„CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST

DOCTORAL SCHOOL

FIELD OF MEDICINE

MOLECULAR, BIOLOGICAL AND CLINICAL RISK FACTORS ANALYSIS IN SEVERE HEMOPHILIA A AND B: THEIR IMPACT ON THE CHOICE OF THERAPY AND EVOLUTION

PhD THESIS SUMARY

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INTRODUCTION

Hemophilias represent hereditary coagulopathies characterized by prolonged bleeding, occurring spontaneously or after trauma/surgery. They are diseases with X-linked transmission that manifest themselves mainly in males. The pathognomonic element is represented by joint bleeding (hemarthrosis) [1-2]. It has a repetitive character, leading to locomotor disabilities and decreased quality of life. The evolution of the disease is heterogeneous, being influenced by a series of factors: the level of factor VIII/IX activity, the bleeding phenotype, the type of causative mutation and other coexisting mutations [3].

During the last decades, great progress has been made both in the diagnosis and monitoring of hemophilias, as well as in their treatment. Paraclinical evaluation methods have evolved at the same pace, offering the possibility of monitoring treatment response. At the same time, new tools were developed to evaluate outcomes and therapeutic effectiveness. These tools are useful in assessing both health status and degree of disability, as well as the impact of disease on functionality, activity and participation.

The purpose of this paper is to analyse adult patients diagnosed with severe hemophilia A and B in the evidence of Fundeni Clinical Institute, a reference center in this field. The retrospective analysis of enrolled patients sought to demonstrate the impact of certain clinical characteristics on the disease phenotype, joint status, degree of activity and functional independence and quality of life. At the same time, we demonstrated the usefulness of genetic testing in hemophilia as a prognostic factor of the disease severity and evolution and as a risk factor for inhibitors development. This information is useful in medical practice, especially in guiding prophylactic therapy and monitoring patients.

I believe that real-life experience plays a significant role in identifying people with a more severe bleeding phenotype and adapting their therapy in such ways as to preserve joint health for as long as possible and to prevent the onset of locomotor disabilities. A comprehensive, multidisciplinary approach is essential in improving the quality of life and ensuring functional independence.

GENERAL PART

Chapter 1. Generalies in hemophilia

Hemophilia is a rare X-linked hereditary coagulation disorder [2], caused by the deficiency of a coagulation factor (VIII in hemophilia A, IX in hemophilia B). Hemophilia A occurs with an incidence of 1-2 cases per 10,000 male births and represents ~80-85% of cases [2, 4]. The disease is caused by an abnormality at the level of the specific gene (F8 or F9) on the long arm of the X chromosome, causing the deficient synthesis of factor VIII, respectively IX protein [2, 4-6].

Depending on the residual level of factor activity, hemophilias are classified as severe (factor <1%), moderate (factor 1-5%) and mild (factor >5%) [4]. The clinical manifestations are represented by profuse and prolonged bleeding that can occur spontaneously, early in life (in severe forms) or later, after trauma or surgical interventions (moderate and mild forms) [4, 7].

Hemarthrosis is the most frequent manifestation in hemophilia (70-80%), followed by muscle hematomas (10-25%) [4, 8]. Other bleeding can occur at the oropharyngeal, urinary, digestive or intracranial level. The latter, although very rare in frequency, have an increased mortality in the absence of prompt diagnosis and treatment [9].

The clinical manifestations are heterogeneous and vary from one individual to another, depending on the bleeding phenotype, F8/F9 mutations [5], mutations of the regulatory genes of the inflammatory/immune system [10], as well as the coexistence of other disorders of hemostasis (thrombophilias) [5, 11]. Identifying the bleeding pattern has an important role in choosing the optimal dose and moment to initiate prophylaxis.

The diagnosis can be established early in the prenatal period, through the genetic analysis of the product of conception, after genetic counseling and informed consents [12]. Postpartum, the laboratory tests that establish the diagnosis of hemophilia are prolonged APTT and low level of factor VIII or IX activity measured by one-stage or chromogenic tests [13]. The same hemostasis tests are used later, in order to monitor the response to the administered treatment (recovery and pharmacokinetic tests).

Inhibitor testing is done periodically (from the initiation of prophylaxis) or whenever we suspect a decrease in the therapy effectiveness in preventing/treating bleeding. The testing is done by Bethesda or Nijmegen methods and uses the principle of measuring the activity of the factor in serial dilutions from the mixture of patient plasma with normal plasma incubated for 2 hours at 37°C [14]. A result above 0.6 BU is considered positive.

Genetic testing in hemophilia is useful in establishing the genotype-phenotype relationship, in predicting the risk of inhibitors and the response to immune tolerance induction therapy and in confirming the carrier status [13]. Up to now ~3500 F8 mutations have been identified and over 1200 mutations of F9 gene [4, 15]. Approximately half of severe hemophilia A cases are determined by inversions of Introns 22 and 1 [4, 15]. The genetic testing protocol is dictated by the type and severity of hemophilia and family history. In severe hemophilia A, testing begins with Int22 and Int1 inversions; in negative severe HA cases, in moderate and mild hemophilia A and all HB patients gene sequencing is required.

Chapter 2. Therapeutic strategies in hemophilia

2.1. History

Hemophilia treatment has evolved significantly, from the administration of fresh plasma or cryoprecipitate and freeze-dried plasma concentrates (with the disadvantage of transmitting HCV, HBV or HIV viral infections) [16], to recombinant products obtained using complex viral inactivation techniques [16]. New factor concentrates have recently been developed, with extended half-life, to help patients with poor venous access, low adherence or the need for intensive prophylaxis [17].

For inhibitor patients who no longer respond to the administration of factor VIII/IX, bypass agents (recombinant activated factor VII and activated prothrombin complex) have become available [16]. These two products made possible for these patients to undergo major surgical interventions (especially orthopedic), previously contraindicated do to bleeding risks [18]. Immune tolerance induction therapy (ITI) remains the only way to eradicate inhibitors [17, 19], its success depending on the

inhibitor titre (historical peak level and pre-ITI level), time interval between inhibitor detection and the start of ITI, genetic mutation in F8/F9 and patient compliance.

Recent research has made available to hemophiliacs new, non-substitutive therapies, which have the advantage of subcutaneous administration, at intervals of 1-2 or 4 weeks, some being effective both in HA and HB, with or without inhibitors [19].

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2.2. Therapeutic options in hemophilia

In the management of hemophilia there are two therapeutic strategies: on demand treatment, in case of bleeding and prophylactic therapy. Episodic treatment aims to stop the hemorrhagic event as soon as possible, by administering the optimal doses of the factor in the first 2 hours after the onset of bleeding [20]. Prophylaxis is the standard treatment in hemophilia, recommended by international guidelines, to prevent bleeding at any time, improve the quality of life, preserve joint status and avoid long-term complications [13].

Depending on the time of initiation, prophylaxis is primary, secondary or tertiary [2, 13], all of which have clear benefits compared to episodic therapy, as evidenced by the extensive studies carried out. Prophylaxis can be initiated in small doses with increasing frequency (escalated regimen) or in large doses (intensive regimen). The differences lie in the risks of bleeding (higher in the low-dose regimen) and the inhibitor development (increased in the intensive regimen [13]). Regardless of the regimen chosen and the time of initiation, international guidelines recommend the individualization of therapy according to bleeding phenotype, joint status, pharmacokinetics and patient preferences [13, 21]. Other factors to consider are age, venous access, physical activity level and the presence of target joints.

Chapter 3. Chronic complications in hemophilia

3.1. Disease complications

The main cause of morbidity and disability in hemophilia is chronic hemophilic arthropathy, a consequence of repeated hemarthroses [22]. From the first episodes of joint bleeding, structural changes appear in the synovium [22], cartilage and bone tissue [13]. They are triggered by the formation of toxic iron radicals (resulting from

blood degradation), the release of pro-inflammatory cytokines and the direct harmful action of blood. Chronic hemophilic arthropathy is often multi-articular and affects especially the knees, elbows and ankles, interfering with daily activities [2].

In advanced stages of arthropathy, following loss of mobility and antalgic flexion, extensor muscle atrophy sets in. Each episode of joint bleeding should be followed by recovery sessions and physical therapy.

Another complication is represented by the decrease in bone mineral density and the increased risk of fractures [23]. They can appear spontaneously or after trauma.

Isufficient treatement of muscle hematomas can lead to the formation of pseudotumors [24], encapsulated formations that grow slowly and can cause pain, tissue necrosis or vascular-nervous damage.

3.2. Treatment complications

The main complications of hemophilia therapy are viral infections with hepatitis B and C virus or HIV infection and the development of inhibitors.

As for viral infections, they are due to the administration of blood products (plasma or cryoprecipitate) or lyophilized plasma concentrates [16].

The inhibitors development (allo-antibodies against the administered factor) remains a serious complication of hemophilia therapy [2, 5, 19]. It occurs at a rate of ~25-30% among people with severe HA and 3-5% in severe HB. Approximately 80% of inhibitors appear in the first 10-20 days of exposure, therefore careful and frequent monitoring is recommended [25].

The mechanism behind these antibodies formation is a complex one involving mediators of the immune system, cytokines and cells specialized in defense (B and T_H lymphocytes, APC antigen-presenting cells) [26]. The final result is the activation and differentiation of B lymphocytes and the production of polyclonal antibodies of the IgG class against exogenous factor VIII or IX [27].

The risk factors involved in the development of inhibitors are classified in [28]: a) non-modifiable factors (type of F8/F9 mutation, family history, severity of hemophilia, HLA genotype, mutations in IL10, TNFα, $CTLA_4$ genes) [5, 27, 29-32]; b) modifiable factors (type of factor concentrate, age at initiation of therapy, number of exposure days, intensity of treatment) [27, 33-34] and c) danger signals (infections,

vaccinations, intensive treatments) [35]. Considering that people with inhibitors have a poorer quality of life and increased morbidity and mortality compared to those without inhibitors, it becomes increasingly important to identify these predisposing factors and adopt preventive strategies for high-risk patients [28].

Chapter 4. Evaluation of outcomes in hemophilia

In the context of the availability of a wide range of therapeutic options in hemophilia, it was necessary to implement outcome evaluation tools [36]. The classic evaluation methods, using laboratory tests or annual bleeding rates, are not sufficient, especially since the aim of new therapies is "zero bleeding" and the new drugs are difficult to monitor [36]. Also, the bleeding rate does not provide information regarding the impact on functionality and activity [37] and many subclinical bleeding events are not reported. In order to optimize the therapeutic schemes, to justify the use of resources, generic and specific methods were introduced to measure the state of joint health (clinical and imaging examination), the degree of activity and functional independence and the quality of life [13, 36] The physical examination uses scores to quantify the degree of joint damage: the Gilbert score and the Hemophilia Joint Health Score [37]. The imaging evaluation completes the clinical examination and is performed using ultrasound (for early joint changes) [13], radiography (for bone structure changes) [38] or MRI (the gold standard in joint evaluation) [36].

In order to evaluate activity and participation, a series of questionnaires were developed: Hemophilia Activity List (filled by the patient) [39] and Functional Independence Score in Hemophilia (filled by the doctor after observing the patient) [40]. Both scores reflect the patients possibilities to perform certain activities that involve the function of the arms and legs, thus measuring the impact of hemophilia on the patients' functional abilities.

II. ORIGINAL PART

Chapter 5: Hypothesis and general objectives

Classification of severity in hemophilia is no longer done solely on the basis of factor VIII or IX plasma level, as it has been shown that there is a heterogeneity of the bleeding phenotype among hemophiliacs. As a result, increased attention is paid to the identification of the factors that influence the severity of manifestations in hemophilia: genetic mutation F8/F9, joint status, age of first bleeding, frequency of bleeding, degree of physical activity, mutations of other genes involved in hemostasis (Factor V Leiden, Prothrombin) or in the regulation of the inflammatory response.

The development of allo-antibodies against exogenous factor VIII or IX remains the most feared complication of hemophilia treatment, with a negative impact on joint status, bleeding management and quality of life. The predisposing factors involved in the occurrence of inhibitors are genetic or environmental. The identification of these factors gives the doctor the opportunity to anticipate the appearance of this complication and intervene on the therapeutic approach of the patient at risk.

Another aspect that must be taken into consideration is that of monitoring outcomes in hemophilia, more precisely the identification of tools for evaluating the disease evolution and the treatment response. In the context of intensive and personalized prophylactic regimens (targeting a trough level of at least 3-5% and zero bleeding), of non-factor therapies, the usual monitoring methods (annual bleeding rate or FVIII/IX plasma level) they are not sufficient. New instruments must be used to measure the overall state of health, the degree of activity, participation and integration in social life, functional independence and quality of life.

In this context, the present doctoral research aims to study the clinical, biological and molecular characteristics and their impact on disease evolution and in choosing the optimal therapy. The paper aims to demonstrate the correlation between the bleeding phenotype and the degree of joint damage, the age of the first clinical manifestations and diagnosis, the genetic mutation, the presence of comorbidities; the relationship between the type of prophylaxis and the degree of arthropathy, as well as the benefits of prophylaxis on joint functionality and activity by using the HAL and

FISH questionnaires. In addition, this thesis aimed to demonstrate the impact of hemophilia, the type of treatment and comorbidities on the quality of life. It is necessary to implement genetic testing in the investigation of people with hemophilia due to its role, not only in genetic counseling and prenatal diagnosis, but also in disease management (some mutations are associated with a significant risk of developing inhibitors).

Statistical analysis

For the statistical analysis of the study data, we used the IBM SPSS Statistics for Windows software, Version 29.0. (30-day trial version) Armonk, NY: IBM Corp. We also performed a descriptive analysis of frequencies using Office 365. We presented the nominal data as absolute frequency and percentage, and the continuous variables were expressed by mean, median, standard deviation, minimum and maximum, Q25 (percentile 25), Q75 (75th percentile) and IQR (interquartile range).

We analysed the association between categorical variables using crosstabs and the $\chi 2$ (chi-square) test. If the results of the chi-square test were sufficiently altered that they could not be taken into account we used Fisher's exact test. To compare the means according to the dichotomous variables in the study, we used the t-test for independent samples. To compare three or more group means where the participants are the same in each group, we used the ANOVA test. A p-value of less than 0.05 was considered significant.

Chapter 6 - Study 1: Clinical features of hemophilia patients analysis and their impact on evolution and treatment

In this study, we aimed to identify the demographic and clinical characteristics of hemophilia patients and establish the association between disease characteristics, comorbidities and evolution and therapeutic implications.

6.1. Materials and methods

We performed a retrospective, observational, cohort study, which included a number of 201 cases of adults diagnosed with severe hemophilia A and B (Factor VIII/IX <1%) from Fundeni Clinical Institute.

6.1.1. The observation period

Patients were followed from 2018 to 2023 or until death.

6.1.2. The investigated parameters

Data were collected from admission records, the hospital's electronic database, patient history and documentation. The main data collected were: the age at enrollment, height, weight, family history, the age of the first hemorrhagic manifestations and the first joint bleeding, the age at which the diagnosis of hemophilia was established; virological status (HCV, HBV, HIV), comorbidities, number of target joints, presence of inhibitors, venous access, type of therapy at enrollment, age at initiation of prophylaxis, dose of prophylaxis, average annual joint bleeding rate and history of orthopedic interventions.

6.2. Results

The study group included 201 patients with severe hemophilia, 89.6% type A and 10.4% type B, with an average age of 39.89 years (18-80 years). 72.1% of patients were physically active. 55.2% of the subjects had HCV infection secondary to transfusions of blood products (incidence increasing with age, 95% of those over 60 being HCV+), but only 25.9% received antiviral treatment. In terms of phenotype severity, 67.7% had a severe bleeding phenotype of bleeding.

In our group of patients, 43.3% report the absence of hemophilia cases in family.

The age at the onset of symptoms was on average 6.12 months (SD±3.295), the first joint bleeding occurred on average at 12.82 (SD±4.129). The hemophilia diagnosis was established on average at 21.58 months (SD±20.573), relatively late compared to the data reported in the literature [41].

Regarding the treatment followed, 21.4% of patients received factor only on demand and 78.6% were in prophylactic treatment (16.9% continuous, 61.7% intermittent). Prophylaxis was initiated late, on average at 33.91 years (9-68 years).

Most patients were diagnosed in the context of post-traumatic hematomas (34%) or bleeding in the oral cavity (20%). Hemarthrosis was a reason for presenting to the doctor and for diagnosis in 15% of the patients. Only 4% of patients were diagnosed from birth due to the declared antecedents of hemophilia in family.

The mean number of target joints was 3.23 (SD 1.023), the value being significantly higher in those with severe phenotype (3.58) than those with moderate phenotype (2.49) ($t(199)=8.1$, $p< .00001$).

Using the t-test for independent samples we demonstrated the absence of a significant difference between those with a moderate phenotype and those with a severe phenotype in terms of age at diagnosis ($p=0.360$), but we found significant differences ($p<0.001$) in terms of the age of onset of first symptoms and age of first joint bleeding. In those with a severe phenotype, the age of onset of first symptoms and age of first joint bleeding were significantly lower than in those with a moderate phenotype.

I tried to demonstrate the existence of an association between joint status and the presence of B, C, HIV co-infections. Among these, only the presence of HCV hepatitis made a significant difference (and the group of HIV+ and HBV+ patients beeing small), in the sense that HCV-infected patients had more affected joints than those without infection (figure 6.1).

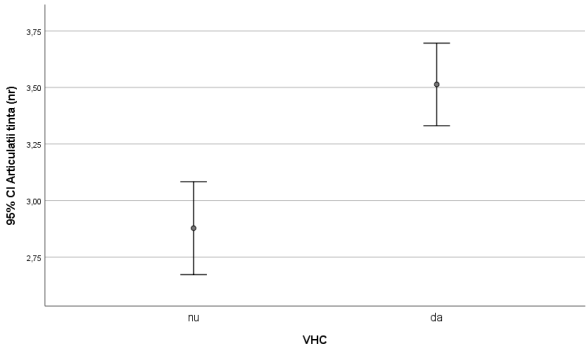

Figure 6.1 Distribution of the number of target joints by HCV status

The proportion of patients with HCV and with a severe phenotype is significantly higher (78.4%) than that of patients without HCV and with a severe phenotype (54.4%) ($\chi2=13.012$; $df=1$; $p<0.001$; Cramer's $V=0.254$).

Using the chi-square test, I was able to demonstrate that orthopedic interventions are encountered with a higher proportion of patients with severe phenotype (41.9%), compared to patients with a milder phenotype who have orthopedic interventions in a proportion of 21.5% ($\chi 2=7.990$; df=1; p=0.005). Similarly, the late age of onset of prophylaxis had a negative impact on joint status, with these patients more frequently requiring orthopedic surgery (p 0.002).

Orthopedic interventions in those who received prophylaxis from a younger age (9-17 and 18-29 years) are represented by synovectomies (in 3 out of 3 cases, respectively 8 out of 32), while in people with late onset of prophylaxis total arthroplasties (36%) and fractures (8%) favored by osteoporosis predominate.

6.3 Conclusions

1. In our study group, the prevalence of severe HA was 89.6% and of severe HB only 10.4%. 43.3% of hemophilia cases were classified as de novo;

2. In our group of patients, although the factor deficiency was severe (below 1%), the diagnosis was established late, on average at 21.58 months;

3. 67.7% of the evaluated patients present a severe phenotype of the disease, characterized by an earlier onset of bleeding, a higher annual rate of hemarthrosis and a greater number of target joints. This category of patients should benefit from more intensive treatment;

4. Prophylaxis was introduced late, on average 33.91 years (9-68 years);

5. More than 30% of patients over 60 years of age have undergone at least one joint replacement intervention. Synovectomy was a temporary solution to reduce bleeding for ~16% of 18- to 35-year-olds;

6. Hepatitis C virus (HCV) infection is found in ~55% of enrolled patients, the incidence increasing with age (reaching 95% in those over 60). In the studied group, we observed a negative effect of HCV co-infection on joint status (a higher rate of bleeding and a higher number of affected joints).

Chapter 7 - Study 2: Biological risk factors analysis – the utility of pharmacokinetics in personalizing therapy

Prophylactic therapy with factor VIII or IX concentrates is the standard of treatment in severe (FVIII<1%) or moderate hemophilia A and B with severe bleeding phenotype. It aims to maintain a trough level high enough to prevent bleedings. A number of factors can influence the dose and frequency of factor administration. Among them we mention: the pharmacokinetic profile, the bleeding phenotype, the joint status, the level of physical activity, the adherence to the treatment, the venous access and the type of factor concentrate used (SHL or EHL).

7.1. Obiectives

- Application of pharmacokinetic analysis in the individualization of the prophylactic regimen
- Demonstrating the benefits of personalized therapy using pharmacokinetic analysis

7.2. Materials and methods

We conducted a prospective, observational, interventional study that included 30 adult patients with severe HA (Fact VIII<1%) from Fundeni Clinical Institute, who were previously on a standard prophylactic regimen (dose/kg) and in whom it was necessary to customize the prophylactic therapy. The patients were followed between 2019-2023, with a follow-up of a minimum of 1 year and a maximum of 4 years.

7.2.1. The investigated parameters

Data were collected from patient observation sheets, the hospital's electronic database, patient diaries and medical history.

The following parameters were followed:

- the annual joint bleeding rate (AjBR) before and 1 year after therapy personalization;

-pharmacokinetic analysis and trough levels during both treatment schemes;

-the number of patients who achieved "zero bleeding" with both treatment schemes.

7.2.2. Methods

One-stage or chromogenic tests were used, according to the specifications in the summary of product characteristics.

For the standard FVIII products, we took blood samples (in tubes with sodium citrate) before the administration of the factor, at 1 hour, 24h and 48h (before the next administration - the trough level), and for FIX, 2 samples were needed at 24h interval (between 24-36h and 48-60h after injection). For the products with extended half-life (EHL) of FVIII we added a sample collected at 60-84h, and for FIX a sample at 5-12 days. The pharmacokinetic curves were made with the help of online programs: Wapps-hemo, myPKfit®.

7.3. Results

The main reason for personalization of prophylactic therapy was the need to ensure increased protection against bleeding (73.3% of cases, 22/30). In 16.7% of patients (5/30) the therapeutic scheme was changed due to the very difficult venous approach; in 6.7% (2 out of 30) low adherence to the administrations 3 times/week required the adjustment of the administration frequency.

We observed that the personalized therapy provided a significantly higher trough level (4.7) compared to the previous scheme (2.1) (figure 7.1).

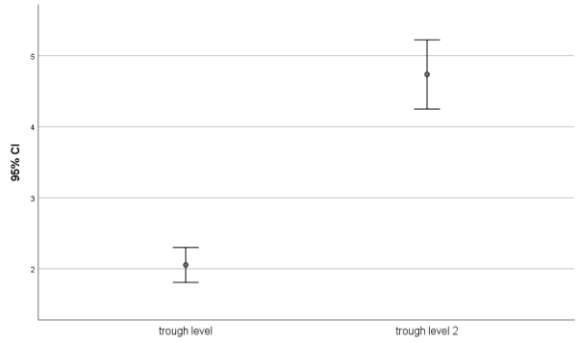

Figure 7.1 The minimum trough level provided by standard prophylaxis vs personalized prophylaxis

If we compare the prophylaxis with EHL products and that with rFVIII we can see that the average trough level is higher in those switched to EHL (5.1) than to rFVIII (3.9), even if the administration is less frequent.

The differences are also significant in terms of the annual joint bleeding rate. This decreased from a maximum of 19.0 (under the on-demand regimen), to 5.4 (under

standard prophylaxis) and reached a minimum under the personalized prophylaxis regimen (1.3) (figure 7.2).

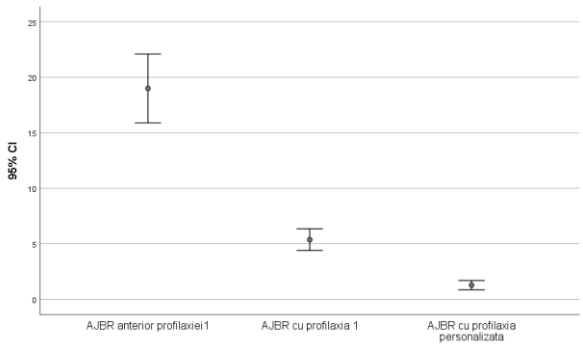

Figure 7.2 Comparison of AjBR between treatment regimens

Another important aspect that we demonstrated was the superior effectiveness of personalized prophylaxis in the prevention of bleeding, 26.7% of patients whose treatment regimen was changed reached the goal of "zero bleeding" (vs 0% in standard prophylaxis).

7.4. Conclusions

1. in our group of patients, 73.3% required increased protection (so a higher trough level) and 16.7% had a very difficult venous approach;

2. through personalized therapy we obtained a higher trough level (4.7%) compared to the previous prophylactic therapy (2.1%);

3. EHL therapy ensures a higher trough level (5.1%) than SHL (3.9%), even with less frequent administrations;

4. the joint bleeding rate decreased from an average of 19 (prior to any prophylaxis regimen) to 5.4 after the introduction of standard prophylaxis and reached a minimum of 1.3 with the help of personalized therapy;

5. 26.7% of patients in personalized treatment achieved the goal of "zero bleeding".

Chapter 8 - Study 3: Molecular risk factors analysis in severe hemophilia A

The main objective of this study was to evaluate the prevalence of inversions Int22 and Int1 in the studied group and to compare the results with those in the literature. The secondary objective was to assess the impact of these inversions on clinical phenotype and inhibitor development.

8.1. Materials and methods

We conducted a transversal, cross-sectional, observational study in which a number of 180 adults with severe hemophilia A from Fundeni Clinical Institute were included. Of these, 162 were genetically tested for Inv22 and Inv1. To evaluate the prevalence of these inversions, 24 related patients were excluded, the analyzed group totaling a number of 138 unrelated patients with severe HA.

8.1.1. The investigated parameters

Data were collected from admission records, the hospital's electronic database, patient history and documentation. Molecular testing results were provided by the Molecular Biology Laboratory of Fundeni Clinical Institute.

Evaluated variables: bleeding phenotype, number of target joints, age at onset of symptoms, age at first joint bleeding, age at diagnosis and type of genetic mutation.

8.1.2. Methods

Peripheral blood was collected from each patient in 3 EDTA tubes. The samples were processed in the Molecular Biology department of Fundeni Clinical Institute.

After the isolation of DNA from the blood and the qualitative and quantitative verification of the extracted DNA, we used Inverse Shifting-PCR (IS-PCR) and the protocol described by Rossetti et al. [42]. The next steps were genomic DNA digestion and enzymatic inactivation to obtain circular DNA [15]. For each patient, 3 polymerization reactions were performed using circular DNA, 2 reactions for Inv22 (diagnostic and complementary test) and one reaction for the Inv1 [15]. Amplification products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide under a UV lamp [15].

8.2. Results

8.2.1 Prevalence analysis of Inv22 and Inv1 in severe HA

The presence of the Inv 22 was identified in 66 of the 138 unrelated patients with severe HA (47.83%): 51 of the patients with the distal Inv22 type I (36.9% of the total, 77.27% of those with Inv22) and 15 with the proximal Inv22 type II(10.8% of the total, 22.73% of those with Inv22). Inv1 was detected in 7 unrelated patients with severe HA (5.07%). A total of 73 patients (52.9%) were detected with inversions Int22 and 1 (table VIII.1).

Table VIII.1 Prevalence of Inv22 and Inv1 in the studied group

	N (%)
Inv Int22	66 (47.83%)
Inv Int22 tip I	51 (77.27%)
Inv Int22 tip II	15 (22.73%)
Inv Int1	7 (5.07%)

The data are consistent with the literature and the results of other studies indicating a frequency of Inv22 and Inv1 of 40-50% and 2-5%, respectively, among people with severe HA [15, 43-44]. We compared our results with the reports of other studies carried out in Eastern Europe, the percentages being similar (table VIII.2).

Table VIII.2 Frequency of Inv22 and Inv1 among patients with severe HA from Eastern Europe

Country	**Number of patients tested for Inv22**	**Inv22**	**Inv22 I**	**Inv22 II**	**Number of patients tested for Inv1**	**Inv1**
Hungary [45]	104	54 (52%)	43 (80%)	11 (20%)	NA	NA
Hungary [46]	34	20(58.8%)	16 (80%)	3 (15%)	NA	NA
Romania [15]	156	65 (41.7%)	56 (86.2%)	9 (13.8%)	156	5 (3.2%)
Romania	**138**	**66 (47.8%)**	**51 (77.3%)**	**15 (22.7%)**	**137**	**7 (5.07%)**
Serbia [47]	50	21 (42%)	17 (81.8%)	4 (18.2%)	50	3 (6%)
Poland [48]	113	57 (50.5%)	47 (82.5%)	10 (17.5%)	NA	NA
Czech Republic [49]	162	71 (44%)	NA	NA	162	7 (4.3%)

8.2.2. Analysis of the impact of Int22 and Int1 inversions on the bleeding phenotype and inhibitor development

We compared the age at onset of symptoms, age at first joint bleed and age at diagnosis in patients with Inv22 positive and those with Inv22 negative. The differences are significant for all three cases. For the age at the onset of symptoms: $t(146)=8.86$, $p<0.0001$, so the age at the onset of symptoms is significantly lower in those with the Inv22+. Inv22+ patients also have a younger age at first joint bleeding and diagnosis than Inv22- patients.

To test the relationship between bleeding phenotype and Inv1 or Inv22 mutation, we used the chi-square test. We found that severe phenotype was found in a significantly higher proportion of patients with a positive Inv22 or Inv1 mutation (89.5%, respectively 100%) than in those with a negative Inv22 or Inv1 mutation, (50%, respectively 66.7%) ($\chi2=29.144$; $df=1$; $p<0.001$, respectively $\chi2=4.378$; $df=1$; $p=0.036$) (figure 8.1 and 8.2).

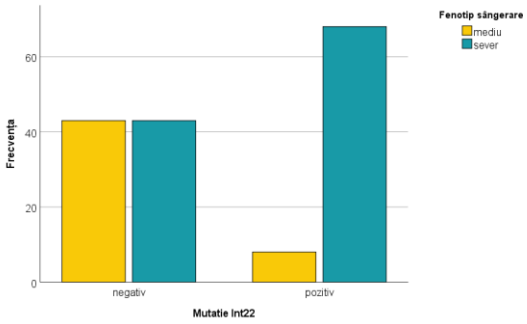

Figure 8.1 Bleeding phenotype according to Inv22 mutation status

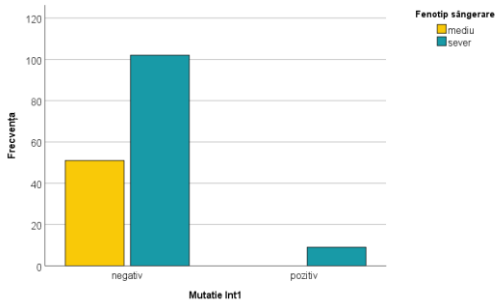

Figure 8.2 Bleeding phenotype according to Inv1 mutation status

We compared Inv22+, Inv1+ patients with those with both negative inversions in terms of the number of target joints and observed a significant difference between the 3 groups, with Inv22 and Inv1 mutations being associated with a higher mean number of target joints than those negative for both mutations. The confidence intervals overlap, but when we take into account that the categories are related, in ANOVA, the difference becomes significant: $F(2, 159)=3.345$, $p=0.038$ (<0.05).

Comparing the average annual joint bleeding rates in Inv22+ or Inv1+ patients with those with negative ones, we observed that those positive for Inv22 or Inv1 have a much higher AjBR before prophylaxis than the negative ones (20.37 vs 14.93, respectively 20.89 vs 17.28). After prophylaxis all have much lower AjBR and, in addition, there are no more differences between positive and negative in the case of Inv22 (4.75 vs 4.37).

In the group of 11 patients with hemophilia A and inhibitors we observed that 54.54% (6 out of 11) of the inhibitors were detected in the context of the loss of efficacy of factor therapy, 27.27% (3 out of 11) were diagnosed at the annual follow-up routine and 18.18% (2 out of 11) were detected in the context of the perioperative evaluation.

We subsequently evaluated the possible factors associated with the development of inhibitors in these patients. Int22 and Int1 inversions are found in 81.81% (9/11) of inhibitor patients, underlining the impact of the genetic factor in the development of alloantibodies. 4 out of 11 patients (36.36%) had collateral antecedents of inhibitors or had less than 50 days of exposure. 3 patients (27.27%) developed inhibitors after intensive doses of factor (in the context of surgery or major bleeding), and 2 of 11 (18.18%) patients had an active infection at the time of diagnosis.

8.3. Conclusions

1. The prevalence of inversions in the studied patients was 47.82% for Inv22 and 5.07% for Inv1. The data are consistent with the literature and the results of studies indicating a frequency of Inv22 and Inv1 of 40-50% and 2-5%, respectively, among people with severe hemophilia A [15, 57-58];

2. Patients with Inv22 or Inv1 have a younger age at onset, at the first joint bleeding and at diagnosis than people without these mutations, the difference being statistically significant ($p<0.05$). Also, the positive ones have a more severe bleeding phenotype, a higher number of target joints (the most affected being those with Inv22+) and a higher average annual joint bleeding rate than the negative ones. There were no significant differences (p 0.839) between the phenotype of patients with Inv22+ and those with Inv1+, the two mutations having a similar impact on the phenotype, both being null type mutations;

3. 54.54% were tested for inhibitors due to reported loss of effectiveness of the substitution therapy, 27.27% came out positive during the annual routine testing, and ~18% were detected during the preoperative evaluation;

4. Genetic mutation represented the major risk factor in the development of inhibitors (in 81.81% of cases);

5. The results of the study emphasize the need for genetic testing in hemophilia (preferably from the moment of diagnosis) and its role in establishing the bleeding phenotype, in choosing the optimal therapeutic approach (type of product used, intensity of treatment) and careful monitoring of patients at risk of inhibitors.

Chapter 9 - Study 4: Assessment of the impact of hemophilia and comorbidities on function, activity and quality of life

The aim of this study is to evaluate patients with severe hemophilia using the HAL, FISH and EQ-5D-5L questionnaires and analyze the impact of the disease, comorbidities and treatment on them. This research starts from the need to introduce new instruments in the assessment of people with hemophilia not only from the point of view of disease and structure, but also of functionality, activity and participation.

9.1. Materiala and methods

We performed a prospective, observational, cohort study (for the dynamic analysis of HAL and FISH) simultaneously with a transversal, cross-sectional, observational study (for the assessment of quality of life). We included adult patients with severe hemophilia from Fundeni Clinical Institute. The follow-up period was between 201 and 2023.

9.1.1 The investigated parameters

Data were collected from admission records, the hospital's electronic database, patient history and documentation. The HAL (Hemophilia Activity List), FISH (Functional Independence Score in Hemophilia) and EQ-5D-5L questionnaires were completed.

The collected data were: type of hemophilia, age at the time of enrollment, age at the start of prophylaxis, bleeding phenotype, type of prophylaxis (intermittent/ continuous/absent), viral infection status (HCV, HBV, HIV), number of affected joints, results of questionnaires administered at 4-year interval (HAL and FISH) and the quality of life questionnaire (EQ-5D-5L).

9.1.2. Methods

The HAL and FISH questionnaires were completed by patients at 4-year intervals, to assess any changes under prophylaxis; the FISH test was not administered to patients who had acute joint bleeding or within the previous 2 weeks.

9.2. Results

From the analysis of the results of the HAL questionnaires, we observed the following aspects:

- the best results were obtained for the activities of the upper limbs UPPER (63.98), followed by the basic activities of the legs LOWBAS (47.64), the lowest score being recorded for the complex activities of the lower limbs LOWCOM (30.66).
- at 4 years, an improvement of the results is observed, both of the normalized scores on all activities (increasing values, therefore improved functionality) and of the total score (decreasing from 137.34 to 116.4). UPPER still remains the best-scoring composite domain, while LOWCOM remains the lowest-scoring.

From the analysis of the results of the FISH questionnaires, we observed the following aspects:

- the most affected are domains C8 (running), B5 (kneeling), C7 (stair climbing) and C6 (walking), with the lowest average scores. Running (C8) and kneeling (B5) were the only activities that some patients could not perform;
- after 4 years, the most affected areas were still B5, C8 and C7. Domains B5 and C8 still remain impossible for some patients, in a slightly diminished percentage.

From the analysis of the results of the EQ-5D-5L questionnaires, we observed the following aspects:

- the percentage of patients with severe problems: 16.5% pain, 13.9% mobility, 8.9% activity, 1.3% self-care, 1.3% anxiety;;

➢ the average EQ-VAS score was 77.85 (SD±14.77), with a minimum of 35 and a maximum of 100. Patients aged 18-24 years have scores comparable to the general population (91.82 vs 90.6). After the age of 25 the scores decrease progressively, the difference being significant after the age of 35 years.

To demonstrate the correlations between the results of HAL, FISH and EQ-5D-5L (EQ-VAS) and the age of initiation of prophylaxis, type of prophylaxis, the presence of viral infections and bleeding phenotype we performed a multivariate analysis and used T-test. We observed significantly higher values in patients with:

• moderate (versus severe) phenotype (figure 9.1, figure 9.2)

• continuous (versus intermittent) prophylaxis

• do not have HCV (compared to those who have HCV)

We observed that prophylaxis, regardless of whether it is continuous or intermittent, has benefits that are demonstrated by improved scores, thus improved joint functionality.

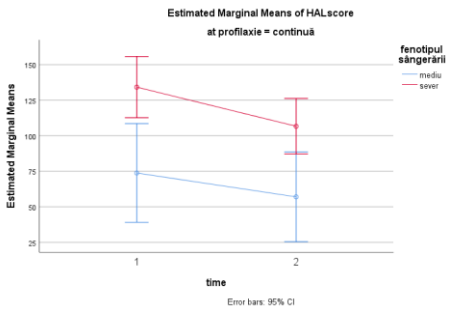

Figure 9.1 HAL 2019-2023 score by bleeding phenotype

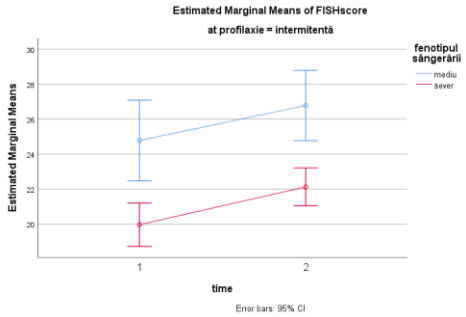

Figure 9.2 FISH 2019-2023 score by bleeding phenotype

9.3. Conclusions

1. In our group of patients, both the HAL questionnaire and the FISH score revealed significantly greater problems in the degree of functionality and activity in the lower limbs;

2. Hemophiliacs who received continuous prophylaxis (52 weeks/year) scored significantly better on the HAL and FISH questionnaires than those on intermittent prophylaxis (maximum 45 weeks/year), both in 2019 and in 2023.

3. The presence of HCV infection proved to have a negative influence on the activity and functionality, but also on the quality of life of hemophiliac patients;

4. Compared to the general population, hemophiliacs scored lower on the EQ-VAS questionnaire, with differences starting to be significant after 35 years.

Chapter 10: Original contributions

This doctoral thesis represents the most extensive study on hemophilia in Romania, with a cohort of 201 patients and a simultaneous analysis of clinical, biological and molecular parameters.

The purpose of this thesis is to provide useful and practical informations about the general population with severe hemophilia A and B, necessary in the optimal management of these patients. The four presented studies aimed to identify the factors that influence the clinical evolution in hemophilia and the in-depth understanding of their impact on the therapeutic approach.

All four studies achieved their objectives, through their results, contributing to a better understanding of:

1. stratification of patients according to bleeding phenotype (not only based on factor level) and individualization of therapies according to risk category;

2. the usefulness of pharmacokinetic tests in monitoring and customizing prophylactic regimens so that they meet the patient's needs and reach the goal of "zero bleeding";

3. the need to implement molecular testing in hemophilia in Romania, in order to confirm the diagnosis, establish the genotype-phenotype relationship, predict the risk of inhibitors and the response to immune tolerance induction therapy;

4. the management of the elderly patient with hemophilia and the need for a multidisciplinary approach to comorbidities and complications occurring in this category of patients;

5. the usefulness of the HAL and FISH questionnaires in monitoring the degree of joint damage, the impact of the disease phenotype, the prophylactic regimen and viral infections on the degree of activity and functionality in hemophilia.

Future Perspectives

1. Complete genotype-phenotype analysis using also the results of sequencing tests of F8 and F9 genes (the rest of almost 50% of the severe hemophiliac population);

2. Research of other factors that can influence the bleeding phenotype: thrombophilia mutations, Protein C, Protein S, antithrombin III;

3. Evaluation of the bleeding phenotype using the Thrombin Generation Assay (TGA), a useful instrument in assessing bleeding risk, in differentiating bleeding phenotypes (both in patients with inhibitors and those without).

Selective bibliography

1. Berntorp E, Fischer K, Hart DP, Mancuso ME, Stephensen D, Shapiro AD, et al. Haemophilia. Nat Rev Dis Primers. 2021 Jun 24;7(1):45. doi: 10.1038/s41572-021-00278-x. PMID: 34168126

2. Srivastava A, Brewer AK, Mauser-Bunschoten EP, Key NS, Kitchen S, Llinas A, et al. Treatment Guidelines Working Group on Behalf of The World Federation Of Hemophilia. Guidelines for the management of hemophilia. Haemophilia. 2013 Jan;19(1):e1-47. doi: 10.1111/j.1365-2516.2012.02909.x. Epub 2012 Jul 6. PMID: 22776238.

3. Pavlova A, Oldenburg J. Defining severity of hemophilia: more than factor levels. Semin Thromb Hemost. 2013 Oct;39(7):702-10. doi: 10.1055/s-0033-1354426. Epub 2013 Sep 11. PMID: 24026911

4. Napolitano M, Kessler CM, Hemophilia A and Hemophilia B, Consultative Hemostasis and Thrombosis (Fourth Edition), Elsevier, 2019, Pages 39-58, ISBN 9780323462020, https://doi.org/10.1016/B978-0-323-46202-0.00003-0.

5. Pezeshkpoor B, Oldenburg J, Pavlova A. Insights into the Molecular Genetic of Hemophilia A and Hemophilia B: The Relevance of Genetic Testing in Routine Clinical Practice. Hamostaseologie. 2022 Dec;42(6):390-399. doi: 10.1055/a-1945-9429. Epub 2022 Dec 22. PMID: 36549291; PMCID: PMC9779947.

6. Karim, Md Anwarul & Jamal, Chao. (2013). A Review on Hemophilia in Children. Bangladesh Journal of Child Health. 37. 10.3329/bjch.v37i1.15349.

7. Franchini M, Mannucci PM. Past, present and future of hemophilia: a narrative review. Orphanet J Rare Dis. 2012 May 2;7:24. doi: 10.1186/1750-1172-7-24. PMID: 22551339; PMCID: PMC3502605.

8. Alcalay M, Deplas A. Rheumatological management of patients with hemophilia. Part II: Muscle hematomas and pseudotumors. Joint Bone Spine. 2002 Dec;69(6):556-9. doi: 10.1016/s1297-319x(02)00451-7. PMID: 12537262

9. Stieltjes N, Calvez T, Demiguel V, Torchet MF, Briquel ME, Fressinaud E, et al. Intracranial haemorrhages in French haemophilia patients (1991-2001): clinical presentation, management and prognosis factors for death. Haemophilia. 2005 Sep;11(5):452-8. doi: 10.1111/j.1365-2516.2005.01090.x. PMID: 16128887.

10. de Alencar JB, Macedo LC, de Barros MF, Rodrigues C, Cadide RC, Sell AM, et al. Importance of immune response genes in hemophilia A. Rev Bras Hematol Hemoter. 2013;35(4):280-6. doi: 10.5581/1516-8484.20130095. PMID: 24106448; PMCID: PMC3789435.

11. Nichols WC, Amano K, Cacheris PM, Figueiredo MS, Michaelides K, Schwaab R, et al. Moderation of hemophilia A phenotype by the factor V R506Q mutation. Blood. 1996 Aug 15;88(4):1183-7. PMID: 8695835.

12. Kitchen S, Signer-Romero K, Key NS. Current laboratory practices in the diagnosis and management of haemophilia: a global assessment. Haemophilia. 2015 Jul;21(4):550-7. doi: 10.1111/hae.12639. Epub 2015 Feb 16. PMID: 25688866.

13. Srivastava A, Santagostino E, Dougall A, Kitchen S, Sutherland M, Pipe SW, et al. WFH Guidelines for the Management of Hemophilia panelists and co-authors.

WFH Guidelines for the Management of Hemophilia, 3rd edition. Haemophilia. 2020 Aug;26 Suppl 6:1-158. doi: 10.1111/hae.14046. Epub 2020 Aug 3. Erratum in: Haemophilia. 2021 Jul;27(4):699. PMID: 32744769.

14. Verbruggen B, van Heerde WL, Laros-van Gorkom BA. Improvements in factor VIII inhibitor detection: From Bethesda to Nijmegen. Semin Thromb Hemost. 2009 Nov;35(8):752-9. doi: 10.1055/s-0029-1245107. Epub 2010 Feb 18. PMID: 20169511.

15. **Brinza, M.;** Grigore, A.; Dragomir, M.; Jardan, D.; Jardan, C.; Balanescu, P.; et al. Large Intron Inversions in Romanian Patients with Hemophilia A—First Report. Medicina 2023, 59, 1821. https://doi.org/10.3390/medicina59101821.)

16. Franchini M, Mannucci PM. Past, present and future of hemophilia: a narrative review. Orphanet J Rare Dis. 2012 May 2;7:24. doi: 10.1186/1750-1172-7-24. PMID: 22551339; PMCID: PMC3502605.

17. Marchesini E, Morfini M, Valentino L. Recent Advances in the Treatment of Hemophilia: A Review. Biologics. 2021 Jun 15;15:221-235. doi: 10.2147/BTT.S252580. PMID: 34163136; PMCID: PMC8214539.

18. Rangarajan S, Austin S, Goddard NJ, Négrier C, Rodriguez-Merchan EC, Stephensen D, et al. Consensus recommendations for the use of FEIBA(®) in haemophilia A patients with inhibitors undergoing elective orthopaedic and non-orthopaedic surgery. Haemophilia. 2013 Mar;19(2):294-303. doi: 10.1111/hae.12028. Epub 2012 Sep 19. PMID: 22989234.

19. **Brinza M,** Uscatescu V, Hemcinschi M, Chiriac E, Gherghe G, Coriu D. Challenges in prophylactic therapy with Emicizumab in patients with hemophilia A: Focus on monitoring tests. Rev Romana Med Lab. 2024;32(1):89-96. DOI:10.2478/rrlm-2024-0003

20. Saulyte Trakymiene S, Steen Carlsson K. On-demand treatment in persons with severe haemophilia. Eur J Haematol Suppl. 2014 Aug;76:39-47. doi: 10.1111/ejh.12373. PMID: 24957106.

21. Valentino LA. Considerations in individualizing prophylaxis in patients with haemophilia A. Haemophilia. 2014 Sep;20(5):607-15. doi: 10.1111/hae.12438. Epub 2014 Apr 8. PMID: 24712891.

22. Lafeber FP, Miossec P, Valentino LA. Physiopathology of haemophilic arthropathy. Haemophilia. 2008 Jul;14 Suppl 4:3-9. doi: 10.1111/j.1365-2516.2008.01732.x. PMID: 18494686.

23. Caviglia H, Landro ME, Galatro G, Candela M, Neme D. Epidemiology of fractures in patients with haemophilia. Injury. 2015 Oct;46(10):1885-90. doi: 10.1016/j.injury.2015.06.034. Epub 2015 Jul 3. PMID: 26190631.

24. Caviglia H, Candela M, Landro ME, Douglas Price AL, Neme D, Galatro GA. Haemophilia pseudotumours in patients with inhibitors. Haemophilia. 2015 Sep;21(5):681-5. doi: 10.1111/hae.12632. Epub 2015 Feb 16. PMID: 25688657.

25. Tabriznia-Tabrizi S, Gholampour M, Mansouritorghabeh H. A close insight to factor VIII inhibitor in the congenital hemophilia A. Expert Rev Hematol. 2016 Sep;9(9):903-13. doi: 10.1080/17474086.2016.1208554. Epub 2016 Aug 16. PMID: 27367203.

26. Franchini M, Mannucci PM. Inhibitors of propagation of coagulation (factors VIII, IX and XI): a review of current therapeutic practice. Br J Clin Pharmacol. 2011 Oct;72(4):553-62. doi: 10.1111/j.1365-2125.2010.03899.x. PMID: 21204915; PMCID: PMC3195733.

27. Astermark J. Why do inhibitors develop? Principles of and factors influencing the risk for inhibitor development in haemophilia. Haemophilia. 2006 Jul;12 Suppl 3:52-60. doi: 10.1111/j.1365-2516.2006.01261.x. PMID: 16683997.

28. Coppola A, Santoro C, Tagliaferri A, Franchini M, DI Minno G. Understanding inhibitor development in haemophilia A: towards clinical prediction and prevention strategies. Haemophilia. 2010 Jan;16 Suppl 1:13-9. doi: 10.1111/j.1365-2516.2009.02175.x. PMID: 20059564.

29. Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. Haemophilia. 2006 Dec;12 Suppl 6:15-22. doi: 10.1111/j.1365-2516.2006.01361.x. PMID: 17123389.

30. Astermark J, Oldenburg J, Pavlova A, Berntorp E, Lefvert AK; MIBS Study Group. Polymorphisms in the IL10 but not in the IL1beta and IL4 genes are associated with inhibitor development in patients with hemophilia A. Blood. 2006 Apr 15;107(8):3167-72. doi: 10.1182/blood-2005-09-3918. Epub 2005 Dec 27. PMID: 16380445.

31. Astermark J, Oldenburg J, Carlson J, Pavlova A, Kavakli K, Berntorp E, et al. Polymorphisms in the TNFA gene and the risk of inhibitor development in patients with hemophilia A. Blood. 2006 Dec 1;108(12):3739-45. doi: 10.1182/blood-2006-05-024711. Epub 2006 Aug 22. PMID: 16926287.

32. Astermark J, Wang X, Oldenburg J, Berntorp E, Lefvert AK; MIBS Study Group. Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. J Thromb Haemost. 2007 Feb;5(2):263-5. doi: 10.1111/j.1538-7836.2007.02290.x. PMID: 17269936.

33. van den Berg HM, Fischer K, Carcao M, Chambost H, Kenet G, Kurnik K, et al. PedNet Study Group. Timing of inhibitor development in more than 1000 previously untreated patients with severe hemophilia A. Blood. 2019 Jul 18;134(3):317-320. doi: 10.1182/blood.2019000658. Epub 2019 Jun 11. PMID: 31186271.

34. Astermark J, Altisent C, Batorova A, Diniz MJ, Gringeri A, Holme PA, et al. European Haemophilia Therapy Standardisation Board. Non-genetic risk factors and the development of inhibitors in haemophilia: a comprehensive review and consensus report. Haemophilia. 2010 Sep 1;16(5):747-66. doi: 10.1111/j.1365-2516.2010.02231.x. Epub 2010 Apr 14. PMID: 20398077.

35. Matzinger P. The danger model: a renewed sense of self. Science. 2002 Apr 12;296(5566):301-5. doi: 10.1126/science.1071059. PMID: 11951032.

36. Manco-Johnson MJ, Warren BB, Buckner TW, Funk SM, Wang M. Outcome measures in Haemophilia: Beyond ABR (Annualized Bleeding Rate). Haemophilia. 2021 Feb;27 Suppl 3:87-95. doi: 10.1111/hae.14099. Epub 2021 Jan 4. PMID: 33398908.

37. Recht M, Konkle BA, Jackson S, Neufeld EJ, Rockwood K, Pipe S. Recognizing the need for personalization of haemophilia patient-reported outcomes in the prophylaxis era. Haemophilia. 2016 Nov;22(6):825-832. doi: 10.1111/hae.13066. Epub 2016 Aug 31. PMID: 27581872.

38. Fischer K, Poonnoose P, Dunn AL, Babyn P, Manco-Johnson MJ, David JA, et al. participants of the International Symposium on Outcome Measures in Hemophilic Arthropathy. Choosing outcome assessment tools in haemophilia care and research: a multidisciplinary perspective. Haemophilia. 2017 Jan;23(1):11-24. doi: 10.1111/hae.13088. Epub 2016 Sep 15. PMID: 27633342.

39. van Genderen FR, van Meeteren NL, van der Bom JG, Heijnen L, de Kleijn P, van den Berg HM, et al. Functional consequences of haemophilia in adults: the development of the Haemophilia Activities List. Haemophilia. 2004 Sep;10(5):565-71. doi: 10.1111/j.1365-2516.2004.01016.x. PMID: 15357785.

40. Poonnoose PM, Manigandan C, Thomas R, Shyamkumar NK, Kavitha ML, Bhattacharji S, et al. Functional Independence Score in Haemophilia: a new performance-based instrument to measure disability. Haemophilia. 2005 Nov;11(6):598-602. doi: 10.1111/j.1365-2516.2005.01142.x. PMID: 16236109.

41. Pollmann H, Richter H, Ringkamp H, Jürgens H. When are children diagnosed as having severe haemophilia and when do they start to bleed? A 10-year single-centre PUP study. Eur J Pediatr. 1999 Dec;158 Suppl 3:S166-70. doi: 10.1007/pl00014347. PMID: 10650861.

42. Rossetti LC, Radic CP, Larripa IB, De Brasi CD. Developing a new generation of tests for genotyping hemophilia-causative rearrangements involving int22h and int1h hotspots in the factor VIII gene. J Thromb Haemost. 2008

May;6(5):830-6. doi: 10.1111/j.1538-7836.2008.02926.x. Epub 2008 Feb 12. PMID: 18284600.

43. Schröder J, El-Maarri O, Schwaab R, Müller CR, Oldenburg J. Factor VIII intron-1 inversion: frequency and inhibitor prevalence. J Thromb Haemost. 2006 May;4(5):1141-3. doi: 10.1111/j.1538-7836.2006.01884.x. PMID: 16689771.

44. Peyvandi F. Carrier detection and prenatal diagnosis of hemophilia in developing countries. Semin Thromb Hemost. 2005 Nov;31(5):544-54. doi: 10.1055/s-2005-922226. PMID: 16276463.

45. Andrikovics H, Klein I, Bors A, Nemes L, Marosi A, Váradi A, Tordai A. Analysis of large structural changes of the factor VIII gene, involving intron 1 and 22, in severe hemophilia A. Haematologica. 2003 Jul;88(7):778-84. PMID: 12857556.

46. Klein I, Andrikovics H, Bors A, Nemes L, Tordai A, Váradi A. A haemophilia A and B molecular genetic diagnostic programme in Hungary: a highly informative and cost-effective strategy. Haemophilia. 2001 May;7(3):306-12. doi: 10.1046/j.1365-2516.2001.00497.x. PMID: 11380636.

47. Ilić, N.; Krstić, A.; Kuzmanović, M.; Mićić, D.; Konstantinidis, N.; Guć-Šćekić, M. Identification of intron 1 and intron 22 inversions of factor VIII gene in Serbian patients with hemophilia A. Genetika 2013, 45, 207–216.

48. Sawecka J, Skulimowska J, Windyga J, Lopaciuk S, Kościelak J. Prevalence of the intron 22 inversion of the factor VIII gene and inhibitor development in Polish patients with severe hemophilia A. Arch Immunol Ther Exp (Warsz). 2005 Jul-Aug;53(4):352-6. PMID: 16088320.

49. Habart D, Kalabova D, Hrachovinova I, Vorlova Z. Significant prevalence of the intron 1 factor VIII gene inversion among patients with severe hemophilia A in the Czech Republic. J Thromb Haemost. 2003 Jun;1(6):1323-4. doi: 10.1046/j.1538-7836.2003.t01-4-00326.x. PMID: 12871342.

LIST OF PUBLISHED SCIENTIFIC PAPERS:

1. Batorova A, Boban A, **Brinza M,** Lissitchkov T, Nemes L, Zupan Preložnik I, Smejkal P, Zozulya N, Windyga J. Expert opinion on current and future prophylaxis therapies aimed at improving protection for people with hemophilia A. J Med Life. 2022 Apr;15(4):570-578. doi: 10.25122/jml-2022-0103. PMID: 35646171; PMCID: PMC9126455. Impact factor **3.9**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9126455/

2. Dirzu N, Hotea I, Jitaru C, **Brinza M,** Urian L, Peters MC, Gal K, Popescu L, Blag C, Marian M, Pal E, Stanescu M, Cenariu D, Tarniceriu C, Serban M, Dima D, Coriu D, Tomuleasa C. Mobile Health Technology for the Personalized Therapy of Hemophilia. Front Med (Lausanne). 2021 Aug 10;8:711973. doi: 10.3389/fmed.2021.711973. PMID: 34447770; PMCID: PMC8382969. Impact factor **3.9** https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8382969/

3. Zimta AA, Hotea I, **Brinza M,** Blag C, Iluta S, Constantinescu C, Bashimov A, Marchis-Hund EA, Coudsy A, Muller-Mohnssen L, Dirzu N, Gulei D, Dima D, Serban M, Coriu D, Tomuleasa C. The Possible Non-Mutational Causes of FVIII Deficiency: Non-Coding RNAs and Acquired Hemophilia A. Front Med (Lausanne). 2021 Apr 15;8:654197. doi: 10.3389/fmed.2021.654197. PMID: 33968959; PMCID: PMC8099106. Impact factor **3.9**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8099106/

4. Hotea I, **Brinza M,** Blag C, Zimta AA, Dirzu N, Burzo C, Rus I, Apostu D, Benea H, Marian M, Mester A, Pasca S, Iluta S, Teodorescu P, Jitaru C, Zdrenghea M, Bojan A, Torok-Vistai T, Niculescu R, Tarniceriu C, Dima D, Truica C, Serban M, Tomuleasa C, Coriu D. Current therapeutic approaches in the management of hemophilia-a consensus view by the Romanian Society of Hematology. Ann Transl Med. 2021 Jul;9(13):1091. doi: 10.21037/atm-21-747. PMID: 34423003; PMCID: PMC8339806. Impact factor **3.932**

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8339806/

5. **Melen B**, Uscatescu V, Gheorghe G, Chiriac E, Ciobanu C, Orban H, et al. Thromboelastography in pre-surgery monitoring in Hemophilia A with high inhibitor titer: case report and literature review. Rev Romana Med Lab. 2020; 28(2):217-24. DOI:10.2478/rrlm-2020-0021. Factor de impact **0.5** (capitolul 1, pagina 6; capitolul 3, pagina 19; capitolul 8, pagina 85); https://sciendo.com/it/article/10.2478/rrlm-2020-0021

6. Vladareanu AM, Marinescu C, Vasile D, Gaman M, Onisai M, Giovani AM, **Brinza M**, Uscatescu V – The subtlety of a rare disorder – acquired Hemophilia B; Documenta Haematologica. 2017, vol XXXVII, Nr 3-4: 12-15

7. **Brinza M,** Uscatescu V, Hemcinschi M, Chiriac E, Gherghe G, Coriu D. Challenges in prophylactic therapy with Emicizumab in patients with hemophilia A: Focus on monitoring tests. Rev Romana Med Lab. 2024;32(1):89-96. DOI:10.2478/rrlm-2024-0003. Factor de impact **0.5** (capitolul 2, pagina 10)

https://sciendo.com/it/article/10.2478/rrlm-2024-0003

8. **Brinza M,** Grigore A, Dragomir M, Jardan D, Jardan C, Balanescu P, Tarniceriu CC, Badulescu OV, Blag C, Tomuleasa C, et al. Large Intron Inversions in Romanian Patients with Hemophilia A—First Report. Medicina 2023, 59, 1821. doi: 10.3390/medicina59101821. Factor de impact **3.3** (capitolul 1, pagina 2, 8, capitolul 8); https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10608589/

9. Grigore A, Dragomir M, Călugăru OT, Jardan D, Jardan C, **Brinza M,** Bălănescu P, Coriu D, Mutational Profile in Romanian Patients with Hemophilia A. Int. J. Mol. Sci. 2024, 25, 8366. https://doi.org/10.3390/ijms25158366. Factor de impact **4.9**; https://www.mdpi.com/1422-0067/25/15/8366