RoFAR
Foundation for Anemia Research

Bi-annual report

December 2006

RoFAR is an independent foundation run by an international Board of Trustees and funded by an unrestricted grant from Roche. All submitted applications are peer reviewed by an independent Scientific Advisory Board.

Mission

The Roche Foundation for Anemia Research (hereinafter "the RoFAR") is a registered Medical Research Charity with the mission of "encouraging innovative research that will open new avenues of exploration in the study of anaemia, its mechanisms and outcomes." Individuals eligible for grants are members of academic staff in universities, dialysis centres and research institutes.

The RoFAR was established by the Roche Group in 2004 under Swiss law and incorporated in Basel, Switzerland. The Roche Group is committed to providing funding of CHF four million annually for at least four years from inception to a total of at least CHF 16 million. The RoFAR is a non-profit, autonomous and legally independent charitable organisation.

The RoFAR encourages the exploration of new research in areas associated with the study of anaemia, its mechanisms and outcomes. The Board of Trustees will set the focus of research for the specific cycle.

In addition to focusing on anaemia related to kidney disease and oncology, the RoFAR also will encourage research into:

- Anaemia of chronic disease
- Anaemia related to congestive heart failure and stroke
- Effects of erythropoietin and erythropoietin-like substances as protective drugs in various target organs
- Central resistance to erythropoietin
- Biology of anaemia and outcomes

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1 *Preface*

In the second half of 2006, ROFAR has awarded grants of a total of over 2 million CHF to outstanding research projects dedicated to advancing knowledge in the field of anaemia, its associated complications, erythropoietic agents and outcomes.

Six applications made during the fifth cycle of competition have been selected to receive RoFAR research grants of up to 200,000 CHF each distributed over two years. Once again, the high expectations for the quality of research projects and applicants have been met. Since 2004, RoFAR has awarded thirty-three regular grants totalling over 7 million CHF or 5.4 million USD.

There are two cycles of RoFAR awards each year. Timelines for the cycles and the submission deadline for application of an award are published on the Foundation's website. The first step in the application process is to submit a Letter of Intent (LOI) which once submitted, is reviewed by our Scientific Advisory Board (SAB). Applicants who are considered by the SAB to have submitted the most compelling LOIs are then invited to proceed to the next stage and submit a full application. Full applications are considered in detail by the SAB, and final decisions on award winners are confirmed by the Board of Trustees (BT) which undertakes to notify applicants of their decision six months after submission of the LOI.

In addition to the regular competition cycles, RoFAR recently invited scientists and institutions to submit applications for a special grant to support ground-breaking scientific work, both basic and clinical, which topics included investigation of anaemia and erythropoietin ranging from hypoxia-sensing to the organ-protective role of erythropoietin and understanding of iron metabolism. RoFAR was particularly interested in innovative proposals, involving established and junior researchers, which provide proof of principle and/or translational research, particularly studies which have potential for results to be transferred into clinical practice. The submission and selection procedure – similar to the one in use for regular grants – has resulted in an award of a grant of 800,000 CHF. The special grant recipient is announced in this report.

To inform the broad scientific community about the funding opportunities RoFAR provides, our promotional campaign has included

- advertisements in major scientific journals and on web portals
- distribution of brochures to major cardiology, oncology and nephrology centres
- distribution of leaflets and brochures at national and international scientific congresses

- information booths at selected international congresses
- public announcement of awarded applicants at important international congresses.

In the future, RoFAR plans to continue advertising its programme both through announcements in major scientific journals and websites and by selected activities at a number of scientific congresses. In particular, selected RoFAR award winners will have the opportunity to present their results during an evening symposium at the World Congress of Nephrology in Rio de Janeiro on 24 April 2007.

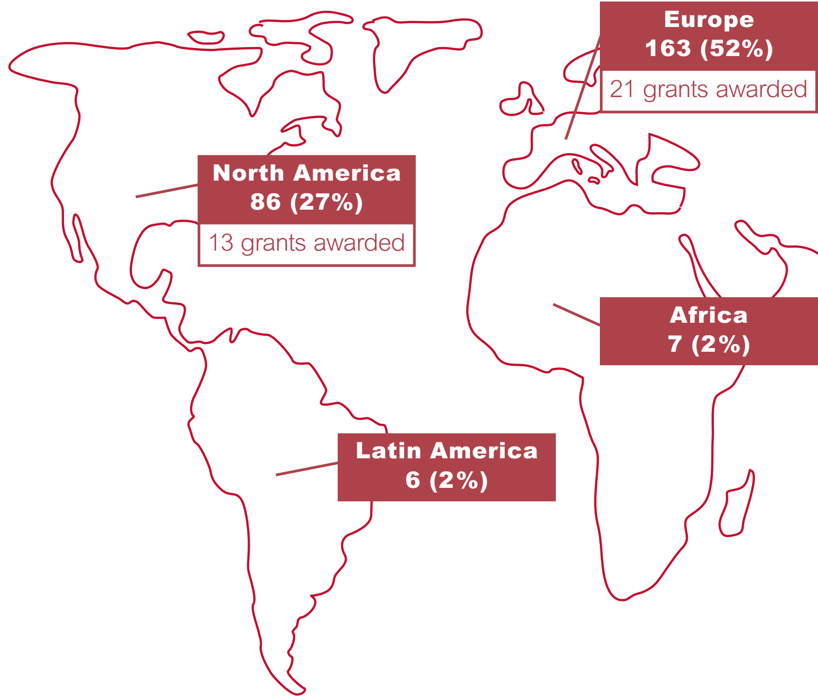
RoFAR is committed to its mission of fostering innovative anaemia-related research, and sincerely hopes to make a major contribution to the scientific community by encouraging scientists to apply their skills and intellect to furthering knowledge and understanding in this field. The BT and the SAB of the RoFAR all join in expressing their gratitude to F. Hoffmann-La Roche Ltd. for its generous gift to the anaemia research community and for Roche's enduring commitment to anaemia and related avenues of research.

RoFAR welcomes any feedback or suggestions to assist us in accomplishing its stated mission.

On behalf of the Board of Trustees

Dr Nathan W. Levin
Chairman of the Board of Trustees
Roche Foundation for Anemia Research

Geographical breakdown of submitted research proposals



Applicants in the first six cycles of competition including a special cycle represent a range of institutions in 42 countries. More than half (52%) of all the LOI applications have been submitted from Europe, primarily from Germany, UK, Italy and Switzerland. About one quarter of the applications (27%) have been submitted from the United States and Canada. About 25% of the applicants are female scientists. The great majority (98%) of applicants work in universities or university-affiliated institutions. Research proposals are distributed among clinical studies (52%), animal trials (38%) and basic science projects. Submitted projects focus on nephrology and diabetology (42%), haematology (41%), oncology (17%) and cardiology (14%) with some overlap between areas.

Asia and Oceania
52 (17%)

In cycles I to V, twenty-one regular grants have been assigned to European applicants, twelve to North American applicants. One special grant has been awarded to a North American applicant. Cycle VI grants have not yet been awarded.

Submitted research proposals by

Study type

Human trials	(52%)
Animal studies	(38%)
Others	(10%)

Research focus

Nephrology	(42%)
Haematology	(41%)
Oncology	(17%)
Cardiology	(14%)
Others	(22%)

(multiple allowed)

Gender of main applicant

Males	(75%)
Females	(25%)

Institution type

Universities & related	(98%)
Others	(2%)

3 Overview of awarded grants

Cycle I	***Nancy C. Andrews***	*Children's Hospital Boston, USA*
	Martin W. Bergmann	*Franz Volhard Clinic, Charité University, Berlin, Germany*
	Andrew McKie	*King's College, London, UK*
	Marco Merlano	*S. Croce General Hospital, Cuneo, Italy*
	Peter Mertens	*University Hospital Aachen, Germany*
	Chris D. Vulpe	*University of California, Berkeley, USA*
Cycle II	***Hans Ulrich Bucher***	*University Hospital of Zurich, Switzerland*
	Edward Debnam	*Royal Free & University College Medical School, London, UK*
	Diana Gilligan	*Puget Sound Blood Center, Seattle, USA*
	Alexander Maxwell	*Queen's University Belfast, UK*
	Jun-ichi Nishimura	*Duke University Medical Center, Durham, USA*
	Radek Skoda	*University Hospital Basel, Switzerland*
	Carole Soussain	*Oregon Health and Science University, Portland, USA*
	Christina Warnecke	*University Erlangen-Nürnberg, Germany*
Cycle III	***Max Gassmann***	*Vetsuisse, University Hospital of Zurich, Switzerland*
	Peter J. Kirkpatrick	*University of Cambridge, UK*
	Véronique Lefebvre	*Cleveland Clinic Foundation, USA*
	Stephen Leib	*University of Berne, Switzerland*
	Barbara Scheiber-Mojdehkar	*Medical University of Vienna, Austria*
	Jürg Schifferli	*University Hospital Basel, Switzerland*
	Marcela Votruba	*Cardiff University, UK*

	Grant awarded	Progress report published	Final report published
Hepcidin regulation in the anaemia of chronic disease	11/2004	07/2006	
Effect of 5,000 IU erythropoietin beta once weekly subcutaneously administered for six months in patients subjected to percutaneous coronary intervention displaying reduced LV- ejection fraction due to regional left ventricular wall motion defects	11/2004	12/2006	
Characterisation of a novel intestinal haem transporter	11/2004	07/2006	12/2006
In vitro analysis of tumor response to radiation in oxic and hypoxic conditions	11/2004	07/2006	
Mechanisms for erythropoeitin resistance in transformed and non-transformed cells	11/2004	07/2006	
Characterisation of a family of putative mammalian haeme chaperones	11/2004	07/2006	
Erythropoietin reduces brain, eye and lung damage in very preterm infants: Proof-of-concept study	05/2005	07/2006	
Is inflammation an important factor in the anaemia of chronic renal failure?	05/2005	07/2006	
Regulation of gene expression during erythropoiesis	05/2005	12/2006	
Investigation of the role of JUNE-1 in erythropoiesis	05/2005	12/2006	
Innovative drug design using RNA aptamers for various anaemias	05/2005	12/2006	
The role of SMAD4-dependent signalling in anaemia	05/2005	12/2006	
Neuroprotective effect of erythropoietin on chemo- and radiotherapy-induced toxicity	05/2005	07/2006	
Molecular mechanisms underlying the hypoxic induction of erythropoietin by HIF-2α	05/2005	12/2006	
The impact of erythropoietin on the hypoxic ventilatory response of mouse and man	11/2005	12/2006	
Effects of systemic erythropoietin therapy on cerebral auto-regulation and the incidence of delayed ischaemic deficits in patients with aneurysmal subarachnoid haemorrhage	11/2005		12/2006
Roles of Sox6 in erythropoiesis	11/2005		
Effect of erythropoietin on brain injury and regeneration in bacterial meningitis	11/2005		
Recombinant human erythropoietin: A new treatment for Friedreich`s ataxia	11/2005		
Erythropoietin or erythrocyte transfusion for anaemia?	11/2005		
Erythropoietin neuroprotection in retinal neurodegeneration	11/2005		

3 Overview of awarded grants

Cycle IV	***Christof Dame***	*Charité University of Berlin, Germany*
	Ricarda Diem	*University of Göttingen, Germany*
	Tomas Ganz	*University of California, Los Angeles, USA*
	Dirk Hermann	*University Hospital of Zurich, Switzerland*
	Stéphane Picot	*Claude Bernard University of Lyon, France*
	Jerôme Rossert	*Georges Pompidou European Hospital, Paris, France*
Cycle V	***Anne Angelillo-Scherrer***	*University Hospital of Lausanne, Switzerland*
	Margaret H. Baron	*Mount Sinai School of Medicine, New York, USA*
	Michael Bulger	*University of Rochester, USA*
	Sandra Juul	*University of Washington, Seattle, USA*
	Herbert Y. Lin	*Massachusetts General Hospital, Boston, USA*
	Stefano Rivella	*Weill Medical College of Cornell University, New York, USA*
Special cycle	***Nicoletta Eliopoulos***	*Lady Davis Institute for Medical Research (McGill University), Montreal, Canada*

	Grant awarded	Progress report published	Final report published
Role of GATA transcription factors in regulating erythropoietin and its receptor in the heart	*05/2006*		
Efficacy and safety of erythropoietin as an add-on therapy in subjects with acute autoimmune optic neuritis	*05/2006*		
Pathogenesis of anaemia of chronic infection	*05/2006*		
Effects of human erythropoietin on brain plasticity and functional recovery following stroke	*05/2006*		
Randomised trial of erythropoietin to prevent death from cerebral impairment during severe malaria	*05/2006*		
Study of the characteristics and fate of erythropoietin-producing cells	*05/2006*		
Role of growth arrest-specific gene 6 in anaemia of chronic disease	*11/2006*		
Regulation of red blood cell enucleation	*11/2006*		
Function of Sox6 in ß-globin gene silencing and definitive erythropoiesis	*11/2006*		
Mechanisms of erythropoietin-mediated neuroprotection	*11/2006*		
Regulation of iron metabolism by soluble haemojuvelin, Fc fusion protein	*11/2006*		
Identification of the genes responsible for the pleiotropic effects observed in ß-thalassaemia	*11/2006*		
Cell and gene therapy with erythropoietin-secreting marrow stem cells for kidney repair	*11/2006*		

Dr Anne Angelillo-Scherrer

University Hospital of Lausanne, Switzerland

Role of growth arrest-specific gene 6 in anaemia of chronic disease

We intend to study the novel role of the growth arrest-specific gene 6 (Gas6), its mechanism of action and possible diagnostic and therapeutic use in anaemia of chronic disease, the second most prevalent anaemia after iron-deficiency anaemia. Anaemia results from insufficient production, excessive destruction or loss of red blood cells. By limiting the capacity of the blood to carry oxygen, anaemia may lead to multiple organ malfunction. Anaemia of chronic disease is associated with numerous disorders promoting inflammation such as rheumatoid arthritis and cancer. Correction of anaemia in these patients reduces their morbidity and therefore improves their quality of life.

Gas6 is a cell survival factor that amplifies the response to erythropoietin (EPO) during anaemia and is a down-regulator of inflammation. In addition, Gas6 insures normal iron levels in blood. Iron is necessary for the generation of new red blood cells in response to anaemia, but also to continuously replace old red blood cells with new red blood cells. In anaemia of chronic disease, iron is diverted from blood into storage tissues (bone marrow, liver, spleen), considerably reducing its availability for the generation of new red blood cells. We therefore hypothesise that Gas6 deficiency favours anaemia of chronic disease by inducing a resistance to EPO, creating a proinflammatory environment and impairing iron metabolism.

Consequently, we plan to investigate the role of Gas6 in iron metabolism and its potential use to treat anaemia of chronic disease in experimental models. We will also determine whether Gas6 levels in blood might have a diagnostic value for anaemia of chronic disease.

Results of this work could open promising perspectives including clinical trials implying recombinant Gas6 alone or in combination with EPO to prevent and treat anaemia of chronic disease.

Dr Margaret H. Baron

Mount Sinai School of Medicine, New York, USA

Regulation of red blood cell enucleation

The objective of this research is to capitalise on novel transgenic mouse and cell culture models developed in our laboratory to deepen our understanding of the mechanisms underlying erythroid (red blood cell) maturation, with the ultimate goal of developing better methods for the production of red blood cells for transfusion therapies for anaemias of various etiologies. Primitive erythroid cells (EryP) are the first differentiated cell types to form in the mammalian postimplantation embryo and play a vital role in oxygen delivery, detoxifying reactive oxygen species, and in maintaining shear forces necessary for normal vascular development. In the mouse, large, nucleated EryP are produced in huge numbers within the blood islands of the yolk sac and begin to circulate around embryonic day (E)10, when connections between the yolk sac and embryonic vasculature mature. Two to three days later, small cells of the definitive erythroid lineage (EryD) begin to differentiate within the fetal liver and rapidly replace EryP in the circulation. Despite their abundance and indispensable functions, the development and maturation of EryP remains poorly defined. EryP form in a synchronous wave in the yolk sac, a feature that provides a major advantage in studying their maturation. We have found that terminal steps in primitive erythroid maturation, including enucleation, occur in the erythroblastic islands of the fetal liver and may require adhesive interactions with macrophages. Using innovative tools and experimental approaches, we will build on extensive preliminary studies to explore the biology of primitive erythroid development. The aims of this proposal are (1) to determine whether primitive erythroblast (EryP) enucleation is determined cell-autonomously or driven by interactions with macrophages; and (2) to determine whether cell-adhesion interactions within the fetal liver are required for enucleation of primitive erythroblasts.

Dr Michael Bulger (principal applicant)
Dr James Palis (co-applicant)

University of Rochester, USA

Function of Sox6 in β-globin gene silencing and definitive erythropoiesis

In normal red blood cells, oxygen is carried by haemoglobin, which in turn consists of individual globin molecules. Disorders of globin function, which include sickle-cell anaemia, represent the most common genetically inherited disorders worldwide. A hallmark of these disorders is that they can theoretically be treated simply by providing a victim's red blood cells with a normal globin. In practice, however, this has proven very difficult. An alternative approach is suggested by the presence, even in afflicted individuals, of intact globin genes that are normally inactivated during development. If we could find a method to re-activate these globin genes, the effects of globin gene disorders could be greatly ameliorated. Our work centers on a regulatory factor, termed Sox6, which has been shown to be required for the normal inactivation of some globin genes. We are performing experiments designed to elucidate the mechanism by which Sox6 represses these genes. In addition, we will identify other factors that work togheter with Sox6 to achieve gene repression, in the hope that these factors will provide additional targets for the development of drugs that can re-activate globin gene expression.

Dr Sandra Juul[1] (principal applicant)
Dr Robert Lane[2] (co-applicant)

[1]University of Washington, Seattle, USA
[2]University of Utah, Salt Lake City, USA

Mechanisms of erythropoietin-mediated neuroprotection

Perinatal asphyxia occurs in 2-4 of every 1,000 live-born term infants, and accounts for 23% of neonatal deaths worldwide. In recently published hypothermia trials, between 55 and 62% of infants diagnosed with perinatal asphyxia treated with conventional therapy died or survived with significant neurodevelopmental disability. Effective treatment strategies to improve neurodevelopmental outcome remain elusive; so new approaches to this problem are desperately needed. Our laboratory has used unilateral carotid artery ligation with oxidative stress to study brain injury in mice. Administration of high-dose erythropoietin (EPO) after the acute injury improves cerebral histology and long-term behavioural measures in a gender-dependent manner. RNA and protein expression are necessary. The underlying molecular mechanisms through which EPO induces these changes in gene expression are unknown. We hypothesise that high-dose EPO will decrease neonatal hypoxic-ischaemic brain injury and will produce gender-specific epigenetic modifications. Our specific aims are to determine the gender-specific short- and long-term epigenetic modifications associated with EPO treatment (5000 U/kg x 3 doses) following neonatal hypoxic-ischaemic brain injury. Specific genes targeted for analysis include: BRM, an ATPase of the SWI/SNF complex involved in chromatin remodelling and cell cycle; MDM2, a target of p53 that functions to degrade p53; pMDM2 Ser-166, an activated form of MDM2; GR, the glucocorticoid receptor, and pGR Ser-211, the activated but short-lived version of GR. Based on our preliminary results, we anticipate that EPO will provide significant neuroprotection, and that this protection will be mediated by early and persistent changes in gene methylation and acetylation in the injured hemisphere. Our findings suggest that the EPO-treated brains are phenotypically different from vehicle-treated controls, which is consistent with our previous findings of histological and functional improvement in these animals.

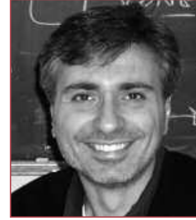
Dr Herbert Y. Lin

Massachusetts General Hospital, Boston, USA

Regulation of iron metabolism by soluble haemojuvelin, Fc fusion protein

The secreted liver protein hepcidin is now recognised as the "iron hormone". When hepcidin levels are abnormally high, iron absorption from the gut and iron mobilisation from body stores are impaired, leading to low serum iron levels and to iron deficiency anaemia. When hepcidin levels are abnormally low, iron absorption and iron mobilisation are excessive, leading to high serum iron levels and to iron overload disorders such as juvenile haemochromatosis. Consequently, lowering the high levels of hepcidin seen in iron deficiency anaemias should allow increased absorption and mobilisation of iron, leading to increased serum iron levels. Correcting the iron deficiency seen in many types of anaemias such as anaemia of chronic disease and anaemia of end-stage renal failure should be beneficial to patients. Haemojuvelin is a protein in the liver that regulates hepcidin production. We will study the novel therapeutic potential use of soluble haemojuvelin-Fc fusion protein to inhibit liver hepcidin expression and thus increase serum iron levels in mice. These studies will have important implications as a new type of therapy for iron deficiency anaemias to complement intravenous iron and erythropoietin.

Dr Stefano Rivella



Weill Medical College of Cornell University, New York, USA

Identification of the genes responsible for the pleiotropic effects observed in β-thalassaemia

β-thalassaemia major or Cooley's anaemia is a genetic disorder that arises as a result of mutations in the β-globin gene and which affects production of the oxygen carrier molecule, haemoglobin. In β-thalassaemia, decreased or absent production of the β-globin chain leads to increased erythropoiesis, albeit ineffective, augmented intestinal iron absorption, extramedullary haematopoiesis and osteopaenia. Our hypothesis is that reduction of β-globin expression triggers indirect modification of the expression levels of genes that contribute to the β-thalassaemia phenotype. In β-thalassaemia, a process called ineffective erythropoiesis triggers anaemia and is often lethal. This phenomenon is poorly understood but is thought to be caused by the augmented number of erythroid cell precursors that, however, fail to generate normal erythrocytes. In addition, in β-thalassaemia the erythroid precursor cells leave the marrow, and invade and damage other organs such as the spleen and liver. We generated the first mouse model of adult lethal β-thalassaemia major and generated data that suggest that the erythroid precursor cells behave as cancer cells, since these cells have an increased rate of proliferation, decreased mortality and actively invade other organs. In other words, this would be the first case of tumour-like behaviour with no mutations in a tumour-related gene. Studying the gene expression profile of erythroid cells from mice affected by β-thalassaemia, we identified genes that might be responsible for this behaviour. The goal of this project is to modify the expression of genes that might be responsible for the abnormal erythropoiesis in β-thalassaemia. We believe that the characterisation of genes that are altered under these pathological conditions will contribute to the development of new tools to predict the prognosis of this disease, and to discover new pharmacological and genetic approaches for the treatment of β-thalassaemia and other forms of acquired anaemia characterised by ineffective erythropoiesis.

5 RoFAR grant awards in the Special Cycle

In 2006, RoFAR invited investigators to submit applications for special grants with a volume up to 2.4 million Swiss Francs for innovative projects holding the promise to transfer study results into clinical practice.

We are very proud to announce that a grant of over 800,000 Swiss Francs has been awarded to Dr Nicoletta Eliopoulos for a three-year project aimed at coupling cell therapy and gene therapy to the protective effect of erythropoietin in recovering kidney function after acute renal failure. Mouse models, which have been designed to possibly translate into clinical experimentation, will be used to perform the studies.

Dr. Eliopoulos graduated from McGill University in Montreal in 1990 with a BSc, and obtained her MSc and PhD degrees at the University of Montreal in 1993 and 1999, respectively. After that she worked as a post-doctoral fellow in the group of Dr Jacques Galipeau at the McGill University-affiliated Lady Davis Institute for Medical Research, where she has been a project director since 2005. Recently she has been investigating the delivery of EPO by genetically engineered cells in the treatment of anaemia of chronic renal disease.

In 2007, RoFAR would like to re-extend the invitation to investigators to submit applications for special grants of up to 1.6 million Swiss Francs each. Projects must include innovative basic and clinical topics. Studies providing proof of principle and/or translational research at the forefront of research, particularly studies holding the promise to transfer study results into clinical practice are preferred. Projects must be submitted by 30 June 2007 as letters of intent via the RoFAR website (www.rofar.org).

Scientists interested in applying can obtain further information by contacting the RoFAR Secretariat (admin@rofar.org).

Dr Nicoletta Eliopoulos[1] (principal applicant)
Dr Jacques Galipeau[2] (co-applicant)
Dr Raymond F. Gagnon[3] (co-applicant)

[1]Lady Davis Institute for Medical Research (McGill University), Montreal, Canada
[2]Jewish General Hospital, Montreal, Canada
[3]Montreal General Hospital, Canada

Cell and gene therapy with erythropoietin-secreting marrow stem cells for kidney repair

Acute renal failure (ARF) is a common and serious disease with a high mortality rate exceeding 50% that has not decreased in the last 40 years. Various experimental studies in ARF have indicated that bone marrow-derived mesenchymal stromal cells (MSCs) can participate in the protection and repair of kidneys through their ability to differentiate into renal tubular and renal endothelial cells, but mainly through their secretion of factors protecting renal cells from death, and/or through their inducing proliferation of surviving tubular cells or differentiation of endogenous renal stem cells. Erythropoietin (EPO), an essential cytokine for erythropoiesis, has also been determined to be a multifunctional cytokine, with its receptor shown to be expressed on a variety of cells that include renal cells. EPO has been found to exert a reparative and protective effect on damaged kidney through an increase in proliferation and decrease in death of renal tubular cells, as well as an accelerated recuperation of kidney function and structure. The goal of our proposed research is to test a novel approach combining cell therapy and gene therapy for ARF, one coupling the kidney reparative/protective effects of MSCs with those of EPO. Therefore, we propose a clinically translatable cell and gene therapy study where MSCs will be genetically engineered to secrete EPO and implanted in two mouse models ARF. One mouse model will be induced by ischaemia and the other by the nephrotoxic drug cisplatin. Animals will be monitored over time, and numerous analyses will be conducted to assess kidney function and structure. The effect will be compared with several control groups, such as ARF mice administered with non-EPO-secreting MSCs or recombinant mouse EPO, alone and combined. We expect that kidney injury will be repaired/protected significantly by our novel, easily clinically-translatable strategy combining the beneficial effects of MSCs with those of EPO for the treatment of ARF.

Dr Andrew McKie (principal applicant)
Dr Robert J. Simpson (co-applicant)
Prof. Robert C. Hider (co-applicant)
(Cycle I)

King's College, London, UK

Characterisation of a novel intestinal haem transporter

Haem is a highly bioavailable source of iron which is absorbed from the diet 4 to 5 times more efficiently than non-haem iron. The transporter responsible has recently been identified as HCP1[1]. Hereditary haemochromatosis (HH) is one of the most common inherited disorders in Caucasians, affecting nearly 5 per 1000 people of Northern European extraction. Previous work has suggested that patients with HH, as well as absorbing too much non-haem iron from the diet, have a similar defect in haem iron absorption[2]. Thus patients with HH are often advised to reduce the consumption of red meat in their diet. More than 80% of HH patients harbour the Hfe mutation which results in a cysteine-to-tyrosine conversion at amino acid 282 (C282Y) in a region of the protein (Hfe) corresponding to the $\alpha 3$ domain. This mutation results in the loss of interaction with β_2-microglobulin and decreased cell surface expression of Hfe. These patients have increased dietary iron absorption resulting in excessive iron accumulation in parenchymal cells particularly in the liver and heart. Despite increasing information regarding Hfe structure and expression, the mechanism by which Hfe regulates non-haem and haem absorption remains undefined.

However, anaemia, either due to dietary iron deficiency or genetic factors such as found in atransferrinaemia in humans (hypotransferrinaemia (HPX) in mice), is a serious condition with the former affecting millions of people worldwide. Haem iron may represent an important source of dietary iron in anaemic individuals. However, little is known about the regulation of dietary haem absorption by anaemia. The aim of this project was to determine the influence of anaemia, either due to genetic conditions or dietary iron deficiency, as well as to investigate the effect of Hfe mutations on ^{59}Fe-labelled duodenal haem absorption. We used HPX mice; mice fed an iron-deficient diet and Hfe knock-out mice as models and developed an *in vivo* assay to measure haem absorption from duodenum.

Radiolabelled haem (100 µM ^{59}Fe haem conjugated with arginate) was injected into a pre-washed tied-off duodenal segment of mice under anaesthesia. In homozygous

Hfe knock-out mice *($Hfe^{-/-}$)*, haem absorption was significantly increased as compared to a wild-type (wt) group. *$Hfe^{-/-}$* mice had a significant increase in intestinal mucosal uptake (TMU) due to increases in both duodenal uptake and mucosal transfer of the radioiron into the plasma as compared to wt group (Fig. 1). Haem was efficiently absorbed by the intestine despite significant hepatic iron overload in *$Hfe^{-/-}$* (liver non-haem iron was 3.89 ± 0.04 nmol/mg in *$Hfe^{-/-}$* mice compared to 0.37 ± 0.16 nmol/mg for WT $p<0.04$, $n=4$).

These results indicate that the Hfe mutation results in increased dietary haem iron absorption, which further adds to the iron loading of liver and other tissues in patients with haemochromatosis.

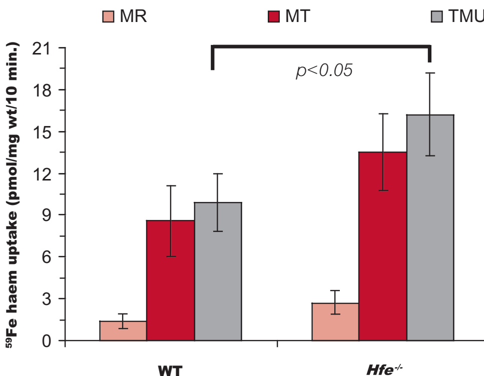


Figure 1. *Intestinal ^{59}Fe haem absorption in $Hfe^{-/-}$ (n=6) and (n=4) mice. Uptake was measured after 10 min. Data are means ± SD.*

In homozygous HPX mice ($hpx^{-/-}$) haem iron absorption by the duodenum was increased approximately twofold (Table 1), despite increased liver iron stores approximately tenfold higher than wt mice. This indicates that increases in erythropoetic activity can upregulate the rate of dietary haem iron absorption even in the face of massively increased iron stores. Iron deficiency increased dietary haem absorption to a lesser extent than HPX. However, we found that the increase in iron deficiency could be blocked by incubation of the tissue with HCP1 antisera (Table 1). This indicates that the haem carrier HCP1 is likely to be responsible for the upregulation of haem absorption observed in iron deficiency. Our results indicate Hfe, iron and erythropoesis all regulate dietary haem absorption with the latter appearing to have the greatest effect. Our data also indicate that HCP1, at least in the case of iron deficiency, mediates this regulation. Haem absorption via HCP1 is therefore an important pathway of entry of iron into the body, which will effect body iron stores and has important implications for the treatment of diseases resulting in anaemia and iron overload.

		^{59}Fe Haem uptake (pmol/mg/min)			Liver non-haem iron (nmol/mg)
	n	MR	MT	TMU	
wt	6	5.3±3.4	10.7±2.4	16.0±4.9	2.79±1.6
hpx$^{-/-}$	6	11.0±6.7	24.7±11.6	35.8±15.9	24.5±3.1
		p=0.09	*p=0.02*	*p=0.02*	*p=0.0001*
CD1 +Fe	4	5.7±2.6	12.9±3.6	18.6±4.5	2.06±0.31
CD1 -Fe	4	9.8±4.0	15.0±3.9	24.8±7.1	0.43±0.15
CD1 -Fe anti HCP1	4	7.7±2.6	7.2±1.6	14.9±3.2	
		p=0.4	*p=0.01*	*p=0.04*	*p=0.0001*

Table 1. *In vivo ^{59}Fe haem uptake (10 min) and liver non-haem iron levels in HPX and wt mice, iron-replete (Fe+) versus iron-deficient CD1 mice (Fe-, mice fed an irondeficient diet for 3 weeks from weaning) and in Fe- mice treated with HCP1 antiserum (CD1+Fe and CD1-Fe were treated with preimunne serum as control). Results are mean ±SD.*

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Dr Peter J. Kirkpatrick (principal applicant)
Dr Ming-Yuan Tseng (co-applicant)
(Cycle III, one-year project)

University of Cambridge, UK

Effects of systemic erythropoietin therapy on cerebral autoregulation and the incidence of delayed ischaemic deficits in patients with aneurysmal subarachnoid haemorrhage

Background

Erythropoietin (EPO) has been demonstrated to have neuroprotection, similar to ischaemic preconditioning. In this phase-II randomised controlled trial, we explored potential neuroprotection from exogenous supplement of high-dosed EPO following aneurysmal subarachnoid haemorrhage (aSAH), and hypothesised that these effects were associated with multiple mechanisms.

Methods

Eighty aSAH patients (age mean 58.6, range 24-82 years) were randomised to receive intravenous EPO (30,000 IU EPO-β) or 0.9% saline on days 0, 2, 4 following admission starting within 72 hours from the haemorrhage. Primary endpoints were the incidence and duration of cerebral vasospasm and impaired autoregulation estimated from transcranial Doppler (TCD). Secondary endpoints were the incidence of vasospasm-related delayed ischaemic deficits (DIDs), scores of the modified Rankin scale (MRS) and National Institutes of Health Stroke Scale (NIHSS) at discharge. Laboratory data included haematology, inflammatory and lipid profiles measured every 3 days. Data were compared between the trial groups, and between patients with/without unfavorable outcome using repeated measurement ANOVA and Dunnett's correction.

Results

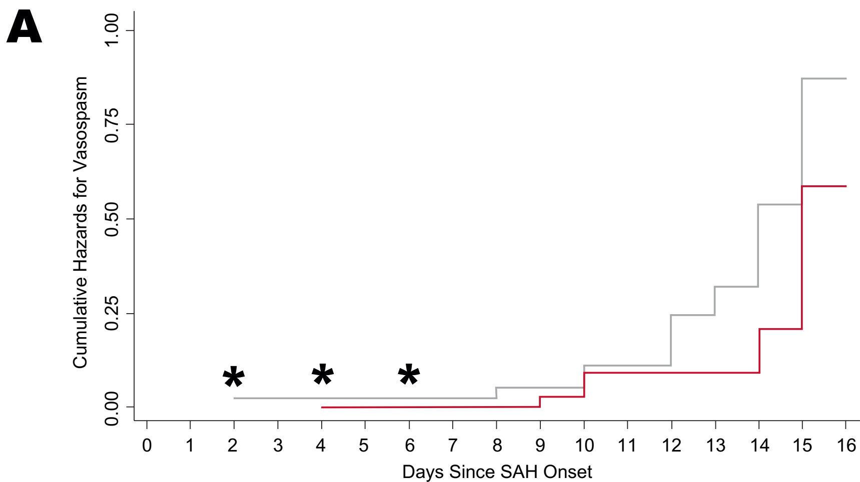
No trial-related complications were observed, including uncontrolled hypertension and systemic thromboembolism. Pre- and post-randomisation characteristics were well balanced except for age, where the EPO group was older (mean age 59.8 compared with 53.8 years, $p=0.036$).

There were significant reductions of incidence and duration of vasospasm in the EPO group from 65 to 40% (χ^2 test *p=0.025*) and from 4.0 ± 4.7 days (95% CI 2.5 to 5.5 days) to 1.6 ± 2.5 days (95% CI 0.8 to 2.4 days, t-test *p=0.007*) respectively (Figure 1). The incidence and duration of severe vasospasm were reduced in the EPO group from 27.5 to 5.0% (Fisher's exact test *p=0.013*) and shortened from 1.1 ± 2.9 days (95% CI 0.2 to 2.1 days) to 0.1 ± 0.4 day (95% CI 0 to 0.2 day, t-test *p=0.024*) respectively. Impaired autoregulation on the ipsilateral side was shortened by 3.2 days (*p<0.001*) and vasospasm-related DIDs was reduced from 40.0 to 7.5% (*p=0.001*) in the EPO group (Figure 1). However, no difference was seen in mortality. Patients in the EPO group demonstrated a more favourable outcome (MRS 1 & 2, 67.5 compared with 40%, *p=0.014*), and achieved lower NIHSS scores (5.1 compared with 11.7, *p=0.005*).

Baseline laboratory data were similar between the trial groups. Subsequent measurements revealed that reticulocyte counts were significantly higher in the EPO group (day 9, relative reticulocyte count 3.67% compared with 1.96%, *p<0.01*, Figure 2), but no difference was observed in haemoglobin or haematocrit between the trial groups. Progressive haemodilution can be seen in the placebo group only (day 15 haematocrit -3.89%, *p<0.05*, Figure 2). Furthermore, irrespective of the allocated treatment, patients with unfavourable outcome had progressive haemodilution, decreased high-density lipoprotein cholesterol and significant increments in inflammatory profile.

Conclusions

Acute systemic treatment with high-dosed EPO is safe, reduces physiological and clinical manifestations of cerebral vasospasm, and improves early outcome after aSAH. Neuroprotective mechanisms may involve direct cerebrovascular protection, haematopoiesis, anti-inflammation and beneficial lipid profile.



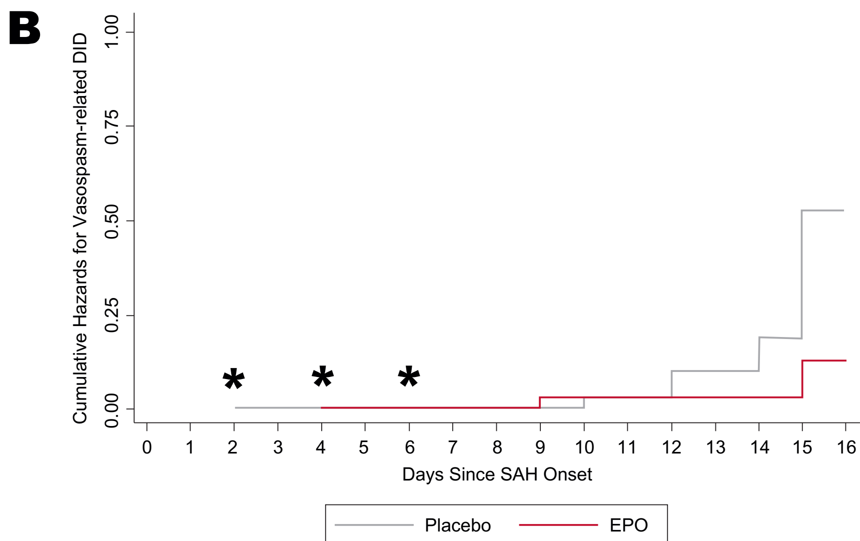


Figure 1. *The Nelson-Aelan cumulative hazard estimates for (A) vasospasm and (B) vasospasm-related DIDs. * indicates the time when trial medications were given.*

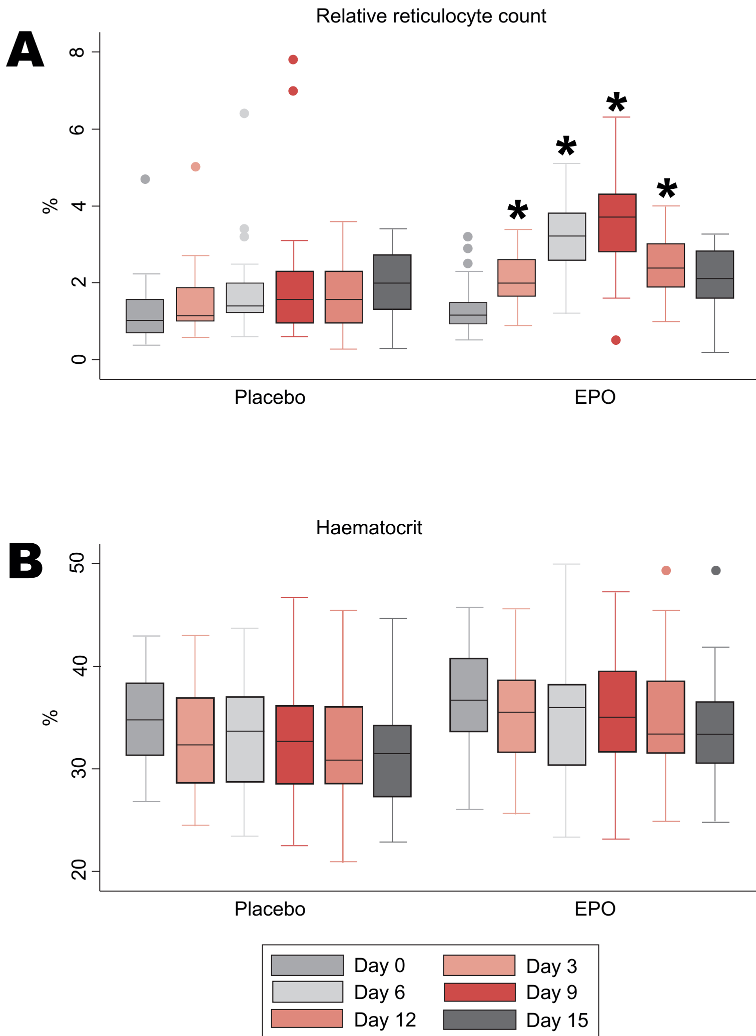


Figure 2. *Comparison of relative reticulocyte counts (A) and haematocrit (B) between the trial groups. Each box represents the median value. Each whisker represents the 75th (upper hinge) and the 25th percentiles (lower hinge). *p<0.05 after repeated measurement ANOVA and Dunnett's adjustment.*

Dr Martin W. Bergmann (principal applicant)
Prof. Rainer Dietz (co-applicant)
(Cycle I)

Franz Volhard Clinic, Charité University, Berlin, Germany

Effect of 5,000 IU erythropoietin beta once weekly subcutaneously administered for six months in patients subjected to percutaneous coronary intervention displaying reduced LV-ejection fraction due to regional left ventricular wall motion defects

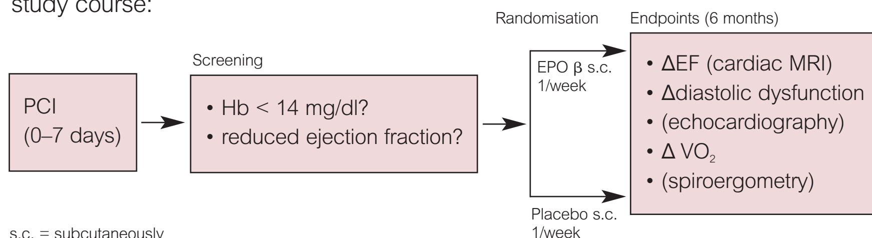
Background

Since approval of the grant several additional experimental studies have suggested erythropoietin (EPO) to be a good candidate for enhancing the recovery of cardiac function in ischaemic cardiomyopathy. However, the clinical studies in this field focusing on anaemic patients and treatment with EPO or its derivatives to correct anaemia failed to reach their primary endpoint, although anaemia was sufficiently corrected[1, 2]. This may be interpreted as anaemia being associated with, but not causal to heart failure. Clearly, new approaches must be tested, one of which is to use EPO in ischaemic heart failure patients independent of haemoglobin levels.

Several effects known to be exerted by EPO directly in the heart independent of haemoglobin levels could be of value immediately after revascularisation procedures in ischaemic cardiac remodelling: the generation of new capillaries is enhanced by the mobilisation of endothelial progenitor cells from the bone marrow[3]. EPO is neuro- and cardio-protective after ischaemia/reperfusion[4]. Administration of EPO enhances neuronal progenitors to differentiate into functional neurons[5]; this observation may also be valid for the cardiac compartment. The concept of organ-specific effects independent of haemoglobin levels is supported by the analysis of EPO analogues lacking haematopoietic activity[6]. Currently, this concept in humans, can only be tested by the use of EPO-doses that do not affect haemoglobin levels. The concept is valid as clinical trials have been performed showing that doses as low as 5,000 IU EPO once weekly increase the levels of endothelial progenitor cells in blood[7].

Study outline

This investigator initiated, double-blind, placebo-controlled study is testing the hypothesis that low doses of EPO ß started within 7 days after a successful percutaneous coronary intervention (PCI) enhance left ventricular remodelling as determined by comparison of two cardiac MRIs over a course of 6 months. Secondary endpoints include changes in diastolic dysfunction as measured by echocardiography and VO_2 measured by spiroergometry. The diagram depicted below summarises the study course:



After final approval of the study by the authorities, the first patient was included at the beginning of June 2006 and recently finished the 6-month study course uneventfully. Meanwhile, a second centre was activated at the beginning of November 2006 at the Department of Cardiology, Charité Campus Virchow, Berlin, which is headed by Prof. Rainer Dietz. With both centres together, the aim of recruiting 60 patients for this study in an 18-month timeframe after initiation of the study may be achieved.

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Dr Diana Gilligan
(Cycle II)

Puget Sound Blood Center, Seattle, USA

Regulation of gene expression during erythropoiesis

We are studying the regulation of gene expression during haematopoiesis. In particular, we are interested in how the expression of adducin genes may differ among the different lineages during haematopoiesis. In mature human red blood cells, only α (ADD1) and β (ADD2) adducins are detected by western blot[1] and γ-adducin (ADD3) is not present. In contrast, in mature human platelets, only α and γ-adducins are detected by western blot and β-adducin is not present[2]. This suggests very different mechanisms for regulation of expression of the adducin genes. In our preliminary data, we identified a novel erythroid exon 1 for ADD2. The genomic sequence immediately 5' to this exon contains predicted binding sites for GATA and EKLF transcription factors, suggesting that it functions as an erythroid specific promoter.

We have made considerable progress in the analysis of the putative erythroid promoter for ADD2. Figure 1 shows a diagram of the promoter region of ADD2 that we are currently analysing with the luciferase reporter system. A 960 bp fragment of genomic DNA is being analysed initially for promoter activity. PCR primers were designed to amplify this region from human chromosome 2 and create restriction sites for cloning into the multi-cloning site of pGL3 luciferase reporter vector (Promega). After analysis of the full-length fragment, two smaller fragments can be generated by cutting the vector with *Sma*I or with *Hind*III and reannealing. The multi-cloning site of the vector contains *Sma*I and *Hind*III restriction sites downstream of the *Nhe*I site. In addition, the region from *Sma*I to *Hind*III can be cut out from the vector and then ligated into vector cut with *Sma*I and *Hind*III for analysis of this region by itself.

Figure 2 shows the resulting constructs that are currently being tested for promoter activity. Once we have determined which regions are critical to the promoter activity, we will pursue mutational analysis of putative transcription factor binding sites. If there are no previously identified transcription factor binding sites in the

critical regions, then we will pursue a yeast one-hybrid library screening to identify binding proteins for the critical DNA sequence.

It is important to test the function of the critical promoter sequences in stable transfections as well as in the transient transfections and luciferase assays. Therefore, we will also test critical regions using the RMCE (recombinase mediated cassette exchange) approach that was outlined in the original proposal. We have obtained the appropriate vector for these constructs and a previously established cell line will be used for this analysis. We hope to complete these studies and submit a report for publication within the next year.

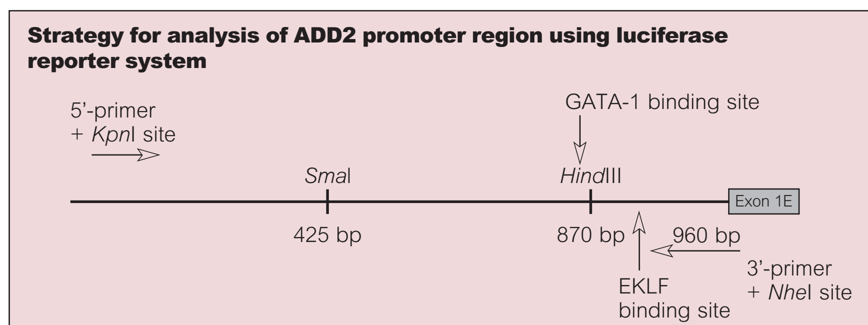


Figure 1.

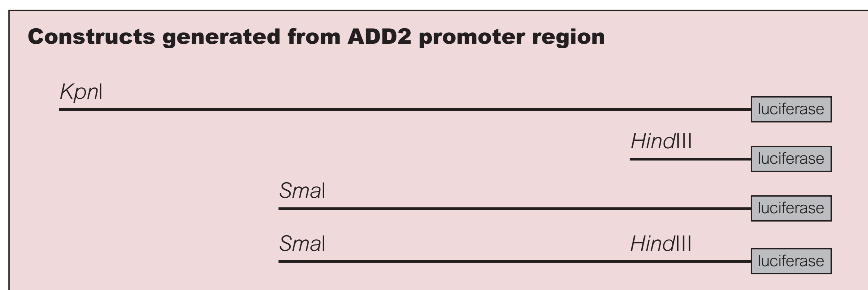


Figure 2.

Prof. Alexander Maxwell (principal applicant)
Prof. Terence Lappin (co-applicant)
(Cycle II)

Queen's University, Belfast, UK

Investigation of the role of JUNE-1 in erythropoiesis

This project is designed to fully characterise a new erythropoietin-regulated gene, *JUNE-1*. The original proposal described four main aims to be achieved over the 2-year cycle of the grant. Significant progress has been made in achieving the research objectives within the first year.

Aim 1: Examine the expression levels of the *JUNE-1* splice variant

A. The splice variant has a widespread expression pattern similar to full-length *JUNE-1* although mRNA levels of the splice variant are consistently lower than full-length *JUNE-1* (~50-60%).

B. Rat anti-mouse *JUNE-1* monoclonal antibodies have now been raised. Human *JUNE-1* was cloned into pTracer mammalian expression vector. Mouse *June* has been cloned into pEF6 mammalian expression vector. Both vector systems permit successful, reproducible mammalian expression of *JUNE* in 293T cells (assessed using V5 tag by Western blotting) to enable validation of antibody sera.

Aim 2: Determine the cellular site of localisation of *JUNE-1*

A. MEL cells, initially proposed in the application, proved difficult to transfect. Therefore we have switched to using K562 cells. The K562 model is now established with differentiation using haemin. Haemoglobin production was confirmed using orthodianisidine staining.

B. Prior to the development of specific *JUNE-1* antibodies (Aim 1, part B), we performed localisation studies using JUNE-GFP. Human *JUNE-1* and human splice variant *JUNE-1* were cloned into the Invitrogen pcDNA3.1 NT-GFP TOPO vector. This was used for mammalian studies resulting in expression of *JUNE-1* with N-terminal GFP fusion protein. Expression studies have been performed in the K562 model as well as in 293T cells. In both cell systems, human *JUNE* demonstrates nuclear localisation (counterstaining with DAPI to stain the nucleus and phalloidin red to stain for actin).

Aim 3: Determine the effect of overexpression and underexpression of *JUNE-1* (siRNA knockdown) in a cell culture model of erythroid differentiation

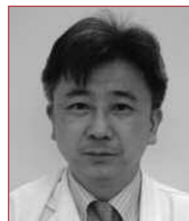
A. Overexpression studies are to be completed. Further optimisation of the transfection efficiency of the K562 cell model is in progress (using the Amaxa nucleofect system). The splice variant has been cloned into a mammalian expression vector (pcDNA3.1 V5/HIS TOPO) for overexpression analysis.
B. *JUNE-1* siRNA tested alongside appropriate scrambled control. Transfections performed using Amaxa nuclear transfection system. Q-PCR demonstrates ~60% knockdown of *JUNE-1* mRNA (protein levels to be confirmed with antibodies described in Aim 1, part B). Results demonstrated a reduction in proliferation of K562 cells following *JUNE-1* knockdown.

Aim 4: Identify proteins that interact with *JUNE-1*

A. GST pull-down assays: A system for the *in vitro* translation of *JUNE-1* has been successfully developed. *JUNE-1* was cloned into a modified pCITE vector (additional 5' FLAG and 3' HIS tags), transcribed using a Promega RiboMax kit and *in vitro* translated using rabbit reticulocyte lysate. Protein translation confirmed by Western blot analysis using anti-FLAG antibodies. Protein produced using this method can be used as bait for pull-down assays.
B. Co-Immunoprecipitation assays (IP): Preliminary IP data suggests that *JUNE-1* binds to VHL.

Further work is now in progress to fully characterise the role of *JUNE-1*, an erythropoietin-induced gene, in normal cell biology.

Dr Jun-ichi Nishimura (principal applicant)
Dr Marilyn J. Telen (co-applicant)
(Cycle II)



Duke University Medical Center, Durham, USA

Innovative drug design using RNA aptamers for various anaemias

To prevent or treat vaso-occlusion in sickle cell disease (SCD), we have targeted three important adhesion molecules, including αVβ3, P-selectin, and B-CAM, using RNA aptamers. These therapeutic strategies were presented at the Research Retreat and Review of the Duke-UNC Comprehensive Sickle Cell Center on 9 February 2006.

Integrin αVβ3

We have synthesised a high-affinity aptamer clone 17.16 (UUCAACGCUGUGAA-GGGCUUAUACGAGCGGAUUACCC) that binds to human integrin αVβ3. To measure its anti-adhesion activity, an *in vitro* flow chamber assay was adopted. We first induced enhanced expression of αVβ3 on the immortalised HUVEC cell line EC-RF24, using each of the following: TNF-α (ng/mL for 18.5 h at 37° C), thrombin (1 nM for 5 min at 37° C), or histamine (25 mM for 12 min at RT), and αVβ3 expression by treated and nontreated cells was measured by flow cytometry. The anti-adhesion activity of aptamer clone 17.16 was then confirmed using HUVEC treated with thrombin (1nM for 5 min at 37° C) using a flow chamber assay. (Figures 1, 2).

P-selectin

Aptamer clone PF377 (ACGCUCAACGAGCCAGGAACAUCGACGUCAGCAAAC GCGAGCGCAACCAGUAACACC) that binds to human P-selectin has been synthesised, and its high-affinity to P-selectin was confirmed by the binding assay. However, we found it hard to detect the enhanced expression of P-selectin on the immortalised EC-RF24 by flow cytometry under various stress conditions, including TNF-α (10nm/mL for 4 or 13.5 or 18.5 h at 37° C), thrombin (1nM or 0.1U/mL for 5 min at 37° C), or histamine (25μM for 12 min at RT), because of the short transient expression on the surface. Therefore, we are currently testing the use of IL-4 (20 ng/mL) and/or IL-13 (5ng/mL) for 48 h, followed by stimulation with histamine immediately prior to studying in a flow chamber assay. Expression of P-selectin in such cytokine-stimulated cells has been reported to be apparent at 24 h and to be

maintained for at least 48 h[1]. Simultaneously, we are generating recombinant human P-selectin[2], which will be directly coated on slides, so that we can perform continuous flow assays to test how adherent ssRBCs are to the pre-coated slides in the presence and absence of aptamer.

B-CAM

After more than 10 rounds of SELEX, we have failed to identify aptamers that specifically bind to human B-CAM. Interestingly, Dr Telen (co-applicant) recently found that epinephrine acted through erythroid signalling pathways (cAMP-dependent protein kinase A) to activate sickle erythrocyte adhesion to endothelium via ICAM-*4* (LW, CD242) αVβ3 interactions[3]. Since stress is a potential initiation factor for vaso-occlusion, epinephrine modulation of adhesion provides a powerful biological link between intraerythrocytic signalling pathways and the external milieu. Therefore, ICAM-4 has replaced B-CAM as a therapeutic target and recombinant ICAM-4 is currently being prepared for use with the SELEX methods.

Thus, we have made significant advances in spite of various difficulties. Most notably, αVβ3 aptamer clone 17.16 has reached a stage suitable for modification to enhance stability and bioavailability. In addition, *in vivo* experiments in mice will employ intravital microscopy to measure anti-adhesion activity *in vivo.*

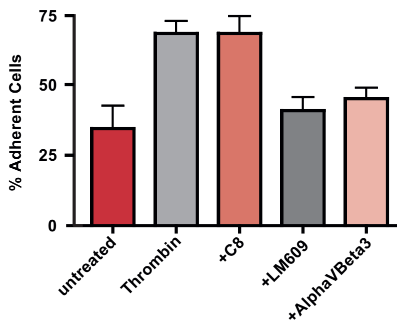


Figure 1. Anti-adhesion activity of αVβ3 binding aptamer clone 17.16. *Aptamer clone 17.16 (30nM) had anti-adhesion activity similar to LM609(an inhibitory antibody to αVβ3), whereas human complement 8 aptamer[4] (negative control, 30nM) did not have anti-adhesion activity. Error bars show SD of different experiments (≥3) measuring adhesion at a shear stress of 2 dynes/cm².*

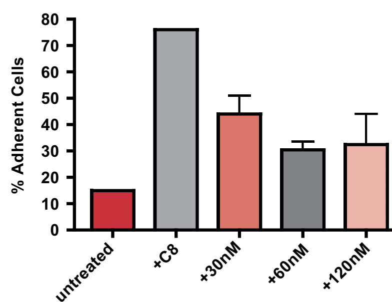


Figure 2. Enhanced anti-adhesion activity of αVβ3 binding aptamer clone 17.16 by a dose increase. *The anti-adhesion activity of aptamer clone 17.16 (30nM) was enhanced by a dose increase to 60nM and 120nM. At 2 dynes/cm², maximal inhibition of adhesion was 60%. Error bars show SD of 2 different experiments measuring adhesion at a shear stress of 2 dynes/cm².*

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Prof Radek Skoda
(Cycle II)

University Hospital, Basel, Switzerland

The role of SMAD4-dependent signalling in anaemia

We proposed to elucidate the connection between the SMAD4-dependent signalling pathway and the development of anaemia in a conditional SMAD4 knockout mouse. Our preliminary data suggested that the presence of SMAD4 is necessary for maintaining normal transferrin (Tf) serum levels. However, injection of Tf protein could not rescue the anaemia phenotype. We therefore re-examined the Tf serum levels in induced Mx-Cre x $SMAD4^{fl/fl}$ mice using an ELISA specific for mouse Tf and we found no difference in Tf levels with normal controls (healthy control: 1.88 ± 0.86, anaemia: 1.86 ± 0.16). Thus, our initial findings of low Tf could not be confirmed and are in retrospect due to the lower sensitivity of the human Tf ELISA assay for the mouse Tf protein. Despite only slightly decreased serum iron levels, we found a marked decrease of iron in the liver of SMAD4-deficient mice 5–6 weeks after induction (healthy control: 11.5 ± 3.97 nmol/g, anaemia: 2.64 ± 0.34). This suggested that iron stores were depleted and transported to the bone marrow to maintain a hyperactive erythropoiesis. Consistently, serum erythropoietin levels and reticulocytes were strongly elevated. The easiest explanation for the phenotype at this point became occult blood loss through the gastrointestinal tract. We therefore collected stool over several weeks and found by haemoccult assay that mice deficient in SMAD4 indeed displayed bleeding at various intervals after Cre-*lox*-mediated deletion (Figure 1). Histopathology revealed polyps in the stomach and colon (Figure 2). Stomach histopathology was characterised by elongated and dilated tubular structures with stromal cell expansion. In the colon, hyperplasia, dysplasia and inner surface erosion was observed. These morphological changes are likely the cause of the observed bleeding. Consistently, duodenum quantitative RT-PCR showed an increased level of iron absorption-related genes such as Dctb, DMT1 and TfR2, which might, in part, compensate for the loss of iron via internal bleeding. The hepcidin mRNA expression in the liver of Mx-Cre x $SMAD4^{fl/fl}$ mice was markedly decreased (< 5% or undetectable), but these mechanisms are unable to compensate for the blood loss and depletion of iron stores.

These data together with the results of our bone marrow transplantation assays establish that the severe anaemia observed in the SMAD4-deficient mice is caused by blood loss and the subsequent iron deficiency. Although we cannot further pursue the original hypothesis, our data demonstrate that SMAD4 is (surprisingly) not required for adult haematopoiesis.

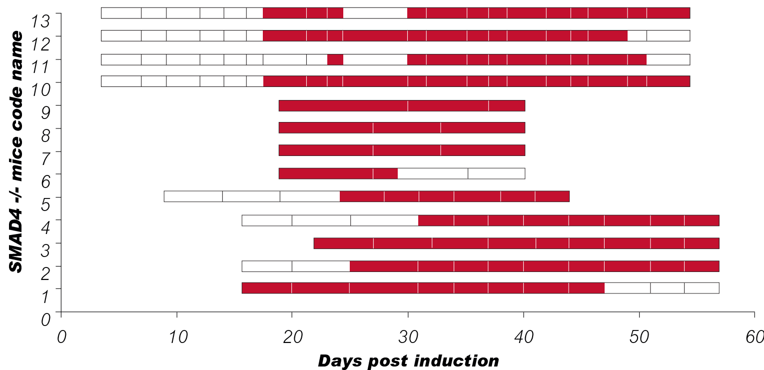


Figure 1: *Faecal occult blood test in SMAD4$^{-/-}$ anaemia mice. A total of 13 mice were analysed (x-axis). Time after first PolyIC injection in days is shown on the y-axis. Horizontal bars represent the overall duration of the stool collection. Empty box: negative haemoccult tests; filled boxes: positive haemoccult tests.*

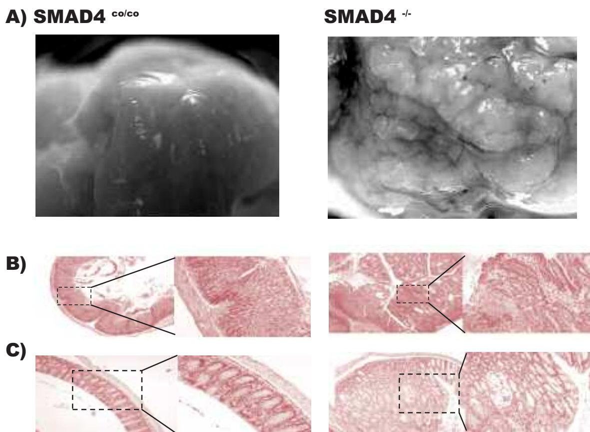


Figure 2: *Stomach and colon polyp formations in SMAD4$^{-/-}$ anaemia mice. The pictures on the left-hand side show a control SMAD4$^{co/co}$ mouse (conditional allele), and the pictures on the right side a SMAD4$^{-/-}$ anaemia mouse. Stomach (A), HE staining of stomach(B) and colon(C) and corresponding magnified area.*

Dr Christina Warnecke (principal applicant)
Prof. Kai-Uwe Eckardt (co-applicant)
(Cycle II)

University Erlangen-Nürnberg, Germany

Molecular mechanisms underlying the hypoxic induction of erythropoietin by HIF-2α

The first step was the identification of further hypoxia-inducible factor 2α (HIF-2α) target genes and the comparison of their regulation in different HIF-α-expressing cell lines. This approach was based on the observation that erythropoietin (EPO) is only weakly inducible and HIF-1α-dependent in HepG2 cells, suggesting that HIF-2α is "defective" or essential cofactors are lacking in this cell line. We used Affymetrix Gene Chip analysis combined with siRNA-mediated HIF-α knock-down in Hep3B cells, and found many novel HIF-1α and several HIF-2α targets, such as CITED2, IGFBP1, EGFR and PTPRM, which were confirmed to be HIF-2α-dependent in Hep3B cells. Some of the putative HIF-2α targets also revealed to be HIF-2α targets in HepG2 cells. However, none of them could be confirmed in HeLa cells, despite hypoxic accumulation of HIF-2α suggesting the existence of a cell-type-specific repressor of HIF-2α function. Furthermore, in Hep3B cells, HIF-2α-dependent gene regulation was affected by cell culture conditions such as the serum type used; e.g. EPO expression levels were markedly reduced after 2 weeks passaging in the presence of endotoxin-free fetal bovine serum. However, HIF-2α mRNA and protein levels were not altered by cell culture conditions indicating that not HIF-2α, but rather its co-factors or repressors are subject to modulation by stimulus-dependent and cell- type-dependent signalling pathways. None of the novel HIF-2α targets were as markedly affected by HIF-2α knock-down as EPO, which may indicate that HIF-2α only indirectly participates in gene regulation.

For the identification of factors interacting with HIF-2α, we established co-immuno-precipitation assays (IP), and are currently investigating whether NEMO[1] or c-Myc[2] bind to HIF-2α. IP are performed on the endogenous HIF-2α protein using a rabbit anti-HIF-2α antiserum, thus avoiding the use of the HA-tagged overexpressed HIF-2α. This precludes confounding effects of illegitimate protein

interactions encountered with HIF-α overexpression[3]. In parallel, siRNAs directed against the putative modulating factors were designed to investigate the effect of the knock-down on HIF-2α target gene induction.

To identify transcription factors binding to the EPO enhancer and activating EPO in a manner possibly synergistic with HIF-2α, we tested a series of EPO-enhancer mutants in luciferase reporter assays with HIF-α knock-down. Only inactivation of the HNF-binding site interfered significantly with the hypoxic transactivation of the EPO enhancer (Fig. 1A,B). The results, which were identical with two different siRNA pairs, also suggested that HIF-1α may also be involved in the transactivation of the EPO enhancer, at least in the context of a plasmid reporter construct which lacks chromatin structure. In general, the relative effect of HIF-2α knock-down was more pronounced under hypoxia than in the presence of the hypoxia-mimetic 2,2'-dipyridyl and less pronounced than the HIF-2α knock-down effect on the endogenous EPO gene. This may indicate that, in addition to the enhancer (HRE), HIF-2α regulates EPO expression both in the kidney and the liver also through sequence elements outside of the enhancer and the proximal promoter.

References

1 Bracken CP, *et al.* Activity of hypoxia-inducible factor 2α is regulated by association with the NF-κB essential modulator. *J Biol Chem* 2005; 280(14):14240
2 Gordan JD, *et al.* HIF-2α promotes cellular proliferation and growth under hypoxia by enhancing c-Myc transcriptional activity. Keystone Symposium "Hypoxia and Development, Physiology and Disease", January 16–21, 2006, Breckenridge, Colorado, U.S.A.
3 Warnecke C, *et al.* Differentiating the functional role of hypoxia-inducible factor (HIF)-1α and HIF-2α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells. *FASEB J* 2004;18:1462

Data presented at the Keystone Symposium "Hypoxia and Development, Physiology and Disease", January 16–21, 2006, Breckenridge, Colorado, U.S.A., and are submitted for publication.

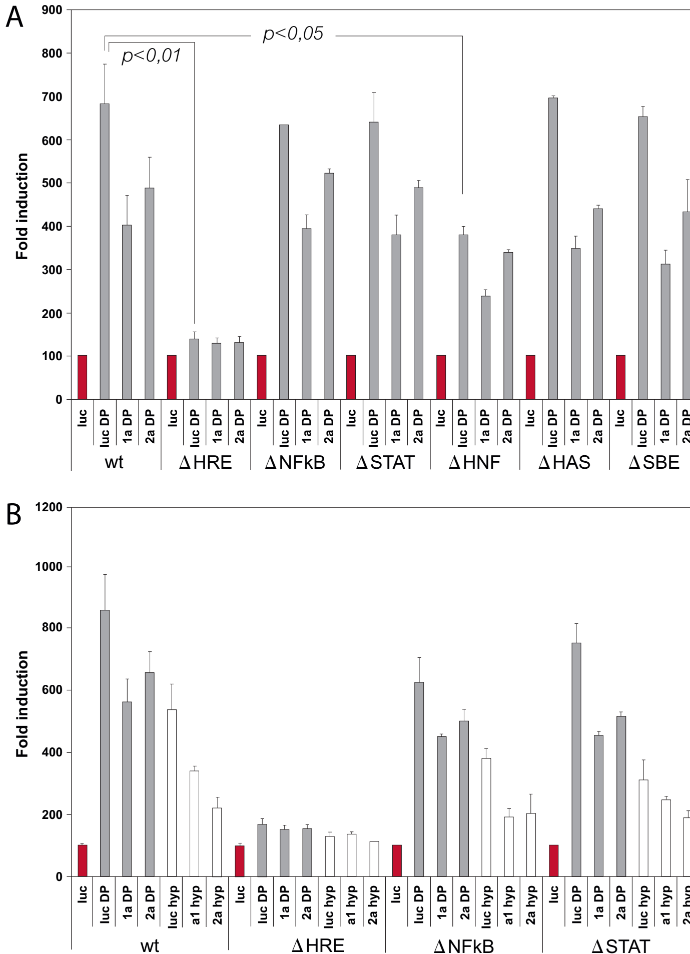


Figure 1. Luciferase activities driven by the 223-bp EPO enhancer and enhancer mutants
A. *EPO enhancer/SV40 promoter pGL2 luciferase constructs were co-transfected with HIF-1α (1a) and HIF-2α (2a) or, as a control, pGL3 luc siRNAs and stimulated with the hypoxia mimetic 2,2'-dipyridyl. Inactivation of HRE almost completely abolished this effect. In addition, inactivation of the hepatocyte nuclear-factor-(HNF)-binding site significantly reduced hypoxic induction. The inactivation of the NFkB-binding-site, the STAT-binding site, the HIF-1 ancillary sequence (HAS) and the SMAD binding element (SBE) had no significant effect.*
B. *The effect of the HIF-2α knock-down was more pronounced under hypoxia (white bars) than in the presence of DP (grey bars), black bars = unstimulated cells, wt = wild-type EPO enhancer.*

Prof. Max Gassmann
(Cycle III)

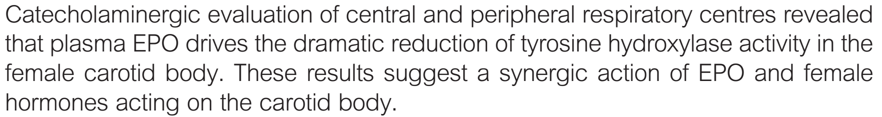
Vetsuisse, University of Zurich, Switzerland

The impact of erythropoietin on the hypoxic ventilatory response of mouse and man

Apart from enhancing red blood cell numbers, erythropoietin (EPO) has been found to exert neuroprotective functions upon injuries such as stroke. Little is known, however, on EPO's non-erythropoietic impact in a non-pathological but physiological context.

We postulated that EPO is capable of modulating the neuronal control of ventilation upon exposure to reduced oxygenation (hypoxia) - as physiologically occurring at high altitude. The response to hypoxia is a complex ability of the organism in which activation of the neural respiratory system (central and peripheral) leads to increased ventilation, and the erythropoietic response ultimately elevates the oxygen-carrying capacity.

Despite the tight physiological relationship of these systems, no functional interaction between them has been described so far. We recently demonstrated that the mouse carotid body (the main peripheral sensor for hypoxaemia) expresses the EPO receptor[1]. This suggested that apart from EPO's direct (most probably endocrine and/or paracrine) impact in the brain, circulating EPO also indirectly modulates the neural control of hypoxic ventilation. To test this notion, we measured basal ventilation and hypoxic ventilatory response in males and females from three mouse models: Tg21, a transgenic mouse line overexpressing EPO only in neural tissue; Tg6, a transgenic mouse line showing dramatic increase of both cerebral and plasma EPO levels; and wild-type mice upon intravenous injection with recombinant human EPO (rhEPO). We found that brain-derived EPO stimulation of hypoxic ventilation is restricted to severe hypoxia. However, plasma-derived EPO resulted in stimulation of normoxic and hypoxic ventilatory response in female but not in male animals.



Catecholaminergic evaluation of central and peripheral respiratory centres revealed that plasma EPO drives the dramatic reduction of tyrosine hydroxylase activity in the female carotid body. These results suggest a synergic action of EPO and female hormones acting on the carotid body.

To test whether EPO has a similar impact on humans, 13 male and 7 female volunteers were given rhEPO prior to being exposed to 10% oxygen. The results show that rhEPO significantly increased tidal volume in both men and women. However, this increase was compensated with a significant decrease of respiratory rate. We conclude that plasma EPO participates in the neural control of hypoxic ventilation in mice and men, most probably in a gender-dependent manner.

References

1. Soliz *et al., J Physiol.* 2005

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10 FAQs

Who is eligible for LOI submission?
RoFAR funds established members of academic institutions, dialysis units, and research centres. There are no age or geographical restrictions.

What kind of projects are RoFAR interested in?
RoFAR supports both clinical and basic science projects focused on anaemia related to kidney disease and oncology, effects of erythropoietin and erythropoietin-like substances as protective drugs in various organs, central resistance to erythropoietin, anaemia of chronic disease, anaemia related to congestive heart failure and stroke, biology of anaemia and outcomes. Especially, RoFAR encourages innovative research that will open new avenues of exploration in the study of anaemia, its mechanisms and outcomes.

What will I need to provide RoFAR with if my project is funded?
Funds are paid in three instalments over a maximum of 2 years and are dependent on the delivery of an interim and a final report for public use. Additionally, RoFAR must be acknowledged in publications, on posters, etc. Applicants may be asked to attend events organised by RoFAR and present their results.

Are budget indications approximate or am I committed to them?
RoFAR assigns funds to awarded projects based on provided budget details. It is not possible to renegotiate the amount after project approval. Indirect costs (institutional overheads, insurance, etc.) are the responsibility of the applicant. A maximum of 10% of the assigned funds can be used for the indirect costs.

Am I allowed to submit more than one project to RoFAR?
Applicants are allowed to hold only one grant at a time. Furthermore you may not submit more than one LOI in the same cycle. This rule holds both for main applicants and co-investigators.

www.rofar.org

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Foundation for Anemia Research

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Our Mission

The Roche Foundation for Anemia Research is a registered Medical Research Charity with the mission of "encouraging innovative research that will open new avenues of exploration in the study of anemia, its mechanisms, erythropoetic agents and outcomes." Those eligible for grants are members of academic staff in universities, dialysis centres and research institutes.

RoFAR announces a new special competition cycle

In addition to its regular research grant awards, RoFAR now invites scientists and institutions to submit applications for a special grant of up to 1.6 million CHF. RoFAR is particularly interested in innovative proposals, involving established researchers, which provide proof of principle and/or translational research, particularly studies which have the potential to be transferred into clinical practice. Letter of Intent submission will start on 30 November 2006 and end on 30 June 2007. Applications must be submitted via the RoFAR website.
For more information, please download the PDF leaflet.

How to apply for regular grants?

Letters of Intent submission for Cycle VI has closed. The deadline for submission in Cycle VII is **24 June 2007**. Submissions will be possible soon from the submission area.

RoFAR bi-annual reports available for download

Discover more on the award winners, their projects and progresses in the donwload section.

Upcoming congress activities

Visit RoFAR's exhibition booth at following congresses:

- ASH 2006, December 9-11, Orlando FL, USA (booth number 2441)
- WCN 2007, April 21-25, Rio de Janeiro, Brasil (booth number not assigned yet)

For more information and if you have any questions, please contact the RoFAR Secretariat.

Status of ongoing cycles

Cycle VI - LOI are being evaluated

Letters of Intents are being evaluated. Applicants will be notified in January 2007 and selected applicants will be invited to submit a full application within 4-5 weeks (a deadline will be provided).

What kind of assistance is RoFAR giving to awarded applicants?

The purpose of RoFAR is to provide awarded applicants with funds for the submitted project and to share outcomes with the scientific community. RoFAR will not provide any administrative assistance or scientific consultancy, nor recommend any preferential channels for the purchase of drugs or machinery necessary for the completion of the study.

Where can I find relevant information about RoFAR?

The RoFAR website (www.rofar.org) is the main information channel. There you can find important announcements, future deadlines, submission forms, charters and regulations, as well as reports on awards and on funding history. If you have any specific questions, please do not hesitate to contact the secretariat (admin@rofar.org).

11 *How to apply*

Projects are submitted electronically via our website

Projects are submitted as Letters of Intent (LOI)
Submissions twice per year (June and November)

You are asked to provide your personal details, indications about the budget, a short description of your experience and of the submitted project (latter two limited to 750 words). No figures, tables or extensive literature list can be submitted at this stage.

LOI are evaluated by a Board of Scientific Advisors
6–9 weeks

LOI are thoroughly reviewed by 3 members of the Advisory Board and judged by considering relevance to RoFAR, originality, scientific excellence and feasibility. Applicants are informed of the outcome 6–9 weeks after submission. Declined applications are not provided with any feedback from the reviewers.

Top-ranked applicants are invited to submit a full application
4–6 weeks

Based upon the Scientific Advisors' evaluation, top-ranked applicants are invited to submit a full application with an approximate 50% chance of funding. Sample forms and guidelines are available in the Download section of the RoFAR website. Usually, 4–6 weeks are given for submission. Only completed applications are accepted and the stated deadline is final.

Full applications are evaluated by a Board of Scientific Advisors
8–10 weeks

Applications are thoroughly reviewed by at least 3 Scientific Advisors and judged by considering relevance to RoFAR, originality, scientific excellence and feasibility. The Board of Trustees selects the projects to be granted based upon the evaluations made by the Scientific Advisors. Applicants are informed about the outcome 8–10 weeks after submission of the full application.

Notes

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