A Novel Fibrinogen Mutation p.BβAla68Asp Causes an Inherited Dysfibrinogenemia

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Abstract

Objective Our study aimed to analyze the phenotype and genotype of a pedigree with inherited dysfibrinogenemia, and preliminarily elucidate the probable pathogenesis.

Methods The one-stage clotting method was used to test the fibrinogen activity (FIB:C), whereas immunoturbidimetry was performed to quantify the fibrinogen antigen (FIB:Ag). Furthermore, DNA sequence analysis was conducted to confirm the site of mutation. Conservation analysis and protein model analysis were performed using online bioinformatics software.

Results The FIB:C and FIB:Ag of the proband were 1.28 and 2.20 g/L, respectively. Gene analysis revealed a heterozygous c.293C > A (p.BβAla68Asp) mutation in *FGB*. Bioinformatics and modeling analysis suggested that the missense mutation could potentially have a deleterious effect on fibrinogen.

Conclusion The BβAla68Asp mutation in exon 2 of *FGB* may account for the reduced FIB:C levels observed in the pedigree. To our knowledge, this point mutation is the first report in the world.

Keywords
- *FGB*
- novel mutation
- dysfibrinogenemia
- genetic analysis

Introduction

Fibrinogen is a 340-kDa glycoprotein, composed of two sets of three polypeptide chains (Aα, Bβ, and γ) that are joined by multiple disulfide bonds.[1] The Aα, Bβ, and γ-chains are encoded by three related genes (*FGA*, *FGB*, *FGG*), respectively, which are located at the long arm of chromosome 4 (4q32.1).[2] These genes are composed of 6, 8, and 10 exons, respectively.[2] Fibrinogen exhibits a typical trinodal structure, with a central E-domain and two peripheral D domains.[3] The D domains consist of the COOH-terminal regions of the Bβ and γ chains, whereas the central E-domain is formed by amnio-terminal regions of three chains.[4] The COOH-terminal region of the Aα chain is positioned close to the central E-domain.[4] Owing to its critical role in the process of hemostasis, wherein active thrombin catalyzes the conversion of soluble fibrinogen into insoluble fibrin, fibrinogen is commonly referred to as coagulation factor I.[5] Furthermore, fibrinogen also plays an essential role in inflammation, wound healing, and other biological functions.[1]

Congenital fibrinogen deficiency is a rare disease that can be classified into two types of disorders: quantitative and qualitative. Quantitative disorders comprise afibrinogenemia (complete absence of functional and antigenic fibrinogen) and hypofibrinogenemia (proportional decrease of functional and antigenic fibrinogen).[6] Qualitative disorders

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include dysfibrinogenemia (normal antigenic fibrinogen and decreased functional fibrinogen levels) and hypodysfibrinogenemia (discrepant decrease of functional and antigenic fibrinogen levels).[6] The molecular abnormalities of congenital dysfibrinogenemia are frequently located in exon 2 of *FGA* and exon 8 of *FGG*.[7]

In this study, we have detected a heterozygous mutation in the *FGB* gene that resulted in the substitution of BβAla68Asp amino acid. To investigate the underlying pathogenic mechanism, we employed various methods including coagulation test, DNA sequence analysis, and bioinformatics analysis.

Materials and Methods

Materials

The proband is a 48-year-old woman, who was admitted to our hospital due to persistent symptoms of regurgitation and gastric distension for 6 months. She had a regular menstrual cycle with normal volume and no history of hemorrhage or thrombosis. Routine tests were conducted before undergoing gastroscopy and colonoscopy exams, which revealed normal results except for the coagulation test indicating prolonged thrombin time (TT) and low fibrinogen activity (FIB:C). The fibrinogen antigen (FIB:Ag), activated partial thromboplastin time (APTT), and prothrombin time (PT) were all within normal range. None of the nine members of the three-generation family (►Fig. 1A) had any history of abnormal bleeding or thrombosis. The present study selected 150 healthy individuals aged between 20 and 54 years as control groups to exclude the polymorphism. The control group's standard consisted of healthy individuals who exhibited no abnormality in hepatic and renal function and had no history of bleeding or thrombotic diseases. Our study was approved by the Ethics Committee of the First Hospital Affiliated of Wenzhou Medical University (China).

Coagulation Tests

After obtaining informed consent, the peripheral blood samples were extracted from the family. Routine coagulation tests such as PT, APTT, TT, and FIB:C were tested by a clotting assay. D-dimer (D-D) and fibrin degradation products (FDPs) were measured using immunoturbidimetry on the STAGO-STAR analyzer (Diagnostica Stago, Asnières-Sur-Seine, France), while the level of FIB:Ag was determined using immunoturbidimetry on the Beckman Coulter AU5800 Chemistry Analyzer (Beckman Coulter, California, United States). All operations were performed according to the manufacturers' protocols.

DNA Sequence Analysis

Following informed consent, genomic DNA was extracted from blood samples of family members following the manufacturer's protocol (Tiangen, Beijing, China). Twenty-six pairs of primers for all fibrinogen genes (*FGA*, *FGB*, and *FGG*), including all exons and flanking regions, were designed based on the published sequence (GenBank accession

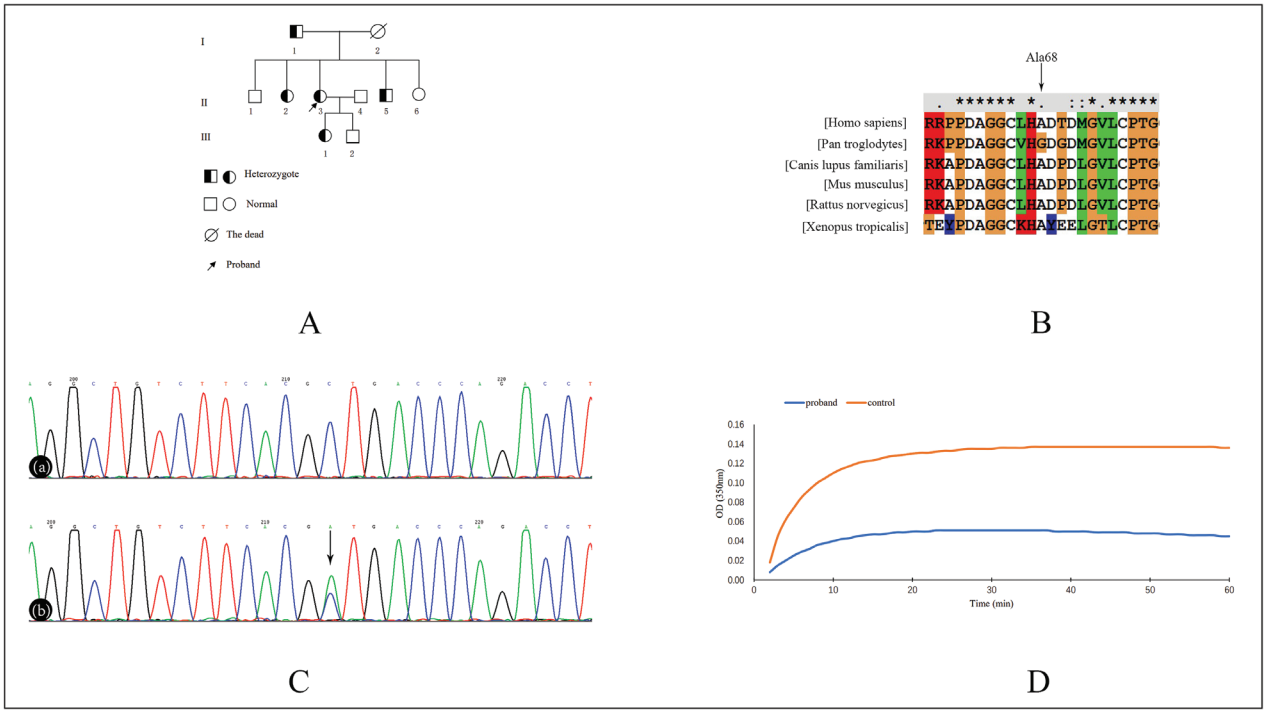

Fig. 1 (**A**) Pedigree chart of the congenital dysfibrinogenemia family. (**B**) Conservation analysis diagram. "*" means conserved; ":" means highly conserved; "." means lowly conserved. The Ala68 is lowly conserved between six homologous species after the analysis of software ClustalX-2.1-win. (**C**) The sequence diagram of c.293C > A. Part (a) is the forward sequencing of wild type; Part (b) is the forward sequencing of heterozygous c.293C > A. The position of the mutational base is indicated with an arrow. (**D**) The curves of thrombin-catalyzed fibrin polymerization. The blue line showed the variation trend of the proband in thrombin-catalyzed fibrin polymerization; the orange line showed the variation trend of the healthy control in thrombin-catalyzed fibrin polymerization. The curve of the proband showed a decreased slope of thrombin-catalyzed fibrin polymerization and decreased maximum aggregation rate.

numbers: M64982, M64983, and M10004). The polymerase chain reaction (PCR) was amplified under standard conditions on Applied Biosystem Thermal Cycler 2720 (ABI, Foster City, California, United States). The PCR products were purified and sequenced by Sunny Biotechnology Corporation (Shanghai, China). The sequencing results were compared with the sequence published by the NCBI gene bank and mutation sites were identified using the software Chromas.

Thrombin-Catalyzed Fibrin Polymerization

First, the concentration of plasma fibrinogen in both the family members and the control groups was diluted to 0.5 g/L through the process of blood coagulation analysis dilution (Barbital sodium-buffered saline solution, Diagnostica Stago). Subsequently, 140 µL of the diluted samples and 10 µL of human calcium thrombin (Diagnostica Stago) were added into a 96-well plate to maintain the concentration at 1 NIH U/mL. The control groups were the first to be assessed, and the increased in turbidity at 350 nm was continuously monitored every second for 60 seconds using the multi-well plate reader (Thermo Scientific Varioskan Flash).

Conservation Analysis and Bioinformatics Analysis

Conservation analysis was conducted using multisequence alignment software ClustalX-2.1-win (Science Foundation Ireland) with six homologous species as listed in ►Fig. 1B (HomoloGene, http://www.ncbi.nlm.nih.gov/homologene).

To assess potential adverse effects on fibrinogen function, four online bioinformatics software (MutationTaster, PolyPhen-2, PROVEAN, and SIFT) were utilized. Besides, to examine the molecular structure of the mutation, a mutant protein model was constructed using Swiss-Pdb Viewer 4.0.1 and Protein Interactions Calculator programs (PIC, http://pic.mbu.iisc.ernet.in) based on the three-dimensional (3D) structure in the Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do, PDB ID: 3GHG).

Results

Coagulation Tests

The phenotypic characterizations of the proband and her family members are shown in ►Table 1. The APTT, PT, D-D, and FDP of the proband were within the normal range, although her TT value was slightly elevated at 22.5 seconds (normal range: 14.0–20.0 seconds). The TT levels of her father, sister, younger brother, and daughter showed varying degrees of increased levels. In addition, the proband's FIB:Ag level was within the normal range, while her FIB:C level was decreased to 1.28 g/L. Her father, sister, younger brother, and sister also exhibited decreased FIB:C/FIB:Ag levels, with values less than 0.7. On the other hand, the results of her brother, spouse, younger sister, and son presented normal results.

DNA Sequence Analysis

According to the results of sequence analysis, the presence of a heterozygous c.293C > A point mutation in exon 2 of *FGB* has led to the emergence of the p.BβAla68Asp missense

Table 1 Phenotypic and genetic analysis of the pedigree of family members

Family	PT (s)	APTT (s)	TT (s)	D-D (mg/L)	FDP (mg/L)	FIB:C (g/L)	FIB:Ag (g/L)	FIB:C/FIB:Ag	c.293C > A
Proband (II_3)	14.2	35.4	22.5	0.40	1.20	1.28	2.20	0.58	Heterozygote
Father (I_1)	13.9	36.1	23.4	0.45	1.70	1.15	2.11	0.55	Heterozygote
Brother (II_1)	14.1	35.5	17.5	0.50	1.30	2.38	2.67	0.89	Wild type
Sister (II_2)	12.6	39.2	24.6	0.37	1.80	1.22	2.28	0.54	Heterozygote
Spouse (II_4)	13.5	35.2	18.3	0.41	2.22	2.73	2.84	0.96	Wild type
Younger brother (II_5)	12.0	37.3	23.7	0.42	2.07	1.43	2.18	0.66	Heterozygote
Younger sister (II_6)	13.2	38.5	16.3	0.31	1.56	2.55	2.67	0.96	Wild type
Daughter (III_1)	13.5	37.0	23.9	0.33	1.70	1.17	2.03	0.58	Heterozygote
Son (III_2)	12.2	36.7	15.5	0.35	2.88	2.81	3.05	0.92	Wild type
Reference range	11.5–14.6	29.0–43.0	14.0–20.0	0.00–0.50	0.00–5.00	2.00–4.00	2.00–4.00	>0.7	

Table 2 Bioinformatics prediction results of Ala68Asp mutation

Software	Prediction results	Score
MutationTaster	Disease causing	1.000
PolyPhen-2	Possible damaging	0.859
PROVEAN	Tolerable	−1.740
SIFT	Tolerable	0.056

Notes: The meanings of scores are as followed: (1) MutationTaster—a result closer to 1.000 indicates higher confidence in the prediction. (2) PolyPhen-2—on a scale from 0.000 to 1.000, the closer to 0, the less harmful, and the closer to 1, the more harmful. (3) PROVEAN—the predefined threshold of score is −2.5, the score ≤ −2.5 (deleterious), > − 2.5 (neutral). (4) SIFT—range from 0 to 1; the score less than 0.05 means damaging; others show tolerated.

mutation (as illustrated in ►Fig. 1C). The DNA sequence analysis of the pedigree has been presented in ►Table 1, revealing that the heterozygous BβAla68Asp mutation was apparent in the proband and her father, sister, younger brother, and daughter. Conversely, her brother, spouse, younger sister, and son were identified as wild types. After consulting the related database, genome aggregation database (http://gnomad-sg.org/), and polymorphism database (http://www.ncbi.nlm.nih.gov/snp/), it was observed that the mutation of c.293C > A (p.BβAla68Asp) of *FGB* has not been previously reported.

Thrombin-Catalyzed Fibrin Polymerization

Upon the addition of human calcium thrombin, the proband demonstrated a reduced thrombin-catalyzed fibrin polymerization slope and a decreased maximal aggregation rate in comparison to the control group (►Fig. 1D). The slope and maximal aggregation rate represent the velocity of aggregation. The observed reduction in slope and maximal aggregation rate signifies impaired functionality of fibrinogen in the proband.

Conservation Analysis and Bioinformatics Analysis

According to the four software, namely MutationTaster, PolyPhen-2, PROVEAN, and SIFT, the predicted results of the BβAla68Asp were "disease-causing," "possible damaging," "tolerable," and "tolerable," respectively (►Table 2). Additionally, the software ClustalX-2.1-win has demonstrated that Ala68 is conserved among six homologous species (►Fig. 1B). In the wild type, Ala68 is a nonpolar amino acid, which formed a hydrogen bond with the positive-charged polar amino acid His67. However, the substitution of nonpolar Ala68 with negatively charged polar Asp68 leads to the formation of an additional hydrogen bond between Asp68 and Asp69, which results in the extension of the side chain of the amino acid, ultimately affecting the stability of the spatial structure (►Fig. 2).

Discussion

The clinical presentation of congenital fibrinogen deficiency is diverse and includes various symptoms such as asymptomatic cases, bleeding, thrombosis, recurrent miscarriages in women, and so on.[8] In contrast to the bleeding tendency observed in afibrinogenemia, approximately 55% of patients with dysfibrinogenemia remain asymptomatic, while 25% exhibit a mild bleeding tendency, and nearly 20% are at risk of developing arteriovenous thrombosis.[9] The mechanisms for the risk of thrombosis in patients with dysfibrinogenemia include increased thrombin levels due to defective binding with fibrinogen (fibrinogens Malmo, Naples, New York I, Pamplona II, and Poitiers), altered structure, and stability of fibrinogen, as well as the impaired function of the tissue plasminogen activator-medicated fibrinolysis (fibrinogens Argenteuil, Chapel Hill III, Date, New York I, Nijmegen, Pamplona II, and Paris V).[10,11] Previous research has demonstrated that specific fibrinogen fragments in the E region, namely, Aα27–50 and Bβ15–42 portions, as well as the BβAla68 site participate in interaction with thrombin. Thrombophilic dysfibrinogenemia is characterized by defective non-substrate thrombin binding, particularly in fibrinogen New York I (des Bβ9–72) and fibrinogen Naples (BβAla68Thr). In the study conducted by Al-Mondhiry et al, a 54-year-old female possessing the fibrinogen New York I presented with a 23-year history of repeated life-threatening thrombosis.[12] Fibrinogen New York I lacks a

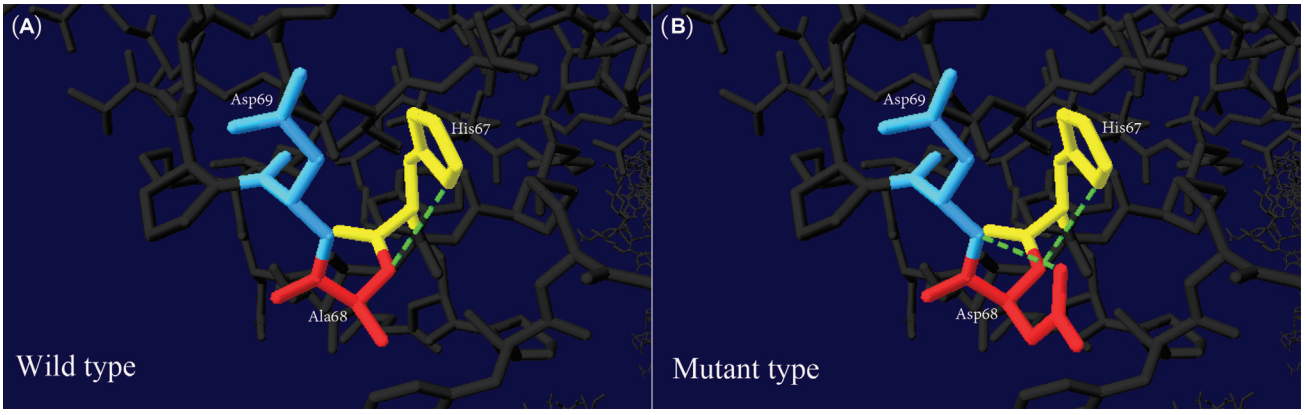

Fig. 2 Model analysis diagrams. (**A**) Wild type. (**B**) Mutant type. The green line shows the hydrogen binds; the yellow amino acids are His67; the red amino acids are Ala68 or Asp68; the blue amino acids are Asp69. After the substitution of Ala68 to Asp68, there formed an additional hydrogen bond between Asp68 and Asp69 and the side chain of Asp68 was prolonged.

major portion of the binding between fibrinogen and thrombin, which is associated with recurrent deep venous thrombosis (DVT) and fatal pulmonary embolism.[13]

The mutations in *FGB* are less common since Bβ-chain is regarded as a rate-limiting factor in the production of fibrinogen hexamer within the liver. Missense mutations in *FGB* are primarily observed in the highly conserved C-terminal domain of the β-chain.[14] In the present investigation, we examined a pedigree afflicted with congenital fibrinogen deficiency. The proband carried a heterozygous mutation c.293C > A in exon 2 of the *FGB* gene, leading to the BβAla68Asp amino acid substitution. The proband presented normal FIB:Ag but low FIB:C, thus was diagnosed with dysfibrinogenemia. Thrombin-catalyzed fibrin polymerization showed a decreasing trend for impaired fibrinogen aggregation. According to the bioinformatics analysis, the BβAla68Asp mutation was identified as a "disease-causing" mutation. Furthermore, the model analysis revealed an additional hydrogen bond due to the missense mutation. Hence, it is speculated that the BβAla68Asp heterozygous mutation could negatively affect both the structure and function of fibrinogen.

Ceznerová et al[15] have reported that the BβAla68 is located in the sequence of BβHis67-Pro70, which forms a β-turn with high conservation. Replacement of BβAla68 with BβAsp68 introduced a natural hydrogen bond which altered the partial conformation of fibrinogen. Multiple alignments had indicated that BβAla68 is conserved among various species, indicating its importance for fibrinogen structure. According to the findings of Koopman et al, the mutation of BβAla68Thr (fibrinogen Naples) reduced the interaction of thrombin to fibrinogen and impaired the release of fibrinopeptides catalyzed by thrombin.[13,16,17] In the central E domain, BβAla68 leads to the hydrophobic interior of the interface. The mutation of BβAla68Thr led to the replacement of the nonpolar group with a polar one, leading to steric problems and disrupting the fibrinogen–thrombin binding. In addition, Mullin et al[18] have discovered that the BβAla68Thr mutation led to the subtle conformation changes, affecting the enzymatic phase of the polymerization process and resulting in decreased lateral aggregation.

The study by Koopman et al[16] presented a case of consanguineous marriage, wherein three homozygotes for the BβAla68Thr mutation had a severe medical history that involved both arterial and venous thrombosis. Two of the individuals suffered from strokes at the young age of 21 and 25 years, primarily due to thrombotic occlusion of the artery, and the third individual developed deep vein thrombosis. Similarly, Yoshida et al[19] reported a 17-year-old Japanese male with homozygous BβAla68Thr mutation, who suffered from superior sagittal sinus thrombosis, bilateral pulmonary embolisms, and DVT in the bilateral lower extremities. These findings indicate that the homozygous BβAla68Thr mutation could be a significant contributor to thrombophilia. One possible mechanism could be the regulation of active thrombin in the plasma through thrombin binding to the fibrinogen, which helps sequester thrombin and decrease the feedback reaction that enhances thrombin production and has been referred to as "antithrombin I."[20] However, reduced thrombin binding to fibrinogen could lead to an increase in active thrombin levels, which could eventually cause excessive coagulation or even thrombosis.[8,16]

In a study by Ceznerová, a 17-year-old female presented with mild posttraumatic cutaneous bleeding.[15] After conducting DNA sequencing, a heterozygous mutation was found in position c.292G > T in exon 2 of the *FGB* gene, which resulted in the amino acid substitution BβAla68Ser. This mutation was named fibrinogen Svetec. Although the patient's brother also showed mild bleeding, he did not have the BβAla68Ser mutation, leading Ceznerová to believe that their bleeding tendency may not be linked to the said mutation.[15]

The BβAla68Asp mutation has a similar effect on the amino acid site as the mutation found in fibrinogen Naples (BβAla68Thr), where both threonine and aspartic acid are polar. This suggests that both alterations may result in similar mechanisms for thrombosis and decreased polymerization. Homozygous individuals with the BβAla68Asp mutation may experience thrombosis due to impaired thrombin binding to fibrinogen, while heterozygous carriers may exhibit asymptomatic.[13] However, heterozygous carriers with the BβAla68Asp mutation may increase the risk of thrombosis when combined with other thrombogenic factors such as malignancy, obesity, trauma, and pregnancy, among others.

In conclusion, we discovered a novel heterozygous mutation in exon 2 of the *FGB* gene (p.BβAla68Asp) caused the dysfibrinogenemia. We hope to provide insights into the early detection and treatment of thrombosis associated with *FGB* gene mutations.

What is known about this topic?

Congenital fibrinogen deficiency is a rare disease that presents with varying clinical manifestations. Of those diagnosed with dysfibrinogenemia, more than half (55%) are always asymptomatic, whereas approximately 25% exhibits a mild bleeding tendency, with the remaining approximately 20% having a risk for thrombosis. The BβAla68Thr mutation is one of the most common mutations associated with thrombosis due to the defective thrombin binding to fibrinogen.

What does this paper add?

We probed into a pedigree with congenital fibrinogen deficiency, found a novel BβAla68Asp missense mutation, and preliminarily analyzed the possible pathogenesis. Based on our research, the BβAla68Asp mutation possibly influences the interaction of thrombin between fibrinogens, leading to higher levels of active thrombin in plasma. The homozygous mutation maybe associated with thrombosis, while heterozygous carriers might be asymptomatic. Additionally, the mutation may decline fibrinogen lateral aggregation.

Ethical Approval

Our study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (China).

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Erratum: In this article, the letter "ß" has been replaced by the Greek letter "Beta" (10.1055/a-2218-6919).