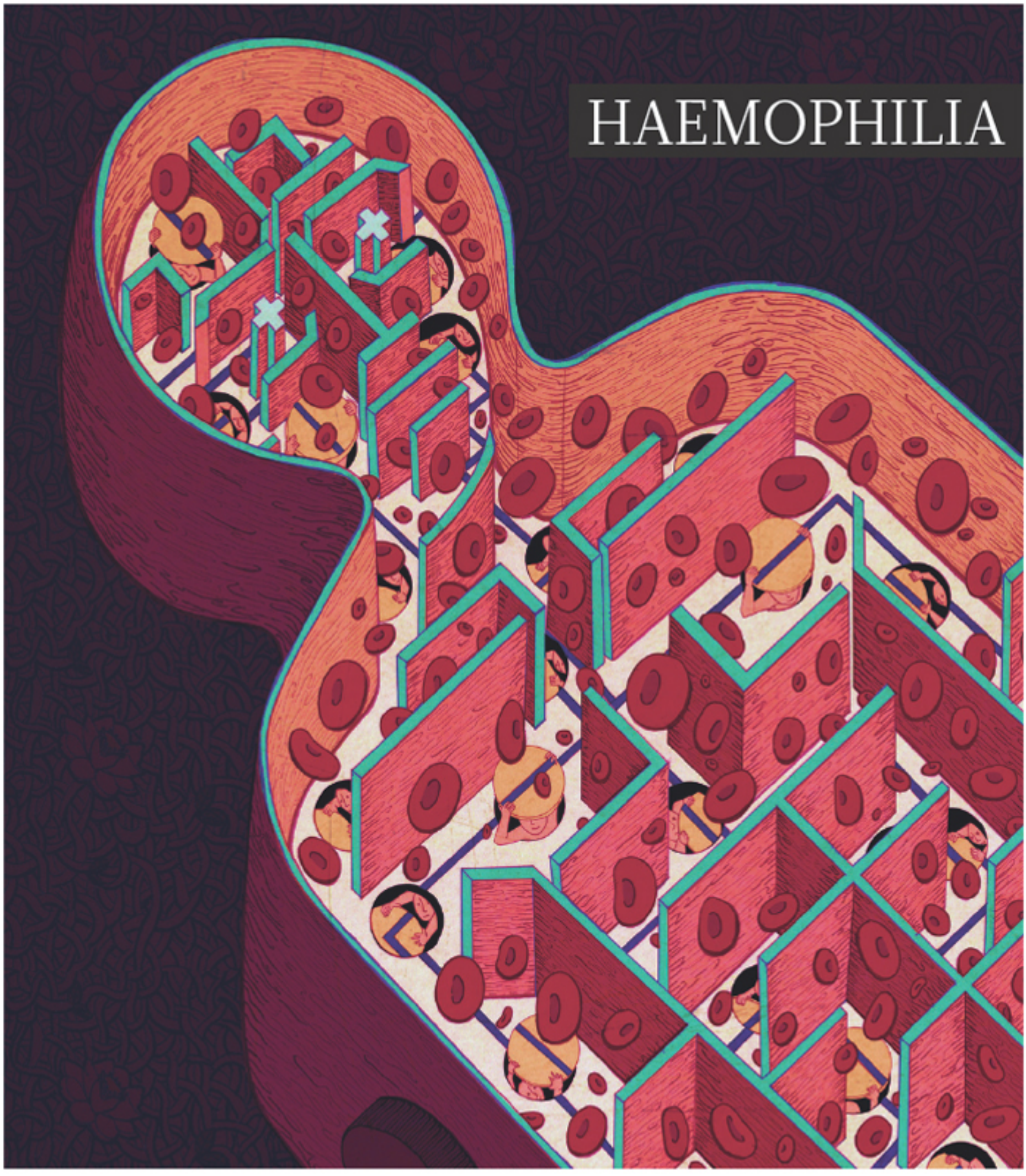
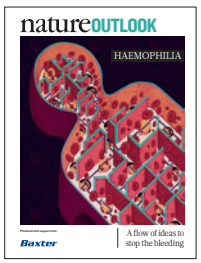
nature OUTLOOK



nature OUTLOOK

HAEMOPHILIA

27 November 2014 / Vol 515 / Issue No 7528



Cover art: Jessica Fortner

In any complex machine, the lack of a single part can lead to big trouble. That is the problem faced by the 170,000 people globally who have the bleeding disorder known as haemophilia. A genetic mutation (usually inherited) suppresses the production of proteins that make blood coagulate (see page S158). Internal bleeding into the joints causes bone degradation and excruciating pain (S170), and even mild injuries can be life-threatening.

The standard therapy is frequent infusions with blood-clotting promoters. These treatments are uncomfortable and expensive, so it is welcome news that several longer-lasting clotting factors have been developed (S162). Many people develop an immune resistance to these infused factors, but relief may be on the way in the form of anti-inhibitory pills made from plants (S166). Development of these pills depends on colonies of haemophilic dogs that serve as cooperative test subjects (S172).

Clotting-factor infusions treat symptoms of haemophilia, but gene therapy could provide a cure (S160). Research is also moving ahead on an alternative treatment strategy to remove or disable the body's anticoagulants (S168) rather than adding clotting factors.

The haemophilia community is still haunted by the traumas of blood supplies that were contaminated with HIV and hepatitis C. These experiences have led to reluctance to accept the good news that may soon be on offer, says medical historian Stephen Pemberton (S165).

To maximize its impact, this Outlook is being published in both *Nature* and *Scientific American*.

We are pleased to acknowledge the financial support of Baxter Healthcare Corporation in producing this Outlook. As always, *Nature* has sole responsibility for all editorial content.

Herb Brody
Supplements Editor

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CITING THE OUTLOOK
Cite as a supplement to *Nature*, for example, Nature **Vol. XXX**, No. XXXX Suppl., Sxx–Sxx (2014).

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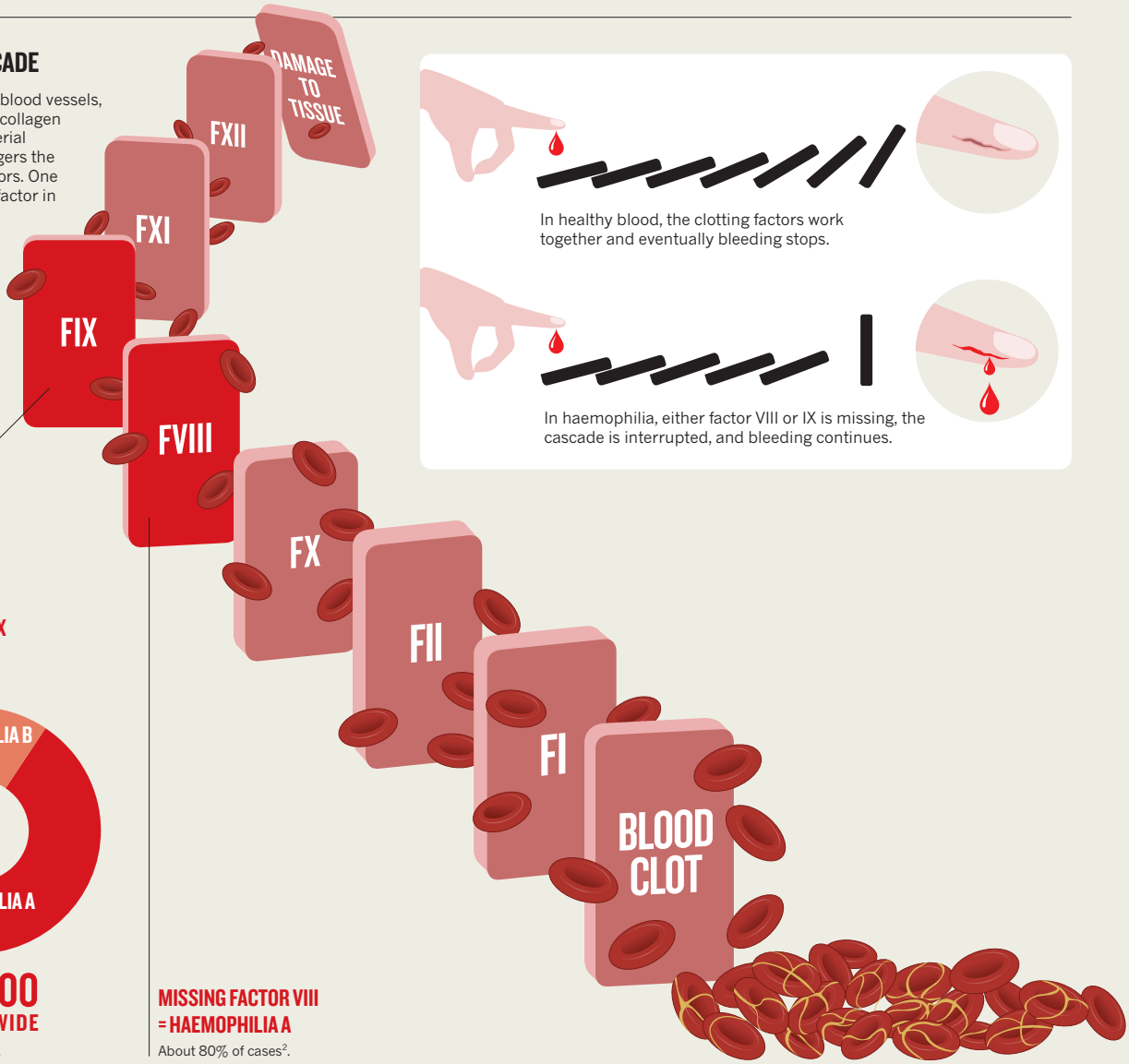
BORN IN THE BLOOD

People with the inherited bleeding disorder haemophilia lack factors that cause the blood to clot. The disease affects thousands of people around the world and has even played a part in historic events. By **Neil Savage**.

COAGULATION CASCADE

When damage occurs to blood vessels, exposure of the blood to collagen in the cell walls and material released by the cells triggers the activation of clotting factors. One factor activates the next factor in a series of events (some not depicted here) that eventually produces fibrin. Fibrin forms a mesh to hold together a plug of platelets to form a clot (platelets are a type of cell that circulates in the blood to help coagulation)[1].

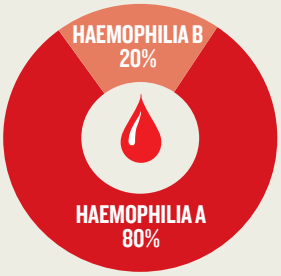


In healthy blood, the clotting factors work together and eventually bleeding stops.

In haemophilia, either factor VIII or IX is missing, the cascade is interrupted, and bleeding continues.

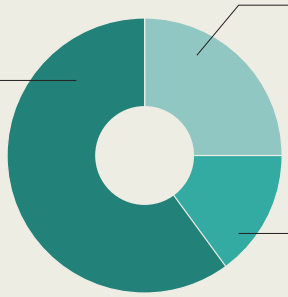
MISSING FACTOR IX = HAEMOPHILIA B
About 20% of cases[2].

MISSING FACTOR VIII = HAEMOPHILIA A
About 80% of cases[2].



AT LEAST 172,000 PEOPLE WORLDWIDE HAVE HAEMOPHILIA

OF ALL THE PEOPLE WITH HAEMOPHILIA...



60%
Have severe haemophilia (clotting factor in the blood is less than 1% of the level in normal, healthy people)[3]
Bleeding after injury, spontaneous bleeding, risk of joint damage. Median age at diagnosis is one month.

25%
Have mild haemophilia (clotting factor levels of 6–30%)[3]
Bleeding only after serious injury, accidents or surgery. Heavy menstrual bleeding in women. Median age at diagnosis is 36 months, though it is often not diagnosed until after a serious injury.

15%
Have moderate haemophilia (clotting factor levels of 1–5%)[3]
Bleeding after injury, some spontaneous bleeding, risk of joint damage. Median age at diagnosis is eight months.

TREATMENT TIMELINE

About two-thirds of the world's population lacks access to prophylaxis with clotting factors because the cost of treatment is too high.

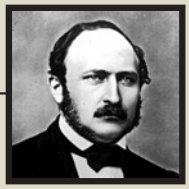
1840

1840s
First blood transfusions for bleeding episodes.

THE ROYAL DISEASE

Queen Victoria became a carrier of haemophilia through what is believed to have been a spontaneous genetic mutation[4].

Queen Victoria
1819–1901



Prince Albert
1819-1861

Victoria passed it on to one son, who died of bleeding, and two daughters.

Victoria

Edward VII

Alice
1843–1878

Alfred

Helena

Louise

Arthur

Ludvig IV

Leopold
1853–1884

Beatrice
1857–1944

Haemophilia spread throughout European royalty, including Victoria's great-grandson, Alexei Romanov, son of the last Russian tsar, Nicholas II.

The 'mad monk' Rasputin claimed to be able to treat Alexei's haemophilia, and his influence with the tsar's family is credited with contributing to the Russian revolution.

In 2009, DNA tests on Alexei's remains showed that Victoria carried haemophilia B.

Alexandra
1872–1918

Nicholas II
of Russia

Alexei
1904–1918

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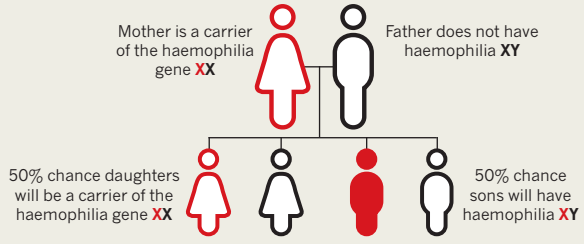
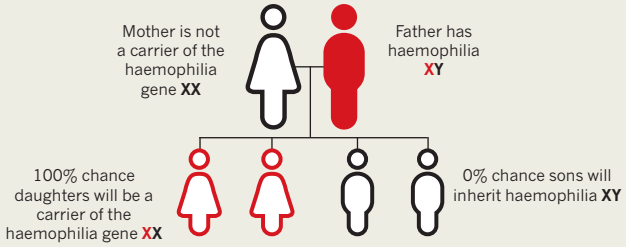
INHERITANCE PATTERN

The mutations causing haemophilia are carried on the X chromosome. Woman are usually carriers, with a 50% chance of having sons with haemophilia or daughters who are carriers. Men with haemophilia will have no sons who are haemophiliacs, but all their daughters will carry the gene. In rare cases, female carriers or girls with both X chromosomes affected will have haemophilia; fewer than 10% of cases occur in females. Approximately one-third of cases arise from spontaneous mutation[5].

Does not have haemophilia
Carrier of haemophilia gene
Has haemophilia





An estimated 40,000 haemophiliacs worldwide were infected with HIV, including 70–90% of those with severe haemophilia[6].

40,000 HIV

The cost of prophylaxis treatment could reach $300,000 per child per year[7].

$300,000

1900

1923 First use of plasma replacement therapy

1950s–1960 Fresh frozen plasma requires large volume transfusions

Mid-1960s Cryoprecipitate (a concentrate of blood components made from frozen plasma) allows smaller transfusions

1970 Freeze-dried clotting factors allow home-based prophylaxis

1970s–early 1980s Haemophiliacs contract hepatitis and HIV from blood products

1987 Heat-treated factor VIII reduces risk of blood-born pathogens

1989 Genetically engineered factor VIII, no risk of HIV or hepatitis

2000

1997 Genetically engineered factor IX

Future Gene therapy

2014 First extended-life clotting factors approved

References: 1. Pipe, S. W. (Ed.) The Hemophilia Report (2014) available at: www.hemophiliareport.com 2. 2012 World Hemophilia Foundation Survey, covering 91% of the world's population; 3. US National Hemophilia Foundation 4. Rogaev, E. I. *et al. Science* **326,** 817 (2009). 5. US Centers for Disease Control and Prevention 6. Starr, D. *Blood: An Epic History of Medicine and Commerce*, p346, Harper Perennial (2000). 7. Manco-Johnson, M. A. *et al. N. Engl J. Med.* **357,** 535–544 (2007).

JESSICA FORTNER

GENE THERAPY

Genie in a vector

Repairing the faulty genes that cause haemophilia could ultimately cure the disease, but it will be a tough challenge.

BY JULIE GOULD

Martin never learned to ride a bike, could not play football with his friends and wore a crash helmet when playing in the garden, just in case he bumped his head. His parents had good reason to be protective: his severe haemophilia B meant that the gentlest touch could lead to a serious, debilitating bleed. "It's very frustrating, growing up with haemophilia," says Martin. "You want to be like the other kids, but you can't."

As a result of an inherited genetic mutation, people with haemophilia B lack a protein called factor IX that is crucial for forming blood clots (see page S158). Currently, patients are treated several times a week with infusions of a concentrated version of the protein. This stops the bleeding, but it does not address the underlying cause of the disease nor does it fully remove its debilitating symptoms.

A few years ago, Martin had to stop his work as a truck driver. "I was letting the company down because I couldn't make it into work," he says. "The bleeding into my joints had made it very painful for me to move." In 2011, after 37 years of pain and joint degeneration caused by internal bleeding, Martin signed up for a clinical trial of a gene-therapy treatment at the Royal Free Hospital in London, hoping that it would provide some relief.

Rather than infusing functional clotting factors, the therapy aims to get the body to create its own. DNA with a functional factor IX gene was bundled into the molecular wrapper of a virus — known as AAV8 — then shuttled into liver cells, where factor IX is normally made.

Of the six patients who enrolled, four were able to discontinue their infusion treatments after the therapy[1]. Martin was one of them: his factor IX levels increased significantly, taking him out of the severe haemophiliac range and into the moderate group. His clotting factor levels have remained stable ever since.

The success was a crucial stepping stone for Edward Tuddenham, emeritus professor of haemophilia at University College London, who led the clinical trial. He wants to find a treatment not just for haemophilia B but for the much more common haemophilia A — but that is turning out to be a challenge.

FREEDOM OF EXPRESSION

The viral vehicle AAV8 is ideal for treating haemophilia B, but it works less well for haemophilia A. This is because the DNA encoding the clotting factor that is missing in the latter — factor VIII — is about six times larger than for factor IX, so it doesn't fit into AAV8. To make it fit, researchers often cut 4,500 base pairs out of the factor VIII gene sequence. The section they delete encodes a specific region of the protein — called the B-domain — that ensures efficient secretion of factor VIII. In its place, Tuddenham and his colleagues tried inserting a DNA sequence that is one-fiftieth of the size, but has the same function. But in a 2010 study of haemophiliac mice, these B-domain-modified treatments did not increase the level of factor VIII expressed in the blood[2]. Since then, Tuddenham has not only been trying to fix the gene but also to improve its expression.

The rate at which the factor VIII gene produces its protein is affected in part by the placement of the triplets of DNA bases — codons — that dictate where translation of the genetic material into protein should start and stop. The start and stop codons in the DNA sequence of a normal mouse or human factor VIII gene, did not promote vigorous protein production. "So we replaced them with better ones," says Tuddenham. When that was done, expression levels in a mouse model of haemophilia went from about 2% of that found in healthy mice to about 2,000%. The increase produced by the codon optimization was "enormous, truly stunning", he says.

In 2015, Tuddenham and his team hope to lead trials to test safety

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Meet people living with haemophilia in our film:
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and efficacy of their optimized factor VIII gene therapy for people with haemophilia A. The number of people in the trials is likely to be between 10 and 20, but even if the factor is expressed effectively in humans, there are still hurdles to overcome.

DELIVERY ON A PLATELET

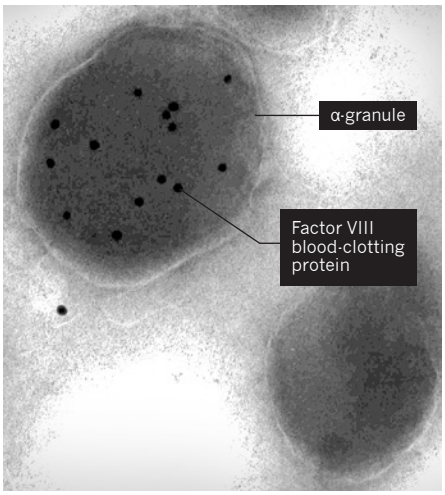
One hurdle is that AAV8 can be administered only once, because the virus triggers a strong immune response. "After one treatment with AAV8, you can't ever have a repeat dose. You are immunized against it," says Tuddenham. So although gene therapy could be a one-off cure, if the immune response is triggered before the therapy reaches the target, it is useless.

David Wilcox at the Children's Hospital of Wisconsin Research Institute in Milwaukee, hopes to get around this problem by using the body's own cells to deliver factor VIII. He is developing a way to insert functional factor VIII into structures called α-granules, which are found inside blood cells called platelets (see image, right). Platelets are the first cells to arrive at a wound site, where they rapidly begin to help form blood clots by releasing chemical messengers. Wilcox is working on modifying platelets to also release functioning factor VIII. "This removes the problem of having AAVs and factor VIII proteins floating around the rest of the body," says Wilcox, "thus avoiding any immune reactions."

"We've still got a lot to learn about gene editing in large animals before we even think about trying it in adult humans."

First, however, Wilcox has to harvest blood stem cells from the patient. He uses growth factors to coax stem cells in the bone marrow out into peripheral blood vessels, where they can easily be collected. The stem cells, which make up 2–5% of the peripheral blood sample, are then separated out in a procedure called peripheral blood stem cell apheresis and undergo gene therapy so that they contain the working factor VIII. The patient then has chemotherapy to partially suppress their existing bone-marrow stem cells before receiving a transfusion of the engineered stem cells into the blood. These cells find their way back to the bone marrow, where they will eventually produce platelets that contain functioning factor VIII.

In 2013, Wilcox tested the procedure on three dogs with severe haemophilia A, using a human factor VIII gene — and two of the dogs no longer require the usual treatment with infused factor VIII[3]. As predicted, none of the dogs showed signs of developing antibodies to the human factor VIII proteins — when the dogs received a cut, blood clots formed faster than they had without the gene therapy. "We think that the factor VIII is secreted from the platelets so quickly at the trauma site that the immune system does not have time to react before the factor VIII can start repairing the vascular injury," says Wilcox. Like Tuddenham, Wilcox's team hopes to start clinical trials inext year.



DAVID A. WILCOX & PAQUITA NURDEN

Researchers are modifying platelets to release factor VIII from α-granules at the site of injury.

But even if platelets can offer an alternative delivery vehicle, it could be an unpleasant one for patients. "I think they have a viable approach for patients with antibodies to AAVs or those affected by HIV and hepatitis," says Tuddenham, "but the doses of chemotherapy treatment before the stem-cell transplant aren't a walk in the park".

CORRECTING IN PLACE

So far, gene-therapy trials have focused on adults with the disease, but haemophilia is an inherited disease, affecting a person from birth. Unfortunately, the technique is not a viable option for children. If a child's liver were to be infused with factor VIII genes introduced through AAV, there would be an initial increase in the levels of clotting factor in the blood, as with the adults in Tuddenham's 2011 trial. But as the child grows, the expression levels would decrease when new liver cells are produced without the functioning factor VIII gene are produced, says haematologist Katherine High, at the Children's Hospital of Philadelphia in Pennsylvania.

In theory, Wilcox's method might work in children because the functional clotting-factor genes have been integrated into the stem cell's genome and will be passed on to daughter cells. In practice, however, no responsible physician would expose an infant or child with a non-lethal disease such as haemophilia to chemotherapy.

A promising way to avoid these problems is *in vivo* genome editing, in which mutant genes are corrected *in situ* rather than replaced. This could potentially work at any age — but the earlier in life such a treatment is available, the better, as the benefit would be lifelong.

Conceptually, this approach is as simple as setting up a biological tool to cut out the mutated area of the genome, then another to insert a corrected template, says Merlin Crossley of the University of New South Wales, Sydney, Australia. Crossley sees gene-editing therapies as the best potential tool for curing haemophilia.

This could be particularly beneficial for children: as the liver grows, the new daughter cells would contain the functioning clotting-factor gene. The clotting factor would then be recognized as part of the body, and could ultimately eliminate the child's haemophilia. "The replacement template is cloned from healthy patients and wouldn't be attacked by the immune system because it isn't considered as foreign," Crossley says.

A 2011 study in mice[4] by High provided strong evidence that genome editing is a viable option. Immediately after birth, one set of mice was given Tuddenham's style of gene-transfer therapy; a second set was given the genome-editing treatment. High discovered that the levels of functioning clotting proteins in mice receiving the genome-editing treatment stayed high even after a portion of their liver was surgically removed; in the mice receiving gene therapy, by contrast, factor levels decreased. "This is the advantage of this treatment, especially for children," says High.

Genome editing has to be precisely targeted to the mutation to be repaired, and the sheer number of mutations for haemophilia A — more than 2,000 — makes this a challenge.

Both High and Tuddenham believe that in the short term, genome editing is not the answer. "The gap between proof-of-principle experiments in mice to clinical trials in humans for gene-transfer therapy was 14 years," says High. "And we've still got a lot to learn about gene editing in large animals before we even think about trying it in adult humans, let alone infants."

Having his haemophilia reduced to a moderate level has improved Martin's quality of life tremendously. He has needed the standard infusion treatment fewer than ten times since the gene therapy, and says that "only one of those occasions was a serious bleed". He says that signing up for the trial was not an easy decision, because there were not any other similar trials on which to base his decision. But he believes that his successful experience should help to encourage people to participte in future studies. "You go from a position of knowing what you are, how you are and how to deal with it, to a position of complete uncertainty," he says. "So I hope that the uncertainty is reduced for other patients when they hear about our experiences." ■

Julie Gould *is the editor of Naturejobs.*

1. Nathwani, A. C. *et al. N. Engl. J. Med.* **365,** 2357–65 (2011).
2. McIntosh J. *et al. Blood.* **117,** 798–807 (2011).
3. Du, L. M. *et al. Nature Comm.* 4, 2773 (2013).
4. Li, H. *et al. Nature* **475,** 217–21 (2011).

WORLD FEDERATION OF HEMOPHILIA

Boys with haemophilia receive a blood-clotting factor by intravenous injection (also referred to as an infusion).

CLOTTING FACTORS

Stretching time

Extending the life of clotting factors may improve quality of life for people with haemophilia.

BY NEIL SAVAGE

For the parents of a child born with haemophilia, the diagnosis comes with both good and bad news. The good news is that the child, at least if he (or rarely, she) is born in the developed world, can expect a near-normal lifespan, up from a mere 20 years in 1970. The bad is that the parents must teach themselves to find their child's veins, insert a needle and infuse him with a clotting factor to replace what he lacks. Parents must infuse a toddler as often as every other day, and children with haemophilia will have to continue that treatment for the rest of their lives.

But treatment is getting easier. Down the road, gene therapy and other approaches look likely to bring longer-term treatments for patients with the rare bleeding disorder. For now, improvement in treatment lies in the emergence of new, longer-lasting replacements for the blood-clotting factors missing from the blood of people with the condition. These therapies could stretch the time between infusions to days or even weeks. The first two such treatments were approved by the US Food and Drug Administration (FDA) earlier this year, and more are in the pipeline, with some expected to be approved in 2015. As these therapies emerge, dealing with haemophilia will become less troublesome (see 'Drugs to help the blood'). This could increase compliance with treatment, reduce complications — and perhaps even allow some people to live almost as if they were free of the disease.

Replacing the clotting ability lacking in haemophilia has been the treatment since the 1840s, when attempts were made to treat people with the disease by transfusion with whole blood from people with normal clotting. By the end of the 1960s, freeze-dried concentrates of clotting factors were available for home use, to prevent spontaneous bleeding. In the 1990s, treatment leapt forward again, with donated plasma being replaced by clotting factors manufactured through recombinant DNA-technology, eliminating the transmission of viral diseases that had devastated the haemophiliac community in the 1970s and 1980s.

But prophylactic treatment still has its problems. The clotting factors do not last very long in the body. Depending on the person, the amount of factor VIII — the protein missing in haemophilia A — in the bloodstream drops by half in a mere 8–12 hours. Factor IX — which people with haemophilia B lack — lasts longer, 18–24 hours. Those short half-lives mean that most people with haemophilia must transfuse themselves every two or three days. And inserting a needle directly into a vein can be difficult. "Adherence to therapy is not great, because you have to inject yourself, and it's a hassle," says David Lillicrap, a professor of pathology and molecular medicine at Queen's University in Kingston, Ontario, Canada.

One 2001 study suggested that up to 40% of

people with severe haemophilia do not follow the prophylactic schedule[1]. Those people are more likely to develop spontaneous bleeding that causes joints to fill with blood and results in progressive damage similar to arthritis. They can also develop intracranial bleeding, which can cause brain damage and even death.

Drug companies have responded with clotting factors that last longer, making the time between infusions greater. Biogen Idec, based in Cambridge, Massachusetts, has two such factors approved by the FDA this year. Eloctate, for haemophilia A, was approved in June and is recommended for an initial infusion once every four days, with a physician adjusting that up to five days or down to three as appropriate. Alprolix, the company's treatment for haemophilia B approved in March, promises infusions once a week, and perhaps every ten days or two weeks in some patients. Other versions of the clotting factors from other drug developers are showing similar extensions of lifetimes.

"It's a big improvement," says Timothy Nichols, a cardiologist and pathologist who studies haemophilia at the University of North Carolina at Chapel Hill. "It's not *no* treatment, but it is a lot easier than sticking a needle in your kid three times a week."

Steven Pipe, a paediatric haematologist at the University of Michigan's C. S. Mott Children's Hospital in Ann Arbor, agrees that the progress is significant. In particular, work that is stretching the lifetime of factor IX by three to five times is "really transformative", he says. And because half-lives can vary between patients, "at high doses, you could probably in some individual cases get a month's worth of factor IX," Pipe says.

BORROWED TIME

The trick to extending the half-lives of clotting factors is to interfere with the body's natural mechanisms for flushing them away. There are three very similar approaches, each of which extends half-life by about the same amount for the respective clotting factors. The only real difference is between factor IX, for which the techniques are offering extensions long enough to make a substantial difference in treatment, and factor VIII, for which the improvement has been more modest. Unfortunately, haemophilia A, which is caused by factor VIII deficiency, is about four times as common as haemophilia B.

Two of the techniques piggyback on the half-lives of other longer-lived proteins that occur naturally in the body. One such is immunoglobulin, a large Y-shaped protein with a half-life of about three weeks. The stem of the Y is known as the Fc region. When a clotting protein is fused to an Fc region, the body treats the clotting factor more like an immunoglobulin, and allows it to stick around for longer, although not for as long as a complete immunoglobulin molecule.

DRUGS TO HELP THE BLOOD

A number of treatments to aid blood clotting are in clinical trials or have been approved this year.

	Product	Approach	Company	Half-life (hours)	Status
Factor VIII infusions (for haemophilia A) Conventional infusion half-life: 8-12 hours	Eloctate	Fc fusion protein	Biogen Idec	20	FDA approved in June 2014
	BAX 855	PEGylation	Baxter International	19	Submission for approval planned for late 2014
	BAY94-9027	PEGylation	Bayer	19	Submission for approval planned for mid-2015
	N8-GP	PEGylation	Novo Nordisk	19	Submission for approval planned for 2018
Factor IX infusions (for haemophilia B) Conventional infusion half-life: 18–24 hours	rIX-FP	Albumin fusion	CSL Behring	92	In clinical trials
	N9-GP	PEGylation	Novo Nordisk	110	Submission for approval planned for 2015
	Alprolix	Fc fusion protein	Biogen Idec	87	FDA approved in March 2014

FDA, US Food and Drug Administration.

For factor VIII, Fc fusion extends the half-life from a maximum of about 12 hours to about 18 hours. Factor IX, which has a longer half-life to begin with, shows a more dramatic increase, from one day to five days.

Both the approved Biogen drugs are based on Fc fusion. Similar fusion drugs have been on the market to treat other diseases for many years, for example the rheumatoid arthritis drug Etanercept, which was approved by the FDA in 1998. Jerry Powell, the retired director of the Hemophilia Treatment Center at the University of California, Davis, says that the success of those drugs suggests that this is a safe approach to altering the clotting factors.

BIOGEN IDEC

Coagulation factor IX, used to treat haemophilia B

A similar approach, which is being pursued by CSL Behring, based in King of Prussia, Pennsylvania, is to fuse the clotting factors with albumin. Albumin is a major protein of blood plasma and, like immunoglobulin, has a half-life of about 20 days. Phase I safety studies of factor IX fused to albumin showed a fivefold increase in half-life, up to about four days. Unfortunately, attempts to do the same with factor VIII have been unsuccessful. Powell says that the albumin seems somehow to interfere with the normal activity of that clotting factor.

"These are really big molecules," he says. The activity of factor VIII in action, he adds, is so complex that it resembles a dancing elephant — too easily thrown off its rhythm when something else is attached. "If you put the wrong kind of contraption on the elephant, it doesn't dance as well."

The third strategy takes a slightly different approach. Instead of marrying the clotting proteins to a natural substance in the body, they are attached to synthetic polyethylene glycol (PEG) molecules (see 'PEGylation protection'). The PEG forms a sort of 'watery cloud' around the protein, protecting it from various mechanisms that would break it down; for instance, PEG prevents the clotting factors from binding to protein-specific receptors that would normally clear them away. PEG is eventually flushed from the body through the kidneys and liver, but before then it gives the clotting factors a new lease of life. Three large drug companies — Bayer in Leverkusen, Germany, Baxter International in Deerfield, Illinois, and the Danish company Novo Nordisk in Bagsvaerd — have all developed PEGylated factor VIII with a half-life of roughly 19 hours. Baxter expects to submit its product for regulatory approval by the end of this year, Bayer next year, and Novo Nordisk by 2018.

Novo Nordisk is also testing a PEGylated factor IX that has shown a half-life of 110 hours in clinical studies. The company says that it hopes to submit that drug for approval next year.

Up to now, tests have not shown much difference, in safety or effectiveness, between the three approaches. There are concerns that PEG might accumulate in the liver or kidneys over years of use, but studies of PEG have found it to have very low toxicity, and Powell thinks that those fears are exaggerated[2]. "PEG's been around a long time, there's a lot of toxicology and all the toxicology indicates no concern," Powell says. And if, as he expects, gene therapy replaces these treatments in the next decade, patients will in any case not have lifetime exposure to PEG.

One barrier to haemophilia therapy is the tendency of factor VIII to prompt the body into producing anti-factor VIII antibodies,

known as inhibitors. For a person with haemophilia A, factor VIII is a foreign substance, and the immune system can see it as a threat. About 30% of people with haemophilia A develop inhibitors, and once they do, treating their bleeding becomes much more difficult. Only about 4% of people with haemophilia B develop inhibitors to factor IX.

There is a lot of worry, Pipe says, that altering factor VIII to extend its half-life could make the inhibitor problem worse. "Everyone treads lightly in the factor VIII field, because there is such a fear of immunogenicity with any change of the molecule," he says. "There's no question with the current strategies that all of them have sort of hit a ceiling. If we're really going to overcome that ceiling, you are going to have to accept more dramatic changes to the molecule."

PEG may prove helpful in that regard. Studies dating back to the 1970s have shown that PEGylation can reduce the chances of a foreign protein stimulating an immune reaction, although the effect has not yet been proved in people with haemophilia. "That'd be a huge breakthrough if that were true," Powell says.

CONSTANT CASCADE

One researcher might have worked out a way to avoid the inhibitor issue almost entirely, by developing a different molecule to take the place of factor VIII in the clotting cascade.

Normally, once activated by previous steps in the cascade, factor VIII grabs hold of both factor IX and factor X, bringing them together to perform the next steps in the cascade. Midori Shima, director of the Hemophilia Center at Nara Medical University in Japan, has created a 'bispecific' antibody to do the same job.

Antibodies are immunoglobulins, and the upper arms of these Y-shaped proteins are designed to bind specifically to another molecule. Shima has created an antibody with one arm that binds to factor IX and the other to factor X, pulling the two together so that the clotting cascade can continue. The bispecific antibody has a half-life of about 30 days, much longer than the 12-hour upper limit of factor VIII, Shima says. Chugai Pharmaceuticals, based in Tokyo, and Hoffman-La Roche, based in Basel, Switzerland, are working on developing his findings into a treatment.

The researchers have not yet released the results of their phase II initial clinical trials, but Shima says that in the patients with haemophilia they looked at, bleeding frequency decreased dramatically. Among six people receiving the lowest dose of the treatment, who had each had 20–60 episodes of bleeding in the 12 weeks before the trial, two had no bleeding episodes at all during the 12 weeks of the trial. And out of 64 patients, only one developed an inhibitor. The team is planning a larger, phase III trial.

One bonus of this treatment is that because of the nature of the antibody, it does not have to be delivered intravenously, but instead can be injected under the skin, like insulin. "We think we can change the whole concept of haemophilia treatment," Shima says.

Lillicrap agrees. "That bispecific antibody would be hugely disruptive if it works," he says. "We'll know within the next couple of years whether it delivers on the promise which so far it's shown."

Treatments with extended half-lives may provide benefits beyond the convenience of less-frequent infusions and the potential increase in the number of people who stick to their treatment regime. If people under treatment now keep to their current schedule with the extended-life products instead of taking fewer infusions, the increased concentration of clotting factors in their blood could improve their quality of life even further.

"It is a lot easier than sticking a needle in your kid three times a week."

When patients have an infusion of clotting factor every 48 hours, the concentration of clotting factor initially reaches 100% of normal levels and stays there for about 12 hours. For the next 36 hours, it is high enough to be useful, but below normal. For the last 6 to 8 hours, the level is very low, less than 5%, Pipe says. Physicians try to keep the lowest level, the trough, from falling below 1% of the amount a non-haemophiliac person has circulating in their blood, enough to prevent spontaneous bleeding.

But if the trough level can be higher, it might make life easier for the patients, allowing them to, for instance, take up athletics with less fear of injury. "Ideally, you'd like to have zero bleeding," Pipe says. "What is the threshold for that I don't think anybody knows." Still, there would be substantial benefit from a less-than-perfect level of clotting factor. "If you could maintain a level of 10% or 15%, you would probably eliminate all joint disease," he says.

Lillicrap hopes that the emergence of several therapies means that it will make economic sense for drug companies to provide treatments to poorer parts of the world that have not been able to afford them. "No longer are people thinking about these therapies being only Western European and North American therapies," he says. If pharmaceutical companies are pouring money into this research, he thinks that it is at least in part because they can see a worldwide profit benefit.

For all the advantages of these extended-life molecules, the researchers predict that they will be supplanted in perhaps a decade by advances in gene therapy, which will enable people with haemophilia to produce their own clotting factors. But in the meantime, trading current therapies for longer-lasting ones can improve patients' lives. "As a bridging therapy between the really good outcomes we have currently and maybe a cure down the line," says Pipe, "I think the extended-half-life molecules are a perfect transition." ■

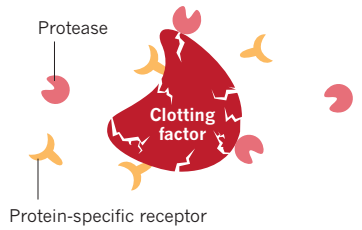
PEGylation protection

A key advance in haemophilia treatment is to prolong the effectiveness of the injected coagulation-promoting proteins (clotting factors) by shielding them from destruction.

BEFORE

Unprotected molecule

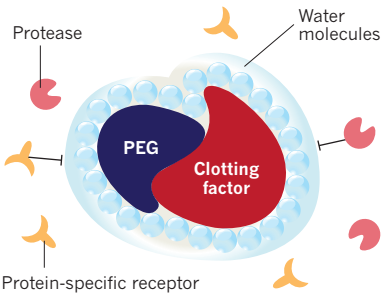
Under normal circumstances, proteases and protein-specific receptors break up the clotting factor and rapidly clear it from the bloodstream.



AFTER

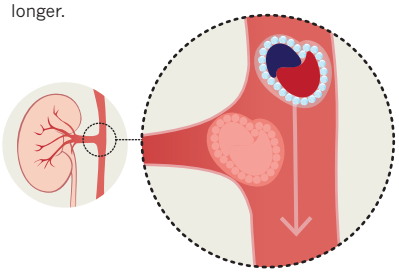
Microscopic shield

In PEGylation, molecules of polyethylene glycol (PEG) are attached to the clotting factor. The PEG molecules bring with them water molecules, which shield the clotting factor from attack.



Too big to discard

The watery cloud makes the factors too big for the kidneys' filtration mechanism, so the molecule circulates in the bloodstream for longer.



Neil Savage *is a freelance writer based in Lowell, Massachusetts.*

1. Hacker, M. R., Geraghty, S. & Manco-Johnson, M. *Haemophilia* **7,** 392–396 (2001).
2. Webster, R. *et al. Drug Metab. Dispos.* **35,** 9–16 (2007).

PERSPECTIVE


HEATHER VAN UXEM LEWIS

The fix is in

History explains why people with haemophilia, and their physicians, are cautious to believe that a cure is in sight, says **Stephen Pemberton**.

In 2011, a remarkable study[1] in the *New England Journal of Medicine* detailed the successful treatment of six adults with haemophilia B, which is caused by a deficiency in the coagulation protein known as factor IX. All of the participants were able to eliminate or reduce the frequency of clotting-factor-replacement injections — the current standard treatment for the disease — after their livers began producing functional levels of factor IX. The experimental therapy came in the form of an adeno-associated virus (AAV) carrying a gene that encodes instructions for production of normal levels of human factor IX. Three trials of AAV-mediated gene transfer in patients with haemophilia B are ongoing, with high expectations.

After more than 20 years of research on gene transfer, it is a promising time for haemophilia therapies. It now seems likely that a single-dose treatment for haemophilia B using an AAV or another gene-transfer technique will be a viable option for many people in the next decade or two.

Yet haemophilia researchers are not inclined to speak enthusiastically of a cure. Part of that caution comes from recognition that there are still problems to solve. For example, some 40% of people with haemophilia B would find no refuge in an AAV treatment because they produce antibodies that attack and neutralize this virus[2].

And even if that problem were solved, the treatment would apply only to those with haemophilia B. The more common form of the condition, haemophilia A, stems from a deficit in another protein — factor VIII — and the gene for that protein is a more difficult target. Regardless of the type of haemophilia, researchers remain hesitant about gene therapy owing to the unresolved ethical issues that arose decades ago.

RESEARCHERS ARE HESITANT ABOUT GENE THERAPY OWING TO THE UNRESOLVED ETHICAL ISSUES.

The unfettered optimism that characterized the early years of gene-therapy research came to a screeching halt in 1999, when 18-year-old Jesse Gelsinger died in a phase I clinical trial at the University of Pennsylvania in Philadelphia. Gelsinger had undergone an experimental gene transfer for his otherwise treatable metabolic disorder. His death, along with a series of other harmful events in early gene-therapy trials for a variety of diseases, threatened the whole field.

Haemophilia specialists who were engaged in gene-transfer studies were more guarded than most of that era's self-proclaimed gene doctors[3]. The source of their reserve goes beyond the cautious optimism that characterized such research after 1999; it is grounded instead in the long and troubled experience that the haemophilia community has had with technological fixes.

By the late 1970s, a therapeutic revolution had transformed haemophilia from an obscure hereditary malady into a manageable disease[4]. But the glory of this achievement was tragically short-lived. The same clotting-factor-replacement therapies that delivered a degree of normality to the lives of people with haemophilia brought unexpected and fatal results: tens of thousands of people with haemophilia were diagnosed with transfusion-related HIV/AIDS in the 1980s and with hepatitis C virus (HCV) in the 1990s.

The memory of tainted transfusions still haunts those who have, or work with, haemophilia. Add Gelsinger's death into the mix and it is clear why specialists are debating thorny ethical problems, such as when to try out AAV-mediated gene transfer on children. Gene therapy is not even the most promising treatment for haemophilia on the immediate horizon. The biotechnology industry is producing recombinant-clotting-factor products for both haemophilia A and B that can limit bleeding episodes with less-frequent injections (see page S162).

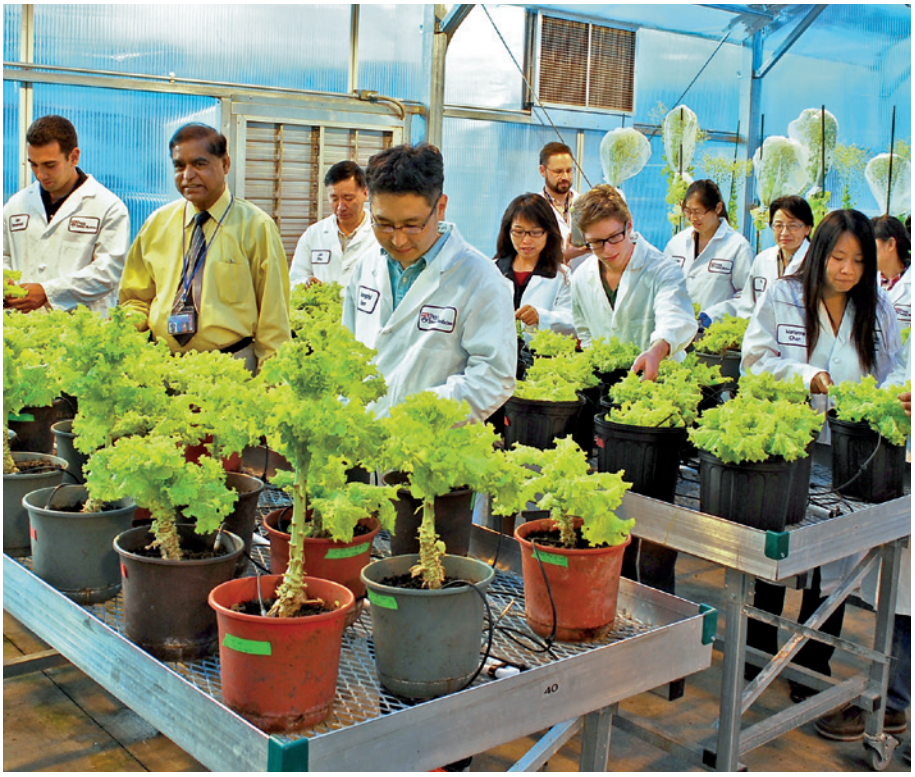
But the lure of a less-intrusive form of treatment raises a historical spectre of its own. It was this same desire for convenience that led many haemophilia physicians and patients in the United States in the 1980s to continue using clotting-factor concentrates that had a high risk of HIV contamination rather than switch back to older, more cumbersome but less risky forms of plasma-replacement therapy. Thousands of people with haemophilia contracted HIV and HCV because of this acculturated preference[4].

Finally, there is the difficulty of making costly treatments available to the vast majority of the world's haemophilia patients who live in low income countries. About 75% of people with haemophilia still receive inadequate treatment, particularly in less-developed nations where clotting-factor therapy is limited[5]. An effective gene therapy could well offer these underserved patients their first chance at effective intervention[6].

History suggests that the fix will not lie in just one solution, but will be contextual and messy. The wants and needs of people with haemophilia in the developed world might not be the same as for those in low income countries. Yet social justice demands that there be equity in access to treatment. The transfusion scandals of the past remind us of the importance of bringing together patients and treatment professionals with stakeholders from industry and public health to weigh the various technological fixes. If such discussions had taken place in the 1970s and 1980s about the known problem of transfusion-related hepatitis B, the haemophilia community would not have been blind-sided by the emergence of HIV and HCV. ■

Stephen Pemberton *is a medical historian at the New Jersey Institute of Technology in Newark, and author of* The Bleeding Disease: Hemophilia and the Unintended Consequences of Medical Progress.
e-mail: stephen.pemberton@njit.edu

1. Nathwani, A. C. *et al. N. Engl. J. Med.* **365,** 2357–2365 (2011).
2. High, K. H., Nathwani, A., Spencer, T. & Lillicrap, D. *Haemophilia* **20** (Suppl. 4), 43–49 (2014).
3. Wailoo, K. & Pemberton, S. *The Troubled Dream of Genetic Medicine: Ethnicity and Innovation in Tay-Sachs, Cystic Fibrosis, and Sickle Cell Disease* (Johns Hopkins Univ. Press, 2006).
4. Pemberton, S. *The Bleeding Disease: Hemophilia and the Unintended Consequences of Medical Progress* (Johns Hopkins Univ. Press, 2011).
5. Mannucci, P. M. *Haemophilia* **17** (Suppl. 3), 1–24 (2011).
6. High, K. A. & Skinner, M. W. *Mol. Ther.* **19,** 1749–1750 (2011).



KWANG-CHUL KWON, JIN SU & HENRY DANIELL, UNIV. PENNSYLVANIA

Blood-clotting factors produced by these lettuce plants could eliminate the problem of immune rejection.

IMMUNOLOGY

Oral solutions

Pills made from lettuce leaves could help to prevent one of the most serious complications of haemophilia treatment.

BY ELIE DOLGIN

The food in Anita's bowl is not your average dog chow. Although the dish contains pellets and wet food, there is also a sprinkling of green powder — the product of a trailblazing experiment to address a potentially lethal complication of haemophilia treatment. Anita, so named because her red coat reminded breeders of the character from the animated film *One Hundred and One Dalmatians*, is a keagle (a mix of a beagle and a Cairn terrier) with haemophilia B.

Like people with this rare genetic disorder, Anita is naturally deficient in factor IX, a protein that helps the blood to form clots. When treated with replacement coagulation proteins, the dog naturally develops antibodies, or inhibitors, against the therapy — a problem that is also seen in some 5% of humans with haemophilia B. In these people, the immune system identifies the therapeutic protein as dangerous, causing the body to stop accepting the protein as a normal part of the blood, and destroys it before it can stop the bleeding. Continuing to take factor-replacement therapies can result in life-threatening allergic reactions, such as anaphylaxis.

The problem is even worse with haemophilia A, a disease that is four times more common than haemophilia B and in which the missing link in the coagulation chain is a protein called factor VIII. Around 30% of people with haemophilia A develop antibodies against replacement factor VIII.

Therapies are available to eliminate these antibodies. Some people, for example, undergo an intensive treatment called immune tolerance induction therapy, which involves regular intravenous administration of coagulation factors. But this is time consuming and costly (around US$1 million for an average five-year-old patient), and the treatment works in only about three-quarters of patients. "The challenges of treating haemophilia with inhibitors are just staggering," says Timothy Nichols, director of the Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill, which maintains the colony of haemophiliac dogs to which Anita belongs (see page S172).

Inducing immune tolerance in people who have developed inhibitors is one approach. But avoiding the problem altogether would be even better. "If you can prevent antibody formation in the first place, by finding some way of producing immunological tolerance that gets around that type of protocol, that would be a major advantage," says David Lillicrap, a clinician and researcher who specializes in bleeding disorders at Queen's University in Kingston, Ontario, Canada.

The green powder in Anita's dish might do just that. The oral treatment is a concentrate of freeze-dried lettuce-leaf cells, each containing around 10,000 chloroplasts — the organelles responsible for photosynthesis — that have been genetically engineered to produce factor IX. These proteins cannot themselves be used to prevent bleeding episodes, because the cellular machinery found in plants cannot package the human clotting factors into the biologically active form. What they can do, however, is prevent the immune system from mounting an attack against subsequent therapy.

The researchers behind the bioengineered lettuce have shown that inhibitor formation and severe allergic reactions can be prevented in mice by feeding the animals with a product based on these plants[1,2]. If the strategy also works in Anita and her kennel mates — and ultimately in humans — it could form the basis of the first product to protect against the immune responses associated with haemophilia treatment.

Anita is one of only two dogs to have received the bioengineered lettuce. "So far, it's going very well," says lead researcher Henry Daniell, director of translational research at the University of Pennsylvania School of Dental Medicine in Philadelphia.

AN ACT OF TOLERANCE

In 2006, Lillicrap demonstrated that a simple oral treatment could train the immune system not to produce inhibitors. Working with a mouse model of haemophilia A, he and his colleagues gave the mice a purified fragment of the human factor VIII protein, through the nose or mouth. The researchers found that the treatment afforded some protection against antibody development after factor VIII replacement therapy[3]. But the approach did not deliver sufficient amounts of the factor to immune cells in the gut or nasal passage to fully quash inhibitor formation.

Daniell came up with an improved delivery system. He focused first on haemophilia B. Adapting a technique[4] that he had previously developed to delay the onset of type 1 diabetes, Daniell and his group genetically modified tobacco plants to express human factor IX in their chloroplasts. (Daniell has since switched to using lettuce.)

Chloroplast DNA is separate from the genome DNA in the plant nucleus, and the large numbers of these tiny organelles in the

cell allow huge volumes of the coagulation protein to accumulate in each tobacco leaf. Once ingested, the plant cell wall protects the coagulation protein from being destroyed by stomach acid. Gut microorganisms farther down the digestive tract then chew away at the cell wall, releasing the clotting-factor protein.

To target the proteins to the immune system, Daniell then attached a second protein that has high binding affinity for a receptor found on the inside of the human gut. With this fused construct tethered to the intestinal wall, the coagulation protein could be absorbed into the body and processed by the specialized cells in the immune system that induce tolerance.

Working with Roland Herzog, a molecular biologist at the University of Florida in Gainesville, Daniell then tested the plant-based product in animal models. In 2010, they showed that oral delivery of factor IX expressed in chloroplasts in this way led to almost undetectable inhibitor levels in mice, and no sign of anaphylactic shock[1]. "The mice are healthy, they show no allergic responses and they don't form the inhibitors," Herzog says. "That's pretty exciting."

Daniell then modified the tobacco leaves to express factor VIII and shipped powders of the leaves to Herzog. Earlier this year, the two researchers and their teams documented[2] suppression of inhibitor formation and even reversal of pre-existing inhibitors in mouse models of haemophilia A.

INHIBITORY CONTROL

Other strategies being pursued to prevent the formation of inhibitors of clotting-factor therapy include immunosuppressants and drugs that deplete specific immune cells. However, these therapies have many side effects, including increased susceptibility to infection.

A potentially safer option comes from Selecta Biosciences, a company in Watertown, Massachusetts. Selecta has developed a nanoparticle delivery system in which an immune-modifying compound is contained in biodegradable plastic particles just 150 nanometres across. When injected together with factor VIII into mouse models of haemophilia A, the nanoparticles deliver their payload to cells in the lymphoid tissue that are responsible for initiating immune responses. These cells, in turn, instruct factor-VIII–specific immune cells to become tolerant to the coagulation protein, resulting in suppression of misdirected antibody responses to the replacement therapy — all without affecting the rest of the immune system.

David Scott and his colleagues at the Uniformed Services University of the Health Sciences in Bethesda, Maryland, teamed up with Selecta to show that inhibitors remained undetectable for at least six months after treatment with the nanoparticle formulation[5]. "This underscores the point that we're actually teaching the immune system to become tolerant to factor VIII," says Selecta's chief scientific officer, Takashi Kei Kishimoto.

The nanotechnology approach that is being tested for inhibitor control could also improve the haemophilia treatment that is now at the cutting edge of clinical research: gene therapy. Using the standard gene-therapy approach, researchers have shown that they can achieve long-term expression of factor IX in adults with haemophilia B at sufficiently high levels to convert the bleeding disorder into a mild disease (see page S160). There has so far been no reported evidence of inhibitor formation in the small number of human participants in clinical trials for this viral therapy[6].

KWANG-CHUL KWON, JIN SU & HENRY DANIELL, UNIV. PENNSYLVANIA

Green power: from leaf to powder to capsule.

Still, the standard form of liver-targeted gene therapy carries a range of potential complications, including the risk of harmful mutations and of the body mounting an immune response against the viral vectors used to carry the correct forms of the defective genes responsible for haemophilia. That is why several research groups are attempting to replace viral vectors with nanoparticles that can deliver gene therapies as 'DNA pills'.

PILL PROTECTION

DNA pills combine DNA plasmids — circular pieces of bacterial DNA containing the gene encoding either factor VIII or factor IX — with nanoparticles made of chitosan, a tough polymeric carbohydrate found in the exoskeleton of crustaceans. Chitosan protects the therapeutic gene product and chaperones it through the gut. "The oral route has significant appeal," says Gonzalo Hortelano, a gene-therapy researcher at McMaster University in Hamilton, Canada. "The key is to achieve a system of delivery that's persistent, effective and completely safe."

Independent studies by Hortelano's group and other research teams in Germany and the United States have shown that this oral gene therapy does not activate the immune system. Indeed, exposure of the protein produced by the nanoparticle-based gene therapy to the gut mucosa prevents inhibitor development and restores clotting-factor activity in mouse models of both haemophilia A[7,8] and B[9]. "This approach really could hold big benefit for patients," says Jörg Schüttrumpf, a transfusion-medicine specialist who led one of the studies performed at the German Red Cross Blood Donor Service in Frankfurt.

Kam Leong, a biomedical engineer at Columbia University in New York City whose team was the first to demonstrate success with this approach in mice[7], has even tried feeding the chitosan–DNA nanoparticles to dogs with haemophilia A. Leong found some evidence of gene transfer and a reduction in inhibitors in the animals. But bleeding times were not reduced, which would be expected if sufficient levels of factor VIII were being produced. "It is still a very inefficient process," Leong says, "so it requires continued optimization."

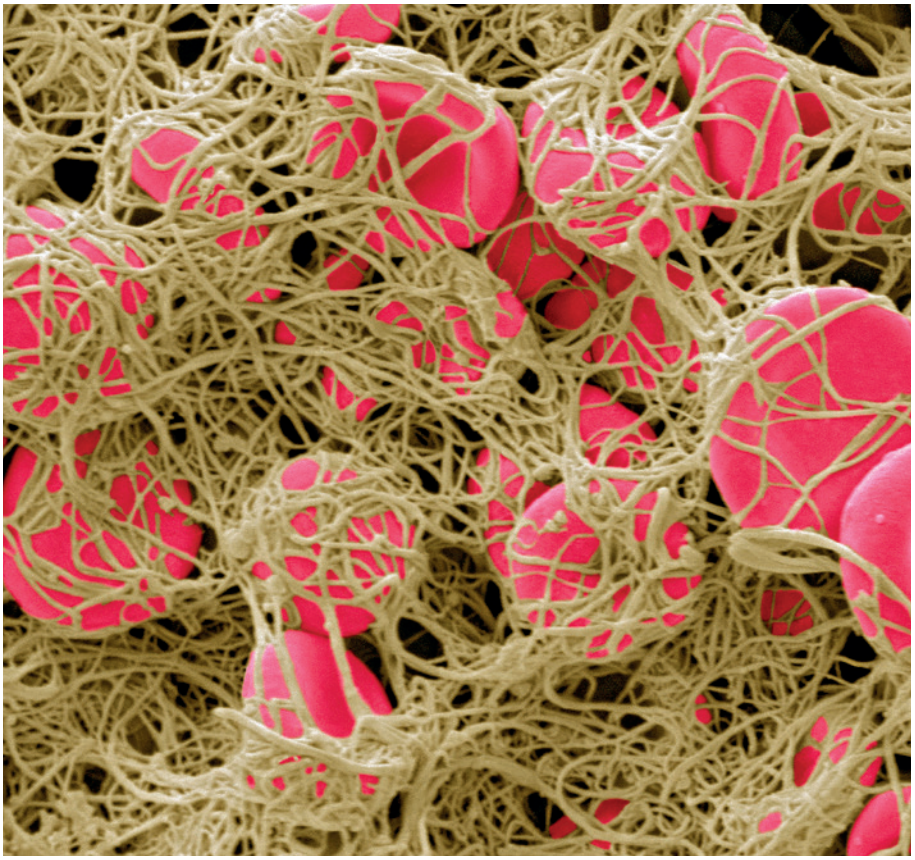
Although the ideal remains a gene therapy that both corrects the disease and offers immune tolerance, some scientists have focused on treating inhibitor formation, without worrying about fixing the disease. Under this strategy, people would still need to take factor-replacement therapies, but they could do so without fear of inhibitor development.

With this in mind, independent teams led by Scott and Herzog took the conventional viral-vector approach to inducing tolerance through gene therapy. But rather than delivering the entire gene for the clotting-factor proteins to cells, as most gene therapies do, the researchers used the viruses to engineer immune-regulating B cells to express a fragment of the clotting factor fused to an immune molecule called an immunoglobulin. This led to long-lived tolerance in mouse models of haemophilia A[10] and B[11].

Pursuing such gene-therapy approaches offers a degree of bet hedging, says Herzog. "Each strategy has potential advantages and disadvantages," he points out, "and we do not really know yet what will work or may work best in people." With so many therapeutic tactics moving through the preclinical pipeline, scientists and clinicians remain hopeful that at least one will ultimately succeed, eliminating the problem of inhibitor formation for people with haemophilia altogether. ■

Elie Dolgin *is a science writer in Somerville, Massachusetts.*

1. Verma, D. *et al. Proc. Natl Acad. Sci. USA* **107,** 7101–7106 (2010).
2. Sherman, A. *et al. Blood* **124,** 1659–1668 (2014).
3. Rawle, F. E. *et al. J. Thromb. Haemost.* **4,** 2172–2179 (2006).
4. Ruhlman, T., Ahangari, R., Devine, A., Samsam, M. & Daniell, H. *Plant Biotechnol. J.* **5,** 495–510 (2007).
5. Zhang, A.-H. A. *et al. Blood* **122,** 2337 (2013).
6. High, K. A. *Blood* **120,** 4482–4487 (2012).
7. Bowman, K., Sarkar, R., Raut, S. & Leong, K. W. *J. Control. Release* **132,** 252–259 (2008).
8. Dhadwar, S. S., Kiernan, J., Wen, J. & Hortelano, G. *J. Thromb. Haemost.* **8,** 2743–2750 (2010).
9. Quade-Lyssy, P. *et al. J. Thromb. Haemost.* **12,** 932–942 (2014).
10. Lei, T. C. & Scott, D. W. *Blood* **105,** 4865–4870 (2005).
11. Wang, X. *et al. Mol. Ther.* **22,** 1139–1150 (2014).



STEVE GSCHMEISSNER/SPL

The control of blood clotting treads a fine line between promotion and inhibition.

THROMBOSIS

Balancing act

A promising therapy curtails clotting inhibitors rather than replacing proteins that promote blood clotting.

BY CASSANDRA WILLYARD

Kanjaksha Ghosh has seen more than a thousand people with haemophilia since he became a physician. But he has always wondered why some patients bleed spontaneously and develop crippling joint damage whereas others barely seem to be affected.

Ghosh, who heads the National Institute of Immunohaematology in Mumbai, India, remembers a soldier who had been fighting insurgents in the northeast of the country. The man's brother was almost bedridden by haemophilia, but the soldier's symptoms were so mild that he did not even realize that he had the disease until he was shot on the battlefield.

In the 1990s, Ghosh began trying to work out why such discrepancies existed by studying families like the soldier's. When he delved into the genomes of those with a milder disease, he often saw not just a mutation in the affected clotting-factor gene, but also a mutation in another gene — the first causing haemophilia, the tendency to bleed, and the second causing thrombophilia, the tendency to clot. Ghosh's research leads to the conclusion that a patient with haemophilia who co-inherits a thrombophilic gene bleeds less than one without that mutation.

Blood coagulation is regulated by one set of proteins that causes clotting and another set that prevents it (see 'Perfect balance'). Too little clotting ability leads to bleeding disorders. Too much leads to vessel-blocking clots that can cause strokes and heart attacks. Existing haemophilia treatments tip the balance towards clotting by adding what the body lacks — the clotting factor that is missing or defective. But natural human experiments such as Ghosh's soldier suggest an alternative strategy to treat the disease. Rather than boosting the factors that promote clotting, researchers might instead disable the anticoagulation machinery that prevents clotting.

In the past few years, three drug companies have moved compounds aimed at inhibiting anticoagulation into clinical trials. The hope is that these therapies will be as effective as existing treatments and much more convenient. Rather than receiving multiple infusions of protein replacement each week, patients might be able to control their bleeding with long-lasting injections.

TARGET PRACTICE

The complex cascade that results in the formation of a clot begins when a blood vessel is injured. Several proteins hold the process in check to prevent clots from forming where they are not needed. One such protein, tissue factor pathway inhibitor (TFPI), impedes the initiation of coagulation. Studies published over the past two decades suggest that blocking this protein can promote clotting, which could curb bleeding in people with haemophilia.

The Danish pharmaceutical company Novo Nordisk in Bagsvaerd began working on an antibody designed to inhibit TFPI in the 1990s. Its researchers showed that this antibody could speed up clot formation in blood plasma from people with haemophilia[1]. They also found that it could shorten bleeding time and hasten clotting in rabbits with induced haemophilia. These results seemed promising, but Novo Nordisk began pursuing other strategies to treat haemophilia, and research to develop an anti-TFPI antibody was halted.

In 2006, Novo Nordisk decided to look for therapies that could be injected under the skin and revived the programme. By 2010, the company had launched a clinical trial in Europe and Asia to test the safety of an anti-TFPI monoclonal antibody called concizumab. The researchers administered the antibody either intravenously or subcutaneously to 28 healthy volunteers and 24 people with haemophilia. Preliminary results presented in 2013 at the International Society on Thrombosis and Haemostasis meeting in Amsterdam suggest that concizumab is safe, and that it can improve coagulation. Participants did not report any severe adverse events, although one of the healthy volunteers in the group receiving the highest dose of concizumab developed a small blood clot that disappeared on its own.

The company hopes to launch a second study in mid-2015 to determine the appropriate dose before moving on to test the efficacy of the treatment. "We have liked TFPI as a target for a long time," says Ida Hilden, scientific director of Novo Nordisk's concizumab project.

Drug company Baxter International, based in Deerfield, Illinois, sells recombinant clotting factors for treating haemophilia and also has its sights on TFPI. In the same year that Novo Nordisk launched its concizumab trial, Baxter struck a deal to purchase a suite of haemophilia-related assets from the former therapeutics company Archemix. Those assets included a therapy designed to inhibit TFPI that had already entered a safety study in the United Kingdom. This therapy was an aptamer, a small strand of nucleotides

designed to inhibit TFPI's activity by binding to it, much like an antibody.

The compound, known as BAX 499, performed well in animal studies but failed to deliver in humans[2]. In 2012, Baxter halted the trial due to an increased number of bleeding events. The failure came as a shock. "We did extensive safety studies in monkeys," says Fritz Scheiflinger, vice-president of research and innovation at Baxter BioScience in Vienna. "We gave huge amounts of aptamer over six months", yet there were no signs that the compound was unsafe, he says.

Scheiflinger and his colleagues think that they now have an explanation for this strange effect. TFPI lasts no more than a couple of hours in the bloodstream, but BAX 499 has a longer half-life. When BAX 499 binds to TFPI, it allows the protein to persist for longer and, over time, to accumulate. And although the drug binds to TFPI, it does not completely deactivate it. So, as partially active TFPI piles up, the balance eventually tips from a pro-clotting effect to an anti-clotting effect. The problem seems to be confined to this particular compound, but nonetheless, the company has shifted its focus away from aptamers.

Baxter is now concentrating on peptides — short strings of amino acids that can be tailored to block part of the TFPI protein — a strategy that Scheiflinger and his colleagues first considered in 2005. The company has identified several promising candidates, but has not yet decided whether it will move them into clinical trials.

TFPI is not the only target for companies hoping to hamper the anticoagulant system. Alnylam Pharmaceuticals in Cambridge, Massachusetts, has set its sights on antithrombin — a protein produced by the liver that hinders clotting. "Antithrombin is probably one of the most potent natural anticoagulants we have in the body," says Benny Sorensen, medical director of clinical research and development at Alnylam. But rather than inhibiting antithrombin's activity, the company plans to block its expression by using short strands of RNA to silence the messenger RNA that carries the code for antithrombin — an approach called RNA interference.

The company is testing its therapy, called ALN-AT3, in a safety study, and the initial results were presented at the World Federation of Haemophilia annual meeting in Melbourne, Australia, in May. After giving healthy volunteers a single low dose of the drug, expression of antithrombin was reduced by 28–32% — an outcome that Sorensen says left the researchers "very surprised". They had thought that it would take higher doses to achieve such a result.

But Sorensen believes that they can do even better. In that first phase, the researchers were not allowed to exceed a 40% reduction in antithrombin because of the safety risks to healthy volunteers. The next phase of the study will include people with haemophilia, and there will not be the same limitation. So the researchers plan to administer multiple doses of the drug. Sorensen thinks that if they can achieve a 50–80% reduction in antithrombin, ALN-AT3 may be able to control bleeding in people with haemophilia without infusions of clotting factor.

PERFECT BALANCE

The body must maintain a delicate equilibrium to ensure that blood flows freely most of the time but clots when necessary. Haemophilia tips the scale towards bleeding, but researchers are looking for new ways to restore the equilibrium.

HAEMOPHILIA

People with haemophilia do not produce enough factor VIII or factor IX, proteins that play a crucial part in clotting.

FACTOR REPLACEMENT TREATMENT

To prevent and staunch bleeding, physicians typically give patients with haemophilia infusions of the factors they lack. Adding these extra factors restores the balance between bleeding and clotting.

ANTICOAGULANT INHIBITION TREATMENT

An approach under development restores balance instead by inhibiting the proteins that prevent clotting – natural anticoagulants such as tissue factor pathway inhibitor (TFPI) and antithrombin.

CAUTIOUS OPTIMISM

All of these therapies have one major advantage over protein replacement: antibodies, peptides and RNA can be effective even when injected under the skin, in part because they are so much smaller than the proteins used for factor-replacement therapy. Novo Nordisk envisages putting its antibody into a 'pen' like the one that people with diabetes use to administer insulin. This would be much more convenient than the intravenous infusions required for existing therapies. "Haemophilia patients are pestered from when they are one or two years old for the rest of their lives with intravenous injections," Sorensen says. "If we can achieve a correction of this haemostatic imbalance that would prevent spontaneous bleeds, then we've really offered an unbelievable change in the lives of these haemophilia patients."

If compounds such as concizumab and ALN-AT3 prove effective, they will undoubtedly be a boon for at least one group of people with haemophilia: those who develop inhibitory antibodies against the blood-clotting factors VIII and IX, and who can no longer receive this standard therapy. Roughly 5% of those with haemophilia B fall into this category, and 30% of those with haemophilia A (see page S166). Baxter, Novo Nordisk and Alnylam think that their products will appeal to other people with haemophilia. But whether these therapies will be safe and effective enough to replace infusions of clotting factor "is the million-dollar question", Scheiflinger says. Sorensen is the most optimistic. He speculates that a once-a-month dose of ALN-AT3 might control bleeding without the need for prophylactic infusions of clotting factor. Even if patients cannot completely forgo factor replacement, he adds, ALN-AT3 might allow them to use less, which could reduce the risk of developing inhibitors.

But many of the physicians who treat patients with haemophilia are not convinced. "The common thinking among haemophilia treaters is that these new strategies can never replace treatment with factor VIII and IX in non-inhibitor patients," says Erik Berntorp, a haematologist at Lund University in Malmö, Sweden. David Ginsburg, a geneticist at the University of Michigan, Ann Arbor, is equally cautious. "In the case of a genetic deficiency, it's pretty hard to improve on replacing the missing factor," he says.

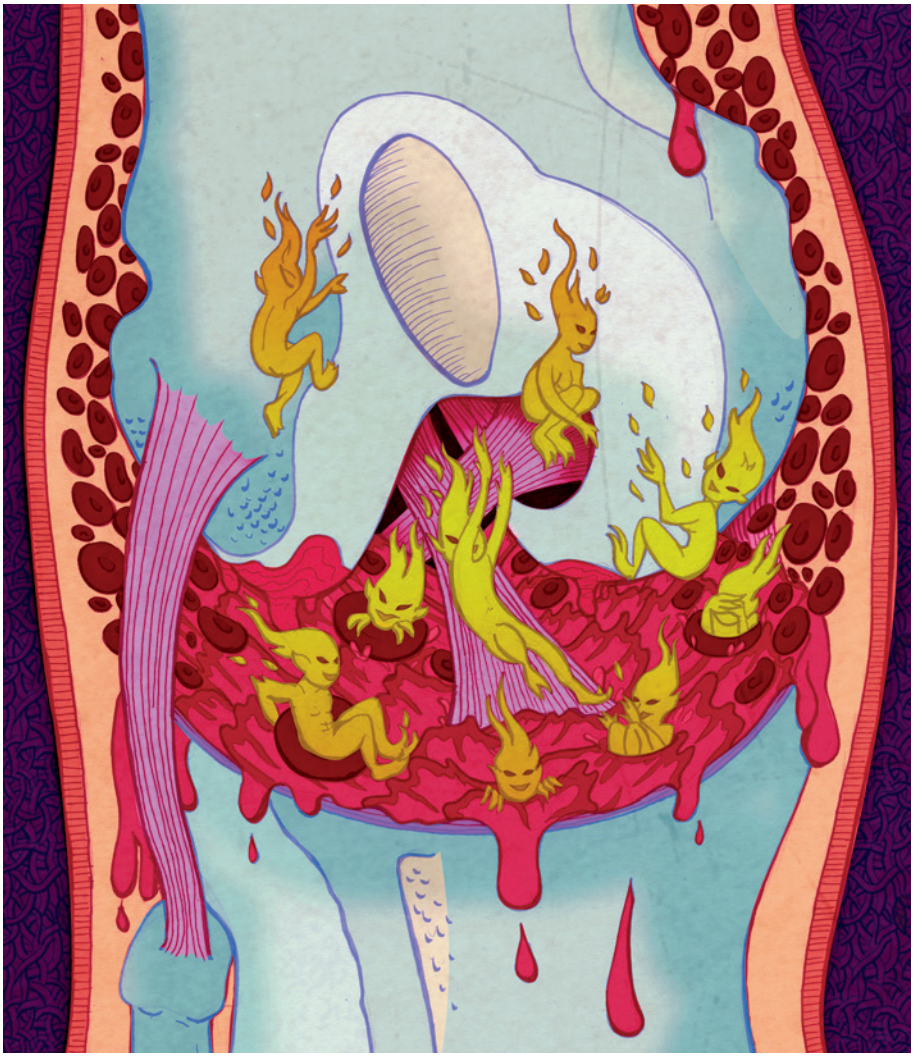
Kenneth Mann, a biochemist at the University of Vermont in Burlington, does not doubt that blocking these anticoagulant pathways will increase the production of thrombin, a key protein in clotting, but he does not think that these therapies will necessarily work for everyone. People with haemophilia "are more heterogeneous than we'd like to admit," he says. And companies will have to work out how to stratify patients on the basis of their real bleeding risk to determine who will benefit from these new approaches. "I don't mean to throw a wet blanket on this," he says, "but caution is required."

One risk is that these therapies will work too well, tipping the balance towards clotting. In a person without haemophilia, Ginsburg says, a total lack of antithrombin "seems to be disastrous". Mice that lack either antithrombin or TFPI die *in utero*. Although the antithrombin-based therapies for haemophilia are not designed to completely block their targets, "knocking them down is not without risk", he says. And as the failure of BAX 499 shows, the risks posed by any new medication can be hard to predict.

Jakob Back, vice-president of the concizumab project at Novo Nordisk, understands the scepticism. Protein replacement has been the go-to therapy for haemophilia for decades. Concizumab and similar therapies represent "a completely different way of approaching haemophilia compared to anything we've been doing for the last 50 years", he says. "We are moving into unknown territory." ■

Cassandra Willyard *is a freelance writer in Madison, Wisconsin.*

1. Nordfang, O., Valentin, S., Beck, T. C. & Hedner, U. *Thromb. Haemost.* **66,** 464–467 (1991).
2. https://ash.confex.com/ash/2012/webprogram/Paper51012.html



JESSICA FORTNER

ORTHOPAEDICS

Joint effort

The hunt is on for ways to diagnose and treat the joint problems that are now the main chronic problem in haemophilia.

BY KATHARINE GAMMON

As a physician who cares for adults with haemophilia, Annette von Drygalski sees patient after patient with bulging, painful knees and elbows caused by bleeding into the joint. The rise in cases of this crippling condition, which can lead to arthritis and disability, drives the work of von Drygalski and her team at the University of California's San Diego Medical Center — part of a growing body of researchers studying haemophilic joint disease and the pain that it causes.

Before clotting factor became widely available as a treatment (see page S162), people with haemophilia rarely reached adulthood, so haemophilic joint disease was not on the radar of most research programmes. But now that people with the disease have a life expectancy similar to that of the general population, arthritis caused by the disorder has emerged as a serious medical problem.

A bleed inside a joint leads quickly to stiffness and pain. The residual iron from pooled blood causes inflammation of the joint lining, a condition known as synovitis. Physicians can remove the inflamed tissue surgically (which, for people with haemophilia, comes with a high risk of bleeding) or by injecting radioisotopes into the joint. These emit radioactive particles that destroy the cells in the joint lining and prevent further bleeding. Such surgeries are delicate procedures, says Mauricio Silva, an orthopaedic surgeon at the University of California, Los Angeles, who specializes in haemophilic joint operations. "The deformities are much more severe than someone with arthritis," he says.

The basic remedy for bleeding into the joint has been for patients to self-administer more clotting factor when they believe they are having a bleeding episode. But this is expensive, and does not help everyone. "This field will require lots of new thoughts, beyond administering clotting factor for joint health, over the next decade to improve the life of those with haemophilia," says von Drygalski.

Researchers are tackling the problem from multiple directions: through better imaging, by using novel biomarkers that might be able to reveal even minor joint bleeds, and by applying knowledge from other types of arthritis. It will take research in all of these areas to work out new ways to diagnose and treat haemophilic joint disease and understand its causes.

JOINT INSPECTION

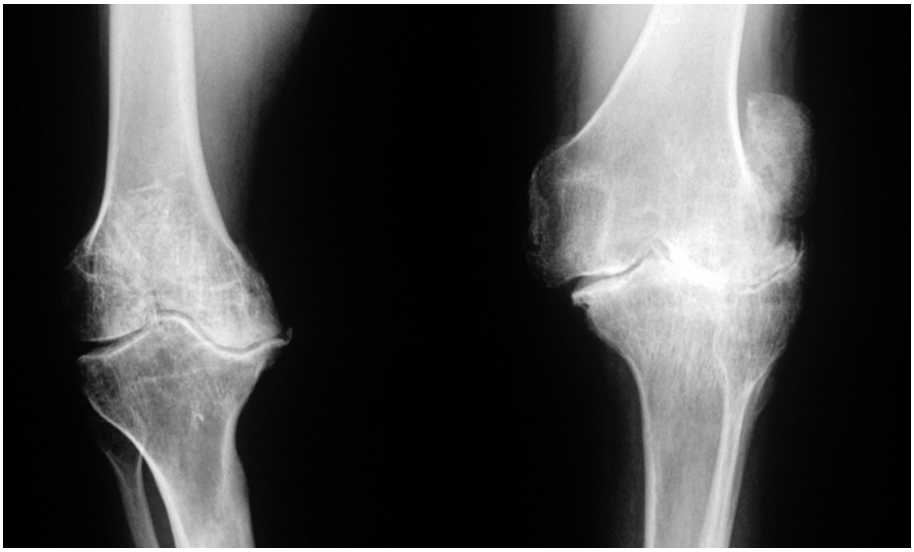
One problem is that there is no definitive way for physicians to distinguish between normal arthritic joint pain and that caused by a bleed. Von Drygalski's research shows that only one-third of painful episodes reported by people with haemophilia are associated with bleeding into the joint[1]. Similarly, physicians find it hard to determine the cause of joint pain: in one small study[1], von Drygalski and her colleagues found that physicians' assessments, based on patient interviews and physical examinations, were incorrect in 18 of 40 instances.

Imaging technologies can help. The highest-quality pictures come from magnetic resonance imaging (MRI), but these systems are slow, bulky and costly to run, and so are not commonly used in haemophilia clinics.

With an eye on those drawbacks, von Drygalski and her colleagues developed a clinical tool that uses ultrasound. The musculoskeletal ultrasound (MSKUS) system — featuring a hockey-stick-shaped ultrasound probe — can distinguish between bleeding and inflammation during painful episodes. As part of a large initiative in Europe sponsored by pharmaceutical giant Pfizer, staff at about 10–15 haemophilia treatment centres are currently being trained to use the technology. The same initiative is in the planning stages in the United States, where training will be given at 10 centres.

MSKUS checks the crevices of joints for inflammation or bleeding, and is less costly than MRI but just as accurate, says von Drygalski. In particular, she says, ultrasound provides greater detail on what is happening in acutely and chronically painful haemophilic joints, where bleeding has caused both synovitis and inflammatory changes to soft tissue.

MIKE DEVLIN/SPL



An X-ray of the knees of a person with haemophilia, both damaged from bleeding inside the joints.

MOLECULAR MARKERS

The molecular basis of how haemophilia results in joint pain is still not clear. One hypothesis is that the blood of patients with the disease is a poor activator of a key protein called thrombin activatable fibrinolysis inhibitor (TAFI), which controls clot stability and reduces inflammation. For example, administering additional TAFI relieves discomfort in non-haemophiliacs with inflammatory arthritis. Because the protein stops blood clots from breaking down, it helps people with haemophilia to form clots and maintain them. Von Drygalski, in collaboration with Laurent Mosnier, an assistant professor of molecular medicine at the Scripps Research Institute in La Jolla, California, is studying how treating patients with extra TAFI might help to relieve haemophilia joint problems.

Mosnier, for his part, is doing basic molecular studies to better understand the contribution of clot breakdown in bleeding, and to investigate whether TAFI can be genetically modified to make it more potent and diminish bleeding complications.

To tease out TAFI's clotting and anti-inflammatory roles — and to find out why TAFI may not be fully functional in people with haemophilia — both researchers are using haemophilic mouse models as well as mice that have been engineered to lack the gene that encodes TAFI. Von Drygalski hopes that this will lead to treatments beyond the standard infusions of clotting factor. If it is established that poor TAFI activation in haemophilia contributes to joint disease and inflammation, researchers could develop engineered versions of TAFI with high potency that persist for longer in the body. The researchers hope that such agents could eventually mitigate or even prevent haemophilic joint disease.

DRUG SEARCH

Ideally, physicians would like to have a test that determines which people with haemophilia have the highest risk of developing joint disease. At Rush University in Chicago, Illinois, molecular biologist Narine Hakobyan has found about half a dozen biomarkers in the blood of haemophilic mice[2] that could signal very minor bleeds before damage occurs in the joint.

She and her colleague Leonard Valentino (who now works at health-care company Baxter International in Deerfield, Illinois) set out to create animal models for haemophilic joint degradation in 2001. They made one mouse model that had joint bleeds after injury and another that bled into the joint even in the absence of trauma. They also created a scoring system to evaluate how well drugs stopped bleeding in the joints, which could be used to rank the effectiveness of new drugs.

Hakobyan's study[2] revealed biomarkers that could be detected after injecting just 25 microlitres of blood into the joints of mice that lack clotting factor — showing that even tiny bleeds have markers that could be used to predict joint deterioration. These could guide scientists' search for new drugs to treat haemophilic joint disease, and could point to the fundamental mechanisms underlying the illness. "It would be helpful to know at which point joint disease is reversible, and where we can act to use drugs as therapeutic agents," says Hakobyan. Other markers are likely to be found for different stages of the disease, Hakobyan says.

BEYOND CLOTTING FACTORS

To better understand the joint and its response to bleeding, researchers are studying changes to the bone around it. This may require creative thinking about mechanisms beyond clotting factors, says Paul Monahan, a haematologist at the University of North Carolina in Chapel Hill, who has studied whether rheumatoid arthritis drugs can improve mobility and reduce inflammation in haemophilic mice.

Monahan thinks that treatment with infusions of clotting factor, known as prophylaxis, is not a good way to treat all patients with haemophilia, especially those who have breakthrough bleeding — bleeds that happen in between their infusions of clotting factor. For instance, previous research[3] has shown that regularly giving extra doses of clotting agent beyond what is needed for primary prophylaxis adequately controls joint bleeding in less than 40% of people with haemophilia.

He likens this approach to giving only one therapy to patients with asthma. "You wouldn't treat an asthmatic with just a bronchodilator — you need to address both the acute spasm and the underlying inflammation," he says. Likewise, patients with haemophilia could potentially be treated with drugs that reduce inflammation as well as being given clotting factor.

Another potential therapy is the use of special radioisotopes to attack the inflamed joint lining. In July, Navidea Biopharmaceuticals of Dublin, Ohio, announced a partnership with the start-up firm Rheumco to develop a tin radioisotope technology that blasts out inflamed joint tissue. The idea is to inject a colloidal suspension of tin-117 particles into the joints of children with haemophilia. This radioisotope was selected because it has a small, focused area of radiative impact, so there is less chance of radiation damaging nearby tissue — an important consideration for children whose bones are still growing.

Navidea and Rheumco are completing animal testing for the tin-isotope project and are optimizing the technology for use in people. Being able to treat children with the method would be a boon because early treatment is key for these disorders, says Mark Pykett, formerly chief executive of Navidea and now chief executive of Agilis Biotherapeutics in New York. Physicians have identified joint microbleeds in patients as young as two years old. "If you can prevent that, 10 or 20 years down the road, they will be better off," he says.

The limited treatment options for haemophilic children and adults with joint pain strongly motivates researchers. Only a few decades ago, patients with haemophilia did not have the chance to grow old; now they are feeling the effects of living for longer with the disease. "Joints are so important," says von Drygalski, "because people are living to 60 or 70 years old — just trying to live normal lives." ■

Katharine Gammon *is a freelance science writer in Santa Monica, California.*

1. Ceponis, A., Wong-Sefidan, I., Glass, C. S. & von Drygalski, A. *Haemophilia* **19,** 790–798 (2013).
2. Hakobyan, N. *et al. J. Thromb. Haemost.* **12** (suppl. 1), 1 (2014).
3. Greene, W. B., McMillan, C. W. & Warren, M. W. *Clin. Orthop. Relat. Res.* **343,** 19–24 (1997).

T. C. NICHOLS, B. J. Q. NICHOLS & S. A. GALLAGHER

Haemophilic dogs at the University of North Carolina's blood-research laboratory are helping researchers to learn about the disease and develop treatments.

ANIMAL MODELS

Dogged pursuit

In the study of haemophilia, man really does have a best friend.

BY EMILY SOHN

Austin, a fluffy white-and-black Old English sheepdog, was still a puppy when his owners called the University of North Carolina's Francis Owen Blood Research Laboratory in Chapel Hill four years ago. After deciding that the children were finally old enough to get a dog, the family had quickly bonded with the rambunctious pup. But within six months of bringing Austin home, they had spent US$10,000 on veterinary bills to deal with extreme bleeding from small scrapes. Austin was also suffering from spontaneous bleeding into his joints and uncontrollable nosebleeds caused simply by overexcitement. The family loved him, but could not take care of him.

Timothy Nichols, director of the North Carolina lab, gets enquiries about haemophilic dogs from around the world four or five times a year. Sometimes he offers advice and information. Other times, he goes and gets the dog. After blood tests confirmed that Austin had haemophilia, two of Nichols' lab members flew to the family's home in New Orleans, Louisiana, where they rented a car, packed it with a cool box full of medication and drove Austin back to Chapel Hill. There, the dog joined a colony that for nearly seven decades has been quietly transforming understanding of haemophilia.

Unlike the rats favoured as animal models for many other diseases, dogs develop haemophilia naturally, have enough blood to contribute to research studies and live long enough to reveal long-term outcomes of treatments. "We have a 60-year track record now showing that if it works well in dogs, it's likely going to work well in humans," says Nichols.

LIKE HUMAN, LIKE DOG

The earliest recognized cases of haemophilia in dogs were documented in 1935 in three related Scottish terriers. About a decade later, a lawyer in New York contacted the North Carolina blood-research lab to discuss two Irish setters that were bleeding frequently, both inside and out. Already eager to acquire an animal model of haemophilia, the lab's then-director, Kenneth Brinkhous, adopted the aristocratic, long-haired dogs and began searching for breeding partners for them. Since then, colonies of haemophilic dogs have sprung up at Queen's University in Kingston, Canada; the University of Alabama at Birmingham; and Nara University in Japan. There are also a few dogs at Cornell University in Ithaca, New York. Today, these colonies breed both haemophilic and healthy dogs to maintain populations with specific variants of the disease.

It did not take long for dogs to become pivotal to scientists' understanding of the disorder in humans: the disease works in the same way in both species. Early breeding efforts in the 1940s, for example, made it clear that in dogs, the genes responsible for haemophilia lie on the X chromosome — which later proved to be true for people, too. Except in rare cases, only males get the disease; females are carriers. "The genetic and laboratory studies from breeding these dogs and testing their blood helped establish the classic parallel example of humans

and animals having the same genetic defects," says Jean Dodds, a veterinary surgeon in Santa Monica, California, who has been working with haemophilic dogs since 1959.

More recently, gene-sequencing studies have revealed that identical genes with parallel mutations account for many cases of the disease in both dogs and humans. Both species can have either haemophilia A or haemophilia B, versions of the condition caused by defects in the genes that produce the clotting proteins factor VIII and factor IX, respectively. Symptoms are remarkably similar across species: both people and dogs with the disease are unable to form clots, so cuts can bleed uncontrollably. Bleeding in the bowel can lead to diarrhoea. And lumps of blood can form in joints and muscles.

Dogs are also good models for practical reasons. Most of them are bigger than small children. They react to medicines much like humans do, allowing researchers to look to dogs first as they calculate doses. And the animals cooperate well. "The dogs here are around people all the time," says Nichols. "If you need to draw blood, they put their paws out."

DOGS FIRST

Dogs in haemophilia colonies often win researchers' hearts. Veterinary surgeon Clint Lothrop of the University of Alabama at Birmingham has adopted several from his colony, and he treats them at home when they bleed. The Queen's University dogs run, climb and play with balls and other toys every afternoon, says Queen's haematologist David Lillicrap. The North Carolina dogs have access to an outdoor play area. With severe haemophilia, animals can bleed simply from wearing collars, so handlers are careful to prevent fights or rough play.

Between play sessions, dogs give blood for research. Those donations have allowed scientists to make key discoveries about why the disease develops.

By the 1950s, researchers knew that normal blood could correct clotting defects, but they were not sure which components of blood mattered most. With the help of dog blood, Brinkhous and others deduced[1] that clotting factors were in the plasma rather than mixed in with platelets or blood cells. Giving healthy plasma to haemophilic dogs made them better. Once scientists had identified factors VIII and IX, and could distinguish between healthy and haemophilic dogs, Brinkhous and his colleagues were able to develop a test for measuring levels of the factors in plasma on the basis of how long it took for clots to form in test tubes.

In the 1940s, life expectancy for humans with haemophilia had been about 20 years, often plagued by painful bleeding into muscles and joints, says Nichols. Plasma-replacement therapy transformed the quality — and duration — of life, as did the ability to concentrate the factor in plasma, developed by the mid-1960s.

In the 1970s and early 1980s haemophilia treatment went through a dark period: contaminated plasma infected many recipients with hepatitis or HIV. Dogs helped people out of this tragic stage.

Scientists thought that they had found the light at the end of the tunnel in 1984, when the cloning of the gene for factor VIII allowed them to make artificial factor in the lab[2]. But after years of dealing with blood-borne infections and a cultural fear of such genetically modified products, it was hard to get people to try the synthetic factor. Then studies[3] in dogs showed that the treatment worked without complications, and a 43-year-old North Carolina state legislator agreed to be the first person to sign up. "He knew of Brinkhous's work and he knew of the dogs at Chapel Hill and it helped him to know that it had really helped the dogs and was safe," says Nichols.

To everyone's relief, the treatment worked. In fact, infusion of the factor was so uneventful that the recipient, known as GM, pretended to be a hamster during the procedure (the product had been produced in hamster cells). After the treatment was licensed, GM spoke at a celebration at the Genetics Institute in Cambridge, Massachusetts. "After slowly and painfully climbing to a balcony half way up the stairs, he delivered a powerful story about what it was like to grow up with hemophilia without adequate treatment, how as a child he had lost a beloved older brother from a bleed, and how important the development of safe recombinant factors was to him and all people with hemophilia," wrote Gilbert White, director of the Blood Research Institute at the BloodCenter of Wisconsin in Milwaukee, in a paper[4] describing 35 years of advances in haemophilia research. "His comments had the entire company in tears."

> ***"We have a 60-year track record showing that if it works well in dogs, it's likely going to work well in humans."***

POINTING THE WAY

Research in canines often foreshadows what is coming for humans. Over the years, more than 25 products that had been tested in dogs have been licensed for clinical use in people. One of the first studies to show the feasibility of gene therapy[5], published in 1993, involved three factor-IX-deficient dogs and an extremely invasive procedure, in which researchers removed two-thirds of each dog's liver. Over the course of three days, they injected the regenerating organs with a potentially dangerous HIV-like virus loaded with the healthy gene. The procedure boosted levels of factor IX from zero to 1% of normal — enough to fuel optimism that a more efficient procedure might one day be possible.

By 1999, dog studies[6] began to show that one injection with a much safer vector called an adeno-associated virus could deliver a healthy factor IX gene, boosting levels of the clotting factor to 2% — enough to reduce spontaneous bleeds. "We were able to move past that rapidly and have had levels of 10% for a long time," says Katherine High, a haematologist at the Perelman School of Medicine at the University of Pennsylvania in Philadelphia. Dogs can now get simple, 10- to 15-minute infusions of factor-bearing viral vectors. Similar work with factor VIII is close behind, says Nichols.

Some of the first dogs to receive factor IX gene therapy with just a single injection have lived full and happy lives. Brad and Semi were two basenjis — African hunting dogs — who lived in the Alabama colony. After receiving the treatment, one died at 13, the other at 14, neither from haemophilia-related causes. Several clinical trials are now assessing gene therapy with factor IX in humans (see page S160).

Other studies are testing the possibility of administering factors VIII and IX orally instead of with an injection — a technique that has been shown to work in mice and is now being tested in dogs. And ongoing work by Lothrop and his colleagues suggests that replacement factors might become available as longer-lasting, less-invasive subcutaneous shots instead of intravenous injections.

Dogs are also helping scientists to develop strategies for combating the inhibitor antibodies that many patients develop in response to factor-replacement therapy. One approach[7] gives dogs a gene to express another clotting factor, factor VIIa, completely bypassing the need for factors VIII and IX. The technique can reduce the number of bad bleeds each year from five or ten to one or even none.

In other lines of work, dogs have undergone bone-marrow transplants to express factor VIII in their platelets, shielding them from inhibitors. And Nichols' team has acquired a strain of dogs deficient in clotting factor VII, allowing it to test therapies for rare bleeding disorders that may not occur in enough humans to allow large clinical trials.

It is unlikely that any of these next-generation approaches would have been possible without canine models. "The role of haemophilic dogs in the preclinical development of novel therapies for haemophilia during the past three decades has been enormous," says Lillicrap. The disease once seemed insurmountable, but in the years ahead, he says, dogs will continue to provide insights that will make life better for humans. ■

Emily Sohn *is a freelance journalist in Minneapolis, Minnesota.*

1. Brinkhous, K. M., Penick, G. D., Langdell, R. D., Wagner, R. H. & Graham, J. B. *AMA Arch. Pathol.* **61,** 6–10 (1956).
2. Wood, W. I. *et al. Nature* **312,** 330–337 (1984).
3. Giles, A. R. *et al. Blood* **72,** 335–339 (1988).
4. White, G. C. *Trans. Am. Clin. Climatol. Assoc.* **121,** 61–75 (2010).
5. Kay, M. A. *et al. Science* **262,** 117–119 (1993).
6. Herzog, R. W. *et al. Nature Med.* **5,** 56–63 (1999).
7. Margaritis, P. *et al. Blood* **113,** 3682–3689 (2009).