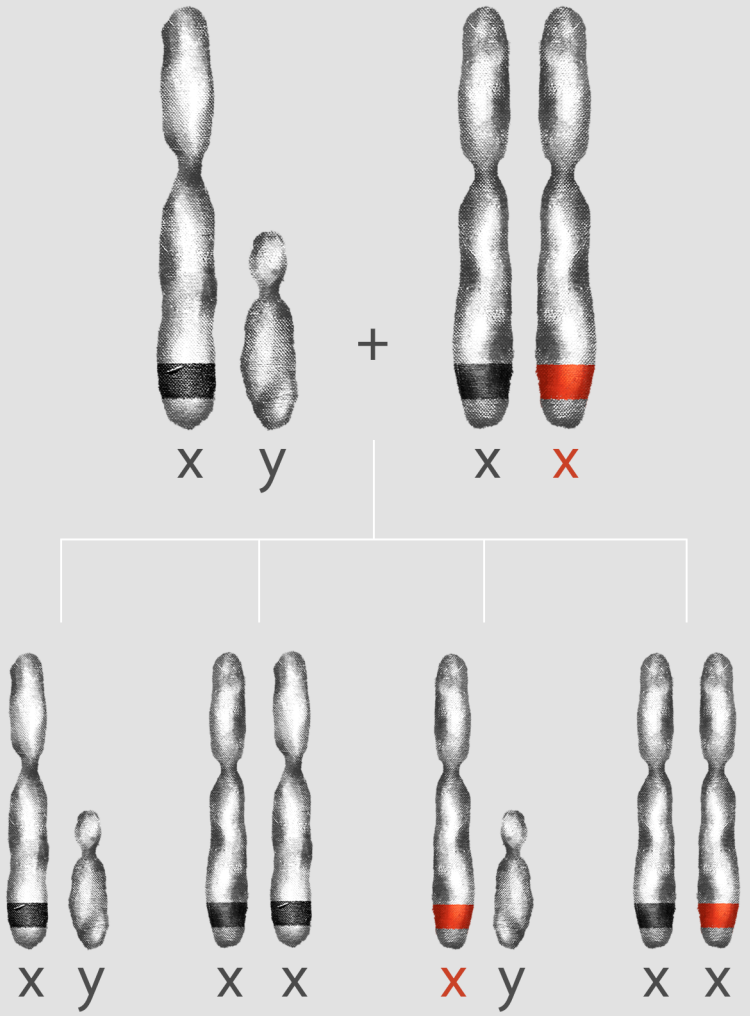
BASIC GUIDE *of* HEMOPHILIA

Clinical manifestations, diagnosis and treatment



asanhemo
ASOCIACIÓN ANDALUZA DE HEMOFILIA

ANTONIO LIRAS

Antonio Liras (Segovia, Spain, 1955) holds a PhD in Pharmacy and a graduate degree in Chemical Science from Madrid's Complutense University (UCM). Dr Liras has devoted over thirty years of his life to university teaching and research. The long path that has brought him hither started in Milan, at the Carlo Besta Neurological Institute, where he cut his teeth in the gene therapy arena.

He is currently the coordinator of UCM's Advanced Gene and Cell Therapy Group as well as a member of different Spanish and international scientific societies and committees. He also belongs to the Medical Advisory Board of the Spanish Federation of Rare Diseases and is a trustee at the Victoria Eugenia Royal Hemophilia Foundation, whose Scientific Committee he chaired. His work has included the organization of specialization and postgraduate courses, the development of several research projects that have benefited from both Spanish and international funding, the supervision of several PhD theses, and the publication of over 50 articles in international impact factor journals and numerous scientific books. He has also delivered a myriad of lectures at scientific conferences and has received several scientific awards for his research.

Dr. Liras has for many years now cooperated with the Andalusian Hemophilia Association, of which he is a collaborating member. Asanhemo has always highly valued his interest, sensitivity and support not only for the Association but particularly for patients living with hemophilia or other congenital coagulopathies.

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PREFACE

This guide is dedicated to patients with hemophilia and well as their families and friends. It is aimed at a fundamental educational and informational goal, which is no other than providing a series of basic concepts and illustrations that may contribute to a better understanding of the nature of blood, the system that makes it circulate throughout our body and the things that happen when something goes wrong.

The guide is made up of four parts. The first one introduces some fundamental concepts that provide the reader with a basic understanding of blood and its functions. The purpose of this initial section is to lay the foundations needed for making sense of what follows in the subsequent chapters. The second part covers hemophilia, its causes, types, clinical symptoms and hereditary transmission. This is followed by a discussion on the treatment of the disease, the development of inhibitors and the potential side effects of treatment such as viral infections and emerging pathogens. The last section is the most promising and hopeful for any chronic patient that is under treatment as it provides an update on the new advanced therapies and biotechnological treatments.

In order to promote and increase the guide's impact and international reach, it is published in a bilingual (Spanish/English) flip format.

The book, published and promoted thanks to the Andalusian Hemophilia Association's commitment with education, would not have been possible without the contribution of a devoted team of individuals who selflessly dedicate their time and efforts to improving the quality of life of people with hemophilia.

Lola Camero

President of the Andalusian Hemophilia Association

BLOOD AND ITS FUNCTION

Blood is a fluid tissue that circulates throughout the body forced by the constant pumping of the heart. As it flows through an intricate network of blood vessels, this precious resource brings life to all the different organs in the body. The heart delivers the blood to large vessels called arteries, which branch out into a tree of progressively smaller vessels until it is fed into the hundreds of millions of capillaries that irrigate all the cells of the body, from where it makes its way back to the heart through the veins.

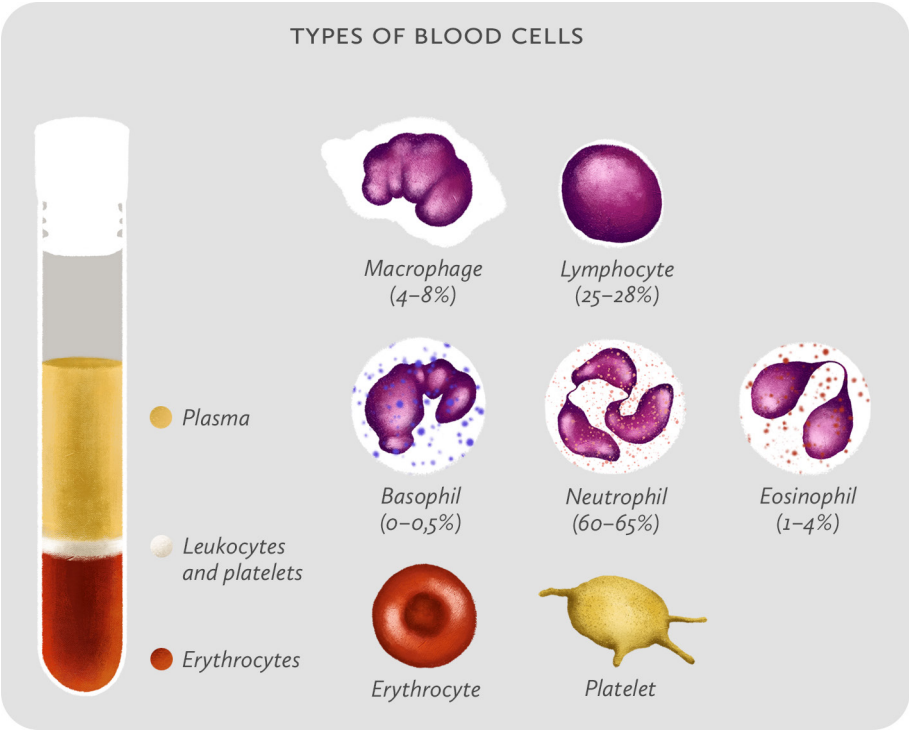
But, what purpose does the blood serve? Blood is a fluid that allows communication within a living being. Thus, the life-sustaining substances manufactured by different organs travel in the blood and are delivered to the cells providing them with nutrients they require, enabling them to recognize certain physiological situations and constantly informing them about the needs of our body. For example, after digesting a meal, the vessels that irrigate the digestive system absorb the nutrients obtained from the food consumed. In the same way, the red blood cells in the lungs fill with oxygen through a gas exchange mechanism and the oxygen-and the nutrient- rich blood is distributed throughout the body, i.e. to all the cells that make up the different tissues, to all the tissues that make up the different organs and to all the organs that make up each system. The nutrients and the oxygen are all that the cells need to live, create energy and maintain the balance of all vital functions. This process, called cell metabolism, creates waste products that are taken up by the blood and transported to the liver for detoxification or directly to the kidneys for elimination.

Blood is formed by a fluid part called plasma and a solid component, the blood cells. The cell component comprises erythrocytes or red blood cells, leukocytes or while blood cells and platelets or thrombocytes. Erythrocytes are by far the most numerous. They possess millions of hemoglobin molecules, which give blood its red color. Because of their structure, erythrocytes can combine with oxygen, which they transport to the tissues of all organs, thus fulfilling their most important function: oxygenating the body.

As regards leukocytes, these blood cells are responsible for providing immunity against infection. The most numerous leukocytes are the neutrophils, followed by lymphocytes. Neutrophils are phagocytic cells, that is, they are capable of phagocyting or ingesting strange molecules, such as pathogenic microorganisms, and kill them with

lethal digestive enzymes before the occurrence of disease. These immune responses are present from birth. Lymphocytes, on the other hand, act when infection has occurred. They learn the best way to attack each pathogen, develop a memory of the foreign substance and produce antibodies in case future attacks. This dual immune protection, innate immunity (present at birth) and acquired immunity (developed in response to a specific pathogen) act in a coordinated manner to fight any infection.

Finally, platelets or thrombocytes, as their name implies, are blood cells whose function is to promote blood coagulation by producing thrombi or clots. Platelets are very small as they are really fragments originated in the cytoplasm of a stem cell, and they make up the largest part of the clots that are formed by the body to prevent blood loss following a vascular lesion.



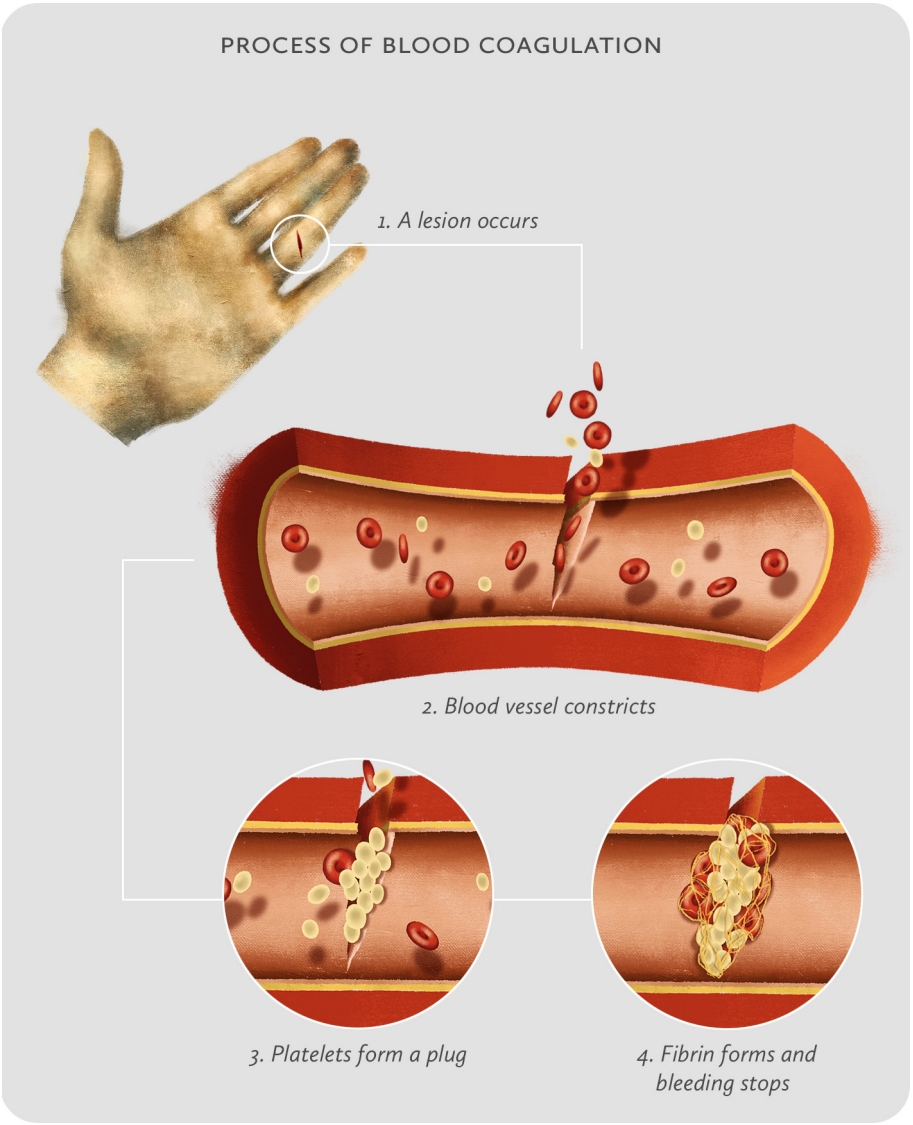
The renewal of blood cells is essential as these cells gradually age and are destroyed. Such renewal takes place in the bone marrow through a process called hematopoiesis. The bone marrow contains the progenitor cells of each of the cell types mentioned, which differentiate to functional cells and are subsequently released into the bloodstream.

On the other hand, plasma, i.e. the fluid portion of blood, contains different types of proteins and many water soluble molecules such as sugars, hormones, enzymes, antibodies and other proteins such as albumin, globulins and fibrinogen.

A hemorrhage occurs when blood leaks out of the blood vessels. The lack of blood will impair circulation and result in decreased oxygen supply to tissue (anoxia), with symptoms such as pallor, sweating, nausea, vomiting, faintness, cramps, etc. Tissues may become damaged and suffer irreversible sequelae; even death may ensue. Hence the vital importance of blood transfusions.

Damage of a blood vessel triggers the activation of certain physiological mechanisms aimed at stemming the hemorrhage; this is what is called hemostasis. When the lining of a vessel breaks, the collagen proteins in the vessel wall are revealed (collagen is an important constituent of the vessel wall), which unleashes three independent but overlapping hemostatic mechanisms: vasoconstriction, platelet plug formation, and coagulation of blood through the creation of a fibrin mesh (red thrombus) that entraps the platelet plug. In the absence of vascular damage, i.e. when the vessel lining is intact, platelets repeal each other, but when a vascular lesion occurs the collagen in the vessel walls becomes exposed, and the platelets bind to it. When this binding occurs, platelets become activated and release a series of granules. These granules in turn release substances that stimulate constriction (narrowing) of the damaged vessel (thus decreasing the bleeding) and, at the same time, attract other platelets that will bind to the ones already adhered to the collagen. These platelets will release further granules, and the process continues through successive iterations. A platelet plug (white thrombus) is thus created in the damaged vessel, which is reinforced by fibrin, an insoluble protein derived from fibrinogen that creates a network of tough fibrin fibers that form a stable hemostatic clot. Thus, blood clots contain platelets and fibrin, and often entrapped red blood cells that give the clot its red color. Finally, contraction of the platelet mass forms a more compact and efficient plug that will prevent bleeding in the event of a mild hemorrhage.

How does fibrinogen turn into fibrin? This is where the coagulation factors circulating in the blood come into play. Fibrinogen itself is one of those coagulation factors, but it needs the help of another twelve clotting factors in order to turn into fibrin. In a vascular lesion the phospholipids of the damaged vessel wall, which are released together with the collagen, activate a first coagulation factor. This factor activates a second clotting factor, which in turn activates a third one, and so forth. This activation cascade ends with the activation of fibrinogen to form fibrin.



The absence or deficiency or any of these coagulation factors –which is what happens in hemophilia– disrupts the aforementioned chain of reactions, inhibiting the production of fibrin and thereby making it impossible to stabilize the clot. Conversely, an excessive increase in the plasma levels of coagulation factors would lead to a decrease in blood fluidity, hampering normal circulation and raising the serious risk that an unstabilized thrombus may steal into the bloodstream and occlude a vessel causing an embolism. Nonetheless, there are natural endogenous anticoagulants that promote blood flow and prevent blood from coagulating when this is not required. This means that when blood vessels have been "repaired," another blood protein called plasminogen activates to plasmin, which degrades fibrin promoting the dissolution of clots that are no longer required.

Therefore hemostasis is all about striking a very delicate balance between the mechanism of fibrin formation through the successive activation of the different coagulation factors and the fibrinolysis process whereby fibrin fibers are degraded.

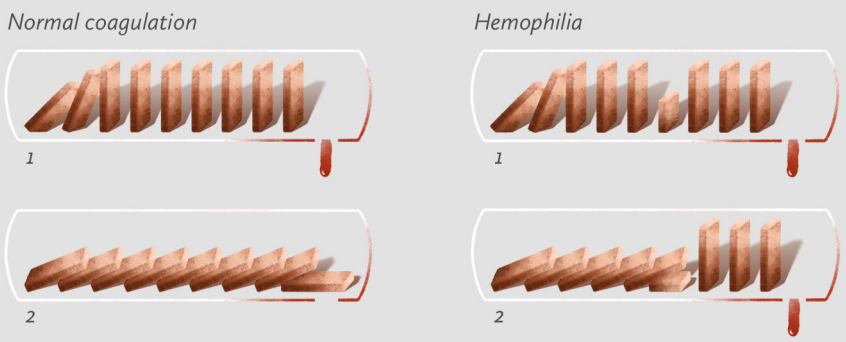
WHAT IS HEMOPHILIA? CAUSES, TYPES, SYMPTOMS AND HEREDITARY TRANSMISSION

Hemophilia is a disease that affects the clotting of blood. It is characterized by the absence or deficiency of one of the elements, called factors, required for blood coagulation. In hemophilia A it is factor VIII that is nonexistent or deficient, whereas in hemophilia B the absent or insufficient factor is factor IX. As, the number of patients with hemophilia is low, it is considered a rare disease (hemophilia A occurs in 1 in every 6,000 live births whereas hemophilia in B in 1 in every 30,000.

The first mention to hemophilia dates to the Babylonian Talmud of the 2nd century AD, which discusses the predisposition of males in certain families, sometimes connected with one another, to profuse bleeding following circumcision. Such was the concern that the laws were changed to exempt these young men from the ritual. In the 21st century we have learnt that some persons are born with certain malfunctioning molecules that predispose them to hemophilia. The history of the disease has been marked by two fundamental milestones. One of them was the discovery by Patek in 1937 of some of the proteins that participate in blood clotting; the other was the discovery of the structure of deoxyribonucleic acid (DNA) by Watson and Crick in 1953. From the times of King Alfonso XIII of Spain whose eldest son was born a hemophiliac just over 100 years ago, things have changed so much that today we know that the disease is not caused by damage by syphilis bacteria to the endothelium of blood vessels; that people with hemophilia no longer die young; and that egg-whites, peanut flour and snake poison do not cure the disease.

Blood clotting can be conceived of as a row of domino tiles one after another. If you flick your finger against the first domino, all the others will fall. If we did this inside a tube with a hole that was plugged by the last tile, we would have succeeded in preventing the fluid going through the tube from leaking out. Hemophilia constitutes the situation where one of the tiles in the middle is shorter than the others and is not capable of pushing the following dominoes in the row. Thus the hole stays open and the blood leaks out. This is a crude description of the coagulation cascade, the dominoes standing for the clotting factors and the last tile for the fibrin clot.

FIGURATIVE COAGULATION CASCADE MODEL



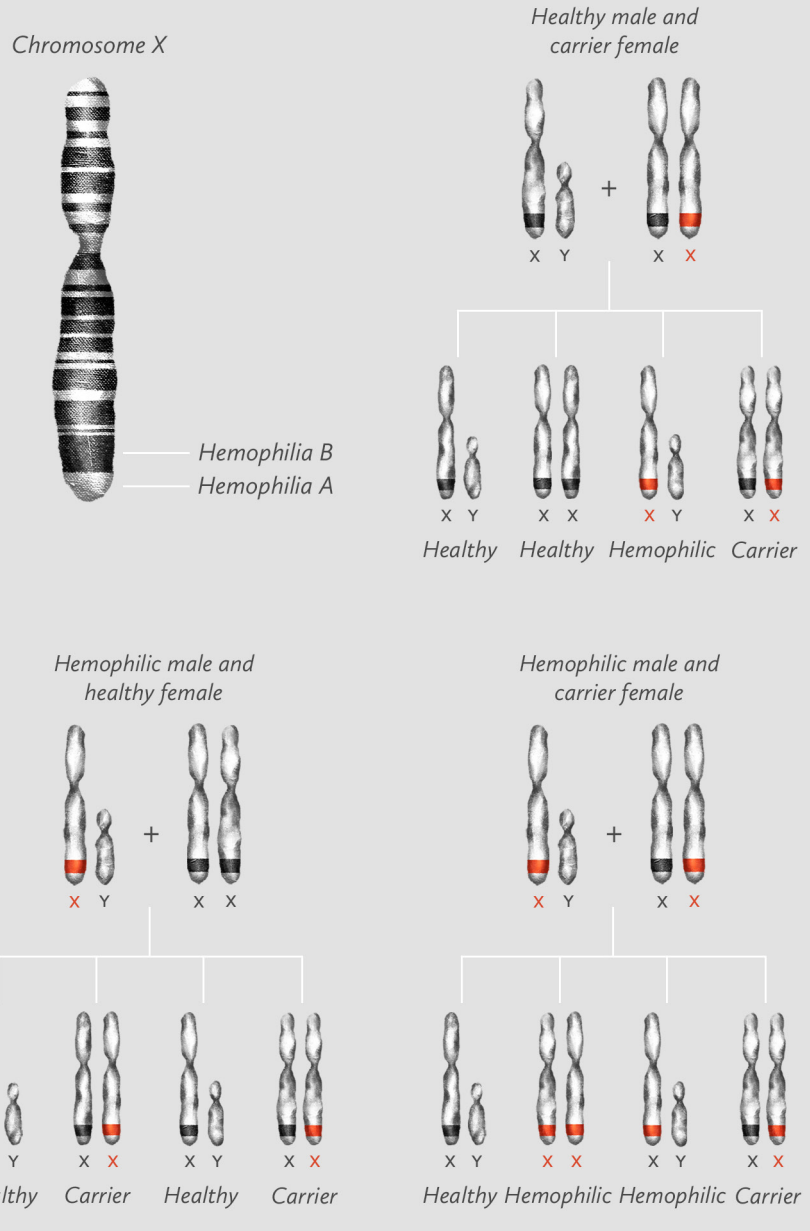
Both types of hemophilia are characterized by bleeding episodes and both result in joint damage. What sets them apart is the tile that is defective in each of them. Thus, in hemophilia A the deficiency is in the factor VIII tile whereas in hemophilia B it is in the factor IX one. Some hemophiliacs have a certain quantity of their affected coagulation factor, while in others the clotting factor is altogether absent. In other words, hemophilia can occur in different phenotypes or degrees of severity. Thus, some patients present with a mild phenotype of the disease, i.e. they have 5-40% of normal levels of clotting factor; others present with a moderate phenotype, i.e. their factor levels are 1-5% od normal; and the last group present with a severe phenotype with less than 1% of normal levels. The last group includes a not-so-unusual group of patients with no clotting factor at all.

Hemophilia is a hereditary disease that is transmitted from parents to their children and to successive generations. It is caused by a faulty gene located on the x chromosome. This chromosome is a sex-determining chromosome, which means that it is transmitted by females, who themselves have no symptoms, and suffered by males. Females have two x chromosomes whereas males have one x and one y chromosome. Transmission of hemophilia is said to be recessive and non-dominant since the disease may not appear in the next generation (generation skipping). Indeed, all daughters born to a man with hemophilia will be carriers but none of his sons will have hemophilia. In effect, hemophilia only becomes an issue when the daughters are considering starting a family.

Abnormalities in coagulation factors arise as a result of defective synthesis of such clotting factors by the human body. Hemophilia being a hereditary condition, the defect is located in the gene that

produces the clotting factor. A coagulation factor is defective as a result of changes in the gene that coded for that factor. The gene, which is actually a fragment of DNA, is like a book made up of a huge amount of words, where a defect would be equivalent to changing one letter, one paragraph or tearing off a whole page from the book. The result of these changes is the development of abnormalities that can range from very minor to extremely serious, depending on the different degrees of factor deficiency (mild, moderate, severe) described above. In the case of hemophilia A the most common factor viii defect is intron 22 inversion, which in our example would be equivalent to moving the pages in the second half of the book to the beginning. But the defect may also be due to changes in a single

INHERITANCE PATTERN IN HEMOPHILIA

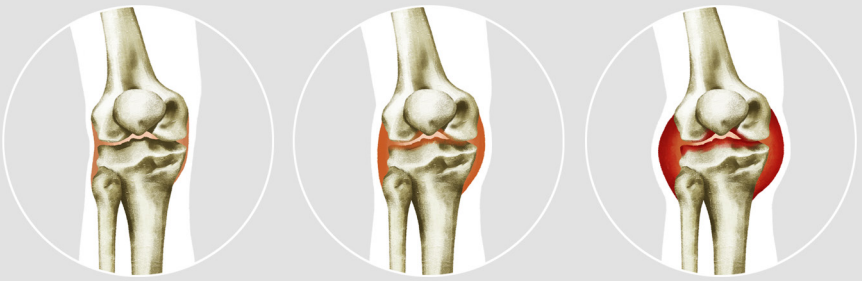


letter (point mutations), the removal of a few sentences (deletions) or the introduction of random words or phrases into a page (insertions). In the case of hemophilia B, errors are also due to point mutations, deletions and insertions, but also to the tearing of a few pages off the book, or the exchange of pages of one book for those of another one that bears no relationship with the former.

Hemophilia, whether type A or type B, is characterized by either spontaneous hemorrhagic manifestations or profuse bleeding following some kind of trauma. It is also important to distinguish between joint, muscle and other bleeding episodes which, on some occasions, can be severe.

To better understand the meaning and significance of joint bleeding for a hemophilic patient, a few basic concepts must be considered about these anatomical structures. Bones are partially held together by a joint capsule that is in turn lined by the so-called synovial membrane with its vast network of capillaries (small blood vessels). The synovial membrane produces a viscous fluid that facilitates smooth joint movements and prevents rubbing between the articulating bones. It acts like a lubricant that reduces friction at the connecting rod in an engine. Injury to the capillaries in the synovial membrane will cause bleeding. But bleeding can also occur spontaneously from an uninjured capillary as a result of the natural movements of a joint. In a person without hemophilia, the clotting mechanism instantly stems the hemorrhage; but in hemophiliacs the bleeding persists. This causes joint swelling and pain. The onset of joint bleeding, also known as hemarthrosis, is characterized by tingling and a burning sensation in the involved articulation. As the capsule fills with blood, the joint will appear swollen and the pain will intensify until all mobility is virtually lost. Without appropriate treatment, repeated bleeding into the same joint causes the synovium to swell and bleed more easily, the blood accumulating in the joint results in progressive tissue damage, production of synovial fluid decreases and friction between the bones leads to partial or total destruction of the joint. This situation, which causes varying degrees of joint deterioration, is known as osteoarthritis or hemophilic arthropathy and may be highly disabling. The joint becomes stiff, painful on movement and unstable. And it becomes even more unstable as the muscles around it start weakening. These hemorrhages occur chiefly in the knee (44% of cases), the elbow (25%), the ankle (15%), the shoulder (8%), the hip (5%), with other locations accounting for only 3% of cases.

GENERAL SYMPTOMS OF EVOLUTION OF AN ARTICULAR BLEEDING OR HEMARTRO



- *Creaking*
- *Tingling*
- *Burning sensation*

- *Inflammation*
- *Pain*
- *Burning sensation*

- *Viscous sensation and gelling touch*
- *Inflammation*
- *Muscle weakening*
- *Morning stiffness*
- *Chronic pain*
- *Limited movement*

Nowadays, –if treated appropriately– patients with hemophilia do not die of a mild, moderate or even severe bleed. The real problem, from a clinical and health policy perspective, is hemophilic arthropathy.

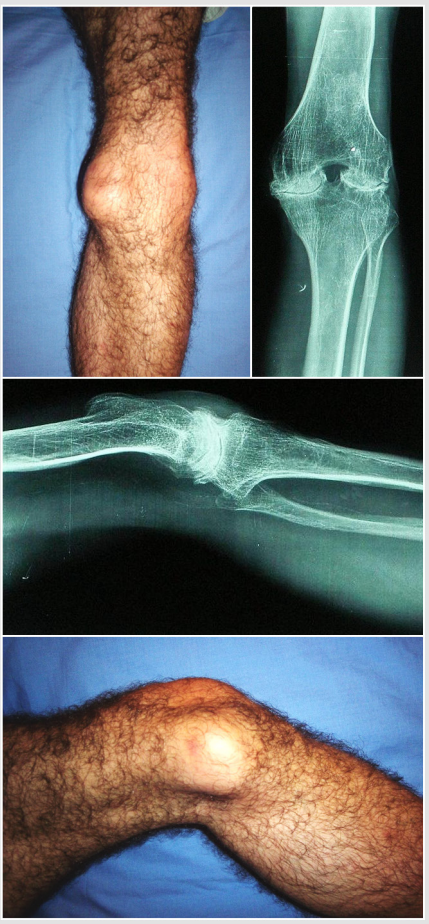
Muscle bleeding, for its part, occurs following a lesion to the muscle capillaries. Sometimes the cause of such lesions is known, but at other times no apparent reason can identified. Further to the bleeding, the muscle becomes stiff and painful. It then becomes swollen, hot on palpation and painful. Bruises develop if bleeding is superficial, but if the hemorrhage occurs in the deeper muscle layers, pressure could be exerted on nerves and/or arteries resulting in tingling and numbness. The final result is usually muscle spasm, a protective muscle contraction that affects the joints pulled by that muscle. Muscle bleeding often occurs in the calves, thighs and the upper portion of the arms.

Bleeding into the psoas muscle (in the front of the hip) or into the muscles of the forearm (both of them fairly frequent occurrences) may affect nerves and arteries and therefore cause irreversible and permanent damage.

Other severe bleeding episodes are those affecting the head, which generally result from injury and may lead to death, especially in children. These hemorrhages may cause pain, nausea, vomiting, drowsiness, confusion, clumsiness, weakness, convulsions and loss of conscience. Hemorrhage from the throat may result from some infections, injuries, dental injections or surgery, and they may cause inflammation as well as swallowing and breathing difficulties. Other potentially very serious hemorrhages, although they do not generally

pose a threat to the patient's life, include hemorrhages in the eyes, the dorsal spine and in the psoas muscle. Hematuria, or blood in the urine, although seldom dangerous, is common in severe hemophilia.

HEMOPHILIC ARTHROPATHY



Nowadays, hemophilia is treated by means of intravenous administration of the deficient factor, VIII or IX, at the appropriate dose depending on age and degree of severity of the bleeding episode. Factor concentrates may be plasma-derived or recombinant. Both are subject to viral inactivation procedures. Although factor administration is the standard of care, some patients develop an immune response against the administered exogenous coagulation factor. The greater the clotting factor deficiency, the stronger the immune response. Patients who develop the so-called "inhibitors" are treated with combined clotting factor concentrates or with activated factor VII.

Nowadays, treatment of hemophilia has come a long way, and patients can treat themselves at home and achieve a high degree of autonomy. Self-treatment requires training by healthcare professionals.

Hemophilia can currently be diagnosed by prenatal screening during the fetal stage. Prenatal tests include amniocentesis (taking a sample of amniotic fluid by a needle inserted into the abdomen); chorionic villus sampling; and cell-free fetal DNA analysis, which involves taking a blood sample from the mother. In the case of assisted reproduction, parents may opt for preimplantation genetic diagnosis whereby embryos created in vitro are screened for inherited diseases. These diagnostic tests provide couples of childbearing age genetic advice as

to whether to embark on a pregnancy or not. Post-natal tests include mutation analysis and determination of clotting factor concentrations.

In summary, notwithstanding the risks inherent in deficient blood coagulation, it can be said that nowadays if the right treatment is administered (both in terms of safety and efficacy) and particularly if such treatment is a prophylactic one, patients with hemophilia can enjoy a very good quality of life and a close-to-normal life expectancy

ACQUIRED HEMOPHILIA

Acquired hemophilia is a bleeding disorder which, although rare, may place the patient's life at risk. Between 0.2 and 1.9 cases are reported for every 1,000,000 inhabitants every year.

This type of hemophilia results from the development of autoantibodies (inhibitors) in persons without hereditary hemophilia or a family history of the disease. These antibodies are normally directed against factor VIII in both adult men and women. Rare cases of autoantibodies against factor IX have also been identified. Therefore the main difference between acquired and congenital hemophilia is that the former is not transmitted from parents to their children, although transmission of a predisposition to form autoantibodies is a possibility.

Although the clinical manifestations of the disease are highly variable across the patient population, symptoms are related, in 80% of subjects, with spontaneous bleeding into the skin, muscles, soft tissue and the mucosae. Hemarthrosis, which is so common in hereditary hemophilia, is very rare in patients with acquired hemophilia. All in all, bleeding episodes can at times be severe, as in the case of retroperitoneal and intramuscular hematomas and of brain hemorrhage. Another manifestation is prolonged bleeding following childbirth, a serious accident or a surgical procedure. Statistics show that in 50% of cases, the disease can be lethal if the appropriate therapeutic measures are not adopted.

The presence of inhibitors is transient in 14 to 35% of cases and disappears without treatment within 20 months. In 54% of cases these result from prenatal and postpartum conditions, immune disorders (autoimmune diseases), certain infections, some drug-to-drug interactions, presence of neoplasms o to drug consumption. The remaining 46% of cases are of idiopathic origin, i.e. the cause is

unknown.

Diagnosis of the disease is simple as it is based on detection of abnormalities in typical coagulation parameters such as an increase in bleeding and coagulation time, a decrease in the number of platelets, longer prothrombin time and longer activated partial thromboplastin time (APPT).

There are two possible kinds of treatment. One aims at getting rid of the antibodies (inhibitors); the other focuses on stopping the bleeding.

Inhibitor eradication is achieved through the use of immunosuppressive drugs such as prednisone and cyclophosphamide aimed at dampening the immunity response of the body. The so-called immunomodulators, such as immunoglobulins, have also shown themselves to be effective at high doses. More recently, a new drug called rituximab has been introduced. It is a monoclonal antibody directed against the CD20 antigen located on the membrane of B lymphocytes, which play a key role in the formation of inhibitors. This drug is also used in patients with congenital hemophilia and inhibitors.

Treatment of bleeding episodes depends on the titer (amount) of antibodies in the patient's plasma. If the titer very low, i.e. below 5 Bethesda units, very high doses of intravenous factor VIII or high doses of immunoglobulin G or desmopressin must be used. If the inhibitor titer is higher than 10 units, porcine factor VIII or activated prothrombin complex can be administered. Although a much more costly alternative, recombinant activated factor VII is also extremely effective. In patients with a very high titer of anti-factor VIII antibodies that have been refractory to immune tolerance induction treatment for 20 months, plasmapheresis therapy is recommended. Plasmapheresis consists in withdrawing the patient's blood, filtering out the antibodies and finally reinfusing the blood back into the patient's bloodstream. However, plasmapheresis is a transient remedy as it is usually not long before autoantibodies start accumulating again.

To conclude, although the circumstances surrounding acquired hemophilia seem very similar to those observed in cases of congenital hemophilia with inhibitors, the former is a distinct clinical entity as it is brought about by autoimmune antibodies, i.e. the body itself produces antibodies against its own normal and properly-functioning coagulation factor. Both conditions may have similar, and sometimes overlapping, consequences in terms of the bleeding they originate,

and the treatment protocols for both may also coincide, but the two must never be confused, especially as regards the hereditary component of congenital hemophilia.

TREATMENT OF HEMOPHILIA

The main goal of hemophilia treatment is to replace or supplement the patient's deficient coagulation factor with normal coagulation factor in order to prevent or address an acute bleeding episode. Exogenous coagulation factors are always administered intravenously, abiding by strict cleaning and disinfection procedures.

Antihemophilic factor concentrates may be classified into two large groups according to their source. Those obtained from human plasma —plasma-derived antihemophilic factors— and those obtained by means of genetic engineering techniques in cultured cells - recombinant factors.

Antihemophilic factors can also be classified in terms of other criteria such as their purity, i.e. clotting activity per milligram of total protein in the final product; the degree of inactivation of viral pathogens; their stability during the preparation process; the presence or absence of animal or human proteins in the culture medium (only in the case of recombinant factors); and the presence or absence of such proteins in the final stabilization of the product.

The fundamental criteria that govern the choice of a certain antihemophilic factor rather than another for treating a patient include the safety of the product as determined by its capacity of preventing the transmission of viral or other pathogens and the ability not to induce the development of inhibitors. Other criteria have been cited such as the clinical/analytical condition of the patient, availability of the product, ease of preparation and administration, cost and the manufacturing laboratory.

CRITERIA FOLLOWED TO CLASSIFY ANTIHEMOPHILIC FACTOR CONCENTRATES

- Derived from human plasma
- Derived from mammalian or human cells through genetic engineering techniques
- Degree of purity (amount of contaminating proteins)
- Inactivation of viral pathogens
- Stability during the preparation process
- Presence or absence of animal or human proteins in the culture medium
- Presence or absence of animal or human proteins as stabilizers
- Half-life

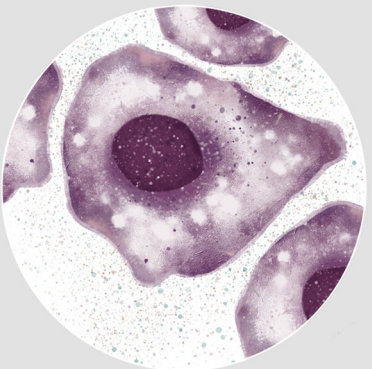
An important advantage of recombinant factors is that, unlike plasma-derived factors, they are susceptible of improvement. It is precisely for this reason that recombinant factors hold so much promise for the future of hemophilia treatment. In fact, it is still possible to further improve their safety, efficacy, their (already low) capacity to produce inhibitors, and their ease of administration. In terms of safety, recombinant factors are growing safer and safer as the amount of human and animal proteins used to stabilize them during their manufacturing process and those present in the final

product are being significantly reduced. Actually, these proteins are no longer used in the new recombinant factors. As regards efficacy, longer half-life recombinant products are being tested through the use of pegylation, liposomes and fusion proteins, as well as other methods such as the alteration of the cleavage sites in the factor molecules or the binding sites of these molecules to hepatocytes or collagen proteins, which inactivate them. This will decrease the amount of factor concentrates needed to treat each bleeding episode, both on demand and prophylactically. Induction of inhibitors will be further diminished with the recombinant factors of the future as this technology will make it possible to modify the sites in the exogenous factor molecule that the body perceives as extraneous and accordingly sets out to attack by getting the immune system to generate inhibitors. Lastly, the new recombinant factors will be much more convenient as a result of their greater stability at room temperature and their potential to be dissolved in a small amount of water given their lower protein content. This will make them easier to transport and easier to prepare and administer, even in continuous perfusion regimens.

Manufacturing plasma-derived factors requires the use of human blood obtained from a large number of donors. Approximately 150 donors are required to obtain 1000 units of factor VIII, which means that the blood comes from a very heterogeneous source. Conversely, recombinant factors are obtained from selected and generically manipulated mammalian cells (infused with the human factor VIII o IX gene) that are cultured in the lab. This type of factor is thus obtained from a much more homogeneous, controlled and safe source.

FACTOR TYPES

PLASMA-DERIVED FACTOR
Human plasma from thousands donors



RECOMBINANT FACTOR
Mammalian cells or human

The higher or lower amount of proteins present in factor concentrates indicates the higher or lower risk posed by such products. Specifically, the more contaminating proteins, which are not factor proteins, the higher the risk to suffer undesirable effects. The content of extraneous proteins in plasma-derived factors is much higher than in recombinant factors, and within the group of recombinant factors, second generation factors contain more of these proteins than third and fourth generation ones. The amount of extraneous proteins present in the final product, the viral inactivation method used and the source where the factor was initially obtained are what determines the level of safety of each product with respect to infections caused by viral or other pathogens.

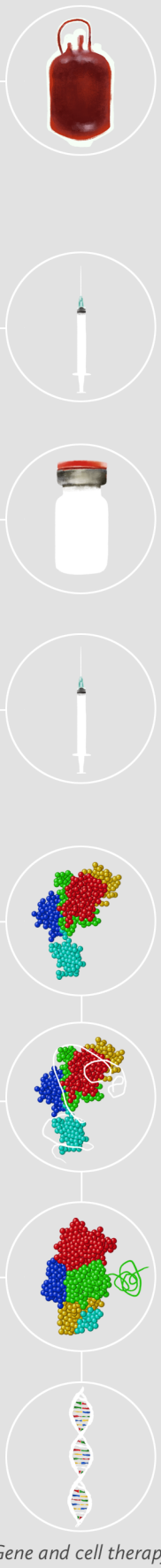
HISTORY OF INFECTIONS IN THE LAST 24 YEARS

- Plasma-derived factors: HIV/HCV/HBV/VHA/PV B19/ Prions
- Recombinant factors: have been no infections

The viral inactivation methods used in the manufacturing process of both plasma-derived and recombinant factors eliminate viruses with a lipid (fatty) envelope around them such as HIV (human immunodeficiency virus), HCV (hepatitis C virus), HBV (hepatitis B virus), the West Nile virus, the virus causing SARS (severe acute respiratory syndrome) and the avian influenza virus, among others. However, if we consider protection against non-lipid enveloped viruses such as parvovirus B19, HAV (hepatitis A virus) and other viruses without an envelope, or against other types of pathogens such as the prions causing vCJD (variant Creutzfeldt-Jakob disease, or "mad cow" disease in humans), plasma-derived the degree of safety provided by plasma-derived factors could be lower.

EVOLUTION IN THE TREATMENT OF HEMOPHILIA

		Injection volume (mL/2000 UI)
< 1930	*Immovilization, ice, rest, analgesia*	
1930	*Blood transfusions*	> 4000
1940	*Plasma*	
1960	*Low-purity Factor VIII concentrate*	2000
	Cryoprecipitate	
1970	*Intermediate-purity Factor VIII concentrate*	400
1980	*High-purity Factor VIII concentrate*	80
1990	***Recombinant Factor VIII (1st generation)***	20
2000	***Recombinant Factor IX***	
	Recombinant Factor VIII (2nd generation)	5
	Recombinant Factor VIII (3rd generation)	
> 2000	*Biosimilars Factor VIII, Factor IX*	5
	Fusion proteins, PEG, Factor VIII, Factor IX	
	PEG-rFVIIa	
	Future therapies	



Gene and cell therapy

Throughout the medical history of the disease, hemophilia has been treated with both plasma-derived and recombinant factors. Around 1970 the first plasma-derived antihemophilic factors became available. In their first 11 years of existence, these factors were responsible for the infection of the hemophilic population with HIV, HCV, Parvovirus B19 and HAV giving rise to the a notorious pandemic. At the beginning of the 90's, the first recombinant factors appeared, and their use has been gradually replacing that of plasma-derived factors. Nonetheless, recombinant antihemophilic factors are not the only recombinant proteins use for the treatment of disease.

REASONS FOR USING PLASMA-DERIVED FACTORS

- Patients' individual and personal wish
- Previous history of inhibitor formation
- Inhibitor deletion
- Unavailability of recombinant factors
- Developing countries
- Lower cost
- Personal interests of prescribing physician

Other examples are insulin, interferon alpha and vaccines. These proteins have also been widely used in modern medicine for many years. The safety of recombinant factors is amply supported by the 24 years during which they have been used for treating hemophilia without resulting in one single adverse effect.

In spite of all the clinical and scientific evidence in favor of the use of recombinant factors as the treatment of choice for hemophilic patients, they are not used to the same degree in every country.

While in countries like Canada, the United States and Australia they are available to every patient, in Europe they are used by 75% of hemophilic patients, and in some Asian countries like Japan and Korea 50% of patients benefit from treatment with those products. Nonetheless, in less prosperous regions their use barely hits the 10% mark.

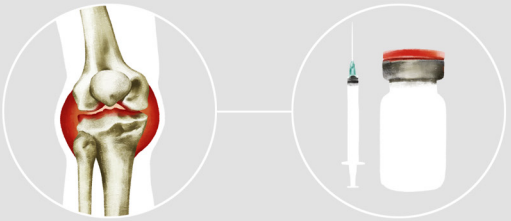
Use of recombinant factors as first choice treatment for the hemophilic population is currently supported by several internationally recognized organizations such as the US National Hemophilia Foundation, the

Canadian Hemophilia Society, Hemophilia Foundation Australia, the United Kingdom Haemophilia Centre Doctors' Organization and the Scientific Commission of the Victoria Eugenia Royal Hemophilia Foundation (Spain).

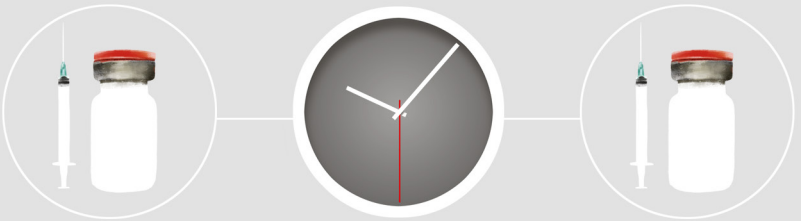
GUIDELINES FOR TREATMENT OF HEMOPHILIA

ON-DEMAND TREATMENT

1. Treatment is applied once the bleeding has occurred



2. Coagulation factors are perfused every 12 or 24 hours



PROPHYLACTIC TREATMENT

1. Treatment is usually administered 2 or 3 times a week

Monday *Wednesday* *Friday*

Those who advocate the use of plasma-derived rather than recombinant factors for treating hemophilia claim that these products are less expensive. However, it should not be forgotten that plasma-derived products can lead to other healthcare expenses as a result of the fact that their level of safety is not always very high. Such expenses are related with anti-HIV, HCV or HBV, treatment, psychological treatment for patients and their families, reproductive treatments for serodiscordant couples (to prevent contagion and transmission to descendants), treatment of the sequelae of HIV and HCV infection, economic compensation, work absenteeism, etc. But, more importantly, there is a high cost in terms of human lives as many of these infections have been, and still are, fatal.

CRITERIA USED TO DECIDE WHICH KIND OF FACTOR TO USE IN PATIENTS WITH HEMOPHILIA

- Safety regarding transmission of viral or other kinds of pathogens
- Capacity to prevent the development of inhibitors
- Clinical/analytical conditions of the patient
- Product availability
- Ease of preparation and administration
- Cost
- Brand (manufacturer)

Despite the above, some medical/clinical circumstances may on some occasions justify the use of plasma-derived factors. For example, a personal wish of the patient; a previous history of inhibitor formation following use of plasma-derived or recombinant factors; the need to delete an inhibitor (immune tolerance induction) or the unavailability of recombinant factors. In developing countries such as those in Africa and Latin America, the use of plasma-derived factors is justified because of the scarcity of resources affecting these countries.

Regardless of the type of factor used, treatment of hemophilia can be approached in two ways. First of all, there is treatment on demand, which involves administration of the deficient coagulation factor when a bleeding episode occurs. The other type of treatment is prophylaxis, whereby the clotting factor is preventively administered two or three times even if no bleeding occurs.

ADVERSE EFFECTS OF HEMOPHILIA TREATMENT

INHIBITORS IN HEMOPHILIA

Although currently available treatments for hemophilia are very safe and efficient, they are associated with a few complications such as the potential development of the so-called inhibitors of coagulation factors. This means that a certain percentage of patients treated with (plasma-derived or recombinant) antihemophilic factor concentrates will develop inhibitors. These are antibodies that neutralize or "inhibit" the function of factors in the coagulation cascade.

Development of these antibodies impairs patients' quality of life as the treatment available for inhibitor eradication is unsatisfactory. In addition, it is associated with significant socio-economic consequences as it substantially increases the cost of antihemophilic treatment as much higher amounts of clotting factor, or the use of other products, may be required to treat each bleeding episode.

The likelihood that a patient may develop inhibitors depends on multiple factors, among them whether the patient has received previous antihemophilic treatment; whether there has been a change in the type of factor administered or in the manufacturer of such a factor (although this aspect has been shown to exert a decreasing influence); the severity of the disease; the type of hemophilia suffered (up to 30% for hemophilia A and up to 8% for hemophilia B); the patient's age (inhibitors usually appear following the first few factor VIII or IX concentrate infusions, which generally coincides, in moderate and severe cases, with the first few years of life); a genetic predisposition (race-related factors), etc. But apart from the frequency of inhibitor development, it is also important to know the inhibitor titer, that is, the amount of inhibitors in the bloodstream of each patient. Thus when the inhibitor titer is low, their presence is usually transient and they disappear after continued treatment with coagulation factors or following treatment with increasing infusions of the factor that led to their development. Nonetheless, if the inhibitor titer is very high the antibodies tend to display such a high level of activity that they neutralize the effect of the administered factors.

It the last few years there has been a lot of speculation as to the causes behind the development of inhibiting antibodies. It seems that genetic factors could be at play, chiefly those related with the type of genetic alteration that gives rise to hemophilia. Thus, for example, in hemophilia A, patients with mutations affecting a significant portion of the factor VIII gene develop inhibitors more frequently (around 35% incidence) than patients where the genetic deficiency in the factor is due to small mutations (around 5% incidence).

As regards hemophilia B, patients with mutations affecting a significant portion of the factor IX gene have a 50% chance of developing inhibitors. This probability decreases to 20% when mutations arise from less significant alterations in the gene. Furthermore, a higher risk of developing inhibitors is related with being a first or second degree relative of a patient with inhibitors and with the major histocompatibility complex.

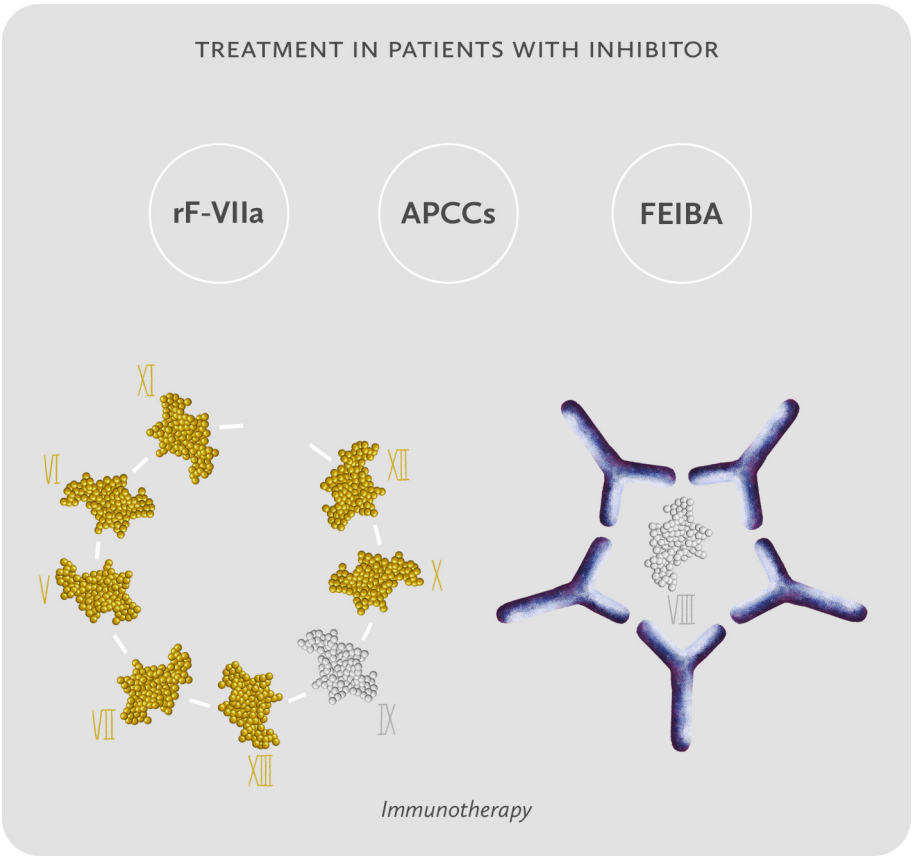
In the same way as it reacts against any extraneous molecule, our immune system also launches a response to the presence of an exogenous antihemophilic factor (from outside the patient's body) by creating inhibitors against that factor. This immune response is mediated by blood cells such as B and T lymphocytes.

The same patient may present with more than one kind of inhibitor. The most common are igG class inhibitors, most of them targeted against specific sites in the factor molecule. For example, anti-factor factor VIII inhibitors bind to the sites required by factor X; factor IXa; factor IX, or by vVF (von Willebrand factor) and factor Xa. They may target just one of these sites or many of them simultaneously. Interaction of inhibitors with the binding sites for other factors leads to a disruption of the coagulation cascade, such that treatment with an antihemophilic factor becomes ineffective.

The presence of inhibitors is generally confirmed by a so-called Bethesda assay, which quantifies the amount of antibodies found in the patient's blood, expressed in Bethesda units. According to this assay, patients may display a low immune response (with a low inhibitor titer) or a high immune response (with a high inhibitor titer) to the exogenous factor. Thus, if their immune system reacts rapidly and intensely, the inhibitor titer may rise above 5 Bethesda units. Such inhibitors are classified as high-responding inhibitor. However, the immune system may produce a slower and weaker response, with the inhibitor titer staying below the 5 Bethesda unit mark. These inhibitors are known as low-responding inhibitors.

Patients presenting with low-responding inhibitors can benefit from immune tolerance protocols (frequent infusions of high doses of clotting factor), whether they have hemophilia A or hemophilia B. Moreover, patients with high antibody titers are usually subjected to plasmapheresis (exchange of all of the patient's blood, following an immune adsorption procedure to lower the inhibitor titer) before receiving antihemophilic factor treatment. Nevertheless, plasmapheresis therapy is just a temporary solution as a new factor infusion will again stimulate the development of inhibitors.

At present, several more or less conventional —and more or less effective— strategies are used to treat hemophilic patients with high-responding inhibitors. The best results have been obtained with recombinant activated factor VII and with activated prothrombin complex concentrates (APCCs), albeit effectiveness of the latter

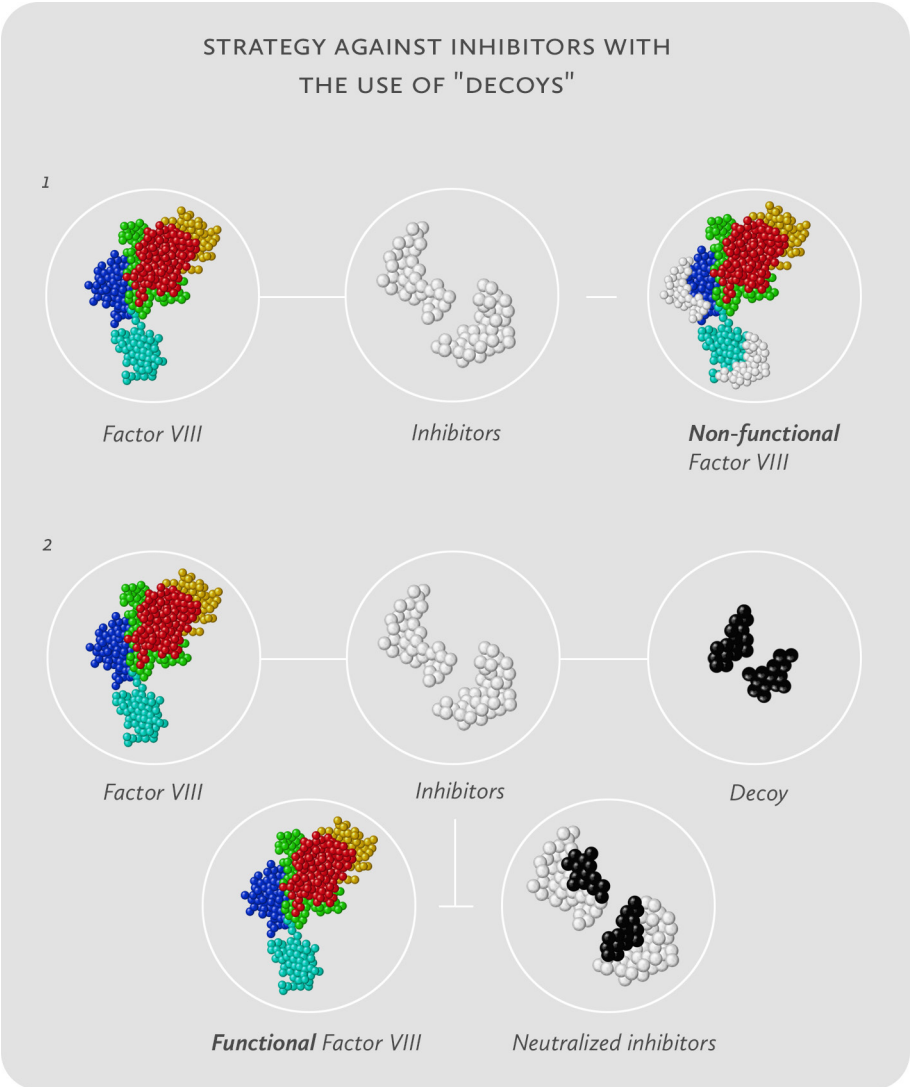


is slightly lower. Therapeutic combinations such as recombinant activated factor VII with APCCs or with antifibrinolytics (compounds that prevent the destruction of fibrin); and FEIBA®, a complex that combines several coagulation factors, have shown themselves to be equally effective —and much less costly. However, even if these treatments do partly address the severe bleeding experienced by patients with inhibitors, all of them are plagued by two shared issues: their low coagulating efficacy as compared with factor VIII or IX concentrates and the fact that the results obtained across patients are highly variable.

In the last few years, the possibility to "hoodwink" immune cells getting them to alter their immune response has gained ground. This idea, first introduced for diabetes mellitus and myasthenia gravis, is based on using antibodies against the lymphocytes that participate in the inhibitor production process or in using specific molecules to "kidnap" the inhibitors themselves. Thus specific antibodies against the CD4 T-lymphocyte receptors have been developed. In mice, simultaneous administration of such antibodies, together with the antihemophilic factor, has resulted in a significant decrease in the generation of inhibitors. However, this strategy is unspecific as it affects all lymphocytes globally and could lead to a dangerous reduction of CD4 T-lymphocytes in the patient's plasma.

The most promising studies focus on strategies specifically targeted to curbing the development of inhibitors. These strategies are based on infusing a series of molecules containing sequences similar to the sites in the factor molecule that produce inhibitors. Inhibitors bind to these "decoy" molecules (are "kidnapped" by them) leaving the actual factor undisturbed. As the amount of unbound inhibitors decreases significantly, the coagulation factor is now available to perform its coagulating function.

This treatment would be highly specific as it would be targeted exclusively to inhibitors and would not alter general immunologic mechanisms, which are often seriously impaired in hemophilic patients, especially given the high levels of HIV/AIDS-induced immunosuppression in those patients. More recently, tests are being conducted with new recombinant factors containing modifications at those highly immunogenic sites that induce the development of inhibitors. Also, clinical trials are already under way on a new much less immunogenic recombinant factor of human origin.



INHIBITOR DELETION PROTOCOLS. IMMUNE TOLERANCE INDUCTION

Immune tolerance induction is a valuable procedure to eradicate inhibitors. It involves administration of factors FVIII or FIX until inhibitor levels cannot be detected by the Bethesda assay and the half-life of the coagulation factor in plasma goes back to normal. Prothrombin complex concentrates and factor VII must be used to resolve or prevent acute bleeding episodes and their complications in case immune tolerance fails. Sometimes supplementary administration of immunosuppressive drugs and/or extracorporeal plasmapheresis is recommended. The success rate of immune tolerance is 90% at 6 to 12 months for inhibitors against factor VIII. Success of ITI depends on such variables as time of treatment initiation, which should be as soon as possible following inhibitor detection; use of immunosuppressors; a low response of the inhibitor below 5 Bethesda units. ITI protocols are variable, some using high doses (200 IU/kg/day) of von Willebrand factor-rich plasma-derived factor VIII while others use very low doses (25 IU/kg/day) when inhibitor levels are very low. In any event, once inhibitors have disappeared long-term prophylactic treatment is recommended with regular factor administration (three times a week).

Protocols aimed at deleting inhibitors against factor IX have been less successful and can, in addition, result in severe anaphylactic or nephrotic syndromes. These may occur when the defective factor IX gene contains large deletions, with treatment of acute bleeding episodes with recombinant activated factor VII being usually required to avoid overexposure to factor IX.

SIDE EFFECTS OF HEMOPHILIA TREATMENT: VIRAL AND EMERGING INFECTIONS

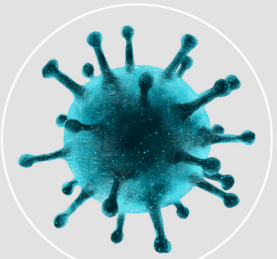
The side effects of hemophilia treatment include, first of all, anaphylactic reactions against coagulation factors. This side effect is nevertheless negligible nowadays thanks to the availability of high-purity concentrates. Of much greater concern is the risk of infection by pathogenic agents, particularly by viruses and prions. The development of inhibitors against exogenously infused factors VIII or IX is not, strictly speaking, a side effect of hemophilia treatment, as such antibodies are considered a natural rejection of extraneous molecules by the body. However, as mentioned above this does not make inhibitors any less of a problem for the efficacy of antihemophilic

treatment.

Nowadays, infections in hemophilic patients are caused chiefly by the so-called emerging pathogens or pathogens that have become resistant to certain (generally antibiotic or antiviral) drugs. The reasons for this are related with adaptation of microorganisms and the dramatic increase in demographic movements. Indeed, when people move from one place to another they disseminate diseases that until then had been circumscribed to specific regions of the planet. By pathogens, we refer chiefly to viruses, bacteria and, more recently, also to proteins (prions).

As explained above, the possibility of a hemophilic patient to become infected is closely connected with the source where the different factor concentrates are obtained. Thus, factors prepared from human plasma will always pose a higher risk of infection whereas recombinant factors are free from the risk of infection as they are obtained from animal or human cells in a laboratory.

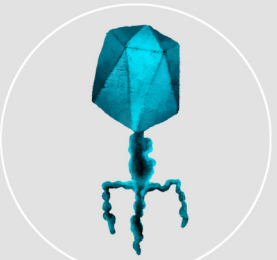
POTENTIAL PATHOGENS
AND POSSIBILITIES OF INACTIVATION



ENVELOPED VIRUS

- *HIV*
- *HCV*
- *HSB*
- *West Nile virus*
- *SARS*
- *Avian flu virus*

INACTIVATION
Solvents–Detergents
60–80 °C/10–72 hours
138 °C/2 seconds



NAKED VIRUS

- *HAV*
- *Parvovirus B19*
- *Others?*

PROTEINS

- *Prions*

INACTIVATION
134 °C/18 minutes
121 °C/30 minutes
Caustic soda 1N
15 minutes

One of the most accurate definitions of emerging disease has been proposed by the US Institute of Medicine. According to that definition, an emerging disease is a new disease, a reemerging old disease or a disease caused by drug resistance, whose incidence in humans has increased in the last two decades, or threatens to increase in the near

future. As mentioned above, one of the main factors behind disease emergence is the great ease with which human beings can nowadays move from one place of the globe to another. Indeed, in 1850, over 350 days were needed to travel round the world and the world population was 24 million people; today (2014), it takes just a few hours to travel round the world and the world population exceeds 7 billion people.

There are already almost one-hundred diseases that fit the definition above. Some of these are HIV-AIDS, tuberculosis, severe acute respiratory syndrome, poliomyelitis, parvovirus B19, hepatitis C, new variant Creutzfeldt-Jakob disease (vCJD), avian influenza, West Nile Virus, the Ebola virus, etc.

The possibility of viral infection through plasma-derived products has been drastically reduced thanks to the modern viral inactivation methods. Nonetheless, it is well known that these methods are not 100% reliable. But why is it that viral inactivation methods are not 100% effective? The first reason is that not all viruses are the same in terms of structure and function. Thus, some viruses are covered with a lipid envelope, whereas others are not. Naked viruses are sensitive to heat and detergent inactivation treatments while those not equipped with an envelope are heat-resistant. Enveloped viruses include HIV, the hepatitis C virus, the hepatitis B virus, the West Nile virus, the SARS virus and the avian influenza virus; naked viruses include HAV, parvovirus B19 and probably others we are not aware of because they have not yet emerged. These viruses are all highly resistant.

The question is then what as-yet unknown emerging viruses, or which known non-enveloped viruses are amenable to successful inactivation. One example is parvovirus B19 which although it is not deadly in the short term may result in the development of autoimmune diseases that produce autoantibodies against molecules of the subject's own body, which can cause serious long-term effects or even autoimmune polyarthritic processes that usually further intensify severe hemophilic arthropathies.

Until a few years ago, the idea that proteins could be infectious agents was unthinkable for microbiology and for the classical theories of infection, as infections had always been held to be caused by viruses and bacteria. But today, we have come to the groundbreaking realization that an infection can be produced by proteins, which are not really living organisms but mere molecules. This conceptual change implies that it is possible to transmit isolated molecules and

provoke a deadly disease because these molecules (eg. prions) are capable of aggregating and precipitating in vital organs like the brain.

Prions are the proteins that cause the "mad cow" disease in cattle as well as its human version (variant Creutzfeldt-Jakob disease [vCJD]). They are transmitted by ingestion of infected-meat and by blood transfusion. Prion-induced disease affects the structure of the brain and bind to the lymphocytes in the blood. Prions pose a major public health risk in connection with the use of plasma-derived coagulation factors as they are currently impossible to detect by means of blood tests. Moreover, prions cannot be inactivated using standard antiviral methods; they are difficult to filter and to separate; and cannot be destroyed by conventional pasteurization as they have to be heated at 134°C for 18 minutes or at 121°C for 30 minutes for successful inactivation. These conditions would break up the structure of the coagulation factors and make them non-functional. Also, prions are endogenous (they exist in our body with a very similar structure) so we do not recognize them as extraneous and we do not generate an immune response to fight them. Finally, prion-induced disease may remain asymptomatic for as long as 40 years.

The fact is that prion-contaminated plasma has been used in many countries in the world, including Spain, to prepare coagulation factors. Although it has been estimated that the risk of contracting a prion-induced disease through plasma-derived concentrates is very low, we cannot today say that these products are risk free as many uncertainties and unknowns remain with respect to prions. Neither the mechanisms of action nor the factors that produce prion-induced disease are known. Nor has it been established whether a very small amount of prions is enough to develop the disease, although it seems to be dose-dependent.

Although at the beginning, not many people had confidence in the first generation of recombinant drugs, today, in the second decade of the 21st century, their wide availability, high efficacy and, above all, their excellent safety profile have turned these products into the treatment of choice.

The question is then how to tackle the threat posed by emerging pathogens. The answer is conceptually easy but complicated in practice. It is internationally known that prevention of known viral infections requires the use of the safest factors possible, which is achieved by applying highly effective inactivation methods to plasma-derived products or using recombinant factors to prevent any kind of infection, including prion-induced infections. Although this seems easy enough to do, many practical difficulties stand in the way. One of these difficulties has to do with the fact that many developing countries cannot afford the inactivation methods or the recombinant factors; also, there are patients that could avail themselves of recombinant factors but do not use them either because there is currently no recombinant product to treat their condition (von Willebrand disease), or because they have a history of inhibitor development.

As regards prion-induced infection, the best alternative from a safety perspective (at least until a test is developed to detect prions in the donors' blood) is the use of recombinant products.

Nowadays, many recombinant proteins have become part of our therapeutic arsenal. Some of them, like recombinant factors, have shown, over their 20+ years of clinical history, an excellent safety profile and a high level of efficacy.

For the effective implementation of the World Hemophilia Federation's "treatment for all" principle, prion-induced infection must be addressed through the development of strategies targeted at carefully selecting both donors (on the basis of their personal characteristics and their region of origin [there are geographical areas with a higher incidence of Creutzfeldt-Jakob disease]) and the plasma used. Other alternatives include leukodepletion, i.e. removing leukocytes with prions attached to them, and sophisticated new techniques to separate prions from plasma, although application of such techniques would increase in the cost of plasma-derived products to such an extent that they would become as costly as recombinant products.

To conclude it can be said that the potential of becoming infected by emerging pathogens is an inescapable fact in the treatment of hemophilia with plasma-derived products. Moreover, an effort should be made to address the problem of emerging pathogens by public

health measures and, specifically, through inactivation and purification of plasma-derived pharmaceutical products. This issue must be tackled both from a medical and a socio-political perspective, and medical practitioners should understand the potential risks of each type of product and inform patients in an appropriate and effective way.

BIOTECHNOLOGICAL TREATMENTS AND ADVANCED THERAPIES

RECOMBINANT FACTORS: THE SAFEST TREATMENT?

Discovery of DNA made possible what is nowadays known as "modern" biotechnology. Indeed, these days it is possible to introduce a portion of ADN into an animal, human or plant-derived bacterial or eukaryotic cell and obtain a certain, so-called recombinant, protein that has functional activity. This means nothing less than that it is now possible to create proteins such as insulin, the growth hormone and plasminogen activator to be used therapeutically n the clinical setting. Today, in the second decade of the 21st century, hundreds of therapeutic proteins have already been produced by means of recombinant technology.

RECOMBINANT FACTORS.
THE FUTURE OF HEMOPHILIA TREATMENT

- Greater safety
- Higher efficacy
- Fewer inhibitors
- More convenient
- Open to improvement

But before these advances became a reality, humanity suffered some devastating iatrogenic events such as the appearance of Creutzfeldt-Jakob disease in children treated years before with growth hormone obtained from the pituitary glands of deceased persons; and the massive and lethal human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections, in the 1980's and 1990's respectively, in hemophiliacs treated with plasma-derived factors.

Important as it is to learn from the lessons of the past, there is no doubt today that "recombinant medicine" holds high hopes for the future. Recombinant products, given their peculiar manufacturing procedure based the expression of a "therapeutic" gene from a fragment of DNA in a cell in order to produce a functional protein that is free from any human or animal component, are particularly "clean". For that reason, they are the therapy of choice to treat many of today's diseases. But recombinant therapy has not yet gained unanimous support, especially among the more conservative physicians. Far from that, the first recombinant proteins used in clinical practice, such as somatostatin in 1976, insulin in 1982, erythropoietin in 1986, tissue plasminogen activator in 1987, and antihemophilic factors in 1990, aroused significant skepticism among clinicians due to their fear of as-yet unknown disciplines such as Molecular Biology and Genetic Engineering.

In spite of that, a large number of biotechnology companies were created. To the extent that a new kind of pharmaceutical company came into scene: the biotechnological company. Even some traditional pharmaceutical companies started gearing their activities toward genetic pharmacology. Investments in this sector are ongoing and expert estimations indicate that in the medium and long term over 30% of the world pharmaceutical market will be made up of these products.

Nowadays, many recombinant proteins have become part of our therapeutic arsenal, some of them with a history of over 20 years of clinical use, an excellent safety profile and proven efficacy. Others are at an initial phase of development, such as those that may be useful to treat conditions such as Graves' disease, multiple sclerosis, myasthenia gravis, scleroderma, phenylketonuria, galactosemia, hemoglobinopathy, etc.

Vaccines, all of which are now recombinant, are used in the general population but also in children, the elderly and immunodepressed individuals because of their excellent safety profile. Of course recombinant interferon for long-term dose-intensive treatment of hepatitis C, for example, is still highly effective. But recombinant products are not only therapeutic, they are also used in the spheres of nutrition, dietary supplements and cosmetics, where recombinant peptides are common.

Without realizing it, we have entered the era of recombinant solutions where man is capable of manufacturing virus and prion-free albumin, the most abundant protein in blood, which could up to now only be obtained from human plasma.

If we compare plasma-derived and human and animal tissue-derived products with recombinant products in terms of their pharmacological efficacy, no significant differences are observed. But when the comparison is made with respect to patient safety the situation is clearly different. In hemophilia treatment, for example, recombinant products have never in their 24 years' history (1990-2014) of clinical use produced one single severe or even mild adverse event, and these are naturally not expected to occur with third and fourth generation recombinant products, which are completely free from human or animal proteins. On the other hand, plasma-derived products, even if their safety profile has improved, have caused thousands of deaths of hemophilic patients throughout the world as a result of HIV and HCV infections. In the years to come these products will continue to compromise the quality of life of many patients as a result of the side effects of anti-HIV drugs, particularly heart disease and renal and psychiatric damage, or of diseases like cirrhosis and cancer of the liver resulting from HCV infection.

Plasma-derived products, and in general all products of human origin, have a good safety profile, albeit only with respect to lipid-enveloped viruses such as HIV, HCV and HBV. Nevertheless, they are not exempt from the growing risks resulting from the well-known profound social changes taking place at a global scale related to the transmission of new diseases including prion diseases, such as Creutzfeldt-Jakob disease (CJD) or "mad cow" disease, which is transmitted through the blood. They are also exposed to the risk of transmitting the so-called emerging diseases, whose causative agents are known but which have nevertheless crossed the "inter species barrier" to become epidemics and even pandemics in areas where they were unknown. This was the case of HIV and other viruses that may be resistant to viral inactivation.

There is therefore an almost universal consensus in favor of recombinant products. Thus, in Europe their use is recommended in the "European Guidelines for Recombinant Therapies" and several countries, such as the United Kingdom, Australia and even Spain (through the Scientific Commission of the Victoria Eugenia Royal Hemophilia Foundation) advocate their use.

The question that comes to mind is "why aren't recombinant products used more widely taking into account their high efficacy and excellent safety profile?" It is difficult to answer this question without going beyond the teachings of Hippocratic medicine. One could say that the problem, at least partly, is a fear of the unknown, but there are also purely economic reasons that result in a lack of solidarity in the distribution of healthcare expenditure. It must be acknowledged that recombinant technology entails high costs both regarding the treatment itself and the investment made by pharmaceutical companies and by governments, whose efforts to produce equitable healthcare budgets often fail to satisfy physicians and even patients themselves. Finally, we cannot rule out potential conflicts of interest in physicians prescribing the products of manufacturers they may have financial ties with.

In any event, although clinical practice has always been a high risk endeavor, now that safer and more effective products exist that are at the same more costly, the picture has complicated even further. In this situation, treating clinicians have the herculean task of prescribing the safest, most efficient and most cost-effective product within a very narrow scope of practice.

In short, we are faced with an interesting pharmacological paradox —avoidable for some, irresoluble for others, namely that the greatest advances in therapeutic medicine – even if often safer - are not always wholeheartedly embraced for the simple reason that they tend to be more costly. Have we forgotten that patient safety should prevail over any cost consideration?

NEW RECOMBINANT FACTORS

The short- and medium-term future of hemophilia treatment will be based on the use of modified recombinant factors, whereas longer term it will be characterized by the use of cell therapy and gene therapy strategies. Recombinant factors are now being modified to achieve three kinds of improvements. Firstly, reduce the capacity to form inhibitors; secondly, promote the efficacy of infused factors by increasing their half-life and, thirdly, achieve higher levels of safety by eliminating contaminating animal and human proteins.

The current strategy to minimize inhibitor formation is immune system screening. The idea is that inhibitors should recognize "decoy"

molecules, whose structure is similar to that of the clotting factor, and target these instead of the infused factor itself, thus preventing factor inactivation. Also, some modifications are being introduced into the tridimensional structure of the factor in order to reduce the development of inhibitors, making it more stable and less immunogenic.

With respect to obtaining longer-acting factor VIII molecules, strategies such as the use of pegylated liposomes and polysialic acid polymers, and the inhibition of factor catabolism in the body are being developed. More recently, fusion protein technology has shown much promise, owing specifically to the development of a new type of factor IX for hemophilia B, already marketed in the United States (ALPROLIX® de Biogen Idec), with a half-life up to 5 times higher than the currently available product. The factor VIII product manufactured by Baxter (Advate®) and Pfizer's factor IX (Benefix®), both third generation factors, as well as Pfizer's factor VIII (ReFacto® AF), also of third generation, which has no traces of animal proteins or mouse monoclonal antibodies, are all recombinant and boast excellent safety profiles. In addition, the upcoming launch in Spain, is the new recombinant factor of human origin of Octapharma (Nuwiq®), which will decrease the incidence of inhibitors in patients.

ADVANCED THERAPIES IN HEMOPHILIA

Future therapeutic products based on advanced therapies such as gene therapy and cell therapy may offer myriad potential clinical applications for the treatment of various monogenic disorders including hemophilia. Although hemophilia is particularly amenable to treatment with these techniques given its monogenic nature and the low levels of deficient coagulation factor required to achieve a moderate or even phenotype, research in the field is still in its infancy and more work must be undertaken to determine whether advanced therapies can be safely applied to this patient population, which presents with specific clinical characteristics. There is much reason for optimism, but caution is imperative in order not to raise false expectations in our patients.

The development of biotechnology has resulted in the emergence of new therapies bound to change medical practice. Advanced gene-, cell-, and tissue-based therapies (gene therapy, cell therapy and regenerative medicine) constitute new strategies for the treatment of

some conditions. Their purpose is either the regeneration of tissue or the restoration of function. Cell therapy is based on transplantation of living cells into an organism in order to repair tissue or restore a lost or a deficient function; gene therapy, in turn, involves the transplantation of genetically modified cells.

Cells are useful for these therapies because of their ability to differentiate into the specific lineages required for repairing a given type of tissue. However, only 20% of stem-cell-related studies in the literature constitute a genuine advancement in scientific knowledge. This can be attributed to the high cost of this kind of research and the multiplicity of issues that remain to be solved such as the need to develop new best practice guidelines for the management of cell cultures and transplant procedures; and guarantee the genetic stability of stem cells before and after transplantation, their quantity and quality when used therapeutically, as well as their safety, specifically regarding teratogenicity (tumor formation).

Most clinical and pre-clinical trials conducted to date on the effects of cell therapy and gene therapy on hemophilia, using both viral and non-viral vectors, have shown no adverse effects, although the immune response against vectors' viral envelopes, the transgenes encoded and hepatotoxicity represents the limit to the clinical application of these therapies.

Gene therapy strategies for hemophilia have been based on the use of (chiefly) lentiviral and adeno-associated virus vectors based on adult stem cells and autologous fibroblasts from the same patient, as well as platelets and hematopoietic stem cells. Non-viral gene transfer and chimeric oligonucleotide-based mutation repair have also been used. Studies on cell therapy for hemophilia have used transplanted healthy cells, such as hepatic sinusoidal cells or iPSC-derived endothelial progenitor cells, to repair or substitute for deficient function.

Of particular interest for the development of advanced therapies are the results obtained by High et al., who used zinc finger nucleases and adeno-associated vectors to correct hemophilia B mutations by "editing" B mutated DNA sequences. Although in this case factor IX expression is only 5% of normal levels, the advantage of the strategy is that it allows strict control of the integration of normal sequences into DNA, thus preventing the development of tumors induced by insertional mutagenesis.

The most significant problems that remain are related to the increasing the efficacy of factor expression levels and maintaining them constant in the long term, and controlling the immune response against vectors and transgenes.

Nathwani et al., completed an interesting clinical trial in patients with hemophilia B. The trial included patients with severe hemophilia B (<1% factor IX) who were injected with a special adeno-associated vector that expressed factor IX and was capable of easily transducing (infecting) hepatocytes. Results showed that this is a more efficient vector and that patients expressed between 3% and 11% of the normal factor IX levels. Another encouraging finding was that no inhibitors (anti factor IX antibodies) were detected. These results must be considered taking into account, first of all, that the expression of factor IX corresponds to a mild-to-moderate phenotype of the disease and, secondly, that concomitant glucocorticoid therapy is required in order to prevent immune rejection and elevation of liver transaminase levels

Generally speaking, the results obtained to date constitute the foundations of the future application of advanced therapies to the treatment of hemophilia. The number of patients included in the clinical trials on advanced therapies in hemophilia conducted so far, including the one by Nathwani, has been very low. In addition, results have been highly variable. Although Nathwani's study is the first one to show a substantial expression of factor IX in humans, it does not clarify whether there is a relationship between liver toxicity and the immune response it generates.

Almost two decades ago, discussions began on the potential benefits of gene therapy for the treatment of hemophilia. At the time, prominent experts envisioned that the cure of the disease would be possible by the first decade of the 21st century. These predictions fueled the hopes of both hemophilic patients and the physicians treating them. But unfortunately those hopes were not fulfilled. Although significant advances have been achieved since the first clinical trials on gene therapy began in the 1990's, especially regarding the design of transfer vectors, researchers came across many problems, especially with respect to biosafety. To date, many of the problems posed by these strategies (immune response, insertional mutagenesis, efficacy and length of gene expression, patient recruitment for clinical trials and large-scale vector production) have not been resolved.

The first question what must be asked is whether the time and financial investment required to establish advanced therapy protocols that may in the future be applicable to the treatment of hemophilia is at all warranted. Although current treatment of hemophilia is optimal, the answer is clearly in the affirmative since hemophilia is a chronic condition and current high-frequency treatment —especially in prophylaxis regimens—. The second question is whether advanced therapies are at all feasible. In this regard, hemophilia is considered an optimal candidate for such treatments as it is a monogenic disease; achieving relatively low levels (1-5%) of coagulation factor already places patients in the moderate phenotype category; a large amount of target cells are available for study; there is no need to regulate gene expression; and a large amount of animal models are available for experimentation.

Other more general —though no less important— questions include, for example, whether it will be possible to extrapolate the safety- and expression level-related outcomes obtained in small-scale animal models to human beings; whether the combination of cell therapy/ gene therapy with the use of mesenchymal stem cells will be the most efficient tool; and whether protocols will have to be restricted to adeno-associated and non-viral vectors.

The problems to be addressed will be the immunogenicity and biosafety of the therapies, as well as the maintenance of factor levels and length of expression. Also, even if most research is currently focused on viral vectors non-viral methods should also be taken into consideration.

In any event, the main criterion to be considered, should be the ratio between efficacy and safety, taking into account that these are highly sensitive issues for both patients and physicians, especially in the face of the lethal consequences of viral infections of the past (HIV/ HCV) on the hemophilic population; and the special immunologic conditions of these patients. In this respect, we may have to settle for less stringent expression requirements (lower coagulation factor levels) in return for greater safety.

Furthermore, caution must be exercised when bringing to light the results of the studies on this issue in order to avoid raising any false expectations in the patient population with respect to advanced therapies, which in spite of the promise they hold as potential therapeutic strategies are only at an initial stage of development. In

the longer term, after overcoming the challenges mentioned above, advanced gene- and cell-based therapies may become a plausible alternative for patients with hemophilia; in short, optimism is in order, but fantasy is best avoided.

ADVANCED FUTURE THERAPIES STRATEGIES IN HEMOPHILIA

GENE THERAPY

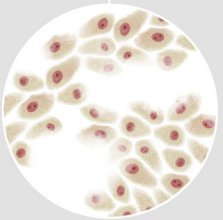
- *Lentiviral vectors*
- *Adenoassociated vectors*
- *Non-viral vectors*

Healthy individual

CELL THERAPY

- *Adult stem cells*
- *Fetal stem cells*
- *Embryonic stem cells*
- *iPSCs (induced pluripotent stem cells*

Transplant
Effectiveness, safety



Transfected or differentiated therapeutic cells

Hemophilic patient

- *Rare disease*
- *Monogenic disease*
- *Mild phenotype (5–40% factor)*
- *Gene expression does not require regulation*
- *Many experimental animal models*

- *Factor VIII or IX expression*
- *Correction of coagulation disorder*

FOR MORE INFORMATION...

• ***Textbook of Hemophilia, 3rd Edition, 2014. Ed. Wiley – Blackwell.***

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• ***Guía sobre Hemofilia. Ministerio de Sanidad, Servicios Sociales e igualdad. 2012.***
Available in: http://www.msssi.gob.es/profesionales/saludPublica/medicinaTransfusional/publicaciones/docs/Hemofilia_AspectosOrganizativos.pdf

• ***La hemofilia en imágenes. Federación Mundial de Hemofilia. 2004.***
Available in: http://www1.wfh.org/sp/index.html

• ***La Hemofilia. Conceptos básicos. Wyeth, 2005.***

• ***Factor VIII therapy for hemophilia A: current and future issues.***
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• ***Current management of hemophilia B: recommendations, complications and emerging issues.***
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ASANHEMO

The Andalusian Hemophilia Association was legally established in 1990 as a not-for-profit governmental entity. In 2009 it was declared a public utility institution by the Spanish Ministry of the Interior. Such official recognition of the work carried out by the Association was granted in view of the fact that Asanhemo's overarching goal is to promote the public interest and achieve the goals set forth in its bylaws. It should be mentioned that the Association's activities are not aimed at benefiting only its members but the whole hemophilic community.

To celebrate its 25th birthday, Asenhemo is publishing this Basic Guide to Hemophilia, clinical manifestations, diagnosis and treatment. And we believe we could not do it in a more auspicious way than by rigorously complying with two of our paramount goals: facilitate the education of carriers, patients and their friends and families, and disseminate information on hemophilia among society at large.

asanhemo
ASOCIACIÓN ANDALUZA DE HEMOFILIA