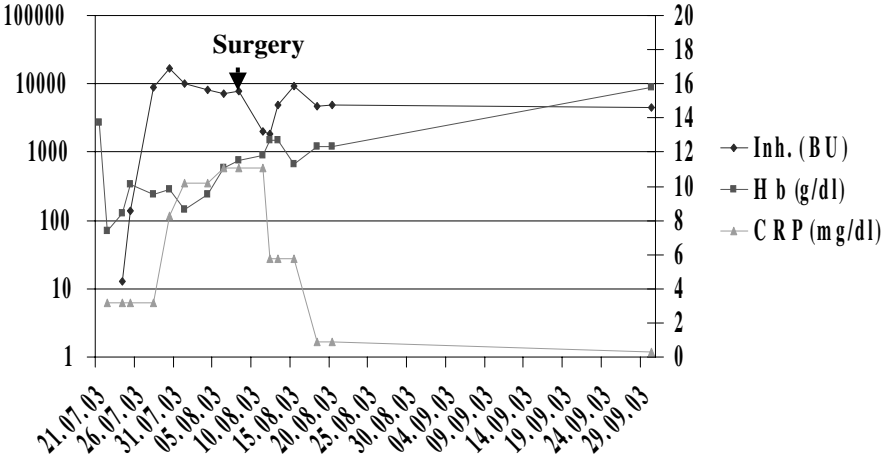
BU. FVIII substitution was stopped, treatment with FEIBA (2 x 6000 IU/d) initiated. During the next two days FEIBA dose rate was reduced (1 x 4000 IU/d) due to rising d-dimers (677µg/l). The FVIII inhibitor titer rose up to 16000 IU.

Our patient developed high body temperature up to 39°C, antibiotic treatment was started with Zienam (Imipenem, Cilastatin), Vancomycin (Vancomycin) and Sobelin (Clindamycin).

Due to recurrent fever (39°C) the hematoma was surgically removed under NovoSeven substitution. There was no bleeding complication during the peri- and postoperative period and NovoSeven was changed to FEIBA (2 x 4000 IU/d). The patient was discharged with prophylaxis 3 x 4000 IU FEIBA /week. One month later the hematoma was totally absorbed and FEIBA prophylaxis was changed to on demand therapy. Inhibitor titer was 4424 IU. ITT, plasmapheresis or Immunadsorption was refused by the patient, a Rituximab (Mabthera) therapy is planned.




## Discussion

Our patient has an uncommon history concerning a hemophilia A treatment in Germany. Possible treatment options were refused by the patient, therefore he had very few ED compared with other severe hemophilia A patients at his age. Immobilisation due to hemophilic arthropathy and disablement could have been avoided with optimal treatment.

Our patient is not the typical PTP of a western country but rather like a patient in developing countries with less than optimum factor concentrate and blood component usage. Therefore the development of an inhibitor to FVIII is not uncommon in our patient, especially with the mutation type with high risk for inhibitor development.

Nevertheless this case and a literature search caused us to ask for the situation of inhibitor development in PTPs in Germany today.

### Introduction of a multicenter PTP-inhibitor study

In September 2003 we started a retrospective study to record data about inhibitor development in PTPs in Germany. A questionnaire with the following questions was sent to 83 hemophilia treating facilities.

**Table 1.**

| Diagnosis (Hemophilia A/B, Severity) | Way of application |
|---|---|
| Gene mutatiton | Concomitant medication |
| Exposure days | Concomitant diseases |
| Factor VIII concentrate | Concomitant blood transfusion |
| Reason for FVIII substitution at inhibitor development | Concomitant vaccination |
| Amount of given FVIII concentrate Before inhibitor development | Inhibitor characteristic |
| Former change of product | Immune Tolerance Theapy |

So far 43 centres answered the questionnaire, 30 reported that no inhibitors in PTPs were detected during the last five years. 13 centres registered altogether 34 FVIII inhibitor developments in PTPs in the mentioned time period. Their data showed that there exist differing definitions for a PTP in Germany, referring to age of patient, number of ED and former change of product. Further data will be collected and published.

In conclusion it can be said that inhibitor development in PTPs is still a serious and underestimated problem in hemophilia treatment today. A definition for »PTP« from the SSC of the ISTH would be helpful.

Secondly a prospective, not product related study on inhibitor development in PTPs should be conducted.

# First Data of a Prospective Study About Incidence of Inhibitors During and After Continuous Infusion of Different Factor Concentrates Given During and After Surgical Procedures in Hemophilia A or B and von Willebrand Disease

G. Auerswald, T. Spranger and S. Meister

## Introduction

Inhibitor development against F VIII / IX or VWF is one of the most severe complication in treatment of patients with hemophilia A, B or von Willebrand disease (VWD). There are publications about increased inhibitor development in patients treated with continuous infusion (C.I.) during surgery or after severe bleeding episodes. In an earlier retrospective study with 118 children and young adults with hemophilia A or B and von Willebrand disease treated with C.I. for surgical reasons we could show about 30–50% less use of factor concentrate compared to bolus infusions.

In addition the factor level could can be kept within the target range without unnecessary high peaks or dangerous low levels. There was no evidence for increased inhibitor development during this study. In a new prospective study we investigated weather there is any increased risk for inhibitor development (especially low titre inhibitors) in patients treated with C.I. for surgical procedures.

## Patients and Investigations

All included patients of our pediatric hospital and associated departments (ENT, oral surgery, urology, orthopedics and gynecology) with hemophilia A, B and VWD were treated with continuous infusion during surgical therapy. Inclusion criteria were: negative history for inhibitors, at least 50 exposure days prior inclusion and a surgical procedure requiring a C.I. for at least 3 days. Patients and / or parents gave informed consent to the study. In patients with hemophilia A or B the molecular defect was considered. 29 patients were included into the study so far: 18 patients with hemophilia A, 4 patients with hemophilia B and 7 patients with VWD.

Coagulation tests and estimation of hemoglobin, hematocrit and platelets were done before every surgical procedure. Specific coagulation tests included prothrombin time, APTT, thrombin time, fibrinogen, residual activity of F VIII or IX as well as a inhibitor test.

In patients with VWD additionally the bleeding time, VWF activity and VWF:Ag were measured. Recovery and half life was done in all patients.

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## Surgical Procedures and Therapy

The kind of surgery in patients with hemophilia A and B and VWD can be seen in Table 1–3. The age of the patients at the moment of surgery was 2,6 to 24 years for hemophilia A and 2,9 to 16,3 for hemophilia B. 8 patients with hemophilia A were treated with high purity pd concentrates and 3 patients with pd VWF containing concentrates. In 5 patients therapy was done with full molecule rFVIII concentrates and in 2 with B-domain deleted FVIII concentrate. All hemophilia B patients were treated with pd FIX concentrates. Therapy was started with a bolus dose of 30–50 IU/kg followed by continuous infusion with 3–4 IU/kg/h. The dose of C.I. was adapted to the factor levels which were measured minimum once a day.

6 patients with VWD were treated with Haemate HS, 1 patient was treated with Immunate.

**Table 1.** Surgical procedures in Hemophilia A

| | |
|---|---|
| • abdominal surgery | (3 patients) |
| • oto-laryngeal surgery | (7 patients) |
| • orthopedic surgery | (1 patient) |
| • neurosurgery | (1 patient) |
| • Port-a-Cath-implantation | (1 patient) |
| • oral surgery | (3 patients) |
| • accident surgery | (2 patients) |

**Table 2.** Surgical procedures in Hemophilia B

| | |
|---|---|
| • abdominal surgery | (1 patient) |
| • oto-laryngeal surgery | (1 patient) |
| • oral surgery | (1 patient) |
| • accident surgery | (1 patient) |

**Table 3.** Surgical procedures in VWD

| | |
|---|---|
| • abdominal surgery | (1 patient) |
| • oto-laryngeal surgery | (2 patients) |
| • oral surgery | (2 patients) |
| • accident surgery | (2 patients) |

## Results

All 29 patients underwent the surgical procedures without a major blood loss. The time of continuous infusion was between 3 and 9 days (median 5.8 days). The consumption with continuos infusion was up to 48 % lower compared to the calculated consumption of factor concentrates with bolus doses. No thrombotic events or postoperative wound infection was seen. The determination of the inhibitor activi-

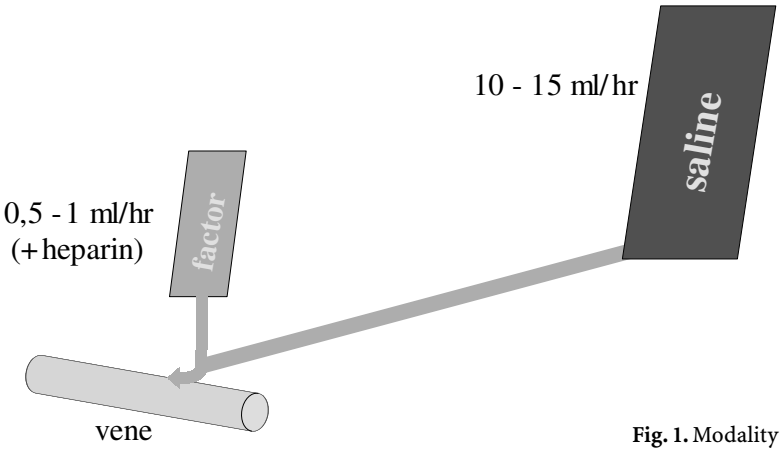


**Fig. 1.** Modality of infusion

ty as well as the half-life was done latest before demission from the hospital. We found values within the estimated range for all patients also the inhibitor activity at that time was negative. A follow-up examination was done in all patients after 2–4 weeks after finishing continuous infusion. At this time again there was no inhibitor development detectable. 6 patients with hemophilia A had an intron 22 inversion, 4 patients had a large and 3 patients had a small deletion in the F VIII gene. The molecular diagnosis of the other patients is not available at the moment.

The group of investigated patients is still to small and heterogeneous to make a clear statement about incidence of inhibitor development during surgical procedures done under continuous infusion of factor con1es. This study will be continued.

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## *VIIc. Thrombophilia*

# Acquired Thrombophilia in Patients on Hemodialysis with Recurrent Vascular Access Thrombosis

R. Klamroth, O. Eike, F. Seibt, S. Gottstein, H. Rimpler and H. Landgraf

## Introduction

Vascular access site thrombosis in patients receiving hemodialysis is a major cause of hospital admission and recurrent surgery [2]. The underlying pathologic cause is often stenosis of the venous vessel due to fibromuscular hyperplasia [5]. But in the case of early failure occasional studies have investigated that hypercoagulability could play an important role in this context [6]. The presence of antiphospholipid antibodies seems to be correlated with increased risk for thrombotic complications of vascular hemodialysis access [9].

## Aim of the Study

Is there a higher prevalence of hereditary and acquired thrombophilic risk factors in patients with vascular access thrombosis in comparison to patients vascular access thrombosis?

## Patients

In 2002 we examined 40 consecutive patients receiving hemodialysis. 22 patients had a history of vascular access site thrombosis (group 1) and 18 patients had not and a vascular access for longer than one year (group 2). All patients in group 1 had a history of at least two occlusions of vascular access. All 10 patients with prosthetic

**Table 1.** Patients

| | All patients | Group 1 | Group 2 |
|---|---|---|---|
| n | 40 | 22 | 18 |
| Age (years) | 65,8 | 64,4 | 67,6 |
| Women/men | 20/20 | 11/11 | 9/9 |
| Vascular access thrombosis | 22 | 22 | 0 |
| Arteriovenous fistula | 30 | 12 | 18 |
| PTFE prosthetic graft | 10 | 10 | 0 |

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grafts in group 1 had a history of thrombosis of arteriovenous fistula before implantation of PFTE graft. Patients with known systemic lupus erythematodes were excluded.

## Methods

In every patient hereditary and acquired thrombophilic risk factors were determined including antithrombin, protein C, protein S, factor V-G1691A-mutation, prothrombin-G20210A-mutation, homocysteine, lipoproteine (a), lupus anticoagulant, cardiolipin antibodies IgG and IgM , fibrinogen and factor VIII.

Platelet hyperreactivity was studied by light transmittance aggregometry in platelet rich plasma (Aggregometer PAP 4, Fa. Mölab). Aggregation was recorded as the maximum percentage change in light transmittance from baseline using platelet poor plasma as a reference. We defined »sticky platelets« as platelet aggregation > 30% after induction with different concentrations of ADP (10, 1 and 0,5 µmol) in platelet rich plasma [8].

## Results

The results of thrombophilia evaluation are presented in the in Table 2. We found in 50% of patients with vascular access site thrombosis antiphospholipid antibodies in comparison to only 12% in patients without thrombosis. The patients in group 1 showed more activated platelets than patients in group 2. There were no significant differences in the number of hereditary risk factors in both groups.

**Table 2.** Thrombophilic risk factors

| | Group 1 (n=22) | Group 2 (n=18) |
|---|---|---|
| Lupus anticoagulant positive | 11/22 | 2/18 |
| Cardiolipin antibodies IgG positive | 6/22 | 0/18 |
| Sticky platelets syndrome | 9/22 | 3/18 |
| Homocysteine (< 15 µmol/l) | 31,6 ± 21 µmol/l | 21,8 ± 10 µmol/l |
| Fibrinogen (1,8 -3,5 g/l) | 4,3 ± 1,0 g/l | 4,9 ± 1,4 g/l |
| Factor VIII (80-150%) | 224 ± 100% | 213 ± 44% |
| Factor V-G1691A-mutation | 2/22 | 1/22 |
| Lipoprotein (a) (< 300 mg/l) | 286 ± 301 mg/l | 286 ± 219 mg/l |
| Antithrombin (80 – 150%) | 102 ± 13% | 106 ± 19% |
| Protein C (70 – 150%) | 101 ± 17% | 96 ± 28% |
| Protein S (70- 140%) | 94 ± 27% | 91 ± 21% |
| Prothrombin-G20210A-mutation | 0/22 | 0/22 |

## Discussion

We found in all 40 patients thrombophilic risk factors like elevated factor VIII, homocysteine and fibrinogen concentrations. The higher concentrations of homocysteine are due to end stage renal disease. One possible cause for higher concentrations of fibrinogen and factor VIII, activated platelets and antiphospholipid antibodies could be a chronic inflammational process in patients receiving hemodialysis [4].

In the etiology of recurrent vascular access thrombosis only higher platelet activation and antiphospholipid antibodies play an important role [3, 7]. The influence of homocysteine levels on vascular access thrombosis remains controversial [2, 10]. We found slightly higher concentrations of homocysteine in patients with thrombotic complications of vascular access. The hereditary thrombophilic risk factors in comparison to the acquired factors were less important. In our small group we could not find any relevant differences.

## Conclusions

In patients receiving hemodialysis we found a high prevalence of acquired thrombophilic risk factors. There seems to be causal relation between vascular access site thrombosis and especially antiphospholipid antibodies and activated platelets. The evaluation of thrombophilic risk factors in patients with recurrent vascular access site thrombosis could lead to an improved antithrombotic therapy.

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# Is Travelling a Risk Factor for Venous Thrombosis in Individuals with Factor V Leiden in Heterozygous Form?

P. Dulíček, J. Malý and M. Beránek

## Introduction

Venous thromboembolism (VTE) is a serious disorder with an incidence about 1 per 1000 individuals per year [1]. It is a multifactorial disease that is defined by multiple interactions between genetic and environmental components. In 1993, Dahlbäck et al. identified a new mechanism causing inherited thrombophilia, characterized by a poor anticoagulant response to activated protein C resistance (APC–R) [2]. The molecular defect underlying this phenomenon was identified as an amino acid substitution at the cleavage site of the factor V gene (1691 G to A) [3]. Among Caucasian populations, F V Leiden is the most common inherited cause of VTE currently known and it is considered to be a mild risk factor. VTE usually manifests in risk situation for VTE. Travelling is one of these risk factors, but fair risk of VTE after travelling in individuals with FV Leiden has not been clearly assessed yet.

## The aim of the study

We have assessed the frequency of clinical manifest and objectively confirmed (by Doppler (ultrasound, phlebography or lung perfusion scan) VTE in individuals with FV Leiden in heterozygous form in association with travelling. VTE was assessed after travelling by plane (> 6 hrs.) and by bus (> 12 hrs. – overnight ) within period of 4 weeks after journey. The other risk factors for VTE, which could contribute to VTE were assessed as well. Inclusion criteria: Age > 18 years, individuals without pharmacological thromboprophylaxis before this travel. Exclusion criteria: Patients with oral anticoagulation treatment, patients with known malignancy, pregnancy and patients with history of surgery and administration of plaster cast within period of one month before journey.

## Method

We compare the frequency of VTE in 2 cohorts. Both groups are comparable by age and number. Everybody in these two groups underwent such a travel at least once in life after the age of 18 year.

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1. cohort contains 251 individuals with FV Leiden in heterozygous form (age range 18–72, mean age 41 years). Average age of these patients at the time of travel was 37 years in this group. 116 patients of them have already had VTE, 135 individuals are still asymptomatic.
2. cohort-control contains 230 individuals (age range 18–69, mean age 38 years) without FV Leiden (1st degree relatives of p. with FV Leiden). 2 individuals from this cohort have also already had VTE. The average age of these individuals was 33 years in this group. None of the patients with personal history of VTE had pharmacological prophylaxis before travelling in both cohorts.

## Results

1. cohort: VTE occurred in 7 cases (in 7 individuals). All VTE were diagnosed within first 2 weeks after travelling. Distal vein thrombosis was diagnosed in 5 cases (one woman also took oral contraceptives), proximal DVT complicated by pulmonary embolism occurred in two cases. In any of these 7 patients were identified any other inherited or acquired risk factor for VTE. On the other hand, VTE did not occurred in 5 women in spite of using of oral contraceptives.
2. cohort: Distal DVT developed in one man in this group. 4 women were taking oral contraceptives in this group. The results were statistically analyzed by Fisher's exact test ($p < 0.05$) and frequency of VTE in the first group reached statistically significance (0.070119 – the bordeline statistical significance).

## Discussion

Whether long – distance travel and symptomatic VTE are associated is debated. On the basis of current available literature a fair risk estimate cannot be obtained. However, the consensus at a meeting of experts convened in March 2001 by the WHO to review the evidence is low and mainly involves passengers with additional risk factors for VTE [4]. In the recent study [5] the pooled odds ratio for the association between any travel and symptomatic VTE was 0.9 (95% CI: 0.6–1.4). The median travel time was 7 h. Separate analyses performed for different types of transport (plane, car, bus or rain) yielded comparable odds ratios. The analysis for duration of travelling showed an increased odds ratio of 2.5 (95% CI: 1.0–6.2) in the category of 10–15 hrs. of travelling. The possible contribution of inherited thrombophilia to onset of VTE in association with travelling is not clearly assessed. In one small retrospective study [6] 30% of patients with flight – related DVT had FV Leiden.

Travelling by bus highly prevailed in both our cohorts (more than 95%). It is due to travelling by bus to the Mediterranean Sea. Frequency of VTE was higher in the 1st cohort and reached mild statistical significance. Except one woman (with OC), VTE occurred in individuals older than 50 years.

## Conclusion

In our study the travelling was found as a mild risk factor for VTE in individuals with factor FV Leiden in heterozygous form. We recommend pharmacological thromboprophylaxis (by LMWH) before the travelling (by bus more than 12 or by plane more than 6 hrs.) in these situations :

- In all individuals with FV Leiden and personal history of VTE
- In all individuals with FV Leiden and other inherited thrombophilia or with other acquired risk factor (mainly in the case of a several risk factors )
- LMWH is individually assessed in all persons with FV Leiden older than 50 years

We are aware, that for more precise recommendation the larger well – designed studies are needed.

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# Popliteal Artery Embolism in Abdominal Aortal Thrombus with Liver Abscesses Caused by Heterozygous Prothrombin Mutation with Protein S Deficiency and Factor VIII Elevation

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We report on a 64-year-old woman who was admitted to a peripheral hospital with known cholecystolithiasis complicated by acute abdominal pain. Her past medical history included pulmonary embolism and deep vein thrombosis *(DVT)* 3 years ago, arterial hypertension and obesity. The following day, laparoscopic cholecystectomy was performed because of gallbladder hydrops. Postoperatively, she complained of progressive right-sided abdominal pain. A CT scan was performed 2 weeks postoperatively showing multiple hepatic and splenic abscesses as well as right-sided thoracic effusion. Furthermore, an extensive aortal thrombus of about 10 cm above the coeliac trunc was visible without radiological signs of dissection or infection. Intravenous therapeutical heparinization was started aiming at an anti Xa level of around 0.6 U/ml. During the following days, further CT scans revealed progressive liver necrosis, and severe impairment of liver function developed necessitating transfer to ICU.

The patient was transferred to our hospital and directly admitted to the intensive care unit on day 42. She presented in poor general condition with confusion, severe dyspnea and sepsis without signs of shock. Biochemistry showed signs of systemic infection (leucocytosis of 62000/µl, C-reactive protein of 230 mg/l), a disturbed liver function with signs of hepatocellular necrosis (prothrombin time 50%, cholinesterase 2.4 kU/l, AST 750 U/l) and impaired respiratory parameters (blood gas analysis under oxygen administration of 8 l/min: pO2 63, pH 7.46, lactate 3.4 mmol/l). Imaging procedures (ultrasound, CT scan) confirmed the aortal thrombus, extensive hepatic and splenic abscesses and a right-sided thoracic empyema as well as basal pneumonia.

Immediate therapy with a total of three percutaneous thoracic and hepatic abscess drainages was initiated under ultrasound-guidance. In addition to other germs (Enterococcus faecalis, Candida spp.), a multiresistant strain (Enterobacter cloacae) was detected in cultures taken from the drainages. Therefore, quadruple antibiotic therapy was administered.

The hepatic drainages rested in place (with several changes) for several months. Internal drainage by a stent placed by ERCP was ineffective, the stent had to be removed by a second ERCP. During these months, several problems complicated the clinical course (relapse of thoracic empyema necessitating repeated thoracic drainage, severe episodes of arterial hypertension, recurrent AV-reentry tachycardia, repeated vomiting, symptomatic transitory psychotic syndrome).

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Initially, no aortal embolectomy was intended due to the high risk of the procedure, the complicated course of the disease and a thrombus size stability as seen on repeated CT scans. Despite adequate heparin therapy, the patient developed acute occlusion of the right popliteal artery requiring emergency embolectomy on day 164. Therefore aortal embolectomy was performed on day 175. Finally, the patient was discharged from hospital on the 212th postoperative day. At that time, she was fully mobilized and under anticoagulation by a low molecular weight heparin (targeting at anti Xa levels of 0.4 U/ml). Her liver abscesses were completely resolved.

Extensive coagulation analyses (after resolution of the septic episode) revealed a heterozygous prothrombin mutation (G20210A; factor II activity 132%; normal range: 70–120%), protein S deficiency (free protein S 46%; normal range: 57–114%) and a sustained factor VIII elevation (>500%; normal range: 61–185%).

This is the first reported case of an extensive aortal thrombus due to a combination of three coagulation disorders representing three independent risk factors for venous thromboembolism aggravated by exogenous risk factors. Although the relevance for arterial occlusion is plausible and probable, especially for combination defects, little is known about the clinical relevance of multiple risk factors of hereditary thrombophilia together with exogenous risk factors on arterial thrombosis. In this case, the aortal thrombus caused an aggravated course of the disease by multiple embolism (212 days of in-patient treatment).

# Effect of the new Direct Thrombin Inhibitor Melagatran in Cord and Adult Plasma: an in-vitro Examination

M. Köstenberger, S. Gallistl, G. Cvirn, B. Leschnik and W. Muntean

## Abstract

The aim of our study was to compare the effects of the new thrombin inhibitor melagatran on markers of thrombin generation in cord and adult plasma activated with either high or low amounts of tissue factor (TF). The new direct thrombin inhibitor melagatran acts, in contrast to the most frequently administered anticoagulant heparin, independent of the antithrombin content in plasma. In the absence of melagatran adult plasma clotted significantly prior to cord plasma when activated with high amounts of TF, while in contrast clotting of adult plasma is significantly delayed when compared to clotting times of cord plasma under low activation. Under both high and low activation of plasma clotting times dose-dependently increased when melagatran concentrations were successively elevated. Increasing amounts of melagatran resulted in dose-dependently decreased F 1.2 and TAT generation in both cord and adult plasma under both high and low amounts of TF.

Our in vitro results cannot be used for determination of clinical efficacy but assessing the degree of thrombin generation inhibition may be useful for selecting effective doses of melagatran for in vivo studies in newborn infants with thromboembolic complications.

## Introduction

The hemostatic system of neonates has unique features when compared with adults [1, 2]. In the newborn, plasma concentration of the vitamin k-dependent coagulation factors, i.e. prothrombin, and of the anticoagulants antithrombin (AT), protein C (PC), protein S (PS), and tissue factor pathway inhibitor (TFPI) are reduced to approximately 50 % of adult amounts [3–6]. Physiological low levels of anticoagulant proteins in neonatal plasma might compensate for low levels of coagulation factors and allow sufficient thrombin generation.

Infants less than one year old constituted the age group at highest risk for thromboembolic complications among children, mainly due to serious associated conditions such as central venous lines [7]. Until there is convincing data to the contrary they do require anticoagulant therapy. Usually newborns with thromboembolic complications are treated with heparin. It is uncertain whether the relative deficiency of AT in neonatal plasma limits the antithrombotic effects of heparin in

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this age group. On one hand inhibition of thrombin in heparinized plasma was suggested to be less effective than in adult plasma due to neonatal AT deficiency [8], on the other hand Chan et al. [9] demonstrated that reduced concentrations of UH and LMWH were required to inhibit thrombin generation in cord compared to adult plasma.

Direct thrombin inhibitors (DTI), such as melagatran, were developed to improve the efficacy/bleeding ratio to that of heparin and to overcome the inability of the heparin/antithrombin complex to inactivate bound thrombin [10]. DTIs produce a more predictable anticoagulant response than heparin because, unlike heparin, they do not bind to plasma proteins or require antithrombin as a cofactor. Therefore DTIs may be of particular interest in newborns with thromboembolic complications. We, therefore, investigated the potential significance of the physiological AT deficiency in neonatal plasma for the antithrombotic effects of melagatran when compared to adult plasma.

The lower levels of coagulation factors in newborns are reflected in standard assays as a prolonged prothrombin time (PT) and prolonged activated partial thromboplastin time (APTT). However, prolongation of PT and APTT in neonatal plasma give only a simplified picture of the relative importance of various components of the coagulation process. Davie et al. [11] suggested that coagulation activation via the extrinsic pathway with low amounts of TF is probably more physiological than the plasma activation commonly used in standard assays. Low TF concentrations have been shown to be suitable for sensitive detection of the effects of different levels of pro- and anticoagulants on thrombin generation [12].

We, therefore, investigated the effect of melagatran on prothrombin activation, thrombin-antithrombin generation, and clotting time activated with either high or low amounts of tissue factor in cord versus adult plasma.

## Materials and Methods

Melagatran, the active form of the oral direct thrombin inhibitor ximelagatran (Exanta), was kindly supplied by AstraZeneca R&D (Mölndal, Sweden). The Imubind Tissue Factor ELISA Kit for quantitative determination of TF concentration was obtained from American Diagnostica Inc. (Greenwich, CT, USA). H-Gly-Pro-Arg-Pro-OH (GPRP; Pefabloc) was purchased from Pentapharm LTD (Basel, Switzerland). Testkit prothrombin fragment 1.2 (F 1.2) micro for determination of F 1.2, and testkit thrombin-antithrombin (TAT) micro for determination of TAT were purchased from Behring Diagnostics (Marburg, Germany). Stopping solution for F 1.2 and TAT determination consisted of Trasylol/EDTA/Na-citrate 8:1:1 and 110 µmol/l PPACK (D-Phe-Pro-Arg chloromethyl ketone) from Sigma (Vienna, Austria). Trasylol from Bayer (Vienna, Austria), contained the protease inhibitor aprotinin. Buffer A contained 0.05 mol/L Tris-HCl (pH 7.4), 0.1 mol/l NaCl and 0.5 mol/l bovine serum albumin.

**Collection and Preparation of Plasma**

Cord blood was obtained immediately following the delivery of 24 full term infants (38-42 weeks gestational age). Newborns with Apgar scores of 9 or less five minutes after delivery were excluded from the study. Blood (2.7 ml) was collected into pre-citrated S-Monovette pre-marked tubes from Sarstedt, Nümbrecht, Germany, containing 300 µl 0.106 M citrate, centrifuged at room temperature for 15 min at 2800 x g, pooled and stored at -70°C in propylene tubes until assayed. The hematocrit of cord blood was slightly, but not significantly, elevated over adult values. FV and FVIII activities were elevated over the respective adult values, other pro- and anticoagulant factors were in the normal range for neonates. I.e., cord plasma contained 162 µg/ml AT. In the same way blood from 18 healthy adults was collected from the antecubital vein, prepared, and checked. I.e., adult plasma contained 264 µg/mL AT.

**Activation of Plasma**

Four-hundred µl plasma were incubated with 30 µl of buffer A containing melagatran at desired concentration for 2 minutes at 37°C in flat-bottomed plastic tubes while stirring. After subsequent incubation with 20 µl buffer A containing H-Gly-Pro-Arg-Pro-OH (Pefabloc FG, 1.0 mg/mL final concentration) to inhibit fibrin polymerization, plasma samples were activated by addition of 50 µl of an activation-mixture composed of 0.5 M $CaCl_2$ and diluted Thromborel S as a source of TF. Final concentration of TF were 1.84 ng/ml TF for »high coagulant challenge« and 4.6 pg/ml TF for »low coagulant challenge«, respectively.

**Determination of AT Plasma Concentrations**

Determination of the AT content of cord plasma was performed by means of a standard chromogenic method on a BM/Hitachi 917 from Boehringer Mannheim (Vienna, Austria).

**Determination of TF Concentrations**

TF concentrations were quantitatively determined via an enzyme-linked immunoassay. TF levels were determined by measuring solution absorbency's at 450 nm and comparing the values with those of a standard curve.

**Determination of Clotting Time**

Clotting times were determined by means of the optomechanical coagulation analyzer Behring Fibrintimer II from Dade Behring (Marburg, Germany), which applies the turbodensitometric measuring principle. Plasma samples were prepared as described above except that Pefabloc was replaced by buffer A.

### Determination of F 1.2

Plasmas were prepared and activated as described above. Prothrombin activation was determined by subsampling 10 µl aliquots, taken from plasmas 10 minutes after explosive thrombin generation has occurred, into 490 µl stopping solution. After subsequent 1:10-dilution in stopping solution, the amount of F 1.2 generated was quantified by using an immuno-enzymatic kit.

### Determination of TAT

Plasmas were prepared and activated as described above. TAT activation was determined by subsampling 10 µl aliquots, taken from plasmas 10 minutes after explosive thrombin generation has occurred, into 490 µl stopping solution. After subsequent 1:20-dilution in stopping solution, the amount of TAT generated was quantified by using an immuno-enzymatic kit.

### Statistical Analysis

The effects of different concentrations of melagatran on TAT and F 1.2 generation were analyzed using paired t-test. For comparison of TAT and F 1.2 values after activation of plasma at high and low coagulant challenge Mann-Whitney U test was performed. The significant level of P-values was set at $P<0.05$.

## Results

All results obtained in cord and adult plasma are expressed as means (± SD) of five experiments.

### Effect of Melagatran on Clotting Time in Cord and Adult Plasma

Under high coagulant challenge (cc) adult plasma clotted significantly prior to cord plasma in the absence of the direct thrombin inhibitor melagatran (Fig. 1a). In contrast, under low coagulant challenge clotting of adult plasma is significantly delayed when compared to clotting times of cord plasma in the absence of melagatran (Fig. 1b).

Under both high and low cc clotting times dose-dependently increased when melagatran concentrations were successively elevated. The clotting-time-prolonging effect of melagatran in cord plasma was comparable to that in adult plasma.

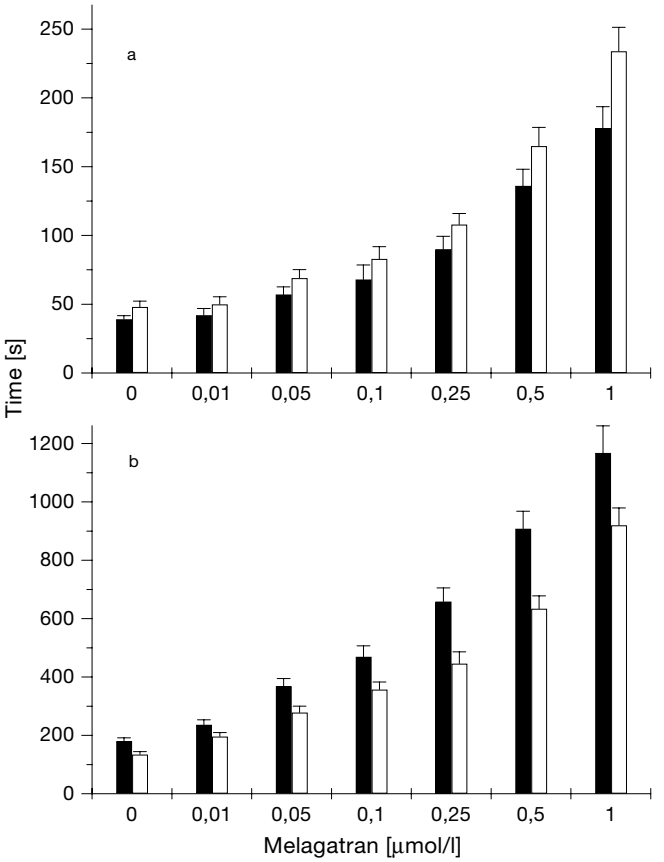


**Fig. 1a, b.** Effect of various melagatran concentrations on prolongation of clotting time in cord and adult plasma under high coagulant challenge **a)** and low cc **b)**. Black columns: Melagatran, adult plasma. White columns: Melagatran, cord plasma. Results shown are expressed as means (n = 10).

**Effects of Melagatran on Prothrombin Fragment 1.2 Values in Cord and Adult Plasma**

Addition of increasing amounts of melagatran resulted in dose-dependently decreased F 1.2 generation in both cord and adult plasma under high (Fig. 2a) and low (Fig. 2b) coagulant challenge.

Melagatran revealed comparable capability to suppress F 1.2 formation in both cord and adult plasma under high (Fig. 3a) and low (Fig. 3b) coagulant challenge, calculated in percent inhibition referring to F 1.2 values in the absence of the drug.

**Effects of Melagatran on TAT Generation in Cord and Adult Plasma**

Addition of increasing amounts of melagatran resulted in dose-dependently decreased TAT generation in both cord and adult plasma under high (Fig. 4a) and low (Fig. 4b) coagulant challenge.

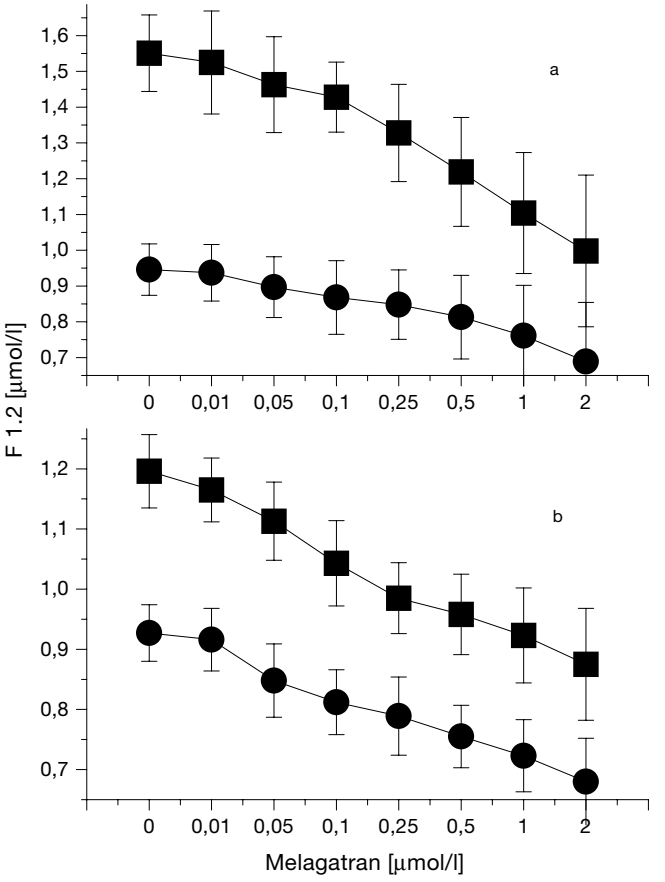


**Fig. 2a, b.** Prothrombin fragment 1.2 (F 1.2) values after high and low coagulant activation of cord and adult plasma. Melagatran dose dependently decreased F 1.2 values in both cord and adult plasma under high **a)** and under low coagulant challenge **b)**. Square: Melagatran, adult plasma. Circle: Melagatran, cord plasma.

Melagatran revealed comparable capability to suppress TAT formation in both cord and adult plasma under high (Fig. 5a) and low (Fig. 5b) coagulant challenge, calculated in percent inhibition referring to F 1.2 values in the absence of the drug.

## Discussion

The coagulation system in full term neonates is immature at birth and evolves postnatally toward the mature adult system. The plasma concentration of both procoagulants and inhibitors significantly differ in healthy neonates compared with adults [1]. The capacity to generate thrombin is decreased and delayed in plasmas from healthy newborns compared with adults [2]. Despite physiological low levels of procoagulant proteins hemostasis in the neonate is as least as good as in adults. Physiological low levels of inhibitors might compensate for low levels of procoagulants, and, thus, allow sufficient thrombin generation. Results from our previous stu-

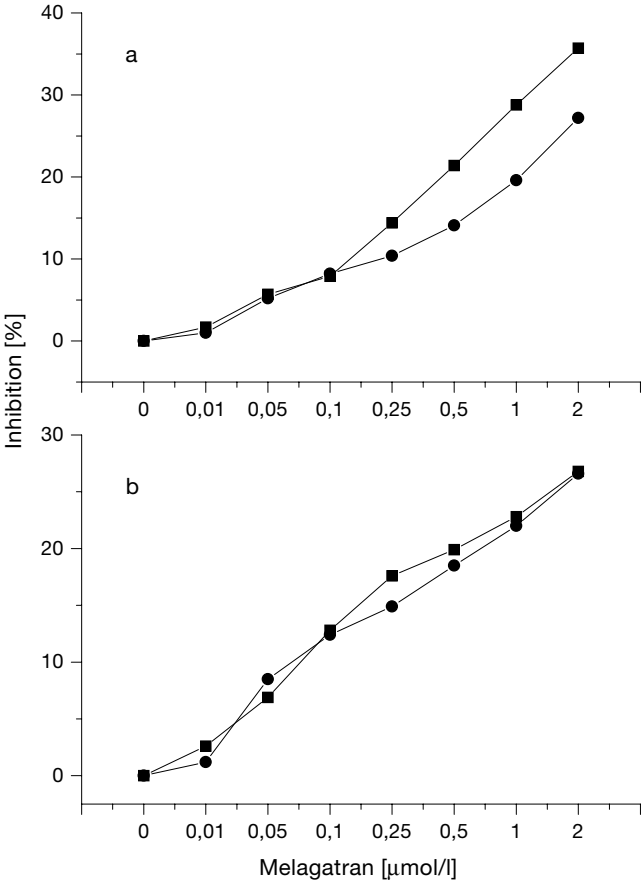


**Fig. 3a, b.** To compare the relative effect of different melagatran concentrations on cord versus adult plasma per cent inhibition referring to the control F 1.2 values in the absence of drug was calculated. Results in percent inhibition are shown under high a) and under low coagulant challenge b). Square: Percent inhibition, adult plasma. Circle: Percent inhibition, cord plasma.

dies confirm this assumption: combined physiological low levels of TFPI, PC, and AT markedly facilitated free thrombin generation in TF-activated plasma samples [5-6].

Usually newborns with thromboembolic complications are treated with heparin followed by oral anticoagulant therapy. Data about use of low molecular weight heparin in pediatric patients with thrombotic disease do also exist [13]. It is speculated that on one hand newborns may be resistant to heparin therapy of thromboembolic disease but, on the other hand, lower plasma heparin levels may be required to prevent formation of thrombin activity in newborns than in adults. The ideal anticoagulant to be used in newborns or adults, is one that can be administered orally, subcutaneous, and intravenously, requires minimal monitoring and has few side effects. The oral DTI ximelagatran is suggested to be an advance in antithrombin therapy because of its oral administration, which results in a rapid conversion to melagatran with low interindividual variability in melagatran plasma levels [14]. The binding to thrombin is competitive and reversible [15]. In animal

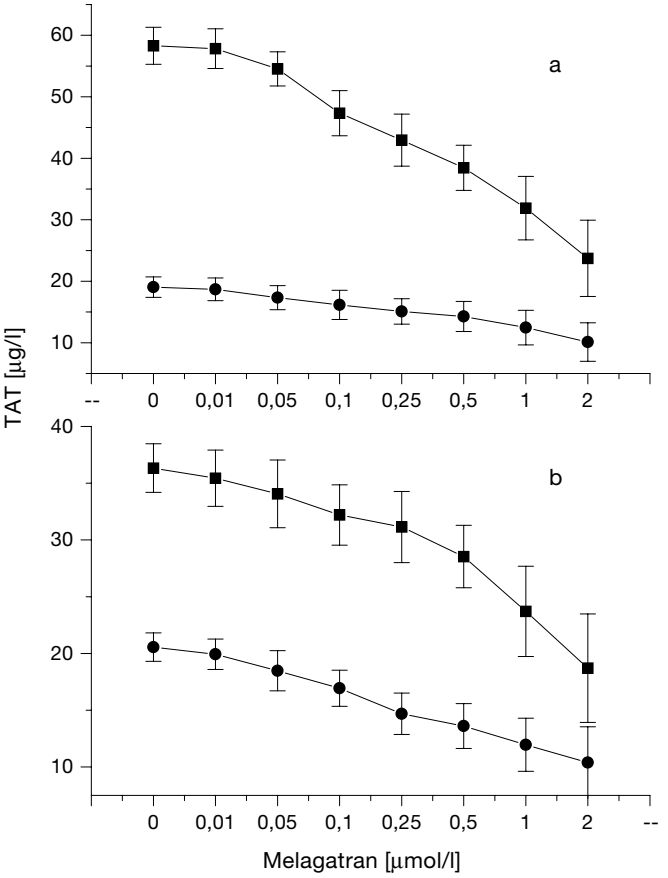


**Fig. 4a, b.** Thrombin-antithrombin (TAT) formation after high and low coagulant activation of cord and adult plasma. Melagatran dose dependently decreased TAT formation in both cord and adult plasma under high a) and under low coagulant challenge b). Square: Melagatran, adult plasma. Circle: Melagatran, cord plasma. Results shown are expressed as means (n = 5).

models of experimental thrombosis [16] and in patients with venous thrombosis [17,18], melagatran seems to be a potent inhibitor. Based on these data, a therapeutic plasma concentration of 0.05 to 0.5 µmol/L was predicted [16]. In thrombosis prevention studies of patients undergoing orthopedic surgery, the risk-benefit ratio of ximelagatran is described [19]. It was suggested by Eriksson et al. that there is no association between dose of melagatran and the incidence of excessive bleeding administered to patients for prophylaxis of thromboembolism after hip or knee replacement [20].

However, there are no in-vitro data about use of melagatran in newborns. Prior to performing clinical trials of melagatran anticoagulant therapy in newborns, the aim of the study was to compare the effects of melagatran on markers of thrombin generation in cord and adult plasmas in vitro. Endogenous thrombin generation can be assessed by assays that measure either activation fragments following activation of coagulation, or enzyme inhibitor complex formation. We, therefore, used F 1.2 and TAT as markers of thrombin generation in adult and cord plasma in the

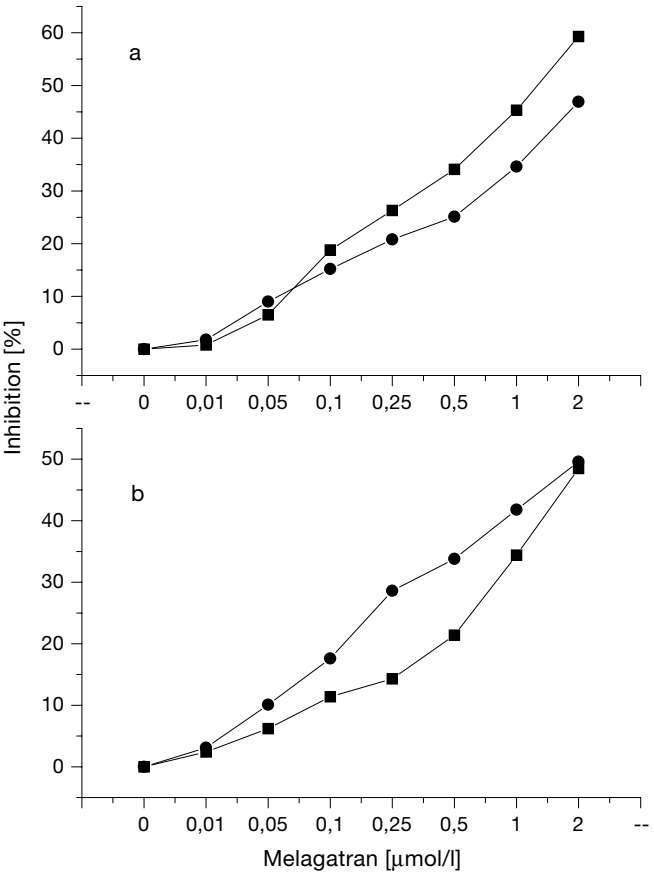


**Fig. 5a, b.** To compare the relative effect of different melagatran concentrations on cord versus adult plasma percent inhibition referring to the control TAT values in the absence of drug was calculated. Results in percent inhibition are shown under high a) and under low coagulant challenge b). Square: Percent inhibition, adult plasma. Circle: Percent inhibition, cord plasma. Results shown are expressed as means (n = 5).

presence of increasing melagatran concentrations. Before measurements of thrombin generation, differences in clotting time was determined. Using a high coagulant challenge, that might exist in conventional assays, adult plasma clotted earlier than cord plasma in the absence and presence of increasing melagatran concentrations. This is in best agreement with the literature showing prolonged PT and APTT in neonates [1]. In contrast, under our low coagulant challenge a complete »change« was observed. Clotting time of adult plasma is significantly delayed compared to cord plasma in the absence or presence of melagatran. As demonstrated in previous studies [5–6], the low anticoagulant capacity of the three inhibitors APC, TFPI, and AT in cord plasma allows enhanced thrombin formation associated with shorter clotting times compared to adult plasma when low amounts of TF are applied to initiate clot formation. As described in several studies, F 1.2 and TAT generation in our study were significantly impaired in cord compared to adult plasmas.

In conclusion, effects of melagatran on cord and adult plasma depend on the coagulant challenge, that is hardly to estimate in clinical situations. Whether our in-

vitro results are significant in-vivo has to be determined in clinical trials, suggesting that similar target ranges for melagatran use in newborns and adults might be justified. Before randomized, controlled trials are initiated, studies in neonates assessing pharmacokinetics and safety/bleeding ratio of melagatran are required.

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# Higher Concentrations of Heparin and Hirudin are Required to Inhibit Thrombin Generation in Tissue Factor-Activated Cord Compared to Adult Plasma

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## Abstract

In standard clotting assays plasma samples are markedly diluted and high amounts of initiator are added to trigger clot formation. Under these conditions, adult plasma clots faster than neonatal plasma and only 30–50 % of peak adult thrombin activity can be produced in neonatal plasma. It was reported that decreased concentrations of heparinoids are required to inhibit thrombin generation in plasma from newborns to the same extend as in adults due to reduced neonatal thrombin potential (TP). Plasma activation by addition of low amounts of lipidated tissue factor (TF) in combination with only slight dilution of plasma samples probably better reflects the conditions in vivo and allows sensitive detection of the effects of anticoagulants on thrombin generation. We have shown recently that under these conditions cord plasma clots faster and higher amounts of thrombin are generated compared to adult plasma. In accordance, we show in the present study that higher amounts of the anticoagulants heparin and hirudin are required in cord plasma for effective inhibition of thrombin generation compared to adult plasma: Prolongation of clotting time and suppression of TP were significantly more pronounced in adult plasma under low coagulant challenge compared with that in cord plasma. In contrast, prolongation of clotting time and suppression of TP were significantly more pronounced in cord plasma under high coagulant challenge. Our results suggest that in neonates effects of anticoagulants very much depend on the type of activation used to initiate clotting and doses of anticoagulants can not be derived from studies done in adults, and that higher doses of thrombin inhibitors may be required.

## Introduction

The hemostatic system of neonates is different to that of children and adults. The risk for thromboembolic complications is considerably less for newborns and children than for adults for any given insult, because neonates have lower levels of procoagulant factors, especially prothrombin and the other factors of the prothrombin complex. This is reflected in prolonged prothrombin time (PT) and activated partial thromboplastin time (APPT). Physiological low levels of anticoagulant proteins in neonatal plasma might compensate for low levels of coagulation factors and allow

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sufficient thrombin generation. Neonatal plasma levels of antithrombin (AT) are reduced to approximately 60 % of adult values [1]. It has been shown that these physiological low AT levels result in an impaired ability of neonatal plasma to inhibit thrombin compared to adult plasma [2].

It has been suggested that decreased concentrations of heparinoids are required to inhibit thrombin generation in plasma from newborns to the same extent as in adults due to reduced neonatal thrombin potential (TP) [3]. However, thrombin generation is only decreased and delayed in newborn plasma compared to adults when plasma samples are markedly diluted due to addition of high amounts of activator, as is usually done in the conventional clotting assays activated partial thromboplastin time (APTT) or prothrombin time (PT). Under these conditions only 30-50% of peak adult thrombin activity can be produced in neonatal plasma [4].

Plasma activation due to addition of low amounts of tissue factor (TF) probably better reflects the conditions *in vivo* [5] and allows sensitive detection of the effects of pro- and anticoagulants on thrombin generation [6]. In previous work we have shown that under such a low coagulant challenge, in contrast to the findings in the presence of high amounts of activator, thrombin generation starts faster than that in adult plasma and higher amounts of free thrombin can be generated in cord compared with adult plasma [7]. This elevated thrombin-forming capacity of cord compared with adult plasma is caused by the low anticoagulant action of the natural inhibitors tissue factor pathway inhibitor (TFPI), antithrombin (AT), and activated protein C (APC) in newborns [7, 8]. Thus, one might assume that higher concentrations of anticoagulants might be required in neonates to inhibit thrombin generation to the same extent as in adults in the presence of low amounts of initiator of clot formation due to increased neonatal thrombin potential (TP).

Therefore, it was the aim of our study to evaluate the anticoagulant action of the anticoagulants heparin and hirudin under low coagulant challenge (better reflecting the conditions *in vivo*) in comparison to that under high coagulant challenge (reflecting the conditions prevailing in standard clotting assays). We investigated both the effect of an indirect and a direct thrombin inhibitor. We measured the effects of increasing amounts of heparin or hirudin on prolongation of clotting time and on suppression of TP in both cord and adult plasma in the presence of high (30 μM final concentration) or low (30 pM final concentration) amounts of TF; in a effort to better understand the mechanisms of anticoagulation with indirect and direct thrombin inhibitors in neonates and provide a rationale for dose finding in neonates.

## Material and Methods

### Reagents

Buffer A contained 0.05 M Tris-HCl at pH 7.4, 0.1 M NaCl and 0.5 M bovine serum albumin. Buffer B was analogous to buffer A but contained in addition 20 mM EDTA. Full length lipidated recombinant human tissue factor (a stock solution was

prepared by dissolving 50 ng of the lyophilized lipoprotein in 4.5 ml buffer A, aliquots of 500 µl were stored at –70°C), and Actichrome TF activity assay were obtained from American Diagnostica Inc. (Greenwich, CT, USA). Heparin (1000 IE/mL) was obtained from Baxter Hyland Immuno, Vienna, Austria. A stock solution of 10 IU/ml was prepared by 1:100 dilution with distilled water. Hirudin was obtained from Sigma, Vienna, Austria. 500 IE were dissolved in 1 mL of distilled water and subsequent 1:10 dilution in buffer A. For strong activation of plasma samples we used Thromborel S as a source of TF, obtained from Behring Diagnostics GmbH (Marburg, Germany), containing human placental thromboplastin and calcium chloride. The fibrin polymerization-inhibitor H-Gly-Pro-Arg-Pro-OH (GPRP, Pefabloc FG) was purchased from Pentapharm LDT (Basel, Switzerland). The chromogenic substrate used for thrombin determination was H-D-Phe-Pip-Arg-pNA.2HCl (pNAPEP 0238) from CoaChrom Diagnostics (Vienna, Austria). Twenty-five mg of the lyophilized substrate were dissolved in 7 ml of distilled water to a concentration of 5.7 mM.

**Collection and Preparation of Plasma**

Cord blood was obtained immediately following the delivery of 16 full term infants (38–42 weeks gestational age). Newborns with Apgar scores of 9 or less five minutes after delivery were excluded from the study. Blood (2.7 ml) was collected into precitrated S-Monovette premarked tubes from Sarstedt (Nümbrecht, Germany), containing 300 µl 0.106 M citrate, centrifuged at room temperature for 10 min at 4100 x g, pooled and stored at –70°C in propylene tubes until assayed. The hematocrit of cord blood was similar to that of adult blood. FV and FVIII activities were elevated over the respective adult values, other pro- and anticoagulant factors were in the normal range for neonates, i.e. cord plasma contained 49 µg/ml prothrombin, 22 ng/ml TFPI and 172 µg/ml AT. In the same way plasma from 14 healthy adults was collected from the antecubital vein, prepared, and checked. Adult plasma contained 103 µg/ml prothrombin, 58 ng/ml TFPI, and 294 µg/ml AT.

**Determination of TF Concentration**

TF procoagulant activity was quantitated by means of the Actichrome TF activity assay.

**Activation of Plasma**

Three hundred eighty µl of plasma were incubated with 20 µl of buffer A containing different amounts of heparin (0–0.5 IU/ml) or hirudin (0–2.5 IU/ml) for 1 min at 37°C. Subsequently, 20 µl of buffer A containing H-Gly-Pro-Arg-Pro-OH (Pefabloc FG, 1.0 mg/ml final concentration) to inhibit fibrin polymerization [12] were added. Subsequently, 40 µl of buffer A were added containing TF (30 pM final concentra-

tion) or Thromborel S (30 μM final concentration of TF) for 1 minute at 37°C. Finally, 20 μl of 0.5 M $CaCl_2$ were added to trigger clot formation.

### Determination of Clotting Time

Plasma was activated as described above except that Pefabloc FG was replaced by buffer A. Clotting times were determined by means of the optomechanical coagulation analyzer Behring Fibrintimer II from Behring Diagnostics GmbH (Marburg, Germany), which applies the turbodensitometric measuring principle.

### Determination of Thrombin Generation

We used a subsampling method derived from a recently described technique [4–5, 14]. Plasmas were prepared and activated as described above. At timed intervals 10 μl aliquots were withdrawn from the activated plasma and subsampled into 490 μl buffer B containing 255 μM pNAPEP 0238. Amidolysis of pNAPEP 0238 was stopped after 6 min by addition of 250 μl 50% acetic acid. The amount of thrombin generated was quantitated by measuring the absorbency by double wave length (405–690 nm) in the Anthos microplate-reader 2001, from Anthos Labtec Instruments GmbH, Salzburg, Austria. The total amidolytic activity measured is caused by the simultaneous activity of free thrombin and the alpha 2-macroglobulin-thrombin complex. Free thrombin generation curves were calculated by mathematical treatment of total amidolytic activity curves using a method developed by Hemker et al.

The area under the free thrombin generation curve has been called »thrombin potential (TP)«. The TP has been shown to be a suitable parameter to reflect the thrombogenic potency in a given plasma sample [9].

### Statistical Analysis

Results obtained in cord and adult plasma were compared by means of Mann Whitney U test. The effects of different concentrations of heparin or hirudin on clotting time, and TP were analyzed using Wilcoxontest. The significance level of p-values was set at 5%. Calculations were performed using Winstat 3.1 (Kalmia Co. Inc., Cambridge, MA, USA).

## Results

### Effect of Increasing Amounts of Heparin on Clotting Time

Under both low and high coagulant challenge (30 pM and 30 μM of TF were applied to trigger clot formation, respectively) clotting time was dose-dependently prolonged in both cord and adult plasma in the presence of increasing amounts of

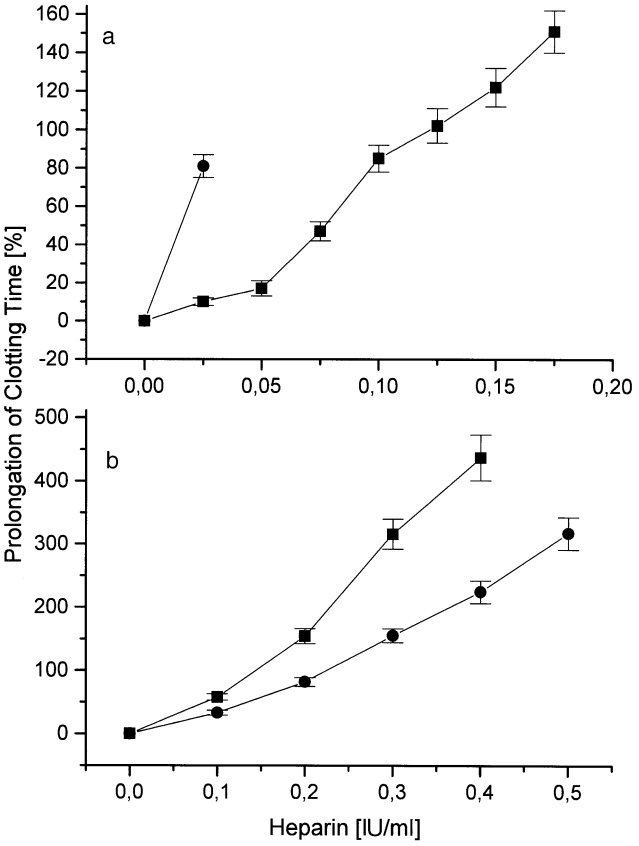


**Fig. 1a, b.** Effect of addition of heparin on prolongation of clotting time in cord (■) and adult plasma (●) in the presence of 20 pM (panel a) and 30 μM (panel b) of tissue factor to induce clot formation. No clot formation within 15 min was observed in adult plasma under low coagulant challenge at heparin concentrations exceeding 0.025 IU/ml and in cord plasma at heparin concentrations exceeding 0.125 IU/ml (panel a). No clot formation within 15 min was observed in adult plasma under high coagulant challenge at heparin concentrations exceeding 0.5 IU/ml and in cord plasma at heparin concentrations exceeding 0.4 IU/ml (panel b).

heparin (Fig. 1). Whereby, prolongation of clotting time was significantly more pronounced in adult plasma under low coagulant challenge compared with that in cord plasma (P of differences < 0.01, panel A). On the contrary, prolongation of clotting time was significantly more pronounced in cord plasma under high coagulant challenge compared with that in adult plasma (P of differences < 0.01, panel B).

### Effect of Increasing Amounts of Heparin on Thrombin Potential

Under both low and high coagulant challenge suppression of thrombin generation dose-dependently increased in the presence of increasing amounts of heparin in both cord and adult plasma (Fig. 2). Whereby, the anticoagulant action of heparin was significantly more pronounced in adult plasma under low coagulant challenge compared with that in cord plasma (P of differences < 0.01, panel A). On the contrary, suppression of thrombin generation due to addition of heparin was significantly more pronounced in cord plasma under high coagulant challenge compared with that in adult plasma (P of differences < 0.01, panel B).

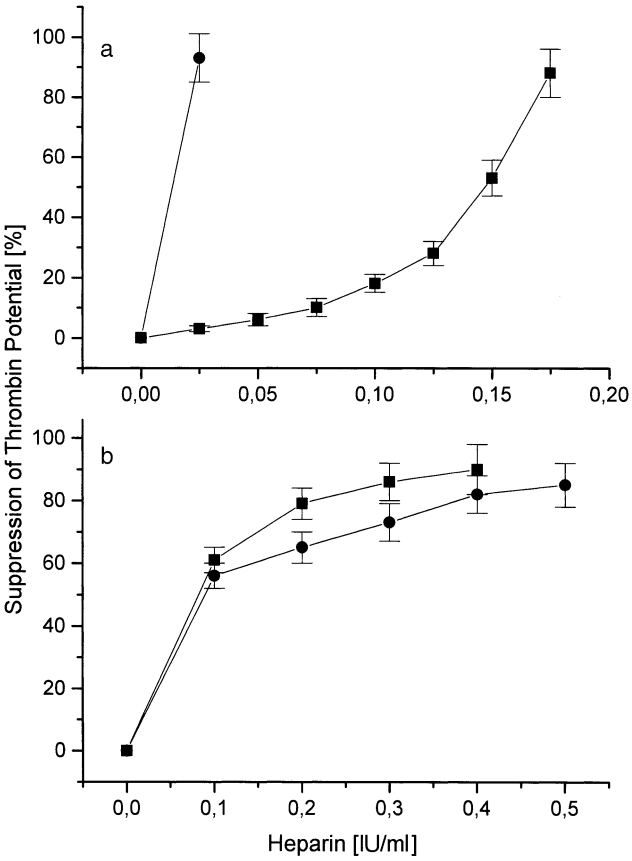


**Fig. 2a, b.** Effect of addition of heparin on suppression of thrombin potential in cord (■) and adult (●) plasma in the presence of 20 pM (panel a) and 30 μM (panel b) of tissue factor to induce clot formation. Virtually no thrombin formation within 15 min was detected in adult plasma under low coagulant challenge at heparin concentrations exceeding 0.025 IU/ml and in cord plasma at heparin concentrations exceeding 0.175 IU/ml (panel a). Virtually no thrombin formation within 15 min was observed in adult plasma under high coagulant challenge at heparin concentrations exceeding 0.5 IU/ml and in cord plasma at heparin concentrations exceeding 0.4 IU/ml (panel b).

**Effect of Increasing Amounts of Hirudin on Clotting Time**

After activation with high and small amounts of TF the clotting time was dose-dependently prolonged in both cord and adult plasma in the presence of increasing amounts of hirudin (Fig. 3). Whereby, prolongation of clotting time was significantly more pronounced in adult plasma after activation with small amounts of TF compared with that in cord plasma (P of differences < 0.01, Fig. 3 panel a). On the contrary, prolongation of clotting time was significantly more pronounced in cord plasma after activation with high amounts of TF compared with that in adult plasma (P of differences < 0.01, Fig.3 panel b)

**Effect of Increasing Amounts of Hirudin on Thrombin Potential**

After activation with high and small amounts of TF suppression of thrombin generation dose-dependently increased in the presence of increasing amounts of hiru-

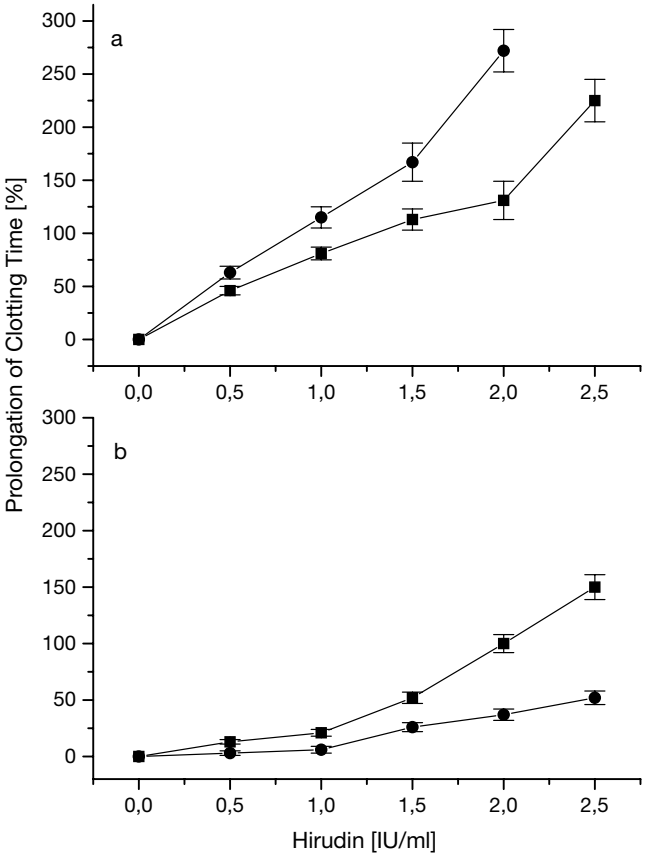


**Fig. 3a, b.** Effect of addition of hirudin on prolongation of clotting time in cord (■) and adult plasma (●) in the presence of 20 pM (panel a) and 30 μM (panel b) tissue factor to induce clot formation. No clot formation within 15 min was observed in adult plasma under low coagulant challenge at hirudin concentrations exceeding 2.0 IU/ml and in cord plasma at hirudin concentrations exceeding 2.5 IU/ml (panel a). Both cord and adult plasma clotted within 15 min under high coagulant challenge when hirudin concentrations up to 2.5 IU/ml were added.

din in both cord and adult plasma (Fig. 4). Whereby, the anticoagulant action of hirudin was significantly more pronounced in adult plasma after activation with small amounts of TF compared with that in cord plasma (P of differences < 0.01, Fig. 4 panel a). On the contrary, suppression of thrombin generation due to addition of hirudin was significantly more pronounced in cord plasma after activation with high amounts of TF compared with that in adult plasma (P of differences < 0.01, Fig. 4 panel b).

## Discussion

The excellent hemostasis in neonates despite physiological low levels of procoagulant proteins is not completely understood so far [10]. Physiological low levels of inhibitors might compensate for low levels of procoagulants, and thus allow sufficient thrombin generation. Results from our previous studies confirm this assumption: the combined physiological low anticoagulant action of the three inhibitors of

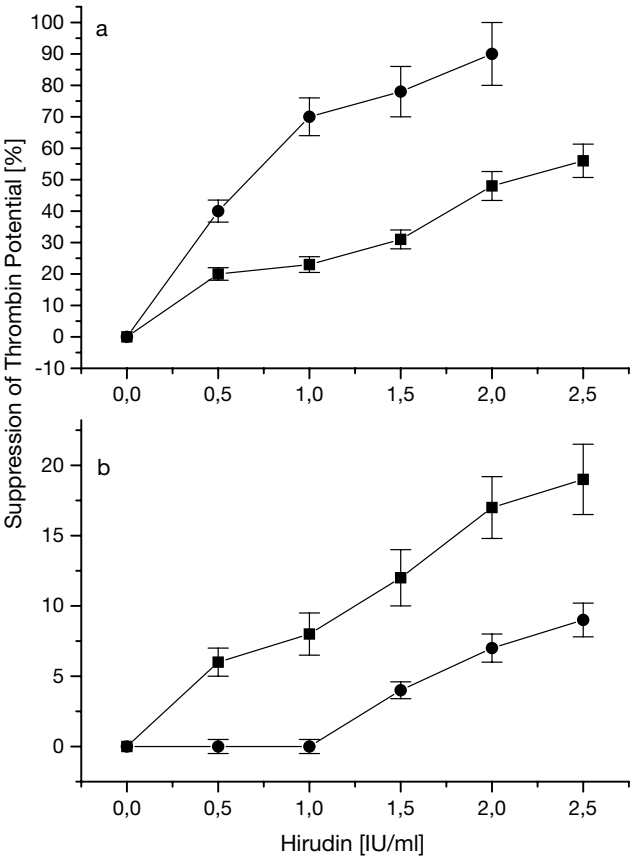


**Fig. 4a, b.** Effect of addition of hirudin on thrombin generation in cord (■) and adult plasma (●) in the presence of 20 pM (panel a) and 30 μM (panel b) tissue factor to induce clot formation. Virtually no thrombin formation within 15 min was observed in adult plasma under low coagulant challenge at hirudin concentrations exceeding 2.0 IU/ml and in cord plasma at hirudin concentrations exceeding 2.5 IU/ml (panel a).

TFPI, AT and activated protein C (APC) in cord plasma allows enhanced thrombin formation associated with shorter clotting times compared with adult plasma when low amounts of TF are applied to initiate clot formation [11]. These findings are in good agreement with the clinically observed well- functioning hemostasis.

In conventional clotting assays plasma samples are markedly diluted and high amounts of initiator are applied to trigger clot formation. Under these conditions adult plasma clots faster than neonatal plasma and the capability of neonatal plasma to generate thrombin is delayed and reduced as compared with that in adult plasma, due to decreased prothrombin and increased $\alpha_2$-macroglobulin [1-2, 12]. It has been shown that under these conditions lower amounts of anticoagulants are required in neonatal plasma to suppress thrombin generation to the same extent as in adult plasma [3].

The high coagulant challenge applied in standard clotting assays probably does not reflect the conditions prevailing in the clinical situation. Marked dilution of plasma samples completely alters the kinetics of the enzymatic reactions on which the blood coagulation process is based on [13]. Additionally, the high amounts of

initiator (thromboplastin applied in conventional clotting assays contains TF in the micromolar range) might not be present in the clinical situation. Thus, activation of plasma samples by means of low amounts of initiator (i.e. TF in the picomolar range) is applied in the majority of laboratory investigations in last years [6, 7, 13]. It has been shown recently that under this low coagulant challenge, in contrast to the findings under high coagulant challenge, cord plasma clots faster than adult plasma and higher amounts of thrombin are generated in cord compared to adult plasma [4]. We show in the present study that under this low coagulant challenge significantly higher amounts of the anticoagulants heparin (indirect thrombin inhibitor) and hirudin (direct thrombin inhibitor) are required to inhibit thrombin generation in cord plasma to the same extent as in adult plasma. Completely contradicting the dosage requirements derived from experiments performed in the presence of high amounts of initiator [3].

However, a reservation has to be stated. Our experiments were performed in cord and not in neonatal plasma due to ethical reasons. The coagulation system has been shown to be at an activated state immediately after birth. I.e., elevated FV, FVII, and FVIII activities have been detected in cord plasma [14], not present in neonatal plasma. Additionally, cord and venous blood differ in their hematocrit [15, 16], influencing the concentrations of citrate and both pro- and anticoagulant proteins in plasma.

In conclusion, whereas decreased amounts of anticoagulants are required in neonatal plasma to suppress thrombin generation to the same extent as in adults under high coagulant challenge, results from our in vitro study suggest that increased amounts of anticoagulants are required to suppress thrombin generation to the same extent as in adults under low coagulant challenge. If we assume that low coagulant challenge reflects in vivo situation better, our study might suggest that higher doses of thrombin inhibitors, both direct and indirect, may be required in neonates.

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# Molecular Genetic Analysis in Patients with Inherited Thrombophilia and Antithrombin, Protein C or Protein S Deficiency

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## Introduction

Thrombophilia is a disorder associated with an increased tendency to either inherited or acquired venous thrombosis. The three classic single-gene disorders causing inherited thromboembolic disease affect the coagulation inhibitors- antithrombin (AT), protein C (PC), and protein S (PS), each being responsible for roughly 5% of familial thromboses. The three genes are located on chromosome 1, 2 and 3, respectively (Fig. 1). The mature AT protein of 432 aa residues is encoded by exons 2 to 6 of AT gene [4]. Nine exons code the PC protein that comprises 461 aa residues [2, 3]. The PS protein consists of 636 amino acids encoded by 15 exons [1]. Studies of the molecular basis of hereditary AT, PC and PS deficiencies showed a great variety of mutations occurring throughout the genes. The heterogeneity of the mutations hampered rapid genetic analysis in affected patients.

## Materials and Methods

Twelve families – 2 with protein C, 1 with protein S, and 9 with antithrombin deficiency, have been examined. All the exons and the 5'flanking region of each of the three genes were amplified and the PCR samples screened by Denaturing High Performance Liquid Chromatography (DHPLC) followed by direct sequencing of the fragment showed abnormal pattern (Fig. 2).

## Results and Discussion

By this approach we identified 9 mutations, 2 in PC, 1 in PS and 6 in AT, which have been found by examination of patients from eleven families with clinically expressed deficiencies of PC [1], PS [1] and AT [8]. In AT deficiency we found four missense mutations (S116P, L99F, K114E, S162R) and one stop mutation (W49Stop).Two of the patients had one and the same mutation (S116P). All four missense mutations affected the binding site of heparin and have been previously reported. In three of the families with AT deficiency no mutation has been found. The mutations found in PC and PS deficiencies patients were a 4 bp insertion (CCTG) and 1 missense mutation (R230S) in exon 9 of the PC gene and an 1 bp (T) deletion in exon

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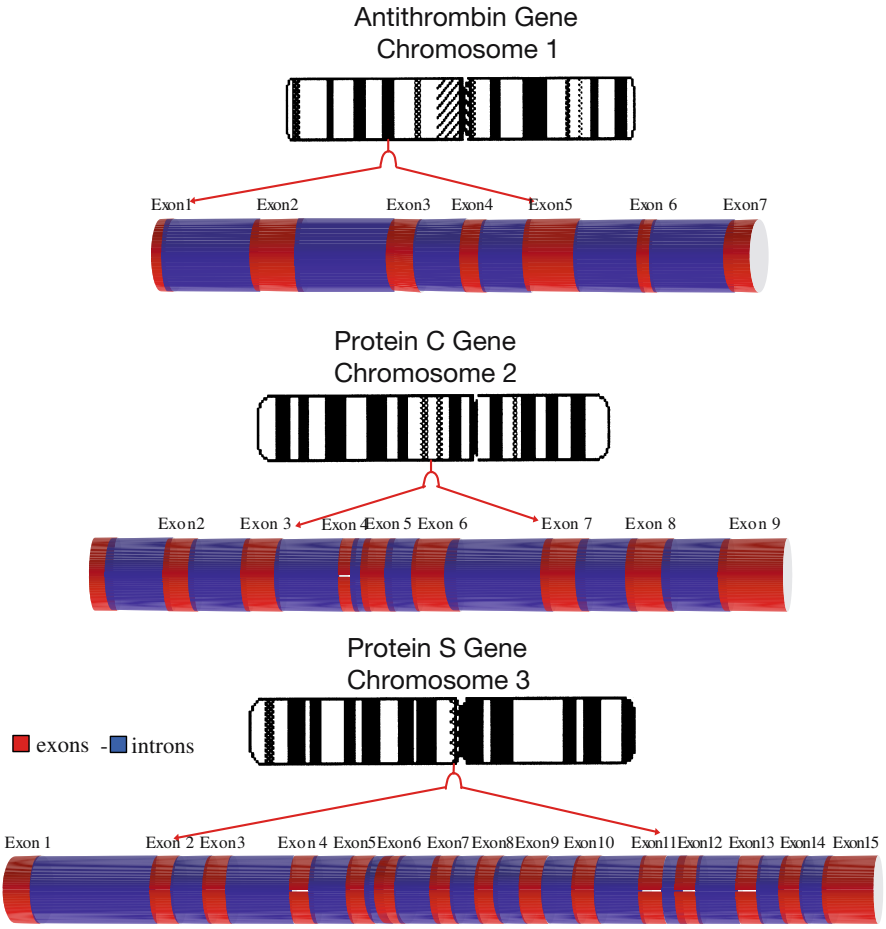


**Fig. 1.** Schematical presentation of the chromosomal location and structure of AT, PC and PS genes

15 of the PS gene. Both, the deletion and the insertion led to a frame shift with a subsequent stop codon and therefore should be regarded as causative for the phenotype (Table 1) [5].

## Conclusion

We conclude that the strategy of using DHPLC as a mutation screening method followed by direct sequencing of a single fragment with abnormal pattern represents rapid and reliable approach for the mutation analysis for AT , PC and PS genes.

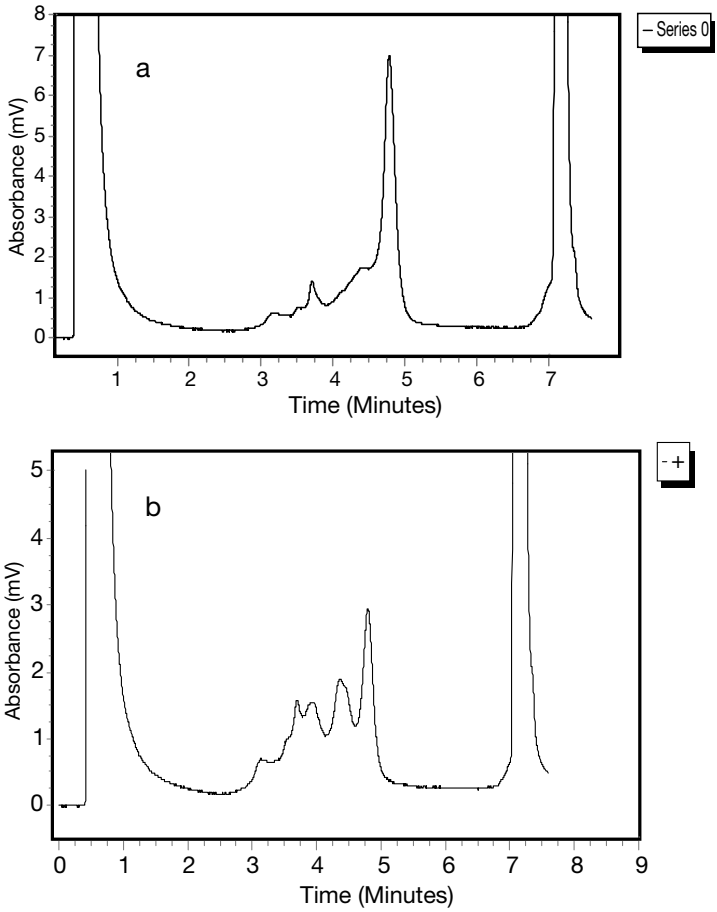


**Fig. 2a, b. a)** DHPLC chromatogram of exon 15 of wild type protein S sample; **b)** DHPLC chromatogram of exon 15 of mutated Protein S sample (T del 618)

**Table 1.** Overview of the detected mutations

| gene | number of mutations | type of mutation | location of mutation |
|---|---|---|---|
| protein C | 1 | small insertion | CCTG ins 372 |
| | 1 | missense mutation | R 230 S |
| protein S | 1 | small deletion | T del 618 |
| antithrombin | 5 | stop codon | W49Stop |
| | | missense mutation | S116P |
| | | missense mutation | L99F |
| | | missense mutation | K114E |
| | | missense mutation | S162R |

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# Analysis of Thrombophilic Risk Factors in Patients Suffering from Ocular Thrombotic Complications

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## Objective

Non-arthritic ischemic optic neuropathy or ocular venous thrombosis may cause sudden unilateral loss of vision sometimes complicated by contralateral thrombotic events some years later. Retinal hemorrhages may occur due to thrombosis, but hemodilution, administration of corticosteroids and inhibitors of platelet aggregation is standard therapy prior to effective anticoagulation [1].

Effectivity of the therapy has been discussed controversially. Minor therapeutic results of this regimen could be related to the patients` underlying hypercoagulable state and impaired plasmatic coagulation.

We report preliminary results of hemostaseologic screening for thrombophilic risk factors in 12 consecutive patients suffering from acute ocular thrombotic complications.

## Patients Characteristics

12 patients (17 to 78 years old) suffered from acute, almost complete loss of vision due to thrombotic occlusions either of central vein (n=6), minor ocular veins(n=3), ischemic optical neuropathy(n=2), or arterial thrombosis(n=1), respectively. In 4 patients, loss of vision had been recognized more than 72 hours prior to admission.

We performed the following hemostaseologic examinations for thrombophilic risk factors: coagulation factor analysis, lupus anticoagulant, anticardiolipin antibodies, procoagulant factors (protein C, protein S, antithrombin), and indicators of fibrinolysis (PAI-1, tissue factor, D-dimers).

Molecular analysis was done for MTHFR C677T, Factor V Leiden G1691A, or prothrombin gene G20210A mutations. The patients were checked for autoimmune vasculitis, diabetes mellitus, and hyperlipoproteinemia.

## Hemostaseologic Results

Thrombophilic diathesis was diagnosed in almost all patients: MTHFR gene mutation C677T was found in patients, one of them homozygous; Factor V Leiden G1691A in 2; and prothrombin gene mutation G20210A in 2 patients. We found dia-

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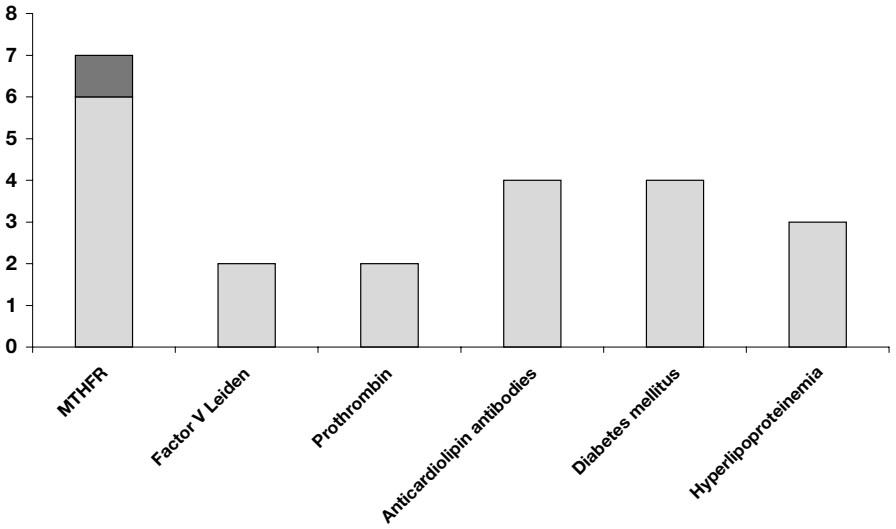


**Fig. 1.** Hypercoagulable state, characterization of risk factors

betes mellitus in 4 patients, hyperlipoproteinemia in 2 patients. Anticardiolipin antibodies were seen in 4 patients (Fig. 1).

## Conclusions

Concerning our hemostaseologic data ocular thrombosis seems to resemble hypercoagulable state due to plasmatic disorders of coagulation [2]. Therefore, anticoagulation with low-molecular weight heparin additional to standard therapy might be a useful therapy in patients suffering from acute ocular complications.

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# Protein C Concentrate for the Treatment of Veno Occlusive Disease in a boy with Nephroblastoma

K. Kentouche, H.J. Mentzel, J. Hermann and F. Zintl

Veno occlusive disease of the liver is a severe complication of intensive chemotherapy. Most often seen in the setting of stem cell transplantation, it sometimes occurs in the high dose chemotherapy courses of conventional tumor treatment protocols. Clinical signs are painful hepatomegaly, weightgain due to ascites and renal insufficiency which is believed to be of prerenal origin like in the hepato-renal syndrome. Diagnosis is based on clinical criteria: hyperbilirubinemia > 2 mg/ dl, painful hepatomegaly, fluidretention, weightgain > 5% or ascites. Ultrasound-Doppler-sonography may reveal a retrograde flow in the portal veins. Although the mechanism is not fully understood there is sinusoidal endothelial damage of the liver acinus with hepatocyte necrosis and denudation of the subendothelium with consecutive thrombosis. Narrowing of the sinusoidal lumen is augmented by secondary fibrosis. Inflammatory, thrombotic and fibrotic processes stand side by side in the pathogenesis of VOD [2]. Severity of VOD is graded according to the outcome. Mild VOD is selflimited and the patient should be treated with analgesics only for painful hepatomegaly, whereas moderate VOD requires additional diuretics for fluid-balance. Severe VOD is defined as causing death within the first 100 days after SCT. About 50 % of patients with VOD after SCT die with this condition. Low levels of protein C (PC) have been reported in patients with VOD [1]. This report is on treatment of a patient with VOD using a PC concentrate.

A 6.5-year-old boy was diagnosed with clear cell sarcoma of the right kidney. Treatment followed the treatment protocol SIOP Nephroblastom 2001 (Fig. 1). Tumornephrectomia followed a four week course of preoperative chemotherapy. Postoperative treatment consisted of actinomycine D, vincristine and doxorubicine.

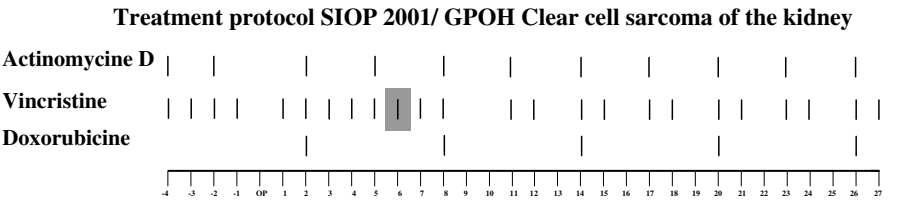


**Fig. 1.** VOD ■ developed in the sixth week of the postoperative treatment after the second application of actinomycine D. The following Vincristine was delayed for one week and actinomycine was reduced to 66 % of the original dosage. All further treatment was applied according to the treatment protocol, without any hint for reoccurrence of VOD.

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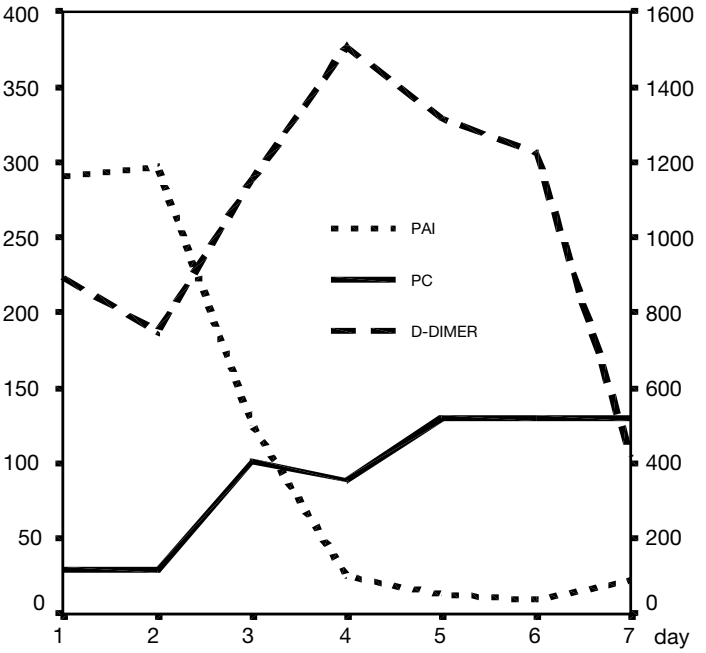


**Fig. 2.** PC-concentrate was infused with 60 U/kg BW from the second day on every 12 hours. Fig. 3. shows the course of PC-activity (%) and PAI-1-activity (U/ml) on the left axis and d-dimer levels (µg/l) on the right axis. After restoration of PC-activity inhibitory activity of plasminogen activator inhibitor decreases. Fibrinolysis is enhanced as shown by elevation of d-dimers.

After the second application of actinomycine D platelet count dropped from 860 to 7 x $10^9$/l within one week. There was an increase in the patients weight of 10 %, hyperbilirubinemia of 2 mg/dl and an 1.5-fold increase in serumcreatinine. Sonographic findings were hepatomegaly, ascites and a retrograde flow in the portal veins (Fig. 3). With the diagnosis of VOD of the liver established heparin dosage was increased to 200 IU/ kg/day. Antithrombin was administered to keep activity at 100 %. PC-activity was decreased to 29 %. From the 2$^{nd}$ day of treatment on the patient received 60 IU/ kg of a plasma derived PC concentrate (Ceprotin) twice daily to keep PC-activity above 100%. Within 4 days the boy recovered from VOD. Elevated PAI-1-activity (297,1 U/ ml) decreased to normal and fibrinolysis measured by D-Dimers was restored (Fig. 2). Dopplersonography revealed a normal flow in the V.portae (Fig. 3). On the 5$^{th}$ day he developed epistaxis which could be stopped by tamponation. Therapy with PC was stopped on the 6$^{th}$ day. After recovery chemotherapy was continued. 6 months after VOD and 2 months after completion of the treatment protocol the patient is well and there is no hint for tumor-recurrence or long term sequelae of the VOD.

VOD of the liver is a life-threatening disease, in which the outcome is not predictable. Therapeutic options, derived from studies in stem cell transplant patients, include prophylactic administration of heparin, antithrombin, ursodiole and

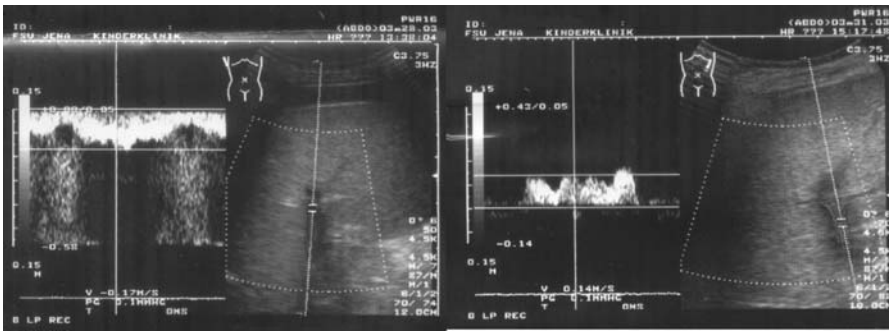

**Fig. 3a, b.** Sonographic demonstration of the change of blood flow direction in the portal vein. **a)** shows retrograde flow before treatment. **b)** normal antegrade flow after three days of PC administration.

n-acetyle glucosamine and therapy with tissue plasminogen activator, prostaglandine E or defibrotide [3]. Our patient differs from patients in the aforementioned studies, as he did not receive a myeloablative therapy. VOD developed solely by a conventional chemotherapy. Nevertheless it was felt to be a life threatening event. In our patient VOD led to an acquired PC deficiency. The anticoagulant and fibrinolytic properties of PC were the rationale to administer PC concentrate. The fibrinolytic response to PC measured by PAI-1 and D-Dimer levels supports it's beneficial effect. Treatment resulted in full recovery from VOD and was well tolerated except for controllable slight nose bleed.

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## *VIId. Molecular Diagnostics*

# Homozygous Factor X Gene Mutation Gly380Arg is Associated to Perinatal Intracranial Hemorrhage

F. H. Herrmann, M. Navarette, L. Salazar-Sanchez, J. M. Carillo, G. Auerswald and K. Wulff

Factor X (FX) deficiency is a very rare hemostatic disorder inherited as an autosomal recessive trait. The factor X gene (F10) is located on chromosome 13q14 and contains eight exons [1]. The variable severity manifested by individuals with FX deficiency correlates poorly with laboratory phenotype [2]. We have analyzed in an international research study more than 102 subjects with reduced FX level from 34 different centres in order to detect the molecular defect and to characterize the phenotype-genotype relationship. Central nervous system (CNS) bleeds are rare but one of the most severe symptoms of FX deficiency, that occurs perinatally or in early childhood. This study reports data from homozygous patients with CNS bleeds, which enable us to associate this severe clinical phenotype to the homozygous condition of specific mutations.

## Subjects and Methods

### Patients

The identification of patients with FX deficiency registered in the Greifswald study of FX deficiency have been clinically evaluated and hemostasiologically analyzed by the local hospitals. The here described patients were from different places from Costa Rica. The analysis of hemostasiological parameters and treatment was done in Children Hospital of CCSS, San Jose (Costa Rica). The CNS bleeds were analyzed by computerized tomography.

### Molecular genetical study

EDTA-blood was collected from patients with FX deficiency. The molecular basis of FX deficiency was analyzed by sequencing of the F10 gene (all coding regions, the exon–intron boundaries and the 5'flancing region containing the promotor) in probands with reduced FX level. All sequences were analyzed at least twice [3, 4].

The haplotype of FX alleles were determined by the following polymorphisms: hexanucleotide (TTGTGA) deletion in nt-343 to nt-348 (Symbol A), base changes in the promotor region –222C>T (symbol B) and –220C>A (symbol C), polymorphisms in intervening regions IVS1-55ins16bp (E); IVS2-16C>T (G); IVS3+98A>C

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(H) as well as the exon 7 polymorphism Thr224Thr (817T>C, symbol D). The frequent alleles of the studied polymorphisms are marked by 1, the rare alleles by 2. The polymorphisms were identified by sequencing [5] or in cause of deletion TTGTGA by heteroduplex analysis [6]. The FX haplotypes were detected by analysis of homozygosity of the mutations.

## Results and Discussion

In the Greifswald research study of FX deficiency 102 subjects (patients and family members) with reduced FX activity are characterized by DNA sequencing. 29 different FX gene mutations are detected among these 102 patients: 26 missense mutations, 2 deletions, one splice site mutation and one double mutation. 17 of these mutations are novel mutation, previously unreported.

42 of 102 patients with analyzed F10 gene defects have clinical symptoms (Fig. 1).

One of the most severe symptom of FX deficiency is central nervous systems hemorrhage (CNS bleed). CNS bleeds occurred in 6 homozygous FX deficient patients: 5 patients are homozygous for the missense mutation Gly380Arg. One patient with CNS bleed is homozygous for the FX gene deletion Tyr163delAT.

The clinical symptoms of homozygous patients for the missense mutation Gly380Arg are summarized in Table 1. Family histories were negative for bleeding diathesis. The heterozygous parents were asymptomatic. 5 of these 6 homozygous patients suffered from CNS bleeds. Further bleeding symptoms are gastrointestinal (GI) bleeds, epistaxis, gum bleeds, easy bruising, hematomas, hematuria, smooth tissue bleeds.

In all cases the CNS bleeds occured perinatally. The patients were treated with FIX complex concentrates.The sequelae to intracranial hemorrhage in patient 1 are blindness, deafness and learning problems. Patient 2 suffers from partial paralysis

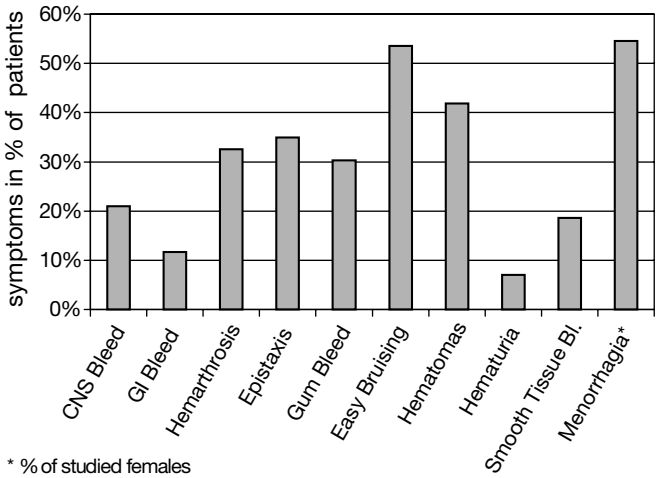


**Fig. 1.** Bleeding symptoms of symptomatic FX deficiency patients with analyzed F10 gene mutations. (Greifswald research study of FX deficiency)

**Table 1.** Characterization of homozygous patients of the Gly380Arg substitution of FX

| Case-Number | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| DNA-Number | 11648 | 11632 | 11653 | 11651 | 11661 | 11683 |
| Sex | m | m | m | f | m | m |
| born | 15.9.89 | 26.9.92 | 23.12.98 | 24.12.97 | 24.7.01 | 9.6.98 |
| FX: C | <1 | <1 | <1 | <1 | <2 | <1 |
| FX:Ag | | <1 | <1 | | | <1 |
| Symptoms: | | | | | | |
| CNS bleed | + | + | + | + | + | no |
| days after birth | 7 | 7 | 5 | 27 | 16 | – |
| GI bleed | + | – | + | – | – | + |
| Hemarthrosis | – | – | – | – | – | – |
| Epistaxis | – | + | – | – | – | – |
| Gum bleed | – | – | – | – | + | + |
| Easy bruising | – | – | + | – | + | + |
| Hematomas | + | + | – | – | + | – |
| Hematuria | – | + | – | – | – | – |
| Smooth tissue bl. | + | – | – | – | – | – |

and he attends a special school. Patient 3 is in a special school too with adequate learning results. Patient 4 presents with epilepsy and developmental retardation. Patient 5 suffered from hydrocephalus and died in the age of 15 month. Patient 6 has no severe problems because he was early diagnosed and treated with prothrombin complex concentrates.

The mutation Gly380Arg was recently described in Italy in a compound heterozygous patient without bleeding manifestation [7]. In our international research study of FX deficiency were included patients from Germany, Costa Rica, Poland, Slovakia, Sweden and Venezuela. We could detected this mutation only in Costa Rica, and here in homozygous conditions in 6 patients with FX deficiency. This is the first description of homozygosity for the Gly380Arg mutation.

The haplotype of the studied Arg380 alleles is in all cases the same (A1, B1, C2, E1, G1, H1, D1), indicating that the mutation is probably of the same origin. The prevalence of 6 homozygous patients for Gly380Arg in Costa Rica seems to be caused by a founder effect . All these 6 homozygous patients show severe FX deficiency and 5 of them suffer from CNS bleeds. The CNS bleeds seem to be associated specifically to the Gly380Arg mutation (genotype-phenotype-correlation).

In human FXa protein Gly380 is located immediately after the active site 379. As shown by molecular modeling the substitution of nonpolar Gly with the polar Arg leads to the formation of new hydrogen bonds with Ala 234. The very large side chain of Arg causes alteration of the protein folding and could lead to secretion problems [7], which explains the phenotype. In vitro expression for the mutation is under investigation.

The homozygous girl for the novel deletion Tyr163delAT (born 02.06.1992) has FX:C 1% (FX:Ag <1). Five days after delivery she suffered from CNS bleed. She presents now light motor problems and attends regular school without learning difficulties. With four years a gastrointestinal bleed was observed. Now she has severe menstrual bleeds for which she is treated with PCCs in higher dosis.

The novel deletion Tyr163 del AT interrupts the reading frame and leads to a stop in codon 163 (T*AT* CAT>TCAT, the new stop codon is underlined) in exon 6 of F10 gene. This premature termination of the FX protein caused the severe FX deficiency, because no active FX can be produced. The clinical manifestation results from this molecular defect.

Probably this mutation correlates to CNS bleeds too. Because until now only this one homozygous case is described, for final conclusion more homozygous patients with the same mutation are needed.

Severe bleeders with FX deficiency are classified as those who had spontaneous and /or life-threatening bleeding episodes, such as hemarthrosis, muscle hematomas, GI and CNS hemorrhage [8]. The prevalence of CNS bleeds was in our study 21% and 9% in symptomatic patients from Iran (3/32) [2] . In the few described case reports of FX deficiency with intracranial hemorrhage [9–11] the molecular defect of F10 gene was not determined. Phenotype-genotype-correlation for CNS bleeds in FX deficiency was not done in previously reported studies. For reliable phenotype-genotype correlations more homozygous patients for the causative mutation are needed. The here reported prevalence of perinatal CNS bleeds in five FX deficient patients with the homozygous causative mutation Gly380Arg indicates the association of this symptom to this mutation. The relationship of CNS bleed to the causative mutation Tyr163delAT should be confirmed by other homozygous patients for the same mutation.

On the basis of these molecular biological results genetic counseling and prenatal diagnosis in the corresponding families are possible.

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# Mutation Analysis of the C1 Inhibitor Gene

T. Förster, A. Kocot, J. Schröder, E. Aygören-Pürsün, I. Martinez-Saguer, W. Kreuz, K. Bork, I. Scharrer, C. Müller and J. Oldenburg

## Introduction

The C1 inhibitor (C1INH), a member of the serpin family of proteinase inhibitors, is involved in the activation of the complement system, the contact system of kinin generation and the intrinsic coagulation pathway. It is the sole inhibitor of the C1r and C1s components of the classical complement pathway and the major regulator of factors XI and XII and of plasma kallikrein.

The importance of the C1 inhibitor is illustrated by hereditary angioedema (HAE, MIM#106100), which develops in individuals who are heterozygous for a deficiency or dysfunction of C1INH [4, 6]. Clinically, HAE presents as edema of the extremities, face, trunk, airways or abdominal viscera, often triggered by psychological and/or physical stress. If not treated properly, swelling of the larynx can be fatal. According to the antigenic plasma level of the C1INH protein two types of HAE are distinguished. Type I HAE is evident in 85% of patients and is characterized by reduced levels of C1INH protein and function (5-30 % of normal). Reported mutations resulting in this type of disease include large deletions, duplications and single-base changes. Type II HAE is evident in 15% of HAE patients and is characterized by normal or raised antigenic levels of C1INH that have diminished function. Mutations causing Type II HAE, primarily point mutations, are typically found within the active site or in the proximal hinge region, which is involved in the proper folding and presentation of the reactive loop [1].

The C1INH gene maps to chromosome 11q12-q13.1 and consists of 8 exons distributed over a DNA stretch of 17 KB, with most introns particularly rich in repetitive Alu sequences [3]. Deletions and duplications caused by these repeats account for approximately 12% of all mutations. 40 % of the mutations are described as missense mutations, 31 % as small deletions and insertions, 8 % as splice site mutations and 7% as nonsense mutations. 2% of all published mutations are reported to be promotor mutations [2].

## Patients and Methods

Patients suspicious for hereditary angioedema were sent from specialized outpatient clinics in Frankfurt and Mainz.

All 8 exons of the C1 Inhibitor gene and the flanking intronic regions were sequenced.

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The high percentage of large deletions and insertions caused by the frequent intronic Alu-Sequences was analyzed by Southern Blotting after digestion of genomic DNA with BclI [5].

## Results

So far 90 families comprising 151 members have been analyzed by direct sequencing of all 8 exons. In 64 families with 98 members, we found 50 different mutations that have not been published before (Fig. 1). In 19 families with 41 members we detected mutations that were already known from the literature. In 7 families with 12 members we detected large deletions by Southern Blotting. The distribution of the different kinds of mutations in our sample corresponds well to the published data (Fig. 2).

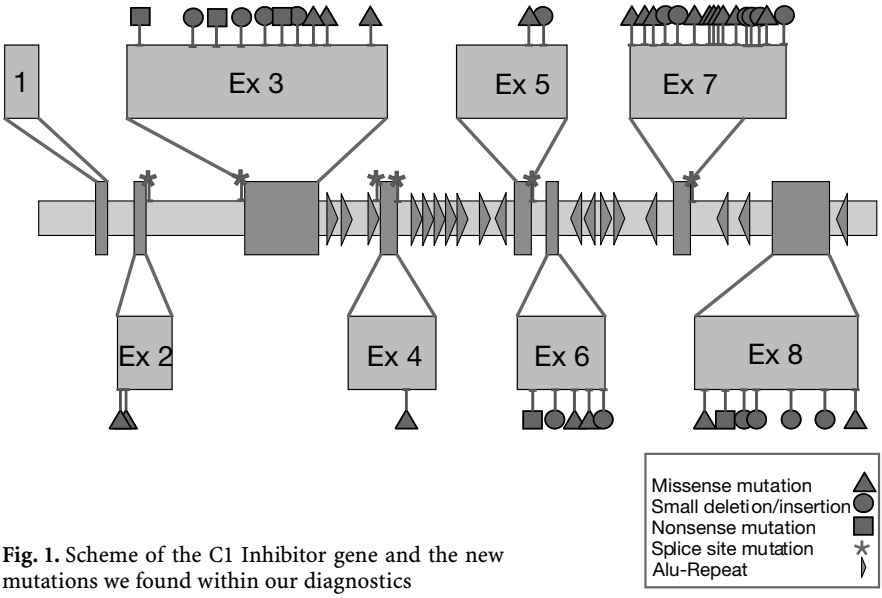


**Fig. 1.** Scheme of the C1 Inhibitor gene and the new mutations we found within our diagnostics

To date 106 different mutations of the C1INH gene are reported in the literature (Fig. 3). We are now able to expand this list by one third to a number of 156.

## Conclusion

The knowledge of the genotype will be of importance for the understanding of the clinical course of patients and may allow in the future to identify patients at high risk for developing acute and life threatening edema. Furthermore, mutation analysis is a fast and safe tool for diagnosing mutation carriers in affected families prior to clinical manifestation.

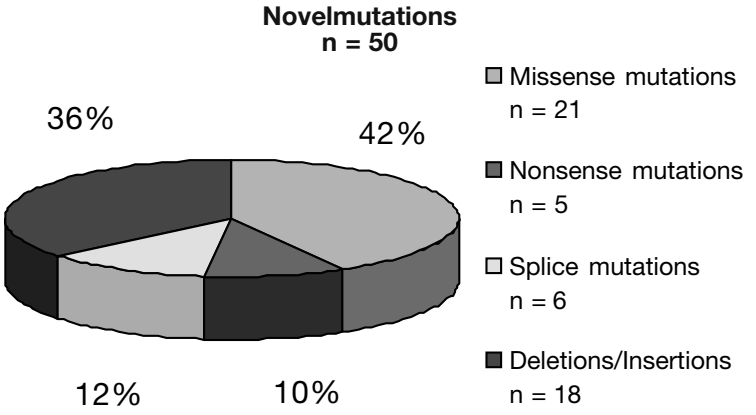


**Fig. 2.** Diagram of the distribution of the new mutations we found within our diagnostics

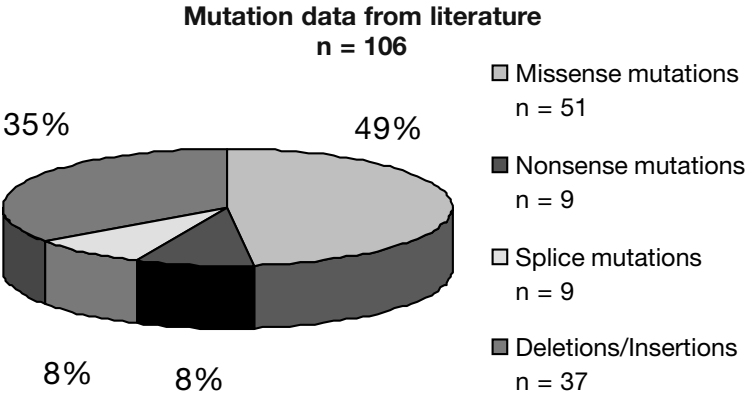


**Fig. 3.** Diagram of the distribution of mutation data from literature

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# Novel and Recurrent Mutations in the Gamma-Glutamyl Carboxylase (GGCX) Gene

S. Rost, C. Geisen, A. Fregin, D. Koch, M. Compes, E. Seifried, C. R. Müller and J. Oldenburg

## Introduction

Familial multiple coagulation factor deficiency is a very rare bleeding disorder which is caused either by a genetic defect of the gamma-glutamyl carboxylase (GGCX) gene or a defect in one of the components of the vitamin K epoxide reductase complex (VKOR) [4, 3]. So far, only two different mutations in the GGCX gene have been described [1, 2].

Last year we reported on a patient (patient Z) with two heterozygous mutations, representing the first case of compound heterozygosity in the GGCX gene. Now we describe a second patient (patient W) compound heterozygous for mutations in the GGCX gene, having one missense mutation with the described patient Z in common.

## Patients and Methods

Both patients showed a mild deficiency of all vitamin K dependent coagulation factors with activities ranging from 20 to 40% of normal (Tab. 1).

**Table 1.** Vitamin K dependent factor levels in the index patients (*not determined).

| | Factor II | Factor VII | Factor X | Protein C | Protein S |
|---|---|---|---|---|---|
| Patient Z | 21% | 42% | 36% | –* | –* |
| Patient W | 38% | 47% | 24% | 26% | 35% |

High vitamin K doses could increase these activities only to 50-60% in both patients. Genetic analysis was performed by sequencing of all exons and flanking intronic regions of the GGCX gene.

## Results and Discussion

The patient Z described last year revealed a splice site mutation of exon 3 (IVS2-1 G>T) and a missense mutation in exon 11 (R485P). The new patient W showed a

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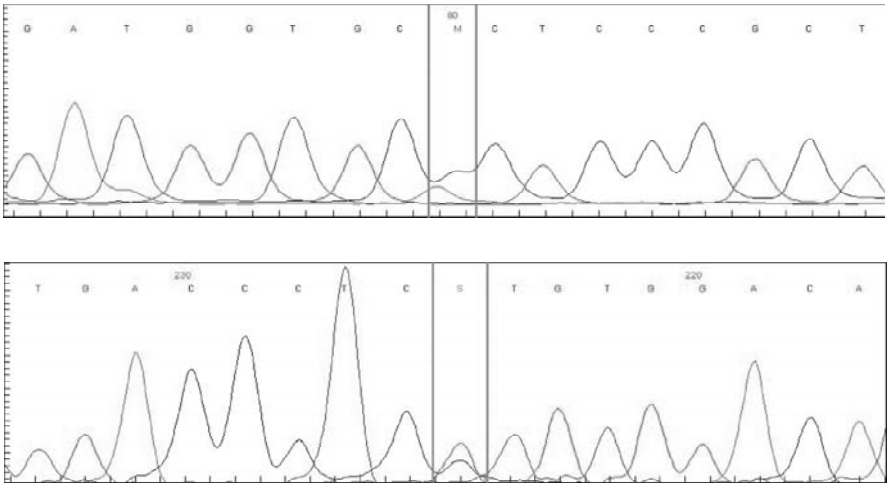

**Fig. 1.** Sequence analysis of exon 9 (above) and exon 11 (below) of the GGCX gene in patient W. A heterozygous A to C mutation in exon 9 leads to an amino acid exchange of histidine to proline and a heterozygous G to C transversion in exon 11 results in the conversion of arginine to proline at residue 485 in the protein.

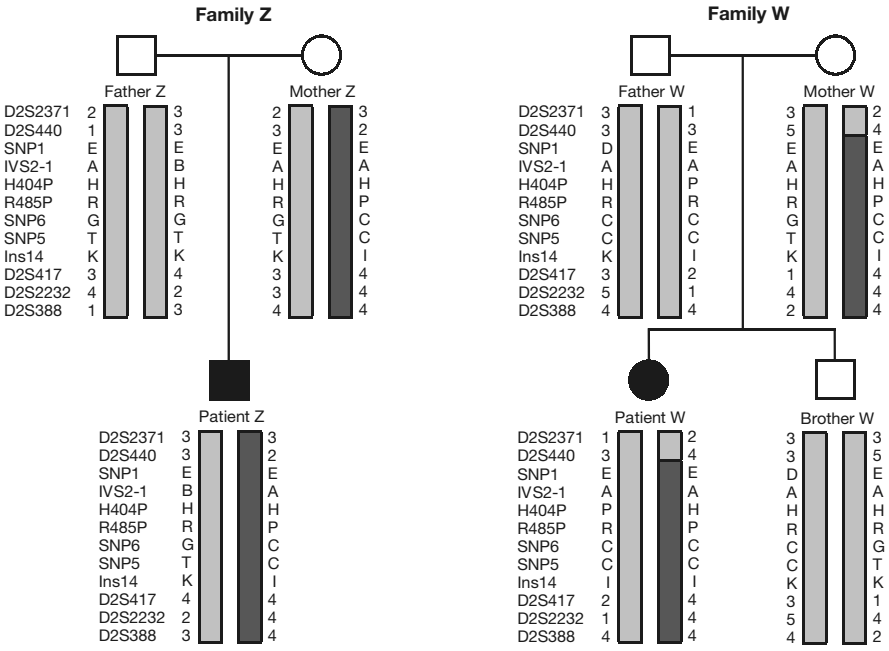


**Fig. 2.** Haplotype analysis of families W and Z. The genomic order of markers is given to the left of the bars. Black bars indicate alleles in common to both patients and their mothers.

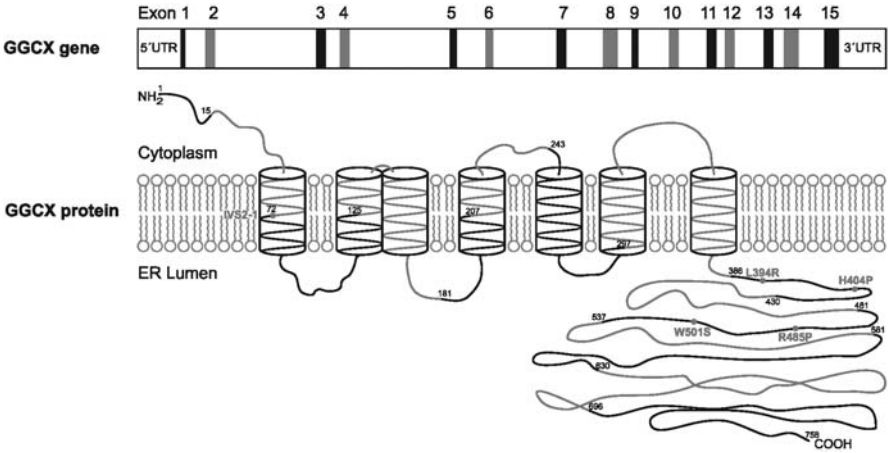


**Fig. 3.** Model of the GGCX gene (above) and protein (below). Exons are shown in alternating colours. All five known mutations are stressed by red colour.

missense mutation at amino acid 404 (H404P; Fig. 1) and the same missense mutation in exon 11 as patient Z (R485P; Fig. 1). Since the probability for the occurrence of the same mutation in two unrelated patients is very low we suspected a founder effect. To test this hypothesis we examined four polymorphisms within the GGCX gene and five microsatellite markers surrounding this gene in both families. Results of this haplotype analysis (Fig. 2) provide evidence that a founder effect is responsible for the missense mutation R485P in both patients. The localization within the GGCX protein of the previously reported mutations (W501S by Spronk et al. and L394R by Brenner et al.) and those found by our group are shown in Fig. 3.

## Conclusion

Patients Z and W are the first cases being compound heterozygous for mutations in the GGCX gene. Both patients show the same missense mutation in exon 11 (R485P) which could be proven by haplotype analysis of the two families to be due to a founder effect.

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# First Case of Compound Heterozygous Mutations in the Kininogen Gene Causing Severe High Molecular Weight Kininogen Deficiency

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## Introduction

High molecular weight kininogen (HK), prekallikrein (PK) and Factor XII (FXII) form the plasma kallikrein/kinin system (KKS) which has been traditionally understood as the »contact activation system« of blood coagulation [1, 2]. Autoactivation of FXII on negatively charged surfaces results in activation of PK to plasma kallikrein. Further activation of FXII and subsequent activation of factor XI initiates the intrinsic pathway of blood coagulation [1]. Recent data on the physiological role of the plasma KKS suggest that it may be relevant in vascular biology, contributing to blood pressure control and constitutive anticoagulant nature of the intravascular compartment [1, 3-5]. Plasma and tissue kallikreins cleave HK and LK releasing bradykinin, a potent vasodilating peptide, leaving a kinin-free kininogen (Hka). HK and Hka, respectively were shown to exhibit anticoagulant, anti-proliferative, anti-angiogenic, anti-aggregatory, and anti-inflammatory activities [4-6].

The two forms of kininogens HK and Low-molecular-weight kininogen (LK) are encoded by a single gene (KNG) localized on chromosome 3 at 3q26-qter. KNG spans about 27 kb and contains 11 exons [7, 8]. Alternative splicing of exon 10 results in a unique mRNA for HK and LK, respectively. Consequently, HK and LK share the first three domains of the heavy chain which are encoded by the first nine exons. Domain 4 is coded by part of exon 10. It consists of the bradykinin sequence and the first 12 amino acids of the light chains of HK and LK. The remainder of exon 10 codes for the HK light chain, which consists of domain 5 (D5H) and domain 6 (D6H). D5H represents an artificial surface binding region; D6H contains the PK and Factor XI (FXI) binding regions. Exon 11 codes for the unique light chain of LK (D5L) [9, 10].

HK deficiency represents an extremely rare autosomal-recessive entity and has been reported in only 13 families to date [11-19]. Despite a marked prolongation of the activated partial thromboplastin time (APTT), HK deficiency does not lead to a hemorrhagic phenotype in these families [2]. In fact, a prothrombotic phenotype has been observed in this condition and, also in a »natural knockout« strain of rats [11, 16, 20]. In three of the reported families (e. g. F. and W. trait) the genetic basis of the HK-deficiency has been described as resulting in a nonsense mutation, a small frame shift deletion and an intronic mutation, respectively [16, 21]. Here, we

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report on the first case of compound heterozygous mutations in the kininogen gene. A nonsense mutation and a splice site mutation cause severe HK deficiency in a 19-year-old female. Beside the markedly prolonged APTT no abnormal clinical phenotype has been observed. There is no evidence for a thrombotic risk, neither in laboratory nor in clinical data.

## Study Design

### Case report

The proband is a 19-year-old female of German origin. Repeated preoperative coagulation testing revealed prolonged activated partial thromboplastin time (APTT) values between 71 to 113 s and a moderate prolongation of the r-time of the thrombelastogram. FXI and PK activity were mildly reduced while all the other coagulation tests were within the normal range (Table 1). Upon family testing, the non-consanguineous parents were found to have normal coagulation parameters except a borderline decreased HK-activity and the entire family history was negative for both, hemorrhagic and thromboembolic diathesis. The study was performed in accordance with the Declaration of Helsinki. All subjects gave their informed consent.

### Analysis of Genomic DNA

Genomic DNA was prepared from EDTA blood leucocytes by standard procedures and amplified by PCR [22]. The 11 exons of the kininogen gene, including the flanking regions, were screened for mutations by PCR and subsequent sequencing on an automated sequencing system (ABI Prism 310, Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's recommendations. The presence of mutations in the normal population was tested by denaturing high performance liquid chromatography (dHPLC) [23]. All aberrant DNA fragments detected by dHPLC were amplified again and purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) for subsequent sequencing. Scores for the donor splicing sites of exon 8 were calculated using a web-based software: http://www.fruitfly.org/seq_tools/splice.html. Scores range from 0 to 1 (lowest to highest probability of splicing).

## Results and Discussion

In the present study we report on the clinical and genetic findings in a family with severe HK deficiency (Table 1). Sequence analysis of the kininogen gene revealed a nonsense mutation in exon 6 (c.718C>T, p.Arg240Stop) and a mutation of the exon 8 donor splice site (c.1008+1G>C [c. IVS8+1G>C]). Family studies confirmed compound heterozygosity since the father was carrier of the nonsense mutation and the mother exhibited the splice site mutation. Both mutations are suggested to repre-

**Table 1.** Coagulation profile of propositus and family members

| | Normal range | Proband | Mother | Father |
|---|---|---|---|---|
| APTT, s | 30-45 | 71-113 | 36 | 35 |
| Prothrombin time, s | 11-16 | 12 | 12 | 12 |
| Thrombin time, s | 8-12 | 11 | 9 | 10 |
| Fibrinogen, g/L | 2.0-4.5 | 2.9 | 3.2 | 2.7 |
| Factor II:C, % | 75-110 | 128 | 109 | 111 |
| Factor V:C, % | 70-140 | 111 | 115 | 116 |
| Factor VII:C, % | 65-130 | 140 | 131 | 134 |
| Factor VIII:C, % | 70-170 | 112 | 166 | 147 |
| Factor IX:C, % | 70-120 | 149 | 131 | 123 |
| Factor X:C, % | 75-115 | 115 | 95 | 115 |
| Factor XI:C, % | 75-110 | 62 | 80 | 87 |
| Factor XII:C, % | 70-150 | 155 | 142 | 163 |
| Factor XIII:C, % | 70-120 | 98 | 101 | 134 |
| VWF:Ag, % | 50-160 | 82 | 78 | 109 |
| VWF:CBA, % | 45-160 | 110 | 90 | 111 |
| Prekallikrein:C, % | 75-150 | 52 | 85 | 88 |
| High molecular weight kininogen:C, % | 70-180 | <1 | 68 | 61 |
| Antithrombin, % | 80-120 | 103 | 95 | 118 |
| Protein C activity, % | 70-120 | >100 | 91 | >100 |
| Protein S activity, % | 60-140 | 76 | 69 | 61 |
| Lupus inhibitor (LCA,DVV) | negative | negative | negative | negative |
| Factor V Leiden | negative | negative | negative | negative |
| Prothrombin 20210G>A | negative | negative | negative | negative |
| D-Dimer, µg/mL | <0.3 | <0.1 | 0.1 | 0.2 |
| FDP, µg/mL | <10 | <10 | <10 | <10 |
| Thrombelastography: | | | | |
| r-time, min | 10-16 | 24/31 | 16 | 15 |
| maximum amplitude, mm | 90-150 | 72/99 | 102 | 111 |

C indicates coagulant activity; VWF:Ag, von Willebrand factor, antigen concentration; VWF CBA, von Willebrand factor, collagen binding activity; LCA, lupus anticoagulant activity; DVV, diluted Russel viper venom test; FDP, fibrin(ogen) degradation products; and r-time, reaction time

sent severe molecular gene defects resulting in the lack of HK protein. The C>T exchange at position c.718 generates a premature termination codon (PTC), which is followed by an exon-exon junction more than 50–55 nucleotides downstream, thus being prone to nonsense-mediated mRNA-decay (NMD) [24]. NMD, also known as mRNA surveillance, prevents the production of a truncated protein. The G>C mutation at c.1008+1 affects the highly conserved first intronic nucleotide of the splicing consensus sequence. Using a web-based software (http://www.fruitfly.org/seq_tools/splice.html), the wild type donor splice site calculates a splicing score of 0.62, while for the mutated sequence no splice site is predicted. The two

mutations discovered in the proband represent the fourth and the fifth mutation described thus far within the kininogen gene and the first case showing compound heterozygosity.

Until now three distinct mutations of the kininogen gene have been reported in consanguineous families (F., W. and a 6-year-old boy trait). In W. trait a nonsense mutation in exon 5 (p.Arg195X) prevents synthesis of both HK and LK [21]. In the F. case a 17 base pair mutation in intron 9 was found which may lead to a truncated HK lacking epitops of domain 6 (positions 543 to 595). The corresponding level of LK antigen was 40% [16]. Recently, in a 6-year-old boy with severe HK-deficiency a single nucleotide deletion at position 1492 of the cDNA leading to a premature stop codon after amino acid position 532 was detected. Since the mutation is located after the side of alternative splicing in exon 10, normal LK-levels could be determined [16]. Additionally, in a Japanese patient, who displayed HK deficiency with normal LK antigen concentration, a partial deletion of intron 7 was found [15]. Whether this defect is causative for HK-deficiency is not known.

Kininogens have been shown to possess anti-adhesive, anticoagulant, and profibrinolytic properties and can inhibit platelet activation at low thrombin concentrations. Therefore it has been hypothesized that HK may function as an antithrombotic protein. Because contact system factor activation directly or indirectly initiates fibrinolysis, patients deficient in individual contact factor proteins may paradoxically be at increased risk of thrombosis [25, 26]. Indeed, two of the previously

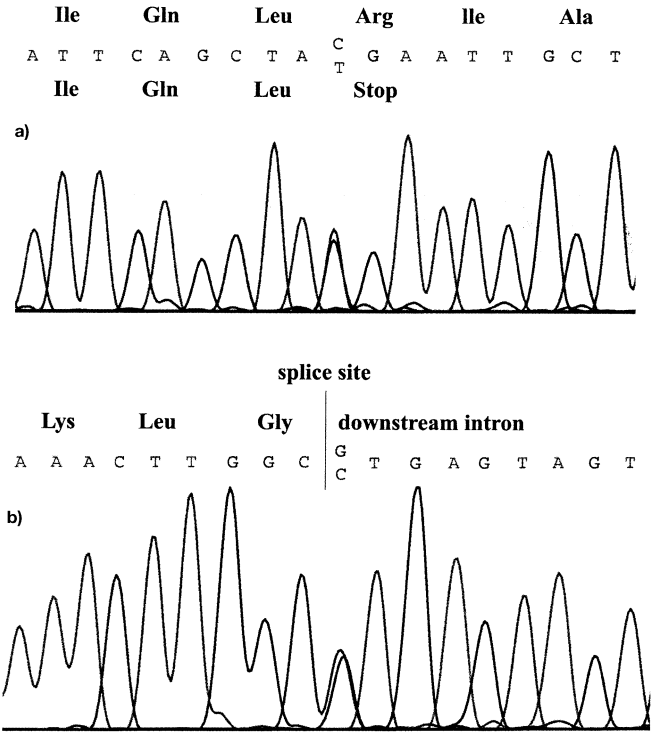


**Fig. 1a, b.** Mutation analysis. The electropherograms show a C to T transition at position c.718 in exon 6 leading to a nonsense mutation (p.Arg240X) in one allele (a) and a G to C transversion at position c.1008+1 of the donor splice site of exon 8 in one allele (b).

reported index patients with total HK-deficiency, both the 64-year-old woman (W.) and the 6-year-old boy showed thromboembolic events, e. g. deep vein thrombosis with pulmonary embolism and cerebral artery thrombosis, respectively [11, 16]. Moreover, experimental data including animal studies using a »natural knockout« strain of rats in a vascular injury model have shown that severe HK-deficiency leads to a prothrombotic phenotype [4-6, 20].

However, our propositus has neither experienced a thromboembolic event to date, nor did we find laboratory evidence for an increased procoagulant activity, as D-dimer and fibrin(ogen). degradation products (FDP) concentrations were within the normal range (Table 1). Because of the rare number of patients with severe HK deficiency and discordant reports on thromboembolic events it remains uncertain whether total HK deficiency leads to a prothrombotic phenotype. Further studies of HK deficiency in patients might provide a better understanding of the physiological role of kininogens in hemostasis.

## Summary

High molecular weight kininogen (HK) is the pivotal component of the plasma kallikrein/kinin system also known as the »contact activation system« of blood coagulation. In the present study severe HK deficiency with no detectable HK activity (< 1%) was diagnosed in a 19-year-old female. Sequence analysis of the kininogen gene revealed two null-mutations, a nonsense mutation in exon 6 (c.718C>T, p.Arg240X) and a donor splice site mutation of exon 8 (c.1008+1G>C [c. IVS8+1G>C]). Family studies confirmed compound heterozygosity. Until now three different mutations of the kininogen gene have been reported in consanguineous families (e. g. F. and W. trait). Here, we report on the first case of compound heterozygous mutations in the kininogen gene. A prothrombotic phenotype that has been suggested in severe HK-deficiency by experimental data and previous case reports, however has not been observed so far in the proband neither in laboratory nor in clinical data.

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# Sequence of the rat Factor VIII cDNA

M. Watzka, C. Geisen, E. Seifried and J. Oldenburg

Factor VIII functions as an essential cofactor in the blood coagulation cascade for the factor IXa-mediated activation of factor X. Here we report the cDNA corresponding to the rat homologue of the human factor VIII gene.

## Materials and Methods

To find sequences containing the rat FVIII gene, DNA data of the rat genome project were aligned with mouse and human FVIII cDNAs. Alignment of the most homologue contig with mouse cDNA revealed several exon-intron boundaries. These data were used to design primers to span the complete rat FVIII cDNA. PCR was performed using expand long template PCR System (Roche) under standard conditions including 1 µl human or rat liver cDNA (corresponding to 100 ng total RNA). Column or gel purified fragments of appropriate length were sequenced.

## Results

Sequencing of rat FVIII cDNA with primers derived from the rat genome project resulted in 6777 bp of the complete rat FVIII cDNA (NM_183331). Overall similarity of this cDNA compared to human and mouse cDNA is 61% and 68 %, respectively (Table 1). Most posttranslational modification sites of the human factor VIII are

**Table 1.** Nucleotide and Amino Acid Homology

| | nucleotide homology | | amino acid homology | |
|---|---|---|---|---|
| domain | rat/mouse | rat/human | rat/mouse | rat/human |
| A1 | 70 % | 68 % | 70 % | 67 % |
| a1 | 53 % | 57 % | 56 % | 57 % |
| A2 | 74 % | 73 % | 76 % | 71 % |
| a2 | 66 % | 58 % | 45 % | 41 % |
| B6 | 2 % | 49 % | 41 % | 26 % |
| a3 | 40 % | 34 % | 29 % | 22 % |
| A3 | 70 % | 68 % | 69 % | 69 % |
| C1 | 78 % | 72 % | 76 % | 76 % |
| C2 | 79 % | 72 % | 73 % | 66 % |
| overall | 68 % | 61 % | 59 % | 51 % |

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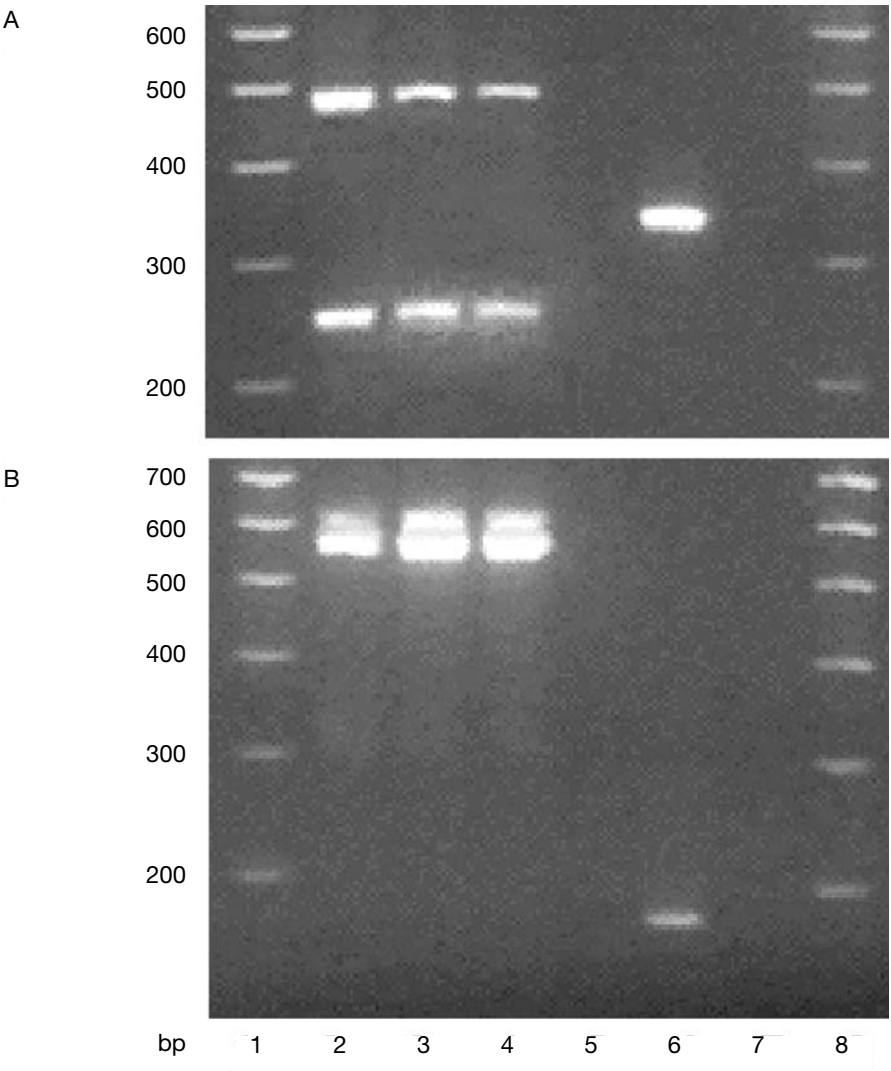


**Fig. 1.** Characterization of rat and human Factor VIII alternative mRNAs. Lanes 1-3 rat liver cDNA, lane 4, 6 negative controls, lane 5 human liver cDNA. In human liver, neither exon skipping of exon 17, nor alternative splicing of exon 20 is observed.

conserved in the rat factor VIII. A consensus polyadenylation signal was identified at nt 8142. Screening 203 bp 5´ of the ATG start codon revealed a potential transcription initiation point at nt -29 with a basic promoter consisting of a TATA box (26 bp), CAAT box (78 bp), and GC box (115 bp) situated upstream of this point.

While investigating rat liver FVIII cDNA, extra bands appeared when amplifying exons 16-22. Sequencing these bands, we found exon skipping of exon 17 and

**Table 2.** Variations of the Rat FVIII cDNA

| Exon | nt exchange | aa exchange | domain |
|---|---|---|---|
| 14 | AAC>AAT | Asn>Asn | B |
| 14 | CCC>CCT | Pro>Pro | B |
| 14 | GAA>GAG | Glu>Glu | B |
| 14 | GCC>GCT | Ala>Ala | B |
| 14 | GAA>AAA | Glu>Lys | B |
| 14 | ATG>ATC | Met>Ile | B |
| 14 | AGA>AGG | Arg>Arg | B |
| 20 | CTC>CTT | Leu>Leu | C1 |
| 23 | CTG>CTT | Leu>Leu | C1 |
| 24 | CCG>CTG | Pro>Leu | C2 |
| 25 | GGC>GGT | Gly>Gly | C2 |
| 3´UTR | G>C | – | – |
| 3´UTR | | ins A | – |

an alternative spliced exon 20 containing 26 additional bp (Fig. 1). While sequencing FVIII cDNA 13 polymorphisms in 5 alleles of 3 rats could be detected (Table 2).

## Discussion

The rat FVIII nucleotide and the resulting amino acid sequence show significant similarity to human and mouse FVIII through the A and C domains, but not in the B and a domains. The low degree of conservation and high frequency of detected polymorphisms in the B domain supports the current view that the B domain does not significantly contribute to procoagulant activity.

The alternative transcript lacking exon 17 is formed due to a weak wt acceptor splice site flanking this exon. This is underlined by the relatively high expression of this transcript. The transcript results from alternative splicing of exon 20 because of a second acceptor splice site situated 26 bp upstream. Since the alternative splice site is weaker, expression of wt mRNA is clearly higher.

Protein variants derived from stop mutations in the human FVIII gene are not secreted in general [1]. Thus also the predicted alternative rat FVIII proteins are not expected to be secreted due to the phenomenon of nonsense mediated mRNA decay, a mechanism described for human FVIII and other genes [2].

This mechanism selectively degrades nonsense mRNAs with premature stop codons. Recently, regulated unproductive splicing and translation (RUST), a mechanism of alternative splicing coupled with NMD and nonsense associated altered splicing was discussed to be an ubiquitous mechanism in regulating protein expression [3–4]. However, the biological relevance of the alternative rat FVIII RNA variants with subsequent downregulation of the wt FVIII protein expression is not known. The diversity of rat FVIII is also reflected by 13 polymorphisms found in only 5 alleles of three animals. Four polymorphisms are found in the functionally important C1 and C2 domains, even one with an aa exchange from the sterically demanding Prolin to Leucin. The majority of changes represent C to T transitions

in CpG dinucleotides (7 of 13).With the discovery of the rat FVIII cDNA, the rat now becomes available as an animal model for further studies on the function of the FVIII protein. Through its highly divergent amino acid sequence, rat FVIII might provide new insights into the importance and function of particular amino acids, cleavage sites and sites of posttranslational modification, as the FVIII molecules from other species can do.

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# Mutation Profiling in Congenital FXIIIA Deficiency: Detection of 6 Novel Mutations

V. Ivaskevicius, R. Seitz, R. Hilgenfeld, T. Sicker, H.-H. Brackmann, W. Eberl, K. Kurnik, U. Göbel, E. Seifried and J. Oldenburg

## Introduction

Mutations of the factor XIII A-subunit gene (F13A) have been found in patients with XIIIA deficiency, a rare autosomal recessive bleeding disorder. F13A gene is located on chromosome 6p24-25 and contains 15 exons [1]. The corresponding protein stabilizes the fibrin clot and increasing its resistance to fibrinolysis.

## Materials and Methods

The F13A gene of 13 patients (5 Turkish, 4 German, single patients from France, Grait Britain, Spain and Lithuania) was analysed on denaturing high performance liquid chromatography (DHPLC) and sequencing. The three – dimensional structure of the mutant amino acides were analysed by a molecular protein model based on the X-ray sructure of FXIIIA [2].

**Table 1.** List of identified mutations in 13 FXIIIA deficient patients.

| Type of Mutation | Mutation | Amino Acid Exchange | Exon | Domain |
|---|---|---|---|---|
| Missense | c.232C>T | R78C | 3 | Beta-sandwich |
| | c.646G>A* | G216R | 5 | Core |
| | c.888C>G | S296R | 7 | Core |
| | c.980G>A | R327Q | 8 | Core |
| | c.1261G>A | G421S | 10 | Core |
| | c.1687G>A | G563R | 12 | Barrel 1 |
| Splice Site | IVS5-1 G>A | – | – | – |
| | IVS12+1 G>A* | – | – | – |
| | IVS14-2 A>G* | – | – | – |
| Small Deletions | c.617-625del* | In-frame | 5 | Core |
| | c.748delC* | Frameshift | 6 | Core |
| | c.1475-1476delGA* | Frameshift | 12 | Core |
| Small Insertion | 1201insC | Frameshift | 9 | Core |

*novel mutations

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**Table 2.** Haplotype analysis of 13 patients that were homozygous for IVS5-1 G>A mutation (patients 1-3) and homozygous or compound heterozygous for further different mutations (patients 4-10) within F13A gene.

| SNP | Patient | | | | | | | | | | | | | MAF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |
| F13A01 STR [AAAG]n | **6/6** | **6/6** | **6/6** | 5/6 | 6/5 | 6/5 | 6/6 | 5/5 | 6/6 | 5/5 | 5/5 | 5/5 | 6/6- | – |
| 2281G>A | **1/1** | **1/1** | **1/1** | 1/2 | 1/2 | 1/1 | 1/1 | 2/2 | 1/1 | 2/2 | 1/1 | 1/1 | 1/1 | 0.2175 |
| c.103G>T, V35L | **1/1** | **1/1** | **1/1** | 1/2 | 1/1 | 1/2 | 1/1 | 1/1 | 1/1 | 1/1 | 2/2 | 1/1 | 1/1 | 0.2575 |
| c.614A>T, Y205F | **1/1** | **1/1** | **1/1** | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 2/2 | 0.0275 |
| c.996A>C, P332P | **1/1** | **1/1** | **1/1** | 1/1 | 1/1 | 1/1 | 2/2 | 1/1 | 1/1 | 1/1 | 2/2 | 1/1 | 1/1 | 0.1775 |
| IVS9-24C>T | **1/1** | **1/1** | **1/1** | 1/1 | 1/2 | 1/1 | 1/1 | 1/1 | 1/1 | 2/2 | 1/1 | 1/1 | 1/1 | 0.1600 |
| IVS9-23C>T | **1/1** | **1/1** | **1/1** | 1/2 | 1/2 | 1/1 | 2/2 | 2/2 | 1/1 | 2/2 | 1/1 | 1/1 | 1/1 | 0.3450 |
| c.1694 C>T, P565L | **1/1** | **1/1** | **1/1** | 1/1 | 1/2 | 1/1 | 1/1 | 1/1 | 1/1 | 2/2 | 2/2 | 1/1 | 1/1 | 0.2075 |
| c.1707A>G, E568E | **1/1** | **1/1** | **1/1** | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 2/2 | 0.0825 |
| c.1950G>A, V651I | **1/1** | **1/1** | **1/1** | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 0.0400 |
| c.1953G>C, E652Q | 2/2 | 1/1 | 1/1 | 1/2 | 2/2 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 1/1 | 0.2350 |

MAF= allele frequency of minor allele. [AAAG]n – a highly sensitive STR (Short tandem repeat) marker in F13A gene, SNP= single nucleotide polymorphism

## Results and Discussion

A total of thirteen different mutations in 13 unrelated patients comprising 6 missense, 3 splice site, 3 small deletions and one small insertion could be elucidated (Table 1). Splice acceptor site mutation in intron 5 (IVS5–1 G>A) was found in 3 families originated from Germany, U.K. and Turkey. Haplotype analysis (Table 2) revealed an ancient founder effect, that likely has occurred some thousand years ago since it was spread at least in central and southern Europe as well as in Asia (Turkey). Six mutations were described for the first time. The protein modelling of novel missense mutation in exon 5 (G215R) demonstrated that R215 is located at the interface of two beta strands and the barrel 1 and barrel 2 domains. Substitution of the small Gly by the large Arg obviously affects the three dimensional configuration substantially. Two novel splice site mutations (IVS12+1 G>A, IVS14-2A>G) cause splicing error of exon 6 and exon 15 respectively, while two novel small deletions (c.748delC, c.1475-1476delGA) result in premature termination of FXIIIA protein. A novel 9bp del in exon 5 (c.617-625del) results in frame deletion of amino acids V206, L207 and N208.

## Conclusions

1. DHPLC has proven to be a fast and highly sensitive method for the mutation analysis of the F13A gene.
2. The identified novel mutations will help better to understand the functionally important sites of the FXIIIA protein.
3. IVS5-1 G>A mutation seems to be the most common F13A gene defect in FXIIIA deficient patients, due to an ancient founder effect.

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# Functional Analysis of the Factor VIII B Domain

O. El-Maarri, J. Schwalbach, U. Herbiniaux, P. Hanfland and J. Oldenburg

## Introduction

Factor VIII is a part of the tenase complex where it acts as a cofactor for factor IX. This cofactor activity has a 300.000 fold increase on the activation of factor X by factor IXa. Factor VIII is synthesized as single chain of 2332 aa that has 3 distinguishable domains in the order A1-A2-B-A3-C1-C2. The one chain form of the protein is cleaved intra-cellularly in the B domain at two positions aa 1313 and aa 1648 (Fig. 1). The rest of the B domain aa 741 till 1313 is cleaved out by thrombin in the plasma prior to activation. The B domain was shown to be dispensable for the coagulation activity of factor VIII. However its heavy N-glycosylation suggests the interaction with some glycosylation dependent intracellular transporters that would guide the mature correctly folded factor VIII from the ER to the Golgi and eventually for secretions. Such proteins are ERGIC-53, MCFD2, Immunoglobulin-binding protein, calnexin and calreticulin. To shade more light on the function of the B domain we applied a yeast two hybrid approach to screen for interactions with unknown proteins.

## Materials and Methods

We used the Matchmaker two-hybrid system 3 to screen for protein interactions of the factor VIII B domain with liver proteins. Three baits were constructed in the pGBKT7 vector containing either the complete B domain (aa 741-1648) or part 1 (aa 741-1313) or part 2 (aa 1313-1648). Each one of these baits were screened against the same liver library. The positive clones were isolated on medium stringency culture plates (-Trp, -Leu, -His). Plasmids containing the candidate interacting protein was isolated and the insert was sequenced. The identity of sequences were determined by Blast search.

Isolated plasmids containing the candidate interacting insert (in pGADT7 vector) were co-transformed with the corresponding bait to reconfirm the interactions. The resulting positive clones were plated on X-Gal plates (Fig. 2), The blue clones were further analyzed for true interactions by measuring the levels of the secreted alpha-galactosidase assays (Fig. 3).

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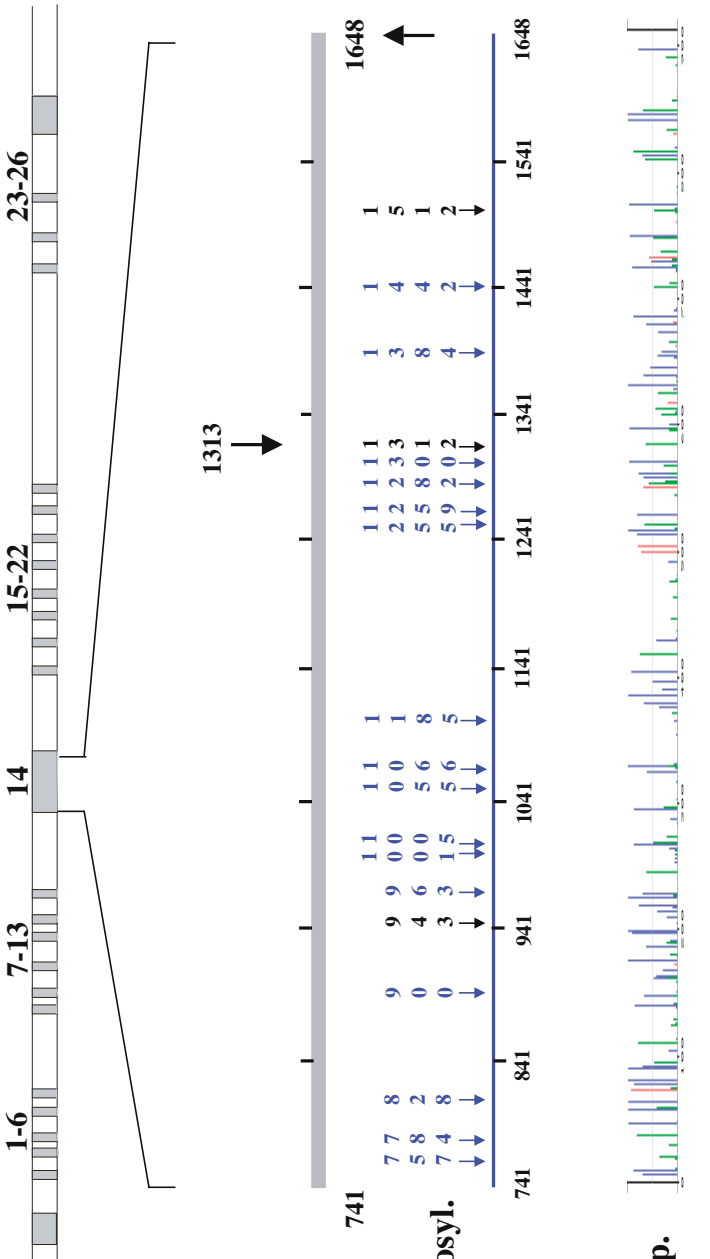


Fig. 1. The factor VIII gene genomic structure. The two parts of the B domain is shown together with the potential N-glycosylation and phosphorelation sites.

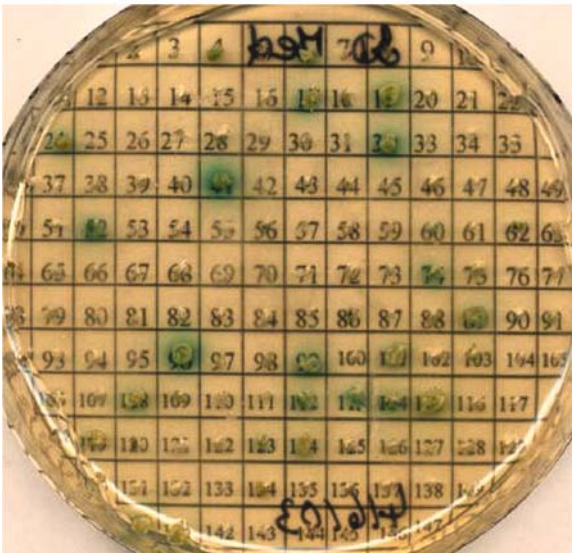

**Fig. 2.** To reconfirm the interactions. The positive clones were re-plated in an array format on medium stringency plates containing X-a-Gal (SD/-His/-Leu/-trp/X-α-Gal).

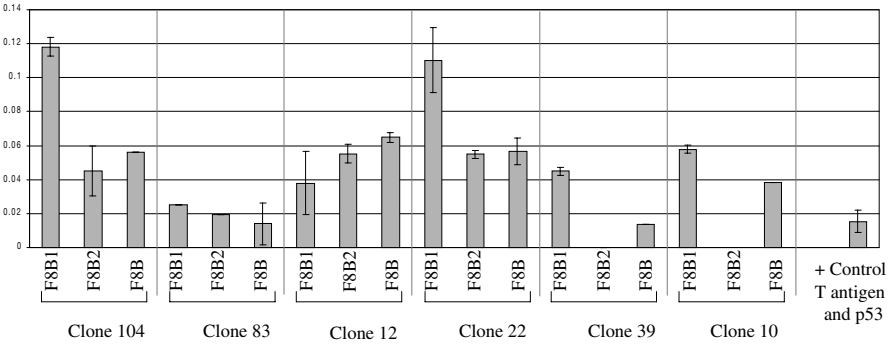


**Fig. 3.** Alpha-Galactosidase assay showing the relative intensity of activating the reporter genes through the interaction between the bait and the candidate protein. All measurements were done three times.

## Results and Discussion

The role of the B domain is only partly understood. It was shown to be dispensable for the coagulation activity of the active FVIII molecule and a B deleted recombinant FVIII was successfully used to correct the bleeding disorder in Hemophilia A patients. No homology to the B domain is known from the human genome sequence. Moreover, the B-domain had extensively diverged between different species. However, within humans it has maintained a high degree of conservation, thus pointing to an important function at least in humans. The B-domain contains 19 sites for N-Glycosylation, and it has been suggested that it is involved in the intra

cellular trafficking of FVIII molecule. As a result of our yeast two hybrid screening we were able to identify few clones that interact either with both parts 1 and 2 of the B domain (clones 12, 22, 83 and 104) or with the part 1 (clones 10 and 39) (Fig. 3). These interactions may represent candidates for some functions related to either the intracellular trafficking of the FVIII molecule or to modulating its function in the blood stream (outside the cell). Further studies, like co-immunoprecipitation, to verify the interactions are under way.

# Expressing Recombinant Coagulation Factors in Yeast and Insect Cells

J. Schwalbach, O. El-Maarri, U. Herbiniaux, P. Hanfland and J. Oldenburg

## Introduction

Coagulation activity is controlled by a complex network of proteins. This cascade of proteins activation and deactivation is delicately controlled by protein interactions and by positive and/or negative feedbacks from end products. In this study we are expressing several coagulation factors (Factor II, VII, IX, X, AT3, factor V B domain and factor VIII domains) in e.coli, mammalian cells, yeast and insect cells based vector systems. The proteins will be used to select for specific aptamers against the surface of the protein molecules. As part of a novel microwave-array mass spectrometry device these aptamers will be used for the qualitative and quantitative study of protein-protein interactions. RNA aptamers against factor IX have been shown as potential candidates for therapeutical use in anticoagulation (Rusconi et al. Nature 2002). Most interestingly, the antidot of the aptamer was represented by its antisense sequence.

## Materials and Methods

We choose to use the Gateway system because of the easiness to shuttle the insert from one vector to another. The cDNAs were amplified from a human liver library using primers attached to attB1 and attB2 sequences (Fig. 1). After purification the PCR product was inserted by an enzymatic reaction (with BP enzyme mix from Invitrogen) into a pDONR Gateway vector (Invitrogen); the reaction produce more that 90% positive clones, after e.coli transformation, with the right insert orientation. This would tremendously save time and efforts that would otherwise be needed when using normal cloning with restriction enzyme, especially when large cDNAs has to be cloned.

Once the cDNA were successfully inserted in the pDONR vector, the inserts were sequenced, following standard procedure (using ABI 310 machines), to verify the sequence integrity. The inserts were shuttled to pDEST 17 (e.coli), pBAD-DEST 49 (e.coli), pDEST 26 (mammalian), Baculovirus (SF9 insect cells) and pPICZα (yeast) and some to pGBKT7 (from Clonetech: converted to a gateway compatible vector) for protein interaction studies (Fig. 1).

The successful expression of the protein were than tested by both SDS page and western blot analysis. For western blot an antibody recognizing penta-His was used from QIAGEN (Fig. 2).

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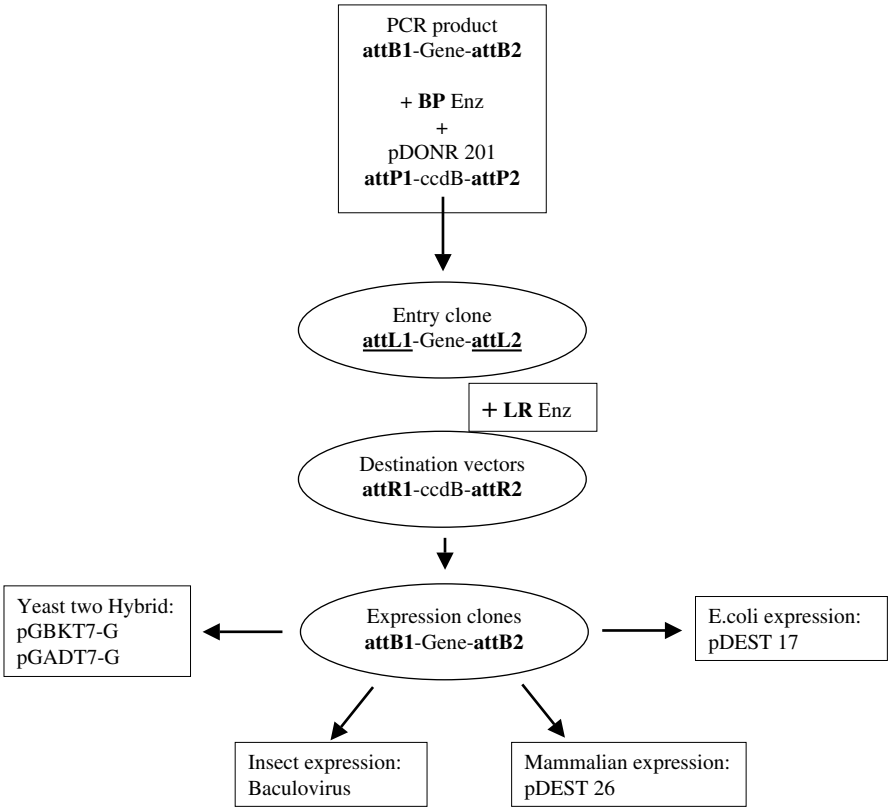


**Fig. 1.** general scheme for vectors formations, the vectors used are mentioned.

## Results and Discussion

We found that the Gateway platform is an easy system that would allow the shuttling of a given insert to different expression vectors. This is particularly useful when many functional studies are to be performed.

The expression of human proteins in E.coli is not an easy and straightforward process as many eukaryotic proteins are not expressed by an e.coli vector. When using the pDEST 17 vector we were unable to get a signal on western blots, however by fusing the human protein with a protein that is expressed in both prokaryote and eukaryote (Thioredoxin in pBAD-DEST49) we were able to get good signals on Western blots, from the factor VIII domains, that correspond to the expected size of the fused protein. Moreover the pBAD-DEST49 expression can be tightly controlled by different concentrations of arabinose; we found that for most proteins a 0.02 to 0.002% arabinose concentration was optimum for the highest signal on western blots. Experiments with pDEST 26 in CHO cells are still underway as well as the yeast and the insect cell expression, however it is expected that the later approaches will give higher yields.

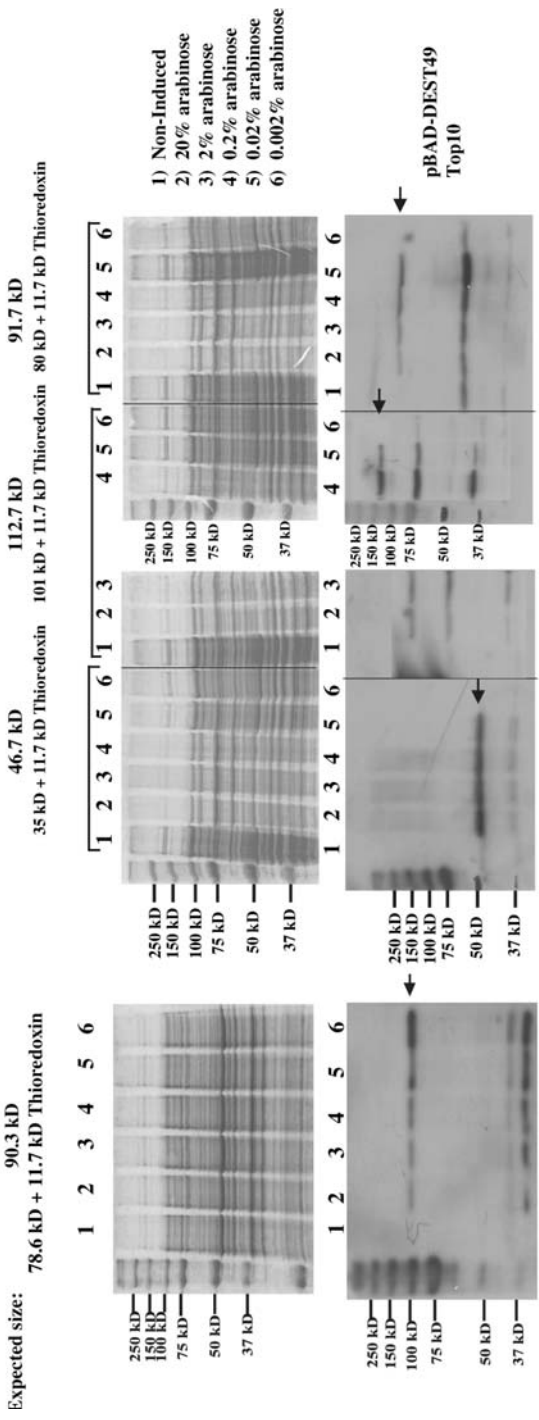


**Fig. 2.** SDS-PAGE gels (upper part) and western blots (lower parts) of different domains of the factor VIII protein expressed in pDAB-DEST 49 vector that produce a hybrid protein fused with Thioredoxin at the N-terminal.

# Long Term Optimisation of F8 Gene Mutation Screening by DHPLC

C. Klein, H. Singer, M. Lim-Eimer, M. Watzka, V. Ivaskevicius, J. Schröder, O. El-Maarri, J. Graw, H.-H. Brackmann, W. Schramm, E. Seifried, R. Schwaab, P. Hanfland, C. Müller-Reible and J. Oldenburg

## Introduction

Hemophilia A is caused by a great variety of different mutation types that are distributed throughout the factor VIII gene (FVIII). We have applied Denaturing High Performance Liquid Chromatography (DHPLC) technique for mutation screening of the FVIII gene in those patients that have escaped mutations detection by DGGE. In order to optimize our DHPLC protocol for sensitivity and throughput we retrospectively evaluated our data.

## Patients, Materials and Methods

In our investigation, DHPLC mutation screening was used to detect FVIII mutations in 242 hemophiliacs. By this technique DNA heteroduplices are separated from homoduplices by ion-pair reverse phase liquid chromatography according to their differences in melting behavior. Partial heat denatured DNA fragments interact length and sequence specific with a non-porous poly(styrene-divinyl-benzene) matrix. Elution from the matrix was achieved by a linear acetonitrile-gradient, resulting in length and sequence specific retention times of the hetero- and homoduplex species (Fig. 1). Initially, we used the original protocol established on the 'Stanford' recommendations comprising 33 PCR fragments that were analyzed in 54 DHPLC runs, because for most of the fragments more than one temperature had to be applied (http://insertion.stanford.edu/melt.html).

## Results and Discussion

A total of 99 different mutations, comprising 52 missense mutations, 11 stop mutations, 5 splice site mutations and 31 small deletions/insertions could be identified. All 99 mutations were also identified by an alternative DHPLC protocol comprising only 46 runs, because some of the formerly used temperatures yielded no additional information. Thus the speed of DHPLC testing could be improved by 15% without any deficit in sensitivity. Five different missense mutations in the exons 7 (2 cases), 17 (2 cases) and 23 (1 case) initially were not detected by DHPLC (even not with the more extended original protocol), but discovered later during sequencing of the complete coding region of the FVIII gene.

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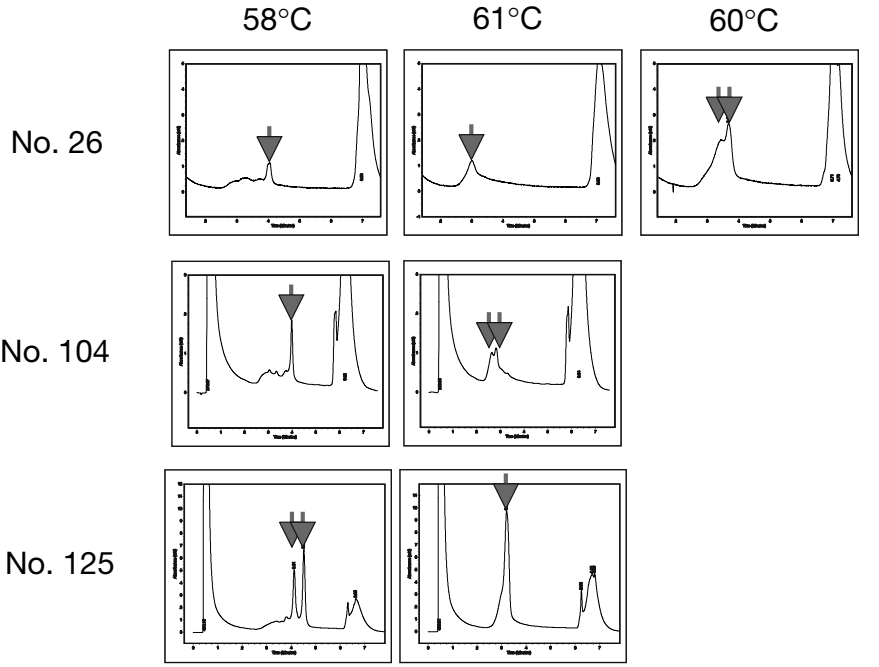


**Fig. 1.** Exon 7 mutation detection by optimized DHPLC conditions

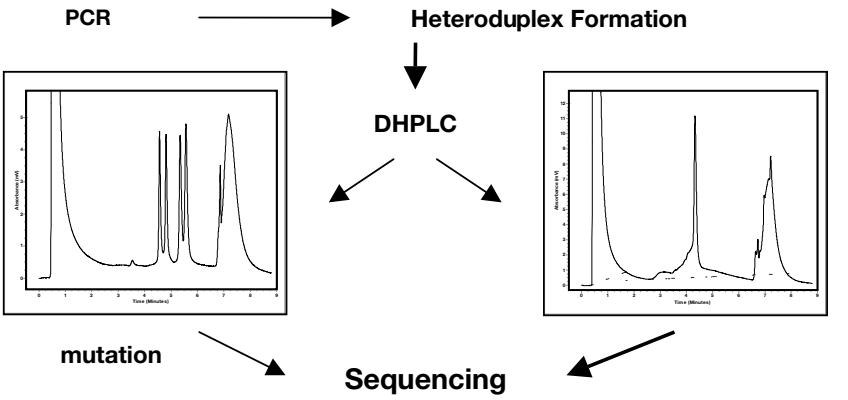


**Fig. 2.** DHPLC screening strategy for FVIII mutation detection

However, these mutations could be clearly identified by analyzing these samples on DHPLC at three additional temperatures. The obtained information was merged towards an optimized testing: The optimized protocol with 46 runs was used as a first line DHPLC analysis that exhibited a sensitivity of 95% (99 of 104 mutations

**Table 1.** Optimized DHPLC conditions for FVIII screening

| Exon | original temperature | optimized temperature | additional temperature |
|---|---|---|---|
| 4 | 57 / 59°C | 58°C | – |
| 7 | 58 / 61°C | 58 / 61°C | 60°C |
| 11 | 56 / 58°C | 57°C | – |
| 13 | 57 / 59°C | 57°C | – |
| 14.7 | 57 / 59°C | 58°C | – |
| 14.8 | 57 / 59°C | 58°C | – |
| 15 | 56 / 58°C | 56°C | – |
| 17 | 57 / 59°C | 59°C | 60°C |
| 23 | 57 / 60°C | 57 / 60°C | 59°C |
| 26 | 60 / 61°C | 62°C | – |

detected). If no mutation was identified, three additional temperatures were applied during a second line protocol (Table 1) increasing the sensitivity to 100% (based on our current data set).Furthermore we optimized the testing for two frequent polymorphisms (Glu1241Asn and Ser1269Ser) within the B–Domain that are present at a prevalence of 27% and 7%, respectively. The presence of these polymorphisms often produces ambiguous melting patterns leading to frequent sequencing. Therefore, we developed a strategy utilizing heteroduplexformation with a wild-type control and homozygous controls for the each type of polymorphism. This approach allowed us to identify the presence of mutations and the genotype for both polymorphisms in three DHPLC runs without the need of sequencing.

## Conclusion

In conclusion, by optimizing the FVIII DHPLC protocol we improved the throughput by 15% at excellent sensitivity rates for mutations detection of 95% (first line) and 100% (second line), respectively.

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# Expression Studies of Recombinant FVIII Proteins Exhibiting Mutations in the B-Domain

K. Klempau, H. Singer, C. Klein, H.-H. Brackmann, P. Hanfland, T. Tonn, R. Schwaab and J. Oldenburg

## Introduction

The phenotype of hemophilia A is due to the deficiency or absence of coagulation factor VIII (FVIII) caused by a great number of heterogeneous mutations within the large FVIII gene, including various point mutations, inversions and deletions or insertions. Missense mutations have been of special interest because they point to functional important regions of the FVIII molecule. Notably, missense mutations are significantly underrepresented in the middle part of the B-domain, indicating that this part of the FVIII molecule may be less important for its function. Furthermore, the successful treatment with a B-domain deleted recombinant FVIII-concentrate, shows that the B-domain might be dispensable for a functional FVIII protein. However, in some hemophilia A patients a missense mutation was the only sequence variation found within the FVIII gene, even by sequencing the complete coding region (Fig. 1).

## Materials and Methods

Missense mutations within the B-domain were identifying in hemophilia A patients by mutation screening and sequencing of FVIII gene. Using oligonucleotide site directed mutagenesis, we constructed FVIII mutants with missense mutations within the B-domain to study the effect of these mutations. To investigate the causality of such missense mutations expression studies were performed in Chinese hamster ovary cells (CHO), transfected with vector constructs encoding the FVIII protein variants (Fig. 2).

## Results and Discussion

In the present study five mutated rFVIII-proteins Pro928Arg, Val993Leu, Asp1241Glu, Arg1310Gly and Asn1441Lys were expressed in CHO cells and characterized for FVIII activity (FVIII:C), FVIII antigen (FVIII:Ag)-level and the specific activity (FVIII:C[%]/FVIII:Ag[%]). The results were compared to the expression of wildtype FVIII protein. The protein variant Arg1241Glu has also been reported in the normal population thus representing a polymorphism. Four of the five missense mutations (Pro928Arg, Val993Leu, Asp1241Glu, Arg1310Gly) showed signifi-

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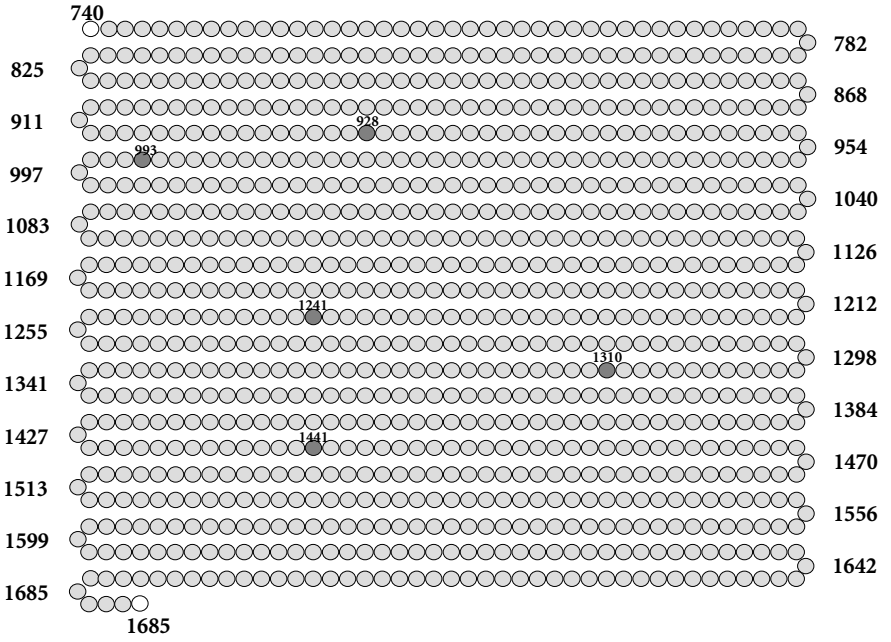


**Fig. 1.** Amino-acids of the B-Domain subjected to mutagenesis

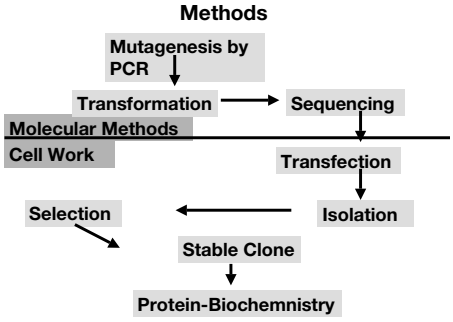


**Fig. 2.** Methods for the expression of recombinant FVIII protein variants

cantly expression of functional FVIII protein thus probably not being causative for a hemophilic phenotype. However, there were some differences in the specific activity and expression/secretion levels, which may point to a FVIII:C modulating effect. The Arg1310Gly variant showed secretion and specific activity (~1) very similar to normal FVIII expression. The rFVIII-proteins Asp1241Glu and Pro928Arg exhibited a higher specific activity (~1,7-2), but secretion- and expression levels were only 50% of the wild type protein. The rFVIII-protein Val993Leu showed nearly the same specific activity (~0,9) as normal rFVIII, but slightly higher expression and secretion levels compared to wildtype FVIII. The expression studies of the Asn1441Lys variant gave inconclusive results and need further evaluation (Table 1).

**Table 1.** Characterization of recombinant FVIII protein variants

| Sample | AK tat. [%] | AK tat. [U/ml] | AG tat. [%] | AG tat. [ng/ml] | AK/AG | spec. AK [U/mg] | Mass cells [mg] | spec. AK/ mg/cells [U/mg*mg] |
|---|---|---|---|---|---|---|---|---|
| wt D2 | 0.75 | 0.0075 | 0.67 | 1.005 | 1.12 | 7.46 | 240 | 0.03 |
| wt D2 | 0.74 | 0.0074 | 0.67 | 1.005 | 1.10 | 7.36 | 240 | 0.03 |
| wt D2 | 1.08 | 0.0108 | 0.9 | 1.35 | 1.20 | 8.00 | 240 | 0.03 |
| wt D2 | 1 | 0.01 | 0.9 | 1.35 | 1.11 | 7.41 | 240 | 0.03 |
| wt D2 | 0.79 | 0.0079 | 0.9 | 1.35 | 0.88 | 5.85 | 240 | 0.02 |
| wt D2 | 1.26 | 0.0126 | 0.9 | 1.35 | 1.40 | 9.33 | 240 | 0.04 |
| wt D2 | 5.75 | 0.0575 | 5.17 | 7.755 | 1.11 | 7.41 | 240 | 0.03 |
| wt D2 | 6.25 | 0.0625 | 5.78 | 8.67 | 1.08 | 7.21 | 240 | 0.03 |
| wt E11 | 0.64 | 0.0064 | 0.43 | 0.645 | 1.19 | 9.92 | 190 | 0.05 |
| wt E11 | 0.47 | 0.0047 | 0.43 | 0.645 | 1.09 | 7.29 | 190 | 0.04 |
| wt E11 | 0.64 | 0.0064 | 0.67 | 1.005 | 0.96 | 6.37 | 190 | 0.03 |
| wt E11 | 0.74 | 0.0074 | 0.67 | 1.005 | 1.10 | 7.36 | 190 | 0.04 |
| wt E11 | 0.58 | 0.0058 | 0.43 | 0.645 | 1.35 | 8.99 | 190 | 0.05 |
| wt E11 | 0.47 | 0.0047 | 0.43 | 0.645 | 1.09 | 7.29 | 190 | 0.04 |
| wt E11 | 5.05 | 0.0505 | 5.11 | 7.665 | 0.99 | 6.59 | 190 | 0.03 |
| wt E11 | 5 | 0.05 | 4.78 | 7.17 | 1.05 | 6.97 | 190 | 0.04 |
| 1241 F5 | 0.64 | 0.0064 | 0.43 | 0.645 | 1.49 | 9.92 | 90 | 0.11 |
| 1241 F5 | 0.74 | 0.0074 | 0.43 | 0.645 | 1.72 | 11.47 | 90 | 0.13 |
| 1241 F5 | 1.08 | 0.0108 | 0.67 | 1.005 | 1.61 | 10.75 | 90 | 0.12 |
| 1241 F5 | 1 | 0.01 | 0.67 | 1.005 | 1.49 | 9.95 | 90 | 0.11 |
| 1241 F5 | 0.69 | 0.0069 | 0.43 | 0.645 | 1.60 | 10.70 | 90 | 0.12 |
| 1241 F5 | 0.74 | 0.0074 | 0.43 | 0.645 | 1.72 | 11.47 | 90 | 0.13 |
| 1241 F5 | 2.74 | 0.0274 | 1.55 | 2.325 | 1.77 | 11.78 | 90 | 0.13 |
| 1241 F5 | 1.25 | 0.0125 | 1.22 | 1.83 | 1.02 | 6.83 | 90 | 0.08 |
| 1310 (G) E8 | 0.64 | 0.0064 | 0.67 | 1.005 | 0.96 | 6.37 | 280 | 0.02 |
| 1310 (G) E8 | 0.74 | 0.0074 | 0.67 | 1.005 | 1.10 | 7.36 | 280 | 0.03 |
| 1310 (G) E8 | 0.96 | 0.0098 | 1.14 | 1.71 | 0.86 | 5.73 | 280 | 0.02 |
| 1310 (G) E8 | 1 | 0.01 | 1.14 | 1.71 | 0.88 | 5.85 | 280 | 0.02 |
| 1310 (G) E8 | 0.69 | 0.0069 | 0.67 | 1.005 | 1.03 | 6.87 | 280 | 0.02 |
| 1310 (G) E8 | 0.74 | 0.0074 | 0.67 | 1.005 | 1.10 | 7.36 | 280 | 0.03 |
| 1310 (G) E8 | 5.86 | 0.0586 | 5.17 | 7.755 | 1.13 | 7.56 | 280 | 0.03 |
| 1310 (G) E8 | 6.5 | 0.065 | 5.56 | 8.34 | 1.17 | 7.79 | 280 | 0.03 |
| 928 E10 | 0.4 | 0.004 | 0.19 | 0.285 | 2.11 | 14.04 | 230 | 0.06 |
| 928 E10 | 0.47 | 0.0047 | 0.19 | 0.285 | 2.47 | 16.49 | 230 | 0.07 |
| 928 E10 | 0.98 | 0.0098 | .043 | 0.645 | 2.28 | 15.19 | 230 | 0.07 |
| 928 E10 | 1 | 0.01 | 0.43 | 0.645 | 2.33 | 15.50 | 230 | 0.07 |
| 928 E10 | 0.58 | 0.0058 | 0.43 | 0.645 | 1.35 | 8.99 | 230 | 0.04 |
| 928 E10 | 0.74 | 0.0074 | 0.43 | 0.645 | 1.72 | 11.47 | 230 | 0.05 |
| 928 E10 | 3.99 | 0.0399 | 2.87 | 4.305 | 1.39 | 9.27 | 230 | 0.04 |
| 928 E10 | 3.75 | 0.0375 | 2.56 | 3.84 | 1.46 | 9.77 | 230 | 0.04 |

**Table 1.** Continue

| Sample | AK tat. [%] | AK tat. [U/ml] | AG tat. [%] | AG tat. [ng/ml] | AK/AG | spec. AK [U/mg] | Mass cells [mg] | spec. AK/ mg/cells [U/mg*mg] |
|---|---|---|---|---|---|---|---|---|
| 993 F9 | 0.98 | 0.0098 | 1.14 | 1.71 | 0.86 | 5.73 | 170 | 0.03 |
| 993 F9 | 1 | 0.01 | 1.14 | 1.71 | 0.88 | 5.85 | 170 | 0.03 |
| 993 F9 | 1.19 | 0.0119 | 1.39 | 2.085 | 0.86 | 5.71 | 170 | 0.03 |
| 993 F9 | 1.26 | 0.0126 | 1.39 | 2.085 | 0.91 | 6.04 | 170 | 0.04 |
| 993 F9 | 0.92 | 0.0092 | 0.9 | 1.35 | 1.02 | 6.81 | 170 | 0.04 |
| 993 F9 | 1 | 0.01 | 0.9 | 1.35 | 1.11 | 7.41 | 170 | 0.04 |
| 993 F9 | 6.22 | 0.0622 | 6.16 | 9.24 | 1.01 | 6.73 | 170 | 0.04 |
| 993 F9 | 10 | 0.1 | 6.78 | 10.17 | 1.47 | 9.83 | 170 | 0.06 |
| 1441 F3 | 0.52 | 0.0052 | 0.19 | 0.285 | 2.74 | 18.25 | 120 | 0.15 |
| 1441 F3 | 0 | 0 | 0.19 | 0.285 | 0.00 | 0.00 | 120 | 0.00 |
| 1441 F3 | 0.98 | 0.0098 | 0.67 | 1.005 | 1.46 | 8.75 | 120 | 0.08 |
| 1441 F3 | 0.47 | 0.0047 | 0.67 | 1.005 | 0.70 | 4.68 | 120 | 0.04 |
| 1441 F3 | 0.46 | 0.0046 | 0.043 | 0.0645 | 10.70 | 71.32 | 120 | 0.59 |
| 1441 F3 | 0.21 | 0.0021 | 0.043 | 0.0645 | 4.88 | 32.56 | 120 | 0.27 |
| 1441 F3 | 1.14 | 0.0114 | 0.039 | 0.0585 | 29.23 | 194.87 | 120 | 1.62 |
| 1441 F3 | 0.5 | 0.005 | 0 | 0 | 0.00 | 0.00 | 120 | 0.00 |

## Conclusion

Expression studies represent a useful tool for studying the causality of missense mutations in the B-domain. The protein variants investigated in our study seemed not to be responsible for a hemophilic phenotype.

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# Genetic Variability of the Factor VIII Gene in the Normal Population

H. Singer, R. Ahmed, V. Ivaskevicius, M. Watzka, J. Schröder, O. El-Maarri, E. Seifried, R. Schwaab, P. Hanfland and J. Oldenburg

## Introduction

The factor VIII protein (FVIII) is involved in two hereditary conditions. Deficiency of FVIII leads to hemophilia A while increased levels of > 150% are known to represent a risk factor for thromboembolic complications (odds ratio about 5). The mechanisms that lead to inherited increased levels of the FVIII protein are still unknown. In order to analyze the genetic variability of the F8 gene in the normal population we screened 560 alleles from male and female blood donors for the presence of sequence variations by DHPLC.

## Materials and Methods

The genomic DNA of 330 healthy blood donors has been amplified by PCR and screened using denaturing high performance liquid chromatography (DHPLC) (Fig. 1). The abnormal peaks obtained by DHPLC have been further sequenced by automated sequencing. From the underpresentation of missense mutations in the B-domain in hemophilic patients it was concluded that the B-domain is functionally less important. Furthermore, the successful treatment of hemophiliacs with a recombinant B-domain deleted FVIII also demonstrates that the B-domain might

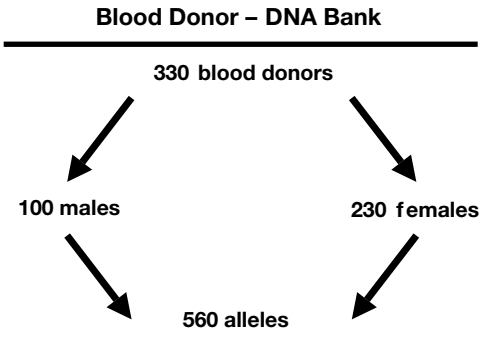


**Fig. 1.** Screening strategy for polymorphism detection.

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be dispensable for the FVIII clotting function. However, in the 560 alleles of blood donors only 6 exhibited a SNP beside the known SNPs in codons 1241 and 1269 within the 3 kb encoding the B-domain, thus indicating a genetically highly conserved region.

## Results and Discussion

In so far 29 of 33 analyzed fragments of the F8 gene we found 11 polymorphisms (Table 1, Fig. 2). The already reported frequent SNPs in the B-domain, Asp1241Glu and Ser1269Ser, showed frequencies of 27 and 7%, respectively, thus corresponding

**Table 1.** Polymorphisms within the human factor VIII gene. red color indicates those with amino acid exchange.

| Exon | codon | nt exchange | aa exchange | frequency |
|---|---|---|---|---|
| 1 | 15 | nt 102 C>T | Asp>Asp | 2/560 |
| 1 | 21 | nt 120 C>T | Leu>Leu | 1/560 |
| 9 | 408 | nt 1279 A>G | Lys>Glu | 1/560 |
| IVS 9 | – | IVS 9-5 T>C | – | 1/560 |
| 14 | 794 | nt 2439 G>A | Leu>Leu | 1/560 |
| 14 | 831 | nt 2550 G>T | Leu>Leu | 1/560 |
| 14 | 981 | nt 2998 C>T | Pro>Ser | 2/560 |
| 14 | 1038 | nt 3170 A>C | Glu>Ala | 1/560 |
| 14 | 1241 | nt 3780 C>G | Asp>Glu | 151/560 |
| 14 | 1269 | nt 3864 A>C | Ser>Ser | 39/560 |
| 14 | 1667 | nt 5058 C>T | Thr>Thr | 1/560 |

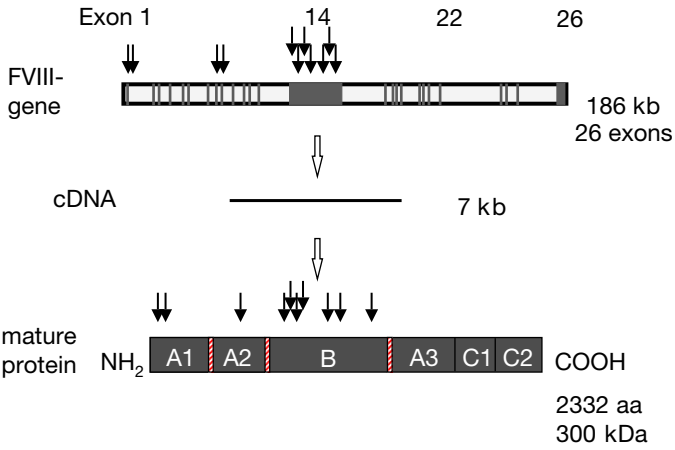


**Fig. 2.** Localization of polymorphism within the FVIII gene and FVIII protein.

very well to those reported in the literature. Beside these known polymorphisms only 9 more rare SNPs were found, that together altered 11 of 560 analyzed alleles. While 5 SNPs (codon 15, 21, 794, 831, and 1667) showed neutral amino acid exchanges, 3 SNPs (coden 408 Lys>Glu, codon 981 Pro>Ser, and codon 1038 Glu>Ala) led to an exchange of amino acids and one alters a splice site consensus sequence (intron 9).

## Conclusion

Since SNPs are very rare within the F8 gene, the hereditary increased FVIII activity levels in the normal population cannot be explained by polymorphic variants of the FVIII protein but instead should be due to other mechanism regulating the FVIII activity.

# Three Novel Microdeletions and the First Insertion / Deletion in Patients with Factor X Deficiency

K. Wulff, M. Gnad, M. Navarette, L. Salazar Sanchez, J. M.Carrillo, A. Batorova, W. Kreuz, R. Strey and F.H. Herrmann

## Summary

In the »Greifswald study congenital FX deficiency« the molecular defect underlying congenital factor X (FX) deficiency were investigated in 102 subjects. 29 different factor X gene lesions including three novel microdeletions and the first indel (insertion/deletion) mutation were analyzed. The F10 lesions Tyr163delTA, Gly319delG and the indel mutant Glu329Lys330insTTdelGA are nonsense mutants. The homozygosity of mutants Tyr163delTA and Glu329Lys330>Asp329stop330 is associated with severe bleedings diathesis. The novel FX gene lesion Ile269delTCA represents an inframe mutant with loose of amino acid Ile269 of FX. Problems in the substrate recognition by prombinase in this mutant FX are likely.

## Introduction

Factor X (FX) is a vitamin K-dependent plasma protein which plays a central role in blood coagulation. FX, the zymogen of the serine protease FXa is synthesized by the liver and circulated in the plasma as a two-chain protein. FXa, in complex with is cofactor Va, forms the prothrombinase complex and is the important physiologic activator of prothrombin. The FX gene (F10) contains eight exons and is located on chromosome 13q34 [9]. The exons of F10 encodes the specific protein domains: signal peptide, propeptide, GLA domain, two EGF domains, activation peptide and the serine protease domain with the catalytic triad His236, Asp228 and Ser379. Severe FX deficiency is a rare autosomal recessive disorder with an estimated prevalence of 1:1,000,000 in the general population (Perry et al., 2002). To day, 55 different F10 mutations have been reported in the HGMD database F10, 2003, include 48 nucleotide substitutions, 4 small deletions (<30bp) and 3 gross deletions. We investigated the molecular defects underlying congenital factor X (FX) deficiency. In the »Greifswald study congenital FX deficiency« the F10 gene of 59 unrelated families with FX deficiency or strong reduced FX levels were analyzed on naturally occurring mutations. Three novel small deletions (<30bp) and one indel mutant in coding regions of the FX gene were detected in patients and there families. The novel F10 gene lesions and the effect of the FX function were discussed.

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## Material and Methods

102 patients with FX deficiency or strong reduced FX levels and her family members were studied. The measurement of specific FX clotting factor activity was performed by one-stage clotting assays in patient's home hospitals.

DNA was isolated from whole blood samples by standard methods (Miller et al. 1988). All exon regions, the exon/intron boundaries and the 5' flanking region including the promotor were amplified by PCR under standard conditions, using primer pairs designed from flanking intron regions (genbank, accession number FX 13628312). Heteroduplex analysis were used for the identification of gene variations in the F10 gene [11]. The sequencing system of ABI and the sequencer ABI 377 were used for the mutation detection. All variations was detected least two times.

## Results

The first F10 indel mutant and three novel microdeletions in exon regions of the F10 were identified in the »Greifswald study congenital FX deficiency« by heteroduplex analysis and sequencing.

A first compound mutant (insertion/deletion) cause a substitution of the two base pairs GA to TT (Glu329Lys330 > Asp329stop330) in exon 8. This lesion (named FX Regensburg) was detected homozygous in a German patient with FX:C <2% and FX:Ag 7 % with symptoms of GI bleedings, smooth tissue bleedings and cephalic hematomas. The patient receives prophylactic PCC (prothrombinase complex concentrates). The heterozygous parents of this patient are asymptomatically. The low FX activity and antigen level represent a $CMR^-$phenotype.

Three novel FX microdeletions were detected in exons 6 and 8.
The two base pair deletion (named FX San Jose) was identified homozygous in a patient from Costa Rica with FX:C 1% ; FX:Ag <1% and his heterozygous parents. The deletion 607-608delTA interrupts the reading frame in exon 6 and caused a stop codon 163. The homozygous female patient shows five days after delivery CNS bleedings, with four years gastrointestinal bleeding symptoms and with 13 years severe menorrhagia.

The small deletion 1077delG in Gly319 named FX Frankfurt II represents a one base pair deletion in exon 8 with stop in codon 321. This novel F10 mutant was detected heterozygous in an German proband with reduced FX level (FX:C 41%).

The novel F10 lesion, deletion 926-928delTCA in exon 8 (named FX Bratislava) was identified heterozygous in a female patient of Slovakia with FX:C 30% to 50% and symptoms of bleedings (severe menorrhagia). This small deletion caused a loose of codon Ile269 of F10 and represents the first identified inframe deletion in a exon region of F10. The protein modeling shows, that the loose of the amino acid Ile269 could leads to changes of the molecular surfaces in the adjacency of the relevant residue. Wilkens and Krishnaswamy [10] demonstrated that the residues 262–271 and 282–296 are involved in the substrate recognition by prothrombinase.

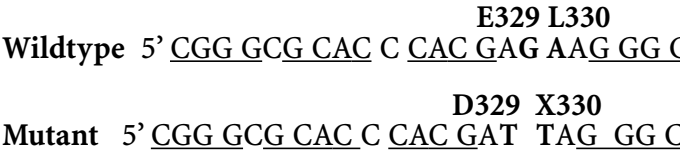


**Fig. 1.** DNA sequence context of the compound mutant insTT/delGA in exon 8 of the FX gene in a homozygous patient. The two base pair substitution are marked in bold. The location of the imperfect symmetric elements are denoted by underline.

## Discussion

The »Greifswald study congenital FX deficiency« demonstrate, that microdeletions of one or more nucleotides are not rare in F10 gene. Four of the 29 different causative FX mutants analyzed in the »Greifswald study congenital FX deficiency« were microdeletions or an indel mutant. Symmetric elements in the DNA (Fig. 1) were found to be prevalent in the vicinity of gene deletions and indel mutants (Chuzhanova et al. 2003). These motifs may serve to promote instability by facilitating the formation of secondary structure intermediates [2].

Until now, only few nonsense mutants of the F10 gene have been discovered world wide [3] include three microdeletions. In all these previously reported patients are heterozygous or a nonsense mutation was combined with a other F10 gene lesion in the genotype compound heterozygous. We reported the first two unrelated patients with FX deficiency and severe clinical symptoms of bleedings which are homozygous for the nonsense mutations (1105-1106insTT;1105–1106delGA) or (607-608delTA) in F10. The homozygosity of these nonsense mutants enabling to define the clinical phenotype associated with these novel lesions.

This work was supported in part by the German Federal Ministry for Education and Research (NBL3 program, reference 01 ZZ 0103).

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# SNP Map of the Protein C Gene

A. Pavlova, C. Geisen, M. Lim-Eimer, M. Watzka, E. Seifried and J. Oldenburg

## Introduction

Protein C is a vitamin K dependent anticoagulant protein that plays an important role in the regulation of the hemostatic system. Activated protein C exerts its anticoagulant effect by inactivating factor Va and VIIIa and thereby reducing thrombin generation. Deficiency of protein C is associated with an increased risk for venous thrombosis. The human protein C gene maps to chromosome 2q13-14 and comprises a coding region (exons 2 to 9) and a 5' untranslated region encompassing exon 1 (Fig. 1a) [2, 4]. Protein C is a member of the vitamin K-dependent family and contains a phospholipid-binding Gla domain, 2 epidermal growth factor (EGF) domains (the light chain) and a serine protease (SP) domain (the heavy chain) (Fig. 1b) [1, 5]. The aim of the present study was to investigate the genetic variability of the protein C gene in the normal population.

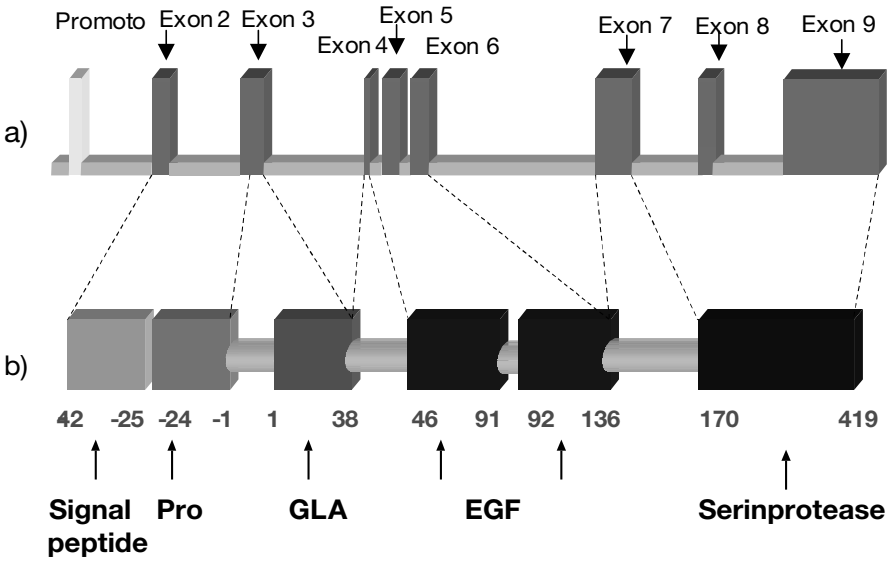


**Fig. 1a, b.** Structure of human Protein C gene and protein. **a)** Humen Protein C gene -Exons -Intros -Promotor. **b)** Human Protein C protein -presentation of the domain structure of the protein

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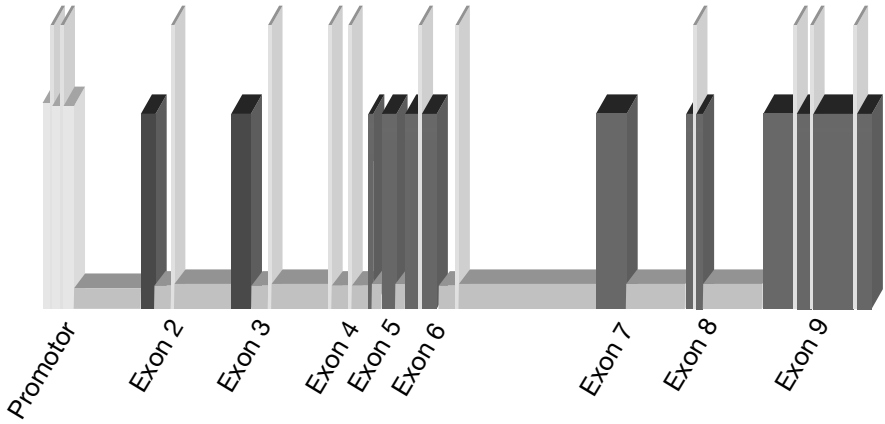


**Fig 2.** Schematic presentation of the polymorphisms in human Protein C.
□-Exons ■-Introns Promotor -Polymorphisms

## Materials and Methods

200 healthy blood donors (100 males and 100 females), representing 400 alleles, were screened for the presence of polymorphisms in the Protein C gene. Denaturing High Performance Liquid Chromatography (DHPLC) was applied as a screening strategy followed by direct sequencing of the fragments with abnormal patterns.

## Results and Discussion

In total, 12 different polymorphisms were identified in examination of 400 alleles – 2 in Promotor (untranslated region) , 5 in the intronic parts of the gene and 5 in the protein coding exons 2 to 9 (translated region). Eight of them, with relatively rare frequency were described for the first time – #2,3,4,5,6,10,11,12 (Table 1). From the other four polymorphisms, three (655A>T, 5472 T>G, 9385 T>C) were common with a frequency of 0.42, 0.37 and 0.28, respectively [3]. Two of them were in the exon 6 (5472 T>G) and 8 (9385 T>C) and 1 was in Promotor (655A>T). Only one rare polymorphism (10830 A>G) in exon 9 exhibited an amino acid substitution (N329D).

## Conclusion

In conclusion, only a single polymorphic allele with an amino acid exchange could be found in Protein C in 400 alleles, suggesting that this protein is highly conserved in the normal population. Thus no polymorphic candidates for the modulation of protein C activity could be identified so far.

**Table 1.** Distribution of polymorphisms within the human protein C gene

| # | Contig Position | Exon/ Intron | Nucleotide position | Nucleotide Exchange | Amino Acid Exchange | Frequency |
|---|---|---|---|---|---|---|
| 1 | 1237489 | 5' UTR | 655 | A>T | - | T-0.427 |
| 2 | 1237571 | 5' UTR | 737 | G>A | - | A-0.010 |
| 3 | 1239132 | Intron | 2294 | T>G | - | G-0.005 |
| 4 | 1240491 | Intron | 3647 | T>A | - | A-0.002 |
| 5 | 1241878 | Intron | 5030 | C>G | - | G-0.002 |
| 6 | 1241904 | Intron | 5055 | C>T | - | T-0.002 |
| 7 | 1242323 | Exon | 5472 | T>G | S141S | G-0.374 |
| 8 | 1242483 | Intron | 5643 | G>T | - | T-0.027 |
| 9 | 1246221 | Exon | 9385 | T>C | D256D | C-0.285 |
| 10 | 1247692 | Exon | 10826 | G>A | P327P | A-0.002 |
| 11 | 1247696 | Exon | 10830 | A>G | N329D | G-0.002 |
| 12 | 1247884 | Exon | 11018 | C>T | G401G | T-0.002 |

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## *VIIe. Miscellaneous*

# Characterization of Factor VIII-Von Willebrand Factor (FVIII-VWF)-Complex Concentrates Under Shear Stress

J. Siekmann, H.P. Schwarz and L. Turecek

## Introduction

Human von Willebrand Factor (VWF) is an adhesive glycoprotein present in human plasma [1, 2]. The mature subunit consists of 2050 amino acid residues [3] and is built in a series of multimers. The smallest of the von Willebrand multimers is the primary dimer with a molecular mass of 540 kDa, which is composed of two C-terminal disulfide-linked subunits. This structural element is assembled into larger multimers with molecular masses up to 10,000 kDa by N-terminal disulfide linkages [4].

Von Willebrand factor has at least two functions in vivo, the stabilization of coagulation factor VIII (FVIII) and in primary hemostasis it serves as a bridge between platelets and specific components of the extracellular matrix, such as collagen. Quantitative or qualitative alterations of VWF lead to von Willebrand disease (VWD), the most frequent inherited bleeding disorder in humans [5].

Von Willebrand disease can be treated by replacement therapy with high and intermediate purity FVIII concentrates [6–8]. Although VWF in FVIII-VWF concentrates lack the highest molecular weight multimers, they can control bleeding in patients with different VWD types [9, 10]. Currently characterization of VWF is based on methods such as ristocetin cofactor and collagen binding assays done under static conditions [11]. To assess whether FVIII-VWF concentrates can promote platelet adhesion under shear stress we used six therapeutic products with different multimeric structures and characterized them by simulating in vivo flow conditions.

## Materials and Methods

### Structural and Functional Characterization of VWF

The analysis of VWF multimers was done by SDS-agarose gel electrophoresis using a 1 % gel [12] with minor modifications. FVIII activity was measured by the two-stage clotting method [13]. VWF antigen (VWF:Ag) was determined with a sandwich commercial ELISA system (Asserachrom vWF, Boehringer Mannheim, Germany). Ristocetin cofactor (VWF:RCo) activity was measured according to standard procedure [14]. Collagen-binding activities (VWF:CB) were determined as described [15].

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**Perfusion Studies**

A parallel plate perfusion chamber was manufactured according to Sakariassen et al. [16] and used for all perfusion experiments. For all experiments the cover slips were coated with type I collagen from equine tendon (collagen reagent Horm; Nycomed, Munich, Germany) and inserted in the chamber. Perfusates (artificial whole bloods) were prepared by mixing erythrocytes, platelets and VWF-containing sample to give a hematocrit of 40 % and a number of platelets of approximately 200,000/µl and perfused at 37° C as described [17, 18]. After perfusion the coverslips were removed and fixed with glutardialdehyde and methanol and stained with May-Grünwald solution. The degree of platelet adhesion (mean of two cover slips) to the collagen surface was determined by light microscopy and video image analysis. For each coverslip 50 fields were selected and evaluated. In addition aliquots of the freshly prepared perfusates were applied to ADP cartridges of the platelet function analyzer, PFA-100 (DADE, Munich, Germany) [19] and the closure times were determined by the standard procedure according to the instructions of the manufacturer.

**Structural and Functional Characterization of FVIII-VWF Concentrates**

Six different plasma-derived FVIII-VWF concentrates (5 high purity and 1 intermediate purity) were used in this study. The multimer analysis (Fig. 1) shows that all concentrates have less multimers than normal plasma (22 multimers) ranging from 14 (concentrate D) to 20 multimers (concentrates B and E). On the basis of the multimer pattern the different concentrates can be classified into two groups:
Group A: 14–15 multimers (concentrates A, D and F) and group B: 18–20 multimers (concentrates B, C and E).

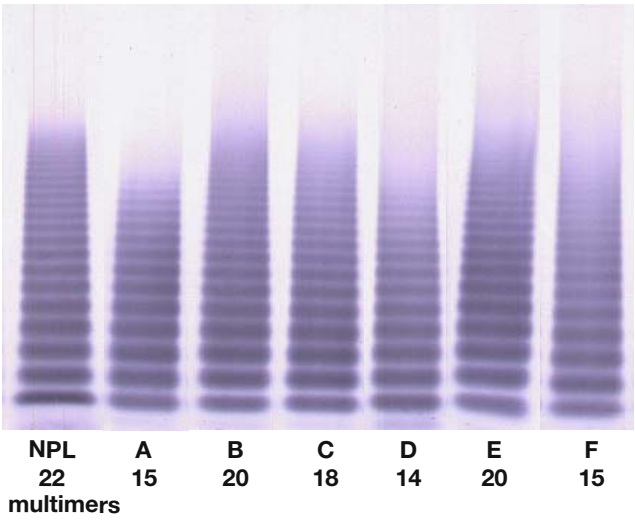


**Fig. 1.** VWF multimer composition of VWF in six different FVIII-VWF concentrates: (A–E) high purity concentrates; (F) intermediate purity concentrate; (NPL) normal human plasma (control sample).

**Table 1.** Functional characterization of different therapeutic FVIII-VWF concentrates

| Concentrate | | Ratio FVIII 2-stage/ VWF:Ag | Ratio VWF:RCo/ VWF:Ag | Ratio VWF:CB/ VWF:Ag | Ratio VWF:CB/ VWF:RCo | Multi-mers (1% gel) |
|---|---|---|---|---|---|---|
| High purity | A | 0.53 | 0.39 | 0.73 | 1.87 | 15 |
| High purity | B | 0.39 | 0.88 | 0.90 | 1.02 | 20 |
| High purity | C | 0.02 | 0.81 | 0.90 | 1.11 | 18 |
| High purity | D | 0.76 | 0.50 | 0.79 | 1.58 | 14 |
| High purity | E | 0.52 | 0.89 | 0.74 | 0.83 | 20 |
| Intermediate purity | F | 0.23 | 0.39 | 0.64 | 1.64 | 15 |

In addition these concentrates were characterized according to their functional activities (Table 1). Different concentrates are shown to have different FVIII levels indicated by the ratio FVIII two-stage assay/VWF:Ag. Concentrate C contains practically no FVIII. The specific activity of the VWF in the concentrates is given by the ratios VWF:RCo/VWF:Ag and VWF:CB/VWF:Ag. The ratio VWF:CB/VWF:RCo reveals that the collagen-binding activity is substantially higher than the ristocetin cofactor activity in preparations with a lower number of multimers (group A, n = 14–15; concentrates A, D, F). In contrast to this the collagen-binding activity was essentially the same in preparations of group B containing 18–20 VWF multimers (concentrates B, C, E).

## Characterization of FVIII-VWF Concentrates Under shear Stress Conditions

Artificial bloods composed of platelets, erythrocytes and VWF from FVIII-VWF concentrates in various concentrations (expressed in RCo activity units per total volume perfusate) were perfused in the parallel plate perfusion chamber (Fig. 2) and the VWF-mediated adhesion of platelets to coverslips coated with collagen was measured. The degree of platelet coverage on the collagen surface was evaluated by light microscopy and video image analysis. Figure 3 shows the typical microscopic structures obtained with different concentrations of high purity concentrate D. Perfusion experiments at various shear rates in the range of 400 to 3000 $sec^{-1}$ reveal that the platelet adhesion to a collagen surface mediated by FVIII-VWF concentrates increases with increasing VWF concentration (Fig. 4).

Figures 5 and 6 show a comparison of all concentrates in a series of perfusion experiments. In Figure 5 the results are expressed as the function of ristocetin cofactor activity. All concentrates are shown to mediate platelet adhesion in a dose-dependent manner. The degree of platelet adhesion obtained with concentrate D seems to be substantially higher than for the other concentrates although this preparation belongs to group A with the lowest number of multimers. Similar results were obtained by calculating the adhesion curves on the basis of VWF antigen (Fig. 6).

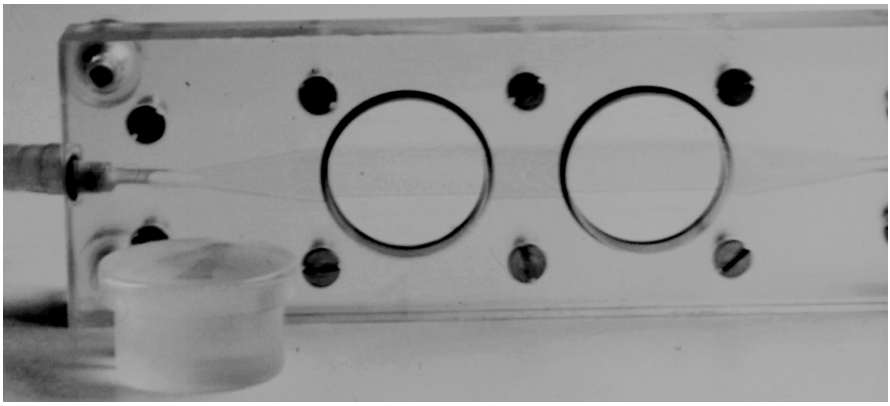

**Fig. 2.** Design of the parallel plate perfusion chamber. The chamber (length: 175 mm/ flow slit: 10 mm width and 0.6 mm height) is designed for perfusion experiments with two coverslips (18 mm x 18 mm) inserted in series.

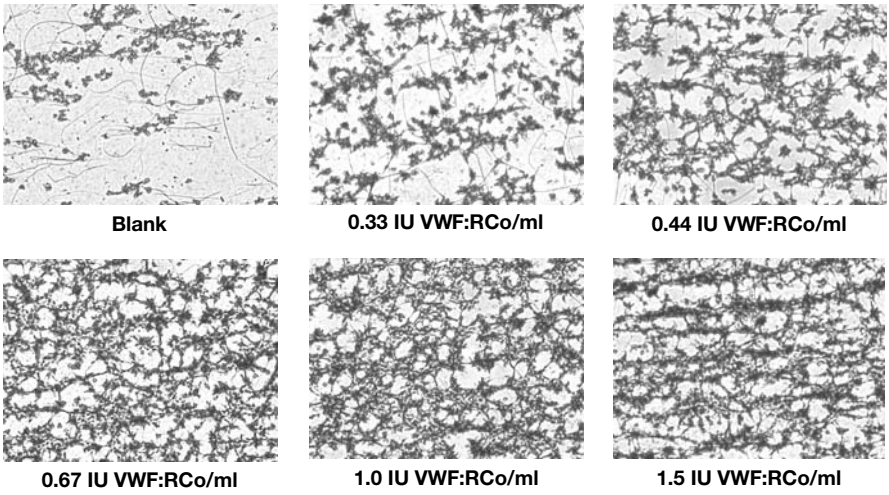


**Fig. 3.** Microscopic structures of platelets adhered to collagen at high shear rate (2500 $sec^{-1}$) mediated by FVIII-VWF concentrate (magnification 400X).

Our results were confirmed by applying the same artificial whole bloods to the platelet function analyzer, PFA-100, equipped with ADP-cartridges. For the FVIII-VWF concentrate D, showing the highest efficacy in the parallel plate perfusion chamber, definite closure times, although above the normal range (71-118 sec), could be measured when VWF concentrations higher than 1.0 VWF:RCo IU per ml of total perfusate were applied (Table 2). With the other concentrates no closure time could be determined. For mixtures of erythrocytes and platelets supplemented with platelet-poor plasma a closure time of 87 sec within the normal range was measured.

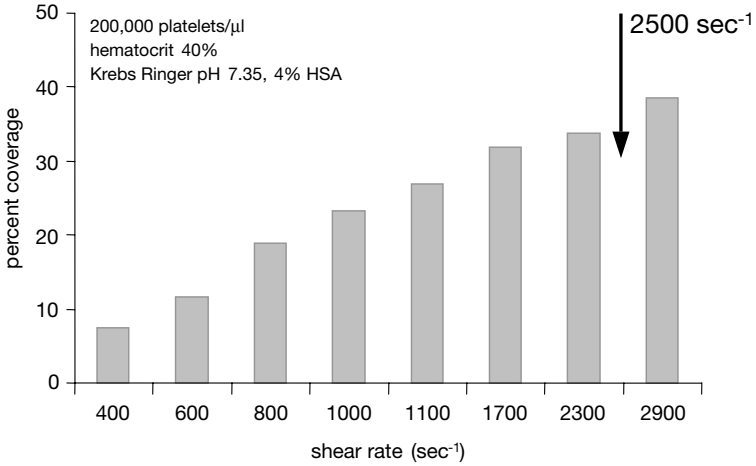


**Fig. 4.** Platelet adhesion to collagen surface mediated by VWF in high-purity FVIII-VWF concentrate D at different shear rates.

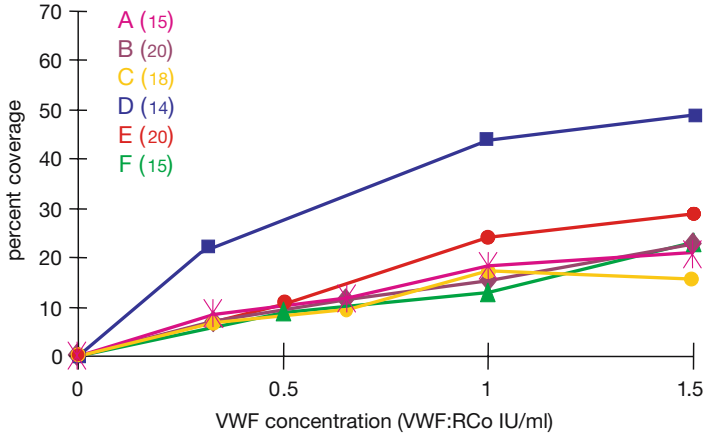


**Fig. 5.** Platelet adhesion to collagen surface mediated by VWF in six different FVIII-VWF concentrates at high shear rate (2500 $sec^{-1}$).

## Conclusions

In summary our results show that FVIII-VWF concentrates promote platelet adhesion under flow at high shear stress in a dose-dependent manner. In addition our results indicate that platelet adhesion is effectively promoted by FVIII-VWF concentrates even if they lack the highest molecular weight multimers. There was no correlation between the VWF-mediated platelet adhesion to collagen and the number of multimers.

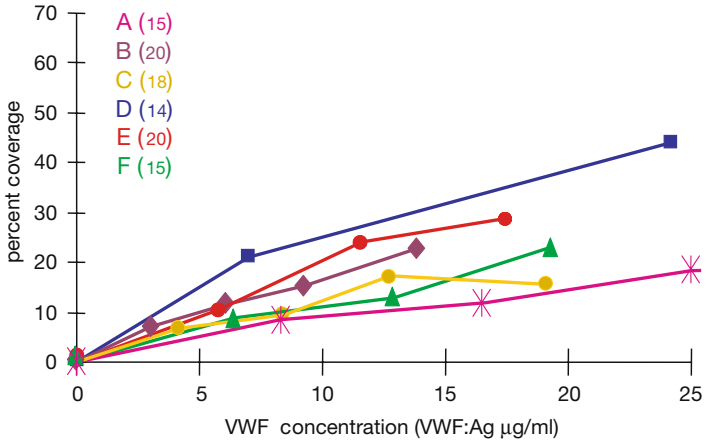


**Fig. 6.** Platelet adhesion to collagen surface mediated by VWF in six different FVIII-VWF concentrates at high shear rate (2500 $sec^{-1}$) as a function of antigen concentration (1 IU VWF:Ag/ml was regarded as 10 µg/ml).

**Table 2.** Closure times measured with the PFA-100 system.
The samples were composed of erythrocytes (Hematocrit 40 %), platelets (200,000 cells/µl) and FVIII-VWF concentrate. VWF-deficient artifical blood containing no VWF and a perfusate composed of erythrocytes and platelets supplemented with platelet-poor plasma (PPP) were used as control samples

| | | VWF:RCo (IU/mL) | Closure time ADP (sec) |
|---|---|---|---|
| Concentrate | A, B, C | 1.5<br>0.7 | <br>no closure time |
| | D | 1.5<br>1.0<br>0.7 | 137<br>194<br>no closure time |
| | E, F | 1.5<br>0.7 | no closure time |
| Control | PPP | 0.25 | 87 |
| | VWF-deficient artificial blood | – | no closure time |
| | normal range (citrated whole blood) | 1.0 | 71–118* |

* according to instruction leaflet

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# Procedural Rules of a National Hemophilia Register in Germany

R. Zimmermann, H.-H. Brackmann, M. von Depka Prondzinski, H. Lenk, W. Schramm, I. Scharrer and Members of the Medical Advisory Council of the German Hemophilia Association (DHG)

The Medical Advisory Council of the German Hemophilia Association (DHG), the supervisory Board of the Hemophilia Commission of the GTH, the managing Board of the DHG as well as the political committees demand the institution of a register of the patients with hemophilia and other hemorrhagic diatheses. Consequently, the medical Advisory Council of the German Hemophilia Association and the Hemophilia Commission of the GTH have set themselves the task to compile a national hemophilia register. A set of procedural rules has been formulated as the basis for the collection, central storage, processing, evaluation and publication of data. The activities for creating such a register are aimed at the following objectives:

Production and care of a register of patients with hemophilia and other hemorrhagic diatheses in Germany; to create the conditions for establishing such a register, especially in terms of data privacy and data security; Presentation of the data and publication of important aspects to provide an incentive for the planning of multicenter studies; to raise funds for the care of the hemophilia register; Promotion of the cooperation among the hemophilia centers; solution of clinical and health-policy problems as well as to optimize patient care.

The procedural rules define the organs such as the Steering Committee and the Data Safety and Monitoring Committee. Appendix I of the agenda describes the rules for publication. Appendix II sets forth the utilization of the data. In Appendix III, all of the participants involved in establishing the hemophilia register have to declare their consent to the procedural rules by giving their signatures. Appendix IV provides an in-depth explanation of the data processing and the data privacy and security. In another appendix, the patients are informed about the objectives of the hemophilia register and about data privacy and security; in addition the agenda contains a proposal for the patients informed consent.

Medical Advisory Council of the German Hemophilia Association: K.-H. Auerswald, H.-H. Brackmann, M. von Depka Prondzinski, B. Eifrig, L. Gürtler, C. Heinrichs, F.H. Herrmann, L. Hovy, W. Kreuz, K. Kurnik, H. Lenk, H. Pollmann, I. Scharrer, G. Syrbe, H.-H. Wolf, R. Zimmermann. Assessors: M. Siller, W. Kalnins.

I. Scharrer/W. Schramm (Ed.)
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# Quality Management of Clotting Factor Replacement Therapy in Medical Institutions – Advantage or Obligation

K.H. Beck and M. Mohrmann

## Background

Besides the costly and time consuming care of patients with hemorrhagic diatheses the treating physicians are exposed to increasing administration efforts.

According to § 137 SGB V medical institutions are obliged to perform a quality management system. The German transfusion law stipulates to document the patient applied clotting factor units. The Health Insurances scrutinize the clotting factor supply according to patients' needs (§ 12 SGB V). The controlling process by insurances means time consuming and troublesome work for the hospital staff.

A quality management tool that could help to reduce the considerable effort for providing the patients' data requested by the medical service of the insurances (MSI) may possibly help to solve this problem.

## Methods

### Process-Analysis

Structure: Insurances are bound to control the services rendered by the medical institutions with regard to prerequisite, type and extent by their medical service (§ 275 SGB V).
Core process (Fig. 1): The hospitals invoice the insurances the special charges for factor replacement therapy. The MSI controls certain quality indicators recorded in the file of the patient by order of the insurances. The control act usually necessitates a copy of the patient file. With a standard request letter which considers the individual case the MSI requests this file from the different hospitals.
Problem: Time delays (six months and longer) combined with an additional work effort are accruing for all parties.

The reasons are :
- The documents are not complete despite a detailed letter of request.
- The standard letter of request has erroneously been sent to the department where the patient's main disease was treated even though the hemostaseologic consultant is responsible for the management of the bleeding diathesis.

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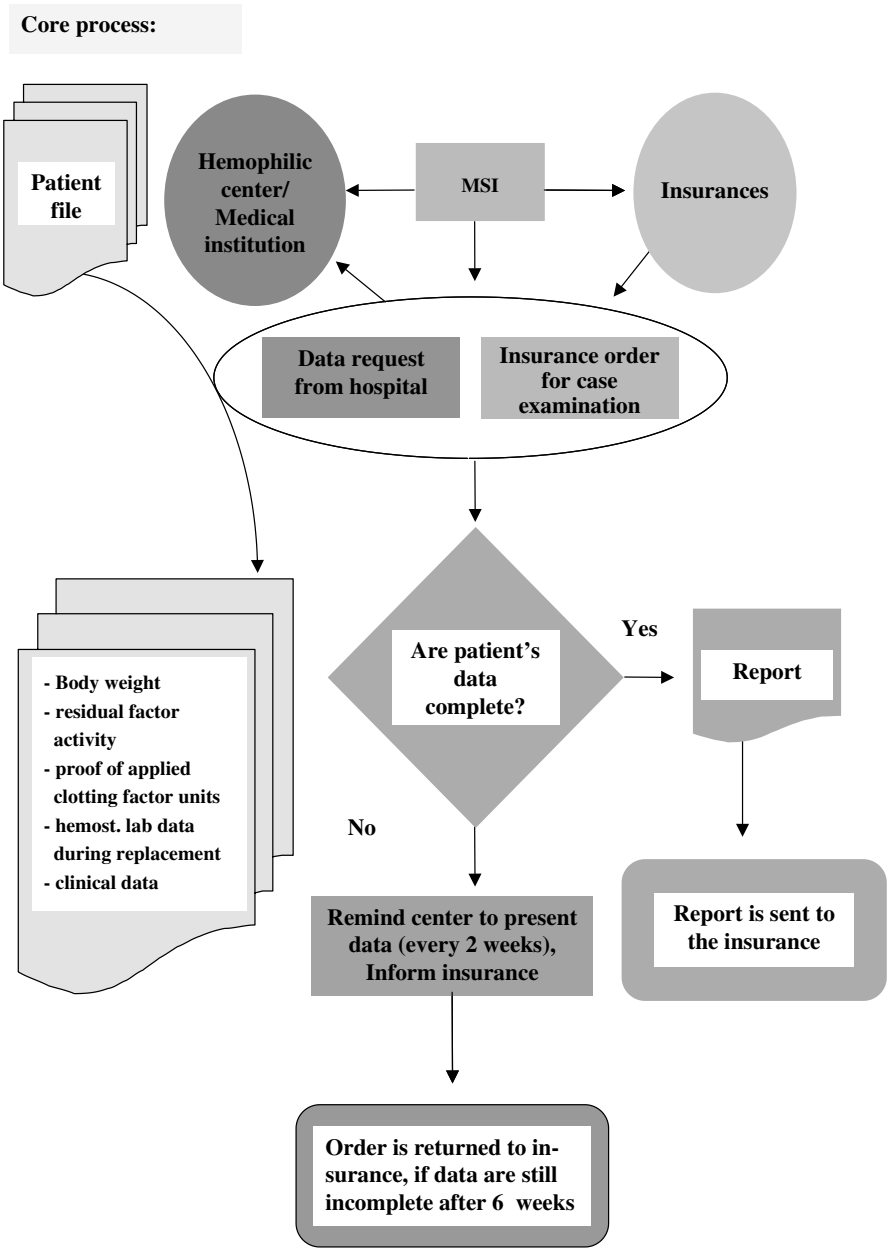


**Fig. 1.** Core process of MSI report generation according to § 275 SGB V

– Discrepancies between prescribed and documented amount of factor concentrate or between the documented amount of factor concentrate and the effective costs.

**Change Management**

The Department of Hemostaseology should provide a contact person for the MSI who coordinates the replacement therapy in the different departments where the patient has been treated during one hospital stay. This person exactly knows the different wards where the patient was for a special time period, because he/she supervises the patient's clotting situation during the whole hospital stay. In this way recurrent requests by the MSI regarding patient data and references from one department to the other thereby could be avoided.

A separate documentation schedule (Table 1) should be provided where the most essential quality indicators are listed, i.e.

– date and time of the replaced amount of factor concentrate, and related data of the lab as well as
– patient's personal data like body weight and residual factor activity and
– clinical informations regarding the course of the bleeding under the replacement therapy.

The documentation schedule should also be available in an electronic form.

**Table 1.** Documentation schedule for replaced clotting factor units

| Clotting factor replacement / products: | | | | | | | |
|---|---|---|---|---|---|---|---|
| Patient: | | | | Bleeding localization: extent: residual act.: (Mutation): Body weight: | | Diagnosis (clotting deficiency and actual diagnosis),bleeding history: | |
| Date | Time | Units replaced | Act. (%) before / after replacement | aPTT before / after replacement | PT before / after replacement | Inhibitor (BU) | Act. course of hemorrhage (i.e. reduction, additional, resorption state), application modus (bolus injection, continuousinfusion), side effects |
| | | | | | | | |
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## Results

Doubts about the real applied factor amount caused by differences in prescribed and documented amounts should no longer occur, because only the applied amount will be recorded on that schedule.

Separate documentation schedule in combination with the letter of discharge should normally be sufficient for the MSI assessment

A contact person of the MSI has the following advantages:

- He/She can quickly get the documents in paper form or as a floppy-disk and closely refer to the MSI. If both documents (letter and documentation schedule) are stored separately in an electronic form, they could be provided even faster. He/She is available for the MSI for being consulted on specialized questions.
- He/She can remove possible arising interface problems, because he/she is familiar with the course of the process and is familiar with the involved parties.

## Conclusion

- The quality adopted management of the inquiries by the insurances leads to a quicker processing of documents requested with a reduced expense of time for all parties.
- The small additional effort for the separate documentation which has to be done for legal reasons anyway (German Transfusion Law § 14) is justified by the reduced administration effort.
- The documentation schedule could be the basis for a continual improvement of the patient related data transfer from hospital to the MSI regarding the SGB V associated data examination.

# Proteus Syndrome: Successful Therapy of Severe Migraine Symptoms with low Molecular Weight Heparin

V. Aumann, G. Lutze, A. Neumann, K. Mohnike and U. Mittler

## Introduction

Proteus syndrome is a sporadically occurring dysplasia syndrome presumably caused by a lethal point mutation where only the carrier of a genetic mosaic is capable of surviving.

The clinical picture is characterized by a high variability. Symptoms include a partial or complete hemihyperthrophy of the body, partial gigantism of the hands or feet, ossification disorders of individual bones, subcutaneous and abdominal tumors (lipoma, hemangioma), pigmented moles and varicosis of the lower extremities with hardly any anomalies of the deep veins.

From the perspective of a differential diagnosis there is a close relationship to the Klippel-Trenaunay-Weber syndrome which is characterized by a triad of symptoms consisting of hypertrophy of soft tissue and bones, cutaneous tumors and varicosis. The most important characteristics are anomalies of the deep venous system with atresia, hypoplasia, valvular incompetence, and aneurysmal dilatation. Thrombophlebites and thromboembolic events such as pulmonary embolism have been frequently described.

## Case report

A now 16-year-old boy shows a mostly right-sided partial hemihypertrophy with the feet, hand and shoulder being affected (Fig. 1). Naevus flammeus and superficial varices are also found on the lower legs and the distal upper legs. Syndactylia between the 3rd and 4th left toe were removed surgically. Radiological tests (Fig. 2) confirmed the varicosis and hypertrophies of the 1st and 3rd ray of hand and foot and of additional bone segments. MRT tests of the skull did not show any significant results. Since there were no anomalies of the deep veins, the Klippel-Trenaunay-Weber syndrome was considered unlikely and a Proteus syndrome was diagnosed instead.

The patient who attends high school with good credentials does not show acute symptoms of an illness. Severe migraine-like headaches have occurred since the age of seven with up to three attacks per week; this considerably impairs his life and his school attendance frequencies. An MRT excluded intracranial vascular malformations (Fig. 2).

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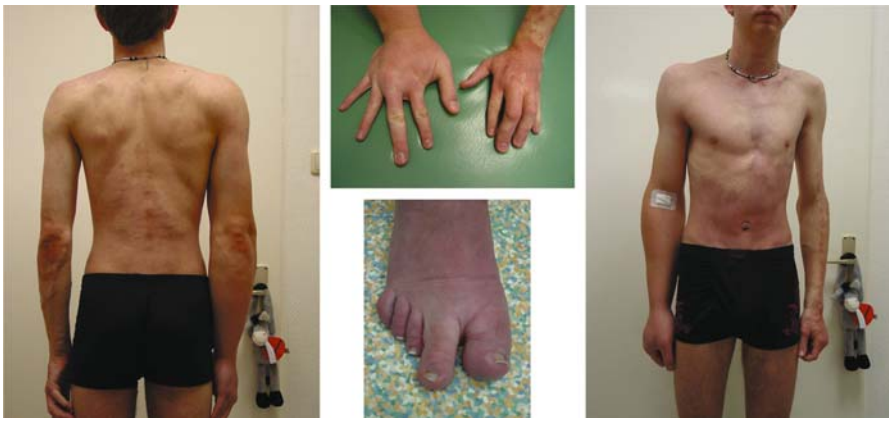

**Fig. 1.** Clinical signs of the patient

## Coagulation Diagnostics

Several coagulation tests did not reveal the presence of an inherited thrombophilia. However, an increase in the prothrombin fragment 1+2 (F 1+2) and in D-dimer as well as elevated platelet aggregation indicated continuous coagulation activation. Given an expected range of < 1.4 nmol/L, the prothrombin fragment 1+2 was permanently elevated at a value of above 2.0 nmol/L (Fig. 3). The D-dimer levels were also constantly above 2.0 mg/L (expected value < 0.5 mg/L). Given the induced platelet aggregation with ADP at various concentrations, the pronounced effect of low ADP concentrations indicated an increased platelet aggregation or hyper-reactivity (Fig. 4).

## Therapy

Under the assumption that the increased platelet aggregation may be considered the cause for the migraine-like pain attacks in addition to the existing vascular disturbances, acetyl salicylic acid (ASA) was first administered followed by a treatment with clopidogrel (Plavix). Both platelet aggregation inhibitors did not show any clinical success although the desired inhibition of the ADP-induced aggregation could be demonstrated under laboratory conditions. The treatment with Plavix was discontinued following a pain attack that lasted an entire day accompanied by vomiting and impaired vision. Treatment in a pain center and orthopedic, physiotherapeutic and paramedical treatment were not successful either.

Following a painful swelling of the right lower leg with a suspected venous thrombosis of the lower leg, low molecular weight heparin (Clexane) was administered since the thrombophilic situation had already been established. Surprisingly, the headache attacks disappeared almost completely. Currently the patient is administered Clexane 60 on a daily basis. Tests done before and after a Clexane 60 injection show that the hypercoagulability largely disappears (Table 1).

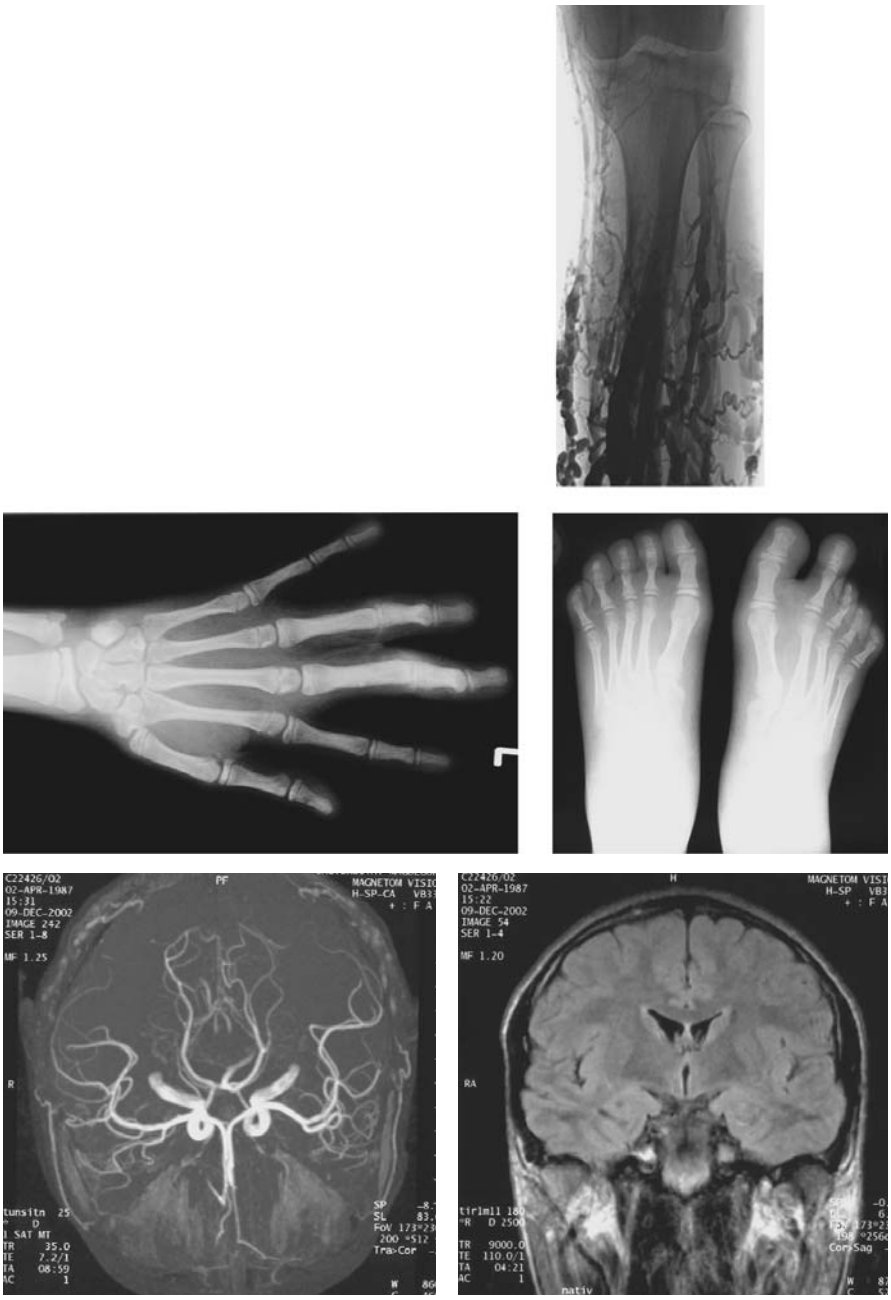


**Fig. 2.** Radiological results
*Phlebography:* Varicosis without arteriovenous shunts and clot formation
*X-ray examination of the hand and foot skeleton:* Hypertrophic anomalies
*Skull-MRT and Angio-MRT:* no significant signs

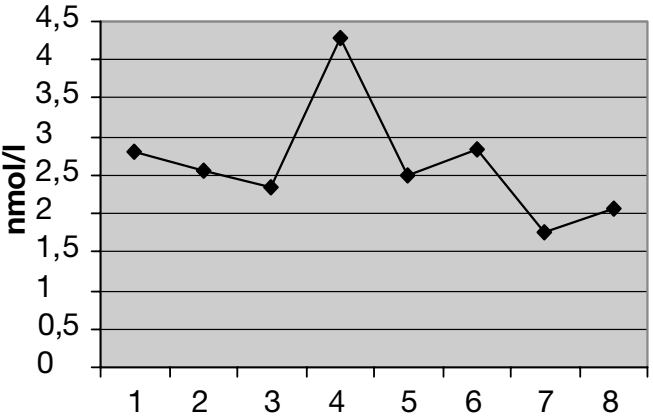


**Fig. 3.** Prothrombin fragment 1+2
Course over time

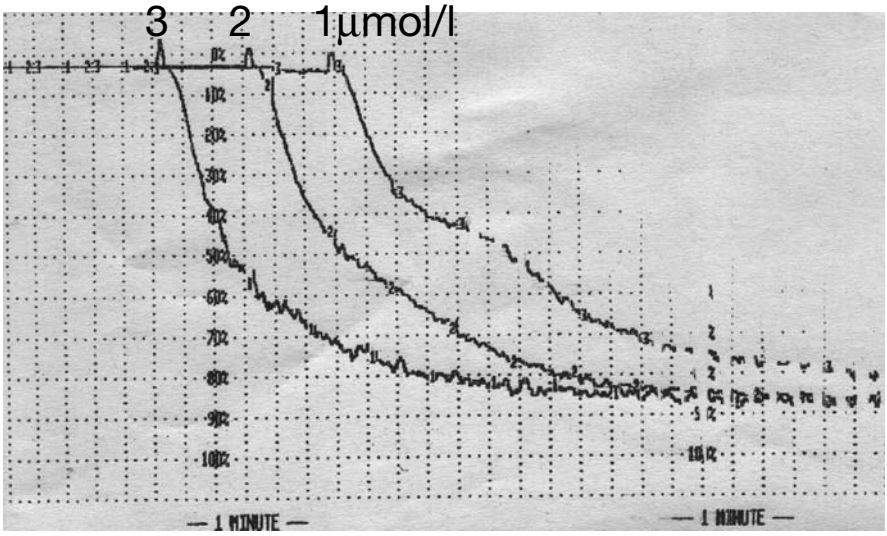


**Fig. 4.** Induced platelet aggregation
Addition of ADP (final concentrations of 1, 2, and 3 µmol/L)

**Table 1.** Laboratory findings before and after Clexane 60 injections

| Parameter | Before injection | 2 hours after injection | 4 hours after injection |
|---|---|---|---|
| F1+2 (nmol/L) | 1.55 | 1.29 | 1.23 |
| D-dimer (mg/L) | 0.60 | – | 0.50 |
| Clexane 60 (U/mL) | < 0.10 | 0.48 | 0.56 |
| Aggregation | normal | normal | normal |

## Summary and Discussion

- In addition to the partial hemihypertrophy, other changes which are also indicative of Proteus syndrome could be observed.
- The patient has suffered from migraine-like headache attacks since the age of seven. Literature references mention in particular headache and thromboembolies when there are overlaps with the Klippel-Trenaunay-Weber syndrome.
- Coagulation tests revealed in particular increased concentrations of the activation markers prothrombin fragment 1+2 and D-dimer and an increased platelet aggregation.
- Following the administration of low molecular weight heparin, the patient was almost completely symptom-free. The reason for this may be that the pronounced varicosis caused an increased platelet activation together with an increase in plasmatic coagulation. The aggregate formation of the hyper-reactive platelets triggers the headache attacks. Since the effects of the drugs administered did not relieve the patient from his complaints, the increased plasmatic coagulation had to be considered the main cause. Heparin hence is currently the best-possible therapy option.

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# Administration of Recombinant Factor FVIIa (NovoSeven, NovoNordisk) in a Patient with Glanzmann Thrombasthenia

A. Zyschka, Z. Elanjikal, C. Escuriola Ettingshausen, I. Martinez Saguer, R. Linde and W. Kreuz

## Introduction

Glanzmann thrombasthenia is a rare autosomal recessive disorder caused by a functional defect of the GP IIb- IIIa complex which is important for the binding between platelets and fibrinogen.

Clinical characteristics are severe mucosal bleeding symptoms (epistaxis, menorrhagia in women), prolonged bleeding time along with normal platelet count. A diagnosis of Glanzmann`s thrombasthenie is based on the following findings: normal or slightly decreased platelet counts; prolonged bleeding time, deficient platelet aggregation to collagen, thrombin, epinephrine and ADP and decreased expression of platelet membrane glycoprotein IIb and IIIa.

Platelet transfusion is the standard treatment for severe bleedings and for surgical support. Anti-HLA or anti GP IIb-IIIa alloantibodies frequently limit the efficacy of platelet transfusions in this condition. In such cases, the use of recombinant human Factor VIIa (rh FVIIa, NovoSeven,) has been reported. Furthermore, platelet transfusions have the risk for adverse reactions including virus transmission.

## Case report

We report on a 7-year-old boy, born in Afghanistan, the diagnosis of Glanzmann thrombasthenia has been established there. Since he was a few months old he had repeated nose bleedings. In June 2003 the patient presented with severe nose bleeding and had to admitted to our hospital.

The bleeding could not be controlled by using conservative measures such as the use of local pressure, nasal packing and tropical use of tranexamic acid. The hemoglobin concentration drooped to 8.3 mg/dl. Desmopressin and platelet transfusions were admitted immediately. The nose bleeding stopped and the boy was discharged the next day.

24 hour after discharge the nose bleeding started again from both nostrils. The bleeding could not be stopped and he was admitted once again to our hospital. There was no evidence of trauma or infection.

The bleeding did not resolve despite platelet transfusions and Desmopression, therefore we decided to give recombinant Factor VIIa (NovoSeven, NovoNordisk).

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The patient received a bolus dose of 100 µg/kg bw. After injection the nose bleeding decreased immediately. After 1 hour interval, NovoSeven in the same dose was repeated again. One hour and 30 minutes after the first injection the bleeding had finally ceased, however hemoglobulin was only 7.6 mg/dl and packed red blood cells were given. On this regimen the bleeding was stopped permanently and the boy was discharged two days later in good health.

## Discussion

Epistaxis in patients with Glanzmann thrombasthenia may be extremely difficult to arrest. Factor VIIa is an attractive alternative to platelet transfusions for the treatment of Thrombasthenie Glanzmann. Our experience from the present case is that NovoSeven was more sufficient than platelet transfusion to decrease the nose bleeding.

# Combined Heterozygous Factor XIII-Deficiency in a Family Case Report

E. Rusicke, V. Ivaskevicius, D. Klarmann,
C. Escuriola Ettingshausen, J. Oldenburg and W. Kreuz

## Introduction

Factor XIII is the last enzyme in the clotting cascade. Its function is the conversion of loose fibrin polymer into a highly organized structure [1]. Factor XIII deficiency is a bleeding disorder associated with characteristic hemorrhages and a life-long bleeding diathesis [2]. The bleeding tendency of the congenital factor XIII deficiency depends on the residual factor XIII activity as well as the gene-mutation [3]. The hemorrhage can occur spontaneously or after trauma. Characteristic bleeding sites are subcutaneous tissue, mouth and gum, intracranium, muscles and joints. As the first manifestation of the bleeding disorder umbilical bleedings occur in about 80% of all affected newborns [1].

The standard therapy of acute bleeding episodes or to cover surgical procedures consists of the i.v. application of a plasma derived factor XIII-concentrate (Fibrogammin, Aventis Behring). In selected patients with a high bleeding tendency a long-term prophylactic treatment of factor XIII-concentrate may be indicated.

The standard laboratory clotting tests (aPTT, PT) are within normal ranges in factor XIII deficient patients.

## Case Report (Index Patient- 31-year-old male)

Since early childhood the index patient showed easy bruising. At the age of 2 years he developed a large hematoma at his forehead after a minimal trauma. Local surgical interventions lead to bleeding complications with severe blood loss. Hemoglobin levels dropped to 7.6 g% and blood transfusions became necessary.

### *Coagulation Parameters*

The standard clotting tests (aPTT, PT) as well as platelets, platelet function, fibrinogen, von-Willebrand-parameters and in vivo bleeding time (Simplate I: 4 min) were normal in our index patient. A FXIII residual activity of 2% was assessed (Table 1).

**Table 1.** Laboratory report

| | index patient | father | mother | sister | niece |
|---|---|---|---|---|---|
| aPTT [sec.] | 32 | 30 | 29 | 30 | 29 |
| TPZ [%] | 106 | 98 | 96 | 100 | 103 |
| Factor XIII act. [%] | 2 | 48 | 46 | 51 | 65 |

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The patient developed recurrent spontaneous and traumatic bleedings into muscles and joints (knee, ankle) which were effectively treated with 25 IU/kg bw of a plasma derived factor XIII-concentrate (Fibrogammin Aventis Behring). In order to prevent the development of arthropathy a prophylactic treatment with12 IU/kg bw factor XIII-concentrate given every 4 week. Because of recurrent bleedings the treatment schedule had to be intensified up to 3-weekly intervals. Under this regimen relevant bleeding events could be prevented successfully. FXIII trough levels before each infusion were 7%.

## Family Case History

The index patient was the first child of a Greek, non-consanguineous relation.

Mother and father also showed decreased factor XIII-activities without any clinical relevance (bleeding tendency).

**Table 2.** Gene-analysis

| father | mother | sister | index-patient |
|---|---|---|---|
| Small Insertion exon 9 (Cins, codon 400) heterozygous | | Small Insertion exon 9 (Cins, codon 400) heterozygous | Small Insertion exon 9 (Cins, codon 400) heterozygous |
| | Splice-site-mutation in intron 5 (IVS 5-1 G>A) heterozygous | | Splice-site-mutation in intron 5 (IVS 5-1 G>A) heterozygous |

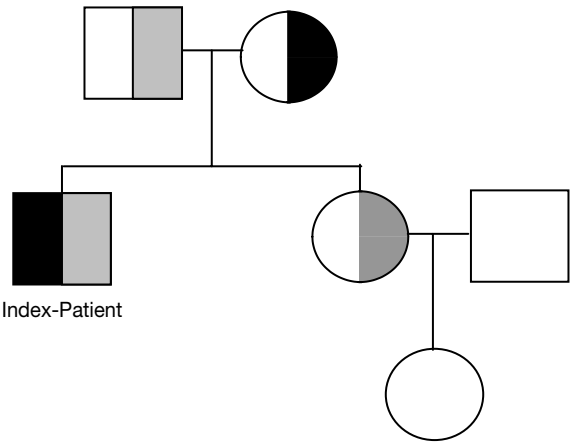


**Fig. 1.** Family tree

The younger sister (29 yrs.) had also low levels of factor XIII-activity. Bleeding complications occurred after tonsillectomy. Consecutively a prophylactic treatment with 31 IU/kg bw Fibrogammin was given before delivery and epidural anesthesia. No bleeding complications were observed.

The newborn niece showed normal factor XIII-activity at the age of 5 months.

Gene analysis in the family showed that the index-patient is combined heterozygous for a small insertion in exon 9 and a splice-site mutation in intron 5. Father and sister are both heterozygous for a small insertion in exon 9. The mother is heterozygous for a splice-site mutation in intron 5 (Table 2).

## Discussion

We report about the clinical picture of a combined heterozygous factor XIII-deficiency in a family.

The combination of heterozygous insertion in exon 9 (Cins, codon 400) and a splice site mutation in intron 5 (IVS 5-1 G>A) causes substantially decreased levels of factor XIII activity (2%) with severe bleeding tendency in our index-patient. A prophylactic treatment with factor XIII-concentrate is indicated in this patient. In the opposite to our index-patient his father, mother and sister who were heterozygous for one of this mutations had only a mild factor XIII deficiency with a factor XIII-activity about 48%. Even they have nearly the same factor-XIII-activity there is a different between clinical outcome of the sister with the small insertion in exon 9, who had bleeding complications after tonsillectomy and the mother with the splice-site-mutation who has no bleeding complication even after birth of two children.

Bleeding complications were successfully treated with factor XIII-concentrate (Fibrogammin, Aventis Behring). Concentrate was well tolerated. No seroconversion of HIV, HBV, HAV;HCV were observed.

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# Resistance to Coumarin in a 55-year-old Patient with Chronic Arrhythmia

W. Miesbach, J. Oldenburg, M. Krause, Ch. von Auer and I. Scharrer

Retrospective studies suggest that patients with continuous chronic arrhythmia have an increased risk of stroke and, for this reason, it is recommended that they receive oral vitamin K antagonists for the long-term treatment, such as coumarin derivatives to achieve an international normalized ratio (INR) range within the therapeutic level.

In most clinical situations, therapeutic doses of coumarins are sufficient to control this disease and prevent further thromboembolism.

Coumarins target blood coagulation by inhibiting the vitamin K epoxide reductase multiprotein complex (VKOR), which is involved in the carboxylation of several blood coagulation factors [1].

Presumably intestinale malabsorption or interactions with some other drugs are the most common causes of ineffective low INR levels.

Acquired resistance to Warfarin is also described in patients with enteral feedings [2] or with mechanical heart valve [3]. Several enteral feeding products may bind warfarin and reduce the bioavailability of the drug.

In contrary, the term warfarin failure is used in patients who develop thromboembolic complications despite an apparently stable INR at therapeutic levels.

Hereditary resistance to coumarin is a very rare phenomena which has previously been described in the literature by very few cases.

We report on a male patient, born in 1947, who appeared in our ambulance after he could not be treated sufficiently by oral anticoagulation in an external hospital.

In that hospital they could not see any prolongation of the thromboplastin time in the patient while regularly taking phenprocoumon. The patient had even occasionally taken considerable doses such as two tablets in the morning, three tablets in the midday and five tablets in the evening (a daily dose of 30 mg), without any effect on coagulation system being observed.

However since chronic continuous arrhythmia was diagnosed in March 2002 it was necessary to carry out efficient anticoagulation since the arrhythmia could not be converted to a regular sinus rhythm by electric or pharmaceutical means. We were also aware that the patient was suffering from chronic heart failure and hyperlipidemia. The medical therapy consisted of acetyldigoxin, spironolacton, amiodaron and atorvastatin. Besides the daily dose of one or two tablets of phenprocoumon the patient received also enoxaparin.

The measurement of INR was 1.02 and as a result the dose of enoxaparin was altered and a plan for dosing with phenprocoumon was set up. At his next con-

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sultation the measurement of the quantitative phenprocoumon level in blood was in the highest theapeutical range with 2.4 mg/l (therapeutical range: 0.16–3.6 mg/l, Prof. Dr. G. Kauert, University Frankfurt). The same time we measured an INR of 1.07. After changing the treatment from phenprocoumon to coumadin and acenocoumarol we were unable to achieve any effect on coagulation. Additional examinations gave negative results in terms of a dysfunction of abdominal resorption or liver function. A previously carried out nephrological examination also resulted in normal values. The other investigations of the blood were apart from slightly higher liver transaminases unremarkable, as were the values of ferrum or vitamin B12.

Under treatment with enoxaparin 60 mg twice daily the anti-Xa level remained continuously within the therapeutic range and the patient did not developed any thromboembolic or bleeding complications until now.

In conclusion, we suspect that the patient may have a genetic defect in the metabolism of vitamin K/coumarin since other causes could be excluded. This defect could be confirmed by Rost et al. as a genetic mutation of the first component of the VKOR complex [4].

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# Investigation of the Tendency to Bleeding in Patients with low Activity of Plasminogen Activator Inhibitor-1 (PAI-1)

W. Miesbach, N. Noormalal, T. Vigh and I. Scharrer

The relationship between increased values of plasminogen activator inhibitor-1 (PAI-1) and atherosclerosis as well as thrombotic disease, especially of the cardiac system has been well established.

The risk for ischemic heart disease might be due to impaired thrombolysis after plaque ruptur [1].

The progression of atherosclerosis is supposed by inhibiting the clearance of fibrin incorporated into atherosclerotic plaques [2]. PAI-1 may also affect cellular migration, matrix remodeling, and the activation of growth factors [3].

PAI-1 is elevated in many solid tumors, too and is associated with a poor prognosis in cancer [4].

The proteolytic activity of fibrin (fibrinolysis) is mediated by plasminogen and its activators, the serine proteases, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA).

PAI-1 was initially isolated and purified from the medium of vascular wall endothelial cell cultures by van Mourik et al in 1984 [5].

PAI-1 is a member of the serine protease inhibitor super-family (Serpin), the primary inhibitor of t-PA in plasma and an important physiologic factor which regulates fibrinolytic activity by inhibiting fibrinolysis.

Platelets, vascular endothelial cells, and vascular smooth muscle cells also contain PAI-1, suggesting that it is an important regulator of fibrinolysis at sites of vascular injury and thrombus formation.

The balance between serin proteases and serpins may affect the clinical manifestation of hemorrhage.

PAI deficiency leads to increased fibrinolysis. Individual reports of sometimes dramatically bleeding events in patients with low PAI levels have already been published [6, 7].

Congenital PAI-1 deficiency has been reported in very few cases [8].

Considerable information regarding the in vivo function of PAI-1 has been obtained from analyses of PAI-1 deficient mice generated by homologous recombination in embryonic stem cells [9].

The aim of this study was to identify patients with PAI-1 deficiency and characterize the typical manifestations of bleeding.

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## Material and Methods

We investigated retrospectively clinical manifestations in 216 patients with PAI activity of 0 U/ml to test the relationship between lower PAI levels and a tendency to bleeding.

Testing of PAI activity was ordered due to a history of unexplained bleeding or a recently thrombotic disorder.

PAI activity was assayed by means of the Bioimmunoassay Chromolize TM PAI-1. PAI antigen was assayed by means of the enzyme-linked immunosorbent assay (ELISA) TintElize PAI-1 and t-PA-antigen by TintElize tPA.

## Results

Of the 216 patients 77 % were female and 23 % male. The median age was 42 years (9–91 years). The median PAI antigen-level was 6.1 ng/ml (0.5–68.7 ng/ml, normal value: 3–25 ng/ml). The median t-PA antigen-level was 4.3 ng/ml (0.9–31.7 ng/ml, normal value: 1.9–8.4 ng/ml). 71 patients reported a tendency to bleeding, of which 96 % (68/71) were female. 13/71 patients were receiving anticoagulation therapy or treatment with aspirin due to a recently thrombotic disorder. In patients with tendency to bleeding and a PAI activity of 0 U/ml the values of PAI antigen and t-PA antigen were significantly lower than in patients without any tendency to bleeding (4.8 ng/ml vs. 7 ng/ml and 3.6 ng/ml vs. 4.6 ng/ml). Other possible causes of bleeding such as von Willebrand syndrome, deficiency of coagulation factors or dysfunction of platelets could be excluded in patients with a history of bleeding.

The majority of the patients (57 %) reported bleeding after surgical procedures or traumata (Fig. 1). They also mentioned easy bruising (18 %), abnormal long or heavy menstrual bleeding (13 %), noise bleeding (8 %) or gum bleeding (4 %). The extent of bleeding was reported by 52 % as being very high, by 25 % as high. It was possible to cure it by the application of tranexamic acid.

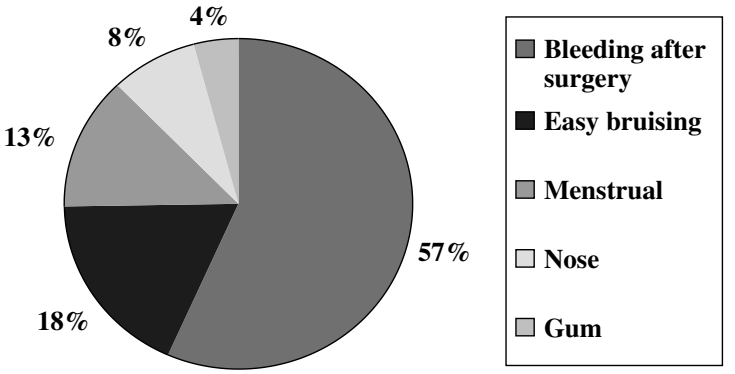


**Fig. 1.** Type of bleeding

## Discussion

PAI-1 plays an important role in haemostasis and is considered as a critical regulator of the fibrinolytic system. Elevated and reduced levels of PAI-1 may cause severe complications.

In this retrospective investigation of patients with a PAI activity of 0 U/ml we have seen especially in patients with additionally lower values of t-PA and PAI antigen a particularly severe tendency to bleeding. It was striking that mostly women were affected by hemorrhagic symptoms.

The characteristic clinical manifestations of these patients comprised prolonged abnormal bleeding after trauma, dental extractions and surgical procedures. Spontaneous bleeding such as petechiae or intra-articular hemorrhages could not be found in these patients. After initially stop of bleeding there was a common tendency toward rebleeding after a few hours at the wound site.

The administration of tranexamid acid clearly improved the hemorrhagic symptoms in these patients. But also other bleeding symptoms were reported, such as easy bruising, gum bleeding or menstrual bleeding. In the literature we found only one report of a female patient with PAI deficiency and menorrhagia [8].

PAI-1 deficiency can be homozygous or heterozygous. Mostly heterozygous PAI-1 deficiency does not result in a increased tendency of bleeding indicating that a single functional PAI-1 gene probably is sufficient for normal hemostasis.

It can also be suspected that some patients contain a mutation in the PAI-1 gene or an altered PAI-1 expression. There are cases in the literature with decreased functional activity of PAI-1 where the molecular basis for PAI-1 deficiency could not be determined. For example Schleef et al. described a 76-year-old man with a lifelong history of severe bleeding after trauma or surgery but normal plasma PAI-1 antigen, but low PAI-1 activity [10].

A spectrum of abnormal bleeding can be suspected in patients with PAI-1 deficiency. Although bleeding occurs mostly in response to trauma or surgery, it can be severe and even life-threatening.

Therefore, it appears reasonable that PAI-1 deficiency should be classified as a moderate bleeding disorder. Correct diagnosis is important because this disorder can be treated effectively with fibrinolysis inhibitors. In our ambulance patients with PAI deficiency get an emergency document with exact therapeutic recommendations. Oral fibrinolysis inhibitors, such as tranexamid acid and e-aminocaproid acid are effective in preventing and treating patients with bleeding events and PAI-1 deficiency.

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# The Importance of Pre-Analytic Conditions on the Determination of VWF Parameters

S. Täschner, M. Böhm, I. Stier-Brück, G. Asmelash, B. Putz, T. Vigh, C. Rabenstein, V. Meyer, G. Eckert and I. Scharrer

## Introduction

It is well established that cold promotes a shortening of PT by FXII mediated activation of FVII in whole blood and in plasma [5]. The effect of low temperature on Von Willebrand Factor (VWF) parameters has there against not been studied in detail. We therefore conducted a study to investigate the pre-analytic processing of blood samples on VWF:Ristocetin-Cofactor activity (VWF:RCo), VWF:Antigen (VWF:Ag) and FVIII activity (FVIII:C).

## Patients and Methods

We collected 50 ml citrated blood from 10 apparently healthy individuals and from 8 patients with Von Willebrand Disease (VWD), type I and from 2 patients with VWD, type II. Individuals with conditions associated with increased VWF:Ag-levels such as surgery in the last 6 months, diabetes mellitus, renal insufficiency, chronic coronary heart disease, malignancy [4] and other severe disorders were excluded from the study. All participants gave their informed consent for blood sampling and research use. 30 ml of the blood was directly centrifuged (40 min, 4°C and 4000 g) and the separated plasma was either immediately frozen at –80°C (normally processed sample) or stored at Room Temperature (RT) or on crashed ice for 3 and 6 hours, respectively. The remaining citrated whole blood (CWB) was either stored at RT or on crashed ice for 3 and 6 hours prior centrifugation and storage at –80°C. All samples were tested for VWF:RCo, VWF:Ag and FVIII:C. VWF:RCo was measured with the BC von Willebrand Reagent from Dade Behring (Marburg, Germany) on the Behring Coagulation Timer (Dade Behring, Marburg, Germany) according to the manufacturers instructions. VWF:Ag was determined by enzyme immunoassay using rabbit anti-human VWF A0082 (DAKO A/S, Glostrup, Denmark) for capture and peroxidase-conjugated rabbit anti-human VWF P0226 (DAKO A/S, Glostrup, Denmark) for detection. FVIII:C was measured with FVIII deficient plasma from Instrumentation Laboratory (Lexington, USA). Results are expressed as percentage of the normally processed sample. Wilcoxon-matched pairs-test (Bias 7.01 software, Frankfurt, Germany) was used to analyse the difference between the test results of the various storage conditions. Values of $p<0.05$ were considered to be statistically significant.

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## Results

Storage on crashed ice as citrated whole blood induced in the samples from healthy individuals and patients with VWD, type I a drastic time dependent decrease of VWF:RCo, VWF:Ag and FVIII:C (Figs. 1–3, Table 1). The cold-induced loss of

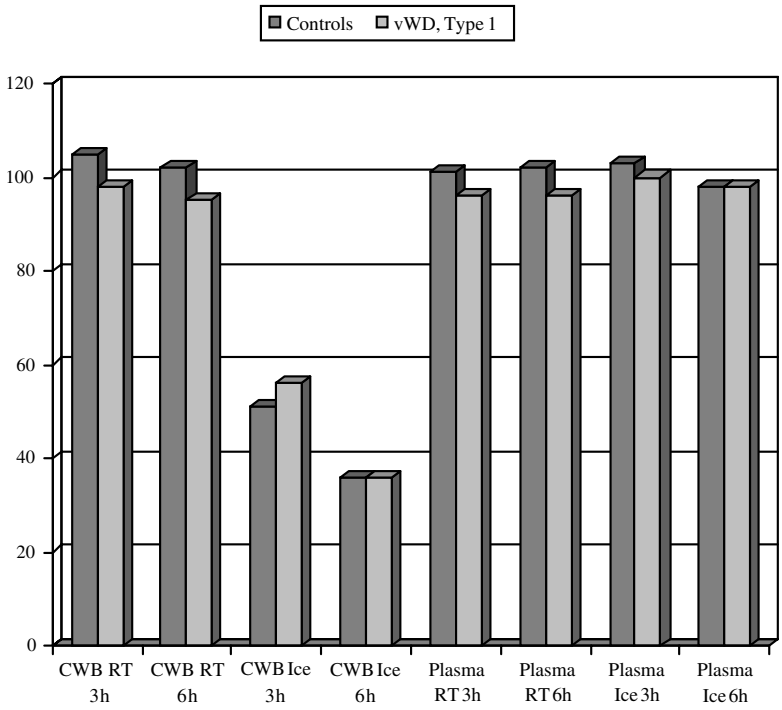


**Fig. 1.** VWF:RCo after storage of whole blood and plasma at RT and on ice
Results are expressed as percentage of the normally processed sample, the columns depict the mean of the 8 investigated individuals

**Table 1.** Storage of citrated whole blood on crashed ice. Results (mean +/-SD) are shown in % of the normally processed sample

| | VWF:RCo | | VWF:Ag | | FVIII:C | |
|---|---|---|---|---|---|---|
| | 3h | 6h | 3h | 6h | 3h | 6h |
| Controls (n=10) | 51% +/–30 | 36% +/–22 | 53% +/–21 | 50% +/–17 | 73% +/–22 | 57% +/–18 |
| VWD, type I (n=8) | 54% +/–23 | 34% +/–15 | 69% +/–21 | 54% +/–20 | 60% +/–16 | 48% +/–12 |
| VWD, type II (n=2) | n.m. | n.m. | 93% +/–20 | 115% +/–14 | 86% +/–4 | 79% +/–13 |

n.m. = not measurable

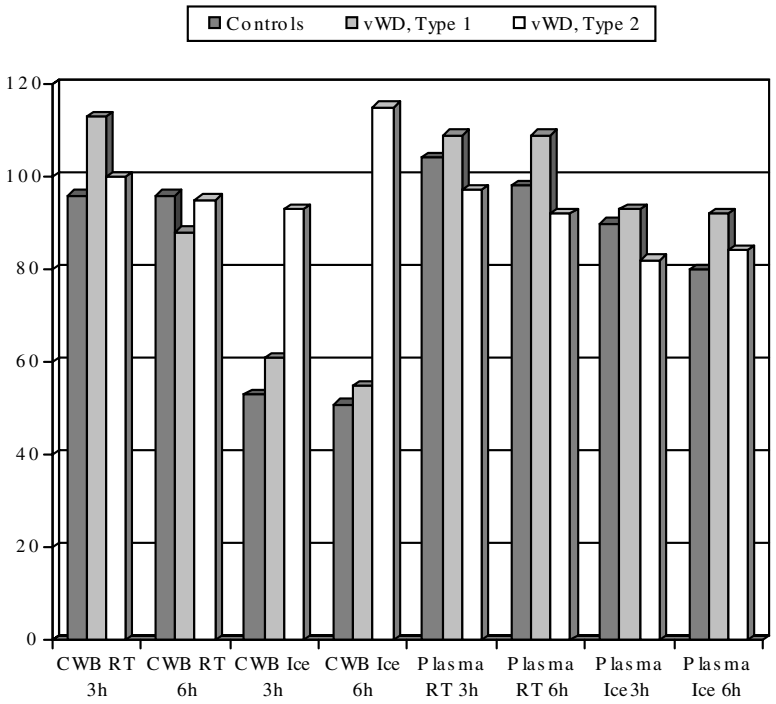


**Fig. 2.** VWF:Ag after storage of whole blood and plasma at RT and on ice
Results are expressed as percentage of the normally processed sample, the columns depict the mean of the respective individuals

VWF:RCo was significantly correlated to the loss of VWF:Ag and FVIII:C (Spearman-Rang-Correlation: r=0.72; p=0.002 and r=0.70; p=0.002 respectively). The two patients with VWD, type II showed in the normally processed sample VWF:RCo of <5%, VWF:Ag of 20 and 40% and FVIII:C of 16 and 53%, respectively. Storage of citrated whole blood on crashed ice from these two patients did not induce a significant loss of VWF:Ag and only a minor reduction of FVIII:C (Figs. 2–3, Table 1).

Statistical analyses confirmed the significant loss of VWF:RCo and VWF:Ag after storage as whole blood on ice (Table 1). Storage of whole blood at RT or storage of plasma at RT and on ice did there against not significantly change VWF:RCo and VWF:Ag-levels except for storage of plasma from healthy individuals for 6h on ice (Table 2). However, the decrease of VWF:Ag in the latter group was much lower than after storage of whole blood on ice (average reduction from baseline level was 20% and 49% respectively, Fig. 2).

FVIII:C was significantly reduced under all investigated storage conditions except for storage of whole blood from controls at RT for 3 hours (Table 1). The average reduction from baseline levels after storage of whole blood at RT and storage of plasma was always < 27%, whereas storage of whole blood on ice induced an average decrease of 40 and 52% (Fig. 3).

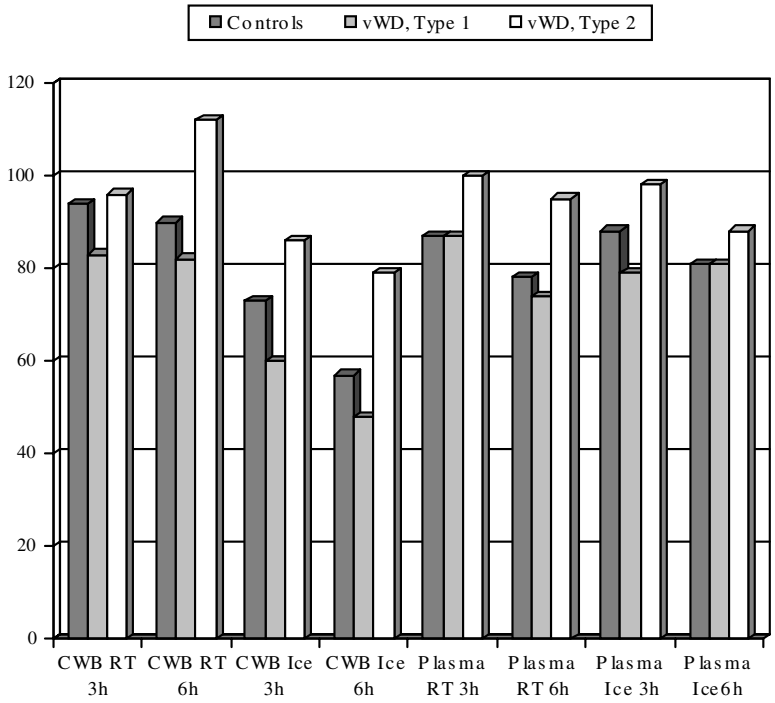


**Fig. 3.** FVIII:C after storage of whole blood and plasma at RT and on ice
Results are expressed as percentage of the normally processed sample, the columns depict the mean of the 8 investigated individuals

**Table 2.** Statistical comparison of the different storage conditions
Results from the stored samples were compared to the results of the normally processed samples with Wilcoxon-matched-pairs-test

| Parameter | Cohort | CR3 | CR6 | CE3 | CE6 | PR3 | PR6 | PE3 | PE6 |
|---|---|---|---|---|---|---|---|---|---|
| VWF:RCo | Controls (n=10) | n.s. | n.s. | p=0.02 | p=0.02 | n.s. | n.s. | n.s. | n.s. |
| | VWD, type I (n=8) | n.s. | n.s. | p=0.008 | p=0.008 | n.s. | n.s. | n.s. | n.s. |
| VWF:Ag | Controls (n=10) | n.s. | n.s. | p=0.002 | p=0.002 | n.s. | n.s. | n.s. | p=0.04 |
| | VWD, type I (n=8) | n.s. | n.s. | p=0.008 | p=0.008 | n.s. | n.s. | n.s. | n.s. |
| FVIII:C | Normals (n=10) | n.s. | p=0.04 | p=0.006 | p=0.02 | p=0.04 | p=0.02 | p=0.01 | p=0.04 |
| | VWD, type I (n=8) | p=0.03 | p=0.02 | p=0.008 | p=0.008 | p=0.02 | p=0.008 | p=0.008 | p=0.008 |

n.s. = not significant (p>0.05)

## Discussion

We found a drastic time-dependent loss of about VWF:RCo, VWF:Ag and FVIII:C after storage of citrated whole blood on crashed ice for healthy individuals as well as for patients with VWD, type I. Since we did not find a cold induced loss of the VWF in plasma and in samples from patients with VWD, type II (VWF:RCo < 5%) we assume, that the drastic cold-induced loss of VWF:RCo, VWF:Ag and FVIII:C is dependent on the presence of platelets and of high–molecular-weight-VWF. It is well established, that cold induces an extensive platelet shape change by intracellular cytoskeletal rearrangement [1]. Hoffmeister et al. [3] recently found, that chilling of platelets induces a clustering of the Glycoprotein Ib (GPIb) receptor on the cell surface. We hypothesise, that the cold-induced loss of VWF in citrated whole blood is due to cold-promoted binding of VWF to platelets probably due to increased susceptibility of GPIba subunit for VWF.

Our results demonstrate that blood destined for analysing VWF parameters should be stored at RT rather than at 4°C.

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# Recombinant FVIIa in Chemotherapy Related Thrombocytopenic Bleedings

H. H. Wolf, O. Dorligshaw and H. J. Schmoll

Thrombocytopenia is a severe, life threatening complication in cytotoxic therapy of hematologic as well as oncologic malignancies. Usually, substitution of thrombopheresis products is sufficient to prevent and treat bleeding complications in thrombocytopenic patients. However, either due to malignancy or chemotherapy hemostaseologic complications may occur which might afford additional treatment.

## Patients

We report two 41 and 42 years old male patients who underwent aggressive chemotherapy regimens for extragonadal germ cell tumor or Hodgkin's lymphoma (HD), respectively.

In the patient suffering from germ cell tumor bone marrow examination prior to chemotherapy revealed hypoplastic hematopoiesis due to long term methotrexate administration for psoriasis over a 8 years period. Platelet count was 89 Gpt/l. The patient presented with supra- and infradiaphragmatic lymphomas as well as gastric metastases.

Following cisplatin based induction chemotherapy tumorlysis of gastric metastases induced massive gastrointestinal bleeding. Platelet count was below 20 Gpt/l for more than 10 days despite substitution of thrombopheresis products. Furthermore, antiidiopathic platelet antibodies were detected after repeated platelet substitution.

Hemostaseologic therapy was intensified with packed red cells (n=8), fresh frozen plasma (n=7), and coagulation factor concentrates (activated prothrombin complex 4800 IU per day). The patient was admitted to intensive care unit for hemorrhagic shock. There was no chance for surgical or endoscopic intervention. After administration of recombinant factor VIIa (rFVIIa) 240 kIU every 6 hours for 2 consecutive days bleeding stopped and hemorrhagic shock could be managed.

The second patient suffered from liver infiltration of Hodgkin's disease with ascites, hypoproteinemia, and impaired synthesis of coagulation factors. He presented nodal as well as pulmonary involvement and hemorrhagic pleural effusion. After administration of cytotoxic therapy, plasmatic disorders of coagulation and thrombocytopenia were seen. The patient underwent artificial respiration for systemic candida infection. Acute hemorrhage after pleurocentesis was treated with

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rFVIIa 240 kIU every 6 hours for 3 consecutive days. Bleeding stopped after first administration of rFVIIa, and the patient's condition improved subsequently.

## Conclusions

In patients with combined thrombocytopenic and plasmatic disorders of coagulation life threatening hemorrhagic complications of cytotoxic therapy may occur despite substitution of platelets and plasma derived coagulation products. rFVIIa is able to activate coagulation cascade by interaction with tissue factor on platelets and endothelial cells, coagulation factor VIII and IX, and von Willebrand factor. Therefore, rFVIIa may induce thrombin burst and is able to improve hemostyptic effects at the side of bleeding [1, 2]

Therefore, there may be an indication to rFVIIa therapy in thrombocytopenic patients with acquired plasmatic disorders after cytotoxic therapy.

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