Hemostasis and the kidney: an unforeseen initial opportunity to create a small research group that grew into an institute

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ABSTRACT

This is the story of how, in the mid-1970s, a small group of young doctors and scientists in Bergamo began studying hemostasis and thrombosis in kidney diseases in collaboration with hematology researchers at Mario Negri Institute in Milan. At a small laboratory that was set up in the Nephrology and Dialysis division of Bergamo Hospital, their collaborative earlier research focused on platelet-vessel wall interactions, prostaglandins and bleeding in uremia, prostacyclin and prostacyclin-stimulating plasma factors in pre-eclampsia and thrombotic microangiopathies, and platelet hyperaggregability in nephrotic syndrome. As the group grew, so did the need to address patients' nephrological problems through more efficient laboratory research. This led to the foundation of the Mario Negri Bergamo Institute in 1984. Since then, their research work has expanded to include mechanisms of proteinuria and disease progression, organ transplantation and rejection, tolerance, autoimmune disease, glomerulonephritis, molecular biology, and more. In 1992, the Aldo and Cele Daccò Clinical Research Center for Rare Disease was established as an integral part of the Institute.

Introduction

Our group started working on hemostasis and kidney diseases by chance. Giuseppe Remuzzi, the person who later became the creator and head of the group was a young hematologist working as a fellow at Bergamo Hospital in the division of Medicine. He was particularly interested in platelet dysfunction in pregnancy. As no hematologist position was available, in 1975 he was hired in the Nephrology and Dialysis division, while continuing to cultivate his interest in hematology. He was drawn to the importance of integrating clinical practice and research and began to study the relationship between renal diseases and thrombosis and hemostasis, focusing on the mechanisms underlying bleeding tendency in uremic patients, the occurrence of thrombosis in arteriovenous fistulas in dialyzed patients, the pathogenesis of acute renal failure associated with thrombocytopenia, and microangiopathic hemolytic anemia. However, it soon became clear that these investigations necessitated access to a research institute where laboratory experiments and analyses could be performed. A meeting with Giovanni de Gaetano and Maria Benedetta Donati therefore proved to be highly productive. Both were medical doctors who headed a Laboratory of Hemostasis and Thrombosis Research and a Laboratory of Cardiovascular Clinical Pharmacology, respectively, at the Istituto di Ricerche Farmacologiche Mario Negri in Milan. Both researchers had previously spent several years as PhD students at the Laboratory of Professors Marc Verstraete and Jos Vermylen, University of Leuven, Belgium, specializing in platelets and blood coagulation. This meeting led to a fruitful and long-lasting collaboration that was further strengthened by the involvement of Manuela Livio, a biologist from Bergamo, who was on the Mario Negri staff. Her contributions were instrumental in shaping and developing Remuzzi's research group.

In the mid-1970s, there was not yet a tradition of conducting research at the Bergamo Hospital, so it was considered somewhat bizarre for a young doctor to dedicate himself not only to patient care but also to studying platelet dysfunction. However, Giuliano Mecca, the chief of the division of Nephrology and Dialysis, appreciated this enthusiasm and dedication and facilitated the creation of a small laboratory (so small we called it the 'metabolic cage') that was initially equipped with two aggregometers to study platelet function, and a few basic instruments, such as a balance, a centrifuge and a pH-meter. The laboratory

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was located just in front of the dialysis rooms (Figure 1a). Encountering patients who had to spend many hours every week attached to the dialysis machine and learning about their problems and poor quality of life, was a strong motivator for the young scientists in the lab to collaborate with nephrologists to research the mechanisms driving disease progression to renal failure and to explore strategies to prevent the need for dialysis. From 1979 onwards the group of young doctors, biologists and medical students who worked in the laboratory grew, and within the following few years 25 people were working there. Some had the opportunity to gain research experience abroad in leading medical centers in Cambridge, London, New York, Strasbourg or Leuven, and then to return to Bergamo with new techniques, collaborative programs and original ideas.

Platelet-vessel wall interactions in uremia

Between 1977 and 1979 nine papers regarding bleeding, a common complication in patients with renal failure,[1-9] were co-authored and published by Bergamo and Mario Negri Milan scientists. The focus was on the interaction between platelets and vessel walls, a critical event in the hemostatic process, and on the discovery - made during that time - that human vascular tissues produce prostacyclin, an unstable metabolite of arachidonic acid, which is a potent inhibitor of platelet aggregation.[10,11] In an article published in 1977 in the prestigious journal *Lancet*,[2] the Bergamo-Milan team showed for the first time that venous specimens from patients with renal failure and very prolonged bleeding times generated higher prostacyclin-like activity than venous tissue from control subjects. The prostacyclin-like activity was assessed in terms of platelet aggregation, as proposed by Salvador Moncada and Sir John Vane, two pioneers in the field of prostaglandins[10,11] For the experiments, vascular specimens were cut into rings and washed with 'tris' buffer. The supernatant was then incubated with platelet-rich plasma from healthy donors, and platelet aggregation initiated with ADP. After repeated washings of the rings, when the inhibition of platelet aggregation could hardly be detected in control tissue samples, potent inhibitory action was still evident in samples containing uremic venous tissue.[2] Studies involving a larger number of patients with renal failure confirmed the initial data in the *Lancet* paper,[2] showing that longer bleeding times were associated with higher prostacyclin-like activity.[3,4] Moreover, platelets from patients with uremia and prolonged bleeding times exhibited a reduction in malondialdehyde generation, a reliable parameter of platelet activation, in response to different aggregating agents.[3,4] Malondialdehyde is a product of the platelet arachidonic acid metabolism, which is considered to reflect the generation of prostaglandin endoperoxides,[12] which are potent aggregating agents. In uremic patients, prostaglandin metabolism was disrupted in opposite ways in platelets and vessel walls, both contributing to impaired primary hemostasis. Further studies showed that plasma from uremic patients inhibited prostaglandin formation in platelets, as indicated by reduced malondialdehyde levels, and stimulated prostacyclin-like activity in 'exhausted' rat aortic rings, as indicated by platelet aggregation inhibitory potency.[8] It was therefore hypothesized that one or more non-dialysable endogenous factors, which regulate prostaglandin synthesis, could accumulate in the uremic plasma and be responsible for the different disturbances in the arachidonic acid metabolic pathways in uremia.[8] A heat-labile non-dialysable plasma factor that powerfully stimulated prostacyclin synthesis by cultured pig aortic endothelial cells had already been described by MacIntyre *et al*.,[13] and the existence of this plasmatic activity was then confirmed by other groups using different experimental systems.[14,15] Later, the prostacyclin-regulating plasma factor was partially purified and identified as a low, stable and polar molecule capable of reactivating prostacyclin generation by rat aortic rings that have been 'exhausted' by prolonged washing in buffer.[16] This plasma factor acted as a reducing cofactor for the cyclo-oxygenase peroxidase, and its main physiological role was probably to protect the vascular prostacyclin-forming system from exhaustion in chronically irritated blood vessels.[16] Studies on platelets, prostaglandins and bleeding in uremia continued in the 1980s, and our group's first publication in the *Journal of Clinical Investigation* was on a study on reduced platelet thromboxane formation in uremia.[17]

In 1979 an international symposium titled 'Endothelium, Platelets and Prostaglandins: Fresh Insight into Renal Diseases' was organized in Bergamo (Figure 2). It was a great success and represented an invaluable opportunity for growth for our group. For the first time, internationally renowned experts from seemingly unrelated areas of research, such as hemostasis, prostaglandins and nephrology, could meet to exchange data and formulate hypotheses on the role of platelet-vessel wall interaction and their mediators in the pathogenesis of conditions such as uremic bleeding, pre-eclampsia, microangiopathic hemolytic

Figure 1. a) 1977. The small laboratory at Bergamo Hospital. b) 1984. The first installation of a research institute in a sixteenth-century building. c) 2010. The relocation of the Institute to new laboratories at the Kilometro Rosso.

INTERNATIONAL SYMPOSIUM

ENDOTHELIUM, PLATELETS AND PROSTAGLANDINS: FRESH INSIGHT INTO RENAL DISEASES

Divisione di Nefrologia e Dialisi, Ospedali Riuniti, Bergamo, Italy
Istituto di Ricerche Farmacologiche "Mario Negri" Milan, Italy
Under the auspices of
Associazione Bergamasca per lo Studio delle Malattie Renali, Bergamo, Italy
Mario Negri Institute Foundation, New York, N.Y., U.S.A.

Bergamo, 13-15 September, 1979 Seminario Giovanni XXIII Via Arena, 11

INVITED SPEAKERS

P.M.Nachman (New York)	L.Morel Maroger (Paris)	B.B.Vargaftig (Paris)
G.V.R.Born (London)	C.G.Cochrane (La Jolla)	J. Vermylen (Leuven)
C.L.Pirani (New York)	M.J.Silver (Philadelphia)	T.L.Dormandy (London)
A.Parbtani (London)	S.Moncada (Beckenham)	J.L.Gordon (Cambridge)
J.S.Cameron (London)	P.M.Bolger (Washington)	M.Levy (Paris)
J.Hawiger (Boston)	A.R.Morrison (St. Louis)	R.Habib (Paris)
P.Verroust (Paris)	P. Needleman (St. Louis)	C.R.M. Prentice (Glasgow)

Figure 2. The International Symposium organized in Bergamo, in 1979.

anemia, and glomerular disease. One year later, an extensive book based on the proceedings of this international Symposium was published by Raven Press under the title "Hemostasis, Prostaglandins and Renal Disease" (Figure 3).

Prostacyclin and pregnancy

Our research group and the Mario Negri Milan staff jointly studied whether prostacyclin and prostacyclin-stimulating plasma factor could play a role in pre-eclampsia, a major cause of morbidity and death for pregnant women and fetuses. Pre-eclampsia is characterized by edema, hypertension and proteinuria. Renal failure and consumptive coagulopathy can also develop, and histopathological examination may reveal that placental vessels and glomerular capillaries are occluded by fibrin deposits. In papers published in 1979 and 1980, we showed that healthy fetal blood vessels have a high capacity to synthesize prostacyclin, suggesting it plays an important role in the regulation of fetal circulation.[18] We also demonstrated that, in contrast, prostacyclin formation was significantly reduced in the umbilical and placental vascular tissues of patients with severe pre-eclampsia.[19,20] This deficiency in prostacyclin may contribute to reduced blood flow, leading to impaired fetal nutrition. It could also account for thrombi in the placental and renal circulation. Next, we measured the plasmatic activity of prostacyclin-stimulating factor in early and late normal pregnancies and in late pregnancy complicated by severe pre-eclampsia.[21] In early pregnancy, plasmatic activity was comparable with activity

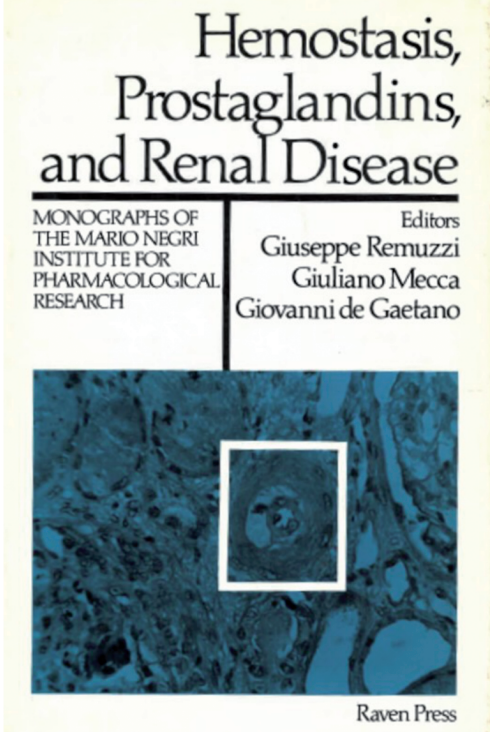

Figure 3. Front cover of the proceedings book from the International Symposium organized in Bergamo, in 1979.

in control non-pregnant women. In late normal pregnancy there was a significant decrease, whereas in severe pre-eclampsia it was relatively normal. These results suggested that the activity of prostacyclin-stimulating factor present in normal plasma is depressed in late normal pregnancy due to a feedback mechanism that limits an excessive increase in vascular prostacyclin. In severe pre-eclampsia, when vascular prostacyclin synthesis is reduced,[20] normal activity could act as a reactive defense mechanism.[21]

Platelets in nephrotic syndrome

A nephrological problem that was addressed by the Bergamo/Milan team regarded the thrombotic tendency in nephrotic syndrome. In 1979 it was proposed that hypoalbuminemia could render platelets hypersensitive to arachidonic acid and an inverse relationship between serum albumin concentration and platelet aggregation was demonstrated.[22] Platelet hypersensitivity to arachidonic acid was corrected by the addition of albumin to nephrotic platelet-rich plasma (PRP) *in vitro*, and by intravenous albumin infusion and prolonged treatment with corticosteroids *in vivo*. A further study showed that platelet malondialdehyde and thromboxane B2 production was greater in nephrotic PRP than in normal PRP, and that this defect was corrected by the addition of normal plasma.[23]

Thrombotic microangiopathies

An area of research we have been involved since the beginning of our work, and remained involved in, regards hemolytic uremic syndrome (HUS) and the related disorder thrombotic thrombocytopenic purpura (TTP).[24] Both syndromes are characterized by thrombocytopenia, hemolytic anemia and renal involvement. The fundamental pathological lesion, thrombotic microangiopathy, is identical in HUS and TTP, although it can involve different organs. Platelet aggregates are present in the microcirculation of the kidney and other organs, possibly because of uncontrolled intravascular platelet aggregation and fibrin deposition. Endothelial cell damage is the trigger for intravascular platelet aggregation.[25] Through initial studies we demonstrated that vascular specimens from HUS patients had a reduced capacity to generate prostacyclin,[26] and that the deficiency of a plasma factor-stimulating prostacyclin synthesis[27] could be the cause. This is an abnormality shared by TTP.[28] More recently, the Bergamo group's focus regarding HUS and TTP has been moved to the genetic determinants of microvascular lesions that include members of the complement system, specifically. In this area, the group has contributed to original studies and to the discovery of novel genes responsible for disease manifestation.[29] A nice anecdote linking the Bergamo group's interest and *The Lancet* was a letter of rejection from the then deputy editor David Sharp that we will never forget (Figure 4). This was the only time in our scientific careers that we felt just as happy when a paper was rejected as we would have had it been accepted. Today, the relationship between authors and scientific journals feels impersonal and cold. We truly miss the days when editors could express things with such grace, as David did.

The growing need to integrate laboratory research with clinical work, and the well-established collaboration between Mario Negri in Milan and the Nephrology division in Bergamo led Remuzzi, de Gaetano and Donati to propose to Professor Silvio Garattini, founder and Director of Mario Negri Institute in Milan, the establishment of a new biomedical research laboratory in Bergamo. A proposal accepted by Garattini who actively committed himself to the realization of what only seemed to be our dream. That's how in December 1983 the Negri Bergamo Laboratories were set up in a beautiful sixteenth-century build-

21st June 2000

Dear Beppe,

You give such a lot of useful help to The Lancet *that it pains all of us when, as must happen occasionally, we have to say NO to you. Your detailed study of familial HUS clearly deserves publication but the feeling amongst the editorial team here is that it is a bit too specialised for our readers. I know you are going to find it easy to find an editor better placed to accept this one and I can assure that there are no scientific criticisms from* The Lancet.

David Sharp

Figure 4. A gracious rejection letter from the *Lancet*.

ing called the Conventino (Figure 2b). Ten years later, the staff had already grown to include over 160 researchers and other staff, and over 450 scientific papers had been published.

Laboratory extension and clinical integration

The original research on platelets, thrombosis and hemostasis that we had started in the small laboratory in the hospital progressively became a marginal part of our research. During the last 30 years, indeed, our main areas of research included mechanisms of proteinuria and the progression of kidney disease, with the aim of finding new molecules and/or strategies to slow down or even induce the regression of the disease. This was pursued by setting up animal models that reproduced kidney diseases and using *in vitro* renal cells in culture. The group's work then expanded to organ transplantation and rejection, tolerance, autoimmune diseases, glomerulonephritis, molecular biology and many other areas (selected publications from the group are reported in the *Supplementary Material*).

In 2010, the laboratories were relocated from the Conventino to a new structure, also in Bergamo, the Centro Anna Maria Astori at the Kilometro Rosso Scientific Park, where over 100 people are currently working (Figure 1c). Alongside the growth of the team in the laboratory, a number of clinical scientists working with Remuzzi came together at the Aldo e Cele Daccò Clinical Research Center for Rare Diseases in Ranica, near Bergamo, which now employs 100 people. We have to mention that in 2016 Professor Barry Brenner, following a visit to the two facilities, kindly wrote to us: 'I can honestly state that Mario Negri is the place to be in Italy, and perhaps in all of Europe, for the serious and focused study of renal biology today.'

COVID-19 and coagulopathy

More recently, in the years following the outbreak of the COVID-19 pandemic, during which the Bergamo area was one of the hardest-hit regions in the world, our group invested significant effort in studying this field. In addition to developing serological tests for diagnosing SARS-CoV-2 infection, we conducted a highly controlled study to assess genetic predisposition to severe forms of the disease. This was facilitated by the extensive viral spread in the area. This study led to the identification of genes inherited from Neanderthals that may influence the development of severe COVID-19.[30] As previously mentioned, we had almost abandoned since many years research on platelets and coagulation. However, to our slight embarrassment, the COVID-19 pandemic forced us to rediscover just how crucial this field truly is. The hypercoagulability observed in severe COVID-19 cases drew renewed attention to the role of platelets and the coagulation cascade in disease progression. Several studies have demonstrated that SARS-CoV-2 infection can trigger endothelial dysfunction, platelet hyperactivation, and an imbalance in pro-thrombotic and anti-thrombotic factors, leading to an increased risk of thrombosis and microvascular complications. This has not only deepened our understanding of the pathophysiology of COVID-19 but has also highlighted the need to reassess the role of platelets in inflammatory and infectious diseases.[31]

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Online supplementary material:
List of Selected Publications from the Group.