



RoFAR

Foundation for Anemia Research

Bi-annual report

July 2008

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

On behalf of the RoFAR Board of Trustees, it is my pleasure to announce that RoFAR has awarded an additional 1.2 million Swiss Francs to fund ground-breaking anaemia research in Germany, Israel and in the US in the first half of 2008.

Six applications received during the eighth cycle of competition have been selected for RoFAR funding of up to 200,000 CHF each, distributed over two years. The progress shown in the previously- awarded RoFAR projects reflects the expertise of the investigators and meets the high quality of research that RoFAR expects. Since 2004, RoFAR has awarded fifty-two regular grants and three special cycle grants, totalling over 12.5 million CHF or 11.43 million USD.

There are two cycles of RoFAR awards each year. Timelines for the cycles and the submission deadlines for applications – usually in June and November – are published on the Foundation's website (www.rofar.org). The first step in the application process is to submit a Letter of Intent (LOI), which 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 within six months after submission of the LOI.

To inform a broader scientific community about the funding opportunities RoFAR provides, our promotional campaign in the first half of 2008 has included

- distribution of brochures to major nephrology, oncology and cardiology centres
- media releases about the eighth regular cycle
- advertisements in major scientific journals

RoFAR hopes to make a major contribution to the scientific community through its investment in innovative anaemia-related research and in encouraging scientists to apply their expertise in expanding knowledge and understanding in this field.

RoFAR has been administering an initial donation of 16 million CHF made by F. Hoffmann-La Roche in 2004. As a sign of Roche's commitment to RoFAR, a further donation of 3 million Swiss Francs has been provided for 2008. For its

generous gift to the anaemia research community and enduring commitment to anaemia and related avenues of research, the BT and the SAB of RoFAR join in expressing their gratitude to Roche.

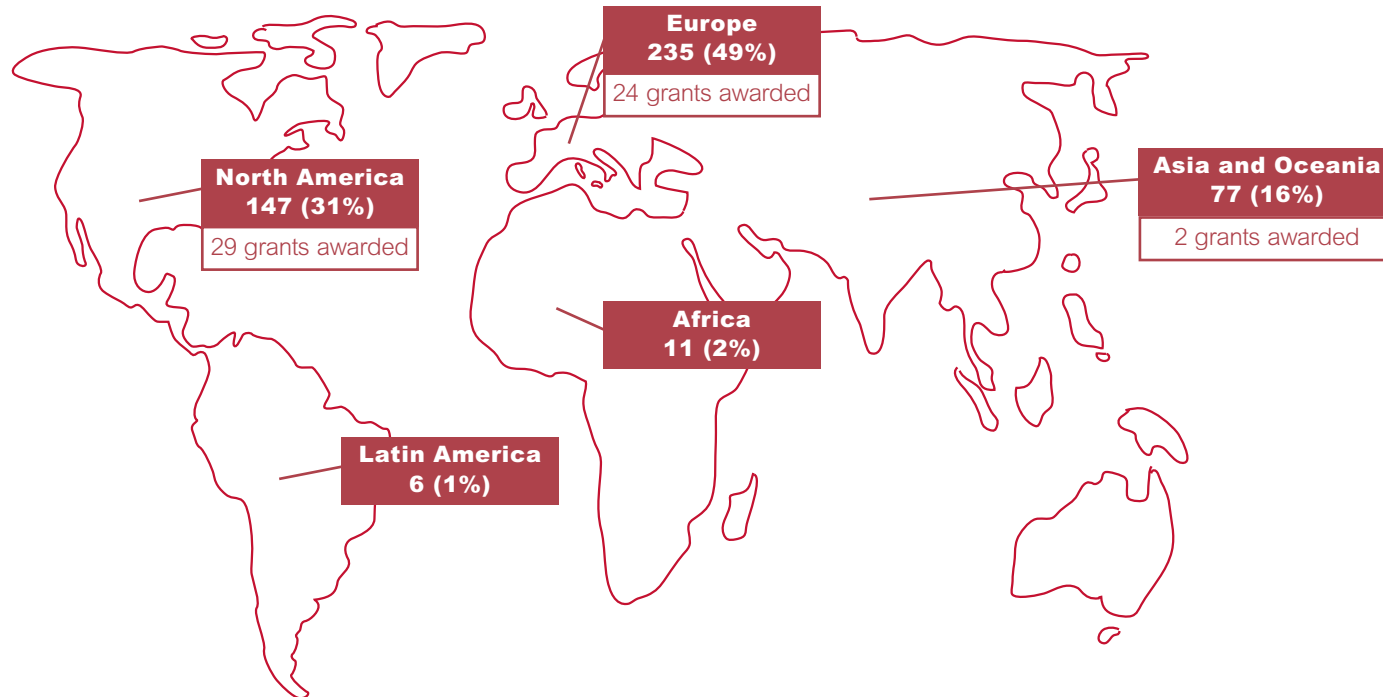
RoFAR welcomes any feedback or suggestions to assist us in accomplishing our stated mission.

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



Submitted research proposals by

Study type

Human trials	(47%)
Animal studies	(44%)
Others	(41%)

Research focus

Nephrology	(41%)
Haematology	(44%)
Oncology	(16%)
Cardiology	(12%)
Others	(22%)
(multiple allowed)	

Gender of main applicant

Males	(73%)
Females	(27%)

Institution type

Universities & related	(99%)
Others	(1%)

Applicants in the first nine regular competition cycles and two special cycles represent a range of institutions in 48 countries. Approximately half (49%) of all the LOI applications have been submitted from Europe, primarily from Germany, UK, Italy, Switzerland and Israel. Over one-third (31%) of the applications have been submitted from the United States and Canada. About 27% of the applicants are female scientists. The great majority (99%) of applicants work in universities or university-affiliated institutions. Research proposals are distributed among clinical studies (47%), animal trials (44%) and basic science projects. Submitted projects focus on nephrology and diabetology (41%), haematology (44%), oncology (16%) and cardiology (12%) with some overlap between areas.

In cycles I to VIII, twenty-four regular grants have been assigned to European applicants, twenty-seven to North American applicants and one to Oceania (Australia). Special grants have been awarded to two North American applicants and one to an applicant from Australia. Cycle IX grants have not yet been awarded.

				Grant awarded	Progress report published	Final report published
Cycle I	Nancy C. Andrews	Children's Hospital Boston, USA	Hepcidin regulation in the anaemia of chronic disease	11/2004	07/2006	07/2007
	Martin W. Bergmann	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	11/2004	12/2006	
	Andrew McKie	King's College, London, UK	Characterisation of a novel intestinal haem transporter	11/2004	07/2006	12/2006
	Marco Merlano	S. Croce General Hospital, Cuneo, Italy	In vitro analysis of tumor response to radiation in oxic and hypoxic conditions	11/2004	07/2006	07/2007
	Peter Mertens	University Hospital Aachen, Germany	Mechanisms for erythropoietin resistance in transformed and non-transformed cells	11/2004	07/2006	07/2007
	Chris D. Vulpe	University of California, Berkeley, USA	Characterisation of a family of putative mammalian haeme chaperones	11/2004	07/2006	07/2007
Cycle II	Hans Ulrich Bucher	University Hospital of Zurich, Switzerland	Erythropoietin reduces brain, eye and lung damage in very preterm infants: Proof-of-concept study	05/2005	07/2006	12/2007
	Edward Debnam	Royal Free & University College Medical School, London, UK	Is inflammation an important factor in the anaemia of chronic renal failure?	05/2005	07/2006	12/2007
	Diana Gilligan	Puget Sound Blood Center, Seattle, USA	Regulation of gene expression during erythropoiesis	05/2005	12/2006	12/2007
	Alexander Maxwell	Queen's University Belfast, UK	Investigation of the role of JUNE-1 in erythropoiesis	05/2005	12/2006	07/2007
	Jun-ichi Nishimura	Duke University Medical Center, Durham, USA	Innovative drug design using RNA aptamers for various anaemias	05/2005	12/2006	12/2007
	Radek Skoda	University Hospital Basel, Switzerland	The role of SMAD4-dependent signalling in anaemia	05/2005	12/2006	07/2007
	Carole Soussain	Oregon Health and Science University, Portland, USA	Neuroprotective effect of erythropoietin on chemo- and radiotherapy-induced toxicity	05/2005	07/2006	07/2007
	Christina Warnecke	University Erlangen-Nürnberg, Germany	Molecular mechanisms underlying the hypoxic induction of erythropoietin by HIF-2 α	05/2005	12/2006	12/2007
Cycle III	Max Gassmann	Vetsuisse, University of Zurich, Switzerland	The impact of erythropoietin on the hypoxic ventilatory response of mouse and man	11/2005	12/2006	12/2007
	Peter J. Kirkpatrick	University of Cambridge, UK	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
	Véronique Lefebvre	Cleveland Clinic Foundation, USA	Roles of Sox6 in erythropoiesis	11/2005	07/2007	07/2008
	Stephen Leib	University of Berne, Switzerland	Effect of erythropoietin on brain injury and regeneration in bacterial meningitis	11/2005	07/2007	
	Barbara Scheiber-Mojdehkar	Medical University of Vienna, Austria	Recombinant human erythropoietin: A new treatment for Friedreich's ataxia	11/2005	07/2007	07/2008
	Jürg Schifferli	University Hospital Basel, Switzerland	Erythropoietin or erythrocyte transfusion for anaemia?	11/2005	07/2007	07/2008
	Marcela Votruba	Cardiff University, UK	Erythropoietin neuroprotection in retinal neurodegeneration	11/2005	07/2007	

Overview of awarded grants

				Grant awarded	Progress report published	Final report published
Cycle IV	Christof Dame	Charité University of Berlin, Germany	Role of GATA transcription factors in regulating erythropoietin and its receptor in the heart	05/2006	12/2007	
	Ricarda Diem	University of Göttingen, Germany	Efficacy and safety of erythropoietin as an add-on therapy in subjects with acute autoimmune optic neuritis	05/2006	12/2007	
	Tomas Ganz	University of California, Los Angeles, USA	Pathogenesis of anaemia of chronic infection	05/2006	07/2007	07/2008
	Dirk Hermann	University Hospital of Zurich, Switzerland	Effects of human erythropoietin on brain plasticity and functional recovery following stroke	05/2006	07/2007	
	Stéphane Picot	Claude Bernard University of Lyon, France	Randomised trial of erythropoietin to prevent death from cerebral impairment during severe malaria	05/2006		
	Jerôme Rossert	Georges Pompidou European Hospital, Paris, France	Study of the characteristics and fate of erythropoietin-producing cells	05/2006	07/2008	
	Kai-Uwe Eckardt	University of Erlangen-Nürnberg	Study of the characteristics and fate of erythropoietin-producing cells	03/2008		
<small>Will continue project from Prof. Jérôme Rossert</small>						
Cycle V	Anne Angelillo-Scherrer	University Hospital of Lausanne, Switzerland	Role of growth arrest-specific gene 6 in anaemia of chronic disease	11/2006	07/2008	
	Margaret H. Baron	Mount Sinai School of Medicine, New York, USA	Regulation of red blood cell enucleation	11/2006	12/2007	
	Michael Bulger	University of Rochester, Rochester (New York), USA	Function of Sox6 in β -globin gene silencing and definitive erythropoiesis	11/2006	12/2007	
	Sandra Juul	University of Washington, Seattle, USA	Mechanisms of erythropoietin-mediated neuroprotection	11/2006	07/2008	
	Herbert Y. Lin	Massachusetts General Hospital, Boston, USA	Regulation of iron metabolism by soluble haemojuvelin. Fc fusion protein	11/2006	12/2007	
	Stefano Rivella	Weill Medical College of Cornell University, New York, USA	Identification of the genes responsible for the pleiotropic effects observed in β -thalassaemia	11/2006	07/2008	
Special cycle 2006	Nicoletta Eliopoulos	Lady Davis Institute for Medical Research (McGill University), Montreal, Canada	Cell and gene therapy with erythropoietin-secreting marrow stem cells for kidney repair	11/2006	07/2008	
Cycle VI	Nancy C. Andrews	Children's Hospital Boston, USA	Regulation of hepcidin expression	05/2007		
	Mark D. Fleming	Children's Hospital Boston, USA	The genetics of erythroid haem and iron metabolism	05/2007	07/2008	
	David Johnson	Princess Alexander Hospital, Brisbane, Australia	Haemoglobin levels in patients with erythropoietin-resistant anaemia treated with oxpentifylline (HERO Trial)	05/2007	07/2008	
	Zvonimir S. Katusic	Mayo Clinic, Rochester (Minnesota), USA	Role of antioxidant enzymes in vasculoprotective effect of erythropoietin	05/2007		
	Frank S. Lee	University of Pennsylvania School of Medicine, Philadelphia, USA	Prolyl hydroxylase domain protein 2, a physiologic regulator of erythropoietin	05/2007		
	Tonia S. Rex	University of Tennessee Health Science Center, Memphis, USA	Analysis of rhEPO processing in mouse tissue - implications for gene therapy of retinal degenerations	05/2007		

Overview of awarded grants

				Grant awarded	Progress report published	Final report published
Cycle VII	Clara Camaschella	University Vita-Salute San Raffaele, Milan, Italy	GLRX5 deficiency as a model of anaemia responsive to iron chelation	12/2007		
	Madeleine Carreau	Laval University, Quebec, Canada	Fanconi anaemia proteins as regulators of genes involved in haematopoietic stem cell function	12/2007		
	Wenbin Deng	University of California, Davis, Sacramento, USA	Protective effects of erythropoietin against hypoxic-ischaemic injury to developing oligodendrocytes	12/2007		
	Adam Goldfarb	University of Virginia School of Medicine, Charlottesville, USA	Iron regulation of erythropoiesis: Characterisation of a novel signalling pathway	12/2007		
	Véronique Lefebvre	Cleveland Clinic Foundation, USA	Erythropoiesis control by Sox6 and erythropoietin signalling	12/2007		
	John G. Quigley	University of Illinois at Chicago, USA	FLVCR protein trafficking and the regulation of haem export	12/2007		
	Li Zhong	University of Florida College of Medicine, Gainesville, USA	Recombinant parvovirus vectors for gene therapy of Fanconi anaemia	12/2007		
Special cycle 2007	Tomas Ganz	University of California, Los Angeles, USA	Regulation of the hepcidin-ferroportin axis in anaemia of inflammation	12/2007		
	Stephen M. Jane	Bone Marrow Research Laboratories, Parkville, Australia	Developing small molecule inhibitors of PRMT5 for treatment of thalassaemia and sickle cell disease	12/2007		
Cycle VIII	M. Amin Arnaout	Massachusetts General Hospital, Charlestown, USA	Role of the zinc finger transcription factor ZBP-89 in haematopoiesis	05/2008		
	David M. Briscoe	Children's Hospital Boston, USA	Erythropoietin and vascular endothelial cells	05/2008		
	Saghi Ghaffari	Mount Sinai School of Medicine, New York, USA	Foxo3 regulation of erythropoiesis	05/2008		
	Esther Meyron-Holtz	Technion – Israel Institute of Technology, Haifa, Israel	Macrophage's reaction to erythrophagocytosis	05/2008		
	Heike L. Pahl	University Hospital Freiburg, Germany	Role of transcription factor NF-E2 in mediating anaemia and erythrocytosis	05/2008		
	Mitchell J. Weiss	Children's Hospital of Philadelphia, USA	Regulation of erythropoiesis by microRNAs	05/2008		

Prof. M. Amin Arnaout



Massachusetts General Hospital, Charlestown, USA

Role of the zinc finger transcription factor ZBP-89 in haematopoiesis

Haematopoietic stem cells (HSCs) sustain the production of blood cells throughout the lifetime of an individual. HSCs also possess the capacity for inducing immune tolerance to engrafted solid organs (such as the kidney) when combined with HSC transplantation from the same donor. Despite the therapeutic benefits of being able to increase the number of HSCs as blood-forming and immune-modulatory cells, the key factors that control formation of HSCs during embryonic/foetal development and regulate their sustained capacity to generate all blood elements in the adult remain incompletely understood.

We have identified the DNA-binding protein ZBP-89 as master regulator of haematopoiesis during embryonic life in zebra fish, as well as in murine embryonic stem cell cultures differentiated into HSC subsets *in vitro*. In preliminary studies, we also found that newborn mice that are homozygous for a hypomorphic *ZBP-89* allele die within 1-2 days after birth with severe anaemia, reflecting a critical role for *ZBP-89* in foetal erythropoiesis. We now propose to: 1) examine the requirement for *ZBP-89* in foetal as well as adult haematopoiesis using engineered mice in which *ZBP-89* is temporally and selectively inactivated in haematopoietic tissues; 2) assess the effects of *ZBP-89* over-expression in bone marrow-derived HSCs on blood lineage commitment *in vivo* as well as *in vitro*.

The results from these studies will define the role of a previously unrecognised master regulator of blood cell development, and will likely provide novel approaches for expanding the use of bone marrow stem cells for gene transfer and bone marrow reconstitution. The additional role of *ZBP-89* as a key regulator of foetal erythropoiesis may also provide new ways of enriching the red blood cell population from less differentiated precursors to treat anaemia.

Dr David M. Briscoe



Children's Hospital Boston, USA

Erythropoietin and vascular endothelial cells

Erythropoietin (EPO), as its name suggests stimulates the proliferation and differentiation of erythroid (red blood cell) lineage progenitor cells. It is widely used as a treatment for anaemia in several chronic disease states including chronic renal disease. However, a growing body of evidence indicates that EPO has effects beyond the correction of anaemia. In addition, while it has been known for some time that vascular endothelial cells (EC) lining blood vessels express EPO receptors, only recently has the effect of EPO on EC been studied. Following inflammatory insults (where vascular EC are the targets of injury), damaged cells slough into the circulation. Replacement of EC occurs via the proliferation of neighbouring EC and/or recruitment of endothelial progenitor cells (EPCs) from the circulation. In the absence of microvascular repair, nutrient and oxygen deprivation to cells results in cellular dysfunction and ultimately in tissue death. Protection and/or repair of the vasculature have the potential to prevent cellular injury. To our knowledge, little is reported on the effects of EPO on human EC, and no study has evaluated its biology in human EPC. In this research proposal, we plan to identify the effects of EPO on human EPC and mature human EC in established *in vitro* models. We will assess the effect of EPO on angiogenic as well as protective intracellular signalling in EC, and we will determine its effect on the maintenance of vascular integrity *in vivo*. The ability of EPO to interact with vascular EC suggests that it has the potential to be a novel agent to induce microvascular protection and vascular repair before (such as in kidney organ donors) or following tissue injury (such as in acute renal injury). Also, understanding this biology is likely to give insight into the potential pro-angiogenic, pro-tumourigenic effects of EPO in humans.

4 Grant awards in Cycle VIII

Dr Saghi Ghaffari



Mount Sinai School of Medicine, New York, USA

Foxo3 regulation of erythropoiesis

In red blood cells, haemoglobin carries oxygen and is therefore essential for life. However, the process of haemoglobin formation and its interaction with oxygen are a source of production of oxygen radicals that are toxic for red blood cells. We have identified a nuclear factor (Foxo3) that detoxifies oxygen radicals in red blood cells, and through this process, extends the lifespan of these cells. Experiments described here build upon findings in our laboratory suggesting that this regulatory factor may control the rate of red blood cell formation by modulating the levels of oxygen radicals. In our research, we will use both red blood cell precursors derived from genetically modified animals and from human embryonic stem cells to investigate how Foxo3 modulates genes involved in oxygen radical detoxification. We will identify additional genes that are regulated by Foxo3 and factors that, together with Foxo3, control the rate of red blood cell formation. These factors may provide new targets for the treatment of anaemia.

Dr Esther Meyron-Holtz



Technion – Israel Institute of Technology, Haifa, Israel

Macrophage's reaction to erythrophagocytosis

Macrophages (MPs) are a heterogeneous family of phagocytosing cells which play a major role in growth and development, the immune system and systemic iron recycling in mammals. Phagocytosis of pathogens initiates a range of pro-inflammatory responses, while phagocytosis of apoptotic cells induces anti-inflammatory responses. Many factors such as receptor recognition and substances released from the phagocytosed particle determine the signal transduction and response of the MP to phagocytosis.

Senescent red blood cells (sRBCs), similar to pathogens and apoptotic cells, are recognised and phagocytosed by MPs, a process called erythrophagocytosis (EPC). EPC is a regular activity of MPs that eliminates about 3 million RBCs per second in humans. However, neither an inflammatory nor an anti-inflammatory response of MPs to physiological EPC has been elucidated.

Our research will focus on the molecular characterisation of the MP response to EPC. We hypothesise that both the MP receptors involved in the recognition of sRBCs, and the large amount of haem and its breakdown products released from decaying sRBC, may play a role in determining the MP response to EPC.

Specific aims:

1. Characterisation of cellular events following sRBC phagocytosis and breakdown.
Approach: MPs before and after EPC of aged RBCs will be analysed *in vivo*. Changes in MP activation status at different stages of haem catabolism will be characterised by analysing candidate cytokines and by microarray.
2. Identification of the receptors and additional factors, such as haem breakdown products involved in the signal transduction induced by physiological EPC.

4 Grant awards in Cycle VIII

Approach: Specific blocking of MP receptors, receptor deletions with RNAi and genetic deletions will be used to identify the key players in the MP response to EPC.

A decreased RBC lifespan and changes in RBC surface markers are reported in diabetes mellitus, haemoglobinopathies and other conditions. This implies that these pathologies may change MP activation status and immune homeostasis. Future studies will include the effect of this response on systemic iron recycling and the immune system.

Prof. Heike L. Pahl



University Hospital Freiburg, Germany

Role of transcription factor NF-E2 in mediating anaemia and erythrocytosis

The generation of red blood cells is a continuous process in the body. In a healthy person, millions of red blood cells are produced every day, just as millions are destroyed because they have aged or become used. Since red blood cells are the main cell type in blood, the number of red blood cells in the blood determines the cell/fluid balance of the blood.

It is vital that there are sufficient cells in the blood to supply oxygen, since red blood cells carry oxygen to all parts of the body. There are various conditions in which there are too few red blood cells in the blood. These are referred to collectively as “anaemia”. Symptoms of anaemia include fatigue and listlessness, as the body tries to preserve valuable oxygen, which is in short supply. In contrast, if the percentage of red blood cells is too high, the blood becomes viscous, flows more slowly and becomes more likely to form a blood clot. This dangerous condition is known as “polycythaemia” – too many cells in the blood.

Since it is so important for the body to maintain the optimal number of red blood cells in the blood, the production of red blood cells is tightly regulated and controlled. However, to date, this process is not understood at a molecular level. Due to this lack of understanding, it is also unclear what changes take place when the system malfunctions, such as in patients who develop anaemia or polycythaemia.

In this project, we will investigate the role of a single protein, called “transcription factor NF-E2” in regulating red blood cell production. We will determine its role in causing anaemia and polycythaemia in patients affected by a certain group of blood disorders, called “myeloproliferative disorders”. We hope to use our understanding to develop better therapies for these patients.

Prof. Mitchell J. Weiss**Children's Hospital of Philadelphia, USA***Regulation of erythropoiesis by microRNAs*

MicroRNAs are a newly discovered class of tiny RNAs that regulate gene expression, tissue development and carcinogenesis. We discovered a microRNA gene, termed *miR144/451*, which is important for red blood cell development. We showed that *miR144/451* is conserved in evolution and expressed at high levels specifically in mature red blood cells and their precursors. In zebra fish, loss of *miRNA144/451* function causes severe anaemia. Now, we will study the functions of *miRNA144/451* further by ablating the gene in mice and determining the consequences on blood formation. We will use these mice, along with methods to manipulate *miRNA144/451* expression in cultured cells, to better define the genetic pathways through which this important microRNA gene exerts its effects. If successful, our studies will illustrate new principles in the basic biology of red blood cell formation and function. In addition, defining the actions of *miRNA144/451* could illustrate molecular pathways that could be manipulated pharmacologically for the treatment of various anaemias.

5 Final reports of RoFAR award winners

Dr Véronique Lefebvre
(Cycle III)



Cleveland Clinic Foundation, USA

Roles of Sox6 in erythropoiesis

Summary of results

This RoFAR grant allowed us to uncover important, unique roles for Sox6 in erythropoiesis. Sox6 belongs to the family of transcription factors with an Sry-related DNA-binding domain, and like many members of its family, Sox6 controls cell fate and differentiation in specific lineages. We have shown that mouse fetuses lacking Sox6 feature a large proportion of nucleated definitive red blood cells and are anaemic despite an elevated erythropoietin level and enlarged erythropoietic tissue. Adult mice lacking Sox6 in erythroid cells look normal under physiological conditions, but exhibit compensated anaemia, with abnormal reticulocytes, and short-lived red blood cells. Many of these mice die a few days after injection of phenylhydrazine, an oxidative agent that causes acute anaemia. The mice that survive this treatment are delayed in recovering a normal haematocrit because of severely impaired erythroblast maturation.

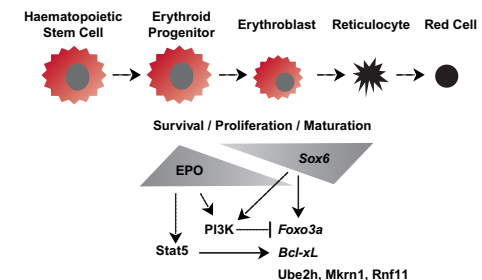
Reticulocytes are also maturation impaired, thereby causing dramatic, often lethal hypoxia. We proved that Sox6 acts cell-autonomously in erythroid cells by showing that it is highly expressed in these cells and that mice lacking Sox6 solely in these cells have the same erythroid phenotype as Sox6-null mice. Using primary erythroid cell cultures, we showed that Sox6 enhances the ability of erythropoietin signalling to stimulate early erythroid cell survival and proliferation. However, defects at these levels are not readily detected in mutant mice, likely because of compensation by erythropoietin signalling.

In addition, Sox6 has a critical, uncompensated role both *in vivo* and *in vitro* in promoting erythroblast terminal condensation and enucleation. Gene expression microarray screening with RNA from control and Sox6-null erythroid cells and tissues revealed that Sox6 is needed to silence embryonic globin genes and to enhance expression of genes for important erythroid maturation factors, such as

the anti-apoptotic factor Bcl-xL and the transcription factor Foxo3a. Our data suggest that Sox6 cooperates with the erythropoietin target Stat5 in activating the Bcl-xL gene. Sox6 is also needed to enhance expression of the genes for several components of the ubiquitination machinery, such as Ube2h, Mkrn1 and Rnf11. The *in vivo* roles of the latter genes are still unknown, but ubiquitination is known to control erythroblast remodelling into mature red cells, indicating that Sox6 acts at least in part by promoting ubiquitination.

In conclusion, our study provides compelling evidence that erythropoietin signalling and Sox6 control erythroid cell development both cooperatively and sequentially (see model). Erythropoietin signalling synergises with Sox6 to stimulate survival and proliferation of early erythroblasts. Later, downregulation of erythropoietin signalling and upregulation of Sox6 expression promotes erythroblast terminal maturation and red blood cell survival. Maintaining a proper balance between erythropoietin signalling and Sox6 activity is thus critical to produce fully functional red blood cells in a timely manner during both basal and stress erythropoiesis. We are now aiming at identifying how Sox6 controls its target genes and how its own gene expression is controlled in erythroid cells. We believe that our studies are key to better understanding anaemia diseases and designing new or improved erythropoietin-based therapies for all diseases characterised by or associated with severe anaemia.

Model. Sox6 and erythropoietin signalling control erythroid cell development both cooperatively and sequentially.



References

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2. Dumitriu B, *et al.* Generation of mice harboring a Sox6 conditional null allele. *Genesis* 2006; 44:219-224.
3. Lefebvre V, *et al.* Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. *Int. J. Biochem. Cell. Biol.* 2007; 39:2195-2214.
4. Dumitriu B, *et al.* Essential roles for Sox6 in stress erythropoiesis (in revision).

5 Final reports of RoFAR award winners

Prof. Jürg Schifferli (principal applicant)

Dr Christoph Hess (co-applicant)
(Cycle III)



University Hospital Basel, Switzerland

Erythropoietin or erythrocyte transfusion for anaemia?

The hypothesis that vesicles released by ectocytosis from erythrocytes have immunosuppressive properties was studied and demonstrated to be true, particularly for vesicles (ectosomes) released by erythrocytes stored before transfusion¹. The support of RoFAR allowed us to establish a model for the analysis of the biological activities of ectosomes on dendritic cells in general, a model that is now being tested for erythrocyte-derived ectosomes¹. As the model took longer than expected to establish, the projected work in mice has not yet begun. Firstly, an *in vitro* model had to be developed that showed similar activities for ectosomes of mice erythrocytes. It became apparent that mice erythrocytes did not release ectosomes under the same conditions that cause ectosome release from human erythrocytes. However, the model is now established and ready to be tested. The project supported by RoFAR will continue and has allowed us to enter new grounds, with much remaining to be explored.

Summaries of the two main areas of the work supported by RoFAR are provided below:

Erythrocyte ectosomes are immunosuppressive¹

Several clinical studies have suggested that blood transfusions are immunosuppressive. Although there have been reports describing immunosuppression induced by leukocytes or their fragments, the possibility that microparticles released by erythrocytes during storage are also involved was not investigated. In this manuscript, we present evidence that such microparticles (E-ecto) have all the properties of ectosomes including size, the presence of a lipid membrane, and the specific sorting of proteins. They express phosphatidylserine (PS) on their surface membrane, suggesting that E-ecto may react with and down-regulate cells of the immune system. *In vitro*, E-ecto were taken up by macrophages and significantly inhibited macrophage activation by zymosan and lipopolysaccharide

(LPS). In addition, the effects of E-ecto were not transient, lasting for at least 24 hours. In summary, E-ecto may interfere with the innate immune system / inflammatory reaction. E-ecto transfused with erythrocytes may therefore account for some of the immunosuppressive properties attributed to blood transfusions.

Polymorphonuclear neutrophil (PMN) ectosomes interfere with the maturation of dendritic cells²

PMN activation leads to the release of potent antimicrobial agents through degranulation. Simultaneously, PMNs release cell-surface derived ectosomes (PMN-Ect). PMN-Ect are rightside-out vesicles, expose PS and down-modulate monocyte/macrophage activation *in vitro*. We studied the effects of PMN-Ect on the maturation of human monocyte-derived dendritic cells (MoDCs). Intriguingly, exposing immature MoDCs to PMN-Ect modified their morphology, reduced their phagocytic activity and increased the release of transforming growth factor (TGF)- β 1. When immature MoDCs were incubated with PMN-Ect and stimulated with LPS, the maturation process was partially inhibited as evidenced by reduced expression of cell surface markers, inhibition of cytokine release and a reduced capacity to induce T cell proliferation. Taken together, these data provide evidence that PMN-Ect have the ability to modify MoDC maturation and function. PMN-Ect may therefore represent an as yet unidentified host factor that influences MoDC maturation at the site of injury, thereby possibly impacting on downstream MoDC-dependent immunity.

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Dr Barbara Scheiber-Mojdehkar

(Cycle III)



Medical University of Vienna, Austria

Recombinant human erythropoietin: A new treatment for Friedreich's ataxia

Background

Friedreich's ataxia (FRDA), the most common inherited ataxia, affects one in 50,000 people. FRDA, caused by a GAA-trinucleotide expansion in the frataxin gene, results in reduced expression of the mitochondrial protein frataxin. The exact function of frataxin is unknown, but it may be involved in mitochondrial iron homeostasis, assembly of iron-sulphur proteins and haem synthesis. Clinically, intramitochondrial iron accumulation, reduction of mitochondrial DNA, FeS cluster-containing subunits of the electron transport chain and aconitase activity have been observed.

Recently we showed that recombinant human erythropoietin (rhuEPO) increases frataxin levels in isolated lymphocytes from FRDA-patients, and cardiac and neuronal cells¹. Based on these findings we initiated a clinical trial to investigate the effect of rhuEPO in FRDA patients.

Study design

This open-label trial was performed at the Medical University Innsbruck (primary investigator Dr. Sylvia Bösch) and consisted of a proof-of-concept phase, an interim analysis and an extension phase (Figure 1).

Results

As primary outcome, frataxin-levels in isolated lymphocytes were assayed and were significantly increased in the proof-of-concept phase². Mean increase compared with individual pre-treatment frataxin levels was 27%, varying from 15 to 63%. In the follow-up phase, the frataxin levels showed a 24% overall increase ($p=0.017$).

Neurological outcome was rated before treatment and at the end of the follow-up using two ataxia-rating scales, Friedreich Ataxia Rating Scale (FARS) and Scale for the Assessment and Rating of Ataxia (SARA). FARS improved from 58.9 ± 15.4 points before treatment to 50.5 ± 16.6 points at the end of the trial ($p=0.0063$). SARA also significantly improved (20.3 ± 3.6 points before treatment versus 15.1 ± 5.4 points at the end of the trial, $p=0.0045$).

Oxidative stress parameters: 8-Hydroxydeoxyguanosine, the "gold standard" for oxidative DNA damage, was significantly decreased (mean \pm SD 22.8 ± 13 ng/mg creatinine before treatment versus 5.52 ± 1.33 ng/mg creatinine at study endpoint, $p=0.012$). In addition, peroxides were significantly reduced (mean \pm SD 137.0 ± 57.3 μ M before treatment versus 40.1 ± 36.5 μ M at study endpoint, $p=0.028$).

Quality of life (SF-36): There was a significant change in the Mental Component Score ($p=0.043$), whereas self-estimation of the Physical Component Score remained unchanged ($p=0.345$), suggesting that rhuEPO has effects on motor and cognitive functions in FRDA patients. All patients reported feeling physically stronger, less tired and having more endurance, especially during physiotherapy.

Safety and tolerability: RhuEPO was well-tolerated and blood pressure remained within normal limits in all patients.

Haematopoietic response: There was a gender-dependent increase of haemoglobin in all FRDA patients. Four out of eight patients had a haemoglobin increase beyond pre-defined values and were phlebotomised during the extension phase.

Iron metabolism: Serum ferritin levels significantly decreased (mean \pm SD 95.9 ± 69.0 μ g/L before treatment versus 14.9 ± 7.5 μ g/L at study endpoint, $p=0.017$). Transferrin levels increased significantly (mean \pm SD 265.0 ± 38.0 mg/dL before treatment versus 315.1 ± 24.4 mg/dL at study endpoint, $p=0.012$).

Conclusion

Treatment with rhuEPO resulted in a stable increase in frataxin levels in FRDA patients. Two different ataxia-rating scales revealed a significant neurological improvement. Two markers of oxidative stress were significantly reduced. In parallel with haematopoietic changes, we observed changes in iron metabolism. Whether iron relocation from areas of accumulation is one of rhuEPO's effects in

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FRDA remains to be established. Future studies to define human pharmacokinetics and dose-response characteristics of rhuEPO are highly warranted and clinical efficacy should be tested in a placebo-controlled trial.

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Prof. Tomas Ganz

(Cycle IV)



Study design

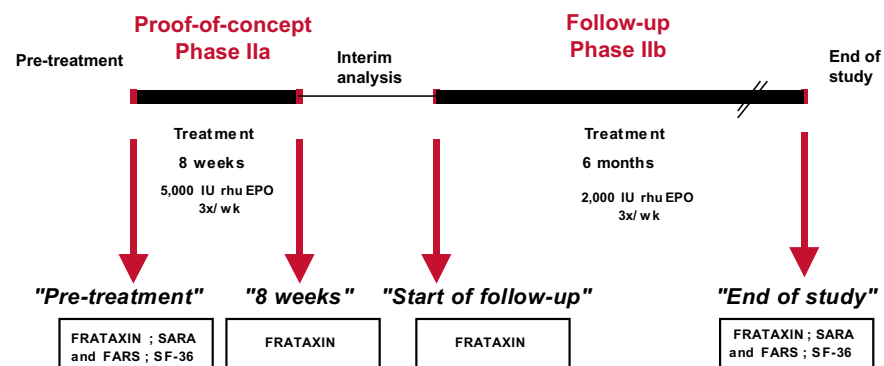


Figure 1. Study design

University of California, Los Angeles, USA

Pathogenesis of anaemia of chronic infection

We have examined the molecular pathogenesis of anaemia of inflammation (AI), with these specific aims:

- 1: Develop and characterise a simple non-surgical model of anaemia of chronic infection**
- 2: Determine the role of hepcidin in anaemia of chronic infection**
- 3: Characterise the role of IL-6 in anaemia of chronic infection**

Successful completion of these aims represented a significant advance in the study of AI.

Specific Aim 1: We have developed two models of sterile, inflammatory peritonitis that are similar to mycobacterial and brucella peritonitis. In the mycobacterial model, mycobacterial cell wall emulsified in oil (complete Freund's adjuvant, CFA) was injected into the peritoneal cavity. Within two weeks there was a trend towards anaemia and by the three weeks 92% of mice experienced a fall in haemoglobin (average decrease in haemoglobin of 25.1 g/L for CFA-treated mice compared with increase of 4.8 g/L for diluent-treated, $p < 0.001$). Consistent with anaemia of chronic infection, these mice had evidence of iron restriction (mean corpuscular volume [MCV] 45 compared with 49 in control mice, $p < 0.001$) despite preserved iron stores, as measured by liver and splenic iron content. The mice also had evidence of a hepatic acute phase response, as measured by expression of serum amyloid A1 (SAA-1) and fibrinogen- γ .

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The brucella model is not yet as thoroughly evaluated as the CFA model. In this model *Brucella abortus* antigen (BA) was injected intraperitoneally into mice. These mice developed anaemia more quickly than the CFA-treated mice, but at three weeks they remained anaemic. At three weeks, 88% of all mice experienced a fall in haemoglobin (average decrease in haemoglobin of 44.1 g/L for BA-treated mice compared with increase of 0.6 g/L for diluent-treated, $p < 0.001$). These mice also had iron restriction (MCV 42 compared with 49 in control mice, $p < 0.001$) with preserved iron stores. In addition, these mice had evidence of a hepatic acute phase response.

Significance: These two models of AI are the first simple murine models of this condition (previous models required surgery, e.g. cecal ligation and puncture or were unreliable, e.g. turpentine injections).

Specific Aim 2: We used hepcidin-deficient (knockout, HepKO) mice to determine the contribution of hepcidin to the development of AI in the CFA model described above. HepKO mice were polycythemic at baseline and their final haemoglobin at 3 weeks after CFA was significantly higher than that of wild type (WT) mice (146 vs. 120 g/L, $p = 0.01$). Although the difference in the mean haemoglobin fall in HepKO mice compared with WT mice (11.8 g/dL vs. 25.1 g/dL, respectively, $p = 0.2$) did not reach significance, fewer HepKO mice developed anaemia compared with WT (50% vs. 92%). Unlike WT mice, HepKO mice treated with CFA did not develop microcytosis, indicating that iron restriction did not contribute to the small fall in haemoglobin seen in these mice. In summary, hepcidin is essential for the development of anaemia in the CFA model but a small fall in haemoglobin occurs in HepKO mice treated with CFA by an unknown alternative mechanism not involving iron restriction. Future studies are planned to elucidate this mechanism. We will also explore whether the baseline polycythemia in HepKO mice can be avoided by low-iron diet and whether these mice will still be resistant to CFA-induced anaemia.

Significance: These are the first data describing the contribution of hepcidin to the development of AI.

Specific Aim 3: To determine the importance of IL-6 in anemia of chronic infections, we utilised interleukin (IL)-6 deficient (IL-6 KO) mice and the CFA model described above. IL-6 KO mice were not protected from anaemia in the CFA model and, in fact, had worse anaemia than WT mice (82.6 vs. 102.7 g/L, respectively, $p = 0.01$; diluent-treated IL-6 KO and WT mice were equivalent). However, the pattern of the anaemia was distinctly different in IL-6 KO mice, as

they did not develop evidence of iron-restricted anaemia (MCV 45 vs. 43 in CFA- and diluent-treated mice, respectively, $p = 0.1$) and hepcidin levels were not increased. There was no significant increase in hepatic expression of SAA-1 or fibrinogen- γ in IL-6 KO mice, indicating a less intense hepatic acute phase response. IL-6 plays an important role in regulating hepcidin and therefore contributes to iron restriction in anaemia of chronic infections. We interpret our results to indicate that IL-6 also plays another role in either supporting erythropoiesis or prolonging erythrocyte lifespan, since IL-6 KO mice develop more severe anaemia in response to inflammation. Further studies are underway to study the specific mechanism of anaemia in IL-6 KO mice treated with CFA.

Significance: This was the first study exploring the role of IL-6 in the development of AI. Total and life-long deficiency of IL-6 in IL-6 KO mice appears to have complex effects on the development of CFA-induced anaemia, on one hand negating the iron-restriction component, but on the other hand causing worse anaemia by an alternative mechanism. We are exploring a different anti-IL-6 intervention in which specific antagonists of murine IL-6 or IL-6 receptor would be used to suppress the effect of IL-6 acutely.

Assessment: The goals of the project have been met and led to significant achievements. Publications are in preparation.

Partial results have been presented at the following scientific meetings:

1. 73rd Annual Scientific Assembly of the American College of Chest Physicians (CHEST 2007), 20-25 October 2007 in Chicago, Illinois, USA
2. 49th Annual Meeting of the American Society of Hematology (ASH), 8-11 December 2007 in Atlanta, Georgia, USA
3. Experimental Biology 2008, 5-9 April 2008 in San Diego, California, USA

6 Progress reports of RoFAR award winners

Prof. Jérôme Rossert (principal applicant)

Dr Patrick Bruneval (co-applicant)

Dr Marc Froissart (co-applicant)

Dr Jean-Paul Duong-Van Huyen (co-applicant)

Dr Patrick Mayeux (co-applicant)

(Cycle IV)



Georges Pompidou European Hospital, Paris, France

Study of the characteristics and fate of erythropoietin-producing cells

The initial aim of our research project was to generate knock-in mice expressing the gene encoding the enhanced green fluorescent protein (eGFP) or the Cre recombinase gene, under the control of the very same regulatory elements than the erythropoietin gene. We believe that these mice will represent valuable tools (1) to precisely characterise the renal cells that produce erythropoietin, and study their fate during the course of CKD, (2) to study the fate of liver erythropoietin-producing cells after birth, and (3) to characterise the non-renal cells that produce erythropoietin.

In order to produce the above-mentioned knock-in mice, the first step was to generate constructs suitable for injection into ES cells. The two constructs have been successfully generated using an IRES-Cre-Hygro-FRT vector kindly provided by Dr. Weil (Pasteur Institute, Paris). However, the initial IRES cDNA has been replaced by an IRES-SUR cDNA, which has been shown to be effective in erythropoietin-producing cells. In addition, the Cre cDNA that was present in the initial vector has been replaced by an eGFP cassette (generated by PCR and fully sequenced) to produce the eGFP knock-in construct. Both homologous arms have been generated by PCR using SV129 genomic DNA, and fully sequenced. The size of the 5' arm is 5.6 kb, and of the 3' arm is 3.4 kb. Figure 1 provides a schematic representation of both constructs.

While first nucleofection of the Cre construct did not produce positive clones, a second nucleofection experiment allowed us to select five positive clones. One of them has been shown to have integrated a single copy of the construct, without unexpected 3' or 5' recombination. This clone has been used to produce chimeric mice. Once sexually mature, these mice will be used to generate heterozygous knock-in mice, and then homozygous ones.

Regarding the eGFP construct, nucleofection of this construct has led to the identification of eight positive clones. Southern blot analyses are being performed for these clones. Once a positive clone will have been selected, the corresponding ES cells will be used for generation of chimeric mice.

In conclusion, we have successfully generated the two constructs needed for production of knock-in mice. These constructs have been used to generate embryonic stem (ES) cells harbouring either the Cre cDNA or the eGFP cDNA within the 3' untranslated region of the erythropoietin gene. A clone of ES cells in which the Cre cDNA has been inserted into the erythropoietin gene has been used to generate chimeric knock-in mice, while a clone of ES cells in which the eGFP cDNA has been inserted into the erythropoietin gene is being selected for injection.

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Dr Anne Angelillo-Scherrer

(Cycle V)



University Hospital of Lausanne, Switzerland

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

The protein product of gene 6 (*Gas6*) is important for cell survival across cell types. In response to erythropoietin (EPO), murine erythroblasts release *Gas6* and this enhances EPO receptor signalling in these cells. In the absence of *GAS6*, erythroid progenitors and erythroblasts are hyporesponsive to the survival activity of EPO and fail to restore haematocrit levels in response to anaemia. In addition, *Gas6* may influence erythropoiesis via paracrine erythroblast-independent mechanisms involving macrophages. *Gas6*, alone or in combination with EPO, increases haematocrit levels in murine models of acute and chronic anaemia¹.

We pursued our investigations on the role of *Gas6* pathway in erythropoiesis and anaemia:

Physiological clearance of expelled nuclei from erythroblasts by bone marrow macrophages¹

Late in erythropoiesis, nuclei are expelled from erythroblasts and engulfed by macrophages located in the blood island. Expelled nuclei expose phosphatidylserine (PS) on their surface, which is used as an “eat me” signal for their engulfment by macrophages. The PS opsonins milk-fat-globule EGF8 (MFG-E8) and *Gas6*, together with their respective receptors $\alpha v \beta 5$ (and $\alpha v \beta 3$) and *Axl/Mertk/Tyro3*, are involved in the phagocytosis of apoptotic cells. As foetal liver and bone marrow macrophages do not express MFG-E8, the *Gas6-Mertk* pathway might constitute the main pathway for the engulfment of nuclei expelled from erythroblasts. To test this hypothesis, we isolated nuclei from late-stage erythroblasts from the spleens of phlebotomised mice, and tested the capacity of bone marrow-derived macrophages (BMDMs) from mice deficient either in *Gas6* (*Gas6*^{-/-}), *Axl* (*Axl*^{-/-}), *Mertk* (*Mertk*^{kd}) or *Tyro3* (*Tyro3*^{-/-}) to internalise these nuclei. Phagocytosis was determined by counting the number of BMDMs with ingested

nuclei and the phagocytosis index, which revealed the number of engulfed nuclei per macrophage. We found that *Gas6*^{-/-} BMDMs cleared 30% fewer nuclei than wild-type (WT) BMDMs. We observed a slight decrease of internalisation capacity for *Axl*^{-/-} BMDMs, whereas *Tyro3*^{-/-} BMDMs engulfed the nuclei as efficiently as WT BMDMs. In contrast, *Mertk* deficiency almost abolished nuclei phagocytosis. When *Axl/Tyro3*^{-/-} and *Axl*^{-/-}/*Mertk*^{kd} BMDMs were compared with WT BMDMs and single knockouts, there were no cumulative effects when compared with single knockouts. *Mertk* was therefore critical for the phagocytosis of nuclei from erythroblasts, whereas the role of *Axl* and *Tyro3* appeared to be negligible. In conclusion, we have shown that *Gas6* and its *Mertk* receptor were involved in late erythropoiesis when nuclei are expelled from the erythroblasts and engulfed by BMDMs in the blood island. Indeed, *Gas6* binding to nuclei exposing PS on their surface might form a bridge between PS and the *Mertk* receptor on BMDMs, allowing their efficient clearance.

The *in vivo* localisation of unphagocytosed nuclei in *Mertk*^{kd} mice is currently being studied. In addition, the signalling of BMDMs during nuclei phagocytosis is under investigation.

Iron metabolism

Bone marrow iron stores were comparable in WT and *Gas6*^{-/-} mice, but were reduced in the liver, spleen and duodenum of *Gas6*^{-/-} and *Mertk*^{kd} mice when compared with WT. Iron stores in BMDMs were comparable in *Gas6*^{-/-}, *Mertk*^{kd} and WT BMDMs at steady state. However, in response to endotoxin, there was a greater increase in iron stores in *Gas6*^{-/-} and *Mertk*^{kd} BMDMs than in WT BMDMs. These data suggest that the absence of *Gas6* or *Mertk* is responsible for abnormal iron metabolism in mice. Investigations on iron metabolism are currently being performed in mice with deficiency in other *Gas6* receptors or with combined deficiency in *Gas6* receptors. In addition, iron metabolism will be investigated at the cellular and molecular level in all genotypes.

Parts of this work have been presented at the following scientific meeting

49th Annual meeting of the American Society of Hematology (ASH), 8-11 December 2007 in Atlanta, USA.

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6 Progress reports of RoFAR award winners

Dr Sandra Juul¹ (principal applicant)

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(Cycle V)



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Mechanisms of erythropoietin-mediated neuroprotection

Hypothesis: High-dose erythropoietin (EPO) will decrease neonatal hypoxic-ischemic brain injury and will produce gender-specific epigenetic modifications.

Specific Aims: To determine the gender-specific short- and long-term epigenetic modifications associated with high-dose EPO treatment (5000 U/kg x 3 doses) following neonatal hypoxic-ischemic brain injury.

The purpose of these experiments is to improve understanding of the molecular mechanisms by which EPO provides neuroprotection following neonatal brain injury.

We initially planned to use BALB-c mice for all experiments. However, we noted wide variation in control injury scores after hypoxia-ischemia (HI), possibly due to known variability in BALB-c cerebrovascular organisation. We therefore switched to C57/black 6 mice to see if we could establish more uniform injury. In addition, we extended the duration of our hypoxia episodes by 5 minutes each in an effort to increase brain injury without increasing mortality.

For our new protocol, P10 C57/black 6 mice undergo right common carotid artery ligation by cauterization, recover from anesthesia for 30 minutes, and are then returned to their dams for 1 hour. The mice are next placed in an incubated chamber (36°C) and exposed to 3 alternating episodes of hypoxia (8% oxygen for 20 minutes) and hyperoxia (100% oxygen for 5 minutes). Although mortality is less than 10%, there is still considerable variability in gross injury scores. C57/black 6 mice have the same average life span as BALB-c mice (approximately 30 months). In our protocol, animals that undergo neonatal brain injury at P7 are allowed to survive for 60 days. At that time, animals are euthanized

for further epigenetic studies. We have not found that animals die spontaneously before scheduled death. Importantly, we found that gross scoring of the degree of brain injury by inspecting the whole brain surface underestimates the brain injury present. This is because injury may include sub-cortical regions even when no cortical injury is evident on the surface. Thus for an accurate assessment of injury we have adopted a new scoring system that takes into account the cortex, hippocampus, white matter, and basal ganglia.^{1,2,3}

It is increasingly common in the literature to do a more extensive assessment of the brain using a numeric scale that includes a variety of regions. In the case of unilateral brain injury, not only are the slides blinded to treatment group, but it is possible to compare the uninjured side to the injured side. Thus an internal control is always present. The system we have implemented for evaluating neuropathologic injury is modified from previously described techniques. Two investigators blinded to experimental protocol evaluate the injury in cresyl violet-stained sections from four brain regions (cortex, hippocampus, striatum, thalamus). Brain injury is scored 0–4 using standardised photos of brain injury for comparison (0: no injury, 1: few small isolated groups of injured cells, 2: several larger groups of injured cells, 3: moderate confluent infarction, or 4: extensive confluent infarction). This process is repeated with GFAP-stained slides to detect extent of gliosis. The total score is summed for each stain. The average of two investigator's scores is used for the statistical analysis.

We have now collected $n=86$ C57/black 6 mice randomised to 5 groups and sacrificed at 48 hours for epigenetic analysis:

1. Control
2. Sham + placebo (Sham V)
3. Sham + EPO (Sham E)
4. HI/H + placebo (HI V)
5. HI/H + EPO (HI E)

Treatment Group	F	M	Grand Total
HI E	11	9	20
HI V	17	12	29
Sham E	9	10	19
Sham V	9	9	18
Grand Total	46	40	86

Brains were divided by hemisphere, snap frozen, and sent to the University of Utah.

Analysis of histone methylation and acetylation is ongoing.

We have also started to produce animals in each treatment group that will be studied long-term. Thus far we have 4 litters of animals that are aging for further study.

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Using the C57/Black 6 mice, we continue to see variability in the degree of brain injury both within a single experiment and between experiments, despite controlling experimental variables as much as possible, particularly temperature. In reading the literature and speaking with other researchers in this field, this variability in response to injury is a common feature of all models of unilateral brain injury in neonatal animals. Such variability occurs in the P7 rat model as well as in various strains of mice. Indeed, this is also a feature of human response to brain injury.

Our vehicle-treated animals have gross injury scores ranging from 0 to 4, with a mean score of 2.4, and median score of 2. The brains sent for epigenetic studies were separated by hemisphere, frozen, and sent for further analysis. We therefore cannot comment on the internal injury scores in this group.

We will be able to complete the study as planned because we have appropriate control groups. Thus the epigenetic effects of EPO treatment in sham-treated animals and brain-injured animals will be determined by comparing these animals to sham-vehicle and brain-injured-vehicle treated animals. Analysis is underway.

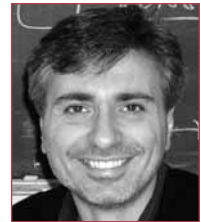
In other studies in our lab, we have been using the more detailed method of scoring brain injury, and have been able to define specific areas of response to injury that were not apparent by scoring gross injury alone. We scored two separate areas of cortex, the internal capsule, hippocampus, and thalamus separately with scores of 0-4. This allowed us to identify a higher susceptibility to injury following neonatal stress of the internal capsule.

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Dr Stefano Rivella

(Cycle V)



Weill Medical College of Cornell University, New York, USA

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

In β -thalassaemia, the mechanism driving ineffective erythropoiesis (IE) is insufficiently understood. Our objective is to characterise genes that are upregulated in thalassaemic erythroid cells that have a role in IE and other pathophysiological effects observed in this disorder. As a first step, we analysed mice affected by β -thalassaemia intermedia (*th3/+*) and major (*th3/th3*). Using these models, we have previously shown that the severity of anaemia (haemoglobin [Hb] as low as 1 g/dL) inversely correlates with the total number of nucleated erythroid cells (100-fold compared with wild-type [WT] mice). However, the percentage of apoptotic cells and the level of haemolytic markers (e.g. bilirubin and lactic acid dehydrogenase), slightly increased or were not different compared to WT mice. While not excluding a role for apoptosis, our observations suggest that control of the cell cycle and maturation of erythroid precursors play an important role in IE.

We sought to determine whether IE could also be characterised by limited erythroid cell differentiation. In thalassaemic mice, we observed that a greater than normal percentage of erythroid cells was in S-phase, exhibiting an erythroblastic-like morphology. Binding of EPO to its receptor (EPO-R), activates anti-apoptotic and cell cycle promoting genes, through the activation of Jak2 and Stat5. Analysis of thalassaemic erythroid cells indicated that EPO-R, Stat5, the anti-apoptotic protein BclXL, and proteins that promote the cell cycle (e.g. CycA and Cdk2) are upregulated compared with control cells. Furthermore, a larger number of thalassaemic cells exhibited Jak2-phosphorylation. Freshly purified thalassaemic erythroid cells proliferate faster *in vitro* than normal cells, a phenomenon blocked by AG490, a Jak2 inhibitor, which suggests a potential

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physiological role for Jak2 in IE and splenomegaly. The use of Jak2 inhibitors might prevent splenomegaly and limit IE-related damage due to excessive iron absorption, including secondary osteoporosis.

Significantly, we have been able to reproduce results from our animal studies in humans when comparing normal and thalassaemic blood and spleen specimens. We analysed erythroid cells from the peripheral blood of normal and thalassaemic subjects using the two-phase liquid culture¹. While β -globin mRNA and Hb production were reduced in thalassaemic patients as expected, the levels of cell cycle related mRNAs such as *Jak2*, *Ki-67*, *CycA*, *Bcl-XL*, and *EPO-R* were significantly higher than normal. In addition, spleen sections from control and thalassaemic patients undergoing splenectomy were stained with Ki-67 and the erythroid glycoprotein C and α -spectrin antibodies to qualitatively evaluate the number of replicating erythroid cells (Ki-67+). Our data indicated that thalassaemic splenic tissue has more red pulp and a greater number of Ki-67+ erythroid cells than that of a normal patient (splenic rupture). With this observation, we have demonstrated, for the first time that a considerable number of erythroid cells in an enlarged thalassaemic spleen were actively proliferating despite the patient being transfused.

In conclusion, we propose that IE in β -thalassaemia is likely to be the result of altered cell proliferation and impaired cell differentiation, which in turn limit apoptosis, thereby mimicking tumour-like behaviour.

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Dr Nicoletta Eliopoulos¹(principal applicant)

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(Special Cycle 2006)



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

Acute renal failure (ARF) is a common and serious disease caused by toxic and/or ischemic insult, such as from chemotherapy. Cell therapy may substantially improve prognosis, as studies in rodent models of ARF have revealed kidney-protective and -reparative actions of bone marrow-derived mesenchymal stromal cells (MSCs). In order to observe the renoprotective effects of MSCs in mice with ARF, we prepared polyclonal populations of MSCs from male donor mice (C57Bl/6 strain) to allow traceability of these cells by the Y chromosome following their implantation in female mice. We tested these cells *in vitro* by flow cytometry analysis and confirmed the phenotype characteristic of MSCs. More specifically, these MSCs were CD31⁻, CD34⁻, CD44⁺, CD45⁻, CD105⁺, MHC I⁺, and MHC II⁻. We then evaluated the effect of these male donor-derived MSC preparations in female recipient mice with cisplatin-induced ARF. After obtaining promising preliminary results in a syngeneic model, we investigated allogeneic MSCs for kidney protection/repair because of the potentially enhanced interest in these cells if effective in ARF as universal donors.

We have previously reported that MSCs are immune rejected in MHC mismatched recipient mice, but we believe this occurrence may not interfere with a beneficial action of MSCs when an immediate and not chronic effect is sought, such as in ARF. We obtained very promising results. Briefly, we tested the intravenous implantation of C57Bl/6-derived MSCs (mixed in RPMI media) in MHC-mismatched female Balb/c recipient mice and noted that these cells significantly protected mice from cisplatin-induced toxicity compared with mice injected with RPMI only, as seen by significantly lower plasma urea nitrogen levels and mortality (Figures 1 and 2).

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Other than the beneficial action of MSCs, several studies have also shown the renoprotective effects of erythropoietin (EPO) administration in experimental rodent models of ARF. Coupling the kidney protective/reparative effects of MSCs with those of EPO through the use of MSCs gene-enhanced to secrete EPO may, therefore, produce a greater and more significant therapeutic effect in ARF-induced kidney damage. In order to test our hypothesis, we generated several populations of murine MSCs expressing the cDNA for murine EPO (EPO-MSCs) by retroviral-mediated gene-transfer. We measured the *in vitro* secretion of EPO by EPO-MSCs using an ELISA specific for mouse EPO, and determined that our preparations of EPO-MSCs secrete up to 49 units of EPO per 10^6 cells per 24 hours. We likewise produced control vector (i.e. not expressing EPO) gene-modified MSCs. We have begun testing these genetically engineered MSCs in mice with cisplatin-induced ARF in order to evaluate and compare their effects on the kidney.

Our findings on the renoprotective action of allogeneic mouse MSCs were submitted as an abstract and will be presented as a poster at the annual meeting of the International Society for Cellular Therapy in May 2008.

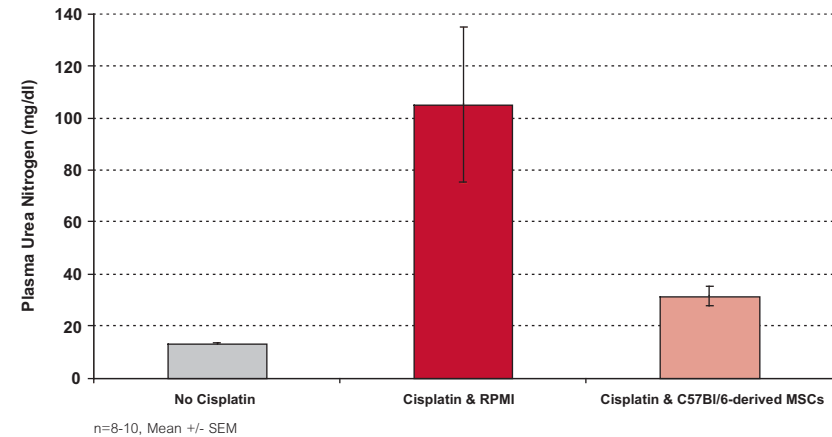
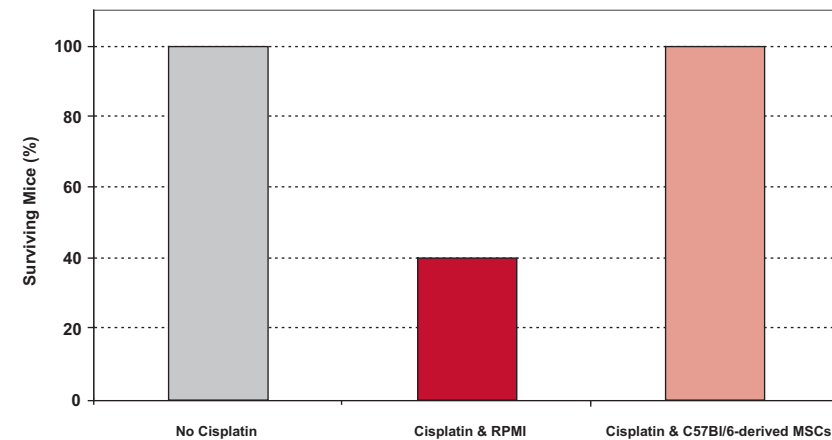


Figure 1. Plasma urea nitrogen levels in Balb/c mice 4 days post administration of cisplatin and next day intravenous injection of MHC-mismatched MSCs



Figures 2. Percentage of Balb/c mice alive at 11 days post administration of cisplatin and next day intravenous injection of MHC-mismatched MSCs

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Dr Mark D. Fleming
(Cycle VI)



Children's Hospital Boston, USA

The genetics of erythroid haem and iron metabolism

The proposal evolved from the phenotypic characterisation and positional cloning of an ENU-induced mouse hypochromic, microcytic anaemia mutation called *hem6*. We had already determined that the pathophysiology of the anaemia was likely due at least in part to alterations in haem biosynthesis and that that mutation gene was *Hem6* - one of a family of four genes encoding proteins of previously unknown function, having no homology to any proteins other than themselves. The original specific aims of the grant were the following:

Specific Aims:

Aim 1. To utilise RNAi technology to discover the phenotypic, functional, and gene expression consequences of loss of the *Hem6* gene *in vitro*.

- 1a. Evaluate the phenotypic and functional effects of RNAi-mediated knock-down of *Hem6* in erythroid cells.
- 1b. Determine the effect of RNAi-mediated knock-down of *Hem6* on gene expression *in vitro* in erythroid cells.

Aim 2. To identify proteins which interact with HEM6.

- 2a. Explore the possibility that HEM6 interacts in a complex with nuclear and/or cytoplasmic components.
- 2b. Use co-immunoprecipitation combined with proteomic technology to identify factors that interact with HEM6.

At the time the proposal was submitted, we had narrowed the haem biosynthetic defect to early in the pathway, before the assembly of the porphyrin ring. Since addition of δ -aminolevulinic acid (ALA), the first intermediate in the pathway, could partially correct the haem biosynthetic defect in *hem6* reticulocytes, we

hypothesised that the expression of the enzyme responsible for the synthesis of ALA, erythroid aminolevulinic acid synthase (ALAS2), was defective. Indeed enzymatic assays showed that ALAS activity was decreased and quantitative RT-PCR demonstrated that the *Alas2* mRNA was likewise altered. Together with our previous phenotypic and mapping data, this result will be published in a manuscript entitled "*hem6*: an ENU-induced recessive hypochromic microcytic anaemia mutation in the mouse", already accepted in the journal *Blood*.

With regard to aim 1, we successfully performed mRNA profiling experiments in mouse erythroleukemia (MEL) cells treated with *Hem6*-specific siRNAs and developed a gene expression signature of the knocked-down cells. However, based upon results we obtained in aim 2, it became clear that the ideal cells for performing expression profiling were reticulocytes from wild type and *hem6* animals; we have proceeded with expression profiling accordingly (see below).

Aim 2 focused on identifying the pathway in which HEM6 functions through cloning interacting proteins. Our primary approach was to be co-immunoprecipitation followed by mass spectroscopy; however, it soon became clear that our antibody against the native HEM6 protein was not sufficiently specific to perform these assays and that over-expression of an epitope-tagged protein resulted in mislocalisation (or possibly aggregation) and often killed the cells. We have begun to make additional polyclonal antibodies against HEM6 in order to circumvent this problem.

Nonetheless, we succeed in identifying several interacting partners by using our secondary strategy, a yeast two-hybrid screen using HEM6 as bait. In this screen, we identified several proteins, including the poly-adenylation binding protein PABP, which we isolated four times as two independent clones. Since PABP has been reported to stabilise globin mRNAs through maintenance of the poly-A tail length, we chose to follow up on this protein first.

We first demonstrated using co-immunoprecipitation that HEM6 does indeed interact with PABP and that in our yeast two-hybrid assay the *hem6* mutant allele does not interact with PABP. As suggested by the known function of PABP, when we examined the poly-A tail length of *Alas2* as well as several other erythroid specific genes in reticulocytes from wild type and *hem6* mutant animals, we found that the *hem6* poly-A tails were on average shorter for any given mRNA. Furthermore, we examined the persistence of erythroid-specific mRNAs by microarray and by qRT-PCR in *hem6* reticulocytes aged *in vitro*, and found that the loss of HEM6 had little effect on the stability of non-erythroid-specific mRNAs,

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but that the erythroid-specific mRNAs decayed at a more rapid rate. As the experiments proposed in aim 1 utilised live nucleated cells that are continuously transcribing mRNA (see above), it is highly likely that we would have missed such an mRNA decay effect. At the present time, we are reconfirming these results with additional replicates as well as with a time course to examine the temporal nature of the decay.

As evidenced above, I believe that we have made substantial progress on this project and have developed some very interesting results.

Prof. David Johnson

(Cycle VI)



Princess Alexandra Hospital, Brisbane, Australia

Haemoglobin levels in patients with erythropoietin-resistant anaemia treated with oxpentifylline (HERO Trial)

Significant progress has been made in the first year of funding for this study. Ethical approval has been obtained at several sites and clinical trial agreements (CTA) have been signed with participating sites. The trial was registered, study medication and placebo obtained and web-based data management systems developed. Patient recruitment will occur in the last quarter of 2008.

Interested sites

In late 2006, a questionnaire was sent to all renal units in Australia and New Zealand (ANZ) to determine support for and feasibility of the trial. Twenty sites have agreed to participate, and we are collating responses to a recent communication with units to determine whether additional sites will join the trial.

Ethics Submissions

Ethical approval has been obtained at several sites, with more sites finalising their ethics submissions. A multi-centre ethics application is in development for all New South Wales (NSW) sites, with John Hunter Hospital acting as lead site.

Clinical Trial Agreements (CTA) and Clinical Trials Notification (CTN)

A CTA has been fully executed for one site, and all remaining sites will have CTAs executed during 2008. A CTN template for notifying the Therapeutic Goods Administration (TGA) of the trial has been established, which all sites will receive in September 2008.

Medication

The study medication is Oxpentifylline (Trental®). A placebo medication is not available from any pharmaceutical company therefore the Australasian Kidney Trials Network (AKTN) has arranged for a placebo to be manufactured. This is

currently in production. It is expected that AKTN will receive the medication in September 2008, which will then be repackaged and delivered to sites prior to the randomisation of the first patient.

Case Report Forms (CRF)

Online electronic CRFs (eCRF) are being used for data management. The 'Inform' software system facilitates trial monitoring through stringent data checks and data audit trails. Paper-based CRF templates for the current study have been produced and finalised, and production of the electronic eCRFs will be in place by the time of patient recruitment. Patient randomisation is also being performed online, using the Flexetrial randomisation software.

Study initiation meetings

A study initiation on 8 October 2008 in Sydney, will include Principal Investigators and Study Coordinators from all trial sites. Professor David Johnson will present the scientific basis for the trial and outline the trial protocol. Other key AKTN staff members associated with the conduct of the trial will also be present.

Safety and Data Monitoring Committee (SDMC)

The AKTN has a SDMC which will oversee the conduct of the HERO trial. This committee consists of two internationally-recognised nephrologists and two experienced and respected clinical trial / statistical experts. This group held its first meeting in August 2008.

Methods Paper

A manuscript detailing the proposed study methods was successfully submitted to *BMC Nephrology*, an electronic, peer-reviewed journal.

We have made significant progress towards enrolling our first patients in the HERO study. We anticipate that the trial will be completed by late 2009 with presentation and publication of results in 2010.

Dr Nathan Levin Chairman	Medical and Research Director Renal Research Institute New York, USA
Dr James O. Armitage	Department of Internal Medicine University of Nebraska Medical Center Omaha, USA
Dr Lars Birgerson	Global Head of Medical Affairs F. Hoffmann-La Roche Inc. Nutley, USA
Dr Charles Herzog	Director of Cardiovascular Special Studies Center United States Renal Data System Professor of Medicine Hennepin County Medical Center, University of Minnesota Minneapolis, USA
Dr Adeera Levin	Professor of Medicine, Division of Nephrology Co-Director, Clinical Investigator Program University of British Columbia Director, Kidney Function Clinic St. Paul's Hospital Vancouver, Canada
Prof. Jean-François Morere	Professor of Medicine, Service d'Oncologie Médicale Hôpital Avicenne University of Paris Paris, France
Dr Neil Powe	Professor of Medicine, Epidemiology, Health Policy and Management Johns Hopkins University Baltimore, USA
Prof. Eberhard Ritz	Department of Internal Medicine Renal Center University of Heidelberg Heidelberg, Germany

Prof. Inder Anand	Professor of Medicine University of Minnesota Medical School Minneapolis, USA
Dr Fred Appelbaum	Director of the Clinical Research Division Fred Hutchinson Cancer Research Center Seattle, USA
Prof. Gavin Becker	Director of the Nephrology Department The Royal Melbourne Hospital, Australia
Prof. Nicole Casadevall	Professor of Medicine Haematology Laboratory, Hôpital Saint Antoine Paris, France
Prof. Franco Cavalli	Director IOSI, Oncology Institute of Southern Switzerland Ospedale S. Giovanni Bellinzona, Switzerland
Prof. Adriana Di Polo	Associate Professor Department of Pathology and Cell Biology, Department of Ophthalmology University of Montréal, Canada
Prof. Ronald Falk	Head of the Division of Nephrology and Hypertension University of North Carolina Chapel Hill, USA
Prof. Patrick Maxwell	Professor of Nephrology University College London London, UK
Dr William McClellan	Assistant Professor Department of Epidemiology Emory University Atlanta, USA
Dr Jerry Spivak	Professor of Medicine and Oncology Johns Hopkins University Baltimore, USA
Prof. Gary Striker	Professor of Medicine and Nephrology Mount Sinai School of Medicine New York, USA

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 is 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, resistance to erythropoietin, anaemia of chronic disease, anaemia related to congestive heart failure and stroke, biology of anaemia and outcomes. RoFAR especially 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 budget details given. It is not possible to renegotiate the amount after project approval. Indirect costs (institutional overhead, 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 principal and co-applicants.

The screenshot shows the RoFAR website homepage. At the top left is the URL www.rofar.org. The RoFAR logo is at the top right. A navigation menu on the left includes: Home, Information, Regulations, Regular grants, Special grant, Grant Awards, Downloads, Secretariat, Links, and Q & A. The main content area features several sections:

- 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, erythropoietic 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 page.
- RoFAR bi-annual reports available for download:** Discover more on the award winners, their projects and progress in the 'download' section.
- Upcoming congress activities:** Visit RoFAR's exhibition booth at following congresses:
 - ASH 2006, December 9-11, Orlando FL, USA (booth number 2441)
 - WON 2007, April 21-25, Rio de Janeiro, Brazil (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 Intent 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 does RoFAR give 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 channel of information, where you can find important announcements, future deadlines, submission forms, the RoFAR charter and regulations, as well as progress reports and funding history. If you have any specific questions, please do not hesitate to contact the secretariat (admin@rofar.org).

10 *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.

LOIs are evaluated by a Board of Scientific Advisors

6–9 weeks

LOIs are thoroughly reviewed by 3 members of the Scientific Advisory Board and judged based on 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

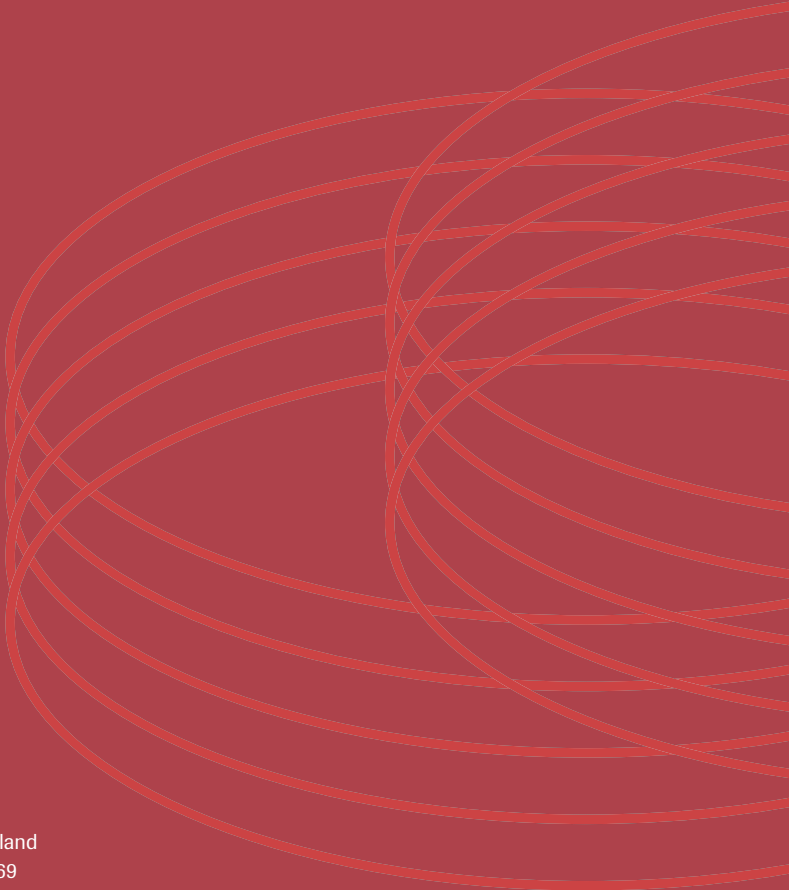
4–6 weeks

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 based on 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.



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