The memoirs of Désiré Collen

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A HEART'S DESIRE
BLENDING ACADEMIC RESEARCH AND BUSINESS ENTERPRISE

Désiré Collen

Translation into English from the Dutch original

Memoires Désiré Collen

Een hart voor onderzoek en ondernemen

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Preface

The development of rt-PA (recombinant tissue plasminogen activator) into a successful drug is a fine example of successful collaboration between academia and industry. Some even refer to it as a paradigm. I never felt that this success was the consequence of meticulous planning, at least not at my end. The success of rt-PA resulted from a conjunction of circumstances and a substantial dose of serendipity, even though supported by hard work in our laboratories in Leuven as well as in Genentech, and in several clinical centers in Europe and the USA. Anyhow, I take pride in the t-PA story and look back on its success with particular feelings. Within less than ten years, a biochemical concept - the fibrin selectivity of specific proteins that dissolve blood clots – was translated into the most successful thrombolytic drug to treat heart attacks. Only eight years separate the very first experiment with t-PA in our laboratory (at that stage the natural protein) and the approval of the biotechnologically produced recombinant t-PA by the American *Food and Drug Administration* (FDA). Anyone familiar with the pharmaceutical industry will realize that this is an exceptionally short time span. It has become a textbook example of 'from bench to bedside' or ' translational research' (as it is fashionably called) into development of new drugs.

rt-PA became an important drug: in spite of its high price tag, it became the drug of choice to treat patients suffering from myocardial infarction; at least for patients who could be hospitalized within a few hours. Rapid intervention during a heart attack indeed was and still is crucial, even with the use of percutaneous coronary interventions (dottering and stenting). During its heyday, every year more than a quarter of a million heart patients were treated with rt-PA. Before the availability of thrombolytic drugs, up to a quarter of these patients did not survive. Towards the end of the eighties, this death rate had dropped to below 10% thanks to the new developments in cardiovascular medicine, including thrombolytic therapy. Tens of thousands of patients owe their lives to rt-PA.

t-PA was patented by *Leuven Research and Development, a* non-profit organization for technology transfer within the K.U.Leuven. The ensuing significant income from royalties - supplemented by regular and competitive research funding - provided for the expansion of the Center for Molecular and Vascular Biology at the university of Leuven into a research center of international renown, and enabled us to partially fund the Center for Transgene Technology and Gene Therapy of the Flanders Institute for Biotechnology ("VIB") in 1994. In addition, we had the opportunity to timely explore new research areas, including angiogenesis (the formation of blood vessels), gene therapy and neuronal development, leading to potential new treatments for solid tumors and neurodegenerative diseases such as amyotrophic lateral sclerosis.

The rt-PA royalties also allowed to support four to five young academics yearly to acquire relevant expertise and experience in the USA, initially with fellowships from the K.U.Leuven and later under the auspices of the *Belgian American Educational Foundation*. Finally, we also contributed to the expansion of the biomedical industry in Flanders. I believe that *Thromb-X* and its successor, *ThromboGenics*, have highlighted Flanders on the map of industrial biotechnology. Being one of the most recent Stock Exchange listed companies in this sector, I hope that ThromboGenics will follow in the footsteps of *Genentech*.

Serving as a successful role model, the cooperation on rt-PA between K.U.Leuven and *Genentech* probably also contributed to the foundation of the Flanders Institute for Biotechnology ("VIB"). This crownjewel among Flemish research institutes is well underway to establish itself within the top league of European research centers in Life Sciences. Over the years the VIB has progressed to the major league of research organizations - of course, within its boundaries of size and resources - such as the German *Max Planck Gesellschaft zur Fördering der Wissenschaften*, the French *Centre National de la Recherche Scientifique* or the Swiss *Federal Institute of Technology*.

Not all my projects have been as successful. Staphylokinase - the t-PA for the "poor" - did not turn out to be a success, despite meticulous planning and with the track record experience with rt-PA in support. Also my relationship with some members of the K.U. Leuven management as well as some colleague professors was not always as smooth as it could have been. Not everybody was happy with the manner in which I managed and prioritized the flow of income from royalties, although I always proceeded in consultation and in agreement with the K.U.Leuven, as I was contractually obliged. Some even believed that the university, being the owner of the t-PA patent and all the associated legal and economic rights via its university-industry interface of K.U.Leuven Research and Development (LR&D), should have the right to centrally determine the allocation of the income. I managed to counteract this efficiently.

In contrast, the vast majority of my scientific collaborators reflect positively on our collaborations and I have made many friends in my professional career and (exceptionally) few enemies. I am very pleased with the poster presented to me by my current staff members in Leuven at the occasion of my new status as emeritus professor in 2008. On the background of the poster, which announces the farewell symposium (*Heart for the Future*) at the occasion of my retirement is a symbolized t-PA structure, as adapted from the *Nature* publication of Pennica et al, but with the amino acid building blocks substituted by the circular photographs of my staff members. Since there are 527 amino acids (circles) in the natural t-PA and since I had in total 'only' 274 staff members up to 2008, a little artistic freedom had to be taken in the poster design.

On publication of these memoirs, I will be emeritus for one year and will have had time to reanalyze certain events. Not only the t-PA story itself, but also what preceded it and what were the consequences and changes that it caused. The idea of publishing my personal experiences in a book is not new and was not even my own. I have been regularly faced with the issue, mostly during conversations over a meal or at a reception. It was however clear to me that I would have to address sensitive issues, such as the personalities of various actors in the t-PA story, the dominant influence of industry on research, the strained relationships within the university, and so on. A couple of times, I actually started to write my story, just to stop soon thereafter. I realized that I would touch on too many sensitive issues and that the time was (then) not yet right. To paraphrase Leo Tindemans (a former Belgian prime minister): by writing this book too early, I would 'lose friends', while by writing it too late, I would 'have no more readers left'. Therefore, I think that now is the right time. Time has seasoned certain facts with leniency, while, on the other hand, the story still contains enough relevant elements to evoke interest from a broad readership.

It has turned out to be a book with many facets: setting the stage for t-PA in Section I, the unfolding of the t-PA story in Section II, and the ensuing biopharmaceutical drug development between academia and industry in Section III. It covers not only my positive experiences as professor, researcher and entrepreneur, but also my less successful ventures.

It is neither my intention to throw a late punch with this book nor to cover up anything. If that were to be the case, I should have gone fishing (or in my case flying) instead. Those with whom I am acquainted, know that I am not rancorous, but rather that I wear my heart on my sleeve. When I disagree with somebody's point of view I openly contest this, regardless of the openent's identity. I am fully aware that some of the players in this story would tell certain sections differently. Perhaps their memory stored a different version or perhaps different people interpret the same facts differently. I have based this narrative on my own recollection and on my - rather limited - correspondence, on the comprehensive scientific library on thrombolysis, the patents and contracts, as well as on the testimonies that were delivered at the symposium '*Heart for the future*' and those in the '*Anthology of Scientific Collaborations*', both produced on the occasion of my retirement from the K.U.Leuven. However, I still had the feeling that an independent person should doublecheck some matters, and I therefore entrusted Peter Raeymaekers with some research and the manuscript drafting. I found him to be an excellent soundboard, because of his fifteen years of experience in biomedical research combined with ten years as science author and communicator. Nevertheless, the accounts in the book are my impressions and my interpretations and, consequently, I accept full responsibility.

The scientific staff of the Center for Thrombosis and Vascular Research (K.U.Leuven) around 1985 with from left to right Professor Vermylen, Professor Verstraete (head of department), Professor Collen, Professor Lijnen and Professor Verhaeghe

I was fortunate enough to be able to join the research-oriented Laboratory for Blood Coagulation, founded by Professor Dr. Marc Verstraete in 1955 as a one-man business. The laboratory was later renamed as the 'Center for Thrombosis and Vascular Research" and then as the 'Center for Molecular and Vascular Biology' of the K.U.Leuven and presently employs a staff of around eighty members. I owe a lot to Professor Dr. Marc Verstraete, for his continued support of my research, even when it sometimes went beyond his own field of expertise. I was fortunate to work with many young Belgian and international MDs and PhDs, and also with a host of loyal and very competent laboratory assistants and technicians. Furthermore, the t-PA story would never have been possible had it not been for the very close collaboration with many other research groups, both within universities and the industry. To all these people, I convey my most cordial gratitude.

This book deals with my experiences as researcher and entrepreneur and, as explicitly requested by my family, does not include personal or family details, except for a few anecdotes, which are an intricate part of this account. My sincere gratitude goes to my wife Louisa for her devotion and endurance. She was always on standby, ready to pack our things on short notice for New York, Stockholm, Burlington VT, San Francisco or London. Being in charge of most family duties and responsibilities it was not possible for her to retain a job from 1968 onwards. This is the one area where I do not claim any progressive attitude or balanced distribution of responsibilities for myself.

Désiré Collen

t-PA and thrombolysis ... the context of a life's story

A simple experiment

February 1979. The experiment was extremely simple. We mixed a few drops of 'conditioned' growth medium from the ‘Bowes’ melanoma cell line with purified fibrinogen, the precursor to fibrin which is also responsible for the formation of blood clots, and we allowed the mixture to clot. We already knew that the cell line originating from the malignant melanoma of patient Bowes secretes a so-called plasminogen activator, which activates a mechanism that dissolves blood clots. The aim of the experiment was to determine to what extent the plasminogen activator from the Bowes cell line would bind to the fibrin. Other plasminogen activators, like urokinase or streptokinase known at that time, did not bind, while the activator that circulates in our blood in very low concentrations does. We were overjoyed to see that the activator secreted by the 'Bowes' melanoma cell line adsorbed very strongly to the fibrin clot. The experiment was utterly simple in its setup, but would have far-reaching consequences.

Lifesaving option against heart attack

Based on these results, I was convinced that if the plasminogen activator from the Bowes cell line could be purified, characterized and produced on a large scale, it could have an impact on the increasing burden of heart attacks. Finally, we would be able to save the life of thousands of people that succumb in hospitals to heart attacks each year, instead of being largely helpless. Waiting and hoping that the patient would survive, was one option; an aspirin was the second option. Our department director, Prof. Dr. Marc Verstraete, worked on a third option: thrombolysis. This technique aimed at dissolving the blood clots, which are blocking the arteries during an acute heart attack. As early as 1969, Verstraete published the results of a clinical study in which he and three other European cardiologists administered streptokinase to patients with a heart attack[1]. In 1971 and 1979, under his leadership, the "*European Working Party on Streptokinase*', published additional

[1] Amery A, Roeber G, Vermeulen HJ, Verstraete M. Single-blind randomised multicenter trial comparing heparin and streptokinase treatment in recent myocardial infarction. Acta Med Scand Suppl. 1969; 505: 1-35.

studies in the *British Medical Journal*[2] and the *New England Journal of Medicine*[3] respectively, two top medical journals.

Based on the simple experiment with fibrinogen and the culture medium of the Bowes cell line, it appeared to me that the plasminogen activator from that cell line today known as tissue-type plasminogen activator, or just t-PA) could be a much better drug than streptokinase. However, we still had to prove this and convince the rest of the world (see "t-PA story").

Thrombolytic therapy eventually became a routine treatment worldwide. Over a 20 year period more than a quarter of a million patients would be treated every year. In just one decade, the mortality rate among patients, admitted to hospitals with acute heart attacks, decreased by more than half: from 25% in the mid seventies to 10%, thanks also to the introduction of recombinant t-PA (rt-PA) by *Genentech*. [4] My only frustration is that we could have saved many more people had t-PA been more readily available, also to people who were not as fortunate to live in the prosperous western world. Still, it would take much more time, lots of research, thousands of air miles, sweat and for some even tears before t-PA would become readily available to patients. But that one simple basic experiment in our laboratory on the Gasthuisberg campus of the K.U.Leuven set the stage for the development of t-PA as a drug to dissolve blood clots.

Heart and vascular diseases - a heavy toll

In the western world, heart and vascular diseases are still a main cause of death, illness and disability. They are responsible for a quarter of the mortality among people over the age of forty. Three main categories are to be distinguished: diseases of the coronary blood vessels (the coronary arteries supplying the heart with blood) as a result of atherosclerotic degeneration or commonly, 'calcification of the arteries', which may eventually cause acute myocardial infarction (AMI);
affected blood vessels in the brain (cerebrovascular accident, CVA) which cause strokes or the formation of blood clots in the deeper veins of the legs (deep vein thrombosis, DVT), which can migrate via the heart to the lungs and cause pulmonary embolism (PE).

[2] Streptokinase in recent myocardial infarction: a controlled multicenter trial. European working party. Br Med J. 1971; 3: 325-31.

[3] Streptokinase in acute myocardial infarction. European Cooperative Study Group for Streptokinase Treatment in Acute Myocardial Infarction. N Engl J Med. 1979; 301: 797-802.

[4] Delude C. Clot-Busters !! – Discovery of thrombolytic therapy for treating heart attack and stroke. Breakthroughs in Bioscience, FASEB 2004, 6.

An estimated 80 million Americans suffer from cardiovascular disease. Annually 7 million of them are hospitalized, and each year about one million Americans die because of cardiovascular disease. Among the casualties 52% suffer from a coronary blood vessel disease and 17% die from a stroke. The price tag for the American Society amounts to 475 billion dollar per year.[5] Europe is not doing any better: the corresponding numbers (normalized to the size of the population) in most West European countries closely match those of the USA, and in Eastern Europe the prevalence is even considerably higher.

Plaques and clots

Today, we realise that myocardial infarction or stroke are predominantly caused by the formation of a blood clot which occludes a narrowed blood vessel. The narrowing of a blood vessel is caused by atherosclerosis, which in turn is caused by small damages in the smooth muscle-lining of the blood vessel. The body tries to repair these damages and during this process white blood cells clump together with blood platelets at the site of the injury. In addition, other blood substances, such as cholesterol, are deposited at this site. A foam-like mass forms, which is then covered by a layer of cells that causes the blood vessel to narrow (atherosclerotic plaque). Because of this narrowing of the vessel, the blood supply to organs and body parts can gradually fail. When this occurs in the coronary arteries, angina pectoris (AP) develops, whereas in the brain transient ischemic attacks (TIA) occur. When a blood clot suddenly occludes a coronary or cerebral artery, the distal tissues no longer receive blood and become necrotic. When this occurs in the heart, we refer to it as a heart attack or an acute myocardial infarction (AMI), whereas in the brain it causes a stroke or a cerebrovascular accident (CVA).[6]

[5] Lloyd-Jones D et al. Heart disease and stroke statistics – 2009 update: A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2009; 119: e21-e181.

[6] Dutch Heart Foundation, http://www.hartstichting.nl/go/default.asp?mID=5544

"Open-Artery" hypothesis

Early 1979, the debate on the mechanism underlying a heart attack was not yet decided.[7] Though the first elements in this discussion were proposed by the pathologist Ludwig Hektoen and the internist Sir William Osler. In a JAMA editorial[8] in 1899, Hektoen wrote "in most cases, a myocardial infarction is caused by thrombosis, which occurs secondarily after sclerotic changes in the coronary blood vessels". In 1892, Osler was even more specific[9]: "The obstruction of a blood vessel by a thrombus or embolus leads to a disorder, which we call anemic necrosis or white infarct. This is most commonly observed in the left ventricle and in the septum, which are perfused by the anterior coronary artery."

At the beginning of the 20th century, physicians still accepted that an acute myocardial infarction was fatal. Hardly any research was done, neither towards therapy, nor into the clinical symptoms. Therefore, the mechanisms that cause a heart attack were not understood very well. The Russian physicians Obraztsov and Strazhesko changed this situation in 1910 by describing the clinical symptoms of a patient who was struck by a heart attack but survived.[10] In 1912, James Herrick described in a lecture to the *Association of American Physicians* the classic signs and symptoms of a so-called acute coronary artery occlusion, the obstruction of a coronary artery. Although the work of Herrick is now recognized as a milestone achievement, his colleagues at that time were not enthusiastic. An updated version of his research in 1918, again presented to the same association, received more attention and determined that the term 'coronary thrombosis' be coined to designate acute myocardial infarction. Of importance was Herrick's emphasis that coronary thrombosis was not necessarily fatal. He wrote[11]: "I am surprised by the preserved bodily power of some patients, who survived a heart attack. Some are capable to walk around within hours and continue with their usual lives after a couple of days. Just as with many other diseases, we must abandon the prejudice that an autopsy is the only way to diagnose a heart attack."

[7] Maroo A and Topol EJ. The early history and development of thrombolysis in acute myocardial infarction, J Thromb Haemost 2004; 2: 1867-70.

[8] Hektoen L. Infarction of the heart, JAMA 1899; 33: 919.

[9] Osler W. The Principle and Practice of Medicine. New York: D. Appleton, 1892.

[10] Maroo A and Topol EJ. The early history and development of thrombolysis in acute myocardial infarction, J Thromb Haemost 2004; 2: 1867-70.

[11] Herrick JB. Clinical features of sudden obstruction of the coronary arteries. JAMA 1912; 59: 2015–20.

The awareness of the strong association between coronary thrombosis and heart attack, and the notion that not all heart attacks are fatal, paved the way for a new therapeutic model: the 'open-artery' hypothesis. According to this hypothesis, the survival and cure of heart patients could be achieved by restoration of the blood flow to the heart muscle.

Cause or consequence?

In the 1940's, however, most cardiologists changed their opinion. The controversy started in 1939 when C. K. Friedberg and H. Horn published their paper '*Acute myocardial infarction not due to coronary obstruction*'.[12] Based on autopsies, the authors concluded that only 31% of the patients that died of a heart attack showed indications of coronary thrombosis.

Through these and other studies, the 'open-artery' hypothesis was questioned and the issue arose whether coronary thrombosis was the cause or the consequence of a heart attack. William C. Roberts, Head of the *Cardiac Pathology Heart Institute* at the *National Institutes of Health* (NIH) was one of the most prominent protagonists in this controversy. Roberts favored the view that coronary thrombosis was the consequence of myocardial necrosis and not the other way around, as claimed by the 'open-artery' hypothesis[13]: "Although it may be an important factor in the development of atherosclerosis, coronary thrombosis only plays a minor role - or even none at all - in triggering a fatal coronary accident. It is evident that myocardial necrosis precedes coronary thrombosis."

Roberts thus supported the hypothesis that a heart attack is caused by uncontrollable muscle spasms. As a result, the development of thrombolytic drugs unfortunately was not further pursued.

Endurance of the stubborn

Nonetheless, some stubborn and headstrong people continued to believe in thrombolytic treatment of a heart attack. Sol Sherry was one of them. He was convinced that the protein streptokinase, produced by hemolytic streptococci, was capable of dissolving pathologic

[12] Friedberg CK, Horn H. Acute myocardial infarction not due to coronary artery occlusion. JAMA 1939; 112: 1675–9.

[13] Roberts WC, Ferrans VJ. The role of thrombosis in the etiology of atherosclerosis (a positive one) and in precipitating fatal ischemic heart disease (a negative one). Semin Thromb Hemost 1976; 2: 123–35.

blood clots[14]. Without knowing exactly how streptokinase worked, Sherry administered it in the second half of the 40's to patients with hemothorax (accumulation of blood in the pleural cavity) and empyema (accumulation of pus in the pleural cavities)[15] [16] [17]. Sherry was particularly interested in streptokinase as a potential thrombolytic drug. In 1957, after *Lederle Laboratories* managed to provide a purified form of streptokinase, Sol Sherry, Tony Fletcher and Norma Alkjaersig compiled a protocol to dissolve blood clots in human coronaries. First, they administered a 'loading' dose of streptokinase, followed by a continuous infusion with a lower dose. This protocol paved the way for the first clinical trials with heart attack patients. Interestingly, the authors already then suggested that only the timely administration of streptokinase (within 14 hours after the beginning of the heart attack) can effectively reduce the mortality rate. Patients, who received the treatment at a later stage, hardly had a better chance to survive.[18] This observation - the earlier the treatment, the more effective the therapy - was confirmed for the first time in a controlled study by the European study group head by Marc Verstraete (see later on) and would become one of the cornerstones of thrombolytic therapy.

Little enthusiasm

These first (limited) successes did however not immediately translate into across-the-board applications in the hospitals in the 1960's and -70's. The positive clinical results of the *European Working Party on Streptokinase*[19] [20] did not convince the majority of cardiologists, to some extent because other studies yielded less convincing results. When the most important side effect of streptokinase - the increased risk of potential fatal bleeding - was taken into account some cardiologists openly questioned whether thrombolysis really provided a clinical benefit to the patient. In retrospect, these researchers did not sufficiently appreciate the importance of the early start of the treatment.

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[14] Sherry S. The origin of thrombolytic therapy. J Am Coll Cardiol 1989 ; 14: 1085-92

[15] Tillet WS and Sherry S. The effect in patients of streptococcal fibrinolysin (streptokinase) and streptococcal desoxyribonuclease on fibrinous, purulent, and sanguinous pleural exudations, J Clin Invest 1949; 28: 173-90.

[16] Sherry S, Tillet WS, Read CT. The use of streptokinase-streptodornase in the treatment of hemothorax. J Thorac Surg 1950; 20: 393–418.

[17] Tillet W, Sherry S, Read C. The use of streptokinase-streptodornase in the treatment of chronic empyema. J Thorac Surg 1951; 21: 325–41.

[18] Sherry S, Fletcher A, Alkjaersig N, Smyrniotis FE. An approach to intravascular fibrinolysis in man. Trans Assoc Am Physicians 1957; 70: 288–95.

[19] European Working Party. Streptokinase in recent myocardial infarction: a controlled multicenter trial. Br Med J 1971; 3: 325–31.

[20] Streptokinase in acute myocardial infarction. European Cooperative Study Group for Streptokinase Treatment in Acute Myocardial Infarction. N Engl J Med. 1979; 301: 797-802.

Burton Sobel later emphasized[21]: "We tried to dissolve blood clots of patients whose heart muscle had already died. We did not sufficiently realize how vital early treatment was to achieve clinically positive results. In fact, we were all out of pace and thought that something was wrong with the hypothesis that heart attacks were caused by blood clots. Our poor understanding was the reason that we did not make significant progress and that research on the thrombolytic treatment of a heart attack was discontinued."

Renewed interest

Only early 1980 interest in thrombolytic therapy was revived. Marcus DeWood and his colleagues detected coronary occlusions and blood clots red-handed! DeWood published his research in the prestigious journal *The New England Journal of Medicine*[22]. Using coronary angiography - a technique whereby via a catheter that is inserted in the groin and advanced into the aorta, a contrast agent is injected into the coronary arteries and X-ray pictures are made - he witnessed how in 110 of the 126 (87%) examined patients a heart attack was caused by total obstruction of a coronary artery. With 59 patients, the obstruction was clearly caused by a blood clot. With 52 patients, he was even able to remove the thrombus via the catheter. Today this has become a routine procedure but in those days it stood out as a remarkable example of interventional skills.

In addition, the research on reperfusion by E. I. Chazov[23] and later by K. Peter Rentrop[24] again boosted the research on thrombolytics: they administered streptokinase to patients with a heart attack via the coronary artery during a diagnostic angiography. Their research confirmed that re-opening of the coronary arteries could be achieved in an acute setting, and that it led to an improvement of the symptoms and to recovery of the heart function.

Today physicians in training would be amazed that the concept of coronary obstruction was one of the most discussed hypotheses during most of the 20th century. Now we readily accept that, in case of a heart attack, the flow of blood to the heart must be restored as quickly as possible, either with thrombolytic drugs, which dissolve the blood clot, or by angioplasty, a procedure whereby the blood vessel is mechanically dilated with a small balloon followed by the insertion of a stent to keep the blood vessel open.

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21 Delude C. Clot-Busters !! – Discovery of thrombolytic therapy for treating heart attack and stroke. Breakthroughs in Bioscience, FASEB 2004, 3.

22 DeWood MA et al. Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. N Engl J Med. 1980; 303: 897-902.

23 Chazov EI et al., Intracoronary administration of fibrinolysin in acute myocardial infarct. Ter Arkh 1976; 48: 8–19.

24 Rentrop KP et al. Acute myocardial infarction: intracoronary application of nitroglycerin and streptokinase. Clin Cardiol. 1979; 2: 354–63.

The availability today of cathlabs in the Flemish hospitals has made this last technique even to be preferred, that is when the intervention can take place within 3 hours - preferably within 90 minutes - after the first occurrence of symptoms.

It was in this historical context that we discovered towards the end of 1970 that the Bowes melanoma cell line secreted the protein t-PA, providing us with a source for this thrombolytic protein, which was probably more efficient than streptokinase for the dissolution of blood clots and possibly much more specific, thus causing less bleeding complications. An additional advantage is that t-PA is endogenous to the human body, so that in contrast to streptokinase it does not activate the immune system preventing the generation of neutralizing antibodies.

Evidently, all these 'assumptions' remained to be proven.

Section I.
The pre-history of the t-PA story

Double qualifications

It is best to start my story as physician, scientist and entrepreneur in October 1961. My life's journey of thousands of miles started at the age of eighteen when I took my first step onto the campus of the Catholic University of Leuven, my *alma mater*. I enrolled for the study program that would lead to the title of doctor in medicine, surgery and obstetrics. I had seven years of study ahead of me. This choice was certainly no vocation, but rather a pragmatic consideration: being a medical doctor at that time was still considered as a respectable occupation. My father influenced my choice. As a son of an unskilled worker, he never had the opportunity for a higher education and with his high school diploma he was the highest qualified member of the previous generation in our family. In a family where, to my knowledge, nobody ever obtained a university degree, the prospect of having a doctor in the family filled my parents with immense pride. The choice of Leuven was pretty obvious: it had a solid reputation and I knew the city well. After all, I spent seven years there in boarding school during my high school education. Leuven was only just an hour away by train from my hometown Sint-Truiden in the province of Limburg, where I was born in 1943.

First disciplined, then undisciplined and finally hard working

My first year in Leuven can be briefly summarized. I was quite a 'good' student, I attended all my lectures, practical classes and tutorials and commuted back and forth between Sint-Truiden and Leuven. I passed with distinction in July. During my second year, I stayed in a student room in Leuven and changed my tactics. I realized that I had invested unnecessary effort in my first year. I attended only the compulsory practical classes and tutorials and started spending more time in the student clubs and cafés than at my desk. The results were accordingly: I barely managed to pass the 1963 June/July examinations.

In my third pre-clinical year, I realized that university education meant more than hanging around and doing the bare minimum to pass the examinations. I looked around in the Faculty of Medicine for a job in one or the other research project and found one as student researcher in the Physiology Laboratory of Prof. J.P. Bouckaert. He was to receive his emeritate the following year. The next year, rather by coincidence than planning, I ended up in the Laboratory for Blood Coagulation under the direction of Marc Verstraete. Verstraete seems to have a good recollection of our first meeting[25]: "We were at that time doing experiments with coumarin derivatives. To calibrate the clotting time of patients'

[25] Verstraete M. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 119-21.

blood, we needed blood of healthy test persons on a daily basis. At the end of a lecture, I recruited volunteers among the students. Désiré was challenged by some friends, which ended in a betting contest. The next day Désiré was present, he gave his permission to be pricked, won the bet and got interested in our research."

I had no particular preference for coagulation or cardiology, but I was stimulated by the personality of Guido Tytgat, a young physician specializing in gastroenterology. Guido studied the rate at which the important coagulation protein fibrinogen is cleared by the liver of healthy people and of patients diagnosed with liver cirrhosis. That would become the research topic for his aggregate thesis. Guido was an inspiring person: he was a workaholic, with a strong personality and a broad view on the medical sciences. In addition to the research for his thesis, he also studied the regulation of the acidity level in the stomach, the diagnostic value of biopsies of the ileum, the exchange-transfusion in acute liver atrophy, experimental liver cirrhosis in dogs and much more. I was impressed by his intelligence, his diligence and perseverance and thanks to him, I became an adept of biomedical research. However, this had no positive influence on my studies. Though I traded the pubs for the lab, I did not devote more time to studying. I still had to bestir myself in order to get through my examinations. But this time I felt better about the situation as I had done something useful instead of only studying basic courses.

First research experience

After he unraveled the clearance of fibrinogen in liver patients and the contribution of blood coagulation and fibrinolytic degradation in the blood vessels, Guido Tytgat turned to the study of the turnover of plasminogen. Plasminogen is not an active protein, but a so-called 'pro-enzyme' that in its active form is capable of dissolving blood clots. Because neither Guido nor I had any practical biochemical background, we called in the help of René De Vreker, the biochemist in the laboratory of Marc Verstraete. He would isolate plasminogen from human plasma for us.

René quickly provided us with a protein solution that appeared to be homogeneous at first sight and which dissolved blood clots after activation. Guido, René and I were convinced that we isolated 'the real thing'. After we had labeled the protein with radioactive iodine, we first injected ourselves, and subsequently two dozen volunteers and two dozen liver cirrhosis patients.

The first results were most promising. After nine days, half of the radioactivity had disappeared, and we thought we could deduce valuable experimental results from that. But a lot of other observations did not comply with our working hypotheses.

Gradually we realised that our 'the real thing' was in fact merely a mixture of proteins, at best contaminated with plasminogen. Guido Tytgat did not take this matter too seriously and dropped the project. He was too preoccupied with preparing his departure for Seattle in the US to continue his training as gastroenterologist. Guido would later settle in Amsterdam and pursue an exceptional research career as first professor in gastroenterology in the Netherlands and as head of the world-renowned Department of Gastroenterology of the Academic Medical Center.

Medicine and chemistry

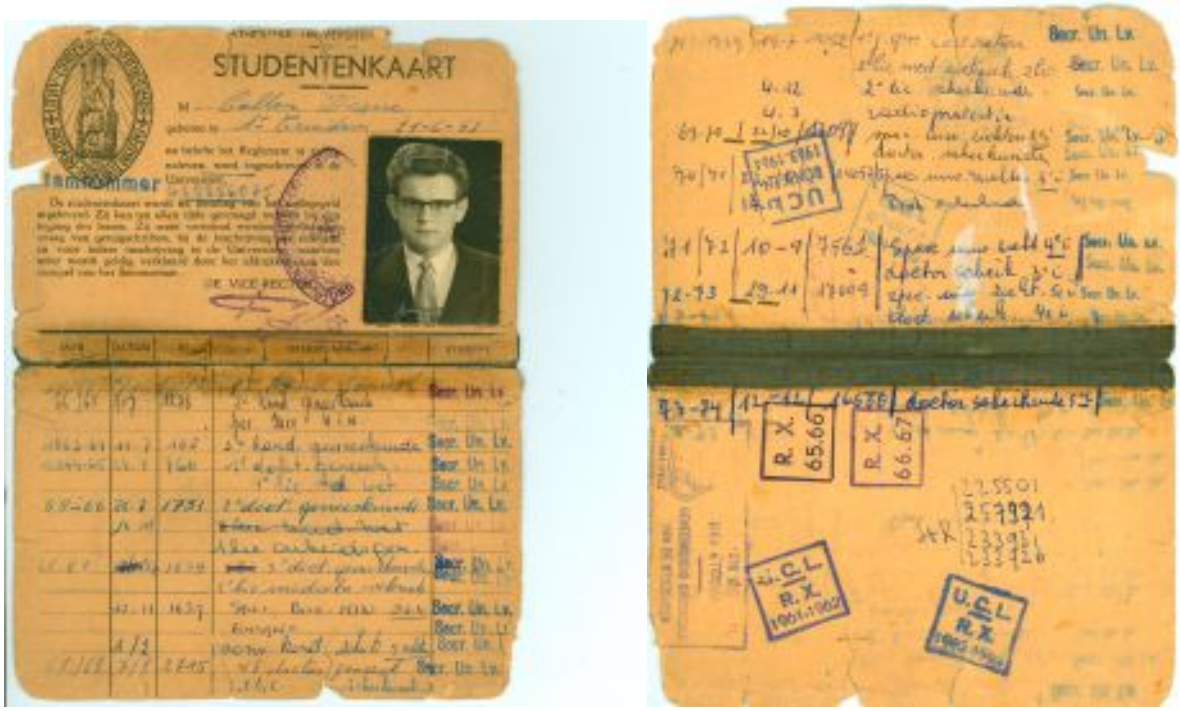
It became more and more unlikely to me that I would ever become a practicing general physician or specialist. I felt more for a career as biomedical researcher. A first goal would have to be a thesis on plasminogen research. Needless to say that the departure of Guido and the lack of research results disappointed me immensely. To further advance my research, I needed to learn more about biochemistry.

I asked myself why I would not combine my training in the Faculty of Medicine with an additional training in the Department of Chemistry in the Faculty of Sciences. I discussed this with Marc Verstraete and he found it to be an excellent idea. My wife - we got married in the summer of 1966 - unconditionally accepted the fact that we would not be able to go on holiday for the next couple of years.

I indeed would have to roll up my sleeves: the Faculty of Sciences was not overly 'keen' with its new student and hardly made any concessions. I had the opportunity to complete all courses in Chemistry for which I did not receive an exemption on the basis of my medical education in the September session of 1967. I successfully crossed the examination's hurdle of September 1967. However for the last two years of the programme I was not granted any privileges. I had to take examinations for all courses, including the attendance of all the compulsory practical classes throughout the year. Those practical classes were time consuming and took up the best part of the week.

1968 turned out to be a terribly hectic year. I hardly participated in the student uprisings of 1968. I was only occupied by medicine and chemistry. Throughout the academic year, I worked all day in the laboratory for the chemistry practicals. Eventually, I completed the final exams in medicine in February and July (I never attended some of the classes). The chemistry exams are reserved for the second session in September.

In the meantime, I had become an expert in the completion of examinations and received the degree of Doctor in Medicine in 1968, as well as master in the Medical Sciences and the Licentiate in Sciences, section Chemistry, in 1969 - every time summa cum laude. My student card of the K.U.Leuven is, with all the additional registrations for specialization courses in Medicine and the Doctorate in Sciences (Chemistry), perhaps one of the longest in all of Leuven's history.



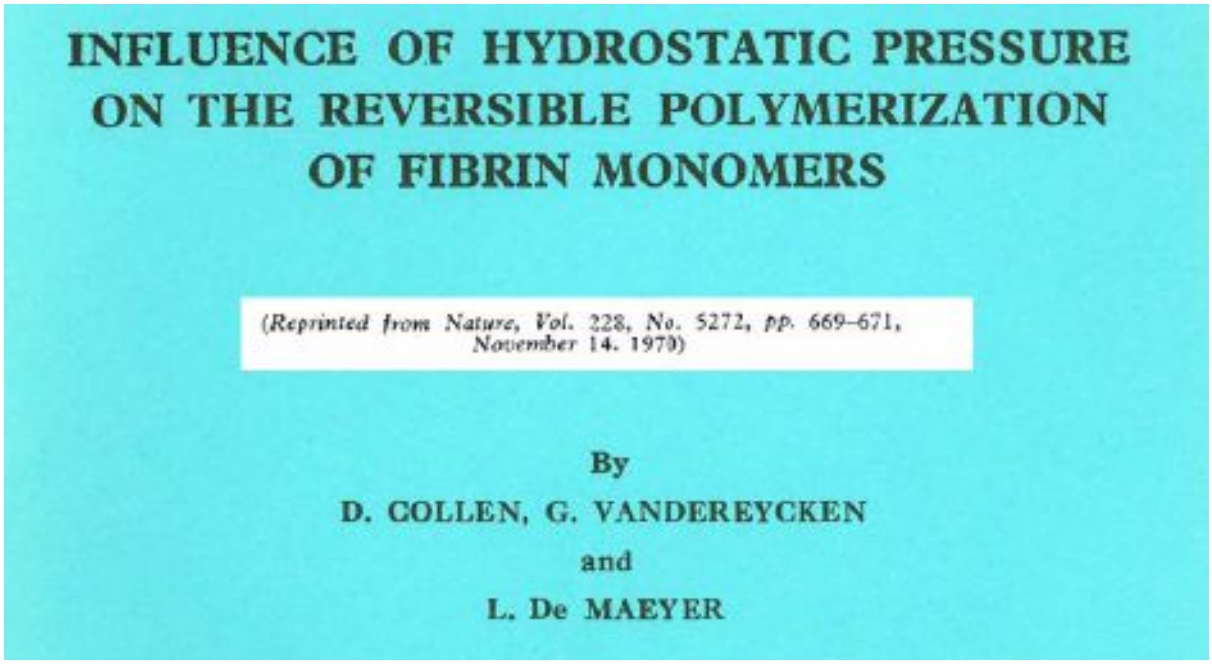
Student card of Désiré Collen of the K.U.Leuven.

In the grip of research

In July 1968, with the title of physician acquired and with the examinations for the first license in chemistry still ahead of me, I was appointed as research assistant (first “stagiair”, then “aspirant” and finally appointed researcher) at the National Fund for Scientific Research ("NFWO"), which is currently active under the name Fund for Scientific Research Flanders ("FWO"). My workplace was the laboratory of Marc Verstraete. Again, I had to call on his flexibility and support, in order to do a licentiate thesis in chemistry (simultaneously with my mandate) under the supervision of Professor Leo De Maeyer. The latter only had a part-time appointment at the K.U.Leuven. He was head of the Laboratory for Physical Chemistry and also held a position in Göttingen at the Max Planck Institute (Germany). Leo De Maeyer developed and built instruments to determine the initial velocity ('Transient Kinetics') of specific chemical reactions in the first microseconds. Later Manfred Eigen would receive the Nobel Prize for Chemistry thanks to, among other things, the instruments of De Maeyer.

De Maeyer had no real interest in blood coagulation. Still, he allowed me to start a research project on the polymerization of fibrin. I performed the purification of fibrin and the chemical part of the investigation in the laboratory of Marc Verstraete. Furthermore, I could use the hydrostatic pressure equipment in the laboratory of Professor Putzeys, a biochemist in the last year before his emeritate. By the end of the year, we had promising results. They were interesting enough to be published in the Journal *Nature*[26]. I was very proud, for as far as I knew then, it was quite exceptional for a research project at licentiate level to be published in *Nature*. I could then not anticipate that this publication would end up in a little niche within biochemical high-pressure research and would yield only about twenty citations. In any case, I felt that my career as scientific researcher was launched.

[26] Collen D, Vandereycken G, De Maeyer L. Influence of hydrostatic pressure on the reversible polymerization of fibrin monomers. Nature. 1970; 228: 669-71.



INFLUENCE OF HYDROSTATIC PRESSURE ON THE REVERSIBLE POLYMERIZATION OF FIBRIN MONOMERS

(Reprinted from Nature, Vol. 228, No. 5272, pp. 669–671, November 14, 1970)

By

D. COLLEN, G. VANDEREYCKEN

and

L. De MAEYER

Clinical training hampered by revalidation and reorientation

The goal of a "NFWO" research assistantship in a clinical discipline is to combine research with formal training in a medical specialty. It happened to be internal medicine in my case. A research career without any clinical responsibility, as I aspired, was then highly unusual for a trained physician. The regular five-year program only allowed one year of full-time research, whereas the other four years should be used to gain clinical experience. Most of the students schedule the research year somewhere towards the end or, at the earliest, half way of their training. In my case, the research quota was already reached after the first year. I started in July 1969 with my clinical rounds in internal medicine in the University Hospital St. Rafael in Leuven.

However, three months later fate struck. It was caused by a badly conceived experiment. We were looking for a method to treat patients with serious blood coagulation problems. During that time, the potential use of organ transplantation as a treatment for patients with Hemophilia A, was fiercely debated. This is a genetic coagulation disease characterized by impaired blood clotting. The patients (mostly boys) risk dying due to internal bleeding or serious joint malformations because of bleeding. These hemophilia patients lack a specific protein, Factor VIII, which is crucial for coagulation. We intended to investigate whether a spleen transplantation would possibly help. Marc Verstraete came up with a simple experiment, which would provide the 'proof of concept' for the therapy: we would perfuse the blood of a patient with severe hemophilia (less than one percent Factor VIII) through the artery of an isolated spleen of a deceased patient to observe whether the blood collected on the venous side contained Factor VIII activity.

The whole experiment turned out to be a gigantic fiasco. In the first place, the spleen came from a patient with serious splenomegaly or enlarged spleen. The veins of the spleen were almost impenetrable because of the swelling. Even with 'brute force' it was not possible to perfuse blood through. Moreover, the hemophilia patient whose blood was taken, was admitted into hospital due to aggressive chronic hepatitis. The experiment totally failed and ended with a severe Hepatitis B infection (serum hepatitis) for three of the researchers. I stayed in bed for 6 weeks with extreme fatigue. I could not even read a book, and the infection nearly caused my death. This accident happened during the time that the Hepatitis B virus was identified as the cause of serum hepatitis and the Australian antigen was developed as diagnostic biomarker. The patient who donated blood for our spleen experiment afterwards appeared to have an extremely high titer of circulating hepatitis B virus. The Rega Institute even used it for several years as a reference sample for the development of Hepatitis B analyses.

A disadvantage, however, sometimes turns into an advantage. I had to undergo a six-month period of revalidation and hence could not enter any hospital ward. Out of necessity - but not asked for - I was compelled to limit my activity to the laboratory.

Around March 1970, I had fully recovered from the Hepatitis B infection. The high titer of circulating antibodies against the virus indicated that I had completely recovered. I subsequently resumed my clinical training with three-monthly rotations in the department of cardiology (Professor J. W. Joossens), endocrinology (Professor P. De Moor), hepatology (Professor J. De Groote), bleeding and vascular diseases (Professor M. Verstraete), infectious diseases (Professor L. Eyckmans) and in the consultations internal medicine (Professor A. Amery). In retrospect, this was actually quite an instructive experience. Although I eventually did not become an internist, I did gather a lot of expertise, which turned out to be useful for a bridging function in a typical multidisciplinary research environment with physicians on the one side and doctors in sciences on the other. I therefore do not regret to have passed this training. Back on full force again, I combined these rounds with work on my doctor's thesis in chemistry and my aggregate thesis in medicine. I took care of all my clinical files and made sure that the patients and my supervisors were satisfied with me. Thus, I passed these eighteen months without receiving any serious complaint or reprimand. Since I then managed to combine clinical routine work with research for two theses, there was no time left for clinical specialization, because I just could not attend seminars or discussions of hospital cases and could not read clinical literature. Gradually it became clear to me that I would not become a competent clinical internist.

New York

In September 1971, after eighteen months of clinical training, I left together with my wife and two children for New York, to work for a year with Alan Johnson as 'aspirant' of the Belgian "NFWO". Marc Verstraete arranged this for me. Johnson and Verstraete knew each other from the streptokinase research project. Johnson had successfully developed a number of biochemical tests and was also involved in experimental thrombolysis with laboratory animals and patients. Johnson was a nice guy, he was amiable, pleasant and well mannered, but his laboratory was a mess - mainly due to a lack of continuity in staff and research projects.

I took it for granted that my scholarship from the "NFWO" - exactly $260 per month - would at least cover a part of our subsistence costs in New York. With some panic, I discovered that only the rent of the apartment in Manhattan amounted to $259 per month. It was not far away from the laboratory, but had only one bedroom.

When Alan announced, after the first couple of days, that I would earn $12,000 per year as research assistant from the funds he had available from the *National Institutes of Health* (NIH), I was pleasantly surprised and especially relieved. Two days later even more, when he realized from reading through my curriculum that I also had a Masters degree in Chemistry. That allowed me to be promoted to the level of associate research scientist with a salary of $16,000 per year. The champagne corks flew in all directions when six weeks later I received notice from Belgium that I had been awarded an additional research scholarship of $8,000 by IBM. This scholarship could be combined with financial support from other sources. Financially we were fine and my wife and the two children spent a fantastic year in New York.

Professionally, however, New York was a disappointment. My direct supervisor in the laboratory was a Chinese-American PhD with very little creative thinking and even less persistence. He was more of a burden than a help. The output of one New York year was exceptionally meager: 60 to 80 hours work per week produced only one paper on the conversion of a degrated form of plasminogen. The paper would be published only two years later in a journal with low impact factor[27]. Johnson was so kind to allow me to use all my New York data for my doctoral thesis in Leuven.

[27] Collen D, Ong EB, Johnson AJ. Human plasminogen: in vitro and in vivo evidence for the biological integrity of NH2-terminal glutamic acid plasminogen. Thromb Res. 1975; 7: 515-29.

To conclude my New York experience, one more personal anecdote. Married as a student in July 1966, my family was supported until my departure for New York by a 'subsidy' from my father until I completed my studies as physician in 1968. From then on I was supported by a scholarship from the "NFWO". Moreover, my wife did the bookkeeping of her father's business until 1971.

At the time of our departure for New York our family fortune accrued to approximately 185,000 BEF, which I changed into American Travelers Checks for the amount of $3,700 (approximately 50 BEF for a $). At the end of 1971 the value of the dollar depreciated further to 40 BEF per $, because the Americans abandoned the gold standard. I was then left with about $3,000 in American Travelers Checks, which meant a loss of 20 percent. A year later, when I had only approximately $1,500 left, the dollar dropped to 30 BEF and I again lost more than 20 percent. It was quite evident to me then that I had not quite mastered the tactics of Wall Street.

Stockholm

My stay in Stockholm (Sweden) in 1972-1973 in the laboratory of Birger Blombäck at the renowned *Karolinska Institutet* was in many respects the opposite of my stay in New York. Birger accepted me in Stockholm on condition that I was able to support myself financially. Finally, this succeeded with a "Navo" travel grant on top of my "NFWO" mandate as 'aspirant'. The research in Stockholm - determining the protein sequence of isolated fragments of the protein fibrinogen - was a wonderful experience. Two good publications followed, which contributed sifnificantly to the elucidation of the protein structure of fibrinogen[28] [29], and I learned advanced protein chemistry.

There I also discovered the downside of a career as head of an academic laboratory, a role that I also envisaged for myself: Birger had to constantly deal with a demanding administration, engage into fights with the bureaucrats of the university and keep the suppliers at bay whose bills remained unpaid. Furthermore, he lived a life consisting of a hectic flight schedule between Stockholm and New York where he also led a laboratory in the *New York Blood Center*. Yet this eccentric boss, when he was present, was particularly inspiring. I learnt the most from him during our almost daily evening visits (together with his

[28] Kudryk BJ, Collen D, Woods KR, Blombäck B. Evidence for localization of polymerization sites in fibrinogen. J Biol Chem. 1974; 249: 3322-5.

[29] Collen D, Kudryk B, Hessel B, Blombäck B. Primary structure of human fibrinogen and fibrin. Isolation and partial characterization of chains of fragment D. J Biol Chem. 1975; 250: 5808-17.

four postdocs and doctoral students) to the pub where we, while drinking a few 'mellanöls' (somewhat better than alcohol-free, but still by far no Stella Artois), learned from his scientific and philosophic escapades. Fortunate for my family life, but still somewhat regrettable for my world view, he was in Stockholm only for about a third of the time, and always at eleven o'clock in the evening a wooden plate with 'Stängt' (closed) appeared on the bar counter.

My wife was less happy with our stay in Stockholm. She was stuck with (in the meantime) three toddlers in Solna, a suburb of Stockholm, where nothing much happened during the day because everybody had gone to work. To be back in Leuven was a true blessing for her.

Back in Leuven

In September 1973, we returned to Leuven. In order to complete my specialization in internal medicine, I still needed to do one final clinical year as assistant. Not really a prospect to look forward to. After all, I had only a minimum of clinical hospital experience. My turns in New York and Stockholm each were counted for a specialization year, although I never saw a single patient, not even remotely so. In addition, I previously missed a number of months of clinical practice due to my Hepatitis B infection. Moreover, due to my personal preference for research, I did not consider my specialization training as internist as a high priority. Instead of the usual four years of experience for a 'senior resident', I had approximately one and a half years of experience, which dated back to three years ago. Almost everybody around me was more experienced. I always, during my limited clinical training, performed my tasks and responsibilities correctly and to the satisfaction of the supervisors. However, I did not really want to take up the position of 'senior resident'. How would I deal with this?

The solution came from applying for a certificate for specialization in clinical chemistry on the basis of my double training in medicine and chemistry. In other words, I exchanged my specialization in internal medicine for one in clinical chemistry. In this way, I could start as clinical biologist in the Laboratory for Bleeding and Vascular Diseases of the Academic Hospitals of the University of Leuven - a position of which I did know something about.

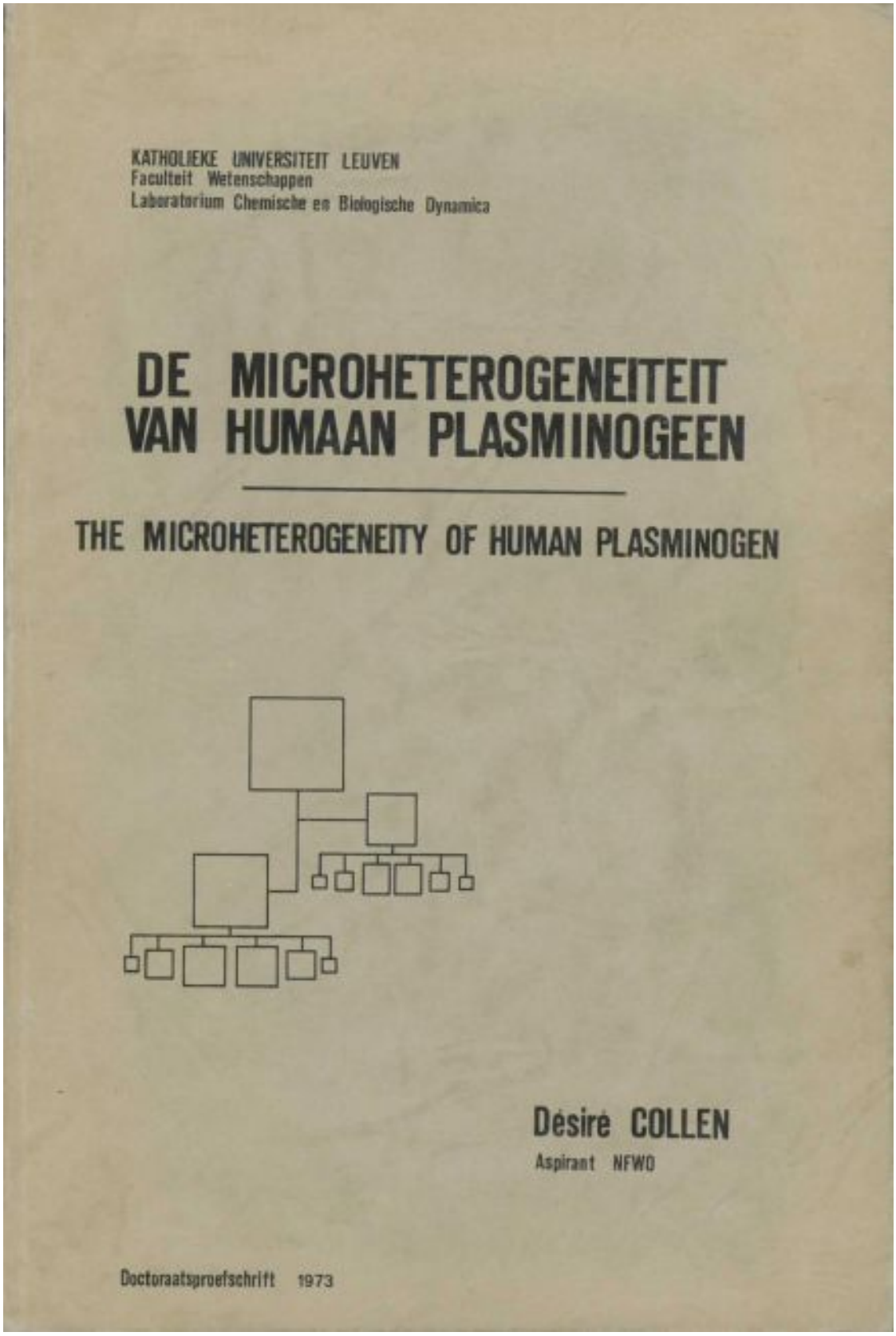
Yet, also this certification was not obvious: I had no formal training whatsoever in microbiology or clinical chemistry. Today, fortunately this would not be possible anymore. Indeed, when I replaced the clinical biologist in a routine clinical laboratory in Brussels in the summer of 1980, it became apparent that I was inadequately qualified for the position.

It was a real deception: with the equivalence of more than twenty years of university training (seven years medicine, five years specialization, seven years sciences (chemistry) and two years licentiate in medical sciences), I was in fact insufficiently qualified to practice medicine outside the university.

Concluded

After returning from Stockholm, I could further concentrate on my doctoral thesis in chemistry, which dealt with the microheterogeneity of human plasminogen, and on my doctoral thesis in medicine on the turnover of plasminogen and prothrombin in humans. I defended the first thesis in January 1974 and the second in July 1974. Although my chemistry thesis did not contain many new elements, it allowed me to introduce a number of biochemical techniques and procedures in the Laboratory for Blood Coagulation, which later turned out to be crucial. My thesis in medicine laid the basis for the discovery of α2-antiplasmin and the investigation that lead to t-PA.

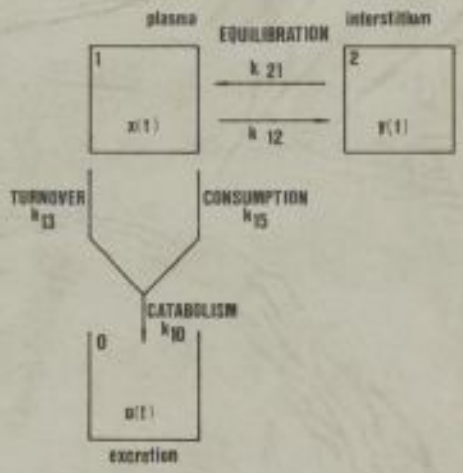
After an additional delay of fifteen months, because of my military service (from September 1974 until November 1975) and a temporary appointment at the University, I finally was appointed as Lecturer (Docent) in the Faculty of Medicine and as Adjunct Head of Clinic in the Section Bleeding and Vascular Diseases in the Department of Internal Medicine. It was exactly fifteen years ago that I first entered a lecture hall of the K.U.Leuven. My training was now completed, and it was time to start an academic career.



Doctoral thesis in chemistry (left) defended in January 1974 and doctoral thesis in medicine (right) defended in July 1974.

UNIVERSITY OF LEUVEN, BELGIUM
FACULTY OF MEDICINE
DEPARTMENT OF MEDICAL RESEARCH
LABORATORY OF BLOOD COAGULATION

PLASMINOGEN AND PROTHROMBIN METABOLISM IN MAN



Désiré COLLEN
Aangesteld navorser N.F.W.O.

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
"GEAGGREGEERDE VAN HET HOGER ONDERWIJS"

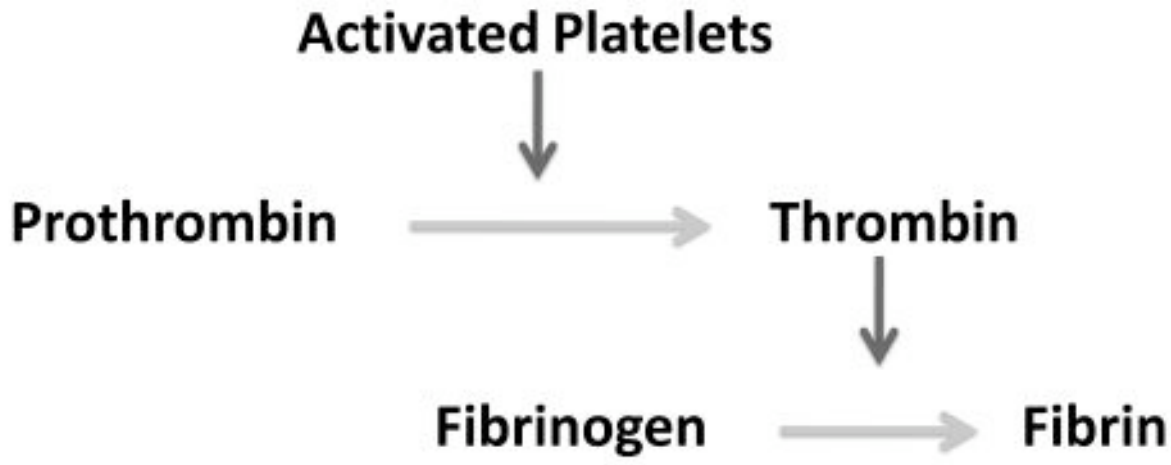
1974
acco
LEUVEN

Molecular mechanism of fibrinolysis elucidated

The blood coagulation system is a good example of how our body maintains a delicate balance between opposing activities. On the one hand, blood must clot as soon as a tissue or a blood vessel is damaged otherwise we would bleed to death at the slightest infliction of a wound. On the other hand, blood clots must also be broken down (fibrinolysis) and cleared to allow growth of new tissues. For both processes, coagulation and fibrinolysis, the body has developed a molecular system, comprising many controls and balances. When the equilibrium is broken illness is the consequence.

Life and death of a blood clot

The most important participants in blood coagulation are the blood platelets and the proteinsthrombin and fibrin. If a blood vessel is damaged, blood platelets accumulate at the site of the injury and a temporary plug is formed. Simultaneously, the blood platelets release signal compounds resulting in conversion of the inactive protein prothrombin into thrombin. Thrombin in turn transforms the protein fibrinogen into fibrin. These fibrin proteins clump together locally to form a fine network consisting of long fibrils, which completely seal the damaged blood vessel. These blood platelets and fibrin strands are the main components of a blood clot.



The mechanism of clotting, as proposed here, is an oversimplification of the real process. In fact, more than a dozen different proteins are involved in the regulation of blood coagulation, whereby one protein activates or inhibits the other. The first participants in this complex network were already described by the end of the 19th century, among others by

Rudolf Virchow. Most of the other proteins were known before 1970[30].

A variant of the 'normal' blood coagulation is thrombosis (see the chapter 't-PA and thrombolysis, the context of a life's story'). Here, essentially the same molecular participants are involved: blood platelets, thrombin and fibrinogen; this time they do not react to a trauma but to an atherosclerotic plaque in a coronary artery or to a hypercoagulability (thrombophilia) in a deep vein, i.e. deep vein thrombosis. Oversimplified, thrombosis could be considered an unwanted by-product of physiological blood coagulation.

The lytic trio: plasminogen activator, plasminogen and fibrin

It was described for the first time in the 18th and 19th century that the blood of mammals contains a protein system to dissolve blood clots. The proteins involved were largely identified between 1930 and 1950[31]. In this search the protein streptokinase, produced by streptococci, played an important role.

Already in 1933, it was discovered that some streptococci strains were capable of dissolving blood clots[32]. It seems to be a peculiar twist of nature that bacteria are capable of dissolving human blood clots, but at closer look, it is an adaptation of the bacteria against our defense systems. In local bacterial infections, our body indeed reacts by clotting the blood around the core of the infection. In that way, the in- and outflow routes are shut off, the bacteria are trapped and confined locally so that they cannot infect other tissue areas. Hemolytic streptococci have developed the protein streptokinase as a remedy: the protein activates our own fibrinolytic system so that the sealing network of blood clots is dissolved and the bacteria can migrate.

Streptokinase was a useful tool to researchers in the middle of the 20th century to identify the other proteins involved in fibrinolysis. The researchers simply had to discover with which proteins streptokinase reacts. In this manner, plasminogen was discovered in 1944 and 1945 together with its active derivative, plasmin.[33] [34] [35]

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[30] Collen D. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost. 1980; 43: 77-89. Review.

[31] Collen D. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost. 1980; 43: 77-89. Review.

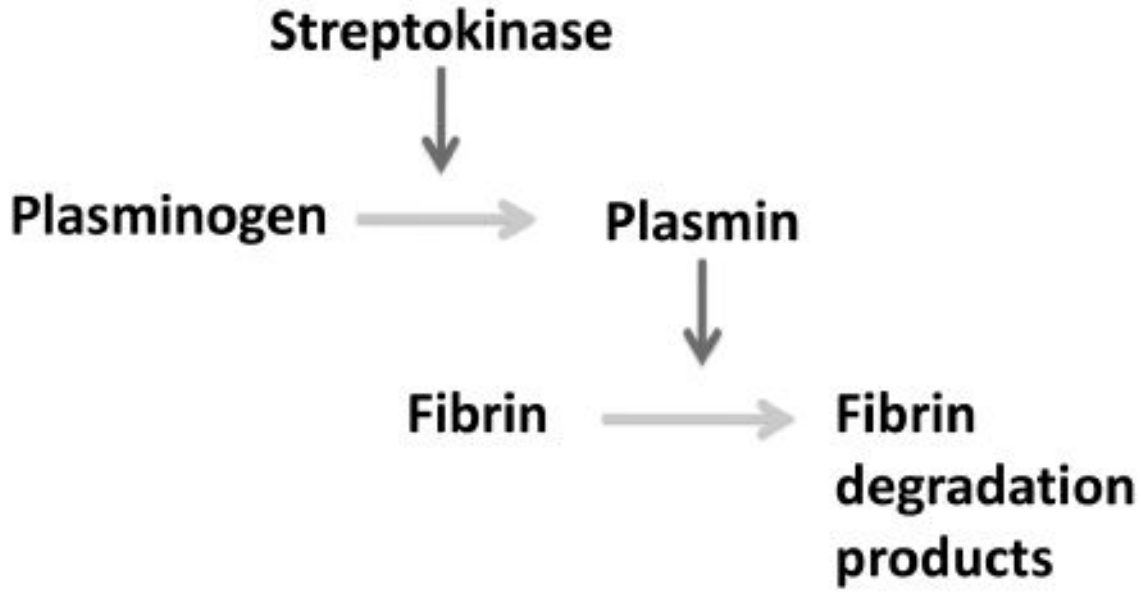
[32] Tillett WS, Garner RL. Fibrinolytic activity of haemolytic streptococci. J Exper Med. 1933; 58: 485-502.

[33] Milstone H. Factor in normal blood which participates in streptococcal fibrinolysis. J Immunol. 1941; 42: 109-16.

[34] Kaplan MH., Nature and role of the lytic factor in haemolytic streptococcal fibrinolysis. Proc Soc Exper Biol Med. 1944; 57: 40-3.

[35] Christensen LR, Macleod CM. Proteolytic enzyme of serum: characterization, activation, and reaction with inhibitors. J Gen Physiol. 1945; 28: 559-83.

Plasmin is a protein that degrades fibrin strands. Plasmin itself is derived from plasminogen, a precursor protein, which circulates in a free form in the blood, just like fibrinogen. The conversion of plasminogen into plasmin by streptokinase involves a proteolytic cleavage: streptokinase bound to plasminogen cuts a piece of another plasminogen molecule, which is thereby activated.



In 1947 Tage Astrup and Per Permin were the first to describe a protein existing in animal tissue with characteristics closely resembling those of streptokinase[36]. It would take another five years before Astrup would isolate the protein[37], although never in a completely pure form. Initially, it was given the name fibrinokinase, but it was later modified to tissue plasminogen activator.

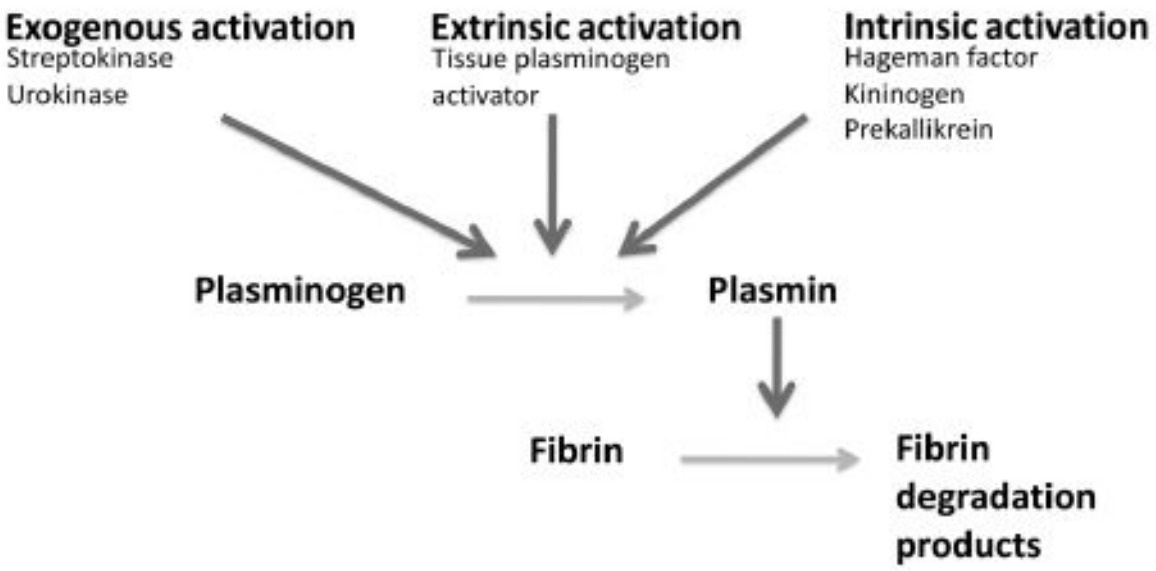
In 1947, it was discovered that urine also contains substances capable of dissolving blood clots[38]. It concerns another plasminogen activator, namely the protein urokinase.[39] Finally, a third activating pathway was identified in the period from 1950 to 1960, besides the exogenous activation (via urokinase or streptokinase) or the extrinsic activation (via tissue plasminogen activator): the intrinsic pathway.This involves the contact of plasminogen with a series of proteins - among others, Hageman factor, kininogen and prekallikrein. A detailed overview of these reaction chains would take us too far.

[36] Astrup T, Permin PM. Fibrinolysis in the animal organism. Nature. 1947; 159: 681-682.

[37] Astrup T, Stage A. Isolation of a soluble fibrinolytic activator from animal tissue. Nature. 1952; 170: 929.

[38] Macfarlane RG, Pilling J. Fibrinolytic activity of normal urine. Nature. 1947; 159: 779.

[39] Williams JRB. The fibrinolytic activity of urine. Brit J Exper Pathol. 1951; 32: 530-37.



An unknown inhibitor

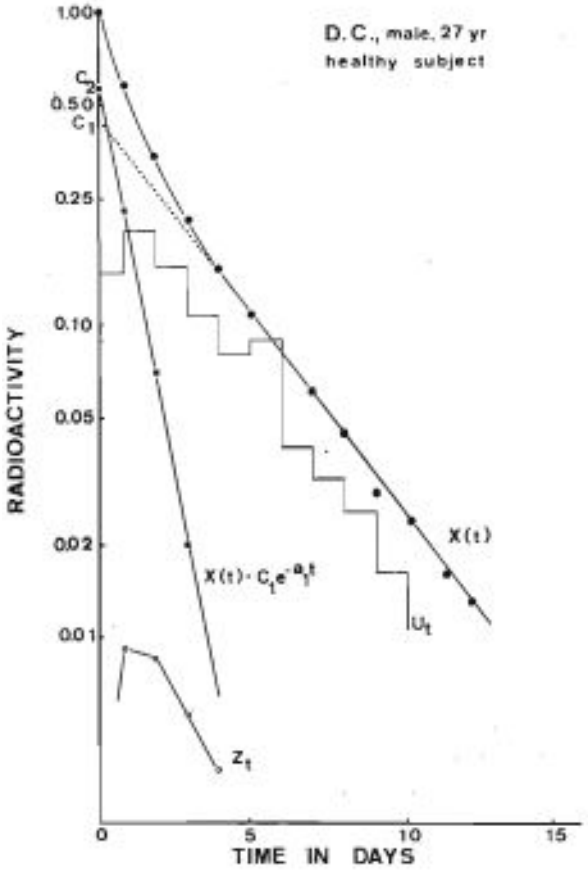
When I started researching fibrinolysis, much was already known about the activation of plasmin. Much less was known about the neutralization of plasmin and the turnover of plasminogen. It is clear that plasmin cannot remain in its active form. If that would be the case, then each blood clot in the body would be dissolved too rapidly. In a first phase, I therefore focused my research on the turnover of plasminogen and the contribution of the conversion into plasmin.

In a first experiment from 1970, published in 1972, we studied the rate at with which our body cleared plasminogen and plasmin[40]. Homogeneous plasminogen - purified in collaboration with Bjorn Wiman - was labeled with radioactive iodine and injected into volunteers. Subsequently, we traced how fast the radioactivity - and thus also the plasminogen - disappeared from the plasma and appeared as breakdown products in the urine.

In a 'healthy' masculine volunteer of 27 with initials 'D. C.', half of the radioactivity appeared to have disappeared from the blood after 2.5 days. In those days, researchers quite often acted as their own laboratory animals. Today a similar experiment would first have to be

[40] Collen D, Tytgat G, Claeys H, Verstraete M, Wallén P. Metabolism of plasminogen in healthy subjects: effect of tranexamic acid. J Clin Invest. 1972; 51: 1310-8.

approved by a multidisciplinary ethical committee.



The metabolism of plasminogen in a test person ('D.C.') : x(t) = radioactivity in plasma; u_t = fractional daily excretion of the radioactive marker in urine; z_t = radioactivity in plasma, which is not precipitable with trichloroacetic acid (From Collen D., Tytgat G., Claeys H., Verstraete M., Wallén P. Metabolism of plasminogen in healthy subjects: effect of tranexamic acid. J Clin Invest. 1972; 51: 1310-8).

Sometimes we went a little too far with the experiments on our own bodies. The moment I arrived in New York for my stay with Alan Johnson, I had to undergo a medical examination. When the doctor saw my arms, he raised his eyebrows and asked all sorts of suggestive questions. The doctor took it for granted that I was (had been) a junkie. It took me a while to explain him where the prick marks actually came from.

Those were, however, the kind of experiments that enabled us to obtain a better insight into the function and mechanisms of the proteins involved in blood coagulation and fibrinolysis. Nowadays, we have at our disposal for similar kinds of experiments computational models, cell cultures, specific animal models - whether or not transgenic or knockout - from worms via flies and zebra fishes to mice. Previously, we had only our own bodies.

Discovery of α2 antiplasmin

When Jos Vermylen of the K.U.Leuven and I together determined the turnover of labeled plasminogen in thrombosis patients that were being treated with streptokinase, we initially were puzzled[41]: on the one hand, streptokinase quickly converted free circulating plasminogen into plasmin, but in turn this was neutralized very rapidly. On the other hand, the radioactivity disappeared only slowly (with a half-life of approximately 12 hours) from the blood. We concluded that plasmin is inactivated by binding to another protein, rather than by clearance of free plasmin from the blood, which would have been a much slower process.

We thus had to isolate the complex of plasmin with the unknown inhibitor. Actually, everyone in the research field accepted that the inhibiting protein must be α1-antitrypsin or α2-macroglobulin, because it was suspected that these proteins were involved in the neutralization of plasmin. We purified a protein complex from the blood that, at least in its size, was compatible with a complex plasmin-α1-antitrypsin.

Later on it turned out that the complex only reacted with antisera against plasminogen, but not with antisera against α1-antitrypsin or any other known inhibitor of plasmin. We were dealing with a new protein. We knew its function but we still had to isolate the protein itself. The protein was named α2-antiplasmin[42] and we would be able to isolate it soon after.

The accidental discovery of α2-antiplasmin in 1973 would be followed by a series of systematic studies on the protein, mainly regarding its biochemical and kinetic properties. Bjorn Wiman was our most important partner for these studies. Bjorn was working in Stockholm and joined our Leuven laboratory for one year (see also chapter ‘A difficult protein’ in part II ‘the t-PA story). Later, Roger Lijnen joined us as a post-doctoral fellow and continued this research. We also initiated a collaboration with Willem Holmes from San Francisco, when he was preparing his PhD thesis in Leuven.

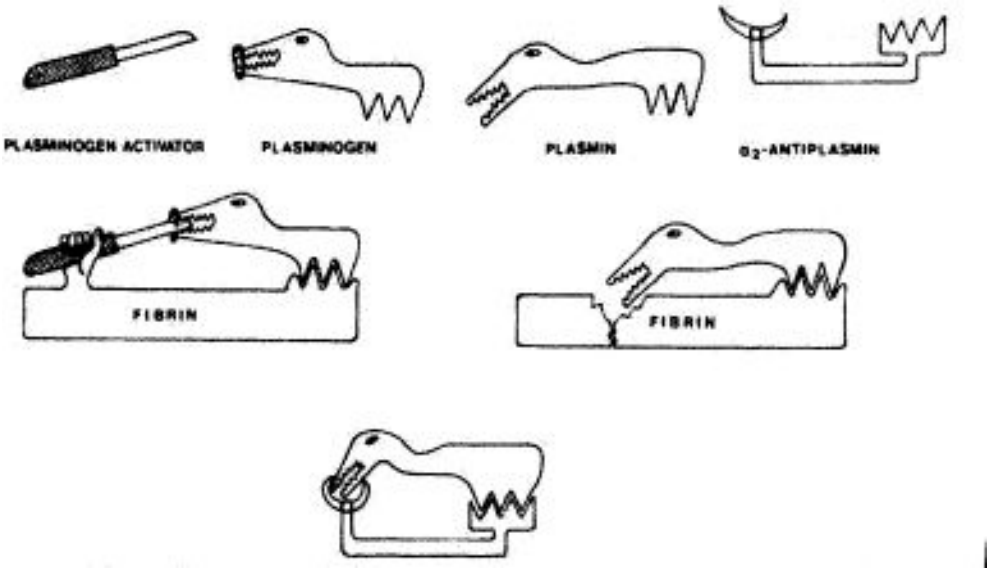
In 1978 all these experiments resulted in the following functional model[43]: α2-antiplasmin is a very fast inhibitor of active plasmin circulating freely in the plasma. However, when the 'lysine-binding sites' of plasmin are occupied - these are the structures by which plasmin binds to fibrin and are indicated in the figure by the little feet - the inhibitory action of α2-

[41] Collen D, Vermylen J. Metabolism of iodine-labeled plasminogen during streptokinase and reptilase therapy in man. Thromb Res. 1973; 2: 239-50.

[42] Collen D. Identification and some properties of a new fast-reacting plasmin inhibitor in human plasma. Eur J Biochem. 1976; 69: 209-16.

[43] Wiman B, Collen D. Molecular mechanism of physiological fibrinolysis. Nature. 1978; 272: 549-50.

antiplasmin is much slower.



Schematic representation of the molecular interactions which regulate the degradation of fibrin. Plasminogen activator transforms inactive ('muzzled') plasminogen into the proteolytic active enzyme plasmin. This conversion can only take place in an efficient way if both proteins are bound to fibrin in the blood clot. Free plasmin in the blood is rapidly inactivated by α2-antiplas*min. Plasmin bound to the fibrin surface is partially protected against inactivation. From Collen D. Regulation of fibrinolysis: plasminogen activator as a thrombolytic agent, in 'Pathobiology of the endothelial Cell'. Eds. H.L. Nossel and H.J. Vogel. Academic Press, New York, 1982, p.183-189.*

There is a clear biological rationale for that mechanism: free circulating plasmin is a 'danger' to our body: it not only cleaves fibrin, but it is also capable of cleaving a variety of other proteins. Active plasmin circulating freely in the blood must be neutralized as soon as possible. Plasmin should only remain active when bound to a blood clot, in other words, bound to fibrin. A system in perfect balance.

The principle of the differential neutralization of active plasmin, depending on whether it is bound to a blood clot or freely present in the plasma, will in the years to come remain a focus of our research. It will also be a milestone in the discovery of t-PA as thrombolytic agent. Indeed, fibrin binding is crucial also in the activation of plasminogen to plasmin.

The discovery of α2-antiplasmin was undoubtly a first important breakthrough for our laboratory. It resulted in invitations to give important lectures, to write reviews and to mix with the 'hot shots' in the area of cardiology[44] [45] [46].

[44] Collen D, Wiman B. Fast-acting plasmin inhibitor in human plasma. Blood. 1978; 51: 563-9. Review.

[45] Collen D, Wiman B. The fast-acting plasmin inhibitor of human plasma: The Prix Servier Lecture 1978. In: 'Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 4'. Ed.: J.F. Davidson. Churchill Livingstone, Edinburg, 1979; 11-19.

[46] Collen D. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost. 1980; 43: 77-89. Review.

Future entrepreneur?

Following the discovery of α2-antiplasmin, I also applied for my first patent (see Appendix 1). The patent was entitled 'Thrombosis test'. The first version was submitted in the Netherlands with 19 September 1975 as the priority date. The patent was submitted in the US on 13 September 1976 and was adapted a couple of times.

The idea was to protect the commercial exploitation of α2-antiplasmin. The patent actually covered more than α2-antiplasmin. In addition, the use in a thrombosis test of other specific enzyme inhibitor-complexes in blood plasma, including plasmin-antiplasmin, plasmin-α2-macroglobulin and thrombin-antithrombin III, was protected by the patent application.

I was convinced that these enzyme complexes were suitable biomarkers to monitor activation of the fibrinolytic system in blood. It could show whether a person has a thrombosis, or rather, had one. The idea of the biomarkers was conceptually confirmed by subsequent research in collaboration with Ed Plow of the *Scripps Institute* in La Jolla (US)[47]. In principle, the test worked, but one had to take into account that no monoclonal antibodies were available at that time. The antisera that we used consisted of polyclonal mixtures of antibodies obtained from rabbits that were immunized with plasmin-antiplasmin complex. The mixtures not only contained antibodies against the complex, but also against plasmin and antiplasmin itself. The trick was 'to purify' the antisera first by absorbing them successively with plasmin and antiplasmin. The hope was that there were still antibodies left, which were exclusively directed against the complex plasmin-antiplasmin. In principle, it succeeded, apart from the fact that this method did not produce antisera suitable to be used in a repoducible commercial diagnostic test. Scientists from Organon, who obtained a license of the patent, reached the same conclusion. The project was not further developed and the patent would not yield more than a reasonable research funding for a couple of years.

Under contract with LR&D

At that time, an agreement was concluded with the K.U.Leuven concerning the valorization of research. There were many questions concerning the submission of patents: in case this research project would lead to commercial applications, who would be the owner, how would a possible financial 'return' be divided, who could claim which part of the cake, et cetera.

[47] Plow EF, de Cock F, Collen D. Immunochemical characterization of the plasmin-antiplasmin system. Basis for the specific detection of the plasmin-antiplasmin complex by latex agglutination assays. J Lab Clin Med. 1979; 93: 199-209.

The university was aware of these problems and on 18 January 1973 established a technology transfer office for the valorization of research results by scientists of the K.U.Leuven. That organization was named *Leuven Research and Development* (non-profit organization), shortened to LR&D.

Early February 1976, I reached an agreement with LR&D (see Appendix 2). This agreement explicitly mentioned: 'Dr. D. Collen and his colleagues, employed by the Catholic University of Leuven, transfer all juridical, commercial and financial privileges, as well as the execution thereof, of research results, which they accomplish directly or indirectly in the framework of their teaching and research assignments at the Catholic University Leuven, in favor of the non-profit organization *Leuven Research and Development.*

Royalty income generated by LR&D, based on our research results, would be divided on the following basis: the K.U.Leuven received 10%, LR&D 7%, and after the deduction of all expenses in the execution of its mandate, LR&D reserved a minimum of 50% of the remaining part for continued research in our laboratory. The remaining part could be used as personal compensation to the inventors and/or colleagues. However, the document concludes with: 'the concrete distributing modalities shall be determined at a suitable time after mutual deliberation between LR&D and Dr. D. Collen for submission to the Management Board of LR&D for approval'. The Management Board of LR&D can at all times adapt its viewpoint in this regard to the circumstances.'

That document was signed on 9 February 1976 by Guido Declercq, then the General Manager of K.U.Leuven and Jos Bouckaert, the Director of LR&D. I signed the agreement on 11 February 1976 with the approval of my Head of Department, Marc Verstraete.

Farmer-entrepreneur

My first activities as an entrepreneur were not so devoted to providing licenses of research results and inventions to companies, but to the production and supply of reagents for immunological tests. In fact, this is a funny but at the same time slightly embarrassing story.

After my agreement with LR&D and after the first patent on the thrombosis test, Jos Bouckaert stimulated my enthusiasm for patents. In the following months, more patent applications followed without any decent return. Instead of financial returns to our laboratory, I was faced with an enourmous amount of patent costs with LR&D. I had to find a way to settle my account with LR&D. Therefore, I became a producer of antisera.

In fact, a euphemism for the 'farmer activity' that we developed mainly in my garden in Winksele. I was no stranger to farm life, because I spent part of my childhood in the Limburg countryside in Sint Truiden. I used to stay with my grandmother on my mother's side. She became a widow early on and had to struggle together with her five children with the produce of four milk cows, a dozen chickens and a cherry orchard. Now and then a pig was slaughtered for own use. We were never hungry, but a bathroom or central heating was not available. The toilet was a separate construction in the backyard, the yield of which found its way back to fertilize the vegetable garden. During winter evenings, we gathered round the 'Leuvense Stoof' (a typical Flemish pit coal stove) and went to bed with a hot-water bottle.

But let us return to the production of antisera. The family home that my wife and I bought in Winksele - near Leuven - in 1975, had a garden that quickly expanded into a parcel of meadowland, which we purchased from the church council. The small garden was quickly transformed into a rabbit hutch. In our laboratory we were short of specific antisera for the isolation of proteins active in blood coagulation and fibrinolysis. Nowadays these antibodies and antisera are available from specialized companies, or if need be, you have them produced by the supporting services of the university. To provide us with the necessary antisera, we started a colony of rabbits in the garden shade. They could serve as antibody producers. We injected the rabbits with the proteins against which we wanted to produce antibodies and then some weeks later we drained the blood. What worked for my own laboratory, could be translated on a commercial basis. I started delivering the antisera to a supplier of antibodies in the Benelux. The benefits were sufficient to settle our account with LR&D and to cover research related expenses including representation costs.

Rabbits, however, are small animals with relatively little blood, and as such they produce relatively small quantities of antisera for commercial application. Therefore, some goats were bought for the same purpose and later even a pony. In addition, colleagues from other research departments occasionally made use of the 'Winksele production facilities'. The practical management of this facility was mostly managed by my wife and children (the pony also served for horseback riding). My family cared for all the quadrupeds, which supplied a section of the biomedical research at the K.U.Leuven with essential antibodies. One of our first and most faithful 'customers' was Alfons Billiau of the Rega Institute. He tells of his first-hand experiences in Winksele[48]: "My research at the Rega Institute was focused on human interferon. At one point we wished to prepare a potent and specific anti-

[48] Billiau A. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 17-9.

interferon antiserum that we might need for characterization studies and for affinity chromatography. We wanted to immunize goats, so as to be able to obtain sizable quantities of serum. However, large animals were not available in our University's Experimental Animal Unit, where they only produced antisera using small animals. The Director advised us to contact Désiré Collen who, faced with a similar problem had elaborated a private solution. In fact, it appeared that Mrs. Louisa Collen had already become an expert and loving goat keeper, and we found her kind enough to keep two more animals for us, one to be immunized with partially pure interferon-β, the other with interferon-β that we believed was completely pure. Professor Jo Van Damme, then a PhD student, still nostalgically remembers chasing the goats in the green and hilly landscape of Winksele trying to catch goats and draining blood. Both antisera would become critical reagents in the successful cloning, in 1980-1981, of interferon-β by a consortium of researchers from our Rega Institute, the Pasteur Institute of Brussels and the University of Ghent. More importantly, the difference between the two sera was to become a critical asset in the cloning of a protein co-produced with interferon and recognized by the first but not the second antiserum. This protein would turn out to be nothing less than interleukin-6."

Later Alfons Billiau would play an important role in my t-PA story. I could always count on him, especially in times when my relationship with other members of the Rega Institute had gone sour. Alfons became a dear friend, and, still is. Together with Jos Vermylen, he belongs to a small, separate class of real 'gentlemen'. I was fortunate to work with them at the K.U.Leuven.

Section II.
The t-PA story

A difficult protein

Bjorn Wiman took the first major step in the t-PA research in Leuven. Until recently he conducted his research at the *Karolinska Institutet* in Stockholm (Sweden). My collaborations with Bjorn focused specifically on the purification and the biochemical characteristics of antiplasmin and the reaction of antiplasmin with plasmin (see Section I). Bjorn was visiting postdoc in the 'new' laboratory of Marc Verstraete on Gasthuisberg from August 1977 until September 1978.

Before his residency in Leuven, Bjorn did research on the protein tissue plasminogen activator, or short t-PA. The Danish researcher Tage Astrup and his colleagues were the first to isolate the protein as early as 1952[49]. It was known that t-PA converts non-active plasminogen into active plasmin, which in turn cleaves fibrin, the main blood clot component. Upon cleavage of the long fibrin strands, the clot falls apart and is dissolved. In other words, t-PA does not directly dissolve blood clots, but rather triggers a dissolving mechanism.

Fibrin specific

In the summer of 1975, Bjorn Wiman, together with Per Wallen in the Swedish Umea°, started the purification of t-PA from the heart muscle of pigs[50]. “I tried to find a way to measure the activity of t-PA", Wiman recollects[51]. “Just like it was at that time usual for urokinase, also a plasminogen activator, I was looking for, at first, a test based on degradation of casein, the most important protein in milk”.

t-PA itself is a 'protease' or a 'protein cutter'. In other words, t-PA activates plasminogen by cutting off a piece. The intention of the test was to measure the extent to which caseins are cleaved by the formed plasmin. Wiman: "Unfortunately, we did not obtain any reaction. That was strange, since the same sample of t-PA was quite active in the dissolution of blood clots via the activation of plasminogen. I then remembered that the colleagues of Astrup at some stage suggested that t-PA had an affinity for fibrin. They could not really prove this statement then, because they did not have any purified t-PA.

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49 Astrup T and Stage A. Isolation of a soluble fibrinolytic activator from animal tissue. Nature. 1952;170: 929.

50 Wallén P, Wiman B. Proceedings: Purification of tissue activator using affinity adsorption on fibrin and hydrophobic interaction chromatography. Effect of fibrin on the enzymatic properties of the activator. Thromb Diath Haemorrh. 1975; 34: 609.

51 As informed orally, Wiman B. ‘The t-PA story as told by Bjorn Wiman’ on occasion of ‘Heart for the Future’, 6 October 2008 and Wiman B. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 125-6.

That is why I added some washed fibrin to the casein solution, and immediately the milk protein was broken down. It was clear: t-PA must first be bound to the surface of fibrin before it can effectively activate plasminogen."

The rolling snowball

When Bjorn arrived in Leuven, we were concentrating mostly on antiplasmin. He convinced me, however, of the fibrin specificity of t-PA. That turned out to be a revelation. This new insight made me realize the potential of t-PA. It was already evident at that moment that the low specificity of streptokinase could become a problem in its further use as thrombolytic agent. When streptokinase is injected into the bloodstream of heart patients to dissolve a blood clot in a coronary blood vessel, it simultaneously triggers the activation of plasminogen elsewhere in the body. This process seriously disturbes the blood coagulation system. If, on the other hand, t-PA would be active only when bound to the fibrin strands, it would make out a much more specific thrombolytic drug.

Bjorn Wiman deserves a lot of credit for his contribution to the t-PA story. Although he minimalizes his own contribution as follows: “Coming from the North, I rather would like to compare the t-PA story with a snowball and what I did was to convince Désiré about the remarkable specificity of t-PA, I made a little snowball. Desiré started rolling the snowball and he directed its motion so that it got bigger and bigger and bigger.”

A drug against cancer?

The second milestone in my t-PA story came from the United States and the Rega Institute in Leuven. Around 1975, Ed Reich and his colleagues from the *Rockefeller University* in New York established a correlation between the malignant phenotype of tumor cells and the secretion of plasminogen activators. [52]

[52] Ossowski L, Biegel D, Reich E. Mammary plasminogen activator: correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. Cell. 1979; 16: 929-40.

Since 1976, I had been examining the effect of plasma on the fibrinolytic activity of malignant cells[53] together with Alfons Billiau of the Rega Institute. We discovered that plasma suppressed the capacity of tumor cells to dissolve blood clots and that the presence of α2-antiplasmin played an important role. From these findings, we advanced the rather presumptuous hypothesis that, for the development of specific inhibitors against proteases in tumors, one must use the primary structure of α2-antiplasmin as a lead point. We hoped that these inhibitors would have anti-tumoral effects and that we would be able to identify new drugs against cancer. Looking back, it was rather a naive way of thinking.

In order to proceed with those investigations, we needed a source of such proteases. I explored different possibilities to get hold of suitable cell lines or culture media and eventually ended up with Grant Barlow of *Abbott Laboratories* in Chicago (US). He sent us a culture medium of a human melanoma cell line. Grant Barlow got the cell line directly from Ed Reich's laboratory. The cell line originated from a lung metastasis of a certain Mrs. Bowes, who already passed away in 1974 of the consequences of her melanoma (a particularly malignant type of skin cancer).

We soon realised that we would need much larger quantities of the medium for our experiments, than Barlow could provide. For that reason, we had to cultivate the cell line ourselves. Towards the end of 1978, I obtained it via Dan Rifkin of the *New York University Medical Center*.

Alfons Billiau of the Rega Institute still remembers the day when I entered his office with the Bowes cells[54]: "One winter's day, It must have been winter time when one day, out of the blue, Désiré stepped into my office, handing me a Falcon flask: 'Could I take care of keeping and culturing a melanoma cell line'. He explained that the line was called 'Bowes' and he also gave me an initiation course on thrombolysis and a background on plasminogen activators. For our lab it was easy to grow the cell line and to produce enough growth medium and deliver it to Désiré. At the Rega we had a pilot system for the large scale culture of fibroblasts. We used those fibroblasts to produce interferon. As usual Désiré did not like half work; he immediately recruited a technician and he let her scale-up the culture of the Bowes-cell line at the Rega Institute. "

[53] Collen D, Billiau A, Edy J., De Somer P. Identification of human-plasma protein which inhibits fibrinolysis associated with malignant cells. Biochim Biophys Acta. 1977; 499: 194-201.

[54] As informed orally, Billiau A. 'The t-PA story as told by Alfons Billiau' on occasion of 'Heart for the Future', 6 October 2008 and Billiau A. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008; 17-19.

Production champion

It was with this culture medium that we showed in February 1979 that the plasminogen activator, produced by the Bowes cell line, only became active when bound to fibrin and that we probably were dealing with t-PA (see also the story's beginning in the introductory chapter). The fact that we chose the Bowes cell line was quite a fortunate coincidence. Only later on it became clear that most of the cancer cell lines produce a urokinase-like plasminogen activator that does not bind to fibrin. Until this very day, as far as I know, the Bowes cell line is still the best production machine for t-PA - made by nature.

Because it produced so much plasminogen activator, the Bowes cell line was already cultured in numerous laboratories before it came to Leuven. In spite of all those years of research, nobody identified the real plasminogen activator, even though Lynn Wilson and Eugene Dowdle did publish in 1980 that some tumors in culture produced a non-urokinase-like plasminogen activator[55]. Nobody had the idea to develop this plasminogen activator as a thrombolytic agent. Once more a lucky coincidence in my t-PA story.

Run out of luck

Soon after Alfons Billiau started the large-scale culture of the Bowes cell line, it appeared as if we had run out of luck. In order to purify the t-PA from the culture medium of the Bowes cell line, we used Bjorn Wiman's protocol. By chromatography on fibrin-Sepharose, lysine-Sepharose, arginine-Sepharose and butyl-Sepharose we could show that the plasminogen activator from the Bowes cell line behaved in the exact same manner as natural t-PA. Moreover we lost, as was described, a large amount of protein during the purification process through adsorption on glass, gels, dialysis membranes, ultrafilters etc. That confronted us with huge problems. Although we were making some progress, we still had not succeeded in the summer of 1979 to obtain a homogeneous product. We were stuck.

[55] Wilson EL, Becker ML, Hoal EG, Dowdle EB. Molecular species of plasminogen activators secreted by normal and neoplastic human cells. Cancer Res. 1980; 40: 933-8.

Assumptions become facts

Rescuer from the Netherlands

To purify t-PA remained a major challenge. The protein was sticking to every surface and we did not succeed in its purification to homogeneity. The solution for our problem would come from the Netherlands. In September 1979, Dingeman (Dick) Rijken started this three year postdoctorate in Leuven. He just completed his PhD at the *Gaubius Institute* of TNO in Leiden and succeeded in isolating one milligram of pure t-PA from five kilograms of human uterus tissue[56]. After years of struggle Dick solved the problem concerning the adsorption loss by using the detergent Tween80. In Leuven, Dick applied a simplified version of his protocol. Using Tween80, it only took him two months to isolate pure and homogeneous t-PA from the culture medium of the Bowes cell line[57].

"At first Désiré did not ask me to isolate t-PA for him", Dick Rijken says[58]. "Pieter Brakman, my supervisor in Leiden at that time, and Marc Verstraete, Désiré's boss, had arranged my stay in Leuven. I was pleased to go and work for a "science star" like Désiré. Désiré and Björn Wiman received the "Prix Servier" at the Karlovy Vary-meeting in 1978 for their research on α2-antiplasmin. However, I was a little bit disappointed in Désiré when he did not show up at my presentation on the biochemical and immunological characterization of t-PA during that meeting. He probably had other, more important concerns."

While finishing his PhD, Dick Rijken was actually doubting whether he would continue his t-PA research during his postdoc. He believed that all relevant information on t-PA was already described in 1978-79. Actually, nobody could foresee that thousands of papers on t-PA remained to be published in the years to come. Dick Rijken visited me for the first time

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56 Rijken DC, Wijngaards G, Zaal-de Jong M, Welbergen J. Purification and partial characterization of plasminogen activator from human uterine tissue. Biochim Biophys Acta. 1979; 580: 140-53.

57 Rijken DC and Collen D. Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. J Biol Chem. 1981; 256: 7035-41.

58 As informed orally, Rijken D. 'The t-PA story as told by Dingeman Rijken' on occasion of 'Heart for the Future', 6 oktober 2008 and Rijken D. An anthology of scientific collaboration compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008:91-3.

in September 1978. It was a Saturday morning. “I presented the results of my research from Leiden and we talked about what I could do during my stay in Leuven. We agreed that I would come to Leuven after my PhD defence. The plan was to study the kinetics of the activation of plasminogen by t-PA” said Rijken.

“Désiré changed these plans though. In the summer of 1979, some weeks before my arrival in Leuven, I met Désiré in London during the ISTH (International Society on Thrombosis and Haemostasis) congress. Désiré told me about the melanoma cell culture, producing at least 500 units t-PA per ml. But there were some problems with the purification. Could I solve these issues as soon as I arrived in Leuven?

Eventually it turned out that one milliliter of medium contained only 20 units instead of the promised 500 units t-PA, still a huge amount compared with other t-PA sources. We managed to purify one milligram of t-PA from 10 liters of conditioned medium and that was a huge success in comparison to one milligram out of 5 kilogram uterus tissue during my Leiden-period.”

Based on Dick Rijken's original protocol, which we would further simplify later on, my colleagues at Gasthuisberg isolated a total of two to three grams of pure t-PA from the Bowes cell line culture medium[59]. This was sufficient to fully elucidate the biochemical, biological and thrombolytic characteristics of t-PA. This research was conducted in collaboration with Roger Lijnen, Marc Hoylaerts, Irène Juhan-Vague and Christian Korninger. Numerous other collaborations were established and the first clinical trials were performed. This paved the way to turn recombinant t-PA into a successful drug.

A short sprint

At this point the research progressed at tremendous speed. Osamu Matsuo from Myasaki (Japan) joined our research group in September 1979 for a one year period. He examined the specific thrombolytic activity of t-PA in plasma[60]. More important is the fact that he succeeded in testing the purified t-PA in an animal model: rabbits with an experimental

[59] Collen D, Rijken DC, Van Damme J, Billiau A. Purification of human tissue-type plasminogen activator in centigram quantities from human melanoma cell culture fluid and its conditioning for use in vivo. Thromb Haemost. 1982; 48: 294-6.

[60] Matsuo O, Rijken DC, Collen D. Comparison of the relative fibrinogenolytic, fibrinolytic and thrombolytic properties of tissue plasminogen activator and urokinase in vitro. Thromb Haemost. 1981; 45: 225-9.

pulmonary embolus[61]. Matsuo worked 24/7. Sometimes I took him home in the evening so that he at least got a decent meal. A sick and exhausted Japanese postdoc would not be productive anyway. For Matsuo these invitations mostly implied a dilemma, because it was always in the middle of an experiment. As a compromise he very regularly took a rabbit home and placed it in my bath-tub. In between dishes or whilst enjoying a glass of *Old Smuggler*, Matsuo would jump up to take blood samples from his test rabbit. In an amazingly short time span, my Japanese friend successfully completed the first convincing pre-clinical studies with t-PA.

At the same time, Marc Hoylaerts studied the kinetics of plasminogen activation by t-PA and elucidated the role of fibrin[62]. His paper, published in the *Journal of Biological Chemistry,* would eventually be cited more than a thousand times.

All of this contributed to the fact that throughout 1980 we were able to obtain an excellent view of the functioning and characteristics of t-PA from the melanoma cell line. Moreover, we established a link between t-PA research and the results of the α2-antiplasmin research, which was equally well on track. In the chapter '*Plasminogen activator as a thrombolytic agent*' of the book, '*Pathobiology of the Endothelial Cell*', I could consequently write the following with conviction:

> *"Extrinsic plasminogen activator has a weak affinity for plasminogen in the absence of fibrin (K_M = 65 μM) but a much higher affinity in the presence of fibrin (K_M between 0.15 and 1.5 μM). This increased affinity appears to be the result of a 'surface assembly' of plasminogen activator and plasminogen on the fibrin surface. In this reaction plasminogen binds to fibrin primarily via specific structures called the 'lysine-binding site'. Thus one way of regulating fibrinolysis is at the level of plasminogen activation localized at the fibrin surface.*
>
> *Plasmin is extremely rapidly inactivated by α2-antiplasmin ($k1 \sim 10^7\ M^{-1}\ sec^{-1}$); the half-life of free plasmin in the blood is therefore estimated to be approximately 0.1 sec. Plasmin with an occupied lysine-binding site is however inactivated 50 times more slowly by α2-antiplasmin. Reversible blocking of the active site of plasmin with*

[61] Matsuo O, Rijken DC, Collen D. Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. Nature. 1981; 291: 590-1.

[62] Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J Biol Chem. 1982; 257: 2912-9.

substrate also markedly reduces the rate of inactivation by α2-antiplasmin. From these findings one can extrapolate that plasmin molecules generated on the fibrin surface, which are bound to fibrin through their lysine-binding sites and involved in fibrin degradation, are protected from rapid inactivation by α2-antiplasmin. Plasmin released from the fibrin surface would, however, be rapidly inactivated by α2-antiplasmin. These interactions are schematically visualized in Fig. 2".

The illustration (see page 37) as drawn by my former co-worker Frans DeCock, became a classic and was shown in many lectures - not only mine. What were only 'assumptions' during our first rudimentary experiment in February 1979, had in one year become a viable case study with a sound theoretical background. It became clear to everyone that we now, more than ever, should promote the concept of a fibrin selective thrombolytic therapy. In the same chapter of that book, I thus also stated:

"The molecular model for the regulation of fibrinolysis described above has important consequences for the development of thrombolytic agents. Indeed, the presently available thrombolytic agents streptokinase and urokinase have no specific affinity for fibrin and therefore activate circulating and fibrin-bound plasminogen relatively indiscriminately. Consequently, plasmin formed in circulating blood will initially be neutralized very rapidly by α2-antiplasmin and be lost for thrombolysis.

Once the inhibitor becomes exhausted, residual plasmin will degrade several plasma proteins (fibrinogen, factor V, factor VIII, etc.) and cause a serious bleeding tendency. This may explain why treatment with streptokinase or urokinase has only a limited efficiency and is associated with serious, sometimes life-threatening side effects.

From this reasoning it appears that specific thrombolysis will be possible only if the activation process of plasminogen can be localized at and confined to the fibrin surface. According to the present concepts, this can only be adequately achieved with the use of an activator that, like the physiological activator, adsorbs to the fibrin surface and becomes active in loco."

A patent and a cont (r) act … with the alleged daughter of a professor

Good advice from the Rector, Pieter De Somer

On 22 May 1980, I gave a lecture at the Rega Institute providing a comprehensive overview and update of the biochemical, biological and thrombolytic properties of t-PA. After the presentation Pieter De Somer, founder of the Rega Institute and at that time the Rector of the K.U.Leuven for 10 years, encouraged me to look for an industrial partner. He was strongly in favour of a patent application for t-PA through *Leuven Research and Development,* LR&D, a non-profit organization, the 'technology transfer office' of the K.U.Leuven.

Pieter De Somer actually also helped me obtaining the first industrial contact. As early as May 1980, I would sign an agreement on confidentiality with the German Bayer AG. The company acquired all information on t-PA (including non-published material) and had a six month deliberation period to decide whether it wanted to proceed with t-PA. The collaboration with Bayer did not really take off. It never felt as if Bayer believed in the potential of t-PA. The collaboration just petered out.

A first patent

On 11 June 1980, LR&D submitted a patent application for t-PA (see Appendix 3). As inventors were listed: Désiré Collen, Dick Rijken and Osamu Matsuo[63]. The first application was submitted in the Netherlands, afterwards the rest of Europe and the US would follow. Of critical importance for my t-PA future was the first submission date of 11 June 1980. One day later I presented a lecture at the *Fifth Congress on Fibrinolysis* in Malmö (Sweden). I was going to disclose the thrombolytic potential of t-PA for the first time. The fate of t-PA was to take a decisive turn, though a rather unexpected one.

[63] The first submission was made in the Netherlands on 11 June 1980 and was included in the US Patent 4,752,603, jun. 21, 1988, Collen et al. Plasminogen activator and pharmaceutical composition having thrombolytic activity.

Too early, too late … or just in time

"So there were rumours back in 1980 about a blood clot dissolving substance called t-PA and Genentech asked me to go to a meeting in Sweden to learn more about it." Remembers Diane Pennica. [64] "I had been at Genentech only one month, I barely knew what cloning was but I was young, excited, I had a passport and I told my boss "Sure, I'll go to Sweden".

"So I arrived in Sweden to check out the meeting a day in advance because I didn't want to be late. I wanted to check out the meeting and I got to the hotel to check in and the hotel clerk said "oh, the meeting of the doctors started today" and I got so upset because I thought I missed the first day of the meeting. Genentech sent me halfway around the world, so I threw my suitcases in my room, I ran to the meeting and I peeked in the room and I saw about thirty guys, sitting around a big table and I was so upset because I thought I had the wrong day. And just as I sat down, Désiré walked up to the podium, and started to talk about t-PA. And I got really excited because he said he had a cell line, he said he had an antibody and he had purified protein and this is exactly what I came here to hear."

A piece of cake

"It was only during a break that, after introducing myself to several of the participants including Désiré, I realized that I'd stumbled into a very exclusive pre-conference meeting restricted to a small elite group of researchers. The actual conference was, indeed, scheduled for the next day.

One of the men told me that they just assumed I was one of the speakers' daughter, otherwise I would have been asked to leave that small pre-conference meeting. Instead, the

64 As informed orally Pennica D. 'The t-PA story as told by Diane Pennica' on occasion of 'Heart for the Future', 6 oktober 2008 and Pennica D. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008:81-3.

scientists invited me to join them for dinner, where I struck up a conversation with Désiré, and explained my desire to clone t-PA.

He told me that it would be very difficult because it was such a huge protein. To be honest, I didn't know much about cloning at that time. I knew Genentech scientists had cloned human insulin and growth hormone, so I didn't see any reason why we couldn't clone t-PA, as well. I told him, “Sure, we can do it!”

With more than 300 attendees, the chance was slim that I would have met any of these leading scientists, let alone Désiré.”

A new industry

Diane Pennica portrayed without doubt the enthusiasm and the voluntarism, which characterized the first days of the brand-new Californian biotech industry. *Genentech* was one of the most important exponents of this industry. Bob Swanson and Herbert Boyer founded Genentech in 1976. Swanson only 28 at that time, had a *Bachelor of Science* in Chemistry and a *Masters in Business Administration,* both from the prestigious *Massachusetts Institute of Technology* in Boston (US). After his education, he worked in San Francisco for a *‘venture capital’* business. He got inspired by a book on the discovery of the DNA structure by Francis Crick and James Watson in 1953 and wanted to start a biotechnology company. Herbert Boyer had more '*credentials*'. He had a solid reputation as a geneticist and biochemistry professor at the *University of California / San Francisco* (UCSF)[65].

In April 1976, both founders contributed $500 as starting-up capital for the new company. During the first nine months of *Genentech*, the venture capital investor *Kleiner, Perkins, Caulfield & Byers*, the former company of Swanson, invested another $100,000. Since they did not have their own laboratory they used this money to sub-contract their research, mainly to scientists at the UCSF and the City of Hope University.

Within a year, they succeeded to isolate the human gene of somatostatin, to transfer it into a bacteria and to produce the human protein. This procedure is what scientists call 'cloning'. In 1977, with two scientists and their own laboratories, Swanson and Boyer succeeded to clone human insulin. In 1979, they built their headquarters in the south of San Francisco and by spring of 1980 - about the time when I was ‘overwhelmed’ by Diane Pennica – *Genentech*

[65] Diebold J. The innovators. The discoveries, inventions, and breakthroughs of our time. Truman Talley Books/Plume, New York, 1990: 227-32.

carried, besides somatostatin and insulin, α-interferon and human growth hormone in their portfolio.

In the fall of 1980 *Genentech's* initial public offering was announced. Genentech brought one million shares to the American stock market on 14 October. The offering opened at $35 per share. Within twenty minutes of trading, the shares skyrocketed to $88 per share. The closing price that day was $71.25. The initial public offering of *Genentech* is still a memorable event in the history of Wall Street[66].

LR&D on a mission

Upon her return from Malmö, Diane Pennica enthused her Genentech bosses about t-PA. Soon after I had several telephone conversations with Herbert Heyneker of *Genentech*. He repeatedly expressed his interest in a collaboration with us, but at the same time one could feel a slightly threatening tone: if Leuven continued its restraint, *Genentech* would consider searching for alternatives to make a thrombolytic drug out of t-PA. At that point we only had the patent application which clearly isn't the same as an awarded patent on t-PA.

In August 1980, I asked Jos Bouckaert, manager of LR&D, to visit *Genentech.* He returned with a proposal of a non-exclusive cooperation between *Genentech* Inc and LR&D. According to this agreement, samples of the Bowes cell line, purified t-PA and antiserum against t-PA would be transferred to *Genentech.* In return we would obtain access to recombinant (cloned) t-PA, involvement in all further research of *Genentech* on recombinant t-PA (rt-PA) and a royalty of 1% on any commercial returns on rt-PA. In September 1980, this agreement (see Appendix 4) was signed by LR&D with my approval. The complete contract was one and a half pages long, including the signatures. Nowadays contracts with such far-reaching implications for the K.U.Leuven, as well as for companies like *Genentech*, would count numerous pages. An army of lawyers and *business developers* would scrutinize the contract clauses for weeks in a row. But back then scientific collaboration was often based on mutual trust and, I admit, we were all still quite naive and amateurish at this level.

[66] Glick BR and Pasternak JJ. The molecular biotechnology revolution in *Molecular Biotechnology, Principals and Applications of Recombinant DNA*, Third Edition, ASM Press, Washington DC, 1998, 3.

Later on I realized that the contract also contained a *right of first refusal* clause. This clause implies that all future agreements involving t-PA that we could have reached with other partners had to be submitted to *Genentech* first. Genentech was entitled the right to block these agreements and to acquire the contracts for themselves at the same conditions. Fortunately, we always had a smooth collaboration with Genentech, but I would certainly not adapt such a naïve attitude in future negotations again.

18/24, 7/7

In the fall of 1980 it dawned on Diane Pennica that cloning t-PA was no 'piece of cake' at all. For many months she worked 18/24 and 7/7 skipping all holidays. After all, other companies also had set their mind on rt-PA. The only way to stay in front position was to work harder than the others. Many months of disappointment and failed experiments followed. Finally, in October 1981, when she looked at a partial DNA sequence of one of so many clones, there was a common point of recognition: a very unusual sequence of five amino acids, which she recognized as belonging to the t-PA protein. The first clone with a human t-PA gene, or at least a large part of it, was identified[67].

A beautiful protein

In the following months, the *Genentech* scientists fully unraveled the structure of the t-PA gene and the protein. The successful cloning was announced during the *Sixth Congress on Fibrinolysis* in 1982 in Lausanne (Switzerland) and published in the journal *Nature* on 20 January 1983 (see Appendix 5)[68].

For those interested: the protein t-PA consists of 527 amino acids and has 35 cysteine residues through which it can form seventeen potential disulfide bridges. There are four potential N-glycosylation sites, localized on the amino acids Asn117, Asn184, Asn218 and Asn448. Afterwards, it would become evident that t-PA isolated from the Bowes cell line was a derivative of the natural t-PA of which the first three amino acids

[67] As was conveyed orally, Pennica D. 'The tPA story as told by Diane Pennica' on occasion of 'Heart for the Future', 6 October and Pennica D. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 81-3.

[68] Pennica D. et al. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. Nature. 1983 Jan 20;301(5897):214-21.

were cleaved off. Natural t-PA thus actually contains 530 amino acids, but the original numbering from our *Nature* article was generally maintained.

Later on, the genetic engineers of *Genentech* succeeded in transferring the gene to a system of mammalian cells, which ultimately was used for the production of the drug version' of recombinant t-PA.

With the successful cloning and expression, *Genentech* secured the pole position for itself in the race to develop t-PA into a successful thrombolytic drug.

A headwind

Fat in the fire

As mentioned previously, I gave a lecture at the Rega institute in Leuven on 22 May 1980, just a couple of weeks prior to my departure for Malmö. The somewhat lukewarm reactions and the fact that Rector De Somer explicitly suggested that I should find an industrial partner, convinced me to collaborate with *Genentech*. On top of that, some experts advised us that the cloning and expression of t-PA could well be a very long term and very expensive project. LR&D and I agreed in all honesty that we would better collaborate with a partner, holding strong references in the field of cloning. At that time, *Genentech* was the world champion in this discipline.

However, at the end of 1980 the fat got in the fire. Herbert Heyneker of *Genentech* had been invited to give a lecture at the Rega Institute on recombinant interferon. Heyneker also mentioned the collaboration with LR&D on the cloning of t-PA. Bouckaert and I were summoned by Rector De Somer to justify our agreement with *Genentech.*

During this discussion, we were informed that the Rega had initiated its own research group for recombinant DNA technology and that they wanted to use t-PA as test case to introduce and master the technology. The new ambitions of the Rega Institute clearly originated after Guido Volckaert and Ghislain Opdenakker had joined the Rega Institute in the summer of 1980.

Loyalty in collaborations

Both Bouckaert and I then explained our agreement with *Genentech* elaborately and expressed our desire for an open and fair relationship with both *Genentech* and the Rega Institute. Moreover, we believed that our collaboration with the American company would in no way interfere with all other obligations we had towards the university. In addition, I explicitly confirmed that I would honor all previously made agreements, just as I always did. And this is exactly what I did. We always supported Ghislain Opdenakker during his PhD work, we supported him technically with activity tests, and we provided him with all requested crucial reagents, which included antibodies against t-PA and purified t-PA.

I believe that he would not have succeeded to isolate messenger-RNA for t-PA, as described in the *European Journal of Biochemistry*, without these reagents, in such a short period of time[69]. Nonetheless, he found it necessary to declare on oath during the legal procedures later issued in the U.S. and in England relating to the patents on t-PA and rt-PA, that he executed all work independently, except for some standard tests. He therein ignores the crucial role of these essential reactants.

Considering my relationship with *Genentech* and the own intentions of the Rega Institute, all further interactions with the Rega Institute since the end of 1980 were conducted by my colleague Roger Lijnen in order to avoid any possible conflict of interest. My biggest frustration from that period is that my smooth and very collegial collaboration with Alfons Billiau came to an end.

The last important contribution of our research group towards the t-PA research at the Rega Institute took place in the second half of 1981. On explicit request we transferred 12 mg purified t-PA (batch 110, purified in the period August - September 1981) for the determination of the partial amino acid sequence. The material was treated by my colleague Roger Lijnen on 10 October 1981 with trypsin, after which 11 peptides - protein fragments - were isolated and dispatched to Wilfried Rombauts of the Department of Biochemistry. Rombauts was equipped with more sensitive methods and materials for protein sequence determination than our own.

During our rather lively discussion with Rector De Somer at the end of 1980 we made a very clear agreement that the Rega Institute would have free access to all non-confidential information for research purposes. I was, however, never informed that the Rega Institute had contact with the British biotechnology company Celltech, until Guido Volckaert, during a reception, it must have been sometime in 1985, mentioned that he had obtained oligonucleotides for the identification of cDNA-clones of t-PA originating from Celltech. I was also totally in the dark about the fact that the Rega Institute, together with Celltech, intended to publish t-PA research results, until their joint publication appeared[70]. To make matters even worse, it appeared that they made use of the same pentapeptide sequence than Diane Pennica a few years before. I had then had a hard time to convince *Genentech*, using the data provided by Roger Lijnen and Wilfried Rombouts, that I had absolutely no hand in that publication myself.

[69] Opdenakker G, Weening H, Collen D, Billiau A, De Somer P. Messenger RNA for human tissue plasminogen activator. Eur J Biochem. 1982; 121: 269-74.

[70] Harris TJ, Patel T, Marston FA, Little S, Emtage JS, Opdenakker G, Volckaert G, Rombauts W, Billiau A, De Somer P. Cloning of cDNA coding for human tissue-type plasminogen activator and its expression in Escherichia coli. Mol Biol Med. 1986; 3: 279-92.

Till then, I was unaware that information, to which my research group had contributed, could have been used in a collaboration that could possibly lead to competition with *Genentech*.

Collaboration with *Genentech* continues

By the middle of 1981, it became apparent that *Genentech* would succeed in cloning t-PA (see previous chapter). Faster than what we ever expected in Leuven. On request from *Genentech* and with my approval, a new partnership agreement was signed between *Genentech* and LR&D. This agreement would form the framework of the intense collaboration between our laboratory at the K.U.Leuven and the American biotechnology company and form the basis for the exclusive licensing agreement of t-PA in 1983. Thomas Kiley of *Genentech* and I worked out the details of the agreement from 1981, but various interim versions of the contract were read and amended by Jos Bouckaert, Manager of LR&D.

This agreement stipulated that LR&D (and thus in *casu* my laboratory and me) would be part of an exclusive joint venture for two years with *Genentech* on the area of t-PA. The agreement took effect on 1 November 1981. This however did not mean that *Genentech* obtained exclusive rights to the possible commercial return of rt-PA. After the two-year period we basically could join forces with other partners - *Genentech* indeed only had the right of pre-emption. The issue of total exclusivity remained to be negotiated.

The collaboration stipulated that *Genentech* would pay $200,000 annually for the production of 200 mg t-PA by our laboratory. Because the agreement would be valid for two years, it meant that Genentech would pay $400,000. Afterwards, the agreement was extended for another two years.

The produced t-PA, originating from the Bowes cell line, would be used to set up *in vitro* and *in vivo* studies. Moreover, we would perform a series of pre-clinical and clinical trials with t-PA, and inform *Genentech* of the outcome.

Before the agreement was signed by LR&D, with the approval of the responsible persons of the K.U. Leuven, a copy was submitted to Pieter De Somer in his capacity as Rector of the University. The storm seemed to have subsided.

A first patient

Attending a Rotterdam conference

The first time t-PA was used as a thrombolytic drug involved Alfons Billiau of the Rega Institute. Billiau attended a congress in Rotterdam and met the Dutch nephrologist Willem Weimar of the Dijkzigt Hospital. Billiau and Weimar tell the story[71] [72].

"With mixed feelings I drove to the Annual Interferon Conference in Rotterdam on April 21, 1981 together with Pieter De Somer", Billiau writes. "Our interferon project at the Rega Institute was at a critical stage. At that time our interferon, isolated from fibroblast cultures, was tested in clinical studies in patients with cancer, multiple sclerosis and viral infections. With one of these studies Willem Weimar was involved. The results of these studies were not overly positive. In fact, our enthusiasm and that of our mentor, Pieter De Somer, faded day after day. Moreover, recombinant DNA technology was booming and promised to become the method of choice for producing human interferons. Hence, we felt that we had reached the point were we would have to abandon our plans to develop commercial production of interferon from cultured human fibroblasts. The contrast between the ease of producing milligram amounts of t-PA by Désiré compared to the pains to obtain as little as microgram quantities of interferon, made our discouragement even greater. To make matters worse Willem Weimar didn't show up at the opening reception of the conference".

A critically ill kidney transplant patient

Weimar: "I arrived late for the opening reception at the Town Hall of Rotterdam, because I was worrying about a patient with a renal transplant. The patient was developing an ascending thrombosis in her iliac vein (the main vein in the pelvis). This thrombosis threatened to be fatal for the transplanted kidney. Kidney function was already deteriorating and graft loss was expected within a day".

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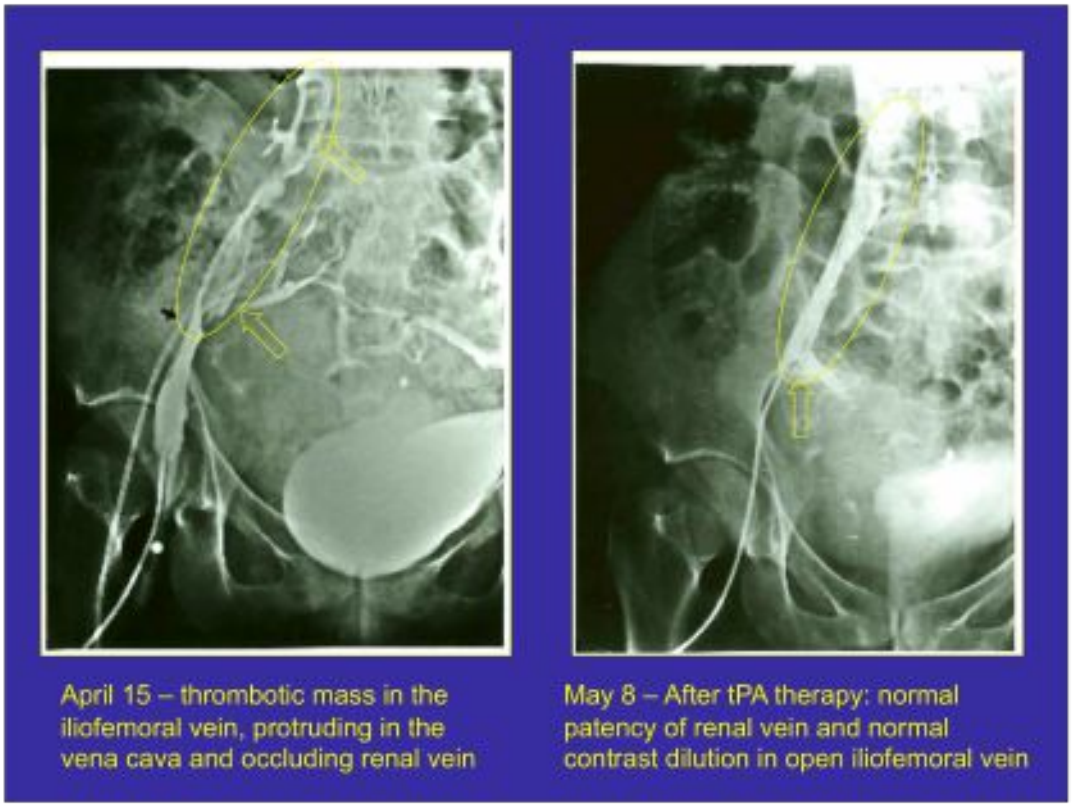
[71] As was conveyed orally, Billiau A 'The t-PA story as told by Alfons Billiau' on occasion of: 'Heart for the Future', 6 October 2008 and Billiau A. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 17-9.

[72] Weimar W. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 123-4.

“Ever heard of t-PA, tissue plasminogen activator?”, Fons asked me, “Désiré Collen is working on it. In the Rega we just produced a first batch for clinical application”. “I immediately understood that this could be the only way to save my patient”.

Billiau: “Instead of attending the congress, I returned to Leuven the next morning. I called Désiré and explained the problem. Désiré immediately agreed, I brought the t-PA sample to Rotterdam and before noon the first batch of t-PA was administered to the patient.”

Weimar: “It resulted in a dramatic effect. The clot completely dissolved, as we proved radiologically. The kidney graft function rapidly improved, we wrote a Lancet paper and Désiré became famous. [73]. Now, 27 years later, the patient is still alive with a perfectly functioning renal allograft.”



X-rays of the pelvis of a kidney transplant patient before and after the treatment with t-PA.
Left is to be seen how the iliac vein is occluded by a blood clot, right the vein is completely open (radiography taken three weeks after the treatment).

[73] Weimar W, Stibbe J, van Seyen AJ, Billiau A, De Somer P, Collen D. Specific lysis of an iliofemoral thrombus by administration of extrinsic (tissue-type) plasminogen activator. Lancet. 1981; 2: 1018-20.

Willem Weimar would later treat a second kidney patient with t-PA - for this patient Dick Rijken's car was crucial. Dick wanted to combine a trip to Rotterdam as t-PA courier with a visit to family and friends.

That a complete thrombolysis with infusions of only 5 and 7.5 mg t-PA occurred with the first patient could be considered as a miracle. The standard thrombolytic treatment of heart attack would later on be 100 mg t-PA. In hindsight, we can only conclude that such mini-doses were effective, because in these kidney patients the blood clots had an exceptional fragile architecture.

There is no doubt that these patients strengthened my conviction and that of my colleagues that we were on the right path with t-PA as potential thrombolytic agent.

Preclinical t-PA research

We doubled our input into the research on t-PA . Our own group in Leuven, *Genentech* in San Francisco as well as numerous other collaborators all over the world, put tremendous energy in the t-PA research. As a result of the agreement with *Genentech* in 1981, my research group obtained sufficient financial means to continue exploring the biochemistry, as well as the physiological characteristics of t-PA from the Bowes cell line. Various *in vitro* and *in vivo* studies were performed in preparation of preclinical and clinical trials with rt-PA the moment this would become available.

American dogs

A very important collaboration in this period was with Steven Bergmann and Burton Sobel of the *Washington University School of Medicine* in Saint Louis (US). Today Burton Sobel is Director of the *Cardiovascular Research Institute* at the *University of Vermont* in Colchester (US).

Using a canine model of coronary thrombosis, we wanted to demonstrate that t-PA was just as good, or even a better thrombolytic agent than streptokinase, and would have less side effects. We also wanted to examine to what extent t-PA would be effective when injected intravenously instead of directly into the coronary artery using an arterial catheter. The main advantage of an intravenous injection is that this is a quicker way to administer t-PA to patients. Preparing a patient in a cathlab environment indeed is time consuming. An additional advantage of the research in Saint-Louis was that we could use positron emission tomography (PET) in addition to an electrocardiogram and an angiography to measure damages to the heart. Afterwards, it became evident that this technology is not only useful for experimental animals, but also delivers good diagnostic results in humans with heart attack.

We performed experiments on 24 anesthetized dogs. A blood clot was formed in a coronary artery by means of a copper coil inserted through an arterial catheter. Following this procedure, the animals were treated with streptokinase or t-PA, either intracoronary or intravenously.

Upon intracoronary administration, recanalization of the blood vessel took an average of 31 minutes with streptokinase, as compared to an average of 85 minutes upon intravenous administration. t-PA performed much better: intracoronary as well as intravenous administration resulted in recanalization within on average 8 minutes.

Furthermore, no systematic activation of the fibrinolytic system occurred in the dogs treated with t-PA, because t-PA activates only plasminogen at the site of the blood clot and not elsewhere in the body. The experiments by Bergmann and Sobel were a major success, and were published in *Science*[74].

Recombinant t-PA, indistinguishable from natural Bowes melanoma t-PA

At the end of 1982, we obtained our first batch of rt-PA from *Genentech*. Research in the US and in our own laboratory quickly illustrated the similarities between t-PA from the Bowes cell line and rt-PA. Not only their sequences were identical, also biochemically they displayed the same characteristics[75] [76] [77].

However, that in itself does not prove that rt-PA also would have thrombolytic effects *in vivo*, in an experimental animal model, for example. A first series of experiments was performed on dogs by Burton Sobel in Saint Louis and by Frans Van der Werf in Leuven. Both of them compared rt-PA with urokinase. With the nine dogs treated with rt-PA, the blood flow was restored after 13.7 minutes on average. Of the ten dogs treated with urokinase, thrombolysis occurred in only seven dogs and only after an average of 19.3 minutes. Furthermore, two dogs suffered from serious bleeding and in all ten the coagulation profile was markedly affected[78]. This research provided a solid basis to start clinical studies with rt-PA.

Taking no risks

Nevertheless, *Genentech* and I did not want to be on thin ice and decided to bring in a third research team in parallel for the preclinical research. The choice fell on Herman Gold and Tsunehiro Yasuda of the *Massachusetts General Hospital,* associated with the *Harvard Medical School* in Boston (US).
Tsunehiro Yasuda tells how the preclinical research on dogs was performed in two

[74] Bergmann SR, Fox KA, Ter-Pogossian MM, Sobel BE, Collen D. Clot-selective coronary thrombolysis with tissue-type plasminogen activator. Science. 1983; 220: 1181-3.

[75] Pennica D et al., Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. Nature. 1983 Jan 20; 301(5897): 214-21.

[76] Zamarron C, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by natural and recombinant tissue-type plasminogen activator. J Biol Chem. 1984; 259: 2080-3.

[77] Collen D et al. Biological properties of human tissue-type plasminogen activator obtained by expression of recombinant DNA in mammalian cells. J Pharmacol Exp Ther. 1984; 231: 146-52.

[78] Van de Werf F et al. Coronary thrombolysis with intravenously administered human tissue-type plasminogen activator produced by recombinant DNA technology. Circulation. 1984; 69: 605-10.

phases[79]. "We wanted to check in the first place what the optimal rt-PA doses would be for a successful restoration of the blood flow in dogs. In each dog a stable blood clot was formed in a side-branch of the left coronary artery. We used doses of 5, 10, 15 and 25 microgram rt-PA per minute and per kilogram body weight. Each of these doses led to a restoration of the blood flow, but the higher the dose, the faster the recovery."

Following these experiments the investigators set up a randomized and blinded study. [80]. A number of dogs would receive rt-PA and other animals a placebo in the form of a salt solution. The second group served as a control group. The researchers themselves did not know which animals received rt-PA or placebo. Only one technician, Bob Holt, knew the randomization key. The first experiments started in March 1983 and the procedure was applied on two or three dogs a week. "The days Désiré was in Boston, he would be in the lab already at 7 in the morning and he would stay for the whole day. He wanted to follow up the experiments himself. By December 1983 Genentech wanted to have results. The data were analyzed one late evening about 2 weeks before Christmas. To our amazement, t-PA treated dogs had 100% reperfusion rate and control saline dogs had none. This completely blinded study gave me a tremendous confidence in rt-PA.", was the conclusion of Tsunehiro Yasuda.

A lost friend

Herman Gold, 'Chip' for his friends, together with his family would become very dear friends. Not only was our joint research, the preclinical as well as the clinical studies (see the next chapter), successful, but also in daily life Chip and I got along very well. I became so to speak a part of the Gold family. Tsunehiro shares this views on my dear friendship with Chip: "Désiré's other important contribution was convincing Dr. Gold to purchase a house. His residence was a small 2-bedroom apartment in the Prudential Center in Boston. His wife, Barbara Nath, a gastro-enterologist, was very anxious to move to a 'normal' house since they had a 5 year old girl, Lisa.

[79] As was conveyed orally, Yasuda T. 'The t-PA story as told by Tsunehiro Yasuda' on occasion of 'Heart for the Future', 6 October 2008 and Yasudo T. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 127-9.

[80] Gold HK et al. Coronary thrombolysis with recombinant human tissue-type plasminogen activator. Circulation. 1984; 70: 700- 7.

The Prudential Center is a sky scraper with mostly offices, not exactly a tempting environment for a 5 year old. Barbara set her sights on a house in Brooklyn, on the West side of Boston but Chip was not really keen on the idea. Finally Désiré's opinion was asked. Désiré persuaded Chip to buy the house. Chip's objection that a 6 bedroom house was too big for the three of them was quickly countered by Désiré, stating that the remaining rooms would rapidly be filled with all kinds of junk. Finally, Dr. Gold agreed to purchase it and he transformed the third floor of the house into a semi-permanent stay for Désiré.

Indeed, this third floor would become my home base during my numerous visits to Boston, varying from a couple of days to even weeks. The Gold family installed a television (which I never used) and equipped a bathroom (which I used at least a hundred times). I spent more time there than in any specific hotel. Not long after they moved, their second child was born, Jonathan Gold, who in the meantime has become a smart young fellow.

Sadly, Chip passed away on 2 March 2008, after a long illness. I have lost one of my very best friends.

The first clinical studies with t-PA

Double record

20 January 1983, three pm. The cathlab in the University Hospital in Leuven is overcrowded. Everybody is stressed. Twenty to thirty people attend the procedure, as I did. For the first time a patient, B.R. 58 years old with a myocardial infarction, is to be treated with t-PA under supervision of cardiologist, Frans Van de Werf. At that time, we still used t-PA purified from the Bowes cell line. Only later on would rt-PA become available for human use.

The procedure was a disaster: neither intravenous nor intracoronary administration of t-PA did dissolve the clot. Fortunately, the patient survived his heart attack, even without successful fibrinolysis. Some twenty years later, he will receive a pacemaker, because of heart rhythm disturbances. Finally, he passed away at the age of 83, 25 years after the very first, but unfortunately failed, t-PA thrombolysis therapy. Eventually, Frans Van de Werf successfully treated four other myocardial infarction patients in Leuven. Thank God. The time it took to dissolve the blood clots of these four patients, was 30, 37, 22 and 19 minutes, respectively. In addition, Burton Sobel of the *Washington University School of Medicine* in Saint-Louis (US) successfully treated two patients.

The data of both teams were jointly published in the *New England Journal of Medicine* (see Appendix 6)[81]. Frans Van de Werf remarked that the publication covered the smallest study - in terms of the number of treated patients - to ever be published in the *New England Journal of Medicine*. Moreover, the paper achieved a world record for the number of citations per patient[82].

First randomized study

The first randomized studies with rt-PA would soon be conducted. After the successful cloning of t-PA, *Genentech* invested heavily in the upscaling of the production in so-called CHO cell lines (*Chinese Hamster Ovary*).

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81 Van de Werf F et al. Coronary thrombolysis with tissue-type plasminogen activator in patients with evolving myocardial infarction. N Engl J Med. 1984; 310: 609-13.

82 As was conveyed orally, Van de Werf F. 'The t-PA story as told by Van de Werf F.' on occasion of the 'Heart for the Future', 6 October 2008 and Van de Werf F. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen. Leuven 2008: 113-4.

The company wanted to produce the first batches of rt-PA for clinical research as soon as possible. After all, the experiments on dogs demonstrated equivalence of rt-PA with t-PA from the Bowes cell line. Moreover, the first patients were successfully treated with t-PA in Leuven and Saint Louis.

We were now faced with the difficult task to show that rt-PA would survive the critical test of the randomized clinical studies. We were all aware of the fact that this is the phase in which most of the potentially innovative drugs fail.

In 1983, The *Food and Drug Administration* (FDA) approved a prospective, randomized and placebo-controlled trial of rt-PA with a maximum of 50 patients. The trial took place in three top hospitals in the United States: the *Washington University* (Saint Louis), the *Massachusetts General Hospital* (Boston) and the *Johns Hopkins University* (Baltimore) under the supervision of Burton Sobel, Herman 'Chip' Gold and Myron Weisfelt respectively. The protocol of the study was relatively complex, but at the same time very clever because it would convincingly demonstrate the power of rt-PA[83]: two thirds of the patients were to be treated with rt-PA immediately and one third with a placebo. The placebo patients, whose blood flow was not restored, would be treated with rt-PA as well. Five additional patients would not be randomized, but treated with rt-PA, anyhow.

In 25 of the 33 patients treated with rt-PA (75%), the blood flow recovered completely or partially within a period of 90 minutes after start of the treatment. Of the fourteen patients treated with placebo, the coronary occlusion disappeared in only one patient (7%). The thirteen patients, originally in the placebo group and without signs of reperfusion, also received t-PA treatment. The blood flow of 9 of them (69%) would also be restored. Six patients, who did not react to rt-PA, were subsequently treated with intracoronary streptokinase, however without any result.

In spite of the success of the study, it also caused animosity among the researchers. The first patient in this study was treated by Eric Topol, a young specialist-in-training in the department of Myron Weisfelt in Baltimore. Topol would become one of the leading clinical researchers in the area of new treatments for myocardial infarction.

[83] Collen D et al. Coronary thrombolysis with recombinant human tissue-type plasminogen activator: a prospective, randomized, placebo-controlled trial. Circulation. 1984 Dec; 70: 1012-7.

After the first patient was treated, the public relations department of the *Johns Hopkins University* released a grandiloquent press communication, which led to fierce protest by the other two centers. It probably cost Topol the first author's position in the publication, because, after the manuscript was completed based on the first draft, which I wrote together with Elliott Grossbard, at that stage the *Director of Clinical Research* of *Genentech,* the clinical researchers could not agree on the order of the authors (the first and the last positions are considered to be the most important). In the end, it was decided that I should be the first and Grossbard the last author. The study was published in the prestigious cardiology journal *Circulation* in December 1984. Although I actively participated in the writing of the manuscript, I never saw any of those patients and I even had no official medical accreditation in the USA.

After this open study, time had come for blind studies.

European ECSG and American TIMI Studies

During the Seventies Marc Verstraete coordinated a consortium of cardiology researchers under the name *European Working Party on Streptokinase* to perform clinical studies on thrombolysis (for an overview)[84].
After the encouraging experiments with t-PA and rt-PA in dogs and baboons[85] and the positive experiences with the first treated patients in Leuven and Saint Louis[86], Marc Verstraete set up a new European consortium to perform studies on rt-PA. The consortium performed its research under the name *European Cooperative Study Group (ECSG) for Recombinant Tissue-type Plasminogen Activator* and more than 30 cardiologists and referral centers from hospitals all over Western Europe participated.

The European study group started with a double-blind, placebo controlled study with 129 myocardial infarction patients: 64 patients received rt-PA, intravenously administered, and 65 patients a placebo. After 90 minutes, in 61% of the rt-PA group the occluded coronary artery was recanalized, as compared to only 21%[87].

In a second study, rt-PA would be compared to streptokinase. That protein already had an established reputation as a useful blood clot dissolving agent. The American authorities had already in 1982 approved through the FDA the intracoronary administration of streptokinase for the treatment of acute myocardial infarction. The FDA had not yet taken a decision on the use of intravenously administered streptokinase, because at that stage not enough convincing data were available on the efficiency of the drug administered in this way.

In a new blinded, randomized European study, 64 patients were treated with rt-PA and 65 with streptokinase[88]. After 75 to 90 minutes the occluded coronary arteries in 75% of the rt-PA treated patients were recanalized. For the patients treated with streptokinase this was the case in 55%. The difference between both groups is however not statistically significant (p=0,054). Moreover, mortality rates were eventueally not different. There, however, was a

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84 Verstraete M. Trials of the European Working Party on streptokinase and of the European Cooperative Study Group on alteplase in patients with acute myocardial infarction. European Investigators. J Interv Cardiol. 1995; 8: 611-21. Review.

85 Flameng W, Van de Werf F, Vanhaecke J, Verstraete M, Collen D. Coronary thrombolysis and infarct size reduction after intravenous infusion of recombinant tissue-type plasminogen activator in nonhuman primates. J Clin Invest. 1985; 75: 84-90.

86 Van de Werf F et al. Coronary thrombolysis with tissue-type plasminogen activator in patients with evolving myocardial infarction. N Engl J Med. 1984; 310: 609-13.

87 Verstraete M et al. Double-blind randomised trial of intravenous tissue-type plasminogen activator versus placebo in acute myocardial infarction. Lancet. 1985; 2: 965-9.

88 Verstraete M et al. Randomised trial of intravenous recombinant tissue-type plasminogen activator versus intravenous streptokinase in acute myocardial infarction. Report from the European Cooperative Study Group for Recombinant Tissue- type Plasminogen Activator. Lancet. 1985; 1: 842-7.

difference in the fibrinogen level in the blood: it remained higher in the rt-PA treated patients (61% of the start level) than in the patients with streptokinase treatment (12% of the start level). This shows that rt-PA affects the blood coagulation system to a lesser extent. As such one could conclude that the risk of serious bleeding is lower with rt-PA treatment.

Between 1984 and 1992, the ECSG consortium would perform six different clinical studies in which a total of 2121 patients with acute myocardial infarction were enrolled. These studies would result in six 'princeps' publications and eighteen supporting publications (for an overview)[89]. It was tested, among other things, to what extent an infusion over a long period of time with rt-PA prevented 're-occlusion' i.e. the recurrent occlusion of the coronary blood vessel[90], whether angioplasty (the opening of the blood vessel with a balloon) after thrombolysis presented additional benefits[91], and whether the co-administration of heparin did have an effect[92]. These trials, together with the American studies, would lead to the desgn of the optimal protocol for the use of rt-PA in cardiovascular medicine.

NIH joins, TIMI-1

Across the Atlantic Ocean, the American *National Institutes of Health* (NIH), already showed interest in clinical studies on thrombolysis at the end of 1981. That was even before the FDA approved the intracoronary administration of streptokinase.

On 20 May 1982, the *Advisory Council* of the *National Hearth, Lung and Blood Institute* (NHLBI) approved the protocol of a first study, the TIMI 1 study[93]. Initially it involved three groups of patients: one patient group would receive intracoronary streptokinase treatment, another group intravenous streptokinase and the last group a placebo. The TIMI 1 study was supervised by Eugene Braunwald of the *Harvard University* and thirteen clinical centers

89 Verstraete M. Trials of the European Working Party on streptokinase and of the European Cooperative Study Group on alteplase in patients with acute myocardial infarction. European Investigators. J Interv Cardiol. 1995; 8: 611-21. Review.

90 Verstraete M et al. Acute coronary thrombolysis with recombinant human tissue-type plasminogen activator: initial patency and influence of maintained infusion on reocclusion rate. Am J Cardiol. 1987; 60: 231-7.

91 Simoons ML, Verstraete M, Wood D. Thrombolytic therapy and percutaneous coronary angioplasty. Lancet. 1988; 1: 1056.

92 de Bono DP et al., Effect of early intravenous heparin on coronary patency, infarct size, and bleeding complications after alteplase thrombolysis: results of a randomised double blind European Cooperative Study Group trial. Br Heart J. 1992; 67: 122-8.

93 Brody BA. Ethical issues in drug testing, approval, and pricing. The clot dissolving drugs. Oxford University Press, 1995, 29.

participated. Among the participants was the research group of Chip Gold at the *Massachusetts General Hospital*. Besides a steering committee with all participants and representatives of the NHLBI, the study was monitored by a *Policy Advisory and Data Monitoring Board*, an independent advisory board with William Hood Jr presiding. This complex steering and monitoring structure is quite common in multicenter trials designed by the NIH.

rt-PA versus streptokinase first round

In 1983, the original protocol of the study was drastically modified, because of the arrival of rt-PA. The TIMI 1 trial would develop into a comparative study between streptokinase and rt-PA. The American cardiologists indeed considered it useless to perform a large clinical thrombolytic study without the inclusion of rt-PA.

In this adapted version of TIMI 1, the first patients were treated as from 20 August 1984. It was the intention to include 340 patients in the study, of which one half would be treated with rt-PA and the other half with streptokinase. The main clinical endpoint was the restoration of blood flow (complete or partial reperfusion as monitored by angiography). The coronary angiography was performed 90 minutes after onset of the treatment.

The study would be evaluated twice 'ad interim', after 50% and 75% of the patients had received treatment. These interim evaluations should allow to stop the study, if it turned out that too large a difference existed between the two study arms. For example, in the event that one drug would be more effective than the other or because unacceptable side effects would occur. Such interim analyses and early termination procedures are standard elements in clinical studies today.

On 5 February 1985, after 316 patients received treatment, the NHLBI indeed terminated the study on advice of the *Policy Advisory and Data Monitoring Board.* The difference between the two arms of the study was larger than expected: 70% of the patients treated with rt-PA (100 of the 143) experienced total or partial reperfusion after 90 minutes; in the streptokinase group, this was 43% (63 of the 147). The numbers are even more striking when only the group of 232 patients with a complete occlusion of a coronary artery at the onset of the treatment, is considered. After 30, 60 and 90 minutes the blood flow was completely or partially restored in respectively 24%, 48% and 62% of the patients in the rt-PA group, in contrast to 8%, 23% and 31% in the streptokinase group.

NEJM and Lancet

A complete analysis of the TIMI 1 trial would only be published in 1987 in three different papers[94] [95] [96]. The prestigious *New England Journal of Medicine* would publish an interim update of the TIMI1 results on 4 April 1985. This is a highly unusual strategy for this journal[97]. Usually the journal publishes only final research results with a new medicine, but the *editorial board* found the rt-PA results so significant that they allowed the exception.

About a week later, on 13 April 1985, the *Lancet* would publish the previously quoted article by Marc Verstraete and the ECSG[98] (see page 77). Although this study in itself did not show statistically significant differences between the rt-PA and the streptokinase group, it did substantially contribute to the general perception that rt-PA is a better thrombolytic agent than streptokinase. The NEJM and Lancet papers created a real hype around rt-PA. The future drug was presented in the common press as the new miracle drug to treat myocardial infarction. The expectations for rt-PA were set very high, too high perhaps.

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94 Mueller HS, Rao AK, Forman SA. Thrombolysis in myocardial infarction (TIMI): comparative studies of coronary reperfusion and systemic fibrinogenolysis with two forms of recombinant tissue-type plasminogen activator. J Am Coll Cardiol. 1987; 10: 479-90.

95 Chesebro JH et al. Thrombolysis in Myocardial Infarction (TIMI) Trial, Phase I: A comparison between intravenous tissue plasminogen activator and intravenous streptokinase. Clinical findings through hospital discharge. Circulation. 1987; 76: 142-54.

96 Sheehan FH et al., The effect of intravenous thrombolytic therapy on left ventricular function: a report on tissue-type plasminogen activator and streptokinase from the Thrombolysis in Myocardial Infarction (TIMI Phase I) trial. Circulation. 1987; 75: 817-29.

97 The Thrombolysis in Myocardial Infarction (TIMI) trial. Phase I findings. TIMI Study Group. N Engl J Med. 1985; 312: 932-6.

98 Verstraete M et al. Double-blind randomised trial of intravenous tissue-type plasminogen activator versus placebo in acute myocardial infarction. Lancet. 1985; 2: 965-9.

Statistically significant, but also clinically relevant?

However, in spite of the enthusiasm, there was also some criticism on the first TIMI trial. Some cardiologists were questioning whether restoration of the blood flow after 90 minutes was indeed a clinically sound endpoint. To what extent has it been proven that the rapid recanalization of an occluded blood vessel is also clinically relevant? That it would lead to an improved heart function and a prolonged survival? What would the perception of the TIMI trial have been, had other endpoints been investigated? These were questions which needed to be answered in the years to come.

Meanwhile, from a more in-depth analysis of the TIMI 1 results, it could not be concluded that rt-PA in comparison to streptokinase does lead to an improved ventricular heart function the moment the patient leaves the hospital. There are at least two possible explanations why rt-PA was not superior to streptokinase in the endpoint of this study. Either immediate reperfusion had much less influence on the final heart function and the survival of the patient, or the protocol of the first TIMI study was too limited to demonstrate a benefit for clinically relevant endpoints. Both hypotheses would be elaborately tested in the years to come.

Ethical debate concerning placebo

In the US, the NIH initiated an ethical debate on the desirability to continue clinical thrombolytic studies using a placebo group. Initially, intentions were to continue with a placebo group in TIMI 2, the successor of TIMI 1, but this was opposed by some investigators, as well from ethical circles as NIH representatives[99]. According to them, sufficient indications of streptokinase's superiority over placebo were available, even when administered intravenously. They referred, among others, to a meta-analysis by NIH researcher Salim Yusuf, who combined all clinical studies on intravenously administered streptokinase versus placebo of the past 25 years and concluded that the drug reduced mortality by 22%[100].

[99] Brody BA. Ethical issues in drug testing, approval, and pricing. The clot dissolving drugs. Oxford University Press, 1995, 33.

[100] Yusuf S, Collins R, Peto R, Furberg C, Stampfer MJ, Goldhaber SZ, Hennekens CH. Intravenous and intracoronary fibrinolytic therapy in acute myocardial infarction: overview of results on mortality, reinfarction and side-effects from 33 randomized controlled trials. Eur Heart J. 1985; 6: 556-85.

This conclusion was substantiated in 1986 and 1987 by large thrombolytic studies in which mortality and recovery of the heart function were the central endpoints. These were mainly the Italian GISSI study (*Gruppo Italiano per lo Studio della Streptochinasi nell'infarto Miocardico*)[101] [102] [103], wherein 12,000 patients participated and the ISIS-2 study (*Second International Study of Infarct Survival)*[104] [105] with 20,000 patients.

A new series of rt-PA studies

One could assume that rt-PA would perform at least as well as intravenous streptokinase with respect to the mortality rate. This assumption was substantiated between May 1986 and November 1987 by the ECSG consortium in a double-blind, randomized and placebo controlled study in which the efficiency of rt-PA was determined from the size of the myocardial infarction, the functional recovery of the heart function and survival. Besides the University Hospital Gasthuisberg in Leuven, 25 other clinical centers participated in this trial. The intention was to administer patients intravenously with rt-PA (355 patients) or placebo (366 patients) within four hours after the onset of the first symptoms. In addition, all patients received 250 mg aspirin and 5000 units of heparin at the onset of the procedure and during the first days after the treatment.

After fourteen days, the mortality rate in the rt-PA group reached 10%, and 21% in the placebo group. After three months, the mortality rate was respectively 18% and 29%. In other words, rt-PA reduced mortality by 36% after three months. For patients who received treatment within three hours after the first symptoms, the differences were even more remarkable: after two weeks, 2% of the patients in the rt-PA group had died as compared to 13% in the placebo group; after three months the mortality rate was respectively 6% and 17%, a reduction by 59%. rt-PA also performed better than placebo regarding the size of the infarct zone and the recovery of the heart function. However rt-PA treatment was accompanied by a higher frequency of cerebral hemorrhage (1.4% of the treated patients).

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101 Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. Gruppo Italiano per lo Studio della Streptochinasi nell'Infarto Miocardico (GISSI). Lancet 1986; 1: 397-402.

102 Long-term effects of intravenous thrombolysis in acute myocardial infarction: final report of the GISSI study. Gruppo Italiano per lo Studio della Streptochi-nasi nell'Infarto Miocardico (GISSI). Lancet. 1987; 2: 871-4.

103 Rovelli F, De Vita C, Feruglio GA, Lotto A, Selvini A, Tognoni G. GISSI trial: early results and late follow-up. Gruppo Italiano per la Sperimentazione della Streptochinasi nell'Infarto Miocardico. J Am Coll Cardiol. 1987; 10: 33B-39B.

104 Intravenous streptokinase given within 0-4 hours of onset of myocardial infarction reduced mortality in ISIS-2. Lancet. 1987; 1: 502.

105 Randomised trial of intravenous streptokinase, oral aspirin, both, or either among 17,187 cases of suspected acute myocardial infarction: ISIS-2. ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Lancet. 1988; 2: 349-60.

Frans Van de Werf, coordinator of this study, concluded in the *British Medical Journal*[106]: "rt-PA combined with heparin and aspirin decreases the size of the infarct zone, preserves the left ventricular function better, and reduces complications and deaths due to heart disease, but, is associated with a slightly increased risk for bleeding."

The same year, these conclusions were confirmed by the ASSET study (*Anglo-Scandinavian Study of Early Thrombolysis*) undertaken in the United Kingdom, Norway, Sweden and Denmark. In this 'rt-PA versus placebo' study, 5011 patients participated. One month after the treatment, mortality rate in the rt-PA group was 7.2% and 9.8% in the placebo group. The researchers believed that the higher rate of complications (mainly hemorrhage) did not counter balance the obvious clinical advange of rt-PA treatment[107].

Simultaneously with the ECSG and the ASSET studies, three smaller placebo-controlled studies were performed in the US and Australia. These studies focused more on the pump function of the heart than on the mortality rate. The heart function can be quantified by the so-called 'ejection fraction': the quantity of blood ejected expressed as a percentage of the quantity in the filled heart. These studies were performed by Alan Guerci of the *Johns Hopkins University*[108], the *TICO* group (*Group for the Study of Thrombolysis in Acute Coronary Occlusion*)[109] and the *National Heart Foundation of Australia*[110].

In view of the GISSI data - which demonstrated that intravenous streptokinase is much more effective than placebo - and based on interim analyses of the studies, the Guerci and the TICO studies were terminated prematurely. The researchers found it to be unethical to administer a placebo to patients with myocardial infarction. In their opinion, the clinical advantage of streptokinase and rt-PA was convincingly demonstrated. In the study of the *National Heart Foundation of Australia,* it was decided that patients who could be treated within two hours of the onset of symptoms, should not be allocated to the placebo group.

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106 Van de Werf F, Arnold AE. Intravenous tissue plasminogen activator and size of infarct, left ventricular function, and survival in acute myocardial infarction. BMJ. 1988; 297: 1374-9.

107 Wilcox RG, von der Lippe G, Olsson CG, Jensen G, Skene AM, Hampton JR. Trial of tissue plasminogen activator for mortality reduction in acute myocardial infarction. Anglo-Scandinavian Study of Early Thrombolysis (ASSET). Lancet. 1988; 2: 525-30.

108 Guerci AD et al. A randomized trial of intravenous tissue plasminogen activator for acute myocardial infarction with subsequent randomization to elective coronary angioplasty. N Engl J Med. 1987; 317: 1613-8.

109 O'Rourke M et al. Limitation of myocardial infarction by early infusion of recombinant tissue-type plasminogen activator. Circulation. 1988; 77: 1311-5.

110 Coronary thrombolysis and myocardial salvage by tissue plasminogen activator given up to 4 hours after onset of myocardial infarction. National Heart Foundation of Australia Coronary Thrombolysis Group. Lancet. 1988; 1: 203-8. Erratum in: Lancet 1988; 2: 519.

They were to be treated directly with rt-PA. Only patients who could only receive treatment after two to four hours, would still be randomized.

80, 100 or 150 mg - back to TIMI

The TIMI group would eventually never perform a placebo-controlled study with mortality as endpoint, neither with rt-PA, nor with streptokinase. Between August 1985 and March 1986, the TIMI group did investigate a new form of rt-PA. *Genentech* had adapted its production method in order to produce larger quantities of rt-PA in the future. That transformation however caused the structure of the protein to be altered: instead of a 'bicatenary' protein (two chains), it became a 'monocatenary' protein (one chain). The old form was available as a liquid, the new form as a lyophilized powder to be dissolved by the physician.

Pilot studies[111] showed that the new, single chain form was less effective. The dose had to be increased from 80 milligrams to at least 100 milligrams. Perhaps more was even better and some even suggested a dose of up to 150 milligrams, and perhaps even more. When the TIMI 2 study was launched, this 150 milligram dose was used. This however led to an unacceptable high number of cerebral hemorrhages and as a consequence the dose was again reduced to 100 milligrams during the study. The TIMI 2 trial would eventually examine to what extend the combined use of thrombolysis and coronary angioplasty (the mechanical dilation of an occluded coronary blood vessel) and the administration of beta-blockers after the treatment would lead to better clinical results[112].

The above summarized series of parallel and consecutive clinical trials would result in the approval of rt-PA as a thrombolytic drug by the FDA in 1987. However, this approval would still require a fierce battle.

[111] Mueller HS, Rao AK, Forman SA. Thrombolysis in myocardial infarction (TIMI): comparative studies of coronary reperfusion and systemic fibrinogenolysis with two forms of recombinant tissue-type plasminogen activator. J Am Coll Cardiol. 1987; 10: 479-90.

[112] Immediate vs delayed catheterization and angioplasty following thrombolytic therapy for acute myocardial infarction. TIMI II A results. The TIMI Research Group. JAMA. 1988; 260: 2849-58.

1987, the FDA approves rt-PA

The main objective of clinical trials is to monitor on a standardized and scientifically sound basis to what extent a new clinical practice - a new treatment, a new drug, an alternative surgical intervention et cetera – better is and less side effects has than the best existing treatment. It is obvious that a new drug should be thoroughly tested before it is commercialized. It is the government's duty to make sure that this happens. In the United States, the governmental agency is the FDA (*Food and Drug Administration)*, whereas in Europe it is mainly the EMEA (*European Medicines Evaluation Agency*).

Friday, 29 May 1987

Before rt-PA was allowed on the American market, it first needed approval by the FDA. This process comprises several steps: the manufacturer submits a file which will then be examined by one or several independent advisory commissions. After careful examination by several experts, a decision on its approval is taken by the FDA staff.

Both the files on intravenously administered streptokinase and on rt-PA would be examined first by the *Cardio-Renal Advisory Committee* of the FDA. The *Committee* would meet on 29 May 1987 to first discuss the intravenous streptokinase case in the morning and in the afternoon the rt-PA file. This was a very tight and busy schedule. The day would actually cause such commotion that none of the involved parties would ever forget it. I was present at the public session as a spectator together with a crowd of journalists. The public session was held in a crowded auditorium in Bethesda (MD), where the FDA is located.

Baruch Brody, the ethics specialist, gives a comprehensive account of the day's events in his book '*Ethical Issues in Drug Testing, Approval and Pricing, the Clot-dissolving drugs*'[113], which is based on the minutes of the meeting and the testimonies given by the participants. In a nutshell: the morning session proceeded rather smoothly and the advisory committee approved intravenous streptokinase as thrombolytic therapy for the treatment of acute myocardial infarction (the coronary administration of streptokinase was already approved previously).

[113] Brody BA. Ethical issues in drug testing, approval, and pricing. The clot dissolving drugs. Oxford University Press, 1995; 46-54.

The afternoon session was more chaotic and the committee requested additional research from *Genentech*. This stopped the approval procedure of rt-PA. The decision left us dumbfounded. Not only the staff members of *Genentech*, everyone who had experience with rt-PA, including the cardiologists who administered it - were stunned. They were really convinced that rt-PA was at least as good, probably even better than streptokinase. The decision by the *Cardio-Renal Advisory Committee* could cost thousands of lives, they indicated. Eugene Braunwald, the cardiologist who led the TIMI trials, came to this conclusion already during the meeting of 29 May. He told the members of the *Committee*: *"I think you have to be very careful that you are not rejecting a drug that is twice as effective as a drug that you approved a few hours ago",* [114] *referring to Streptokinase.*

Also in the media there was a lot of disapproval with the decision of the *Cardio-Renal Advisory Committee*. In an editorial of the *Wall Street Journal t*he members of the committee and the FDA were badly criticised. The editorial concluded with the open question *"Are American doctors going to let people die to satisfy the bureau of drug's chi-square studies?"* [115]

The scientific press also critized the decision. Daniel E. Koshland, the editor of Science, was very specific: "When a circus clown steps on his toes and falls on his face, it is a cause for laughter. When a regulatory agency that licences drugs for heart attacks stumbles, it may have not only egg on its face but blood on its hands. Complex questions seen in an oversimplified way, however, can make good intentions look like bureaucratic bungling. The recent decision by the Food and Drug Administration (FDA), or lack thereof, in the tissue plasminogen activator (t-PA) controversy, is an interesting case in point." [116]

A balanced view of the facts

Twenty years later, it is probably possible to have a more nuanced view on those stormy days of May 1987. For sure, the FDA made a couple of mistakes. But the same goes for *Genentech* as well as the investigators of the *National Heart, Lung and Blood Institute* (NHLBI), a subdivision of the NIH.

[114] In Brody BA. Ethical issues in drug testing, approval, and pricing. The clot dissolving drugs. Oxford University Press, 1995, 46.

[115] In "Human Sacrifices", Wall Street Journal, June 2, 1987.

[116] Koshland DE. TPA and PDQ. Science. 1987; 237: 341.

Perhaps it is appropriate now to consider the context. Firstly, the members of the advisory committee did not have the results of all the clinical studies discussed in the previous chapter. They were unaware of the results of the ECSG and the ASSET studies, which evaluated rt-PA in comparison to placebo for mortality reduction and other clinically relevant endpoints. The results of the GISSI trial in which streptokinase was compared with placebo with mortality as the most important endpoint were readily available.

The most important data in favour of the efficiency of rt-PA were based on the trials in which the reperfusion rate was examined (i.e. the first TIMI trial). An additional source of confusion for rt-PA was the still ongoing TIMI 2 study. That study started with an rt-PA dose of 150 milligrams which was altered to 100 milligrams because of the high frequency of cerebral hemorrhage. The members of the committee were well informed on the side effects caused by the 150 milligrams dose, but were unaware if the 100 milligrams dose of the 'single chain' form, was equally effective and as safe as the 80 milligrams form of the ‘two chain' form which was used in all the previous studies.

Based on all these uncertainties, the advisory committee decided to put the approval of rt-PA on hold with the following three considerations[117]:

• There is insufficient proof to support the effectiveness of the proposed dose of 100 milligrams, at least as far as other parameters than thrombolysis and blood flow are concerned. For future studies, an improvement in the ventricular function would be considered as sufficient. Mortality studies were not requested.

• Concern prevails over the occurrence of cerebral hemorrhages. An unacceptable high incidence of bleeding took place with the 150 milligrams rt-PA dose in the TIMI 2 trial. The committee would have liked to see the data on the side effects with the 100 milligrams dose.

• The interpretation of all the data provided was complicated by the fact that *Genentech* changed the form in which the drug was presented (two chain in solution, single chain in powder form), as well as the dose (80, 100 and 150 milligrams) when the crucial clinical studies were ongoing.

[117] Kowey PR, Fisher L, Giardina EG, Leier CV, Lowenthal DT, Messerli FH, Pratt CM. The TPA controversy and the drug approval process. The view of the Cardiovascular and Renal Drugs Advisory Committee. JAMA. 1988; 260: 2250-2.

A lot of uncertainty

Genentech felt that it had been wronged by the outcome. Partly correct, because another division of the FDA, the *Biologics Division,* previously led *Genentech* to believe that the effectiveness of rt-PA demonstrated on the basis of the reperfusion rate would suffice for approval. The unexpected switch made by the *Cardio-Renal Advisory Committee* when requesting also other endpoints besides reperfusion came as a surprise to *Genentech. Genentech* felt trapped between two American government institutions: the NHLBI-NIH that raised ethical concerns on placebo-controlled studies with as endpoint mortality or ventricular function and the FDA that expected exactly these data before it would consider the approval of rt-PA. Anyhow, the FDA should never have discussed both streptokinase and rt-PA on one and the same day - and especially not on a Friday. That was already apparent on that day, because, during the rt-PA session several members of the advisory committee had to leave the meeting to catch a plane. The entire procedure was conducted in an atmosphere of chaos, which did not favor a sensible decision making[118].

On the other hand, also *Genentech* or the NIH, represented respectively by Elliot Grossbard and Eugene Braunwald, were not without blame. In his presentation, Grossbard did provide the results of the *Genentech*-sponsored placebo controlled study undertaken by Guerci (the study performed at the *Johns Hopkins University* that was terminated early due to the positive GISSI results), but the committee members did not receive the results of this study long enough in advance. Therefore the results were not interpreted in an in depth manner by all the members.

Moreover, Grossbard and Braunwald were not inclined to release data on the TIMI-2 trial and the side effects of rt-PA at a dose of 100 milligrams. There was after all an agreement with the NIH that the unpublished data of the TIMI-2 study, even when they were available internally, would not be released and would not be made available to the advisory committee of the FDA. Undoubtedly, a most remarkable decision, for it means that one American government agency withholds information from another American government institute, which may endanger hundreds if not thousands of human lives. Very strange, indeed.

[118] Brody BA. Ethical issues in drug testing, approval, and pricing. The clot dissolving drugs. Oxford University Press, 1995, 53.

All's good that ends well?

Eventually, *Genentech* and the FDA would collaborate intensively in the following months. Drawing from the numerous ongoing studies, Genentech convinced the FDA that rt-PA is an excellent thrombolytic agent and that, administered in the appropriate dose of 100 milligrams, the side effects would be acceptable. The final approval of rt-PA took place on 13 November 1987. Diane Pennica recollects how Genentech held a grand celebration accompanied by fireworks over the South San Francisco Bay[119]. Meanwhile, the production of rt-PA at *Genentech* was scaled up and immediately after the approval, the entire marketing and sales departments were geared into the selling of rt-PA. 'Activase' became the brand name for rt-PA. The expectations were high: the new 'clot buster' just had to become a financial 'block buster'.

In a time span of just 7 weeks (until the end of 1987) Genentech realized a turnover of $58 million. Bob Swanson, the CEO of *Genentech,* proudly announced to analysts that Activase "was the most successful new medicine ever to be launched."

[119] Pennica D. An anthology of scientific collaborations compiled at the occasion of the 65th birthday of Désiré Collen, Leuven 2008: 81-3.

Just as good or even better, but in any case more expensive

With the approval of both streptokinase and rt-PA by the FDA, cardiologists had a choice as to which thrombolytic agent they would use. Three studies from 1988, collectively reported that more than 90% of the American hospitals taking part in the poll, applied thrombolytic therapy, but only on 12% to 17% of the patients admitted with a myocardial infarction[120] [121]. As most important reason was quoted that many patients did not qualify, because they were hospitalized more than six hours from the onset of the first symptoms. One generally accepted that under these circumstances thrombolysis would have no benefit, because the threatened heart tissue must have died off already.

Striking was also that more than half of the hospitals definitely preferred, rt-PA. In only 19% to 39% (dependent on the study) the preference was exclusively for streptokinase and in 4% to 25% it alternated. That the majority of cardiologists in 1988 chose rt-PA is surprising, because the price tage of rt-PA was about ten times higher than that of streptokinase. For a thrombolytic treatment of 100 mg rt-PA *Genentech* charged $2,200, while the cost of a dose of streptokinase was about $200. This price difference triggered a lot of debate.

Dichotomy private-public

Physicians who chose rt-PA justified the high cost by stating that rt-PA acted faster and more efficient in dissolving occluding thrombin (see, for example, the first TIMI study) and because it had less impact on the coagulation system, so that less bleeding occurred. Cardiologists who resolutely preferred streptokinase, argued that the superiority of rt-PA had not been proven at all and thus they choose the cheapest drug.

There was a clear difference between physicians in private, and public hospitals. In private hospitals, 65% preferred rt-PA, in the public hospitals it ranged from 31% (in *federal public hospitals*) to 53% (in *non-federal public hospitals*). The difference is explained by the financing system of the American health care. American hospitals are paid by external health insurances for their services.

[120] Grasela TH Jr, Green JA. A nationwide survey of prescribing patterns for thrombolytic drugs in acute myocardial infarction. Pharmacotherapy. 1990; 10: 35-41.

[121] Brody B, Wray N, Bame S, Ashton C, Petersen N, Harward M. The impact of economic considerations on clinical decisionmaking: the case of thrombolytic therapy. Med Care. 1991; 29: 899-910.

For a part these are private insurers, but for Americans older than 65 there is, for instance Medicare, Americas largest health insurance program, covering more than 40 million Americans.

The more expensive private insurers are inclined to refund innovative treatments for their customers - they consider it as a competitive business principle. Organizations, such as Medicare, are rather more restricting in the refunding of expensive treatments. This is even more the case since a new refunding system, introduced in 1983, stipulates that a hospital only receives a fixed fee for the treatment of a patient. In 1988, an American hospital received between $3,579 and $4,200 to treat a Medicare patient with a non-complicated myocardial infarction, and between $5,449 and $6,007 for a patient with a myocardial infarction with complications. The extra expense of $2,200 for rt-PA would have to be carried by the hospital, because Medicare does not pay extra for cardiology services which prefer rt-PA above streptokinase. As such rt-PA meant a significant additional burden on the hospital budget when a Medicare patient was treated[122].

Goose with the golden eggs?

Genentech had however invested heavily in rt-PA. They absolutely wanted to turn rt-PA into a blockbuster. Early 1988 the periodical *Fortune* boosted that '*Genentech* has the goose that lays golden eggs in house'[123]. According to *Fortune* a new era had begun for the treatment of heart disease, the number 1 killer in the industrialized world. Stock exchange specialists watching the evolution of the *Genentech* shares predicted in *Fortune* that the sales of rt-PA would reach $350 million in 1988 and $600 million the next year, to become one of the most successful drugs ever. In the same article, they predicted a golden future for *Genentech:* in 1987 the total volume of trade amounted to only $231 million and the profit to $42 million; in 1988 it would increase to $500 million and $130 million respectively; in 1989 *Genentech* would break through the $1 billion ceiling of trade volume and thus become the fastest growing company in the US, still according to the *Fortune* crystal ball.

[122] Brody BA. Ethical issues in drug testing, approval, and pricing. The clot dissolving drugs. Oxford University Press, 1995, 55-58.

[123] Bylinsky G. *Genentech* has a golden goose. The blood-clot-busting drug t-PA is throwing off profits that will underwrite a host of new products. Watch for the company to join the big boys in pharmaceuticals. Fortune. 9 May 1988. http://money.cnn.com/magazines/fortune/fortune_archive/1988/05/09/70518/index.htm

However, it turned out to be different. In the fall of 1988, the *Wall Street Journal* reported that *Genentech* stopped production of rt-PA temporarily, because sales dipped and stock was piling up[124]. In September, the sales of rt-PA hit a dramatic low of $11.2 million, down from $13 million in August and $15 million in July. Instead of a rising trend, sales dropped. The year's sales figures would probably still total between $175 and $180 million, instead of the predicted $350 to $400 million. It became so clear that the 'golden eggs' of the rt-PA-goose were not real yet. If Genentech wanted to turn rt-PA into the forecasted market success they absolutely had to prove that it was superior to streptokinase, or adapt the pricing policy and make rt-PA more affordable.

Towards 'head-to-head'?

The controversy over streptokinase versus rt-PA remained front-page material between 1987 and 1990 in the scientific press[125]. In a review article in the *Annals of Internal Medicine*[126], I agreed with the proposal to perform comparative studies between rt-PA and streptokinase. Although personally I was convinced that rt-PA was the better drug. In the review, I considered the known literature data on the treatment of acute myocardial infarction with streptokinase and rt-PA. If rt-PA could be administered early after the onset of the first symptoms, it clearly had a higher thrombolytic potential. From numerous experimental and clinical studies, large as well as small, it was always evident that the clinical advantage of thrombolysis is determined by the fast recovery of the blood flow in the occluded coronary artery. Whether the higher efficacy of rt-PA will really result in a better recovery of heart function and a lower mortality rate, could however only be clarified by prospective comparative clinical studies, in which streptokinase and rt-PA would be compared directly with each other. Head to head.

On the other hand, I was definitely in favor of a reduced price for rt-PA. I regretted, during the entire period that the drug was commercialized under patent, that rt-PA in fact became the thrombolyticum of the rich. Due to its high cost the use of rt-PA remained restricted to the industrialized countries like the US, Canada, Western Europe and Japan. For the vast majority of East Europeans, South Americans, Asians and Africans the drug remained

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[124] Chase M. *Genentech* halts TPA production for rest of '88. Wall Street Journal. 14 October 1988. http://aidsinfobbs.org/articles/wallstj/88/335.txt

[125] Marx J.L. Which clot-dissolving drug is best? Science. 1988; 242: 1505-6.

[126] Collen D. Coronary thrombolysis: streptokinase or recombinant tissue-type plasminogen activator? Ann Intern Med. 1990; 112: 529-38.

unavailable. In our Leuven laboratories I would personally invest heavily in a plasminogen activator for the ‘poor’. But that is another story to be told later on in this book.

rt-PA tested in the courtroom

rt-PA not only had to deal with streptokinase as competitive thrombolytic agent, but also with itself. Other companies were trying to market their own variants of rt-PA. LR&D and *Genentech,* however, had their own rights on rt-PA in the US protected by three patents. The first (U.S. Patent 4,752,603 awarded on 21 June 1988) was a "continuation in part" of the very first patent that LR&D had submitted in the Netherlands with Désiré Collen, Dick Rijken and Osamu Matsuo as inventors (Appendix 3). In addition there were two patents on rt-PA, submitted by *Genentech* - the so-called 'Goeddel-patents' (US Patent 4,766,075 awarded on 23 August 1988 and US Patent 4,853,330 awarded on 1 August 1989). Both had been submitted in 1982. It is striking that it took more than 6 years between submission and award. This is just another example of the inertion in some American administrative departments, especially the *American Patent Office*.

Before the people's jury

Based on these patents, *Genentech* in 1988 started a lawsuit against *Genetics Institute* and *Burroughs-Wellcome*, the American subsidiary of the British *Wellcome Foundation*. According to *Genentech*, the intention of both companies to bring their versions of rt-PA was an infringement against the protection that *Genentech* enjoys via our and their own patents.

The counter parties argued that their version of rt-PA is not completely identical to that of *Genentech*. There were, indeed, a couple of DNA bases different in the t-PA genes, which they used and as compared to the Bowes cDNA clone from which *Genentech* produced its rt-PA protein. Moreover, the counter parties were resolute in the attack: *Genentech* was summoned with a claim that it intended in a fraudulent manner to monopolize the rt-PA market and the market for thrombolytic drugs in general. The case would serve before a court of law in Wilmington in the American state of Delaware. The head office of *Genentech* was registered in this state.

In March 1990 the judge of the *District Court* of the state of Delaware ruled that *Genetics Institute* and *Burroughs-Wellcome* had strictly not made any legal infringement against the patents of *Genentech* , because the sequence of the various rt-PA clones differed in a few sites. The judge allowed the jury to decide whether it was an infringement. The question he asked was whether the various rt-PA's were 'equivalent' in the sense that they 'do the same thing', overall 'lead to the same result' and whether 'they operate in the same way according to the same mechanism'. This question on 'equivalence' homed in directly on the core of the protection provided by the patent law, and is central in many lawsuits with

biotechnological products as input. The rt-PA case was, in other words, an important test case for many lawsuits to come in the biotechnology industry.

Three weeks in Wilmington

Because I was an important witness, I had to appear in court. For three weeks, I attended the trial, presided by Judge Joseph J. Farnan. Together with other '*testimonial witnesses*' of the three parties, I had to explain to among others, Joe the plumber and Sue the school teacher - members of the jury - what biotechnology was, how we isolated t-PA from the Bowes cell line, what thrombolysis is, etc.

Several times the lawyers of the opposition tried to discredit me and my laboratory. We were actually nearly accused of having isolated t-PA in a fraudulent manner from the Bowes cell line. Some of my (former) colleagues and I were extensively questioned by the lawyers of Burroughs-Wellcome. Among others, Dick Rijken, but also Ghislain Opdenakker and Guido Volckaert of the Rega Institute had to make declarations under oath. Laboratory notes were copied and all abstracts and papers on t-PA were scrutinized from cover to cover for any mistakes. The whole lawsuit produced a truckload of paperwork and more than 300 pages of court transcripts.

All along the line

On 6 April 1990 the jury gave its verdict in favor of *Genentech* (See Appendix 7)[127]. The jury found that the slightly altered t-PA genes and the resulting rt-PA proteins of *Burroughs-Wellcome* and *Genetics Institute* were equivalent with the products under protection of the patents of LR&D and *Genentech*. *Genentech* and LR&D, however, received no damages, presumably because the rt-PA of the opponents was not commercialized at that point. Furthermore, the jury found no indication that *Genentech* had monopolized the market in an illicit manner. In other words, it became a victory all along the line for us and Genentech.

Burroughs-Wellcome announced a few weeks later that they would stop further development of their rt-PA.

[127] United States District Court for the District of Delaware, Judgment in a civil case, Case number CA 88-330/89-407 JJF, 6 April 1990.

Major crisis

Between 1988 and 1990, thrombolytic reperfusion therapy was fully developped. An increasing number of cardiology centers treated their myocardial infarction patients with a thrombolytic agent. The medical and scientific literature bulged in that period with articles on thrombolysis. Even a third drug, APSAC (*anisoylated plasminogen streptokinase activator complex*) was launched on the market and became a competitor for rt-PA and streptokinase.

The emphasis was directed increasingly toward rapid intervention for treatment of myocardial infarction. The first preliminary protocols were proposed in order to treat the patient as soon as possible after hospitalization.[128] [129]. Additional studies were performed, i.e. the MITI trials, which monitored to what extent the administration of a thrombolytic agent during the patients' transport, would lead to faster reperfusion and less cardiac damage[130].

However, the billion-dollar question remained to be answered: which thrombolytic drug assures the lowest mortality?

The cold shower of GISSI-2

The GISSI-2 trial was supposed to answer that question. The GISSI-2 started by recruiting patients in Italy and, as from October 1988, was expanded to other countries. Eventually about 20,000 patients participated in the GISSI-2 trial. The trial was largely financed by *Boehringer Ingelheim*, the pharmaceutical company that purchased the European distribution rights of rt-PA from *Genentech*.

In the GISSI-2 trial, patients were randomized and subdivided in a streptokinase or rt-PA arm, with or without heparin. All patients also received beta-blockers and aspirin. In this mega trial with 20,000 patients, mortality was the main endpoint.

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[128] Leizorovicz A, Boissel JP, Robert F. Coronary reperfusion rates in acute myocardial infarction patients after thrombolytic treatment with anistreplase: correlation with the delay from onset of symptoms to treatment: a review of 424 case records of patients admitted to coronary reperfusion studies with anistreplase. J Cardiovasc Pharmacol. 1992; 19: 34-9.

[129] Sharkey SW, Bruneete DD, Ruiz E, Hession WT, Wysham DG, Goldenberg IF, Hodges M. An analysis of time delays preceding thrombolysis for acute myocardial infarction. JAMA. 1989; 262: 3171-4.

[130] Weaver WD, Cerqueira M, Hallstrom AP, Litwin PE, Martin JS, Kudenchuk PJ, Eisenberg M. Prehospital-initiated vs hospital-initiated thrombolytic therapy. The Myocardial Infarction Triage and Intervention Trial. JAMA. 1993; 270: 1211-6.

The results were published in July 1990 in the journal *Lancet*[131] [132]. The mortality with rt-PA was 8.9%, with streptokinase 8.5%. Streptokinase was associated with more bleeding, rt-PA with more strokes. The differences, however, appeared statistically not significant.

These results were definitely a disappointment to everybody who had confidence in rt-PA. In the American media, both rt-PA and the future of *Genentech* were written off. Not a single insurer or patient would still be prepared to pay $2,000 extra for a drug that delivered no better clinical results than the cheaper streptokinase. Some even believed that without rt-PA *Genentech* had become an empty box.

A complex truth

The truth was however more complex and more nuanced. Smaller studies created the impression that the most important clinical advantage of rt-PA - the rapid restoration of coronary blood flow - could only be sustained when simultaneously, or shortly after the onset of the treatment, heparin is administered[133] [134]. Heparin is a drug that prevents the formation of new blood clots. Patients receiving rt-PA appear to have a larger tendency to reocclusion of a recanalized blood vessel than patients receiving streptokinase. There is a clear biochemical explanation for this: rt-PA has a relatively short half-life time and thus disappears from the blood more rapidly, allowing the formation of a new blood clot. In addition, rt-PA affects the normal mechanism of blood coagulation less severely than streptokinase. The clinicians performing the trials therefore had the impression that every clinical advantage of rt-PA was lost due to the higher frequency of new occlusions. In another series of studies, it was shown that an accelerated administration of the normal dose of 100 mg rt-PA (in 90 minutes instead of three hours, as was conventionally done)

131 GISSI-2: a factorial randomised trial of alteplase versus streptokinase and heparin versus no heparin among 12,490 patients with acute myocardial infarction. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico. Lancet. 1990; 336: 65-71.

132 In-hospital mortality and clinical course of 20,891 patients with suspected acute myocardial infarction randomised between alteplase and streptokinase with or without heparin. The International Study Group. Lancet. 1990; 336: 71-5.

133 Hsia J, Hamilton WP, Kleiman N, Roberts R, Chaitman BR, Ross AM. A comparison between heparin and low-dose aspirin as adjunctive therapy with tissue plasminogen activator for acute myocardial infarction. Heparin-Aspirin Reperfusion Trial (HART) Investigators. N Engl J Med. 1990; 323: 1433-7.

134 Bleich SD, Nichols TC, Schumacher RR, Cooke DH, Tate DA, Teichman SL. Effect of heparin on coronary arterial patency after thrombolysis with tissue plasminogen activator in acute myocardial infarction. Am J Cardiol. 1990; 66: 1412-7.

causes a more rapid reperfusion without a significant increase of side effects[135] [136].

During the GISSI-2 study, these data were unknown: heparin was administered only twelve hours after the treatment and the conventional application protocol was followed. Under these conditions, rt-PA had no advantage over streptokinase, but GISSI-2 could not prove whether, when applied under optimal circumstances, rt-PA would not be superior after all.

ISIS-3, a (fatal?) blow on top

In 1992, the results of the *ISIS-3* study with 41,299 patients were published[137]. This trial compared rt-PA with streptokinase and with APSAC. The mortality rate was 10.6% for streptokinase, 10.5% for APSAC and 10.3% for rt-PA. Yet again, the differences are not statistically significant. Despite the large number of patients and the use of mortality as endpoint, this study insufficiently delineated the real clinical potential of rt-PA. Heparin was indeed not administered timely and the outdated protocol of 100 mg rt-PA given over three hours was followed. Moreover, the high mortality rates - compared with other studies like the GISSI-2 - caused questions to be raised over the setup and the validity of the ISIS-3 study.[138] [139]

It is clear that the results of the GISSI-2 and *ISIS-3* were bad news for *Genentech*. This way rt-PA would never win the struggle for the market from streptokinase. Unless *Genentech* played all its cards … the GUSTO.

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[135] Carney RJ, et al. Randomized angiographic trial of recombinant tissue-type plasminogen activator (alteplase) in myocardial infarction. RAAMI Study Investigators. J Am Coll Cardiol. 1992; 20: 17-23.

[136] Cannon CP et al. Comparison of front-loaded recombinant tissue-type plasminogen activator, anistreplase and combination thrombolytic therapy for acute myocardial infarction: results of the Thrombolysis in Myocardial Infarction (TIMI) 4 trial. J Am Coll Cardiol. 1994; 24: 1602-10.

[137] ISIS-3: a randomised comparison of streptokinase vs tissue plasminogen activator vs anistreplase and of aspirin plus heparin vs aspirin alone among 41,299 cases of suspected acute myocardial infarction. ISIS-3 (Third International Study of Infarct Survival) Collaborative Group. Lancet. 1992; 339: 753-70.

[138] Sobel BE, Collen D. After ISIS-3. Lancet. 1992; 339: 1225-6.

[139] Sobel BE, Collen D. Questions unresolved by the Third International Study of Infarct Survival. Am J Cardiol. 1992; 70: 385-9.

The 1% of GUSTO

The basic idea behind the GUSTO study (*Global Utilization of Streptokinase and t-PA for Occluded Coronary Arteries*) was that coronary thrombolysis exerts its maximal effect on the heart and the life of the patient when the coronary occlusion is cleared rapidly and permanently. The GUSTO study encompassed four randomized groups, each consisting of 10,000 patients:

• group 1 was treated with streptokinase and subcutaneously administered heparin;

• group 2 with streptokinase and intravenous heparin;

• group 3 with an accelerated dose of rt-PA and intravenous heparin during the treatment;

• group 4 with a combination of streptokinase and rt-PA plus intravenous heparin.

The most important clinical endpoint was mortality after 30 days.

Mega study
In the field of thrombolysis research, the GUSTO 1 is still considered as the 'mother' of the clinical thrombolysis studies. In total, 41,021 patients were involved in the study, originating from 1,081 hospitals from fifteen countries: the US (with 23,105 patients), Israel (2,944), Canada (2,898), the Netherlands (2,299), Australia (2,287), Belgium (2,030), Germany (1,282), France (1,239), the United Kingdom (1,050), New Zealand (666), Spain (467), Poland (360), Switzerland (209), Ireland (185) and Luxembourg (22).

The first patient was treated on 27 December 1990, the last one on 22 February 1993. The trial was coordinated by cardiologist Eric Topol, who meanwhile moved to the *Cleveland Clinic Center* in Cleveland (US). He had, as mentioned earlier, treated the first patient with rt-PA at the *Johns Hopkins University*. The *Steering Committee* consisted of 30 other prominent cardiologists representing the most important participating centers.

The practical aspects of the trial were coordinated by the *Duke University Medical Center* in the US and by the K.U.Leuven, with Robert Califf and Frans Van de Werf, respectively, acting as the clinical managers. David Stump of *Genentech* also closely monitored the study. In addition, there was a *Data and Safety Monitoring Board,* as well as a *Stroke Review*

Committee. *Bayer*, *CIBA-Corning*, *Genentech*, *ICI Pharmaceuticals* and *Sanofi Pharmaceuticals* financed the trial.

All members of the steering committee, the *Data and Safety Monitoring Board* as well as the *Stroke Review Committee* had to declare in writing that neither they, nor their family members, had any financial interest with any of the sponsoring organizations in the form of shares, honoraria for consultancies or travel and subsistence allowances. Also, the *principal investigators* of all the participating centers had to declare that they owned no shares of one of the companies involved.

In addition to GUSTO, the GUSTO *Angiographic Study* was set up. This trial would determine in a quantitative manner to what extent the recanalization rate contributed to a survival benefit. This study involved a subgroup of 2,431 patients.

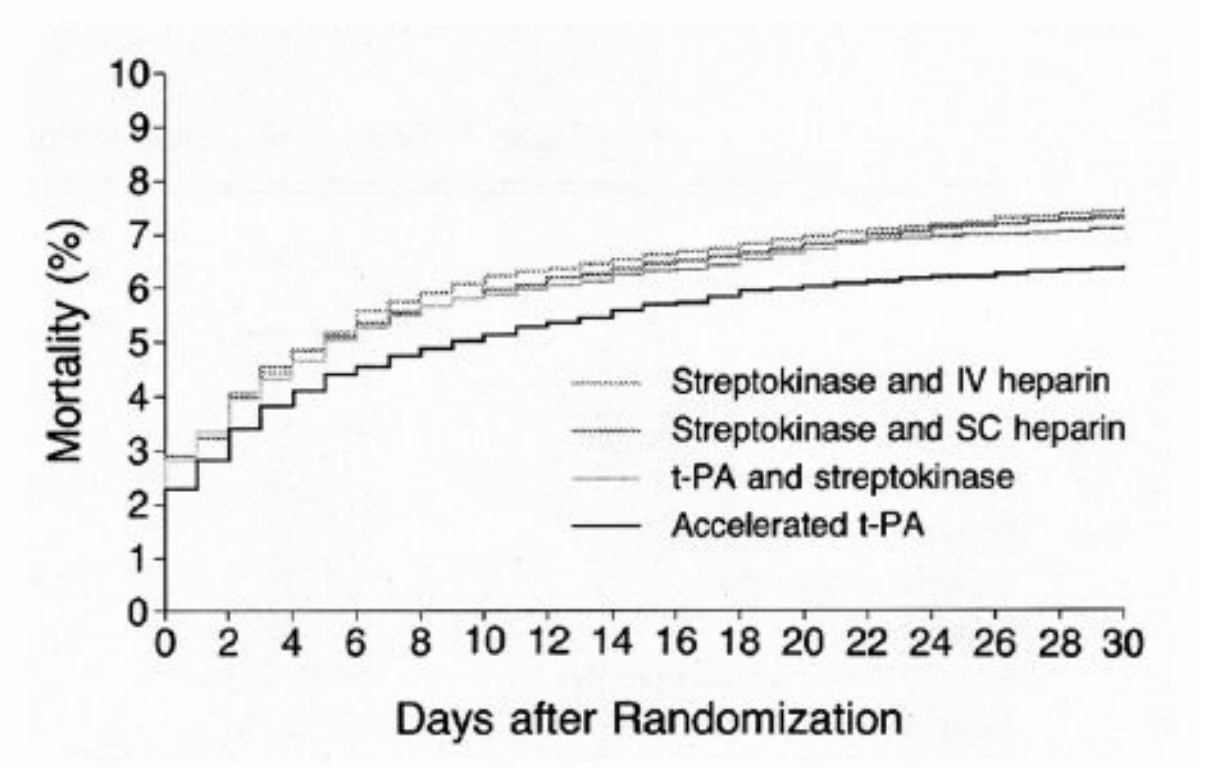
Combined data

The results of both the GUSTO and the GUSTO *Angiographic Study* were published in September and November 1993 in *The New England Journal of Medicine*[140] [141]and can be summarized as follows[142]: the 30 day mortality amounted to 6.3% in the group with the accelerated rt-PA protocol with simultaneous administration of heparin (Group 3); 7.2% in the streptokinase group with subcutaneous heparin (Group 1); 7.4% in the streptokinase group with intravenous heparin (Group 2) and 7.0% in the group with the combination of rt-PA and streptokinase (Group 4). The mortality rate in the rt-PA group is in absolute figures 1% lower (or in relative figures 14% lower) than in the streptokinase groups. This difference was statistically significant (p = 0.001).

[140] An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. The GUSTO investigators. N Engl J Med. 1993; 329: 673-82.

[141] The effects of tissue plasminogen activator, streptokinase, or both on coronary-artery patency, ventricular function, and survival after acute myocardial infarction. The GUSTO Angiographic Investigators. N Engl J Med. 1993; 329: 1615-22.

[142] Sobel BE and Collen D. Controversy and clarification: preliminary results of the GUSTO trial. In: "Coronary thrombolysis in perspective", Eds. BE Sobel and D Collen, Marcel Dekker Inc, New York, N.Y. 1993, p. 303-316.



Results of the GUSTO study: thirty-day mortality in four treatment groups. The group with the accelerated rt-PA administration had the lowest mortality rate.

Furthermore, the *net clinical benefit* - survival without incapacitating stroke - was highest in Group 3 with rt-PA and simultaneous heparin (6.3% deaths with 0.6% strokes) in relation to the other groups (Group 1 - 7.2% deaths and 0.5% strokes, Group 2 - 7.4% deaths and 0.5% strokes, Group 4 - 7.0% deaths and 0.6% strokes). Also with respect to the incidence of various other heart and arterial complications, the Group 3 treatment achieved the best results.

It follows from the GUSTO *Angiographic Study* that mortality was lowest in the group of patients with reperfusion within 90 minutes. Reperfusion occurred fastest in patients with rt-PA treatment. This settled the ongoing controversy: the faster the blood supply is restored the higher the survival benefit.

The results of both GUSTO studies are not only convincing because of their statistical significance, but also because of the internal consistency over the different analyzed subgroups.

1% mortality reduction at a reasonable price

Outsiders might ask whether a difference in mortality of 1% is indeed that relevant. How important is a decrease in the mortality rate of 7.3% to 6.3% for society? In this context, we must consider the high number of people who suffer a myocardial infarction every year and who benefit from rapid thrombolysis.

Based on the published incidence statistics, this mortality rate difference implies that rt-PA saves six lives per day in the US alone, that would not otherwise have been saved with streptokinase. On a yearly basis this means 2,000 saved lives in the USA.

Because rt-PA is more expensive than streptokinase, society pays a price for these saved human lives. A simple calculation learns us that in order to save one more human life with rt-PA than with streptokinase, 100 patients must be treated. The cost of one saved human life thus amounts up to $200,000. This raised the question in the American media whether a human life is actually worth such an amount of money. In 1993, a similar question caused a lot of commotion. For many people no price is too high for a human life and good health is priceless. On the other side, the budgets for health care are limited and the $200,000 that is spent to save a patient with a myocardial infarction can not be used for treating patients with other diseases. In other words, in health policy priorities must be set.

Today health economists frequently raise this question. Also for rt-PA this question required appropriate answers. One of the first relevant pharma-economic analyses of rt-PA was performed by two New Zealanders, L. B. Barradell and K. L. Goa[143]. Based on the GUSTO data, they calculated that, for an American patient, the so-called cost-effectiveness ratio for accelerated rt-PA therapy versus streptokinase amounts to $32,687 per life year gained (dollar rate of 1993). This surcharge falls under the limit of $50,000/life year gained which is accepted in the majority of Western countries as standard for an acceptable investment into the health care system. Today there are many treatments that are much more expensive per life year gained. Thus, rt-PA as a thrombolytic agent is clinically superior to streptokinase and can be administered at an acceptable extra cost.

After the GUSTO study, rt-PA became the drug of choice for most of the cardiologists in the Western world. For *Genentech* the outcome of the GUSTO study was reason enough to bottle a special Chandon 'Cuvée Gusto'.

[143] Barradell LB, Goa KL. Alteplase: a pharmacoeconomic evaluation of its use in the management of myocardial infarction. Pharmacoeconomics. 1995; 8: 428-59.

Château Genentech – Cuvée Gusto 1993.

rt-PA, epilogue

After the publication of the GUSTO trial, the clinical use of rt-PA was to increase markedly. The sales of rt-PA, commercialized by *Genentech* under the name Activase (product name alteplase), increased from $182.1 million dollar in 1992 to $236.3 million in 1993. Also, the market share grew from 50% to 66% in one year's time. The following years would become commercial top years for rt-PA. Especially 1995 stands out with sales amounting to $301 million and a market share close to 80%. The $600 million annual sales predicted by the periodical *Fortune* would, however, never be realized.

Between the approval of rt-PA as trombolytic agent in 1987 and today, *Genentech* will sell more than $4.7 billion's worth of thrombolytic drugs. Since 2005, the use of thrombolytic agents has increased again markedly, not so much for heart patients but for the treatment of stroke and other forms of thrombosis. The *Genentech* sales figures were up again, rising from $180 million in 2002 upto $275 million in 2008[144].

The amount of rt-PA sold in the rest of the world, judging from the annual royalties' report of Genentech to LR&D, nearly equals the amount in the US. The amount on royalties paid to LR&D and its beneficiaries in the period from 1987 to 2006, when the original patents and agreements expired, amounted to $144 million.

The children of rt-PA

Recombinant t-PA in the form of alteplase (Activase) was challenged by a number of derivatives and competitors from the second half of 1990. Reteplase being the first. It is a mutant in which part of the original t-PA protein is deleted. It has the advantage of being more stable when injected intravenously.[145] [146] [147]In contrast to alteplase, that is administered as an infusion over 90 minutes, reteplase can be given in two consecutive

[144] All numbers based on the numbers provided annually by *Genentech between* 1987 and 2009, www.gene.com/gene/ir/financials/earnings-releases/index.jsp

[145] Bode C, et al. Randomized comparison of coronary thrombolysis achieved with double-bolus reteplase (recombinant plasminogen activator) and front-loaded, accelerated alteplase (recombinant tissue plasminogen activator) in patients with acute myocardial infarction. The RAPID II Investigators. Circulation. 1996; 94: 891-8.

[146] Smalling RW, Bode C, Kalbfleisch J, Sen S, Limbourg P, Forycki F, Habib G, Feldman R, Hohnloser S, Seals A. More rapid, complete, and stable coronary thrombolysis with bolus administration of reteplase compared with alteplase infusion in acute myocardial infarction. RAPID Investigators. Circulation. 1995; 91: 2725-32.

[147] Bode C, Kohler B, Moser M, Schmittner M, Smalling RW, Strasser RH. Reteplase (r-PA): a new plasminogen activator. Expert Opin Investig Drugs. 1997; 6: 1099-104.

injections. The GUSTO III trial, with more than 10,000 patients, in which reteplase was compared directly to alteplase, could however not demonstrate that reteplase is superior to alteplase[148].

Another successor developed and marketed by *Genentech* itself, is tenecteplase (also known as TNK-t-PA). This form of rt-PA has three mutations (**T**103, **N**117, **K**HRR296-299) making the protein more stable in the blood, enhancing the fibrin-specificity and delaying the inhibition rate. Because of these modifications, TNK-t-PA therefore can be administered through one single injection instead of an infusion. In a direct comparison, with more than 16,000 patients, tenecteplase appears to be equivalent to its parent molecule alteplase, as far as 30-day' mortality is concerned. In patients that received treatment only fours hours after the onset of the first symptoms, tenecteplase even performed better[149]. The frequency of cerebral hemorrhage is hardly different (0.93% for tenecteplase and 0.94% for alteplase)[150].

The major advantage of tenecteplase over alteplase is the speed at which it can be administered. An experiment in the United Kingdom showed that tenecteplase requires less preparation time (on average 10.5 minutes) whereby the number of patients that receive treatment within 30 minutes of arrival in the hospital rose from 58% to 78 %[151].

Thrombolysis or angioplasty and stenting

Although thrombolytic therapy saved the lives of tens of thousands of heart patients, standard thrombolytic therapy also has important limitations. It, indeed, takes time to dissolve a blood clot (in general 45 to 60 minutes after the onset of the treatment) and a

[148] A comparison of reteplase with alteplase for acute myocardial infarction. The Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO III) Investigators. N Engl J Med. 1997; 337: 1118-23.

[149] Assessment of the Safety and Efficacy of a New Thrombolytic (ASSENT-2) Investigators, Van De Werf F, Adgey J, Ardissino D, Armstrong PW et al. Single-bolus tenecteplase compared with front-loaded alteplase in acute myocardial infarction: the ASSENT-2 double-blind randomised trial. Lancet. 1999; 354: 716-22.

[150] Van de Werf F, et al. ASSENT-2 Investigators. Assessment of the Safety and Efficacy of a New Thrombolytic. Incidence and predictors of bleeding events after fibrinolytic therapy with fibrin-specific agents: a comparison of TNK-tPA and rt-PA. Eur Heart J. 2001; 22: 2253-61.

[151] Leah V, Clark C, Doyle K, Coats TJ. Does a single bolus thrombolytic reduce door to needle time in a district general hospital? Emerg Med J. 2004; 21: 162-4.

complete recanalization of the vessel is only achieved with 50% to 60% of the patients[152]. Moreover, in 5% to 15% of the patients a reocclusion occurs[153] and the risk for cerebral hemorrhage remains, especially in older patients.

Cardiologists in the 21st century agree that timely performed percutaneous coronary intervention (PCI) yields better results than thrombolysis. PCI is also known as 'angioplasty and stenting': via a catheter, inserted in the groin, the thrombus is removed from the obstructed blood vessel, which can eventually be dilated with a little balloon, and the interventional cardiologist then places a stent - a metal feather which prevents the artery from collapsing.

Percutaneous coronary intervention, at least when it is performed by an interventional cardiologist with extensive experience, is associated with less mortality on the short term than thrombolysis (7% versus 9%), a lower risk of a recurrent infarct (3% versus 7%) and a lower frequency of cerebral hemorrhage (1% versus 2%). Also over a longer period of time, PCI leads to a lower mortality rate[154].

The major problem, however, is that PCI requires a specialized cathlab and is therefore not always timely available to all patients. Patients must indeed be treated between 90 and 120 minutes after the first onset of symptoms. In a country like Belgium, with its well developed network of hospitals with a cardiology center and a good road infrastructure, it is generally possible to perform PCI in a timely manner. Precious time is often lost because the patient does not recognize the symptoms and calls in medical assistance too late. In other countries, where the distances between peripheral hospitals and a specialized cardiology center are much larger, timely PCI is not always possible and thrombolysis remains the treatment of choice.

A second life

While thrombolytic therapy for myocardial infarction patients is increasingly being replaced by PCI, the focus of clinical thombolysis research is now more on the treatment of

[152] Van de Werf FJ, Topol EJ, Sobel BE. The impact of fibrinolytic therapy for ST-segment-elevation acute myocardial infarction. J Thromb Haemost. 2009; 7: 14-20. Review.

[153] Topol EJ. Acute myocardial infarction: thrombolysis. Heart. 2000; 83: 122-6. Review.

[154] Keeley EC, Boura JA, Grines CL. Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials. Lancet. 2003; 361: 13-20. Review.

stroke or CVA (cerebrovascular accidents). There are two kinds of strokes: in more than 80% of the cases obstruction of the blood flow in a part of the brain occurs, caused by a blood clot (ischemic stroke, CVA). The remaining 20% of the cases concern cerebral hemorrhage, which results from rupture of a blood vessel, whereby blood flows into the brain (hemorrhagic CVA)[155].

Just as with myocardial infarction, the lack of oxygen in the downstream tissues affects the survival of brain cells. Again, it is crucial to restore the blood flow as quickly as possible[156]. On the other hand, one has to realize that thrombolytic therapy is contra-indicated in the case of a hemorrhagic CVA.

At first, thrombolytic treatment of stroke was not really succesful. Mainly because the imaging techniques did not yet allow to distinct an ischemic from a hemorrhagic CVA, or to show that the symptoms may be caused e.g. by the presence of a tumor. Furthermore, some patients in these trials received treatment very late, sometimes days or even weeks after the first onset of symptoms[157].

In the middle of the 1990's, several randomized and placebo-controlled clinical trials are published in which various thrombolytic drugs, including rt-PA, were tested[158]. Based on the results of this study by the American NIH, rt-PA was in 1996 approved by the FDA, as the only thrombolytic agent for the treatment of ischemic CVA.

Many other studies would follow. From the pooled data, it seems that rt-PA reduces morbidity as a consequence of stroke on the long term with 33%. It is however necessary that thrombolysis is started as quickly as possible - preferably within an hour after the onset of the stroke, but definitely within three hours[159]. rt-PA treatment indeed also has a

[155] Albers GW, Amarenco P, Easton JD, Sacco RL, Teal P. Antithrombotic and thrombolytic therapy for ischemic stroke: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest. 2004; 126: 483S-512S. Review.

[156] Siesjo B. Pathophysiology and treatment of focal cerebral ischemia: I. Pathophysiology. J Neurosurg 1992; 77: 169–84.

[157] Brott T, Broderick J, Kothari R. Thrombolytic therapy for stroke. Curr Opin Neurol. 1994; 7: 25-35. Review.

[158] Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med 1995; 333: 1581–7.

[159] Albers GW, Amarenco P, Easton JD, Sacco RL, Teal P; American College of Chest Physicians. Antithrombotic and thrombolytic therapy for ischemic stroke: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8thEdition). Chest. 2008; 133: 630S-69S.

downside: in 6% of the patients additional cerebral hemorrhage occurs[160], possibly, due to late administration of rt-PA. The blood vessels and the neuronal tissues distal from the blood clot can already be severly damaged due to the lack of oxygen and the release of stress hormones. For the damaged tissues, rt-PA and other thrombolytic agents (possibly with the exception of microplasmin - later more on that issue) could be toxic, because the plasmin that they generate also degrades proteins that connect the nerve cells with the extracellular matrix. Disruption of these connections causes increased apoptosis of the already stressed cells[161] [162] [163].

The rigorous clinical guideline that rt-PA should not be administered later than three to maximally four hours after the onset of the first symptoms, means in practice that only 3% to 5% of the CVA patients qualify for a thrombolytic treatment[164].

Besides being a 'clot buster' for myocardial infarction and stroke, rt-PA is also used to dissolve blood clots in the lungs (pulmonary embolism)[165] [166] and deep for vein thrombosis[167]. Recently, American researchers discovered that rt-PA can restore blood flow in the frozen limbs of mountaineers, resulting in a spectacular decrease in the number of amputations[168].

Who knows which lives rt-PA will still save in the future.

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[160] Hacke W, Donnan G, Fieschi C, et al; ATLANTIS Trials Investigators; ECASS Trials Investigators; NINDS rt-PA Study Group Investigators. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. Lancet. 2004; 363: 768-74.

[161] Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA. Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. Nat Med. 1998; 4: 228 –31.

[162] Chen ZL, Strickland S. Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. Cell. 1997; 91: 917–25.

[163] Nagai N, Vanlinthout I, Collen D. Comparative effects of tissue plasminogen activator, streptokinase, and staphylokinase on cerebral ischemic infarction and pulmonary clot lysis in hamster models. Circulation. 1999; 100: 2541-6.

[164] Delude C. Clot-Busters!! – Discovery of thrombolytic therapy for treating heart attack and stroke. Breakthroughs in Bioscience, FASEB 2004, 13.

[165] Wan S, Quinlan DJ, Agnelli G, Eikelboom JW. Thrombolysis compared with heparin for the initial treatment of pulmonary embolism: a meta-analysis of the randomized controlled trials. Circulation. 2004; 110: 744-9.

[166] Dong B, Jirong Y, Liu G, Wang Q, Wu T. Thrombolytic therapy for pulmonary embolism. Cochrane Database Syst Rev. 2006; 2: CD004437. Review.

[167] Watson LI, Armon MP. Thrombolysis for acute deep vein thrombosis. Cochrane Database Syst Rev. 18; 4: CD002783. Review.

[168] Bruen KJ, Ballard JR, Morris SE, Cochran A, Edelman LS, Saffle JR. Reduction of the incidence of amputation in frostbite injury with thrombolytic therapy. Arch Surg. 2007; 142: 546-51.

Looking back on the t-PA story

Much has happened since the experiment in February 1979, in which we demonstrated for the first time, the fibrin-specificity of the plasminogen activator secreted by the Bowes melanoma cell line. More than three million heart patients have been treated with rt-PA and the drug continues to save lives.

Often I am asked whether I perhaps regret selling the rights on t-PA to *Genentech* and if I should not have founded my own 'Collen Pharmaceutica' instead. The development of drugs was already in the 1980's a complex business, and today even more so. Moreover, with t-PA, we faced a new era, namely that of the biotechnological drugs. In my opinion, we did not have the expertise to clone a protein like t-PA in Flanders at that time, nor the technology to produce sufficient quantities of rt-PA in mammalian cells. I never egretted the agreement between LR&D and *Genentech*. On the contrary, *Genentech* was and still is a technological 'power house' and has done every effort to turn t-PA into a success story. Moreover, they remained a loyal partner during the entire period from the cloning to the marketing, and largely kept their promises.

Section III.
Business between Academia and Industry

From LR&D via Innovi to Thromb-X and ThromboGenics Ltd

Recombinant t-PA turned *Genentech* into a major biotechnology company and it gave our laboratory and myself a certain financial independence. Everybody knows that, and I have never denied it. The royalty flow from rt-PA - in total $144 million - was however largely invested in new research projects. This always happened, as was agreed to in 1976, after consultation and in agreement with the Managing Board of LR&D, mandated by K.U.Leuven and owner of the patent rights.

Yet, this did not stop some colleagues and officials from ventilating their displeasure. One of the objectives that I want to achieve with this book is to demonstrate in a transparent manner that the rt-PA royalties were used in the best possible way. They enabled world-class research in Leuven, and even the development of industrial biotechnology.

Basic documents

Three documents are important as the basis for my discussion on the way the royalties were used. They were already discussed earlier in this book:

1. The agreement which I reached in February 1976 with *Leuven Research & Development* , in which the rights of my research were transferred to LR&D and in which it is clearly stated that the modalities for distribution of potential income would be determined after mutual consultation between LR&D and myself (Appendix 2). Therein the following was explicitly stipulated: *'Dr. D. Collen and his colleagues, employed by the Catholic University of Leuven, relinquish all juridical, commercial and financial rights, as well as the execution thereof, of research results, which they accomplish directly or indirectly, in the framework of their teaching and research at the Catholic University Leuven'.*
   Income that LR&D would generate based on our research would be divided on the following basis: the K.U.Leuven receives 10% of the income accrued, LR&D 7%, and after deduction of all expenses related the execution of its mandate, LR&D will keep available a minimum of 50% of the remaining part for future research in our laboratory. The remaining part could be used as personal indemnification to the inventors and/or colleagues.

The document concludes, however, with: *'The specific distributing modalities shall be determined at a suitable time after mutual deliberation between LR&D and Dr. D. Collen and submitted to the Management Board of LR&D for approval. The Management Board of LR&D can at all times change its opinion according to the circumstances.'*
This means that, for K.U.Leuven, LR&D is the only owner of the legal, commercial and financial rights on inventions made in our laboratory, and that we by mutual agreement, but subject to approval by the Management Board of LR&D, determine how the income on these inventions will be divided. I explicitly draw attention to this contractual agreement because the transfer of means to, among others, the *D. Collen Research Foundation* and the *D. Collen Trust,* could not even partially be considered by the treasury as donations to myself, that might be subject to capital transfer taxes or even to limitations to the indicated goals under the Belgian inheritance legislation.

2. The patent on t-PA was submitted on 11 June 1980 through LR&D with as inventors Désiré Collen, Dick Rijken and Osamu Matsuo (Appendix 3). The patent was first submitted in the Netherlands with patent application number 8003402, and would later be extended to the US and awarded under the patent number 4,752,603. Without this patent, we would never have received royalties on rt-PA.

3. The initial non-exclusive cooperation agreement (*Cooperative Letter of Agreement*) signed by Jos Bouckaert, Director of LR&D, in September 1980 with *Genentech*, in which LR&D claimed a royalty of 1% from all income that *Genentech* would ever generate from rt-PA. At that stage, our collaboration with *Genentech* was not yet exclusive, but *Genentech* already had the right of pre-emption (Appendix 4).

These three documents are the basis of all later agreements and contracts that will be concluded between the parties.

A new player – Innovi

In February 1982, LR&D npo (non-profit organization) signed an agreement with the 'innovations-management-association' Innovi NV (Inc) to transfer control of some LR&D files. Innovi was established a year earlier in the broader context of the DIRV (Third Industrial Revolution in Flanders) action. DIRV was established at the beginning of the Eighties by the chairman of the Flemish Community ('Vlaamse deelregering'), Gaston Geens. In the same period and in a similar context, the Flemish Regional Investment Fund (GIMV) was also founded.

The quiet intention of the politicians and the Flemish industry was to turn Innovi into a kind of LR&D, but then involving all the universities. Innovi was supposed to support the valorization of academic research in Flanders and, as far as possible, to allow Flemish companies to benefit from this research activity. Officially, the goal of Innovi was to support businesses by providing services dealing with identification of inventions, technologies, know-how; the acquisition of intellectual property, the placing and controlling of development contracts and the signing of licensing agreements with interested industrial and commercial parties.
Innovi would play a role in the foundation of the Flemish biotechnology companies *Plant Genetic Systems* and *Innogenetics*.

It was the intention to transfer also the t-PA files to Innovi so that they could manage the administrative and technical aspects. The transfer took place officially on 12 July 1982 and LR&D instructed Innovi:

• to duly execute the existing agreements with *Genentech*;

• to conclude potentially new agreements on plasminogen activator with *Genentech*, or a third party;

• to accept responsibility for the maintenance of patent applications and to follow up the procedure to the point of approval.

The agreement also stated that Innovi would receive a compensation of 12% on the revenues of t-PA contracts and patents. LR&D transfered its 7% from the agreement of February 1976 to Innovi and the K.U.Leuven would thus transfer half of its 10% share. K.U.Leuven would keep 5% of the rt-PA revenues.

A pat on the back

At that time, I thought there were good reasons to transfer the management of the t-PA contracts to Innovi. In the first place because Jos Bouckaert was going to leave LR&D to take up the position of Managing Director of Innovi. He knew the t-PA file particularly well, and I thought at the time that not enough expertise was left within LR&D to take proper care of my files - a feeling that Jos Bouckaert gave me. Moreover, I thought that Jos could use a helping hand in starting up Innovi, certainly since the input from other universities was only marginal.

The helping hand that Innovi thus received increasingly turned out to be nothing but the sequestration of limited available research resources. One example: between July 1982 and October 1985, Innovi was allowed to charge $78,000 (12% of $650,000) based on

the existing contract between LR&D and *Genentech* of November 1981. That was easy money since the only actions Innovi had to take, was sending an invoice to *Genentech* every three months and to check whether the revenues were devided according to the agreements.

Exclusive agreement with *Genentech* - 1983 version

On 18 March 1983, a new agreement was reached with *Genentech* - this time between Innovi and *Genentech*, with approval of LR&D and myself. To describe it as 'concluded between Innovi and *Genentech*' is quite an overstatement! I personally negotiated the new contract with Jim Gower of *Genentech* and Innovi's sole contribution consisted therein that Jos Bouckaert gave his approval after one single reading session during a dinner at my house.

The contract of 18 March 1983 was mainly an extension of the contract of 1981. The main difference was that *Genentech* obtained the exclusive world-wide sales rights on rt-PA, for as long as the patent of LR&D was valid. In exchange for the exclusivity, *Genentech* would pay 3% royalties on sales in the US and 2% in the rest of the world. In other words, *Genentech* doubled or tripled the royalties.

At that point in time, it was still four and a half years to the approval by the FDA (November 1987) of rt-PA as thrombolytic drug and more than ten years to the publication of the GUSTO trial (November 1993). All of us were hoping that rt-PA would become a success based on the first results with patients, but nobody could predict it. I clearly recollect how I, during a conversation with ex-minister Gaston Geens towards the end of the eighties, enthusiastically proclaimed that t-PA would bring about one billion BEF in royalties to Belgium. Mr. Geens congratulated me, but clearly did not believe it.

Over and out with Innovi

Both LR&D and the K.U.Leuven realized that Innovi was quite abundantly rewarded just to execute some simple administrative tasks. On 14 June 1984, reluctantly a new agreement was made in which Innovi agreed to reduce its compensation from 12% to 10% and to transfer 2% to LR&D to cover expenses relating to personnel administration.

Actually, the contribution of Innovi was limited to that of a mere postbox function. Indeed, the new contracts were negotiated by myself while the responsibility for upholding and protecting the t-PA patents was officially transferred to *Genentech* on 23 February 1984. Between July 1982 and October 1985 there was never any significant professional input from Innovi in the t-PA story, nor did it ever take any financial risk.

Nevertheless, as stated earlier, they received $78,000 for occasional administrative tasks.

LR&D and I agreed that this situation would become even worse when rt-PA would really become a successful thrombolytic agent. Innovi would then collect one tenth of the royalties as administrative overhead, which could amount to at least several hundreds of thousands of dollars per year. Needless to say that I was convinced that this money could serve for better purposes. After I learnt that Jos Bouckaert would be leaving Innovi - he left for the vineyards of California where he pursued a successful career as manager of a company involved in genetically improved vines - I agreed that LR&D would cancel the contract with Innovi. This was done on 30 September 1985 by Jacques Vander Eecken, then chairman of the Board of Directors of LR&D.

Innovi bought out

This termination of the agreement was, however, not gracefully accepted by Innovi. I then went to see the chairman and several members of the Management Board of Innovi NV (R. Stouthuysen of Jansen Pharmaceutica, J. Hinnekes of the Boerenbond and G. Declerq of the Kredietbank) and then, when realizing that they would not agree, I got angry with them. It would take until the end of December 1988 before the issue was finally settled. The matter was even taken to court, but turned out to the disadvantage of LR&D.

Finally, the only way out was to buy out Innovi. For that purpose, Innovi established the company NV t-PA, which owned all legal rights and agreements on t-PA. An independent external auditor assessed the value of NV t-PA on 32,001,000 BEF. This was the amount that LR&D had to pay to take over all the shares of NV t-PA in order to regain all the rights on the rt-PA royalties. Collecting the money were the shareholders of Innovi, the top of the Belgian and Flemish financial and business sectors, with among others, the Generale Maatschappij, the GIMV, Ibel, Investco, Prominvest, Agfa Gevaert, Aveve, Bekaert, Bell, Janssen Pharmaceutica and MHO.

My proposal to the Management Board of LR&D to take over the NV t-PA with central LR&D means was, however, declined. Eventually, the NV t-PA was bought by my '*Protein Research Division*' within LR&D. In this way 10% of the future earnings from t-PA would go to our

laboratory. This investment of 32 million BEF later yielded in total approximately 350 million BEF of royalties for our laboratory and *Thromb-X* (see further).

The NV t-PA

The NV t-PA will still exist for several years and remain entitled to 10% of the royalty flow from rt-PA. The NV t-PA will from now on be managed from within LR&D, and the Management Board will consist of representatives of LR&D, including myself. An agreement with K.U.Leuven was concluded which stipulates that the NV t-PA will sponsor the research on therapeutic products for cardiovascular medicine at the Center for Thrombosis and Vascular Research (K.U.Leuven). The first year (from 15 January 1990 to 14 January 1991) the NV t-PA paid 33 million BEF. This amount covered the employment of qualified scientific collaborators, laboratory as well as operational - 'investing' - costs and overhead. This agreement would be renewed three more times and successively 15 million (1991), 22.5 million (1992) and 20 million BEF (1993) would be transfered to the laboratory. Over these four years, this amounts a total of 90.5 million BEF – about € 2.25 million.

At first sight, the NV t-PA seemed to be a perfect system for the direct investment of rt-PA royalties in academic scientific research. But, the fiscal authorities looked with increasing suspicion at these untaxed transfers, because the construction was not really conform to the viewpoint of Belgian tax authorities. The channeling of the royalties through LR&D, a non profit organization, was indeed subject to a tax of 25% on 85% of the amount received. Therefore, it appeared wiser to merge the NV t-PA with Thromb-X on 4 November 1993.

The merger of NV t-PA with *Thromb-X* was approved by all parties, *in casu* the top of LR&D, backed by K.U.Leuven and myself. The merger document (see Appendix 8) was signed by Jacques Vander Eecken and Karel Tavernier on behalf of NV t-PA, (respectively Chairman of LR&D and General Manager of K.U.Leuven). For *Thromb-X* Hans Claes signed. Things did not really change for LR&D and K.U.Leuven. LR&D was after all the main shareholder of NV t-PA and it brought the shares into *Thromb-X* of which it also owned half the shares.

Thromb-X

Thromb-X was established on 20 December 1991 as an NV (see Appendix 9). There were two partners in the company, *Leuven Research & Development* (in other words the *Protein Research Division* of LR&D) and myself, as private-person, both subscribing to half of the

shares. The contribution of each partner amounted to 12.5 million BEF in capital shares of 10,000 BEF and in addition an equal number of founders' shares. The money that I contributed came from my personal share in the rt-PA royalties.

Hans Claes became Managing Director of *Thromb-X*. The objective of the company was *'the execution of all managing and financial activities, more specifically: research in, development of and sales of diagnostic and therapeutic substances for the prevention or removal of thrombosis, and other pharmaceuticals in the area of human medicine.'*

With *Thromb-X,* I had a clear objective in mind and at the same time a dream. My dream was to establish a Flemish mini-*Genentech*; my objective was to develop an alternative thrombolytic drug at least as effective or even better than rt-PA that would sell for a fraction of the rt-PA price. I already had a candidate at that stage: staphylokinase (see chapter Staphylokinase – t-PA for the poor) that was licensed from LR&D, from *Yakult Honsha* in Japan and from the former East German Institute for Sciences in Jena. *Thromb-X* had to enable the development of staphylokinase right upto the end phase, in other words from the concept phase and the biochemical characterization, through the preclinical and clinical research phases up to the market introduction. To channel a drug through these different phases from within an academic environment is impossible. The required expertise or the financial resources are not available. The t-PA story illustrates that an industrial partner is indispensable. *Thromb-X* had to be that ideal partner: on the one hand it could benefit from a steady flow of royalties, thanks to the merger with NV t-PA, and on the other hand it would search for additional investors. Over the next years, I myself progressively bought out the *Protein Research Division* of LR&D from *Thromb-X* at 20,000 BEF for the founders' shares and 30,000 to 60,000 BEF for the capital shares. This increased the virtual value of the company, which was important to limit dilution after eventual future investments.

In 1997, 54.5 percent of future rt-PA royalties were sold for 675 million BEF by LR&D to Thromb-X, and in 1998, the remaining 35.5 percent - in a mutual agreement between LR&D and myself - was brought under the umbrella of a non-profit organization, Biggar Ltd, who subsequently sold them to Thromb-X NV for 600 million BEF (the remaining 10% were already sold to Thromb-X by NV t-PA).

Thromb-X and 4C

Thromb-X performed translational research on stem cells during the second half of the nineties until approximately 2006. In 1996, Luc Schoonjans was sent to the laboratory of Randall Moreadith in Dallas in order to try to generate transgenic rabbits. The procedure

was as follows: first a gene is manipulated in embryonic stem cells, the cells are then inserted into host blastocysts (early stage embryos), out of which chimeric animals are bred that transmit the manipulated gene to the next generation. This technique is a routine procedure with mice since the 1980's, but failed with other species. After many years of intensive effort, Schoonjans succeeded in generating chimeric rabbits, but the transmission of the genetic modification to the offspring failed. He did produce a markedly improved cell culture medium and genetically pure embryonic stem cell lines of presumed 'non-permissive' mouse strains, which proved to be high-quality reagents for stem cell research. In the meantime, these reagents are licensed out by *Thromb-X* to companies active in this area.

Allow me a remarkable anecdote in this context. In order to produce Schoonjans' 'improved' culture medium on a large scale, we engaged with *4C Biotech* (*4C*) in Seneffe, a small biotechnology company founded by Alain Miller, biochemist and professor at the university of Mons. Initially, *4C* wanted to develop a new cell culture technology, but gradually it evolved into a pure 'service provider'. The material that *4C* produced for *Thromb-X* was of a very good quality. When it became apparent that the founder had no future in his own company - a planned reorganization - *Thromb-X* and Alain Miller signed an agreement to take over the shares of Miller (kept by his management company, CIL), and thus the control over *4C* (see appendix 10).

The legal adviser of Miller drew up the agreement in French. During lunch with his son and legal adviser Axel Miller, on the top floor of the headquarters of Dexia in Brussels, the deal was concluded in principle while tasting a sensational bottle of Château Palmer (a top Margaux, that definitely must have cost Dexia € 100). Following a final teleconference with Miller, who was at a meeting in Italy at the time, the agreement was signed in my office Gasthuisberg on Thursday 3 October 2002 by myself for *Thromb-X* and by Mister Poskin, the chief financial officer (CFO) of *4C.* Miller gave his approval by telephone while he was on the line.

Coup de théatre the next Monday morning: I was informed that the purchase of the CIL shares of Miller to *Thromb-X* was cancelled. *GSK* appeared to have purchased *4C* and intended to accommodate it in *Henogen*. I was aware that *GSK* had shown interest in *4C,* but according to Miller it was "totally out of the question" that he would be making a deal with *GSK*. Apparently, over the weekend, after Miller's return from Italy, GSK made a proposal that now was acceptable to him. A telephone conversation with his son, Axel Miller and with Jean Stephenne, CEO of *GSK* in Belgium, did not help, even while I emphasized that we had

a signed sales agreement with Miller. They suggested that I would have to go to court; he probably decided to hide (out of honest shame?).

I did not find it useful to take this matter to court. To be working in a hostile environment in Seneffe, was not really an attractive option to solve our production problem. I eventually hired three staff members of *4C* in Leuven (they had second thoughts about working for GSK) and started our own mammalian cell culture system for the production of potential biotechnological drugs. Looking back, this turned out to be very valuable for *ThromboGenics NV,* and we are still using it. This was actually fortunate because with the money invested in the purchase of equipment and the development of the technology – instead of in the acquisition of the CIL shares - we now achieved the same, but under our own exclusive control. I leave it to the reader to decide on the moral of this story. To me it is obvious that a high-ranking position in the financial/industrial world is no guarantee for high ethical values.

Thromb-X and the trade unions

In the middle of the Nineties, we had a full time accountant in *Thromb-X* who was proficient and had authorization to use the credit card for daily expenses. Unfortunately, it appeared that he occasionally withdrew small amounts for private use. He entered these costs in the books by adjusting hand written invoices and receipts. When we found out, the accountant admitted tha fact and he was immediately discharged on compelling grounds. The trade union, however, was of a different opinion and advised him to deny the allegations, so that we eventually were summoned before labor court. Our request to allow a graphological expert to examine the 'corrected' invoices was rejected by the court and we were sentenced because we did follow the correct procedures for discharge. We had to pay an indemnification of 750,000 BEF. Apparently, the labor unions believe that even when caught red handed, a staffmember cannot be discharged without compensation.

ThromboGenics Ltd

In order to search for venture capital, we later founded *ThromboGenics* Ltd in Ireland with a founding capital of $1 million. At that time, the Irish legislation was more lenient towards attracting venture capital. *ThromboGenics* Ltd will, among others, obtain funds from *East Hill University Spinouts Fund, Biggar Ltd* and the *D. Collen Research Foundation*, but otherwise the search for capital proved to be very difficult.

It eventually became necessary to bring *Thomb-X* under the control of *ThromboGenics Ltd* by converting the *Thromb-X* shares at acquisition value into an equivalent part of *Thrombogenics Ltd* at five Irish pounds per share. Even then it still was not evident to attrack risk capital. The development of t-PA for the poor is not exactly in the interest of *'Venture Capital'* or *'Business Angels',* with the exception of *Landon Clay* and *East Hill.* Finally, we would not succeed to collect € 40 million to fund the critical phase III study with staphylokinase versus streptokinase in 10,000 to 15,000 patients with acute myocardial infarction.

During our 'road shows' we were occasionally warned that *ThromboGenics Ltd* was a 'one-trick pony' and that our only program provided no good investment proposition for venture capital providers who actually are quite 'anti risk'. This painful experience was an important stimulus for us to actively start working on a products portfolio in *ThromboGenics.* On 30 May 2006, we founded, together with the two largest shareholders, *East Hill* and *Biggar Ltd*, the NV *ThromboGenics*. The intention was to acquire via the Brussels Stock Exchange, the necessary funding for the further development of our ever expanding portfolio of candidate drugs. *Thromb-X* and *ThromboGenics Ltd* would eventually transit into *ThromboGenics NV*. More about that later on.

The D. Collen Research Foundation

After the bad experience with Innovi, the K.U.Leuven, now headed by Rector Roger Dillemans, LR&D and I searched for a good alternative to manage the rt-PA royalties. Indeed, some resentment was arising within the K.U.Leuven against a possible claim by inventors and/or colleagues on maximally 41.5 percent of future t-PA earnings. It became increasingly clear that the earnings would be considerable.

We decided to establish a non-profit association named *D. Collen Research Foundation* (DCRF). To avoid previous mistakes, we called in the external expertise of Lawrence Fouraker, *Dean* of the renowned *Harvard Business School* in Boston, USA. Thanks to my good old friend, Herman 'Chip' Gold, Fouraker accepted a position as director in the *D. Collen Research Foundation* and gave us valuable advice. Fouraker once suffered a heart attack and was treated by Chip with rt-PA. Fouraker found that he had the opportunity to 'return a favor' for the inventor of the drug that saved his life.

Managed jointly

As such, on 2 July 1988 in the Rector's chambers of the K.U.Leuven the following persons founded the *D. Collen Research Foundation* (see Appendix 11):

• I, who was appointed as statutory chairman;

• Rector Roger Dillemans and General Manager Karel Tavernier represented the K.U.Leuven, each with a seat on the Board of Directors;

• Jacques Vander Eecken, Chairman of Leuven Research & Development;

• Lawrence Fouraker represented the *Harvard Medical School,* and was later replaced on the Board of Directors by the Harvard cardiologist, Herman Gold (see also chapter 'pre-clinical t-PA research').

The goal of the foundation was: *'with exclusion of any commerical objective, the execution, promotion and support of scientific research, in general and biomedical and biotechnological research in particular, by making available research grants, research positions, travel bursaries, by organizing scientific congresses and symposia, providing financial support for publications and all related activities that support the advancement of science. Therefore, it may also perform any other economic activities, including the acquisition of any kind of property, on condition that the revenues will be used exclusively to achieve the main goal.'*

The founding of the D. Collen Research Foundation in the chambers of the Rector of K.U.Leuven with from l. to r. Rector Roger Dillemans, Prof. Karel Tavernier, Prof. Désiré Collen, Prof. Lawrence Fouraker and Prof. Jacques Vander Eecken.

In the bylaws, we would formulate the following objectives:

• Granting scholarships to Belgian researchers for further specialization abroad in the areas of biotechnology and biomedical sciences;

• Attracting outstanding researchers in the field of molecular biology to lecture at the K.U.Leuven;

• Contribute to the development of biotechnological infrastructure at the K.U.Leuven and in Flanders;

• Establish a scientific collaboration in the field of molecular biology between the K.U.Leuven and *Harvard Medical School*.

As long as DCRF was active, between 1988 and 2006 (when DCRF was transformed into *Life Sciences Research Partners*), the major part of the t-PA royalties was also divided, according to previously agreed-upon criteria, and in mutual consultation between the K.U.Leuven, LR&D and myself: 5% went directly to the K.U.Leuven, 2% to LR&D, 10% initially went to the NV t-PA (see previous chapter) and was transferred to *Thromb-X* from 1994 onwards, while 41.5% was for the Foundation. Half of the remainder (20.75% of the total) was used for direct support of the Center for Thrombosis and Vascular Research. The other half was due to D. Collen and to the laboratories of Dick Rijken in Leiden and Osamu Matsuo in Osaka, Japan. After all, Dick and Osamu were co-inventors on the t-PA patent and were entitled to

a part of the royalties. In 1997 and 1998, the future t-PA royalty rights were sold to *Thromb-X* for a total amount of 1.275 billion BEF.

The activities of DCRF

The activities of the *D. Collen Research Foundation* are reported in an open transparent manner in the annual reports. These activities cover a broad spectrum. A few examples of the Foundation's activities are:

- Over the years approximately 100 young researchers were able to follow further specialization with the support of the Foundation. Initially, only graduates from the K.U.Leuven were selected, but from 1993 onwards the Foundation joined forces with the *Belgian American Educational Foundation (BAEF)* and also students from other universities were considered. At the moment, and for the next four years, four young researchers per year will be awarded a scholarship from the Foundation, to further develop their skills in the United States.

- DCRF granted substantial support to the *International Society on Thrombosis and Haemostasis.* The *International Society for Fibrinolysis and Proteolysis* was founded with a subsidy of $50,000 from the Foundation.

- Numerous congresses and symposia, organized by professors from Leuven, were sponsored by DCRF.

- My mentor, Marc Verstraete, who after his emeritate collected 'chairs' for the K.U.Leuven (currently to a total amount of over 1 billion BEF) persuaded me to fund two chairs of five million BEF each. One of these apparently went to the laboratory of Alfons Billiau. This seems to me only a small compensation for his contribution to the t-PA story.

- Important national symposia in the area of the life sciences and biotechnology, including '*Life, a Noble Story'* organized by the Section Biotechnology of the KVCV and '*Knowledge for Growth*' organized by *FlandersBio*, received substantial support.

- For severl years, the research by Dr. Jean-Marie Saint-Remy, Dr. Nobuo Nagai and Dr. Bart De Geest from the Center for Molecular and Vascular Biology at the K.U.Leuven was supported by the DCRF.

- The ninth floor of the research building on the campus of the University Hospital Gasthuisberg was built with financial support of the DCRF. During the construction of the Central Service building, financed by the National Health secretary, we took the opportunity to fund an additional floor on the top of the building, on condition that

75 million BEF was donated to the K.U.Leuven for the additional fortification of the foundations, as well as the finishing construction works for the top floor of 3600 m^2. In exchange, 1/3 of the surface was to be made available to the DCRF (the total interior construction would cost 225 million BEF), 1/3 to our university research laboratory (Center for Molecular and Vascular Biology) in exchange for the space previously allocated to use, on the 7th floor in the 'old' building. The remaining 1/3 was initially not assigned, but afterwards DCRF acquired half of that as laboratory space in exchange for a donation of 38 million BEF. The other half was used to build a state of the art '*Specific Pathogen Free Animalium Marc Verstraete*' at a cost of about 200 million BEF.

- On request by some members of the board of the DCRF, the university received some financial support, to help, for example, the University Orchestra (New Belgian Chamber Orchestra, afterwards the Beethoven Academy), to support the library of Theology, to extend the journal subscription of the Faculty of Medicine or to help the fruit culture center of the K.U.Leuven through a difficult period. As usual, a university is always short of financial means, and eagerly looks for potential sources of support.

The most important activity of the *Protein Research Division* of LR&D and of the *D. Collen Research Foundation* was the support of scientific research, initially in our own university laboratory (previously Center for Thrombosis and Vascular Research, and later Center for Molecular and Vascular Biology), and later also our VIB department (Center for Transgene Technology and Gene Therapy, recently renamed to Vesalius Research Center). For several years, up to 30 researchers and technicians were financed. In the early nineties about one million dollars were used to transfer, with Peter Carmeliet, the transgenic mice technology from the *Whitehead Institute* to Leuven.

In 2005, the legislation on non-profit organizations was amended, and the bylaws of the Foundation had to be adapted. The use of the words 'Foundation' or 'Trust' was no longer allowed in the name of non-profit organizations. Consequently, a new name was chosen, namely '*Life Sciences Research Partners, VZW*. In addition, because of the passing away of both Lawrence Fouraker and Chip Gold, *Harvard University* no longer serves as member or director of the new association.

A 'not so' rich, but headstrong professor?

My private jet

In an interview with the periodical Knack, I incidently mentioned that university colleagues eagerly gossiped about my 'wealth'. They thought that I was the best paid professor at the K.U.Leuven. Sometimes the gossiping became really hilarious. The best story that circulated concerned the small second-hand aircraft I bought in the US in 1992. In Gasthuisberg word was spread that I bought myself a private jet so I could fly to meetings in the USA without loosing too much of my precious time. My prive jet actually was a second-hand 1982 model Cessna 210 Centurion. The centurion is a single-prop engine plane suitable for pleasure flights. With its flight range of about 1000km it certainly is not suited to cross the Atlantic. It had a price tag in the range of a decent car, certainly less than a Ferrari or a Bently. The plane had the call sign OO-T-PA. The OO being the call sign for Belgium and TPA referred to the financing source.

The Cessna Centurion at its arrival at Zwartberg (EBZW,) after we crossed over from the US under the American call sign (N9808Y). The pilots are Désiré Collen and the experienced co-pilot Michel Notelaers in their survival gear.

It is true that I bought it in the US. We replaced the backseats by a temporary fuel tank and I crossed the ocean together with my instructor. Without an experienced pilot I would never have been given clearance for a transatlantic flight with the Cessna. The flight over Goose Bay in Canada to Keflavik in Iceland using the autopilot and the GPS was not particularly exciting and I even could get some sleep during our eight-hour flight.

Travelling to meetings with the Centurion is actually not a good idea. I did it twice, to Stockholm and Geneva, but weather conditions often prevented me from taking-off in due time. The Cessna was honestly never intended for that purpose.

The same Cessna, after its registration in Belgium, with the new call sign 00-TPA.

When *East Hill* became share holder of *ThromboGenics* in 2001 with an investment of $12.8 million, they prompted me to take a Key Man Insurance for $10 million in addition to the regular Director's Insurance. The reason for this was that in case something would happen to me, sufficient finances would be available to attract an appropriate replacement. Eventually, two American insurance companies were willing to endorse this policy, but to limit the annual premium to one hundred thousand US dollars, I had to contractually commit myself to fly only when a second '*safety pilot*' was on board. This clearly limited my freedom and independence to fly. As a consequence, my flying hours decreased from 70 before 2001 to only 15. Because of this limited time in the cockpit my hobby was becoming quite an expensive affair. The *Key Man Insurance* policy has been stopped after the IPO of *ThromboGenics* in 2006, but nowadays the lack of routine prevents me from making solo flights abroad.

Sometimes I reflect on my flying adventures in 1997. Together with my colleague pilot and friend Albert Degens we participated in the '*International Air Rally of Malta*' and much to our surprise ended second out of 16 participants in the general classification and first in the timeflight.

Van Roger: flight against the clock?

Solidarity cashdesks at the K.U.Leuven

The gossiping about my private jet and other stupidities did not really affect me. However, the prejudice against the 'rich professor' certainly caused some harm to me and my colleagues. It was frequently suggested that Collen and his research team had ample private funding - and as such they should not compete for research grants from the university, the Flemish or the federal government. Some even stated that I should be ashamed to compete for these limited funds. Several colleagues actually gave me this message quite unambiguously.

Most of the time, I did not take these “well intended" advices seriously. I still fail to see why our research team should not be allowed to compete for alternative funding. In the first place, because the royalties from rt-PA would run dry. After all, the patent had an expiry date. Moreover, I needed additional funds to attract talented researchers so that our laboratory could compete on an international level. Turning talented researchers into productive scientists indeed has its price. Not so much because of the salary of the researcher itself, but for the required infrastructure. High quality level, at least in the 'life sciences', only thrives today where the best possible facilities (with up to date equipment and technology) are available, supported by experienced and trained technicians (many of my former technicians and laboratory assistants had more than twenty years of research experience). In addition, instead of being solitary individualists, scientists today have to be real team players. There has to be sufficient critical mass in the team, which is very expensive to achieve.

Moreover, money is not the only concern when applying for research grants, but also the label of quality and the networking. The Interuniversity Attraction Poles (IUAPs), from the Belgian Science Policy ('Federaal Wetenschapsbeleid') are a good example. The goal of these programs is to support excellent research teams from the different communities of our country, which perform basic research in a network context. From 2007 to 2011 forty four such networks are supported in the fields of biology, medicine, chemistry, physics, applied sciences, historical sciences, social sciences and the humanities. The network P6/30, for example, 'Blood vessel formation and vessel wall biology in pathology and medicine' depends strongly on the expertise of Peter Carmeliet and his team. If he were to be excluded from this network, because of the fact that his group already has sufficient alternative funding, the international research community would consider Belgium’s research policy as rather curious.

It would also have been rather ridiculous if the IUAP project of 10 years ago - 'An integrated approach to study the underlying mechanisms of atherothrombosis'[169]- had been approved without our team.

Solidarity fund - round 1

I realize that my stubbornness also has downsides. My participation amongst others in IUAP projects caused other researchers to drop out of the funding because only a limited number of Leuven participants is allowed. As a compensation, I have generously contributed to the 'IUAP Solidarity Fund' of the K.U.Leuven on several occasions. This allowed financial compensation for excellent researchers who did not obtain these grants. I want to illustrate that this was not always handled in an elegant manner.

My first participation in the IUAP was in the second round (in 1990) with a group consisting of our university laboratory, the cardiology group (Frans Van de Werf) in Leuven, and two American groups (cardiology with Chip Gold from the *Massachusetts General Hospital*, *Harvard University*, Boston and structural chemistry with Al Tulinski from Minnesota). Foreign groups could then participate in the network, but were not funded with Belgian means. Rector Roger Dillemans and Research Coordinator Herman Vanden Berghe imposed a condition for participation. This condition implied that I could 'only' request an annual budget of five million BEF (normally provided for projects from the Humanities) instead of twelve million BEF (provided for more expensive projects from the Biomedical Sciences).

Our project was positively evaluated in Brussels, but since it was considered as a biochemical project, it should receive a financial support of twelve million BEF annually. However, Leuven was eager to remind me that I had agreed to a lower funding. On the other hand, I could not refuse the extensive support from Brussels. Eventually, I paid 35 million BEF (five years a rato of seven million BEF) from the t-PA rights of our laboratory into the cash fund of the research council.

[169] Ischaemic damage of the endothelium, oxidation of LDL and atherothrombosis, research project P4/34 (IUAP research network section P4) , http://www.belspo.be/belspo/fedra/proj.asp?l=nl&COD=P4/34

Solidarity fund - round 2

The rules for the next round in the IUAP had been changed: instead of allowing foreign groups, only Belgian groups could participate in the networks. Because it was not possible to line up a competitive critical mass in our field of fibrinolysis and thrombolysis in Belgium, we changed tactics.

We submitted a new project (P4/34), 'Ischemic damage to the endothelium, oxidation of LDL and atherothrombosis', in which my colleague Paul Holvoet would play an important role and in which we formed a network with three French-speaking research laboratories, Notre *Dame de la Paix* in Namur with José Remacle and two laboratories of the *Université Catholique de Louvain* with Claude Remacle and Yves-Jacques Schneider. At first, another group from Antwerp participated, but the dean of the University of Antwerp clearly suffered from a 'Collen trauma' and threatened to boycott the career of his researcher if he would join this project. This talented Antwerp colleague, therefore, withdrew from the project, much to his regret, just a couple of hours before submission.

Our project was again approved and this time for the full finding. I felt comfortable that this was a closed deal. Two days later, however, the former research coordinator of the K.U.Leuven, a neighbour of mine both private as for our laboratories, told us that we may well be recognized as IUAP network in Brussels, but not yet in Leuven. He alleged that the UCL considered the two participating laboratories as one, because they were localized on the same floor and shared some equipment, although employing different staff members and being involved in different research projects. If the UCL laboratories were to be considered as one, we would not have the minimum required number of laboratories to constitute an IUAP network.

I was infuriated - one of the very few times - and demanded an interview with the senior official in the office of the Prime Minister. My colleague was startled and said that he would try to arrange the matter personally. He clearly did not like the prospect of me causing a public riot concerning an important funding source for universities as the IUAP. A couple of days later he triumphantly announced that the UCL had now accepted that its two participating groups would be recognized as independent laboratories. They would receive IUAP funding as UCL satellites, although at the cost of other groups of the UCL.

Meanwhile, other dark clowds gathered in the Leuven skies. The funding intended for the group of Holvoet was, apparently, in the meantime awarded to a group of the human sciences. If I would finance this group, everybody would be happy, we would keep our IUAP

label and network and the other group would receive my financial compensation. This seems a fishy solution, but finally I did transfer 25 million BEF from our division in LR&D to the research council, because I believed that the project, which would thus be funded, indeed had important intrinsic qualities.

I never made this story public and I even presume that the 'sponsored' colleague never knew the source of his/her funding. I briefly explained the situation to colleague Holvoet as the decision on our IUAP project was positive first, then negative and finally positive after all. The other participants in our network have, as far as I remember, never known about this.

Coup de théatre and unfair insinuations

However, there was a sequel to this story. In 2004, Holvoet wrote a letter to the academic authorities in which he stated that he was deprived of participation in the structural resources in our laboratory. According to Holvoet, I even channeled his IUAP funding to other groups. He first showed a copy of this letter to both Jos Vermylen and myself.

When I reminded him of the 'ransom' of 25 million BEF that I paid for his IUAP and that all 'his' resources were being used under 'his' supervision, he was more or less flabbergasted. The damage was, however, done. Holvoet and his group had to find another home base for their research, and he was entitled to take all his financial resources.

All's good that ends well? Not really. With the next IUAP round we, again, had to change tactics due to the departure of Holvoet and his research program. A new research proposal was set up around Peter Carmeliet in the area of angiogenesis (blood vessel formation). The proposed satellites were Michel Foidart / Agnes Noel from Liège, Marc Mareel from Ghent, Dirk Brutsaert from Antwerp and Jean-Marie Boeynaems from the ULB.

Although the project was of great scientific value, the “comedia dell’arte” started all over again. Dirk Brutsaert and Marc Mareel, although internationally recognized researchers, were at less than five years from their emeritate and were disqualified on these grounds. Dirk Brutsaert’s closest collaborator moved to Ghent, where they claimed not to know him well enough to provide IUAP funding. In Liege, one picked up a signal from Leuven that they did not want to invest in Collen anymore, because he diverted public university funding to private industrial activities.(Sic!!!). When Michel Foidart, the Liège participant, phoned me

while I was waiting for a return flight in Rome, to inform me what he was told by his Rector, I was completely taken aback. According to Foidart, this was not a real problem for his group because the University of Liège, that highly respected and recognized his research work would, if necessary, reclassify him to another IUAP.

The real problem in Leuven appeared to be that there was no alternative funding available for Paul Holvoet in his new environment, to compensate for his expiring IUAP. Therefore the story was concocted that his participation in 'our' IUAP would make up for the lack of groups after the disqualification of Mareel and Brutsaert. Consequently, I refused to continue as spokesman and withdraw from the next IUAP, thinking 'enough is enough and too much is too much'.

The unfunded insinuation that I transferred public money to private industrial goals was too much for me to accept. A couple of years earlier (January 2000), I changed my position from full professor into a part-time appointment (with 25% of the full salary), and I resigned as "adjunct head of clinic" (becoming "consulent" 1/11 ,with 9% residual salary). Thereby, I wanted to prevent the perception of exaggerated cumul with my involvement with *Thromb-X* and *ThromboGenics*. The funding, which thus would become available, could then be used in our laboratories. After this unfair insinuation, I immediately initiated my return from the extraordinary to the regular staff, and this after four years, instead of the planned six years or permanently.

All things considered, the K.U.Leuven was not all that generous in funding our department, even more so when one takes the output of our laboratory into account. On numerous occasions, when I did not get things I was entitled to, I got angry and upset, but in the end I just paid for it myself – often to the frustration of the academic authorities, because they had no hold on me. I had made myself independent and that suited me well.

I must admit that things improved in the post-Oosterlinck period. My successors obtained with Methusalem, Odysseus and Excellence funding, besides European and other international funding. Fortunately, because the t-PA royalties meanwhile have dried up.

No chair and …

During my entire career as professor, I was never given the opportunity to display my skills as lecturer in the Faculty of Medicine. With my two qualifications - a PhD in chemistry and a

MD/PhD in medicine - I did not really get a chance. The Faculty of Medicine considered me to be a chemist and the Faculty of Sciences thought I was a physician. They had their own people to give classes. The only place where I was ever welcome to teach was in the "faculty of physical education" ('sportkot'), where I lectured in chemistry and biochemistry. In the sciences or medicine, my didactic qualities were never recognized. When I once mentioned this to Rector Dillemans, he responded with a hush: "Dear Désiré, stick to your research, you are so good at it. The K.U.Leuven has many colleagues who can teach."

In other universities, my talents were apparently more appreciated and respected: Between 1984 and 2005, I was '*Professor of Biochemistry and Medicine*' at the *University of Vermont College of Medicine* in Burlington (US) and between 1987 and 1994 '*Visiting Professor of Medicine*' at the *Harvard Medical School* in Boston (US). Occasionally I wonder whether declining a potential career at *Harvard* really was in my best interest.

… no rector

In the spring of 1995, I had the, in retrospect perhaps 'stupid', idea to announce my candidacy for the position of rector. In fact, the idea was inspired by frustration. At that time, I frequently visited Harvard where I met young talented scientists from Leuven, like Peter Carmeliet, Wim Robberecht, Stefan Janssens and others. They were offered the opportunity to stay on in the Harvard system after the completion of their post-doctoral training in the USA. In contrast, they would have very limited resources to develop their research careers at home - in Leuven or elsewhere in Belgium. The three colleagues mentioned above did eventually succeed. That frustrated me, hence my candidature. I thought I could make an impact as rector, and transform our university into a real research institute.

The rector's election, however, was no success. With 15% of the votes, I did not make it through the first round. Of course, I did say the wrong things to some people. I was also not such a 'promiser'. When the student representatives came to inquire whether they could obtain more seats on the Management Board of the University, I replied that it was already bad enough that a majority of professors was on the Board. Furthermore, when I was asked for my vision on the expansion of the Kortrijk CampusI replied that this campus should be closed as soon as possible. In a debate with students, I stated that there were 7,000 students in Leuven who actually did not achieve a university level. When I circulated my curriculum - with some hundred research articles in scientific journals and about 100 reviews - it was interpreted by many colleagues as an attempt to boast, instead of showing my commitment to make the K.U.Leuven a strong research university. Also, the emphasis

that I put on the University Hospitals as economic regional engine and service center was not well received by the academics in the 'softer' sectors. I had another clash with the students, because 'I was unaware of the specific problems in the social sector', all the more because I thought that a 'tuition fee' of less than € 500 contrasted sharply with the real investment of society in university training, even though the graduates afterwards will pay social security and taxes.

Technical college and/or research institute?

I always urged that the Belgian universities are having too much of a 'technical college' profile[170]. We train good physicians, economists, lawyers, engineers etc., but on the other side, we have little tradition in creative and competitive scientific research and are therefore, no true research institute. The university wants to be the university for everybody, but that is not compatible with the ambition to produce excellent research. This viewpoint once was even confirmed by Rector Oosterlinck at a Management Board meeting of the VIB, which I attended as an observer for the Scientific Directors: "I must as Rector not only care for the prima donnas of research but also for Tom, Dick and Harry." I had to restrain myself from saying that at the K.U.Leuven I was never a 'Prima Donna', but rather a 'Cinderella'. If you try to pull down everybody who excells, while pampering and supporting those who do not, then our universities have a real problem. I have to admit, however, that Oosterlinck did seriously attempt to address the second problem, at least in the most obvious cases.

The problem is that we strive to please everybody. I agree that universities should perform broad and basic research, in many disciplines, but one must also dare to define programs in which you want to excell, backed up by adequate funding. I do not believe this view is undemocratic, although linearly applied democracy in my opinion a guarantee is for mediocrity. Otherwise, I am a fierce supporter of democracy on regional, national and European level. In this manner, autocratic excesses become less probable and countries become more and more familiar with each other. It is very unlikely that our Low Countries would stage a war as was the case for centuries. Borders are fortunately progressively disappearing, even though we still lack the advantages of a common language. The question

[170] Prionet E. Leuven does not want to be a mini Harvard. Interview in Knack, 1 October 2008, 50-53.

is rather whether we want to keep advancing our knowledge. Do we, in Flanders and Europe, in future decades only want to apply the medical developments from Harvard, Stanford or Johns Hopkins, or do we want to develop things ourselves? Do we consider ourselves as the composer or as the musician? That is the choice. I always believed that the K.U.Leuven could become a small Harvard. Well, the majority of the academics clearly do not want this, because it treathens their confortable position.

Fortunately - and possibly out of necessity - times are changing. Under pressure from outside, concepts such as 'evaluation', 'audit', 'inspection' gradually make their way into the Belgian universities. I consider it normal that academics are evaluated on a regular basis and that their output - especially at the level of basic research and teaching - is compared with that of others. It is evident that also academics, at regular intervals, account for his or her performances.

In any case, at the time of the rector's election in 1995, the K.U.Leuven was not yet ready to promote excellence. Whe I left the race, I supported the candidacy of André Oosterlinck, because his program correspondend best with my ideas. Of the other candidate at that stage, one could rather expect the opposite.

To London

In 2000, I terminated my professorship and moved to London, in order to run *ThromboGenics* Ltd from there. Also in 2000, I became 'Extraordinary Professor' - a position with a limited (25%) salary. In 2004, I took up my regular professorship again and returned my domicile to Belgium, because of the IUAP problems and insinuations. Of course I was still heading the Center for Molecular and Vascular Biology and the VIB department (later on more on that). I kept my office at Gasthuisberg, since it is located on the floor that I had co-financed.

Transfer of LR&D to DCRF

Initiated by Rector Oosterlinck, LR&D stopped all collaborations with me, our laboratory and *Thromb-X.* Ten years of productive (for all parties involved) cooperation with LR&D ended on 20 June 2001 with the transfer of the entire t-PA and *Thromb-X* file

to the *D. Collen Research Foundation*. Both files were managed by the *Protein Research Division* of LR&D, and this section was no longer welcome within LR&D.

The transfer and the termination of the *Protein Research Division* occurred in the context of (according to the minutes of the meeting held on 20 June[171]): '*on short-term scheduled reactivation of risk-bearing valorization activities within LR&D, and this independent of the existing activities of the Protein Research Division, which constituted at that time the most important activity of LR&D. This transfer involved all joint activities of the Protein Research Division including coworkers and personnel, equipment, patents and patent-applications, a part of the operating funds, the current research and license agreements with and for the K.U.Leuven and with third-party contract beneficiaries'*. Since then, LR&D has been reduced to a dormant npo without any noteworthy activities.

The Management Board of LR&D further states: '*the objective of the D. Collen Research Foundation corresponds, on the one hand, with that of LR&D, and moreover, because of the specific emphasis on biomedical and biotechnological research better adapted to the specific activities of the Protein Research Division of LR&D'*. In view of the non-profit states of both npo's, the management board decided unanimously to approve of the transfer of the *Protein Research Division* of LR&D to the npo *D.Collen Research Foundation* and this free of costs. The transfer was signed by Raymond De Bondt, as chairman of LR&D, and by the LR&D managers G. Langouche, G. Declercq, D. Collen and Rector A. Oosterlinck (who as power of attorney holder, also signed on behalf of the members, V. Goedseels, G. Mannaerts and R. Bouillon who were not present).

After signing , I of course, stepped down as manager of LR&D. That was what it was all about after all. In my opinion, the real reason behind it all was that I as manager had problems with some of the transactions within LR&D. I will not discuss this in more detail, in order not to distract from the main themes of the book. Moreover, I do not want to discredit LR&D, which under the direction of Koen De Backere, has become a highly professional, valuable and efficient technology transfer organization. I only want to emphasize that I did not 'walk away' with the *Protein Research Division* of LR&D, but was “excommunicated” to the *D. Collen Research Foundation*.

[171] Leuven Research & Development, Minutes of the meeting of the Management Board of 20 June 2001.

Anchor not lifted after all

From the preceding account, it is clear that between 1995 and 2005, I had a kind of 'LAT' (living-alone-together) relationship with the University management. Still, I was director of the Center for Molecular and Vascular Biology and of the VIB Department of Transgene Technology and Gene Technology (now the Vesalius Research Center). That brought enough responsibilities to stay in Leuven - especially towards all my colleagues who rather wanted me to stay than to see me go. From my experience in 1980, as described in the first part of this book, I knew that a career as a clinical biologist in the periphery was no option for me.

I always tried to maintain a loyal partnership with our most important employer-financiers. With the VIB after a somewhat bumpy start, this worked out just fine. But with the K.U. Leuven the relationship was far more difficult.

Perhaps the impression might arise from the above that the issues around the rt-PA royalties just caused worry and torment, strife and dispute. That is absolutely not the case. The royalties enabled us to perform groundbreaking research in Leuven - research that led to more than 650 original research papers and almost 200 review articles. Many of those were published in high impact journals. More than 140 papers obtained 100 or more citations.

We not only performed groundbreaking research in the area of fibrinolysis and thrombolysis, but also in domains such as transgenesis, angiogenesis, heart and blood vessel disorders, cancer, neuronal disorders, gene therapy and others. Many of our projects could eventually lead to the development of new drugs, that may help a multitude of patients in comparison with those treated with rt-PA. This includes drugs to treat heart attacks (staphylokinase), eye diseases and strokes (microplasmin), deep vein thrombosis and pulmonary embolism (anti-FVIII), cancer (anti-PlGF) and others. Their development will be described later on in this book.

Staphylokinase - t-PA for the poor

A déjà vu

At the Tokyo congress of the *International Society on Thrombosis and Haemostasis* in August 1989, Osamu Matsuo told me about some *in vitro* experiments that he had performed with the bacterial plasminogen activator from *Staphylococcus aureus*. To his amazement this protein - known as staphylokinase - was capable of dissolving a fibrin clot in plasma without affecting the free-circulating fibrinogen. This indicated that staphylokinase, just like t-PA, only activates plasminogen when it is bound to the blood clot.

I immediately wondered whether staphylokinase could be a valid successor of t-PA. Staphylokinase is a bacterial protein and its production would be a lot cheaper than a human protein such as t-PA. The high cost for rt-PA is due in part to the fact that the protein is produced in mammalian cells. A disadvantage of staphylokinase is that it is foreign to the human body, and that after the first application the body probably reacts by producing antibodies against the protein. In cases of reinfarction, the patient could possibly not be treated with staphylokinase again.

As was the case with t-PA, we were not the first to work on staphylokinase. The fibrinolytic properties of certain strains of *Staphylococcus aureus* were already described around 1950[172] [173] and the protein itself was isolated in 1963[174]. At the time of my conversation with Osamu in Tokyo, the staphylokinase gene was actually already cloned [175] and expressed in various bacteria, including *Escherichia coli*, *Bacillus subtilis* and *Streptococcus sanguis*[176].

Less favorable results had been published on the fibrinolytic properties of the protein. In 1964, Jessica Lewis and John Wilson in Pittsburgh[177] [178] treated a number of dogs with staphylokinase. In four of the six animals the blood clot was entirely or partly dissolved,

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[172] Lack CH. Staphylokinase; an activator of plasma protease. Nature. 1948; 161: 559.

[173] Lewis JH, Ferguson JH. A proteolytic enzyme system of the blood. III. Activation of dog serum profibrinolysin by staphylokinase. Am J Physiol. 1951; 166: 594-602.

[174] Glanville KL. A simple method of purifying staphylokinase. Biochem J. 1963; 88: 11-4.

[175] Sako T, Sawaki S, Sakurai T, Ito S, Yoshizawa Y, Kondo I. Cloning and expression of the staphylokinase gene of Staphylococcus aureus in Escherichia coli. Mol Gen Genet. 1983; 190: 271-7.

[176] Behnke D, Gerlach D. Cloning and expression in Escherichia coli, Bacillus subtilis, and Streptococcus sanguis of a gene for staphylokinase--a bacterial plasminogen activator. Mol Gen Genet. 1987; 210: 528-34.

[177] Lewis JH, Kerber CW, Wilson JH. Effects of fibrinolytic agents and heparin on intravascular clot lysis. Am J Physiol. 1964; 207: 1044-8.

[178] Lewis JH, Shirakawa M. Effects of fibrinolytic agents and heparin on blood coagulation in dogs. Am J Physiol. 1964; 207: 1049-52.

but this was accompanied by serious bleeding. From this experiment they concluded that the protein is highly toxic, which limited the enthusiasm of many researchers to study staphylokinase as potential thrombolytic agent.

Lab in overdrive

One year later, Matsuo and his Japanese colleagues would publish their results in the journal *Blood*[179]. In the meantime, my colleague Roger Lijnen and I had already started an intensive collaboration on staphylokinase with Matsuo. Roger and his team put in a lot of work, and nearly every series of experiments resulted in a publication.

In a first series of experiments, we elucidated the mechanism of plasminogen activation by staphylokinase [180] [181] [182]. It appeared that, unlike t-PA, staphylokinase itself has no enzymatic activity[183]. This means that staphylokinase does not activate plasminogen by cleaving it. Both proteins bind to each other, resulting in a confomational change in plasminogen, exposing the active site. This staphylokinase/plasminogen - complex activates other plasminogen molecules by cleaving them into plasmin.

The activation mechanism of staphylokinase strongly resembles that of streptokinase, except for the fact that streptokinase is much less fibrin selective than staphylokinase. When streptokinase is mixed with human plasma to which a blood clot is added, it forms a complex with all the plasminogen, both the free circulating and the fibrin-bound plasminogen. Thus, a chain reaction starts whereby all the plasminogen is activated after some time. The physiological inhibitor of plasmin, α2-antiplasmin, becomes completely exhausted and the excess plasmin will cleave, besides fibrin, also other proteins including fibrinogen. This results in disturbation of the normal blood coagulation system.

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[179] Matsuo O, Okada K, Fukao H, Tomioka Y, Ueshima S, Watanuki M, Sakai M. Thrombolytic properties of staphylokinase. Blood. 1990; 76: 925-9.

[180] Lijnen HR, Van Hoef B, De Cock F, Okada K, Ueshima S, Matsuo O, Collen D. On the mechanism of fibrin-specific plasminogen activation by staphylokinase. J Biol Chem. 1991; 266: 11826-32.

[181] Lijnen HR, Van Hoef B, Matsuo O, Collen D. On the molecular interactions between plasminogen-staphylokinase, alpha 2- antiplasmin and fibrin. Biochim Biophys Acta. 1992; 1118: 144-8.

[182] Lijnen HR, De Cock F, Matsuo O, Collen D. Comparative fibrinolytic and fibrinogenolytic properties of staphylokinase and streptokinase in plasma of different species in vitro. Fibrinolysis. 1992; 6: 33-7.

[183] Collen D, Schlott B, Engelborghs Y, Van Hoef B, Hartmann M, Lijnen HR, Behnke D. On the mechanism of the activation of human plasminogen by recombinant staphylokinase. J Biol Chem. 1993; 268: 8284-9.

When staphylokinase is mixed with plasma containing a fibrin clot, it hardly reacts with free-circulating plasminogen, but very strongly with plasminogen that is bound to the fibrin surface. As long as the staphylokinase/ plasminogen-complex remains bound to fibrin, it is protected from inhibition by α2-antiplasmin. However, when it is released into solution it rapidly becomes neutralized.

With staphylokinase, we thus had a protein which dissolves blood clots following the same mechanism as streptokinase, but with the fibrin specificity of t-PA. The molecular interactions between staphylokinase, plasminogen, plasmin, α2-antiplasmin and fibrin are unique, and have until now not been observed for any other thrombolytic protein[184] [185] [186].

Clone and gene

Because we could not rely on a constant and adequate supply of staphylokinase from Japan, we cloned the gene directly from a lysogenic strain of *Staphylococcus aureus*[187] to set up our own production system. This resulted in a whole series of studies on the gene and protein structure of staphylokinase. The gene codes for a protein of 163 amino acids, which is transformed to a mature protein with 136 amino acids in a single protein chain without disulphide bridges[188]. The three-dimensional structure of the protein will be published only in 1997[189].

[184] Lijnen HR, Van Hoef B, Vandenbossche L, Collen D. Biochemiscal properties of natural and recombinant staphylokinase. Fibrinolysis. 1992; 6: 412-25.

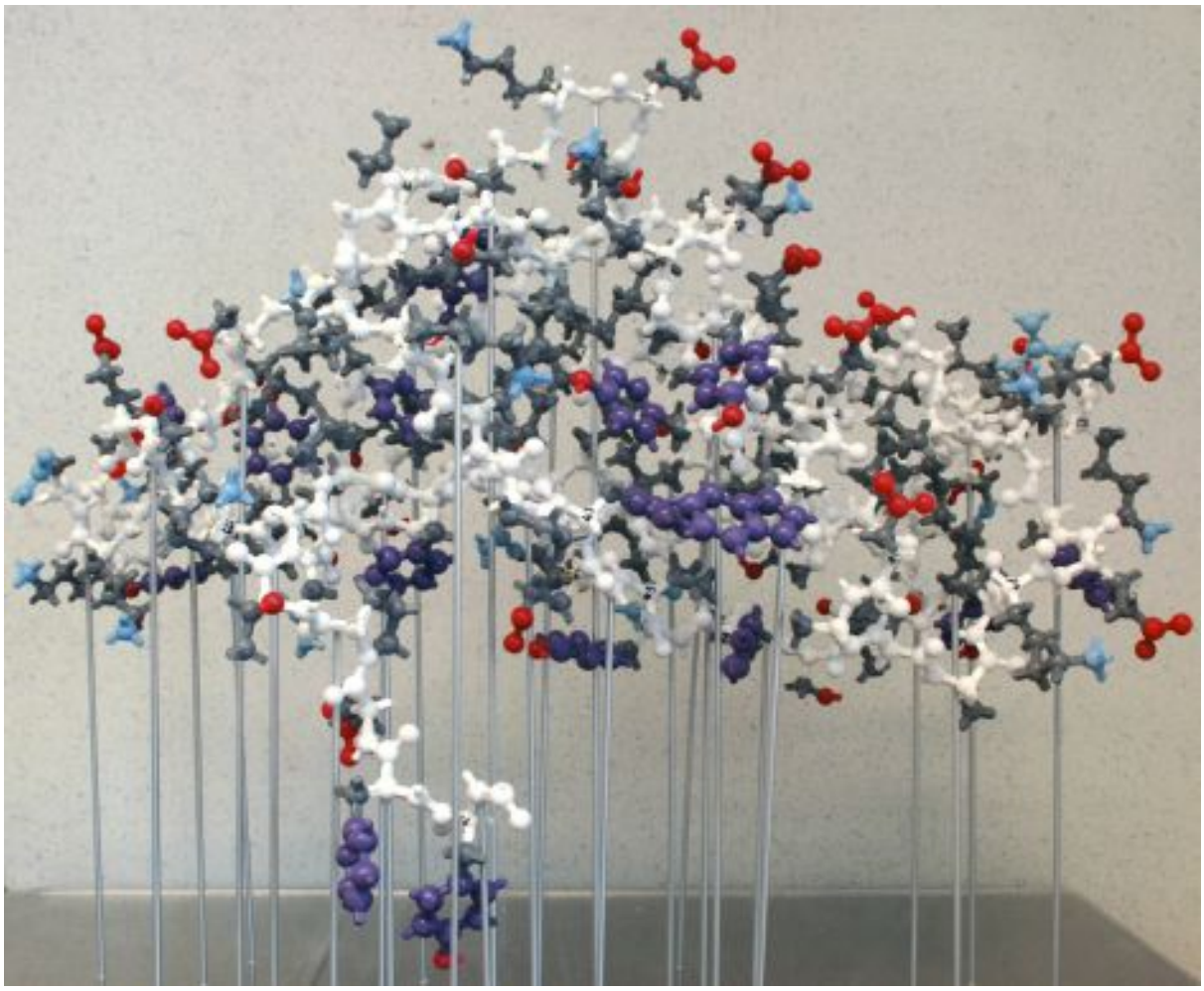
[185] Silence K, Collen D, Lijnen HR. Regulation by alpha 2-antiplasmin and fibrin of the activation of plasminogen with recombinant staphylokinase in plasma. Blood. 1993; 82: 1175-83.

[186] Silence K, Collen D, Lijnen HR. Interaction between staphylokinase, plasmin(ogen), and alpha 2-antiplasmin. Recycling of staphylokinase after neutralization of the plasmin-staphylokinase complex by alpha 2-antiplasmin. J Biol Chem. 1993; 268: 9811-6.

[187] Collen D, Silence K, Demarsin E, De Mol M, Lijnen HR. Isolation and characterization of natural and recombinant staphylokinase. Fibrinolysis. 1992; 6: 203-13.

[188] Collen D., Zhao ZA, Holvoet P, Marijnen P. Primary structure and gene structure of staphylokinase. Fibrinolysis 1992; 6: 226-231.

[189] Rabijns A, De Bondt HL, De Ranter C. Three-dimensional structure of staphylokinase, a plasminogen activator with therapeutic potential. Nat Struct Biol. 1997; 4: 357-60.



Structural model of staphylokinase.

Preclinical research

Immediately after the meeting with Matsuo in Tokyo, we compared the thrombolytic and pharmacokinetic properties of staphylokinase and streptokinase in two experimental venous thrombosis models - hamster and rabbit[190]. The first results indicated that staphylokinase is a potent thrombolytic agent comparable to streptokinase. These promising results justified an investment in additional research.

[190] Lijnen HR, Stassen JM, Vanlinthout I, Fukao H, Okada K, Matsuo O, Collen D. Comparative fibrinolytic properties of staphylokinase and streptokinase in animal models of venous thrombosis. Thromb Haemost. 1991; 66: 468-473.

Experiments in vitro with human, baboon, rabbit, hamster, rat and dog plasma yielded surprising results (see[191]): the fibrinolytic system in plasma of baboons, rabbits and hamsters appears to react on staphylokinase in the same way as that of humans. The rat system seemed to be resistant - staphylokinase hardly succeeded in dissolving the blood clots. With dogs, staphylokinase acted in a very strange way: it was a very potent plasminogen activator, however, without any fibrin-specificity. Further research showed that canine α2-antiplasmin inactivated the complex staphylokinase/plasmin at ten times lower rate than human α2-antiplasmin. This experiment thus explains the less favorable thrombolysis results of Jessica Lewis and John Wilson in 1964 [192] [193] in dogs. The hypothesis that staphylokinase would cause serious bleeding also in humans based on the experience with dogs, thus appeared to be wrong. Dogs are simply unsuitable as a preclinical model to investigate the thrombolytic activity of staphylokinase. Unfortunately, research on staphylokinase was discontinued for over 25 years.

In our laboratory, Jean-Marie Stassen convincingly demonstrated the *in vivo* thrombolytic potential of staphylokinase in several animal models. In hamsters with pulmonary embolism, the thrombolytic potency of staphylokinase was comparable to that of streptokinase[194]. Towards blood clots enriched in blood platelets (1.5 million platelets per microliter), staphylokinase was even five times more active. This is relevant, because thrombi that occlude the coronary artery are platelet-rich.

Furthermore, also with baboons, Jean-Marie Stassen could demonstrate that intravenously administered staphylokinase dissolves blood clots as efficiently as streptokinase[195]. Also in this case, platelet-rich blood clots were dissolved better by staphylokinase than by streptokinase. Moreover, staphylokinase is less immunogenic and causes fewer allergic reactions.

The first patient

Mid 1992, less than three years after my conversation with Matsuo, we were ready to treat the first patient with staphylokinase. Within three years, we unravelled in our laboratory the mechanism of action of staphylokinase and its biochemical properties, and we elucidated

[191] Lijnen HR, De Cock F, Matsuo O, Collen D. Comparative fibrinolytic and fibrinogenolytic properties of staphylokinase and streptokinase in plasma of different species in vitro. Fibrinolysis. 1992; 6: 33-7.

[192] Lewis JH, Kerber CW, Wilson JH. Effects of fibrinolytic agents and heparin on intravascular clot lysis. Am J Physiol. 1964; 207: 1044-8.

[193] Lewis JH, Shirakawa M. Effects of fibrinolytic agents and heparin on blood Coagulation in dogs. Am J Physiol. 1964; 207: 1049-52.

[194] Collen D, De Cock F, Vanlinthout I, Declerck PJ, Lijnen HR, Stassen JM. Comparative thrombolytic and immunogenic properties of staphylokinase and streptokinase. Fibrinolysis. 1992; 6: 232-42.

[195] Collen D, De Cock F, Stassen JM. Comparative immunogenicity and thrombolytic properties toward arterial andvenous thrombi of streptokinase and recombinant staphylokinase in baboons. Circulation. 1993; 87: 996-1006.

the interactions with all its binding partners – fibrin, plasminogen, plasmin, α2-antiplasmin. The gene was cloned and expressed, a series of preclinical studies was conducted, and pure recombinant protein was produced for use in clinical studies[196]. That is an achievement to be really proud of.

On 25 June 1992, Frans Van de Werf treated the first myocardial infarction patient with recombinant staphylokinase in Leuven - we called the product SakSTAR. In the same prospective study, four more patients would be included[197]. Each patient received 10 mg SakSTAR intravenously over a period of 30 minutes. In four of the five patients, complete or partial thrombolysis occurred within 40 minutes. The plasma fibrinogen and α2-antiplasmin levels were unaltered after the treatment. However, after two weeks, antibodies were found in the blood of all patients, an observation that had to be followed up. These first clinical tests were an unexpected success (see Appendix 12).

Thromb-X and ThromboGenics

To further develop staphylokinase, the *Protein Research Division* of *Leuven Research & Development* and I founded *Thromb-X* NV (see also previous chapter). We started as equal partners and each brought in half of the capital. Initially, the funds that *Thromb-X* had at its disposal for research on staphylokinase came from the right on 10% of the rt-PA royalties, but it was my intention to involve other partners so that we could, on reasonable terms, finance the development of staphylokinase into a drug. In order to search for additional funding, I founded, together with a few partners, ThromboGenics *Ltd.* in Dublin, Ireland.

Help from outside

Although we ourselves had done a tremendous job, I realized that we needed help from outside in order to develop staphylokinase further. We were indeed stretching our own resources: our production method for recombinant staphylokinase was not robust enough to produce sufficient protein for the planned clinical studies.

[196] Collen D, De Mol M, Demarsin E, De Cock F, Stassen JM. Isolation and conditioning of recombinant staphylokinase for use in man. Fibrinolysis. 1993; 7: 242-7.

[197] Collen D, Van de Werf F. Coronary thrombolysis with recombinant staphylokinase in patients with evolving myocardial infarction. Circulation. 1993; 87: 1850-3.

I sought a solution with the former group of Detlev Behnke from the *Institute for Molecular Biology (IMB)* in Jena (Germany) who developed an expression system in *E. coli,* allowing exceptionally high yields of staphylokinase.[198]

Initially, I was reluctant when I found out that Detlev Behnke lost his job after the unification of Germany because of his Stasi past. His closest colleagues, who were still employed at the institute, assured me that Behnke had never been a Stasi collaborator, but that he only pretended to in order to have more priviliges, among other things to travel abroad.

Moreover, it appeared that *IMB* had already licensed out the staphylokinase expertise and patent application to the pharmaceutical company *medac GmbH* in Hamburg. After a period of negotiations, a collaboration's agreement was reached between *Thromb-X* and *medac* in December 1992 in which ” *medac Gmbh accepts to transfer in full property all its present and future staphylokinase patent applications to Thromb-X NV. In exchange, medac GmbH shall become the owner of 5% of all shares of Thromb-X NV and shall obtain an irrevocable exclusive marketing and distribution license for an indefinite period in Germany, at a royalty rate of 3% of its net sales”.*

The collaboration with the Germans originally went very smooth. The material produced in Jena was used for several additional biochemical and pilot clinical studies[199] [200] [201] [202]. In October 1993 the inventors in Jena, however, felt that they were not getting their fair share. They had concluded an agreement with *medac*, in which a royalty was promised on the sales by *medac*, and they wished now to extend this outside the *medac* territory. Finally, the more because rapid progress was made, we reached a compromise whereby I offered them, from my own shares' stock, 5% of all *Thromb-X* shares free of charge. They were very pleased indeed.

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[198] Schlott B, Hartmann M, Gührs KH, Birch-Hirschfeid E, Pohl HD, Vanderschueren S, Van de Werf F, Michoel A, Collen D, Behnke D. High yield production and purification of recombinant staphylokinase for thrombolytic therapy. Biotechnology 1994; 12: 185-9.

[199] Collen D, Van Hoef B, Schlott B, Hartmann M, Gührs KH, Lijnen HR. Mechanisms of activation of mammalian plasma fibrinolytic systems with streptokinase and with recombinant staphylokinase. Eur J Biochem. 1993; 216: 307-14.

[200] Collen D, Schlott B, Engelborghs Y, Van Hoef B, Hartmann M, Lijnen HR, Behnke D. On the mechanism of the activation of human plasminogen by recombinant staphylokinase. J Biol Chem. 1993; 268: 8284-9.

[201] Lijnen HR, De Cock F, Van Hoef B, Schlott B, Collen D. Characterization of the interaction between plasminogen and staphylokinase. Eur J Biochem. 1994; 224: 143-9.

[202] Schlott B, Hartmann M, Gührs KH, Birch-Hirschfeld E, Gase A, Vettermann S, Collen D, Lijnen HR. Functional properties of recombinant staphylokinase variants obtained by site-specific mutagenesis of methionine-26. Biochim Biophys Acta. 1994; 1204: 235-42.

In March 1996, while we were negotiating with a big pharmaceutical company on an exclusive license on staphylokinase, it appeared that, all along, they had an ace up their sleeve. In May 1993 *medac* and the inventors signed an agreement, in which they were promised 4% royalties on the net sale and *"Sollte medac den Vertragsgegenstand an Dritte veraussern, so hat medac für die Übernahme der in diesen Vereinbarung enthaltenen Rechten und Pflichten Sorge zu tragen". (*"should medac sell the subject of the contents of the agreement to a third party, then medac had to take care of the transfer of the rights and duties contained in this agreement"*)* To our amazement, it appeared that the inventors as well as *medac,* when signing our agreements and the transfer of shares, had withheld this crucial information, making it impossible to sell the staphylokinase project. I then bought back the royalty rights and the *Thromb-X* shares from the Jena inventors for a total of 26 million BEF. In an attempt to rescue the staphylokinase project, I dropped their exorbitant royalty claim against Thromb-X*.* Trust was however lost and in August 1999, *Thromb-X* bought back the rights of *medac* for $ 1 million and the *DCRF* bought all their shares in *Thromb-X* for 14.8 million BEF. This termination agreement contained a clause *"medac irrevocably guarantees that it shall hold Thromb-X NV harmless of any proprietary or financial claim relating thereto and including inventor rights from the patents and applications transferred to Thromb-X".*

In my opinion, I thus had a claim of 26 million BEF, with which I had bought out the guys from Jena, which by way of this agreement could be held against *medac*, and which I also tried to enforce. It was submitted to arbitration in which the arbitrator decided that the claim of D. Collen on *medac* was not part of the termination agreement between Thromb-X and *medac*. I even started proceedings against *medac* through the commercial court in Leuven, but when it appeared that *medac* wanted to involve *Thromb-X* as a third party in this case, I decided to drop the case, also on advice of our main shareholders in *ThromboGenics* Ltd (in the meantime 100% mother company of *Thromb-X*). From this story it might appear that dishonesty is sometimes rewarded, at least when one has no scruples or principles. Naturally, we have, since this shocking experience, broken all contact with *medac* and Jena. The further development of staphylokinase, of which we at least now owned all the expertise and intellectual rights, was performed under our own direction. After all these tribulations, I remained convinced that staphylokinase could be an affordable lifesaving drug.

Clinical studies

A first randomized study[203] with staphylokinase with 100 patients took place in seven Belgian hospitals. Besides the UZ Gasthuisberg, AZ Middelheim (Antwerp), AZ Imelda (Bonheiden), AZ St-Jan (Genk), AZ St.-Elisabeth (Ukkel), AZ St-Jan (Brugge) and the AZ Virga Jesse (Hasselt) participated. Of the 100 patients with myocardial infarction a control group of 52 received rt-PA; 25 patients received 10 mg SakSTAR and 23 patients 20 mg SakSTAR. The conclusion of the trial was that recombinant staphylokinase in patients with myocardial infarction was as effective for early thrombolysis, monitored as recovery of blood flow, as rt-PA. In addition, staphylokinase appeared to be much more fibrin specific than rt-PA. Afterwards, we treated five more patients with a dose of 40 mg SakSTAR, with even more convincing results.

Subsequently, a pilot study was performed with thirteen patients, receiveing a 'bolus' injection of staphylokinase instead of a continuous infusion[204]. This was followed up with a comparative study between rt-PA (52 patients) and a double bolus of 15 mg staphylokinase (50 patients) given 30 minutes apart[205]. Complete reperfusion within 90 minutes was obtained in 68% of the patients that received staphylokinase, as compared to 57% with rt-PA.

In addition, we also performed a clinical trial on a group of 30 patients with peripheral arterial occlusion[206]. Complete reperfusion was achieved in 25 patients and partial reperfusion in two.

Immunogenicity

Staphylokinase is a heterologous protein to our body. This implies that we will produce antibodies against the bacterial protein. Moreover, people never before treated with staphylokinase can have antibodies although at low titer, because of a previous

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203 Vanderschueren S, et al. A randomized trial of recombinant staphylokinase versus alteplase for coronary artery patency in acute myocardial infarction. The STAR Trial Group. Circulation. 1995; 92: 2044-9.

204 Vanderschueren S, Collen D, van de Werf F. A pilot study on bolus administration of recombinant staphylokinase for coronary artery thrombolysis. Thromb Haemost. 1996; 76: 541-4.

205 Vanderschueren S, Dens J, Kerdsinchai P, Desmet W, Vrolix M, De Man F, Van den Heuvel P, Hermans L, Collen D, Van de Werf F. Randomized coronary patency trial of double-bolus recombinant staphylokinase versus front-loaded alteplase in acute myocardial infarction. Am Heart J. 1997; 134: 213-9.

206 Vanderschueren S, Stockx L, Wilms G, Lacroix H, Verhaeghe R, Vermylen J, Collen D. Thrombolytic therapy of peripheral arterial occlusion with recombinant staphylokinase. Circulation. 1995; 92: 2050-7.

Staphylococcus aureus infection. This could cause allergic reactions to treatment with staphylokinase or neutralization of the administered staphylokinase. The induction of such antibodies also occurs with streptokinase, and yet this has been successfully administered to hundreds of thousands of patients. Parallel to the biochemical, preclinical and clinical tests we also set up a research project to investigate the immunogenic character of staphylokinase.

First of all, we noticed that in the general population the prevalence of antibodies against staphylokinase is lower than is the case with streptokinase [207] [208]. Moreover, the clinical trials on 300 patients indicated that serious allergic reactions against recombinant staphylokinase did not occur or at least very rarely. On the other hand, it was shown that most of the treated patients produced neutralizing antibodies, so that 're-treatment', because of a second or a third infarct, would be less efficient. Therefore, we attempted to suppress the immunogenic character of staphylokinase via 'site-directed mutagenesis', the introduction of site-directed mutations in the staphylokinase gene. We succeeded in producing several variants of SakSTAR which were less immunogenic without affecting the activity[209] [210] [211] [212] [213].

[207] Declerck PJ, Vanderschueren S, Billiet J, Moreau H, Collen D. Prevalence and induction of circulating antibodies against recombinant staphylokinase. Thromb Haemost. 1994; 71: 129-33.

[208] Vanderschueren SM, Stassen JM, Collen D. On the immunogenicity of recombinant staphylokinase in patients and in animal models. Thromb Haemost. 1994; 72: 297-301.

[209] Collen D, Bernaerts R, Declerck P, De Cock F, Demarsin E, Jenné S, Laroche Y, Lijnen HR, Silence K, Verstreken M. Recombinant staphylokinase variants with altered immunoreactivity. I: Construction and characterization. Circulation. 1996; 94: 197-206.

[210] Collen D, Moreau H, Stockx L, Vanderschueren S. Recombinant staphylokinase variants with altered immunoreactivity. II: Thrombolytic properties and antibody induction. Circulation. 1996; 94: 207-16.

[211] Collen D, De Cock F, Demarsin E, Jenné S, Lasters I, Laroche Y, Warmerdam P, Jespers L. Recombinant staphylokinase variants with altered immunoreactivity. III: Species variability of antibody binding patterns. Circulation. 1997; 95: 455-62.

[212] Collen D, Stockx L, Lacroix H, Suy R, Vanderschueren S. Recombinant staphylokinase variants with altered immunoreactivity. IV: Identification of variants with reduced antibody induction but intact potency. Circulation. 1997; 95: 463-72.

[213] Laroche Y, Heymans S, Capaert S, De Cock F, Demarsin E, Collen D. Recombinant staphylokinase variants with reduced antigenicity due to elimination of B-lymphocyte epitopes. Blood. 2000; 96: 1425-32.

Thus, by the turn of the century we were ready to intiate larger clinical trials. The search of *Thromb-X* and *ThromboGenics Ltd* for partners and additional financial resources was, however, not as successful as we had hoped. We would have to change tactics, and eventually raising the funds via the stock exchange seemed to be a last resort.

The Flemish (Interuniversity) Institute for Biotechnology

The early nineties was not a golden era for Belgian scientists. I already discussed the poor funding for research activities in Belgium and Flanders earlier on. Those days hardly any decent funding for research was available and innovation was an unknown notion for Flemish politicians. Many politicians believed that the best way to keep scientists at work was to stick them between a rock and a hard place. A lack of financial means would keep them alert and creative.
In 1992 researchers got fed up with the lack of financial support and about 3,000 scientists, PhD students and eminent professors held a protest march in Brussels. It is very unusual for scientists to demonstrate. The Vice Rector of the K.U.Leuven, Herman Vanden Berghe, made a parody of the motto of the Flemish government during a news broadcast. He turned the slogan ‘what we do ourselves, we do better’ into ‘what we destroy ourselves, we destroy better’.

VLAB (Flemish Action Program Biotechnology)

However, there seemed to be light at the end of the tunnel. The 'Flemish Action Program Biotechnology' (VLAB) was established and financed some '*centers of excellence*'. Among others, the research groups of Walter Fiers and Marc Van Montagu in Ghent, Herman Vanden Berghe and my own group in Leuven, together with some smaller teams. The VLAB was managed through the IWT (Innovation by Science and Technology), a product of the DIRV programs of the Flemish regional Minister-President Gaston Geens.

Geens’s successor, Luc Van den Brande, used the concept of the VLAB as a starting point to found the Flemish Interuniversity Institute for Biotechnology (VIB). This institute would be established in analogy with the IMEC (the Interuniversity Microelectronics Center) in Leuven and the VITO (Flemish Institute for Technological Investigation) in Mol. Van den Brande's argument was that biotechnology in Flanders already had a broad basis and that there were some eminent specialists. This was also confirmed by the evaluations of the VLAB centers of excellence. Moreover, Van den Brande regularly undertook economic missions abroad and so it happened that he, occasionally on a flight, ended up in the company of Fiers, Van Montagu, Vanden Berghe and myself. Our lobbying undoubtedly contributed a lot to the foundation of the VIB.

Van den Brande would allocate one billion Belgian Franc to the VIB for the next five years. However, many questions remained unanswered. What would VIB look like? Would it be located in a central building like IMEC and VITO? Where would VIB be located? In Ghent, with a focus on plant biotechnology? Alternatively, in Leuven, with focus on red biotechnology? Or elsewhere?

Compromise 'à la belge'

My gut feeling was that the government intented to locate VIB in Ghent. This way, Leuven would have its IMEC and Ghent its VIB. Flanders still suffered from the accrued debt in the seventies and eighties and financial freedom was as such very limited. There were insufficient funds to build a completely new research campus for biotechnology. Moreover, Leuven was not in favor of the idea that VIB would be located in Ghent.

This called for a compromise: the VIB became a virtual institute without walls. A central administrative structure would be situated in Ghent, but the researchers would remain on their local campuses. In this way, they remained embedded within their own university laboratories, they could continue using the existing infrastructure and *brain drain* from the universities to the VIB could be avoided. The flow of knowledge would remain intact and the VIB could use the university as a permanent 'database' to recruit talented young researchers.

A bank guarantee!?

The next issue was who would be eligible to enjoy the VIB funding. Which groups could participate besides the '*big four* ' - Vanden Berghe, Collen, Van Montagu and Fiers? Also within the K.U.Leuven the question caused disparity. What about the Rega Institute, for example? After all, it was a large research center, renowned in the *life sciences*, especially in virology and immunology. From a political point of view Leuven could not claim VIB funding for three large research groups. Moreover, the Rega Institute was not part of the VLAB and thus was not recognized as a center of excellence. The fact that Rega was never recognized as a center of excellence was mainly caused by internal problems. Rega failed to show themselves as a tight unit to the outside world and as such missed the VLAB participation. It seemed, however, inconceivable that Rega would now also miss the VIB train.

This was also Rector Roger Dillemans' opinion. During his term in office, Dillemans quite often took on the role of compromiser and social mediator. Dillemans eventually, after intense discussions, pursued a give-and-take policy in which Vanden Berghe and I agreed to share a part of our VIB funding with the Rega Institute. We had no alternative but to agree

with this compromise. A few days later, I received a letter from Jan De Smyter, chairman of the Rega Institute, in which he requested a bank guarantee to seal the achieved compromise. That was a bridge too far for me. Also for Rector Dillemans. The redistribution scenario was abandonned forever.

In hindsight, such a compromise in Leuven would never have been accepted by the VIB administration. The financial and budgeting control by the VIB is indeed quite strict, and the criteria to access the VIB funds particularly high.

'Administrative office' and 'peripheral executants'

Finally, VIB started after its trial year in 1995 with 9 groups. The 'famous big four' - the groups of Vanden Berghe, Fiers, Van Montagu and our group – as well as 5 smaller research teams. They all originated from the VLAB and also received excellent evaluations. It concerns the teams of: Christine Van Broeckhoven (UAntwerp), Danny Huylebroeck (K.U.Leuven), Rene Hamers (VUB), Joël Vandekerckhove (UGent) and Nicolas Glansdorff (VUB).

As one of the largest centers, 16.5% of the total VIB subsidy came our way. Since we were highly ranked in the five-year evaluations, our share slightly increased throughout the years.

However, the VIB was not a cash cow that freely distributed funding over avaricious professors, regardless of their international reputation. VIB was headed by Jo Bury and Rudy Dekeyser, and their central management had a clear vision, but seemed – according to the eminent professors – too nosy and meddlesome. I must admit that my attitude in those days was also one of just receiving the financial means without wanting too much interference in the way we did our research and how we spent the funding. After all, we know best how we have to do research and on what to spend money. Bury and Dekeyser, however, saw matters slightly differently: they prioritized well defined core activities and objectives and even interfered in the daily management of the research groups - they imposed a personnel management that in many respects was different from that implemented at the universities.

During the first years, we had many discussions. I invariably referred to the main office as the 'administrative office in Ghent' and in turn, Bury and Dekeyser usually referred to the directors of the research groups as ‘peripheral executants’.

Unique institute with focus on scientific excellence

Meanwhile, the 'big four' are retired, and I let go of my, rather limited, scepticism about the VIB. Not out of necessity, but rather out of respect for the inspiring vision and the unabated efforts of Jo Bury, Rudy Dekeyser and their VIB colleagues. VIB has known a remarkable evolution from 1994 till now. If the expression, 'from bad to worse' should have an antonym in the sense of 'from good to better', then it would certainly apply to the VIB.

The executive committee of the Flemish Institute for Biotechnology in 2008. Standing from l. to r. Prof. Rudy Beyaert (represents Prof. Frans Van Roy), Prof. Lode Wyns, Dr. Rudy Dekeyser (managing director VIB), Dr. Jo Bury (managing director VIB), Prof. Joël Vandekerckhove, Prof. Bart De Strooper.
Seated from l. to r. Prof. Désiré Collen, Marijke Lein (VIB human resources director), Prof. Johan Thevelein, Wim Goemaere (VIB chief financial officer), Prof. Dirk Inzé. Absent: Prof. Christine Van Broeckhoven, Prof. Frans Van Roy.

Whereas fifteen years ago the VIB started as a loose and virtual association of nine research departments, today it is an institute with solid foundations structured around 70 research groups, most of them top quality.

The financial means made available by the VIB provided stability. Because of these financial injections - recently supplemented by the so-called Methusalem, Odysseus and Hercules programs of the Flemish government -, we can keep talented top researchers at our universities, and even attract foreign talented researchers. Without the VIB, research in the *life sciences* in Flanders would not have such a bright future. I must admit myself that, without the VIB, I would not have reached my emeritate at the K.U.Leuven.

There is of course a price to pay for the financial stability offered by VIB. VIB sets high standards for its researchers: world-class achievements are expected. If the criteria are not met, the research groups loose their VIB funding. Each group leader is evaluated on the basis of measurable output in terms of publications and successful patent applications. In addition, an external scientific committee performs an on site visit and evaluation every five years. We had to simply roll up our sleeves. Originality, creativity, productive collaboration and excellence were the relevant criteria. '*Me too*' research was not accepted. Publications in top journals were a priority. In short, to work for the VIB is '*not a free lunch*'.

On top of this, the Flemish government closely evaluates the VIB activities every five years. This forces the institute to be self-critical and to perform better year after year. A challenge well met up to now.

Therefore, I found the recent formal and very negative criticism on VIB in a document entitled 'Flanders in action, a reaction' by colleague Zeger Debyser, inappropriate. The author states that he did not write the document out of 'personal frustration', but out of a 'chronic concern about scientific research in Flanders'. I phoned colleague Debyser to remind him of the fact, that I, about a year ago, fully supported his project-request for VIB financial support and that this project was evaluated very thoroughly and compared with competing projects. The project was not accepted solely on the basis of strict quantitative and qualitative criteria. With 'spontaneous' magnanimity, colleague Debyser declared that his initiative was not inspired in the hope of forcing his way inside VIB, but in the general interest, even if he was excluded from participating on the basis of objective evaluations.

Climate for entrepreneurship

Thanks to VIB the climate for biotech companies in Flanders also improved. Proof of that are the numerous VIB spin off companies noted on the stock exchange. In an important way the VIB contributed to securing a place in the sectors of industrial biotech and *life sciences* for Flanders.

The VIB does what I did in the eighties and nineties with t-PA. The concept of technology transfer was previously unknown at our universities. Many professors considered 'patent', 'license' and 'valorization', as unethical concepts because they were associated with making profit or just 'commercial'. An academic was supposed to stand above the mêlee of the labor field and to stay clear of the business world. A true academic served only the pure spirit of 'Science' and 'Truth'.

Because of this attitude we missed on the added value of some important discoveries, such as creating job opportunities in our own region. VIB has created a structural bridge between academic research in the *life sciences* and the business world. VIB succeeded where organizations like Innovi failed. The inventions of the VIB researchers, which form the basis for new social and industrial applications, are timely spotted and thanks to a well-defined strategy become available to society. With a limited team, the VIB managed to protect valuable inventions and to compile an important patent portfolio, to round up profitable licenses and to support successful start-up companies. Examples are *Devgen*, *CropDesign* (now BASF), *Ablynx*, *Pronota* (previously Peakadilly) and *Actogenix*.

VIB can compete with reputable institutions such as EMBL (*European Molecular Biology Laboratory*), the German Max Planck Institute or the French FNRS. It is still too early to place VIB in in the same league as the MIT (*Massachusetts Institute of Technology*) in Cambridge (US). The VIB is still too young for that and lacks the resources available to the MIT. But to become a European MIT would be a noble challenge for the VIB.

A pat on the back for the politicians

The Flemish politicians in the department of sciences should also receive a well deserved pat on the back. Gaston Geens, Luc Van den Brande and Dirk Van Mechelen as well as Fientje Moerman and Patricia Ceysens all realized the importance of science and technological innovation for Flanders' prosperity. The founding, permanent support and regular expansion of the resources of the VIB were, definitely as far as biotechnology and the *life sciences* are concerned, an important milestone. Our neighbouring countries often envy us for that. My deepest respect for their vision in these matters.

VIB3 - Department for Transgene Technology and Gene Therapy

One of the most talented researchers to cross the ocean with the support of a fellowship from the *D. Collen Research Foundation* is without doubt Peter Carmeliet. I met Peter rather accidentally during a presentation in Leuven somewhere in the beginning of 1989. He had completed his medical studies at the K.U.Leuven and obtained a PhD. Just a few months later, he was bound for the *Harvard Medical School* for a post-doc of one year.
I realized that Peter Carmeliet would be the right person to transfer the new (at that time) '*gene-targeting*' technology to Leuven. This technology allows to generate knockout mice. A specific gene is switched off in these mice, allowing to study its function. To master the technology, Peter went to the laboratory of Richard Mulligan at the *Whitehead Institute for Biomedical Sciences* between 1990 and 1991. Richard Mulligan's lab is a subdivision of the *Massachusetts Institute of Technology* in Cambridge (US). In order to speed up things, I invested about 1 million dollar to cover the salary of two Belgian laboratory technicians as well as the operating costs for our knockout project at the *Whitehead Institute*.

First knockouts in Belgium

This way, we succeeded in mastering the *gene-targeting* technology right from the start. Peter succeeded to generate three knockout mice models during his stay at the *Whitehead*: a t-PA, a urokinase and a PAI 1 knockout (plasminogen activator inhibitor 1).
I personally carried the first t-PA knockouts in my hand luggage from Boston to Leuven. I thus manually introduced the first knockout mice in Belgium. These knockouts were to become the subject of a whole range of successful fibrinolytic and thrombolytic studies, resulting in a number of important publications[214] [215] [216] [217].

[214] Carmeliet P, et al. Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. J Clin Invest. 1993; 92: 2746-55.

[215] Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, De Mol M, Mulligan RC, Collen D. Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. J Clin Invest. 1993; 92: 2756-60.

[216] Carmeliet P, et al. Physiological consequences of loss of plasminogen activator gene function in mice. Nature. 1994; 368: 419-24.

[217] Dewerchin M, Nuffelen AV, Wallays G, Bouché A, Moons L, Carmeliet P, Mulligan RC, Collen D. Generation and characterization of urokinase receptor-deficient mice. J Clin Invest. 1996; 97: 870-8.

On his return in Belgium, Peter and I put all our effort (both work as well as funding) in building a *state of the art* pathogen-free mouse facility, a laboratory for the characterization of mouse phenotypes and to attract a core team of excellent colleagues. In no time we built a 'knock-out mice center', unrivalled in Europe! In this 'knockout factory', many new mice models would be generated, including models for the Zellweger syndrome[218], factor VII deficiency [219] and thrombomodulin.[220]

VEGF at the origin of a new research direction

While he was still in training in the *Whitehead*, Peter Carmeliet developed an interest in blood vessel formation (angiogenesis) and the idea to produce knockouts for VEGF, or vascular endothelial growth factor. Angiogenesis was in every respect a totally new direction of research for our laboratory.

Carmeliet nearly bit off more than he could chew with his VEGF knockouts. He knew that VEGF must have an influence on the growth of the endothelial cells that line the interior surface of the blood vessels, but to eliminate the VEGF gene in mice seemed virtually impossible. Nonetheless, knockouts seemed to be a very reasonable manner to learn more about the function of VEGF. However, the experiments failed time and again. Even when only one of the two VEGF genes was eliminated - a mouse inherits of each gene one copy from the father and one from the mother - the embryos died prematurely in the womb. Somehow, there had to be a mistake in our protocol. Carmeliet even moved to the *Lunenfeld Institute* in Toronto (Canada) for a short stay to attempt to find a solution for the problem together with Andras Nagy.

Despite all the problems, they were on the verge of a breakthrough. When they performed a detailed examination on the dead embryos, they discovered that all embryos had serious defects in their blood vessels, which prevented them from developing further. Even with one healthy VEGF gene left further development appeared impossible. VEGF is so important that you need two healthy genes to develop beyond the embryonic phase. A unique conclusion, for no single other gene is so important during the embryonic phase of development.

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[218] Baes M, et al. A mouse model for Zellweger syndrome. Nat Genet. 1997; 17: 49-57.

[219] Rosen ED, et al. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature. 1997; 390: 290-4.

[220] Conway EM, et al. Structure-function analyses of thrombomodulin by gene-targeting in mice: the cytoplasmic domain is not required for normal fetal development. Blood. 1999; 93: 3442-50.

With this observation, Carmeliet made it to the headlines of the entire scientific press. The paper (see Appendix 13) which was published in *Nature* is still considered to be one of the most important publications in angiogenesis. The article was cited more than 1500 times by other scientists.[221]

Shortly after Carmeliet's paper in *Nature*, a similar article was published by N. Ferrara, a pioneer in the VEGF research, and his team at *Genentech*. I would like to share an anecdote here: around April 1995, when Peter Carmeliet gradually became desperate after successive failures to generate a VEGF knockout mouse, I was at *Genentech* in South San-Francisco for one of my last visits there. I told Dr. Simon, then *Director of Research* at *Genentech*, about the problems with the VEGF knockout. He confirmed the problems and told me that Ferrara's group at *Genentech* had given up. A few months later, when it appeared that the heterozygous form of the VEGF knockout dies off *in utero,* because it is impossible to form a heart and blood vessel system with only one gene, thus explaining the problem, the late Werner Risau, a colleague of Peter Carmeliet, visited *Genentech* and discussed the solution to the problem. I suspect, but do not know for sure, that this caused *Genentech* to re-boost their VEGF knockout project. Fact remains that to my knowledge, our group in Leuven was the first to demonstrate independently that inactivation of one of the two VEGF genes resulted in embryonic lethality. And that was a real first. That this was confirmed by another group, independently or with inside information, definitely facilitated the publication as well as the acceptance of the conclusions.

VIB 3

At the start of the VIB between 1994 and 1995, our group was named ‘VIB department 3’. I renamed VIB3 to the Center for Transgene Technology and Gene Therapy. I became Director of the Department and offered Peter Carmeliet the position of Adjunct Director. After my emeritate, Peter would take over from me and rename the department into the Vesalius Research Center. I will get back to the story behind this new name later on.

[221] Carmeliet P, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996; 380: 435-9.

Angiogenesis explored

Angiogenesis is a crucial process. An imbalance between too much or too less blood vessel formation leads to disease, either way. Excessive blood vessel formation occurs in cancer, eye diseases and inflammatory disorders . Impaired blood vessel formation contributes to ischemic heart disease, peripheral vascular disorders, deficient wound healing et cetera. Over the past years, about 35,000 scientific papers were devoted to angiogenesis and a variety of drugs for the treatment of cancer and eye diseases based on the principles of angiogenesis were developed[222]. In spite of fierce competition, Peter Carmeliet and his team managed to keep their leading position. Even better, he surpassed his ambitions in angiogenesis. The paper by his group in *Nature* was followed by several others in *Nature Medicine, Nature Genetics* or *Cell*, covering topics in the cardiovascular field, but also in cancer and neurology.

Within a few years, the following breakthroughs were published:

• in *Nature* that HIF proteins play an important role as an oxygen sensor to trigger angiogenesis in tumors; [223]

• in *Cell* that vascular endothelial cadherin promotes the adhesion between endothelial cells in the blood vessels via binding to the VEGF receptor 2, beta catenin and PI3 kinase;[224]

• in *Nature Medicine* that a relation exists between the different isoforms of VEGF and their specific function in the angiogenic process; [225]

• in *Nature Genetics* that mutations in the promoter sequence of the VEGF gene can cause motor neuron disorders; [226]

[222] Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005; 438: 932-6. Review.

[223] Carmeliet P, et al. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature. 1998; 394: 485-90.

[224] Carmeliet P, et al., Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGFmediated endothelial survival and angiogenesis. Cell. 1999; 98: 147-57.

[225] Carmeliet P, et al. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med. 1999; 5: 495-502.

[226] Oosthuyse B, et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet. 2001; 28: 131-8.

• in *Nature Medicine* that VEGF and placental growth factor (PlGF) contribute in a synergistic manner to the angiogenic processes; [227]

• in *Nature Medicine* that the loss of HIF2 and the inhibition of VEGF impair fetal lung maturation and that treatment with VEGF is a potential treatment for prematures with respiratory problems;[228]

• in *Cell* that antibodies against PlGF inhibit solid tumor growth[236]

Each of these papers (except for the latest Cell publication, which is still too recent) has a citation index of more than 200; the *Nature* paper already exceeds 1000. In the same period, Peter Carmeliet wrote several reviews, of which one in *Nature Medicine*[229] and one in *Nature*[230] were cited more than 2000 and 3000 times, respectively.

Cure for ALS?

Sometimes one is faced with surprises during a research project. For instance, the fact that mice whose promoter sequence of the VEGF gene has been altered so that less VEGF is produced, develop neuronal disorders. On closer inspection, it appeared that these mice showed pathological characteristics of amyotrophic lateral sclerosis (ALS): this is a degeneration of motor neurons in the spinal cord and the brain stem[231]. Also in humans, VEGF plays a role in the onset of ALS[232]. Five out of 100,000 people are affected by ALS. It is an incurable disease that affects people in the prime of their lives. Initially, the muscles get weaker, it is increasingly difficult to move and paralysis occurs. Finally, the muscles used for basic functions such as swallowing, speaking and breathing fail. There is no effective cure for ALS and approximately half of the patients die between the third and the fifth year after diagnosis. A patient rarely survives more than 10 years.

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227 Carmeliet P, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med. 2001; 7: 575-83.

228 Compernolle V, et al. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med. 2002; 8: 702-10.

229 Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med. 2000; 6: 389-95. Review.

230 Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000; 407: 249-57. Review.

231 Oosthuyse B, et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet. 2001; 28: 131-8.

232 Lambrechts D, et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet. 2003; 34: 383-94.

Carmeliet's research indicates that VEGF has a double effect on motoric nerves: it allows a good blood flow providing the neuronal cells with sufficient oxygen and nutrients, and also directly protects the cells.

The next step was to find out whether VEGF would offer therapeutic perspectives for the treatment of ALS. Two strategies were tested extensively on experimental animals: gene therapy - in collaboration with *Oxford Medica*[233] - and the use of an osmotic pump which delivers VEGF directly into the brain.[234] Both methods were successful, but the use of the pump appears to be more appropriate for the time being. Rats with ALS that receive VEGF through a pump, performed better in a series of motoric tests, and their life expectancy also increased considerable. Moreover, the advantage of the osmotic pump is that the dosages can be adjusted and the treatment can be interrupted immediately when side effects occur.

In December 2008, the first clinical rials were started at the K.U.Leuven in collaboration with the Swedish biopharmaceutical company *NeuroNova*. Coordinated by neurologist Wim Robberecht (UZLeuven and Vesalius Research Center), the controlled trials should demonstrate that VEGF can be administered safely, and that it is tolerated by the patients. In a next phase, clinical trials will be conducted to establish whether VEGF indeed has a beneficial effect on ALS in humans. Even with positive results, it will take several more years before a drug can be developped.

Cancer research

Through angiogenesis, Peter Carmeliet brought also cancer research into our research group. Tumors indeed grow quicker than normal tissue and thus need more oxygen and nutrients. Therefore, tumors try to stimulate the growth of new blood vessels through signal molecules such as VEGF and its homologue placental growth factor (PlGF). These molecules

[233] Azzouz M, Ralph GS, Storkebaum E, Walmsley LE, Mitrophanous KA, Kingsman SM, Carmeliet P, Mazarakis ND. VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. Nature. 2004; 429: 413-7.

[234] Storkebaum E, et al. Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. Nat Neurosci. 2005; 8: 85-92.

bind to receptors on the endothelial cells of adjacent blood vessels and initiate the formation of new blood vessels[235].

By using antibodies that specifically bind to VEGF and PlGF, the interaction of these proteins with their receptors can be blocked, angiogenesis is halted and the tumors starve[236]. Various cancer therapies based on inhibition of tumor angiogenesis are being developed, but most of them are directed against VEGF or its main receptor. Until now, it appeaed that such antibodies have a significant effect on the survival of cancer patients, however only limited in time. Apparently, the tumor cells compensate by generating other growth factors that stimulate angiogenesis. The drugs also have serious side effects. There is clearly a need for additional targets and PlGF seems to be the most obvious one.

From a first series of pre-clinical experiments, it appeared that an antibody against PlGF in mice with tumors causes fewer side effects than anti-VEGF. Moreover anti-PlGF in starving tumor cells does not switch on a 'rescue operation' by generation other growth factors.[237] [238] The favorable evaluation of anti-PlGF as potential drug against cancer kindles hope for a more efficient cancer therapy with fewer side effects - also for children and pregnant women. Anti-PlGF not only increases the efficiency of chemotherapy and of present anti-angiogenesis therapy, but it also inhibits the growth and spreading of tumors that are resistant to existing drugs. *ThromboGenics* and the pharmaceutical company *Roche*, that meanwhile has purchased *Genentech*, will invest in the clinical development of anti-PlGF (more about that in the next chapter).

Vesalius

VEGF and ALS are not the only link between the blood vessel and the nervous system. Peter Carmeliet and his colleagues showed that blood vessels and axons use the same pattern and the same molecular signals during embryonic development. That blood vessels and axons

[235] Carmeliet P, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med. 2001; 7: 575-83.

[236] Luttun A, et al., Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med. 2002; 8: 831-40.

[237] Fischer C, et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor- resistant tumors without affecting healthy vessels. Cell. 2007; 131: 463-75.

[238] Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGFB and PlGF: drug targets for antiangiogenic therapy? Nat Rev Cancer. 2008; 8: 942-56. Review.

find their way via the same 'road map' through the developing body, is a phenomenon that Andreas Vesalius had already observed in the 16th century: during his anatomical analysis he found it remarkable that important axons lay next to important blood vessels. In other words, he already observed four hundred years ago what Carmeliet and his eam would explain on a molecular level in the 21st century.

In honor of this 'father of anatomy' who was educated in Leuven, the VIB Department for Transgene Technology and Gene Therapy was renamed into the Vesalius Research Center in the spring of 2008. I hereby wish the Center a brilliant future.

ThromboGenics, stock exchange and future

Since 7 July 2006, *ThromboGenics* NV is listed on the *Euronext Brussels*. 7.77 million shares were issued at € 4.50 and our IPO provided us with € 35 million. This enabled us to further finance research into the development of new drugs. We failed in collecting venture capital but clearly succeeded in our IPO. As acting CEO of *ThromboGenics*, I was particularly delighted with the outcome of our IPO. The successful introduction to the stock exchange indeed indicated that *ThromboGenics* is a scientifically as well as economically credible business with a range of promising products in the pipeline. Although all our products are related to the vascular system, they have an unexpected broad area of application.

A stock exchange listing has more advantages than the mere input of capital. The company becomes more visible to investment funds, analysts and potential employees. Moreover, the value of the enterprise is much clearer. An alternative would have been to sell *ThromboGenics* to the highest bidder. This would take a lot of burden from our shoulders, but of couse the challenges would also be gone.

In 2009 *ThromboGenics* became a biopharmaceutical company with European stature. It devotes itself to the research and development of innovative biotechnological drugs for a range of serious disorders, like diseases of the back of the eye, cardiovascular disorders and cancer. We put strong emphasis on products that create a high value for both our shareholders and for society. We moved to the bio-incubator in Leuven, a more efficient location for our staff. Subsequently, I passed command on to Patrik De Haes. He became CEO of *ThromboGenics* in August 2008. I remain Chairman of the Management Board.

ThromboGenics has already achieved a couple of significant milestones, but I am convinced that the best is yet to come. *ThromboGenics* is one of the few Belgian companies to succeed in taking a potential drug to phase III clinical trials. This concerns the protein microplasmin for the treatment of eye diseases. Moreover, *ThromboGenics* concluded an important agreement with *Roche* for the further development of an antibody against the placental growth factor (anti-PlGF) as anti-angiogenic cancer drug. This agreement provides *ThromboGenics* with a steady flow of income by means of an important up-front payment, milestone payments and potential *'double digit'* royalties. This agreement created more strategic flexibility and a stable financial basis for *ThromboGenics*. This strong financial position allows us to invest more in our own clinical trials. *ThromboGenics*' portfolio indeed contains more than microplasmin and anti PlGF.

Microplasmin in 'pole position'

Microplasmin is a shorter and more stable form of plasmin, the enzyme that dissolves the proteins in blood clots and that is formed naturally in our body (See chapter 'Molecular basis of fibrinolysis unraveled'). The Center for Molecular and Vascular Biology was already interested in the development of microplasmin as a potential thrombolytic drug. In 2000, we succeeded to make a recombinant and stable form of this truncated protein in the yeast *Pichia pastoris*[239]. We extracted 500 mg microplasminogen from 1 liter yeast culture. Microplasminogen is a precursor protein that is easily converted to plasmin.

At first, microplasmin was successfully tested by my Japanese colleague Nobuo Nagai as a thrombolytic drug in an experimental stroke model[240] [241] [242]. *ThromboGenics* took microplasmin under license to develop it further clinically. In March 2004, *ThromboGenics* started a collaboration with American *NuVue*[243]. It appeared that microplasmin also breaks down proteins in the eye which connect the vitreous material (the clear gel in the eyeball) with the retina. This property of microplasmin can change the treatment of diseases at the back of the eye. Some of the affections that could be treated with microplasmin are problems with vitreomacular traction, diabetic retinopathy, diabetic macular edema, macular holes and age related macular degeneration. The current treatment requires a surgical intervention whereby the vitreous gel in the eyeball is detached from the retina and subsequently fully drained. This procedure is rather expensive and some patients suffer from side effects such as blurred vision, bleeding, retinal detachment, and development of

[239] Patent UK 0031196.9. Recombinant human plasminogen and plasmin: Methods for high yield production, stabilization and use for treatment. Collen D. (inventor); Thromb-X, NV (assignee); December 21, 2000.

[240] Nagai N, De Mol M, Van Hoef B, Verstreken M, Collen D. Depletion of circulating alpha(2)-antiplasmin by intravenous plasmin or immunoneutralization reduces focal cerebral ischemic injury in the absence of arterial recanalization. Blood. 2001; 97: 3086-92.

[241] Nagai N, Demarsin E, Van Hoef B, Wouters S, Cingolani D, Laroche Y, Collen D. Recombinant human microplasmin: production and potential therapeutic properties. J Thromb Haemost. 2003; 1: 307-13.

[242] Suzuki Y, Chen F, Ni Y, Marchal G, Collen D, Nagai N. Microplasmin reduces ischemic brain damage and improves neurological function in a rat stroke model monitored with MRI. Stroke. 2004; 35: 2402-6.

[243] Press release ThromboGencis 10 March 2004: ThromboGenics and NuVue Technologies to Collaborate on Development of Plasmin-Based Drugs for Visual Disorders.

glaucoma and cataract. From the current clinical trials[244] [245], it appears that microplasmin detaches the vitreous gel in the eye ball from the retina in 30% to 40% of the patients, without surgical intervention[246] [247] [248] [249]. For other patients, the surgical intervention is facilitated because the adhesion of the vitreous gel to the retina is decreased.

To phase III

Following a successful '*end of phase II meeting*' with the FDA upon completion of the phase II clinical trial. *ThromboGenics* started with phase III in the clinical development of this potential drug. The project consists of two studies of which one will take place in the United States[250] and a second one in both Europe and the United States[251]. In each of the studies 320 patients will be recruited in 40 centers. The initial clinical picture for the phase III studies is the non-surgical treatment of focal vitreomacular adhesion. In this disorder, the vitreous gel in the eyeball shows abnormally strong adhesion with the retina. This adhesion can lead to the deformation of blood vessels and retina, which results in deterioration in the patient's vision. Moreover, vitreomacular adhesion plays an important role in back of the eye affections, such as macular holes and particular forms of macular edema.

Both studies are randomized, double-blind controlled trials, which will evaluate a dose of 125 µg microplasmin versus placebo. The primary endpoint of both studies is the

[244] ClinicalTrials.gov, A Dose-Escalation Clinical Trial of Intravitreal Microplasmin in Patients Undergoing Surgical Vitrectomy for Vitreomacular Traction Maculopathy, NCT00123279

[245] ClinicalTrials.gov, A Randomized, Sham-Injection Controlled, Double-Masked, Ascending-Dose, Dose-Range-Finding Trial of Microplasmin Intravitreal Injection for Non-Surgical PVD Induction for Treatment of Vitreomacular Traction, MIVI-IIt, NCT00435539

[246] Press release ThromboGenics, 4 December 2007: ThromboGenics release results of the Vitreomacular Traction Trial-Study (MIVI IIT) on occasion of the annual meeting of the "American Society of Retinal Specialists".

[247] Press release ThromboGenics, 23 May 2008: ThromboGenics release more results of the Vitreomacular Traction Trial-Study (MIVI IIT) on occasion of the annual Euretina congress in Vienna.

[248] Press release ThromboGenics, 30 June 2008: ThromboGenics announce positive results from its Phase IIb trial-study of Microplasmin in Vitrectomy (MIVI III).

[249] Press release ThromboGenics, 14 November 2008: ThromboGenics announce very promising results from its Phase IIb trial-study (after 6 months) of Microplasmin in Vitrectomy (MIVI III) for the treatment of eye disorders.

[250] ClinicalTrials.gov, A Randomized, Placebo Controlled, Double-Masked, Multicenter Trial of Microplasmin Intravitreal Injection for Non-Surgical Treatment of Focal Vitreomacular Adhesion**,** The MIVI-TRUST (TG-MV-006) Trial, NCT00781859.

[251] ClinicalTrials.gov, A Randomized, Placebo Controlled, Double-Masked, Multicenter Trial of Microplasmin Intravitreal Injection for Non-Surgical Treatment of Focal Vitreomacular Adhesion, The MIVI-TRUST (TG-MV-007) Trial, NCT00798317.

resolution of the adhesion within one month without a surgical intervention. We expect to terminate these trials by the end of 2010. In addition, phase II studies with microplasmin will be initiated for the treatment of diabetic macular edema[252] and age related macular degeneration.

Microplasmin for ischemic stroke

Microplasmin is also a potential drug for the dissolution of blood clots, in particular for the treatment of acute ischemic stroke. Microplasmin is a directly acting thrombolytic agent with fewer side effects than other thrombolytics and has the potential to be effective for a longer time after the stroke, possibly for up to twelve hours. A thrombolytic drug, like t-PA, can only be administered maximally three to four hours after the onset of the stroke, which means that less than five percent of the ischemic stroke patients are treated with t-PA.

The results of a phase II study[253] with microplasmin for the treatment of acute stroke indicated that it is not only well tolerated but also shows some superiority at the level of activity[254]. Approximately 25% of the patients treated with microplasmin showed a recovery of the blood flow within eight hours of the treatment, in comparison to 10% of the patients with placebo treatment. Moreover, in 33% of the patients with more serious vascular occlusions treated with microplasmin, reperfusion occurred, in comparison to 14% of the patients who received placebo. Because of small sample size, none of these results was statistically significant. Nonetheless, this research showed that patients treated with microplasmin, had significantly less damage to the blood - brain barrier, as compared to placebo treated patients.

Based on the promising results of these phase II studies, *ThromboGenics* decided to look for a financially strong partner for the further development of this drug against ischemic stroke. In spite of the promising phase II results, this is not evident because the earlier development of drugs against stroke often turned into a 'major disaster'. Except for the use of rt-PA,

[252] Press release ThromboGenics, 6 March 2009: ThromboGenics end recruiting of patients for Phase II trial-study of microplasmin for the the treatment of diabetic macular edema (MIVI II DME).

[253] ClinicalTrials.gov, A Multicenter, Double-Blind, Placebo-Controlled, Ascending-Dose, Clinical Trial of Intravenous Microplasmin Administration in Patients With Acure Ischemic Stroke, NCT00123305.

[254] Press release Thrombogenics, 29 September 2008: ThromboGenics announce very promising results of MITI IV Phase II trial-study on the treatment of acute ischemic stroke with microplasmin.

which must be administered within the first hours after the onset of the thrombosis, a multitude of promising drugs were tested out - without success.

Staphylokinase

Staphylokinase is a bacterial protein that dissolves blood clots and can be used in the treatment of cardiovascular diseases, including myocardial infarction (see chapter staphylokinase, 't-PA for the poor'). Phase II trials demonstrated that staphylokinase is at least as effective as rt-PA[255]. Staphylokinase could eventually be made available to patients at a much lower cost.

Definitely, the biggest disillusion of my career is that I could not develop staphylokinase as the 't-PA for the poor' in the Western world. I am absolutely convinced that staphylokinase could have been an inexpensive life-saving drug, but in the present context of regulations it is economically not viable. Due to the commotion around the 'mega trials' (GISSI, ISIS and GUSTO), it is no longer sufficient to convincingly show that a thrombolytic agent works. One must also demonstrate that it saves at least as many lives as the present standard therapy, being mostly TNK-t-PA or tenecteplase. Because the thirty-day' mortality for patients with myocardial infarction currently lies at 8%, it is necessary to conduct a phase III trial with at least 15,000 patients, of which half is to be treated with the control drug. The cost of only TNK-t-PA would then amount to approximately €15 million, and the full trial cost to $100 million. We have no alternative but to develop staphylokinase for countries where t-PA is not available (being too expensive) and where the efficacy in comparison to streptokinase as endpoint will be accepted.

Therefore, *ThromboGenics* signed an agreement in 2006 with *Bharat Biotech International Ltd.* (India) for the clinical development, production and commercialization of staphylokinase. *Bharat Biotech* has the capacity to produce staphylokinase and at present negotiates with the regulating authorities in India for the launch of a phase III trial.[256] [257]

[255] Vanderschueren S, et al. Randomized coronary patency trial of double-bolus recombinant staphylokinase versus front-loaded alteplase in acute myocardial infarction. Am Heart J. 1997; 134: 213-9.

[256] Press release ThromboGenics, 6 December 2006: ThromboGenics sign a licence agreement with Bharat Biotech for a new blood clot dissolvent.

[257] Press release ThromboGenics, 4 September 2007: ThromboGenics announce the successful conclusion of the technology transfer to Bharat Biotech for the production of a new blood clot dissolvent.

THR-174 is a second generation staphylokinase. The drug has an improved activity and safety profile. These are based partially on its reduced immunogenicity in comparison with the natural versions of staphylokinase and other established thrombolytics, like streptokinase. In 2007, *ThromboGenics* signed a licensing agreement with *Rhein Minapharm* (Egypt) for the production, the clinical development and the marketing of THR-174 in amongst others, the Middle East and Africa.[258]

Antibody against factor VIII (TB-402)

Another potential drug in *ThromboGenics'* pipeline is TB-402. This is an antibody against coagulation factor VIII (FVIII), an important component in the coagulation cascade. With TB-402, *ThromboGenics* wants to prevent the formation of unwanted blood clots, for example in deep vein thrombosis. Such blood clots form in a deep vein, mostly in the lower leg. These clots are life threatening since they could embolize via the heart to the lungs and cause pulmonary embolism. Deep vein thrombosis is considered to be a serious general health problem. It is estimated that annually, in the US alone, more than 350,000 people are affected by deep vein thrombosis or pulmonary embolism. Both diseases are fatal to more than 100,000 people per year in the US.[259]

Moreover, increasingly more people undergo knee and hip replacement surgery, which are frequently associated with deep vein thrombosis (up to 40% in the absence of anticoagulation). All these patients are eligible for prophylactic treatment with an anticoagulant drug to reduce the risk of blood clot formation.

TB-402 is an anticoagulant with prolonged activity (half-life of approximately three weeks). This means that the patient requires only one dose after surgical intervention resulting in at least one month of safe anticoagulation. It is important that the action of TB-402 is reversible. This means that a patient, who received TB-402 treatment, can safely undergo a surgical intervention. These are all advantages over the existing anticoagulants. TB-402 is developed by *ThromboGenics* in collaboration with the Swedish *BioInvent.*[260]

[258] Press release ThromboGenics, 4 October 2007: ThromboGenics and Rhein Minapharm announce an agreement on next generation thrombolytic medicines.

[259] Press release Office of the Surgeon General, US Dept. of Health and Human Services, 15 September 2008: Acting Surgeon General Issues 'Call to action to prevent deep vein thrombosis and pulmonary embolism'.

[260] Press release ThromboGenics, 27 September 2004: BioInvent and ThromboGenics strike alliance to jointly develop antibody-based therapeutics.

A phase II trial is currently ongoing[261]. It is a prospective, randomized, multi-center, *'open label'* study. This study evaluates escalating doses of TB-402, administered as a single bolus injection after knee replacement surgery. This trial will enroll 300 patients, divided across 36 centers, mainly in central Europe. The primary target is to evaluate the safety and efficacy of the three escalating doses of TB-402. As reference, one group of patients will receive heparin as anticoagulant. This trial will be completed towards the end of 2010.

ThromboGenics and *BioInvent* have the intention to out-license TB-402 to a partner with the necessary infrastructure and financial means for its clinical development. This partner should also guarantee the successful commercialization of TB-402, considering its enormous market potential.
Another application for which TB-402 has considerable potential is that of atrial fibrillation. These are heart rhythm disturbances that develop because the upper chambers contract irregularly. The consequence is that the blood is not completely pumped from the heart and could clot. These clots could migrate to the brain via the blood circulation and cause stroke. Atrial fibrillation occurs relatively frequent in elderly patients: approximately seven million people in Europe and the United States are affected. The anticoagulant properties of TB-402 could constitute a promising new treatment for the prevention of stroke in patients with atrial fibrillation.

Antibody against PlGF

In June 2008 *ThromboGenics* reached an important agreement with *Roche*, as partner for its unique anticancer drug TB-403[262]. TB-403 is a humanized monoclonal antibody against PlGF (placental growth factor) that blocks the formation of new blood vessels in solid tumors (see also chapter 'VIB3 - Department for Transgene Technology and Gene Therapy').

By blocking the formation of new blood vessels, TB-403 has the potential to reduce the growth and metastasis of cancer cells. Anti-angiogenic agents have already been used clinically, but the present generation, directed predominantly against VEGF, influences the

[261] ClinicalTrials.gov: Single Intravenous Administration of TB-402 for the Prophylaxis of Venous Thromboembolic Events (VTE) After Total Knee Replacement Surgery: A Dose-Escalating, Multicenter, Randomised, Active-Controlled Open Label Study. NCT00793234.

[262] Press release ThromboGenics, 18 June 2008: ThromboGenics and BioInvent announce a strategic alliance with Roche for TB-403, an innovative antibody against cancer.

growth and the formation of new blood vessels in cancer as well as in healthy tissue. Their therapeutic potential is thus limited by side effects. TB-403, in contrast, decreases the growth of new blood vessels in cancer without affecting healthy tissue[263]. In June 2008 *ThromboGenics* announced the positive results of a phase Ia trial with TB-403[264]. It showed that TB-403 was safe and well tolerated and had a pharmacokinetic profile compatible for an anti-cancer drug. At present, TB- 403 is in a phase Ib trial[265] to assess its tolerability, pharmacokinetic and pharmacodynamic properties in patients with advanced cancer. This study was taken over by *Roche*, which is responsible for the further development.

The financial aspect of the agreement with *Roche* attracted attention. The Belgian quality newspaper 'De Standaard' headlined *'thoroughbread biotech company hits jackpot'*;[266] 'The financial newspaper 'De Tijd' writes *'ThromboGenics strikes first deal with heavy weights.'* [267] It is difficult to overestimate the financial value of the agreement. *Roche* paid €50 million up-front and depending on the achievement of milestones will pay a maximum of €450 million additionally. The eventual marketing of the drug will earn *ThromboGenics* and *BioInvent* 'double-digit' royalties, of which *ThromboGenics* is entitled to 60% and *BioInvent* to 40%.

For the sake of comparison: the rt-PA agreement with *Genentech* was worth $144 million, including 3% royalties.

Early in the pipeline

ThromboGenics remains on the lookout for new opportunities. It is in contact with a broad range of research centers and universities in Belgium as well as abroad to continue research on innovative technology and molecules to add to its pipeline. Recently, the anti-VPAC1 project *(vasoactive intestinal peptide/pituitary adenylyl cyclase-activating peptide receptor 1)* was launched for the treatment of thrombocytopenia (low number of platelets in blood).

263 Fischer C et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor- resistant tumors without affecting healthy vessels. Cell. 2007; 131: 463-75.

264 Press release ThromboGenics, June 2008: ThromboGenics and BioInvent announce positive results on the Phase I trial-study with anti-PlGF, TB-403, a medicine against cancer.

265 ClinicalTrials.gov: An Open, Phase I, Dose Escalation Study of the Monoclonal Antibody TB-403 Directed Against PlGF, Given as Multiple IV-Doses to Patients With Solid Tumors. NCT00702494.

266 De Standaard, 'Kassa rinkelt voor biotechvolbloed', 19 June 2008.

267 De Tijd, 'ThromboGenics heeft eerste deal met grote jongens beet', 19 June 2008.

Trombocytopenia is a serious side effect of chemotherapy in cancer patients. The patient risks spontaneous bleeding. Currently, the only available treatment is a transfusion of blood platelets. This is, however, only a temporary solution and implies additional expenses and risks.

A joint research project by *ThromboGenics* and the K.U.Leuven demonstrated that the inhibition of VPAC1 sent-signals, stimulates the production of blood platelets[268]. The VPAC1 receptor was found on the surface of megakaryocytes, the bone marrow cells that produce blood platelets. The inhibition of VPAC1 promotes the production.

A winning team

ThromboGenics is about to become one of the most promising biotech companies in Europe. A highly skilled team forms the backbone for *Thrombogenics'* success. Over the last years and months, this team succeeded to make excellent progress with its clinical pipeline, closed an important license agreement with *Roche* and continues its search for new opportunities. Without doubt, the success of *ThromboGenics* is based upon its excellent scientific knowledge, its rich academic culture and its internal expertise. All these components at *ThromboGenics* are of an international level and are a source of immense satisfaction to me as the founder of the company.

[268] Freson K, Peeters K, De Vos R, Wittevrongel C, Thys C, Hoylaerts MF, Vermylen J, Van Geet C. PACAP and its receptor VPAC1 regulate megakaryocyte maturation: therapeutic implications. Blood. 2008; 111: 1885-93.

The ThromboGenics NV team combines entrepreneurship, scientific excellence and a rich academic culture.

Epilogue:
What's next?

In October 2008, I became emeritus professor at the K.U.Leuven. Headstrong emeritus professors frequently are not welcome on their former campus. They are considered to interfere with the younger generation that after being of patronized, wants to go its own way. I was taught by my predecessor, Marc Verstraete, that the best way to keep up good relations with your successors is to not interfere in their affairs, unless they invite you to do so.

Professor Roger Lijnen is now director of the Center for Molecular and Vascular Biology and Professor Peter Carmeliet became manager of the Vesalius Research Center. Everything appears to be in good hands. If they ask my advice, I will be glad to provide it, if not, it is fine by me. I will keep my office and the adjacent administrative section on the ninth floor of Gasthuisberg together with my secretary, Diane De Wyngaert. This is, after all, an acquired right (until the end of 2035), due to my co-financing of the construction of the floor with t-PA royalties. From there we manage the *Life Sciences Research Partners* npo (LSRP), the successor of the *D. Collen Research Foundation*.

LSRP was mandated by K.U.Leuven to manage the technology transfer and valorization of the Center for Molecular and Vascular Biology, as well as the Vesalius Research Center of VIB/KUL. The current LSRP Management Board consists of four members: Désiré Collen, chairman, Chris Buyse, manager and financial expert, Guy Mannaerts, manager delegated by the K.U.Leuven and Raymond De Bondt, manager delegated by *Biggar Ltd. Biggar Ltd* is the investment tool of a charity institution, financed with t-PA royalties in agreement with K.U.Leuven and D. Collen according to the agreement of February 1976. Through the initial financing of *ThromboGenics* Ltd at 1 Irish Pound per share, *Biggar* still owned more than one third of the *ThromboGenics'* shares after its IPO in 2006. Under pressure of the institutional investors *Biggar* had to partly divest to decrease the overhang of shares. Because of this, *Biggar* has considerable financial resources. Approximately €1.5 million are transferred to LSRP annually, to be used as seed money for start-up companies. Clearly, this is a useful, although unpaid, extra job for an emeritus professor.

In addition, I am also Chairman of the Management Board of *ThromboGenics*. Patrik De Haes, CEO, Chris Buyse, CFO, Landon Clay, currently the main shareholder, Jean-Luc Dehaene, EU parliamentary, Staf Van Reet, chairman of *Movetis* and Luc Philips, CFO of *KBC* constitute the Management Board. Furthermore, as a scientific adviser of *ThromboGenics NV,* I am a member of its '*Preclinical Research Board*' with the goal to supply the pipeline with candidate drugs. I attend their weekly meetings.

Management Board of ThromboGenics NV June 2009. From l. to. r. Staf Van Reet, Désiré Collen, Luc Philips, Chris Buyse, Patrik De Haes and Jean-Luc Dehaene. Insert Landon Clay.

Although my future now largely lies behind me, I still inted to do some useful things, at least as long as I remain in good health and feel that ‘drive’. I indeed do not expect to be punished in the afterlife for my sins or to be rewarded for my good deeds. So I guess it must all happen here. For me the present day is actually worthwhile.

What I definitely refuse are paid side jobs, certainly not in fixed employment. These would indeed imply that I would have to dance to someone else's tune and I have put those days behind me, a long time ago.

APPENDICES

1. Trombosis test, my first patent

First “Collen”-patent, initially submitted in the Netherlands, later extended to the U.S. The patent protects the commercial exploitation of tests of enzym-inhibitor complexes in blood plasma (a.o. plasma-antiplasmin, plasmin-a_2-macroglobulin and thrombin-antithrombin II). The test has never been commercialized because (in those days) specific (monoclonal) antibodies were not available.

United States Patent [19] [11] **4,216,291**

Collen [45] **Aug. 5, 1980**

[54] **THROMBOSIS-TEST**

[75] Inventor: **Desire J. Collen**, Winksele, Belgium

[73] Assignee: **Leuven Research & Development V.Z.W.**, Louvain, Belgium

[21] Appl. No.: **949,631**

[22] Filed: **Oct. 10, 1978**

Related U.S. Application Data

[63] Continuation of Ser. No. 723,187, Sep. 13, 1976, abandoned.

[30] **Foreign Application Priority Data**

Sep. 19, 1975 [NL] Netherlands 7511055

[51] **Int. Cl.**[2] C07G 7/00; C07G 7/02; G01N 31/14; G01N 33/16

[52] **U.S. Cl.** ..... 435/7; 23/230 B; 260/112 R; 424/12; 435/13

[58] **Field of Search** ..... 195/103.5 A, 103.5 R, 195/99; 424/12, 85, 88; 23/230 B; 435/7, 13; 260/112 R

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,912,805 10/1975 Cayzer et al. ..... 195/103.5 X

OTHER PUBLICATIONS

Collen et al., A Tanned Red Cell Hemagglutination Inhibition Immunoassay (TRCHII) for the Quantitative Estimation of Thrombin-Antithrombin III and Plasmin-Alpha-Antiplasmin Complexes in Human Plasma, Thrombosis Research, vol. 7 1975, pp. 235–238.

Collen, D., Emergence in Plasma During Activation of the Coagulation or Fibrinolytic Systems of Neoantigens, Associated with the Complexes of Thrombin or Plasmin with their Inhibitors, Thrombosis Research, vol. 5, 1974, pp. 777–779.

Collen et al., Immunochemical Distinction Between Antiplasmin and Alpha$_1$-Antitrypsin, Thrombosis Research, vol. 7 1975, pp. 245–249.

Merskey et al., A Rapid, Simple, Sensitive Method for Measuring Fibrinolytic Split Products in Human Serum, Proc. Soc. Exp. Biol. Med., vol. 131 1969, pp. 871–875.

Primary Examiner—David M. Naff

Attorney, Agent, or Firm—Lewis H. Eslinger

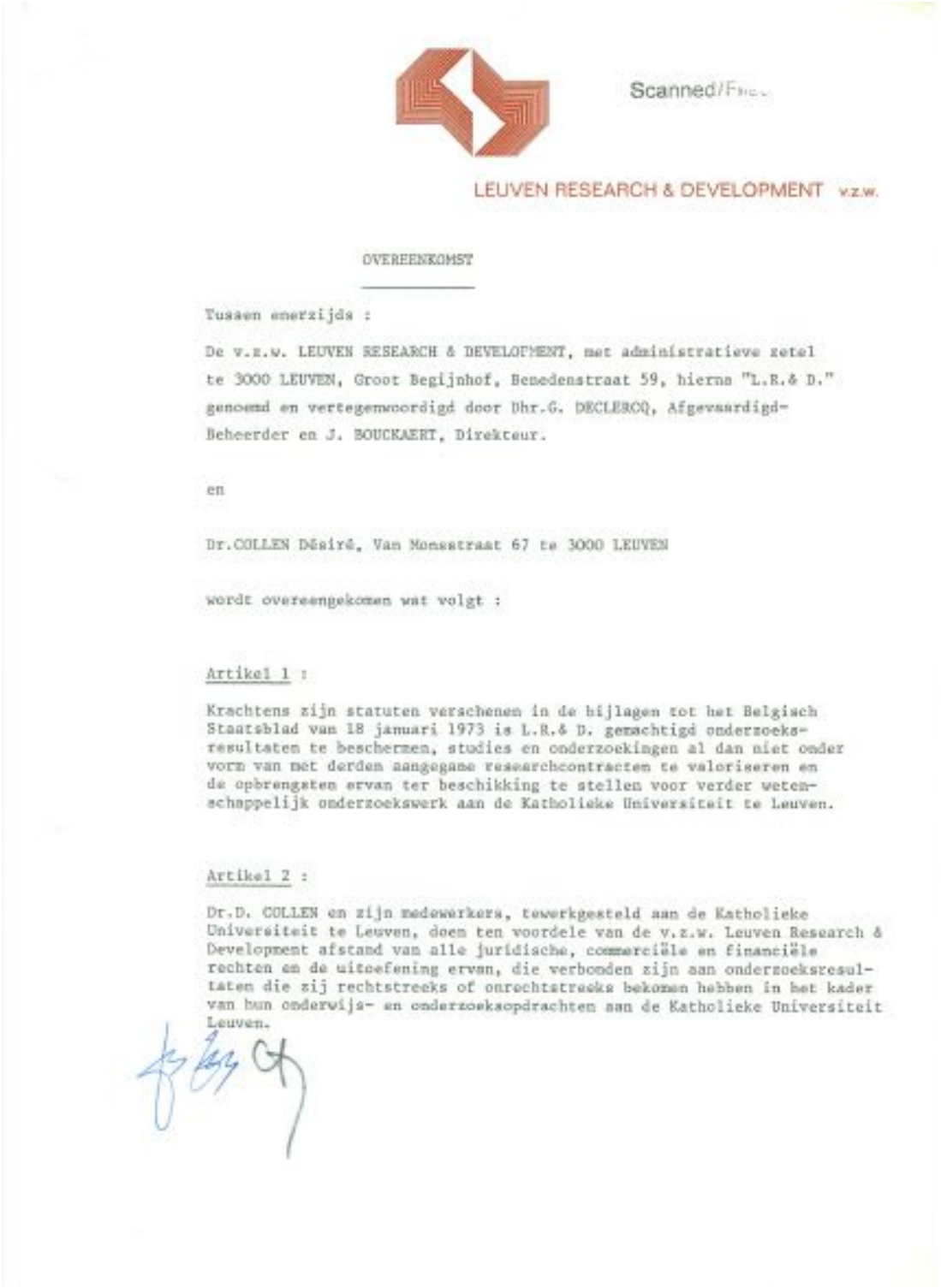
[57] **ABSTRACT**

A thrombosis test is based on the presence of specific, newly-found, enzyme-inhibitor complexes (such as plasmin-antiplasmin, plasmin-α_2-macroglobulin, thrombin-antithrombin-III) in blood samples having an activated blood coagulation and/or fibrinolytic system. The test is immunochemical and uses a purified antiserum which has been generated against the enzyme-inhibitor complex. The antiserum is purified by incubating the antiserum with plasminogen and fresh blood plasma and isolating a gamma globulin fraction containing antibodies specific to the enzyme-inhibitor complex. A reagent for the test is prepared by contacting a blood cell suspension or a latex of particles of synthetic resin with the purified antiserum to obtain a suspension or latex in which antibodies from the antiserum are present on the surface of the cells or particles.

8 Claims, No Drawings

2. Agreement between LR&D and Désiré Collen

The frame agreement with Leuven Research and Development vzw (non profit organization) for the valorization of the research results in the lab of Désiré Collen. The contract was signed in February 1976 and forms the basis for the allocation of the t-PA royalties.



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LEUVEN RESEARCH & DEVELOPMENT v.z.w.

OVEREENKOMST

Tussen enerzijds :

De v.z.w. LEUVEN RESEARCH & DEVELOPMENT, met administratieve zetel te 3000 LEUVEN, Groot Begijnhof, Benedenstraat 59, hierna "L.R.& D." genoemd en vertegenwoordigd door Dhr.G. DECLERCQ, Afgevaardigd-Beheerder en J. BOUCKAERT, Direkteur.

en

Dr.COLLEN Désiré, Van Monsstraat 67 te 3000 LEUVEN

wordt overeengekomen wat volgt :

Artikel 1 :

Krachtens zijn statuten verschenen in de bijlagen tot het Belgisch Staatsblad van 18 januari 1973 is L.R.& D. gemachtigd onderzoeks-resultaten te beschermen, studies en onderzoekingen al dan niet onder vorm van met derden aangegane researchcontracten te valoriseren en de opbrengsten ervan ter beschikking te stellen voor verder weten-schappelijk onderzoekswerk aan de Katholieke Universiteit te Leuven.

Artikel 2 :

Dr.D. COLLEN en zijn medewerkers, tewerkgesteld aan de Katholieke Universiteit te Leuven, doen ten voordele van de v.z.w. Leuven Research & Development afstand van alle juridische, commerciële en financiële rechten en de uitoefening ervan, die verbonden zijn aan onderzoeksresul-taten die zij rechtstreeks of onrechtstreeks bekomen hebben in het kader van hun onderwijs- en onderzoeksopdrachten aan de Katholieke Universiteit Leuven.

LEUVEN RESEARCH & DEVELOPMENT v.z.w.

Artikel 3 :

L.R.& D. is alleen gemachtigd deze rechten uit te oefenen en verplicht zich er toe naar best vermogen de juridische, commerciële en financiële belangen van Dr.D. COLLEN en zijn medewerkers te behartigen en te verdedigen.

Artikel 4 :

De eventuele inkomsten zullen door L.R.& D. als volgt verdeeld worden :

- 10 % komt toe aan de Katholieke Universiteit Leuven
- 7 % komt toe aan de v.z.w. Leuven Research & Development
- Na aftrek van alle kosten verbonden aan de uitoefening van zijn opdracht zal L.R.& D. minimum 50 % van het resterend gedeelte ter beschikking houden voor verder onderzoek in het laboratorium van Dr.D. COLLEN. Het resterend gedeelte kan aan Dr.D. COLLEN en zijn medewerkers als persoonlijke vergoeding uitgekeerd worden.

Artikel 5 :

De concrete verdelingsmodaliteiten zullen ten gepaster tijd in onderling overleg tussen L.R.& D. en Dr.D. COLLEN bepaald worden en ter goedkeuring aan de raad van beheer van L.R.& D. worden voorgelegd.
De raad van beheer van L.R.& D. kan ten allen tijde zijn standpunt hierin aanpassen aan de omstandigheden.

Opgemaakt in Leuven in twee exemplaren.

Dr. D. COLLEN

Leuven, 11 februari 1970

Voor LEUVEN RESERACH & DEVELOPMENT v.z.w.

Leuven, 9. Februari 1970

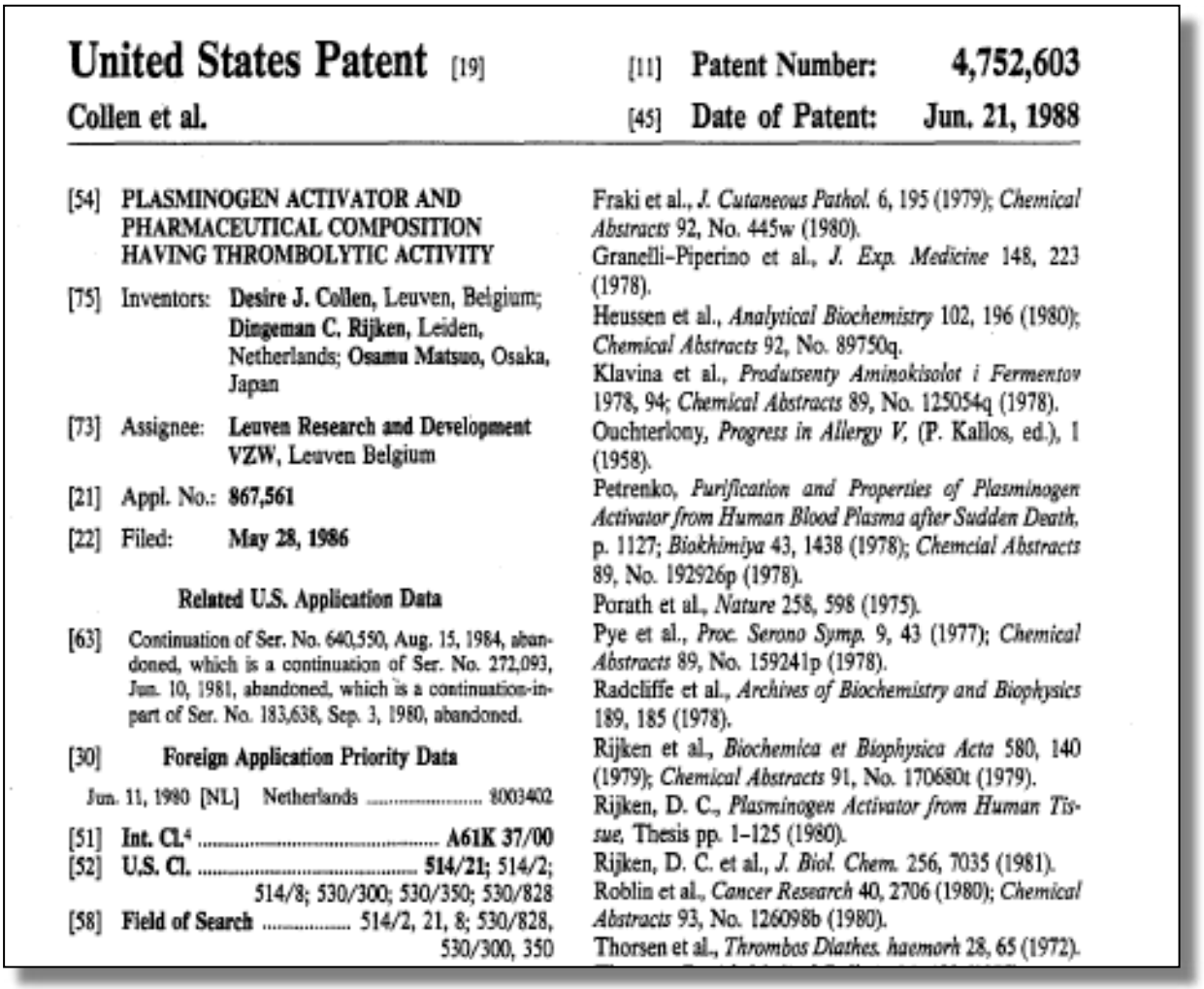
G. DECLERCQ
Afgevaardigd-Beheerder

PROF. M. VERSTRAETE

J. BOUCKAERT
Directeur

3. The t-PA patent

Front page of the t-PA patent, first submitted in the Netherlands on June 11, 1980, later extended to the U.S. The patent was assigned to *Leuven Research and Development* vzw (non-profit organization) mentioning Désiré Collen, Dick Rijken and Osamu Matsuo as inventors.



United States Patent [19]

Collen et al.

[11] **Patent Number: 4,752,603**

[45] **Date of Patent: Jun. 21, 1988**

[54] **PLASMINOGEN ACTIVATOR AND PHARMACEUTICAL COMPOSITION HAVING THROMBOLYTIC ACTIVITY**

[75] Inventors: **Desire J. Collen**, Leuven, Belgium; **Dingeman C. Rijken**, Leiden, Netherlands; **Osamu Matsuo**, Osaka, Japan

[73] Assignee: **Leuven Research and Development VZW**, Leuven Belgium

[21] Appl. No.: **867,561**

[22] Filed: **May 28, 1986**

Related U.S. Application Data

[63] Continuation of Ser. No. 640,550, Aug. 15, 1984, abandoned, which is a continuation of Ser. No. 272,093, Jun. 10, 1981, abandoned, which is a continuation-in-part of Ser. No. 183,638, Sep. 3, 1980, abandoned.

[30] **Foreign Application Priority Data**

Jun. 11, 1980 [NL] Netherlands 8003402

[51] **Int. Cl.**[4] **A61K 37/00**

[52] **U.S. Cl.** **514/21**; 514/2; 514/8; 530/300; 530/350; 530/828

[58] **Field of Search** 514/2, 21, 8; 530/828, 530/300, 350

Fraki et al., *J. Cutaneous Pathol.* 6, 195 (1979); *Chemical Abstracts* 92, No. 445w (1980).

Granelli-Piperino et al., *J. Exp. Medicine* 148, 223 (1978).

Heussen et al., *Analytical Biochemistry* 102, 196 (1980); *Chemical Abstracts* 92, No. 89750q.

Klavina et al., *Produtsenty Aminokisolot i Fermentov* 1978, 94; *Chemical Abstracts* 89, No. 125054q (1978).

Ouchterlony, *Progress in Allergy V*, (P. Kallos, ed.), 1 (1958).

Petrenko, *Purification and Properties of Plasminogen Activator from Human Blood Plasma after Sudden Death*, p. 1127; *Biokhimiya* 43, 1438 (1978); *Chemcial Abstracts* 89, No. 192926p (1978).

Porath et al., *Nature* 258, 598 (1975).

Pye et al., *Proc. Serono Symp.* 9, 43 (1977); *Chemical Abstracts* 89, No. 159241p (1978).

Radcliffe et al., *Archives of Biochemistry and Biophysics* 189, 185 (1978).

Rijken et al., *Biochemica et Biophysica Acta* 580, 140 (1979); *Chemical Abstracts* 91, No. 170680t (1979).

Rijken, D. C., *Plasminogen Activator from Human Tissue*, Thesis pp. 1–125 (1980).

Rijken, D. C. et al., *J. Biol. Chem.* 256, 7035 (1981).

Roblin et al., *Cancer Research* 40, 2706 (1980); *Chemical Abstracts* 93, No. 126098b (1980).

Thorsen et al., *Thrombos Diathes. haemorh* 28, 65 (1972).

4. First agreement between Genentech Inc.and Leuven Research and Development vzw (non-profit organization)

GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CALIFORNIA 94080
(415) 952-0123 TWX: 910371738

COOPERATIVE LETTER OF AGREEMENT BETWEEN
GENENTECH, INC.
AND
LEUVEN RESEARCH & DEVELOPMENT V.Z.W.

Genentech, Inc. and Leuven Research & Development V.Z.W., representing the University of Leuven and Dr. Desire Collen, agree to cooperate with each other in the development of human tissue plasminogen activators.

The points of cooperation are:

A. Leuven Research & Development V.Z.W. via Dr. Desire Collen will provide Genentech with:

1. Appropriate cell line(s) that are producing maximal levels of the tissue activators. These cells will be used for RNA extraction in order to make the complementary DNA necessary for cloning.

2. A quantity of purified tissue activator material to conduct sequencing in order to compare the gene sequence we obtain from the cloned material with your original purified tissue plasminogen activator. Purified activator will also be used as standard in any assays that are developed.

3. A source of antibody to help in the identification of positive clones.

B. In consideration for the above, Genentech will provide Leuven Research & Development V.Z.W. with:

1. Human tissue plasminogen activator when successfully produced from bacterial sources for use in biochemical and pharmacological clinical studies.

2. Recognition of Leuven Research & Development V.Z.W. (Dr. Desire Collen) on any scientific publications that arise from these investigations.

3. A royalty of 1% applicable to Genentech income derived from the sale of product made using any material provided by Leuven Research & Development V.Z.W.

In addition, it is understood that should Leuven Research & Development V.Z.W. obtain patent rights to human tissue plasminogen activators, Genentech shall have a right of first refusal to negotiate for an exclusive, royalty-bearing, worldwide license under any such patents. At the least, Genentech, for the 1% royalty payment mentioned in point B-3 above, shall have non-exclusive worldwide rights under any such patents granted Leuven Research & Development V.Z.W.

ACCEPTED:

Robert F. Byrnes
Genentech, Inc.

Leuven Research & Development V.Z.W.

5. Successful cloning of t-PA

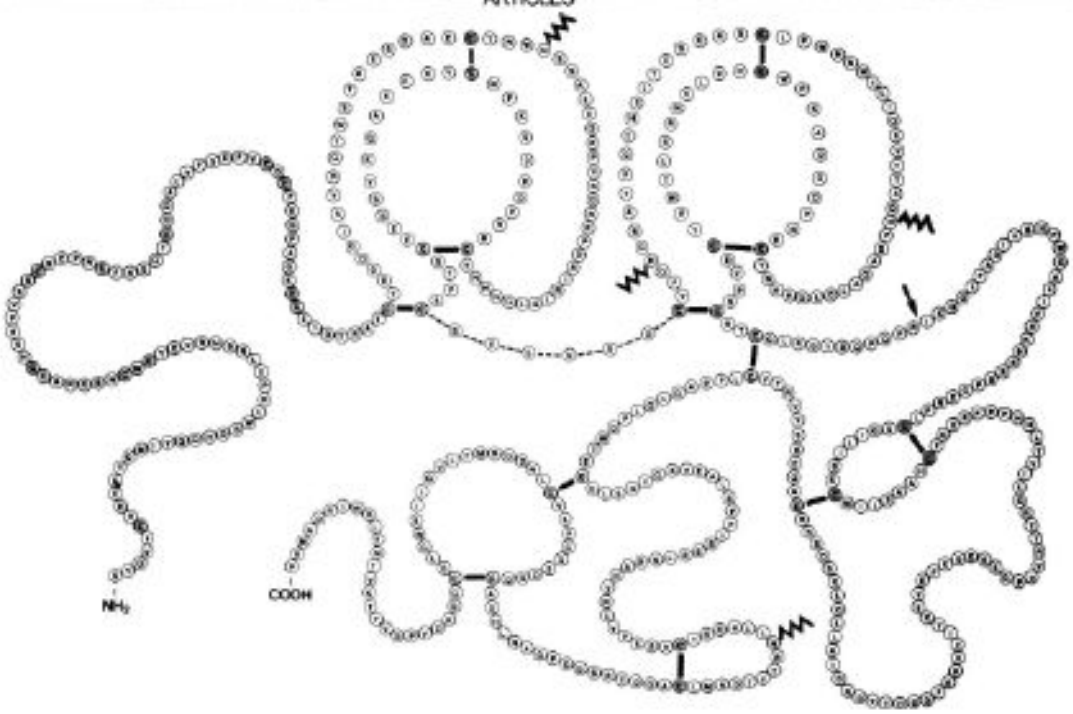


Fig. 6 Schematic diagram of the potential structure of human tissue-type plasminogen activator. The one-letter code for each of the amino acids is given in the open circles. The one-letter abbreviations are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature[34]. The cysteine residues are shaded. The solid black bars indicate the potential disulphide bridges based on homology with other serine proteases. The arrow indicates the potential cleavage site between arginine and the isoleucine which generates the two-chain molecule from the one-chain form. The zig-zag lines indicate potential glycosylation sites. The broken lines connect the six amino acids between the two kringles.

Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*

Diane Pennica*, William E. Holmes*, William J. Kohr†, Richard N. Harkins†, Gordon A. Vehar†, Carole A. Ward†, William F. Bennett†, Elizabeth Yelverton*, Peter H. Seeburg*, Herbert L. Heyneker* & David V. Goeddel*

Departments of *Molecular Biology and †Protein Biochemistry, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

Desire Collen

Center for Thrombosis and Vascular Research, Department of Medical Research, University of Leuven, B-3000 Leuven, Belgium

6. First Publication in *New England Journal of Medicine of results in patients*

CORONARY THROMBOLYSIS WITH TISSUE-TYPE PLASMINOGEN ACTIVATOR IN PATIENTS WITH EVOLVING MYOCARDIAL INFARCTION

FRANS VAN DE WERF, M.D., PHILIP A. LUDBROOK, M.B., B.S., STEVEN R. BERGMANN, PH.D., ALAN J. TIEFENBRUNN, M.D., KEITH A. A. FOX, M.B., CH.B., HILAIRE DE GEEST, M.D., MARC VERSTRAETE, M.D., PH.D., DESIRE COLLEN, M.D., PH.D., AND BURTON E. SOBEL, M.D.

Abstract Tissue-type plasminogen activator is a naturally occurring, clot-selective activator of fibrinolysis. We recently reported that human tissue-type plasminogen activator isolated from a Bowes-melanoma–tissue-culture supernate lysed coronary thrombi in dogs without depleting circulating fibrinogen or α_2-antiplasmin, in contrast to the case with streptokinase and urokinase. In the present study coronary thrombolysis, confirmed angiographically, was induced within 19 to 50 minutes with intravenous or intracoronary tissue-type plasminogen activator in six of seven patients with evolving myocardial infarction. Circulating fibrinogen, plasminogen, and α_2-antiplasmin were not depleted by this agent, in contrast to the case in the two patients subsequently given streptokinase. In the one patient in whom lysis was not inducible with tissue-type plasminogen activator, it was also not inducible with streptokinase.

These observations indicate that clot-selective coronary thrombolysis can be induced in patients with evolving myocardial infarction by means of tissue-type plasminogen activator, without concomitant induction of a systemic lytic state. Definition of its therapeutic benefit must await greater availability of the agent and the performance of appropriate clinical trials. (N Engl J Med 1984; 310:609-13.)

INITIATION of myocardial reperfusion by systemic activation of the fibrinolytic system was demonstrated more than 20 years ago,[1,2] and reperfusion by intracoronary administration of activators in pilot studies was reported as early as 1976.[3] Enthusiasm for the approach was kindled by the observations by Rentrop et al.,[4] who demonstrated that thrombolysis with intracoronary streptokinase restored angiographic patency to the occluded vessels supplying jeopardized myocardium in as many as 80 per cent of patients,[5] and by DeWood et al., who angiographically documented coronary thrombosis soon after the onset of symptoms in patients with evolving acute myocardial infarction.[6] Beneficial effects on global[7,8] and regional ventricular function,[9] perfusion,[10] and myocardial metabolic integrity[11-13] have been observed.

Activators such as streptokinase or urokinase have recently been given for the most part by the intracoronary route, in the hope of providing a high local concentration while minimizing the predisposition to systemic bleeding. Whether administration is intracoronary or intravenous,[14] streptokinase and urokinase activate the fibrinolytic system in the general circulation,[15] inducing what has been called a systemic lytic state.[16] The lytic state increases the risk of bleeding and is reflected by conversion of plasminogen to plasmin in the circulation, proteolytic depletion of circulating fibrinogen, accumulation of fibrinogen-degradation products, and consumption of circulating α_2-antiplasmin.

Since the potential benefit of coronary thrombolysis is markedly dependent on its prompt implementation[11,17] and safety — e.g., during surgery which is frequently needed soon after thrombolysis to correct high-grade residual stenosis — certain properties are desirable in an activator. These include lack of antigenicity, selectivity of action on a clot, avoidance of induction of a systemic lytic state, and a short biologic half-life so that hemostatic integrity can be restored promptly in patients requiring invasive procedures. Accordingly, we have been evaluating the potential utility of tissue-type plasminogen activator for coronary thrombolysis.[12]

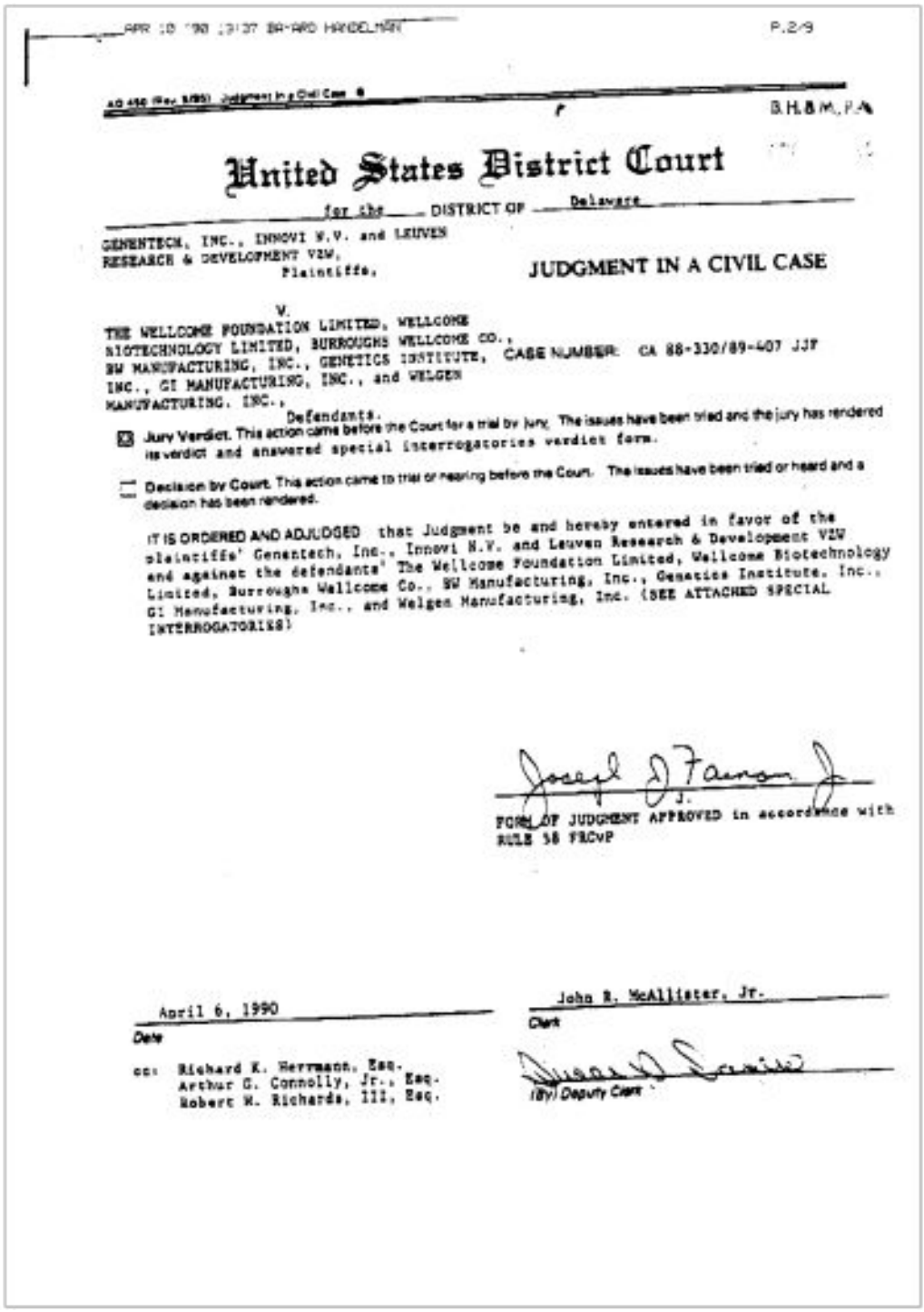
Tissue-type plasminogen activator is a naturally occurring serine protease that activates the fibrinolytic system under physiologic conditions by converting plasminogen to plasmin. It does not bind avidly to circulating plasminogen (K_M = 65 μM) but has a high affinity for fibrin (K_d = 0.16 μM). Circulating plasminogen binds avidly to the tissue-type plasminogen activator–fibrin complex through the plasmino-

From the Washington University School of Medicine, St. Louis, and the University of Leuven, Leuven, Belgium. Address reprint requests to Dr. Sobel at the Cardiovascular Division, Washington University School of Medicine, 660 S. Euclid, Box 8086, St. Louis, MO 63110.

Supported in part by a grant (HL 17646 [Specialized Centers of Research in Ischemic Heart Disease]) from the National Institutes of Health and by the Geconcerteerde Onderzoeksactie (Project 80/85-3).

7. Trial in Wilmington

Verdict of the jury in the case "*Genentech*, *Innovi* and *Leuven Research and Development*" versus "*Burroughs-Wellcome* and *Genetics Institute*" regarding the t-PA patents.



APR 10 '90 13:27 BA-ARD HANDELMAN P.2/9

AO 450 (Rev. 5/85) Judgment in a Civil Case

B.H.&M., P.A.

United States District Court

for the DISTRICT OF Delaware

GENENTECH, INC., INNOVI N.V. and LEUVEN RESEARCH & DEVELOPMENT VZW,
Plaintiffs,

V.

THE WELLCOME FOUNDATION LIMITED, WELLCOME BIOTECHNOLOGY LIMITED, BURROUGHS WELLCOME CO., BW MANUFACTURING, INC., GENETICS INSTITUTE, INC., GI MANUFACTURING, INC., and WELGEN MANUFACTURING, INC.,
Defendants.

JUDGMENT IN A CIVIL CASE

CASE NUMBER: CA 88-330/89-407 JJF

☒ Jury Verdict. This action came before the Court for a trial by jury. The issues have been tried and the jury has rendered its verdict and answered special interrogatories verdict form.

☐ Decision by Court. This action came to trial or hearing before the Court. The issues have been tried or heard and a decision has been rendered.

IT IS ORDERED AND ADJUDGED that Judgment be and hereby entered in favor of the plaintiffs' Genentech, Inc., Innovi N.V. and Leuven Research & Development VZW and against the defendants' The Wellcome Foundation Limited, Wellcome Biotechnology Limited, Burroughs Wellcome Co., BW Manufacturing, Inc., Genetics Institute, Inc., GI Manufacturing, Inc., and Welgen Manufacturing, Inc. (SEE ATTACHED SPECIAL INTERROGATORIES)

Joseph J. Farnan, Jr.
J.

FORM OF JUDGMENT APPROVED in accordance with RULE 58 FRCvP

April 6, 1990
Date

John R. McAllister, Jr.
Clerk

(By) Deputy Clerk

cc: Richard K. Herrmann, Esq.
Arthur G. Connolly, Jr., Esq.
Robert W. Richards, III, Esq.

RULE 49(a) SPECIAL VERDICTS

A. Equivalence

1. Do you find that Genentech has proved by a preponderance of the evidence that the product and process of Wellcome are equivalent to that claimed in the following patents? Mark your answer with an "X" in the appropriate space.

	YES	NO
'603 Collen Patent	✓	
'330 Goeddel Patent	✓	

2. Do you find that Genentech has proved by a preponderance of the evidence that the product and process of Genetics Institute are equivalent to that claimed in the following patents? Mark your answer with an "X" in the appropriate space.

	YES	NO
'603 Collen Patent	✓	
'075 Goeddel Patent	✓	
'330 Goeddel Patent	✓	

3. If you found for Genentech in Question Nos. 1 or 2, what is the amount of damages appropriate to compensate Genentech?

Damages from Wellcome $ None

Damages from Genetics Institute $ None

8. Document that confirms the fusion between nv (Inc) t-PA and Thromb-X

FUSIEVOORSTEL

Op donderdag 4 november 1993 werd, overeenkomstig Artikel 174/2 van de Vennootschappenwet, door de Raden van Bestuur van de navermelde vennootschappen, in gemeen overleg, het volgende fusievoorstel opgesteld, waarvan de tekst hierna volgt.

De aan de voorgestelde fusie deelnemende vennootschappen zijn:

1. de naamloze vennootschap "THROMB-X", met maatschappelijke zetel te 3000 Leuven, Leopoldstraat 1, Bus 21, handelsregister nr. 81 514, BTW-nummer 446.116.955, hier vertegenwoordigd door de heer Hans Claes, gehuisvest te 3140 Keerbergen, Tremelobaan 145, hierna genoemd 'de overnemende vennootschap';
2. de naamloze vennootschap "t-PA", met maatschappelijke zetel te 3000 Leuven, Groot Begijnhof, Benedenstraat 59, handelsregister nr. 76 384, BTW-nummer 438.374.573, hier vertegenwoordigd door de heer Jacques Vander Becken, gehuisvest te 3210 Linden, Jachthuislaan 16, en de heer Karel Tavernier, gehuisvest te 3210 Linden, Jachthuislaan 4, hierna genoemd "de over te nemen vennootschap".

De Raden van Bestuur van voornoemde vennootschappen verbinden zich jegens elkaar om te doen wat in hun macht ligt om tussen deze vennootschappen een fusie tot stand te brengen tegen de navermelde voorwaarden en leggen bij deze het fusievoorstel vast dat ter goedkeuring zal worden voorgelegd aan de respektieve Algemene Vergaderingen van deze vennootschappen.

Het voorstel komt er essentieel op neer dat ten gevolge van de toekomstige verwerving van alle aandelen van de vennootschap t-PA N.V. door de vennootschap THROMB-X NV, de vennootschap t-PA N.V. ontbonden zal worden, zonder vereffening en dat het hele vermogen van de vennootschap, zowel de rechten als verplichtingen, zullen overgaan naar THROMB-X NV. Als redenen hiervoor worden de volgende argumenten gegeven. De onderzoeksprojecten die door t-PA N.V. werden uitgevoerd met als doel de ontwikkeling van nieuwe diagnostische en therapeutische stoffen voor de voorkoming of verwijdering van trombose hebben tot op heden geen concreet resultaat opgeleverd in de zin van een commercialiseerbaar farmaceutisch produkt. Deze onderzoekingen hebben evenwel geleid tot de opbouw van een zeer aanzienlijke kennis en know-how op het gebied van trombolitica. De vennootschap THROMB-X N.V. beschikt terzake over een exclusieve licentie op een aantal octrooiaanvragen op het gebied van ontwikkeling van therapeutische stoffen voor de voorkoming of verwijdering van tromboses THROMB-X NV heeft terzake eveneens reeds overeenkomsten afgesloten met derde partijen voor de ontwikkeling van een pilootfabriek.

9. Memorandum of association of Thromb-X

N. 920109 — 767

« Thromb-X », naamloze vennootschap

Leopoldstraat 1/21
Leuven

OPRICHTING - BENOEMINGEN

Er blijkt uit een akte, verleden voor notaris Paul Kuijpers te Leuven-Heverlee op twintig december negentienhonderd éénennegentig, geregistreerd te Leuven, eerste kantoor der registratie, op vierentwintig december nadien boek 232 blad 69 vak 20, dat er een naamloze vennootschap werd opgericht, met als naam "THROMB-X" en zetel te Leuven, Leopoldstraat 1/21, voor onbepaalde duur, door : a) de heer Désiré COLLEN, hoogleraar, gehuisvest te Herent-Winksele, Schoonzichtlaan 20; b) De vereniging zonder winstoogmerk "LEUVEN RESEARCH & DEVELOPMENT", met zetel te Leuven, Krakenstraat 3.

Het kapitaal bedraagt vijfentwintig miljoen frank, verdeeld in tweeduizend vijfhonderd aandelen zonder nominale waarde, volledig volstort door de oprichters, te weten : 1) door de Heer Désiré COLLEN : twaalfhonderdvijftig aandelen of twaalf miljoen vijfhonderdduizend frank, volstort ten belope van twee miljoen vijfhonderdduizend (2.500.000) frank; 2) door de vereniging zonder winstoogmerk "LEUVEN RESEARCH & DEVELOPMENT" : twaalfhonderdvijftig aandelen of twaalf miljoen vijfhonderdduizend frank, volstort ten belope van twee miljoen vijfhonderdduizend frank.

Er werden eveneens tweeduizend vijfhonderd winstaandelen gecreëerd, waarvan twaalfhonderdvijftig (1.250) aandelen met stemrecht en twaalfhonderdvijftig (1.250) aandelen zonder stemrecht, en allen met deelname in de winst. Zij genieten dezelfde rechten als de kapitaalsaandelen van de vennootschap voor wat betreft de winstverdeling en de verdeling van het vereffeningsoverschot. De winstaandelen met stemrecht worden meegeteld voor de berekening van het kworum op de algemene vergaderingen. De winstaandelen zijn en blijven op naam.

De raad van bestuur is gemachtigd het kapitaal te verhogen ten belope van honderdvijfentwintig miljoen frank tot honderdvijftig miljoen frank, in één of meerdere keren, eventueel mits omzetting van reserves. De raad van bestuur kan binnen de grenzen van het toegestaan kapitaal converteerbare obligaties of warrants uitgeven.

De vennootschap heeft tot doel het uitoefenen van alle beheers- en financiële aktiviteiten en meer in het bijzonder : onderzoek naar, ontwikkeling van en verkoop van diagnostische en therapeutische stoffen voor de voorkoming of verwijdering van trombosen, en andere farmaca in het domein van de menselijke geneeskunde. Zij mag bij middel van inbreng, deelneming, fusie, plaatsing of op iedere andere wijze belangen nemen in alle vennootschappen of ondernemingen die een soortgelijk of gelijkaardig doel hebben. In het algemeen mag zij alle industriële, commerciële, financiële, onroerende en roerende verhandelingen doen die rechtstreeks of onrechtstreeks in verband staan met haar doel of die van aard zijn haar nijverheid of haar handel uit te breiden of te bevorderen.

Het batig overschot van de balans, na aftrek van de algemene onkosten, maatschappelijke lasten met inbegrip van de provisie voor de belastingen op de winst van het boekjaar en de nodige afschrijvingen, vormt de zuivere winst. Van deze winst wordt ten minste vijf ten honderd afgehouden voor het aanleggen van de wettelijke reserve. Deze afhouding houdt op verplichtend te zijn wanneer het reservefonds één/tiende bedraagt van het kapitaal. Deze afhouding dient opnieuw te gebeuren indien dit tiende aangetast wordt. Het eventuele saldo wordt ter beschikking gesteld van de algemene vergadering die zal beslissen over de aanwending ervan. Voor zover het aantal aandelen zonder stemrecht niet méér dan één/derde van het maatschappelijk kapitaal vertegenwoordigt of heeft vertegenwoordigd, geven deze aandelen recht op een preferent en opvorderbaar dividend alsmede op een recht in de uitkering van het winstoverschot, dat niet lager mag zijn dan dat van het winstoverschot van de aandelen met stemrecht.

Na ontbinding zal in voorkomend geval het positief vereffeningssaldo, na aanzuivering van alle schulden, lasten en vereffeningskosten of daartoe gedane consignatie, eerst dienen om de aandelen terug te betalen onder aftrek van het bedrag dat gebeurlijk nog zou verschuldigd zijn voor de volledige volstorting. Het eventueel saldo zal dienovereenkomstig verdeeld worden onder alle aandelen. Voor zover het aantal aandelen zonder stemrecht niet méér dan één/derde van het maatschappelijk kapitaal vertegenwoordigt of heeft vertegenwoordigd, geven deze aandelen een recht op de uitkering van het na vereffening overblijvende saldo, waarvan het bedrag niet lager mag zijn dan dat uitgekeerd aan de houders van aandelen met stemrecht.

Het boekjaar begint op één januari en eindigt op éénendertig december van ieder jaar. Het afsluiten van het eerste boekjaar is vastgesteld op éénendertig december negentienhonderd tweeënnegentig. De gewone jaarvergadering komt bijeen op de eerste maandag van de maand april van ieder jaar om zestien uur in de zetel, of op iedere andere plaats in België die aangeweezen zou worden in de bijeenroepingen. Indien deze dag een wettelijke feestdag is, wordt de vergadering de eerstvolgende werkdag gehouden op hetzelfde uur.

Om toegelaten te worden tot de algemene vergadering moeten de aandeelhouders op naam tenminste vijf dagen voor de vergadering hun aandelen neerleggen op de zetel of op de plaats in België, in de oproepingsbrief vermeld, tenzij de oproepingsbrief anders vermeldt. Ieder aandeel geeft recht op één stem. De aandeelhouders kunnen hun stem schriftelijk uitbrengen. Daartoe moet de stembrief, gedateerd en ondertekend, de vennootschap bereiken ten minste vijf dagen vóór het houden van de algemene vergadering.

De vennootschap wordt bestuurd door een raad van bestuur, bestaande uit ten minste drie leden, vennoten of niet, benoemd voor ten hoogste zes jaar door de algemene vergadering der aandeelhouders en door haar steeds afzetbaar, de uittredende bestuurders zijn dadelijk herverkiesbaar.

De raad van bestuur heeft de meest uitgebreide beheers- en beschikkingsmacht voor het bestuur der zaken van de vennootschap. Alle handelingen die niet uitdrukkelijk door de wet of door de statuten voorbehouden zijn aan de algemene vergadering, vallen onder zijn bevoegdheid. Behoudens bijzondere machtiging gegeven door de raad van bestuur, worden alle akten die de vennootschap verbinden, geldig ondertekend hetzij door de voorzitter, hetzij door de gedelegeerd bestuurder, hetzij door twee bestuurders, die ten overstaan van derden, zelfs tegenover een openbaar funktionaris of een ministerieel ambtenaar alsmede tegenover de hypotheekbewaarder, geen verrechtvaardiging van een voorafgaande beslissing van de raad zullen moeten voorleggen.

Worden geroepen tot de funkties van bestuurder : de heer Désiré Collen, voornoemd; mevrouw Louisa Rita Paula Reniers, zonder beroep, gehuisvest te Herent-Winksele, Schoonzichtlaan 20; de heer Hans Alfons Julien Claes, directeur, gehuisvest te Keerbergen, Tressebaan 145, en de heer Jacques Albert Henri Vander Eecken, voornoemd.

De heer Désiré Collen wordt geroepen tot het voorzitterschap van de raad van bestuur. De heer Hans Claes wordt geroepen tot de funktie van gedelegeerd bestuurder. In deze laatste hoedanigheid wordt hij belast met het bestuur van de vennootschap, hierin onder meer begrepen alle financiële verrichtingen beperkt tot vijfhonderdduizend frank. Voor de noden en binnen de perken van dit bestuur zal zijn handtekening, afzonderlijk gebruikt, geldig de vennootschap verbinden. Onder zijn verantwoordelijkheid kan hij het geheel of een deel van zijn machten van het bestuur overdragen aan derden, hierin onder meer begrepen alle financiele verrichtingen beperkt tot tweehonderdduizend frank.

Voor ontledend uittreksel :

(Get.) Paul Kuijpers,
notaris.

Bijgevoegd : uitgifte van de oprichtingsakte met bankattest en volmacht.

Neergelegd te Leuven, 31 december 1991 (A/7807).

3 3 900 BTW 19 pct. 741 4 641

(4211)

10. Agreement between Thromb-X and 4C

CONVENTION DE CESSION DE PARTS

Entre d'une part :

Monsieur Alain, Othon, André MILLER,

Docteur en Sciences,
Demeurant à 7000 Mons, Bld Albert Elisabeth, 63

Et :

Madame Christine GYSELINGS,

Employée,
Demeurant à 7000 Mons, Bld Albert Elisabeth, 63

Ci-après dénommés « les cédants »

Et d'autre part :

Monsieur Désiré COLLEN, demeurant à SW5 0HN London, 28, Collingham Gardens, UK

agissant pour Thromb-X, NV dont le siège social est sis à Leopold I Straat 1, bus 21, 3000 Leuven

Ci-après dénommé « l'acquéreur »

IL A ETE EXPRESSEMENT CONVENU ET ACCEPTE CE QUI SUIT :

Article 1 : objet

Les cédants cèdent à l'acquéreur qui accepte, les 2.500 actions de capital nominatives , sans mention de valeur nominale, représentant chacune un 2.500e du capital social de la SA Compagnie d'investissements du Larzac, en abrégé « CIL », dont le siège social est sis à 7000 Mons, bld Albert Elisabeth, 63, dont ils sont propriétaires.

Article 12

Sous réserve d'un accord des autres membres du Conseil d'Administration, la CIL s'engage à voter positivement la signature d'un bail de 24 mois permettant la mise à disposition de 2 locaux au rez de chaussée de la "Villa" Rue de la Marlette, 14 7180 Seneffe pour un forfait de 743.68 €ur par mois.

Article 13

Le transfert de propriété s'effectuera par remise du chèque conformément au montant prévu par la convention le .. /10/02 . Rue de la Marlette 14 7180 Seneffe.
A défaut de paiement à la date et au lieu convenu, la présente convention sera résiliée de plein droit sans autre recours pour les parties signataires.

Fait à Seneffe , le

En trois exemplaires originaux. Chacune des parties reconnaissant avoir reçu un exemplaire.

03 OCT 02

- Conditional upon conclusion of shareholder agreement
- confirmation of phone agreement of ...
03/10/02

...

11. Memorandum of association of D. Collen Research Foundation vzw

N. 14130 (29618)

« D. Collen Research Foundation »

3000 Leuven

Identificatienummer : 14130/88

STATUTEN

De hierna genoemde personen :

A. Collen, Désiré, gewoon hoogleraar K.U.-Leuven, Schoonzichtlaan 20, 3009 Winksele (Herent), van Belgische nationaliteit;

B. Katholieke Universiteit Leuven, Oude Markt 13, 3000 Leuven, vertegenwoordigd door haar rector, Dillemans, Roger, gewoon hoogleraar K.U.-Leuven, en door haar algemeen beheerder Tavernier, Karel, gewoon hoogleraar K.U.-Leuven, van Belgische nationaliteit;

C. Leuven Research and Development v.z.w., Groot Begijnhof, Benedenstraat 59, 3000 Leuven, vertegenwoordigd door haar voorzitter, Vander Eecken, Jacques, gewoon hoogleraar K.U.-Leuven, van Belgische nationaliteit;.

D. Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, vertegenwoordigd door Lawrence Fouraker, professor emeritus, Harvard Business School, van Amerikaanse nationaliteit,

zijn overeengekomen onder elkaar en met allen die later zullen toetreden, een vereniging zonder winstoogmerk op te richten overeenkomstig de wet van 27 juni 1921 en onder de hierna volgende voorwaarden :

TITEL I. — *Naam, zetel, doel, duur*

Artikel 1. De vereniging draagt als naam « D. Collen Research Foundation », v.z.w.

Art. 2. De vereniging is gevestigd : Onderwijs en Navorsing, Campus Gasthuisberg K.U.-Leuven, Herestraat 49, te 3000 Leuven.

Art. 3. § 1. De vereniging heeft tot doel, met uitsluiting van enig winstoogmerk, het uitvoeren, bevorderen en ondersteunen van wetenschappelijk onderzoek in het algemeen en biomedisch en biotechnologisch onderzoek in het bijzonder, onder andere door het toekennen van onderzoekstoelagen, onderzoeksmandaten, reisbeurzen, het organiseren van wetenschappelijke congressen en symposia, financiële steun bij publicaties en alle andere aanverwante activiteiten die de bevordering van de wetenschap ondersteunen of doen uitstralen. In die zin mag zij ook op bijkomstige wijze alle andere economische activiteiten uitoefenen, inclusief het verwerven van roerende en onroerende goederen, op voorwaarde dat de opbrengst daarvan uitsluitend besteed wordt aan het hoofddoel.

§ 2. De vereniging kan één of meer afdelingen of vestigingen hebben waar de in de vorige paragraaf bedoelde activiteiten uitgeoefend worden, zulks ook buiten het rechterlijk arrondissement Leuven, waar de hoofdzetel gevestigd is.

Art. 4. De vereniging is opgericht voor onbepaalde duur. Zij kan te allen tijde ontbonden worden.

12. First results of SakSTAR in patients

Coronary Thrombolysis With Recombinant Staphylokinase in Patients With Evolving Myocardial Infarction

Désiré Collen, MD, PhD, and Frans Van de Werf, MD

Background. **Staphylokinase (STA), a protein with known profibrinolytic properties, is produced by transduced *Staphylococcus aureus* strains. In experimental animal models, recombinant staphylokinase (STAR) is less immunogenic and more active toward platelet-rich arterial blood clots than streptokinase.**

Methods and Results. **In the present study, 10 mg STAR given intravenously over 30 minutes was found to induce angiographically documented coronary artery recanalization within 40 minutes in four of five patients with acute myocardial infarction. Plasma fibrinogen and α_2-antiplasmin levels were unaffected, and allergic reactions were not observed. Postinfusion disappearance of STAR antigen followed a biphasic mode with a $t_{1/2\alpha}$ of 6.3±0.6 minutes (mean±SD) and a $t_{1/2\beta}$ of 37±15 minutes, corresponding to a plasma clearance of 270±100 mL/min. Neutralizing antibodies against STAR could not be demonstrated at baseline and up to 6 days after infusion, but STAR neutralizing activity, which did not cross-react with streptokinase, was consistently demonstrable in plasma at 14–35 days.**

Conclusions. **STAR can induce clot-selective coronary thrombolysis in patients with evolving myocardial infarction without concomitant induction of a systemic lytic state. STAR, a small protein that can be easily produced by recombinant DNA technology, may therefore offer promise for thrombolytic therapy in patients with thromboembolic disease. (*Circulation* 1993;87:1850–1853)**

KEY WORDS • **thrombolytic therapy • acute myocardial infarction • staphylokinase • fibrin specificity • immunogenicity • pharmacokinetics**

Thrombolytic therapy in patients with acute myocardial infarction has been shown to recanalize occluded coronary arteries, preserve left ventricular function, and reduce mortality. At present, coronary thrombolysis is primarily performed with streptokinase or with alteplase, a preparation of recombinant tissue-type plasminogen activator (rt-PA).[1] The main advantages of streptokinase are its low cost and relative safety, whereas the main shortcomings are its limited efficacy for coronary recanalization and its immunogenicity, which predisposes to allergic reactions and to resistance to repeated administration. The main advantages of alteplase are its higher efficacy for coronary recanalization and its lack of immunogenicity, whereas its main disadvantage is its high cost. Thus, thrombolytic agents with higher thrombolytic potency, reduced side effects, and/or lower cost would be desirable.

Staphylokinase (STA), a protein of 136 amino acids that does not contain disulfide bridges, is produced by transduced *Staphylococcus aureus* strains and has been known for more than 40 years to have profibrinolytic properties. Its mechanism of action, its in vitro fibrinolytic properties, and its in vivo thrombolytic properties in experimental animal models have been evaluated to some extent.[2] Like streptokinase, staphylokinase is not an enzyme, but it forms a 1:1 stoichiometric complex with plasminogen that then activates other plasminogen molecules.[3,4] However, whereas the streptokinase–plasminogen complex undergoes an intramolecular transition without peptide bond cleavage, which results in exposure of a titrable active site in the plasminogen molecule,[5] activation of the staphylokinase–plasminogen complex requires its conversion to plasmin.[6] Furthermore, the staphylokinase–plasmin complex is very rapidly inhibited by α_2-antiplasmin, whereas the streptokinase–plasmin complex is not.[7,8]

The gene coding for staphylokinase has been cloned and expressed in *Escherichia coli* and in *Bacilus subtilis,* and the biochemical properties of recombinant staphylokinase (STAR) have been studied recently in some detail.[9,10] Although the initial in vivo experiments with staphylokinase in dogs have yielded discouraging results,[11,12] we have recently obtained evidence that STAR is fibrin specific, relatively more potent than streptokinase toward platelet-rich clots, and less immunogenic.[13,14] Our studies[6,8,15] suggested that several mechanisms may contribute to the fibrin specificity and the potency of staphylokinase in a plasma milieu. First, the plasmin–staphylokinase complex is rapidly neutralized by α_2-antiplasmin (apparent second-order rate constant 2×10^6 M^{-1}-sec^{-1}) but this reaction is 130 times slower when the lysine binding sites of plasmin in the complex are either removed or saturated by binding to 6-amino-

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13. Publication on VEGF knock-out of Peter Carmeliet in *Nature*

Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele

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The endothelial cell-specific vascular endothelial growth factor (VEGF)[1–4] and its cellular receptors Flt-1 (refs 6,7) and Flk-1 (refs 8,9) have been implicated in the formation of the embryonic vasculature. This is suggested by their colocalized expression during embryogenesis[10,11] and the impaired vessel formation in Flk-1 (ref. 12) and Flt-1 (ref. 13) deficient embryos. However, because Flt-1 also binds placental growth factor[14,15], a VEGF homologue, the precise role of VEGF was unknown. Here we report that formation of blood vessels was abnormal, but not abolished, in heterozygous VEGF-deficient ($VEGF^{+/-}$) embryos, generated by aggregation of embryonic stem (ES) cells with tetraploid embryos (T-ES)[16,17], and even more impaired in homozygous VEGF-deficient ($VEGF^{-/-}$) T-ES embryos, resulting in death at mid-gestation. Similar phenotypes were observed in F_1-$nVEGF^{+/-}$ embryos, generated by germline transmission. We believe that this heterozygous lethal phenotype, which differs from the homozygous lethality in VEGF-receptor-deficient embryos, is unprecedented for a targeted autosomal gene inactivation, and is indicative of a tight dose-dependent regulation of embryonic vessel development by VEGF.

Targeted inactivation of one ($VEGF^{+/-}$) or both ($VEGF^{-/-}$) alleles in ES cells was accomplished by replacement of the third common VEGF exon with the gene encoding neomycin phosphotransferase (neo), which caused a frameshift in the VEGF coding sequence[5] (unpublished observations), and deleted six of the eight essential cysteine residues[18,19] (Fig. 1*a*, *b*). Absence of exon 3 was confirmed by reverse transcription–polymerase chain reaction (RT–PCR) (Fig. 1*c*). Northern blot (Fig. 1*d*–*g*) and RT–PCR analysis (see Supplementary Information) revealed the presence of two aberrant VEGF transcripts at very low abundance (< 3% of wild type) in $VEGF^{-/-}$ embryos and ES cells, differentiated to cystic embryoid bodies (CEBs), which resulted from splicing into (mRNA-1) or around *neo* (mRNA-2) and contained a stop codon after 49 residues in *neo* (mRNA-1) or after 24 out-of-frame residues in *VEGF* exon 4 (mRNA-2) (Fig. 1*a* and see Supplementary Information). These mutant transcripts were similarly detected in $VEGF^{+/-}$ and in $nVEGF^{+/+}$ (containing a randomly integrated targeting vector) CEBs and embryos, and resulted from residual transcription of the randomly integrated targeting vector. Transient transfection of mRNA-2 (Fig. 1*a*) in COS cells under the control of the cytomegalovirus promoter did not reveal detectable expression of a mutant VEGF peptide, nor dominant-negative inhibition by such peptide on the expression level of wild-type *VEGF* in co-transfection experiments, as evaluated by western blot analysis, metabolic labelling, immunoprecipitation (for data, see Supplementary Information) and by induction of procoagulant activity (not shown), indicating that expression of mRNA-2 does not interfere with wild-type *VEGF* expression.

To distinguish between tetraploid-derived and targeted ES cell-derived embryonic cells, ROSA-26 embryos[21], which ubiquitously express *lacZ*, were used as donors for the tetraploid embryos. The marker confirmed that tetraploid cells were confined to the endoderm of the yolk sac and trophoblast cells in all cases studied (see *LacZ*-positive cells in the yolk sac in Fig. 2*d*, *e*). $nVEGF^{+/+}$, $VEGF^{+/-}$ and $VEGF^{-/-}$ T-ES-cell-derived embryos appeared macroscopically normal at 8.5 days post-coitum (d.p.c.), whereas most of the $VEGF^{+/-}$ and $VEGF^{-/-}$ T-ES-cell-derived embryos were retarded at 9.5 d.p.c. and appeared dead at 10.5 d.p.c. At 8.5 and 9.5 d.p.c., the dorsal aorta was completely normal in the anterior (Fig. 2*a*) and posterior (not shown) part of $nVEGF^{+/+}$ T-ES-cell-derived embryos, but was only poorly developed in the $VEGF^{+/-}$ T-ES-cell-derived embryos, where endothelial cells lined a much smaller lumen than normal, especially in the anterior part of the embryo (Fig. 2*b*, *c*). At 8.5 d.p.c., the dorsal aorta was missing over its entire length in $VEGF^{-/-}$ T-ES-cell-derived embryos (Fig. 2*e*). At 9.5 d.p.c., abnormally enlarged vascular structures, lined by endothelial cells and filled with nucleated blood cells, were observed throughout the anterior part of the necrotic embryo instead of the normal dorsal aorta and other blood vessels in $VEGF^{-/-}$ T-ES-cell-derived embryos (Fig. 2*f*). In the caudal part of the embryo, the dorsal aorta was significantly smaller (not shown). A higher degree of tissue necrosis was observed in the $VEGF^{-/-}$ than in $VEGF^{+/-}$ T-ES cell-derived embryos. RT–PCR analysis for the endothelial cell-specific markers Flt-1, Flk-1 and Tie-2 (also known as Tek) (see Supplementary Information), and *in situ* hybridization for Flt-1 and Flk-1, and immunostaining for Flk-1 and PECAM/CD31 (not shown) at 8.5 and 9.5 d.p.c. revealed fewer and frequently isolated, disorganized and scattered Flk-1- and Flt-1-expressing cells throughout the $VEGF^{-/-}$ embryo at 8.5 d.p.c., and relatively low amounts of Flt-1 and Tie-2 message (which appear later during development) in $VEGF^{-/-}$ embryos at 9.5 d.p.c., suggesting delayed but not aborted endothelial cell development. Thus, at both 8.5 and 9.5 d.p.c., the $VEGF^{-/-}$ genotype resulted in a more severe phenotype than did the $VEGF^{+/-}$ genotype.

No viable F_1 $VEGF^{+/-}$ offspring were obtained at birth, indicating embryonic lethality. F_1 $VEGF^{+/-}$ and F_1 $VEGF^{+/-}$: *tie-1-lacZ*$^{+/-}$ embryos, in which the endothelial cells express *lacZ*

14. Curriculum Vitae Désiré Collen

Education

1968:	Doctor in Medicine (MD), K.U.Leuven, Belgium
1969:	Licentiaat (MSc) in Medical Sciences, K.U.Leuven, Belgium
1974:	PhD in Chemistry, K.U.Leuven, Belgium
1974:	Geaggregeerde "Higher Education in Medicine", K.U.Leuven, Belgium

Residencies and Research Fellowships

1968-1971:	Resident Internal Medicine, University Hospitals K.U.Leuven, Belgium
1971-1972:	Associate Research Scientist, New York University Medical Center, New York, N.Y.
1972-1973:	NATO Research Fellow, Karolinska Institutet, Stockholm, Sweden

Academic Appointments within the University of Leuven

1973-1976:	Aangesteld Navorser NFWO
1975-1976:	Extraordinary ("Buitengewoon") docent, Faculty of Medicine, K.U.Leuven
1976-1981:	Docent, Faculty of Medicine, K.U.Leuven, Belgium
1981-1998:	Professor, (“Gewoon hoogleraar”) Faculty of Medicine, K.U.Leuven, Belgium
1990-2007:	Director of the Center for Molecular and Vascular Biology (previously Center for Thrombosis and Vascular Research) Faculty of Medicine, K.U.Leuven, Belgium
1998-2002:	Extraordinary Professor (“Buitengewoon hoogleraar”), Faculty of Medicine, K.U.Leuven, Belgium
2002-2008:	Professor, (“Gewoon hoogleraar”) Faculty of Medicine, K.U.Leuven, Belgium

Academic Appointments outside the University of Leuven

1984-2005:	Professor of Biochemistry and Medicine, University of Vermont College of Medicine, Burlington, VT, USA
1986-1989:	Visiting Professor, Faculty of Medicine and Pharmacy, Free University Brussels, Belgium
1987-1994:	Visiting Professor of Medicine, Harvard Medical School, Boston, MA, USA
1994-2008:	Center for Transgene Technology and Gene Therapy (presently Vesalius Research Center) Vlaams Interuniversitair Instituut voor Biotechnology, Leuven, Belgium

Appointments in University Hospitals

1975-1976:	Consultant (Consulent), University Hospitals, K.U.Leuven, Belgium
1976-2000:	Adjunct Head of Clinic, University Hospitals, K.U.Leuven, Belgium
1987-2005:	Consultant in Medicine, Massachusetts General Hospital, Boston, MA, USA
1999-2002:	Visiting Professor in the Division of Surgery and Anaesthesia, Guy's King's and St. Thomas' School of Medicine, London, UK
1998-2008:	Consultant (Consulent), University Hospitals, K.U.Leuven, Belgium

Other Appointments

1976-2001:	Division Head, Protein Research Division, Leuven Research and Development VZW, K.U.Leuven, Belgium
1988-2007:	Statutory Chairman of the D. Collen Research Foundation V.Z.W.
1991-2007:	Chairman of the Board of Thromb-X NV (Spin-off company of Leuven Research and Development, K.U.Leuven, Belgium)
1998-2006:	Chief Executive Officer and Chairman, ThromboGenics, Ltd., Ireland
2006-2009:	Chief Executive Officer, ThromboGenics, Ltd., Ireland
2006-2008:	Chief Executive Officer and Chairman, ThromboGenics, NV, Belgium
2007- :	Statutory Chairman of Life Sciences Research Partners, VZW (previously D. Collen Research Foundation, VZW)
2008- :	Chairman of ThromboGenics NV

Awards and Honors

1984:	Francqui Prize (University Foundation), Belgium
1985:	Member of the Royal Academy of Medicine of Belgium
1986:	Prix Louis Jeantet de Médecine (Fondation L. Jeantet), Geneva, Switzerland
1988:	Doctor honoris causa, Erasmus University, Rotterdam, the Netherlands
1990:	Five-yearly Prize of Fundamental Medical Sciences of the Belgian Government (Royal Academy of Medicine of Belgium)
1994:	Bristol-Myers-Squibb Award for Cardiovascular Research, New York, N.Y. (jointly with M. Verstraete)
1994:	Doctor honoris causa, Free University of Brussels (VUB), Brussels, Belgium

1995:	Doctor honoris causa, University of Notre Dame, Notre Dame, IN
1999:	Doctor honoris causa, Université de la Méditerranée, Marseille, France
2005:	Health Prize of the Interbrew-Baillet Latour Fund, Belgium (jointly with P. Carmeliet)
2006:	Member of the European Molecular Biology Organization (EMBO)
2007:	2007 Harvard Leadership Prize by the Harvard Club of Belgium

Research Areas

Molecular biology and pathophysiology of haemostasis and thrombosis

Development of new thrombolytic and antithrombotic agents

Transgenesis, gene targeting and gene transfer studies of the cardiovascular system

Translational research on cardiovascular drug development

Research Output

The scientific output of D. Collen between 1968 and 2008 consists of approximately 650 research papers (in peer-reviewed international journals), 170 survey articles and 28 issued US patents. He ranked among the 100 most cited scientific authors of the 1980's (Current Contents August 31, 1992, p3) and is listed with the highly cited authors of the 1980 and 1990's (http: //www.highlycited.com). Until June 2009 his scientific work, as author or co-author, was cited more than 57 000 times.

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