Springer
Berlin
Heidelberg
New York
Barcelona
Hong Kong
London
Milano
Paris
Tokyo

31st Hemophilia Symposium

Hamburg 2000

Editors: I. SCHARRER, W. SCHRAMM

Presentation:

Epidemiology
Inhibitors in Hemophilia
Therapy and Monitoring of Bleeds
in Acute and Intensive Care Medicine
Pediatric Hemostaseology
Case Reports

Scientific Board:
I. SCHARRER, Frankfurt/Main
W. SCHRAMM, Munich

Chairmen:
G. AUERSWALD (Bremen); H.-H. BRACKMANN (Bonn); W. KREUZ (Frankfurt/Main)
H. LENK (Leipzig); E.O. MEILI (Zürich); I. SCHARRER (Frankfurt/Main)
R. SCHNEPPENHEIM (Hamburg); R. SEITZ (Langen); A.H. SUTOR (Freiburg)
R. ZIMMERMANN (Heidelberg)

Springer

Professor Dr. med. INGE SCHARRER
Hemophilia Center, Dept. of Internal Medicine
University Hospital
Theodor-Stern-Kai 7
D-60590 Frankfurt am Main
Germany

Professor Dr. med. WOLFGANG SCHRAMM
Dept. of Hemostasiology
University Hospital
Ziemssenstr. 1a
D-80336 München
Germany

Mit 107 Abbildungen

ISBN-13:978-3-540-42131-3

Library of Concress Cataloging-in-Publication Data
31st Hemophilia Symposium : Hamburg, 2000 / editors, I. Scharrer, W. Schramm.
p. ; cm.
Includes bibliographical references and index.
ISBN-13:978-3-540-42131-3 e-ISBN-13:978-3-642-59383-3
DOI: 10.1007/978-3-642-59383-3

1. Hemophilia-Congresses. I. Title: Thirty-first Hemophilia Symposium. II. Scharrer, I. III. Schramm, W., 1943-
[DNLM: 1. Hemophilia A-therapy-Congresses. 2. Disease Susceptibility-Congresses. 3. Hemophilia A-epidemiology-Congresses. 4. Hemostasis-Child-Congresses. WH 325 H228z 2002]
RC642, H355 2000
616.1'572-dc21

This work is subject to copyright. All rights are reserved, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilm or in any other way, and storage in data banks. Duplication of this publication or parts thereof is permitted only under the provisions of the German Copyright Law of September 9, 1965, in its current version, and permission for use must always be obtained from Springer-Verlag. Violations are liable for prosecution under the German Copyright Law.

Springer-Verlag Berlin Heidelberg New York
a member of BertelsmannSpringer Science+Business Media GmbH
http://www.springer.de/medizin

© Springer Verlag Berlin Heidelberg 2002

The use of general descriptive names, registered names, trademarks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

Product Liability: The publisher cannot guarantee the accuracy of any information about dosage and application contained in this book. In every individual case the user must check such information by consulting the relevant literature.

Typesetting: cicero Lasersatz, 86424 Dinkelscherben
Druck: Sala-Druck, 10435 Berlin

Printed on acid-free paper SPIN 10833803 21/3130 5 4 3 2 1 0

Contents

Presentation of the Johann-Lukas-Schoenlein-Award 1

I. Epidemiology

HIV Infection and Causes of Death in Patients with Hemophilia in Germany (Year 1999/2000 Survey)
W. Schramm, H. Krebs, on behalf of the GTH Hemophilia Committee . . . 7

Hemophilia 2000 – the Annual Survey of the Austrian Hemophilia Centers
H.K. Hartl, U. Eidher, U. Kunze, P. Arends, J. Falger, N.D. Jonas, M. Kronawetter, P. Kurnik, I. Pabinger, H. Ramschak, E. Reiter, R. Schwarz, W. Streif, H. Türk, H. Wank, W. Zenz, K. Zwiauer 15

II. Inhibitors in Hemophilia

Changes in Epitope Specificity and in Distribution of FVIII Antibodies during Immune Tolerance Therapy (ITT) in Hemophilia A Patients with FVIII Antibodies – a Case Report
A. Kallas, T. Talpsep, H. Everaus 23

Clinical Experience with the Modified Bonn-Malmö Protocol since 1996
L. Hess, C. Unkrig, H. Zeitler, W. Nettekoven, W. Effenberger, P. Hanfland, H. Vetter, H.-H. Brackmann 41

Course of Inhibitors in mild Hemophilia A with and without Immune Tolerance Treatment
H. Lenk, F. Kertzscher 47

III. Therapy and Monitoring of Bleeds in Acute- and Intensive Care Medicine

Management of Bleeding in Surgery and Intensive Care
A. Calatzis, W. Schramm, M. Spannagl 55

IV.. Pediatric Hemostaseology

Frequency and Profile of viral posttransfusional Infections in Patients from Hemophilia Center Timisoara
M. Şerban, C. Petrescu, P. Ţepeneu, M. Pop, S. Jenaru, W. Schramm . . . 69

Symptomatic Onset of severe Hemophilia A in Childhood is dependent on the Presence of Prothrombotic Risk Factors
S. Halimeh, C. Escuriola Ettingshausen, K. Kurnik, R. Schobess, C. Wermes, H. Pollmann, W.D. Kreuz, U. Nowak-Göttl 77

Differences between Neonates and Adults in Plasmin Inhibitory and Antifibrinolytic Action of Aprotinin
M. Ries, M. Zenker, J. Klinge 81

Shorter PFA-100 Closure Times (CT) in Neonates than in Adults: Role of Red Cells, White Cells, Platelets, and von Willebrand Factor
B. Roschitz, K. Sudi, W. Muntean 91

V. Free Lectures

Efficacy and Safety of a High Purity Protein C Concentrate in the Management of Patients with severe Congenital Protein C Deficiency
B. Moritz, S. Rogy, S. Tonetta, H.P. Schwarz, H. Ehrlich and the CEPROTIN Study Group 101

Respective Roles of Factors II, VII, IX, and X in the Procoagulant Activity of FEIBA
S. Gallistl, G. Cvirn, B. Leschnik, W. Muntean 110

Capillary Microscopic and Rheological Dimensions for the Diagnosis of von-Willebrand-Disease in Comparison with other Hemorrhagic Diatheses
J. Koscielny, S. Mörsdorf, C. Mrowietz, H. Kiesewetter, U. Budde, U.T. Seyfert, E. Wenzel, F. Jung 114

Evaluation of Denaturing High Performance Liquid Chromatography (DHPLC) in the Analysis of Hemophilia A
J. Oldenburg, O. El-Maarri, V. Ivaskevicius, S. Rost, B.H.F. Weber, R. Schwaab 130

VIa. Poster: Clinic and Casuistic

Rheumatoid Arthritis in a Patient with Hemophilia Arthropathy – a Case Report
H.H. Eickhoff, G. Raderschadt, F.W. Koch, T. Wallny, H.-H. Brackmann 143

Recurrent Fatal Intracranial Hemorrhage (ICH) in Two Non-Identical Twins suffering from Hemophilia B (Factor IX Activity <1%)
S. Halimeh, H. Pollmann, M. Käse, I. Hörnig-Franz, H. Jürgens, U. Nowak-Göttl 149

Progression of Thrombosis under Low Molecular Weight Heparin without Heparin-Induced Thrombocytopenia in a young Man: a Case Report
R. Knöfler, J. Wendisch, K. Nemat, G. Siegert, S. Gehrisch, S. Schellong 153

Cardiac Tamponade in a Patient with acquired Factor VIII Inhibitor and Chronic Renal Failure
E. Strasser, Y. Lindemann, B. Neidhardt, I. Scharrer, R. Zimmermann, V. Weisbach, R. Eckstein 159

Endoscopic Cholecystectomy in a 55-year-old Patient with Heparin-Induced Thrombocytopenia Type II and Replacement of Mitral and Aortic Valve and Tricuspidal Valve Anular Plasty
J. Gross, D. Heitmann, I. Wojtzyk, G. Lutze, M.U. Heim 161

VIb. Poster: Hemophilia

Successful Orthopedic Operations in Hemophilic Adults with Inhibitors against Factor VIII
G. Ludwig, C. Betz, M. Krause, A. Kurth, L. Hovy, I. Scharrer 169

Evaluation of Clinical Efficacy of rFVIIa in Pediatrics
M. Şerban, P. Ţepeneu, C. Petrescu, D. Mihailov, C. Jinca, W. Schramm . 171

Risk Factors for Thrombosis in Hemophilia – an Analysis
V. Aumann, G. Lutze, U. Mittler 179

Status of Pain in Patients with severe Haemophilic Arthropathies
T. Wallny, L. Hess, A. Seuser, H.-H. Brackmann, C.N. Kraft 181

Results of a 5-year Clinical Study with a B-domain Deleted FVIII Concentrate (rFVIII-SQ)
H. Pollmann, H. Richter and the rFVIII-SQ Study Group 186

Outpatient Treatment with Radiosynoviorthesis in Hemophilic Arthropathy
D. Lasovic, G. Tönshoff, M. Fink, A. Rosenstock, A. Ganser, M. von Depka Prondzinski 197

Requirements for Future Hemophilia Treatment from the Patients' Point of View
G. Schelle, W. Breuer, G. Broil, D. Hohmann 199

VIc. Poster: Hemorrhagic Diathesis

Molecular Basis of von Willebrand Disease Type IIC Miami
R. Schneppenheim, T. Obser, J.U. Wieding, F. Bergmann, E. Drewke,
B. Luxemburg, U. Budde 203

Hemorrhagic Diathesis through acquired Factor XIII Inhibitor
E. Strasser, R. Zimmermann, B. Neidhardt, B. Manger, I. Scharrer,
V. Weisbach, R. Eckstein 208

Liver Transplantation in a Patient with severe von Willebrand Disease Type 3:
Levels of von Willebrand Factor Following Transplantation
E. Lechler, D. Stippel 210

Clincal Course and Laboratory Findings in a Patient
with a New Mutation Causing Wiskott-Aldrich Syndrome
F. Kertzscher, A. Meindl, V. Schuster, U. Sack, M. Koksch, H. Lenk . . . 216

Role of acquired and inherited Prothrombotic Risk Factors
in Pediatric Cerebral Venous Thrombosis –
Preliminary Results of a Multicenter Case-Control Study
A. Kosch, U. Schulze-Horsel, C. Heller, R. Schobess, K. Kurnik,
R. Sträter, U. Nowak-Göttl 219

Analysis of the Fibrinogen Genes of 40 Patients with Suspicion
of Dys-, Hypo- or Afibrinogenemia
A. Czwalinna, N. Bartkowiak, B. Canepa, A. Ganser,
M. von Depka Prondzinski 225

VId. Poster: Thrombophilic Diathesis

Hereditary Antithrombin Deficiency – Results of a Family Study
B. Maak, S. Hutschenreiter, F.H. Herrmann, K. Wulff, A. Siegemund,
W. Christoph 233

Elevated Factor IX and Factor XI as Risk Factors
for Venous Thrombosis and Stroke
A. Siegemund, T. Siegemund, J. Berrouschot, H. Voigt, M. Koksch 239

Thrombophilic Risk Parameters in Juvenile »Idiopathic« Stroke Patients
M. Krause, S. Ehrenforth, E. Aygören-Pürsün, G. Ludwig, I. Scharrer 244

Inquiry into the Significance of Constantly Raised FVIII Values
as a Factor in Thrombophilia
M. von Lieven, M. Krause, T. Vigh, C. Rabenstein, I. Scharrer 252

Do Statins Increase the Homocysteine Level?
M. von Lieven, L. Rössig, T. Vigh, C. Rabenstein, I. Scharrer 257

Alpha 2-Macroglobulin Level regulates the Anticoagulant Cofactor Activity of Protein S in Cord and Adult Plasma
G. Cvirn, S. Gallistl, M. Köstenberger, J. Kutschera, B. Leschnik, W. Muntean 262

Combined Prothrombotic Defects and Contraceptives – Risk Factor for Deep Venous Thrombosis in Adolescence
R. Schobess, A. Jassoy, S. Burdach 273

Role of 23 bp Insertion in Exon 3 of the Endothelial Cell Protein C Receptor Gene in Venous Thrombophilia
M. von Depka Prondzinski, A. Czwalinna, R. Eisert, C. Wermes, B. Canepa, I. Scharrer, A. Ganser, S. Ehrenforth 278

Subject Index 283

List of Participants

ALBERIO, L., Dr.
CH-Bern

ALBERT, T.
Institut für Experimentelle Hämatologie und Transfusionsmedizin der Universität, D-Bonn

ANDERS, O., Prof. Dr.
Klinik und Poliklinik für Innere Medizin der Universität Rostock, D-Rostock

ANDRIPSCHKE, K., Frau
Hämophilieambulanz, Klinikum der Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

ANSTADT, M., Frau Dr.
DRK Blutspendedienst Sachsen gGmbH, Institut f. Transfusionsmed. Plauen, D-Plauen

ARENDS, P., Dr.
Arzt für Kinderheilkunde, A-Güssing

ASPÖCK, G., Prim., Dr.
Allg. Österr. Krankenhaus der Barmherzigen Schwestern vom Heiligen Kreuz, Labor I, A-Wels

ASPÖCK, L., Frau Prim.

AUERSWALD, G., Dr.
Professor-Hess-Kinderklinik, Zentralkrankenhaus St.-Jürgen-Str., D-Bremen

AUMANN, V., Dr.
Zentrum für Kinderheilkunde, Medizinische Fakultät, Otto-von-Guericke-Universität, D-Magdeburg

AYGÖREN-PÜRSÜN, E., Frau Dr.
Hämophilieambulanz, Medizinische Klinik I, Johann-Wolfgang-Goethe-Universität, D-Frankfurt/Main

Balleisen, L., Prof. Dr.
Abteilung Hämatologie u. Onkologie, Innere Medizin,
Evangelisches Krankenhaus, D-Hamm

Barthels, M., Frau Prof. Dr.
D-Hannover

Baum,
Bundesministerium für Gesundheit, D-Bonn

Baumgartner, Ch., Dr.
Facharzt für Kinder und Jugendliche, spez. Hämatologie, CH-Gossau

Beck, Ch., Frau Dr.
Ärztin für Kinderheilkunde, D-Berlin

Beck, K.H., Dr.
Abteilung Transfusionsmedizin, Klinikum der Albert-Ludwigs-Universität,
D-Freiburg

Becker, Th., Dr.
Interessengemeinschaft Hämophiler, D-Bonn

Beesere, H.P., Prof. Dr.
Institute for Quality Management and Standardization in Transfusion Med.
and Haemostaseology, D-Teningen

Beha, L., Frau
D-Oberursel

Beneke, H., Dr.
Sektion Hämostaseologie, Abteilung Innere Medizin III, Medizinische
Universitätsklinik, D-Ulm

Bereuter, L., Dr.
Landeskrankenhaus Feldkirch, Abteilung Pädiatrie, A-Feldkirch

Bergmann, F., Frau Dr.
Gemeinschaftslabor, Dr. Keeser und Prof. Arndt, D-Hamburg

Bergsträsser, E., Frau Dr.
Kinderspital, Hämatologie, CH-Zürich

Berthold, B., Dr.
Hämophiliezentrum, Klinik für Innere Medizin I, Klinikum Neubrandenburg,
D-Neubrandenburg

BLAZEK, B., Dr.
FNsP Ostrava, Childrens' Dept. – Haematology, CZ-Ostrava - Poruba

BLICKHÄUSER, R., Dr.
DRK-Kinderklinik, D-Siegen

BÖTTCHER, D., Prof. Dr.
Abteilung Innere Medizin, Krankenhaus Bethesda, D-Wuppertal

BRACKMANN, H.-H., Dr.
Institut für Experimentelle Hämatologie und Transfusionsmedizin der Universität, D-Bonn

BRACKMANN, CH., Frau
Institut für Experimentelle Hämatologie und Transfusionsmedizin der Universität, D-Bonn

BRAND, B., Frau Dr.
Kantonsspital Chur, Blutspendedienst SRK, CH-Chur

BRATANOFF, E., Frau Dr.
Sozialpädiatrisches Zentrum, D-Erfurt

BRAUN, U., Frau Dr.
Deutsche Hämophiliegesellschaft, D-München

BREUER, W.
Interessengemeinschaft Hämophiler, D-Bonn

BUDDE, U., Prof. Dr.
Gemeinschaftslabor, Dr. Keeser und Prof. Arndt, D-Hamburg

BÜTTNER, M., Frau
Ärztin für Kinderheilkunde, D-Homburg

CALATZIS, A., Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt der Ludwig-Maximilians-Universität, D-München

CLAUSEN, N., Dr.
Barnafdelningen, Skejby hospital, DK-Aarhus N

CVIRN, G., Mag.
Allg.Österr. Landeskrankenhaus Graz, Univ.-klinik für Kinder- und Jugendheilkunde, A-Graz

DITTMER, Frau Dr.
Gemeinschaftslabor, Dr. Keeser und Prof. Arndt, D-Hamburg

DOCKTER, G., Prof. Dr.
Kinderklinik, Universitätskliniken des Saarlandes, D-Homburg

DOMSCH, CHR., Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt
der Ludwig-Maximilians-Universität, D-München

DRESSEL, B., Dr.
Praktischer Arzt, A-Nüziders

EBERL, W., Dr.
Kinderklinik, Städtisches Klinikum Holwedestraße, D-Braunschweig

EDELMANN, H., Dr.
Kinderklinik, Zentralklinikum Suhl gGmbH, Südthüringen, D-Suhl

EFFENBERGER, W., Dr.
Institut für Experimentelle Hämatologie und Transfusionsmedizin
der Universität, D-Bonn

EHRENFORTH, S., Frau Dr.
Hämophilieambulanz, Medizinische Klinik I,
Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

EICKHOFF, H.H., Dr.
Orthopädische Klinik, St.-Josef-Hospital, D-Troisdorf

EIFRIG, B., Frau Dr.
Abteilung Onkologie/Hämostaseologie, Medizinische Klinik,
Universitätskrankenhaus Eppendorf, D-Hamburg

EISERT, Frau Dr.
Medizinische Einrichtungen der Heinrich-Heine-Universität,
Zentrum Kinderheilkunde, D-Düsseldorf

ERDLENBRUCH, W., Dr.
Universitätskliniken d. Saarlandes, Abt. Klinische Hämostaseologie
und Transfusionsmedizin, D-Homburg

ESCURIOLA-ETTINGSHAUSEN, C., Frau Dr.
Zentrum der Kinderheilkunde, Klinikum der
Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

ETZLER, J., Frau
D-Hamburg

Evatt, B., Dr.
Disease Branche-CDC National Center for Infectious Diseases,
MS Atlanta GA, USA

Faessler, H., Dr.
FMH Medicina interna, CH-Chiasso

Falger, J., Frau Dr.
Allg. Krankenhaus der Stadt, Universitätsklinik für Kinder-
und Jugendmedizin, A-Wien

Franke, D., PD Dr.
Schwerpunktpraxis für Gerinnungsstörungen und Gefäßkrankheiten,
D-Magdeburg

Franke, St., Frau
Hämophilieambulanz, Medizinische Klinik I, Klinikum der
Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

Frick, U., Frau Prof. Dr.
Institut für Klinische Chemie der Ernst-Moritz-Arndt-Universität,
D-Greifswald

Fuchs, A., Frau
Allg. Krankenhaus der Stadt Wien, Abteilung für Hämatologie
und Hämostaseologie, A-Wien

Furrer, H.U., Dr.
Arzt für Kinderheilkunde, CH-Sarnen

Gabriel, Dr.
Allg. Österr. Krankenhaus der Stadt Linz, Blutspendezentrale d. Roten Kreuzes,
A-Linz

Gallistl, S., Prof. Dr.
Allg. Österr. Landeskrankenhaus Graz, Univ.-klinik für Kinder-
und Jugendheilkunde, A-Graz

Gastl, G., Prof. Dr.
Universitätsklinik Innsbruck, Hämatologie, A-Innsbruck

Gastpar, H., Prof. Dr.
D-Neusäss

Geib, R., Frau Dr.
Klinikum Saarbrücken gGmbH, Klinik für Kinder- u. Jugendmedizin,
D-Saarbrücken

GEISEN, U., Dr.
Zentrallabor, Medizinische Universitätsklinik
der Julius-Maximilians-Universität, D-Würzburg

GENSER, N., Dr.
Universitätsklinik Innsbruck, Klinik f. Kinder-u. Jugendheilkunde, A-Innsbruck

GENSER, E., Frau Dr.

GERHARDT, A., Frau Dr.
Abt. für Transfusionsmedizin, Medizinische Einrichtungen
der Heinrich-Heine-Universität, D-Düsseldorf

GERSTENBAUER, G., Frau
Biomedizinisches Forschungszentrum, A-Orth/Donau

GILBERG, E., Dr.
Kinderklinik, Klinikum Neubrandenburg, D-Neubrandenburg

GROSS, J., Dr.
Inst. f. Transfusionsmedizin und Immunhämatologie, Universitätsklinikum,
D-Magdeburg

GUTENSOHN, K., Dr.
Chir. Universitätsklinik und Poliklinik, Abteilung für Transfusionsmedizin,
Universitätskrankenhaus Eppendorf, D-Hamburg

HABRDLE, S., Frau DKS,
Sozialmed. Zentrum Ost, Donauspital, A-Wien

HALBMAYER, M., Dr.
Zentrallaboratorium, Krankenhaus der Stadt Wien-Lainz, A-Wien

HALIMEH, S., Frau Dr.
Abteilung für Hämostaseologie, Kinderklinik/ Medizinische Einrichtungen
der Westfälischen-Wilhelms-Universität, D-Münster

HAMPEL, Frau Dr.,
Kurpfalzkrankenhaus Heidelberg und Hämophiliezentrum gGmbH,
D-Heidelberg

HANFLAND, P., Prof. Dr.
Institut für Experimentelle Hämatologie und Transfusionsmedizin
der Universität, D-Bonn

HARTL, H.K., Dr.
Institut für Sozialmedizin der Universität Wien, A-Wien

HARTL, M., Frau
A-Wien

HARTMANN, S., Frau Dr.
Ärztin für Hämatologie und Onkologie, CH-Chur

HASLER, K., Frau Prof. Dr.
Abteilung Hämatologie u. Onkologie, Zentrum Innere Medizin I,
Klinikum der Albert-Ludwigs-Universität, D-Freiburg

HASSENPFLUG, W., Dr.
Abt. Hämatologie/Onkologie, Kinderklinik, Universitätsklinik Eppendorf,
D-Hamburg

HAUSHOFER, A., Dr.
Zentrallabor, Krankenhaus der Stadt Wien-Lainz, A-Wien

HEINRICHS, Ch., Frau Doz. Dr.
Krankenhaus im Friedrichshain, Abt. Klinische Hämostaseologie,
Hämophiliezentrum, D-Berlin

HELLSTERN, P., Prof. Dr.
Institut für Transfusionsmedizin und Immunhämatologie,
Klinikum der Stadt Ludwigshafen, D-Ludwigshafen

HELLSTERN, H., Frau
Institut für Transfusionsmedizin und Immunhämatologie,
Klinikum der Stadt Ludwigshafen, D-Ludwigshafen

HEMPELMANN, L., Dr.
Kinderklinik Lindenhof, Krankenhaus Lichtenberg, D-Berlin

HERRMANN, F.H., Prof. Dr. Dr.
Institut für Humangenetik, Medizinische Fakultät der
Ernst-Moritz-Arndt-Universität, D-Greifswald

HESS, L., Dr.
Institut f. Experimentelle Hämatologie und Transfusionsmedizin
der Universität, D-Bonn

HILBERG, Th., Dr.
Lehrstuhl für Sportmedizin, Friedrich-Schiller-Universität, D-Jena

HILGENFELD, E., Frau Dr.
D-Berlin

HILLER, E., Prof. Dr.
Klinikum Großhadern, Medizinische Klinik III, D-München

HOFMANN, H., Dr.
Facharzt für Transfusionsmedizin, D-Töplitz

HOFMANN, KL., Dr.
Abt. Hämatologie und Onkologie, Klinik für Kinder- u. Jugendmedizin, Klinikum Chemnitz gGmbH, D-Chemnitz

HOHMANN, D.
Interessengemeinschaft Hämophiler, D-Bonn

HOLZHÜTER, H., Prof. Dr.
Hämophiliezentrum Nordwest, D-Bremen

HÖRAUF, Prof. Dr.
Allg. Krankenhaus der Stadt Wien, Univ.-klinik für Anästhesie und Allg. Intensivmedizin, A-Wien

HORNEFF, Frau Dr.
Klinik für Kinderheilkunde der Martin-Luther-Universität, Halle-Wittenberg, D-Halle

HOVY, L., PD Dr.
Annastift, Orthopädie, D-Hannover

HUTH-KÜHNE, A., Frau Dr.
Kurpfalzkrankenhaus Heidelberg und Hämophiliezentrum gGmbH, D-Heidelberg

IFSITZ, A., Frau
Wiener Gebietskrankenkasse, A-Wien

INGERSLEV, J., Dr.
University Hospital Skejby, Haemophilia Centre, DK-Arhus N

JOHS, R., Dr.
Kinderklinik, Städtisches Klinikum Holwedestraße, D-Braunschweig

JULEN, E., Dr.
CH-Zermatt

KALLAS, A., Frau Dr.
University of Tartu, Dept. of Pharmacy, Estland-Tartu

KALNINS, W., Dr.
Deutsche Hämophiliegesellschaft, D-Marmagen

KÄSE, M., Frau
Abteilung Hämatologie/Onkologie, Kinderklinik, Medizinische Einrichtungen der Westfälischen Wilhelms-Universität, D-Münster

KELLER, F., Prof. Dr.
D-Würzburg

KERTZSCHER, F.
Klinik für Kindermedizin, Universität Leipzig, D-Leipzig

KIESEWETTER, H., Prof. Dr. Dr.
Institut für Transfusionsmedizin und Immunhämatologie,
Campus Charité Mitte, D-Berlin

KLAMROTH, R., Dr.
Krankenhaus im Friedrichshain, Abt. Klinische Hämostaseologie,
Hämophiliezentrum, D-Berlin

KLARE, M., Dr.
III. Innere Klinik, Klinikum Berlin-Buch, D-Berlin

KLEIN-FRANKE, A., Dr.
Zentrum Kinderheilkunde, Med. Klinik und Poliklinik
der Georg-August-Universität, D-Göttingen

KNÖBL, P., Prof. Dr.
Allg. Krankenhaus der Stadt Wien, Abteilung für Hämatologie
und Hämostaseologie, A-Wien

KNÖFLER, R., Dr.
Klinik und Poliklinik für Kinderheilkunde,
Universitätsklinikum Carl-Gustav-Carus, D-Dresden

KOBELT, R., Dr.
Arzt für Kinderheilkunde, CH-Wabern

KÖHLER-VAJTA, K., Frau Dr.
Ärztin für Kinderheilkunde, D-Grünwald

KOMRSKA, V., Dr.
II. Detska Klinika, FN Motol, CZ-Praha

KOSCH, A., Frau Dr.
Kinderklinik/Kinderonkologie der Medizinischen Einrichtungen
der Westfälischen Wilhelms-Universität, D-Münster

KOSCIELNY, J., Dr.
Institut für Transfusionsmedizin und Immunhämatologie,
Campus Charité Mitte, D-Berlin

KÖSTERING, H., Prof. Dr.
D-Lemgo

KRAUSE, M., Frau Dr.
Hämophilieambulanz, Medizinische Klinik I,
Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

KREBS, H., Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt
der Ludwig-Maximilians-Universität, D-München

KRETSCHMAR, H.A., Prof. Dr.
Klinikum Großhadern, Abteilung Neuropathologie, D-München

KREUZ, W., PD Dr.
Zentrum der Kinderheilkunde, Klinikum der
Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

KRONBERGER, Frau Dr.
St.-Anna-Kinderspital, A-Wien

KUNZE, M., Prof. Dr.
Institut für Sozialmedizin der Universität Wien, A-Wien

KURME, A., Dr.
D-Hamburg

KURNIK, K., Frau Dr.
Kinderklinik im Dr. von Hauner'schen Kinderspital
der Ludwig-Maximilians-Universität, D-München

KURNIK, P., Dr.
Allg. Österr. Landeskrankenhaus, Abteilung für Kinder- und Jugendheilkunde,
A-Klagenfurt

KURTH, A., Dr.
Orthopädische Universitätsklinik, D-Frankfurt/M.

KUSE, R., Prof. Dr.
Abteilung Hämatologie, Allgemeines Krankenhaus St. Georg, D-Hamburg

KUSSER, B., Frau
Kinderklinik im Dr. von Hauner'schen Kinderspital der
Ludwig-Maximilians-Universität, D-München

KYANK, U., Frau Dr.
Universitätskinderklinik, Medizinische Fakultät, Universität Rostock,
D-Rostock

LECHLER, E., Prof. Dr.
D-Esslingen a. N.

LEHMANN, I., Frau
Med. Klinik und Poliklinik I, Hämophiliezentrum, Universität Leipzig, D-Leipzig

LENK, H., PD Dr.
Klinik für Kindermedizin, Universität Leipzig, D-Leipzig

LENTZE, U., Frau
D-Köln

LESTIN, H.-G., Prof. Dr.
Institut für Labormedizin, Klinikum Schwerin, D-Schwerin

LIGHEZAHN, D., Dr.
Universitatea de Medicina si Farmacie Timisoara, Clinica Medicina Interna II, Spital Municipal, R-Timisoara

LIMBACH, H.G., Dr.
Universitätsklinik für Kinder- und Jugendmedizin, D-Homburg

LORETH, R.M., Dr.
Abt. für Klin. Hämostaseologie, Medizinische Klinik III, Westpfalz-Klinikum GmbH, D-Kaiserslautern

LUDWIG, G., Dr.
Hämophilieambulanz, Medizinische Klinik I,
Klinikum der Johann-Wolfgang-Goethe-Universität, D-Frankfurt/Main

LÜHR, C., Frau
Abt. Hämophilie/Med. Poliklinik, Medizinische Hochschule Hannover, D-Hannover

LUTZ, W., Dr.
Praktischer Arzt, CH-Arbon

LUTZE, W., Prof. Dr.
Institut für Klinische Chemie und Laboratoriumsdiagnostik,
Otto-von-Guericke-Universität, D-Magdeburg

MAAK, B., Prof. Dr.
Thüringen-Klinik Georgius Agricola Saalfeld, D-Saalfeld

MANNHALTER, CH., Frau Prof. Dr.
Allg. Krankenhaus der Stadt Wien, Klinisches Institut für Med.
und Chem. Labordiagnostik, A-Wien

MARBY, P., Frau Dr.
Kinderklinik und Ambulanz, Städtisches Klinikum Dessau, D-Dessau

MAREK, R., Dr.
Wiener Gebietskrankenkasse, A-Wien

MARTINKOVÁ, I., Frau Prim. Dr.
odd. Hematologie, Fakultni nemocnice v Plzni, CZ-Plzen-Bory

MARX, G., Dr.
Gerinnungslabor, Chirurgische Klinik, Universitätskrankenhaus Eppendorf, D-Hamburg

MATYSKOVA, M., Frau Dr.
Department of Hematology, II. Interni Klinika, CZ-Brno

MAURER, M., Prof. Dr.
D-Bernau/Chiemsee

MEILI, E.O., Frau Dr.
Universitätsspital, Gerinnungslabor, CH-Zürich

MELUZINOVA, H., Frau Dr.
Faculty Childrens Hosp. J. G. Mendela, Haematology, CZ-Brno

MENTZER, D., Dr.
Abt. Hämatologie und Ger., Zentrum der Kinderheilkunde, Klinikum der Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

MICHIELS, J.J., Prof. Dr.
Goodheart Institute, Haematology Hemostasis Thrombosis, Research Science / Development Center, NL-AT Rotterdam

MINGERS, A.M., Frau Prof. Dr.
D-Würzburg/Lengfeld

MITTEREGGER, R., Frau Dipl.-Med.
Allg.Österr. Landeskrankenhaus Graz, Klinik für Kinder- und Jugendheilkunde, A-Graz

MONDORF, W., Dr.
Haemostas-Frankfurt, D-Frankfurt/M.

MUNTEAN, E.W., Prof. Dr.
Allg. Österr. Landeskrankenhaus Graz, Univ.-klinik für Kinder- und Jugendheilkunde, A-Graz

MUSS, N., Dr.
Salzburger Gebietskrankenkasse, A-Salzburg

NEIDHARDT, B., Dr.
Abteilung für Transfusionsmedizin, Chirurgische Universitätsklinik, D-Erlangen

NEUMAYR, Dr.
Tiroler Gebietskrankenkasse, A-Innsbruck

NIEBUHR, H., Dr.
Abt. Hämophilie und Onkologie, Medizinische Hochschule Hannover, D-Hannover

NIEKRENS, C., Frau Dr.
Pädiatrie, Städt. Klinik Delmenhorst, D-Delmenhorst

NIMTZ-TALASKA, A., Frau Dr.
Klinikum Frankfurt/Oder, Klinik für Kinder- und Jugendmedizin, D-Frankfurt/O.

NOHE, N., Frau
Kinderklinik im Dr. von Hauner'schen Kinderspital der Ludwig-Maximilians-Universität, D-München

NOVAKOVA, I., Frau Dr.
University Hospital Nijmegen, Department Internal Medicine, NL-GA Nijmegen

NOWAK-GÖTTL, U., Frau Prof. Dr.
Abteilung Hämatologie/Onkologie, Kinderklinik, Medizinische Einrichtungen der Westfälischen Wilhelms-Universität, D-Münster

OLDENBURG, J., Dr.
Institut für Experimentelle Hämatologie und Transfusionsmedizin der Universität, D-Bonn

PETRESCU, C., Dr.
Spitalul Clinic de copii, Louis Turcanu/Clin. Pediatrie III, Compartimentul de Onco-Hematologie, R-Timisoara

PINDUR, G., PD Dr.
Abt. Klinische Hämostaseologie und Transfusionsmedizin, Universitätskliniken des Saarlandes, D-Homburg

PLENDL, H., Dr.
Institut für Humangenetik, Klinikum der Christian-Albrechts-Universität, D-Kiel

PODEHL-KLOSE, J., Dr.
Annastift, Klinik 3, D-Hannover

Poek, Kl.
Deutsche Hämophiliegesellschaft, D-Berlin

Poggensee, Frau
Gemeinschaftslabor Dr. Keeser und Prof. Arndt, D-Hamburg

Pollmann, H., Dr.
Ambulanzzentrum an der Raphaelsklinik, D-Münster

Rabenstein, C.
Hämophilieambulanz, Medizinische Klinik I,
Klinikum der Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

Rager, K., PD Dr.
Caritas-Krankenhaus, Kinderklinik, D-Bad Mergentheim

Ramschak, H., Dr.
Allg. Österr. Landeskrankenhaus Graz, I. Medizinische Universitätsklinik,
A-Graz

Reddemann, H., Prof. Dr.
Verein zur Unterstützung krebskranker Kinder und Krebsforschung
im Kindesalter, D-Greifswald

Ries, M., PD Dr.
Kinderklinik mit Poliklinik der Universität Erlangen-Nürnberg, D-Erlangen

Rinkkamp, H., Frau
Abteilung für Hämostaseologie, Kinderklinik, Medizinische Einrichtungen
der Westfälischen Wilhelms-Universität, D-Münster

Rolf, V., Frau Dr.
Zentrum Kinderheilkunde, Medizinische Hochschule Hannover, D-Hannover

Roosendaal, G., Dr.
Van Creveld Clinic, NL-BN Utrecht

Roschitz, B., Frau Dr.
Allg. Österr. Landeskrankenhaus Graz, Univ.-klinik für Kinder-
und Jugendheilkunde, A-Graz

Scharrer, I., Frau Prof. Dr.
Hämophilieambulanz, Medizinische Klinik I,
Klinikum der Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

Scheel, H., Dr.
Klinische Hämostaseologie, Med. Klinik und Poliklinik I, Universität Leipzig, D-Leipzig

Scheel-Walter, H., Dr.
Abteilung Hämatologie und Onkologie, Universitäts-Kinderklinik, D-Tübingen

Scheibel, E., Frau Dr.
Haemofili centret, Rigshospitalet, DK-Kopenhavn

Schelle, G.
Interessengemeinschaft Hämophiler, D-Bonn

Schimpf, Kl., Prof. Dr.
D-Heidelberg

Schlenkrich, U., Dr.
D-Großlehna

Schmeltzer, B., Frau Dr.
Ärztin für Kinderheilkunde, D-Potsdam

Schmid, L., Dr.
Institut für Klinische Chemie und Hämatologie, Kantonsspital, CH-St. Gallen

Schmidt, O., Dr.
Tagesklinik für Angiologie und Phlebologie, D-Magdeburg

Schmitt, K., Prim. Doz. Dr.
Kinder- u. Infektionsabteilung, Landes-Kinderklinik Linz, A-Linz

Schmutzler, R., Prof. Dr.
D-Wuppertal

Schneider, M.M., Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt der Ludwig-Maximilians-Universität, D-München

Schneppenheim, R., Prof. Dr.
Abt. Hämatologie/Onkologie, Kinderklinik, Universitätsklinik Eppendorf, D-Hamburg

Schneppenheim, S., Frau cand. med.
Abt. Hämatologie/Onkologie, Kinderklinik, Universitätsklinik Eppendorf, D-Hamburg

SCHOBESS, R., Frau Dr.
Klinik für Kinderheilkunde der Martin-Luther-Universität, Halle-Wittenberg, D-Halle

SCHRAMM, W., Prof. Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt der Ludwig-Maximilians-Universität, D-München

SCHRÖDER, W., Frau Dr.
Institut für Humangenetik, Med. Fakultät der Ernst-Moritz-Arndt-Universität, D-Greifswald

SCHUBIGER, G., Prof. Dr.
Pädiatrische Klinik, Kinderspital Luzern, CH-Luzern

SCHULTE-OVERBERG, U., Frau Dr.
Kinderklinik, Campus Virchow Klinikum, D-Berlin

SCHULZ, M., Dr.
Abteilung Blutspende- und Transfusionsmedizin der Ernst-Moritz-Arndt-Universität, Klinikum Sauerbruchstraße, D-Greifswald

SCHULZE-HORSCH, U., Frau
Abteilung Hämatologie/Onkologie, Kinderklinik, Medizinische Einrichtungen der Westfälischen Wilhelms-Universität, D-Münster

SCHUMACHER, R., Dipl.-Med.
Kinderklinik, Station A 2, Klinikum Schwerin, D-Schwerin

SCRIBA, P.C., Prof. Dr. Dr. h. c.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt der Ludwig-Maximilians-Universität, D-München

SEDLAK, M., Dr.
Facharzt für Kinder- und Jugendheilkunde, A-Linz

SEDLAK, W., Dr.
Arzt für Kinderheilkunde, A-Linz-Dornach

SEITZ, R., Prof. Dr.
Paul-Ehrlich-Institut, Abteilung Hämatologie und Transfusionsmedizin, D-Langen

SEKNICKA, R., Frau DKS
Allg. Krankenhaus der Stadt Wien, Abteilung für Hämatologie und Hämostaseologie, A-Wien

Şerban, M., Frau Prof. Dr.
University of Medicine, Clinica I-a Pediatrie, R-Timisoara

Severin, Th., Dr.
Abt. Hämatologie u. Hämostaseologie, Kinderklinik,
Klinikum der Albert-Ludwigs-Universität, D-Freiburg

Siegemund, A., Frau Dr.
Institut für Klinische Chemie, Gerinnungslabor Innere Medizin,
Universität Leipzig, D-Leipzig

Siemens, H.J., Dr.
Hämatologie-Labor, Klinik für Innere Medizin II, Med. Universität zu Lübeck,
D-Lübeck

Siller, M.
Deutsche Hämophiliegesellschaft e.V., D-Berlin

Sirb, H., Dr.
Klinik für Kinder- u. Jugendmedizin, Heinrich-Braun-Krankenhaus, D-Zwickau

Söhngen, D., Dr.
Medizinische Klinik I, Hämatologie / Onkologie, Med. Einrichtungen
der Universität Köln, D-Köln

Spannagl, M., Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt
der Ludwig-Maximilians-Universität, D-München

Steinbeck-Klose, A., Frau Dr.
Ärztin für Allgemeinmedizin, D-Bonn

Stigendal, L., Dr.
Koagulationscentrum, Sahlgrenska sjukhuset, S-Gothenburg

Stöhring, Th., Dr.
Kinderklinik Lindenhof, Krankenhaus Lichtenberg, D-Berlin

Strasser, E., Dr.
Abteilung für Transfusionsmedizin, Chirurgische Universitätsklinik,
D-Erlangen

Subert, R., Frau Dr.
Abt. für Hämatologie u. Onkologie, Klinik für Innere Medizin II,
Klinikum Schwerin, D-Schwerin

Sulovska, I., Frau Dr.
Hämatologische Klinik, Fakultätskrankenhaus, CZ-Olomouc

Sutor, A.H., Prof. Dr.
Abt. Hämatologie u. Hämostaseologie, Kinderklinik,
Klinikum der Albert-Ludwigs-Universität, D-Freiburg

Sykora, K.W., PD Dr.
Zentrum Kinderheilkunde, Medizinische Hochschule Hannover, D-Hannover

Syrbe, G., PD Dr.
Innere Abteilung, Landesfachkrankenhaus Stadtroda, D-Stadtroda

Ţepeneu, M., Dr.
Clinica de Chirurgie si orthopedie, Pediatrica, R-Timisoara

Ţepeneu, P., Dr.
Clinica de Chirurgie si orthopedie, Pediatrica, R-Timisoara

Thürmel, K., Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt
der Ludwig-Maximilians-Universität, D-München

Timr, P., Dr.
Childrens' Department, Nemocnice Ceske Budejovice, Ceske Budejovice

Trieb, K., Dr.
Universitätsklinik für Orthopädie, A-Wien

Tsakiris, D., Dr.
Gerinnungs- und Fibrinolyselabor, Kantonsspital, CH-Basel

Tschol, G.A., Dr.
A-Bludenz

Unkrig, Ch., Dr.
Med. Universitätspoliklinik, D-Bonn

van Loon, J., Dr.
Beatrix Kinderkliniek, University Hospital Groningen, NL-GZ Groningen

Verpoort, K., Dr.
D-Hamburg

Vigh, Th.
Hämophilieambulanz, Zentrum der Inneren Medizin,
Klinikum der Johann-Wolfgang-Goethe-Universität, D-Frankfurt/M.

Voerkel, W., Dr.
Gemeinschaftspraxis für Labormedizin,
Mikrobiologie und Transfusionsmedizin, D-Leipzig

Vogel, G., Prof. Dr.
Institut für Experimentelle Hämatologie, Klinikum Erfurt GmbH, D-Erfurt

Vogt, B.
Universitäts-Kinderklinik Leipzig, Abteilung für ambulante und soziale Pädiatrie, D-Leipzig

Voigt, H., Dr.
Med. Klinik und Poliklinik I, Hämophiliezentrum, Universitätsklinikum Leipzig, D-Leipzig

von Auer, F., Regierungsdirektor
Bundesministerium für Gesundheit, D-Bonn

von Depka Prondzinski, M., Dr.
Abt. Hämophilie, Med. Poliklinik, Medizinische Hochschule Hannover, D-Hannover

von Lieven, M., Frau
Hämophilieambulanz, Medizinische Klinik I, Klinikum der Johann-Wolfgang-Goethe-Universität, D-Frankfurt/Main

Vorlova, Z., Frau Dr.
Institut für Hämatologie und Bluttransfusion, CZ-Praha

Wallny, Th.,Dr.
Orthopädische Klinik, Med. Einrichtungen der Rheinischen Friedrich-Wilhelms-Universität, D-Bonn

Wank, J., Dr.
St.-Anna-Kinderspital, A-Wien

Weissbach, G., Prof. Dr.
Klinik Bavaria Zscheckwitz, Rehabilitationszentrum für Kinder und Jugendliche, D-Kreischa

Weisser, J., Dr.
Abteilung Pädiatrie/Neuropädiatrie, Fachkrankenhaus Neckargemünd gGmbH, D-Neckargemünd

Wendisch, J., Dr.
Klinik u. Poliklinik für Kinderheilkunde, Universitätsklinikum Carl-Gustav-Carus, D-Dresden

Wenzel, E., Prof. Dr.
Abt. Klinische Hämostaseologie und Transfusionsmedizin, Universitätskliniken des Saarlandes, D-Homburg

WERMES, C., Frau Dr.
Abt. Hämophilie, Med. Poliklinik, Medizinische Hochschule Hannover, D-Hannover

WERNY, A., Frau Dr.
Abteilung Hämostaseologie, Medizinische Klinik Innenstadt der Ludwig-Maximilians-Universität, D-München

WIEDING, J.U., Dr.
Abteilung Transfusionsmedizin, Universitätskliniken, D-Göttingen

WINDER, A., Dr.
Landeskrankenhaus Bregenz, Pädiatrie, A-Bregenz

WISCH, Frau
Labor, Allgemeines Krankenhaus Altona, D-Hamburg

WOLF, H.-H., Dr.
Klinik u. Poliklinik f. Innere Med. IV, Medizinische Fakultät, MLU Halle-Wittenberg, D-Halle

WOLLINA, K., Frau Dr.
Klinik Innere Medizin II, Friedrich-Schiller-Universität, D-Jena

WULFF, K., Frau Dr.
Institut für Humangenetik, Med. Fakultät der Ernst-Moritz-Arndt-Universität, D-Greifswald

ZELLER, W., Dr.
D-Hamburg

ZELLHOFER, J.
Allg. Krankenhaus der Stadt Wien, Personalvertretung, A-Wien

ZIEGER, B., Frau Dr.
Kinderklinik, Klinikum der Albert-Ludwigs-Universität, D-Freiburg

ZIMMERMANN, R., Prof. Dr.
Kurpfalzkrankenhaus Heidelberg und Hämophiliezentrum gGmbH, D-Heidelberg

ZUPANCIC-SALEK, S., Frau Dr.
Lug Samoborski, Croatia

Johann Lukas Schoenlein Award 2000

I. SCHARRER

The Johann Lukas Schoenlein Prize was first awarded in 1977, sponsored originally by the Immuno Company, now Baxter, to commemorate J. L. Schoenlein, who gave the name to hemophilia. Schoenlein was made a professor at the early age of 26 and taught in Zurich from 1833 to 1840.

The objectives of the Prize are laid down in the Statutes as follows:
The Prize serves to advance clinical research in the area of chronic blood diseases, particularly hemophilia and related congenital diseases of blood clotting. This is an exclusively charitable foundation and achieves this objective by the award of the J.L. Schoenlein Prize for exceptional scientific works.
The recipient of the award is decided by a curatorium consisting of seven scientists and one representative of the foundation.
The criteria on which the curatorium bases its decision include scientific value, clinical relevance, innovation, originality, effectiveness and presentation.

This year we have had a record number of ten entrants. Never before have there been so many from Germany, Switzerland and England as in this year. Even more exciting is that most of these were of an equally high, even extremely high standard, and the curatorium has had to expend much more time and effort than previously in making its decision.

After a thorough examination, the choice fell on Dr. L. Alberio and his co-workers for the work: »Surface expression and functional characterization of alpha-granule factor V in human platelets: effect of ionophore A23187, thrombin, collagen and convulsin«. This was published in the journal »*Blood*« during the year 2000.

The work was done during a scientific sabbatical in Oklahoma in conjunction with Safa, Clemetson, Esmon and Dale between 1997 and 1999.

The importance of plasma Factor V and its mutants has been known since their discovery in 1993 by Dahlbaeck. Platelet Factor V, however, has remained largely undefined until taken on by Dr. Alberio and his co-workers. They have examined the smallest blood cells, the dust particles of the blood, in their most innermost details, searching for the tiniest trace of Factor V.

If the platelet is happy and satisfied, then FV is found in the alpha granule (Fig. 1) somewhere in the center of the platelet. If however it has been annoyed, or stimulated by thrombin, collagen or convulsin, Factor V slips to the surface (Fig. 2) and becomes ready to attack (Fig. 3).

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

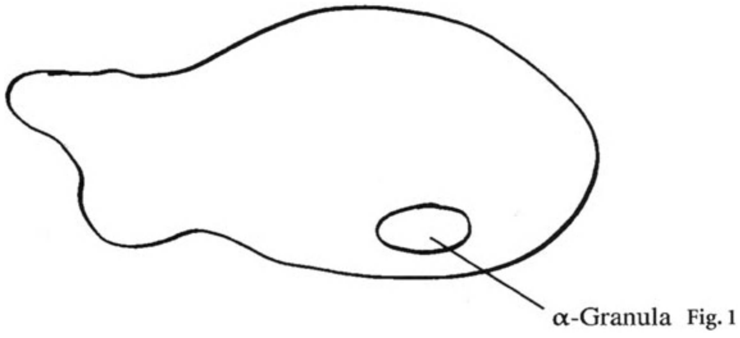


Fig. 1

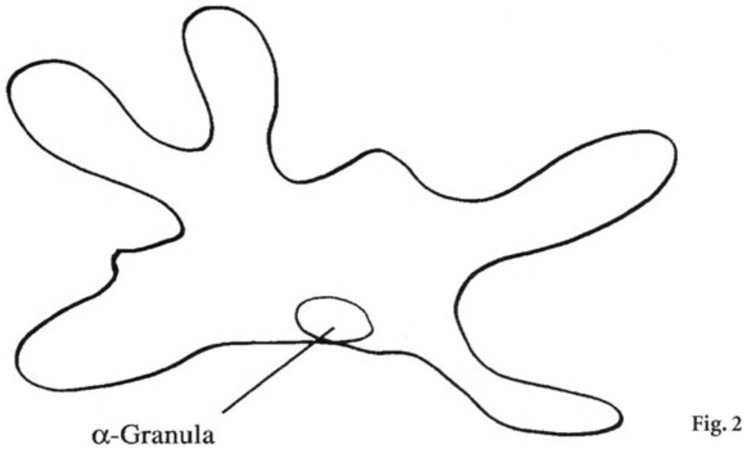


Fig. 2

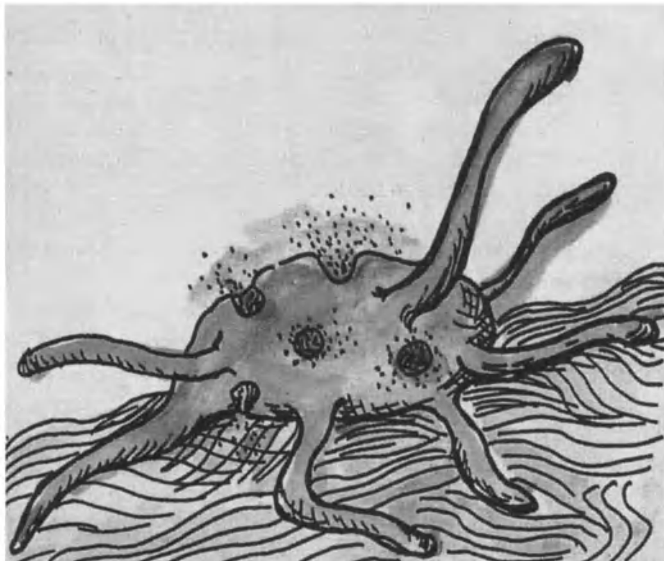


Fig. 3

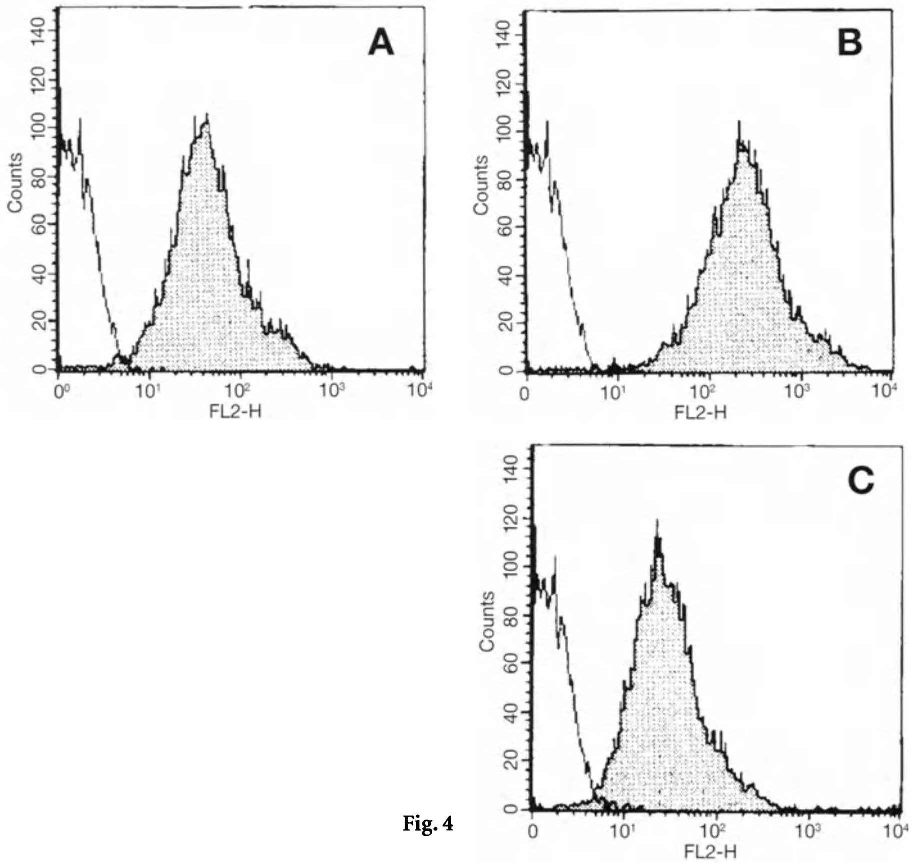


Fig. 4

The research carried out by the successful team shows how important the alpha granules are for the formation of prothrombinase. This has revealed a cellular procoagulatory principle, which was previously unknown. The authors thereby add weight to the importance of cellular hemostasis, which seems to have been somewhat neglected lately.

Young platelets are particularly stimulated by thrombin and collagen to form prothrombinase and thereby to be hemostatically active.

Figure 4 is an original from the publication of Alberio and shows how simply the Swiss can present good scientific evidence. Its summit is the goal for many, as is here for example the Matterhorn (Fig. 5) for the intracellular factor V in peaceful platelets. The similarity is amazing.

With the award of the prize, we are honoring and rewarding scientific value, originality, newness of concept, thorough methodology and the likely clinical relevance of the discovery of focal thrombin production.

The J. L. Schoenlein Prize is to be awarded on November 11, 2000 for the 14th time.

Fig. 5

In the name of the curatorium, I would like to congratulate the prize winner and his colleagues and wish them much further success in research on platelet factor V.

I. Epidemiology

Chairmen:

R. Seitz (Langen)
G. Auerswald (Bremen)

HIV Infection and Causes of Death in Patients with Hemophilia in Germany (Year 1999/2000 Survey)

W. SCHRAMM, H. KREBS, on behalf of the GTH Hemophilia Committee

Basic Facts on the Surveys

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. The early questionnaires used in the survey focused on basic data and were later expanded by additional information particularly about HIV infection and AIDS-related death. Since 1998 more specific data on hepatitis and antiretroviral therapies have been included. Future surveys will be strengthened by data derived from the German Hemophilia Registry that is currently being established on behalf of the GTH Hemophilia Commission.

Participating Centers

Since the first survey the number of participating centers has increased every year, with a particularly huge 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). Although this year the number of reporting hemophilia centers decreased from 93 centers last year to 87 centers this year (Table 1). The total number of patients (including patients with von Willebrand disease) reported from all centers has slightly increased from 7365 to 7548 patients or 2.5% (Table 4).

At this point we would like to thank all colleagues who have contributed data to this survey.

Table 1. Numbers of participating hemophilia centers

	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000
East	47	62	79							
West	18	18	24							
Total	65	80	103	111	119	119	71	75	93	87

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

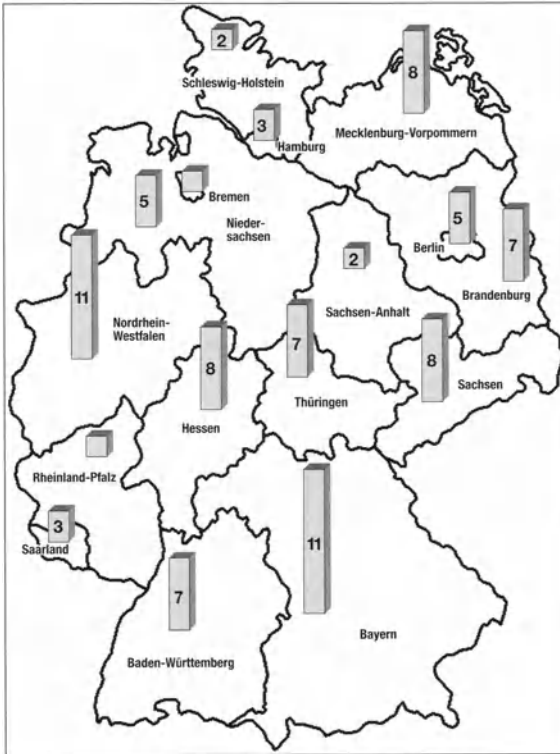


Fig. 1. Distribution of reporting hemophilia centers in Germany

Patients

In 2000, a total number of 7548 patients (including possible double registrations) have been reported from the participating centers. The distribution of patients with hemophilia A (49,47%), B (8,78%) and patients with von Willebrand disease (41,75%) is given in Fig. 2.

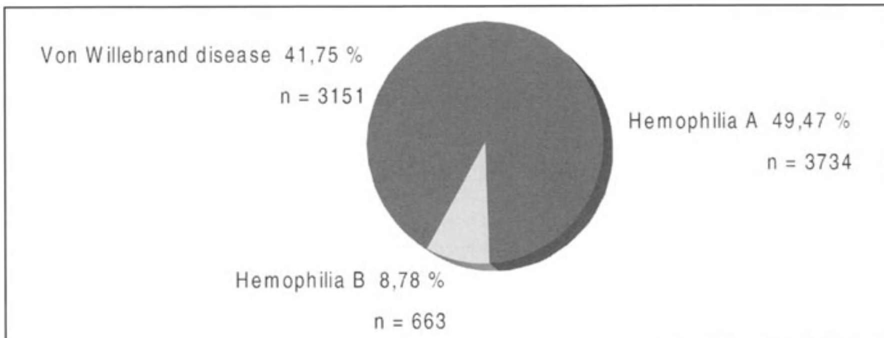


Fig. 2. Overall distribution of diseases

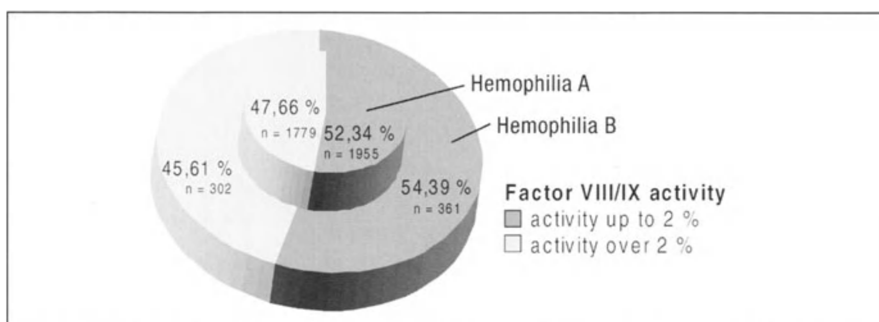


Fig. 3. Distribution of factor VIII/IX activity in patients with hemophilia A and B

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 equal in patients with hemophilia A and B as shown in Fig. 3.

In 4,28% of the patients with hemophilia A and in 2,12% of the patients with hemophilia B an inhibitor was found (see Fig. 4 and Table 2). Nineteen percent of patients with von Willebrand disease showed ristocetin co-factor levels below 30% as demonstrated in Fig. 5 and Table 2.

Table 2. Cumulative data from 87 centers as of 1999/2000

	Hemophilia A		Hemophilia B		Von Willebrand disease		Total
	N	%	*N*	%	*N*	%	*N*
Total	3734	49,47%	663	8,78%	3151	41,75%	7548
Factor activity ≤ 2%	2020	54,39%	347	52,34%	–	–	2367
Factor activity >2%	1694	45,61%	316	47,57%	–	–	1965
Ristocetin Cofactor ≤ 30%	–	–	–	–	633	18,99%	633
Ristocetin Cofactor >30%	–	–	–	–	3093	83,01%	3093
Inhibitor (low responders)	59	1,58%	7	1,06%	–	–	66
Inhibitor (high responders)	100	2,68%	7	1,06%	–	–	107
Total HIV negative	3163	84,10%	579	87,33%	3124	99,14%	6866
Total HIV positive	598	18,90%	84	12,67%	9	0,86%	691
HIV positive, no AIDS	228	38,12%	51	60,71%	7	88,78%	286
HIV positive, CD4<200 cells/µl	152	25,42%	12	14,29%	1	11,11%	165
HIV positive, full-blown AIDS	34	5,69%	5	5,95%	1	11,11%	40
HIV positive, no comment	184	30,77%	16	19,05%	–	–	200

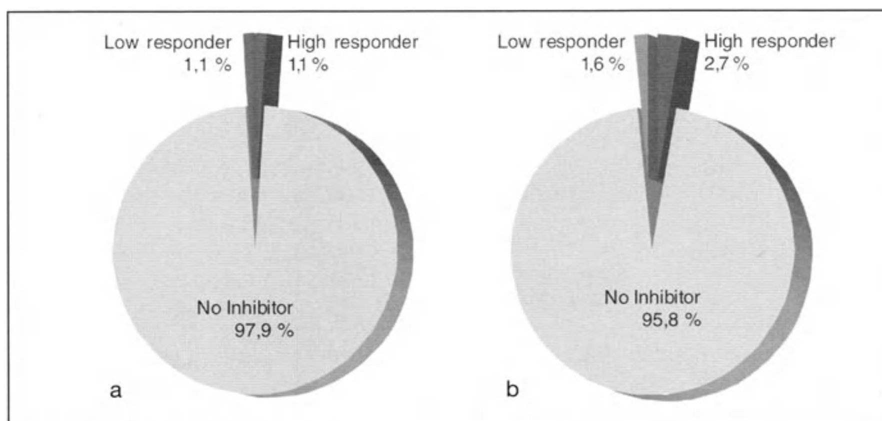


Fig. 4. Distribution of inhibitors in patients with (*a*) hemophilia A and (*b*) hemophilia B

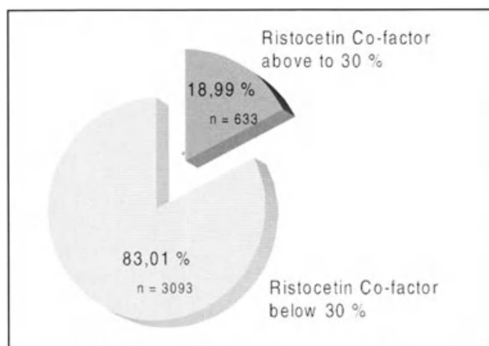


Fig. 5. Distribution of Ristocetin cofactor in patients with von Willebrand disease

HIV Status

Of all reported patients a total of 691 were infected with HIV, equivalent to 9,1%. Analyzed for HIV distribution in subgroups 16% of all patients with hemophilia A, 12,7% of all patients with hemophilia B, and 0,4% of all patients with von Willebrand disease were HIV-infected (see Fig. 6). A total of 40 patients (5,8%) has reached the stage of full-blown AIDS, compared to 286 patients (41,4%) that have up to now not shown severe symptoms of the immune disease (Table 3).

Table 3. HIV status

HIV status	Hemophilia A	Hemophilia B	von Willebrand disease
HIV positive, no AIDS	228	51	7
HIV positive, $CD4^+$ <200 cells/µl	152	12	1
HIV positive, full-blown AIDS	34	5	1
HIV positive, no comment	184	16	0
Total HIV positive	598	84	9

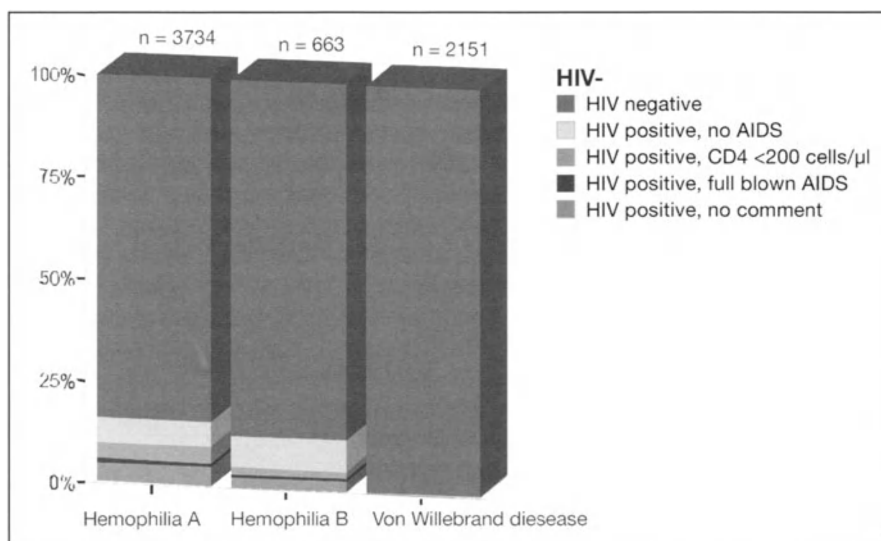


Fig. 6. Distribution of HIV-infected patients

Causes of Death

In the 99/00 period a total of 20 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 654 patients have been reported dead. As compared to the peak of 60 deaths in the 94/95 period a decrease of 66,7% occurred in the 99/00 period. The development of mortality and causes of death since 82/83 are depicted in Fig. 7.

Up to 1995 the number of AIDS-related deaths increased continuously with decline taking place since. Fortunately AIDS-related deaths again receded this year. The main reason for this development can probably be attributed to improved antiretroviral therapies.

Table 4. Distribution of death causes

Patients	*N*	% of dead patients	% of all patients
Living	7528	–	99,7
Died of AIDS	5	25	0,07
Died of other liver disease	3	15	0,04
Died of bleeding	1	5	0,01
Died of cancer	3	15	0,04
Died of other diseases	2	10	0,03
Died, no comment	6	30	0,08
Total number of dead patients	20	100	0,26
Total	7548	–	100

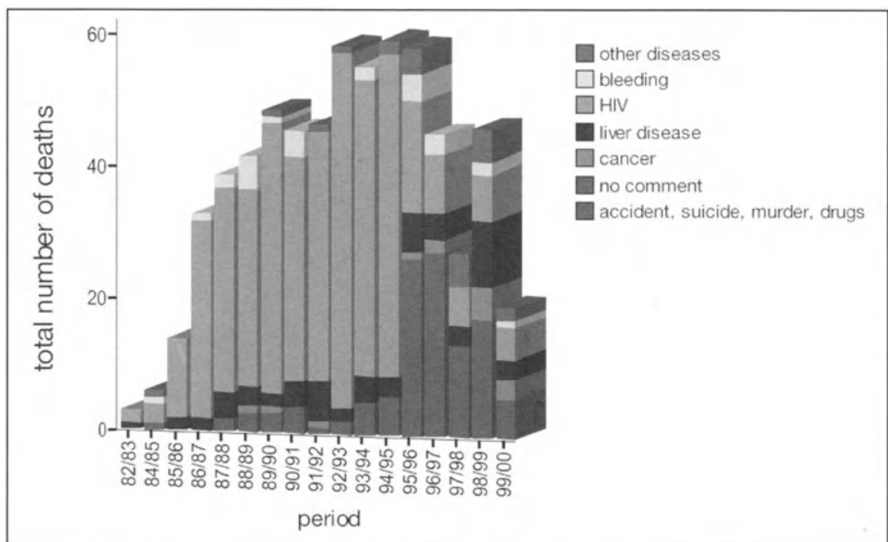


Fig. 7. Causes of death since the beginning of the survey

When analyzing the cumulative data (Fig. 8) it is striking that liver disease has become the second most important cause of death after the still dominating HIV infections. The reason for this may be the increasing number of liver cirrhosis due to hepatitis C. The future development of a possible correlation should be observed carefully.

No indications for Creutzfeld-Jakob disease in our patient collective has been reported since 1978.

Of note, since the 95/96 period the »no comment« section has increased sharply and actually amounts to 30% (Fig. 7).

GTH Hemophilia Registry

The German Society of Thrombosis and Hemostasis (GTH) is currently establishing a central register accessible to all German centers treating patients with bleeding disorders. The goal is to set up a suitable and easy to use system for acquiring and analyzing epidemiologic data of diseases related to bleeding disorders, HIV and hepatitis infection. The basis for the registry is the annual cause of death statistics of patients with bleeding disorders. These statistics have been performed in Germany since 1983. This project is supported by an unrestricted grant from Wyeth Pharma GmbH.

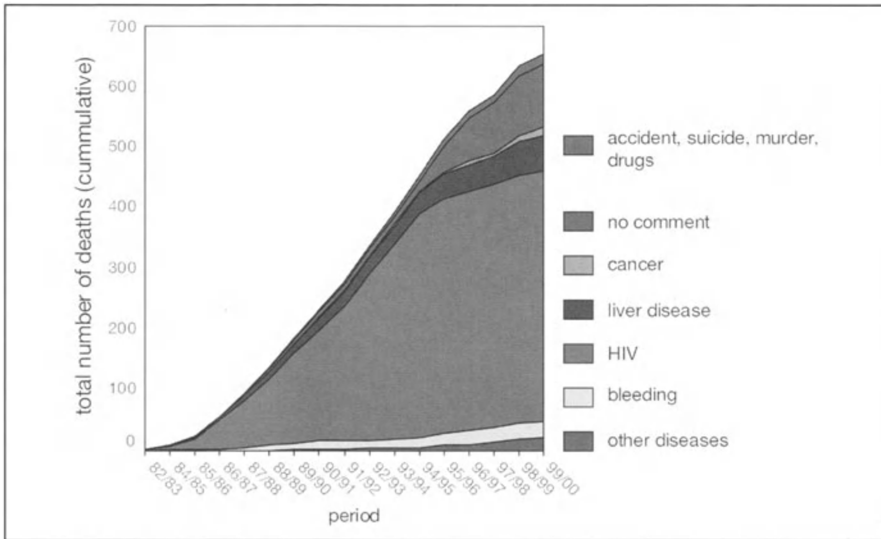


Fig. 8. Cumulative chart of deceased patients, separated for causes of death

Acknowledgements

Augsburg	Boehm	Heidelberg	Zimmermann
Augsburg	Heidemann	Homburg/Saar	Dockter
Augsburg	Schlimok	Homburg/Saar	Wenzel
Berlin	Beck	Jena	Zintl
Berlin	Heinrichs	Jena	Sayer
Berlin	Hempelmann	Kassel	Eggeling
Berlin	Henze	Kiel	Bruhn
Berlin	Koop	Köln (Lindenthal)	Söhngen
Bonn	Steinbeck-Klose	Leipzig	Voigt
Bonn	Brackmann	Leipzig	Lenk
Braunschweig	Eberl	Lübeck	Siemens
Bremen	Auerswald	Magdeburg	Mittler
Bremen	Holzhüter	Magdeburg	Franke
Chemnitz	Hofmann	Marburg	Kretschmer
Cottbus	Möbius	München	Kurnik
Cottbus	Grünhagen	München	Schramm
Delmenhorst	Niekrens	München	Woitinas
Dillingen	Mößeler	Münster	Pollmann
Dresden	Sahr	Neckargemünd	Bittinger
Dresden	Kotte	Nettersheim	Ahrens
Duisburg	Rott	Neubrandenburg	Berthold
Düsseldorf	Scharf	Neubrandenburg	Arndt

Düsseldorf	Göbel	Plauen	Karl
Erfurt	Schubert	Potsdam	Pasold
Erlangen	Klinge	Potsdam	Wedemeyer
Frankfurt/Main	Scharrer	Rostock	Freund
Frankfurt/Main	Kreuz	Saalfeld	Maak
Frankfurt/Main	Mondorf	Saarbrücken	Geib
Frankfurt/Oder	Klinkenstein	Potsdam-Drewitz	Schmeltzer
Frankfurt/Oder	Nimtz	Schwerin	Subert
Freiburg	Hasler	Schwerin	Schumacher
Freiburg	Sutor	Siegen	Göbel
Giessen	Kemkes-Matthes	Stadtroda	Syrbe
Giessen	Blütters-Sawatzki	Suhl	Edelmann
Greifswald	Beck	Tübingen	Niethammer
Greifswald	Herrmann	Ulm	Döhner
Grünwald	Köhler-Vajta	Ulm	Behnisch
Halle-Wittenberg/S.	Schobeß	Wuppertal	Böttcher
Hamburg	Kuse	Würzburg	Keller
Hamburg	Eifrig	Würzburg	Speer
Hamm	Balleisen	Zella Mehlis	Richter
Hannover	von Depka	Zwickau	Schott

Hemophilia 2000 – the Annual Survey of the Austrian Hemophilia Centers

H.K. Hartl, U. Eidher, U. Kunze, P. Arends, J. Falger, N.D. Jones, M. Kronawetter, P. Kurnik, I. Pabinger, H. Ramschak, E. Reiter, R. Schwarz, W. Streif, H. Türk, H. Wank, W. Zenz and K. Zwiauer

Material and Methods

The annual survey of the Austrian Hemophilia Centers is organized by the Institute of Social Medicine of the University of Vienna (ISM) and collects, analyzes and presents the anonymous questionnaires from all collaborating Austrian Hemophilia Centers (AHC).

The data on each patient are collected by the co-authors of this report, according to specially designed questionnaires, very similar to the questionnaire of the »German Survey on Causes of Death among Hemophiliacs«. For this year's survey the AHC received their data reports from 1999 to compare and/or to complete their files, in order to simplify the work in the annual Austrian survey.

The analysis 2000 shows us the distribution of the patients within the Austrian counties in respect to the place where the patients live, the number of patients according to the severity of the disease, and the distribution according to age. The patient overviews of the AHC were used for analyzing the type of disease and the number of HIV-infections among Austria's hemophilia patients.

This time we could receive questionnaires from the Treatment Centers in Graz (Styria), Güssing (Burgenland), Innsbruck (Tyrol), Klagenfurt (Carinthia), Linz (Upper Austria), Salzburg (Salzburg), St. Pölten (Lower Austria) and Wien (Vienna).

Because of a lack of any data from Vorarlberg and concerning adult patients in Upper Austria and Tyrol this presentation cannot show the complete epidemiological situation, but it is quite a representative overview on hemophilia in Austria in the year 2000.

Results

Overall there are 512 patients with hemophilia (PwH) registered and 488 patients' files could be used for presentation of following criteria:

1. Hemophilia type (Table 1) and severity of the disease (Fig. 1)
2. HIV infection among PwH (Fig. 2)
3. Distribution within the Austrian counties (Table 2)
4. Distribution according to age (Tables 3, 4)

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Hemophilia A and B

Hemophilia	384
Hemophilia A	331 (86.2%)
Hemophilia B	51 (11.8%)
Missing	60
Other bleeding disorders	42
Relation to male population	
Hemophilia	1:10.182
Hemophilia A	1:11.813
Hemophilia B	1:73.774

Table 2. Number of patients according to counties

	Total	%	% cumulative
Burgenland	19	3.9	3.9
Carinthia	23	4.7	8.6
Lower Austria	130	26.7	35.4
Upper Austria	31	6.4	41.8
Salzburg	11	2.3	44
Styria	109	22.4	66.5
Tyrol	13	2.7	69.1
Vorarlberg	5	1	70.2
Vienna	145	29.8	100
Total	486	100	

Table 3. Number of patients according to age

Age	Total	%	% cumulative
<10 years	70	14.4	14.6
11–20 years	93	19.1	34.1
21–30 years	88	18.1	52.5
31–40 years	85	17.5	70.3
41–50 years	53	10.9	81.4
51–60 years	35	7.2	88.7
61–70 years	30	6.2	95
>70 years	24	4.9	100
Total	478	98.4	100
Missing	8	1.6	

Table 4. Age of patients according to county

Age (years)	County B	C	LA	UA	S	St	T	Vorarl-berg	Vienna	Total
1 to 10		3	16	8	2	20	5	2	14	70
11 to 20	4	7	25	8	1	15	6	1	28	95
21 to 30	3	3	23	4	2	22	2	1	28	88
31 to 40	5	7	20	2	5	21		1	24	85
41 to 50	1	2	17	4		14			15	53
51 to 60	2	1	10			7	1		14	35
61 to 70	2		11		1	4			12	30
70 to 99	2		6	1		5			10	24
Total	19	23	128	27	11	108	14	5	145	480

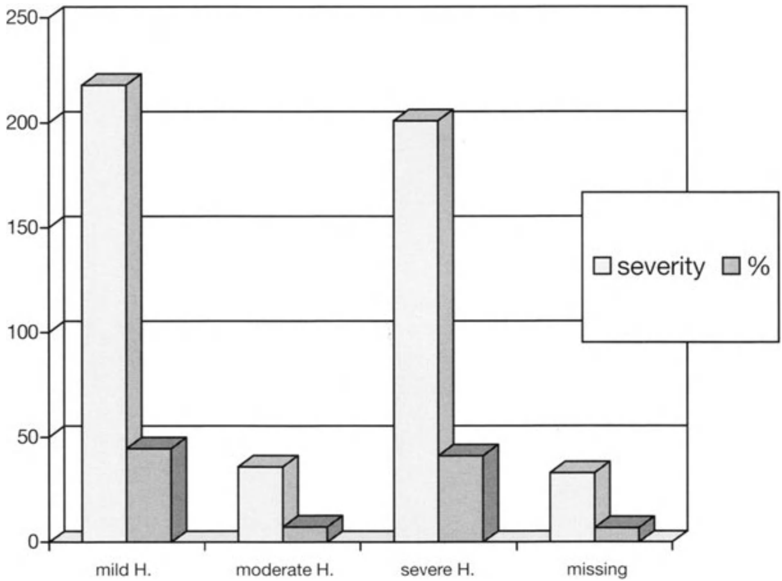


Fig. 1. Severity of the disease

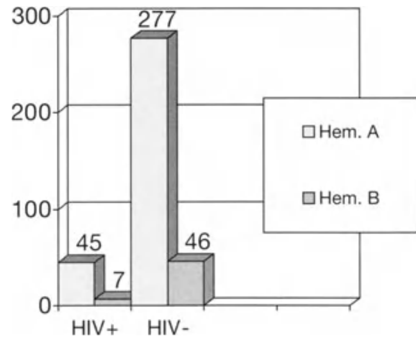


Fig. 2. HIV infections

Severity of the Disease

We received data from 384 PwH, 331 (86.2%) suffer from hemophilia A and 53 (13.8%) from hemophilia B; other bleeding disorders in 42 patients and missing information on type of bleeding disorder in 60 patients.

In comparison to the Austrian population we have one PwH in 10,182 male Austrians, respectively one person with hemophilia A in 11,813, and one with hemophilia B in 73,774 males.

The relation of patients with mild hemophilia to patients with the severe form is, in hemophilia A as well as in B, similar, the relation of hemophilia A to hemophilia B is according to the literature.

Type	Hemophilia A	331 (86.82%)
	Hemophilia B	53 (13.7%)
Severity	Mild	218 (44.7%)
	Moderate	36 (7.4%)
	Severe	201 (41.2%)
	Missing	33 (6.8%)

Distribution Within Austria

The distribution of the patients over the Austrian counties gives information on the registration as well as on participation in the survey itself.

Table 2 shows that counties such as Lower Austria, Styria and Vienna, where most of the Austrian patients are registered, have reached a very high standard of documentation. But it has to be mentioned that, because of missing information from Tyrol, Upper Austria and Vorarlberg, there is a bias, for example in average age etc.

HIV infection

We want to show the status quo in comparison with the survey of 1999 and we found that, probably due to missing information from the above mentioned counties, »only 52« PwH were HIV-infected in 2000, 45 with hemophilia A and 7 with hemophilia B.

Age

The average age is 31,8 years, median 29. The oldest patient is 99, the youngest 1 year. Seventy patients are younger than 10 years and 24 older than 71. The distribution is shown in Table 3, and according to the counties in Table 4.

Discussion

The AHC's annual survey shows a not in all aspects complete but representative overview on hemophilia in Austria and, because of increasing cooperation we come closer and closer to the real epidemiological situation. Because of the use of the German PEI-code and the, in comparison to the years before, very complete patient-files, we could eliminate most of the double registrations.

A challenge for the next survey will be the completion of our data material through integration of the Treatment Centers in Carinthia, Tyrol and Upper Austria (for adult patients, children are documented very well) and Vorarlberg. But we do not expect a big change in the results concerning type or severity of hemophilia or average age.

Concerning HIV-infection we know from the *Unterstützungsfonds für Personen, die durch medizinische Behandlung oder Tätigkeit mit HIV infiziert worden sind, und deren Angehörige*, that there are some nine more HIV-positive PwH receiving monthly payments than there are registered in our files.

The yearly follow ups of the annual survey of the AHC gave us a very representative and more or less complete description of hemophilia in Austria. With these data materials as a basis, now we have the possibility of collecting and documenting other important information, for example on home treatment, use of factor concentrates, frequency of treatment center visits, compliance, psycho-social and quality of life questions etc.

Finally I want to thank all co-workers and their colleagues for their good and successful cooperation.

II. *Inhibitors in Hemophilia*

Chairmen:

W. Kreuz (Frankfurt/Main)
R. Zimmermann (Heidelberg)

Changes in Epitope Specificity and in Distribution of IgG Subtypes of FVIII Antibodies during Immune Tolerance Therapy (ITT) in Hemophilia A Patients with FVIII Antibodies – a Case Report

A. Kallas, T. Talpsep and H. Everaus

Introduction

Replacement therapy of patients with inherited coagulation factor VIII deficiency (hemophilia A) comprises administration of either plasma derived factor VIII concentrates or recombinant factor VIII. Up to 25% of patients may develop antibodies against FVIII (FVIII:Ab). Antibodies arise more often in patients with severe form of hemophilia A caused by inversion, large deletion or stop codon in FVIII gene rather than in patients with mild or moderate forms of the disease (point mutations, small deletion in FVIII gene) [1, 2]. It is not known why only some patients develop FVIII antibodies whereas others do not in spite of extensive replacement therapy.

Factor VIII circulates in plasma as a heterodimer composed of the heavy chain (domains A1-A2-B) and the light chain (domains A3-C1-C2), and forms a complex with von Willebrand factor (vWF) via the light chain [3] (Fig. 1). Von Willebrand factor stabilizes FVIII and can interfere with antibody binding to FVIII either by

Fig. 1. The structure of FVIII molecule. VWF binding sites and antibody binding sites are indicated

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

direct blocking of epitopes on FVIII light chain or by steric hindrance. Most inhibitory antibodies to human factor VIII developed in hemophilia A patients bind to epitopes within A2 domain, C2 domain, and A3-C1 domains [4]. Antibodies reacting with C2 domain of the FVIII light chain possess a lower ability to neutralize FVIII coagulation (FVIII:C) activity, when it is in a complex with vWF (FVIII-vWF) [5]. In a previous study we have shown that the decreased neutralization of FVIII in the presence of vWF by FVIII antibodies was in good correlation with the level of antibodies against FVIII light chain [6]. These in vitro findings suggest that detecting the epitope specificity and the concentration of antibodies can be used for selecting FVIII concentrate for replacement therapy and for immune tolerance induction. Different protocols of immune tolerance therapy (ITT) like the Bonn protocol, the Malmö method, and their modifications demonstrate the similar efficacy in suppressing the production of antibodies to a non-detectable level [7]. While using FVIII concentrate alone for IT therapy [8, 9], the success rate may depend upon the purity of FVIII concentrate used [10].

The effect of different FVIII concentrates on the immune system of hemophilia A patients has been intensively studied both in vitro and in vivo. Lymphocytes respond to FVIII with enhanced cytokine production in vitro [11], but in vivo the situation is more complicated and depends also on the availability of cytokine receptors, which determine the magnitude of immune response to FVIII concentrates. First exposure to FVIII concentrate may cause production of IgM type FVIII antibodies, while isotype switch occurs soon in order to produce IgG isotype antibodies. Distribution of different IgG antibody subtypes depends on whether the Th1 or Th2 phenotype is dominant in a particular patient [12, 13].

We investigated the changes of epitope specificity and distribution of IgG subtypes of FVIII antibodies in hemophilia A patient during ITT with FVIII-vWF concentrate. The IgG subtype distribution of FVIII antibodies was estimated using sensitive ELISA assay and results were compared with those obtained from the samples of hemophilia A patients with persistent FVIII antibody titer, and from the samples taken from the patient with low titer antibodies during on-demand treatment with FVIII concentrates of different purity.

Patients

Patient 1 Undergoing ITT

A 3-year-old severe hemophilia A patient with intron 22 inversion in FVIII gene (type 1) had FVIII antibody titer of 26 BU/ml. Immune tolerance therapy with FVIII concentrate, Haemoctin SDH (Biotest, Germany) was begun when he had had 23 exposure days to FVIII. Haemoctin SDH was the only available FVIII concentrate at that time in Estonia. The specific activity of Haemoctin SDH was 100 IU of FVIII per mg of protein. The immune tolerance therapy comprised continuous infusion (CI, 40 IU/kg/h for 8 days + 8 days via vascular line), followed by bolus injections 100 IU/kg/day for 1 month and thereafter treatment was continued with the dose 100 IU/kg twice a week for 10 months (Fig. 2). After 2 months of ITT, the antibody

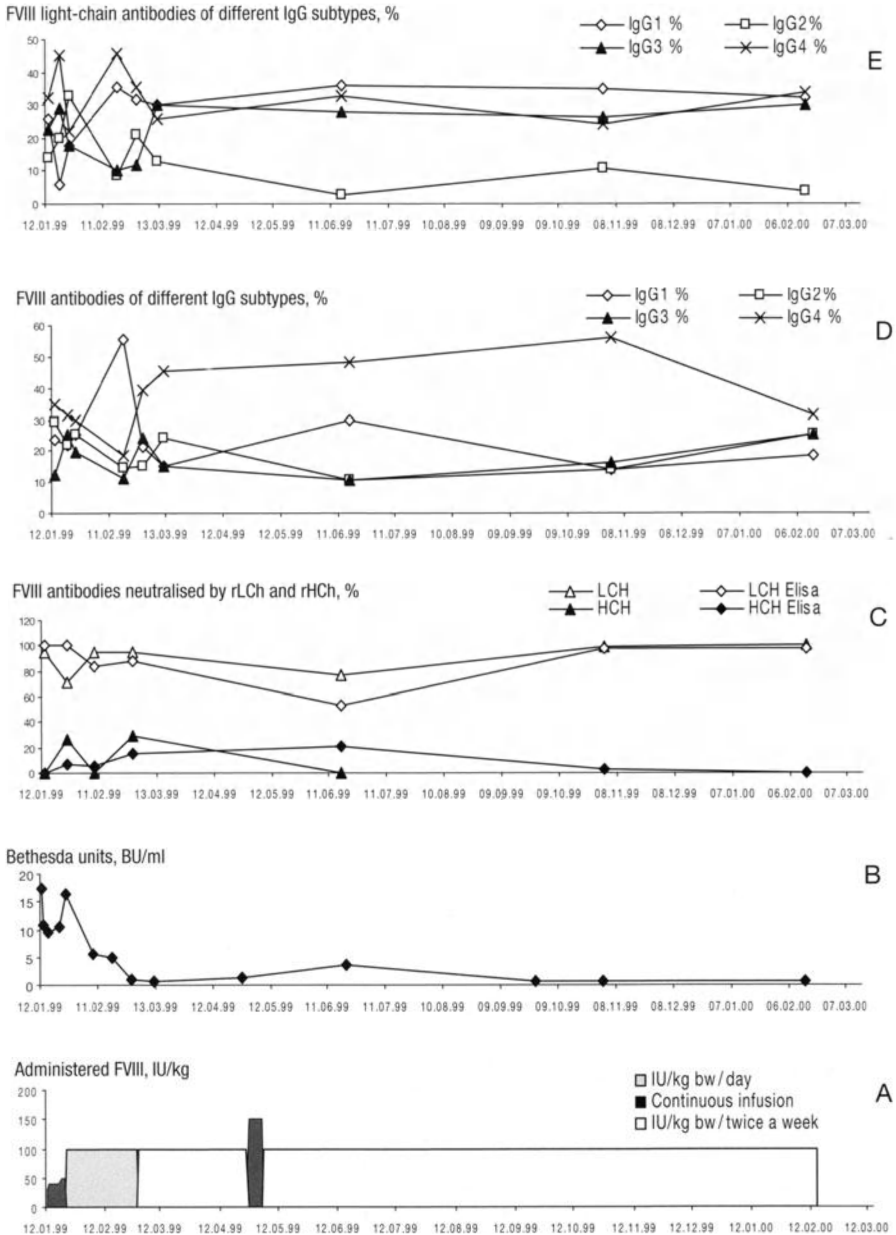


Fig. 2a–e. Immune tolerance therapy in patient 1 with high titer FVIII antibodies. *a* The course of the treatment with FVIII concentrate Haemoctin SDH (Biotest); *b* Changes in FVIII antibody titer measured by Bethesda assay; *c* Relative distribution of FVIII light chain (rLCh) and FVIII heavy chain (rHCh) antibodies measured by an ELISA assay (ELISA) and by a chromogenic method; *d* IgG subtype distribution of FVIII antibodies; **e** IgG subtype distribution of FVIII light chain antibodies

titer had reduced from 17.5 BU/ml to 0.7 BU/ml. During the course of the treatment with a dose of 100 IU/kg twice a week, problems in venous access appeared, which were accompanied by an increase in antibody titer to 1.2 BU/ml. Therefore, the second continuous infusion for 8 days with a dose of 150 IU/kg/h was given. In response to this treatment, the antibody titer peaked to 3.5 BU/ml, and then dropped to 0.8 BU/ml after 9 months of IT therapy. The last three samples contained only antibodies with non-inhibitory activity (Bethesda titer 0.8 BU/ml). In vivo recovery normalized and the patient started prophylactic treatment (dose of 100 IU/kg twice a week). During ITT the patient had no bleeding episodes.

Patient 2 Treated with FVIII Concentrates with Different Purity (On-demand Treatment)

A severe hemophilia A patient with intron 22 inversion in FVIII gene (type 2) developed FVIII antibodies (1.1 BU/ml) at the age of 6 years. Patient's HLA genotype was DR4 (DRB1*04), DR8 (DRB1*08), DQ4 (DQB1*04), and DQ7 (DQB1*0301) as detected by PCR method. He has had 8 exposure days to FVIII concentrates Hemofil-M (Baxter, USA), 8 exposure days to cryoprecipitate and 4 to plasma. The amount of FVIII concentrates used and the characterization of FVIII antibodies in plasma samples taken at different time points are shown in Fig. 3. Treatment of the patient with FVIII concentrates Koate-HP (Bayer, USA) and Kryobulin Tim 3 (Baxter), both of which contain vWF, caused a decrease in FVIII antibodies from 1.0

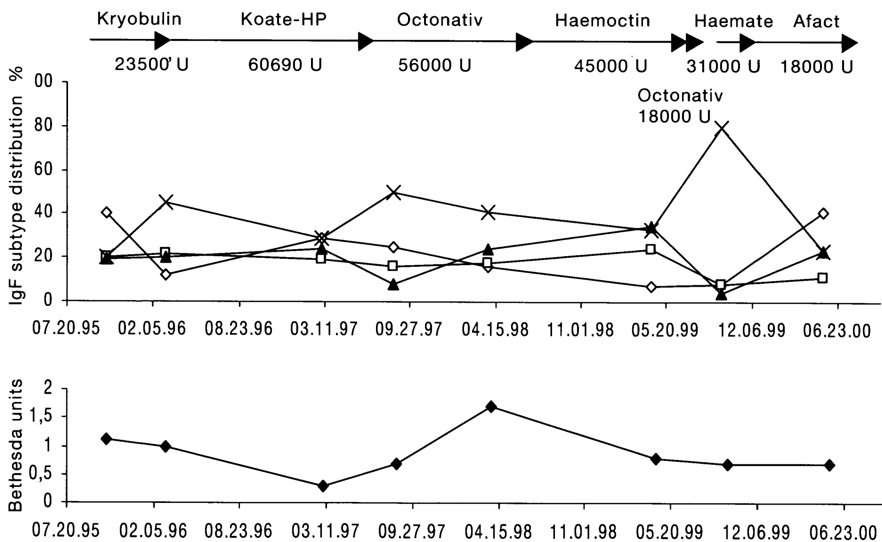


Fig. 3. Changes in IgG subtype distribution of FVIII antibodies of hemophilia A patient 2 during on-demand therapy with FVIII concentrates with different purity. FVIII antibodies of different IgG subtypes are shown as follows: IgG1 (◇), IgG2 (□), IgG3 (▲), IgG4 (×), Bethesda units (Bu/ml) ◆)

to 0.3 BU/ml. However, changing to FVIII concentrate, Octonativ-M (Pharmacia & UpJohn), which was purified by monoclonal antibody, resulted in an increase in FVIII antibodies up to 1.7 BU/ml. The respective antibodies reacted with FVIII light chain and A2 domain. In vivo FVIII recovery was 32.4% after administration of 25 IU/kg of body weight of Octonativ-M. After 15 months of treatment with Octonativ-M, FVIII concentrate was replaced by Haemoctin SDH (Biotest). In September 1998, in vivo recovery was 64.8% (the dosage 25 IU/kg of body weight). Antibody titer had slightly decreased (0.8 BU/ml), and antibodies had reactivity against the light chain and A2 domain of FVIII. Haemoctin SDH was used for on-demand treatment for the next 11 months. The titer of antibodies had decreased to 0.7 BU/ml and antibody reactivity against the light chain and the A2 domain persisted. In vivo recovery with Haemoctin-SDH was 80.5%. Thereafter treatment was switched to FVIII concentrate Octonativ-M for 3 months, followed by treatment with Haemate (Aventis Behring, USA) for 5 months, and then monoclonal antibody purified FVIII concentrate Aafact (CLB, the Netherlands).

Hemophilia A Patients with Persistent FVIII Antibodies

Plasma samples from 12 hemophilia A patients having FVIII antibodies were studied. The study protocol was approved by the Ethics Committee of the University of Tartu, Estonia. Anti-FVIII antibodies had developed in all the patients in response to the on-demand treatment with plasma-derived FVIII concentrates. Plasma samples were drawn in a clinical stable situation before any immune tolerance induction. Plasma samples contained 1 to 300 BU/ml of FVIII antibodies. All investigated FVIII antibodies reacted with A2 domain and the light chain of FVIII as detected by Western blotting using plasma derived FVIII concentrate Haemoctin SDH (Biotest, Germany). Moreover, all plasma samples contained antibodies interfering FVIII interactions with vWF and also interaction with phosphatidylserine measured by ELISA assays, as described by Shima and coworkers [14]. FVIII antibodies competed with murine monoclonal antibody ESH4 (American Diagnostica, USA) for phospholipid binding site at light chain C-terminus of FVIII as detected by an ELISA.

Materials

Recombinant FVIII (rFVIII, Recombinate, Baxter) was used throughout the study. Recombinant FVIII fragments (recombinant heavy chain, rHCh, and light chain, rLCh) were kindly provided by Dr. Mirella Ezban. Bovine serum albumin (BSA) and pNPP (p-nitrophenylphosphate) were purchased from Sigma. Other chemicals were of analytical grade. Majority of in vitro assays was carried through either in TBS (0.02 M Tris buffered saline, pH 7.2) or carbonate buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6).

Methods

FVIII antibodies in plasma samples taken during IT therapy were measured by Bethesda assay and by an ELISA method. Recombinant FVIII (Recombinate) was used as a coating agent for microplates.

Neutralization of FVIII:C Activity in the Presence and Absence of vWF

Plasma-derived FVIII-vWF concentrate (Haemoctin SDH) and recombinant FVIII (Recombinate) were diluted to 1 IU/ml of FVIII:C with TBS buffer containing 1% BSA and incubated with an equal volume of serially diluted (1:2 to 1:1000 in TBS-BSA) FVIII antibody plasma sample for 1 h at 37°C. Residual FVIII:C was determined by chromogenic method according to manufacturer's instructions (Coatest, Chromogenix AB, Italy) on a 96-well microplate. Percentage neutralization was calculated relative to a control. The control had the test plasma sample replaced by buffer or plasma from CRM negative hemophilia A patient without FVIII antibodies. The test plasma sample dilution giving 50% of neutralization of FVIII:C activity was found using both concentrates. The results were used to calculate a ratio of 50% neutralization of FVIII:C with rFVIII to that with FVIII-vWF.

Distribution of FVIII Light Chain and Heavy Chain Neutralizing Antibodies Measured by an ELISA Assay

Microplates (PolySorp, Nunc, Denmark) were coated with rFVIII (Recombinate) diluted to 8 IU/ml in carbonate buffer and incubated overnight at 2–8°C. The sites of nonspecific binding in the wells of the microplate were blocked with TBS containing 3% BSA for 1 h at room temperature and BSA was washed off with TBS-T (washing buffer, TBS with 0.1% Tween 20). Plasma samples with FVIII antibodies were diluted to 4 BU/ml, except test plasma samples with FVIII antibody titer ≤ 4 BU/ml, which were used undiluted. To neutralize FVIII antibodies, plasma samples were incubated with an equal volume of recombinant FVIII fragments diluted from 0.2 to 12.6 IU/ml according to labelled activity, for 1 h at 37°C. After incubation, aliquots of incubation mixture were transferred to antigen in the wells of the microplate and incubated for 2 h at 37°C. Bound FVIII antibodies were detected using rabbit anti-human IgG conjugated with alkaline phosphatase (AP) diluted 1:1000 (Dako, Denmark) and visualized with AP-substrate pNPP (p-nitrophenyl phosphate) and the color change was read at 405 nm. Two controls were included with each test: one mixture that had the antibody plasma sample replaced by buffer (minimal binding), and the second mixture that used buffer instead of recombinant FVIII fragments (maximum binding). The percentage neutralization was calculated as follows: 100 – ([binding with fragment – minimum binding]/[maximum binding – minimum binding]) × 100. The plateau was defined as the minimal concentration of FVIII fragment, which yielded the maximal (plateau) optical density (OD). When FVIII antibodies were partially neutralized by recombinant fragments, all values within the plateau region were averaged.

Distribution Antibodies Neutralized by FVIII Light Chain and Heavy Chain Measured by Chromogenic Method

The plasma samples were diluted to neutralization activity of 4 BU/ml, except plasma samples, which contained ≤ 4 BU/ml of FVIII antibodies and were used undiluted, and incubated with an equal volume of serially diluted recombinant FVIII fragments as described in the previous assay. An aliquot was removed for measuring FVIII antibodies by an ELISA assay. To the remaining mixture an equal volume of rFVIII (Recombinate) diluted to 2 IU/ml was added and incubated for 1 h at 37 °C. Residual FVIII coagulation activity (FVIII:C) was measured by the chromogenic method. Percentage of neutralization was calculated using the above-mentioned formula but binding capacity was substituted with FVIII:C activity. Maximum FVIII:C activity was measured from the mixture of recombinant fragments and rFVIII without an antibody sample, incubated in the same conditions. If the tested plasma contained an FVIII antibody level <10 BU/ml, the plasma sample from the hemophilia A patient (CRM-negative and without FVIII antibodies) was used in the mixture to obtain the maximum FVIII:C activity. Minimum FVIII:C activity was found for each test sample incubated with rFVIII without any competitive FVIII fragments. The plateau value was similarly measured as in the previous assay.

ELISA Assay for Measuring FVIII Antibody IgG1-4 Subtypes

Immunoplates (PolySorp) were coated with 8 IU/ml recombinant FVIII (Recombinate) in carbonate buffer and incubated overnight at 2–8 C. After washing with washing buffer, the wells were blocked with TBS containing 5% BSA and incubated for 1 h at room temperature (RT). Plasma samples were diluted from 1:10 to 1:5000 in TBS, transferred to the wells and incubated for 2 h at 37 °C. Bound FVIII antibodies were detected after washing with sheep monoclonal anti-human IgG1, IgG2, IgG3 or IgG4 antibody (CLB, The Netherlands) diluted 1:1000 in TBS by incubating for 1 h at 37 °C followed by rabbit anti-sheep IgG-biotin conjugate (diluted 1:1000), incubating for 1 h at RT. Streptavidin – AP conjugate was added to the wells at dilution (1:1000) and incubated for 30 min at RT. Between all incubation steps the wells were thoroughly washed with TBS-T. The reaction was visualized using pNPP solution (maximum absorbency was achieved in 30 min) and stopped with 1 M NaOH. Buffer and FVIII antibody free plasma from severe hemophilia A patients were used instead of investigated plasma sample as controls. Cut-off value was defined as a mean value of optical densities measured for controls plus 3 standard deviations. The ELISA titer was defined as the maximal dilution of sample yielding OD exceeding the cut-off value.

ELISA Assay for Estimation of FVIII Light Chain Antibody IgG Subtype Distribution

Immunoplates (PolySorp) were coated with recombinant FVIII light chain diluted 1:1000 in TBS and incubated overnight at 2–8 °C. Afterwards the assay was continued as described in the section above.

Immunoblotting Analysis

Human FVIII (Haemoctin SDH) or recombinant FVIII fragments were cleaved by thrombin (0.01 IU thrombin per 1 IU of FVIII) for 30 min at 37°C and fragments were separated by SDS-PAGE under reduced and unreduced conditions using 10–15% gradient gel.

Inhibition of FVIII Binding to Phospholipids Measured by an ELISA Assay

One hundred microliters of methanol dissolved L-(-phosphatidyl)-l-serine (Sigma) at a concentration of 5 μg/ml was added to each well of polystyrene microplates (PolySorp) and air-dried at room temperature (RT). The wells were blocked by adding TBS containing 5% BSA and incubated for 1 h at RT. After washing with washing buffer, a mixture containing an equal volume of rFVIII (Recombinate) diluted to 2 IU/ml in TBS-BSA (1% of BSA in TBS buffer) and a serially diluted (1:2 to 1:1000) test plasma sample was added to the wells of the microplate and incubated for 2 h at RT with continuous agitation. Bound FVIII was detected by adding mouse anti-human monoclonal antibody ESH5 (against FVIII heavy-chain, American Diagnostica, USA) diluted 1:500 in TBS. After washing goat anti-mouse IgG conjugated with AP (1:1000, Amersham Pharmacia Biotech) was added and incubated at RT for 1 h. The reaction was visualized by adding AP substrate pNPP. The control included in each test contained a mixture in which test plasma sample dilution was substituted with buffer.

Inhibition of FVIII Binding to von Willebrand Factor Measured by an ELISA Assay

Microplate wells (MaxiSorp, Dako) were coated with 100 μl of rabbit anti-human vWF antibody (1:4000, Dako, Denmark) in carbonate buffer and incubated overnight at 2–8°C. After washing, standard plasma (from a plasma pool of 22 plasmas from healthy persons) diluted to 1:20 in TBS-BSA was added and incubated for 1 h at RT. Factor VIII was dissociated from vWF by incubating with 0.4 M $CaCl_2$ for 30 min at RT. After washing, a mixture of a test plasma sample dilution with an equal volume of rFVIII (Recombinate) diluted to 2 IU/ml in TBS-BSA and incubated previously for 30 min at 37°C was added to the wells of the microplate and incubated for 2 h at RT. Bound FVIII was detected as described in the previous assay.

Competitive Enzyme-Linked Immunosorbent Assay

Microplates were coated with rFVIII (Recombinate) diluted to 8 IU/ml in carbonate buffer and incubated overnight. After washing with washing buffer, plates were blocked with 5% BSA in TBS for 1 h at RT. Then a mixture containing an equal volume of test plasma sample dilution (dilutions 1:10 and 1:100) with either of the murine FVIII monoclonal antibodies ESH5 or ESH4 diluted to 25 μg/ml was added

to the wells coated with FVIII and incubated for 2 h at RT with continuous agitation. The bound murine FVIII antibody was detected by using goat anti-mouse IgG diluted to 1:1000 (AP conjugate) and the reaction was visualized with AP substrate pNPP. The competition was calculated relative to the control where test plasma samples were replaced by buffer (no competition of binding to FVIII).

Statistical Analysis

Student *t*-test was used to compare the level of different IgG subtypes. Correlation was calculated according to Spearman rank order correlation coefficient. *P* value of 0.05 was considered significant.

Results

Characterization of FVIII Antibodies on Patient Before ITT

Immunoblotting analysis showed antibody reactivity against thrombin cleaved and non-cleaved FVIII light chain and A2 domain of FVIII. The neutralization activity of FVIII antibodies was reduced in the presence of FVIII-vWF complex (Fig. 4). The ratio of 50% neutralization of FVIII:C with rFVIII to FVIII:vWF complex was 4.5. The extent to which each epitope contributed to FVIII inhibition was determined by neutralization assay. Antibodies directed towards the heavy chain accounted for 30% of the FVIII inhibitory antibodies, while adding a recombinant FVIII light

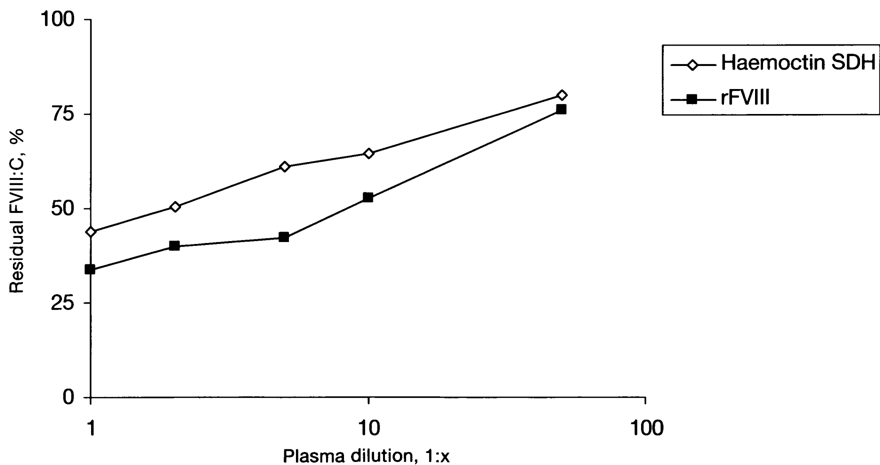


Fig. 4. Neutralization of FVIII:C activity by FVIII antibodies from the plasma sample of patient 1 taken before immune tolerance therapy. Test plasma dilutions were incubated with FVIII concentrate (rFVIII, Recombinate, Baxter) and FVIII concentrate containing vWF (FVIII-vWF, Haemoctin SDH, Biotest). The residual FVIII:C activity was measured by chromogenic method. Each data point represents the mean of two individual experiments

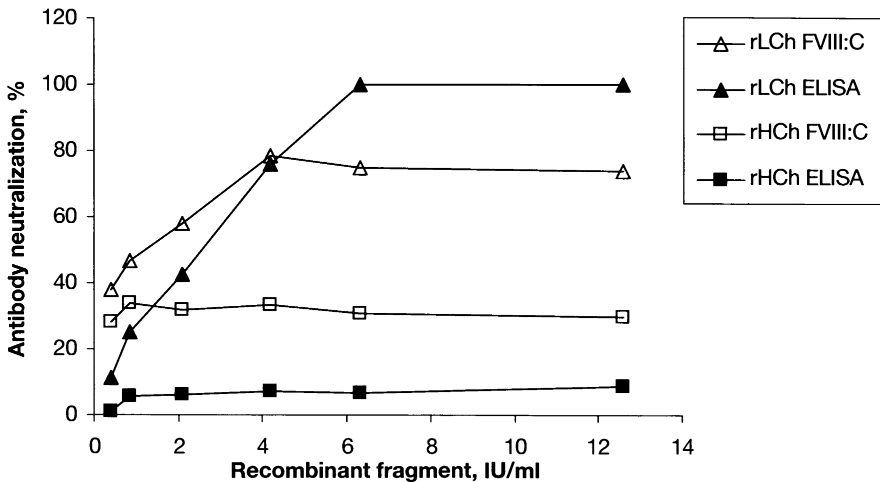


Fig. 5. Neutralization of FVIII antibodies from plasma sample from patient 1 taken before ITT. Plasma sample was diluted to 4 BU/ml and incubated with increasing concentration of recombinant FVIII light chain (rLCh) and heavy chain (rHCh). The neutralization of antibodies was measured by an ELISA assay (ELISA) and by a chromogenic method (FVIII:C) as described in Materials and Methods. Each data point represents the mean of two individual experiments

chain resulted in 78% of FVIII inhibitory antibody neutralization. In an ELISA assay 7% of antibodies were neutralized by recombinant heavy chain and 100% by recombinant light chain (Fig. 5). Antibodies inhibited FVIII interaction with phospholipids (50% of inhibition was achieved at the dilution 1:100) and FVIII interaction with vWF (50% of inhibition was achieved at the dilution 1:50) as measured by ELISA assays respectively [15]. FVIII antibodies of tested plasma samples competed with the murine monoclonal antibody ESH4 for the phospholipid binding site of the C-terminus of the light chain of FVIII as detected by ELISA.

Changes in Epitope Specificity of FVIII Antibodies During ITT

Epitope specificity of FVIII antibodies was measured in samples taken at different time points during IT therapy that contained an inhibitor titer of at least 2 BU/ml. During first continuous infusion (CI) treatment the rHCh neutralizing antibodies reached a non-detectable level measured by chromogenic method and ELISA assay (Fig. 2). However, Western blot analysis using the same recombinant fragments showed the presence of antibodies reacting with the light chain and heavy chain of FVIII in all investigated plasma samples during ITT. The epitope specificity of anti-FVIII light chain antibodies changed towards epitopes without functional activity. Factor VIII heavy chain antibodies were detected mostly by ELISA assay (6–21%) and the concentration of these antibodies reached an undetectable level at the end of the treatment. Theoretically, the total amount of FVIII light chain plus FVIII

heavy chain neutralizing antibodies should be 100%, but it was true for few plasma samples. Samples taken at the early stage of IT therapy contained in summary more than 100% of rHCh and rLCh neutralizing antibodies, while last plasma samples accounted less than 100%. Recombinant LCh or HCh concentration required for the first point of maximum neutralization was significantly different for these plasma samples. It can be explained by the presence of antibodies with different affinities to recombinant fragments. The lower slope of the neutralization curve of recombinant fragments explained this effect.

Changes in IgG Subtype Distribution During ITT

At the beginning of the ITT the patient had 29.8% of IgG4, 25.5% of IgG1, 25% of IgG2 and 19.6% of IgG3 subtype FVIII antibodies. Factor VIII light chain antibodies were present as follows: 38% of IgG4, 24% of IgG1, 21% of IgG3 and 17% of IgG2 subtypes. Results of IgG1–4 subtype distribution are shown on Fig. 2 D, E. During the first continuous infusion changes in FVIII antibody distribution were observed. Thereafter the distribution of IgG subtypes of FVIII:Ab remained unchanged concomitant with an increased level of IgG4 subtype antibodies. FVIII antibodies specific to FVIII light chain (FVIII-LCh:Ab) had a different IgG subtype distribution. During the ITT, factor VIII light chain antibodies of subtypes IgG1, IgG4, IgG3 were almost identically expressed and at a significantly higher level than IgG2 antibodies. At the beginning of treatment a fluctuation in FVIII-LCh:Ab subtype distribution was found to be analogous to that of FVIII:Ab. The amount of antibodies neutralized by recombinant FVIII light chain as measured by an ELISA assay correlated only with IgG2 subtype of FVIII:LCh:Ab (P=0.02). There were two peaks of IgG1 subtype levels. The first was observed after 1 month of treatment with the dose 100 IU/kg per day and another one after 1 month of the second continuous infusion. One of them was accompanied by an increase in the IgG1 subtype of FVIII light chain antibodies and the second one was associated with the appearance of antibodies against epitopes within the FVIII heavy chain.

FVIII Antibody Subtype Distribution in Hemophilia A Patients with Persistent FVIII Antibodies

In order to clarify whether the IgG subtype distribution of FVIII antibodies in hemophilia A patients with persistent FVIII antibodies differs from that detected during ITT, we investigated plasma samples of 12 hemophilia A patients. The characterization of patients is shown in Table 1. We used a sensitive ELISA assay for the detection of IgG subtypes directly from the plasma or serum sample. The estimated cut-off values of optical density for IgG1, IgG2, IgG3, and IgG4 FVIII antibodies were 0.113, 0.103, 0.089, and 0.089 respectively. The results are shown in Table 1. The most abundant were IgG4 type FVIII antibodies and the less abundant were IgG1 antibodies (P=0.02). IgG2 and IgG3 subtype FVIII antibodies were present in almost equal concentration, being significantly lower than IgG4 subtype

Table 1. Characterization of FVIII antibodies (*n.d.* not determined)

Plasma sample	Bethesda units	Distribution of FVIII antibodies				Distribution of FVIII light chain antibodies				Distribution of FVIII antibodies against recombinant light chain (rLCh) and heavy chain (rHCh)				Inversion in intron 22 of FVIII gene
		IgG1 %	IgG2 %	IgG3 %	IgG4 %	IgG1 %	IgG2 %	IgG3 %	IgG4 %	ELISA assay		Chromogenic method		
										rLCh %	rHCh %	rLCh %	rHCh %	
1	140	22	25.2	21	31.6	29.5	23.2	17	33.5	96	7	53	8	Type 1
2	9	25.5	25	19.6	29.8	24	17	21	38	100	7	78	30	Type 1
3	5	25	25	23	26	29.8	10.7	1.8	41.7	97	25	82	42	Rare inversion
4	7	30	14.5	21	33.9	74	7.4	0	18.5	92	20	93	3.5	n.d.
5	8	10.6	19	19	51	17	17	22	43.5	62	20	75	27	Type 1
6	37	32	17	21	32	12.5	12.5	25	50	93	58	82	21	Type 2
7	300	32.8	20.4	19.8	26.9	22.6	19.3	30.4	27.6	90	7	95	7	Type 1
8	280	16.2	34.2	17	32.4	3.4	13.7	31	51.7	95	6	96	6	n.d.
9	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	90	3	83	15	n.d.
10	1	27.8	12.8	11	27.8	27.4	39.6	17.8	14.8	n.d.	n.d.	21	21	Type 2
11	5	26	24.6	24.6	24.6	55	22	11	11	70	74	64	65	n.d.
12	150	30	7	28.9	33.3	24	20	28	28	18	94	26	74	n.d.
Mean		25.3	20.4	20.5	31.8	29.0	18.4	20.1	32.6					
SD		6.8	7.5	4.5	7.1	19.7	8.5	9.1	13.8					

concentration ($P<0.01$ and $P<0.01$ respectively). The amount of antibodies recognizing light chain of FVIII measured by chromogenic method (Table 1) correlated well with the concentration of IgG2 subtype FVIII:Ab ($P<0.01$) in all plasma samples. The results of the Bethesda assay correlated fairly well with the ELISA titers of all four IgG subtypes of FVIII:Ab.

In another ELISA test, recombinant FVIII light chain was used to coat ELISA microplates. The relative distribution of IgG subtype FVIII light chain antibodies (FVIII-LCh:Ab) was compared with the relative subtype distribution of antibodies against a whole FVIII molecule. The estimated cut-off values for IgG1, IgG2, IgG3 and IgG4 were 0.222, 0.217, 0.167, and 0.166 respectively. The results of FVIII-LCh:Ab IgG subtypes are shown in Table 1. The higher relative amount of FVIII-LCh antibodies belonged to IgG4 subtype. The level of FVIII light chain antibodies decreased in the order IgG4>IgG1>IgG3>IgG2 and was similar to subtype distribution of antibodies against a FVIII whole molecule. The amount of FVIII light chain antibodies in all the investigated samples measured by the ELISA assay had a good correlation with IgG2 ($P=0.01$). The results of the Bethesda assay correlated with the results of all IgG subtypes ($P<0.01$).

Changes in FVIII:Ab IgG Subtype Distribution During the Treatment with Different FVIII Concentrates

One low responder hemophilia A patient (patient 2, Fig. 3) was treated with plasma derived FVIII concentrates of different purity for a long time. The samples were drawn at different time points and the subtype distribution of FVIII:Ab was studied. Factor VIII-LCh:Ab was not measured because of the limited amount of plasma. At the beginning of the treatment, IgG1 subtype FVIII antibodies were predominant; during the treatment with Kryobulin TIM 3 the level of IgG1 decreased and was replaced mainly by IgG4. In July 1997, FVIII concentrate was changed to monoclonal affinity purified FVIII concentrate Octonativ-M. A significant increase in IgG4 subtype was accompanied by a decrease in IgG1 subtype FVIII:Ab, while the levels IgG2 and IgG3 subtype remained unchanged. In Sept 1998, treatment was continued with FVIII-vWF concentrate and after 7 months of treatment all subtypes except IgG1 were expressed at almost equal levels. In June and July 1999, FVIII concentrate Octonativ-M was again administered. This caused another profound increase in IgG4 subtype level. Then the patient was treated with Haemate for 5 months and thereafter with monoclonal antibody purified FVIII concentrate (Aafact) for 6 months. As a result of this treatment the levels of FVIII antibodies of IgG1 and IgG3 subtypes increased while the levels of subtypes IgG4 and IgG2 decreased significantly.

Discussion

We investigated the changes in epitope specificity and IgG subtype distribution of FVIII antibodies influenced mainly by the administration of high purity FVIII concentrates containing vWF for immune tolerance induction. In vitro experiments

showed lower neutralization of FVIII concentrate containing vWF by FVIII antibodies of the studied patient and predicted a beneficial outcome using the same concentrate for immune tolerance therapy. The ITT effectively suppressed the production of inhibitory antibodies reacting mainly with FVIII light chain to a non-detectable level. FVIII antibodies of IgG4 subtype dominated over all other subtypes, except IgG1 subtype at two different time points, where the higher level of IgG1 was probably caused by the increased dosage of administered FVIII.

The patient was tolerated using FVIII-vWF concentrate. However, FVIII antibodies recognizing the FVIII light chain were expressed during the whole treatment, but the subtype distribution of FVIII light chain antibodies differed from that of patients with persistent inhibitor antibodies (Table 1). All IgG subtypes, except IgG2 subtype of FVIII light chain antibodies were expressed at equal levels during ITT. This could be due to the fact that the isolated FVIII light chain used in neutralization experiments and ELISA assays possesses a higher number of accessible epitopes on the surface than the form of heterodimer with FVIII heavy chain, as it circulates in plasma. The fact that the ELISA titer of antibodies bound to the FVIII light chain was higher than that bound to the whole FVIII molecule in some samples, suggests that FVIII light chain is more immunogenic than the whole FVIII molecule. Therefore after antigen processing there could be a higher number of small fragments of FVIII light chain presented by antigen presenting cells to T-lymphocytes. It has been shown that the size of epitope fragments capable of binding to the FVIII antibody is rather large (16–66 amino acids) and does not depend on whether the epitopes are linear, sequential or completely denaturated [16–18]. The antibody binding to denaturated plasma-derived FVIII and isolated recombinant FVIII light chain and heavy chain was observed in the investigated patient. The level of FVIII antibody subtypes, and the concentration and affinity of antibodies at different time points indicated the polyclonal antibody response to the treatment.

The results of our study are in agreement with previous reports, where on-demand treatment of hemophilia A patients is associated with the development of FVIII antibodies mainly of the IgG1 or IgG4 subtypes [19, 20]. The level of IgG1 subtype antibodies rises quickly after antigen administration (gene locates in 5'-end), while production of IgG2 and IgG4 subtypes probably needs more support of cytokines, produced by Th2 cells, because the genes encoding these subtypes are located close to 3'-end [12, 13]. FVIII antibodies with inhibitory and non-inhibitory antibodies were of the IgG4 subtype predominantly. During immune tolerance therapy with FVIII-vWF concentrate, the level of FVIII antibodies of IgG1 subtype increased significantly after 1 month of treatment and this rise was accompanied by the peaking of IgG1 subtype antibodies against the FVIII light chain. After the second continuous infusion the sudden rise in IgG1 subtype FVIII antibodies was observed, but the distribution of FVIII light chain antibodies remained unchanged. Surprisingly, antibodies neutralizing the FVIII heavy chain were detected in the same plasma sample. The enhanced level of the IgG1 subtype was observed as a result of the higher dosage of FVIII concentrate and could indicate the novel clone producing FVIII antibodies with different epitope specificity. One report [21] about unsuccessful ITT described an increased level of anti-A2 domain antibodies among heterogeneous antibodies in the patient and the authors suggested using the

epitope mapping as a prediction of the outcome of ITT. We found an increase in the FVIII heavy chain antibody level (IgG1 subtype), but continued ITT suppressed the production of these antibodies to undetectable level. Tolerance induction basis on the clonal selection. Therefore it is important that all FVIII antibody-producing clones have to become tolerant to FVIII administered at sufficiently high doses for a long time.

The effect of different FVIII concentrates used in replacement therapy on hemophilia A patients' immune system has been studied extensively. Results of in vitro studies showed decreased levels of IL-2, TNF, INF-γ and increased levels of IL-4, IL-10 in each different leukocyte subset after stimulation with FVIII concentrate of intermediate purity [11]. The immuno-suppressive effect of these concentrates was explained by the presence of TGF-β [11], which inhibits the proliferation of T-cells, maturation of cytolytic T-cells and the activation of macrophages. In vivo investigations in HIV-negative hemophiliacs have shown increased numbers of CD8+ cells and decreased numbers of CD4+ cells, indicating the immuno-stimulatory effect of treatment caused probably by the presence of TGF-β, which produces some Th2-like effects, like a co-stimulatory effect on CD8+ T-cells [22]. The FVIII concentrates of intermediate purity in general are more prone to decreasing the CD4+ cell count than immuno-affinity purified concentrates used for treatment of HIV-positive hemophilia A patients [23–25]. HIV-negative hemophiliacs who developed FVIII antibodies have been investigated so far only in one multicenter study [26]. This study showed no difference between FVIII-induced IFN-γ and IL-10 production by lymphocytes in hemophilia A patients who developed FVIII antibodies and those who did not.

We studied changes in FVIII antibody subtype distribution in a low responder hemophilia A patient treated with FVIII concentrates with different purities (patient 2). An ELISA assay used for estimating IgG subtypes was sensitive to detecting very low titers of FVIII antibodies. Analogously to patient 1, the higher production of IgG4 subtype FVIII antibodies was more or less pronounced in patient 2 at different time points. Long term treatment with FVIII-vWF concentrates in patient 2 resulted in a decrease of FVIII antibody titer measured in Bethesda units. The change to affinity purified FVIII concentrate Octonativ-M caused an increase in inhibitory antibodies, but isotype distribution of FVIII antibodies remained unchanged. An increase in the production of IgG1 and IgG3 subtype FVIII antibodies was observed during treatment with another immuno-affinity purified concentrate, Aafact. Unfortunately, plasma samples were taken occasionally and therefore it is not absolutely clear whether the observed changes in IgG subtype distribution were caused only by the switch to another concentrate. Apart from the purity of FVIII concentrate used for treatment, many other reasons like the dose and interval between doses could be responsible for different antibody response.

The usage of vWF in FVIII concentrates for ITT suppressed the production of FVIII light chain antibodies with inhibitory activity to non-detectable levels, while antibodies with non-inhibitory activity persisted. The beneficial outcome of using FVIII-vWF concentrates was observed also in patient 2, who had higher in vivo recovery with FVIII-vWF concentrate than with immuno-affinity purified concentrates. Probably after injection of FVIII concentrate of very high purity, both vWF and FVIII antibodies compete for binding sites on FVIII. If the antibodies possess

higher affinity than vWF, the antibody-antigen complex is formed and the FVIII molecule is removed from the circulation. It has been reported, that antibodies reacting with epitopes within the acidic region of the A3 domain (a3 region), C1 [27] and C2 domains [28], inhibit the binding of FVIII to vWF and are able to compete with vWF for binding site. As the intact light chain of FVIII has the maximum vWF binding affinity [29], we used the recombinant full-length FVIII light chain in the neutralization experiment to quantify all antibodies interfering with FVIII-vWF interaction. The vWF in the administered FVIII concentrates covers FVIII antibody binding sites on FVIII light chain and therefore delays the FVIII neutralization. Still half of the FVIII molecules dissociate from the vWF every 10 min [30]. Released FVIII is exposed to FVIII antibodies for forming FVIII-antibody complex, which is more stable than FVIII-vWF complex.

FVIII antibody response to the treatment is associated with the characteristics of the patient. The FVIII gene defect of the patient determines whether a non-functional FVIII molecule is produced or production of any protein is prevented. Hemophilia A patients with mild disease caused by point mutation in the FVIII gene may develop antibodies with higher neutralization activity against administered FVIII than against endogenous FVIII [31]. Investigated patients 1 and 2 had inversion in intron 22 in FVIII gene, which is the most common gene defect among hemophilia A patients and accompanies a higher incidence of FVIII antibody development dominantly against the FVIII light chain and the A2 domain of heavy chain [1, 32].

In addition to a gene defect causing FVIII deficiency, the antibody response to the treatment may be determined by the immune system of the particular patient. HLA genotype of MHC II alleles was characterized only in patient 2. DR8, DQ7 and DQ4 genotypes, as found in our patient, have been reported by Hay and Oldenburg, who detected these genotypes in some patients with inhibitors [33, 34]. However, no correlation between HLA allele genotypes and inhibitor incidence could be established in either of these reports.

So far no efficient treatment option has been found to prevent the development of FVIII antibodies and to achieve successful immune tolerance. However, some reports are more promising in that respect, Rossi et al. have used the blocking of CD40-CD40L interaction in mice [35]. Abolishing another important interaction, CD28-B7, caused less development of inhibitory antibodies, but unfortunately immune tolerance was not gained [36].

Thus it seems that multiple factors are involved in the development and production of FVIII antibodies in hemophilia A patients. Which factor is more relevant depends on the particular patient and further studies are needed to clarify their relative contribution to the success rate of immune tolerance therapy. Our study focused on the presence of vWF in FVIII concentrate used for ITT and we conclude that a beneficial outcome can be achieved using FVIII concentrate containing vWF in hemophilia A patients with antibodies reacting mainly with the FVIII light chain.

Acknowledgements. We gratefully acknowledge Dr. Margus Pooga for a helpful discussion. We thank Prof. Dr. F. H. Herrmann and Dr. R. Ljung for perfoming DNA analyses of haemophilia A patients. The study was in part supported by a Nordic coagulation grant.

References

1. R. Schwaab, H. H. Brackmann, C. Meyer, J. Seehafer, M. Kirchgesser, A. Haack, K. Olek, E. G. Tuddenham and J. Oldenburg. Haemophilia A: mutation type determines risk of inhibitor formation. Thromb Haemost 1995; 74: 1402-6.
2. C. R. Hay, C. A. Ludlam, B. T. Colvin, F. G. Hill, F. E. Preston, N. Wasseem, R. Bagnall, I. R. Peake, E. Berntorp, E. P. Mauser-Bunschoten, K. Fijnvandraat, C. K. Kasper, G. White and E. Santagostino. Factor VIII inhibitors in mild and moderate-severity haemophilia A. UK Haemophilia Centre Directors Organisation. Thromb Haemost 1998; 79: 762–6
3. P. J. Lenting, J. A. van Mourik and K. Mertens. The life cycle of coagulation factor VIII in view of its structure and function. Blood 1998; 92: 3983–96
4. D. H. Scandella. Properties of anti-factor VIII inhibitor antibodies in hemophilia A patients. Semin Thromb Hemost 2000; 26: 137–42
5. T. Suzuki, M. Arai, K. Amano, K. Kagawa and K. Fukutake. Factor VIII inhibitor antibodies with C2 domain specificity are less inhibitory to factor VIII complexed with von Willebrand factor. Thromb Haemost 1996; 76: 749–54
6. T. Talpsep, A. Kallas. Von Willebrand factor in factor VIII concentrates protects factor VIII neutralisation by Factor VIII antibodies of haemophilia A patients. Haemophilia 2001; 7: 375–380
7. D. M. D. Michele. Immune tolerance: a synopsis of the international experience. Haemophilia 1998; 4: 568–573
8. E. P. Mauser-Bunschoten, H. K. Nieuwenhuis, G. Roosendaal and H. M. van den Berg. Low-dose immune tolerance induction in hemophilia A patients with inhibitors. Blood 1995; 86: 983–8
9. E. F. Van Leeuwen, E. P. Mauser-Bunschoten, P. J. Van Dijken, A. J. Kok, E. J. Sjamsoedin-Visser and J. J. Sixma. Disappearance of factor VIII:C antibodies in patients with haemophilia A upon frequent administration of factor VIII in intermediate or low dose. Br J Haematol 1986; 64: 291–7
10. W. Kreuz, E. Ehrenforth, M. Funk, G. Auerswald, D. Mentzer, J. Joseph-Steiner, T. Beeg, D. Klarmann, I. Scharrer and B. Kornhuber. Immune tolerance therapy on paediatric hemophiliacs with factor VIII inhibitors: 14 years follow-up. Haemophilia 1995; 1: 24–32
11. G. Hodge, R. Flower and P. Han. Effect of factor VIII concentrate on leucocyte cytokine production: characterization of TGF-beta as an immunomodulatory component in plasma- derived factor VIII concentrate. Br J Haematol 1999; 106: 784–91
12. M. Mayumi, T. Kuritani, H. Kubagawa and M. D. Cooper. IgG subclass expression by human B lymphocytes and plasma cells: B lymphocytes precommitted to IgG subclass can be preferentially induced by polyclonal mitogens with T cell help. J Immunol 1983; 130: 671–7.
13. P. K. Mongini, W. E. Paul and E. S. Metcalf. T cell regulation of immunoglobulin class expression in the antibody response to trinitrophenyl-ficoll. Evidence for T cell enhancement of the immunoglobulin class switch. J Exp Med 1982; 155: 884–902.
14. M. Shima, H. Nakai, D. Scandella, I. Tanaka, Y. Sawamoto, S. Kamisue, S. Morichika, T. Murakami and A. Yoshioka. Common inhibitory effects of human anti-C2 domain inhibitor alloantibodies on factor VIII binding to von Willebrand factor. Br J Haematol 1995; 91: 714–21.
15. A. Kallas, T. Talpsep and H. Everaus. Von Willebrand factor protects factor VIII against neutralisation by factor VIII antibodies of haemophilia a patients. Haemophilia 2000; 6: 305
16. I. Kuwabara, H. Maruyama, S. Kamisue, M. Shima, A. Yoshioka and I. N. Maruyama. Mapping of the minimal domain encoding a conformational epitope by lambda phage surface display: factor VIII inhibitor antibodies from haemophilia A patients. J Immunol Methods 1999; 224: 89–99.
17. D. S. Palmer, A. K. Dudani, J. Drouin and P. R. Ganz. Identification of novel factor VIII inhibitor epitopes using synthetic peptide arrays. Vox Sang 1997; 72: 148–61
18. M. L. Liu, B. W. Shen, S. Nakaya, K. P. Pratt, K. Fujikawa, E. W. Davie, B. L. Stoddard and A. R. Thompson. Hemophilic factor VIII C1- and C2-domain missense mutations and their modeling to the 1.5-angstrom human C2-domain crystal structure. Blood 2000; 96: 979–87

19. M. Algiman, G. Dietrich, U. E. Nydegger, D. Boieldieu, Y. Sultan and M. D. Kazatchkine. Natural antibodies to factor VIII (anti-hemophilic factor) in healthy individuals. Proc Natl Acad Sci U S A 1992; 89: 3795–9.
20. C. A. Fulcher, S. de Graaf Mahoney and T. S. Zimmerman. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. Blood 1987; 69: 1475–80.
21. H. M. v. d. Berg, J. Voorberg, E.-A. M. Turenhout, G. Roosendaal and E. P. Mauser-Bunschoten. Switch of epitope specificity during immune tolerance in a patient with severe haemophilia. Haemophilia 2000; 6: 308
22. G. Hodge, J. Lloyd, S. Hodge, C. Story and P. Han. Functional lymphocyte immunophenotypes observed in thalassaemia and haemophilia patients receiving current blood product preparations. British Journal of Haematology 1999; 105: 817–825
23. R. de Biasi, A. Rocino, E. Miraglia, L. Mastrullo and A. A. Quirino. The impact of a very high purity factor VIII concentrate on the immune system of human immunodeficiency virus-infected hemophiliacs: a randomized, prospective, two-year comparison with an intermediate purity concentrate. Blood 1991; 78: 1919–22.
24. S. V. Seremetis, L. M. Aledort, G. E. Bergman, R. Bona, G. Bray, D. Brettler, M. E. Eyster, C. Kessler, T. S. Lau, J. Lusher and et al. Three-year randomised study of high-purity or intermediate-purity factor VIII concentrates in symptom-free HIV-seropositive haemophiliacs: effects on immune status. Lancet 1993; 342: 700–3.
25. D. Varon. Prospective clinical trial of high-purity factor VIII preparations in haemophiliacs. Blood Coagul Fibrinolysis 1995; 6 Suppl 2: S82–3
26. G. C. Kaplan J, Secord E. Potential for preventation of inhibitor formation by immune tolerance. Seminars in Thrombosis and Hemostasis 2000; 26: 173–178
27. M. Jacquemin, A. Benhida, K. Peerlinck, B. Desqueper, L. Vander Elst, R. Lavend'homme, R. d'Oiron, R. Schwaab, M. Bakkus, K. Thielemans, J. G. Gilles, J. Vermylen and J. M. Saint-Remy. A human antibody directed to the factor VIII C1 domain inhibits factor VIII cofactor activity and binding to von Willebrand factor. Blood 2000; 95: 156–63
28. E. L. Saenko and D. Scandella. A mechanism for inhibition of factor VIII binding to phospholipid by von Willebrand factor. J Biol Chem 1995; 270: 13826–33
29. E. L. Saenko, K. Loster, D. Josic and A. G. Sarafanov. Effect of von Willebrand Factor and its proteolytic fragments on kinetics of interaction between the light and heavy chains of human factor VIII. Thromb Res 1999; 96: 343–54
30. A. J. Vlot, S. J. Koppelman, H. M. van den Berg, B. N. Bouma and J. J. Sixma. The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. Blood 1995; 85: 3150–7
31. K. Peerlinck, M. G. Jacquemin, J. Arnout, M. F. Hoylaerts, J. G. Gilles, R. Lavend'homme, K. M. Johnson, K. Freson, D. Scandella, J. M. Saint-Remy and J. Vermylen. Antifactor VIII antibody inhibiting allogeneic but not autologous factor VIII in patients with mild hemophilia A. Blood 1999; 93: 2267–73.
32. D. Scandella. Epitope specificity and inactivation mechanisms of factor VIII inhibitor antibodies. Vox Sang 1999; 77: 17–20
33. C. R. Hay, W. Ollier, L. Pepper, A. Cumming, S. Keeney, A. C. Goodeve, B. T. Colvin, F. G. Hill, F. E. Preston and I. R. Peake. HLA class II profile: a weak determinant of factor VIII inhibitor development in severe haemophilia A. UKHCDO Inhibitor Working Party. Thromb Haemost 1997; 77: 234–7.
34. J. Oldenburg, J. K. Picard, R. Schwaab, H. H. Brackmann, E. G. Tuddenham and E. Simpson. HLA genotype of patients with severe haemophilia A due to intron 22 inversion with and without inhibitors of factor VIII. Thromb Haemost 1997; 77: 238–42.
35. G. Rossi, J. Sarkar and D. Scandella. Long-term induction of immune tolerance after blockade of CD40-CD40L interaction in a mouse model of hemophilia A. Blood 2001; 97: 2750–7.
36. J. Qian, M. Collins, A. H. Sharpe and L. W. Hoyer. Prevention and treatment of factor VIII inhibitors in murine hemophilia A. Blood 2000; 95: 1324–9.

Clinical Experience with the Modified Bonn-Malmö Protocol since 1996

L. Hess, C. Unkrig, H. Zeitler, W. Nettekoven, W. Effenberger, P. Hanfland, H. Vetter and H.-H. Brackmann

Acquired hemophilia due to factor VIII inhibitor is a life-threatening disease requiring cost-intensive treatment. It requires a fast and effective lifesaving treatment as well as inhibitor eradication therapy leading to a cure of this disease (2, 5, 6, 7, 11, 12, 13). In April 1996 we combined elements from the Malmö and Bonn protocols, which were mainly developed for the eradication of inhibitors in patients with congenital hemophilia, to create a new treatment protocol for acute and/or chronic acquired inhibitors (1, 3, 14, 15, 16). This protocol is known as the modified Bonn-Malmö protocol (MBM protocol).

Methods

I. Long-term immunoadsorption from day 1 to day 5 (daily adsorption target: 2.5-fold plasma volume)
II. Administration of factor VIII (100 U/kg body weight; in exceptional cases up to 200 U/kg bw) every 6 h. Optional dose reduction on the basis of clinical signs and level of recovery achieved (50–80% factor VIII residual activity after 4–6 h) throughout the treatment cycle
III. Administration of immunoglobulins (i.v. IgG 0.3 g/kg bw/day) on day 5 and day 6 (7)
IV. Administration of cyclophosphamide (2 mg/kg bw/day) in combination with prednisolone (1 mg/kg bw/day) from day 1 to day 7

The treatment cycle (from day 1 to day 7) may be repeated several times depending on coagulation data and clinical response.

Immunoadsorption was performed exclusively using columns with sepharose-bound polyclonal sheep antibodies directed against human immunoglobulins (Ig-Therasorb, PlasmaSelect AG, Teterow Deutschland, Germany).

Patients

Treatment according to the MBM protocol was performed in 20 patients with high antibody titers. In most cases antibodies were directed against factor VIII and in one case against factor V. The mean patient age was 65 (median 69, maximum 89,

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Patients treated according to MBM protocol

Patient (n=20)	Age	Maximum inhibitor titer	Indication for MBM protocol
W.H.	68	59	Extremities, trunk, iliopsoas muscle bleed
S.E.	68	327	Multiple bleeding into extremities
M.G.	70	128	Multiple bleeding into extremities
T.S.	30	16	Postpartum uterine bleeding, emergency hysterectomy
M.E.	53	110	Head and lower leg hematoma, fasciotomy
H.W.	66	76 (FV)	Retroperitoneal bleeding, hematuria
B.H.	72	73	Bleeding in face and lower extremities
M.T.	53	505	Bleeding in face, trunk, extremities, compartment syndrome
R.A.	62	33	Knee joint bleeding, soft tissue, extremities, hematuria
H.W.	34	298	Postpartum bleeding into extremities, OP: hematoma evacuation
P.K.	79	67	Retroperitoneal and intraabdominal bleeding, compartment syndrome
H.G.	35	70	Postpartum retroperitoneal bleeding
M.G.	76	33	Intrathoracic bleeding after jugular vein puncture
S.H.	59	665	Retroperitoneal bleeding, bleeding into extremities
K.F.	60	32	Bleeding into extremities, trunk, retroperitoneal region
G.F.	74	22	Knee joint bleeding and extremities
M.U.	87	8400	OP: pleurodesis because of recurrent malignant pleural effusion
W.S.	89	49	Multiple bleedings into extremities
H.W.	81	15	Bleeding into extremities and trunk
H.E.	76	135	Urogenital bleeding, intrathoracic bleeding after jugular vein puncture

minimum 30). All 9 men and 11 women showed life-threatening bleeding, specifically soft tissue bleeding episodes in the extremities and trunk which led to the development of compartment syndromes or respiratory disorders (see Table 1). The mean inhibitor titer was 556 Bethesda units (maximum 8400, minimum 15, median 68).

Confirmation of inhibitors and exclusion of other coagulation disorders, resp. was done by individual factor assay, plasma exchange testing, lupus-aPTT, DVV testing, modified Bethesda and Nijmwegen antibody assay and factor VIII assay in a one-stage test system with naturally immunodepleted plasma (Immuno), and by chromogenic assay (Baxter).

Therapeutic decisions during treatment depended in particular on plasma factor VIII activities in one-stage testing with natural immunodepleted plasma.

We diagnosed inhibitor-associated diseases in two patients with malignant disease (cancer of the prostate, lung cancer), in three patients with collagen disease, in one patient with Hashimoto's autoimmune thyroiditis, and in three patients with postpartum complications.

Results

One to two apheresis sessions were sufficient to control the bleeding process in all 20 patients. Clinical cessation of bleeding was accomplished and there was no need for blood transfusions.

Four of the 20 patients were unable to complete the MBM protocol due to secondary disease. Two of these patients presented cardiopulmonary instability. A stroke occurred in one patient with serious pre-existing occluded artery disease. One female patient declined to continue with the ongoing treatment protocol when she was diagnosed as having a tumour. Sixteen patients were able to complete treatment as scheduled according to the MBM protocol.

Inhibitor was below the limit of detection in these patients (n=16) after a mean of 5.3 days of apheresis treatment (median 5 days, maximum 12, minimum 1). The application of coagulation factor concentrates was no longer necessary after a mean of 16 days (median 15.5, maximum 35, minimum 8). Twelve patients required continued treatment with immunoapheresis for stabilization of factor VIII activity, giving a mean total treatment duration of 17.5 apheresis days (median 16.5, maximum 36, minimum 10) until long-lasting inhibitor elimination was accomplished (see Fig. 1).

The treatment course of B.H., a female patient with a high antibody titer of 73 Bethesda units, is shown in Fig. 2 as an example. The columns show the total quantity of factor VIII administered over a 24 h period. After 12 days of treatment, patient compliance declined due to an epileptic seizure in this patient with a known history of epilepsy. Immunoapheresis treatment was only performed every second day for a period of time. Despite this setback, we achieved long-lasting antibody elimination after 18 immunoadsorption sessions.

The mean factor VIII consumption in patients on the MBM protocol (*n*=16) was 560,000 I.U. (median 384,000 I.U., minimum 181,000 I.U., maximum 2 million I.U.). We exclusively used plasma derived coagulation factor concentrates in 13 patients.

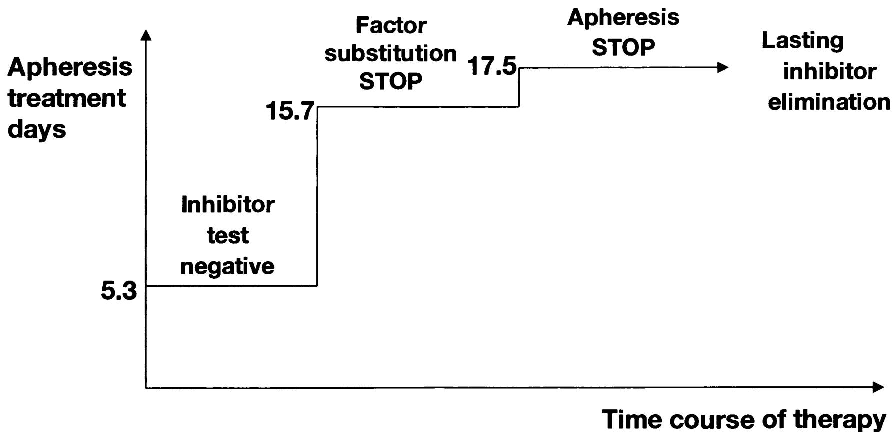


Fig. 1. Therapy by MBM protocol. Average number of apheresis days (n=16)

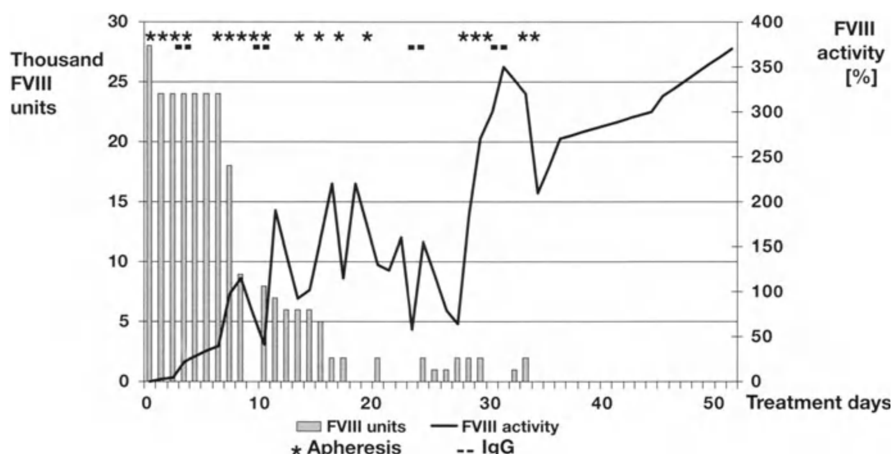


Fig. 2. MBM protocol – case report patient B.H., aged 72

Two patients received recombinant factor VIII. It is not possible at present to evaluate the influence of the type of factor VIII product on treatment duration and consumption of concentrates. One patient with factor V inhibitor received recombinant factor VIIa exclusively.

The mean follow-up period in the 16 patients is currently 29.5 months (median 28, maximum 55, minimum 0.5 months).

Only one patient showed two times reduced factor VIII activities to 10%, and 50%, resp., during follow-up. Immediate further apheresis treatment without factor VIII substitution was performed for a mean of 6 days. This patient's factor VIII levels have now been stable for more than 2 years.

Five patients transferred from other hospitals required pre-treatment with recombinant factor VIIa in order to cover the transfer time.

These patients continued to receive factor VIIa for a median of 3 apheresis treatment days (mean 3.3, maximum 5).

Discussion

The MBM protocol combines features of the Malmö and Bonn protocols providing a rapidly effective and efficient therapeutic modality for the treatment of patients with an acquired inhibitor (7–10, 17).

Within two apheresis treatment days, patients are clinically stabilized regarding their bleeding risk, and they no longer needed factor VIII substitution after a mean of 16 apheresis treatments. The inhibitor elimination achieved can assumed to be permanent, based on the long follow-up period of 29.5 months (7–10, 17).

The rapid and final inhibitor elimination accomplished was, however, associated with a median factor VIII consumption of 384,000 U.

Our experience suggests that continuation of apheresis treatment until achievement of supernormal factor VIII levels in a one-stage test system with natural immunodepleted plasma is a decisive element of treatment, as the cessation of factor substitution causes a decline in factor VIII plasma activities that threatens the outcome of treatment and patient's response.

Furthermore, we consider it empirically proven that the factor VIII quantities administered in the initial phase have an upfront hemostatic and immunological effect. This is reflected in rapid cessation of bleeding and in the negative inhibitor tests after 5 days of treatment.

Optimum cost effectiveness is achieved in our experience by rapid diagnosis and immediate initiation of treatment on the basis of the MBM protocol (7).

In view of the high efficacy of the treatment protocol, our primary goal is now to have these results confirmed in other centres. Validation of the individual elements of therapy (apheresis, factor VIII substitution, application of IVIG and immunosuppressive drugs) in terms of their therapeutic effect should be undertaken in a second step given the low incidence of acquired inhibitor disease ($0.2–1.0 \times 10^{-6}$) (4, 5).

References

1. Brackmann HH (1984) Induced immunotolerance in factor VIII inhibitor patients. Prog Clin Biol 150: 181–185
2. Brackmann HH, Formsen J (1977) Massive factor VIII infusion in hemophilic patients with factor VIII inhibitor. Lancet II 933
3. Brackmann HH, Oldenburg J, Schwaab R (1996) Immune tolerance for the treatment of factor VIII inhibitors – Twenty years 'Bonn Protocol' Vox Sang 70: 30–35
4. Cohen AJ, Kessler CM. Acquired Inhibitors. Baillières Clin. Haematol. 1996; 9: 331–354
5. Green D, Lechner K (1981) A survey of 215 non-hemophilic patients with inhibitors to factor VIII. Thromb Haemost 45: 200–203
6. Kessler, CM (1991) Acquired factor VIII inhibitors in the nonhemophiliac: Historical perspectives, current therapies, and future approaches. Am J Med 91 (5 A): 1S-48 S
7. Hess L, Unkrig C, Zeitler H, Effenberger W, Nettekoven W, Stier S, Hanfland P, Vetter H, Brackmann HH (1998) Modifiziertes Bonn- und Malmö-Protokoll: Behandlung erworbener Hemmkörper bei Nichthämophilen. 29. Hämophilie-Symposium Hamburg 1998, 44–49
8. Hess L, Zeitler H, Unkrig Ch, Nettekoven W, Effenberger W, Hanfland P, Vetter H, Brackmann H-H (1999) Modified Bonn-Malmö Protocol. 30th Hemophilia Symposium Hamburg 1999: 119–125
9. Huth-Kühne A, Zimmermann R (1999) New Therapeutic Options In Acquired Hemophilia. Abstract, Vortrag 43. Jahrestagung GTH Mannheim
10. Huth-Kühne A, Lages P, Zimmermann R (1999) A New Therapeutic Option for Inhibitor Elimination in Patients with Acquired Hemophilia. 30th Hemophilia Symposium Hamburg 1999: 45–52
11. Lottenberg R, Kentro TB, Kitchins CS (1987) Acquired haemophilia: A natural history study of 16 patients with factor VIII inhibitor receiving little or no therapy. Arch Intern Med 147: 1077–1081
12. Ludlam CA, Morrison AE, Kessler C (1994) Treatment of Acquired Hemophilia. Seminars in Hematology 31 2(4): 16–19
13. Morrison AE (1995) Acquired haemophilia and its management. British Journal of Haematology 89: 231–236

14. Nilsson IM, Berntorp E, Zettervall O (1988) Induction of immune tolerance in patients with hemophilia and antibodies to factor VIII by combined treatment with intravenous IgG, Cyclophosphamide, and Factor VIII. N Eng J Med 318: 947–50
15. Nilsson IM, Freiburghaus C (1993) Treatment of patients with factor VII and IX inhibitors. Thromb Haemost 79: 56–9
16. Nilsson IM, Freiburghaus C (1995) Apheresis Inhibitors to Coagulation Factors Inhibitors to Coagulation Factors, Plenum Press, New York
17. Zeitler H, Unkrig C, Brackmann HH, Effenberger W, Hanfland P, Ko Y, Vetter H (1997) Immunomodulatory treatment of acquired hemophilia A with long-term-IgG-immunoadsorption, immunosuppression, and antigen substitution – A modified Bonn protocol inducing immunotolerance. ASH Meeting 39th (Abstract)

Course of Inhibitors in mild Hemophilia A with and without Immune Tolerance Treatment

H. Lenk and F. Kertzscher

Introduction

Mild hemophilia is complicating daily life much less than the severe kind of the disease. The appearance of an inhibitor for such patients therefore is even more intricate than in severe hemophilia because up to this time bleeding problems were very rare.

The patients mostly are already of school age or even adults. The joints are in good shape, because until then joint bleedings were not at all common for them. With the development of an inhibitor the mild hemophilia turns into the severe form. The circumstances of life are changing very radically and muscular as well as joint bleedings may induce chronic joint impairments if the inhibitor persists for a long time.

Frequency of Inhibitors in mild Hemophilia A

In severe hemophilia A 20–50% of the patients develop antibodies to FVIII (3,4). In mild hemophilia this complication is believed to be very rare. Causes for this discrepancy may be different and influenced by more than one fact:

- Diagnosis in most cases is achieved later than in the severe form
- Most children with mild hemophilia very rarely need substitutions of FVIII
- Very often – at least during childhood – they do not reach 20 or even 100 substitution days – critical numbers for inhibitor development in severe hemophilia A
- Most cases of mild hemophilia A are caused by missense mutations and in these mutations inhibitors have an incidence of 5% or less at a rough estimate (1,4)

Nevertheless there is an increasing number of reports about inhibitors in mild and moderate hemophilia A (5,6,7). A third of new inhibitors reported in a survey in the United Kingdom were detected in those patients (2).

Causes for the increase may be:

- A higher frequency of substitutions
- A higher dosage of FVIII
- Characteristics of new FVIII preparations
- New kinds of application, especially continuous infusion
- Frequent changes of the product

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

With the appearance of an inhibitor the FVIII of the patients is reduced mostly to values below 1%. Now bleedings become much more frequent than before and the bleeds are difficult to treat. The question to be answered is, what are the long term treatment options for these patients?

Case Presentation

Case 1

The FVIII level of a boy from a family with known mild hemophilia was 14%. In this family a missense mutation in exon 25 of the factor VIII gene was found to be the cause of hemophilia. After birth, suffering from melena, he was treated once with FVIII concentrate (Haemate). Until the age of 8 years, when he had to undergo adenotomia, he had no further substitution therapy. Then Haemate was infused continuously and as a bolus as well. Four weeks later, the patient presented with a soft tissue bleeding. Substitution had no measurable effect. Using the Bethesda method, an inhibitor of 19,5 BU was detected (Fig. 1). After rFVIIa (NovoSeven) protected implantation of a central venous line (Broviac catheter) ITT was started with 150 U FVIII (Haemate) twice daily. Hemolysis was the reason for changing therapy to a hemolysin-free concentrate (Beriate) but this was answered by an increase in the inhibitor. Therefore we returned to Haemate. The inhibitor ran low and was not detectable after a period of 8 months (Fig. 2). Now the therapy was reduced stepwise. Infections of the Broviac catheter after 1 year of treatment caused a change to a port system and later on also to the removal of the port. At present FVIII recovery is 1.4–1.9% FVIII/U FVIII and kg body weight and level of FVIII 96 h after last substitution is 5–10%.

Case 2

The 19-year-old patient with mild hemophilia A had a level of FVIII of about 10% (5–18%), caused by a missense mutation of the FVIII gene in exon 15. He had FVIII on eight occasions in childhood with altogether 54 exposition days. In 1999, after a period of more than 8 years without any FVIII therapy, he had a large soft tissue bleeding after trauma and was treated intensively with FVIII for another 27 days, 15 days by continuous infusion. At the end of this period no inhibitor was detectable. Four weeks later the patient had a soft tissue bleeding again, but without adequate trauma. An antibody of 7 BU was diagnosed (Fig. 3) and the hematoma disappeared only after treatment with Feiba.

In the next 3 months the inhibitor diminished continuously to 0.7 BU. After 6 months a spontaneous FVIII value of 8% was measured and the bleeding tendency was very low again.

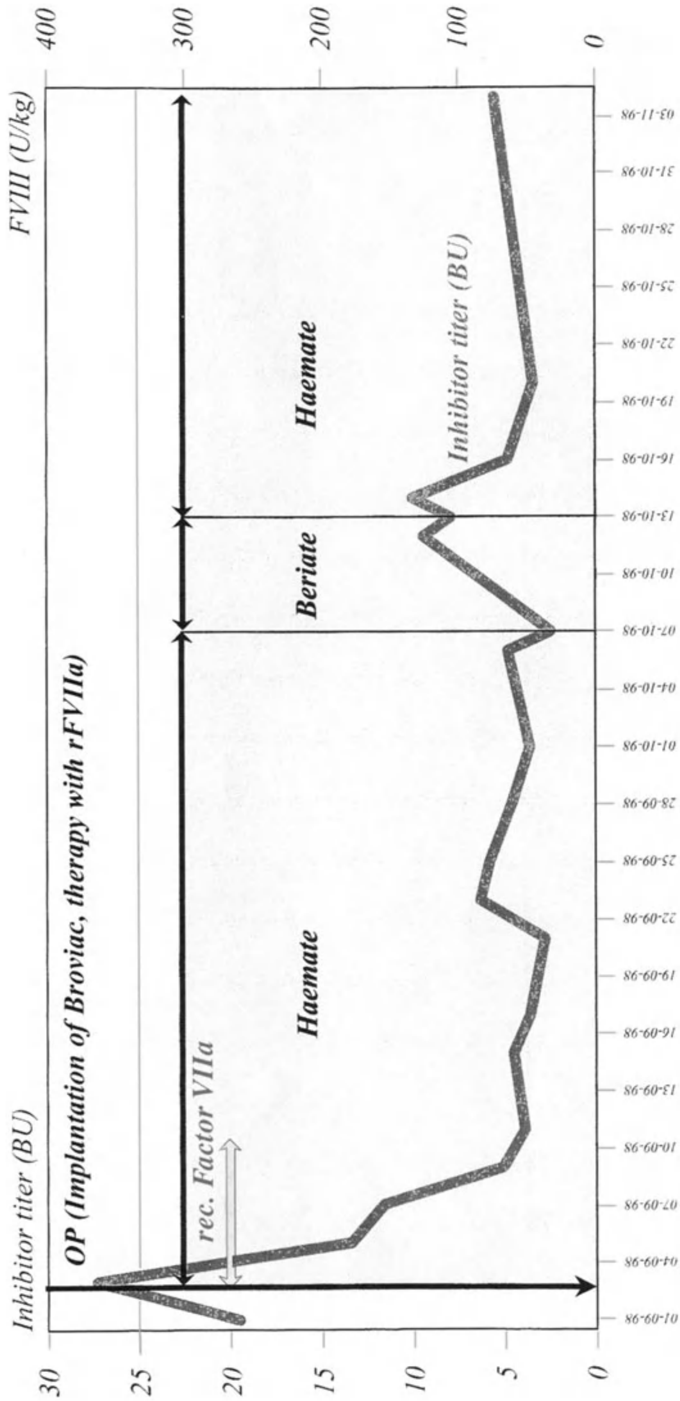


Fig. 1. Start of ITT in a patient with inhibitor to FVIII in mild hemophilia A. Patient 1: 15.9.1998–4.11.1998

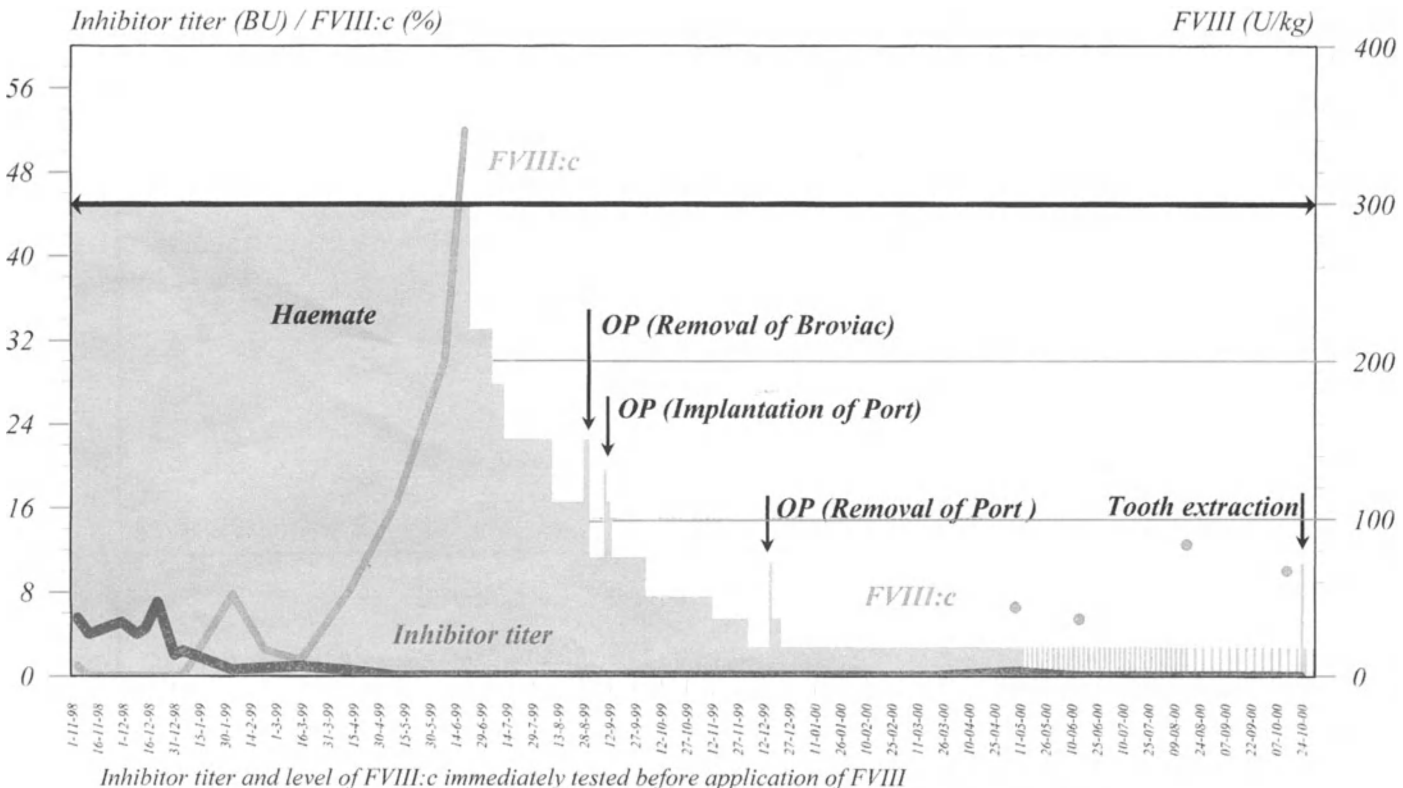


Fig. 2. Continuation of ITT in a patient with inhibitor to FVIII in mild hemophilia A. Patient 1: 5.11.1998–31.10.2000

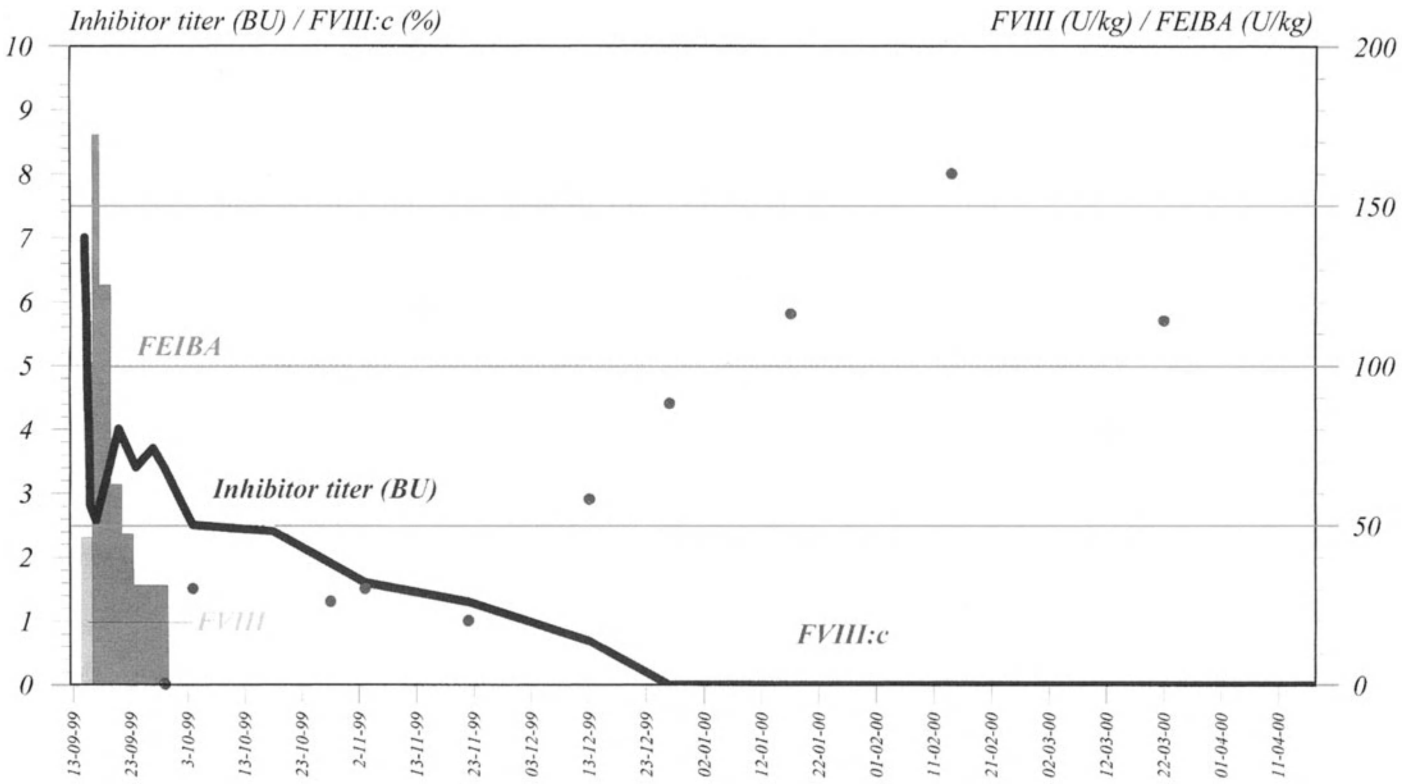


Fig. 3. Spontaneous disappearance of an inhibitor to FVIII in mild hemophilia A. Patient 2: 15.9.1999–17.4.2000

Discussion

The two cases presented here show similarities and differences:

- The antibodies both developed after intensive substitution therapy with FVIII
- FVIII was applied partially as a continuous infusion
- The inhibitors acted against the infused factor and their own FVIII as well
- In the first patient the inhibitor was present already after the 8th substitution day but in the second one only after nine substitution periods with 81 substitution days during 19 years

The first patient was treated successfully by ITT in the same way as in severe hemophilia. Hay et al. reported about 26 patients with inhibitors in non-severe hemophilia, where ITT was done in eight patients, but only in two of them successfully (2).

In patients with successful ITT it remains questionable if a new intensive treatment period again would induce inhibitors, especially if FVIII infusions had been stopped a long time before.

In the second patient the inhibitor increased not higher than 7 BU and it disappeared spontaneously. Afterwards the patient's own FVIII again was detectable.

In the case of new bleedings DDAVP should be considered as an option, but it is necessary to check the effect of DDAVP on FVIII beforehand. If no increase of FVIII is registered, a mild inhibitor still may be present. In these cases a new challenge with FVIII concentrates also can induce the antibody again and Feiba or NovoSeven should be used as an alternative.

References

1. Fakharzadeh SS, Kazazian HH. Correlation between factor VIII genotype and inhibitor development in hemophilia A. Semin Thromb Hemost 2000; 26; 167–71.
2. Hay CRM, Ludlam CA, Colvin BT, et al. Factor VIII inhibitors in mild and moderate-severity Haemophilia A. Thromb Haemost 1998; 79;762–6
3. Lusher JM. Inhibitor antibodies to factor VIII and factor IX: management. Semin Thromb Hemost 2000; 26; 179–88.
4. Schwaab R, Brackmann HH, Meyer C, Seehafer J, Kirchgesser M, Haak A, Olek K, Tuddenham EGD, Oldenburg,J. Haemophilia A: Mutation type determines risk of inhibitor formation. Thromb Haemost 1995; 74; 1402–6.
5. van den Brink EN, Timmermans SM, Turenhout EA, Bank CM, Fijnvandraat K, Voorberg J, Peters M. Longitudinal analysis of factor VIII inhibitors in a previously untreated mild hemophilia A patient with an Arg593→Cys substitution. Thromb Haemost 1999; 81; 723–6.
6. Vermylen J. How do some Haemophiliacs develop inhibitors? Haemophilia 1998; 4; 538–42.
7. White B, Cotter M, Byrne M, O'Shea E, Smith OP. High responding factor VIII inhibitors in mild haemophilia – is there a link with recent changes in clinical practice? Haemophilia 2000; 6; 113–5.

III. *Therapy and Monitoring of Bleeds in Acute- and Intensive-Care-Medicine*

Chairmen:

I. Scharrer (Frankfurt/Main)
E.O. Meili (Zürich)

Management of Bleeding in Surgery and Intensive Care

A. Calatzis, W. Schramm and M. Spannagl

Coagulopathies during the perioperative period and intensive care are caused by different mechanisms (Table 1, Fig. 1) related to patient characteristics, medication and operative procedure. As a consequence, defects in plasmatic as well as in cellular components of the coagulation system can result in severe bleeding complications. Due to the diverse nature of hemostasis disorders and the variety of therapeutic options, a targeted therapeutic management of coagulation is desirable in order to prevent or effectively treat bleeding episodes (1).

Table 1. Causes of coagulopathies in the perioperative field

Preexisting causes of coagulopathies	
Inherited	Acquired
von Willebrand-Juergens Disease	Liver/renal dysfunction
Thrombasthenias and other thrombopathies	Hematological diseases
Inherited coagulation factor disorders	Drugs (anticoagulants, antiplatelet agents, other)
Acute coagulopathies	
Loss of coagulation factors/platelets due to (surgical) blood loss/hemodilution	
Consumption of coagulation factors/platelets due to the intervention/coagulation activation on foreign surfaces/DIC	
Low temperature	
Colloidal solutions	
Anticoagulant therapy (e.g. heparin)	

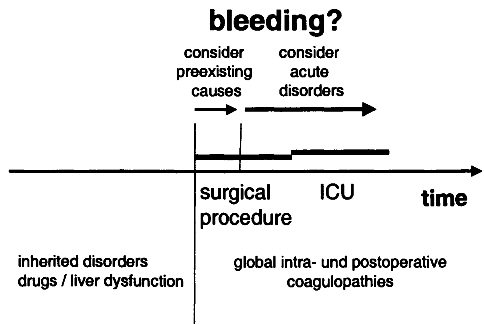


Fig. 1. Preexisting/acute disorders in the perioperative field: preexisting causes of bleeding often initiate bleeding early during the intervention. Bleeding occurring later during the intervention is often caused by secondary changes (loss/consumption of coagulation factors and platelets) or by a combination of predisposing factors and acute coagulopathies

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Preexisting Causes for Coagulopathies

A very important tool for the diagnosis of preexisting causes of coagulopathies is the anamnestic examination (Table 2). Patients with coagulation factor deficiencies (e.g. hemophilia A) usually have a history of bleeding and should be treated in specialized centers. Impaired bone marrow function (resulting in thrombocytopenia, platelet dysfunction and/or anemia) and/or liver and renal dysfunction are risk factors for intraoperative bleeding and should be assessed by the determination of a cell count, liver enzymes and retention parameters.

Table 2. Anamnestic examination of preexisting causes of coagulopathies

History of invasive procedures/trauma associated with bleeding complications? (surgery? tooth extractions? accidents?)
History of invasive procedures/trauma not associated with bleeding complications?
Epistaxis?
Women: prolonged menses?
Bleeding/hematomas following minor trauma?
Family history of bleeding complications?
Intake of non-steroidal anti-inflammatory drugs? (ask for aspirin, it is often not considered as a drug by the patient)
Intake of anticoagulants, antiplatelet drugs? (»any drugs for the heart?«, patient might not know the mechanism)

Laboratory Screening for Preexisting Coagulation Disorders

The preoperative assessment of platelet count, prothrombin time (PT) and aPTT is obligatory in most centers, while the predictive value of these parameters in respect to bleeding and transfusion requirements is relatively low (2–6). One aspect is that a prolongation of the aPTT in asymptomatic patients is often not a sign of a hemorrhagic diathesis.

Prolonged aPTT Not Associated with Bleeding Disorders

Frequent reasons for prolonged aPTT are the so-called lupus anticoagulants or deficiencies of factor XII or kallikrein.

Lupus Anticoagulants

Lupus anticoagulants are a heterogenic group of antibodies against phospholipid structures and/or coagulation factors (mainly prothrombin) (7). In diagnostic assays phospholipids are added to the sample (mainly in the aPTT and PT) as a substitute for platelets (as platelet poor plasma is usually applied for the test procedure). Phospholipids provide binding structures for the clotting factors, which significantly accelerate coagulation activation. By their interaction with the phospholipids lupus anticoagulants can prolong the aPTT and PT. This interference is

highly variable depending on the reagent applied in the laboratory. Despite the prolonged aPTT or PT lupus anticoagulants do not cause a bleeding tendency, but in contrast may predispose for thrombosis. In vivo lupus anticoagulants interact with phospholipid structures on the endothelium (8–9). Endothelial damage arising from this interaction can lead to thrombosis. Therefore, in spite of the prolonged aPTT, patients with lupus anticoagulants must receive perioperative prophylaxis against thrombosis (e.g. using low molecular weight heparin).

Deficiency of Factor XII/Kallikrein

Factor XII (Hageman factor) and kallikrein are part of the contact phase, i.e. of the hemostatic pathway that activates clotting when blood comes into contact with artificial surfaces (dialysis membranes, cardiopulmonary bypass circuit). However, the contact phase is not part of the normal mechanism of hemostasis in the body (10). This can be seen by the lack of bleeding tendency in patients with deficiencies of kallikrein or factor XII. Such patients have a prolonged or even not measurable aPTT but do not have any bleeding tendency and should also receive the usual perioperative thrombosis prophylaxis.

Von Willebrand Disease (vWD)

The most frequent inherited coagulation factor deficiency is the von Willebrand disease (vWD), i.e. a qualitative or quantitative disorder of von Willebrand factor (vWF) (11). VWF is the protein that allows the platelet to attach to the vascular injury under high shear stress (by connecting the platelet's GpIb receptor with subendothelial collagen structures, Fig. 2). VWD is frequently associated with epistaxis and bleeding after tooth extraction or other invasive procedures. VWD may be treated using desmopressin acetate (DDAVP, Minirin, Ferring), which leads to the release of endogenous vWF. The release of vWF by DDAVP depends on whether there are sufficient endogenous pools. This should be evaluated preoperatively by the i.v. application of DDAVP and the analysis of platelet aggregation or PFA-100

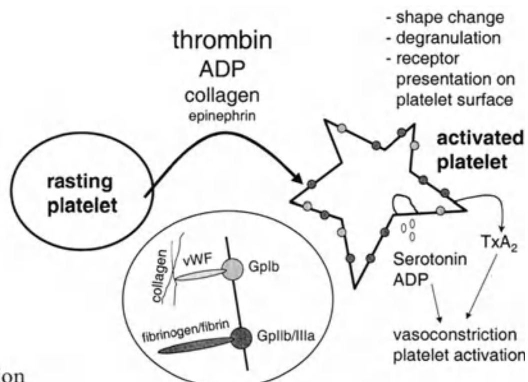


Fig. 2. Mechanisms of platelet activation

analysis (»minirin test«). If the test dosage of DDAVP fails to correct the vWF deficiency/malfunction then the direct application of vWF is necessary (prophylactically or in case of bleeding). This is performed by the application of a factor VIII concentrate rich in vWF (e.g. Haemate HS, Aventis, Liederbach) or by the use of a vWF concentrate (e.g. Facteur von Willebrand, Laboratoire Français de Fractionnement et des Biotechnologies).

Screening/Diagnosis of vWD or Other Primary Hemostasis Disorders

PFA-100 Analysis

The platelet function analyzer (PFA-100, Dade-Behring, Marburg, Germany) is a dedicated instrument for the analysis of platelet function under high shear stress conditions (12). The ability of platelets to close a small aperture in a collagen membrane is assessed (Fig. 3). The parameter determined is the closure time (CT), i.e. the time from start of the analysis until the cessation of blood flow. In addition to the shear stress and the contact of the blood with collagen the platelets are activated using ADP or epinephrine (two different cartridges). The PFA-100 is very sensitive to disorders of vWF and to (using the epinephrine cartridge) ASS intake (13–15). It is less sensitive to the effects of Clopidogrel or Ticlopidine (16). The PFA-100 is mainly useful in the exclusion or confirmation of vWD or ASS intake before invasive procedures. However it cannot be used as a general screening tool before during or after surgical interventions (17–18). The PFA-100 requires at least 100,000 platelets/µl and a hematocrit of 35% for the analysis.

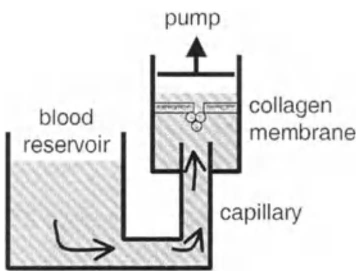


Fig. 3. Measuring method of the platelet function analyzer (PFA-100): by the action of a precision pump citrated blood is suctioned through an aperture in a collagen membrane (diameter 0.15 mm). In the capillary the platelets are activated by high shear stress. Platelets attach on the collagen membrane and close the aperture, resulting in a cessation of blood flow. Disorders of platelet activation (e.g. ASS intake) or of the attachment of the platelet onto the collagen surface (e.g. vWD) are detected by the prolonged closure time

Bleeding Time

In the hands of an experienced examiner the bleeding time is a valuable tool for the examination of primary hemostasis. However variation between different examiners is high and the value of bleeding times performed by less experienced examiners is very low (19–22).

Intra-/Postoperative Coagulation Disorders

The most frequent reason for bleeding in the perioperative period is secondary disorders due to surgical blood loss, consumption of coagulation factors/platelets and

the effects of transfusion solutions, drugs and the contact of blood with artificial surfaces. The predictive value of the PT, aPTT and platelet count in this period has been shown to be low. For the monitoring of coagulopathies in this period the so-called viscoelastic monitoring methods (thromboelastography, ROTEG, Sonoclot) have reached wide application.

Activated Clotting Time (ACT)

The ACT is routinely used to monitor anticoagulant therapy in cardiovascular surgery. However, it should be mentioned that ACT may serve for the control of plasmatic coagulation only and that further shortcomings may limit its use: e.g. the correlation of the ACT with the heparin concentration was reported to be poor (22) and following heparin neutralization by protamine a residual heparin activity of up to 0.7 aXa U/ml can be accompanied by a normal ACT (22). In addition, the ACT does not provide any information on platelet status or on fibrinolysis, which are important factors in the pathogenesis of bleeding in the perioperative period.

Viscoelastic Methods

These monitoring methods assess blood coagulation by the assessment of the elastic properties of the blood clot. In these techniques the coagulation process of a whole blood sample is continuously assessed from the formation of the first fibrin fibers, the formation of the blood clot until the eventual dissolution of the blood clot (in the case of hyperfibrinolysis). Mainly two methods are applied clinically: thromboelastography and Sonoclot analysis.

Thromboelastography

Thromboelastography was presented as early as 1948 by Hartert (23). Since the middle of the 1980s there has been a rising application of TEG especially during the monitoring and treatment of complex intraoperative coagulopathies (24–28). In TEG the firmness of the blood clot is continuously assessed (Figs. 4, 5). Thus, the beginning of clot formation, clot formation kinetics and the maximum firmness of

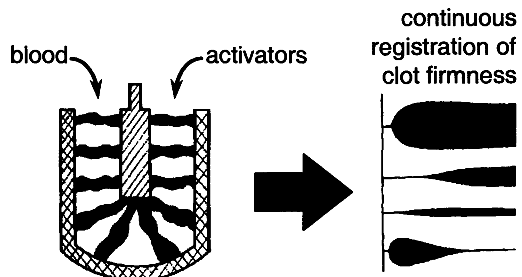


Fig. 4. Measuring method of thrombelastographic systems: the blood sample is placed with or without an activating reagent in a cup. The firmness of the blood clot is continuously assessed by the ability of the blood clot to form a mechanical coupling between the surfaces of the pin and cup over a distance of 1 mm

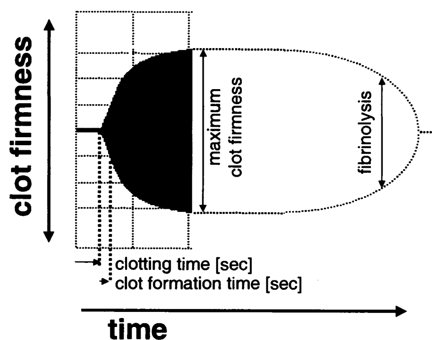


Fig. 5. The thrombelastogram is the continuous registration of blood clot firmness during the entire coagulation process

the blood clot are assessed as well as its stability or lysis (Table 3). Blood clot firmness is a functional parameter which depends on the activation of coagulation, the platelet/fibrinogen content of the sample and the polymerization/cross-linking of the fibrin network. The polymerization process is not assessed by most monitoring methods (e.g. of the aPTT and PT). In the aPTT and PT the analysis ends upon the formation of the first fibrin fibers (Fig. 6).

In several studies TEG has been shown to have a better predictive value in respect to intra- or postoperative bleeding than the aPTT, PT and platelet count

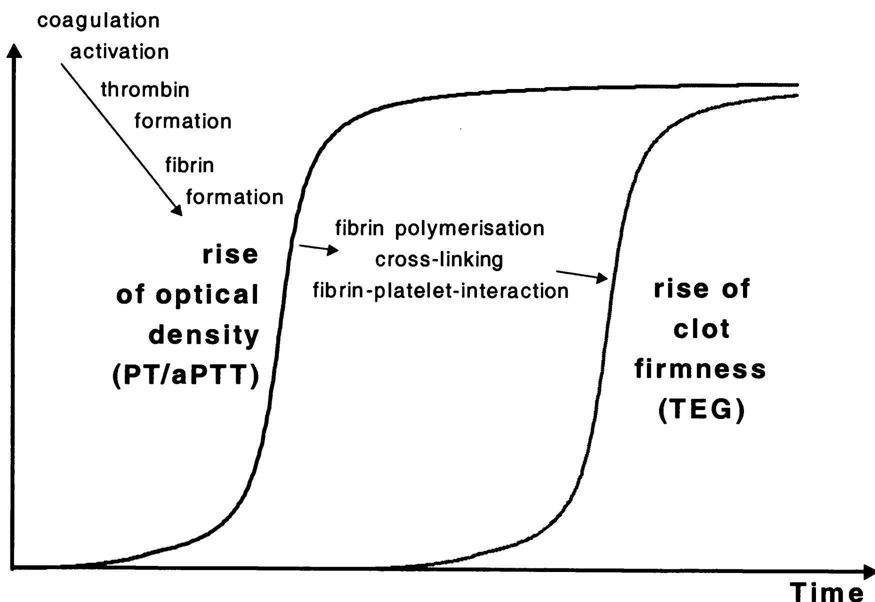


Fig. 6. Comparison of the assessment of blood coagulation in the aPTT/PT and TEG analysis. The usually applied laboratory methods for the aPTT/PT assess coagulation by the rise of optical density upon fibrin formation. Using this method the processes of fibrin polymerization, cross-linking and fibrin-platelet interaction are not assessed. In contrast TEG assesses blood clot firmness, which depends on the complete clot formation

Table 3. Parameters of TEG analysis

Biological event	Parameter	Definition	Abbreviation	Classical abbreviation
Coagulation activation	Clotting time	Time from start of measurement until the beginning of clot formation	CT (s)	r
Clot formation dynamics	Clot formation time	Time from the beginning of clot formation until an amplitude of 20 mm is reached	CFT (s)	k
Maximum clot firmness	Maximum clot firmness		MCF (mm)	MA
Fibrinolysis	Maximum lysis	Reduction of clot firmness during measurement	ML (in % of the MCF)	

(29–32). In some studies reduced transfusion requirements or a reduced reexploration rate by the TEG-assisted coagulation management were also shown (33–34).

The ROTEG analyzer (Pentapharm, Munich, Germany) is a newly developed instrument based on TEG (35–37). The system is easily transportable, provides automatic pipetting and computer analysis and facilitates near-patient assessment of TEG when compared with conventional TEG equipment (Figs. 7, 8). Recently published studies have used the ROTEG analyzer in the assessment of hemostasis during and after cardiac and general surgery (38–40).

Fig. 7. The ROTEG Coagulation Analyzer: a new thrombelastographic analyzer suitable for use in the OR or ICU. Four tests can be assessed in parallel and are shown on the computer display

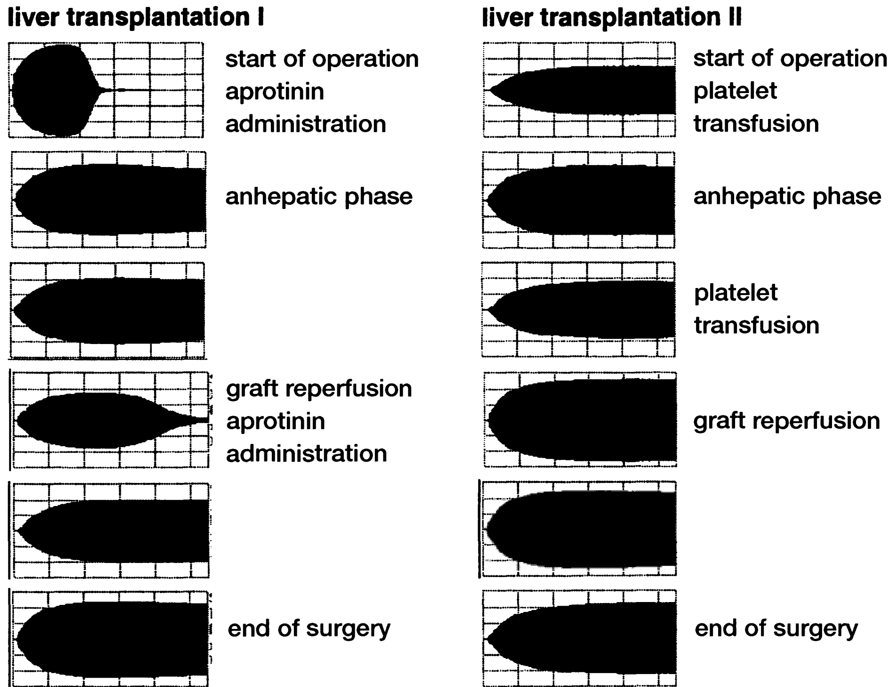


Fig. 8. ROTEG Analysis during two consecutive liver transplantations: during the intervention shown on the left two fibrinolytic events (at the start of operation and during graft reperfusion were treated using aprotinin transfusion. During the second intervention no fibrinolysis was detected, but abnormal clot firmness at the start of operation and during the anhepatic phase was treated using platelet transfusion. This example shows the possibility of targeted coagulation therapy during invasive procedures using whole blood coagulation analysis

Sonoclot Analysis

In Sonoclot analysis the coagulation process is detected by the resonance of the blood clot. The resonance of the blood clot depends on its firmness and thus also changes during the coagulation process (41). Like TEG, Sonoclot has shown to be predictive for the clinical coagulation status during the perioperative period. However, it is less frequently used in comparison with TEG.

The use of thromboelastography, ROTEG or Sonoclot analysis in a near-patient setting is more effort than the application of less complex devices (as POC systems like CoaguCheck® (Roche) or ACT devices) and more training of the staff is required.

Bleeding Due to Heparin/Hirudin Overdose

Anticoagulants are in a large number of hospitalized patients for the prevention or treatment of thromboembolic events as well as for protection of catheters or extra-

corporal circuits against clotting. However, the application of these drugs may also lead to bleeding. Unfractionated heparin may be neutralized by the application of protamine. The antagonization of low molecular weight heparin (LMWH) using protamine is less effective, while no specific antidote is available for hirudin (42).

Accumulation

LMWH and hirudin are mainly excreted by the kidneys and can therefore accumulate in cases of renal dysfunction and the repeated application of the drug (43). The very low sensitivity of the aPTT towards LMWH must be taken into account, i.e. already a slightly prolonged aPTT might be accompanied by hemorrhagic LMWH levels. The determination of LMWH concentration using the anti-factor-Xa activity (chromogenic substrate analysis), the Heptest or Staclot assay provide enhanced sensitivity and specificity. However, these tests are not available in many centers and there is considerable variation among their results (44).

Accidental Exposition

During unexpected bleeding an accidental exposition of the patient with unfractionated heparin should be considered, if the patient has any central venous catheters/port devices. These systems are usually blocked with unfractionated heparin and an accidental exposition of the patient to a high anticoagulant dosage can arise. This can be detected by the prolonged aPTT, ACT or clotting time in the TEG or Sonoclot.

Fibrinolytic Events

Fulminant bleeding in cardiovascular surgery, multiple trauma patients and massive transfusion may be caused by acute hyperfibrinolytic events. Hyperfibrinolysis can only be specifically diagnosed using thromboelastography or Sonoclot. Elevated D-Dimer concentrations are only an indirect sign of fibrinolysis and have a very low specificity in respect to hyperfibrinolysis, as the majority of surgical patients have elevated D-Dimer levels due to the intervention. Hyperfibrinolysis can be treated using aprotinin (Trasylol, Bayer) or tranexamic acid (Cyclocapron/Ugurol).

Summary

Acute coagulopathies can rely on different aspects of hemostasis and on preexisting or acute disorders. The most important screening tool before invasive procedures is the anamnestic examination of previous bleeding episodes. Assessment of the defects of primary hemostasis can be performed by the bleeding time, PFA-100 or more specialized laboratory methods. Acute coagulopathies can be monitored with TEG or Sonoclot analysis which detect disorders of the plasmatic factors, platelets and the fibrinolytic system.

References

1. Despotis GJ, Skubas NJ, Goodnough LT: Optimal management of bleeding and transfusion in patients undergoing cardiac surgery. Semin Thorac Cardiovasc Surg 1999 Apr;11(2):84–104
2. Gravlee GP, Arora S, Lavender SW, Mills SA, Hudspeth AS, Cordell AR, James RL, Brockschmidt JK, Stuart JJ: Predictive value of blood clotting tests in cardiac surgical patients. Ann Thorac Surg 1994 Jul;58(1):216–21
3. Gelb AB, Roth RI, Levin J, London MJ, Noall RA, Hauck WW, Cloutier M, Verrier E, Mangano DT: Changes in blood coagulation during and following cardiopulmonary bypass: lack of correlation with clinical bleeding. Am J Clin Pathol 1996 Jul;106(1):87–99
4. Martin JH, Rosser CJ, Linebach RF, McCullough DL, Assimos DG: Are coagulation studies necessary before percutaneous nephrostomy? Tech Urol 2000 Sep;6(3):205–7
5. Oren A, Breumelhof R, Timmer R, Biesma DH, Hoekstra JB: Abnormal clotting parameters before therapeutic ERCP: do they predict major bleeding? Eur J Gastroenterol Hepatol 1999 Oct;11(10):1093–7
6. Munro J, Booth A, Nicholl J: Routine preoperative testing: a systematic review of the evidence. Health Technol Assess 1997;1(12):i-iv; 1–62
7. Roubey RA: Immunology of the antiphospholipid syndrome: antibodies, antigens, and autoimmune response. Thromb Haemost 1999 Aug;82(2):656–61
8. Rao LV, Hoang AD, Rapaport SI. Mechanism and effects of the binding of lupus anticoagulant IgG and prothrombin to surface phospholipid. Blood 1996 Dec 1;88(11):4173–82
9. Rand JH, Wu XX: Antibody-mediated disruption of the annexin-V antithrombotic shield: a new mechanism for thrombosis in the antiphospholipid syndrome. Thromb Haemost 1999 Aug;82(2):649–55.
10. Rapaport SI, Rao LV: The tissue factor pathway: how it has become a »prima ballerina«. Thromb Haemost 1995 Jul;74(1):7–17
11. Ruggeri ZM: Structure and function of von Willebrand factor. Thromb Haemost 1999 Aug;82(2):576–84
12. Mammen EF, Comp PC, Gosselin R, Greenberg C, Hoots WK, Kessler CM, Larkin EC, Liles D, Nugent DJ: PFA-100 system: a new method for assessment of platelet dysfunction. Semin Thromb Hemost 1998;24(2):195–202
13. Fressinaud E, Veyradier A, Truchaud F, Martin I, Boyer-Neumann C, Trossaert M, Meyer D: Screening for von Willebrand disease with a new analyzer using high shear stress: a study of 60 cases. Blood 1998 Feb 15;91(4):1325–31
14. Lasne D, Fiemeyer A, Chatellier G, Chammas C, Baron JF, Aiach M A study of platelet functions with a new analyzer using high shear stress (PFA 100) in patients undergoing coronary artery bypass graft. Thromb Haemost 2000 Nov;84(5):794–9.
15. Homoncik M, Jilma B, Hergovich N, Stohlawetz P, Panzer S, Speiser W: Monitoring of aspirin (ASA) pharmacodynamics with the platelet function analyzer PFA-100. Thromb Haemost 2000 Feb;83(2):316–21
16. Kottke-Marchant K, Powers JB, Brooks L, Kundu S, Christie DJ: The effect of antiplatelet drugs, heparin, and preanalytical variables on platelet function detected by the platelet function analyzer (PFA-100). Clin Appl Thromb Hemost 1999 Apr;5(2):122–30
17. Wahba A, Sander S, Birnbaum DE: Are in-vitro platelet function tests useful in predicting blood loss following open heart surgery? Thorac Cardiovasc Surg 1998 Aug;46(4):228–31
18. Zaccaria F, Dietrich W, Braun S et al: Does platelet function assessment predict bleeding after cardiopulmonary bypass? AINS 1998 Jul, 33 (Sup 3), PD-E 202.2
19. Boberg KM, Brosstad F, Egeland T, Egge T, Schrumpf E: Is a prolonged bleeding time associated with an increased risk of hemorrhage after liver biopsy? Thromb Haemost 1999 Mar;81(3):378–81
20. Peterson P, Hayes TE, Arkin CF, Bovill EG, Fairweather RB, Rock WA Jr, Triplett DA, Brandt JT: The preoperative bleeding time test lacks clinical benefit: College of American Pathologists' and American Society of Clinical Pathologists' position article. Arch Surg 1998 Feb;133(2):134–9

21. Basili S, Ferro D, Leo R, Juliano L, Alessandri C, Cordova C, Violi F: Bleeding time does not predict gastrointestinal bleeding in patients with cirrhosis. The CALC Group. Coagulation Abnormalities in Liver Cirrhosis. J Hepatol 1996 May;24(5):574–80
22. Murray DJ, Brosnahan WJ, Pennell B et al: Heparin detection by the activated coagulation time: a comparison of the sensitivity of coagulation tests and heparin assays. J-Cardiothorac-Vasc-Anesth. 1997 Feb, 11(1): 24–8.
23. Hartert H: Blutgerinnungstudien mit der Thrombelastographie, einem neuen Untersuchungsverfahren. Klin Wochenschrift 26:577–583, 1948.
24. Kang YG, Martin DJ, Marquez J, Lewis JH, Bontempo FA, Shaw BW Jr, Starzl TE, Winter PM: Intraoperative changes in blood coagulation and thrombelastographic monitoring in liver transplantation. Anesth Analg 1985 Sep;64(9):888–96
25. Spiess BD, Tuman KJ, McCarthy RJ et al: Thromboelastography as an indicator of post-cardiopulmonary bypass coagulopathies. J-Clin-Monit. 1987 Jan, 3(1): 25–30.
26. Martin P, Horkay F, Rajah SM et al: Monitoring of coagulation status using thromboelastography during paediatric open heart surgery. Int-J-Clin-Monit-Comput. 1991, 8(3): 183–7
27. Chandler WL. The thromboelastography and the thromboelastograph technique. Semin Thromb Hemost. 1995;21 Suppl 4:1–6. Review.
28. Mallett SV, Cox DJ. Thromboelastography. Br J Anaesth. 1992 Sep;69(3):307–13. Review.
29. Martin-P; Horkay-F; Rajah-SM; Walker-DR: Monitoring of coagulation status using thromboelastography during paediatric open heart surgery. Int-J-Clin-Monit-Comput. 1991; 8(3): 183–7
30. Davis CL, Chandler WL: Thromboelastography for the prediction of bleeding after transplant renal biopsy. J Am Soc Nephrol 1995 Oct;6(4):1250–5
31. Kaufmann CR, Dwyer KM, Crews JD, Dols SJ, Trask AL: Usefulness of thromboelastography in assessment of trauma patient coagulation. J Trauma 1997 Apr;42(4):716–20
32. Ereth MH, Nuttall GA, Klindworth JT et al: Does the platelet-activated clotting test (HemoSTATUS) predict blood loss and platelet dysfunction associated with cardiopulmonary bypass? Anesth-Analg. 1997 Aug, 85(2): 259–64.
33. Spiess BD, Gillies BS, Chandler W, Verrier E: Changes in transfusion therapy and reexploration rate after institution of a blood management program in cardiac surgical patients. J-Cardiothorac-Vasc-Anesth. 1995 Apr; 9(2): 168–73
34. Shore-Lesserson L, Manspeizer HE, DePerio M, Francis S, Vela-Cantos F, Ergin MA: Thromboelastography-guided transfusion algorithm reduces transfusions in complex cardiac surgery. Anesth Analg 1999 Feb;88(2):312–9
35. Calatzis AN, Fritzsche P, Calatzis AL, Kling M, Hipp R, Stemberger A: A comparison of the technical principle of the roTEG Coagulation Analyzer and conventional thrombelastographic systems. Ann Hematol 72 Sup I P90.
36. Calatzis AN, Calatzis AL, Fritzsche P, Kling M, Hipp R, Stemberger A: An analysis of the correlation of the roTEG Coagulation Analyzer and the Hellige Thrombelastograph D. Ann Hematol 72 Sup I P87.
37. Calatzis AN, Fritzsche P, Kling M et al: A new technique for fast and specific coagulation monitoring. European Surgical Research 28:S1 (89), 1996.
38. Mahla E, Lang T, Vicenzi MN, Werkgartner G, Maier R, Probst C, Metzler H: Thromboelastography for monitoring prolonged hypercoagulability after major abdominal surgery.Anesth Analg 2001 Mar;92(3):572–7
39. Haisch G, Boldt J, Krebs C, Kumle B, Suttner S, Schulz A: The influence of intravascular volume therapy with a new hydroxyethyl starch preparation (6% HES 130/0.4) on coagulation in patients undergoing major abdominal surgery. Anesth Analg 2001 Mar;92(3):565–71
40. Koster A, Kukucka M, Bach F, Meyer O, Fischer T, Mertzlufft F, Loebe M, Hetzer R, Kuppe H: Anticoagulation during cardiopulmonary bypass in patients with heparin-induced thrombocytopenia type II and renal impairment using heparin and the platelet glycoprotein IIb-IIIa antagonist tirofiban. Anesthesiology 2001 Feb;94(2):245–51

41. Hett DA, Walker D, Pilkington SN, Smith DC: Sonoclot analysis. Br J Anaesth 1995 Dec;75(6):771-6
42. Verstrate M. Direct thrombin inhibitors: Appraisal of the antithrombotic/haemostatic balance. Thromb Haemostas 1997;78:357-363
43. Boneu B: Low molecular weight heparins: are they superior to unfractionated heparins to prevent and to treat deep vein thrombosis? Thromb Res 2000 Oct 15;100(2):V113-20
44. Kitchen S, Iampietro R, Woolley AM, Preston FE: Anti Xa monitoring during treatment with low molecular weight heparin or danaparoid: inter-assay variability. Thromb Haemost 1999 Oct;82(4):1289-93.

IV. Pediatric Hemostaseology

Chairmen:

A. H. SUTOR (Freiburg)
H. LENK (Leipzig)

Frequency and Profile of viral posttransfusional Infections in Patients from Hemophilia Center Timisoara

M. Şerban, C. Petrescu, P. Ţepeneu, M. Pop, S. Jenaru and W. Schramm

Introduction

Almost two-thirds of the hemophilia patients all over the world are still either treated with »classic«, non-inactivated products (plasma, cryoprecipitate) or are not treated at all. Posttransfusional infections represent one of the major threats to the patients treated with non-inactivated blood products. There are multiple long-term consequences of an inappropriate replacement therapy in hemophiliacs: high frequency of physical disabilities, posttransfusional infections, psychological burden, difficulties in social and professional integration.

As it is already known, an optimal screening, together with anti-HBV vaccination, can substantially reduce the risk of posttransfusional viral infections. Hepatitis virus infections (HBV, HDV, HCV, HGV) can progress to chronic hepatitis, cirrhosis and hepatocellular carcinoma. HIV infection leaves only the chance of fighting for survival prolongation, before death. The costs incurred in the management of blood-borne infections exceed those of a safe replacement therapy, represented by inactivated factor concentrates. Sustained measures have to be taken for the assurance of a safe replacement therapy, whenever this is really possible.

Objectives

The objectives of the present study were:

- Determination of the frequency and profile of viral posttransfusional infections in patients from the Hemophilia Center Timisoara, in a ten-year period (1990–1999), depending on age, severity, number of therapeutic exposures, modality of treatment
- Evaluation of the donor screening and anti-HBV vaccination impact, by analyzing viral serological markers in different periods of the study interval and in different age-groups of patients

Material and Methods

Our cohort includes 207 non-selected, consecutive hemophilia patients from the Hemophilia Center Timisoara, aged 1–25 years, most of them with severe disease

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

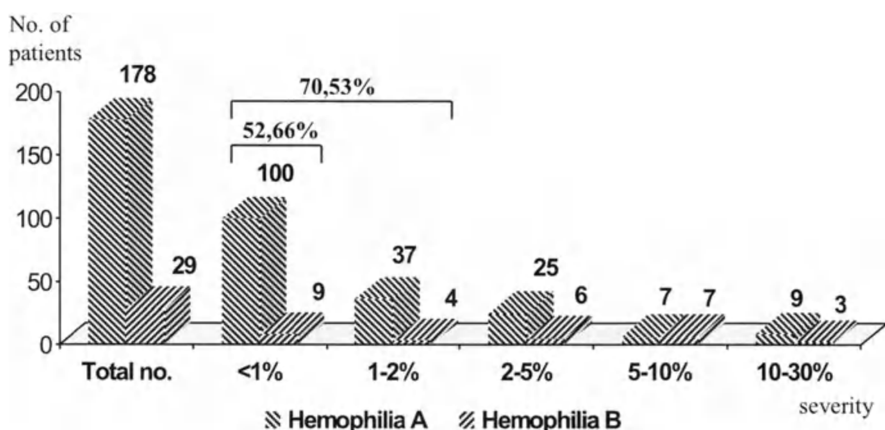
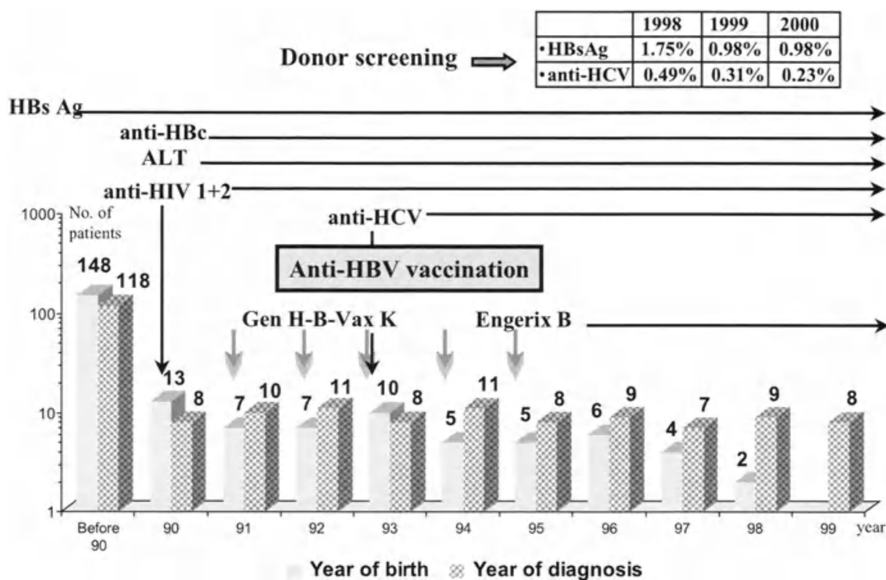


Fig. 1. Hemophilia patients and severity of the form of the disease

(Fig. 1), followed up for a ten-year period, between 1990 and1999. More than two-thirds of the patients (71.50%) were born and about half (57.49%) were diagnosed before 1990 (Fig. 2). Three different groups of treatment were separated: no exposure to blood products (20 patients); treatment with factor concentrates only (7 patients); »mixed« treatment with both »classic« products (plasma and cryoprecipitate) and factor concentrates (180 patients).



	1998	1999	2000
•HBsAg	1.75%	0.98%	0.98%
•anti-HCV	0.49%	0.31%	0.23%

Fig. 2. Donor screening and vaccination of hemophiliacs

Serological markers for different viral infections were tested by means of the ELISA technique of 2nd and 3rd generation as follows: for HBV (HBsAg, HBeAg, anti-HBc, anti-HBe, anti-HBs) and HIV 1 and 2 (anti-HIV 1 and 2), the whole period; for HDV, HCV and HAV (anti-HDV, anti-HCV, anti-HAV total and anti-HAV IgM), since 1993; for CMV and HTLV I and II (anti-CMV total, anti-CMV IgM, anti-HTLV I and HTLV II), since 1996.

In the Transfusion Center Timisoara, donor screening was improved after 1990 (Fig. 2); for HBV infection, HBsAg was combined with anti-HBc, ALT; anti-HIV 1 and 2 have been determined since 1990; HCV has been regularly tested since 1993. In recent years, about 70–80% have been known, iterative, unpaid donors (compared with 50% before 1990).

Anti-HBV vaccination of hemophiliacs was initiated in 1991, using Gen H-B-Vax K (MSD Sharp & Dohme GmbH, Behringwerke AG). Many patients didn't receive the recommended doses or were incompletely vaccinated. In 1995, anti-HBV vaccination of neonates and infants with Engerix B (SmithKline Beecham) was introduced in the National Program of Vaccination. Both are recombinant vaccines with multiple advantages (security, high immunogenity and purity, more rapid production in higher quantities and with lower costs, compared with plasma-derived vaccines).

Results and Discussions

In the first treatment group of 20 patients with no exposure to blood products, 85% of them having factor VIII/IX levels higher than 5%, 3/16 (18.75%) were anti-HBs positive, two of them being born after 1995 and probably anti-HBV vaccinated; all were negative for HCV and HIV. In the second group of 7 patients treated only with factor concentrates, all with factor VIII/IX level under 2%, none was positive for HBV, HCV or HIV.

The great majority of the patients are included in the third group, of »mixed« treatment patients, 78.33% having factor VIII/IX levels under 2%. Most of them have markers of HBV infection (116/159–72.96%) and HCV infection (105/175–60%); 80.95% of these patients have associated HBV and HCV markers.

For the HBV infection, the situation is only apparently satisfactory, since 65.41% (104/159) of the patients recovered (anti-HBs, anti-HBc total positive) (Fig. 3); compared with the known spontaneous recovery rate of about 90% in the natural course of the HBV infection, the lower recovery rate found in these patients (65.41%) could have been influenced by the associated HCV infection. The situation seems more critical if we also take into consideration the fact that 48 of the patients from this group were vaccinated against HBV. For the rest of the patients with persistent HBV infection (6.92% in the replicative and 0.63% in the latency phase) the risk of progression to cirrhosis (15–20%) or hepatocellular carcinoma (5–6%) cannot be neglected.

The association with HDV infection was found in 17.5% (7/40) of the tested patients, all with HBV markers of the recovery phase; this suggests a co-infection (which has a rate of persistent infection of only 2%, compared with 70% in the case of superinfection).

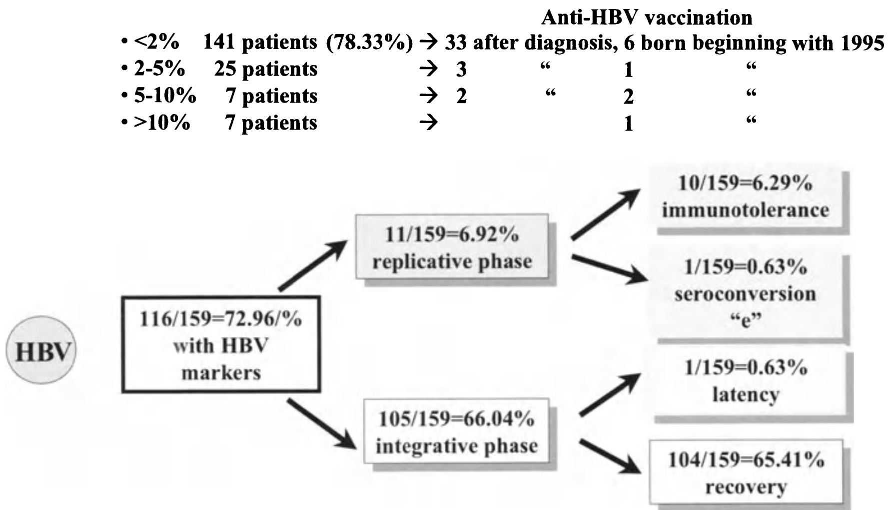


Fig. 3. HBV markers in the group of »mixed« treatment patients

The infection with HCV (Fig. 4) represents undoubtedly a major problem for the future of these patients, since 50–80% of the cases of acute HCV hepatitis are known to become persistent and 38–50% of them will progress to cirrhosis and an as yet unknown percentage to hepatocellular carcinoma in a period of 5–20 years.

The treatment of chronic HBV and HCV hepatitis is very expensive (Interferon, antiviral agents – Lamivudine, Ribavirine) and the recovery rate quite modest; the

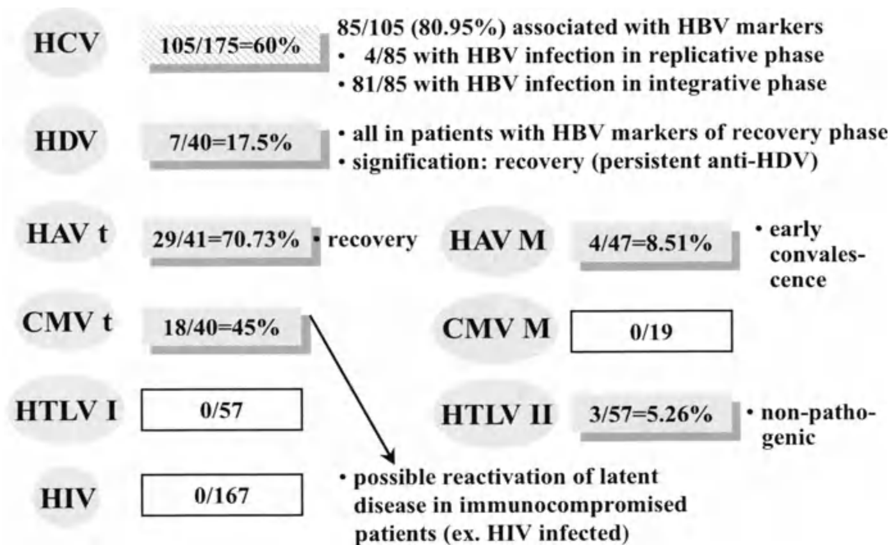


Fig. 4. HCV and other viral markers in the group of »mixed« treatment patients

presence of unfavorable predictive factors in patients from our cohort (associated infections, early infections and depressed immunological reactivity, a.s.o.) could probably alter the recovery chance. The evolution of the chronic infection and the treatment response follow-up (HBV DNA, HCV RNA) represent large supplementary costs. An important issue is that the treatment of chronic hepatitis decreases the risk of progression to cirrhosis and hepatocellular carcinoma.

The absence of HIV infection in our cohort, even in patients treated before 1990, when donors were not tested for HIV, is definitely a very fortunate aspect; the reasons include retaining the modest use of blood products, the great proportion of iterative donors (only one was found to be HIV positive among about 24,000 tested in 1999, none in 2000), the fact that before 1990, HIV infection has not emerged in adults in our country.

From the other viral markers tested in patients who form the third group (Fig. 4), the high frequency of anti-HAV has to be taken into consideration; it suggests an important endemic zone in our country and could serve, taking into account the deleterious effect of multiple hepatic co-infections, as an argument for the anti-HAV vaccination of hemophilia patients. Infections with CMV and HTLV II have no major clinical significance except for the possible reactivation of a latent CMV infection in cases of immunosuppression.

The risk of multiple exposures to non-inactivated blood products is expressed by the fact that 83.2% of the patients with HBV markers and 81.32% of those with anti-HCV have severe forms of hemophilia (factor VIII/IX level under 2%), and were exposed to the frequent use of »classic« products.

	Infection phase / Diagnosed	Before 1990 (Hbs Ag)	Beginning with 1990 (HBs Ag, ALT, anti-HBc) (anti-HBV beginning with 1991)
HBV	**Replicative phase**		
	• Immunotolerance	5/105 = 4.76%	5/74 = 6.75%
	• Seroconversion "e"	1/105 = 0.95%	0
	Integrative phase		
	• Latency	1/105 = 0.95%	0
	• Recovery	79/105 = 75.24%	28/74 = 37.84%
	Total	86/105 = 81.90%	33/74 = 44.59% (only non-vaccinated 40.35%)

	Infection / Diagnosed	Before 1993 (no screening)	Beginning with 1993 (anti-HCV)
HCV	Anti-HCV positive	97/127 = 76.38%	8/48 = 16.67% (all also treated in other counties)

Fig. 5. The efficiency of donor screening

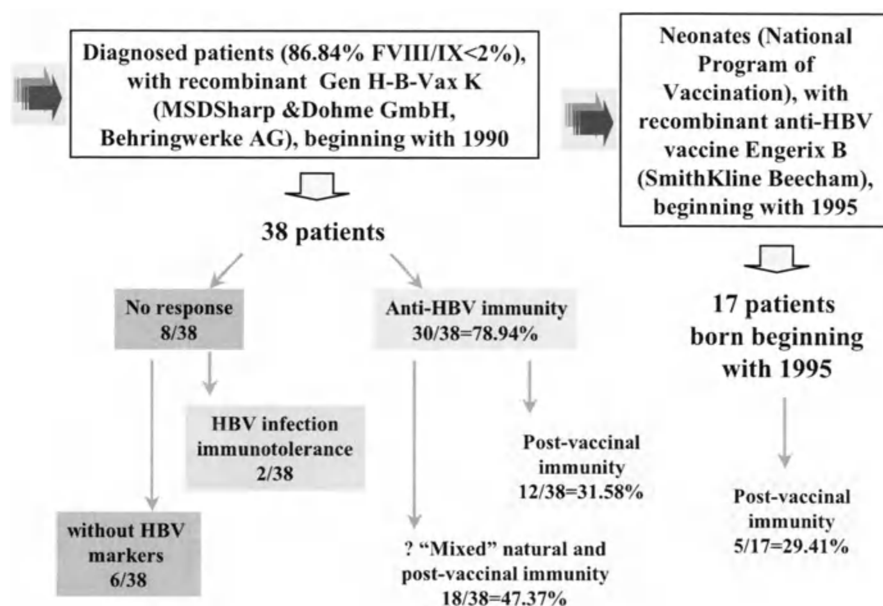


Fig. 6. The response to anti-HBV vaccination

The improvement of donor screening after 1990 proved to be a good modality for reducing the viral posttransfusional risk. For the HBV infection, the frequency of patients with positive markers decreased by 45.56% after 1990; for the HCV infection the efficiency of donor screening was dramatic, with a reduction of 78.17% after 1993 (Fig. 5).

Anti-HBV vaccination (Fig. 6) was applied in 38 diagnosed patients, beginning with 1991 and in 17 patients born from 1995, before diagnosis (National Program of Vaccination). The response rate was not satisfactory in diagnosed patients (31.58% immunized only through vaccination, having only anti-HBs), because in 73.68% of these cases, patients received only one or two doses of vaccine and in 15.79% of cases, the doses were lower than recommended. For the patients born beginning with 1995, the low response rate (29.41%) is probably determined by an incomplete or non-uniform vaccination at the beginning of the national program. An interesting aspect was that »mixed« immunized patients (through vaccination and infection, having anti-HBs but also other markers (anti-HBc and/or anti-anti-HBe), had the highest median titer of anti-HBs (282.2 mIU/ml, compared with 154.68 mIU/ml for the naturally immunized and respectively 109.15 mIU/ml for the patients immunized through vaccination), although with a lower percentage of response maintenance (anti/HBs positive in 81.25% of the patients after a median 6.96-year period, compared with 100% after 5.69 years, in vaccination immunized patients). Together with the fact that anti-HBV vaccination is not contraindicated in cases of HCV infection, this represents an argument for the general anti-HBV vaccination in anti-HBs negative hemophiliacs.

Conclusions

The present study reflects the major risk of viral posttransfusional, mainly hepatitis infections, represented by the use of non-inactivated blood products in hemophilia patients: high frequency of HBV, HDV and HCV infections and many multiple infections. The treatment and follow-up of chronic hepatitis is very expensive and cannot exclude the major long-term risk of progression to cirrhosis and hepatocellular carcinoma. The improvement in donor screening and the anti-HBV vaccination of the patients decreased this risk and have to be constantly applied in the future to all the hemophilia patients in our country who are still treated with »classic« replacement therapy. The only optimal solution is to abandon treatment with non-inactivated blood-products and to totally cover the substitution needs by using plasma derived or recombinant factor concentrates.

References

1. Tegtmeier GE: Infectious diseases transmitted by transfusion: a miscelanea. Vox Sanguinis 1994; 54:179–181.
2. Barbara JAJ: Transfusion-transmitted infections: Epidemiology relevant to blood safety. In Rock G et al: Quality assurance in Transfusion Medicine Vol I, pp419–444; CRC Press-Boca Raton USA, 1992.
3. Sherlock S: Clinical features of hepatitis. In Zuckerman AJ, Thomas HC Viral Hepatitis. Scientific basis and clinical management. New York: Churchill Livingstone, 1993:1–17.
4. Dodd RY: The risk of transfusion-transmitted infection. N Engl J Med 1992; 327:419–421.
5. Nelson KE, Ahmed F, Ness P, Donahue JG: The incidence of post-transfusion hepatitis. N Engl J Med 1993; 328:1280–1281.
6. Barbara JAJ: Hepatitis B: Old virus, new problems? Vox Sang 1993; 45:239–242.
7. Beasley RP: Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 1988; 6(10) 1942–1956.
8. Kleiman S, Busch M, Holland P: Post-transfusion hepatitis C virus infection. N Engl J Med 1992; 327:1601.
9. Donahue JG, Munoz A, Ness PM et al: The declining risk of post-transfusion hepatitis C virus infection. N Engl J Med 1992; 327:369–373.
10. Esteban JI, Gonzales F, Hernandez JM et al: Open prospective efficacy trial of anti-HCV screening of blood donors to prevent posttransfusion hepatitis. In Hollinger FB, Lemon SM, Margolis H Viral Hepatitis and Liver Diseases. Williams and Wilkens, Baltimore, pp396–402.
11. Lemon SM: The natural history of hepatitis A: The potential for transmission by transfusion of blood and blood products. Vox Sang 1994; 67(suppl 4):19–24.
12. Tegtmeier GE: Posttransfusion cytomegalovirus infection. Arch Pat Jab Med 1988; 113:236–245.
13. Gibbs WN, Corcoran P: Improving safety of blood transfusion in developing countries. Vox Sang 1994; 67:61–66.
14. Stivala JFA, Wagstaff W: Quality assurance in blood transfusions. Vox Sang 1994; 67(suppl 5): 37–42.
15. Hewitt PE, Barbara JAJ, Contreras M: Donor selection and microbial screening. Vox Sang 1994; 67 (suppl 5): 14–19
16. Woodfield G: Predonation screening and pretransfusion testing. Vox Sang 1994; 67(suppl 5): 20–27.
17. Blajchman MA, Bull SB, Feinman SV: Post-transfusion hepatitis: impact of non-A, non-B hepatitis surrogate tests. The Lancet, 1995; 345:21–25

18. Aach RD, Szmuness W, Mosley JW et al: Serum alanine aminotransferase of donors in relation to the risk of non-A, non-B hepatitis in recipients: the transfusion-transmitted viruses study. N Engl Med 1981; 304:989–994.
19. Clements CJ, Kane M, Hu DJ et al: Hepatitis B vaccine joints the fight against pandemic disease. World Health Forum 1990; 11:165–168.
20. West DJ, Calandra GB, Ellis RW: Vaccination of infants and children against hepatitis B. Ped Cl of N Am 1990; 37(3):585–601.
21. Jefferson T, Demicheli V: Is vaccination against hepatitis B efficient? A review of world literature. Health Economics 1994; 3:25–37.
22. SmithKline Beecham Biologicals: Engerix-B. Meeting the challenge of hepatitis B, 1994; 1–51.

Symptomatic Onset of severe Hemophilia A in Childhood is dependent on the Presence of Prothrombotic Risk Factors

S. HALIMEH, C. ESCURIOLA ETTINGSHAUSEN, K. KURNIK, R. SCHOBESS, C. WERMES, H. POLLMANN, W.D. KREUZ and U. NOWAK-GÖTTL

Introduction

Hemophilia A and B are X-linked genetic hemorrhagic disorders resulting from deficiencies of blood coagulation factor VIII or IX. Subjects suffering from plasma levels of factor VIII coagulant activity or factor IX below 1% of normal are classified as severe hemophiliacs (1). While mild or moderate hemophilia is not always diagnosed during childhood, severe hemophilia is generally diagnosed at an early age (2–6). Although bleeding symptoms correlate with the levels of the remaining factor activity, it is reported that not all hemophilic subjects with factor levels <1% bleed with the same severity (7,8), and in rare cases of severe hemophilia A (9), thrombotic episodes have been reported also in childhood (9–15).

So far, various molecular defects of different hemostatic components have been established as risk factors for venous thromboembolic diseases in children and adults: deficiencies of protein C, protein S and antithrombin, resistance to activated protein C mostly due to the factor (F) V1691 A gene mutation, and the prothrombin (PT) G20210 A genotype respectively (16–19).

Besides the possibility that the mutation type within the factor VIII gene may influence the clinical severity of hemophilia (20,22), it has been recently suggested that the clinical phenotype of severe hemophilia A is influenced by co-inheritance with the factor V1691 A mutation (21). The present study was therefore conducted to unravel the role of prothrombotic risk factors, i.e. deficiencies within the variant, coinherited with severe hemophilia A with respect to the first symptomatic onset of the disease in pediatric Caucasian patients.

Methods

Inclusion Criteria

Untreated Caucasian infants and children with previously undiagnosed severe hemophilia A (FVIII activity <1%) aged neonate to 16 years who were admitted to the university pediatric hospitals in Frankfurt, Halle, Hanover, Munich and Münster, Germany at the first symptomatic and spontaneous onset of the disease. In the patients enrolled, the classification of reagents and factor VIII-deficient plasma were investigated.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Exclusion Criteria

Pretreated pediatric patients, subjects in whom surgery or major (birth-) trauma-induced bleeding had occurred (5,6). In addition, patients with prenatal diagnosis of hemophilia A (HA) were excluded, as were children with a diagnosis of hemophilia before their first bleeding episode.

Study Endpoint

First symptomatic bleeding to the diagnosis of severe hemophilia A (2,3).

Study Period

From October 1985 to December 1999, 124 consecutive Caucasian pediatric patients with a first symptomatic onset of hemophilia A and B were recruited from different geographic areas of Germany.

Study Population

On the 124 consecutively recruited children, 111 were suffering from HA, and in 13 subjects hemophilia B was diagnosed. The entire study population presented here included 92 children with severe HA. Patients with hemophilia B were not included in the data presented here.

Statistics

The probability of the first bleeding onset as a function of time was determined with the method of Kaplan and Meier including the log-rank risk factors with non-carriers.

Results

Clinical presentation onset: in subjects with severe HA the majority of cases presented with mouth bleeding (28%), bleeding from soft tissues (19%), joint bleeding (18%), large hematomas (17%) or muscle bleeding (14%). In addition, gastrointestinal hemorrhage occurred as leading symptoms in 3% of patients and intraventricular hemorrhage in 1% of patients.

Prevalence of prothrombotic risk factors in German children with severe HA: the prevalence of prothrombotic risk factors in children with severe HA was not different from previously reported data: FV G1691 A 6.5% (6/92), PT G20201 A 3.2% (3/92), and protein C type deficiency 1.1% (1/92). No deficiency states of antithrombin or protein S were found in this cohort of hemophilic patients.

Age at First Symptomatic Bleeding

The first symptomatic bleeding leading to diagnosis of severe hemophilia occurred with a median (range) age of 1.6 years (0.5–7.1) in children carrying defects within the protein C pathway or the PT gene mutation compared with 0.9 years (0.1–4.0) in non-carriers of prothrombotic risk factors (P=0.01).

Discussion

From a small-scale study it has been suggested that coinheritance with the FV G1691 A mutation, especially when sharing the identical factor VIII gene mutation (21), may influence the phenotype of severe HA. In contrast, data reported by Arbini et al. in hemophilic subjects, and very recently data shown by Lee et al. in 137 patients, failed to support these findings, showing that the proportion of severe hemophiliacs whose mild clinical course could be attributed to coinheritance with the FV G1691 A mutation tended to be small (23, 24).

Data from this multicenter cohort study clearly demonstrate that the first symptomatic bleeding onset not associated with surgery or other major trauma which leads to diagnosis of the disease in children with severe HA, occurs significantly later in life in hemophilic subjects carrying additional prothrombotic risk factors than in non-carriers, thereby supporting the hypothesis of Nichols et al. (21) that the hemophilia phenotype is influenced by coinheritance with prothrombotic risk factors.

In this survey 10 of 92 subjects (10.9%) with severe hemophilia A carried prothrombotic risk factors, i.e. the factor V G1691 A mutation (n=6), the PT G202010 A variant (n=3) and protein C type I deficiency (n=1), which is in the reported range for healthy Caucasian individuals (18,19). However, the development of symptomatic thromboembolism diagnosed in two of ten patients carrying additional prothrombotic risk factors (20%) is a rare complication reported in sporadic cases of severe hemophilia.

In conclusion, data of the cohort study presented here suggest that there is at least some evidence that the hemophilic phenotype is influenced by the presence or absence of prothrombotic risk factors.

References

1. Hoyer LW. Hemophilia A N Engl J med 1994;330:38–47.
2. Baehner RL StraussHS. Hemophilia in the first year of life. N Engl J Med 1966: 275:524–8.
3. Conway JH, Hilgartner MW. Initial presentation of hemophiliacs. Arch Pediatr. Adolesc Med 1994;148 589–94.
4. Ljung R, Petrini P Nilssson IM. Diagnostic symptoms of severe and moderate haemophilia A and B. Acta Pediatr.Scand 1990, 79:196–200.
5. Thacker KE, Lim T, Drew JH. Cephalhaematoma: A 10 year review. Aus NZ J Obstet Gynaecol 1987; 27:210–2.
6. Wiswell KE, Lim T, Drew JH. Risk from circumcision during the first month of life compared with those uncircumcised boys. Pediatrics 1989; 83:11011–5.
7. Walsh PN, Rainsford SG, Biggs R. Platelet coagulant activities and clinical severity in hemophilia. Thromb Diath Haemorrh 1973; 29:722–9.

8. Bauer KA, Mannucci PM, Gringeri A, Tradati F, Barzegar S, Kass BL, ten Cate H, Kestin AS, Brettler DB, Rosenberg RD. Factor IXa- factor VIIIa-cell surface complex does not contribute to the basal activation of the coagulation mechanism in vivo. Blood 1992; 79:2039–47.
9. Richie N, Woodman RC, Poon MC. Deep venous thrombosis in hemophilia A. Am J Med 1992; 93:699–700.
10. Vidler V, Richards M, Vora A. Central venous catheter-associated thrombosis in severe haemophilia. Br. J Haematol 1999; 104:461–4
11. Sullivan DW, Purdy LJ, BillinghamM, Glader BE. Fatal myocardial infarction following therapy with prothrombin complex concentrates in a young man with haemophilia A. Pediatrics 1984; 74:279–81.
12. Karayalcin G, Goldberg B, Cherrick I, Kurer C, Bierman F, Lanzkowsksy P. Acute myocardial infarction complicating prothrombin complex concentrate therapy in an 8-year-old boy with hemophilia A and factor VIII inhibitor. Am J Pediatr Hematol Oncol 1993; 15:416–9.
13. Peerlinck K, Vermylen J. Acute myocardial infarction following administration of recombinant activated factor VII (Novo Seven) in a patient with hemophilia A and inhibitor. Thromb Haemost 1999;82:17775–6.
14. Escuriola Ettinghausen C, Martinez Saguer I, Kreuz W. Portal vein thrombosis in a patient with severe hemophilia A and F V G1691 A mutation during continuous infusion of factor VIII after intramural jejunal bleeding - successful thrombolysis under heparin therapy. Eur. J Pediat 1999;158:180–2
15. Olcay L, Gurgey A, Toplaoglu H, Altay S, Parlak H, Firat M. Cerebral infarction associated with factor V Leiden mutation in a boy with hemophilia A. Am J Hematol 1997; 56:189–90
16. Lane DA, Mannucci PM, Bertina RM, Bocchkov NP, Bouljenkov V, Chandy M, Dahlbäck B, Ginter EJ, Miletich JP, Rosendaal FR, Seligsohn U. Inherited thrombophilia: Part 1. Thromb Haemost 1996,76:651–62.
17. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velde PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994;369:64–7.
18. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996;88:3698–703.
19. Junker R, Koch HG, Auberger K, Münchow N, Ehrenforth S, Nowak-Göttl U. Prothrombin G20210 A gene mutation and further prothrombotic risk factors in childhood thrombophilia. Arterioscler Throm Vasc Biol 1999;19:2568–72.
20. Oldenburg J, Schröder J, Schmitt C, Brackmann HH, Schwab R. Small deletion/insertion mutation within poly-A runs of the factor VIII gene mitigate the severe haemophilia A phenotype. Thromb Haemost 1998;79:452–3.
21. Nichols WC, Amano K, Cacheris PM, Figueiredo MS, Michaelides K, Schwaab R, Hoyer L, Kaufman RJ, Ginsburg D. Moderation of hemophilia A phenotype by the factor V R506Q mutation. Blood1996;88:1183–7.
22. Lakich D, Kazaian HH, Antonarkis SE, Gischier J. Inversions disrupting the factor VIII gene are a common cause of severe hemophilia A. Nature Gent 1993;5:236–41.
23. Arbini AA, Mannucci PM, Bauer KA. Low prevalence of the factor V Leiden mutation among »severe« hemophiliacs with a »milder« bleeding diathesis. Thromb Haemost 1995;74:1255–8.
24. Lee DH, Walker IR, Teitel J, Poon MC Ritchie B, Akabatut J, Sinclair GD, Pai M, Wu JWY, Reddy S, Carter C, Growe G, Lillicrap D, Lam M, Blajchman MA. Effect of the factor V Leiden mutation on the clinical expression of severe haemophilia A. Thromb Haemost 2000;83:387–9.
25. Ludlam CA. Treatment of haemophilia Br J Haematol1998;101:13–4.
26. Martinowitz U, Schulman S. Coagulation factor concentrates by continuous infusion. Trans Med Rev 1997;11:56–63.
27. Lindivist PG, Svensson PJ, Dahlbäck B, Marsal K. Factor V Q506 mutation (activated protein C resistance) associated with reduced intrapartum blood loss – a possible evolutionary selection mechanism. Thromb. Haemost 1998;79:69–73.

Differences between Neonates and Adults in Plasmin Inhibitory and Antifibrinolytic Action of Aprotinin

M. Ries, M. Zenker and J. Klinge

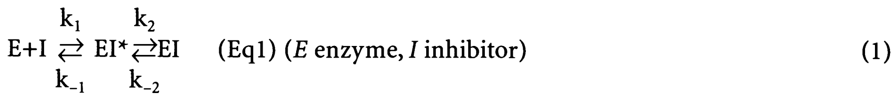
Introduction

The fibrinolytic system is involved in a wide variety of biological phenomena such as dissolution of fibrin blood clots, tissue remodeling, metastasis, angiogenesis, wound healing, embryogenesis and embryo implantation (1). It differs physiologically in neonates from older children or adults. The absolute and relative concentrations of various components differ from adults, and they are dependent on both the gestational age and postnatal age (for review see 2). In addition, the functional behavior of fetal plasminogen and fetal fibrinogen differ from the adult forms, although electrophoretic molecular weight analyses, as well as amino acid composition and partial amino acid sequencing revealed no differences between fibrinogen and between the plasminogen forms 1 and 2 of neonates and adults (3–12). Therefore, variations in functional behavior have been related to differences in carbohydrate composition (5). Oligosaccharides linked to proteins may contribute to receptor-mediated interactions, protein stability, clearance from the circulation, and physiological function (13, 14).

Like adult plasminogen, fetal plasminogen exists in two major glycoforms, plasminogen 1 and plasminogen 2. Both contain an O-glycosylation site at Thr^{345}, while type 1 plasminogen contains an additional N-glycosylation site at Asn^{288}. The difference in glycosylation is possibly responsible for the lower overall activation rates of the two fetal glycoforms when t-PA is used as an activator and CNBr-fibrinogen fragments as a stimulating effector (5). However, despite low plasminogen levels of ~50% of adult values and slower activation kinetics in the fetal plasma, the overall fibrinolytic activity measured by gross functional assays is quite similar to that of adult plasma (15–18).

Plasmin, a serine protease, is generated from its zymogen plasminogen by cleavage of the Arg^{561}-Val^{562} bond. This results in formation of the two-chain, disulfide-bonded plasmin with a heavy chain which contains kringles 1–5 and the lysine-binding sites (LBS), and a light chain which contains the catalytic serine protease site (19). Similar to plasmin-α_2-antiplasmin inhibition (20), the reaction between aprotinin and plasmin proceeds in a two-stage mechanism (21): a fast reaction with a loose complex (EI*), followed by a tightly bound, stable complex (EI), in which enzyme and inhibitor remain largely unchanged (Eq 1).

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002



$$E+I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI^* \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EI \quad \text{(Eq1) } (E \text{ enzyme}, I \text{ inhibitor}) \tag{1}$$

Previously, we have shown that plasmin-α_2-antiplasmin reaction is significantly slower in fetal plasmin type 1 and type 2 compared with the respective adult plasmin types (22).

An understanding of the ontogeny of hemostasis will optimize the prevention, diagnosis, and treatment of hemostatic problems during the neonatal period. A full understanding of the hemostatic system of the neonate includes the characterization of fetal forms of coagulation and fibrinolysis proteins with respect to structure and function.

Although aprotinin has been used in neonates and small infants with congenital heart disease, there have been no investigations into the effect of aprotinin on the neonatal fibrinolytic system. The aim of our study was to look at the possible influence of glycosylation on plasmin activity by determining the reaction kinetics of both adult and fetal plasmin with aprotinin. In addition, we investigated the effect of aprotinin on clot-lysis experiments with neonatal as well as adult plasma.

Patients and methods

Plasma Samples

After obtaining informed consent, blood samples were taken from the placenta of 15 newborn babies by atraumatic puncture of the umbilical vein immediately after the cord was clamped but before separation of the placenta. These infants were full term with no evidence of fetal distress during labor, adequate size for gestational age, had Apgar scores of 8 or more, and no problems for the first 5 days of life. They were born to healthy mothers after uncomplicated pregnancy and delivery. Prior to further analyses, hereditary thrombophilia was excluded by laboratory analyses. Plasmas from ten healthy adults were taken as controls.

The blood was anticoagulated with 0.11 mol/L sodium citrate (1:9, v/v, anticoagulant/blood) and centrifuged at 1500 g for 15 min to obtain the plasma. The platelet poor plasma was quickly frozen and stored at –40°C.

Purification of Plasminogen

Plasminogen was isolated from plasma in the presence of aprotinin by affinity chromatography on lysine-Sepharose (23). This was followed by a gel filtration on Sephadex G-200 to remove aprotinin and EACA. The purified plasminogen was dialyzed against distilled H_2O, and lyophilized. To prevent conversion of Glu-plasminogen into Lys-plasminogen by remaining minimal amounts of plasmin, the plasminogen samples were incubated with aprotinin-Sepharose for 15 min after the gel filtration step. In addition, aprotinin-Sepharose was present during the dialysis steps and finally removed by filtration.

Preparation of Plasmin

Plasminogen was activated to plasmin by use of high molecular weight two-chain u-PA (1st IS, code 87/594 from National Institute for Biological Standards and Control, South Mimms, UK), which was coupled to CNBr-activated Sepharose (all coupling reactions of proteins to CNBr-activated Sepharose 4B were performed in a 0.1 M Borate-buffer, 0.5 M NaCl, pH 8.3, followed by blocking of the residual sites with the same buffer containing 1 M ethanolamine). Active plasmin was flash frozen in liquid nitrogen and stored in aliquots at –40°C for further use. Plasmin concentrations were determined by active site titration with 4-methylumbelliferyl p-guanidinobenzoate hydrochloride, essentially as described (24). K_m and k_{cat} values for each plasmin preparation with the chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251) were calculated from the Michaelis-Menten equation using non-linear regression analysis. This was necessary for the determination of the maximal velocity of plasmin towards S-2251 (v_{max}) in the inhibition studies.

Aprotinin

The concentration of aprotinin was determined by titration against plasmin of known concentration.

SDS-Page

Polyacrylamide gel electrophoresis in the presence of SDS was performed on 7.5% acrylamide gels (Phast System, Pharmacia, Sweden) to demonstrate the purity of the proteins used in the experiments.

Inhibition Studies

All kinetic experiments were performed in a 0.01 M Hepes-buffer, 0.15 M NaCl, pH 7.4, containing 0.01% Tween 20 at 37°C. All experiments were done in duplicate.

The mode of the plasmin-aprotinin interaction was investigated by slow binding kinetics methods (20). To determine inhibition constants, the onset of inhibition was monitored by adding plasmin at a final concentration of 0.5 nM to a mixture of 1 mM S-2251 and aprotinin at concentrations from 0–20 nM. The final volume of each mixture was 250 µl and was overlaid by 50 µl mineral oil to prevent desiccation during the reaction period. Reactions were monitored at 53-s intervals for 5 h in order to establish good estimates of initial reaction rates as well as final steady-state rates. Data were fitted to the integrated rate equation for slow binding inhibition according to Longstaff and Gaffney (20) by nonlinear regression analyses. Fitting generated values for v_o (initial rate), v_s (final rate), k' (apparent rate constant for the transition from v_o to v_s) and A_o (the initial absorbance at 405 nm). Values for v_{max} were calculated from the enzyme reaction rate in the absence of inhibitor. These

values were then used to generate rate constants and inhibition constants. The slope of the line in a plot of v_{max}/v_o versus [I] was used to calculate $K_{i\ initial}$, the initial dissociation constant for the loose complex in equation 1. Similarly, a plot of v_{max}/v_s versus [I] was used to calculate $K_{i\ final}$, the overall inhibition constant for the binding of plasmin and aprotinin. The value of k_{-2}, the dissociation rate constant for the tight complex was calculated from the relationship:

$$k_{-2} = k' \, v_s/v_o$$

In addition, k_2 was calculated from the formula:

$$k_2=[(K_{i\ initial}/K_{i\ final})\ (k_{-2})]-k_{-2}$$

The use of S-2251 in a concentration of 1 mM prevented significant substrate depletion during the whole experiment.

Microtiter Plate Clot-lysis Assay

We used a modification of the microtiter plate clot-lysis assay previously described (18). In brief, clots were formed after adding citrated blood to Tris-HCl-buffer (0.05 M, pH 7.4), thrombin (final concentration 2 IU/ml), calcium chloride (final concentration 10 mM), rt-PA (final concentration 0.2 µg/ml) or urokinase (final concentration 125 IU/ml) and different concentrations of aprotinin (final concentration 0–40 KIU/ml). Clotting and dissolution were monitored as a rise and subsequent fall in sample turbidity measured at 405 nm using a computer-operated microtiter plate reader. Absorbance values were monitored at 1-min intervals for 1 h at 37°C.

Results

Catalytic Constants of Fetal and Adult Plasmin with Chromogenic Substrate S-2251

Values for K_m and k_{cat} of fetal plasmin with S-2251 were lower than the values for adult plasmin with more abundant differences in k_{cat} than in K_m (Table 1).

Table 1. Catalytic constants K_m, k_{cat} and the catalytic efficiency k_{cat}/K_m of fetal and adult plasmin with the chromogenic substrate S-2251

Plasmin type	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (10^4 $M^{-1}s^{-1}$)
Adult plasmin	0.169±0.015	19.8±0.8	11.7±1.1
Fetal plasmin	0.162±0.016	12.1±0.7	7.5±0.9

Kinetics of Fetal and Adult Plasmin Inhibition by Aprotinin

The data obtained for the reactions of aprotinin with both fetal and adult plasmin fitted well to the integrated rate equation for slow binding inhibition

$$A = v_s\, t+(v_o-v_s)\,(1-e^{-k't})/k'+A_o$$

by nonlinear regression analysis, indicating reversible slow binding inhibition to give an initial loose complex followed by a tight complex. This clearly confirms that fetal plasmin reacts with aprotinin in a two-step procedure as has been described for adult plasmin. The raw data for the inhibition of fetal plasmin by different concentrations of aprotinin are given in Fig. 1. No significant differences of the kinetic constants could be observed between fetal and adult plasmin (Table 2).

Table 2. Values for the rate constants of plasmin-aprotinin reaction

	$K_{i\ initial}$ (nM)	$K_{i\ final}$ (pM)	k_{-2} ($10^{-6}s^{-1}$)	k_2 ($10^{-3}s^{-1}$)
Adult plasmin	1.51±0.16	68.4±8.1	55.9±3.1	1.18±0.21
Fetal plasmin	1.40±0.14	68.1±9.0	53.9±3.0	1.05±0.19

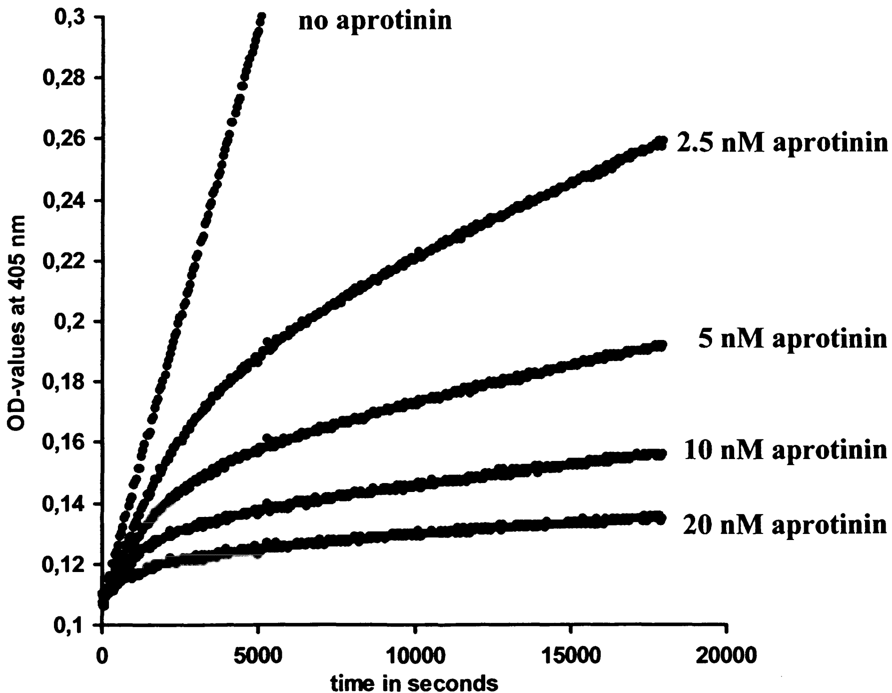


Fig. 1. Raw data for the inhibition of fetal plasmin (0.5 nM) by different concentrations of aprotinin (0–20 nM). Reactions were monitored at 53-s intervals for 5 h. The data obtained fitted well to the integrated rate equation for slow binding inhibition

Clot-lysis Experiments

The 50% clot-lysis times with rt-PA and urokinase (without aprotinin) are listed in Table 3. There were no significant differences between neonates and adults.

To study the effect of aprotinin, we measured the clot-lysis in % after 1 h at aprotinin concentrations ranging from 5–40 KIU/ml (final concentration). Clot-lysis at all tested aprotinin concentrations was significantly less in neonatal plasma compared with adult plasma. The antifibrinolytic action of aprotinin in neonatal plasma had about twice as much effect in adult plasma (Figs. 2, 3).

Table 3. Fifty percent clot-lysis times with rt-PA and urokinase (without aprotinin)

	Urokinase (125 IU/ml)	rt-PA (0.2 μg/ml)
Neonates	21.4±6.9	18.1±3.9
Adults	22.1±7.4	17.7±4.1

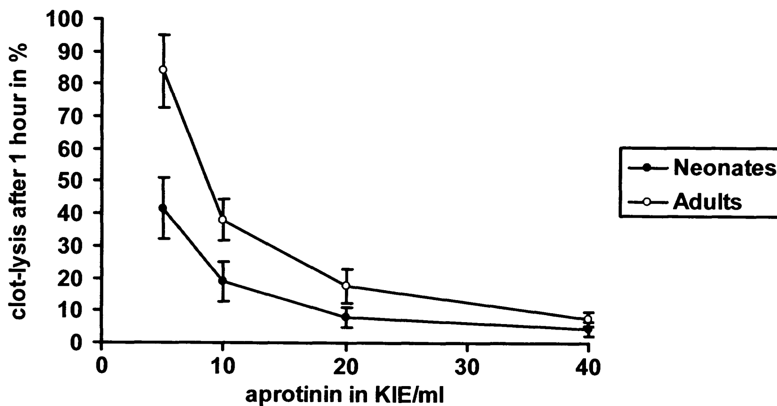


Fig. 2. Clot-lysis in % after 1 h at aprotinin concentrations ranging from 5–40 KIU/ml (final concentration). Clot-lysis experiments were performed with urokinase. Clot-lysis at all tested aprotinin concentrations was significantly less in neonatal plasma compared with adult plasma. The antifibrinolytic action of aprotinin in neonatal plasma had about twice as much effect in adult plasma

Discussion

Throughout childhood, hemostasis is a dynamic, evolving system. Marked differences to that in adults have been reported especially in premature babies and neonates. Most levels of coagulation and fibrinolysis factors are lower than those of the adult (for review see 2). In normal pregnancy and perinatal period, bleeding and thromboembolic complications are rare events, indicating that coagulation and fibrinolysis are well regulated. Therefore, hemostasis in neonates must be considered physiologically adequate.

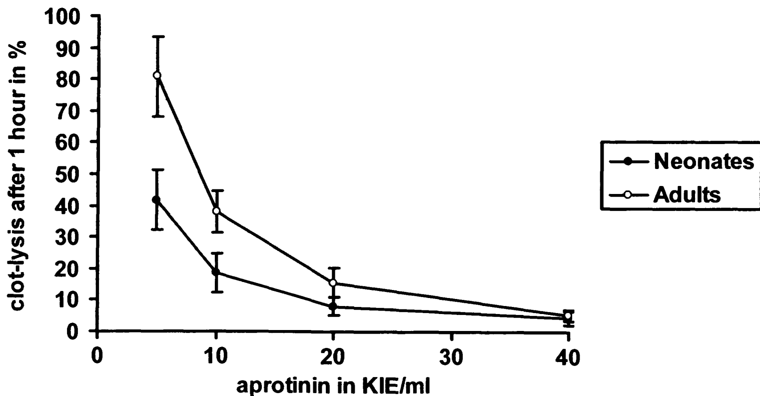


Fig. 3. Similar results for clot-lysis were obtained when rt-PA was used instead of urokinase

In all proteins involved in fibrinolysis and in the coagulation cascade, a number of posttranslational modifications occur. These posttranslational modifications can affect secretion, binding properties and plasma half-life and may be important for functional activity of the protein (13, 14).

Characterization of fetal forms of coagulation and fibrinolysis proteins with respect to structure and function will improve the knowledge of the ontogeny of hemostasis and thus optimize the prevention, diagnosis, and treatment of hemostatic problems during the neonatal period.

Although the two major glycoforms of plasminogen have been extensively studied, the mechanism by which glycosylation alters plasminogen structure and function remains unclear. Plasminogen consists of five kringle regions and a serine proteinase domain. It is a mixture of two major types. Both contain an O-glycosylation site at Thr^{345} while type 1 plasminogen contains an additional N-glycosylation site at Asn^{288} (25–27). Recently, an additional O-glycosylation site has been found at Ser^{248} in plasminogen type 2 (28). Electrophoretic analyses revealed the same molecular weight for fetal and adult plasminogen. In addition, amino acid composition and partial amino acid sequencing revealed no differences between the plasminogen forms 1 and 2 of neonates and adults (5, 10). However, composition analyses of carbohydrate showed marked differences between fetal and adult glycoforms. Fetal plasminogen contained more mannose, N-Acetyl glucosamine and sialic acid as well as less galactose than the respective adult glycoforms while plasminogen activation kinetics with tissue-type plasminogen activator in the presence of CNBr-fibrinogen fragments have been found to be slower for both types compared with adult plasminogen (5). In addition, it could be demonstrated (5) that fetal plasminogen does not bind as well to cellular receptors as adult plasminogen. Previously, we could demonstrate that fetal plasmin (type 1 as well as type 2) is less inhibited by α_2-antiplasmin than the respective adult types (22). Differences in carbohydrate composition could well account for the differences in plasmin-antiplasmin reaction because both carbohydrate and lysine-binding sites are located in the kringle structures of plasmin and it could be shown that the plasmin-antiplasmin reaction is

markedly influenced by the presence of lysine-binding sites (29, 30). In contrast, it could be shown that plasmin-aprotinin reaction is not altered when lysine-binding sites are blocked by lysine-analogues (21). This could readily explain that, in contrast to plasmin-antiplasmin-inhibition, no differences between fetal and adult plasmin could be seen in the catalytic constants of plasmin-aprotinin reaction in our study. When aprotinin reacts with plasmin, it forms a stable stoichiometric 1:1 complex with plasmin. The reaction proceeds in two steps. In the first step, aprotinin rapidly forms a reversible complex with plasmin. The second step results in a tightly bound complex. In our analyses, all curves followed the same pattern, indicating reversible slow binding inhibition. Therefore, fetal plasmin reacts with aprotinin in the same manner as that of adult plasmin with a first complex formation followed by a much tighter second complex in a slow reaction step. Detailed kinetic analyses of plasmin-aprotinin reaction showed no differences between fetal and adult plasmin reactions.

The effect of all tested aprotinin concentrations on the inhibition of plasma clot-lysis was significantly higher in neonatal plasma than in adult plasma. The antifibrinolytic action of aprotinin in neonatal plasma had about twice as much effect in adult plasma (Figs. 2, 3). This clearly indicates that the fibrinolytic action of aprotinin is different in neonates compared with adults. Since we could not find differences in the inhibition kinetics of fetal plasmin compared to adult plasmin, we hypothesize that the results of the clot-lysis experiments are solely related to the lower plasminogen levels in neonates. Aprotinin is a small protein inhibitor of serine proteases belonging to the Kunitz family. It is a single-chain polypeptide with a molecular weight of 6512 Daltons, consisting of 58 amino acid residues. It is naturally occurring and is isolated from bovine lung tissue. Its main pharmacokinetic actions are based on its ability to inhibit proteases such as trypsin, plasmin and tissue kallikrein. In recent years it has been increasingly investigated as a therapeutic agent to reduce blood loss in cardiac and transplant surgery (31–34). Although it has been used in neonates and small infants with congenital heart disease, there have been no investigations into the effect of aprotinin on the neonatal fibrinolytic system. Our results provide a rational basis for dosage regimens of aprotinin therapy in neonates and small infants.

References

1. Collen D, Lijnen HR. Basic and clinical aspects of fibrinolysis and thrombolysis. Blood 1991; 78: 3114–3124
2. Andrew M. Developmental hemostasis: relevance to thromboembolic complications in pediatric patients. Thromb Haemost 1995; 74: 415–425
3. Benavent A, Estellés A, Aznar J, Martinez-Sales V, Gilabert K, Fornas E. Dysfunctional plasminogen in full term newborn – Study of active site of plasmin. Thromb Haemost 1984; 51: 67–70
4. Estellés A, Aznar J, Gilabert J, Parrilla JJ. Dysfunctional plasminogen in full-term newborn. Pediatr Res 1980; 14: 1180–1185
5. Edelberg JM, Enghild JJ, Pizzo SV, Gonzalez-Gronow M. Neonatal plasminogen displays altered cell surface binding and activation kinetics. Correlation with increased glycosylation of the protein. J Clin Invest 1990; 86: 107–112

6. Galanakis DK, Mossesson MW. Evaluation of the role of in vivo proteolysis (fibrinogenolysis) in prolonging the thrombin time of human umbilical cord fibrinogen. Blood 1976; 48: 109–118
7. Francis JL, Armstrong DJ. Sialic acid and enzymatic desialation of cord blood fibrinogen. Haemostasis 1982; 11: 223–228
8. Künzer W. Fetales Fibrinogen. Klin Wochenschr 1961; 39: 536–542
9. Ries M. Molecular and functional properties of fetal plasminogen and its possible influence on clot lysis in the neonatal period. Semin Thromb Hemost 1997; 23:247–252
10. Summaria L. Comparison of human normal, full-term, fetal and adult plasminogen by physical and chemical analyses. Haemostasis 1989; 19: 266–273
11. Witt I, Müller H. Phosphorus and hexose content of human fetal fibrinogen. Biochim Biophys Acta 1970; 221: 402–404
12. Witt I, Müller H, Künzer W. Evidence for the existence of fetal fibrinogen. Thromb Diath Hemorrh 1969; 22: 101–109
13. Cumming DA. Physiological relevance of protein glycosilation. Develop Biol Standard 1992; 76: 83–94
14. Kaufman RJ. Post-translational modifications required for coagulation factor secretion and function. Thromb Haemost 1998; 19: 1068–1079
15. Ekelund H, Hedner U, Nilsson IM. Fibrinolysis in newborns. Acta Paediatr Scand 1970; 59: 33–43
16. Phillips LL, Skrodelis V. A comparison of the fibrinolytic enzyme system in maternal and umbilical-cord blood. Pediatrics 1958; 22: 715–726
17. Ries M, Zenker M, Klinge J, Keuper H, Harms D. Age related differences in a clot lysis assay after adding different plasminogen activators in a plasma milieu in vitro. J Pediatr Hematol Oncol 1995; 17: 260–264
18. Ries M, Klinge J, Rauch R, Trusen B, Zenker M, Keuper H, Harms D. In vitro fibrinolysis after adding low doses of plasminogen activators and plasmin generation with and without oxidative inactivation of plasmin inhibitors in newborns and adults. J Pediatr Hematol Oncol 1996; 18: 346–351
19. Robbins KC, Summaria L, Hsieh B, Shah RJ. The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. J Biol Chem 1967; 242: 2333–2342
20. Longstaff C, Gaffney PJ. Serpin-serine protease binding kinetics: α_2-antiplasmin as a model inhibitor. Biochemistry 1991; 30: 979–986
21. Longstaff C. Studies on the mechanism of action of aprotinin and tranexamic acid as plasmin inhibitors and antifibrinolytic agents. Blood Coag Fibrinol 1994; 5: 537–542
22. Ries M, Egglezou S, Zenker M, Longstaff C, Dell A, Morris HR, Gaffney PJ. Differences between newborns and adults in the reaction kinetics of plasmin and α_2-antiplasmin. Thromb Haemost 1999; 82 (Suppl.): 733
23. Deutsch DG, Mertz ET. Plasminogen: Purification from human plasma by affinity chromatography. Science 1970; 170: 1095–1096
24. Jameson GW, Roberts DV, Adams RWA, Kyle WSA, Elmore DT. Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorometric titration. Biochem J 1973; 131: 107–117
25. Hayes ML, Castellino FJ. Carbohydrate of the human plasminogen variants. I. Carbohydrate composition, glycopeptide isolation, and characterization. J Biol Chem 1979; 254: 8768–8771
26. Hayes ML, Castellino FJ. Carbohydrate of the human plasminogen variants. II. Structure of the asparagine-linked oligosaccharide unit. J Biol Chem 1979; 254: 8772–8776
27. Hayes ML, Castellino FJ. Carbohydrate of the human plasminogen variants. III. Structure of the O-glycosidically linked oligosaccharide unit. J Biol Chem 1979; 254: 8777–8780
28. Pirie-Shepherd SR, Stevens RD, Andon NL, Enghild JJ, Pizzo SV. Evidence for a novel O-linked sialylated trisaccharide on Ser-248 of human plasminogen 2. J Biol Chem 1997; 272: 7408–7411

29. Christensen U, Sottrup-Jensen L, Magnusson S, Petersen TE, Clemmensen I. Enzymic properties of the neo-plasmin-Val-422 (miniplasmin). Biochim Biophys Acta 1979;567:472–481
30. Wiman B, Boman L, Collen D. On the kinetics of the reaction between human antiplasmin and a low-molecular-weight form of plasmin. Eur J Biochem 1978; 87: 143 - 146
31. Mossinger H, Dietrich W. Activation of hemostasis during cardiopulmonary bypass and pediatric aprotinin dosage. Ann Thorac Surg 1998; 65: S45 - S50
32. Peters DC, Noble S. Aprotinin: an update of its pharmacology and therapeutic use in open heart surgery and coronary artery bypass surgery. Drugs 1999; 57: 233–260
33. Rich JB. The efficacy and safety of aprotinin use in cardiac surgery. Ann Thorac Surg 1998; 66: S6 - S11
34. Spray TL. Use of aprotinin in pediatric organ transplantation. Ann Thorac Surg 1998; 65: S71 - S73

Shorter PFA-100 Closure Times (CT) in Neonates than in Adults: Role of Red Cells, White Cells, Platelets, and von Willebrand Factor

B. Roschitz, K. Sudi and W. Muntean

Introduction

Compared with adults, neonates have poor in vitro platelet function and very low levels of some procoagulatory factors (1). Their platelets react hyporesponsively to a variety of physiological agonists, resulting in decreased platelet activation and aggregation (2).

These findings would predispose adults to bleeding, but healthy full term infants do not show any bleeding tendency. Clinical experience does not indicate an increased risk of excessive bleeding during surgical procedures either.

The bleeding time is, in good correlation to clinical experience and in contrast to poor in vitro platelet function, shorter in healthy neonates than in older children and adults (3). Additionally, shorter closure times of cord blood than of adult blood were found by means of the Platelet Function Analyzer, PFA-100 (4, 5, 6).

The PFA-100 is a possible alternative to performing bleeding times, even in young children and neonates, giving a quick and reproducible measure of primary hemostasis. The reasons for shorter CTs in neonates compared with adults and older children have remained unclear, and in various hypotheses they were attributed mostly to increased activity of neonatal von Willebrand factor (vWF) (4, 5), which is elevated in plasma levels and has multimeres of higher molecular weight than in adults (7), and to the high hematocrit present in neonatal cord blood. Other blood constituents such as the very high white cell count possibly also might contribute to the short CT in neonates.

Shorter CTs in neonates might interfere with the use of this method to diagnose platelet disorders and von Willebrand disease, when the short CT is caused by other blood constituents than platelets or vWF.

In an attempt to clarify the cause of short CT in neonates, we investigated CTs in cord blood by modifying red blood cell, white blood cell, and platelet counts, after inhibition of platelet receptors and von Willebrand function.

Materials and Methods

Blood Sampling

Blood was collected from the umbilical vein of 70 full term neonates (gestational age >37 weeks) and from 25 healthy adult controls.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Preparation of Platelet-Rich Plasma, Platelet-Poor Plasma, and Washed Red Blood Cells

Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200 g for 10 min at room temperature. The PRP was carefully aspirated.

Platelet-poor plasma (PPP) was prepared by centrifugation of either whole blood or the remaining layer at 2800 g for 10 min.

After having removed PRP, PPP, and buffy coats red blood cells were resuspended in phosphate-buffered saline. Cells were washed and centrifuged three times at 170 g for 10 min. Cell counts were taken on red blood cells, buffy coats, PRP and PPP before any autologous or heterologous reconstitution was performed.

Preparation of Samples with Reduced Hematocrit or Reduced Platelet Count

Experiments with reconstituted blood were performed according to Dietrich et al. with some modifications (8). In short, reconstitution in the cord blood was performed by adding PRP, PPP, and buffy coats in different proportions to packed red blood cells, either to achieve adult hematocrit levels with neonatal platelet counts or neonatal hematocrit levels with adult platelet counts. Cell counts were checked in reconstituted samples.

Preparation of Leukocyte Free Blood Samples

PRP, PPP, buffy coats, and red blood cells were prepared as described above. The PRP and PPP were added again to the red packed cells to obtain nearly leukocyte-free samples while maintaining the same red cell and platelet counts as in the unaltered whole cord blood samples.

In Vitro Effects of Abciximab

The glycoprotein GPIIb/IIIa antagonist, abciximab (ReoPro®, Eli Lilly GmbH, Vienna, Austria) was added to cord blood to attain final antibody concentrations of 1,75 μg/ml in blood. Each aliquot was mixed by gentle inversion of the tube and incubated at room temperature for 10 min and then tested in duplicate with collagen/epinephrine and collagen/adenosine 5'-diphosphate cartridges.

Inhibition of vWF by Antibody

A polyclonal von Willebrand antibody (goat anti-human vWF, Affinity Biologicals, Incorporated; Hamilton, Ontario, Canada) was used. Cord blood was incubated at room temperature at a variation from 50–100 μg/ml, using the equivalent volume in whole blood, for 5 min prior to the blood being tested in the PFA-100.

Preparation for Heterologous Reconstitution

In order to get samples with adult red and white blood cells and neonatal PRP or vice versa, red and white blood cells were added to the heterologous PRP. To further identify the contribution of PPP to the CT, PPP was prepared either from adult or cord blood and added to the remaining whole blood layer in heterologous fashion.

Statistical Methods

Mann-Whitney's U-test, and analysis of variance (using the Student-Newman-Keuls correction) were used to compare parameters between groups where appropriate. Kruskal-Wallis test was used if variances were not normally distributed. Correlations between variables of interest were calculated using Pearson's correlation coefficient and Spearman's rank correlation. The significance level of *P*-values was set at 5%.

Results

PFA-100 measurements on normal adult subjects conducted in this study indicated a CT from 84 to 150 s for Col/Epi and from 64 to 98 s for Col/ADP.

For cord blood samples the range was from 50 to 112 s for Col/Epi and from 43 to 98 s for Col/ADP. Mean cord blood CTs were significantly shorter than those of adult controls, with respect to hematocrit levels (P=0.01), with respect to platelet count (P=0.00016) and with respect to white blood cell count (P<0.0001).

Effects of Decreased Hematocrit, Platelet Count, and White Blood Cell Count in Cord Blood

Cord blood has a higher hematocrit, higher platelet and higher white blood cell count than adult blood. To account for the importance of these parameters in CTs the following experiments were performed.

Hematocrit

Testing in the PFA-100 showed no significant differences in both cartridge types between the cord blood samples with unaltered cord blood hematocrit and the samples with hematocrit levels adjusted to normal adult range (Col/Epi P=0.29; Col/ADP P=0.46) (Fig. 1).

Platelets

The mean platelet count of the unaltered cord blood samples was about $266\times10^3/\mu l$, in the reconstituted samples (n=10) about $160\times10^3/\mu l$. Mean CTs for both cartrid-

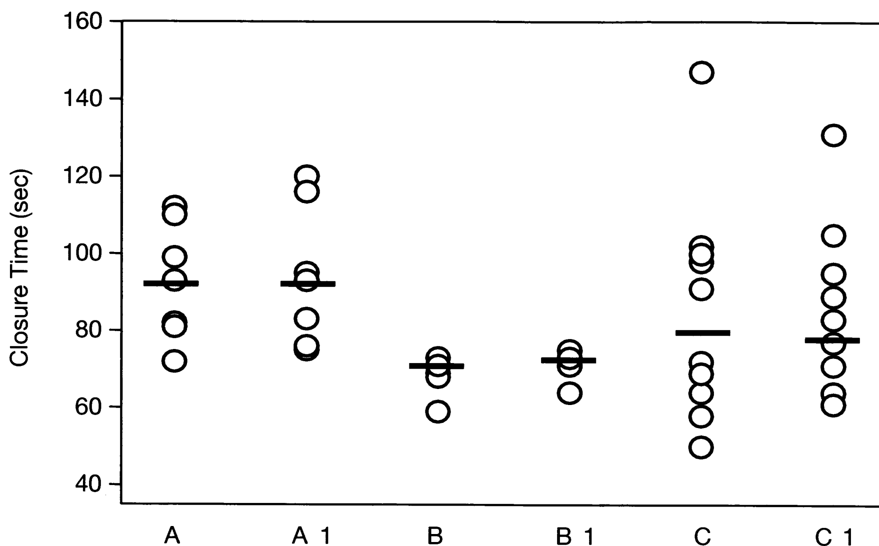


Fig. 1. CTs with the collagen/epinephrine cartridges in normal cord blood (A–C), and reconstituted cord blood samples. A1, nearly leukocyte free blood samples; B1 hematocrit lowered to adult samples; C1, platelets lowered to adult levels. Each symbol represents the median value of duplicate testing. Horizontal bars refer to mean CTs for each group of individuals

ges were not significantly longer after the platelet counts had been adjusted to adult levels (Fig. 1).

White Blood Cells

To show the contribution of white blood cells to CTs PRP and PPP were added to washed red cells to obtain nearly leukocyte free samples (n=7). The white blood cells were lowered from $11.7\times10^3/\mu l$ to $0.9\times10^3/\mu l$ (mean values). The leukocytes did not show any influence on the CT (Col/Epi P=0.4, Col/ADP P=0.28) (Fig. 1)

Heterologous Reconstitution

To further identify the contribution of cord blood constituents to the CT, PRP and PPP were prepared from either adult or cord blood and reconstituted in heterologous fashion.

When analyzing reconstituted blood (remaining adult cellular components and cord blood PRP) (n=5) CTs were significantly shorter than in unaltered adult whole blood samples.

Adult PRP in heterologous reconstituted cord blood (n=9) did significantly lengthen CTs in both cartridges compared to CTs of the unaltered cord blood samples (P=0.001).

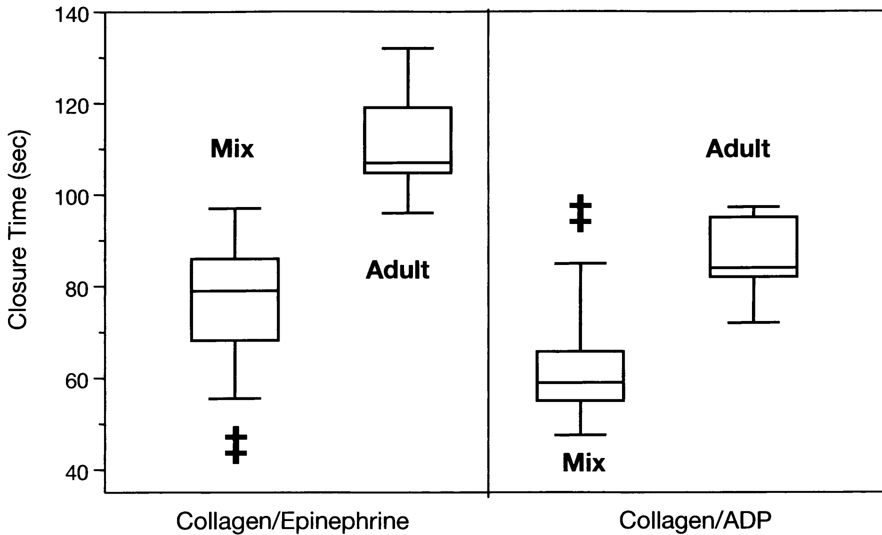


Fig. 2. Box plot of collagen/epinephrine and collagen/ADP CTs of adult controls (Adult) and samples, heterologously reconstituted with adult cellular components and cord blood PPP (Mix). A significant difference was seen between Adult and Mix CTs (C<0.0001)

To assess the importance of PPP, neonatal PPP was added to the remaining layer of adult whole blood (see Materials and Methods). In both cartridges significant differences between the reconstituted and unaltered blood sample (n=15) were found (Fig. 2).

Adding adult PPP to cord blood cellular components significantly lengthened the CTs of both cartridges.

Effect of a GPIIb/IIIa Antagonist, Abciximab

When adding abciximab to cord blood samples, three out of five samples in the Col/Epi, and four out of five samples in the Col/ADP cartridge failed to produce occluding platelet plugs, thus resulting in nonclosures. In the three other samples a prolongation was observed.

Inhibition of vWF by Antibody

After incubating cord blood samples (n=10) with a polyclonal von Willebrand antibody at a concentration of 100 μg/ml in both cartridges three out of five samples showed nonclosures and two a very strong prolongation. At a concentration of 50 μg/ml two nonclosures and three prolongations >200 s were found.

Discussion

In adults, the PFA-100 is a useful tool for diagnosis and therapeutic monitoring of patients with von Willebrand disease and for evaluating platelet function. Neonates have significantly shorter CTs than older children and adults (4). Reasons for these shorter CTs have remained unclear and were mostly attributed to the difference in cellular blood constituents. Hematocrit is higher in neonates than in adults which might influence flow and rheology. In our study the difference between cord blood and adult CTs remained significant after taking respect to higher hematocrit levels. In order to confirm these findings obtained by means of statistical methods, we carried out experiments with cord blood. By modulating hematocrit in reconstituted cord blood samples, we demonstrated that there was no substantial difference in CTs between the unaltered and the modified sample. Adjusting cord blood hematocrit to adult levels did not lengthen the neonatal CT to adult CTs. The results of our experiments showed that the difference in hematocrit between cord blood and adult blood did not account for shortened CTs in cord blood samples.

In adults a dependency of CT on platelet count has been shown. Neonates had slightly higher platelet counts than the adult control group in our study. The difference in platelet counts between cord blood samples and adult controls did not influence CTs in our study significantly, and, therefore, shorter CTs in neonates could not be explained by the slightly higher platelet counts in newborns than in the adult controls.

The most striking difference in cellular components between adults and neonates is the elevated white blood cell count. White cells vary widely in neonates. In the first days of life white blood cell counts up to 30×103/µl can be observed, decreasing within the first 3 months. Leukocytes might be important in platelet aggregation and plug formation. White cells can release prostacyclin and a platelet-activating-factor which modulate platelet function (9). To evaluate the possible role of white blood cells on CTs, PFA-100 measurements were undertaken with nearly leukocyte-free samples. Under these conditions CTs were similar to those of intact whole cord blood. Therefore our study did not provide any evidence that the high number of white cells in neonatal blood did significantly influence the CT.

Overall, our study does not give any support to the notion that the different amounts of cellular components in adult and neonatal blood are responsible for shorter CTs in neonates.

To further assess the possible role of neonatal platelets and plasma in shortening the cord blood CT, we performed mixing experiments with PRP and PPP. Cord blood PRP always leads to shorter CTs when added to adult cellular blood components, whereas neonatal CTs were significantly lengthened when removing its PRP and exchanging it for adult PRP. The fact that neonatal platelets were capable of interacting with plasma vWF very well in the PFA-100 was also supported by our results with abciximab. When ligand GPIIb-IIIa was inhibited, PFA-100 measurements resulted in nonclosures or strong prolongations in neonates, as has already been shown in adults (10). Overall our results suggest that neonatal platelets, despite their poor function in various in vitro tests, work well in the PFA-100.

But neonatal PPP alone added to adult cellular blood components containing adult platelets also shortened adult CTs, suggesting that the blood constituent responsible for shortening the CT has to be part of the PPP. Cord blood PPP did significantly shorten adult CTs, whereas neonatal CTs were lengthened when the cord blood plasma was replaced by adult PPP. It is known that vWF with multimeres of higher molecular weight is present in neonatal plasma (7). Therefore, it is an obvious hypothesis, since vWF has been shown to be important in the formation of the platelet plug at the membrane in the PFA-100 cartridges, that shortened CTs observed in neonates may be due to the elevated levels of their plasma vWF. Shorter CTs in the presence of neonatal PPP might be due to higher vWF multimeres in neonatal plasma allowing increased adhesion of the platelets to the PFA-100 membrane.

In conclusion, our study shows that short cord blood PFA-100 CTs are caused by a constituent of neonatal PPP, most likely the neonatal high multimeric vWF. The shorter CTs in neonates than in adults are not mediated by other cellular blood constituents, such as high hematocrit and high white blood cell counts. Our study demonstrates that the short CT in neonates is dependent on the same components, platelets and vWF, as in adults and it is therefore likely that the PFA-100 can be used in neonates in the same way as in adults to investigate platelet and vWF function. Whether von Willebrand disease and specific platelet disorders can be diagnosed in neonates by means of the PFA-100 remains to be verified in clinical studies.

References

1. Andrew M, Paes B, Milner R, Johnston M, Mitchell L, Tollefsen DM, Powers P. Development of the human coagulation system in the full-term infant. Blood 1987; 70: 165–72.
2. Israels SJ, Daniels M, McMillan EM. Deficient collagen-induced activation in the newborn platelet. Pediatr Res 1990; 27: 337–43.
3. Andrew M, Castle V, Mitchell L, Paes B. Modified bleeding time in the infant. Am J Hematol 1989; 30: 190–1.
4. Carcao MD, Blanchette VS, Dean JA, He L, Kern MA, Stain AM, Sparling CR, Stephens D, Ryan G, Freedmann J, Rand ML. The platelet function analyzer (PFA-100®): a novel in-vitro system for evaluation of primary haemostasis in children. Brit J Haematol 1998; 101: 70–3.
5. Israels SJ, Cheang T, McMillan-Ward E, Cheang M, McNicol A. The PFA-100® platelet function analyser for evaluating primary hemostasis in neonates. Thromb Haemost 1999; Suppl: 562.
6. Praun M, Till H, Glock, Schorp W, Nohe N, Auberger K, De Haan J. Easy testing of platelet function in children by means of the PFA-100®. Thromb Haemost 1999; Suppl: 563.
7. Katz J, Moake JL, McPherson PD, Weinstein MJ, Moise KL, Carpenter RJ, Sala DJ. Relationship between human development and disappearance of unusually large von Willebrand factor multimeres from plasma. Blood 1989; 73: 1851–8.
8. Dietrich G, Kretschmer V, Orth D, Haupt W. Primary hemostasis in hemodilution-1/ Hematocrit. Infusionstherapie 1990; 17: 212–3.
9. Blackwell GJ, Flower RJ, Russel-Smith N, Salmon JA, Thorogood PB, Vane JR. Prostacyclin is produced in whole blood. Br J Pharmacol 1978; 436–41.
10. Kundu SK, Heilmann EJ, Sio R, Garcia C, Ostgaard RA. Characterization of an in vitro platelet function analyzer PFA-100™. Clin Appl Thromb Hemost 1996; 2: 241–9.

V. Free Lectures

Chairmen:

H.-H. Brackmann (Bonn)
R. Schneppenheim (Hamburg)

Efficacy and Safety of a High Purity Protein C Concentrate in the Management of Patients with severe Congenital Protein C Deficiency

B. Moritz, S. Rogy, S. Tonetta, H.P. Schwarz, H. Ehrlich
and the CEPROTIN Study Group

Introduction

Severe congenital protein C deficiency is a very rare disease which is most often caused by a homozygous genetic defect and is inherited as an autosomal recessive trait. The prevalence of homozygous protein C deficiency has been estimated at 1 in every 160,000 to 360,000 births (Miletich, 1987). Double heterozygous defects can also result in severe protein C deficiency. Protein C deficiency is manifested by the occurrence of severe, often life-threatening thromboembolic disease. Affected children typically develop symptoms of purpura fulminans, cerebral thrombosis and/or retinal or vitreous hemorrhage/thrombosis during the first days or weeks of their life. In the absence of an effective replacement therapy, treatment options have been limited and of suboptimal efficacy.

Since the microcirculation is the major site of function of the protein C pathway, the consequences of severe protein C deficiency typically first become manifest in the capillaries of the skin. The clinical signs of purpura fulminans are the result of capillary thrombosis and interstitial bleeding and consist of ecchymotic skin lesions which, if untreated, rapidly develop into hemorrhagic bullae with subsequent gangrenous necrosis which may necessitate amputation. These lesions appear mainly on the extremities but also on other parts of the body (Marlar, 1989). Thrombosis may also occur in larger vessels and multi-organ failure develops in association with severe disseminated intravascular coagulation (DIC). Cerebral thrombotic complications (which may also occur in utero) are responsible for hydrocephalus, mental retardation, delayed development and seizures, and partial or complete blindness may result from retinal thrombosis (Branson, 1983).

Baxter Hyland Immuno has developed a Protein C Concentrate (CEPROTIN) for replacement therapy for prevention and treatment of severe protein C deficiency. CEPROTIN is a monoclonal antibody purified concentrate of protein C. Safety with regard to transmission of blood-borne viruses is provided by two independent virus inactivation steps, intensive detergent treatment (Tween 80) and vapor heating (steam treatment).

The same case reports of patients with severe congenital protein C deficiency treated with CEPROTIN and included in this analysis have already been previously reported (Dreyfus, 1995; Minford, 1995; Richards, 1997 and Sanz-Rodriguez, 1999).

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Demographic data for 22 subjects with a diagnosis of homozygous or double heterozygous protein C deficiency

Sex	Male: 11	Female: 11		
Family history of protein C deficiency	Yes: 9	No: 13		
Age at diagnosis	<1 year: 15	>1 year: 7		
Diagnosis	Homozygous: 17	Double heterozygous: 5		
Genetic analysis available	13 subjects (59%)			
Protein C activity at diagnosis	No. subjects	Age at first symptoms		
		≤ 1 month	>1 month	Unknown
0–3%	6	5		1
4–10%	7	4	3	
>10%	8	4	3	1
Unknown	1	1		
Total	22 (100%)	14 (64%)	6 (27%)	2 (9%)

Methodology

Twenty-two subjects with severe congenital protein C deficiency were treated in an open-label, multicenter study and under compassionate use provisions. Data were collected retrospectively to evaluate the efficacy and safety of CEPROTIN for the treatment of acute symptoms and for prophylactic treatment.

Demographic data for 22 subjects with a diagnosis of homozygous or double heterozygous protein C deficiency are presented in Table 1.

Some data obtained originally were not monitored. In 1998, a retrospective data collection was conducted in Germany, Italy, United Kingdom, Switzerland, Spain, Hungary, Turkey, Canada and the USA. New case report forms were designed to capture crucial information on diagnosis, treatment, treatment outcome and adverse experiences. Centers were contacted and consented to monitoring, and data were collected from subjects' medical records.

Clinical efficacy was evaluated in terms of regression of any skin lesions present at entry, and the dissolution of thrombotic occlusions was evaluated descriptively. Data from subjects who received CEPROTIN for short-term prophylaxis (prior to surgery or in situations of increased risk of thrombosis, such as infection and trauma) or long-term prophylaxis of thrombotic events or complications were evaluated descriptively.

Pharmacokinetic analyses were performed using both compartmental and non-compartmental models. Protein C activity was measured using the amidolytic and/or the anticoagulant test system.

For the safety analyses (adverse experiences and viral transmission) 57 additional subjects, who were on CEPROTIN for other types of protein C deficiency

(simple heterozygous, transient neonatal, acquired, or not specified) were included.

Patients with severe congenital deficiency were also monitored for the development of inhibitory antibodies against protein C using a chromogenic assay based on the principle of the Bethesda assay.

Results – Efficacy

Acute Presentations

Seventeen of 22 subjects were treated for the occurrence of acute symptoms.

A markedly improved or healed purpura fulminans and coumarin-induced skin necrosis could be demonstrated in all cases (Table 2).

Blindness caused by retinal thrombosis or hemorrhage could not be prevented in several subjects. This was probably due to the fact that treatment could not be initiated immediately. Retinal thrombosis occurred in 7 of 22 subjects. Marked improvement could only be achieved in one case, when replacement with CEPROTIN was started before retinal thrombosis was diagnosed.

Table 2. Treatment outcome evaluated by the investigators in a total of 37 acute clinical presentations

Clinical presentation	Marked improvement or healing	Moderate improvement	Slight improvement	No improvement	Not evaluated	Total
Purpura fulminans (10 subjects)	16					16
Coumarin-induced skin necrosis (4 subjects)	6					6
Cerebral thrombosis (1 subject)				1		1
Retinal/vitreous hemorrhage/thrombosis (7 subjects)	1		1	5	1	8
Deep vein thrombosis (1 subject)	1					1
Hematoma (2 subjects)	1				2	3
Catheter thrombosis (1 subject)					1	1
Osteonecrosis (1 subject)		1				1
TOTAL	25	1	1	6	4	37

Table 3. Summary of investigators' evaluations of overall success of treatment with CEPROTIN for 12 short-term treatment courses

Subject ID	Type of treatment	Investigator's evaluation of overall success
PC0024	Resection of infarcted bowel wall	Excellent
	Further resection of small intestine	Excellent
PC0031	Eye surgery	Excellent
	Eye surgery	Excellent
	Eye surgery	Excellent
	Dental surgery	Excellent
PC0036	Repair of ventricular septal defect	Excellent
	Lung infection	Excellent
	Lung infection	Excellent
PC0045	Circumcision	Excellent
PC0056	Endoscopic knee surgery	Excellent
PC0094	Evacuation of intracerebral hemorrhage	Good

Short-term Prophylaxis

Six subjects underwent short-term prophylaxis prior to or during surgery (Table 3). Investigators rated 11 of 12 treatment courses as excellent and one treatment (evacuation of intracerebral hemorrhage) as good. Only one minor thrombotic event occurred (asymptomatic catheter thrombosis).

Long-term Prophylaxis

Nine subjects received long-term treatment with CEPROTIN for a period of up to 7 years. Long-term prophylaxis treatment could be evaluated in seven of these subjects and was graded as excellent in all cases (Table 4). Three of these subjects received the product initially intravenously and subsequently, by independent decision of the investigator, were treated via a subcutaneous route.

Pharmacokinetics

The half-life evaluated for seven subjects using the compartmental model ranged from 1.1–9.7 h (median = 5.6 h), lower than previously described (range 7.8–11 h). Consumption of protein C during the acute phase could result in a shorter half-life. Also, subject-to-subject variability was high and the number of subjects limited. Half-life using the non-compartmental model ranged from 1.7–18.7 h (median = 10.1 h). Median in-vivo recovery was 41.4% (range 6.4–87.1%). These data are presented in Table 5.

Table 4. Duration of treatment with CEPROTIN, outcome evaluation (if available) and thrombotic events during long-term treatment

Subject ID	Long-term treatment duration	Outcome evaluation	Thrombotic episodes during long-term treatment
PC0006	11 January 1993–ongoing	Excellent	Mild thrombotic lesions below the right eye and the right foot probably due to bruising
PC0007	06 September 1992–ongoing	Excellent	None reported
PC0021	25 November 1995–18 April 1996 and 10 June 1996–ongoing	-	Purpura fulminans (interruption of Protein C treatment)
PC0026	25 January 1995–ongoing	Excellent	None reported
PC0030	15 January 1992–14 August 1992 and 31 October 1992–10 July 1994 and 10 October 1995–ongoing	Excellent	Purpura fulminans (interruption of Protein C treatment)
PC0036	14 October 1989–17 July 1992 (intermittent)	Excellent	Catheter thrombosis during hospitalization for pulmonary infection Necrotic ecchymosis of inferior limbs (no Protein C treatment prior to this event) Necrotic ecchymosis of the knees and lung infection
PC0069	30 June 1997–ongoing	Excellent	None reported
PC0102	11 February 1992–ongoing	Excellent	No recurrences after initiation of long-term Protein C treatment
PC4010	06 March 1998–ongoing	–	No recurrent problems

Table 5. Individual half-life values and in-vivo recovery for seven evaluable subjects

Subject ID	Age	Date of PK analysis	$t_{1/2}$ (h)	IVR (%)	Description of thrombotic symptoms	Date of onset
PC0013	15 days	19 November 1993	1.5	15.6	Purpura fulminans	06 November 1993
PC0017	4 days	18 March 1994	1.1	24.3	Purpura fulminans	15 March 1994
PC0028[a]	8 months	18 September 1995	3.9	14.8	Coumarin-induced skin necrosis	September 1995
PC0046	19 years	25 March 1992	8.6	nd	Deep vein thrombosis	February 1992
PC0049	17 years	16 November 1993	7.0	87.1	Coumarin-induced skin necrosis	October 1993
PC0056[a]	39 years	15 April 1997	6.8	nd	–	
PC0069	8 years	30 June 1997	9.7	58.5	–	

[a] Further PK studies were performed and are not presented here. The summary statistics, however, were calculated using the median over all PK parameters for each subject

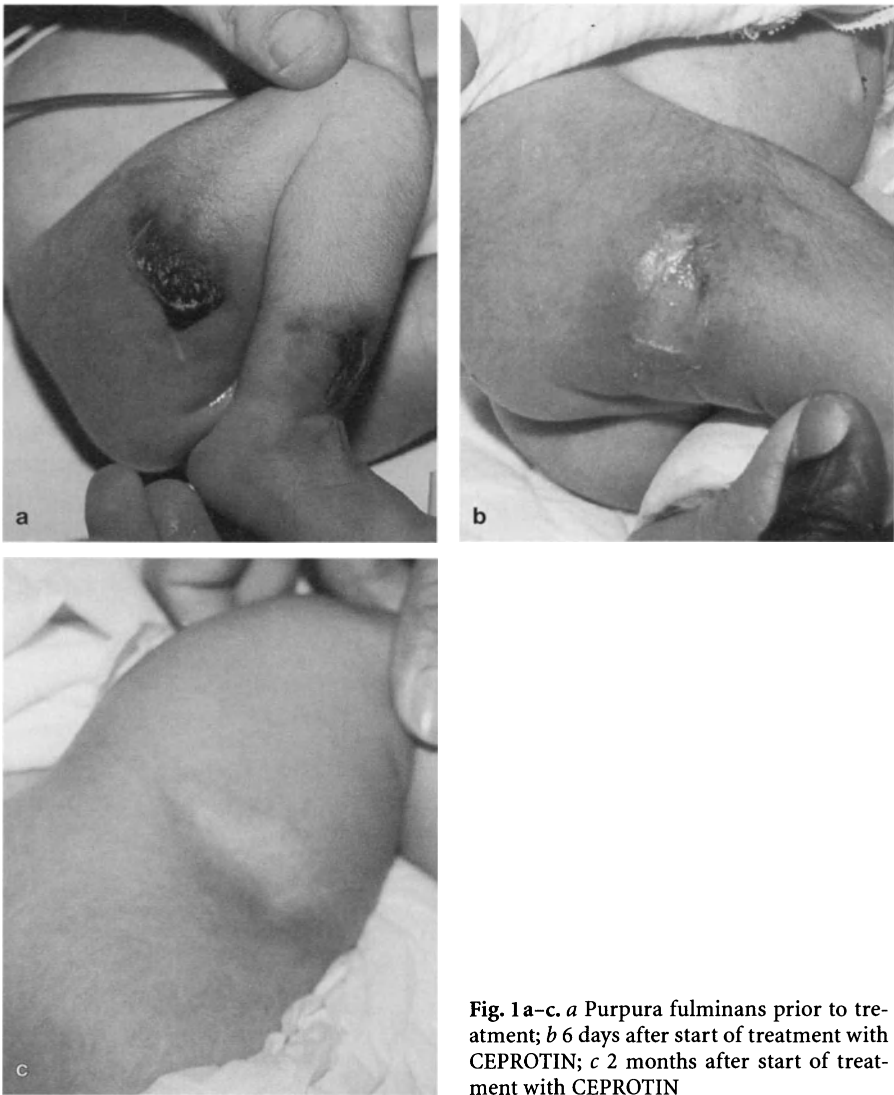


Fig. 1 a–c. *a* Purpura fulminans prior to treatment; *b* 6 days after start of treatment with CEPROTIN; *c* 2 months after start of treatment with CEPROTIN

Results – Safety

More than 7,500 CEPROTIN infusions were administered. No adverse experiences considered related to the product by investigators occurred. No viral transmission or seroconversion was observed in any subject.

Eleven subjects could be monitored for the development of inhibitory antibodies against protein C. These subjects received 31–2386 infusions. Individual total

doses of 22,500–2,180,600 IU CEPROTIN were administered; the treatment duration ranged from 1.5 to 9 years. None of the subjects developed inhibitory antibodies against protein C.

Conclusions

For the 22 homozygous or double heterozygous subjects, who either presented with acute symptoms (purpura fulminans, skin necrosis, thrombosis) or received CEPROTIN prior to surgery for prevention of thrombosis or thrombotic complications, the clinical outcome was rated excellent in 83% of the cases evaluated. A markedly improved or healed purpura fulminans and coumarin-induced skin necrosis could be documented in all 14 subjects and 22 treatment courses evaluated after CEPROTIN administration. (See Fig. 1a–c as an exemplary case).

CEPROTIN could not prevent blindness due to retinal thrombosis or hemorrhage in several subjects, which was probably due to the fact that treatment could not be initiated immediately after diagnosis of severe protein C deficiency or to thrombosis in utero.

The half-life evaluated for seven subjects using the compartmental model ranged from 1.1–9.7 h (median = 5.6 h), lower than previously described (range 7.8–11 h), since consumption of protein C during the acute phase could result in a shorter half-life. Subject-to-subject variability was high and the number of subjects limited.

No adverse experience related to the study drug occurred in any of the >7,500 infusions, no viral transmission, seroconversion or development of inhibitory antibodies against protein C was observed in any of the subjects.

In conclusion, CEPROTIN was shown to be safe and effective in the treatment of subjects with severe congenital protein C deficiency.

CEPROTIN Study Group

J. Allgrove	Newham General Hospital	London	United Kingdom
S. Altfeld/ J.E. Christoph	Kinderkrankenhaus auf der Bult	Hannover	Germany
K. Auberger	Dr. von Haunersches Kinderspital	Munich	Germany
L. Bomgaars	Texas Children's Hospital, Baylor College of Medicine	Houston	United States
J. Cano/ N. Gomez	Hospital Niño Jesus/ Hospital de le Princesa	Madrid	Spain
J. D. Cohen	St. Joseph's Hospital	Phoenix	United States

M. David	Hospital St. Justine	Montreal	Canada
V. De Stefano	Università Cattolica del Sacro »Agostino Gemelli«	Roma	Italy
M. Dreyfus	Hôpital Bicêtre	Paris	France
L. Jardine	The Dr. Charles A. Janeway Child Health Center	Newfound-land	Canada
V.V. Kakkar	Thrombosis Research Institute	London	United Kingdom
S. Kemahli	University of Ankara	Ankara	Turkey
W. Kreuz/ C. Escuriola Ettingshausen	Klinikum der Johann Wolfgang Goethe Universität	Frankfurt	Germany
G.A. Marbert	Kantonsspital Basel	Basel	Switzerland
A. Minford	St. Luke's Hospital	Bradford	United Kingdom
V.E. Mitchell	Leicester Royal Infirmary	Leicester	United Kingdom
K.J. Pasi	Royal Free Hospital	London	United Kingdom
B. Pötzsch	Kerckhoff-Klinik	Bad Nauheim	Germany
W. Schramm/ M. Spannagl	Klinikum Innenstadt der Universität München	Munich	Germany
R.F. Stevens	Manchester Children's Hospital	Manchester	United Kingdom
J. Teitel	St. Michael's Hospital	Toronto	Canada

References

1. Branson HE, Katz J, Marble R, Griffin JH (1983) Inherited protein C deficiency and coumarine: responsive chronic relapsing purpura fulminans in a newborn infant. Lancet ii: 1165–1168
2. Dreyfus M, Masterson M, David M, Rivard GE, Mueller FM, Kreuz W, Beeg T, Minford A, Allgrove J, Cohen J, Christoph J, Bergmann F, Mitchell VE, Haworth C, Nelson K, Schwarz HP (1995) Replacement Therapy with a Monoclonal Antibody Purified Protein C Concentrate in Newborns with Severe Congenital Protein C Deficiency. Seminars in Thrombosis and Hemostasis 21(4): 371–381
3. Marlar RA, Montgomery RR, Broekmans AW (1989) Diagnosis and treatment of homozygous protein C deficiency. Report of the working party on homozygous protein C deficiency of the subcommittee on protein C and protein S. International committee on thrombosis and haemostasis. Journal of Paediatrics 114: 528–534
4. Miletich J, Sherman L, Broze G (1987) Absence of thrombosis in subjects with heterozygous protein C deficiency. NEJM 317(16): 991–996

5. Minford AMB, Parapia LA, Stainforth C, Lee D (1995) Treatment of homozygous protein C deficiency with subcutaneous protein C concentrate. British Journal of Haematology 93: 469–470
6. Richards EM, Markis M, Preston FE (1997) The successful use of protein C concentrate during pregnancy in a patient with type 1 protein C deficiency, previous thrombosis and recurrent fetal loss. British Journal of Haematology 98: 660–661
7. Sanz-Rodriguez C, Gil-Fernandez JJ, Zapater P, Pinilla I, Granados E, Gomez-G. de Soria V, Cano J, Sala N, Fernandez-Ranada JM, Gomez Gomez N (1999) Long-term management of Homozygous Protein C Deficiency: Replacement Therapy with Subcutaneous Purified Protein C Concentrate. Thromb Haemost 81: 887–890

Respective Roles of Factors II, VII, IX, and X in the Procoagulant Activity of FEIBA

S. Gallistl, G. Cvirn, B. Leschnik and W. Muntean

Introduction

Recently we demonstrated increased generation of thrombin in the presence of the activated prothrombin complex concentrate (APCC) FEIBA (2). Determination of thrombin generation has been shown to be able to distinguish between different forms of hyper- and hypocoagulability (7). The observed increased thrombin generation in the presence of FEIBA might be connected with sporadically documented thrombotic complications after its administration (4). FEIBA contains factors II, VII, IX, and X, partially in their activated form. Respective roles of each factor are still a matter of debate. Some authors assume the coagulant activity is mainly carried by factor Xa (6). It has also been claimed that the efficacy of APCC is related to the high content of factor IX or factor IXa (5). It has also been suggested that factor VIIa could by itself exert bypassing activity in patients with inhibitors (3). On the other hand it has been shown that free thrombin generation, at least in newborn plasma, exclusively depended on prothrombin concentration (1). Therefore, we investigated the respective roles of factors II, VII, IX, and X in the procoagulant activity of FEIBA after extrinsic activation of plasma.

Materials and Methods

Nine parts of blood were collected into one part of 0.1 molar citrate using a two syringe technique. Blood was taken from healthy volunteers who were not using drugs known to affect the coagulation system. To obtain platelet-poor plasma (PPP) blood was centrifuged at 3000×g for 15 min. Two hundred microliters of PPP was incubated with 20 µl GPRP dissolved in phosphate buffered saline (final concentration of GPRP: 4 mM) and 15 µl Tris-buffer and incubated at 37°C while stirring. To investigate the effect of FEIBA on thrombin generation Tris-buffer was supplemented with FEIBA to give a final concentration of 0.5 U FEIBA/ml PPP.

The contents of factors II, VII, IX and X were decreased by using immobilized sheep anti-human antibodies, coupled to agarose. Plasmas were activated by the addition of 200 µl Thromborel S.

For the determination of free thrombin generation at timed intervals samples were removed from the activated plasma into 300 µl substrate solution (containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg human serum albumin/ml, and

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

0.22 mM S-2238). After 5 min, 250 µl of 50% acetic acid (v/v) was added, and the absorbency was measured against a blank at 405 nm.

Results

Thrombin generation curves in the absence and presence of FEIBA, before and after the reduction of factors II, VII, IX, and X are shown in Figs. 1–4. The addition of FEIBA elicited thrombin generation to a significant extent.

The reduction of factor II concentration in FEIBA containing plasma (from 160% to 92%) and in normal plasma (from 90% to 52%) resulted in a significant decrease in free thrombin generation (Fig. 1) without influencing the lag phase until the onset of free thrombin generation (clotting time).

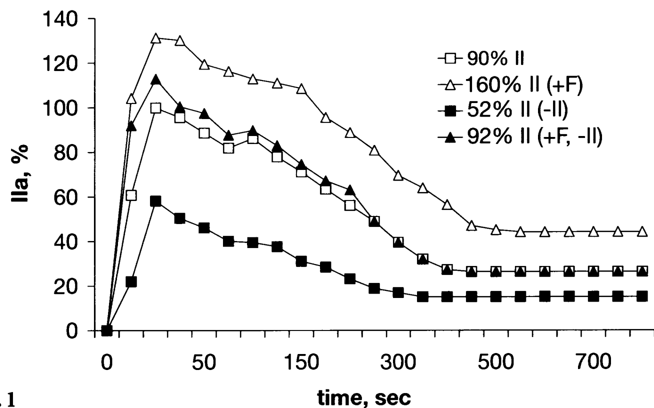


Fig. 1

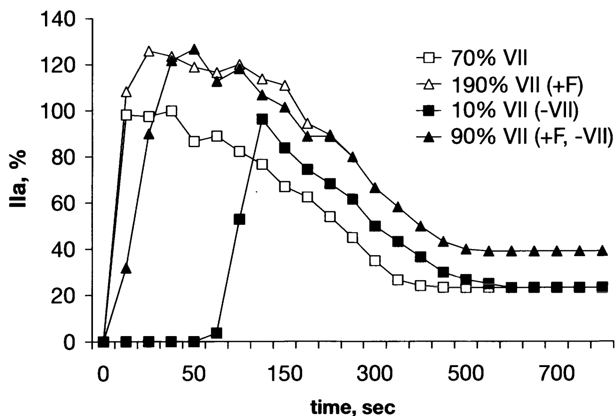


Fig. 2

Figs. 1–4. Thrombin generation curves in control plasmas (*open squares*), in control plasmas after reduction of factor II, VII, IX or X (*closed squares*), in plasmas containing FEIBA (*open triangles*), and in plasma containing FEIBA after reduction of factor II, VII, IX or X (*closed triangles*)

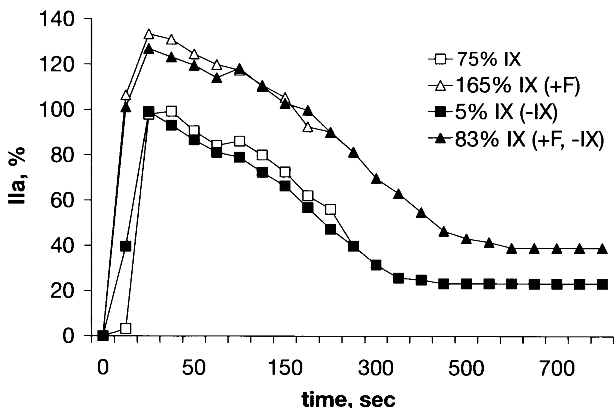


Fig. 3

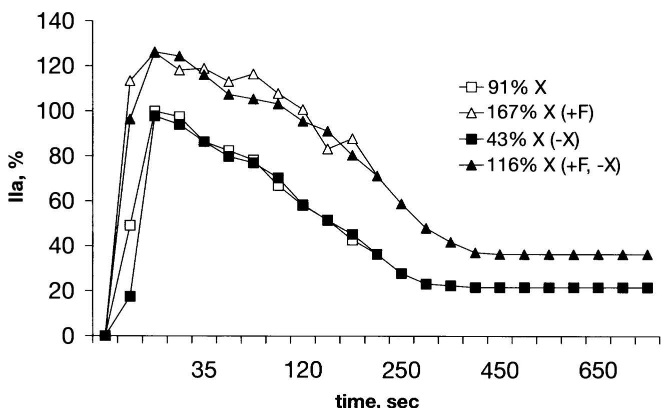


Fig. 4

The reduction of factor VII concentration in FEIBA containing plasma (from 190% to 90%) and control plasma (from 70% to 10%) showed no effect on free thrombin generation (Fig. 2). Factor VII reduction in control plasma resulted in a significant prolongation of the clotting time.

The reduction of factor IX concentration in FEIBA containing plasma (from 165% to 83%) and control plasma (from 75% to 5%) and the reduction of factor X concentration in FEIBA containing plasma (from 167% to 116%) and control plasma (from 91% to 43%) showed no effect on free thrombin generation or on clotting times (Figs. 3, 4).

Summary

Our study demonstrates that under our experimental conditions free thrombin generation critically depends on factor II concentration. This observation was true for control plasmas and plasmas containing FEIBA. Changes in factor VII concen-

tration influenced the clotting time, at least in control plasma, but not the amount of free thrombin generated. Changes in concentrations of factors IX and X had no influence on free thrombin generation or clotting times. Considering that factor II concentrations are normal in hemophilic patients with inhibitors, and that free thrombin generation might reflect hypercoagulability to some extent, our results suggest that the factor II content of APCC might contribute to its observed spontaneous clotting activity. Whether changes in factor II concentration might also influence the hemostatic properties of APCC cannot be answered by our experiments.

Acknowledgements. The study was supported by the Jubilaeumsfonds der Oesterreichischen Nationalbank (grant # 7777)

References

1. Andrew M, Schmidt B, Mitchell L, Paes B, Ofoso F. Thrombin generation in newborn plasma is critically dependent on the concentration of prothrombin. Thromb Haemost 1990; 63: 27–30.
2. Gallistl S, Cvirn G, Muntean W. Recombinant factor VIIa does not induce hypercoagulability in vitro. Thromb Haemost 1999; 81: 245–249.
3. Hedner U. Recombinant coagulation factor VIIa: From the concept to clinical application in hemophilia treatment in 2000. Semin Thromb Hemost 2000; 26: 363–366.
4. Lusher JM. Use of prothrombin complex concentrates in the management of bleeding in hemophiliacs with inhibitors–benefits and limitations. Semin Haematol 1994; 31: 49–52.
5. Philippou H, Adami A, Lane DA, MacGregor IR, Tuddenham EGD, Lowe GDO, Rumley A, Ludlam CA. High purity factor IX and prothrombin complex concentrate (PCC): Pharmacokinetics and evidence that factor IXa is the thrombogenic trigger in PCC. Thromb Haemost 1996; 76: 23–28.
6. Turecek PL, Varadi K, Gritsch H, Auer W, Pichler L, Eder G, Schwarz HP. Factor Xa and prothrombin: Mechanism of action of FEIBA. Vox Sang 1999; 77 (Suppl 1): 72–79.
7. Wielders S, Mukherjee M, Michiels J, Rijkers DT, Cambus JP, Knebel RW, Kakkar V, Hemker HC, Beguin S. The routine determination of the endogenous thrombin potential, first results in different forms of hyper- and hypocoagulability. Thromb Haemost 1997; 77: 629–636.

Capillary Microscopic and Rheological Dimensions for the Diagnosis of von Willebrand Disease in Comparison with other Hemorrhagic Diatheses

J. Koscielny, S. Mörsdorf, C. Mrowietz, H. Kiesewetter, U. Budde, U.T. Seyfert, E. Wenzel and F. Jung

Introduction

There are numerous casuistic reports of angiodysplasia in the large vessels (macrocirculation), especially in the gastrointestinal system in patients with von Willebrand disease (vWD).

Also macroscopic telangiectasia quite comparable to the Morbus Osler were found and impressively described [1, 2].

Early findings of previous studies showed variations in the system of the smallest vessels (microcirculation) in skin [3, 4]. In 1953 O'Brien [3] called it familiarly capillary fragility or a diffuse capillary telangiectasia (Fig. 1). A systematic and quantified examination of the abnormalities within the microcirculation was impossible in those days due to the fact that there were only microscopes with no online documentation or devices for analyzing pictures. In 1961 Blackburn [4] described capillary skin bleedings as a further abnormality of microcirculation, especially in vWD, using the microscopes of his time. After the early sixties the microcirculation in vWD did not attract much attention.

We were already able to differentiate between healthy individuals and patients with vWD [5, 6, 7]. The first video capillary microscopy assisted systematic quantification of the microangiopathy in patients with vWD, especially with type 1 vWD, was done in the nailfold capillaries.

Whilst disorders of single coagulation factors of the plasmatic system and/or thrombocytic defects can quickly be diagnosed, more elaborate work is required for vWD, especially for the more common light to middle-heavy cases based on the predominant intra- and interindividual variance of the laboratory parameters [8]. Nowadays state of the art video capillary microscopy combined with systems to analyze the pictures is a well accepted method in angiology for describing the state of microcirculation. We now raise the question of whether video capillary microscopy is able to improve our diagnostic spectrum concerning vWD, the hemorrhagic diathesis with the highest incidence in the population [9, 10]. Hemorrheological measurements for the screening of hemorrhagic diatheses were used in the past only sporadically.

The present prospective study examined whether patients with vWD differ from patients with other hemorrhagic diatheses by using intravital video capillary microscopic methods. Initially a single parameter analysis is used to determine the microcirculating and hemorrheologic variables. To optimize the selectivity a follow up multiparameter analysis is performed.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

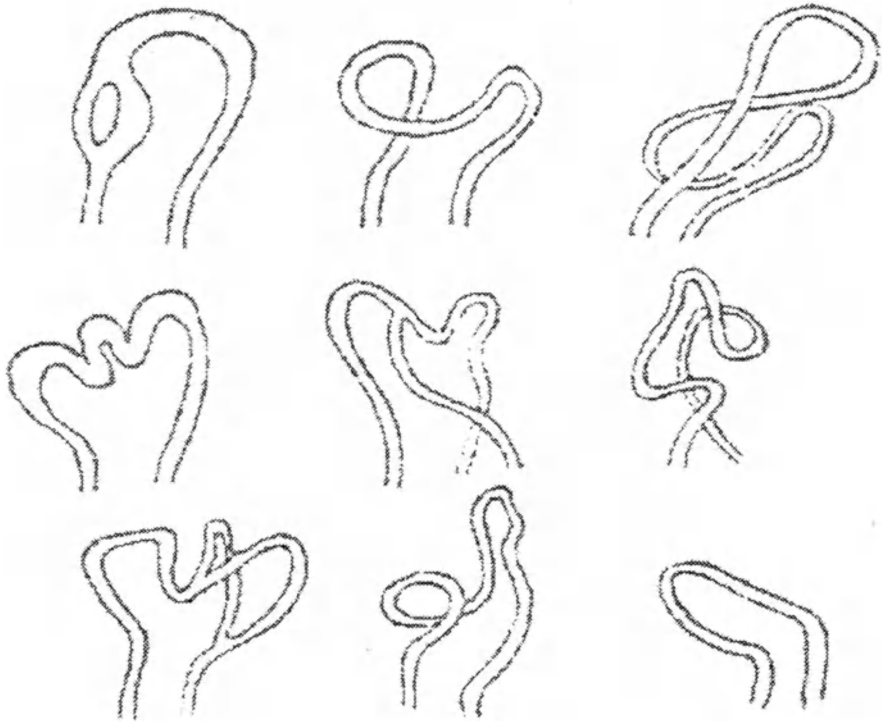


Fig. 1. O'Brien, 1953 (6): abnormal capillaries in patient severely affected with familial capillary fragility (vWD)

Materials and Methods

Patients

All patients gave their informed consent to participate in the study, which was approved by the local Review Board. To include different clinical types of hemorrhagic diatheses and a control group of healthy subjects (n=100), patients expressing the following disorders have been examined: vWD (n=100), thrombocytopathy (n=122); congenital (n=8: one Bernard-Soulier-syndrome, one thrombasthenia, six storage pool diseases) and acquired: (n=112: 49 drug induced during continuous medication - acetylsalicylacid, ticlopidine, diclofenac, clopidogrel - 26 caused by uremia, 37 cases of hepatopathies), thrombocytopenia with an average of 58,000 ± 20,000 platelets per µl (n=101: 34 caused by uremia, 32 cases of hepatopathies, 18 chronic autoimmune thrombocytopenia, 17 myeloproliferative syndromes), severe hemophilia A (n=50), severe hemophilia B (n=20), hereditary dysfibrinogenemia with clinically proven bleedings (n=22) and patients under continuous oral anticoagulation therapy with phenprocoumon (INR: 2.5±1.1) (n=112; indications for anticoagulation: 58 mechanical heart valves, 18 atrial fibrillation after cerebral ischemia, 11 protein-C-deficiency type I with recurrent thromboembolism, 8 protein-S-

deficiency type I with recurrent thromboembolism, 5 antithrombin-deficiency type IIb with recurrent thromboembolism, 4 protein-S-deficiency type II and heterozygous mutation in coagulation factor V Leiden with recurrent thromboembolism, 8 antiphospholipid-syndrome with recurrent thromboembolism). At the time of the examination there was no acute bleeding in any of the groups.

The diagnosis of the vWD was according to the subtype of the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis [11] referring to the multimeric [12]. Ninety-two of the vWD-patients showed a type 1 (definite type 1 vWD: 78 and possible type 1 vWD: 14) and eight patients a type 2 A. The bleeding time (surgicutt) of the vWD-patients was prolonged with an average of 8 min 30 s (± 2 min 10 s). The vWF:Ag was 36±22%, the vWF: RCof was 40±14%, the Factor VIII-activity was 68±17% and the aPTT was 40±6 s. The average duration of the disease or the time since the disease was diagnosed was 8 ± 3 years. The patients' history of having the vWD can be considered as being longer or even since birth. Clinical inspection revealed no pathologic findings. The patients came to the department for preoperative diagnosis or they came for routine controls. Table 1 represents the demographic and clinical data.

Collection of Blood

The blood samples for the analysis of hemostasiologic and rheologic parameters were standardized and venipuncture was performed at the cubital vein without any compression in a sitting position [13]. The rheologic parameters were measured within a 2-h period [14].

Coagulation Assays

The bleeding time was measured with the surgicutt system [15]. The combined measurement of hemoglobin, RBC, WBC, hematocrit, RBC-indices and PC, was carried out thanks to an automated hemoanalyzer system (Coulter Counter).

The induced platelet aggregation were carried out according to Born and Gross [16]. The partially activated thromboplastin time (aPTT) was measured after adding phospholipid-components and an activator [17]. The factors of activity VIII and IX were measured with a single phase test using deficiency plasma as a modified partial thromboplastin time procedure [17]. Factor VIII associated antigen (vWF: Ag) was measured according to the Laurell procedure [18]. The ristocetin factor (vWF: RCof) was determined according to the Mac Farlane procedure [19]. Reagents were used from Dade Behring. The multimeric was carried out according to the Budde procedure [12] and the subtype classification of the subcommittee on the von Willebrand factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis [11].

The fibrinogen concentration was measured according to a Clauss method as a variant of the thrombin time determination [20]. The PT, the thrombin time and reptilase time were measured coagulator with reagents from Dade Behring [21].

Table 1. Demographic and clinical data of patients with hemorrhagic diatheses in comparison with healthy subjects (*AC* anticoagulation, *hem.* hemophilia, *dysfib.* dysfibrinogenemia)

Measuring unit (dimension)		vWD	Thrombo-cytopathy	Thrombo-cytopenia	Hemo-philia A	Hemo-philia B	Dys-fibrino-genemia	Oral anticoa-gulation	Healthy Subjects
Total number:	[-]	100	122	101	50	20	22	112	100
Gender:									
Female	[-]	48	60	48	0	0	7	40	39
Male	[-]	52	62	53	50	20	15	72	61
Age: (years)		33±13	53±11	45±9	28±6	24±3	36±9	48±12	26±6
Bleedings in patients' history:									
Transfer required	[%]	24	14	12	60	80	0	5	0
No transfer required	[%]	100	100	100	100	100	100	100	4
Important atherogenic risk factors:									
Diabetes mellitus	[%]	0	26	31	0	0	0	2	0
Art. hypertension	[%]	3	56	57	0	0	0	20	0
Lipoproteinemia	[%]	5	32	19	0	0	18	33	0
Hyperuricemia	[%]	0	13	10	0	0	0	17	0
Nicotinabuse	[%]	28	32	19	30	20	33	57	13
Obesity (BMI – body mass index >25)	[%]	13	36	24	8	10	17	27	2

Hemorrheological Measurement Dimensions

The hematocrit was measured with the impedance device determining the Ohm's fraction of an alternating current resistance. The variation coefficient was 0.4% in sequence and 2.4% from day to day [14, 22].

Plasma viscosity describing the internal friction or viscosity of blood plasma was a proportional constant. It was determined after centrifugation of venous blood at 1500 revolutions over 5 min by a capillary viscosimeter [23] and calculated from the quotient of shear stress and shear degree. Method of measurement, quality control and the reference ranges (1.19–1.29 mPas) are described in [23]. The variation coefficient amounted to 1.14% in sequence and from day to day 1.8%.

The erythrocyte aggregation was a reversible connection of red blood cells, physiologically conglomerating to form so called coin rolls and clumping in certain pathological cases. Measurement method, reference range (8–21) and implementation of quality control are described in [22]. The variation coefficient was 5.2% in sequence and 7.1% from day to day.

Video Capillary Microscopy

The complete system that was used contains the following items: a brightening microscope with ACM tripod (Company C. Zeiss), an optovar 1.0–2.0 (for a rapid alteration of the extension), a so called cold light source with a green filter (useful for the long wave length of the hemoglobin absorption with approximately 560 nm to have a clear contrast between red blood cells and tissue samples) and a heating filter in order to minimize the warming up of the fingers. A video camera with Newvicon-tubes (Panasonic WV 1550/G), a video timer (to insert a date and a timer with 1/100 seconds-display) a three quarter inch video recorder and a monitor were also used [24].

Depending on the optovar adjustment a final magnification of 1:285 to 1:570 can be achieved. The hand but also the foot to be examined were fixed on an object table heated to 29°C, the measuring place was prepared using ultrasound gel and immersion gel. The capillary density of the first range per mm of the epidermis edge, the diameter of the capillary, the torquation of the capillary as well as the present extravasal blood cells of a bleeding-band were examined. The mean erythrocyte velocity in the nailfold capillaries (in the venolar and arteriolar branch) and the progression of the reactive hyperemia after a 3-min arterial occlusion of the ipsilateral arm were measured to determine the dynamic parameters for the quantification of the capillary circulation. The method and the measuring procedures are described in [24], the biological influence factors and the variation of periungual video capillary microscopy are described in [25]. The erythrocyte velocity was measured by interactive video recording using a picture analyzer system »Cap Image« (Company engineer's office Zeintl, Heidelberg) on Pentium 166 with an image processing card (Company Matrox) [26]. The evaluation was done according to the recommendations of the working group: »Clinical Video Capillary Microscopy« [27]. The reference ranges were given: venolar diameter: 7.0–15.9 μm; arteriolar diameter:

5.3–12.0 μm; torquation index: 0–3; bleeding-bands/extravasates (capillary bleedings): 0; arteriolar capillary RBC-velocity: 0.39–0.81 mm/s; venolar capillary RBC-velocity: 0.31–0.71 mm/s; duration of reactive hyperemia after 3 min of ischemia: 180–300 s. [24, 25].

Statistics

The test on normal distribution was carried out according to Kolmogoroff-Smirnov [28]. Normally distributed spot checks were described with the mean value (mv) and the standard deviations (s) with mv±s, abnormal spot checks are described with median (md) and percentiles (2.5%-percentile and 97.5%-percentile) in the following manner: md (2.5%-p/97.5%-p). The specificity, the sensitivity and the predictive value are shown in 29. For the consideration of several parameters, the evaluation of the predictive value can be extended (equation 1). With this method a predictive value [p(K/Tj)] of a combination parameters (n=j) (a so-called symptom complex) can be measured for the existence of the vWD [29].

Equation 1:

$$p(K/Tj) = \frac{1}{I + \frac{p(K)}{1-p(K)} \prod_{j=1}^{m} \left[\frac{1-p(tj/k)}{p(Tj/K)} \right]}$$

The statistical comparison of samples was performed using the Wilcoxon-Mann and Whitney test [28]. A difference was assumed, if a *P*-value fell below 0.05 (»Stat View-program 4.5«). A Bonferoni adjustment of *P*-values was done.

Results

Patients with vWD (n=100; 92 type 1: definite type 1 vWD and 78 possible type 1 vWD: 14; 8 type 2 A) had in comparison with patients with other hemorrhagic diatheses: thrombocytopathy (n=122), thrombocytopenia (n=101), severe hemophilia A (n=50) and severe hemophilia B (n=20), hereditary dysfibrinogenemia (n=22), oral anticoagulation with phenprocoumon (n=112) and apparently healthy subjects (n=100) a significantly increased capillary torquation (median: 3.5), a venolar and an arteriolar capillary dilatation (median: 16.5 μm; median: 15.1 μm respectively) and the highest level of microscopic bleedings (extravasates) with 40% in the video capillary microscopy as morphological changes (Table 2).

Compared with all other collectives the average diameters of the venolar and arteriolar capillaries were significantly (P<0.001 to P<0.05) larger in patients with vWD. The thrombocytopathy, thrombocytopenia, hemophilia A and B and the oral anticoagulation were comparable in the capillary diameter (arteriolar and venolar) of healthy persons (median: 11.5 μm; median: 8.9 μm respectively). The congenital dysfibrinogenemia showed as well in patients with vWD a raised level of venolar dilatation (median: 14.5 μm) and an arteriolar capillary diameter comparable to a

healthy person. The mean torquation index of patients affected with vWD was significantly larger than for all the other collectives ($P<0.001$ to $P<0.01$). In the other hemorrhagic diatheses there was just a slight difference in the torquation index with a comparable range (2,5 and 97,5% percentile) to healthy persons.

The amount of capillary bleedings (extravasates or bleeding-bands) was significantly higher in patients with vWD compared with all other collectives ($P<0.001$ to $P<0.01$). Extravasates were found in the other hemorrhagic diatheses with a lower frequency between 4% (thrombocytopathy) up to 13% (oral anticoagulation), in healthy persons no bleeding-bands were found. No significant differences were shown in the eight patients with type 2 A (vWD) compared with patients with type 1 (vWD) and in the total group of patients with vWD concerning the morphology of the capillaries. Extravasates were present in four of eight patients (total group with vWD: 40%), the median of the torquation index was 3.8 (total group with vWD: 3.5), the median diameter of the venolar capillaries was 16.2 µm (total group: 16 µm). The capillary density was comparable in all collectives and had an average of between 10.3 and 11.1 capillaries per mm. Differences between the collectives did not appear (these data are depicted in a description).

In Fig. 2a the increased torquation as well as the dilatation of arteriolar and venolar capillary density for a patient with vWD can be seen (magnification 1:285). In comparison, Fig. 2b shows the capillary bed of a clinically healthy subject (magnification 1:570). In Fig. 3, extravasal blood cells (extravasate) in the pericapillary area for a patient with vWD (magnification 1:570) are shown.

Table 2. Capillary morphological parameters for patients with hemorrhagic diathesis in comparison with healthy subjects (median, the 2.5 and 97.5% percentile as well as the percentages are listed) (*Dven* venolar capillary diameter, *Dart* arteriolar capillary diameter, *TI* torquation index, *AC* anticoagulation)

	Dven (µm)	Dart (µm)	TI (-)	extravasates bleeding-bands (%)
vWD (*n*=100)	16.5 (11.4/27.2)	15.1 (10.3/22.4)	3.5 (1.9/11.4)	40
Thrombocytopathy (*n*=122)	10.0 (7.0/13.0)***	8.1 (5.0/9.4)***	2.3 (0.5/3.8)**	6***
Thrombocytopenia (*n*=101)	10.6 (7.5/13.8)***	7.7 (5.0/9.4)***	1.6 (0/5.0)**	4***
Hemophilia A (*n*=50)	12.8 (8.0/17.0)**	9.2 (6.5/12.5)**	1.8 (0/3.5)***	6***
Hemophilia B (*n*=20)	11.9 (7.0/18.0)***	8.0 (6.0/11.7)**	1.3 (0/3.0)***	5***
Dysfibrinogenemia (*n*=22)	14.5 (7.5/19.9)*	10.4 (7.3/15.8)**	1.5 (0/3.6)***	9***
Oral AC (n=112)	12.9 (8.0/17.5)**	9.9 (6.1/13.0)**	2.0 (0/4.2)**	13**
Healthy subjects (*n*=100)	11.5 (7.0/16.9)***	8.9 (5.3/12.0)**	1.6 (0/5.0)***	0***

***$P<0.001$, **$P<0.01$, *$P<0.05$; Bonferoni adjusted

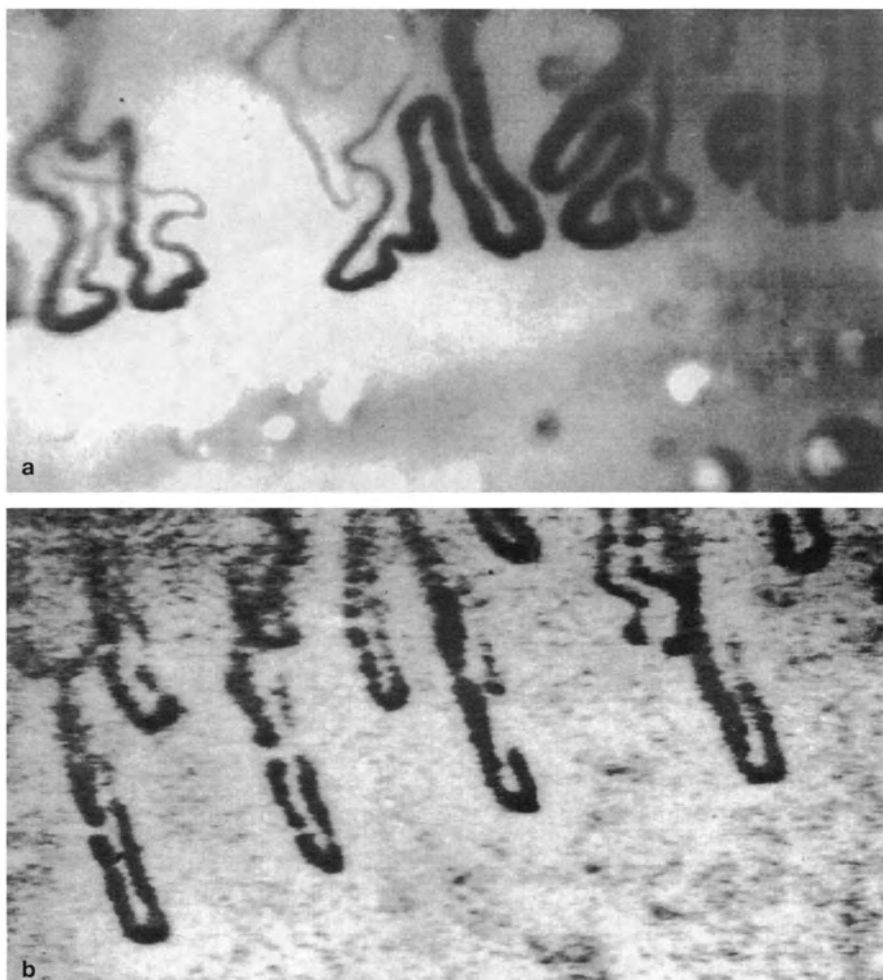


Fig. 2a, b. *a* Increased torquation as well as capillary ectasia in a patient with vWD (age: 32 years, height: 177 cm, weight: 72 kg), magnification: 1:285. *b* For comparable reasons a capillary bed of a clinically healthy subject (age: 35 years, height: 172 cm, weight: 68 kg), magnification: 1:570

Table 3 gives an overall view of the capillary dynamic measuring values for patients with hemorrhagic diatheses, particularly with vWD, in comparison with healthy subjects. The mean red blood cell velocity in the arteriolar and venolar capillaries was within the group of patients with vWD significantly higher, within the group of patients with thrombocytopathy ($P<0.01$, $P<0.05$), patients with thrombocytopenia ($P<0.05$, $P<0.01$) and in the group of patients with dysfibrinogenemia ($P<0.01$, $P<0.001$) it was also significantly higher. There was no significant difference comparing patients with hemophilia A and B, the group of patients under oral anticoagulation and a healthy control group. In patients with vWD the duration of a

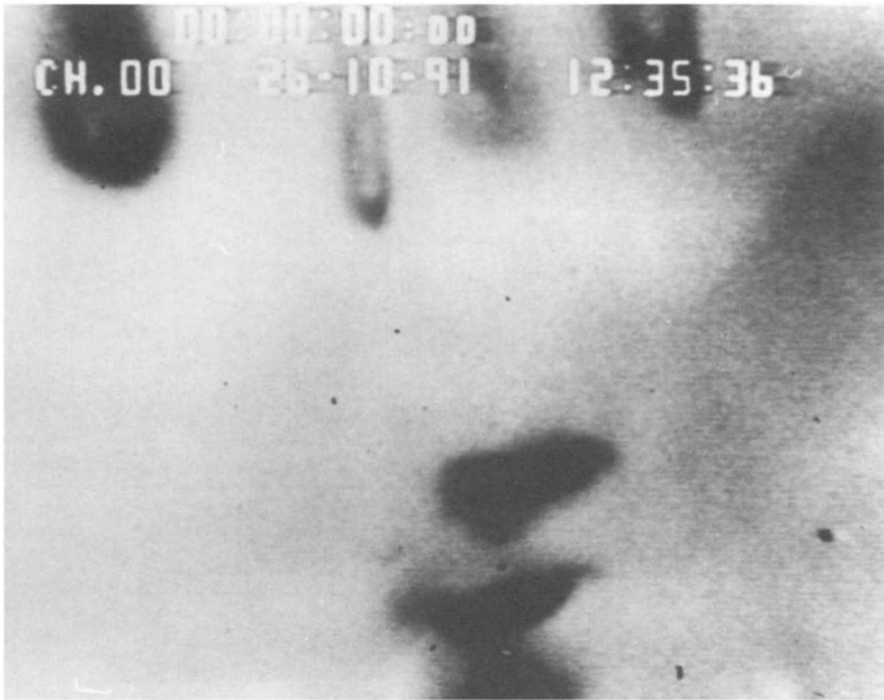


Fig. 3. Extravasal blood cells in the pericapillary region in a patient with vWD (ages: 37 years, height: 168 cm, weight: 66 kg) (8), magnification: 1:570

Table 3. Capillary dynamic parameters (red blood cell velocity in the arteriolar and venolar capillaries, duration of reactive hyperemia) in patients with hemorrhagic diatheses in comparison with healthy subjects (median, the 2.5% and 97.5% percentile are listed) (*Vven* venolar capillary RBC velocity, *Vart* arteriolar capillary RBC velocity, *DrH* duration of reactive hyperemia after 3 min of ischemia, *AC* anticoagulation)

	Vart (mm/sec)	Vven (mm/sec)	DrH (sec)
WD (*n*=100)	0.56 (0.25/1.0)	0.51 (0.22/0.96)	150 (90/240)
Thrombocytopathy (*n*=122)	0.37 (0.21/0.56)**	0.30 (0.09/0.55)*	140 (80/180) n.s.
Thrombocytopenia (*n*=101)	0.37 (0.13/0.61)*	0.24 (0.11/0.59)**	120 (60/190) n.s.
Hemophilia A (*n*=50)	0.61 (0.39/0.81) n.s.	0.53 (0.38/0.89) n.s.	220 (180/280)**
Hemophilia B (*n*=20)	0.65 (0.54/0.82) n.s.	0.55 (0.45/0.68) n.s.	240 (180/300)**
Dysfibrinogenemia (*n*=22)	0.18 (0.10/0.30)**	0.12 (0.06/0.23)***	120 (90/150) n.s.
Oral AC (*n*=112)	0.49 (0.19/0.78) n.s.	0.42 (0.19/0.64) n.s.	170 (90/180) n.s.
Healthy subjects (*n*=100)	0.65 (0.23/1.10) n.s.	0.59 (0.18/1.14) n.s.	240 (180/300)***

***P <0.001, **P<0.01, *P<0.05, n.s. not significant; Bonferoni adjusted

reactive hyperemia (median: 150 s) was significantly reduced compared with patients with hemophilia A ($P<0.01$), hemophilia B ($P<0.01$) and a healthy control group ($P<0.01$): this was the only dynamic change. The duration of the reactive hyperemia was also reduced in the thrombocytopathy (median: 150 s), the thrombocytopenia (median: 120 s), the dysfibrinogenemia (median: 120 s) and in patients with an oral anticoagulation (median: 170 s).

In Table 4 the measuring dimensions of the blood fluidity for patients with hemorrhagic diatheses and for healthy patients are described. The hematocrit was significantly ($P<0.05$) reduced compared with patients with vWD (median: 42%) and in the group of patients with thrombocytopenia (median: 37%). All other hemorrhagic diatheses and (in) healthy persons did not differ compared with patients with vWD. Within in the 1-s-range the plasma viscosity was significantly reduced only in patients with vWD (median: 1.20 mPas). The plasma viscosity was significantly higher in all other groups of patients ($P<0.001$ to $P<0.05$).

The group of eight patients with type 2 A (vWD) showed no differences in comparison with the patients with type 1 (vWD) and also in comparison with the total group of patients with vWD in terms of plasma viscosity (median: 1.19 mPas; total group with vWD: 1.20 mPas).

In the group of patients with thrombocytopenia (median index: 19.2; $P<0.05$) and in patients with dysfibrinogenemia (median index: 28.5; $P<0.01$) the erythrocyte aggregation was significantly higher compared with patients with vWD (median index: 10.2). This tendency can also be seen in the group of patients with a thrombocytopathy (median index: 18.1; n.s.). The hemophilia A and B patients, the group of patients with oral anticoagulation, and the group of healthy persons did not differ in erythrocyte aggregation from patients with vWD.

The capillary morphology, especially the arteriolar and venolar dilatation of the capillaries, the extravasates, the higher grade of capillary torquation (dysplasia) and the hypoplasmaviscosity (1-s-range) showed the highest rate of sensitivity to the vWD (75%, 65%, 40%, 80%), the specificity was also fairly high (74%, 85%, 88%,

Table 4. Parameter of blood fluidity (hematocrit, plasma viscosity, erythrocyte aggregation) in patients with hemorrhagic diatheses in comparison with healthy controls (median, the 2.5% and 97.5% percentile are listed) (*Hct* hematocrit, *PV* plasma viscosity, *EAI* erythrocyte aggregation index, *AC* anticoagulation)

	Hct (%)	PV (mPas)	EAI (-)
vWD (*n*=100)	42 (37/46)	1.20 (1.14/1.24)	10.2 (7.2/13.2)
Thrombocytopathy (*n*=122)	38 (32/45) n.s.	1.36 (1.28/1.48)**	18.1 (12.0/22.3) n.s.
Thrombocytopenia (*n*=101)	37 (28/44)*	1.38 (1.26/1.50)**	19.2 (12.9/24.1)*
Hemophilia A (*n*=50)	40 (35/45) n.s.	1.23 (1.19/1.29)*	9.0 (5.2/13.1) n.s.
Hemophilia B (*n*=20)	41 (35/47) n.s.	1.25 (1.20/1.30)*	11.2 (7.2/14.9) n.s.
Dysfibrinogenemia (*n*=22)	45 (38/50) n.s.	1.48 (1.44/1.51)***	28.5 (21.5/35.5)**
Oral AC (*n*=112)	39 (34/44) n.s.	1.34 (1.21/1.48)**	14.5 (9.1/19.8) n.s.
Healthy subjects (*n*=100)	43 (39/48) n.s.	1.24 (1.19/1.28)*	8.9 (4.1/13.7) n.s.

***$P<0.001$, **$P<0.01$, *$P<0.05$: not significant, Bonferoni adjusted

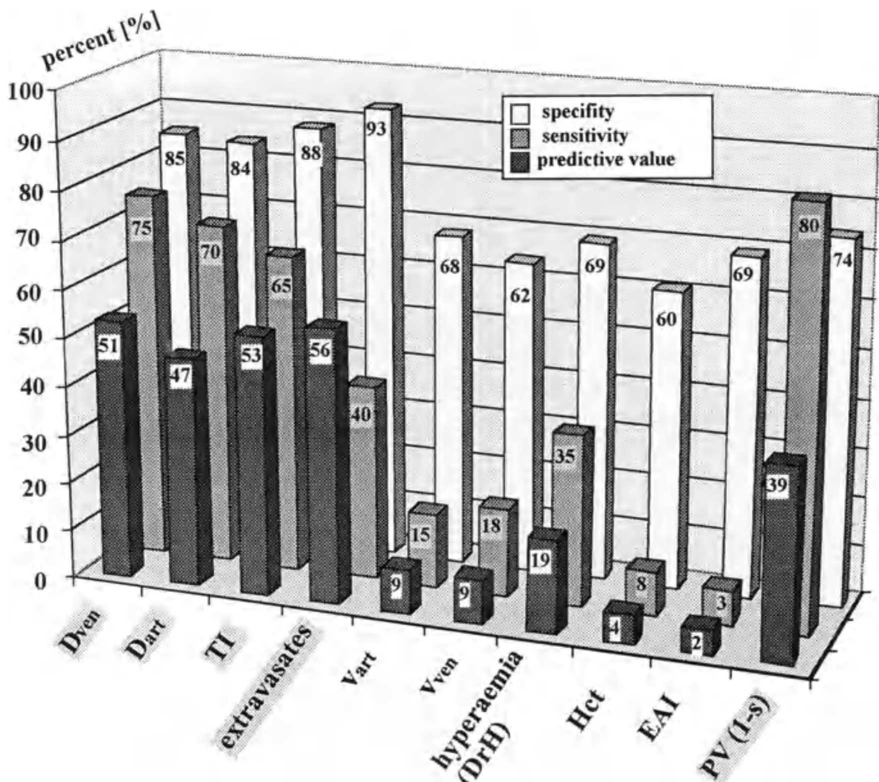


Fig. 4. Specificity, sensitivity and predictive value for the vWD in relation to the capillary morphological and dynamic dimensions, hemorrheological dimensions (grey background: dimensions with high sensitivity)

93%). For these parameters the predictive value for the vWD had a range between 39% and 56% (Fig. 4).

One of these parameters alone does not have a sufficiently high predictive value, although the sensitivity is quite high.

The dynamic parameters of the capillaries, the capillary red blood cell velocity (arteriolar and venolar) and the duration of the reactive hyperemia along with further hemorrheologic parameters, the hematocrit and the aggregation of the red blood cells showed a very low sensitivity, a very low predictive value and a lower specificity. These parameters are not appropriate for a screening procedure in order to differentiate the vWD from other hemorrhagic diatheses.

The overlapping of the distribution concerning the examined variables for the different symptoms of hemorrhagic diathesis, is the essential cause of the diagnostic selectivity. By using a combination of several tests there is a possibility of increasing sensitivities and positive predictive values for the different diseases. The requirements for the validity of such a combination are:

1. Independence of the test
2. Parallel application of the test (30)

Both premises are given in the present case. By using the Bayes-theorem (when the single value is known) the sensitivity and the positive predictive value for a vWD was calculated by a combination of several variables (equation 1 in the chapter statistics). What is striking for patients with vWD is the capillary morphology. A combination of these dimensions are seen in the following form (combination 1):
1. Torquation index (TI >3.5)
2. Venolar capillary diameter (Dven >16.0 μm)
3. Extravasates and/or bleeding-bands (capillary bleedings >0%)

This reaches a positive predictive value of 98% (prevalence 37%) for patients with vWD as compared with other patients with hemorrhagic diathesis. Furthermore, a very high predictive value was obtained when the plasma viscosity (PV), less than 1.25 mPas, was taken into consideration (combination 2): the positive predictive value increased to around 99% (prevalence 28%).

Discussion

First O'Brien and Blackburn described the morphological alteration in the area of cutaneous microcirculation (Fig. 1) for patients with vWD [3, 4], but did not carry out a quantitative analysis. Since then no systematic examination of microcirculation in patients with vWD has been carried out.

Due to the constant follow up of numerous patients with vWD (proven with laboratory diagnostic tools and clinically assured) the question that has been raised since 1991 is whether and in which shape a cutaneous microangiopathy exists for patients with vWD in comparison with patients with other hemorrhagic diatheses or healthy individuals [5, 6, 7]. The earlier described capillary alteration for patients with vWD (types 1 and 2 A), thanks to the present examination, could be confirmed and quantified. The morphology changes of the capillaries, especially with capillary dilatation (median: 16.5 μm), extravasates (microscopic bleedings – median: 40%) and a higher grade of torquation (dysplasia – median index: 3.5) of the nailfold capillaries, have neither been seen in healthy individuals nor in patients with other hemorrhagic diatheses who came for consultation at our hemostasiological outpatients clinics [31, 32, 33, 34, 35, 36].

The thrombocytopathy, thrombocytopenia, hemophilia A and B as well as patients with oral anticoagulation were comparable with a healthy control group (median: 11.5 μm; median: 8.9 μm), in terms of the arteriolar and venolar capillary diameter only the congenital dysfibrinogenemia showed a venolar dilatation (median: 14.5 μm). Microscopic bleedings appeared with much less frequency in the other hemorrhagic diatheses compared with the vWD (types 1 and 2 A), with a range between 4% (thrombocytopathy) and 13% (oral anticoagulation). The dilatation and torquation (dysplasia) of the capillaries, as well as already existing microscopic bleedings, did not change after the administration of DDAVP and con-

centrates of clotting factors. The administration of DDAVP and concentrates of clotting factors can prevent any new appearance of microscopic bleedings. In patients with a type 2 A of the vWD compared with patients with a type 1 of the vWD and the total group of patients with vWD no significant differences in terms of morphology of capillaries and plasma viscosity was found.

The capillary red blood cell velocity (arteriolar and venolar) did not differ between the vWD (types 1 and 2 A), hemophilia A and B, oral anticoagulation and a healthy group. Quite noticeable is the significant reduction of the arteriolar and venolar capillary red blood cell velocity as well as the reduced duration of reactive hyperemia in patients with thrombocytopathy and thrombocytopenia. This could be due to the high number of patients with atherogenic risk factors, among them clinically important angiosclerosis, and in patients with dysfibrinogenemia due to the extreme hyperplasmaviscosity (median: 1.48 mPas) combined with a higher grade of the erythrocyte aggregation (median index: 28.5). The only dynamic change in the vWD (types 1 and 2 A) compared with the hemophilia A and B patients as well as with a healthy group is a significant reduction of the duration of a reactive hyperemia (median: 150 s). This could be a hint as to the loss of flexibility in the precapillary vessels, which are responsible for the hemodynamic resistance. A possible result could be the frequent perivasal (microscopic) bleedings in the vWD. There are no pathological findings of rheological parameters in patients with vWD (types 1 and 2 A). All evaluated rheological data were within the normal reference range; 80% of the patients with vWD (types 1 and 2 A) even showed plasma viscosity at the lower level of the reference range (values below 1.25 mPas; 1-s-range). A change of von Willebrand factor activities could enhance an increase in shear stress within the blood plasma.

The morphology changes of the capillaries, especially with capillary dilatation, extravasates and a higher grade of torquation (dysplasia) of the nailfold capillaries along with the hypoplasmaviscosity are most sensitive for the vWD (75%, 65%, 40%, 80%) with a fairly high specificity (up to 93%). In comparison with patients with other hemorrhagic diatheses definite signs for a good positive predictive value (ppv between 98% and 99%, with reference to the used measured value of sensitivity-analysis) can be elaborated. If the obtained capillary microscopic data of the patients with diabetes type I and type II and of the patients with arterial hypertension class I until III according to the WHO criteria [37, 38, 39] are used for multiparameter analysis, the positive predictive value for the presence of vWD would remain unchanged. A connection between these diseases and the changes of the capillaries can be excluded.

Until now the cause of these capillary alterations in patients with vWD (types 1 and 2 A) remains unclear. Capillary bleedings can be caused by reduced endothelial- and most probably also by reduced endothelial cellular matrix bounds. The capillary dilatation infers local destruction of the extracellular matrix also revealing the growth factor b-FGF with Heparansulfate proteoglykane (b-FGF-HSPG) [40]. Thrombin has the capacity to remove endothelial cellular bonds, to reduce the matrix and to release growth factors with their different functions from the matrix [40]. This chain of observed changes in the morphology within the ungual area in patients with vWD could be based on a different binding of the thrombin to the von

Willebrand factor. Proven results for this hypothesis are not yet available and will be the subject of further studies.

As a conclusion of this work it seems reasonable to discuss the introduction of video capillary microscopy as a possible extension of the differential-diagnostic spectrum as a screening test for hemostasiological and angiological centres.

In principle the results of video capillary microscopic examination are reliable and reproducible [5, 25]. Video capillary microscopy could be performed to distinguish a patient with a hemorrhagic diathesis from someone with vWD (types 1 and 2 A). In particular the quickly performed assessment of the capillary morphology, with its changes in capillary dilatation, its fresh or old capillary bleedings (extravasates and/or bleeding-bands) and the presence of capillary torquations is, according to the estimated results, highly predictive for the presence of vWD (types 1 and 2 A). This screening, under fast optical view diagnosis could help in emergency situations in order to ascertain the required further tests and the therapy.

Due to the limited number of cases, the question between the different types of vWD, in particular type 2B, 3 and acquired forms, in comparison with type 1, remains still unanswered.

References

1. EA. Hereditär Pseudohemofili. Finska Läkarsällskapetes Handl 1926; 67: 7.
2. Armand J, Quick PhD. Teleangiectasia: Its Relationship to the Minot-Von Willebrand Syndrome. The American Journal of the Medical Sciences. 1967; 11: 585–601.
3. O´Brien JR. Familial capillary fragility (Diffuse capillary teleangiectasia). Proc. Int. Soc. Hematology 1953; 20: 546–548.
4. Blackburn EK. Primary capillary haemorrhage (including von Willebrand's Disease). Brit. J. Haemat. 1961; 7: 239–249.
5. Koscielny J, Jung F, Kiesewetter H, Franke RP, Radtke H, Wenzel E. Cutaneous microangiopathy in patients with von Willebrand Jürgens syndrome. Clinical Hemorheology 1995; 15: 817–823.
6. Koscielny J, Jung F, Mrowietz C, Kiesewetter H, Pindur G, Wenzel E. Rheologische und kapillarmikroskopische Größen zur Diagnostik des von-Willebrand-Jürgens-Syndroms. Infusionsther. Transfusionsmed. 1995; 22: 45–47.
7. Koscielny J, Jung F, Seyfert UT, Kiesel J, Groß J, Kiesewetter H, Mrowietz C, Wenzel E. Capillary microscopic findings in patients with von Willebrand-Jürgens syndrome. Annals of Hematology 1992; 64: 17.
8. Blombäck M, Eneroth P, Andersson O, Anvret M. On laboratory problems in diagnosing mild von Willebrand's Disease. Am. J. Hematol. 1992; 40: 117–120.
9. Miller CH, Lenzi R, Breen C. Prevalence of von Willebrand's disease among U.S. adults. Blood 1989; 70: 377–378.
10. Werner E, Broxson E, Tucker E, Giroux D, Shults J, Abshire T. Prevalence of von Willebrand disease in children: Multiethnic study. J. of Pediatrics 1993; 123: 893–898.
11. Sadler J.E. A revised classification of von Willebrand disease. Thromb. Haemost. 1994; 71: 520–525.
12. Budde U, Schneppenheim R, Plendl H, Dent J, Ruggeri ZM, Zimmermann TS. Luminographic detection of von Willebrand Factor multimers in agarose gels and on nitrocellulose membranes. Thromb. Haemost. 1990; 63: 312–315.
13. Alström T, Gräsbeck R, Hjelm M, Skandsen S. Recommendations concerning the collection of reference values in clinical chemistry and activity report. Scand J Clin Lab Invest 1975; 35: 1–8.

14. Jung F, Kiesewetter H, Roggenkamp HG, Nüttgens HP, Ringelstein EB, Gerhards M, Kotitschke G, Wenzel E, Zeller H. Bestimmung der Referenzbereiche rheologischer Parameter: Studie an 653 zufällig ausgewählten Probanden im Kreis Aachen. Klin. Wochenschr. 1986; 64: 37–43.
15. Smith C. Surgicutt: a device for modified template bleeding times. J. Med. Tech. 1986; 3: 229–231.
16. Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; 194: 927–929.
17. Entes K, La Duca FM, Tourbaf KD. Flechter factor deficiency, source of variations of the activated partial thromboplastin time test. Amer. J. Clin. Path. 1981; 65: 626–630.
18. Gruson R, Kernhoff PBA. Diagnostik des von-Willebrand-Jürgens-Syndroms mit Hilfe der quantitativen immunologischen Bestimmung des Faktor VIII-assoziierten Antigens. Dtsch. Med. Wschr. 1974; 99: 1593–1597.
19. Macfarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J. A method for assaying von Willebrand factor (Ristocetin cofactor). Thromb. Diath. Haemorrh. 1975; 34: 306–307.
20. Clauss A. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haemat. 1957; 17: 237–239.
21. Suchmann AL, Griner PF. Diagnostic uses of activated partial thromboplastin time and prothrombin time. Ann. Int. Med. 1986; 104: 810–812.
22. Jung F, Seegert A, Roggenkamp HG, Mrowietz C, Nüttgens HP, Kiesewetter H, Zeller H, Müller G. Gleichzeitige Erfassung von Hämatokrit, Erythrozyten-Aggregation und -Desaggregation: Methodik, Qualitätskontrolle und Referenzbereich. Biomed. Technik 1987: 32: 117–121.
23. Jung F, Roggenkamp HG, Ringelstein EB, Schmidt J, Kiesewetter H. Das Kapillarschlauch-Plasmaviskosimeter: Methodik, Qualitätskontrolle und Referenzbereich. Biomed. Technik 1985; 30: 239–244.
24. Jung F, Wappler M, Nüttgens HP, Kiesewetter H, Wolf S, Müller G. Zur Methodik der Videokapillaroskopie: Bestimmung geometrischer und dynamischer Größen. Biomed. Tech. 1987; 32: 204–213.
25. Jung F, Toonen H, Mrowietz C, Wolf S, Kiesewetter H, Wenzel E, Gersonde K, Müller G. Fehleranalyse, biologische Einflußfaktoren und Varianz der periunguealen Videokapillaroskopie. Biomed. Tech. 1990; 35: 195–204.
26. Klyscz T, Jünger M, Jung F, Zeintl H. Cap Image – ein neuartiges computergestütztes Videobildanalysesystem für die dynamische Kapillarmikroskopie. Biomed. Technik 1997; 42: 168–175.
27. Schmidt J, Caspary L, von Bierbrauer A, Ehrly A, Jünger M, Jung F, Lawall H. Standardisierung der Nagelfalz-Kapillarmikroskopie in der Routinediagnostik. Vasa 1997; 26: 5–10.
28. Sachs L. Angewandte Statistik. Springer Verlag, Berlin – Heidelberg – New York 1984; pp 1–22.
29. Büttner J. Die Beurteilung des diagnostischen Wertes klinisch-chemischer Untersuchungen. J. Clin. Chem. Biochem. 1977; 15: 1–12.
30. Galen S, Gambino SR. Norm und Normabweichung klinischer Daten. Gustav Fischer Verlag, Stuttgart 1979; pp 4–33.
31. Jung F, Waldhausen P, Schwab J, Molter A, Scheffler P, Kiesewetter H, Wenzel E. Einfluß von Naftidrofuryl auf die Mikrozirkulation bei der peripheren arteriellen Verschlußkrankheit. Medwelt 1986; 37: 132–137.
32. Birkel L, Scheffler P, Jung F. Untersuchungen zur Wirkung von Dihydroergotamin auf die venöse Makro- und Mikrostrombahn bei Gefäßgesunden und Patienten mit chronisch venöser Insuffizienz. VASA 1988; 23: 253–256.
33. Kiesewetter H, Jung F, Fladung F, Mrowietz C, Wenzel E. Diagnostik und Therapie des Raynaud-Phänomens. Angio 1991; 13: 221–228.

34. Bach R, Leicht E, Langer HJ, Hartenstein R, Jung F, Berg G, Schätzer-Klotz D, Bonaventura K, Schieffer H, Weinges KF. Kardiale Funktion und kutane Mikrozirkulation bei Akromegalie. Dtsch. med. Wschr. 1992; 117: 483–489.
35. Ohlmann P, Jung F, Rosenbaum P, Hummel B, Schäfer P, Schmidt W. Nichtinvasiver Funktionstest zur Überprüfung der Gefäßreagibilität in der Schwangerschaft und bei schwangerschaftsinduzierter Hypertension. Geburtsh. u. Frauenhlk. 1992; 52: 327–331.
36. Groß J, Seyfert UT, Koscielny J, Wenzel E. Dysfibrinogenämie - Homburg I-VI. In: 22. Hämophilie-Symposion Hamburg 1991. Landbeck G, Scharrer I, Schramm W, eds. Springer-Verlag, Berlin-Heidelberg, 1992; pp 177–183.
37. Kiesewetter H, Körber N, Jung F, Wolf S, Reim M. Die Bedeutung der Fließfähigkeit des Blutes bei diabetischer Retinopathie. In: Diabetische Retinopathie. Lerche W, ed. Schattauer Verlag, Stuttgart, 1984; pp 25–32.
38. Koscielny J, Latza R, Wolf S, Kiesewetter H, Jung F. Early rheological and microcirculatory changes in children with type I diabetes mellitus. Clinical Hemorheology 1998; 19: 139–150.
39. Jung F, Kolepke W, Spitzer S, Kiesewetter H, Ruprecht KW, Bach R, Schieffer, H, Wenzel E. Primary and secondary microcirculatory disorders in essential hypertension. Clin. Investig. 1993; 71: 132–138.
40. Vlodafsky I, Bar-Shavit R, Ishai-Michaeli R, Bashkin P, Fuchs Z. Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? Tibs 1991; 16: 268–271.

Evaluation of Denaturing High Performance Liquid Chromatography (DHPLC) in the Analysis of Hemophilia A

J. Oldenburg, O. El-Maarri, V. Ivaskevicius, S. Rost, B.H.F. Weber and R. Schwaab

Introduction

Hemophilia A is the most common severe bleeding disorder in humans, affecting one in 5000 male newborns. The phenotype is due to deficiency or absence of coagulation factor VIII:C caused by deleterious mutations in the factor VIII (FVIII) gene. Affected individuals develop a variable degree of hemorrhage predominantly into joints and muscles with the severity and frequency of bleeding symptoms correlating well with the residual factor VIII activity in blood plasma. About 50% of the patients exhibit a severe phenotype while 10% show a moderate and 40% a mild expression of the disease [1]. The replacement of human factor VIII derived from plasma or from recombinant sources is the treatment of choice in this disorder. The major complication of this therapy is the development of antibodies against exogenous factor VIII, rendering therapy ineffective.

The FVIII gene maps to the distal end of the long arm of the X-chromosome at Xq28 and spans 186 kb of genomic DNA. It consists of 26 exons [2,3] encoding a mature protein of 2332 amino acids. One large exon, exon 14, covers about 40% of the coding sequence and encodes the B domain that is not essential for FVIII-activity [4]. Intron 22 contains two more genes, designated F8 A and F8B, with so far unknown functions [5,6].

Mutation analysis in the factor VIII gene is valuable with regard to both genetic counseling and medical care of affected families. An important issue in genetic counseling is the determination of the risk of recurrence, in particular given the high frequency of *de novo* mutations [7]. This is complicated by the high mortality from blood borne viruses in previous years which has often led to a situation where an index patient is not available for genetic diagnosis [8,9]. In these instances, it is necessary to directly genotype potential carriers without prior knowledge of the mutation, a situation in which the test sensitivity is of critical importance. For patient care, knowledge of the genotype is helpful as it facilitates the prediction of risk of anti-FVIII antibody development [10]. In the future, this knowledge will become a precondition for entering gene therapy programs [11].

In recent years, the heterogeneity and size of the FVIII gene have hampered mutation testing in hemophilia A patients. In 1991, the first systematic analysis of the complete coding sequence of the FVIII gene was performed by applying denaturing gradient gel electrophoresis (DGGE) after PCR amplification of individual exons [12]. Notably, causative mutations were found in about 90% of non-

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

severe hemophilia A [12] whereas molecular defects could be identified in only 60% of the more severely affected patients [13]. This discrepancy was resolved in 1993 when Naylor et al. (1993) [14] and Lakish et al. (1993) [15] discovered a prevalent intron 22 inversion between the F8 A region in intron 22 and one of two homologous F8 A gene copies located about 300 to 500 kb at the telomeric end of FVIII. This type of mutation was shown to account for most of the remaining severe hemophiliacs and results in a true null allele by interrupting the gene between exon 22 and exon 23.

To date, additional screening methods such as single stranded conformational polymorphism (SSCP), conformational sensitive gel electrophoresis (CSGE) and chemical mismatch cleavage (CMC) have been applied to the factor VIII gene [16–19]. Each of these methods has varying applicability and efficiency, however, all suffer from incomplete detection rates in the range of 70–85%. In addition, each places variable demands on the technical skills and time investment of the investigator. Analysis of mRNA [20,21] reported a close to 100% detection rate, however the technique failed to examine a considerable proportion of patients because of poor quality of RNA preparation.

Recently, Oefner and coworker introduced a novel technique referred to as denaturing high performance liquid chromatography (dHPLC), a rapid and highly sensitive method for the detection of single nucleotide changes [22,23]. To evaluate this technique for the mutation analysis of FVIII, we have developed a detailed dHPLC protocol for the assessment of 33 amplicons representing the entire coding region and exon-flanking sequences. Two patient cohorts comprised of 156 hemophilia A patients with known mutations in FVIII and 27 hemophiliacs in whom the mutations could not be determined by conventional DGGE analysis, were investigated under this protocol.

Methods and Materials

Patients

In order to evaluate dHPLC for its efficiency of mutation detection in hemophilia A, two groups of patients were investigated. One group represents 156 hemophilia A patients with known FVIII gene mutations detected by DGGE and CMC (unpublished results). The second cohort of patients is comprised of 27 hemophiliacs in whom no mutation could be detected in the FVIII gene by conventional mutation analysis (unpublished results). All patients have been analyzed under our institute's established clinical diagnostic protocol which includes Southern blot hybridizations to test for the prevalent intron 22 inversion [15, 19] followed by DGGE and CMC analysis performed according to previously described methods [17]. Blood samples were received in the context of diagnostic testing from hemophilia clinics and genetic counseling units throughout Germany.

Denaturing High Performance Liquid Chromatography (dHPLC)

25 μl PCR reactions for each of the 33 fragments representing the entire coding sequence of the factor VIII gene and including all splice acceptor and donor sequences were prepared using the primer pairs and PCR conditions given in Table 1. Prior to PCR amplification, genomic DNA was diluted to 25–50 ng/25μl reactions to avoid misleading DNA signals in the chromatographic elution profile. Equal volumes of a 25μl PCR reaction product from the patient and a wild type control were mixed, heated for 10 min. at 95°C and followed by incubation at 55°C for 10 min. to allow for heteroduplex formation. Analysis of the heteroduplex and homoduplex mixture was performed on a WAVE™ DNA Fragment Analysis System (Transgenomics, San Jose, USA). By this technique heteroduplices are separated from homoduplices by ion-pair reverse-phase liquid chromatography according to their differences in melting behavior. Partial heat denatured double-stranded DNA fragments interact in a length- and sequence-specific manner with a non-porous poly(styrene-divinyl-benzene) matrix (DNA^{Sep}, Transgenomics, San Jose, USA).

Table 1. Primer pairs and PCR conditions for DHPLC analysis

Exon/ primer	Sequence	Length of PCR amplicon (bp)	Annealing temperature (°C)
1F	5′- TTTGCTTCTCCAGTTGAACAT - 3′	208	53
1R	5′- CGATCAGACCCTACAGGACA - 3′		
2F	5′- TTGAAGTGTCCACCAAAATGAACGACT - 3′	211	54
2R	5′- GATACCCAATTTCATAAATAGCATTCA - 3′		
3F	5′- GTACTATCCCCAAGTAACCTT - 3′	204	54
3R	5′- CATAGAATGACAGGACAATAGG - 3′		
4F	5′- TACAGTGGATATAGAAAGGAC - 3′	296	54
4R	5′- TGCTTATTTCATCTCAATCCTACGCTT - 3′		
5F	5′- CCTCCTAGTGACAATTTCCTA - 3′	188	54
5R	5′- AGCAGAGGATTTCTTTCAGGAATCCAA - 3′		
6F	5′- CATGAGACACCATGCTTAGCT - 3′	224	54
6R	5′- AACTCTGGTGCTGAATTTGGAAGACCCT - 3′		
7F	5′- CAGATTCTCTACTTCATAGCCATAG - 3′	324	54
7R	5′- ATTAAAAGTAGGACTGGATA - 3′		
8F	5′- ATATAGCAAGACACTCTGACA - 3′	338	54
8R	5′- AGAGAGTACCAATAGTCAAA - 3′		
9F	5′- AGAGTTGGATTTGAGCCTACC - 3′	284	54
9R	5′- CAGACTTTTTCTTCTTACCTGACCTT - 3′		
10F	5′- GGATTTGATCTTAGATCTCGC - 3′	204	53
10R	5′- ATTTTAGTTGTTATTGATGA - 3′		
11F	5′- TTGAGCTATTTATGGTTTTG –3′	294	53
11R	5′- GACATACACTGAGAATGAA - 3′		
12F	5′- GCATTTCTTTACCCCTTTCA - 3′	230	54
12R	5′- CTTTATTCACCACCCACTG - 3′		

Table 1. Continued

Exon/ primer	Sequence	Length of PCR amplicon (bp)	Annealing temperature (°C)
13F	5′- GATGTGTCTAAATCTCTTTTC -3′	261	54
13R	5′- ATATAATAACTAACCTGGGTTTTCCATC - 3′		
14(I)F	5′- ATCTGTGTTATGAGTAACCA - 3′	430	54
14(I)R	5′- TCATATTTGGCTTCTTGGAG - 3′		
14(II)F	5′- CATGGGCTATCCTTATCTGA - 3′	479	54
14(II)R	5′- CATGAACTTTCTTGGCTATT - 3′		
14(III)F	5′- TCAAAGTTGTTAGAATCAGG - 3′	441	54
14(III)R	5′- ATTTTGTGCATCTGGTGGAA - 3′		
14(IV)F	5′- GTCCAACAGAAAAAAGAGGG - 3′	481	54
14(IV)R	5′- CTACATTTTGCC - 3′		
14(V)F	5′- CTGGCACTAAGAATTTCATG - 3′	429	54
14(V)R	5′- CCTTCTCATTGTAGTCTATC - 3′		
14(VI)F	5′- GAAACATTTGACCCCGAGCA - 3′	431	54
14(VI)R	5′- TTTTGGGCAAGTCTGGTTTC - 3′		
14(VII)F	5′- CACATACAAGAAAGTTGAGA - 3′	436	54
14(VII)R	5′- CTCATTTATTGCTGCTATTG - 3′		
14(VIII)F	5′- GATACCATTTTGTCCCTGAA - 3′	415	54
14(VIII)R	5′- ACAAGAGCAGAGCA - 3′		
15F	5′- CACCTAGGAAAATGAGGATGT -3′	300	53
15R	5′- ATAGTCAGCAAGAAAATAAA - 3′		
16F	5′- AAGATCCTAGAAGATTATTC - 3′	330	50
16R	5′- TTAGTACACAAAGACCATTT - 3′		
17F	5′- TGATGAGAAATCCACTCTGG - 3′	349	54
17R	5′- GTGCAATCTGCATTTCACAG - 3′		
18F	5′- GTGGAATCCTCATAGATGTCA - 3′	312	53
18R	5′- GAGTAGGTAGAAGAAAGAGCAC -3′		
19F	5′- GCAAGCACTTTGCATTTGAG - 3′	305	52
19R	5′- AGCAACCATTCCAGAAAGGA - 3′		
20F	5′- CCATTTTCATTGACTTACATTTGAG – 3	193	53
20R	5′- ÁGATATAATCAGCCCAGGTTC - 3′		
21F	5′- GAATTTAATCTCTGATTTCTCTAC - 3′	168	53
21R	5′- GAGTGAATGTGAATACATTTCC - 3′		
22F	5′- AAATAGGTTAAAATAAAGTGTTAT	206	53
22R	5′- GACTAATTACATACCATTAAG - 3′		
23F	5′- CTCTGTATTCACTTTCCATG - 3′	250	54
23R	5′- ACAGTTAGTCACCCTACCCA – 3		
24F	5′- GCTCAGTATAACTGAGGCTG - 3′	249	54
24R	5′- CTCTGAGTCAGTTAAACAGT - 3′		
25F	5′- AGTGCTGTGGTATGGTTAAG - 3′	323	56
25R	5′- TTGCTCTGAAAATTTGGTCATA - 3′		
26F	5′- CCAATAAATGCTATCTTTCCT - 3′	217	53
26R	5′- TGACGGCAGTGGCAGGTGCT - 3′		

Elution from the matrix is achieved by a linear acetonitrile gradient [22,23]. The conditions for dHPLC were developed on the basis of exon-specific melting profiles predicted by WAVE-MAKER™ software. Table 2 summarizes the main parameters (temperature and acetonitrile gradient) established for the 33 fragments of the factor VIII gene. To ensure optimal resolution of heteroduplices from known FVIII mutations, the conditions for some amplicons were adjusted for temperature (Table 2, in bold type).

Table 2. Temperatures and acetonitrile gradients given in % buffer B for each of the 33 fragments of the factor VIII gene. Conditions written in *bold type* were added during the evaluation procedure for DHPLC. *T1, T2, T3, T4* = temperature 1, 2, 3, 4

Exon	Temp. (°C)	Acetonitrile gradient (%B)	Exon	Temp. (°C)	Acetonitrile gradient (%B)
1	T1 59	52–60	14(V)	T1 59	56–64
	T2 62	50–58			
2	T1 57	53–61	14(VI)	T1 59	58–66
	T2 60	51–59			
3	T1 60	52–60	14(VII)	T1 60	56–64
	T2 65	**52–60**			
4	T1 57	50–58	14(VIII)	T1 60	56–64
	T2 60	50–58			
5	T1 57	48–60	15	T1 58	55–63
				T2 60	52–60
6	T1 62	50–58	16	T1 57	54–62
				T2 60	51–59
7	T1 56	55–63	17	T1 59	56–64
	T2 59	54–62		T2 61	52–60
	T3 61	51–59			
8	T1 60	55–63	18	T1 58	54–62
				T2 60	51–59
9	**T1 57**	**51–59**	19	T1 59	55–63
	T2 59	51–59		T2 61	48–60
10	T1 57	51–59	20	T1 58	51–59
				T2 60	48–56
11	**T1 58**	**52–60**	21	T1 58	50–58
	T2 60	52–60		T2 60	50–58
12	T1 57	53–61	22	**T1 57**	**52–68**
	T2 58	53–61		T2 58	52–60
	T3 60	50–58		T3 60	52–60
	T4 62	53–61			
13	T1 59	55–63	23	T1 60	51–59
	T2 60	51–59			
14(I)	T1 59	58–56	24	T1 60	53–61
14(II)	T1 58	58–66	25	T1 57	56–64
				T2 60	53–61
14(III)	T1 57	58–66	26	T1 60	51–59
				T2 65	49–57
14(IV)	T1 56	60–68			
	T2 60	52–60			

Results and Discussion

Sensitivity of dHPLC in Hemophilia A Patients with Known Mutations

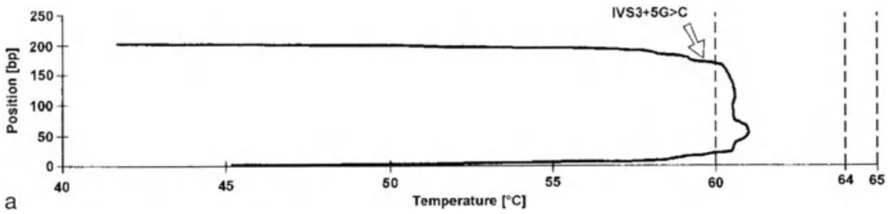
A total of 156 different alleles representing missense mutations, small insertions and deletions, as well as nucleotide alterations in the splice acceptor and donor sites of the FVIII gene (Table 2) were analyzed by dHPLC following a protocol with empirically established parameters for melting temperatures and elution gradients (Table 3). All but six mutations IVS3+5G>C, exon 9: 1293G>T, exon 11: 1569G>T, exon 12: 1804C>T, exon 21: 6193T>G, IVS21−8A>G were easily detectable as heteroduplex peaks, resulting in a detection rate of 96.2%. Subsequently, five of the remaining six mutations were identified by adjusting the melting temperature for each of the fragments (Table 3). Even after varying the melting temperatures over a broad range (50°C - 64°C), the missense mutation 6193T>G (exon 21) remained undetected. Taking together the initial and the optimized dHPLC analyses, a total of 56 injections are required for the 33 PCR fragments (Table 3), facilitating the detection of 155 out of 156 known sequence alterations in the FVIII gene and giving an overall sensitivity of 99.6%.

The identification of small sequence alterations with dHPLC largely relies on the sequence-related melting behavior of DNA domains which can be predicted using computer algorithms (e.g. as provided by the WAVE MAKER program) but needs to be verified experimentally. In Fig. 1a the melting profile of the exon 3 amplicon is shown, suggesting an optimal melting temperature of 60°C. While nine different sequence alterations could readily be identified at this temperature (Table 3), the IVS3+5G>C alteration could not be resolved. Increasing the temperature to 65°C, however, facilitated the resolution of a heteroduplex peak (Fig. 1b). All other conditions did not reveal the presence of the sequence alteration, a finding that is somewhat unexpected considering the nature of the mutation (G>C). Figure 2a provides a melting profile of the exon 9 amplicon. The mutation 1293G>T is located within a low temperature melting domain and escaped dHPLC detection at the empirically determined condition of 59°C. Lowering the melting temperature to 57°C clearly improves the detection of the nucleotide change (Fig. 2b). As shown in Fig. 3a the 6193T>G mutation in exon 21 is located at the peak of a high temperature melting domain. In addition, the nature of the nucleotide change (T>G) may further increase the melting temperature of the heteroduplex fragment. Empirically, a melting temperature as high as 65°C would be required to resolve a heteroduplex peak; however this is not feasible as the DNA strands of the exon 21 amplicon are already melted over its length at 64°C, rendering this mutation undetectable by dHPLC (Fig. 3b).

The above examples illustrate some of the difficulties in establishing optimized conditions for known mutations, difficulties that may be compounded when unknown alterations are the target of analysis. Furthermore, the selection of these known mutations was based on their previous detection by DGGE, a technique also relying on the melting behavior of DNA fragments. This may have biased our sample towards DNA variants particularly amenable to this type of analysis and would lead to an overestimation of the dHPLC detection rate.

Table 3. Nucleotide changes of 156 patients with known mutations in the factor VIII gene. The position of the mutation is given by the exon number and the nucleotide number of the factor VIII gene cDNA starting with the ATG codon that encodes the methionine of the factor VIII signal peptide. The temperatures at which the mutations were identified are given in brackets after each mutation. Mutations written in *bold type* were detected by the use of additional temperatures; one mutation remained undetected

Exon	Nucleotide changes
1	2T>G (59,62), 48-50delCTG (59,62), 103T>C (59,62),121G>T (62)
2	202-207delACTCTG (57,60)
3	IVS2-1G>A (60), 292G>A (60), 316A>G (60), 326A>G (60), 335C>T (60), 350T>G (60), 377A>C (60) 386A>G (60), IVS3+5G>A (60), **IVS3+5G>C (65)**
4	491G>A (60), 525C>A (60), 541G>A (60), IVS4+1G>A (60)
5	665A>T (57), IVS5+1G>T (57)
6	695-698delAGAA (62), 709C>T (62), 755C>T (62), 764G>A (62)
7	871G>T (61), 881C>T (61), 902G>A (59,61), 940A>T (59,61)
8	1063C>T (60), 1171C>T (60), 1174T>C (60), 1184A>G (60), 1226A>G (60), 1228-1230delGAG (60), IVS8+1GC>T (60),
9	**1293G>T (57)**, 1309C>T (59), 1315G>T (59), 1350C>G (59), 1357G>T (59), 1360A>C (59), 1362A>T (59), 1413A>T (59),
10	1477A>C (57), 1481T>C (57), 1492G>A (57), 1511C>A (57), 1537G>A (57)
11	**1569G>T (58)**, 1579-1601del (60), 1615G>T (60), 1636C>T (60), 1648C>T (60), 1649G>A (60), 1745G>T (60), 1751A>C (60), IVS11+5G>A (60)
12	1756A>G (57, 60), **1804C>T (58)**, 1836C>T (60, 62)
13	1965C>G (60), 1976T>C (60), 1941-1944delAGTT (60), 2018-2020delCTC (60), 2059C>T (60), 2059-2062delCTCA (60), 2060T>C (60)
14 (I)	2215G>A (59), 2126T>C (59), 2246T>A (59), 2150G>A (59), 2161A>T (59), 2167G>A (59), 2440C>T (59)
14 (II)	3381G>A (56, 60), 3602delT (56, 60), 3637insA (56, 60), 3637delA (56, 60)
14 (VIII)	5096A>T (60), 5123G>A (60), 5124C>T (60), 5143C>T (60), 5186G>A (60)
15	5251A>T (58, 60), 5301C>A (58, 60), 5305C>A (58, 60), 5306G>A (58, 60), 55323T>G (58,60), 5326G>A (58, 60), 5329C>T (58, 60)
16	5398C>T (57, 60), 5398C>G (57, 60), 5399G>A (57, 60), 5414A>T (57, 60), 5530C>T (57, 60), IVS16+2T>G (57, 60)
17	5594A>C (59, 61), 5600A>G (59, 61), 5603C>T (59, 61), 5622insT (59, 61)
18	5849G>T (58, 60), 5878C>T (58, 60), 5881C>T (58, 60), 5953C>T (58, 60)
19	6028T>C (59, 61), 6046C>T (61), 6093-6094delAC (61), 6103A>G (61), 6107A>G (61), 6113A>G (61), IVS19+1G>A (61)
20	IVS20+1G>A (58, 60)
21	6269T>C (58), **6193T>C (not identified)**
22	**IVS21-8A>G (57)**, 6278A>G (58, 60), 6317A>G (60), 6256A>G (60), 6371A>G (60), 6413C>A (58,60)
23	6439C>T (60), 6448G>A (60), 6482C>T (60), 6505C>T (60), 6506G>A (60), 6506G>T (60), 6515C>A (60), 6515C>G (60), 6532C>T (60), 6537C>A (60), 6544C>T (60), 6545G>A (60), 6548T>A (60),
24	6595delA (60), 6683G>A (60), IVS24+4A>G (60)
25	6760C>T (60), 6769A>G (60), 6781G>T (60), 6797G>A (60), 6828-6830delGAA (57, 60), 6857A>G (60), 6865C>T (57, 60)
26	6920A>C (60, 65), 6932C>A (60, 65), 6955C>T (60, 65), 6967C>T (60, 65), 6977G>T (60, 65)



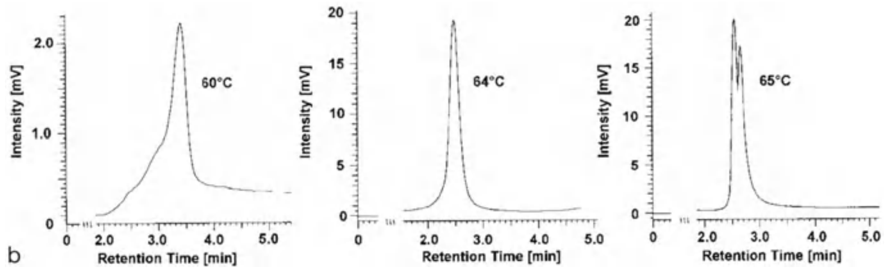
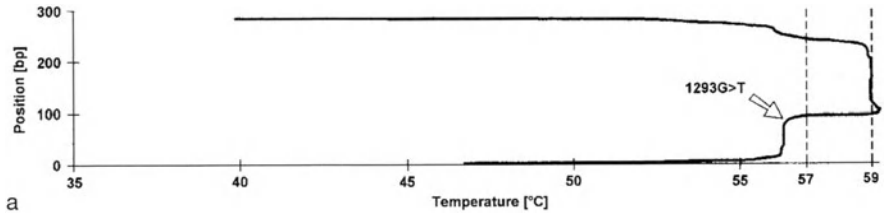


Fig. 1a, b. *a* Empirically derived melting profile of exon 3 of the FVIII gene. The position of the IVS3+5G>C mutation is marked with an *arrow*. The *dotted lines* indicate the temperatures used to premelt the PCR fragment. *b* Retention profiles at 60°C, 64°C, 65°C. In contrast to the predicted 60°C melting temperature, the IVS3+5G>C mutation is not resolved until premelting of the PCR fragment is done at 65°C



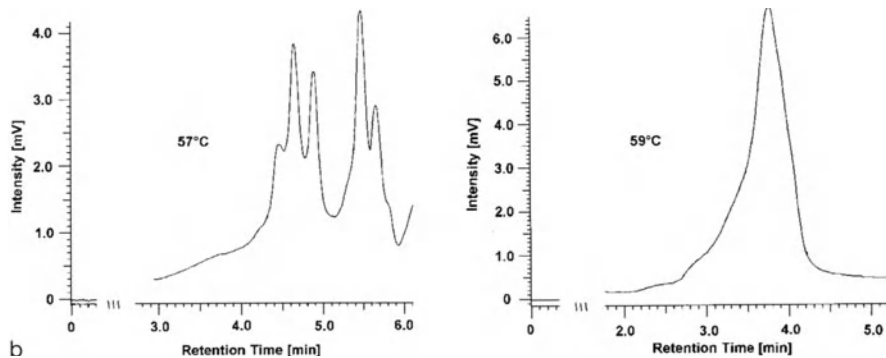


Fig. 2a, b. *a* Empirically derived melting profile of exon 9 of the FVIII gene. The 1293G>T mutation is located within a low temperature melting domain and is marked with an *arrow*. The *dotted lines* indicate the temperatures used to premelt the PCR fragment. *b* The retention profile at 57°C reveals the resolution of the homoduplex/heteroduplex which is not detectable at 59°C

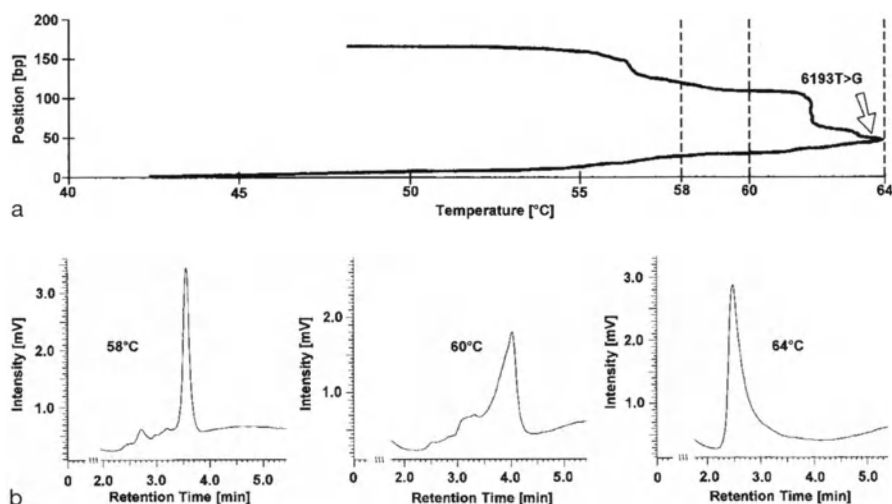


Fig. 3a, b. *a* Empirically derived melting profile of exon 21 of the FVIII gene. The 6193T>G mutation is marked by an *arrow* and located at the peak of a high temperature melting domain. The *dotted lines* indicate the temperatures used to premelt the PCR fragment. *b* The retention profiles at 58°C, 60°C and 64°C fail to resolve the homoduplex/heteroduplex formations. Melting of the double stranded fragments is complete at 64°C

Table 4. List of 19 mutations identified by DHPLC in a cohort of 27 patients in whom the genetic defect remains undetected after screening with conventional methods

No.	Exon	Nucleotide exchange	Amino acid exchange
1	7	901C>T	Arg282Cys
2	7	901C>T	Arg282Cys
3	7	902G>A	Arg282His
4	7	968G>A	Gly304Glu
5	8	784A>G	Lys376Arg
6	11	1673–1680 TGGAGAGA>ATCTCTC	Complex mutation
7	IVS11	IVS11-2delA	Splice site
8	14 (III)	3175A>T	Lys1040Stop
9	14 (VI)	4385–4388delTTTC	Small deletion
10	14 (VII)	4829delC	Small deletion
11	16	5464C>T	Arg1803Stop
12	17	5687C>T	Gln1874Stop
13	18	5825G>C	Gly1923Ala
14	18	5888T>C	Leu1944Pro
15	23	6486C>T	Arg2147Stop
16	23	6547A>G	Met2164Val
17	26	6920A>C	Asp2288Ala
18	26	69256927delTTC	Small deletion
19	26	6967C>T	Arg2304Cys

dHPLC Analysis in Hemophilia A Patients with Unknown Mutations

To test the effectiveness of dHPLC for mutation analysis in detecting unknown mutations, the optimized protocol (Table 2) was applied to a group of 27 hemophiliacs in whom no mutation could be detected by a systematic screen using DGGE and CMC. dHPLC analysis of the 33 amplicons of the FVIII gene revealed probable disease-associated alterations in 19 (70.4%) of these patients (Table 4) while the molecular defects in the remaining 8 patients (29.6%) still remain elusive. The identification of a significant number of additional mutations suggests that the optimized dHPLC protocol provides a greater sensitivity than the previously used methods. The inability to detect the genetic alterations in eight patients may be due to one of three possibilities. First, the nucleotide changes may reside within a complex melting profile unaccounted for by our present protocol. Second, the defects may be located outside the PCR amplicons established for the analysis of the FVIII gene and thus may escape detection. Third, the genetic defect for some of the hemophiliacs may not reside in the FVIII gene but instead may be non-allelic. To resolve this issue the complete sequencing of the FVIII gene in these eight patients is required and is currently in progress.

Future Directions

dHPLC offers a rapid and sensitive technology that is amenable to automation and thus is well suited for routine applications specifically for large genes such as FVIII. Sequencing the complete coding region of the factor VIII gene in those patients where the current protocol is not able to identify the mutation can be used to further optimize dHPLC sensitivity. Reanalyzing newly identified mutations by dHPLC will then lead to the establishment of additional parameters. Following this strategy the protocol can be systematically improved until maximal sensitivity is achieved. This possibility of continuous optimization of the dHPLC protocol is, in our opinion, a major advantage of dHPLC technology; however it may become necessary to develop a multitier strategy to maintain an efficient system when the number of conditions, and consequently the number of injections, increases. Under such a strategy patients would be screened first under a core set of conditions with a specified detection rate, e.g. 96%, before proceeding to a secondary screening under an expanded set of conditions.

References

1. Antonarakis, S.E., Rossiter, J.P., Young, M., Horst, J., de Moerloosw, P., Sommer, S.S., Ketterling, R.P., Kazaian, H.H. Jr., Negrier, C., Vinciguerra, C. et al. Factor VIII gene inversions in severe haemophilia A: Results of an international consortium study. Blood 1995; 86: 2206–2012
2. Toole, J.J., Knopf, J.L., Wozney, J.M., Sulzman, L.A., Buecker, J.L., Pittman, D.D., Kaufman, R.J., Brown, E., Shoemaker, C., Orr, E.C., Amphlett, A.W., Foster, W.B., Coe, M.L., Knutson, G.J., Fass, D.N., Hewick, R.M. Molecular cloning of a cDNA encoding human antihaemophiliac factor. Nature 1984; 312: 342
3. Vehar, G.A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D.P., Rotblat, F., Opperman, H., Keck, R., Wood, W.I., Harkins, R.N., Tuddenham, E.D.G., Lawn, R.M., Capon, D.J. Structure of human factor VIII. Nature 1984; 312: 337–342

4. Pittman, D.D., Alderman, E.M., Tomkinson, K.N., Wang, J.H., Giles, A.R., Kaufman, R.J. Biochemical, immunological, and in vivo functional characterization of the B-domain deleted factor VIII. Blood 1993;81: 2925–2935
5. Levinson, B., Kenwrick, S., Lakich, D., Hammonds, G., Gitschier, J. A transcribed gene in an intron of the human factor VIII gene. Genomics 1990; 7: 1–11
6. Levinson, B., Kenwrick, S., Gamel, P., Fisher, K., Gitschier, J. Evidence for a third transcript from the human factor VIII gene. Genomics 1992; 14: 585–589
7. Strauss, H.S. The Perpetuation of Hemophilia by Mutation. Pediatrics 1967; 39: 186–193
8. Erfle, V., Hehlmann, R., Mellert, W., Krüger, G., Seifried, E., Heimpel, H., Rasokat, H., Lechler, E., Holzer, E., Hellstern, P., Köhler, M., Goebel, F.D. Prevalence of antibodies to HTLV-III in AIDS risk groups in West Germany. Cancer Res 1985; 45 Suppl: 4627–4629
9. Peake, I. Genetic services available for counselling and prenatal diagnosis of haemophilia, Haemophilia 1998; 4 Suppl: 24–25
10. Schwaab R, Brackmann H-H, Meyer C, Seehafer J, Kirchgesser M, Haack A, Olek K, Tuddenham EGD, Oldenburg J. Haemophilia A: Mutation type determines risk of inhibitor formation. Thromb Haemost 1995; 74: 1402–6
11. Kaufman, R.J. Advances toward Gene Therapy for Hemophilia at the Millennium, Human Gene Therapy 1999; 10: 2091–2107
12. Higuchi, M., Antonarakis, S.E., Kasch, L., Oldenburg, J., Economou-Petersen, E., Olek, K., Arai, M., Inaba, H., Kazazian, HH. Molecular characterization of mild-to-moderate hemophilia A: Detection of the mutation in 25 of 29 patients by denaturing gradient gel electrophoresis. Proc Natl Acad Sci USA 1991; 88: 8307–8311
13. Higuchi, M., Kazazian, HH., Kasch, L., Warren, T.C., McGinnis, M.J., Phillips, J.A., Kasper, C., Janco, R., Antonarakis, S.E. Molecular characterization of severe hemophilia A suggests that about half the mutations are not within the coding regions and splice junctions of the factor VIII gene. Proc Natl Acad Sci USA 1991; 88: 7405–7409
14. Naylor, J.A., Green, P.M., Pizza, C.R., Gianelli, F. Analysis of factor VIII mRNA reveals defects in everyone of 28 haemophilia A patients. Hum Mol Genet 1993; 2: 11–17
15. Lakich, D., Kazazian, Jr., Antonarakis, S.E., Gitschier, J. Inversions disrupting the factor VIII gene as a common cause of severe hemopilia A. Nature Genetics 1993; 5: 236–241
16. Lin, S.W., Lin, S.R., Shen, M.C. Characterization of genetic defects of hemophilia A patients of Chinese origin. Genomics 1993; 18: 496–504
17. Schwaab, R., Oldenburg, J., Schwaab, U., Johnson, D.J.D., Schmidt, W., Olek, K., Brackmann, H.H., Tuddenham, E.G. Characterization of mutations within the factor VIII gene of 73 unrelated mild and moderate haemophiliacs. Br J Haematol 1995; 91: 458–464
18. Pienemann, W.C., Deutz-Terlouw, P.P., Reitsma, P.H., Briet, E. Screening for mutations in haemophilia A patients by multiplex PCR-SSCP, Southern blotting and RNA analysis: the detection of genetic abnormality in the factor VIII gene in 30 out of 35 patients. Br J Haematol 1995; 90: 442–449
19. Becker, J., Schwaab, R., Möller-Taube, A., Schwaab, U., Schmidt, W., Brackmann, H.H., Grimm, T., Olek, K., Oldenburg, J. Characterization of the factor VIII defect in 147 patients with sporadic hemophilia A: Family studies indicate a mutation type dependent sex ratio of mutation frequencies. Am J Hum Genet 1996; 58: 657–670
20. Waseem, N.H., Bagnall, R., Green, P.M., Gianelli, F. Start of UK confidential haemophilia A database: Analysis of 142 patients by solid phase fluorescent chemical cleavage of mismatch. Thromb Haemost 1999; 81: 900–905
21. Freson, K., Peerlinck, K., Aguirre, T., Arnout, J., Vermylen, J., Cassiman, J.J., Matthijs, G. Fluorescent chemical cleavage of mismatches for efficient screening of the factor VIII gene. Hum Mut 1998;11: 470–479
22. Oefner, P.J., Underhill, P.A. Comparative DNA sequence by denaturing high performance liquid chromatography (DHPLC). Am J Hum Genet 1995; 57: A266
23. Oefner, P.J., Underhill, P.A. DNA mutation detection using denaturing high-performance liquid chromatography (DHPLC). Current Protocols in Human Genetics 1998; 19 Suppl: 7.10.1–7.10.12

VIa. Poster: Clinic and Casuistic

Rheumatoid Arthritis in a Patient with Hemophilic Arthropathy – a Case Report

H.H. Eickhoff, G. Raderschadt, F.W. Koch, T. Wallny
and H.-H. Brackmann

Prevalence of Hemophilia – Rheumatoid Arthritis

The prevalence of hemophilia in Europe is approximately 1:10,000. According to the literature, the prevalence of rheumatoid arthritis in males is about 1:400 [2]. Therefore the theoretical occurrence rate of a combination of both diseases is 1:4,000,000.

Joint Pattern of Hemophilia – Rheumatoid Arthritis

In hemophilia it is mainly the knee, elbow and ankle joints that are affected. The joint involvement in rheumatoid arthritis usually corresponds to those of the finger, hand, elbow, knee and ankle. However, with the exception of the distal interphalangeal joints, all synovial joints can be affected. An additional extraarticular involvement is frequently observed. In the combination of hemophilic arthropathy with rheumatoid arthritis an additional effect concerning the number and distribution of affected joints as well as the degree of joint involvement can be expected.

Case Report

The case presented is one of a 54-year old patient from the former East Germany with hemophilia A; remaining factor activity was less than 1%. Due to recurrent bleeding episodes severe arthropathy of all major joints developed (knee, hip, ankle, elbow, shoulder) (Figs. 1, 2). In addition, in 1980 the patient developed sero-positive rheumatoid arthritis and the patient's situation deteriorated dramatically (Fig. 3). In 1989 the patient was confined to a wheelchair.

In October 1990 the patient was admitted to the Bonn University Hospital Orthopedic Department for combined hemostatic, orthopedic and rheumatologic therapy.

A cementless total hip arthroplasty was implanted on the right side in October 1990. Postoperatively an antibody developed, which was treated successfully. In January 1991 the second total hip arthroplasty was implanted cementlessly on the left side. Eight years postoperatively the hip implants showed no loosening radiologically and were without any other complications (Fig. 4).

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

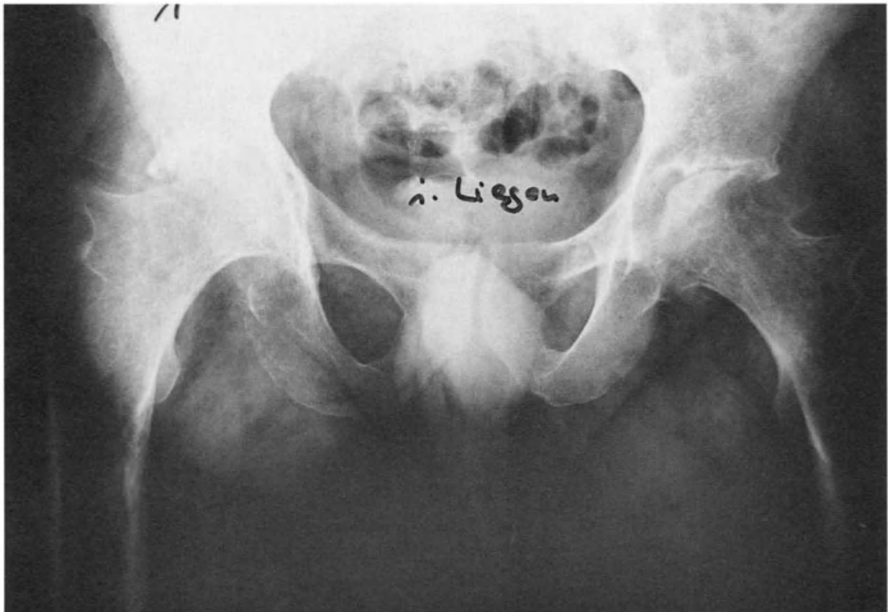


Fig. 1. 05/90: severe hemophilic arthropathy of both hips

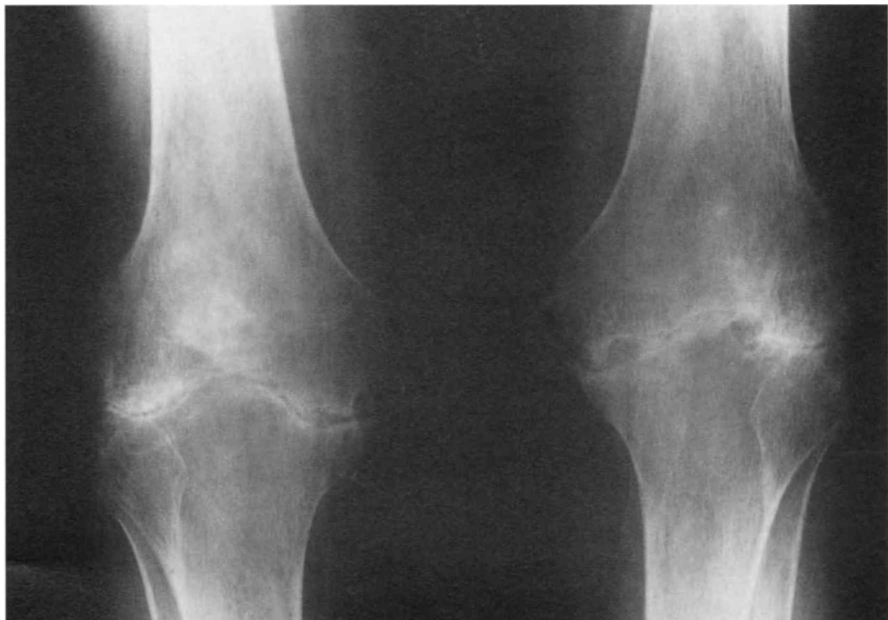


Fig. 2. 05/90: severe hemophilic arthropathy of both knees

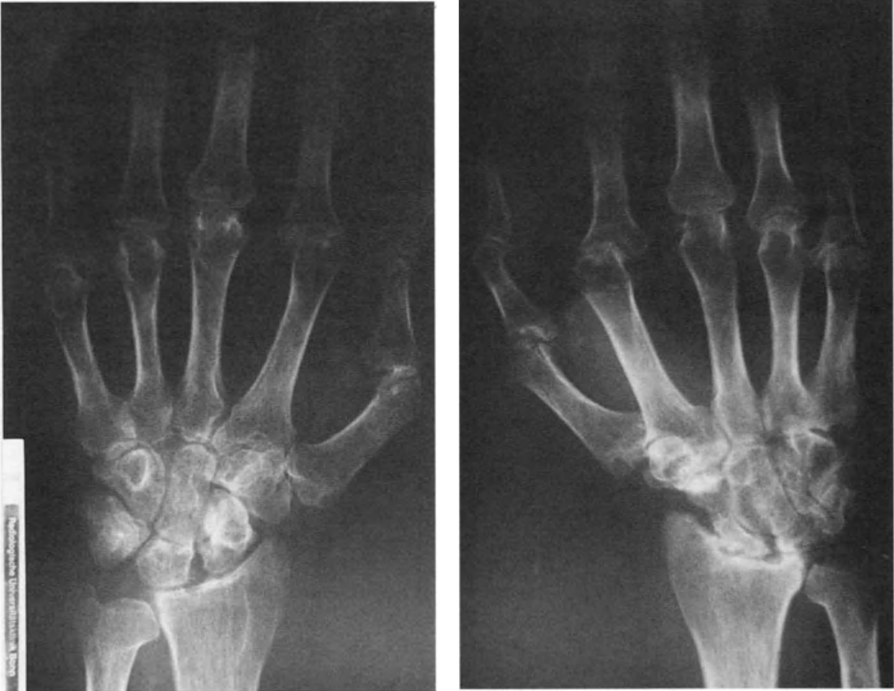


Fig. 3. 05/90: rheumatoid arthritis of both hands

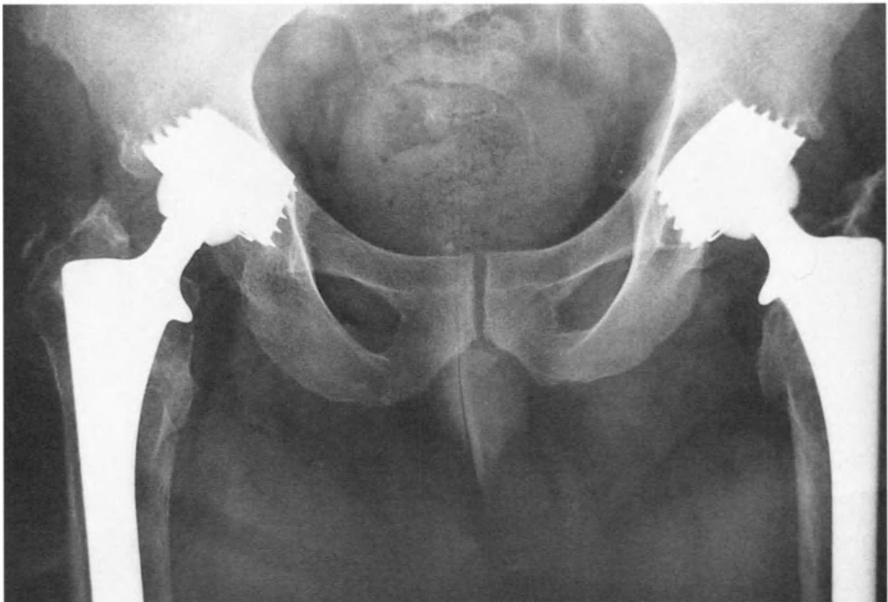


Fig. 4. 10/98: cementless total hip replacement (8 years after operation)

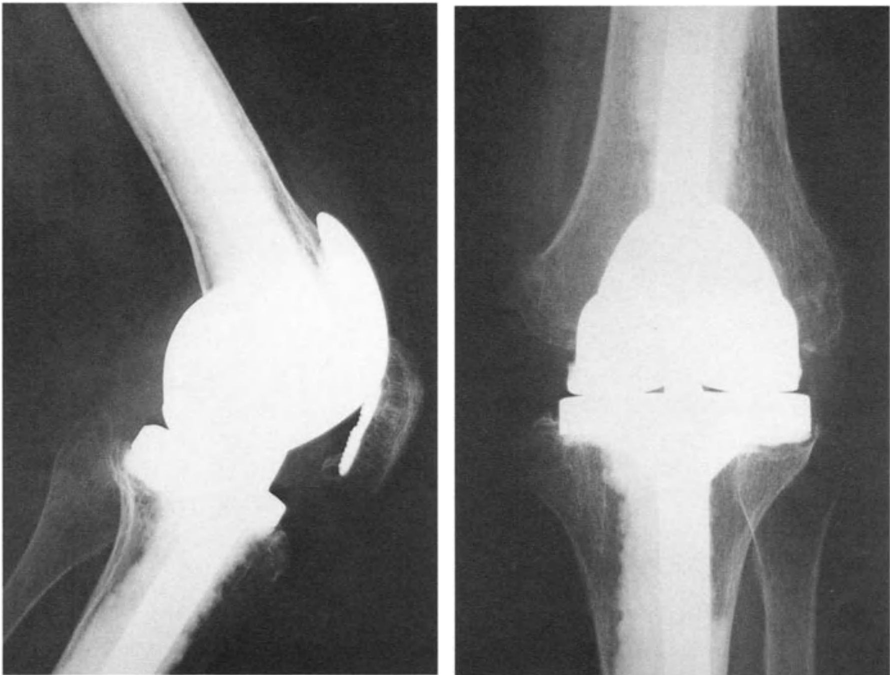


Fig. 5. 11/99: cemented total knee replacement (type GSB) (8.5 years after operation/left side)

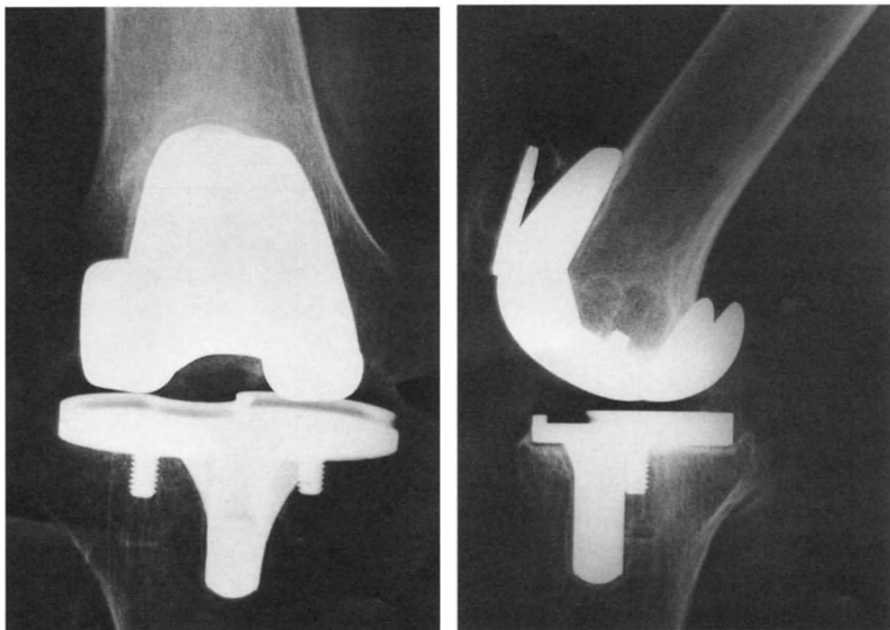


Fig. 6. 05/97: total condylar knee prosthesis (right side). Note the amount of femural resection

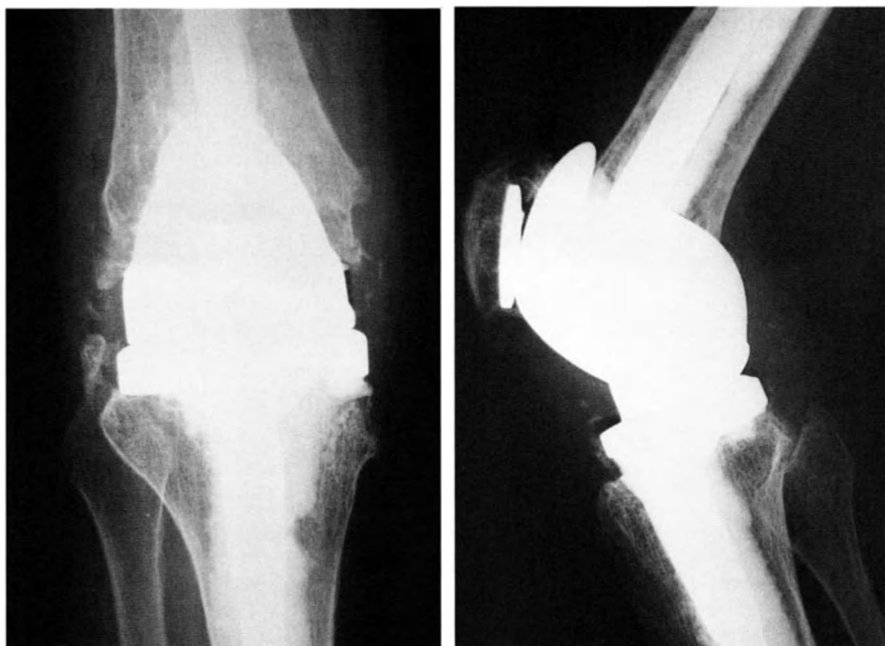


Fig. 7. 11/99: right knee after arthrolysis and revision to a cemented knee arthroplasty (GSB). Note the restored joint line (2.5 years after operation)

A cemented knee arthroplasty (type GSB) was implanted on the left side in April 1991. Follow-up showed no significant problems (Fig. 5).

In February 1992 a total condylar prosthesis was implanted cementlessly on the right side (type Tricon). During follow-up the latter showed a significant loss in the range of movement (Fig. 6). This led to a revision at the Orthopedic Hospital St. Josef, Troisdorf in May 1997 with arthrolysis and exchanging the prosthesis for a cemented GSB-arthroplasty (Fig. 7). The subsequent follow-up was unremarkable.

At the final follow-up in October 1999 the patient walked unaided and was very satisfied with the result.

Summary and Discussion

The combination of hemophilia, particularly hemophilic arthropathy with rheumatoid arthritis, is very rare. Facing the different joint patterns of both diseases there is an additive effect with respect to the number of affected joints as well as the severity of the disease.

Close collaboration of all involved specialities (hemostasiology, orthopedic surgery and rheumatology) is essential.

The necessary orthopedic operations, especially the total joint replacements, are very demanding and should be performed by an experienced surgeon [1]. Due to

the suppression of the immune system by the rheumatoid drugs there is a higher risk of infection peri- and postoperatively. In addition the rehabilitation period is longer and more difficult due to the involvement of multiple joints.

In the presence of a contracture particularly around the knee joint, a wide soft tissue release is required intraoperatively (release of collateral and cruciate ligaments, capsule, popliteus and gastrocnemius tendons). With regard to poor bone quality (osteoporosis) a knee replacement should be done with a cemented prosthesis and at least a tibial stem. In the presented case even a constrained knee implant was necessary.

The hip joint can be replaced, as in the presented case, cementlessly.

As this case report demonstrates, a good result can be achieved with a clear indication and adequate therapy.

References

1. Eickhoff, HH, Koch FW, Brackmann HH (1998) Kniegelenkendoprothetik – Besonderheiten bei der hämophilen Arthropathie. In: Scharrer I, Schramm W (eds) 27. Hämophilie-Symposion Hamburg 1996. Springer, Berlin Heidelberg New York
2. Schattenkirchner M (1986) Erkrankungen des rheumatischen Formenkreises. Einteilung, Klinik, konservative Therapie. In: Jäger M, Wirth, CJ (eds) Praxis der Orthopädie. Thieme, Stuttgart New York

Recurrent Fatal Intracranial Hemorrhage (ICH) in Two Non-Identical Twins suffering from Hemophilia B (Factor IX Activity <1%)

S. Halimeh, H. Pollmann, M. Käse, I. Hörnig-Franz, H. Jürgens
and U. Nowak-Göttl

Introduction

Intracranial hemorrhage is one of the leading causes of serious illness and death in patients with hemophilia. Most reports indicate that the complication is most common in patients of less than 18 years of age. The reported incidence ranges between 2.9 and 7.5% (1–7). The most important factor was trauma (57%), either during birth (30%) or later in life (27%). Seizures were common (63%).There was no significant difference between the prevalence of patients with hemophilia A and B (3.5% vs. 6.3%) and among the groups (8). Hemophilia A and B are sex linked disorders that are due to respective deficiencies in the clotting factors VIII and IX. The frequency of factor VIII deficiency is thought to be approximately 1 per 10,000 male births; for factor IX deficiency, the frequency is approximately 1 per 30,000–50,000 male births (9).

Case Report

Case 1

We report two additional cases of recurrent ICH in two non-identical twins with severe hemophilia B. After an uneventful twin pregnancy not knowing the diagnosis of severe HB forceps delivery was performed after 36 weeks of gestational age. Shortly after birth clinical examination of the first male twin showed a large and progressing cephalohematoma, and generalized seizures followed 10 h later. Immediately performed computed tomography showed a large subdural hemorrhage located in the left occipital lobe. Laboratory evaluation showed prolonged aPTT, with a factor IX activity <1% in the patient and also in his non-affected brother. Factor VIIIa, XIa and XII a showed age-dependent normal values. Substitution therapy in the affected male was started with a virus inactivated factor IX concentrate. The neonate survived and received a prophylactic treatment with recombinant factor IX concentrate thrice weekly for a period of 3 months. Because the first ICH was associated with severe cerebrocranial trauma, i.e. forceps delivery, and the lack of venous access, the treatment regimen was changed to »on-demand« substitution. At the age of 6 months the patient was admitted to the hospital, again with signs of increased intracranial pressure, i.e. vomiting and seizures, and re-

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

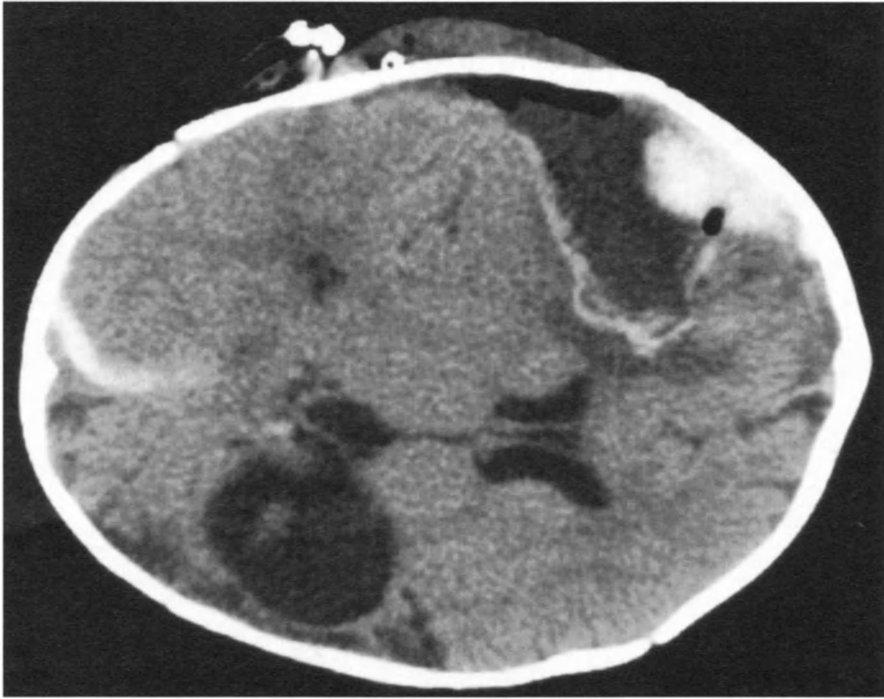


Fig. 1

current contralateral ICH occurring spontaneously was diagnosed at this time point (Fig. 1).

Case 2

At 11 months of age the non-identical twin, free of bleeding so far and therefore not treated prophylactically with factor IX concentrate, was admitted to the pediatric intensive care unit suffering also from spontaneous ICH in the right occipital lobe. Despite factor substitution, craniotomy and immediate intensive care treatment, he died (Fig. 2).

The neuropathology report does not show any hemangioma or an arteriovenous malformation.

Discussion

ICH remain a relatively common complication in hemophilia patients. Silverstein reported the incidence of ICH in various centers in several countries; it ranged between 2.2 and 7.8%. Eyster and de Tezanos Pinto independently reported a median

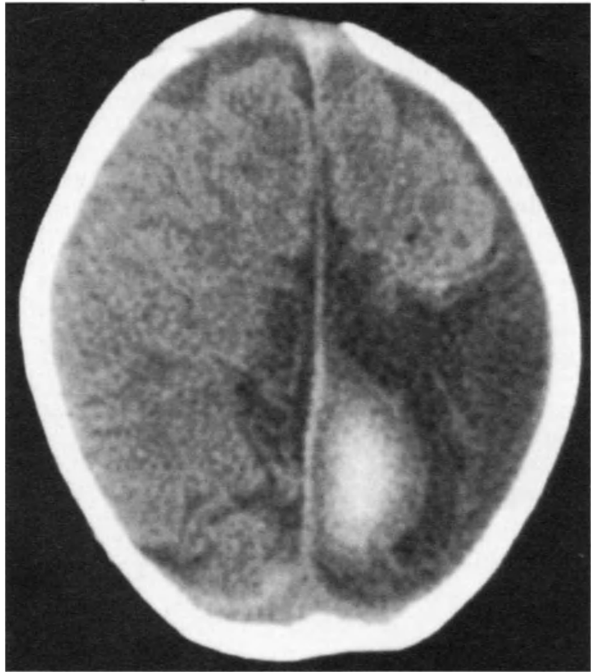


Fig. 2

age of 10 and 10,5 years respectively in patients with hemophilia A and of 4 and 6 years respectively in patients with hemophilia B. In both studies 1/3 of all ICH occurred during the first years of life. Klinge et al. showed in his study the bleeding sites and possible cause and neurological outcome after ICH. He found a median age of 2 years, with 38% occurring within the first week of life. Neurological outcome is still a major problem after ICH, even if prompt and effective therapy is initiated. The study of Klinge et al. showed that only 24% of all patients have neurological deficits. This seems comparable with data published by de Tezanos Pinto et al. Until 1960 nearly 70% of the patients had a fatal outcome; since then the death rate has been approximately 30%. The mortality in the study of de Tezanos Pinto is 29.2%. Eyster reported a 34% death rate.

Based on these two severe ICH cases occurring within 1 year in the same family, a primary prophylactic treatment regimen is discussed in asymptomatic family members carrying the same severe coagulation defect with ICH as a member of the family already known to have shown the first onset of the disease.

These two cases showed us that prospective studies are warranted in order to further evaluate the significance of intracerebral hemorrhage in patients with hemophilia and to provide more information on additional risk factors such as mode of delivery and prophylactic or on-demand treatment.

References

1. de Tezano Pinto M, Fernandez J, Perez Bianco PR (1992) Update of 156 episodes of central nervous system bleeding in hemophiliacs. Haemostasis 22(5): 259–267
2. Eyster ME, Gill FM, Blatt PM, Higartner MW, Ballared JM, Kinney TR, Group atHS (1978) Central nervous system bleeding in haemophiliacs. Blood 51(6):1179
3. Silverstein A (1960) Intracranial bleeding in haemophilia. Arch Neurol 3:141–157
4. Yoffe G, Buchanan GR (1988) Intracranial hemorrhage in newborn and young infants with haemophilia. J Pediatr. 113(2):333–336
5. Petterson H, Mc Clure P, Fitz C. Intracranial hemorrhage in haemophilic Children: CT follow-up. Acta Radiologica 1984; 51:1179–88
6. Martinowitz U, Heim M, Tadmor R. Intracranial haemorrhage in patients with haemophilia. Neurosurgery 1986;18:530–41
7. Kerr CB. Intracranial haemorrhage in haemophilia. J Neurol Neurosurg Psychiatry 1964;27:166–73.
8. Klinge J et al., Prevalence and outcome of intracranial haemorrhage in haemophiliacs –a survey of the paediatrics group of the German Society of Thrombosis and Haemostasis (GTH). Eur J Pediatr 1999 Suppl. 162–165.
9. Haemophilia of Georgia, Protocols for the treatment of haemophilia and von Willebrand Disease. Haemophilia 2000 Suppl. 84–93.

Progression of Thrombosis under Low Molecular Weight Heparin without Heparin-Induced Thrombocytopenia in a young Man: a Case Report

R. Knöfler, J. Wendisch, K. Nemat, G. Siegert, S. Gehrisch and S. Schellong

Introduction

Low molecular weight heparins (LMWHs) have been proven to be efficacious and safe in the treatment and prophylaxis of thrombosis in adults. LMWHs also appear to offer a safe and effective form of anticoagulation in children [5,9,13,15], but in contrast to adults randomized clinical trials comparing LMWHs with unfractionated heparins (UFHs) and vitamin K antagonists for the prevention and treatment of thromboembolic events are lacking. According to clinical experiences with LMWHs in children [5,9,11,15] for prophylaxis of thrombosis plasma anti-Xa activities between 0.2 and 0.4 IU/ml measured 4 h after application are needed for effective prevention of thrombus apposition or recurrence of thrombosis. We report on a 16-year-old boy who suffered from an incomplete thrombosis of the left popliteal vein and developed an extended complete vein thrombosis during secondary thrombosis prophylaxis with nadroparin. At that time the anti-Xa activity was in the prophylactic range, the patient wore a compression stocking and was completely mobilized.

Anamnesis

After surgical care of a laceration on the left lower leg caused by a traffic accident, the 16-year-old boy was immobilized for 4 days without prophylactic anticoagulation. According to the patient's statement the left lower leg was swollen all the time, but pain in this area developed only 3 weeks later, after he was completely mobilized. After admission to a hospital an incomplete thrombosis of the left popliteal vein was detected by phlebography (Fig. 1). He was treated with nadroparin (Fraxiparin®, Sanofi-Synthelabo, Germany) at a dosage of 190 anti-Xa IU/kg/d corresponding to a twice daily subcutaneous injections of Fraxiparin® 0.7 ml. Wearing a compression stocking caused the pain to disappear within a few days. Anti-Xa activity was not determined at that time because the patient was treated in a small hospital with no possibility of measuring this parameter. After 8 days the dose of nadroparin was reduced to 55 anti-Xa IU/kg/d corresponding to the once daily subcutaneous injection of Fraxiparin® 0.4 ml for prophylactic reasons. At that time the patient was symptom-free as far as the thrombosis was concerned. Fifteen days after the start of prophylaxis the patient presented in our outpatient clinic showing

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

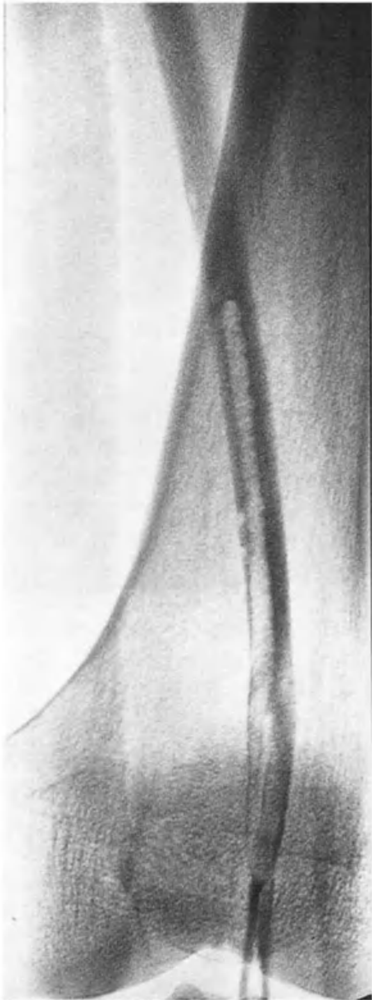


Fig. 1. Phlebographic findings at the time of onset of thrombosis, 3 weeks after surgical care of a laceration on the left lower leg. In the left popliteal vein a vessel wall-adherent thrombus surrounded by contrast media as a sign of an incomplete thrombosis was detected

no clinical signs of thrombosis and an anti-Xa activity (COACUTE® Heparin, Chromogenix, Italy) of 0.22 IU/ml was measured 4 h after nadroparin injection. Three days later pain in the left lower leg occurred again and after admission to our hospital a complete left-sided thrombosis of the distal part of the femoral vein, the popliteal vein, the saphenous parva vein and the soleus veins were detected by Doppler sonography. After exclusion of heparin-induced thrombocytopenia the patient received nadroparin at 190 anti-Xa IU/kg/d s.c. again and was then treated with a vitamin K antagonist (phenprocoumon, Falithrom®, Hexal, Germany) setting the INR value between 2.0 and 3.0. Swelling as well as pain in the left lower leg disappeared again and Doppler sonography performed 5 weeks after clinical manife-

station of thrombosis progression showed no further thrombus apposition but on the other hand also no recanalization.

No thromboembolic events are known in the patient's family history.

Laboratory Data at Time of Onset of Thrombosis Progression

Anti-Xa activities:	0.22 IU/ml measured 3 days before (LMWH dose: 55 anti-Xa IU/kg s.c.o.d.) 0.62 IU/ml measured 2 days later (LMWH dose: 95 anti-Xa IU/kg s.c.b.d.)
D-Dimer concentration:	1.0 µg/ml (reference range: <0.5 µg/ml; 3 days before: 0.3 µg/ml)
Thrombocyte count:	184,000/µl (reference range: 150,000–400,000/µl; 3 days before: 249,000/µl)
Fibrinogen concentration:	5.4 g/l (reference range:1.5–4.5 g/l; 3 days before: 2.6 g/l)
Antithrombin:	108% (reference range: 80–120%; 3 days before: 122%)
HIT antibodies:	Exclusion of heparin-induced thrombocytopenia type II by heparin-induced platelet aggregation test and heparin/platelet factor 4 – ELISA
C-reactive protein:	41.5 mg/l (reference range: <5.0 mg/l)

Results of Thrombophilia Diagnostics

APC ratio:	1.79 (reference range: >2.2)
Molecular genetic analysis:	Heterozygosity for factor V G1691 A mutation, no prothrombin G20210 A variant
Coagulation factor XII:	25% and 33% (reference range: 80–120%)
Normal values:	Antithrombin, protein C act., protein S act. and free protein S, lupus anticoagulant (negative), lipoprotein (a), homocysteine

Discussion

Anticoagulation with heparin is required for the prevention and treatment of thromboembolic complications in children. LMWHs seem to offer many advantages over other anticoagulants currently used in children but randomized controlled trials comparing LMWHs with other anticoagulants are lacking. Up to now only a few reports have been published on the use of LMWHs in pediatric patients. LMWHs were effective and safe for the treatment and prophylaxis of thromboem-

bolic complications [5,13,15], hemodialysis [1,20], for prevention of vascular thrombosis after renal [2,6] and liver transplantation [8] as well as in children with sinovenous thrombosis [4]. In accordance with the literature mentioned above, we also have obtained good clinical results using different LMWHs for the treatment and prophylaxis of thrombosis [9]. As reported by others [11,15], we have set the prophylactic level of plasma anti-Xa activities between 0.2 and 0.4 IU/ml measured 4 h after application. According to our experiences anti-Xa activities in this range are reached by doses of 50 to 100 anti-Xa IU/kg, though it should be noted that newborns and infants require higher doses than older children [13]. The patient reported here received nadroparin at 55 anti-Xa IU/kg s.c. once daily for secondary thrombosis prophylaxis and he is the first one in whom we have seen a thrombosis progression despite the fact that anti-Xa activity (0.22 IU/ml) was in the prophylactic range. From this case report the question that arises is whether we should have used a higher nadroparin dose to prevent thrombosis progression. Lopaciuk et al. [12] administered nadroparin at a higher dose of 85 anti-Xa IU/kg s.c. once daily for secondary prophylaxis in adults and detected a thrombosis recurrence rate of 2% (2 out of 98 patients). In a pediatric setting, Dix et al. [5] used enoxaparin at 110 anti Xa IU/kg s.c. twice daily for secondary thrombosis prophylaxis and found a new or progressive thrombosis in 2.3% patients. Pini et al. [17] administered enoxaparin in adults with a comparable dose of 4000 anti-Xa IU as we have used in our patient (3850 IU) and report a thrombosis recurrence rate of 6% (6 out of 93 patients). Monreal et al. [14] did not detect recurrent thromboses in 40 adults receiving 5000 anti-Xa IU of dalteparin. Concerning the different doses and LMWHs used in these studies, it should be considered that pharmacokinetic profiles differ between the preparations of LMWHs [3]. Therefore, clinical results obtained from studies using different LMWHs cannot be directly compared. However, taking into account the results from the clinical trials and the fact that as far as age (16 years) and body weight (70 kg) are concerned our patient seems to be comparable with the pharmacokinetic profile in a young adult, it is our conclusion that a higher nadroparin dose might have been of advantage for the patient reported here. However, it should be mentioned that even at higher LMWH doses a progression of thrombosis occurs in some patients which may be due to interindividual differences in pharmacokinetics. If the presence of a combination of hereditary thrombophilic risk factors in our patient (heterozygosity for factor V G1691 A mutation and coagulation factor XII deficiency) has contributed to the development of thrombosis progression this constitutes another point for discussion. The importance of combined prothrombotic defects for thromboembolic complications in children has already been proven in a multicenter trial [16]. But it remains an open question whether pediatric patients with combinations of hereditary thrombophilic risk factors need higher LMWH doses for primary and/or secondary thrombosis prophylaxis.

Thrombosis progression may be a clinical sign of heparin-induced thrombocytopenia type II (HIT) which is detected more often in patients who receive unfractionated heparin as opposed to LMWH [19]. Thrombocyte counts typically decrease between day 5 and 10 after the onset of heparin treatment and thromboembolic complications often occur during that time [7]. In a subset of patients, even in children in whom the incidence of HIT is much lower than in adults, throm-

bosis may become clinically manifest without a substantial decrease in thrombocyte count [7,10,18]. Therefore, in our patient HIT was excluded by additional tests (heparin-induced platelet aggregation – HIPA and heparin/PF4-ELISA) despite the fact that the thrombocyte count was only slightly decreased (within 3 days from 249,000/µl to 184,000/µl).

Conclusions

Our case report demonstrates that a nadroparin dose of 55 anti-Xa IU/kg corresponding to an anti-Xa activity of 0.22 IU/ml was not sufficient for secondary thrombosis prophylaxis in a young man. As reported by Lopaciuk et al. from a study using adults, for secondary prophylaxis a higher nadroparin dose of at least 85 anti-Xa IU/kg should be used to reach higher anti-Xa activities than 0.2 to 0.4 IU/ml. To what extent the combination of hereditary thrombophilic risk factors in our patient has contributed to the development of the progressive thrombosis remains an open question.

References

1. Bianchetti MG, Speck S, Müller R, Oetliker OH. Simple coagulation prophylaxis using low-molecular heparin enoxaparin in pediatric hemodialysis. Schweiz Rundsch Med Prax 1990; 79: 730–731
2. Broyer M, Gagnadoux MF, Sierro A, Fischer AM, Révillon Y, Jan D, Beurton D, Niaudet P. Prevention of vascular thromboses after renal transplantation using low molecular weight heparin. Ann Pediatr (Paris) 1991; 38: 397–9
3. Collignon F, Frydman A, Caplain H, Ozoux ML, Le Roux Y, Bouthler J, Thebault JJ. Comparison of pharmacokinetic profiles of three low molecular mass heparins – dalteparin, enoxaparin and nadroparin – administered subcutaneously in healthy volunteers (doses for prevention of thromboembolism). Thromb Haemost 1995; 73: 630–640
4. de Veber G, Chan A, Monagle P, Marzinotto V, Armstrong D, Massicotte P, Leaker M, Andrew M. Anticoagulation therapy in pediatric patients with sinovenous thrombosis: a cohort study. Arch Neurol 1998; 55: 1533–1537
5. Dix D, Andrew M, Marzinotto V, Charpentier K, Bridge S, Monagle P, de Veber G, Leaker M, Chan AKC, Massicotte MP. The use of low molecular weight heparin in pediatric patients: A prospective cohort study. J Pediatr 2000; 136: 439–445
6. Gagnadoux MF, Charbit M, Beurton D, Revillon Y, Niaudet P, Broyer M. Renal transplantation in children under five years of age. Experience at the Hopital des Enfants-Malades. Ann Pediatr (Paris) 1993; 40: 108–111
7. Greinacher A. Heparin-induced thrombocytopenia – pathogenesis and treatment. Thromb Haemost 1999; 82 (Suppl): 148–156
8. Hashikura Y, Kawasaki S, Okumura N, Ishikawa S, Matsunami H, Ikegami T, Nakazawa Y, Makuuchi M. Prevention of hepatic artery thrombosis in pediatric liver transplantation. Transplantation 1995; 60: 1109–1112
9. Hofmann S, Knöfler R, Wendisch J, Siegert G, Müller D, Taut-Sack H, Dinger J, Lorenz N, Tittel B, Kabus M. Clinical experiences with low molecular weight heparins in pediatric patients. Eur J Pediatr 1999; 158 (Suppl 3): S219
10. Klement D, Rammos S, von Kries R, Kirschke W, Kniemeyer HW, Greinacher A. Heparin as a cause of thrombus progression. Eur J Pediatr 1996; 155: 11–14
11. Kosch A, von Kries R, Nowak-Göttl U. Thrombosen im Kindesalter. Monatsschr Kinderheilkd 2000; 148: 387–397

12. Lopaciuk S, Bielska-Falda H, Noszczyk W, Bielawiec M, Witkiewicz W, Filipecki S, Michalak J, Ciesielski L, Mackiewicz Z, Czestochowska E, Zawilska K, Cencora A. Low molecular weight heparin versus acenocoumarol in the secondary prophylaxis of deep vein thrombosis. Thromb Haemost 1999; 81: 26–31
13. Massicotte P, Adams M, Marzinotto V, Brooker LA, Andrew M. Low-molecular-weight heparin in pediatric patients with thrombotic disease: A dose finding study. J Pediatr 1996; 128: 313–318
14. Monreal M, Lafoz E, Olive A, del Rio L, Vedia C. Comparison of subcutaneous unfractionated heparin with a low molecular weight heparin (Fragmin) in patients with venous thromboembolism and contraindications to coumarin. Thromb Haemost 1994; 71: 7–11
15. Nohe N, Flemmer A, Rümler R, Praun M, Auberger K. The low molecular weight heparin dalteparin for prophylaxis and therapy of thrombosis in childhood: a report on 48 cases. Eur J Pediatr 1999; 158 (Suppl 3): S134-S139
16. Nowak-Göttl U, Ehrenforth S, Koch HG, Kreuz W, Münchow N, Scharrer I, Schneppenheim R. Multicenter evaluation of combined prothrombotic defects in childhood thromboembolism. In: 28. Hämophilie-Symposion Hamburg 1997. Scharrer I, Schramm W, Eds. Springer 1999; 105–116
17. Pini M, Aiello S, Manotti C, Pattacini C, Quintavalla R, Poli T, Tagliaferri A, Dettori AG. Low molecular weight heparin versus warfarin in the prevention of recurrence after deep vein thrombosis. Thromb Haemost 1994; 72: 191–197
18. Severin T, Ensenauer R, Wiedensohler A, Fischer KG, Zieger B, Greinacher A, Brandis M, Sutor AH. HIT type II without thrombocytopenia in a 15-year-old boy with protein S deficiency and recurrent deep vein thrombosis. In: 29. Hämophilie-Symposion Hamburg 1999. Scharrer I, Schramm W, Eds., Springer 2001; 114–116
19. Streif W, Mitchell LG, Andrew M. Antithrombotic therapy in children. Curr Opin Pediatr 1999; 11: 56–64
20. Van Biljon I, Van Damme Lombaerts R, Demol A, Van Geet C, Proesmans W, Arnout J. Low molecular weight heparin for anticoagulation during haemodialysis in children – a preliminary study [letter]. Eur J Pediatr 1996; 155: 70

Cardiac Tamponade in a Patient with acquired Factor VIII Inhibitor and Chronic Renal Failure

E. STRASSER, Y. LINDEMANN, B. NEIDHARDT, I. SCHARRER,
R. ZIMMERMANN, V. WEISBACH and R. ECKSTEIN

Factor VIII inhibitors can trigger bleeding complications especially if they remain unrecognized (1). In the following case a prolongation of the activated partial thromboplastin time (aPTT) was found using a global coagulation test that was carried out routinely before an enoral biopsy of a mucous membrane. Further analysis of the coagulation factors influencing the aPTT revealed a factor VIII deficiency caused by an acquired factor VIII inhibitor. This case report describes the rare complication of a cardiac tamponade related to a factor VIII inhibitor and the therapeutic interventions which led to the inhibitor elimination.

We report on a 47-year-old male patient with chronic renal failure who came to the hospital to clarify symptoms of a pemphigoid. The patient showed spontaneous hematomas at this time, which were thought to be clinically irrelevant and to be due to a coagulation disorder caused by azotemia, according to the end stage renal disease. Peritoneal dialysis had regularly been performed without any complications for several months. The aPTT, which was measured before an enoral biopsy, was prolonged to 67 s. Further diagnostic procedures showed an isolated reduction of factor VIII activity (7%) caused by a low-titer factor VIII inhibitor (2.04 BU, Bethesda units). With regard to the case history the diagnosis »acquired factor VIII inhibitor« was established. Immunosuppressive therapy with cyclophosphamide and corticosteroids for the elimination of factor VIII inhibitors was postponed due to pneumonia. Approximately 1 week later severe cardiac failure occurred and a cardiac tamponade caused by serious bleeding into the pericardium was found. Cardiocentesis had to be performed immediately. Preoperative and perioperative substitution of recombinant factor VIIa (NovoSeven) in an overall dosage of 14.4 mg was given. No further bleeding complications occurred. Subsequently, elimination of the factor VIII inhibitor was performed successfully using the following regimen: plasma exchange (3500 ml GFP per day) combined with 500 mg prednisolone intravenously for 5 days, followed by an immunosuppressive therapy with 60 mg prednisolone and 150 mg cyclophosphamide per day. Doses of the immunosuppressive therapy were reduced gradually over the next 4 weeks. The factor VIII levels normalized after 7 days as shown in Fig. 1.

The patient needed daily hemodialysis. Pancytopenia and cytomegalovirus infection appeared under immunosuppressive therapy. This case report confirms the experience, that the level of factor VIII inhibitor titer does not correlate to the severity of bleeding complications (1). Even at comparatively low inhibitor titers a perilous bleeding complication – in this case a cardiac tamponade – can occur. The application of recombinant factor VIIa was an efficient therapy in this situation and enabled the performance of an emergency pericardiocentesis. This is a further

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

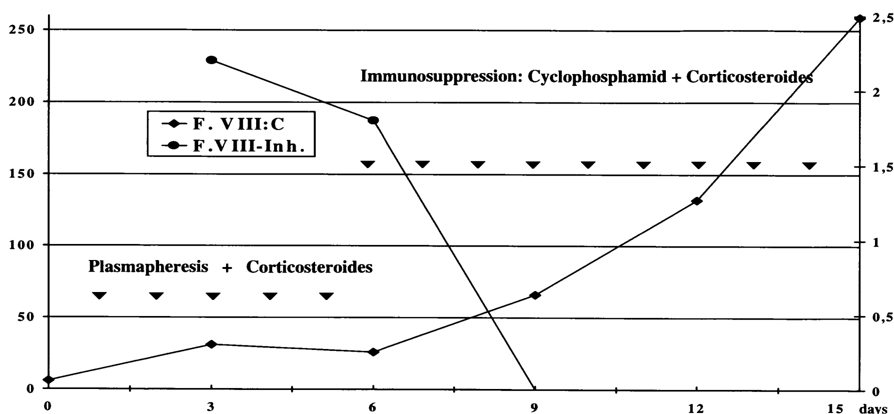


Fig. 1. Normalization of the factor VIII levels

example of the successful use of factor VIIa application in an inhibitor-induced acute bleeding event (2–4). The elimination of inhibitor antibodies by plasma exchange combined with immunosuppressive therapy succeeded within 9 days. Due to the clinical situation of the patient (prolonged artificial respiration after pneumonia) the readily available plasma exchange therapy in combination with glucocorticoids was used. An alternative approach would have been immune adsorption methods (e.g. Therasorb) but they are more expensive and bear the risk of immunoglobulin deficiency (5).

The routinely performed aPTT screening gave the decisive note to findings a hitherto undetected severe coagulation disorder. The result of global coagulation tests prevented the performance of a mucous membrane biopsy, which would have been accompanied by a severe bleeding risk in this case. This underlines the value of global coagulation tests before small routine interventions, even if the case history is inconspicuous.

References

1. Scharrer I., Großmann R., Erworbene Hemmkörperhämophilie. Der Anästhesist, 49: 34–42, 2000.
2. Hay C.R.M., Negrier C., Ludlam C.A., The Treatment of Bleeding in Aquired Haemophilia with Recombinant Factor VIIa: A Multicenter Study. Thrombosis and Haemostasis 78: 1463–1467, 1997.
3. Kessler C.M., New products for managing inhibitors to coagulation factors: a focus on recombinant factor VIIa concentrate. Current Opinions in Hematology 7: 408–413, 2000.
4. Scharrer I., Recombinant factor VIIa for patients with inhibitors to factor VIII or IX or factor VII deficiency. Haemophilia 5: 253–259, 1999.
5. Knöbl P., Derfler K., Extracorporeal Immunoadsorption for the Treatment of Haemophilic Patients with Inhibitors to Factor VIII and IX. Vox Sang 77 (suppl 1): 57–64, 1999.
6. Green D., Rademaker A.W., Briet E. A Prospective, Randomized Trial of Prednisone and Cyclophosphamide in the Treatment of Patients with Factor VIII Autoantibodies. Thrombosis and Haemostasis 70: 753–57, 1993.

Endoscopic Cholecystectomy in a 55-year-old Patient with Heparin-Induced Thrombocytopenia Type II and Replacement of Mitral and Aortic Valve and Tricuspid Valve Anular Plasty

J. Gross, D. Heitmann, I. Wojtzyk, G. Lutze and M.U. Heim

Introduction

Heparin-induced thrombocytopenia (HIT) at present is the most common medicine induced thrombocytopenia. HIT type II, in which activation of platelets is immunologically mediated, represents a serious complication of heparin therapy while severe thromboembolic complications can occur which sometimes result in the loss of extremity if application of heparin is not terminated immediately. Further anticoagulation in patients with HIT type II includes the application of danaparoid-sodium (1) or recombinant hirudin (2). The present study presents the laboratory findings with different doses of hirudin and the clinical course of a 55-year-old patient with HIT type II and replacement of mitral valve, aortic valve and tricuspid valve anular plasty in whom Lepirudin (Refludan) was applied perioperative during endoscopic cholecystectomy.

Methods

Antibodies to platelet factor 4 – heparin – complex: ASSERACHROM HPIA, PF4-ELISA, Diagnostica Stago/France
Prothrombin time: INNOVIN, Dade Behring, Germany (reference range: >70%)
Partial thromboplastin time and thrombin time: PTT-REAGENS and THROMBIN-REAGENS, Roche Diagnostics, Mannheim/Germany (reference range: <35 s and 15.5–20 s, respectively)

Patient

Patient M.R. was female 55 years old and had a body weight (bw) of 47 kg. Until now she had not suffered any thromboembolic events. She had one spontaneous delivery and one abortion in the year 1974. At the age of 14 years the patient suffered from rheumatic fever with swelling of the knee joint and participation of heart valves which required in-hospital treatment over 3 months. In the year 1960 she had a tonsillectomy and in 1994 the patient suffered from torticollis spasticus.

At the age of 54 years she progressively suffered from palpitations and dyspnoea on exertion. Diagnosis of cardiac insufficiency was confirmed combined with insuf-

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

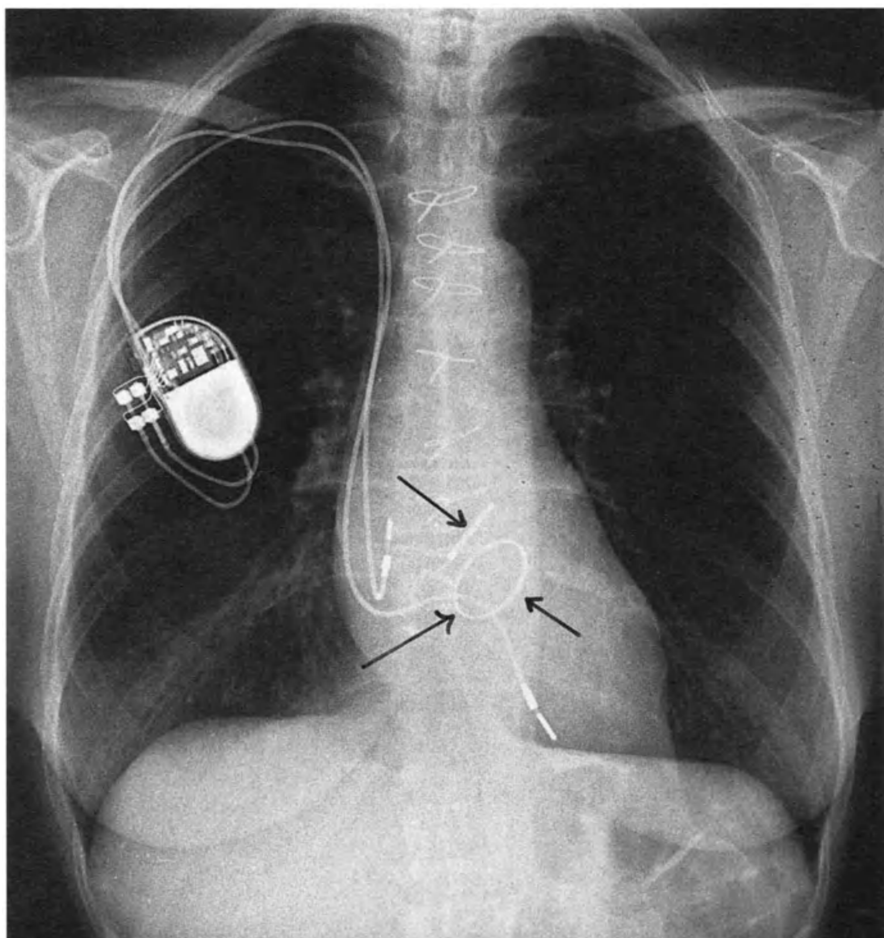


Fig. 1. Replacement of mitral and aortic valve and tricuspid valve anular plasty

ficiency of mitral valve (fourth degree), tricuspid valve (fourth degree) and aortic valve (second degree). Replacement of aortic valve (SJM prosthesis 21 mm), mitral valve (SJM prosthesis 29 mm) and tricuspid valve anular plasty (DURAN ring 31 mm) was carried out. Due to an atrioventricular block of third degree, pacemaker implantation (DDD) was carried out (Fig. 1). During postoperative anticoagulation with unfractional heparin, there was a decrease in platelet count without any thromboembolic events, and diagnosis of HIT type II was confirmed by ELISA.

Clinical Course and Laboratory Findings

Clinical course and laboratory findings are shown in Table 1.

Table 1. Partial thromboplastin time, thrombin time and prothrombin time during therapy with lepirudin (*nd* not determined)

Day	Lepirudin (mg/kg bw/h)	Partial thrombo-plastin time (s)	Thrombin time (s)	Prothrombin time (%)
5 (endoscopic cholecystectomy)	0.05	48	>180	74
	0.02	39	140	74
	0.03	42	172	59
6	0.03	45	84	60
7	0.03	37	167	111
	0.05	39	nd	106
8	0.08	44	>180	92
9	0.09	47	nd	90
10	0.10	59	>180	83
11	0.10	57	>180	83

Day 1

The patient was admitted to the hospital for endoscopic cholecystectomy. Therapy with phenprocoumon was terminated three days before admission. Prothrombin time was 34% and therapy with lepirudin was started with a dose of 0.06 mg/kg bw/h.

Day 5

After normalization of prothrombin time endoscopic cholecystectomy was carried out without any bleeding or thromboembolic complications. The perioperative dose of lepirudin was 0.02 mg/kg bw/h (partial thromboplastin time 39 s, thrombin time 140 s); during the postoperative course lepirudin dose was increased to 0.03 mg/kg bw/h (partial thromboplastin time 42 s, thrombin time 172 s).

Days 6–8

The postoperative course was without any complications; the dose of lepirudin was step by step increased up to 0.08 mg/kg bw/h. Partial thromboplastin time was prolonged 1.3-fold, thrombin time was prolonged more than 9-fold at this dose.

Day 9

Lepirudin dose was 0.09 mg/kg bw/h (partial thromboplastin time 47 s); therapy with phenprocoumon was started again.

Day 10

Partial thromboplastin time was prolonged 1.7-fold, thrombin time was prolonged more than 9-fold, while lepirudin was applied with a dose of 0.10 mg/kg bw/h; prothrombin time was 83%.

Day 13

Therapy with lepirudin was terminated.

Day 15

The patient was discharged from the hospital.

Summary and Conclusions

A 55-year-old patient with replacement of mitral and aortic valve and tricuspid valve anular plasty was admitted to the hospital for endoscopic cholecystectomy. Because HIT type II had been diagnosed at an earlier time, recombinant hirudin (Lepirudin, Refludan) was given as anticoagulant (3). While prothrombin time was normalized after termination of therapy with phenprocoumon, lepirudin was given perioperative with a dose of only 0.02–0.03 mg/kg bw/h; applying this dose regime, bleeding or thromboembolic events were not observed, especially in our patient after complex heart valve surgery with a very strong indication for anticoagulant therapy (4). Laboratory monitoring during the postoperative course revealed up to 1.7-fold prolongation of the partial thromboplastin time while lepirudin was applied with a dose of only 0.10 mg/kg bw/h. So the therapeutical dose in our patient was only two thirds of the one recommended by the manufacturer until now (0.15 mg/kg bw/h). Our data underline that dose recommendations for lepirudin in patients with HIT type II may be discussed. However, ecarin clotting time should be the parameter of choice for monitoring anticoagulation with recombinant hirudin (5).

References

1. Wilde MI, Markham A: Danaparoid: A review of its pharmacology and clinical use in the management of heparin-induced thrombocytopenia. Drugs 1997; 54: 903–924.
2. Greinacher A, Völpel H, Pötzsch B: Recombinant hirudin in the treatment of patients with heparin-induced thrombocytopenia. Blood 1996; 88 (Suppl. 1, Part 1–2): 281 A, p 1113.
3. Amiral J, Bridey F, Wolf M et al.: Antibodies to macromolecular platelet factor 4 – heparin complexes in heparin-induced thrombocytopenia: a study of 44 cases. Thromb Haemost 1995; 73: 21–28.

4. Greinacher A, Völpel H, Janssens U, Hach-Wunderle V, Kemkes-Matthes B, Eichler P, Mueller-Velten HG, Pötzsch B: Recombinant hirudin (lepirudin) provides effective and safe anticoagulation in patients with heparin-induced thrombocytopenia type II - a prospective study. Circulation 1999; 99: 73–80.
5. Pötzsch B, Madlener K, Seelig C, Riess CF, Greinacher A, Müller-Berghaus G: Monitoring of r-hirudin anticoagulation during cardiopulmonary bypass - assessment of the whole blood ecarin clotting time. Tromb Haemost 1997; 77: 920–925.

VIb. Poster: Hemophilia

Successful Orthopedic Operations in Hemophilic Adults with Inhibitors against Factor VIII

G. LUDWIG, C. BETZ, M. KRAUSE, A. KURTH, L. HOVY and I. SCHARRER

A grave complication during the treatment of hemophilia A with blood factor replacement is the development of factor VIII inhibitors. If it is not manageable to reduce the inhibitors with an immune tolerance therapy, bleedings are hard to control. Within the scope of extensive orthopedic operations in the last 12 months, our faculty used recombinant factor VIII peri- and postoperatively on three male adults with high titers of FVIII antibodies. After the factor VIII antibody ascent the patients were treated with the substitution of rFVIIa. In two cases, the initial reduction of the FVIII antibodies was first reached through preoperative Therasorb Immunadsorption over 4 weeks because of the high titer of antibody at the starting point.

Patient 1

A 35-year-old male with severe hemophilia A, factor VIII inhibitor since 1977 (up to 120 BU), chronic hepatitis C 2b, negative HIV test, legible genu varum (20°) with a hindrance of extension and the marked development of a huge cyst in the left femur with severe hemophilic arthropathia, and a clear indication for the provision of a knee prosthesis because of an acute disposition to fracture. The reduction of the inhibitor titer was initially reached with Therasorb-Immunapherese over 4 weeks with a starting point BU of 17. The preoperative inhibitor titer was 0.9 BU.

Patient 2

A 47-year-old male with severe hemophilia A, factor VIII inhibitor since 1998, positive hepatitis C serology, negative HIV test, severe hemophilic arthropathia of various joints with chief complaint in the left ankle joint and an inhibitor titer of 17 BU. After 3 weeks of daily Therasorb treatment the inhibitor titer was 1.5 BU at its starting point.

Patient 3

A 40-year-old male with severe hemophilia A, factor VIII inhibitor since 1980, positive HIV test, positive hepatitis C serology, multiple hemophilic arthropathias in-

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

cluding the left ankle joint, the legible genu varum (20°) which resulted in the immobilization of the patient with a clear indication for a corrective osteotomy and arthrodesis.

A sufficient hemostasis for about 14 days was possible after repeated short-termed measurements of the factor VIII activity and the titer of antibodies. This therapy regimen made further an appropriate physiotherapy of the patient possible. Neither intraoperative nor postoperative bleedings were seen. The preoperative reduction of the factor VIII antibodies with Therasorb Immunadsorption makes a safe perioperative substitution of factor VIII possible. A legible ascent of the antibody titer was seen approximately 5 days after exposition to rFVIII. The substitution was finally continued with rFVIIa factors. This procedure offers the additional factor of safety and proves to be more economical than general substitution with factor VII beginning on the day of the operation.

Conclusion

An appropriate operative therapy for advanced hemophilic arthropathia is possible even with a factor VIII antibody.

Evaluation of Clinical Efficiency of rFVIIa in Pediatrics

M. ŞERBAN, P. ŢEPENEU, C. PETRESCU, D. MIHAILOV, C. JINCA
and W. SCHRAMM

Introduction

One decade ago recombinant activated factor VII (rFVIIa) was introduced as a new therapeutic agent for hemophilia patients with inhibitors (Hedner U, 1988). Very soon, its beneficial effect was also proved in many other diseases (Glanzmann's thrombasthenia, Bernard Soulier thrombopathia, acquired coagulopathy in renal and hepatic failure, congenital deficiency of factor V, VII, XI, acquired hemophilia etc.) (Schulman S, 1998, Martinowitz, 1999). Its clinical use was based on the recognition of the crucial role of FVIIa in hemostasis, initiated both on extrinsic or intrinsic pathway, even in primary hemostasis. The definition of the indications and the optimal therapeutic regimen for rFVIIa is an ongoing process; the establishment of the doses and administration intervals for a minimum hemostatic level is obviously cumbersome.

Objectives

- To assess the hemostatic efficiency of rFVIIa therapy in the situation of minimal regimens (dosage, frequency and duration of administration) in different types of bleeding
- To analyze the cost-efficiency correlation, according to the modality of administration
- To reveal the side-effects of rFVIIa therapy

Material and Methods

This is a retrospective study based on ten patients registered and treated in the IIIrd Pediatric Clinic Timisoara (Table 1): three patients with hemophilia A severe form (FVIII <1%) with high titer of inhibitors (152–600 BU) and seven patients with other coagulopathies (von Willebrand disease, ITP with platelet alloimmunization, aplastic anemia with severe thrombocytopenia, complex hemorrhagic diathesis caused by chronic renal failure, Glanzmann's thrombasthenia).

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Studied material

Ctr. no., name, age	Diagnosis	Clinical indication Type	No.	Associated pathology
1. R.B. 12 years	Severe hemophilia A with inhibitors (580 UB)	Massive extensive muscular hematoma, left thigh and shank[b]	1	Chronic hepatitis with HCV, obesity grade II
		Hemarthroses (shoulder, elbow, ankle)[d]	3	
2. G.E. 9 years	Severe hemophilia A with inhibitors (600 UB)	Hematic pseudocyst of the left calcaneum with superinfection (extensive osteomyelitis left foot)[b]	1	Chronic hepatitis with HCV, complex immunodeficiency, chronic intra-infectious and post-hemorrhagic anemia
		Idem, with necessity amputation of the left shank[b]	1	
		Severe bleeding from postoperative wound[b]	4	
		Idem, with hemorrhagic shock[a]	2	
		Moderate bleeding of postoperative wound[c]	2	
		Osteomyelitis right calcaneum based on a pseudo-hematic cyst[c]	1	
		Preoperative prophylaxis	2	
		Postoperative prophylaxis	14	
3. B.F. 17 years	Severe hemophilia A with inhibitors (152 UB)	Massive bleeding after evacuation of super-infected muscular hematoma of left upper arm[b]	1	Chronic hepatitis with HCV
		Idem, with hemorrhagic shock[a]	1	
		Preoperative prophylaxis	1	
		Postoperative prophylaxis	1	
4. R.A. 15 years	von Willebrand disease	Massive bleeding, hemoperitoneum, hemorrhagic shock, postoperative[a]	1	Acute hepatitis, secondary hyperaldosteronism, acute renal failure, meningo-cerebral bleeding
		Severe, uncontrollable posterior epistaxis, with overflooding of the airways[a]	1	

Table 1. Continued

Ctr. no., name, age	Diagnosis	Clinical indication Type	No.	Associated pathology
5. L.C. 10 years	Severe chronic thrombocytopenia	Prophylactic, before dental extraction for dental abscess with submandibular hematoma	1	Post-transfusional platelet alloimmunization
6. G.A. 13 years	Acute myeloid leukemia	Bleeding of inferior intestinal tract, based on severe pancytopenia[a]	1	Sepsis, mucositis
7. P.D. 14 years	Severe aplastic anemia with severe thrombocytopenia	Severe bleeding from perianal necrotizing wound with perilesional cellulitis; meningo-cerebral bleeding[a]	1	Sepsis, urinary infection with *E. coli*
		Severe gingivo-labial bleeding, epistaxis, macroscopic hematuria massive[b]	1	
8. M.F. 15 years	Complex hemorrhagic diathesis	Severe bleeding during implantation of central venous catheter[b]	1	CGN, multicystic renal dysplasia, chronic renal failure, cervical adenophlegmon
9. O.F. 2 years	Glanzmann thrombasthenia	Uncontrolled severe anterior epistaxis[b]	1	
10. R.L. 12 years	Acute lymphoblastic leukemia	Massive bleeding of inferior intestinal tract, based on severe pancytopenia[a]	1	Sepsis, mucositis, toxic hepatitis

[a] Life-threatening bleeding
[b] Severe bleeding
[c] Moderate bleeding
[d] Mild bleeding

Results and Discussion

The treatment with rFVIIa was applied in 44 situations, either curative (on demand) as in the case of bleeding accidents uncontrollable with other therapeutic measures (25 situations), or prophylactic, as in the case of predictable severe bleedings (19 situations). The hemorrhagic events when rFVIIa was administered were grouped into: life-threatening - 9; severe - 10; moderate - 3; mild - 3.

On demand treatment with rFVIIa was applied in 76% of the situations (19/25) of severe or life-threatening hemorrhages. In over 2/3 of all situations (30/44 - 68,18%) rFVIIa was administered for surgical procedures. Prophylactic administration was given in 94,74% (18/19) of the surgery cases.

The i.v. bolus administration of rFVIIa was the mean modality of administration in the majority of uncontrolled bleeding events that were curatively treated (22/25 - 88%) whereas continuous infusion was used in almost 2/3 of the prophylactic administrations (13/19 - 68,42%) (Fig. 1).

The applied therapeutic regimens have been compared with the conventional regimens as shown in Tables 2 and 3. Economic reasons and the reduced accessibility to this very expensive treatment made it necessary to use smaller doses (minimum 40 μg/kg/dose) or to prolong the intervals between the administration to a

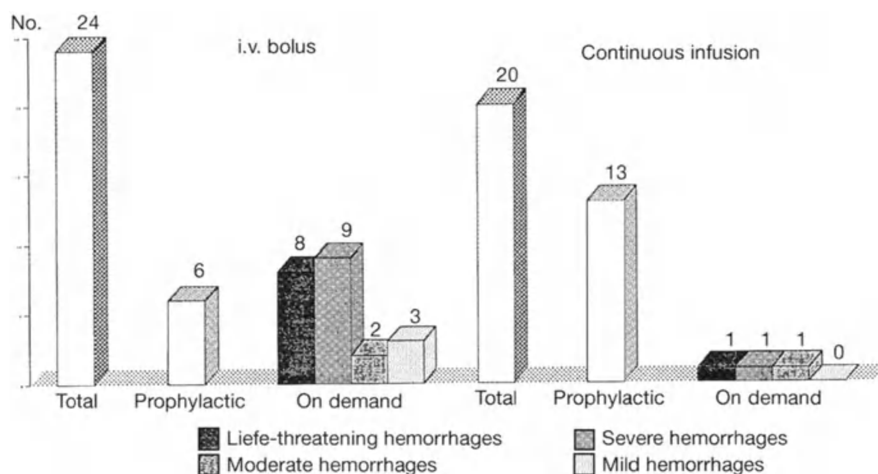


Fig. 1. The situation in which rFVIIa was administered, the modality of administration and the type of hemorrhagic events

Table 2. Therapeutic regimens used in surgical procedures

Therapeutic regimen	Preoperative i.v. bolus	Postoperative continuous i.v. infusion Day I	Day II	Day III	Days IV–VII
Optimal regimen	90 μg/kg	10–20 μg/kg/h	10–20 μg/kg/h		
Regimen used	90 μg/kg	15–18 μg/kg/h	14–18 μg/kg/h depending on the evolution, for 4–22 h		

Table 3. Therapeutical regimens used in non-surgical procedures

Therapeutical regimen	i.v. bolus administration		i.v. continuous infusion	
	Dose	Number of administrations	Dose	Duration of administration
Optimal regimen	60–120 μg/kg	Depending on evolution	7,5–11,5 μg/kg/h	Depending on evolution
Regimen used	40–140 μg/kg	Mean number of administrations 2,45/situation±1,8	13–20 μg/kg/h	Mean duration of administration 10,57/situation±7,49 h

Table 4. Clinical criteria of efficacy evaluation

Type of response	Characteristics of type of response	Score
Very good	Stop of the bleeding for over 48 h after administration	3
Good	Stop of the bleeding, relapse 24–48 h after administration	2
Weak	Stop of the bleeding, relapse at <24 h after administration	1
Absent	Impossible to stop the bleeding	0

maximum of 4 h as opposed to conventional administration patterns. The total period of administration was reduced as well. In the case of surgical procedures, the conventional recommendations were respected as opposed to non-surgical administrations where we used smaller doses. Overall, in 42,66% of the cases, the doses for i.v. bolus administration were below the minimal recommended dose (60 μg/kg). Concerning the number of i.v. bolus administrations, over 2/3 of the patients received only 1–3 doses of rFVIIa (1/3 of the cases received only one administration).

The evaluation of the response to the treatment with rFVIIa was based on clinical criteria (Table 4) and was analyzed according to: indication, doses, time period and modality of administration.

Overall, in 61,36% of the cases, we obtained a very good response, in 27,27% a good response and in 11,36% the response was weak. No failure was encountered. The analysis of the efficiency depending on the type of administration (Fig. 2) showed the absence of failure and high efficiency in almost all cases of continuous i.v. infusion as opposed to i.v. bolus administration (92,8% vs. 66,5%). We mention the fact that in many of the i.v. bolus administrations the doses were smaller than the conventional ones and the number of administrations was reduced.

The overall clinical efficiency of the prophylactic treatment with rFVIIa was very good (mean clinical score 2,9); it is worth mentioning that in general the doses used for prophylaxis met the recommendations.

Concerning the curative treatment, despite the low efficiency and the numerous situations of severe hemorrhages in which larger doses were needed but only smaller were given for economic reasons, we encountered no failure, which represents practically the major consideration (Fig. 3).

The administration of rFVIIa in continuous i.v. infusion allowed an important economy as opposed to i.v. bolus administration (Fig. 4) and also a high level of effi-

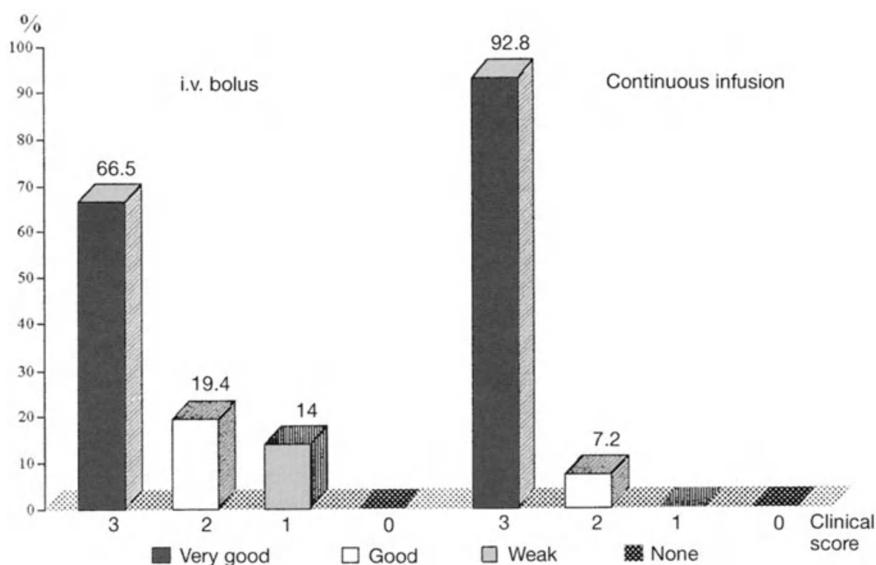


Fig. 2. The efficiency of the treatment with rFVIIa depending on the type of administration

ciency (as shown above). In order to appreciate this issue properly, a comparison was made between the total amount of rFVIIa administered in continuous i.v. infusion and the theoretical recommended amount of i.v. bolus administered for the given period of time.

The use of rFVIIa in the patients with hemophilia A who presented a high titer of anti-FVIII antibodies, contributed to the reduction of the inhibitors titer, probably because of the lack of antigenic stimulation (Fig. 5); during the time of rFVIIa

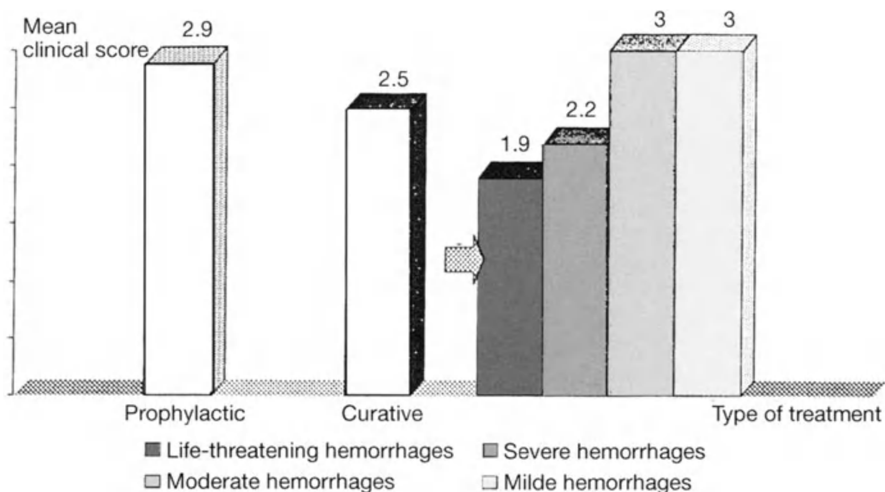


Fig. 3. The efficiency of rFVIIa depending on the type of treatment

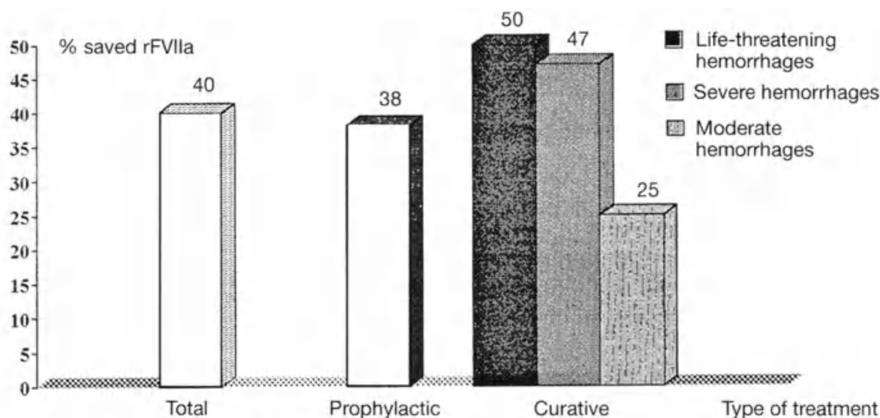


Fig. 4. Saved rFVIIa through continuous infusion administration

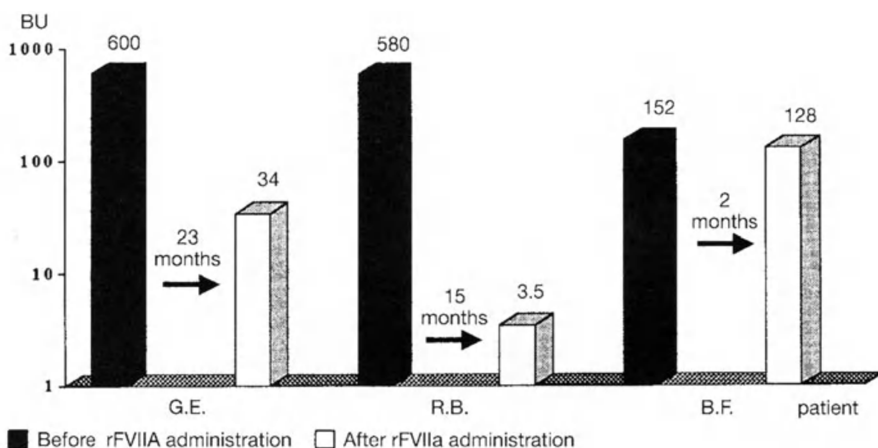


Fig. 5. Reduction in time of inhibitor titer in hemophiliacs treated with rFVIIa

treatment no product containing FVIII was used. In the patient R.B., the first administration of FVIII concentrate after 15 months did not determine any significant clinical response, the titer of inhibitors turning out to be under 5 UB.

Although adverse reactions to the treatment with rFVIIa are mentioned in literature (fever, exanthema, pruritus, nausea, vomiting, blood pressure variations, headache or even more severe reactions like acute renal failure, ataxia, angina pectoris, arrhythmias, cardiogenic shock), we did not encounter any adverse reactions in our study group except one case of mild, transient allergic reaction.

Conclusions

In our experience, the limited access to rFVIIa led to its very economical use from the standpoint of dosage, rate and duration of administration. Despite this situati-

on, the mean clinical score was 2,9 for prophylactic and 2,5 for curative rFVIIa administration; the efficiency of continuous infusion was superior to i.v. bolus administration (clinical score was 3 in 92,8% cases vs. 67,5% cases).

An amount of 25–50% rFVIIa was saved by continuous infusion as opposed to i.v. bolus administration, although high efficiency was achieved. This is a very important issue considering the high price of the product.

Under rFVIIa treatment the inhibitor titer decreased significantly in hemophilia A patients, probably because of no antigenic stimulation.

RFVIIa was well tolerated and highly efficient, even under the conditions of dosage reduction and therapy duration and in bleeding events unresponsive to other therapeutic measures. Continuous infusion had the advantage of reducing the total amount of medication without having a detrimental effect on efficiency.

References

1. Hagen FS, Gray CL, O'Hara P. et al. Characterization of a cDNA coding for human factor VII. Proc Natl Acad Sci USA,1986, 83:2413
2. Hedner U, Bjoern S, Bernvil L et al. Clinical experience with human plasma derived factor VIIa in patients with hemophilia A and high-titer inhibitors. Haemostasis, 1989, 19:335
3. Rapaport SI. Inhibition of factor VIIa/tissue factor-induced blood coagulation: with particular emphasis upon a factor Xa-dependent inhibitory mechanism. Blood, 1989, 73:359
4. Hedner U. Factor VIIa in the treatment of haemophilia. Blood Coag and Fibrinol, 1990, 1:307
5. Hedner U, Ljunberg J, Lund-Hansen T. Comparison of the effect of plasma-derived and recombinant human FVIIa in vitro and in a rabbit model. Blood Coag and Fibrinol, 1990, 1:145
6. Hedner U, Feldstedt S, Glazer S. Recombinant FVIIa in hemophilia treatment. In: Hemophilia and von Willebrand's Disease in the 1990s, Lusher JM, Kessler CM, eds, Elsevier Science Publishers B.V., 1991
7. Schulman S. A therapeutic alternative for haemophiliacs with inhibitors. Acta Paediatr 1992, 81:926
8. Ingerslev J, Knudsen L, Sindet-Petersen S. Hemostatic efficacy of recombinant factor VIIa in haemophilia patients with inhibitors. Abstract presented at the XXth International Congress of the World Federation of Haemophilia, Athens, Greece, 1992, 12–17.10
9. Kristensen J, Killander A, Hippe E et al. Recombinant factor VIIa (rFVIIa) reduces the bleeding time in patients with thrombocytopenia. Abstract presented at the 35th Annual Meeting of the American Society of Hematology, St. Louis, USA, 1993, 3–7.12
10. Rappaport S, Nemerson Y, Kisiel W, Lindhal AK, Morrissey J, Wildgoose P, Lyng Hansen L, Nordfang O, Lund-Hansen T, Lusher JM, Ockelford PA, Ingerslev J, Glazer S, Falch JF. A report on the ISTH Satellite Symposium: Recombinant Coagulation Factor VIIa: Mechanism of action and clinical experience, New York, 10.07.1993, p.1–15
11. Glazer S, Hedner U, Falch JF. Clinical update of the use of recombinant factor VIIa. Presented at the International Symposium about Inhibitors and Coagulation Factors, Univ. of North Carolina, Chapell Hill, 1993, 3–5.11.
12. Ingerslev J. NovoSeven. Clinical Experience and Recommendation, p.1–22, Kontrapunkt A/S, 1995

Risk Factors for Thrombosis in Hemophilia – an Analysis

V. AUMANN, G. LUTZE and U. MITTLER

Introduction

During recent years there has been a considerable increase in the knowledge of the pathophysiology of thromboembolic diseases. Several risk factors for thrombosis, congenital as well as acquired, have been identified. A combination of them increases risk for the patient. The identification of further risk factors has been enabled by establishing molecular biological methods (1). Consequently, the recognition of risk groups has been improved. In the normal population, the prevalence of different risk factors is supposed to be 10–15%. Therefore it is assumed that even in some patients with congenital hemophilia risk factors for thromboses must be present (2). Whether or not these contradictory changes influence the bleeding tendency of the patient is discussed in the following study.

Patients and Methods

Twenty-four patients of the Center for Hemophilia were examined:

- Fifteen patients with severe hemophilia A (s.H.A.)
- Five patients with moderate hemophilia A (m.H.A.)
- Three patients with severe hemophilia B (s.H.B.)
- One patient with vWD type III

The tests were performed in the laboratory of the Institute for Clinical Chemistry at the university. Only commercially available tests were used. Apart from determining global tests ART, PT, aPTT and thrombin time as well as fibrinogen and FVIII-inhibitor, the following risk factors were tested: AT III, protein C, protein S (free and total), plasminogen activity, activity of factor II and XII, lupus anticoagulants, anticardiolipin antibodies (ACA), concentration of prothrombin fragment 1+2, concentration of homocysteine, Factor V Leiden mutation (R506Q), prothrombin gene mutation G20210 A.

Results

Only patients with results varying from the norm are presented (Table 1).

In a relatively high number of patients (13 of 24) changes were found. In most cases, pathological changes were only slight or moderate. The most common change

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Results

Patient	Diagnosis	Factor V Leiden	FII gene mutation	Free protein S	FXII activity	ACA
F.T.	s.H.A.					48 U/ml
F.D.	s.H.A.			50%	50%	
G.D.	s.H.B.			48%		
H.T.	m.H.A.		Proved			
L.M.	s.H.A.			50%	39%	
M.D.	s.H.A.			58%	55%	
M.M.	s.H.A.				61%	
M.K.	s.H.A.				39%	
M.N.	m.H.A.	Proved				
O.O.	s.H.A.				53%	
S.S.	s.H.A.				67%	
S.F.	s.H.A.			50%		55 U/ml
W.M.	s.H.A.			55%	46%	

was a F XII-decrease. It was found in eight patients. This result is not surprising as a combination of FVIII and FXII decrease is quite frequently found. Decreased protein S was determined in six patients. An increase in anticardiolipin antibodies – as found in two patients – may be due to a previous infection. Factor V Leiden mutation and a mutation in the prothrombin gene were only found in one patient respectively. We did not find any connection between the described results and the bleeding tendency or the use of clotting factor concentrates. The two above named mutations were found in two patients with hemophilia A of a moderate type, whose bleeding tendency is usually not very pronounced.

Conclusion

Twenty-four patients with severe or moderate congenital bleeding disorders were examined for thrombophilic risk factors. In 54% of all examined patients changes were found, but only low levels. We were not able to determine a clinical significance.

References

1. Dahlbäck B, Carlsson M, Svensson PJ. Prediction of a cofactor to activated protein C. Proc Natl Acad Sci USA 1993; 90:1004.
2. Pabinger-Fasching I. Epidemiologie der funktionellen Inhibitormängel und weiterer hereditärer Risikofaktoren für venöse Thromboembolien. In: Hämostaseologie (Hrsg. Müller-Berghaus G, Pötzsch B), Springer 1999

Status of Pain in Patients with severe Haemophilic Arthropathies

T. WALLNY, L. HESS, A. SEUSER, H.H. BRACKMANN and C.N. KRAFT

Introduction

Patients diagnosed with haemophilia A before the era of home infusion frequently suffered from severe haemarthropathies. Although it is appreciated that the entity may be associated with pain and deformities, data on the degree of discomfort these disorders cause and the subsequent implications for the haemophiliac do to our knowledge as yet not exist. The aim of our study was therefore to describe the importance of arthritic pain for the haemophiliac and to evaluate the effects of non-surgical treatment methods on the quality of life.

Patients and Method

In 1999, using a questionnaire, we consulted 71 patients suffering from severe haemophilia A (Factor VIII activity <1%) regarding complaints associated with the musculo-skeletal system and the outcome of therapeutic measures taken to alleviate painful joint conditions. Pain in the large joints and the spine was estimated by means of a visual analogous scale (VAS), which ranges from 0–10, 0 being completely pain-free and 10 being the worst pain the patient can imagine. The questionnaire differentiated between regions of serious pain (main regions of pain) and regions of minor pain or slight discomfort (minor regions of pain). Furthermore, patients were asked to subjectively describe the effects of drug treatment and physical measures specifically prescribed to combat joint pain. A further aspect we evaluated was whether the patients' social status was subject to change because of their pain-status or skeletal deformities.

The average age at the time of evaluation was 43,2 years (range: 21–63 years). 78.8% (56/71) of the patients questioned worked full-time, while 21.1% (n=15) were pensioners because of their arthropathies. 87.3% (62/71) of the collective were on prophylactic treatment, meaning that these patients injected at least 3000 IU Factor VIII per week.

Results

Patients claimed to on average have four joints which caused severe pain (main regions of pain) and on average 0,5 joints were defined as minor regions of pain. Back pain distressed ten patients (14.1%), two as the main region of pain, eight as a minor pain region. Main regions of pain were most frequently localised in the ankle

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

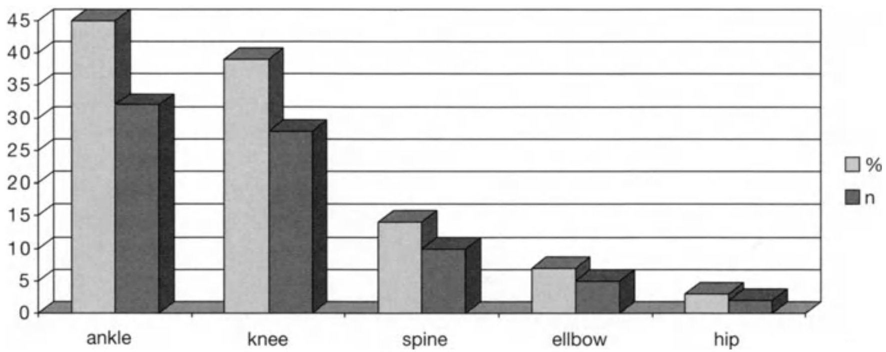


Fig. 1. Painful regions

(45.1%, n=32), followed by the knee (39.4%, n=28) and the elbow joint (7.0%, n=5) (Fig. 1). The most frequently mentioned type of pain was pain of a dull, persistent nature, stabbing pain and sudden-onset pain.

Most of the haemophiliacs interviewed suffered from painful arthropathies in more than one joint. While only 1.4% (n=1) claimed to have one painful joint, 14.1% (10/71) experienced two painful regions and 43,7% (31/71) up to four pain regions. 28.2% (20/71) of the patients stated that they have six and as many as 12.7% (9/71) claimed to suffer from more than six severely painful joints (Fig. 2).

The subjective evaluation on the visual analogue scale averaged five points for the main regions of pain and four points for the minor regions of pain. The elbow joint was subjectively found to be the most painful joint, followed by the ankle and then the knee joint.

The frequency with which the use of Factor VIII was associated with a subjective subsidence of joint pain was particularly astonishing: 49.3% (n=35) of our patients claimed to have pain during the course of the day when no medication was taken, 28.2% (n=20) patients stated that the pain persisted despite the use of Factor VIII and 15.4% (n=11) gave no relief despite the use of Factor VIII and analgetic drugs (see Table 1).

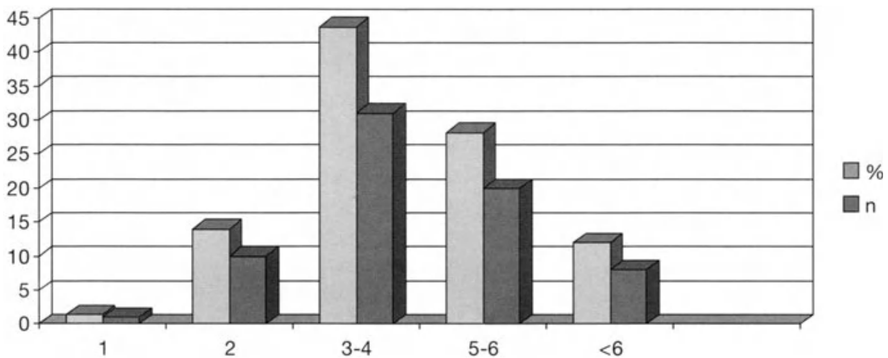


Fig. 2. Number of painful regions

Table 1. Pain and medication

Pain all day long without any medication	
Yes	49% (n=35)
Sometimes	30% (n=21)
No	21% (n=15)
Pain all day long with factor	
Yes	28% (n=20)
Sometimes	34% (n=24)
No	38% (n=27)
Pain all day long with painkillers and Factor VIII	
Yes	15% (n=11)
Sometimes	34% (n=24)
No	51% (n=36)

Some 88.7% (n=63) declared that they are significantly encumbered in the activities of daily life because of constant or recurring pain and 84.5% (n=60) disclosed that their quality of life was moderate due to frequently being in low spirits.

To alleviate pain, patients reported using Factor VIII concentrate, non-steroidal antirheumatic drugs (NSAR), modified orthopaedic shoes, ointments for local application and stabilising bandages (listed in descending frequency; Fig. 3). The most frequently used NSAR were acemetacin and diclofenac. 76.1% (n=54) of all patients needed analgetic medication, only 44.4% (24/54) of these between once to three times per day daily. Of the 54 patients taking analgetic medication regularly 17 (31.5%) were of the opinion that pain was not sufficiently curbed by their medication.

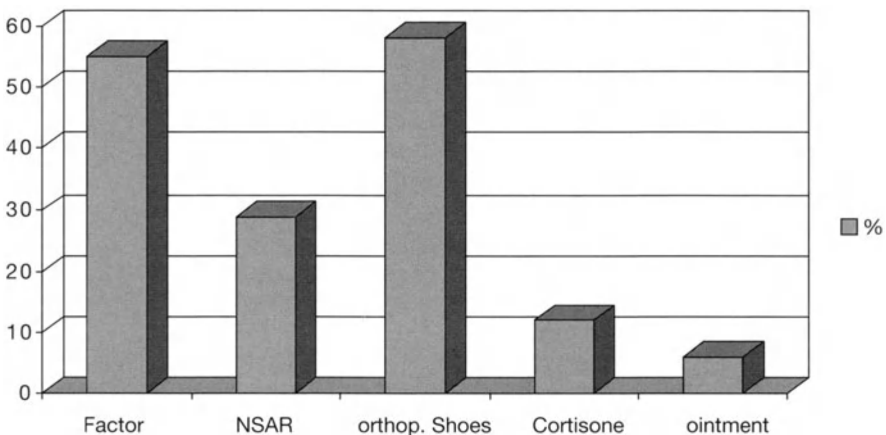


Fig. 3. Type of treatment performed (multiple statements possible)

Discussion

Pain is of particular relevance in the daily life of an haemophiliac. Even haemophilic children, growing up in the era of early prophylaxis usually had some form of experience with painful joints [1]. As newer data published by Aznar et al. [2] emphasise, arthropathically afflicted joints do not necessarily have to be painful. On the other hand it should be considered that sufficient pain therapy is compulsory because pain will lead to a vicious circle with false movements, increased loading of the joints, more bleeding episodes and more pain.

Yet our results underline that therapy for painful symptoms from arthropathies is frequently insufficient. We were surprised to find that although patients regularly attended consultations (average 3–4 times per year) in our Haemophilia Care Unit, almost one-quarter claimed that the pain therapy was inadequate. We believe that by use of a pain questionnaire a tool is at hand which allows early acknowledgement of the patient's pain status and the possibility of offering an adequate and differentiated therapy.

Further therapeutical potential arises by using such a procedure: being urged to consider and confront his symptoms, the haemophiliac may learn to mentally cope more adequately with his handicap and pain status. Bradley et al. [3] were able to show a positive effect of cognitive behaviour therapy on the management of arthritis pain. Varni et al. [4] report on a drug-abusing haemophiliac in whom the intake of analgesics could significantly be lowered solely by means of behavioural treatment.

The axial skeleton of the haemophiliac is rarely involved in episodes of bleeding, primarily due to the relatively small amounts of synovial tissue present. Because of the frequently observed biomechanical changes in severe haemophilic arthropathy caused by leg-length discrepancies, malalignment of leg axis, distortion of the pelvis and hyperlordosis due to flexion-contractures of the hip, secondary lower back pain would nonetheless be expected in these patients. Only 14.1% (10/71) of our patients claimed to suffer from any back pain at all, and interestingly, the majority of these described the back as a »minor region of pain«. Why the haemophiliac's subjective perception of back pain remains secondary and is pushed into the background cannot conclusively be answered by this study. There may possibly be a haemophiliac-typical management of pain processing, which no longer lets the patient perceive regions of low-intensity pain as being disturbing.

We found the amount of patients associating an analgesic effect with the use of Factor VIII noteworthy. It is recognized that the regular i.v. application of factor concentration significantly reduces the risk of joint bleeding [5]. Yet the arthropathic pain from so-called »burnt-out« joints is seldom caused by acute bleeding. Particularly in the chronic pain-plagued patient and even more so in the haemophiliac with a long-standing history of the disease, a psychological factor cannot be denied. Nonetheless, further studies to determine the pathophysiological and pharmacological effects of a possible analgesic effect of Factor VIII seem worthwhile.

Conclusion

Haemophiliacs (Factor VIII activity <1%) suffering from haemarthropathic changes due to poor childhood and adolescent haematogical observation and substitution, should be accepted as and treated like chronic pain-plagued patients. By means of a self-assessment questionnaire particularly those individuals not able to adequately articulate their pain status when consulting their regular physician can be revealed.

References

1. Spitzer A. The significance of pain in children's experiences of hemophilia. Clin Nurs Res 1993; 2:5–18
2. Aznar JA, Magallon M, Querol F, Gorina E, Tusell JM. The orthopaedic status of severe haemophiliacs in Spain. Haemophilia 2000; 6:170–176.
3. Bradley LA, Young LD, Anderson KO, McDaniel LK, Turner RA, Agudelo CA. Psychological approaches to the management of arthritis pain. Soc Sci Med 1984; 19:1353–60
4. Varni JW, Gilbert A. Self-regulation of chronic arthritic pain and long-term analgesic dependence in a haemophiliac. Rheumatol Rehabil 1982; 21:171–4
5. Aledort, LM, Bohn, RL. Blood Coag Fibr 1996; 7 Suppl 1: S35–37.

Results of a Five-Year Clinical Study with a B-domain Deleted FVIII Concentrate (rFVIII-SQ)

H. Pollmann, H. Richter and the rFVIII-SQ Study Group

Introduction

During the 1980s the first steps towards the development of recombinant factor VIII were taken. In 1984, various study groups described the structure of the factor VIII gene [10,23,25]. Several formulations of full-length recombinant factor VIII were developed by different manufacturers.

A common feature of both products is the full length of the factor VIII molecule, including all six domains. For stabilization human plasma derived albumin is used.

In 1988 the first clinical trial with recombinant FVIII for previously treated patients (PTPs) started. In 1989 (Kogenate [14]) and 1990 (Recombinate [6]) the first studies in previously untreated patients (PUPs) started. All studies showed excellent results regarding viral safety, efficacy and immunogenicity. As expected there were no cases of viral transmission in any of the studies. Sometimes it was claimed that the frequency of inhibitor development might be higher for recombinant products than for plasma derived products, but recent studies revealed that this is not the case [1, 7]. In addition, in about half of the PUPs who developed an inhibitor in studies with recombinant factor VIII, the inhibitor was transient, showed no clinical relevance and disappeared without a change in the therapeutic regime.

In recent years the interest in hemophilia treatment has focused on viral safety. Although the viruses with major importance like HIV and hepatitis seem to be disabled by the current virus-inactivation steps, other viruses like Parvovirus B 19 might survive and still be transmitted by plasma derived products. Some publications reported the presence of this virus even in recombinant factor VIII products [8, 12]. Another topic in this debate is the possible transmission of prions, an agent which is supposed to be responsible for the new forms of Creutzfeldt-Jakob disease and bovine spongiform encephalopathy (BSE). This is underlined by a recent publication on the transmission of BSE by blood transfusion in sheep [11]. It might be possible that human blood products or other substances (like bovine insulin or calf serum) that are used for cell culture of Chinese hamster ovary cells or baby hamster kidney cells might infect the cell cultures with the disease, ending in the production of factor VIII contaminated with infectious agents [2].

However, the greatest risk seems to be the fact that most currently available recombinant factor VIII formulations still contain large amounts of human proteins

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

from pooled human donor plasma. The addition of human albumin for stabilization is always a potential infection risk factor. This led to the development of a new generation of recombinant factor VIII which is stabilized without any human substances. Thus a smaller reconstitution volume is achieved. The top of the actual development is the B-domain deleted recombinant factor VIII molecule rFVIII-SQ which is distributed by Wyeth-Pharma, Münster, Germany with the trade name ReFacto. Several studies since its introduction in 1993 have proved its safety and efficacy.

Biochemical Characteristics of B-domain Deleted rFVIII-SQ

Normal factor VIII consists of six domains, called A1-A2-B-A3-C1-C2. For blood clotting effects the B-domain is not necessary. So in rFVIII-SQ the B-domain is eliminated by a fusion of the bases Ser743 and Glu1638. For further details see Fig. 1.

The molecular weight of rFVIII-SQ is 170 kDa, composed of a 90 kDa heavy chain and a 80 kDa light chain, which are bound in a non-covalent metal ion binding. Together they constitute the smallest part of naturally occurring active factor VIII. The structural and functional features of the rFVIII-SQ molecule are nearly equivalent to those of plasma derived FVIII. It has a normal interaction with vWF and thrombin, showing no difference in the mode of interaction. With respect to the interaction with factor IIa, Xa and activated protein C the same functional properties have been found [13,18,19].

The specific activity of this product is 15,000 IU/mg protein in comparison with 4–7,000 IU/mg protein in other rFVIII-formulations, prior to the addition of albumin [5].

According to the manufacturer, the potency of rFVIII-SQ should be assayed with the chromogenic assay, since one-stage assays often understate the real potency. This phenomenon is also known for other highly-purified FVIII preparations, but seems to be especially increased for B-domain deleted rFVIII [3, 4, 15].

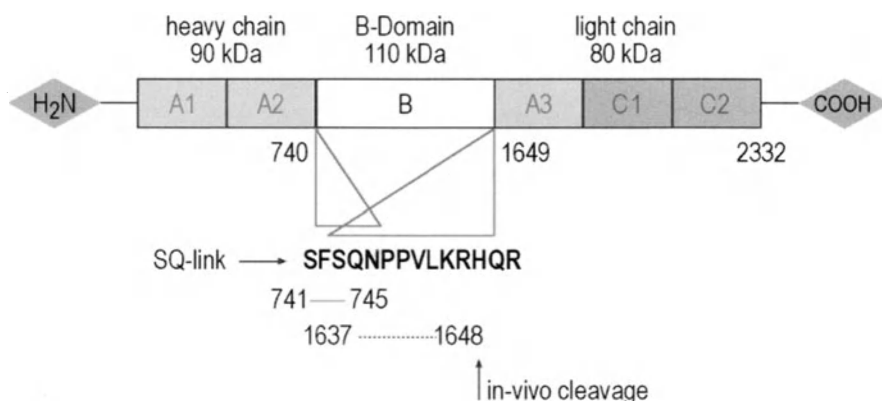


Fig. 1. The B-domain deleted factor VIII molecule

Manufacturing Process

The rFVIII-SQ is produced in Chinese hamster ovary cells, which are being cultured in a medium which contains human serum albumin. The purification process starts with a cationic exchange-chromatography followed by virus-inactivation with a solvent-detergent step using Tri-n-butyl phosphate and Triton-X-100. Immunoaffinity chromatography using murine anti-factor VIII monoclonal antibodies follows (Fig. 2).

Anionic exchange-chromatography as well as hydrophobic interaction chromatography and gel permeation chromatography are the final steps.

At the end of this purification cascade there is the final product in a formulation which needs no stabilization with human serum albumin. As a result of this manufacturing process it does not contain any vWF. For stabilization polysorbate 80 is added, as well as L-histidine, sucrose, sodium chloride and calcium chloride [22].

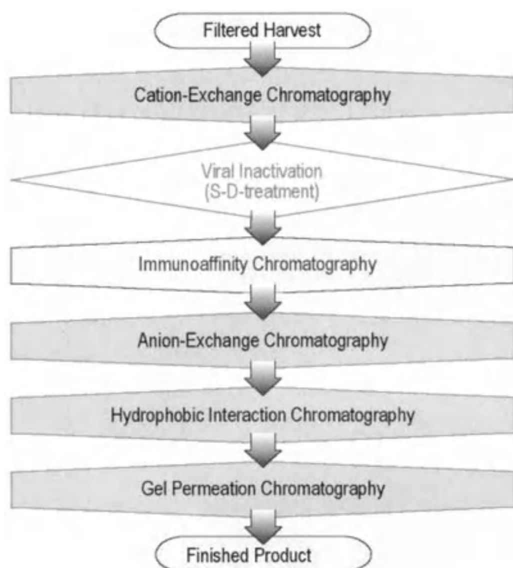


Fig. 2. The manufacturing process of rFVIII-SQ (ReFacto)

Preclinical Studies

To prove the safety of rFVIII-SQ, preclinical studies have been performed in rats and monkeys. The efficacy of this formulation was studied in hemophilic dogs [16, 20]. There was no difference between rFVIII-SQ and plasma derived FVIII.

Study Design

For this open-labeled phase III study patients with severe hemophilia A (FVIII<0.02 IU/ml) have been recruited in ten countries and at 37 hemophilia

centers since 1993. Inclusion criteria were severe congenital hemophilia A (VIII:C <0.02 IU/ml), an age of 7 years or older and at least an average of 30 exposure days/year in the past.

A patient was excluded from participation in the study if he met one or more of the following criteria: detectable inhibitors (≥0.6 units according to the Bethesda method), other coagulation disorders or history of severe adverse reactions to factor VIII concentrates, abnormal renal function (creatinine >1.5 mg/dl), liver cirrhosis or significant ALT increase, anemia (hemoglobin <12 g/dl) or platelet count <75,000 cells/mm^3, lymphocyte subset CD4 counts <300 cells/µl, ethanol or drug abuse or any other condition that may affect patient compliance, participation in another concomitant clinical study (except for the study of rFVIII-SQ in surgical procedures), concomitant treatment with another non-registered drug or other factor VIII-containing products, concomitant treatment with immunomodulating drugs such as interferon or intravenous immunoglobulin (IVIG) – exception: antiretroviral therapy was allowed, body weight >100 kg.

rFVIII-SQ was administered as a prophylactic treatment two to three times a week or as on-demand treatment for bleeding episodes, due to trauma or not. Doses were individually adapted and determined on the basis of previously known response to treatment, usually 20–40 IU/kg at each infusion. Patients recorded each bleeding episode number, infusion number, the time the bleeding episode was first observed, the time of the infusion, the site of each bleeding, the amount of rFVIII-SQ given, the reason for the infusion, any concomitant medication, reactions to the infusions, the patients' comments about the infusion, and assessment of hemostasis. When treatment was administered at the study center, the patient and investigator made the assessment together. Half-life and recovery have been studied in the patients at the first treatment episode and after 6, 12, 18 and 24 months of treatment following the guidelines of Morfini et al. [17]. To eliminate effects from earlier infusions a washout period of 3 days applied. Every 3 months the development of an inhibitor was monitored by Bethesda and ELISA methods. Also the viral safety parameters have been checked every 3 months for the occurrence of hepatitis A, B and C and HIV 1 and 2. Both patient and treating physician had to evaluate the efficacy of rFVIII-SQ when reporting on an injection. Side effects and serious adverse events have been documented carefully.

Results

Overall 113 of 116 patients received the study drug.

Efficacy Results

The 113 PTPs received a total of 32,857 rFVIII-SQ infusions ranging from 4 to 1,530 infusions per patient (median 234) for a cumulative of 68,856,687 IU injected. Whether one or more infusions were required for a bleeding event, the mean dose of rFVIII-SQ for every infusion remained relatively stable during the study period.

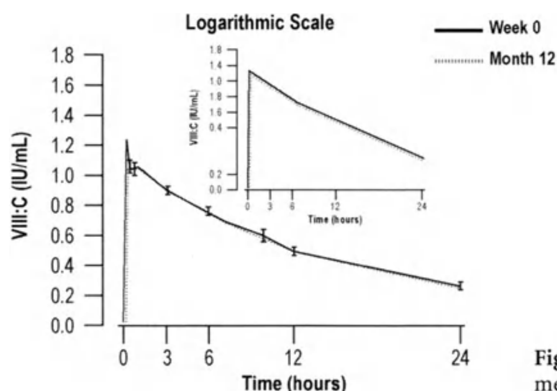


Fig. 3. Recovery and half-life at treatment months 1 and 12

This corresponds to the pharmacokinetic evaluation carried out in treatment months one and twelve. At the beginning of the treatment as well as after 12 months of regular treatment with rFVIII-SQ there was no difference in the recovery and half-life. As can be seen in Fig. 3 the decay curves of the factor VIII activity do not show any significant difference. After 12 months of treatment the recovery remained at a value of 118% (+-17), corresponding to the beginning of the treatment. In an earlier study the bioequivalence of rFVIII-SQ was compared with a plasma derived factor VIII concentrate (Octonativ M) in a single, blind, randomized cross-over study. The patients received about 50 IU/kg bw, adjusted to the nearest package size. The recovery was 111% (±14) in plasma derived FVIII. The half-life of rFVIII-SQ in the PTP-study was 14.1 h (±4.5), while plasma derived FVIII had a half-life of 13.1 h (±3.5) in the previous study. Further details on the pharmacokinetics are shown in Table 1.

The efficacy of a factor concentrate can be measured by the number of infusions needed to stop a bleeding event. Overall 7,310 bleeding episodes were treated with rFVIII-SQ. Eighty percent (5,805/7,310) of the bleeding episodes occurred in joints.

In 71% (5,208 bleeding episodes) one single infusion stopped the bleeding. After the first follow-up substitution another 16% (1,174 out of 7,310 bleeding episodes) were treated successfully. For 6% (461 out of 7,310 bleeding episodes) a total of

Table 1. Pharmacokinetic parameters of rFVIII-SQ vs. pdFVIII

Parameter	ReFacto	pdFVIII
Elimination half-life (h)[a]	14.1±4.5	13.1±3.5
Range	8.3–26.3	8.3–22.3
FVIII activity increase IU/dl per IU/kg[a,b]	2.4±0.4	2.3±0.3
Range	1.9–3.3	1.7–3.0
In vivo recovery (%)[a,b]	118±17	111±14
Range	93–153	85–142

[a]Mean ± SD
[b]FVIII activity determined by chromogenic assay

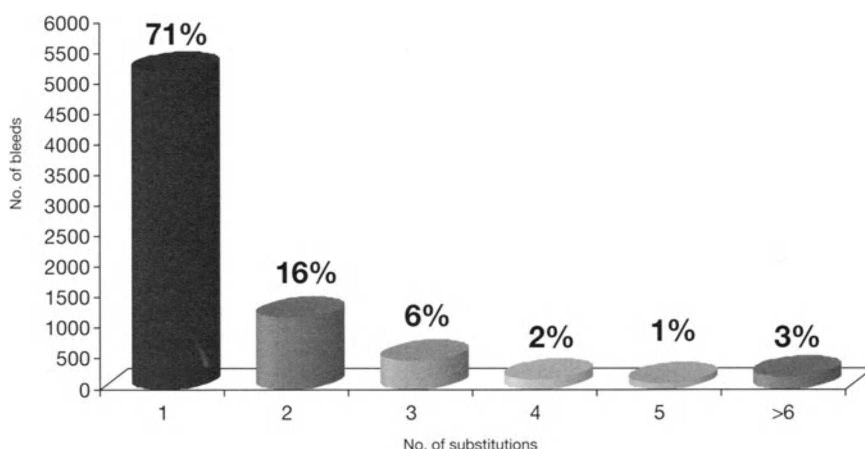


Fig. 4. Number of substitutions per bleed

three substitutions were necessary. Ninety-three percent of all bleeding episodes required not more than three substitutions. Only 3% (205 out of 7,310 bleeding episodes) required more than five substitutions, with several surgical procedures included. Further details are given in Fig. 4.

The mean number of infusions per bleeding episode was less than 2.5 for each year (range 2.0 to 2.4). In addition, the percentage of patients rating the infusions as »excellent« or »good« was similar over the course of the 5 years (range 92% to 96%).

The infusion dose for < 1% (30/7,300) of bleeding episodes was <10 IU/kg, while the dose for 75% (5,478/7,300) of bleeding episodes was ≥10 to 40 IU/kg, and 25% (1,792/7,300) of bleeding episodes was >40 IU/kg. The percentage of bleeding episodes resolved with one infusion by dose group (<10 IU/kg; ≥10 to <40 IU/kg; >40 IU/kg) ranged from 57% to 80%.

Approximately 95% of the infusions (11,655/12,268) were assessed for hemostatic efficacy. Overall, 92% (10,723/11,655) of the infusions were rated by the patient or the investigator as providing »excellent« or »good« response. Figure 5 gives an overview on the rating of efficacy.

Similarly, the response in each of the bleeding sites (joint, muscle/soft tissue/unspecified, and other specified tissues) was consistent with the overall summary assessment, with 86% or more of the responses rated as »excellent« or »good«.

When the hemostatic efficacy was assessed in relation to the number of infusions required to treat a bleeding episode, the more infusions a bleeding episode required, the higher the incidence of »fair« or »no response« ratings. Approximately 98% of the responses for bleeding episodes requiring one infusion were rated as »excellent« or »good,« whereas only 70% of bleeding episodes requiring more than infusions were rated as »excellent« or »good.«

The majority of the ratings of usefulness of rFVIII-SQ treatment over time by both patients (99%, 1204/1222) and investigators (99%, 1214/1226) were rated »very

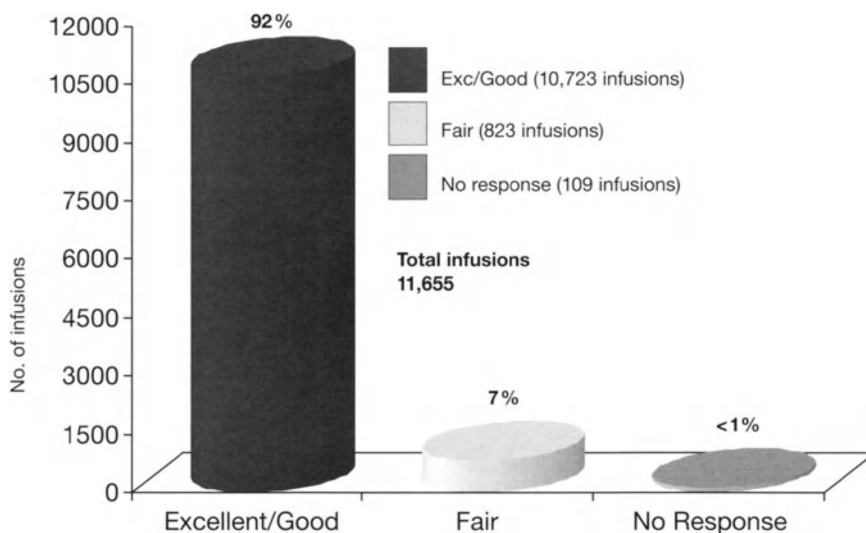


Fig. 5. Rating of efficacy

useful« or »useful.« For patients there were only 16 ratings (1%) of »slightly useful« and 2 ratings (<1 %) of »useless«. For investigators, there were only 12 ratings (<1 %) of »slightly useful« and no rating of »useless«.

A total of 77 patients received prophylaxis during the study. A subset of these patients who had also received prophylactic treatment in the year prior to the study had fewer bleeding episodes per year (10/year) than those who had not received prophylaxis treatment in the year prior to the study (15/year). Nine of the 77 (12%) patients treated on a prophylactic regimen did not report any bleeding episodes.

When the 77 patients receiving routine prophylaxis were analyzed based on the continuous duration of prophylaxis, there was a reduction in bleeding episodes, irrespective of dose, in the patients treated with 1 continuous year of prophylaxis, compared with patients treated on demand. In patients treated for 2 to 4 continuous years of prophylaxis, irrespective of dose, the rate of bleeding episodes per patient ranged from 1.0 to 3.7 per year.

When the numbers of breakthrough bleeding episodes per year were compared, patients receiving 2, 3 and >3 prophylaxis treatments per week experienced similar numbers of episodes (10/year, 10/year and 11/year, respectively). Patients receiving one prophylactic treatment per week had a rate of 21 breakthrough bleeding episodes per year. There is no difference in the rate of bleeding episodes per year when looking at dose per infusion.

Of the 113 patients who received the study drug, 27 were enrolled in an additional surgery study and underwent a total of 37 surgeries. Twenty-two of the 27 PTPs had 27 surgical procedures related to joints (knee, elbow, hip, ankle and toes). The most common single surgical procedure was total knee replacement (five patients). A total of 28 PTPs who had 34 surgeries were not included in the surgery protocol.

The most common surgeries included 13 dental procedures in 12 patients, radioactive synovectomies in four patients, ERCPs in two patients and appendectomies in two patients. A further 16 patients had 23 diagnostic and other simple procedures (i.e. dental treatment, coloscopy, endoscopy, arthroscopy and minor orthopedic surgery) and were treated with a total of between 1,500 IU and 161,082 IU rFVIII-SQ. Blood loss and the need for factor during surgery were rated as adequate to the type of surgery in all cases.

Safety Results

Fourteen patients were withdrawn from the study. Three of the 14 patients were withdrawn before receiving rFVIII-SQ treatment. One of the 14 patients withdrew due to inhibitor development associated with a monoclonal gammopathy.

Overall a total of 1027 adverse events was reported in 104 patients, most of them without any relation to the study drug. In 31 patients 70 adverse events (AEs) were reported to have a possible or probable relationship to the study drug. Most common was nausea in 11 cases, followed by headache in seven cases. Dyspnea and vasodilatation appeared in five cases. Other AEs included taste perversion, asthenia, dizziness, angina pectoris and anorexia.

There were a total of 84 serious adverse events reported in 39 patients. Five SAEs in three patients were thought to be possibly related to the study drug (ALT and AST increase, pain in little finger, weakness on adduction/abduction, bleeding from wound, infected hematoma).

The transmission of infective agents like HIV and hepatitis viruses did not occur during treatment with rFVIII-SQ.

Two deaths occurred, both of which were rated by the investigator as having an unlikely relationship to rFVIII-SQ, as they were related to end-stage AIDS. Both patients were HIV antibody- and hepatitis C antibody-positive when they enrolled in the study.

Conclusions

The second generation of recombinant factor VIII, the B-domain deleted rFVIII-SQ has been undergoing clinical trials since May 1993 and has been released for public use since 1999 in the European Union.

The hemostatic efficacy of rFVIII-SQ was proven in several clinical trials. During the reported study on PTPs 93% of all bleeding episodes were treated successfully with three or less substitutions. This is within the expected range of either plasma derived or recombinant factor VIII products. The safety report is excellent, adverse events occurred in less than 0.2% of injections, all of them being reported to be mild to moderate. A transmission of viral infections did not occur during treatment with rFVIII-SQ.

The development of inhibitors occurred in PTPs only as one single event in a 54-year-old hemophiliac with a monoclonal gammopathy. As this disease is asso-

Table 2. Comparison of inhibitor development

	Kogenate	Recombinate	ReFacto
Inhibitor incidence (severe cases only)	18/64 (28.1%)	22/72 (30.6%)	30/103 (29%)
Surveillance period	1/89–6/96	7/90–10/96	7/94–9/99
Mean rFVIII exposition until inhibitor detection (days)	9 (3–41)	10 (3–69)	12 (3–212)
Formation of inhibitors in high responders (titer ≥5 B.U. with ReFacto)	10/64 (15.6%)	7/72 (9.7%)	16/103 (15%)
Immunotolerance induction	8 (5 successful)	6 (3 successful)	14 (9 successful)

ciated with the development of various autoantibodies, there is no evidence of antibody formation due to the factor VIII formulation. As inhibitors in PTPs are generally rare, a look at the study on PUPs might be more interesting. Under treatment with rFVIII-SQ 30 out of 103 patients (=29%) developed inhibitors. In full length formulations of recombinant factor VIII an inhibitor development of 28.1% and 30.6% has been reported [6, 14]. A comparison of inhibitor development for the various recombinant factor VIII formulations is given in Table 2.

Therefore the incidence of inhibitors seems to be the same in all known recombinant factor VIII products. Moreover, after 1 year of treatment with rFVIII-SQ, the recovery corresponded to that at the beginning of the study. This proves that there is no loss of activity of the formulation due to unwanted side effects like inhibitor development.

rFVIII-SQ is the smallest known active molecule of factor VIII and is therefore better defined than the full length formulations. While other recombinant factor VIII products still need albumin from human pooled plasma, the formulation of rFVIII-SQ is stabilized with polysaccharides, so that another theoretical infection risk is excluded. Especially children benefit from the high virus safety and the exclusion of all contents of human origin. Several organizations concerned with hemophilia treatment like the UK Hemophilia Centre Directors' Executive Committee [24] or the Italian Association of Hemophilia Centres [21] recommend the exclusive use of recombinant factor products in children for this reason. First priority in treatment with recombinant products should be given to PUPs [9], as they have never been exposed to infectious agents by plasma derived products before.

In the final evaluation 99% of both investigators and patients reported rFVIII-SQ to be »very useful« or »useful«, so that rFVIII-SQ is a valuable alternative for hemophilia treatment.

The rFVIII-SQ Study Group. Arkin S, Auerswald G, Barthels M, Battle-Fonrodona J, Berntorp E, de Biasi R, Brackmann HH, Dechavanne M, Derlon A, Ellbrück D, Gazengel C, Gill J, Henrichs C, Hoots WK, Ingerslev J, Kessler CM, Kreuz W, Laurian Y, Lechner K, Ludlam C, Lusher J, Mannucci P, Magallon-Martinez M, Mayne E, Monteagudo-Terres J, Morfini M, Philipp C, Pollmann H, Potron G, Savidge G, Schulman S, Shapiro A, ten Cate JW, Toh CH, Tusell-Puigbert JM, Wenzel E, White G

Study Centers. 37 Centers in total: 8 Centers in US, 29 Centers in Europe: Germany (8), France (5), Spain (4), United Kingdom (4), Italy (3), Sweden (2), Austria (1), Denmark (1), Holland (1)

References

1. Addiego J, Kasper C, Abildgaard C, Hilgartner M, Lusher J, Glader B, Aledort L (1993). Frequency of inhibitor development in haemophiliacs treated with low purity factor VIII. Lancet 342: 462–464
2. Arnold D (1995). Virus safety considerations for recombinant factor VIII (rFVIII, Kogenate). Haemophilia 1 (Suppl. 2): 22–23
3. Barrowcliffe TW (1993). Recommendations for the assay of high-purity factor VIII concentrates. Thromb Haemost 70 (5): 876–877
4. Barrowcliffe TW, Raut S, Hubbard AR (1998). Discrepancies in potency assessment of recombinant factor VIII concentrates. Haemophilia 4: 634–640
5. Berntorp E, Brackmann HH, Dechavanne M, Gazengel CI, Johnsson H, Laurian Y, Tengborn L, Stahl C, Holm R (1995). Prophylactic treatment with a B-domain deleted recombinant factror VIII (r-VIII SQ) in previously treated patients with severe hemophilia A. XVth Cong Int Soc Thromb Haemost, Jerusalem, 1995 (abstract)
6. Bray GL, Gomperts ED, Courter S, Gruppo R, Gordon EM, Manco-Johhnson M, Shapiro A, Scheibel E, White G, the Recombinate study group (1994). A multicenter study of recombinant factor VIII (Recombinate): Safety, efficacy and inhibitor risk in previously untreated patients with hemophilia A. Blood 83: 2428–2435
7. Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B (1992). Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. Lancet 339: 594–598
8. Eis-Hübinger AM, Sosowski U, Brackmann HH, Kaiser R, Matz B, Schneweis KE (1996). Parvovirus B 19 DNA is frequently present in recombinant coagulation factor VIII products. Thromb Haemost 76: 1120
9. Giangrande PL (1997). Who should receive recombinant factor VIII? Blood Coagul Fibrinolysis 8 (Suppl. 1): 25–27
10. Gitschier J, Wood IW, Goralka TM, Wion KL, Chen EY, Eaton DH, Vehar GA, Capon DJ, Lawn RM (1984). Characterization of the human factor VIII gene. Nature 312: 326–320
11. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ (2000). Transmission of BSE by blood transfusion in sheep. Lancet 356: 999–1000
12. Laurian Y, Dussaix E, Rothschild C, Mauser-Bunschoten EP, Gomperts ED, d´Oiron R, Tchernia G (1996). Detection of human parvovirus B 19 DNA by polymerase chain reaction in different factor VIII concentrates: Correlation with infectivity? XXII Int Congr Wld Fed Hemophilia, Dublin, 1996. Haemophilia 2 (Suppl 1): 97 (Abstract)
13. Lind P, Larsson K, Spira J, Sydow-Bäckmann M, Almstedt A, Gray E, Sandberg H (1995). Novel forms of B-domain-deleted recombinant factor VIII molecules. Construction and biochemical characterization. Eur J Biochem 232: 19–27
14. Lusher JM, Arkin S, Abildgaard CF, Schwartz RS, Kogenate previously untreated patient study group (1993). Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. N Engl J Med 328: 453–459
15. Lusher JM, Hillmann-Wiseman C, Hurst D (1998). In vivo recovery with products of very high purity–assay discrepancies. Haemophilia 4: 641–645
16. Mikaelsson M, Eriksson B, Ljungquist A, Löfström A, Sandberg H, Wiken M, Österberg T (1994). Preclinical characterization of recombinant factor VIII SQ. XXI Int Congr Wld Fed Hemophilia, Mexico City, 1994 (abstract)
17. Morfini M, Lee M, Messori A (1991). The design and analysis of half-life and recovery studies for factor VIII and IX. Thromb Haemost 66: 384–386

18. Sandberg H, Bartfai J, Brandt J, Holmquist L, Kene L, Lewin M, Lind P, Nilsson B, Sandberg M, Sebring S, Strömberg S, Alin P (1992). Biochemical characteristics of a recombinant human factor VIII with deleted B-domain. XXth Int Congr Wld Fed Hemophilia, Athens, 1992 (abstract)
19. Sandberg H, Brandt J, Alin P, Strömberg S, Gray E, Bartfai J, Castro V (1995). Glycosylation pattern of a B-domain-deleted factro VIII molecule (r-VIII SQ). XVth Cong Int Soc Thromb Haemost, Jerusalem, 1995 (abstract)
20. Sandberg H, Lind P, Spira J (1991). Characteristics of a new recombinant factor VIII derivative. XIIIth Congr Int Soc Thromb Haemost. Amsterdam, 1991 (abstract)
21. Santagostino E, Mannucci PM, Bianchi Bonomi A (2000). Guidelines on replacement therapy for haemophilia and inherited coagulation disorders in Italy. Haemophilia 6 (1): 1–10
22. Smeds A-L, Ljungquist A, Östlin A, Ljungquist C, Gaal A, Sandberg H, Rosenquist J, Mikaelsson M (1995). The purification process for recombinant factor VIII SQ. XVth Cong Int Soc Thromb Haemost, Jerusalem, 1995 (abstract)
23. Toole JJ, Knopf JL, Wozney JM, Sultzman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown E, Schoemaker C, Orr EC, Amphlett GW, Foster WB, Coe ML, Knutson GJ, Fass DN, Hewick RM (1984). Molecular cloning of cDNA encoding human antihaemophilic factor. Nature 312: 342–347
24. UK Haemophilia Centre Directors' Organisation Executive Committee (1997). Guidelines on therapeutic products to treat haemophilia and other hereditary coagulation defects. Haemophilia 3: 63–77
25. Vehar GA, Keyt B, Eaton D, Rodriguez H, O´Brien DP, Rotblat F, Oopperma H, Keck R, Wood WI, Harkins RN, Tuddenham GD, Lawn RM, Capon DJ (1984). Structure of human factor VIII. Nature 312: 337–342

Outpatient Treatment with Radiosynoviorthesis in Hemophiliac Arthropathy

D. LASOVIC, G. TÖNSHOFF, M. FINK, A. ROSENSTOCK, A. GANSER
and M. VON DEPKA PRONDZINSKI

Purpose

Characteristics of hemophilic arthropathy are recurrent bleedings, chronic synovitis and osteochondral destruction. Prophylactic treatment to prevent intra-articular bleeding is essential. If chronic arthritis has already occurred, operative treatment is considered. Alternatively radiosynoviorthesis (RSO) is discussed. We investigated the effect of outpatient treatment with RSO on advanced hemophilic arthropathic joints.

Methods

From 1996 to 1999 we saw 48 patients with hemophilic arthropathy in our interdisciplinary consulting hours. In 16 joints the symptoms matched our criteria for RSO. It was done in an outpatient procedure with Yttrium-90 or Rhenium-186 after clinical, sonographic, radiographic and scintigraphic evaluation (Table 1). The after-treatment consisted of 48 h of restricted activity, antiphlogistics and 5 days of factor substitution.

Results

Synovitis and frequency of bleeding could be reduced or totally suppressed in all joints (Table 1). Reduction in pain was seen as a major advantage. Complications occurred in one shoulder in the form of a burn as a result of disregarding the directed after-treatment and in one knee due to progressive destruction by overuse.

Conclusions

RSO is usually used for the treatment of hemophilic synovitis to avoid progressive arthropathy with good results. RSO seems also to be a favorable alternative to operative procedures, even in some cases of advanced hemophilic arthropathy (Figs. 1 and 2). It can be done in an outpatient procedure if the compliance is satisfactory and it is cost effective because it reduces the need for factor substitution. It does not reduce the progression of joint destruction but does lead to a reduction in symptoms, especially in pain and joint bleedings.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Summary of data

Period	12/96–12/99
No. of patients	
Seen	48
Selected	9
Age (median and range, years)	40 (30–56)
Hemophilia	
A	7 (<1%)
B	2 (3%, 8%)
Joints	16
Shoulder	2
Elbow	4
Wrist	1
Knee	5
Ankle	4
RSO technique	
Knee	Y90 180 MBq
Other	Re186 70 MBq
Indication	
Bleeding frequency	>3/year
Chronic synovitis	>3 months
Arthropathy	>grade IV
Petterson score	>8
Results	
Bleeding frequency	
None	14
Reduced	2
Assessment	Good to very good

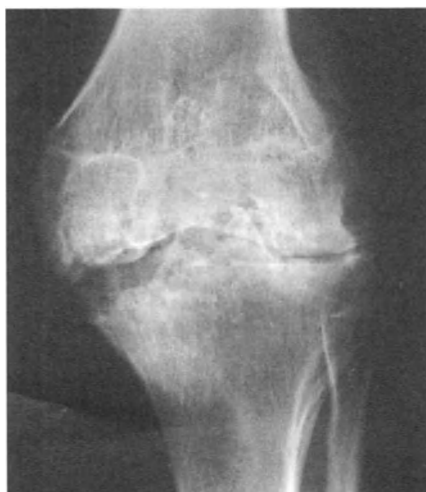


Fig. 1. RSO of the left knee in a 35-year-old patient with severe hemophilia A

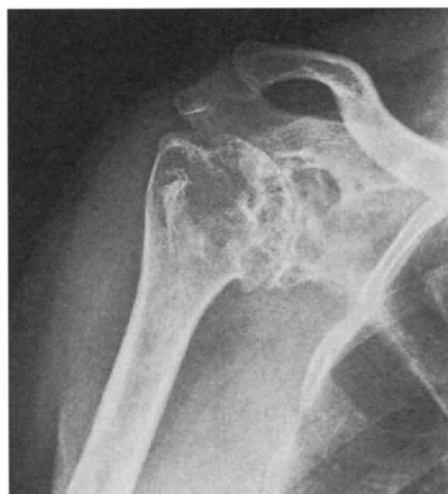


Fig. 2. RSO of the right shoulder in a 54-year-old patient with severe hemophilia A

Requirements for Future Hemophilia Treatment from the Patients' Point of View

G. Schelle, W. Breuer, G. Broil and D. Hohmann

Introduction

Our intention is to look at the requirements for future hemophilia treatment from the patients' point of view.

For that purpose, we developed a questionnaire that was sent to 750 members of our patient organization and also to patients of hemophilia treatment centers.

We asked what was good and where there was room for improvement; furthermore, what requirements there were for hemophilia treatment, for running a hemophilia treatment center, for the coagulation products and finally for recombinant technology and gene therapy.

With this survey, we intend to evaluate the status quo; furthermore, we would like to create a basis which enables us to start a discussion with attending physicians as well as with manufacturers, third party payers and politicians in order to preserve the current status of hemophilia treatment in the future.

Statistics

174 (23.5%) questionnaires were completed.

- Gender
  - Male 85.1%
  - Female 14.9%
- Severity of disease:
  - Severe 70.3%
  - Moderate 17.6%
  - Mild 8.5%
- Groups of participants (%)
  - Hemophiliac 82.4%
  - Family member 12.2%
  - von Willebrand's disease 5.4%

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Summary

Some 23.5% of all members and patients of treatment centers completed the questionnaire. One striking fact is that participation was highest amongst elderly patients with severe hemophilia, whereas only 12.2% of family members responded.

Evaluation of the results revealed that the majority of participants prefer treatment in a hemophilia treatment center with a high reputation, whereas only 2.7% consider treatment in a specialized practice to be sufficient. The reasons for that are the necessity of the 24-h-availability of a physician, regular qualified examination of joints and muscles, documentation of product batches, laboratory tests and good cooperation with other faculties. As many as 68.9% of the patients sometimes travel more than 200 km. What certainly plays a role here is trust in the treatment center and its physicians, for it is noticeable that irrespective of the fact that 74.3% requested standardized treatment regimens for all treatment centers, only about 20% would change to a nearer center.

Treatment with factor concentrates is generally considered to be very safe. That is to say, 58.1% regard recombinant products to be very safe, whereas only 24.3% assume this for plasma products. When compared, there were usually no major differences observed regarding outcome (factor consumption, duration of treatment).

Increase in maximum storage temperature (47.2%) and half-life (73%) were the most frequent answers to the question of what features the products should have. Most participants in the survey (67.5%) desired oral application, i.e. tablets, as an alternative to injections.

Despite the fact that high hopes are placed on gene therapy (cure!), at the moment fears of as yet unknown associated adverse reactions due to the lack of long-term experience still prevail.

VIc. Poster: Hemorrhagic Diathesis

Molecular Basis of von Willebrand Disease Type IIC Miami

R. Schneppenheim, T. Obser, J.U. Wieding, F. Bergmann, E. Drewke, B. Luxemburg and U. Budde

Introduction

Von Willebrand disease type 2 A, subtype IIC Miami (vWd IIC Miami) is characterised by lack of von Willebrand factor (vWF) high molecular weight multimers (HMWM) and the corresponding decrease in platelet dependent function. In contrast to conventional vWd type IIC, vWF:Ag is markedly elevated and inheritance is described as dominant (3). The extreme discrepancy between elevated vWF:Ag and decreased vWF functional activity is the diagnostic mainstay and can be explained by the elevated concentration of functionally deficient vWF low molecular weight multimers and vWF dimers, represented by pronounced low molecular weight electrophoretic bands. Instead of the normal triplet structure of vWF oligomers only single bands are displayed by electrophoresis (Fig. 1).

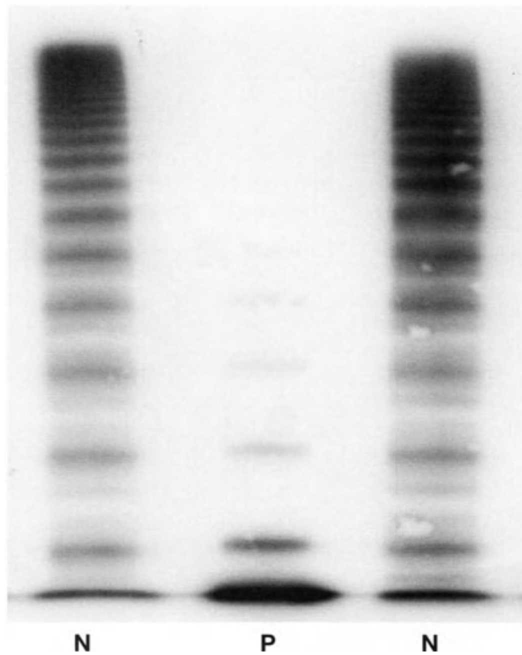


Fig. 1. Von Willebrand factor multimere analysis of a patient (*P*) with vWd IIC Miami compared with normal controls (*N*). Note the lack of HMWM, lack of a defined triplet structure and the pronounced lower bands that correspond to low molecular weight multimeres and the dimer

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Earlier attempts to identify the underlying molecular defect were not successful. In particular, in the region of the vWF pro-peptide, where mutations causing vWd IIC are clustered, no mutations were detected. We report on our molecular study of a family with several affected members and a single unrelated patient with vWd IIC Miami.

Patients, Materials and Methods

Patients

Three generations of a family were available for our study. Among them, eight affected members were identified. DNA samples were available from six patients. An additional unrelated patient from a second family was also analyzed. Hemorrhagic symptoms in the patients mainly occurred under surgery. Spontaneous bleeding was absent. In all patients investigated the discrepancy between elevated vWF:Ag and decreased vWF collagen binding activity (vWF:CB) was present. Multimer analysis was consistent with the vWd IIC phenotype (Fig. 1). The pedigree confirmed the reported dominant inheritance (Fig. 2). In the index patient (II-2) vertebral disc surgery was uneventful after infusion of DDAVP. A brother of the patient (II-4) had to undergo neurosurgery which required optimal hemostasis. Therefore, vWF replacement therapy by a FVIII/vWF concentrate (Haemate HS) was the treatment of choice. Under continuous substitution therapy no serious bleeding complicated surgery. Postoperatively at day 8, when substitution was paused, intracerebral bleed-

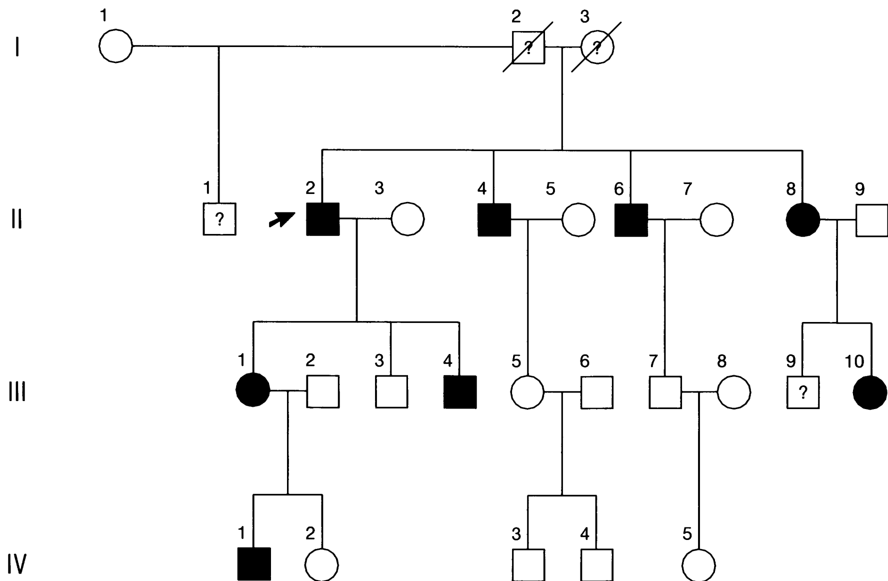


Fig. 2. Pedigree of the family with vWd IIC Miami. The *arrow* points to the index patient

ing occurred which persisted in spite of high dose substitution therapy. In this situation vWF:Ag was 710%, however, vWF collagen binding activity (vWF:CB) was relatively low with 94%.

Diagnosis of vWd

Conventional diagnostic tests were carried out in a reference laboratory (U. Budde) with patient's plasma (0.011 M trisodium citrate) and platelets. The leukocyte buffy coat was separated from whole citrated blood after differential centrifugation, then stored at –20°C for subsequent DNA extraction. The following standard parameters were obtained for each patient: FVIII:C, vWF:Ag and Ristocetin-Cofactor or vWF:CB (1). Multimer analysis was performed in SDS-agarose electrophoresis gels (5) with luminescent visualisation (6).

Molecular Studies

High molecular weight genomic DNA from patients II-2, II-4, II-6, III-1, III-4, IV-1, VH and unaffected family members was prepared from frozen buffy coats by standard techniques and used in the amplification of vWF gene sequences by PCR. PCR was performed in the Trio-Thermoblock (Biometra, Göttingen, Germany) using reagents and Taq polymerase from GIBCO/BRL. The mutation screening was carried out through amplification of single exons flanked by sufficient intron sequences to also allow detection of splice site mutations (7). All PCR products were sequenced by using ^{33}P-labeled ddNTP terminators (Amersham, Braunschweig, Germany).

Results

Diagnostic vWF parameters of the available patients are given in Table 1. The decreased ratio of vWF:CB to vWF:Ag is seen in all patients. Compared with the other patients, vWF:CB of patient VH was relatively high. By our molecular studies we identified a candidate mutation 3296G>A in exon 26 (Fig. 3), predicting the exchange of cysteine 1099 to tyrosine (C1099Y). This mutation was detected in all

Table 1. vWF parameters of the patients who are included in our study. Patient numbers correspond to those in the pedigree (Fig. 2)

Parameter	Patient							
	II-2	II-4	II-6	II-8	III-1	III-4	IV-1	VH
vWF:Ag (%)	225	710	170	156	164	225	221	153
vWF:CB (%)	19	94	3	4	14	19	14	56
Ratio CB/Ag	0.08	0.13	0.02	0.03	0.09	0.08	0.06	0.37

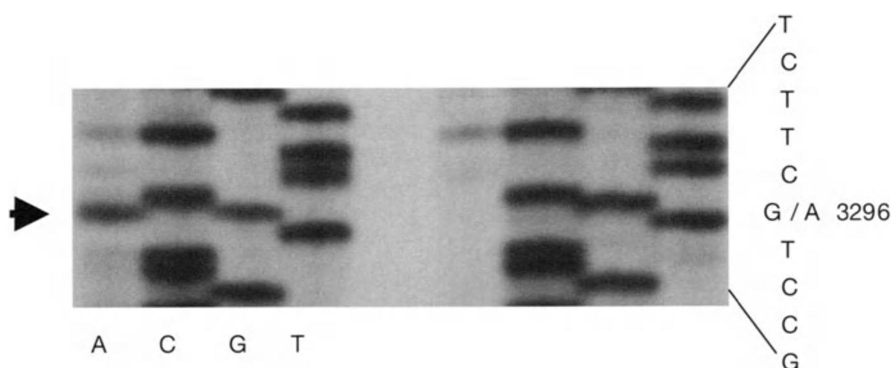


Fig. 3. Sequence analysis of exon 26 that harbors the candidate mutation 3296G>A (C1099Y) compared with a normal control. The *arrow* points to the heterozygous mutation

patients studied, but not in their unaffected family members nor in 50 normal controls. It is located in the D3 domain of vWF where probably several cysteines are involved in the multimerization process, which provides evidence that C1099Y indirectly or directly impairs vWF multimerization.

Discussion

VWd IIC Miami is a peculiar subtype which might escape the screening of vWd in patients with a bleeding tendency, if only vWF:Ag is used as a screening test. This suggests the possibility of under-diagnosis of vWd IIC Miami. Actually, we have identified another four families with elevated vWF:Ag and a decrease in HMWM and vWF:CB to date which are under study now. The correct diagnosis is important since replacement therapy with adequate vWF concentrates does not guarantee cessation of bleeding. It is possible that the patient's highly abundant aberrant vWF inhibits adhesion and aggregation of platelets by competing with the normal vWF from concentrates. This observation in one of the patients requires further clinical studies.

As the result of our molecular study of two families with vWd IIC Miami, we identified the first candidate mutation (C1099Y) in this particular subtype. Although the same mutation was detected in both families the ratio of vWF:CB/vWF:Ag was considerably higher in the single patient VH from the second family. This suggests that other factors might modify the particular phenotype.

The nature of this cysteine mutation suggests an impairment of the multimerization process located in the D3 domain of vWF, since several cysteines in this domain contribute to the intersubunit disulfide bonding at the amino-termini of vWF dimers (4, 2). This mechanism would be analogue to the dimerization defects identified in vWd 2 A subtype IID (7, 8). To date no data are available to explain the elevated vWF:Ag in vWd IIC Miami. It seems possible that the underlying mutations correlate with an elevated expression of mutant vWF. Another possibility could

be a decreased clearance from the circulation either by non-usage due to its functional deficit or due to resistance against proteolysis which has been reported for vWF in conventional vWd type IIC (9). Both explanations can only be verified by expression experiments which are currently in progress.

References

1. Brown JE, Bosak JO. An ELISA test for the binding of von Willebrand antigen to collagen. Thromb Res. 1986;43:303–311.
2. Dong Z, Thoma RS, Crimmins DL, McCourt DW, Tuley EA & Sadler JE. Disulfide bonds required to assemble functional von Willebrand factor multimers. J Biol Chem. 1994;2699: 6753–6758
3. Ledford MR, Rabinowitz I, Sadler JE, Kent JW & Civantos F. New variant of von Willebrand disease type II with markedly increased levels of von Willebrand factor antigen and dominant mode of inheritance: von Willebrand disease type IIC Miami. Blood 1993;821: 169–175
4. Marti T, Rosselet SJ, Titani K & Walsh KA. Identification of disulfide-bridged substructures within human von Willebrand factor. Biochemistry 1987;26: 8099–8109
5. Ruggeri ZM, Zimmerman TS. The complex multimeric composition of factor VIII/von Willebrand factor. Blood. 1981;57:1140–1143.
6. Schneppenheim R, Plendl H, Budde U. Luminography–an alternative assay for detection of von Willebrand factor multimers. Thromb Haemost. 1988;60:133–136.
7. Schneppenheim R, Brassard J, Krey S, Budde U, Kunicki TJ, Holmberg L, Ware J, Ruggeri ZM. Defective dimerization of von Willebrand factor subunits due to a Cys->Arg mutation in type IID von Willebrand disease. Proc Natl Acad Sci U S A. 1996;93:3581–3586.
8. Schneppenheim R, Budde U, Obser T, Brassard J, Mainusch K, Ruggeri Z M, Schneppenheim S, Schwaab R & Oldenburg J. Expression and characterization of von Willebrand factor dimerization defects in different types of von Willebrand disease. Blood. 2001;97: 2059–2066.
9. Zimmerman TS, Dent JA, Ruggeri ZM, Nannini LH. Subunit composition of plasma von Willebrand factor. Cleavage is present in normal individuals, increased in IIA and IIB von Willebrand disease, but minimal in variants with aberrant structure of individual oligomers (types IIC, IID, and IIE). J Clin Invest. 1986;77:947–951.

Hemorrhagic Diathesis through acquired Factor XIII Inhibitor

E. Strasser, R. Zimmermann, B. Neidhardt, B. Manger, I. Scharrer, V. Weisbach and R. Eckstein

Factor XIII inhibitor, a rare cause of coagulopathy, is usually not detected by screening tests, e.g. activated partial thromboplastin time (aPTT) and thromboplastin time (PT) (1). The following report describes the case of a patient with known Coombs-reactive red cell autoantibodies. Factor XIII inhibitor was detected with delay because severe hemorrhagic diathesis was initially attributed to heavy trauma.

After a nocturnal collapse in his apartment a 86-year old man with chronic nephropathy and coronary artery disease was admitted to the hospital with serious symptoms, dyspnea, angina pectoris, pain in the right flank and extensive bleedings from the skin. In the past history hematomas of the skin had been observed for several months. During hospitalization a hematothorax, a hematoma of the Iliopsoas and an extensive retroperitoneal hematoma were diagnosed and a hip fracture was suspected. Extreme anemia with low hemoglobin levels (4.9 g/dl) was supposed to be caused by bleeding combined with a hemolytic disease due to Coombs-reactive red cell autoantibodies. The retroperitoneal hematoma was expanding and the demand for red cell transfusions (4000 ml within 4 days) remained high. However, a hip fracture could not be confirmed. Accordingly, there was a discrepancy between intensity of trauma and severity of bleeding. A coagulation disorder was not detected since the usual laboratory screening tests, aPTT and PT, showed normal results and the platelet aggregation tests did not indicate any sign of a platelet disorder. Further diagnostic procedures revealed an isolated reduction in factor XIII activity to 9%. Substitution of factor XIII (Fibrogammin 7 HS) did not result in the expected increase in factor XIII activity. Subsequently a factor XIII inhibitor (Titer 12.1 Bethesda units) was found, which was thought to be an acquired one. The retroperitoneal bleeding stopped under further substitution of factor XIII (dosage to 71 IU/kg bw/day), though the patient showed only a comparatively low increase in the factor XIII activity (10–15%) (Fig. 1). Further therapy, using immunoadsorption to protein A and processing 8–10 l of plasma a day, was tolerated well but no corresponding increase in factor XIII had been observed after 3 days.

Unfortunately, the patient suddenly died of arrhythmic heart failure. The post-mortem examination did not provide any information about the cause of factor XIII inhibitors but acute bleeding as cause of death was excluded.

Acquired coagulopathy by a spontaneous factor XIII inhibitor is a very rare but clinically serious coagulation disorder. The diagnosis requires the specific determination of factor XIII and factor XIII inhibitor. Global screening tests, e.g. PT and aPTT, normally carried out on suspicion of coagulopathy, are unsuitable, since they

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

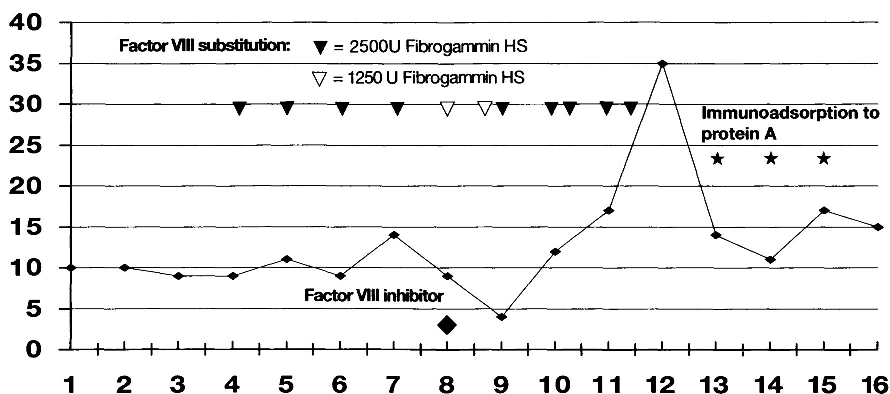


Fig. 1. Factor XIII activity (%)

do not detect a factor XIII defect. In cases of factor XIII deficiency with an inadequate effect of factor XIII substitution a factor XIII inhibitor should be suspected. Although factor XIII substitution did not lead to a dose-adequate increase in factor XIII activity in this case, bleeding was stopped effectively. Similar observations had been published by Dahly et al. (2).

Immunoadsorption to protein A did not lead to a marked increase in factor XIII activity either. However, the shortness of time in our case does not allow a definitive assessment of this method. According to the height of the inhibitor titer (here: 12.1 BU) a much longer therapy might be necessary (3,4). Various methods have been successfully used for the elimination of these inhibitors (5,6). Due to the rarity of this disorder established therapy guidelines are not available.

The reason for the development of factor XIII inhibitor could not be clarified in this case. The coincidence of bleeding with Coombs-reactive red cell autoantibodies suggests the presence of a systemic autoimmune disorder.

References

1. Lechner K., Spezifische Inhibitoren gegen plasmatische Gerinnungsfaktoren: Spontane Faktor XIII Inhibitoren, S. 378. In: G. Müller, B. Pötzsch, Hämostaseologie, Springer Verlag, 1999.
2. Daly H.M., Carson P.J., Smith J.K., Intracerebral haemorrhage due to acquired factor XIII inhibitor – successful response to factor XIII concentrate. Blood Coagul. Fibrinolysis 2: 507–514, 1991.
3. Knöbl P., Derfler K., Extracorporeal Immunoadsorption for the Treatment of Haemophilic Patients with Inhibitors to Factor VIII and IX. Vox Sang 77 (suppl 1): 57–64, 1999
4. Gailani D., An IgG inhibitor against coagulation factor XIII: resolution of bleeding after immunoadsorption with staphylococcal protein A. Am. J. Med. 92: 110–112, 1992
5. Scharrer I., Großmann R., Erworbene Hemmkörperhämophilie. Der Anästhesist, 49: 34–42, 2000.
6. Nakamura S., Kato A., Sakata Y., Aoki N. Bleeding tendency caused by IgG inhibitor to factor XIII, treated successfully by cyclophosphamide. Br.J. Haematol. 68:313–319, 1988.

Liver Transplantation in a Patient with severe von Willebrand Disease Type 3: Levels of von Willebrand Factor Following Transplantation

E. LECHLER and D. STIPPEL

Introduction

Severe von Willebrand disease type 3 (vWD type 3) is a very rare hereditary haemorrhagic diathesis (0.5 to 5/1 million). The most important findings are a (mostly) complete lack of von Willebrand factor (vWF) (functionally as well as antigen), a severe reduction in factor VIII (FVIII) and a severely prolonged bleeding time. The bleeding pattern is haemophilia-like with a preference for the mucous membranes.

Patients previously treated with non-virus inactivated concentrates did run a risk of viral hepatitis and of a hepato-cellular carcinoma.

We report on a patient with vWD type 3 who suffered from a liver cirrhosis (Child A) and a hepato-cellular carcinoma who received a liver transplantation (LTX). So far only one case of an LTX in a patient with vWD type 3 has been reported on (Mannucci et al. 1991). In this patient vWF rose from 0.04 to 1.02% after LTX. In our patient the vWF rose to considerably higher values.

Patient Background

In this patient, born in 1958, no Ristocetin cofactor activity (vWF:RiCof) and no von Willebrand factor antigen (vWF:Ag) was detectable, FVIII was about 6% and the bleeding time was above 15 min. There was a complete deficiency of all vWF multimers in the Western blot (Fig. 1). The diagnosis vWD type 3 was supported by the findings in his son with vWF:RiCof 50%, vWF:Ag 38% and FVIII 56.1% which are compatible with heterozygosity for vWD type 3. The patient had frequent nose bleeds, in recent years intestinal bleeds and since 1977 has undergone oral surgery several times. In 1980 he had a right-sided nephrectomy and pyeloplasty of the left kidney for stricture of the ureter outlet. For treatment, mostly on demand, cryoprecipitate and FVIII concentrates with vWF were used with occasional blood transfusions. In 1979 he was tested positive for hepatitis B and in 1991 for hepatitis C infection (HIV negative). In a routine investigation in 1998 a hepato-cellular carcinoma was suspected and histologically verified. The size and location of the tumour necessitated an LTX.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

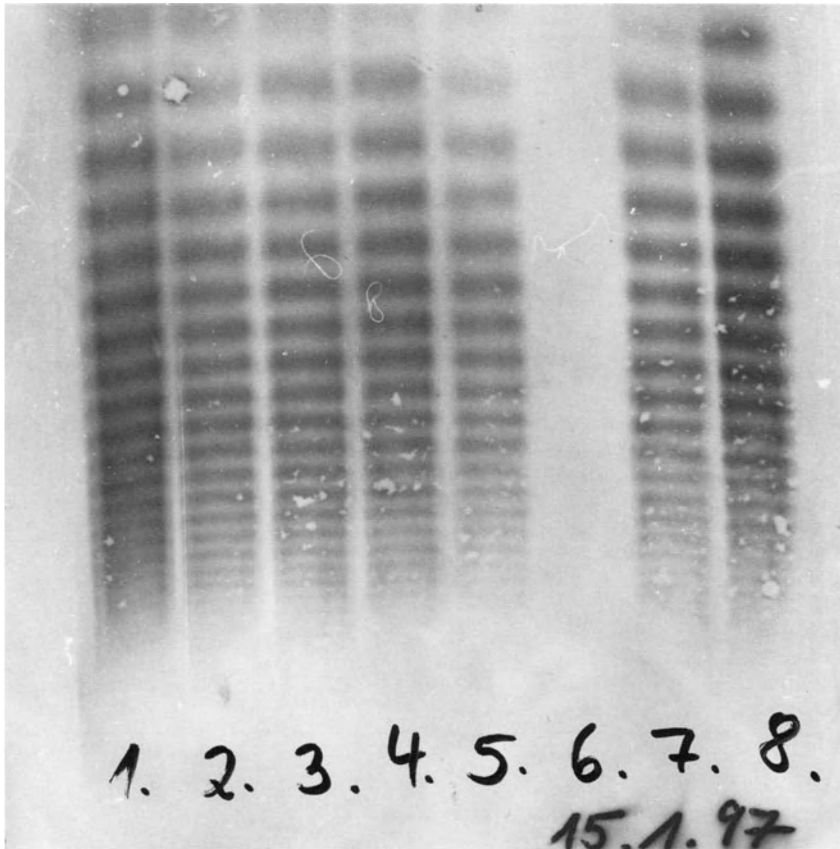


Fig. 1. Western blot of the patient presented: Lane 6

Liver Transplantation

An orthotopic LTX was performed on this 88-kg patient on March 2, 1999. He received preoperatively 3000 U FVIII/vWF concentrate. The substitution was continued with perfusion of 500 U/h during the 5-h-operation. In addition seven erythrocyte concentrates and seven fresh frozen plasmas were infused. The intraoperative blood loss amounted to only 1500 ml, which is low for an LTX. Substitution was continued up to the 15th postoperative day in a decreasing dosage. The total consumption of concentrate amounted to 46,000 U.

The postoperative function of the transplanted liver was good (OT/PT maximum 286/466 U/l). A postoperative biliary leakage was controlled easily with an endoprosthesis. The further course was characterized by a cholestasis which at the beginning could not be explained histologically (increase in bilirubin up to 34 mg/dl). Finally, in a further liver biopsy an unusual progressive fibrosing cholestatic hepatitis was diagnosed which could not be controlled with interferon and ribaverin therapy. The patient died 7½ months after LTX.

Table 1. Course of vWF after LTX with and without substitution of FVIII/vWF

Date	vWF:RiCof %	vWF:Ag % (Laurell)	vWF:Ag % (ELISA)	FVIII:C %	Substitution
02.03.1999					
3:50 a.m.	125.0	133.5	115.0	50.1	Yes
6:00 a.m.	150.0	159.0	155.0	56.5	Yes
0:45 p.m.	125.0	156.0	157.0	74.6	Yes
03.03.1999	100.0	170.0	116.0	72.6	Yes
11.03.1999	100.0	290.0	186.0	115.3	Yes
17.03.1999	32.0	69.0	70.5	134.0	No
19.03.1999	16.0	18.0	40.8	72.3	No
22.03.1999	8.0	12.5	15.5	41.3	No
24.03.1999	8.0	12.5	13.8	36.3	No
26.03.1999	8.0	9.4	12.2	–	No
29.03.1999	8.0	10.7	9.7	30.6	No
31.03.1999	8.0	10.7	9.0	–	No
03.04.1999	4.0	< 6.0	7.0	–	No
06.04.1999	8.0	6.6	8.8	–	No
13.04.1999	125.0	280.0	215.0	–	Yes
23.04.1999	32.0	28.0	–	–	No
03.05.1999	4.0	7.4	9.7	–	No
20.05.1999	6.4	20.5	14.3	–	No
08.06.1999	3.2	7.5	12.4	–	No
15.06.1999	16.0	13.5	18.0	–	No
01.07.1999	16.0	26.0	25.5	–	No
08.07.1999	16.0	23.5	24.0	–	No
17.09.1999					24.0 (Prof. Budde)

Course of the von Willebrand Factor after LTX

The vWF was maintained normal up to the 14th postoperative day. Substitution of FVIII/vWF was terminated on the 15th postoperative day (Table 1). As could be expected in the Western blots under substitution therapy no high molecular vWF multimers were discernible (Fig. 2), but the patient did not bleed.

During periods without substitution the values of the vWF:RiCof ranged from 3.2 to 16% with an average value of about 8% (titration method). Antigen determination of vWF resulted in somewhat higher values up to 26% and FVIII rose several-fold. In the Western blot high molecular multimers were not clearly discernible (Fig. 3), but this may be due to the rather low concentration of the vWF in the plasma samples applied.

Discussion

The vWF is synthesized in the endothelial cells and in the megakaryocytes. Considering the relation of the liver weight to the body weight one might have expected an increase in vWF after LTX to about 2% (provided the density of the endothelial cells in the liver is about the same as in the rest of the body). But the

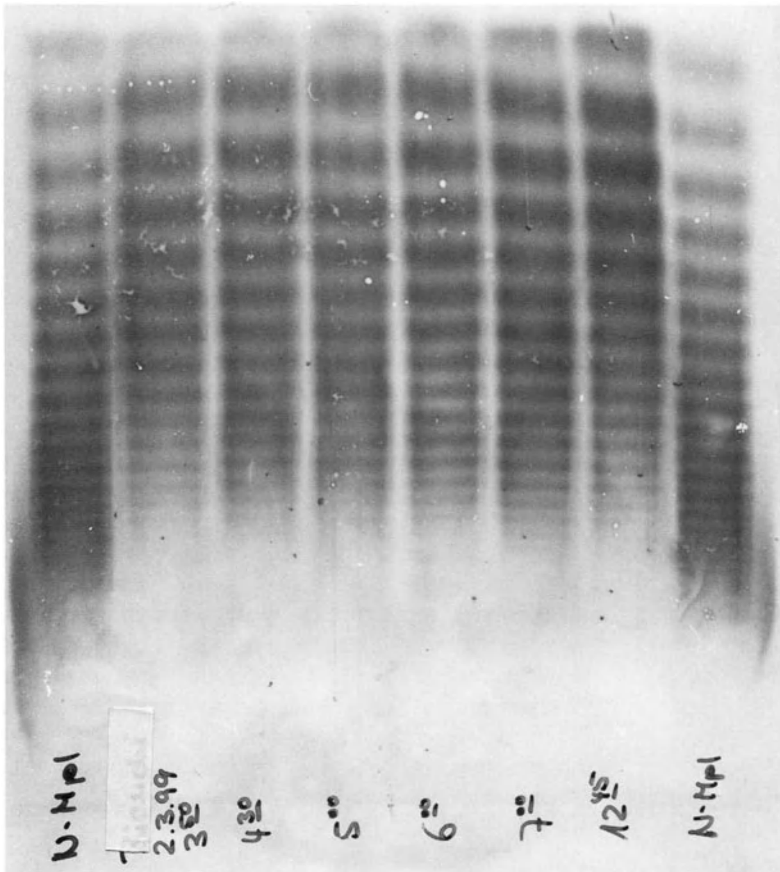
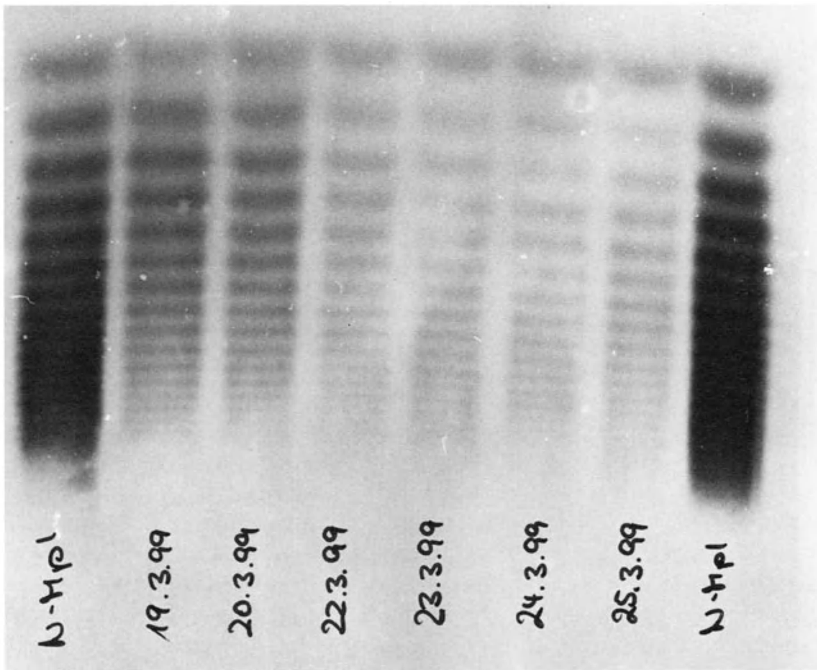


Fig. 2. Western blots in LTX under substitution therapy

values of the vWF we found (vWF:RiCof 3.2 to 16%, vWF:Ag up to 26%) were considerably above the calculated value. Therefore, an increased synthesis and release of the vWF from the endothelial cells through an unknown mechanism may be assumed. One may consider a peritoneal bile irritation in the first weeks after LTX or an effect of the immune suppressant tacrolimus. Later on the high vWF levels might have been a consequence of the inflammatory liver process since it is well known that in chronic hepatitis and liver cirrhosis the vWF and FVIII may increase several-fold (Langley et al. 1985, Maisonneuve and Sultan 1977). We do not know whether the patient might have profited from the increased vWF in respect to his bleeding tendency if he had survived for a longer period of time.

In the first patient with severe vWD type 3 and LTX (Mannucci et al. 1991) the vWF (vWF:Ag) rose to only 1.02% which is considerably less than in our patient. For reasons mentioned above the high levels in our patient may have been a result of the postoperative complications. Therefore, similar high levels probably should not be expected in further vWD type 3 patients with liver transplantation.



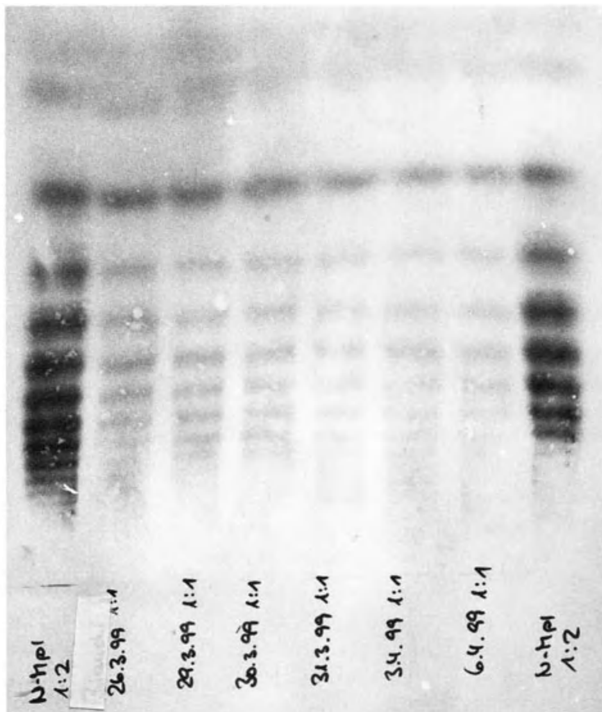


Fig. 3a, b. Western blot after LTX during periods without substitution therapy

Finally, one may speculate that the disturbed fibrinolysis in vWD type 3 (Holmberg et al. 1982, Jeanneau et al. 1987, Korninger et. al. 1981) contributed to the low intraoperative blood loss.

In conclusion, in this patient with severe vWD the vWF rose following LTX even more than one would have expected from the weight of the liver. This is in contrast to the observation of Mannucci et al. In their patient with severe vWD the vWF rose only to 1.02% after LTX. One may speculate that in our patient some stimulatory processes were active (hepatitis?).

References

Holmberg L, Nilsson IM, Wallén P, Astedt B (1982) Studies of the blood plasminogen activator induced by 1-desamino-8-arginine vasopressin with observations in von Willebrand disease. Proc Soc Exp Biol Med 170: 126–132

Jeanneau C, Ounnoughene Bachouchi N, Gorin I, Meimon G, Sultan Y (1987) Absence of functional activity of tissue plasminogen activator in patients with severe forms of von Willebrand's disease. Brit J Hematol 67:79–88

Korninger C, Niessner H, Lechner K (1981) Impaired fibrinolytic response to DDAVP and venous occlusion in a subgroup of patients with von Willebrand's disease. Thromb Res 23:365–374

Langley PG, Hughes RD, Williams R (1985) Increased factor VIII complex in fulminant hepatic failure. Thromb Haemost 54:693–696

Maisonneuve P, Sultan Y (1977) Modification of factor VIII complex properties in patients with liver disease. J Clin Pathol 30:221–227

Mannucci PM, Federici A, Cattaneo M, Fassati R, Galmarini D (1991) Liver transplantation in severe von Willebrand disease. Lancet 337:1105

Clinical Course and Laboratory Findings in a Patient with a New Mutation Causing Wiskott-Aldrich Syndrome

F. Kertzscher, A. Meindl, V. Schuster, U. Sack, M. Koksch and H. Lenk

Introduction

Wiskott-Aldrich syndrome (WAS) is an inherited X-linked disorder caused by mutations of the WAS protein (WASP) gene. Within a family of similar proteins, WASP is responsible for signal transduction from the cellular membrane to actin cytoskeleton. Impaired regulation of actin polymerization in hematopoietic progenitor cells due to altered WASP has been shown to cause the pathophysiology of WAS. Clinical features of WAS are severe disease of platelets (small size, thrombocytopenia, reduced function), of lymphocytes (immunodeficiency) and eczema(1,2,3,5,7). X-linked thrombocytopenia is considered to be a mild form of WAS with isolated thrombocytopenia(2). Prognosis depends on bleeding and immunological complications(1). Stem cell transplantation is nowadays the only successful therapy. The development of gene therapy will be a new therapeutic option, depending on future techniques(6,7).

Clinical Course and Laboratory Findings

A boy was first presented the age of 4 weeks, suffering from a lower gastrointestinal bleeding. At this time he showed petechial bleedings due to a microthrombocytopenia of 72 GPt/l but no eczema. Familial history was normal. Endoscopic investigation of the lower intestines showed nonspecific inflammation. An allergy to cow milk protein was first regarded to be the underlying pathological mechanism, cortisone treatment only slightly cured the thrombocytopenia. In the next 12 months he developed the typical clinical features of Wiskott-Aldrich Syndrome with eczema, severe thrombocytopenia and immunodeficiency. At the age of 1 year, he was hospitalized several times for severe infectious complications like pneumonia and otitis. Thrombocyte count was steady between 10 and 30 GPt/l. Increasing hemolytic anemia required transfusions at nearly 4-week intervals, but no clinical bleeding occurred. Molecular genetic analysis revealed a new, previously not described splice site mutation (Intron 3/+2t→a) of the WASP gene.

Further investigation of the hemostaseological system confirmed the disease of platelets; plasmatic coagulation was normal (Table 1). Platelet flow cytometric analysis was done on two occasions. It confirmed severe dysfunction of platelets. Reticulated platelets as a marker of thrombocytopoiesis were in the normal range.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Hemostaseological and hematological findings

Platelet aggregation	Collagen significantly reduced Adrenaline reduced ADP reduced
Flow cytometric analysis	Disease of platelets (pre-activated platelets, severe dysfunction)
Isoagglutinins	Not found
Thrombocyte size	Microthrombocytopenia
Plasmatic coagulation	Normal
Cytomorphology of the bone marrow	No evidence of malignancy, only few thrombocyte forming megakaryocytic
Haptoglobin	Reduced
Bilirubin (direct/indirect), hemopexin	Normal
Coombs-test	Negative

Expression of the α-granule activation marker P-selectin on non stimulated platelets was increased. Severe reduction of the release of α, δ and lysosomal granules was demonstrated by platelet stimulation using the thrombin receptor activating peptide TRAP 6. Cytomorphology of the bone marrow showed no signs of malignancy.

In the field of immunological function (Table 2), FACS analysis of peripheral blood mononuclear cells (PBMC) was done on four occasions over a period of 12 months. It consistently revealed an increased number of natural killer cells (NK-cells, $CD56^+$, $CD\ 3^-$, $CD\ 16^+$; absolute 2203–3398/μl, normal for age 300–700/μl; relative 56–58%, normal for age 8–15% of peripheral lymphocytes). Another consistent finding was markedly increased cytoplasmatic γ-interferon secretion of the NK and $CD\ 8^+$ cells after stimulation by PMA and ionomycin (46.3% and 50.8% respectively). Phagocytosis of granulocytes and monocytes was reduced. Production of immunoglobulins and subclasses were normal, there was no evidence of active infection by certain viruses like EBV, HSV, CMV, VZV, HHV6. Normal IgG antibodies after vaccination against pertussis, poliomyelitis, diphtheria and *Hemophilus influenzae* were found.

Table 2. Immunological findings

Subpopulations of lymphocytes	Elevated nk-cells, t-cells reduced, normal activation, increased cd4+/cd8+ – ratio
Intracellular cytokines	Elevated production of interferon gamma
Phagocytosis	Reduced functional activity of granulocytes and monocytes
Immunoglobulins, IgG subclasses	Normal (IgG at lower limit)
Serology (CMV, EBV, HSV, VZV, HHV 6)	Negative
Antibodies due to immunization (pertussis, diphtheria, polio, tetanus, HIB)	Normal

Conclusions

In this very young child, a previously not described splice site mutation (Intron 3/+2t→a) of the WASP gene was found to be the cause of a severe clinical course of WAS complicated by severe infections and hemolytic anemia requiring frequent transfusions before the age of 20 months. The rarer intron-mutations are known to lead to quantitatively reduced or absent WASP[7;4]. More extensive molecular genetic investigations (cloning and sequencing of c-DNA) gave evidence for the secretion of a shortened, probably functionally disturbed WASP in this patient leading to a more severe course of the disease. At the age of 21 months, successful bone marrow transplantation (unrelated donor) was performed.

References

1. Kajiwara M, Nonojama S, Eguchi M, Morio T, Imai K, Okawa H, Kaneko M, Sako M, Ohga S, Maeda M, Hibi S, Hashimito H, Shibuja A, Ochs HD, Nakahata T, Yata JI: WASP is involved in proliferation and differentiation of human haematopoietic progenitors in vitro. Br J Haematol 107 (1999) 254–62
2. Kanegane H, Nomura K, Miyawaki T, Sasahara Y, Kawai S, Tsuchiya S, Murakami G, Futatani T, Ochs HD: X-linked thrombocytopenia identified by flow cytometric demonstration of defective Wiskott-Aldrich syndrome protein in lymphocytes. Blood 95 (2000) 1110–11
3. O'Sullivan E, Kinnon C, Brickell P: Wiskott-Aldrich syndrome protein, WASP. Int J Biochem Cell Biol 31 (1999) 382–7
4. Scherbina A, Rosen FS, Remold-O'Donnell E: WASP levels in platelets and lymphocytes of Wiskott-Aldrich syndrome patients correlate with cell dysfunction. J Immunol 163 (1999) 6314–20
5. Snapper SB, Rosen FS: The Wiskott-Aldrich syndrome protein (WASP): Roles in Signaling and Cytoskeletal Organization. Annu Rev Immunol 17 (1999) 905–29
6. Sullivan KE, Mullen CA, Blaese RM, Winkelstein JA: A multiinstitutional survey of the Wiskott-Aldrich syndrome. J Paediatr 125 (1994) 876–85
7. Thrasher AJ, Kinnon C: The Wiskott-Aldrich syndrome. Clin Exp Immunol 120 (2000) 2–9

Role of acquired and inherited Prothrombotic Risk Factors in Pediatric Cerebral Venous Thrombosis – Preliminary Results of a Multicenter Case-Control Study

A. Kosch, U. Schulze-Horsel, C. Heller, R. Schobess, K. Kurnik, R. Sträter and U. Nowak-Göttl

Introduction

Venous thrombosis is an important cause of morbidity and mortality in adult and pediatric patients, and cerebral venous thrombosis is a serious but relatively uncommon diagnosis in children (1, 3, 4, 15–17, 19). It has been associated with underlying systemic conditions, such as dehydration, sepsis, systemic lupus erythematosus, homocystinuria, Behçet disease, acute lymphoblastic leukemia and vascular trauma (8). In addition, various genetic defects of proteins regulating blood coagulation have been discussed as risk factors for cerebral venous thrombosis in childhood (1, 10).

Previously described data indicate that genetic or acquired factors of thrombophilia play an important role in the etiology of venous sinus thrombosis in children (19). To determine to what extent genetic risk factors of familial thrombophilia, including the factor V G1691 A mutation (FV), the prothrombin G20210 A variant (PT), inhibitors of the protein C pathway, and increased concentrations of lipoprotein (Lp) [a], affect the risk of pediatric cerebral vascular accidents, its prevalence was investigated in this prospective, multicenter case-control study.

Patients, Materials and Methods

Study Population

Fifty-six consecutively admitted patients with cerebral venous thrombosis were recruited in a multicenter co-operation over a period of 3 years, starting in January 1998. Mean patient age was 9 years, ranging from newborn to 18 years. With parental consent 168 healthy, age- and sex-matched children served as a control group.

Imaging Methods

In all cases enrolled diagnoses have been confirmed by standard imaging methods (cranial sonography, computed tomography, magnetic resonance imaging) by an independent neuroradiologist.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Blood Samples

With informed parental consent, blood samples in patients were collected 6 weeks to 3 months after the acute thrombotic onset by peripheral venipuncture into plastic tubes containing 1/10 by volume of 3.8% trisodium citrate or into plastic tubes without additives (Sarstedt, Nümbrecht, Germany). Citrated blood (3 ml) was placed immediately on melting ice. Platelet poor plasma and serum were prepared by centrifugation at 3000 *g* for 20 min at 4°C or at room temperature respectively, aliquoted in polystyrene tubes, stored at -70°C and thawed immediately before the assay procedure. For genetic analysis we obtained venous blood (0.5 ml) in EDTA-treated sample tubes (Sarstedt, Nümbrecht, Germany), from which cells were separated by centrifugation at 3000 *g* for 15 min. The buffy coat layer was then removed and stored at -70°C pending DNA extraction by a spin column procedure (Qiagen, Hilden, Germany).

Assays for Genotyping

The FV G1691 A mutation, the prothrombin G20210 A variant and the MTHFR C677 T genotypes were determined by polymerase chain reaction and analysis of restriction fragments as previously described.

Assays for Plasma Proteins

Amidolytic protein C and antithrombin activities were measured on an ACL 300 analyzer (Instrumentation Laboratory, Germany) using chromogenic substrates (Chromogenix, Sweden). Free protein S antigen, total protein S, and protein C antigen were measured using commercially available ELISA assay kits (Stago, France). Lp (a) and anticardiolipin antibodies (IgM and IgG) were determined with ELISA techniques (Chromogenix, Sweden).

Classification of Risk Cut-off

As recently described a heterozygous type I deficiency state (protein C, antithrombin) was diagnosed when functional plasma activity and immunological antigen concentration of a protein were below 50% of the norm for the lower age-related limit. A homozygous state was defined if activity levels and antigen concentrations were less than 10% of the norm. A type II deficiency was diagnosed with repeatedly low functional activity levels 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. Elevated lipoprotein (a) levels were defined as concentrations >30 mg/dl and/or <28 Kringle repeats (11).

Statistics

Prevalences of prothrombotic risk factors in patients and controls were calculated by Chi-square analysis or, if necessary, Fisher's exact test. The significance level was set at 0.05. With respect to the number of different tests applied, a correction according to Bonferroni was performed. In addition, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

Ethics

The study was performed in accordance with the ethical standards laid down in a relevant version of the 1964 Declaration of Helsinki and approved by the medical ethics committee at the Westfälische Wilhelms-University, Münster, Germany.

Results

Thrombotic Locations

Cerebral venous thromboses were localized in the superior sagittal sinus in 43 patients, in the transversal sinus in eight patients, in the inferior sagittal sinus and the internal cerebral vein in two patients, and one child suffered from retinal vein occlusion.

Clinical Symptoms at Onset

In the majority of cases central venous thrombosis appeared with acute headache and vomiting, papilloedema, seizures, hearing loss, nystagmus and in some cases with motor or sensory deficit, confusion or even coma.

Exogenous Triggering Factors

In 28 of the 56 patients with cerebral venous thrombosis consecutively recruited exogenous triggering factors were identified: Steroid administration (treatment of acute lymphoblastic leukemia (n=3), malignant lymphoma (n=3), ulcerative colitis (n=1), induction of fetal lung maturation (n=3) triggered cerebral venous thrombosis. In addition, central venous lines (n=7), infectious diseases, i.e. mastoiditis (n=5) and septicemia (n=4), dehydration (n=1) and cranial trauma (n=1) were reported.

Prothrombotic Risk Factors

Table 1 shows the prevalence rates of prothrombotic risk factors diagnosed in patients and controls. In 31 of the 56 patients investigated at least one prothrombotic risk factor was present (55.3%) compared with 23 of the 168 controls (13.7%). The odds ratio (95% confidence intervals, CI) for prothrombotic risk factors was 7.8 (CI 3.9–15.5, $P<0.001$). Increased Lp (a) concentrations and the heterozygous FV G1691 A mutation were significantly more often diagnosed in patients than in the controls. Prevalence of PT G20210 A variant, protein C deficiency, antithrombin deficiency, acquired ACA IgG antibodies and protein S deficiency was not different in the two groups.

Table 1. Prevalence of genetic risk factors in patients suffering from acute cerebral vein thrombosis (present in 31 out of 56 patients with CVT) (*APS* antiphospholipid syndrome, *AT* antithrombin, *FV* factor V, *Lp* lipoprotein (a), *PT* prothrombin)

Risk factor	Controls (N=168)	Patients (N=56)	Odds ratio (95% confidence interval)	P value
Lp (a)>30 mg/dl	10/168	13/56	4.8 (1.9–11.6)	0.0006
FV GA	8/168	10/56	4.3 (1.6–11.6)	0.005
PT GA	3/168	2/56	2.0 (0.3–12.5)	0.6
Protein C deficiency	1/168	2/56	-	0.15[a]
AT deficiency	0/168	2/56	--	0.06[a]
APS	0/168	2/56	--	0.25[a]

[a] Fisher's exact test

Fatal Outcome

Three patients (5.3%) died during the acute thrombotic event, and two patients developed seizures. However, during the observation period (median 12 months) none of the patients relapsed.

Discussion

Cerebral venous thrombosis in children is a rare disease and either occurs spontaneously or is associated with an underlying systemic disease such as dehydration, septicemia, malignancy or trauma (8, 9). It has rarely been described in patients with systemic lupus erythematosus, homocystinuria, or Behçet disease (2, 18). In addition, dural sinus thrombosis is a known complication of subclavian or jugular vein catheterization (5). The patients may present with non-specific clinical symptoms, for example headache, dizziness and tinnitus, or may suffer from life-threatening events, such as consciousness change with cerebral infarction or hemorrhage with a 10–50% mortality rate reported in children (1, 7).

The data of the multicenter case-control study presented here suggest that beside acquired triggering factors genetic prothrombotic risk factors play an important role in the etiology of venous cerebrovascular accidents in pediatric patients. The overall incidence of familial thrombophilia reported here is higher than in recently published prevalence data in the general population (6, 14): comparable with adult patients with cerebral venous thrombosis we found in 18% of infants and children affected with cerebral vascular accidents the FV G1691 A mutation, or in 23% of cases increased Lp (a). In contrast to the frequency of protein C and protein S deficiency described in adult patients with venous thrombosis (3.8% and 3%), we did not find a significantly higher prevalence of protein C deficiency in patients compared with the controls. The same was true for the prevalence of antithrombin deficiency, which was present in only two patients and in none of the control subjects in our population. Recently Poort et al. reported elevated prothrombin levels associated with a polymorphism in the prothrombin gene (position 20210 A) in 18% of selected patients with venous thrombosis (13). However, in our study population the prothrombin variant was present in only two of 56 patients and in three out of 168 controls. The latter, however, did not reach statistical significance due to the small number of patients recruited so far.

Similar to adults and to pediatric patients suffering from spontaneous ischemic strokes, increased Lp (a) is the most important prothrombotic risk factor in early childhood, followed by the heterozygous FV G1691 A mutation (12). Consistent with these data, in the study presented here, increased Lp (a) was found to be highly significant and more prevalent in pediatric patients with cerebral vascular accidents than in healthy controls.

In summary, data presented here underline the multifactorial etiology of rare cerebral venous thrombosis in neonates and infants, including established prothrombotic risk factors and acquired underlying conditions.

References

1. Barron TF, Gusnard DA, Zimmerman RA, Clancy RR (1992) Cerebral venous thrombosis in neonates and children. Pediatr Neurol 8: 112–116
2. Cocharan FB, Packman S (1992) Homocystinuria presenting as sagittal sinus thrombosis. Eur Neurol 33: 1–3
3. Ehrenforth S, von Depka Prondsinski M, Aygören-Pürsün E, Nowak-Göttl U, Scharrer I, Ganser A (1999) Study of the prothrombin gene 20210 GA variant in FV:Q506 carriers in relationship to the presence or absence of juvenile venous thromboembolism. Arteriosclerosis Thrombosis and Vascular Biology 19: 276–80
4. Egesel T, Buyukasik Y, Dundar SV, Gurgey A, Kirazli S, Bayraktar Y (2000) The role of natural anticoagulant deficiencies and factor V Leiden in the development of idiopathic portal vein thrombosis. Journal of Clinical Gastroenterology 30: 66–71
5. Gebara BM, Goetting FMG, Wang AM (1995) Dural sinus thrombosis complicating subclavian vein catheterization: treatment with local thrombolysis. Pediatrics 95: 138–140
6. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM (1993). Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. Lancet 342: 1503–1506
7. Lee WT, Wang PJ, Young C, Shen YZ (1995) Cerebral venous thrombosis in children. Acta Paed Sin 36: 425–30

8. Lockman LA, Mastri A, Priest JR, Nesbit M (1980) Dural venous thrombosis in acute lymphoblastic leukemia. Pediatrics 66: 943–947
9. Medlock MD, Olivero WC, Hanigan WC, Wright RM, Winek SJ (1992) Children with cerebral venous thrombosis diagnosed with magnetic resonance imaging and magnetic resonance angiography. Neurosurgery 31: 870–876
10. Nowak-Göttl U, Aschka I, Koch HG, Boos J, Dockhorn-Dworniczak B, Deufel T, Jürgens H, Kohlhase B, Kuhn N, Laupert A, Rath B, Wolff JEA, Schneppenheim R (1995) Resistance to activated protein C (APCR) in children with acute lymphoblastic leukaemia - the need for a prospective multicentre study. Blood Coagulation & Fibrinolysis 6:761–764
11. Nowak-Göttl U, Junker R, Hartmeier M, Koch HG, Münchow N, Assmann G, von Eckardstein A (1999) Increased lipoprotein (a) is an important risk factor for venous thrombosis in childhood. Circulation 100: 743–748
12. Nowak-Göttl U, Sträter R, Heinecke A, Junker R, Koch HG, Schuierer G, von Eckardstein A. Lipoprotein (a) and genetic polymorphisms of clotting factor V, prothrombin, and methylenetetrahydrofolate reductase are risk factors of spontaneous ischaemic stroke in childhood. Blood 1999; 94: 3678–3682
13. Poort SR, Rosendaal F, Reitsma PH, Bertina RM (1996) A common genetic variation in the 3' - untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 88: 3698–3703
14. Rees CD (1996) The population genetics of factor V Leiden (arg 506 Gln) Br J Haematol 95: 579–586
15. Seixas CA, Hessel G, Ribeiro CC, Arruda VR, Annichino-Bizzacchi JM (1997) Factor V Leiden is not common in children with portal vein thrombosis. Thrombosis and Haemostasis 77: 258–261
16. Sifontes MT, Nuss R, Jacobson LJ, Griffin JH, Manco-Johnson MJ (1996). Thrombosis in otherwise well children with the factor V Leiden mutation. J Pediatr 128: 324–328
17. Uttenreuther-Fischer MM, Vetter B, Hellmann C, Otting U, Ziemer S, Hausdorf G, Gaedicke G (1997) Paediatric thrombo-embolism: the influence of non-genetic factors and the role of activated protein C resistance and protein C deficiency. European Journal of Pediatrics 156: 227–281
18. Uziel Y, Laxer RM, Blaser S, Andrew M, Schneider R, Silverman ED (1995) Cerebral vein thrombosis in childhood systemic lupus erythematosus. J Pediatr 126: 722–727
19. Zuber M, Toulon P, Marnet L, Mas JL (1996) Factor V Leiden mutation in cerebral venous thrombosis. Stroke 27: 1721–1723

Analysis of the Fibrinogen Genes of 40 Patients with Suspicion of Dys-, Hypo- or Afibrinogenemia

A. Czwalinna, N. Bartkowiak, B. Canepa, A. Ganser
and M. von Depka Prondzinski

Introduction

Fibrinogen is a 340 kDa plasma glycoprotein mainly synthesized by liver parenchymal cells. It is secreted as a dimeric molecule composed of three different polypeptides, Aα, Bβ and γ [2]. Fibrinogen chains are encoded by separate, clustered genes located on chromosome 4q28 in a 50 kb region [5], organized in a γ, α and β sequence with the β gene in opposite transcriptional orientation.

Congenital dys- or afibrinogenemia is a condition which might be caused by mutations in the Aα-, Bβ- or γ-chain of the fibrinogen gene. The causal relationship between dys- or hypofibrinogenemia and clinical symptoms such as thrombosis or hemorrhage has not yet been clarified. Through further molecular genetic analyses a better understanding of the interaction of coexisting mutations in the same or different genes of fibrinogen could be achieved.

Materials and Methods

Patients

Thirty-nine of the investigated patients had suspected dys- or hypofibrinogenemia. One patient suffered from afibrinogenemia (see Table 1: patient 38). The diagnosis of the hypo- or afibrinogenemia was made by the analysis of the fibrinogen levels according to Clauss and Ratnoff-Menzie as well as the determination of the fibrinogen antigen levels by enzyme linked immunosorbent assay and Laurell electrophoresis. Additionally, thrombin time and/or batroxobin time were performed.

Gene analysis

Gene analysis comprised the whole translated region including all exon-intron boundaries and the promoter region of the Aα-, Bβ- and γ-chain of the fibrinogen genes. All investigated regions of the fibrinogen genes were amplified by PCR. Ten microliters of each PCR reaction were used as a control for correct amplification on a 1% agarose gel stained with ethidium bromide. The PCR reactions were purified by QIAquick Multiwell PCR Purification Kit (Qiagen) to reduce contamination.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Mutations and polymorphisms of 40 investigated patients, silent mutations are not listed (*hom* homozygous, *het* heterozygous, *n* negative, *nd* not done, *del* deletion, *A* alpha chain, *B* beta chain)

Patients	Alpha chain	Beta chain	Gamma chain	*Taq*I T1/T2	*Ava*II T/G	*Bcl*I B1/B2	B −1420 G/A	B −854 G/A	B −455 G/A	B −249 C/T	B −148 C/T	A-3 G/A
1	thr312ala het	n	n	het	n	n	n	n	n	het	n	het
2	arg16cys het	n	n	het	hom	nd	nd	nd	n	n	n	nd
3	n	arg448lys het	n	n	het	n	het	n	het	het	het	n
4	n	n	n	het	n	n	n	n	n	n	n	het
5	n	arg448lys het	n	n	het	het	het	n	het	n	het	n
6	thr312ala hom	n	n	hom	n	n	n	n	n	n	n	hom
7	n	arg448lys het 6954–57del GTTT het	arg275cys het	n	het	het	het	n	n	n	het	n
8	thr312ala het	n	n	het	n	n	n	n	n	het	n	het
9	n	n	n	n	n	n	n	het	n	n	n	hom
10	n	n	n	n	n	n	n	n	n	n	n	het
11	n	n	n	het	n	n	n	n	n	het	n	n
12	n	n	n	n	n	n	n	n	n	n	n	n
13	thr312ala het	n	n	het	n	n	n	het	n	het	n	het
14	n	n	arg275cys het	n	n	n	n	n	n	n	n	n
15	thr312ala het	arg448lys het 6954–57del GTTT het	n	het	het	het	het	n	het	het	het	het
16	thr312ala het	n	n	het	n	n	n	n	n	het	n	het
17	thr312ala het	arg448lys het 6954–57del GTTT het	n	het	het	het	het	n	n	n	het	n
18	n	n	n	n	n	n	n	n	n	n	n	het

Table 1. Continued

Patients	Alpha chain	Beta chain	Gamma chain	*Taq*I T1/T2	*Ava*II T/G	*Bcl*I B1/B2	B -1420 G/A	B -854 G/A	B -455 G/A	B -249 C/T	B -148 C/T	A-3 G/A
19	n	n	n	n	n	n	n	het	n	n	n	n
20	n	n	n	n	het	n	het	n	het	n	het	hom
21	n	n	n	n	n	n	n	het	n	n	n	n
22	n	n	n	n	n	n	n	het	nd	n	n	n
23	n	n	n	n	n	n	n	het	n	n	n	het
24	thr312ala het	n	n	het	n	n	n	n	n	het	n	het
25	arg554cys het	arg448lys hom	n	n	het	het	het	n	hom	n	n	n
26	thr312ala het	arg448lys het 6954–57del GTTT het	n	het	het	het	het	n	het	het	het	hom
27	n	n	n	n	n	n	het	nd	hom	n	n	nd
28	n	n	n	n	n	n	n	n	n	n	n	n
29	thr312ala het	n	n	het	het	n	het	n	het	het	het	hom
30	thr312ala het	n	n	hom	n	n	n	n	n	het	n	hom
31	n	n	n	n	n	n	n	n	n	n	n	het
32	n	n	n	n	het	n	het	het	het	n	het	het
33	n	n	n	n	n	n	n	n	n	het	n	het
34	n	n	n	n	n	n	n	het	n	n	n	n
35	n	n	n	n	n	n	n	n	n	n	n	n
36	thr312ala het	n	n	hom	n	n	n	n	n	n	n	hom
37	n	n	n	n	n	n	n	n	n	nd	nd	het
38	n	arg255his hom	n	n	n	n	n	n	n	n	n	hom
39	thr312ala het	n	n	het	n	nd	n	n	n	het	n	het
40	n	n	n	n	n	n	nd	nd	n	nd	n	nd

DNA sequencing was performed on both strands using nested primers for the amplified products, dye-deoxy terminator method and an automated ABI 310 DNA sequencer (Applied Biosystems). The used primers were designed according to the published sequences of the fibrinogen genes (M64982, M64983, M10014, U36503, X05018, U36478; http://www3.ncbi.nlm.nih.gov:80/htbin-post/OMIM).

Results

All detected mutations and polymorphisms of the investigated patients are listed in Table 1. Two silent mutations were detected in patients 3, 5, 7, 15, 25 and 26 in the Bβ chain at the amino acid position 159 Ser and 345 Tyr respectively. Two further mutations were found in intron 4 of the Aα chain (3273 T>C) and in intron 9 of the γ chain (9590 C>T). These mutations are not shown in Table 1.

Only two of the investigated patients (5%) showed no mutation or polymorphism in the investigated regions. No mutation of the translated region was detected in 20/40 patients (50%).

The Thr312Ala polymorphism in the Aα chain occurred in 13/40 patients (32.5%). The Arg448Lys polymorphism in the Bβ chain occurred in 7/40 patients (17.5%). The mutation Arg16Cys in the Aα chain (patient 2) was found only in one patient.

The listed deletion of GTTT (4/40 patients, 10%) in Table 1 is located in intron 6 of the Bβ chain, 9 bp before the start of exon 7. The deletion is strongly associated with the Arg448Lys polymorphism (Table 1).

One further mutation is located in the Bβ-chain in exon 6 at position 6654 (Human fibrinogen beta chain gene, complete mRNA; accession M64983) causing a

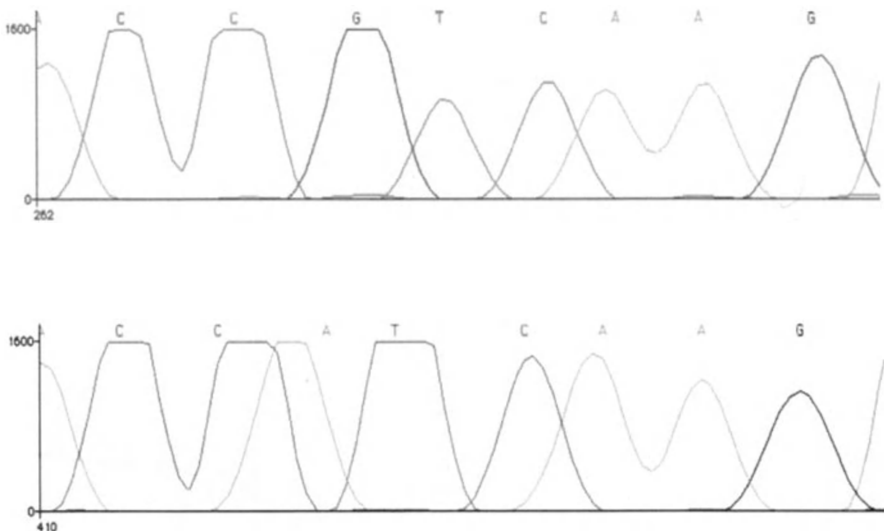


Fig. 2. Gene sequence in exon 6 (6852–6860 bp) of a control person

basepair exchange (Fig. 1) from guanine to adenine which leads to a replacement of arginine by histidine at the position 255 of the amino acid sequence (Aα Arg255His). Besides the –3G>A polymorphism in homozygous form the patient with the afibrinogenemia had only this mutation. It was present in homozygous form.

Apart from known mutations and polymorphisms we were able to detect one new mutation. The –3G>A mutation (A-3, Table 1) is located 3 bp before the major transcription initiation site of the Aα chain. Twenty-three of 40 patients (57.5%) showed this mutation in heterozygous (*n*=15, 65%) or homozygous (*n*=8, 35%) form. In 20/23 (87%) patients with the –3G>A mutation this was the only detectable mutation.

Discussion

The mutation Arg16Cys in the Aα chain (patient 2) is believed to be the most frequent molecular defect in German patients with dysfibrinogenemia [1,7].

The Thr312Ala polymorphism in the Aα chain is involved in the factor XIIIa crosslinking site. Carter et al. showed an association of this polymorphism with venous thromboembolism and a significant interaction with the Val34Leu phenotype of factor XIII [4].

The Arg448Lys polymorphism in the Bβ chain indicates a gender-specific association of increased fibrinogen levels and acute cerebrovascular diseases [3].

The influence on the fibrinogen levels or on the protein function of the Bβ intron 6 deletion of GTTT is not yet clarified.

The –3G>A mutation in the promoter region of the Aα chain could play a potential role in dys- or hypofibrinogenemia because of the high mutation frequency in our investigated patient group. The mutation is located 3 bp before the putative transcription start point and a changed fibrinogen transcription might be the reason for dys- or hypofibrinogenemia in our patients.

The Arg255His mutation was described by Brennan et al. [6] in an abstract. They found that five mutations beneath the Arg255His can result in hypofibrinogenemia inherited in heterozygous state. In the plasma of patients with four of these five mutations the variant fibrinogen chain could not be detected. The non-expression of this particular group of mutations appears to result from perturbation of the five-stranded beta sheet that forms the major structural feature of the D domain. Our patient with afibrinogenemia (patient 38) had the Arg255His mutation in homozygous form.

Further studies are needed to answer the question which of the mutations caused the afibrinogenemia in our patient. Transient transfection experiments with constructs expressing the wild type and mutant fibrinogen need to be performed to investigate the effect of the mutations on the secretion of fibrinogen.

References

1. Meyer, M et al. Dysfibrinogenemia: mutation C1202 T in the FGA gene is the most frequent molecular defect detected in German patients. Annals of Hematology 78 (Supplement 1), A23, FV073 1999
2. Doolittle, RF. Fibrinogen and fibrin, Ann Rev Biochem 1984, 53, 195
3. Carter, AM et al. Gender-specific associations of the fibrinogen B beta polymorphism, fibrinogen levels, and acute cerebrovascular diseases. Arterioscler Thromb Vasc Biol 1997, 17, 589
4. Carter, AM et al. Alpha-Fibrinogen Thr312Ala polymorphism and venous thromboembolism. Blood 2000, 96,1177
5. Marino, MW et al. Chromosomal localization of human and rat Aα, Bβ and γ fibrinogen genes by in situ hybridization. Cytogen Cell Genet 1986, 42, 36
6. Brennan, S et al. Molecular mechanisms in hypofibrinogenaemia. Fibrinogen 2000, 16th International Fibrinogen Workshop
7. Galanakis, D et al. Unusual A alpha Arg16Cys dysfibrinogenaemic family: absence of normal A alpha-chains in fibrinogen from two of four heterozygous siblings. J Clin Invest 1993, 4, 67
8. Masuda, M et al. Structure and function of fibrinogen: insights from dysfibrinogens. Thrombosis and Haemostasis 1999, 82, 283

VId. Poster: Thrombophilic Diathesis

Hereditary Antithrombin Deficiency – Results of a Family Study

B. Maak, S. Hutschenreiter, F.H. Herrmann, K. Wulff,
A. Siegemund and W. Christoph

Introduction

Venous thrombosis or thromboembolism in younger people and especially in children or adolescents is in the majority of cases related to one or more inherited defects of naturally occurring antithrombotic proteins.

The first identified cause of inherited thrombophilia is the deficiency of antithrombin III, described by Egeberg in 1965 (4). During the last 20 years an increasing number of further reasons for a familial thrombotic tendency has been found, e.g. deficiencies of the proteins C and S and the resistance against activated protein C (APC resistance). The latter was described by Dahlbäck and coworkers in 1993 (2).

Hyperhomocysteinemia, elevated factor VIII activities and the mutation G 20210 A in the prothrombin gene are also well known risk factors for a thrombotic tendency. Furthermore a disposition to thrombotic events results also from a factor XII deficiency (5, 11), therefore it is recommended to perform factor XII assays in thrombophilia investigation programs (17). Patients with lowered factor XII activities exhibit a decreased fibrinolytic activity which may be responsible for the development of thrombosis or thromboembolism (9).

The most frequent of the above mentioned risk factors seems to be the APC-resistance which is typically caused by a point mutation (G 1691 A) in the factor V gene. However, there are substantial differences in the prevalence rates reported from different regions around the world (6, 13).

For the antithrombin deficiency, prevalence rates between 0.02% and 0.2% have been estimated (8, 16). Antithrombin deficiency is inherited as an autosomal-dominant trait. According to the levels of antithrombin activity and antithrombin concentration, different types of deficiency states have been described (7). Type 1 is characterized by similarly decreased levels of antithrombin activity and antithrombin concentration; in type 2, however, the concentration is within normal limits and the activity is decreased. In type 3 only the heparin cofactor activity is low. The most common clinical manifestation of heterozygous antithrombin deficiency, especially of the types 1 and 2, is the venous thrombosis of the lower extremities, often followed by pulmonary embolism.

It should be noted that during infancy and childhood the risk of thrombosis in anti-thrombin-deficient individuals is low, after an age of 14–15 years, however, it increases sharply. Because adolescents often are pediatric patients the pediatricians should be familiar with the symptoms, diagnostic procedures and therapeutic

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

measures of thrombotic events. Therefore we report here on a 16-year-old boy who developed venous thrombosis of his right lower limb and the inferior caval vein complicated by multiple pulmonary emboli. The boy and his mother were identified as heterozygous for antithrombin deficiency type 2. As a possible second risk factor we found in the patient and also in the patient's brother a moderate factor XII deficiency.

Case Reports and Results of the Family Study

The patient, a 16-year-old boy, is the first of two children born to nonconsanguinous parents. A respiratory tract infection, presumably of viral origin, showed an unusually prolonged course with intermittent fever and an increasing number of cough attacks.

Multiple bloody expectorations were observed and pneumonia was assumed. A chest radiograph apparently confirmed this diagnosis, antibiotic treatment, however, was without any effect. Two days before hospital admission a slight swelling of the right lower extremity was noted which progressed rapidly during the following hours. After admission to the hospital, emergency diagnostic procedures (ultrasound examination, venography) were performed and as a result a complete occlusion of the deep veins of the right leg and the lower part of the vena cava inferior could be demonstrated. Immediately after completion of the diagnostic procedures the thrombus was removed surgically. Three weeks after the first symptoms a ventilation-perfusion scan of the lungs was performed and multiple perfusion defects in both lungs as a result of pulmonary embolizations were identified (Fig. 1). Retrospectively, the findings in the X-ray examination of the chest were signs of multiple pulmonary emboli rather than pneumonic infiltrations. After discharge from the hospital and under stable anticoagulation with phenprocoumon at an INR 3.0 the patient acquired a common cold and developed a thrombotic occlusion of the left

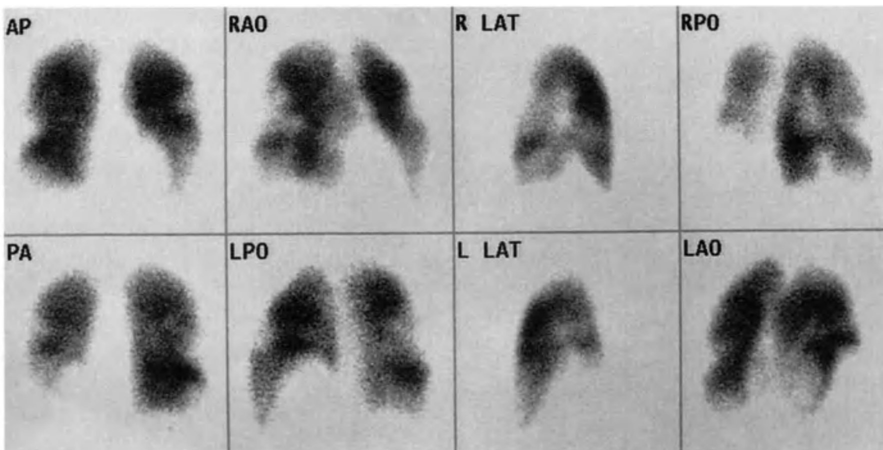


Fig. 1. Ventilation-perfusion scintigraphy, 3 weeks after the beginning of the illness. The bright areas represent distribution or absent perfusion due to multiple pulmonary emboli

external iliac vein which was successfully treated by antithrombin substitution and low molecular weight heparin. After 10 days the phenprocoumon was reintroduced.

The patient's mother showed the first thrombotic event at an age of 18 years shortly after beginning oral contraceptive use. Two years later postoperative immobilization of her right lower limb was followed by extensive swelling. Retrospectively this swelling has been interpreted as a second thrombosis: sonographic studies have been performed, and the results obtained were compatible with the assumption of previously occurred venous thrombotic occlusions. Two pregnancies were without complications.

The results of the coagulation studies are summarized in Table 1. With the exception of an antithrombin activity of 40%, which was estimated before thromb-

Table 1. Results of the laboratory investigations performed in the patient and his family members. From the patient all results were obtained during phenprocoumon treatment. In some instances the results of repeated studies are shown (*n.d.* not done, *WT* wild type)

	Patient	Brother	Mother	Father	Normal range
Antithrombin III:					
activity	59%, 50%, 47%, 55%	107%, 91%	58%, 59%	95%	80–120%
concentration	99%	94%	89%	n.d.	80–120%
APC-ratio	n.d.	2.5	2.5	2.6	>2.0
Protein C (activity)	n.d.	72%	92%	89%	70–140%
Protein S (activity)	n.d.	113%	78%	118%	70–140%
Lipoprotein (a)	41.7 mg/l	54.1 mg/l	33.3 mg/l	n.d.	<300 mg/l
Homocysteine	6.3 μmol/l	5 μmol/l	n.d.	n.d.	4.4–12.4 μmol/l
Factor VIII-activity	118%	80%	76%	n.d.	70–140%
Factor XII-activity	78%, 55%	43%, 39%, 49%, 40%	104%, 105%	127%	70–140%
Factor XIII-activity	135%	97%	100%	n.d.	70–140%
D-dimer	147 μg/l	999.5 μg/l, 685.2 μg/l, 714.0 μg/l, 789.5 μg/l	291.9 μg/l, 62.0 μg/l	257.9 μg/l	<275 μg/l
LA	n.d.	Negative	n.d.	n.d.	Negative
ACA	1.3	<1.0	n.d.	n.d.	<1.0
Mutations:					
Factor V gene	GG 1691 (WT)	GG 1691 (WT)	GG 1691 (WT)	n.d.	WT
Prothrombin gene	GG 20210 (WT)	GG 20210 (WT)	GG 20210	n.d.	WT
MTHFR gene	CC 677 (WT)	CC 677 (WT)	CC 677 (WT)		
Factor XII gene	C 46 T	46 TT	n. d.	n. d.	CC 46 (WT)

ectomy, all other results were obtained during anticoagulation with phenprocoumon. On different occasions the antithrombin activities in the patient were always lower than 60%. In contrast, the antithrombin concentration was in the normal range. Therefore we considered the patient to suffer from a type 2 antithrombin deficiency. The same constellation was found in the patient's mother. Father and brother of the patient exhibited normal antithrombin activities and in the case of the brother the anti-thrombin concentration was also within the normal range. In the family members studied there were normal values for APC-resistance and the activities of the proteins C and S and no mutations were detected in the factor V gene, the prothrombin and the MTHFR gene. In three members of the family the Lp (a) concentrations were not elevated. Surprisingly we measured in the clinically well brother of the patient repeatedly elevated D-dimer concentrations. His factor XII activities were low: between 39 and 49%. The patient showed factor XII activities between 55 and 78% and in the mother and father these values were 105/104% and 127% respectively. On one occasion after the acute illness a slightly elevated concentration of anticardiolipin antibodies in the patient's circulation was demonstrated. The analysis of the factor XII gene revealed a heterozygous state for the 46 C $\rightarrow$ T polymorphism in the 5'-untranslated region in the patient, whereas in his brother the homozygous state (46 TT) could be seen.

Discussion

The laboratory findings presented here are characteristic for an antithrombin deficiency type 2: a reduced antithrombin activity, typically below 60%, contrasts with antithrombin concentrations within the normal range. The autosomal-dominant mode of heredity of this disorder is also evident.

The age at manifestation and the clinical course in our patient and his mother confirm the findings from others (3, 7, 10, 14): in both individuals the thrombotic events occurred at a younger age (before 20 years) and were localized in the lower extremities. In the case of the patient's mother the use of oral contraceptives and later, immobilization after limb surgery, could be identified as triggering factors for her thrombosis manifestations.

In the patient, however, a similarly clear cut situation with regard to the triggering events could not be seen. Respiratory tract infections of probably viral origin preceded the two thrombotic events. Unfortunately there are no results from determinations of the protein C and protein S activities before surgery and also a lupus anticoagulant was not excluded. Mutations in the factor V and prothrombin genes were absent and the concentrations of Lp (a) and homocysteine were within the normal limits. Only the factor XII activity, measured during anticoagulant treatment with phenprocoumon was repeatedly low and on one occasion a reduced factor XII antigen level was found. (The authors thank Prof. Budde, Hamburg, for performing the factor XII-antigen determination.) The second child of the family, at present in good health, exhibited elevated D-dimer concentrations and low levels of the factor XII activity. Other thrombotic risk factors have not been identified up to now. It seems that in both boys a mild factor XII deficiency exists which results

from a polymorphism in the 5'-untranslated region of the factor XII gene. This polymorphism (46 C $\rightarrow$ T) is associated with low translation efficiency (15). Therefore the different factor XII activities in the boys could be ascribed to the heterozygous state (46 C $\rightarrow$ T) in the patient and the homozygous state (46 TT) in the patient's brother. The finding of slightly elevated anticardiolipin antibodies in the patient suggests an additional effect which may be responsible for the different factor XII activities measured on different occasions. In children the synthesis of antiphospholipid antibodies is often induced by infectious diseases (so-called secondary antiphospholipid syndrome). It may be speculated that in the patient the antithrombin deficiency in combination with two other risk factors (mild factor XII deficiency, anticardiolipin antibodies) resulted in the thrombotic events described. The role of the anticardiolipin antibodies in the pathogenetic considerations is in our opinion supported by the fact that viral illnesses preceded the thrombosis manifestations in both instances.

In the patient's brother the repeatedly measured factor XII activities were consistently lower and there were no signs of an inhibitor development. At present we assume that the elevated D-dimer concentrations in this boy may be the result of the factor XII deficiency which is caused by the homozygous state for the polymorphism in the 5'-untranslated region of the factor XII gene.

In summary, we have seen a 16-year-old boy suffering from a dramatic and life-threatening illness. As a consequence questions regarding preventive measures arise. A deficiency of any of the naturally occurring antithrombotic proteins should be suspected in patients who develop thrombosis at a younger age and also in individuals with an appropriate family history. During childhood the risk of thrombosis in patients with antithrombin deficiency or other defects is low because during this period elevated levels of α_2 -macroglobulin may have a protective effect (12). After an age of 14 to 15 years the concentration of α_2-macroglobulin decreases and the risk of thrombosis increases. This observation is impressively confirmed by the first results of the Canadian registry of venous thromboembolic complications in children (1). Therefore a lifelong prophylactic therapy starting immediately seems to be useful; however, such a regimen is not generally recommended (3). In contrast there is no doubt about using an effective prophylaxis when symptom-free individuals are exposed to situations with a high risk of thrombosis development (3, 7). Such situations are surgical interventions, traumatic lesions, especially of the lower limbs, pregnancy and also infectious diseases.

In the patient presented here who suffered from two massive thromboses complicated by multiple pulmonary embolizations we believe that a life-long treatment with vitamin K antagonists is necessary.

References

1. Andrew M, David M, Adams M, Ali K, Anderson R, Barnard D, Bernstein M, Brisson L, Cairney B, De Sai D, Grant R, Israels S, Jardine L, Luke B, Masciotte P, Silva M (1994). Venous thromboembolic complications (VTE) in children: first analyses of the Canadian registry of VTE. Blood 83: 1251-1257

2. Dahlbäck B, Carlsson M, Svensson P J (1993). Familial thrombophilia du to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. Proc Natl Acad Sci USA 90: 1004–1008
3. Demers C, Ginsberg J S, Hirsh J, Henderson P, Blajchman M A (1992). Thrombosis in antithrombin-III-deficient persons. Ann Int Med 116: 754–761
4. Egeberg O (1965). Inherited antithrombin deficiency causing thrombophilia. Thromb Diath haemorrh 13: 516–530
5. Halbmayer W M, Mannhalter C, Feichtinger C, Rubi K, Fischer M (1992). The prevalence of factor XII deficiency in 103 orally anticoagulated outpatients suffering from recurrent venous and or arterial thromboembolism. Thromb Haemostas 68: 285–290
6. Herrmann F H, Koesling M, Schröder W, Altman R, Jimenez Bonilla R, Lopaciuk S, Perez-Requejo J L, Singh J R (1997). Prevalence of factor V Leiden mutation in various populations. Genet Epidemiol 14: 403–411
7. Hirsh J, Piovella F, Pini M (1989). Congenital antithrombin III deficiency. Incidence and clinical features. Am J Med 87 (suppl. 3 B): 34 S–38
8. Lane D A, Olds R R, Thein S L (1992). Antithrombin and its deficiency states. Blood Coagul Fibrinolys 3: 315–341
9. Levi M, Hack C E, de Boer J P, Brandjes D P M, Büller H R, ten Cate J W (1991). Reduction of contact activation relation related fibrinolytic activity in factor XII deficient patients. J clin Invest 88: 1155–1160
10. Mackie M, Bennett B, Ogston D, Douglas A S (1978). Familial thrombosis: inherited deficiency of antithrombin III. Brit Med J 1: 136–138
11. Mannhalter C, Fischer M, Hopmeier P, Deutsch E (1987). Factor XII activity and antigen concentrations in patients suffering from recurrent thrombosis. Fibrinolysis 1: 259–263
12. Mitchell L, Piovella F, Ofosu F, Andrew M (1991). α-2-macroglobulin may provide protection from thromboembolic events in antithrombin III-deficient children. Blood 78: 2299–2304
13. Rees D C, Cox M, Clegg J B (1995). World distribution of factor V Leiden. Lancet 346: 1133–1134
14. de Stefano V, Leone G, Mastrangelo S, Tripodi A, Rodeghiro F, Castaman G, Barbui T, Finazzi G, Bizzi B, Mannucci P M (1994). Clinical manifestations and management of inherited thrombophilia: retrospective analysis and follow-up after diagnosis of 238 patients with congenital deficiency of antithrombin III, protein C, protein S. Thrombos Haemostas 72: 352–358
15. Taisuke K, Takashi O, Koichi O, Mika K, Kazuya S, Naotaka H, Yoshiyuki N (1998). A common genetic polymorphism (46 C to T substitution) in the 5'-untranslated region of the coagulation factor XII gene is associated with low translation efficiency and decrease in plasma factor XII level. Blood 91: 2010–2014
16. Wells P S, Blajchman M A, Henderson P, Wells M J, Demers C, Bourque R, Mc Avoy A (1994). Prevalence of antithrombin deficiency in healthy blood donors: a cross-sectional study. Am J Hematol 45: 321–324
17. Winter M, Gallimore M, Jones D W (1995). Should factor XII assays be included in thrombophilia screening? Lancet 346: 52

Elevated Factor IX and Factor XI as Risk Factors for Venous Thrombosis and Stroke

A. SIEGEMUND, T. SIEGEMUND, J. BERROUSCHOT, H. VOIGT
and M. KOKSCH

Introduction

Recently, elevated levels of the coagulation factors IX and XI as risk factors for venous thrombosis were described by Bertina and Rosendaal (1). Increased levels are associated with a two- to threefold increased risk of venous thrombosis. Reports from several groups indicate that the prevalence of elevated levels in the normal population is about 10% (2). In patients with a first episode of venous thrombosis the prevalence is about 20%. However, the risk of venous thrombosis in connection with elevated F IX and F XI is also age-dependent. Comparable with deficiencies of protein C or protein S, the prevalence is relatively high, which seems to be similar to the prevalence of the well-known APC resistance caused by F V Leiden. Furthermore, a correlation between the levels of coagulation factors and the relative risk of thrombosis has been shown (3). Compared with the risk shown in connection with elevated factor VIII:C, existence of an analogous relation between risk and single factor activity is suggested for the newly described risk factors IX and XI. Data on the association of elevated coagulation factors and arterial occlusions, especially in patients who had strokes, are rare. Only the ARIC study (Arteriosclerosis Risk in Communities) investigated the association between hemostatic variables like F VIII:C, von Willebrand factor and fibrinogen and stroke (4, 5).

In the present study we investigated whether elevated levels of the coagulation factors IX and XI contribute to an increase in risk of thromboembolism in different groups of patients. This question was addressed by comparing coagulation factors IX and XI with other well established thrombophilic coagulation defects in patients with a first thrombotic event, in patients with recurrent events, as well as in young patients who had ischemic strokes.

Patients and Methods

All patients and controls included in this study were admitted to the University Hospital of Leipzig. Collection of data was retrospective. Only patients with objectively confirmed diagnosis of thrombosis (n=160) or stroke (n=70) were included (e.g. computer tomography, sonography or phlebography).

Age and sex matched samples from healthy donors (n=115) were selected for the control group, the age distribution of patients and controls being nearly identical

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

(controls 34.7±11.2 years, patients with thromboembolism 34.7±7.9 years, stroke patients 37.1±6.5 years).

For the evaluation of age-dependency we further investigated 369 thrombosis patients and 371 stroke patients between 30 and 80 years.

Blood was collected in Sarstedt tubes (S-monovette 9NC) containing 0.106 mol/l trisodium citrate. Blood samples from blood donors were taken before blood donation. Plasma preparation was done by centrifugation for 20 min at 2000 *g* by 15°C. If the sample was not assayed within 2 h, the plasma was shock frozen and stored at -70°C.

Factor IX and factor XI were measured by one-stage clotting assay with Pathromtin SL and the corresponding factor deficient plasma at the BCS with reagents from Dade Behring. For excluding other coagulation abnormalities or other thrombogenic risk factors, the global tests PT and APTT were performed and also a thrombophilia screening program.

Statistical Analysis

The software package SPSS for Windows 6.0 (SPSS Munich, Germany) was used for statistical analysis. *P* value of <0.05 was considered significant.

Results

Coagulation factor IX and XI levels showed statistically significant differences between controls and patients with thromboembolism (Table 1). In stroke patients the difference was not significant. Between patients who had a stroke and those with thromboembolism a statistically borderline difference for factor IX ($P=0.043$) was found, but not for F XI ($P=0.134$).

Based on the results of the Leiden Thrombophilia Study (LETS) we have chosen as upper reference limits the levels of 129% factor IX and 123% factor XI (1). Elevated factor IX and factor XI levels above these limits were significantly more frequent in the two patient groups compared with the controls. (Table 2). In the con-

Table 1. Factor IX and factor XI levels in patients and controls

Coagulation factor	Controls *n*=115	Thrombosis patients *n*=160	*P* value Thrombosis vs. controls	Stroke patients *n*=70	*P* value Stroke vs. controls	*P* value Stroke vs. thrombosis
Factor IX (%)						
Mean	106.1	122.4	<0.000	118.5	0.098	0.043
95% CI	104.4–107.7	120.2–124.5		115.6–121.4		
Factor XI (%)						
Mean	98.1	108.6	0.012	106.1	0.57	0.134
95% CI	96.5–99.7	106.7–110.5		103.7–108.5		

Table 2. Frequency of elevated coagulation factors IX and XI in patients and controls

	IX >129% *n* (%)	*P* value	OR (95% CI)	XI >123% *n* (%)	*P* value	OR (95% CI)
Controls (*n*=115)	11 (9.6)			8 (7.0)		
Thrombosis patients (*n*=160)	59 (36.9)	<0.0001	5.5 (2.7–11.1)	36 (22.5)	0.0005	3.9 (1.7–8.7)
Stroke patients (*n*=70)	16 (22.9)	0.013	2.8 (1.2–6.5)	12 (17.1)	0.03	2.7 (1.1–7.2)

trol group a prevalence of 9.6% for elevated factor IX >129% was observed; in young patients with thrombosis the corresponding rate was 36.9%, in stroke patients 22.9%. For elevated factor XI >123% frequency rates of 7.0%, 22.5% and 17.1% were observed in the three investigated groups.

Thus, the risk for thrombotic disease was increased 5.5-fold in individuals with elevated factor IX levels and 3.9-fold in those with elevated factor XI levels. For stroke, the increase in risk was 2.8-fold in connection with elevated factor IX and 2.7-fold in connection with elevated factor XI. In general, in individuals with elevated factor IX or XI the risk with regard to stroke is lower than that for thrombotic disease; nevertheless, the risk of a stroke is significantly higher than in those not affected by elevations in these factors.

From Fig. 1a and b it is obvious, that only the blood donors show a normal distribution, whereas the two groups of patients show a second peak at levels higher than the reference range.

These data were obtained from relatively young patients with a median age of 34.7 years (patients with venous thrombosis). However, there exists an association between age and the levels of F IX and F XI. Levels of both factors increase with age. When comparing thrombosis patients aged 30–40 with thrombosis patients between 60 and 70 years, the mean FXI level increases by about 10% from 103,7% (95% CI 97.7–109.7) in the younger group to 114,7 (95% CI 100.0–119.4) in the older group; for F IX see Fig. 2. The largest increase is observed between the age groups of 30–40 and 40–50 years; after this decade the levels are nearly constant. The age-dependency of F XI in the stroke group is less pronounced, and there exists a tendency for it to decrease in the last two decades.

Discussion

The present study emphasizes the assumption that elevated coagulation factors IX and XI increase substantially the risk of venous thrombosis and stroke. The significantly higher prevalence of elevated coagulation factors in patients compared with controls suggest that both risk factors, factor IX and factor XI, are involved in the etiology of thrombophilia. The relative risk is nearly in the same order of magnitude of elevated levels of F VIII:C and prothrombin respectively.

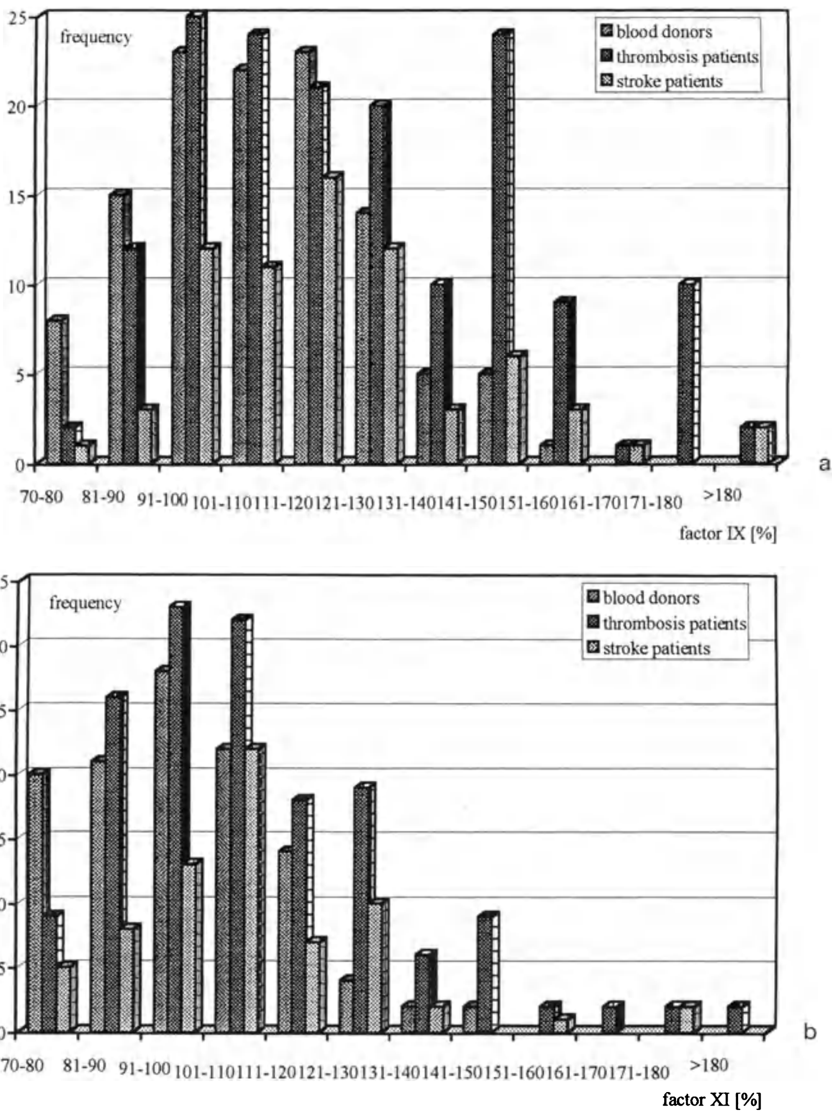


Fig. 1a, b. *a* Distribution of F IX levels in thrombosis patients (n=160), stroke patients (n=70) and controls (n=115). *b* Distribution of F XI levels in thrombosis patients (n=160), stroke patients (n=70) and controls (n=115)

It is described that F XI can be activated by thrombin. Via this pathway F XI contributes to further thrombin generation in addition to the activation that is due to the F X pathway.

F IX levels are age-dependent with higher levels in centenarians. Coagulation factors are known to increase gradually during young adulthood, and increase again

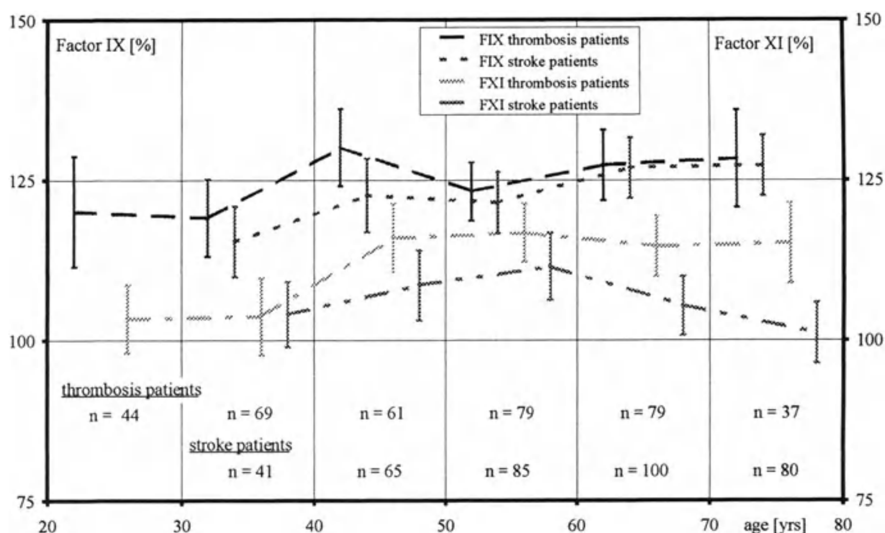


Fig. 2. Age-dependency of coagulation factors IX and XI

a second time in old age, which is a physiological phenomenon of aging. However, this increase may contribute significantly to the age-dependent development of cardiovascular and thrombotic disorders. Kurachi et al. have described a molecular mechanism for the age-associated increase in F IX activity to be related to an age-dependent regulation of the human F IX gene (5). Patients with thrombotic disease exhibit a higher level of 120% and additionally F IX levels increase with age.

F IX and F XI should be involved in the thrombophilia screening program, especially in young patients. Further investigations are necessary to evaluate these risk factors in relation to the well established risk factors like F V-Leiden and prothrombin mutation 20210 GA.

References

1. Meijers JCM, Tekelenburg WLH, Marquart JA, Bouma BN, Bertina RM,Rosendaal FR. Thromb Haemost Suppl.496, 1999.
2. Meijers JCM, Tekelenburg WLH, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. N Engl J Med 2000;342: 696–701.
3. Roosendaal FR. Risk factor for venous thrombotic disease. Thromb Haemost 1999;82:610–9.
4. Koster T, Blann AD, Briet E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. Lancet. 1995; 345:152–55.
5. Folsom AR, Rosamond WD, Shahar E, Cooper LS, Aleksic N, Nieto FJ, Rasmussen ML, Wu KK. Prospective study of markers of hemostatic function with risk of ischemic stroke. Circulation 1999; 100: 736–42.
6. Kurachi S, Deyashiki K, Kurach k. Mechanism underlying homeostasis of blood coagulation: age-associated regulation of the human factor IX gene. Thromb Haemost 1999; Suppl:224.

Thrombophilic Risk Parameters in Juvenile »Idiopathic« Stroke Patients

M. Krause, S. Ehrenforth, E. Aygören-Pürsün, G. Ludwig and I. Scharrer

Introduction

Within the last decade, various genetic defects of proteins regulating blood coagulation, particularly those affecting the physiological anticoagulant systems, have been well established as risk factors of cerebrovascular disease in adults [8;22;23]. Besides the high thrombotic risk reported in patients with homozygous FV:R506Q mutation, homozygous protein C deficiency, homozygous protein S deficiency and homozygous homocysteinuria due to cystathionine-ß-synthase (CBS) deficiency, a high risk of early thrombotic onset is observed in patients with heterozygous antithrombin deficiency, heterozygous protein C deficiency of the so-called dominant type, and heterozygous protein S deficiency [18;29;40].

In contrast, an intermediate or low risk of developing early thromboembolism is observed in patients with heterozygous FV:Q506, heterozygous recessive protein C deficiency and heterozygous defects of the heparin-binding site of the antithrombin molecule, moderate hyperhomocysteinemia (elevated fasting HCY concentrations), plasminogen deficiency and dysfunctional plasminogen or fibrinogen molecules [15;38].

Furthermore, the recently described 20210GA variant of the prothrombin gene seems to be a common but probably mild risk factor of arterial and venous thromboembolism [33]. However, there is only scanty and conflicting information about its role in triggering stroke in inherited or acquired deficiency of heparin cofactor II (HC II), FXII, or elevated levels of histidine-rich glycoprotein (HRGP) respectively [2;13;19].

Since the recent discovery of activated protein C resistance as a highly prevalent hereditary risk factor of venous thromboembolism, evidence has been accumulating that thrombophilia is a multigenetic disorder and that the association of multiple hemostatic defects greatly increases the risk of thrombosis in adults. Although venous thrombosis is being increasingly viewed as a multifactorial disorder as well, information on the role of combined hemostatic defects in juvenile ischemic stroke is limited [32].

Here, we present data on juvenile idiopathic ischemic stroke in patients with regard to the frequency of single and combined hemostatic risk factors for thromboembolic events.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Patients

A total of 344 unrelated patients (170 males/174 females) with a history of ischemic stroke (n=282) or transient ischemic attack (TIA) (n=62) were studied. At clinical onset, patients were aged 18–50 years (median age at first episode: 31 years).

Objective confirmation of cerebrovascular disease (CVD) was performed by angiography, cranial computed tomography and/or magnetic resonance (MR) imaging.

Risk factors predisposing to CVD, such as diabetes mellitus, hypertension, smoking, use of oral contraceptives (OC) etc. were documented in each patient. Patients with severe and/or insufficiently treated hypertension were excluded. None of the patients had diabetes. Use of OC was documented in 40/174 affected women (22.9%).

None of the patients enrolled had overt evidence of cardiologic or systemic disorders known to be associated with an increased risk of stroke/TIA.

Methods

Blood Sampling

With informed consent, blood samples were collected by peripheral vein puncture into 3.8% trisodium citrate (one part anticoagulant: nine parts blood; Sarstedt tubes, Germany) and placed immediately on melting ice. Platelet poor plasma was prepared by centrifugation at 3000 *g* for 20 min at 4°C, aliquoted in polystyrene tubes, stored at -70°C and thawed immediately before the assay. Dade Hepzym (Baxter Diagnostics, Germany) was added to plasma samples to eliminate the influence of heparin.

For genetic analysis, we obtained venous blood in EDTA-treated sample tubes (Sarstedt) from which cells were separated by centrifuging at 3000 *g* for 15 min. The buffy coat layer was then removed and stored at -70°C, pending DANN extraction by standard techniques.

Assays of Hemostatic Factors

- Response to activated protein C: Chromogenix, Sweden (normal range: 1,72–2,94)
- Amidolytic protein C activity: chromogenic substrate, Chromogenix, Sweden (normal range: 77–142%)
- Free PS antigen: (normal range: 64–133%)
- Total PS antigen: (normal range/males: 77–154%, females: 67–158%)
- PC antigen: (normal range: 71–124%): Asserachrom, Stago, France [27, 30, 37]
- Antithrombin activity: chromogenic substrate assay, Chromogenix, Sweden (normal range: 75–112%)
- Fasting plasma homocysteine concentrations: high-performance liquid chromatography (normal range: 3.8–13.8 µmol/l) [33, 38, 39]

- Heparin cofactor II (HCII) antigen: ELISA technique, Asserachrom, Stago, France; HCII-EIA: Diagnostik International, Germany; or Laurell method, Anti HC II: Behringwerke, Germany; (normal range/males: 73–196%; females: 70–170%)
- Plasminogen (Plg) activity: chromogenic substrate, Chromogenix, Sweden
- Plg antigen concentrations: radial immunodiffusion, Behringwerke, Germany (normal range/males: 71–116%; females: 78–137%)
- FXII activity: ACL 300 R; IL, Germany (normal range: 53–135%)
- FXII antigen concentrations: immunoelectrophoresis according to Laurell, Anti FXII: Behringwerke, Germany; Enzym Research Laboratory, USA
- Histidine-rich glycoprotein (HRGP): Laurell method; Anti-HRG: Behringwerke Marburg, Germany (normal range: 50–172%)
- Factor VII activity: one-stage clotting assay (normal range: 59–114%)
- Lupus anticoagulant: was diagnosed according to the criteria of the Subcommittee for the Diagnosis of Lupus Anticoagulants/Antiphospholipid Antibodies of the ISTH
- IgG and IgM anticardiolipin antibodies: ELISA (IgG-normal range: 0.9–11.7; IgM-normal range: 0.3–4.8)
- Prothrombin time (PT), activated partial thromboplastin time (aPTT) and thrombin: conventional methods

Assays for Genotyping

Genetic analysis (prothrombin G20210 A mutation and FV G1691 A) was performed with the use of methods already described [3;33].

Definition Criteria of Prothrombotic Defects

For all plasma-based assays, diagnosis of a prothrombotic defect was established only if the plasma level of a protein was outside the limits of its normal range in at least two different samples.

Results

In our study 27/236 patients (stroke/TIA: 21/6) had the FV:R506Q mutation (including 5 homozygous and 22 heterozygous), corresponding to a prevalence of 11,4%.

12/110 patients (stroke/TIA: 12/0) were heterozygous carriers of the PT A20210 allele (10.9%), 6/97 patients had HHCY (6.2%).

Furthermore, the following prothrombotic states were identified: type I deficiency of FXII (16/301; 5.3%), HCII (6/254; 2.4%), PC (10/341; 2.9%), AT (6/344; 1.7%), PS (6/343; 1.7%), Plg (3/301; 0.3%); elevated levels of FVII (4/83; 4.8%), or HRGP (5/253; 1.9%); dysfibrinogenemia (2/340; 0.6%); presence of anti-phospholipid antibodies (13/279; 4.6%; lupus anticoagulants: 11/279; 3.9%; aCL: 2/279; 0.7%).

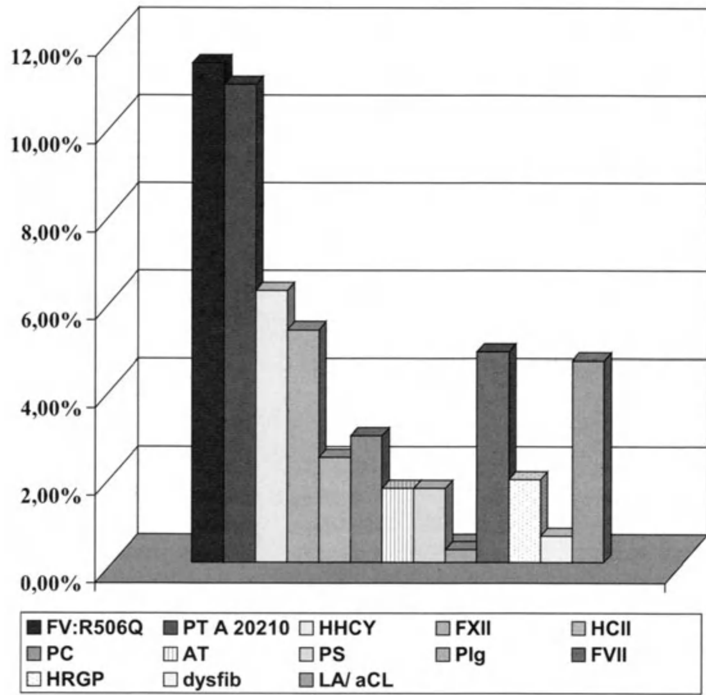


Fig. 1. Thrombophilic defects in patients with stroke/TIA

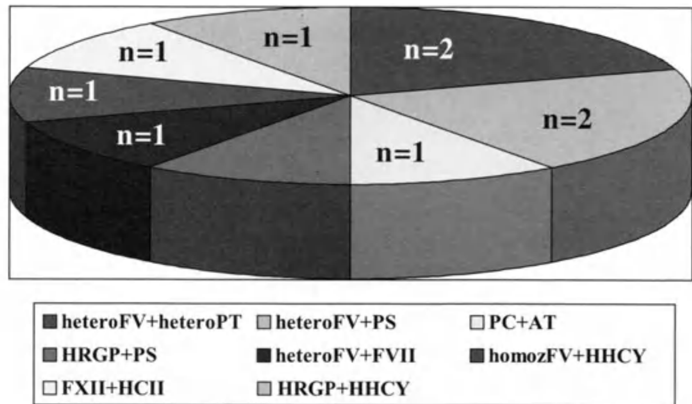


Fig. 2. Combined prothrombotic defects

All deficiencies were of type I with decreased functional activity and antigen concentration (Fig. 1).

In 10 patients (2.9%) combined defects were identified (Fig. 2). Recurrent events occurred in 79/344 patients (22.9%; venous *n*=18, arterial *n*=45, venous + arterial *n*=11, isolated pulmonary embolism *n*=5).

Oral contraceptive use was documented in 40/174 females (22.9%; stroke/TIA: 31/9). In 16/40 (40%) of these women a thrombophilic defect of hemostasis was found.

A positive family history of thromboembolic disorders was documented in 77/344 patients (22.4%; venous n=18, arterial: n=57, venous + arterial n=2).

As controls, we screened 450 healthy persons from the same geographic region for the presence of FV:R506Q mutation and prothrombin G20210 A variant.

In the healthy controls, the FV:R506Q mutation was found in 7.5%, whereas the prothrombin gene variant was identified in 2% of subjects.

Discussion

The etiology of juvenile ischemic stroke remains unexplained in a significant number of patients and the importance of thrombogenic clotting abnormalities in its pathogenesis is still undefined.

APC-resistance due to the FV:R506Q mutation is the most common inherited risk factor for venous thrombosis [3;7;18]. On the other hand the role of APC-resistance (APC-R) in arterial vascular events, in particular cerebrovascular disease, is controversial and still under discussion. There are studies reporting a positive influence on the risk of stroke [25;27] with a FV:R506Q prevalence between 4 and 17% [6;14;28] whereas others did not find an association between the FV:R506Q mutation and stroke [9;16;31;34;35].

Furthermore, the prothrombin G20210 A mutation (PT 20210GA) is another frequent genetic risk factor for venous thrombosis, but its role in CVD is the subject of controversial discussion [17;33]. A study has shown that patients carrying the PT 20210GA genotype have a significantly increased risk of cerebral ischemia (odds ratio: 5.1) [9].

Heterozygous PT 20210GA genotype carriership was associated with a 3.8-fold increased risk of cerebrovascular disease [9]. However, this observation has not been supported in other studies [1;5;10;24;26]. In a prospective study of 14,916 US men, the PT 20210GA genotype was not associated with increased risk of stroke [36].

A recent study has evaluated the effect of the FV:R506Q mutation and the G20210 A mutation in patients with cerebrovascular disease, particularly taking into consideration other vascular risk factors. The data did not suggest a relevant role of the FV:R506Q, neither as a single nor as a combined defect in patients who had had a stroke/TIA [21].

The importance of deficiencies of PC, PS, AT, FXII, HCII and plasminogen, hyperhomocysteinemia, and lupus anticoagulants and anticardiolipin antibodies, high levels of factor FVII, and elevated HRGP in the pathogenesis of ischemic stroke is controversially discussed [4,11,12,20,30,39,41].

In a case-control study with juvenile stroke patients no differences in AT, PS, PC, plasminogen and HCII were observed between cases and controls [41].

The Atherosclerosis Risk in Communities (ARIC) Study has shown that PC was not significantly associated with ischemic stroke, and there was no association between factor FVII, AT and ischemic disease [11].

A recent study has demonstrated that the FV:R506Q mutation was associated with an increased risk of CVD/ischemic stroke in women compared with males [25].

The intake of oral contraceptives is an independent risk factor for thromboembolic disease. The relative risk of ischemic stroke is 2,9 for females who use oral contraceptives. The interaction between oral contraceptives and other risk factors for thromboembolism greatly increases the risk [42].

In our study population of 344 patients who had had idiopathic ischemic stroke we could demonstrate no relevant influence of the FV:R506Q mutation. In contrast, the PT 20210GA genotype was found to be significantly more frequent in this patient group than in the general population (10% vs. 2%). This suggests a significant role of the PT 20210GA genotype in the pathogenesis of ischemic stroke.

Conclusions

- A prothrombotic defect has been found in 31.1% of patients with a history of »idiopathic« juvenile stroke/TIA
- Heterozygosity for the prothrombin G20210 A mutation was diagnosed in 10.9% of cases compared with 2% of controls ($P<0.05$)
- The occurrence of the PT G20210 A mutation might have a more unfavorable effect during the onset of the ischemic disease in comparison with the FV:R506Q mutation, as well as deficiencies of protein S, protein C, antithrombin, factor XII, heparin cofactor II and plasminogen, the presence of hyperhomocysteinemia, lupus anticoagulants, anticardiolipin antibodies, high levels of factor VII and elevated HRGP
- Therefore, thrombophilic defects might play an important role in the etiology of juvenile »idiopathic« arterial cerebral ischemia.

References

1. Bentolila S, Ripoll L, Drouet L, Mazoyer E, Woimant F. Thrombophilia due to 20210 G-A prothrombin polymorphism and cerebral ischemia in the young. Stroke 1997; 28: 1846
2. Bertina RM, Linden van der IK, Engesser L, Muller HP, Brommer EJP. Hereditary heparin Cofactor II deficiency and the risk of development of thrombosis. Thromb Haemost 1987; 57: 196–200
3. Bertina RM, Koelemann BP, Koster T. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 1994; 369: 64–7
4. Bostom AG, Rosenberg ICH, Silberhatz H, Jacques PF, Selhub J, D'Agostino RB, Wilson PW, Wolf PA. Nonfasting plasma total homocysteine levels and stroke incidence in elderly persons: the Framingham Study. Ann Intern Med 1999; 131:352–5
5. Corral A, Gonzalezconejero R, Lozano ML, Rivera J, Heras I, Vicente V. The venous thrombosis risk factor 20210 a allele of the prothrombin gene is not a major risk factor for arterial thrombotic disease. Br J Haematol 1997; 99: 304
6. Cushman M, Bhushan F, Bovill E, Tracy R. Plasma resistance to activated protein C in venous and arterial thrombosis. Thromb Haemost 1994; 72: 647
7. Dahlbäck B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. Proc Nat Acad Sc 1994; 91: 1396–400

8. De Stefano V, Finazzi G, Mannucci PM. Inherited thrombophilia: Pathogenesis, clinical syndromes and management. Blood 1996; 87: 3531-44
9. De Stefano V, Chiusolo P, Paciaroni K, Casorelli I, Rossi E, Molinari M, Servidei S, Tonali PA, Leone G. Prothrombin G20210 A mutant genotype is a risk factor for cerebrovascular ischemic disease in young patients. Blood 1998; 91: 3562-5
10. Ferraresi P, Marchetti G, Legnani C, Cavallari E, Castoldi E, Mascoli F, Ardissino D, Palareti G, Bernardi F. The heterozygous 20210 G/A prothrombin genotype is associated with early venous thrombosis in inherited thrombophilia and is not increased in frequency in artery disease. Arterioscler Thromb Vasc Biol 1997; 17: 2418
11. Folsom AR, Rosamond WD, Shahar E, Cooper LS, Aleksic N, Nieto FJ, Rasmussen ML, Wu KK. Prospective study of markers of hemostatic function with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. Circulation 1999; 100: 736-42
12. Gaustades M, Rudiger N, Moller J, Rasmussen K, Bjerregaard Larsen T, Ingerslev J. Thrombophilic predisposition in stroke and venous thromboembolism in Danish patients. Blood Coagul Fibrinolysis 1999; 10:251-9
13. Halbmayer WM, Mannhalter C, Feichtinger C, Rubi K, Fischer M. The prevalence of factor XII deficiency in 103 orally anticoagulated outpatients suffering from venous and / or arterial thromboembolism. Thromb Haemost 1992; 68: 285-90
14. Halbmayer WM, Haushofer A, Schön R, Fischer M. The prevalence of poor anticoagulant response to activated protein C (APC resistance) among patients suffering from stroke or venous thrombosis and among healthy subjects. Blood coag Fibrinol 1994; 5: 51-7
15. Haverkate F, Samama M. Familial dysfibrinogenemia and thrombophilia. Report on a study of the SSC subcommittee on fibrinogen. Thromb Haemost 1995; 73: 151-61
16. Iniesta JA, Corral J, Fernandez-Pardo J, Gonzalez-Conejero R, Vicente V. Factor-V (Arg506-Gln) mutation in ischemic cerebrovascular disease. Haemostasis 1997; 27: 105-11
17. Kapur RK, Mills LA, Spitzer SG, Hultin MB. A prothrombin gene mutation is significantly associated with venous thrombosis. Arter Thromb Vasc Biol 1997; 17: 2875-9
18. Koster T, Rosendaal FR, de Ronde H, Briet E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden. Thrombophilia Study. Lancet 1993; 342: 1503-6
19. Koster T, Rosendaal FR, Briet E, Vandenbroucke JP. John Hagemann's factor and deep vein thrombosis: Leiden Thrombophilia Study. Br. J Haematol 1994; 87: 422-4
20. Lalouschek W, Aull S, Serles W, Schnider P, Mannhalter C, Lang T, Deecke L, Zeiler K. Genetic and nongenetic factors influencing plasma homocysteine levels in patients with ischemic cerebrovascular disease and in healthy control subjects. J Lab Clin Med 1999; 133: 575-82
21. Lalouschek W, Auell S, Series W, Mannhalter C, Zeiler K. The prothrombin G20210 A mutation and factor V Leiden mutation in patients with cerebrovascular disease. Blood 1998; 92: 704-05
22. Lane DA, Mannucci PM, Bauer KA, Bertina RM, Bochkov NP, Boulyjenkov V, Chandy M, Dahlbäck B, Ginter EK, Miletich JP, Rosendaal FR, Seligsohn U. Inherited thrombophilia: Part 1. Thromb Haemost 1996; 76: 651-2
23. Lane DA, Mannucci PM, Bauer KA, Bertina RM, Bochkov NP, Boulyjenkov V, Chandy M, Dahlbäck B, Ginter EK, Miletich JP, Rosendaal FR, Seligsohn U. Inherited thrombophilia: Part 2. Thromb Haemost 1996; 76: 823-34
24. Longstreth WT Jr., Rosendaal FR, Siscovick DS, Vos HL, Schwartz SM, Psaty BM, Raghunathan TE, Koepsell TD, Reitsma PH. Risk of stroke in young women and two prothrombotic mutations: factor V Leiden and prothrombin gene variant (G20210 A). Stroke 1998; 29: 577-80
25. Margaglione M, D'Andrea G, Giuliani N, Brancaccio V, De Lucia D, Grandone E, De Stefano V, Tonali PA, Di Minno G. Inherited prothrombotic conditions and premature ischemic stroke: sex difference in the association with factor V Leiden. Arterioscler Thromb Vasc Biol 1999; 19: 1751-6

26. Martinelli I, Franchi F, Akwan S, Bettini P, Merati G, Mannucci PM. The transition G to A at position 20210 in the 3' untranslated region of the prothrombin gene is not associated with cerebral ischemia. Blood 1997; 90:3806
27. Mohanty S, Saxena R, Behari M. Risk factors for thrombosis in nonembolic cerebrovascular disease. Am J Hematol 1999; 60: 239–41
28. Mohanty S, Saxena R, Behari M. Activated protein C (APC) resistance in young stroke patients. Thromb Haemost 1999; 81: 465–6
29. Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Audria G, Boers GHJ, Homberg IL, Cerone R, Fowler B, Gröbe H, Schmidt H, Schweitzer L. The natural history of homocysteinuria due to cystathionine -ß-synthase deficiency. Am J Hum Genet 1985; 37: 1–31
30. Munts AG, van Genderen PJ, Dippel DW, van Kooten F, Koudstaal PJ. Coagulation disorders in young adults with acute cerebral ischemia. J Neurol 1998; 245: 21–5
31. Nabari DG, Junker R, Wolff E, Ludemann P, Doherty C, Evers S, Droste DW, Kessler C, Assmann G, Ringelstein EB. Prevalence of factor V Leiden mutation in young adults with cerebral ischemia: a case-control study on 225 patients. J Neurol 1998; 245: 653–8
32. Nowak-Göttl U, Debus O, Findeisen M, Kassenböhmer R, Koch HG, Pollmann H, Postler C, Weber P, Vielhaber H. Lipoprotein(a): its role in childhood thromboembolism. Pediatrics (electronic pages) 1997; 99/6e11
33. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996; 88:3698–703
34. Press RD, Liu XY, Beamer N, Coull BM. Ischemic stroke in the elderly. Role of the common factor V mutation causing resistance to activated protein C. Stroke 1996; 27: 44–8
35. Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Eisenberg PR, Miletich JP. Mutation in the gene coding for coagulation factor V and the risk for myocardial infarction, stroke, and venous thrombosis in apparently healthy men. NEJM 1995; 332: 912–7
36. Ridker PM, Hennekens CH, Miletich JP. G20210 A mutation in prothrombin gene and risk of myocardial infarction, stroke, and venous thrombosis in a large cohort of US men. Circulation 1999; 99: 999–1004
37. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (Activated protein C resistance). Blood 1995; 85:1504–8
38. Sartori MT, Patrassi GM, Theodoridis P, Perin A, Pietrogrande F, Girolami A. Heterozygous type I plasminogen deficiency is associated with an increased risk for thrombosis: a statistical analysis in 20 kindreds. Blood Coag Fibrinol 1994; 5: 889–93
39. Selhub J, D'Angelo A. Hyperhomocysteinemia and thrombosis: acquired conditions. Thromb Haemost 1997; 78:527–31
40. SeligsohnU, Zivelin A. Thrombophilia as a multigenetic disorder. Thromb Haemost 1997; 78(1): 297–301
41. Tosetto A, Ruggeri M, Castaman G, Rodeghiero F. Inherited abnormalities of blood coagulation in juvenile stroke. A case-control study. Blood Coagul Fibrinolysis 1997; 8: 397–402
42. WHO Collaborative study of cardiovascular disease and steroid hormone contraception. Lancet 1996; 348:498–505

Inquiry into the Significance of Constantly Raised FVIII Values as a Factor in Thrombophilia

M. von Lieven, M. Krause, T. Vigh, C. Rabenstein and I. Scharrer

Introduction

APC resistance and FV classification mutation, FII mutation, ATIII, protein S and protein C deficiency are among the known independent risk factors for thrombosis.

Raised post-thrombosis values for the FVIII clotting factor were noted in many patients, so that the FVIII clotting factor will be discussed as an independent thrombosis risk factor.

To investigate the role of FVIII in thrombophilia, a large patient collective (n=66) was compared with a large normal collective, comparable in age and sex (n=100). In order to exclude a wrong interpretation of the raised FVIII level on the grounds of acute phase reaction, the CRP value was determined in addition to the FVIII activity and the vWF antigen.

Patients and Methods

Sixty-six patients between 21 and 78 years of age, as well as 100 volunteers between 23 and 71 years of age, were examined.

Blood was taken from them three times at intervals of at least 3 weeks.

Three of the patients were only able to give two blood samples, and one patient came only once.

FVIII activity, vWF Ag level and the CRP value were measured in the blood samples to exclude any acute inflammation.

A precondition for the patients was the elapse of at least 4 weeks since the last thrombotic event (in most cases about 2 years had elapsed).

Criteria excluding test subjects and patients:

- Pregnancy
- Long-term medication with cortico-steroids
- Chronic active inflammations, especially hepatitis or liver cirrhosis

A thrombophilia defect was already known in 37 patients and no defect had been found in the remaining 29 patients at the time of the investigation.

The venous blood was cooled immediately after extraction, then centrifuged for 40 min at 4,000 rpm/40°C and frozen at –760°C until used, whereby the CRP sample was processed immediately.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

The FVIII activity was determined in a single factor determination test using the ACL analysis system from Instrumentation Laboratory.

The vWF Ag level was determined using the company-owned vWF Ag ELISA.

The CRP value was measured photometrically with a Roche/Hitachi 747.

Statistics

The values were given as median values.

Results

Evaluation revealed mostly normal values in the normal collective.

Raised values were seen in isolated cases, but these were attributable to stress, athletic activity, advanced age or a slight cold with normal CRP (Fig. 1, Normal Collective).

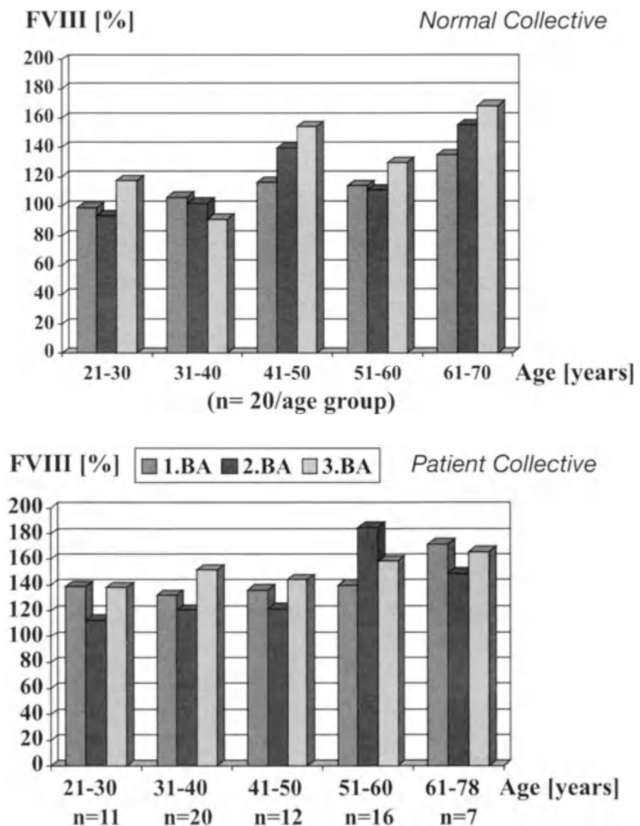


Fig. 1. FVIII values

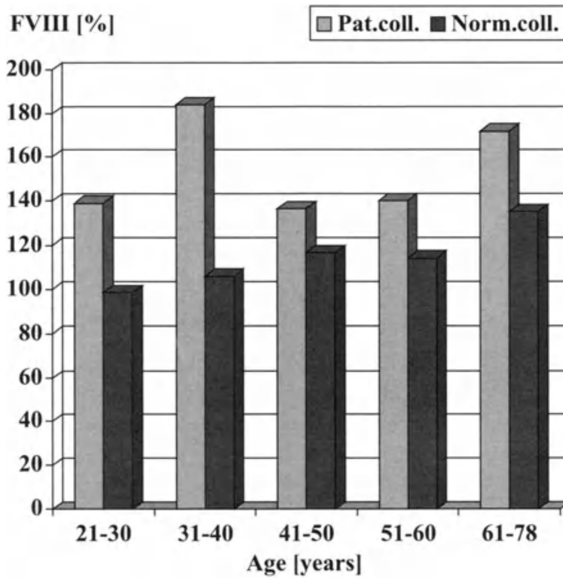


Fig. 2a. Comparison of FVIII values in patients and normal collectives in first blood samples

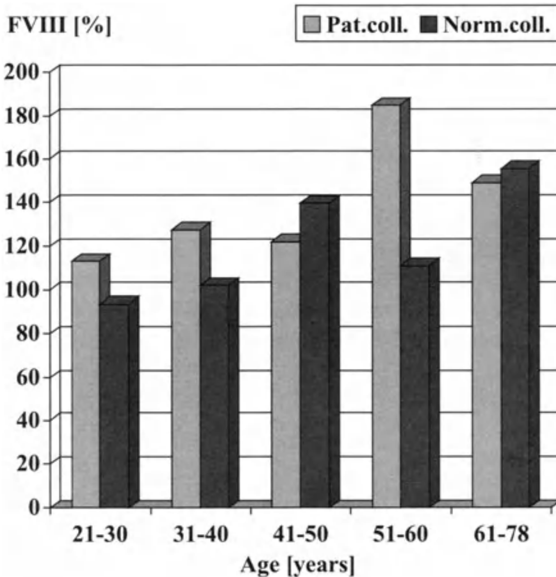


Fig. 2b. Comparison of FVIII values in patients and normal collectives in second blood samples

In the patient collective, on the other hand, single blood samples from 58 patients showed that both FVIII activity (>150%) and vWF Ag (>135%) were raised with CRP values within the normal range. The values were all within the normal range in eight patients (Fig. 1, Patient Collective).

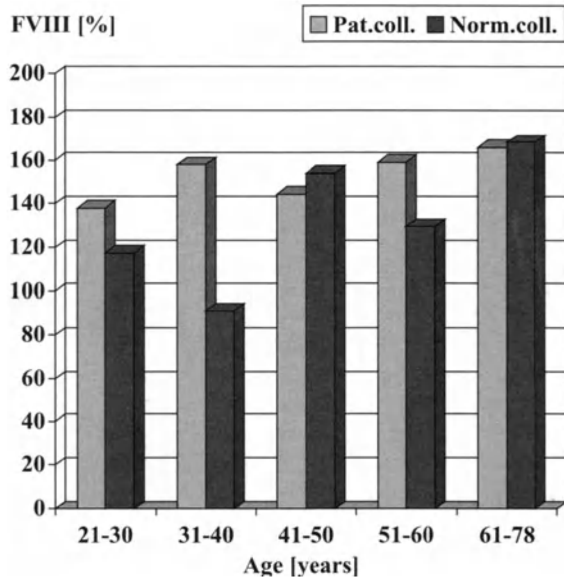


Fig. 2c. Comparison of FVIII values in patients and normal collectives in third blood samples

Fig. 2a–c compares the values from the patients with those of the normal collective for the three blood samples taken.

These illustrations show that the FVIII values, particularly in the case of younger patients between 20 and 40 years of age, are significantly raised, especially in Fig. 2a in the group of 31–40-year-olds.

Fig. 2b, however, shows distinctly raised values in the 51–60 age group.

In the case of 12 of the 66 patients, i.e. in 20% of the cases, the FVIII level in all three blood samples was constantly raised.

Eight of these (75%) were cases of deep leg venous thrombosis (DLVT), and one case was a DLVT with a pulmonary embolism (PE). One female patient had had a pulmonary embolism, and another female patient suffered a brachial venous thrombosis. Thrombophlebitis was diagnosed in a further female patient.

Of these 12 patients three had suffered a relapse (25%).

One female patient suffered a pulmonary embolism, one a deep leg venous thrombosis, and another both a DLVT and a pelvic venous thrombosis.

In 28 of the 66 patients the vWF Ag level was also raised in all blood samples.

A parallel rise in the FVIII and vWF Ag levels was observed in 10 of these 28 patients.

Seven of these patients were suffering from a DLVT and one female patient had a PE. One female patient had both a DLVT and a PE. In this latter case, the female patient was discovered to have a brachial venous thrombosis with a PE.

Two patients had a relapse some time later: one case of DLVT and one of DLVT with pelvic involvement.

The CRP value was largely NAD in all patients.

Discussion

Different studies have shown that raised FVIII values qualify as independent risk factors for venous thrombosis.

Kraaijenhagen et al. have proved in their study that the FVIII level in risk patients is raised for a long period [1], as we have also shown in our studies.

O'Donnell et al. showed that the high values appeared independently of the acute phase reaction [4].

At the same time, no mechanisms have been discovered to explain how thromboses might be caused by raised FVIII levels.

We examined 66 thrombosis patients and 100 other volunteers and compared the values. On the basis of this data, we can state that about 20% of the patients showed constantly raised FVIII values (12 of 66 patients).

Of these 25% (4 of the 12 patients) had suffered a relapse up to 7 years later.

The increased rate of relapse is confirmed by the study of Kyrle et al., who discovered that a relapse occurred in 10.6% of patients with increased FVIII levels following an initial event [2].

The CRP values were largely NAD in all patients.

A pathologically raised CRP level doubles the risk of apoplexy risk and triples the risk of infarction.

An increased thrombosis risk is suspected if, for example, a hormone substitution is done at the same time [3].

There were no increased CRP levels in our study. In contrast to coronary heart disease, the level of CRP in venous thrombosis may thus not be a risk indicator.

Summary

On the basis of the results of our investigation, an FVIII level that is constantly raised over a three-month period can be evaluated as a risk factor for thrombophilia if acute phase reaction, pregnancy or chronic active disease have been excluded.

References

1. Kraaijenhagen et al.: High Plasma Concentration of Factor VIII:C is Major Risk Factor for Venous Thrombembolism. Thromb Haemost 2000; 83: 5–9
2. Kyrle et al.: High Plasma Levels of Factor VIII and the Risk of Recurrent Venous Thrombembolism. N Engl J Med 2000; 343: 457–62
3. Lowe et al.: C-reactive Protein, Idiopathic Venous Thrombembolism and Hormone Replacement Therapy. Thromb Haemost 2000; 84: 730–1
4. O'Donnell et al.: Elevation of FVIII:C in Venous Thrombembolism is Persistent and Independent of the Acute Phase Response. Thromb Haemost 2000; 83: 10–3

Do Statins Increase the Homocysteine Level?

M. von Lieven, L. Rössig, T. Vigh, C. Rabenstein and I. Scharrer

Introduction

Besides their cholesterol-lowering effect, statins are also useful for reducing C-reactive protein (CRP). Patients with higher CRP values have a double risk of apoplexy and a threefold infarct risk compared with patients with low values.

Lipid-lowering measures with statins or fibrates improved the myocardial situation [4, Lipid Study Group, NEJM, 1998]. Nevertheless, it is known from various clinical studies that the homocysteine level rose under fibrate treatment [1, Dierkes et al., Lancet, 1999].

The increase in the blood homocysteine level is considered to be an independent, endothelial-harming risk factor for cerebral and cardiovascular diseases. In order to test whether the level also shows pathological changes under treatment with statins (simvastatin and atorvastatin), we measured the cholesterol, triglycerides, creatinine, CRP and homocysteine levels in five male and two female patients before and 6 weeks after the start of treatment.

Patients and Methods

The patient collective consisted of five male and two female patients between the ages of 56 and 70 suffering from cardiovascular diseases and requiring lipid-lowering treatment. The diseases of the patients:

- CHD, in one patient accompanied by paroxysmal atrial fibrillation and a post-apoplectic condition in another.
- Four of the patients were also hypertensive, and two suffered from pAOD.

Exclusion criteria were previous lipid-lowering treatment, kidney insufficiency and lack of compliance.

Three patients were prescribed 10 mg atorvastatin daily, the others received 10 mg simvastatin.

The following values were ascertained prior to treatment: CRP, homocysteine, cholesterol, triglycerides and creatinine.

The blood sample for homocysteine measurement was immediately cooled on ice, centrifuged within 2 h and frozen until processed. The blood sampling procedure was repeated 6 weeks later and processed in the same way.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Table 1. Values before and after the commencement of treatment with simvastatin and atorvastatin

Values/medication	Before start of treatment	After start of treatment	Differences	Normal range
Triglycerides (mg/dl)				
Simvastatin	188 (112–212)	142.5 (101–189)	24.5 (88–100)	50–150
Atorvastatin	290 (134–390)	234 (234–254)	56 (20–256)	
Cholesterol (mg/dl)				
Simvastatin	241 (226–312)	194 (119–246)	47 (86–127)	130–280
Atorvastatin	219 (219–338)	198 (169–213)	21 (44–119)	
Creatinine (mg/dl)				
Simvastatin	1.15 (0.2–7.1)	1.15 (0.8–8.1)	0 (6.9–7.3)	<0.9
Atorvastatin	0.8 (0.7–0.9)	0.8 (0.6–1)	0 (0.2–0.4)	
CRP (mg/dl)				
Simvastatin	0.3 (0.1–0.6)	0.5 (0.3–1)	0.2 (0.5–0.7)	<1.0
Atorvastatin	0.4 (0.3–1.9)	0.7 (0.8–1.4)	0.3 (0.8–1.6)	
Homocysteine (μmol/l)				
Simvastatin	10.25 (5.8–23.5)	8.75 (6.9–12.1)	1.5 (5.2–17.9)	3.8–13.8
Atorvastatin	7.2 (6.1–12.1)	8.4 (7–10.5)	1.2 (3.5–6)	

The homocysteine value (normal range 3.8–13.8 μmol/l) was measured by enzyme immunoassay (Axis Homocystein EIA). Cholesterol, triglycerides, creatinine and CRP were measured with a Roche/Hitachi 747.

All statistical values are given as median values.

Table 1 describes the median values of patients in the various groups before and after the start of treatment; Figs. 1–3 illustrate the results.

Results

These patients had received no previous lipid-lowering treatment. Six of the patients had normal kidney function; one patient suffered from terminal kidney insufficiency with a creatinine value of 7.1 mg/dl prior to therapy. The medication was taken regularly.

Following the second measurement, the triglycerides had decreased under simvastatin by 10.3%, and indeed by a total of 19.1% under atorvastatin (Table 1, Fig. 2). Similar results were observed for cholesterol, which was reduced by 20.7% under simvastatin treatment and by 22.8% under atorvastatin (Table 1, Fig. 2).

The measured homocysteine values showed that simvastatin effected a 10.2% fall in level, but that atorvastatin had effected a 14.8% rise, although both were within the normal range.

One of the patients receiving simvastatin had terminal kidney insufficiency with raised creatinine and homocysteine values, which were, however, taken into account in the median values.

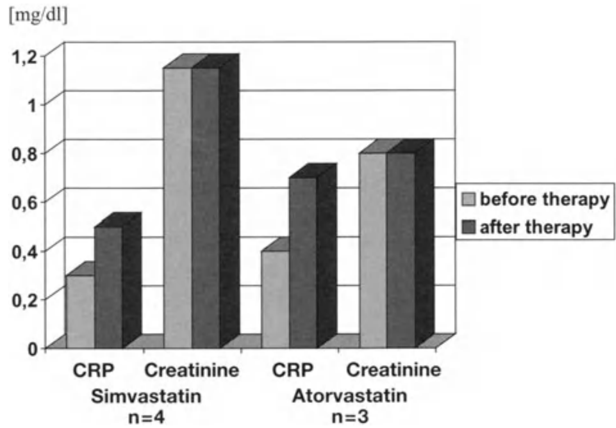


Fig. 1. CRP and creatinine values before and after the therapy with different statins

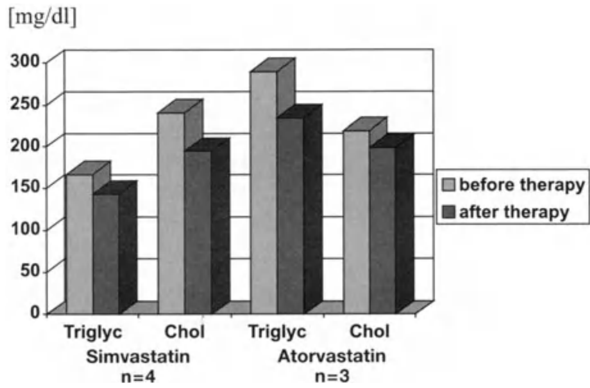


Fig. 2. Cholesterol and triglyceride values before and after the therapy with different statins

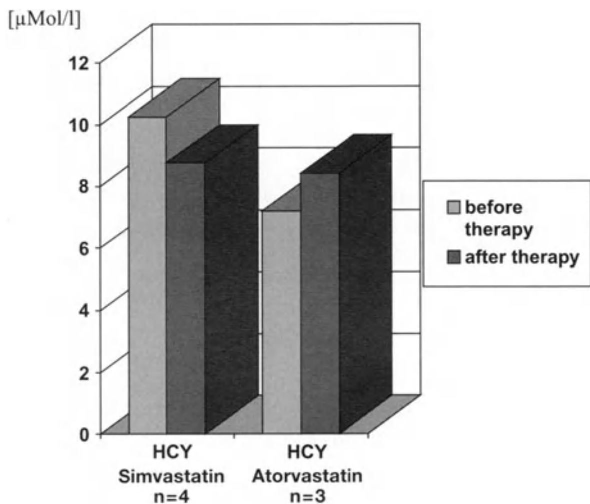


Fig. 3. Homocysteine values before and after the therapy with different statins

The increase in the homocysteine level under atorvastatin (Table 1, Fig. 3) can also be caused by other influencing factors, e.g. one female patient in the atorvastatin group and a female patient from the simvastatin group had type II insulin-dependent diabetes mellitus, which can also lead to increased values (these patients' homocysteine values beforehand: 12.1 µmol/l).

The creatinine values showed a rise of 12% under simvastatin treatment, but showed no changes under atorvastatin. The creatinine values under simvastatin were already outside the limits of normality before the start of the treatment, due to one of the male patients in this group having terminal kidney insufficiency.

The CRP values also showed slight changes, but within the normal range.

Discussion

Statins are milestones in primary and secondary prevention of CHD [5, Lipid Study Group, NEJM 1998; 5, Ross et al., Arch Intern Med 1998]. They not only lower the cholesterol and LDL values, but also have a profibrinolytic/antithrombotic effect [1, Dangas et al., Thromb Haemost 2000]. Since, however, a rise in homocysteine values under fibrate treatment was reported, the question that had to be answered was whether the favorable statin effects were possibly obtained at the price of unfavorable side effects such as a rise in homocysteine levels.

Dierkes et al. discovered a rise in the homocysteine level following fenofibrate treatment (by 44%) and after bezafibrate (by 17.5%) [3]. De Lorgeril et al. also reported a rise of 46% following 6 weeks of treatment with fenofibrate [2]. Jonkers et al., in a double-blind study, described a homocysteine rise of 19% under fibrate treatment [4].

On the basis of the data reported here, we did not ascertain a homocysteine increase into the pathological range in a small patient collective with cardiovascular diseases after 6 weeks of statin therapy.

No significant changes in homocysteine levels were found: while the values in four patients rose only slightly within the normal range, they fell in the other three patients. Why the homocysteine level rose under fibrate treatment and not under statin treatment has not been determined to date.

Jonkers et al. suspected an interaction of homocysteine with the peroxisomal receptor PPAR [4].

Apparently, statins intervene in amino acid metabolism. Lorgeril et al. reported a rise in arginine, a precursor of nitrogen oxide, which in turn plays an important part in the dilation of the coronaries [2].

Summary

In this prospective study, seven patients were treated with either atorvastatin or simvastatin. After six weeks of treatment, the only changes registered were within the normal range of homocysteine levels. A slight rise of 14.8% was recorded in the patients treated with atorvastatin, as was a 10.2% drop in patients treated with simvastatin.

Statins appear to be a good alternative for lipid-lowering treatment, especially for patients who already have a cardiovascular and/or cerebrovascular disease and show increased homocysteine levels.

References

1. Dangas G, Smith D A, Unger A H, Shao J H et al: Pravastatin: an antithrombotic effect independent of the cholesterol-lowering effect. Thromb Haemost 2000; 83: 688–92
2. deLorgeril M, Paillard F, Lacan P, Richard G: Lipidlowering drugs and homocysteine. Lancet 1999; 353: 209
3. Dierkes J, Westphal S, Luley C: Serum homocysteine increases after therapy with fenofibrate or bezafibrate. Lancet 1999; 354: 219
4. Jonkers I J A M, de Man F H A F, Onkenhout W, van der Laarse A, Smelt A H M: Implication of fibrate for homocysteine. Lancet 1999; 354: 1208
5. LIPID Study Group: Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. N Engl J Med 1998; 339: 1349–57
6. Ross SD, Allen IE, Connelly JE et al.: Clinical outcomes in statin treatment trials. Arch Intern Med 1999; 159: 1793–802

Alpha-2-Macroglobulin Level regulates the Anticoagulant Cofactor Activity of Protein S in Cord and Adult Plasma

G. Cvirn, S. Gallistl, M. Köstenberger, J. Kutschera, B. Leschnik and W. Muntean

Introduction

Alpha2-macroglobulin (a2-M) is a broad spectrum protease binding protein. It is known to bind i.e. α-thrombin (1), and activated protein C (APC) (2). The proteinases are physically trapped and thus their active sites are excluded from natural substrates. It has been suggested that a2-M may provide protection from thromboembolic events in antithrombin (AT) deficient children due to complex formation with free α-thrombin (3).

We have recently shown that in cord and adult plasma containing physiological amounts of AT no anticoagulant effect of a2-M addition due to complex formation with free thrombin was observed (4). On the contrary, a2-M exhibits procoagulant activity at physiological AT levels due to complex formation with APC. The anticoagulant APC destroys activated factors V and VIII and thus suppresses feedback-activation of thrombin, reflected in decreased thrombin potential (TP) and prothrombin activation (5). The TP is the area under the free thrombin generation curve and has been shown to be a reliable parameter for assessing the thrombotic state of a given plasma sample (6–8). Prothrombin activation was assessed by determining the time course of prothrombin fragment 1+2 (F1+2) generation. Complex formation between a2-M and APC resulted in decreased anticoagulant action of APC, reflected in enhanced TP and prothrombin activation (5). Nicolaes et al. have shown the effect of APC on TP in normal and APC-resistant individuals (9).

Protein S (PS), a vitamin K-dependent plasma protein, binds to APC and serves as a cofactor for the anticoagulant activities of APC (10). Thus, both a2-M and PS compete for APC. Therefore, at elevated a2-M levels formation of the anticoagulant APC/PS complex might be suppressed. It was the aim of our study to investigate the effects of different a2-M levels on the anticoagulant cofactor activity of PS in cord and adult plasma by determining time-courses of F1+2 and thrombin generation. Decreased formation of the APC/PS-complex due to competition between a2-M and PS for APC might be important particularly in cord plasma. A2-M levels in newborns are elevated over adult values (11) and the antithrombotic protein C pathway is upregulated at birth (12).

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

Materials 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. A2-M concentrate from human plasma was purchased from Sigma, Vienna, Austria. A stock solution was prepared by dissolving 10 mg of the lyophilized glycoprotein in 500 μl of distilled water, dialysis against buffer A for 3 h at 4°C, aliquots of 100 μl were stored at -70°C. Activated protein C (APC) from human plasma was obtained from ICN Biomedicals GmbH, Eschwege, Germany. A stock solution was prepared by dissolving 50 μg of the lyophilized protein in 315 μl of distilled water, dialysis against buffer A for 3 h at 4 °C, aliquots of 30 μl were stored at -70°C. Protein S from human plasma was obtained from ICN Biomedicals GmbH, Eschwege, Germany. A stock solution was prepared by dissolving 100 μg of the lyophilized protein in 250 μl of distilled water, dialysis against buffer A for 3 h at 4°C, aliquots of 30 μl were stored at -70°C. Sheep anti-human protein S was purchased from CoaChrom, Vienna, Austria. Proteinase A, an enzyme from the venom of the Mexican West Coast rattlesnake (*Crotalus basiliscus*) was purchased from Pentapharm LDT, Basle, Switzerland. To 0.175 mg of the lyophilized protein 400 μl of distilled water were added and stored at 4°C. Unitest Assay for a2-Macroglobulin determination in human plasma was obtained from CoaChrom Diagnostics, Vienna, Austria. As a trigger for the extrinsic coagulation pathway we used Thromborel S 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, Basle, Switzerland. The chromogenic substrate used for thrombin determination was H-D-Phe-Pip-Arg-pNA.2HCl (S 2238) from CoaChrom Diagnostics, Vienna, Austria. Testkit F1+2 micro for determination of prothrombin fragment 1+2 generation was obtained from Behring Diagnostics GmbH, Marburg, Germany. Stopping solution for F1+2-determination consisted of Trasylol:EDTA:Na-citrate=8:1:1 and 110 μM PPACK (D-Phe-Pro-Arg chloromethyl keton) from Sigma, Vienna, Austria. Trasylol from Bayer, Vienna, Austria contained aprotinin, a protease inhibitor. Electrophoresis: NuPAGE Tris-acetate SDS running buffer (20X) and NuPAGE sample and transfer buffer from Novex, San Diego, USA, was used. SeeBlue Plus2 pre-stained standard (1X) from Novex, San Diego, USA, was used as molecular weight marker.

Collection and Preparation of Plasma

Cord blood was obtained immediately following the delivery of 14 full term infants (38–42 weeks gestational age). It was collected into 0.1 molar citrate using a two syringe technique, centrifuged at room temperature for 15 min at 2800 *g*, pooled and stored at -70°C in propylene tubes until assayed. Clotting factors, AT, protein C, protein S, and a2-M in the pooled cord plasma were in the normal range for neonates. In the same way plasma from eight healthy adults was collected, prepared, and checked.

Preparation of Plasma with Different a2-M Concentration

The a2-M activity of the pooled neonatal or adult plasma was decreased by the addition of proteinase A, a metalloenzyme that forms a complex with a2-M at a molar ratio of 1:1 (13, 14). To increase the a2-M concentration, to 1 ml of plasma different amounts (0–80 µl) of purified a2-M concentrate were added.

Preparation of Plasma with Different PS Content

The PS content of the pooled cord and adult plasma was decreased by using sheep anti-human protein S coupled to Sepharose 4B. The PS content of plasmas was increased by spiking with different amounts of purified PS concentrate.

Determination of the a2-M Concentration

Determination of the a2-M concentration of pooled cord and adult plasma was performed using the standard test kit Unitest Assay for a2-Macroglobulin determination in human plasma from CoaChrom Diagnostics, Vienna, Austria.

Determination of the PS Content

Determination of the PS content of pooled plasma was performed by means of a standard coagulation method using protein S deficient plasma on a Behring Coagulation Timer from Behring Diagnostics GmbH, Marburg, Germany.

Activation of Plasma

Three hundred and eighty microliters of plasma with different a2-M and PS concentrations were incubated with 20 µl of buffer A (containing different amounts of APC and PS) for 1 min at 37°C. After subsequent incubation with 20 µl buffer A containing H-Gly-Pro-Arg-Pro-OH (GPRP, Pefabloc FG) to inhibit fibrin polymerization and 15 µl Actin-FSL for 1 min at 37°C the plasma samples were activated by addition of 18 µl 0.5 M $CaCl_2$.

Determination of Prothrombin Fragment 1+2

Plasmas were prepared and activated as described above. At timed intervals 5-µl aliquots were withdrawn from the plasma and subsampled into 495 µl stopping solution. After subsequent 1:10-dilution in stopping solution, the amount of F1+2 generated was quantitated by using a standard immuno enzymatic test kit.

Determination of Thrombin Generation

We used a subsampling method derived from a recently described technique (15). 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 S-2238. The reagents were prewarmed to 37°C. Amidolysis of S-2238 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 a2-M/ thrombin complex (6). The amount of free thrombin was determined by two different methods:

A) At timed intervals, aliquots of the extrinsically activated plasma were subsampled into buffer B containing heparin (20 U/ml) and AT (3.4 U/ml) to rapidly and completely inactivate free thrombin in the sample. The residual amidolytic activity was then determined as described above for the total amidolytic activity. The amidolytic activity of the α_2-M/thrombin complex was subtracted from the total amidolytic activity (16).
B) Free thrombin generation curves were calculated by mathematical treatment of total amidolytic activity curves using a method developed by Hemker et al (7).

Electrophoresis

A2-M and PS were incubated for 2 h at 37°C in the presence of 2.5 mmol/l $CaCl_2$. To detect possible complex formation between a2-M and PS we used nondenaturing SDS-PAGE according to Hoogendoorn et al. (2).

Results

Plasma containing different amounts of a2-M was prepared by addition of purified a2-M concentrate or by administration of proteinase A to the pooled plasma. Proteinase A is a metalloenzyme that forms a complex with a2-M at a molar ratio of 1:1. Under our experimental conditions the a2-M protein concentrate did not cleave the chromogenic thrombin substrate S 2238. After addition of different amounts of APC and PS the plasma was intrinsically activated, subsequently F1+2 generation and thrombin potential were determined using subsampling techniques. The conversion of the thrombin substrate S2238 is caused by two different molecular species: free thrombin and the a2-M/thrombin complex. To determine free thrombin generation curves from total amidolytic activity curves we used two different methods as described in Material and Methods. Free thrombin generation curves obtained with both methods were identical within the limits of experimental error. Experiments were done in cord plasma over a concentration range of 1.87–4.43 μM a2-M and in adult plasma over a concentration range of 1.38–4.18 μM a2-M. All experiments were done in triplicate, the average is shown in all figures.

Effect of PS Administration to Cord Plasma Containing 1.45 µg/ml APC in the Presence of Different Amounts of a2-M on Prothrombin Activation

To determine prothrombin activation in plasma containing 1.45 µg/ml APS in the presence of different amounts of a2-M and PS we recorded the time course of F1+2 generation as described in Materials and Methods. The height of the end level of the F1+2 generation curve is an indicator for the total amount of prothrombin split. A typical experiment is shown in Fig. 1. In the presence of 4.43 mg/ml a2-M elevation of the PS content from 2.9 µg/ml up to 5.0 and 7.1 µg/ml respectively, resulted in a slight decrease in prothrombin activation. The height of the end level of the F1+2 generation curve decreased from 0.57 to 0.55 and 0.52 µM respectively, shown in

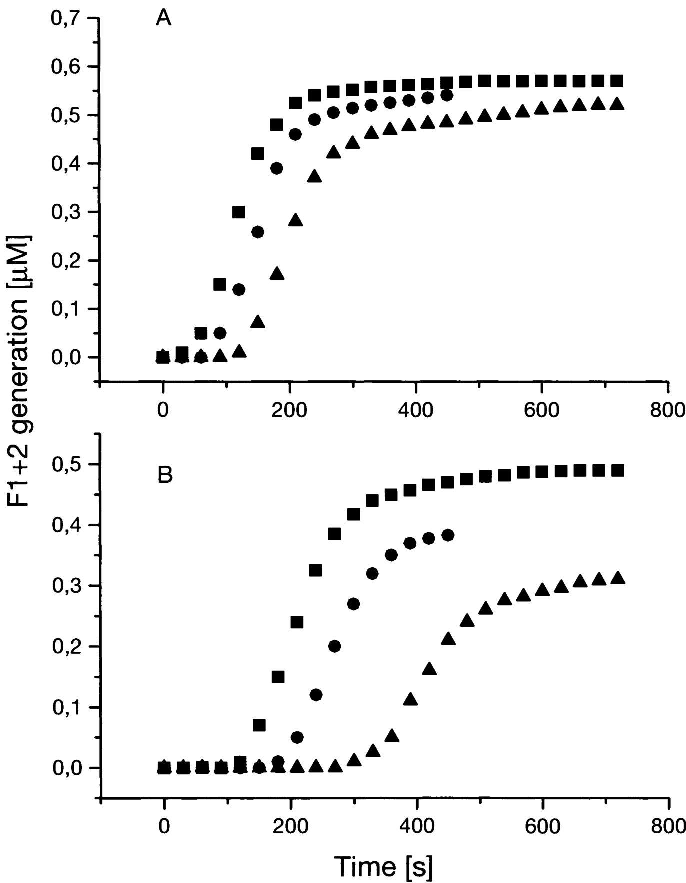


Fig. 1. Effects of different concentrations of protein S (■-*square* 2.9 µg/ml, ●-*circle* 5.0 µg/ml, ▲-*triangle* 7.1 µg/ml) in cord plasma containing 1.45 µg/ml APC in the presence of 4.43 mg/ml a2-M (*a*) and 2.92 mg/ml a2-M (*b*) on generation of prothrombin fragment 1+2. Results are expressed as means (n=3)

Table 1. End levels of prothrombin fragment 1+2 generation curves (*F1+2*) and thrombin potential (*TP*) in cord plasma containing 1.45 μg/ml activated protein C (*APC*) and 2.9, 5.0, and 7.1 μg/ml protein S (*PS*) respectively, in the presence of different amounts of alpha2-macroglobulin. Results are expressed as means (n=3)

a2-M content (mg/ml)	1.45 μg/ml APC + 2.9 μg/ml PS		1.45 μg/ml APC + 5.0 μg/ml PS		1.45 μg/ml APC + 7.1 μg/ml PS	
	F1+2 (μM)	TP (nM/min)	F1+2 (μM)	TP (nM/min)	F1+2 (μM)	TP (nM/min)
1.87	0.44	214	0.30	145	0.16	78
2.25	0.46	222	0.33	161	0.22	108
2.68	0.48	233	0.37	180	0.26	126
2.92	0.49	238	0.41	199	0.31	150
3.20	0.50	247	0.42	214	0.34	172
3.59	0.53	257	0.47	228	0.41	199
4.00	0.54	265	0.49	246	0.45	228
4.43	0.57	276	0.55	267	0.52	252

Fig. 1a. In the presence of 2.92 mg/ml a2-M, the same elevation of the PS content resulted in a marked decrease in prothrombin activation. The height of the end level of the F1+2 generation curve decreased from 0.49 to 0.41 and 0.31 μM respectively, shown in Fig. 1b. Heights of the end level of the F1+2 generation curves for PS administration to plasma containing 1.45 μg/ml APC and 1.87–4.43 mg/ml a2-M are listed in Table 1: the height of the end level increased dose-dependently when the a2-M content was successively elevated.

Effect of PS Administration to Cord Plasma Containing 1.45 μg/ml APC in the Presence of Different Amounts of a2-M on Thrombin Generation

We determined the time course of free thrombin generation as described by Hemker et al. (7). The area under the thrombin generation curve is called thrombin potential (TP). A typical experiment is shown in Fig. 2. In the presence of 4.43 mg/ml a2-M elevation of the PS content from 2.9 up to 5.0 and 7.1 μg/ml respectively resulted in a slight suppression of thrombin generation. The TP decreased from 276 to 267 and 252 nM/min respectively (Fig. 2a). In the presence of 2.92 mg/ml a2-M the same elevation of the PS content resulted in markedly diminished thrombin generation. The TP decreased from 238 to 199 and 150 nM/min, respectively (Fig. 2b). TPs for PS administration to plasma containing 1.87–4.43 μg/ml a2-M are listed in Table 1: the TP increased dose-dependently when the a2-M content was successively elevated.

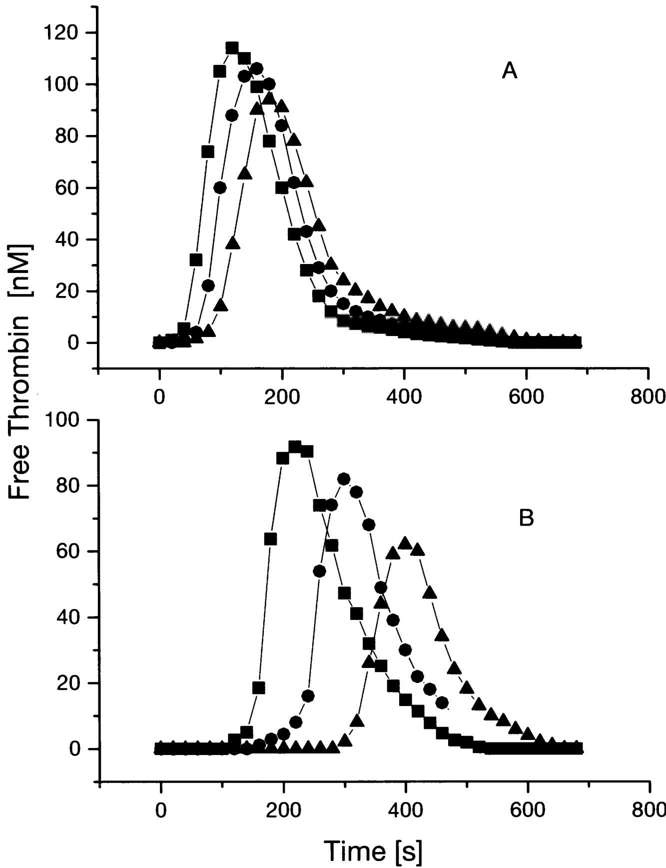


Fig. 2. Effects of different concentrations of protein S (■-*square* 2.9 μg/ml, ●-*circle* 5.0 μg/ml, ▲-*triangle* 7.1 μg/ml) in cord plasma containing 1.45 μg/ml APC in the presence of 4.43 mg/ml a2-M (*a*) and 2.92 mg/ml a2-M (*b*) on free thrombin generation. Results are expressed as means (*n*=3)

Effect of PS Administration to Adult Plasma Containing 3.5 μg/ml APC in the Presence of Different Amounts of a2-M on Prothrombin Activation and Thrombin Generation

We determined the time courses of F1+2 and free thrombin generation as described above. Essentially the same results were obtained in adult as in cord plasma. Administration of PS to plasma containing high amounts of a2-M resulted in slight suppression of prothrombin activation and thrombin generation, reflected in slightly suppressed end levels of F1+2 generation curves and TP, respectively. The same PS administration to plasma containing low amounts of a2-M resulted in a marked suppression of prothrombin activation and thrombin generation, reflected in markedly suppressed end levels of F1+2 generation curves and TP (Table 2).

Table 2. End levels of prothrombin fragment 1+2 generation curves (*F1+2*) and thrombin potential (*TP*) in adult plasma containing 3.5 µg/ml activated protein C (*APC*) and 6.1, 10.0, and 18.8 µg/ml protein S (*PS*) respectively, in the presence of different amounts of alpha2-macroglobulin (*a2-M*). Results are expressed as means (*n*=3)

a2-M content (mg/ml)	3.5 µg/ml APC + 6.1 µg/ml PS		3.5 µg/ml APC + 10.0 µg/ml PS		3.5 µg/ml APC + 18.8 µg/ml PS	
	F1+2 (µM)	TP (nM/min)	F1+2 (µM)	TP (nM/min)	F1+2 (µM)	TP (nM/min)
1.38	0.85	381	0.64	287	0.24	108
1.82	0.92	412	0.75	336	0.39	175
2.96	1.10	493	0.98	439	0.76	340
3.92	1.25	560	1.19	533	1.08	484
4.18	1.27	569	1.22	546	1.12	502

Interaction Between a2-M and PS

No complex formation between a2-M and PS was observed by means of SDS-PAGE under nondenaturing conditions (data not shown).

Discussion

PS serves as a cofactor for APC (10). The APC/PS-complex cleaves activated factors V and VIII and thus suppresses feedback-activation of thrombin (17–19). The function of PS appears to be to alter the cleavage site preferences of APC in FVa, probably by changing the distance of the active site of APC relative to the membrane surface (20). We have recently shown a dose-dependent correlation between APC content of a given plasma sample and thrombin generation: elevation of the APC concentration resulted in prolonged clotting times and suppressed thrombin generation (21). The area under the free thrombin generation curve is called thrombin potential (TP) and has been shown to be a reliable parameter to assess the combined effects of all procoagulant and anticoagulant factors that may influence thrombin generation in a given sample (8).

Hoogendoorn et al. demonstrated a time dependent inhibition of APC by a2-M using an activated partial thromboplastin time (aPTT) based APC assay (2). In accordance, we have recently shown that in plasma containing physiological amounts of AT the anticoagulant action of APC is suppressed in the presence of high amounts of a2-M by determining prothrombin activation and TP in both cord and adult plasma (22).

Thus, a2-M reveals anticoagulant action by binding free thrombin (23), and, on the other hand, acts as a procoagulant by inhibiting APC, associated with augmentation of thrombin generation. Protein C inhibitor (PCI) is the most important APC inhibitor (24). However, it has been shown that a2-M becomes a more important inhibitor of APC when the primary inhibitor PCI is consumed in the face of a

sustained procoagulant challenge. Marked amounts of a2-M/APC complexes were detected during the clinical course of disseminated intravascular coagulation (25).

In the present study we provide further evidence of a possible procoagulant activity of a2-M. APC requires PS as a cofactor to reveal anticoagulant action. Thus, levels of PS co-regulate the amount of APC/PS-complex built in the plasma sample. PS deficiency might contribute to an unfavorable outcome of thromboembolic complications (26, 27). The anticoagulant action of PS is usually determined via the ability of the APC/PS-complex to prolong the aPTT (28, 29). However, features of the thrombin generating process that take place after clotting has occurred are not reflected in the clotting time (8). Therefore, we determined prothrombin activation and free thrombin generation in the presence of different amounts of PS in cord plasma containing 1.5 μg/ml APC and in adult plasma containing 3.5 μg/ml APC. These respective concentrations of APC were applied to simulate activation of the bulk of protein C present in cord or adult plasma. In both cord and adult plasma we found a dose-dependent correlation between PS content and F1+2 or free thrombin generation respectively: F1+2 generation and TP were dose-dependently suppressed when PS concentration was successively elevated and dose-dependently enhanced when PS concentration was successively decreased. We conclude that at elevated PS levels more APC/PS-complex is built, resulting in increased cleavage of FVa and FVIIIa, associated with suppressed thrombin generation.

Additionally, we found that the anticoagulant cofactor activity of PS depends on the a2-M level in cord and adult plasma: the anticoagulant action of PS was decreased at elevated a2-M levels, and enhanced when a2-M levels were successively decreased. One simple explanation for decreased anticoagulant action of PS at high a2-M levels might be complex formation between a2-M and PS. However, we could not detect the formation of an a2-M/PS-complex by incubation of both inhibitors in the presence of 2.5 mM $CaCl_2$. This is in accordance with Hoogendoorn et al., who found that material bound to a2-M in plasma is free of PS (2). Since they have shown that complex formation between a2-M and APC effectively only occurs in the presence of Ca^{2+}-ions (2, 30), we used weak activation of plasma to provide sufficient time for complex formation in all experiments. Our data show that in plasma containing APC and low amounts of a2-M supplementation of PS results in stronger suppression of F1+2 and thrombin generation compared with plasma containing the same amount of APC but high levels of a2-M. Thus, we attribute decreased anticoagulant action of PS at high a2-M levels to competition between a2-M and PS for APC. In the presence of high amounts of a2-M, formation of the APC/PS-complex is decreased.

We suggest that a2-M reveals both anticoagulant and procoagulant action dependent on the AT content of the given plasma sample. In AT deficient plasma a2-M essentially reacts as an anticoagulant by complexing generated thrombin. It has been demonstrated that a2-M complexes up to 49% of generated alpha thrombin in cord plasma (31). When the AT content is successively elevated, the percentage of thrombin bound to a2-M decreases. Thus, more a2-M is available to compete with PS for APC, resulting in decreased formation of the APC/PS-complex.

Our findings have implications particularly for thrombin generation and inhibition in cord plasma. Some clotting factors, especially prothrombin, are much lower in neonates than in adults. However, neonates have no easy bruising and good

wound healing. A2-M levels in newborns are elevated over adult values (11, 32). In addition, it has been shown that PS activity is high due to decreased C4b-binding protein in neonates (33) and that the antithrombotic APC/PS pathway is upregulated at birth (12). We suggest that elevated levels of a2-M might restrict the upregulation of the APC/PS pathway, and thereby help to maintain a balanced clotting system in neonates.

References

1. Sottrup-Jensen L. Alpha 2-macroglobulins: structure, shape, and mechanism of proteinase complex formation. *J Biol Chem* 1989; 264: 11539–11542.
2. Hoogendoorn H, Toh CH, Nesheim ME, Giles AR. Alpha 2-macroglobulin binds and inhibits activated protein C. *Blood* 1991; 78: 2283–2290.
3. Mitchell L, Piovella F, Ofosu F, Andrew M. Alpha 2-macroglobulin may provide protection from thromboembolic events in antithrombin III-deficient children. *Blood* 1991; 78: 2299–2304.
4. Cvirn G, Gallistl S, Muntean W. Effects of a2-macroglobulin and antithrombin on thrombin generation and inhibition in newborn and adult plasma. *Thromb Haemost* 1999; (Suppl. August): 338–339.
5. Cvirn G, Gallistl S, Leschnik B, Muntean W. Effect of alpha 2-macroglobulin on the anticoagulant activity of activated protein C. *Eur J Pediatr* 1999; 158 (Suppl III):217.
6. Hemker HC, Willems GM, Beguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb Haemost* 1986; 56: 9–17.
7. Hemker HC, Wielders S, Kessels H, Beguin S. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. *Thromb Haemost* 1993; 70: 617–624.
8. Hemker HC, Beguin S. Thrombin generation in plasma: its assessment via the endogenous thrombin potential. *Thromb Haemost* 1995; 74: 134–138.
9. Nicolaes GA, Thomassen MC, Trans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and thrombin potential of normal and APC-resistant individuals. *Blood Coagul Fibrinolysis* 1997; 8: 28–38.
10. Esmon CT. The protein C anticoagulant pathway. *Arterioscler Thrombosis* 1992; 12: 135–145.
11. Andrew M, Paes B, Milner R, Johnston M, Mitchell L, Tollefsen DM, Powers P. Development of the human coagulation system in the fullterm infant. *Blood* 1987; 70: 165–170.
12. Petaja J, Fernandez JA, Fellman V, Griffin JH. Upregulation of the antithrombotic protein C pathway at birth. *Pediatr Hematol Oncol* 1998; 15: 489–499.
13. Svoboda P, Meier J, Freyvogel TA. Purification and characterization of three α_2-antiplasmin and α_2-macroglobulin inactivating enzymes from the venom of the Mexican coast rattlesnake (crotalus basiliscus). *Toxicon* 1995; 33: 1331–1346.
14. Rijkers DTS, Wielders SJH, Beguin S, Hemker HC. Prevention of the influence of fibrin and alpha 2-macroglobulin in the continuous measurement of the thrombin potential: implications for an endpoint determination of the optical density. *Thromb Res* 1998; 89: 161–169.
15. Gallistl S, Muntean W, Leis HJ: Effects of heparin and hirudin on thrombin generation and platelet aggregation after intrinsic activation of platelet rich plasma. *Thromb Haemost* 1995; 74: 1163–1168.
16. Andrew M, Schmidt B, Mitchell L, Paes B, Ofoso F. Thrombin generation in newborn plasma is critically dependent on the concentration of prothrombin. *Thromb Haemost* 1990; 63: 27–30.

17. Fulcher CA, Gardiner JE, Griffin JH, Zimmerman TS. Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V. *Blood* 1984; 63: 486–489.
18. Walker FJ, Scandella D, Fay PJ. Identification of the binding site for activated protein C on the light chain of factors V and VIII. *J Biol Chem* 1990; 265: 1484–1489.
19. Lu D, Kalafatis M, Mann KG, Long GL: Comparison of activated protein C/protein S-mediated inactivation of human factor VIII and factor V. *Blood* 1996; 87: 4708–4717.
20. Esmon CT. Regulation of blood coagulation. *Biochim Biophys Acta* 2000; 1477: 349–360.
21. Cvirn G, Gallistl S, Muntean W: Effects of antithrombin and protein C on thrombin generation in newborn and adult plasma. *Throm Res* 1999; 93: 183–190.
22. Cvirn G, Gallistl S, Muntean W. Regulation of thrombin generation in newborn plasma. *Ann Hematol* 2000; 79 (suppl. I): P235.
23. Lambin P, Pochon F, Fine JM, Steinbuch M. The alpha 2-macroglobulin/thrombin interaction in the presence of hydroxylamine. *Thromb Res* 1983; 32: 123–132.
24. Elisen MG, von dem Borne PA, Bouma BN, Meijers JC. Protein C inhibitor acts as a procoagulant by inhibiting the thrombomodulin-induced activation of protein C in human plasma. *Blood* 1998; 91: 1542–1547.
25. Scully MF, Toh CH, Hoogendoorn H, Manuel RP, Nesheim ME, Solymoss S, Giles AR. Activation of protein C and its distribution between its inhibitors protein C inhibitor, alpha1-antitrypsin and alpha2-macroglobulin, in patients with disseminated intravascular coagulation. *Thromb Haemost* 1993; 69: 448–453.
26. Horowitz IN, Galvis AG, Gomperts ED. Arterial thrombosis and protein S deficiency. *J Pediatr* 1992; 121: 934–937.
27. Marlar RA, Neumann A. Neonatal purpura fulminans due to homozygous protein C or protein S deficiencies. *Sem Thromb Hemost* 1990; 16: 299–309.
28. Comp PC. Laboratory evaluation of protein S status. *Semin Thromb Hemost* 1990; 16: 177–181.
29. Preda L, Simioni P, Legnani C, Palareti G, Poggio M, Rossi E, Girolami A. A new global test for the evaluation of the protein C-protein S system. *Blood Coagul Fibrinolysis* 1996; 7: 465–469.
30. Heeb MJ, Gruber A, Griffin JH. Identification of divalent metal ion-dependent inhibition of activated protein C by alpha 2-macroglobulin and alpha 2-antiplasmin in blood and comparisons to inhibition of factor Xa, thrombin, and plasmin. *J Biol Chem* 1991, 266: 17606–17612.
31. Ling X, Delorme M, Berry L, Ofosu F, Mitchell L, Paes B, Andrew M. Alpha2-macroglobulin remains as important as antithrombin III for thrombin regulation in cord plasma in the presence of endothelial cell surfaces. *Pediatr Res* 1995; 37: 373–378.
32. Andrew M, Vegh P, Johnston M, Bowker J, Ofosu F, Mitchell L. Maturation of the hemostatic system during childhood. *Blood* 1992; 8: 1998–2005.
33. Schwarz HP, Muntean W, Watzke H, Richter B, Griffin JH. Low total protein S antigen but high protein S activity due to decreased C4b-binding protein in neonates. *Blood* 1988; 71: 562–565.

Combined Prothrombotic Defects and Contraceptives – Risk Factor for Deep Venous Thrombosis in Adolescence

R. SCHOBESS, A. JASSOY and S. BURDACH

Introduction

Hereditary risk factors for thrombosis in adults are well known. The latest studies (5,6,7,9,10) have shown that the heterozygous factor (F) V G1691A mutation and increased lipoprotein (Lp) (a) are the most commonly found prothrombotic risk factors for spontaneous thrombosis during infancy and early childhood (e.g. in childhood strokes and vena caval thrombosis). In contrast, the prothrombin (PT) G20210A variant is likely to be more important during puberty and adolescence (9).

Pediatric venous thromboembolism is being increasingly viewed as a multifactorial disorder as well (2). However, information on the role of combined hemostatic defects in childhood thrombosis is limited. Very recently we have shown elevated Lp (a), the heterozygous state for the FV G1691A mutation and the PT G20210A mutation, deficiencies of protein C and antithrombin to be risk factors for childhood thromboembolism (1,2,3,4,8).

Case Report

The following case report presents evidence that further additional endogenous and exogenous trigger mechanisms such as oral contraceptives contribute to the early onset of venous thromboembolism in childhood carriers of the PT G20210A variant and elevated Lp (a) levels.

Patient History and Clinical Manifestation

We report the case of a 15-year-old girl with combined prothrombotic risk factors and deep vein thrombosis (DVT) in the lower extremities. The thrombotic event was on October 18, 1999. The girl had been taking hormonal contraceptives for 1 year. She reported pain in the left inguinal region and in the hip for a few weeks. Two days before admission she suffered swelling and blueness of the left leg. It was suspected that the leg and pelvic deep veins were occluded.

Family History

The parents and the brother are asymptomatic. The father has elevated Lp (a), the mother bears the PT G20210A variant heterozygous and the brother has both the elevated Lp (a) and the PT G20210A variant heterozygous.

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

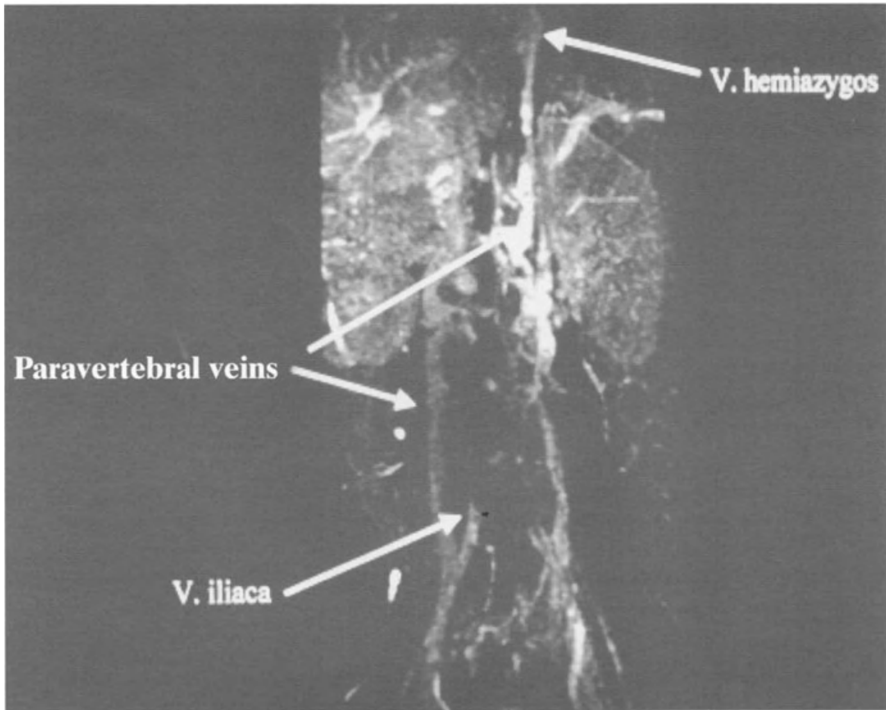


Fig. 1. Initial duplex sonography

Diagnostic Measures

Initial Spiral CT of the Pelvis

The left leg and pelvic deep veins were occluded.

Initial Duplex Sonography

The superficial and common femoral veins and the external and common iliac veins on the left side were completely occluded (Fig. 1)

Initial MRI Abdomen/Pelvis

There were thromboses of the femoral vein and the left external iliac vein, aplasia of the inferior vena cava with blood flow off into the superior vena cava via the azygos/hemiazygos vein system (Fig. 2).

Final Radiological Diagnosis

There was a complete occlusion of the left leg and pelvic deep veins and an aplasia of the inferior vena cava in the caudal part.

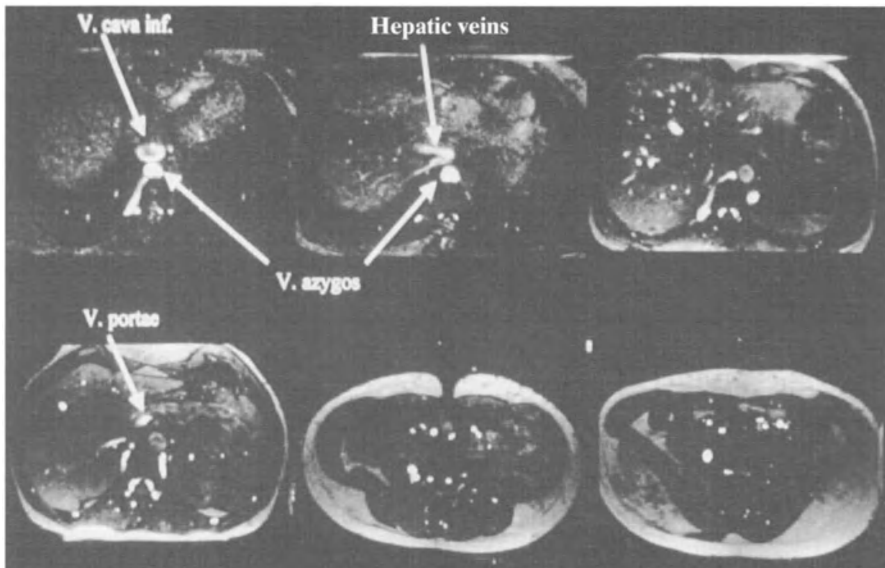


Fig. 2. Initial MRI

Thrombophilia Factors

The normal and pathological results are as follows:

- Normal results: AT III: 109%; FXII: 100%; APC ratio: 2.3; protein C: 100%; protein S: 94%; ANA, AMA,SMA, cardiolipin, AK: negative
- Pathological results: PT: G20210A mutation (heterozygous); Lp (a): 0.75 g/l (normal <0.2 g/l); MTHFR C677T: mutation (heterozygous); fasting homocysteine: 10.4 µmol/l (ELISA) (normal <8.0 µmol/l)

Therapy

Side Effects of Fibrinolysis Therapy

After non-successful thrombolysis and side effects rt-PA was stopped. The side effects were as follows:

- Extensive hematomas of the extremities, especially at the places of vein puncture
- Nose bleeding
- Decrease in hemoglobin
- Decrease in plasminogen: 115% on day 1 and 41% on day 5 of the treatment (fresh frozen plasma infusion was necessary)

LMW heparin was given for another 10 days; cumarin was administered orally (Table 1).

Table 1. Therapy

Therapy	Dose	Laboratory results
Initial heparin (UFH)	400 U/kg/d	PTT 46 s (normal 24–36)
2nd day rt-PA (Actilyse)	0.9–2.0 mg/kg/day	Fibrinogen: 9.3 g/l up to 0.9 g/l on day 5 D-dimers: 0.53 mg/l up to >4.3 mg/l (N <0.25) Plasminogen: 129% up to 41%
From day 5 LMW heparin (Clexane)	1 mg/kg, 2×/day s.c., 2×60 mg/day	Anti Xa level 0.63 U/ml; 0.78 U/l; 0.98 U/ml
From day 15 Marcumar		INR 2.5–3.0

Course and Results

MR angiography was performed 4 weeks after fibrinolysis. Recanalization of the left iliac vein was reached due to anticoagulation with heparin and following Marcumar. Four months after the thrombotic event duplex sonography showed recanalization of the iliac vein and femoral veins on the left side as well as venous drainage by the azygos veins system. One year after the thrombotic event (October 2000) oral anticoagulation was ended.

Risk Factors for Thrombosis

The risk factors for thrombosis are as follows:
- Exogenous: hormonal contraceptives
- Genetic: PT G20210A mutation (heterozygous); Lp(a) increase; C-677-T MTHFR mutation (heterozygous); elevated fasting homocysteine
- In addition: connatal or neonatal thrombosis of the inferior vena cava

Discussion

We conclude a connatal thrombosis of the inferior vena cava in the caudal part and secondary hypoplasia when inheriting two genetic risk factors (elevated Lp (a) and PT G20210A variant). Münchow described that the FV gene mutation or increased Lp (a) concentrations are more common in vena cava occlusion during infancy compared with older children, who predominantly presented isolated vena cava thrombosis concomitant with the heterozygous PT G20210A gene mutation. In adolescence the girl suffered from a recurrent venous thrombosis (rVTE) of the leg and pelvic veins (the triggering factor was oral contraceptives). Data from a prospective multicenter study (8) show that the risk of recurrent VTE in children with a first symptomatic episode of venous thromboembolism is significantly higher in patients carrying two or more prothrombotic risk factors, adjusted odds ratio: 3.3 (301 patients included). The results of a multicenter family study show that

symptomatic children with spontaneous venous thromboembolism have a significantly higher rate of combined genetic prothrombotic risk factors than their first-degree family members (4).

Conclusion

The case report demonstrates the greater importance of PT G20210A mutation for thrombosis during puberty and adolescence and the risk of recurrent venous thrombosis in children with combined prothrombotic risk factors. The combination of prothrombotic genetic risk factors additional to hormonal contraception is to be seen as cause for thrombosis. Before the prescription of hormonal contraception for adolescents prothrombotic risk factors should be excluded.

References

1. Andrew M et al. (1997) Arterial thromboembolic complications in pediatric patients. Thromb Haemost 78:715–25
2. Ehrenforth S et al. (1999) Multicenter evaluation of combined prothrombotic defects associated with thrombophilia in childhood. Eur J Pediatr 158:S97-S104
3. Junker R et al. (1999) Prothrombin G20210A gene mutation and further prothrombotic risk factors in childhood thrombophilia. Arterioscl Throm Vasc Biol 19:2568–72
4. Kosch A et al. (1999) Rate of combined prothrombotic risk factors is significantly increased in children with spontaneous venous thromboembolism compared with their asymptomatic first-degree family members. Blood 94 (abstract)
5. Münchow N et al. (1998) Role of genetic prothrombotic risk factors in childhood caval vein thrombosis. Eur J Pediatr 158:S109-S112
6. Nowak-Göttl U et al. (1999) Increased lipoprotein (a) is an important risk factor for venous thromboembolism in childhood. Circulation 100:743–48
7. Nowak-Göttl U et al. A (1999) Lipoprotein (a) and genetic polymorphisms of clotting factor V, prothrombin, and methylenetetrahydrofolate reductase are risk factors of spontaneous ischemic stroke. Blood 94:3678–82
8. Nowak-Göttl U et al. (1999) Risk of recurrent venous thrombosis in children with combined prothrombotic risk factors. Blood 2001 in press
9. Schobeß R et al. (1999) Factor V G1691A and prothrombin G20210A in childhood spontaneous venous thrombosis – Evidence of an age-dependent thrombotic onset in carriers of factor V G1691A and prothrombin G20210A mutation. Eur J Pediatr 158:S105-S108
10. Vielhaber H et al. (1998) Cerebral venous sinus thrombosis in infancy and childhood: role of genetic and acquired risk factors of thrombophilia. Eur J Pediatr 157:555–60

Role of a 23 bp Insertion in Exon 3 of the Endothelial Cell Protein C Receptor Gene in Venous Thrombophilia

M. von Depka Prondzinski, A. Czwalinna, R. Eisert, C. Wermes, B. Canepa, I. Scharrer, A. Ganser and S. Ehrenforth

Introduction

One of the most frequent causes of morbidity and mortality among Caucasians is the development of thromboembolic disorders. Besides circumstantial influences, patients carrying prothrombotic gene defects are at risk of developing VTE. Among others, the protein C anticoagulant pathway is an important mechanism regulating thrombin generation and the blood clotting response. Therefore deficiencies of members of the protein C anticoagulant pathway may cause severe thrombotic complications. Both protein C and activated protein C (APC) bind to human endothelial cell protein C receptor (EPCR), a newly described member of the protein C anticoagulant pathway [1, 2]. EPCR is located on the surface of endothelial cells of larger vessels, especially of arteries [3, 4], and one of its physiological functions seems to be the enhancement of protein C activation by the thrombin-thrombomodulin complex in an EPCR concentration dependent fashion [5]. On the other hand, EPCR seems to inhibit the APC anticoagulant activity [6], which in turn could cause increased thrombotic tendency [7, 8]. Recently, a 23 bp insertion in exon 3 of the EPCR gene at position 7116 generating a duplication of the 23 bases preceding the insertion point has been described [9]. The mutation generates a STOP codon at the sixth codon downstream of the insertion point. The predicted receptor is truncated missing a part of exon 3 and the whole exon 4. This prompted us to evaluate the frequency of EPCR insertion in patients with VTE.

Materials and Methods

The current study population comprised 640 consecutive patients (384 females, 256 males, Caucasians of the same ethnic and geographical background) who were referred to our outpatient clinics for investigation of thrombophilia. All patients had at least one venous thromboembolic event objectively confirmed by either duplex sonography, ascending phlebography or computed tomography. Patients with evidence of malignancies and/or anti-phospholipid antibody syndrome were excluded. The presence of circumstantial risk factors at the time of any VTE episode (use of oral contraceptives, immobilization longer than 3 days, pregnancy, puerperium, surgery, trauma, or leg cast) was recorded. As controls we screened 395

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000
© Springer-Verlag Berlin Heidelberg 2002

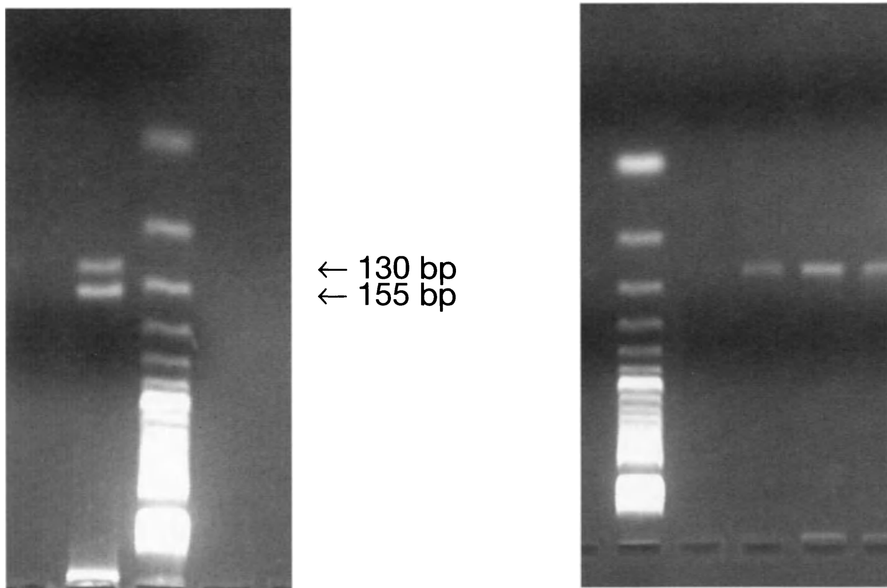


Fig. 1. Ethidium-bromide-stained gel showing two EPCR genotypes. The short fragment (130 bp) corresponds to the non-insertion allele, the longer (155 bp) to the insertion allele

healthy blood donors (216 females, 179 males) without a history of VTE disease, and from the same ethnic and geographic backgrounds as the patients.

For gene analysis, venous blood was collected in EDTA-treated tubes, and genomic DNA was isolated by standard methods. Determination of the PT G20210 A and FV:G1691 A genotypes by polymerase chain reaction was done as described elsewhere [10]. For EPCR genotyping the following primers were designed to amplify the intron 2-exon 3 boundary and part of exon 3 of the EPCR gene: 5'-CTC TGC ACA GTC CCC TGA TC-3' (nt 7064 to 7083) and 5'-CTC CCA TTC ACA GCC ACT TCG AAG-3' (nt 7169 to 7192). Ten pmol of each primer were used in a final volume of 50 µl containing 2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 0.25 mM of each dNTP, one unit of Taq polymerase. Amplification procedure consisted of 33 cycles, denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s. The amplification product is a 130 bp fragment in the absence of the insertion and a 155 bp fragment in the presence of the insertion. The amplification products were electrophoresed on 4% agarose gel and stained with ethidium bromide (Fig. 1).

DNA sequencing was performed in 223 samples from individuals with the wild type genotype and in all samples from the heterozygotes to validate the mentioned PCR protocols using the 310 ABI Prism Genetic Analyzer and BigDye Terminator Sequencing chemistry (Perkin-Elmer, Weiterstadt, Germany). In addition to the EPCR insertion, a polymorphism in exon 3 leading to C to T transition at position 7082 was identified in samples which were sequenced.

Plasma protein C and antithrombin activity as well as free protein S were assayed with commercially available kits excluding 190 (29.7%) patients on oral anticoagulant therapy at the time of blood sampling.

This multicenter study was performed in accordance to the 1996 Declaration of Helsinki. All patients had given informed consent prior to study enrollment.

Statistical Analysis

Genotype frequencies were compared between cases and controls by using the χ^2 test. The odds ratios (OR) for VTE, 95% confidence intervals (CI) and *P*-values (χ^2 test; corrected for multiple testing according to Bonferroni, or Student's *t*-test respectively) were calculated. *P*-values less than 0.05 were considered significant. All calculations were carried out using the SPSS for Windows Release 9.01 statistical package (SPSS, Chicago, Illinois, USA).

Results

Among 640 study patients, the median age at thrombotic onset manifestation was 54 years (range 1–82). Sixty-six percent of all patients suffered from their first VTE before the age of 50 years. The median age of the controls at the time of investigation was 45 years (range 18–65) yielding no significant difference in age distribution between patients and controls.

Four hundred and eighty-three (75.5%) patients suffered from VTE of the lower limb. Twenty-seven (4.2%) subjects had retinal vein thrombosis, 43 (6.7%) subjects had thrombosis in the splanchnic region. Three (0.5%) subjects had VTE of the caval vein, 10 (1.6%) subjects had cerebral VTE, 34 (5.3%) subjects had thrombosis in the upper limbs, and 40 (6.3%) patients had primary pulmonary embolism.

In 51.6% of all cases, the first VTE occurred in the absence of circumstantial factors. In the remaining patients recent major surgery was documented in 5.9%, trauma in 17.2%, and immobilization in 5.9%. In 3.8% of the female subjects the first VTE occurred during pregnancy, and in 4.5% after delivery. Of the female patients 28.7% were on oral contraceptives. In 39 patients (6.1%) more than one circumstantial risk factor was present.

The prevalence of hereditary risk factors found in this study are shown in Table 1. The heterozygous form of the EPCR 7116 23 bp insertion was identified in one patient only (0.2%), compared with three (0.8%) among controls, yielding an OR of 0.2 (95% CI 0.02–2.0; P=0.3). No homozygous carriers were detected.

Discussion

As EPCR augments protein C activation by the thrombin-thrombomodulin complex, and the protein C anticoagulant pathway is a well known mechanism promoting reduced thrombin activation, it is likely that defects or deficiency states of

Table 1. Frequency of EPCR insertion and known prothrombotic defects, and associated risk of venous thromboembolism

Risk factor	Patients, *n* (%)	Controls, *n* (%)	**P*-values, χ^2 test	OR (95% CI)
EPCR 23 bp insertion	1/640 (0.2)	3/395 (0.8)	0.3	0.2 (0.02–1.9)
FV 1961GA	136/640 (35.9)	33/395 (8.4)	0.000	6.1 (4.1–9.1)
FV 1691AA	17/640 (2.8)	0/395 (0%)	0.000	–
Prothrombin 20210GA	49/619 (8.0)	6/357 (1.7)	0.006	5.1 (2.1–11.9)
AT deficiency	13/389 (3.3)	2/202 (1.0)	0.6	1.9 (0.4–8.7)
PC deficiency	9/407 (2.2)	2/195 (1.0)	0.47	2.2 (0.5–10.2)
PS deficiency	30/398 (7.5)	2/192 (1.0)	0.002	7.8 (1.9–33.1)

*Corrected according to Bonferroni

EPCR may predispose to VTE [7, 8]. As a STOP codon the sixth codon downstream of the insertion point is generated by the 23 bp insertion, the predicted receptor misses part of exon 3 and the whole exon 4. Yet, it is unclear whether the truncation generates a loss or decrease in function of EPCR and impairs protein C activation. In an early report, Merati et al.9 found the EPCR mutation in 0.8% of 404 controls compared with 3.4% of 149 patients with VTE, giving a crude OR of 4.6 with a wide 95% CI (1.1–19.7). However, in our study the 23 bp insertion in exon 3 of the EPCR gene was not found to be more prevalent among patients with a history of VTE (0.2%) than in healthy controls (0.8%, OR 0.2; 95% CI 0.02–2.0; *P*=0.3), indicating that the EPCR insertion does not represent a major risk factor for the development of VTE.

Our results might be explained by several findings. First, it could be demonstrated by immunohistochemical investigations that EPCR is located particularly in the aorta and large arteries, whereas it seems to be less present in veins of similar size and to be absent in microvessels [3, 4]. With regard to the vascular location of EPCR, it is therefore unlikely that EPCR plays an important role in the pathogenesis of VTE. Secondly, EPCR binds protein C and activated protein C2, which bind to soluble- and membrane-bound EPCR with similar affinity [11]. When protein C binds to EPCR, EPCR enhances protein C activation by the thrombin-thrombomodulin complex [5], but APC loses its anticoagulant ability when bound to EPCR6. Therefore, it cannot be ruled out that EPCR may inhibit inactivation of activated FV by binding APC, and that this effect may counteract the ability of EPCR to activate protein C. It is not known whether truncated EPCR caused by the insertion may impair the binding of soluble- or membrane-bound EPCR to APC. In this case the mutation would result in an anticoagulant effect as the blocking of the anticoagulant function of APC bound to EPCR is diminished.

In conclusion, our findings failed to support an association between the 23 bp insertion in exon 3 of the EPCR gene and an increased risk of VTE. The low prevalence of this EPCR insertion allele among controls of less than 1% makes estimations of its frequency in the general population difficult. Therefore, analysis of the role of EPCR insertion in VTE, if any, or potential interactions with other risk fac-

tors of VTE will be feasible in large scale studies only. The function of EPCR and the structure function relations of this molecule still need to be further clarified.

References

1. Bangalore N, Drohan WN, Orthner CL. High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells independent of protein S and distinct from known ligands. Thromb Haemost 1994; 72:465
2. Fukudome K, Esmon CT. Identification, cloning and regulation of a novel endothelial cell protein C/activated protein C receptor: cell surface expression and direct ligand binding by the soluble receptor. J Biol Chem 1994; 271:17491
3. Laszik Z, Mitro A, Taylor FB, Jr., Ferrell G, Esmon CT. Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. Circulation 1997; 96:3633
4. Fukudome K, Ye X, Tsuneyoshi N, Tokunaga O, Sugawara K, Mizokami H, Kimoto M. Activation mechanism of anticoagulant protein C in large blood vessels involving the endothelial cell protein C receptor. J Exp Med 1998; 187:1029
5. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci U S A 1996; 93:10212
6. Regan LM, Stearns-Kurosawa DJ, Kurosawa S, Mollica J, Fukudome K, Esmon CT. The endothelial cell protein C receptor. Inhibition of activated protein C anticoagulant function without modulation of reaction with proteinase inhibitors. J Biol Chem 1996; 271:17499
7. Lane DA, Grant PJ. Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. Blood 2000; 95:1517
8. Esmon CT. The endothelial cell protein C receptor. Thromb Haemost 2000; 83: 639
9. Merati G, Biguzzi E, Oganesyan R, Fetiveau R, Qu D, Buciarelli P, Stearns-Kurosawa DJ, Mannucci PM, Esmon CT, Faioni EM. A 23 bp insertion in the endothelial protein C receptor (EPCR) gene in patients with myocardial infarction and deep vein thrombosis (abstract). Thromb Haemost Suppl 507,1999
10. von Depka Prondzinski M, Eisert R, Oberkanins C, Aschermann G, Barthels M, Ganser A. Thrombosis and Prothrombin 20210 G-A Mutation Detected by Rapid PCR and Allele Specific Hybridization. Blood 1997; 90: 653
11. Regan LM, Mollica JS, Rezaie AR, Esmon CT. The interaction between the endothelial cell protein C receptor and protein C is dictated by the gamma-carboxyglutamic acid domain of protein C. J Biol Chem 1997; 272: 26279

Subject Index

Aafact 27
Abciximab 92, 95
abortion 161
accident 12–13
acetylsalicylic acid 115
Acquired Immune Deficiency Syndrome 9–11, 193
ADP [*Adenosindiphosphat*] 57–58, 217
age
- gestational age 81, 149, 263
- postnatal age 81
AIDS [→ *Aquired Immune Deficiency Syndrome)*
albumin 187–188, 194
anticardiolipin antibodies 179–180, 220, 236–237, 246, 249
anticoagulation 115, 117, 119, 121, 123, 125, 153, 155, 161–162, 164, 236, 276
antiretroviral therapy 7, 189,
antithrombin 78, 155, 179, 235, 252, 262–263, 270, 275
- activity 220, 233, 235–236, 288
- concentration(s) 233, 236
- deficiency 77, 116, 222–223, 233–234, 236-237, 248–149, 273
APC [*activated→ protein C*]
Aprotinin 81–86, 88, 263
aPTT [*activated → partial thromboplastin time*]
arthritis 143, 145, 147, 197
arthropathy 143, 147, 169–170, 181–182, 184, 197, 198
- hip arthropathy 144
- knee arthropathy 144
ASA [→ *acetylsalicylic acid*]

BeneFIX [*recombinant → FIX*]
Beriate 48–49
Bernard Soulier syndrome 115, 171
bleeding 11–13, 42–43, 48, 52, 55–56, 58–60, 62–63, 79, 86, 91, 101, 114–117, 120, 125–127, 130, 150, 159–160, 163–164, 170, 172–174, 189, 193, 197, 204, 206, 208–209, 216
- complication(s) 55–56, 159
- disorder(s) 12, 16, 18, 56, 130, 180
- episode(s) 55, 63, 77, 143, 184, 189–193
- frequency 198
- tendency 57, 91, 179–180, 206, 213
- time 58, 63, 91, 116, 210
- → joint bleeding
- → muscle bleeding
blood 92, 95, 110
- clotting 1, 187
- coagulation 59–60, 219
- disease 1
- product(s) 69–70, 75, 186
- transfusion 43, 210
Bonn protocol [→ *protocol*]
bovine spongiform encephalopathy [*BSE*] 186
BSE [→ *bovine spongiform encephalopathy]*

Cancer 11–13
CD_4^+ cell(s) 37, 189
CD_8^+ cell(s) 37
cells
- red blood cells 91–93, 117, 121–126
- white blood cells 91, 93–94, 96
Ceprotin 101–107
chain
- heavy chain (FVIII) 23, 27–30, 32–34, 36–37, 81, 187
- light chain (FVIII) 23–25, 27–29, 31–36, 38, 81, 187
cirrhosis [→ *liver cirrhosis*]
Clopidogrel 58, 115
clot
- lysis 82, 86–88
clotting 57, 63, 84, 278
- factor(s) 56, 125, 149, 180, 263, 270
- time 59, 61, 63, 111–113, 269

coagulation 58–60, 62, 82, 86–87, 270
- activation 56, 61,
- disorder(s) 42, 58, 159–160, 189, 208
- factor(s) 43, 55–56, 58, 114, 155, 159, 240, 242
collagen 1, 57–58, 92, 94–95, 217
coronary artery disease 208
corticosteroid(s) 159, 252
cost [→ *treatment cost*]
Creutzfeld-Jacob disease 12, 186
cryoprecipitate 69, 210
cyclophosphamide 41, 159
cytokine(s) 24, 36, 217
- receptors 24
cytometry
- flow cytometry analysis 216, 217

Dalteparin 156,
DDAVP 52, 57–58, 125, 204
Diclofenac 115, 183
DNA 130, 132, 135, 204, 220, 228, 279
- cDNA 136, 218
domain
- A1-A2-B 23
- A1-A2-B-A3-C1-C2 187
- A2 24, 27, 31, 36
- A3 38
- A3-C1 24
- A3-C1-C2 23
- B 187, 193
- C1 38,
- C2 24, 38
- D 229
- D3 206
drug(s) 12–13, 55–56, 59, 148, 182, 189, 193
- abuse 189

ELISA 28, 35, 71, 155, 162, 189, 246, 275
- assay 24–25, 27–30, 32–37, 220
embolism 247, 255, 280
embryo-
- genesis 81
- implantation 81
endothelium 57
epidemiology 12, 19
Epinephrine 57–58, 92, 94–95
epitope(s) 24, 33, 36, 38
- specificity 23–24, 32, 35

FII 110–111
- activity 179
- gene mutation 180
FIIa 187
FV 1, 4, 41, 262, 269
- alpha-granule FV 1, 2,
- gene 233, 235–236, 276
- Leiden 116, 179–180, 239, 243
- mutation 79, 156, 219–220, 222–223, 249, 252, 273
FVII 110–113, 170, 248–249
- activity 246
- deficiency 171
- gene 136
- recombinant FVIIa 44, 49, 110, 159–160, 169, 170–171, 174–178
FVIII 25, 27, 29–34, 36–38, 41–42, 45, 47–49, 51–52, 71, 73, 77, 116, 131, 159, 169–170, 177, 181–182, 184, 186–187, 189, 194, 210–213, 252, 254–255, 262, 269
- activity 9, 24, 28–29, 31, 42–44, 77, 116, 130, 159,170, 181, 185, 190, 233, 235, 252–254
- antibodies 23–29, 31–38, 169–170
- concentrate 24–27, 31, 35–38, 58, 177, 183, 186, 189–190, 210
- deficiency 23, 28, 149
- gene 23–24, 26, 34, 38, 48, 77, 79, 130–132, 134–135, 137–139, 186
- inhibitor 41, 159, 169, 179
- molecule 35, 38, 186–187
- mutation 79, 134
- recovery 27
- plasma derived FVIII 23, 27–28, 36, 130, 187–188, 190, 193
- recombinant FVIII 23, 27–31, 33, 35, 44, 130, 186–187, 193–194
FVIIIa 149, 229, 270
FIX 71, 73, 77, 110–113, 239–243
- activity 9, 149, 243
- concentrate 149–150
FIXa 110
FX 110–111, 113
FXa 187
FXI 239–243, 246
- deficiency 171
FXIa 149
FXII 56–57, 149, 180, 233, 244, 275
- activity 179–180, 236–237, 246
- antigen 236, 246
- deficiency 156, 233–234, 236–237, 246, 248–249
- gene 235–237
FXIII 208
- activity 208–209
- deficiency 209
- inhibitor 208
Feiba 51, 110–112
fibrinogen 60, 155, 179, 229, 239, 276
- gene(s) 225, 228

- molecule 244
fibrinolysis 82, 86
flow cytometry [→ *cytometry*]
Fraxiparin 153

Gene(s) 36
- analysis 225, 279
- defect(s) 38
- mutation(s) 77,
- therapy 119-200, 216
Gen-H-B-Vax 71, 73
German Hemophilia Registry 7
Gesellschaft für Thrombose- und Hämostaseforschung [GTH] 12
glycoprotein 225, 263
- IIb/IIIa [*GPIIa/IIIb*] 92, 95-96
GTH [→ *Gesellschaft für Thrombose- und Hämostaseforschung* = German Society for Thrombosis and Hemostasis Research]

Haemate-HS 27, 35, 48-50, 58, 204
Haemoctin-SDH 24-25, 27-28, 30-31
HBV [→ *hepatitis B virus*]
HCV [→ *hepatitis C virus*]
HDV [→ *hepatitis D virus*]
HGV [→ *hepatitis G virus*]
hemophilia 1, 7, 15-16, 19, 33, 36-37, 41, 47-48, 51-52, 73, 77, 79, 117, 143, 147, 149-150, 171, 179, 200
A 8-10, 16, 18, 23-24, 26-29, 38, 48, 70, 77-79, 115, 117, 119-123, 125-126, 130-131, 135, 139, 143, 151, 169, 171-172, 176, 178-181, 188-189, 198
- B 8-10, 16, 18, 70, 77-78, 115, 117, 119-123, 125-126, 149, 151, 179, 198
- center(s) 7-8, 15, 69, 188, 194
- treatment 186, 194, 199
hemolysis 48
hemorrhage(s) 78, 101, 103-104, 107, 130, 149-151, 174-175, 177, 222, 225
- hemorrhagic diathesis 114-115, 117, 119-127, 171, 208, 210
- hemorrhagic disorder(s) 77
- hemorrhagic shock 172
hemostasis 3, 55, 57-58, 60, 63, 82, 170, 171, 789, 248
heparin 62-63, 153, 155, 161, 233, 235, 275-276
- cofactor II 244, 246, 248-249
- concentration 59,
- unfractionated heparin 63, 153, 156, 162
hepatitis 7, 12, 69, 72-74, 173, 186, 210-211, 213, 215
- A 189
- - virus 71
- B 189
- - virus [HBV] 69, 71-72
- - - DNA 73
- - - infection 71, 74-75
- C 169, 189
- - virus [HCV] 69,71-72
- - - infection 71, 210
- - - RNA 73
- D virus [HDV] 69, 71, 75
- - infection 75
- G virus [HGV] 69,
hip
- → arthroplasty
- replacement 145,
Hirudin 62-63, 161,
- recombinant Hirudin 161, 164,
histidine 229,
HIT [*heparin-induced → thrombocytopenia*]
HIV [→ *human immunodeficiency virus*]
homocysteine 155, 179, 235-236, 245, 257-260, 276
human immunodeficiency virus 7, 9-13, 37, 73, 169, 186, 189, 193
- infection 15, 17-19, 69, 73
hyperhomocysteinemia 233, 244, 248-249

IA [→ *immunoadsorption*]
IFN [→ *Interferon*]
IgG 24-26, 28, 31, 41, 220, 245
- subtype(s) 29, 31, 33, 35-37, 217
IgM 24, 245
immune-
- response 24
- system 24, 37-38, 148
- tolerance 38, 72-74
- - induction 27, 35, 194
- - therapy 23-26, 28, 31-33, 36-38, 47-48, 50, 52, 169
immuno-
- adsorption 41, 43, 169-170, 208-209
- deficiency 216
- globulin(s) 41, 217
- - deficiency 160
- suppression 73
- suppressive drug(s) 45
- suppressive therapy 159, 160
- intravenous immunoglobulins [IVIG] 45, 189
inhibitor(s) 9, 38, 41-43, 47-52, 110, 171-172, 176-178, 186, 189, 193-194
- elimination 43-44, 159, 209
Interferon 37, 72, 189, 211, 217

intron 22 inversion 24, 26, 34, 38, 131
ischemia 249
ITT [→ *immune tolerance therapy*)
IVIG [*intravenous → immunoglobulin*]

Joint
- bleeding 47, 78, 184, 191, 197
- destruction 197
- replacement 147

Kallikrein 56–57, 88,
knee
- → arthropathy
- arthroplasty 147
- prosthesis 146, 169
- replacement 146, 192
Kogenate 186, 194
Kryobulin-Tim-3 26, 35

Lepirudin 161, 163–164
leucocyte(s) 92, 96
lipoprotein (a) 155, 219–220, 222–223, 235–236, 273, 275–276
- apolipoprotein (a)
liver
- cancer
- cirrhosis 69, 72–73, 189, 210
- disease 11–13
- transplantation 62, 210–213, 215
Lp(a) [→ *lipoprotein (a)*]
Lupus anticoagulants 56–57, 155, 179, 235–236, 246, 248–249
lymphocyte(s) 24, 36, 205, 216–217
- subset 189

Malmö protocol [→ *protocol*]
Marcumar 276
MBM protocol [*modified Bonn-Malmö → protocol*]
Minirin 57
MTHFR [→ *methylentetrahydrofolate reductase*]
murder 12–13
muscle bleeding 78, 191
mutation(s) 130–131, 135–139, 205–206, 216, 225–226, 228–229, 233, 244
- analysis 130, 139,
- deletion
- - large 23
- - small 23
- inversion mutation 23, 34
- missense mutation 47
- point mutation 23, 38
- splice site mutation 205, 216, 218
methylentetrahydrofolate reductase
- gene 235–236
- C667T 220, 275–276

Nadroparin 153–154, 156–157
non-steroid antirheumatic drugs 183
NovoSeven 52, 159
NSAR [→ *non-steroid antirheumatic drugs*]

Occlusion 274
Octonative M 27, 35, 37, 190

Partial thromboplastin time 42, 56–57, 59–60, 63, 116, 149, 159, 161, 163, 208, 240, 246, 269
parvovirus B19 186
Petterson score 198
phenprocoumon 115, 119, 163–164, 234–236
plasmin 81–85,88
plasminogen 81–82, 87–88, 248–249, 275–276
- activation 87
- activity 179, 246
- molecule 244
prednisolone 41, 159
pregnancy 82, 86, 149, 235, 237, 252, 278, 280
previously treated patient [PTP] 186, 189, 193–194
previously untreated patient [PUP] 186, 194
prion(s) 186
protein
- C 79, 101, 103–107, 116, 179, 187, 220, 223, 236, 262–263, 269–270, 275, 278, 280–281
- - activity 102, 155, 235–236, 245
- - antigen 220, 245
- - deficiency 77–79, 101–102, 107, 115, 222–223, 239, 244, 248–249, 252, 273
- activated protein C 155, 233, 235, 245, 262–271, 275, 278, 281
- - resistance 77, 233, 236, 239, 244, 248, 252
- free protein C 179
- total protein C 179
- S 78, 179, 233, 236, 262–267, 269–271
- - activity 155, 235–236
- - deficiency 77, 115–116, 220, 222–223, 239, 244, 248–249, 252, 270
- free protein S 179, 180, 220, 245
- total protein S 179, 220, 245
prothrombin 77, 110, 179, 219–220, 222–223, 236, 248, 263–264, 270, 273
- activation 262, 267–270
- gene 179, 223, 235–236, 248, 276
- mutation 79, 243, 246, 248–249, 273, 275–277

- time 161, 163, 246
protocol
- Bonn protocol 24, 41
- Malmö protocol 24, 41
- modified Bonn-Malmö protocol 41–45
PTP [→ *previously treated patient*]
PTT [→ *partial thromboplastin time*]
PUP [→ *previously untreated patient*]
purpura fulminans 101, 103, 105–107

Radiosynoviorthesis 197–198
Recombinate 27, 29, 30, 186, 194
Refacto 194
Refludan [*recombinant → hirudin*]
replacement therapy 23–24, 37, 69, 75, 204, 206
Ribavirin 211
Ristocetin cofactor 9–10, 205, 210, 212
RNA 131
- mRNA [*messenger RNA*] 131
RSO [→ Radiosynoviorthesis]

Schoenlein, Johann-Lukas
stroke 43, 223, 239–241, 244–249, 273
suicide 12–13
synovectomy 193
synovitis 197

Therasorb 169–170
thrombin 3, 30–31, 57, 60, 110–112, 116, 126, 187, 217, 242, 246, 262–263, 265, 267–270
- activation 280
- deficiency 236
- time 161, 163–164
thrombocyte(s) 217
- count 216
thrombocytopathy 115, 117, 119–123, 125–126
thrombocytopenia 115, 117, 119, 121–123, 125–126, 153–154, 171, 173, 216
- heparin-induced thrombocytopenia 155, 157, 161
- - type II 155, 161–162, 164
thromboembolism 79, 115–116, 233, 239–240, 249
- venous thromboembolism 222, 229, 244, 273, 276–277
thrombophilia 82, 219, 223, 233, 241, 243–244, 252, 275, 278
thrombosis 57, 101–105, 107, 153–157, 179, 225, 233, 235–237, 239–242, 244, 252, 273–274, 276–277, 280
- prophylaxis 153
- venous thrombosis 219, 221, 223, 233–234, 239, 241, 248, 255, 273, 276
Ticlopidine 58, 115
treatment
- cost 69, 71
- on demand 26–27, 151, 174, 189
- prophylactic treatment 151, 175, 181, 192, 197

Uremia 115
UFH [*unfractionated → heparin*]
urokinase 86–87

Vitamin K 153–154, 237, 262
von Willebrand's disease [*vWD*] 8–10, 57, 91, 95, 97, 114–116, 119–121, 124–127, 171–172, 199, 205–206
- type 2 C Miami 203–204, 206–207
- type 3 179, 210, 213, 215
von Willebrand factor [*vWF*] 23–24, 27–28, 30, 37–38, 57–58, 91, 95–97, 116, 127, 187–188, 203, 205–207, 211, 213, 215, 239
- antigen 116, 203, 205–206, 212–213, 252–255
- gene 205
- multimere(s) 203, 210, 212

WAS [Wiskott-Aldrich syndrome] 216
- protein gene 216, 218