



RoFAR
Foundation for Anemia Research

Bi-annual report

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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 first half of 2006, RoFAR has awarded grants of 1.2 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 fourth cycle of competition have been selected to receive RoFAR research grants of up to 200,000 CHF distributed over two years. Once again, the high expectations for the quality of research projects and applicants have been met. In its previous two years of activity, RoFAR has awarded twenty-one grants totalling 3.9 million CHF.

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) who undertakes to notify applicants of their decision six months after submission of the LOI.

In addition to the regular competition cycles, RoFAR invited scientists and institutions to submit applications for a special grant to support ground-breaking scientific work, both basic and clinical, which include investigation of anaemia, erythropoietin and related topics ranging from hypoxia-sensing to the organ-protective role of erythropoietin and 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, and results can be transferred into clinical practice. The submission and selection procedure – similar to the one in use for regular grants – will result in an award to a single research group or institution of a grant of up to 2,400,000 CHF. The special grant recipient will be announced at the end of 2006.

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 web sites and by selected activities at a number of scientific congresses.

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.

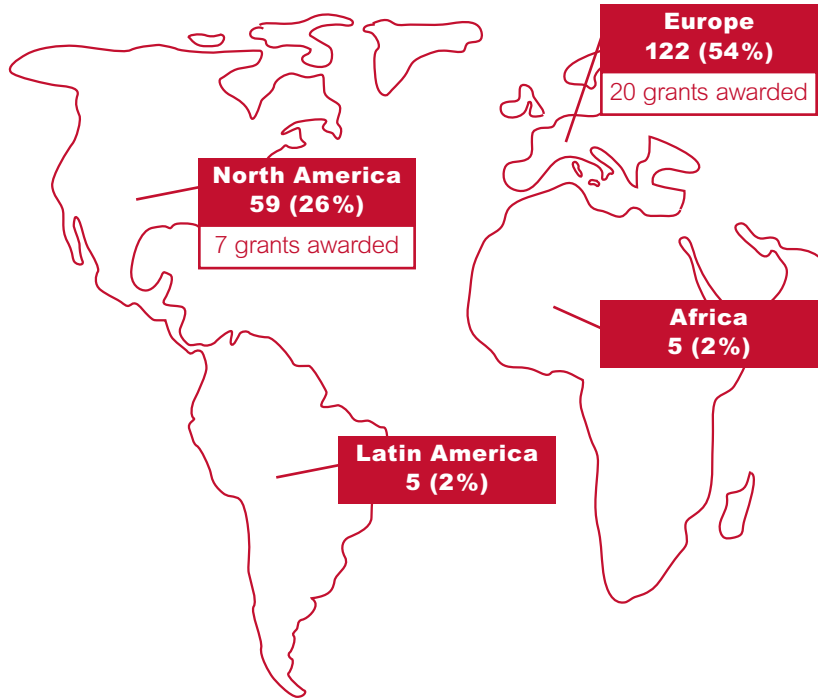
RoFAR welcomes any feedback or suggestions to assist us in accomplishing its stated mission.

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 four cycles of competition represent a range of institutions in 38 countries. More than half (54%) of all the LOI applications have been submitted from Europe, primarily from Germany, Italy, Switzerland and the UK. About one quarter of the applications (26%) 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 (53%), animal trials (34%) and basic science projects. Submitted projects focus on nephrology and diabetology (47%), haematology (42%), oncology (20%) and cardiology (14%) with some overlap between areas.



Asia and Oceania
33 (15%)

Twenty grants have been assigned to European applicants, seven to North American applicants.

Submitted research proposals by

Study type

Human trials	(53%)
Animal studies	(34%)
Others	(13%)

Research focus

Nephrology	(47%)
Haematology	(42%)
Oncology	(20%)
Cardiology	(14%)
Others	(17%)
(multiple allowed)	

Gender of main applicant

Males	(75%)
Females	(25%)

Institution type

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

3 *Grant awards in Cycle IV*

Prof. Christof Dame



Charité University of Berlin, Germany

Role of GATA transcription factors in regulating erythropoietin and its receptor in the heart

Circulating erythropoietin (EPO), which is required for red blood cell production, is primarily produced in the kidney, but also in various other tissues, including the nervous system and the heart. EPO mediates its effects by binding to its specific cell surface receptor (EPO-R). Besides EPO, the EPO-receptor (EPO-R) is also expressed in many non-hematopoietic cell types, including neurons and cardiomyocytes. It has been shown that EPO acts as a potent cell protective and trophic factor. Various animal studies indicated that recombinant EPO, which is available as a pharmaceutical agent, exerts significant cardioprotective effects against infarction and ischemia-reperfusion injury, but also against non-ischemic cardiac dysfunction. Therefore, the understanding of EPO and EPO-R expression has a fundamental implication, both in health and disease. The molecular mechanisms of EPO and EPO-R expression in the heart are not yet known, but increasing evidence is given that both genes are tissue-specifically regulated by transcription factors. The aim of this research project is to elucidate the regulation of EPO-R expression in cardiomyocytes by transcription factors. We will focus on mechanisms of EPO-R expression in acute hypoxicischemic and chronic heart injury. Furthermore, we will analyse the mechanisms by which endogenous EPO expression is silenced in the myocardium. The understanding of these mechanisms will be helpful in optimising the future use of EPO as a cardioprotective agent.

Dr Ricarda Diem (principal applicant)

Prof. Michael Knauth (co-applicant)

Dr Gunther Helms (co-applicant)

Prof. Reinhard A.W. Hilgers (co-applicant)

Prof. Jürgen Petersen (co-applicant)



University of Göttingen, Germany

Efficacy and safety of erythropoietin as an add-on therapy in subjects with acute autoimmune optic neuritis

Optic neuritis is one of the most common and frequently the first clinical manifestation of multiple sclerosis (MS), an inflammatory autoimmune CNS disease. It is mainly characterised by a subacute loss of vision. Visual acuity after an episode of optic neuritis can recover within a few weeks, but this recovery remains incomplete in approximately one third of the patients. Additionally, most patients complain of persistent visual disturbances and show optic nerve atrophy detectable by magnetic resonance imaging (MRI). Further, there is evidence for a degeneration of retinal ganglion cells, the neurons that form the axons of the optic nerve, in patients after an episode of optic neuritis. This neurodegeneration has the strongest impact on the development of persistent visual deficits. Methylprednisolone, the standard therapy for autoimmune optic nerve inflammation, accelerates visual recovery, but does not influence the neurodegenerative component of the disease.

In this double-blind, placebo-controlled clinical trial, we will investigate the neuroprotective potential of erythropoietin (EPO) as an add-on therapy in patients with acute optic neuritis. In animal models of autoimmune optic nerve inflammation, EPO has been shown to protect retinal ganglion cells and lead to a functional improvement of vision when combined with methylprednisolone. Approximately 40 subjects will be randomised in equal numbers into one of the two treatment groups receiving methylprednisolone in combination with EPO or with placebo over three days. The neuroprotective potential of EPO will be assessed by optical coherence tomography measuring nerve fiber loss in the optical nerve head. Atrophy and axonal damage of the optic nerve itself will be monitored by using different MRI techniques. Further endpoints of the study include visual acuity, visual field perception as well as recovery of latency and amplitudes of visual evoked potentials.

3 *Grant awards in Cycle IV*

Prof. Tomas Ganz



University of California, Los Angeles, USA

Pathogenesis of anaemia of chronic infection

Anaemia of inflammation (also called anaemia of chronic disease) is one of the most common kinds of anaemia, but the mechanisms that cause it are not known. Anaemia of inflammation contributes to morbidity, loss of independence, and may increase the mortality of coexisting diseases. Based on our recent work, we propose that infections or other causes of inflammation stimulate the production of cytokines, chiefly interleukin-6, that in turn cause increased synthesis of hepcidin, an iron-regulatory hormone that inhibits the release of recycled iron from macrophages. Decreased delivery of iron to the bone marrow combined with shortened red cell life-span then limits haemoglobin production and causes anaemia. We propose to test this hypothesis in a mouse model of chronic infection. Mouse models have been very useful in the study of many human diseases, but their impact on the study of anaemia of inflammation has been limited due to the lack of a suitable model of chronic inflammation leading to anaemia. We will first refine and characterise a mouse model of chronic infection similar to chronic foreign body-associated infections in humans. We will then compare the anaemia caused by such infections in wild-type mice and mice genetically lacking hepcidin or interleukin-6.

Previous studies have suggested that the production of erythropoietin (EPO) may be suppressed by inflammation and that relative EPO deficiency could contribute to anaemia of inflammation or anaemia of chronic infection. We will compare EPO levels in anaemia of chronic infection to those of similarly anaemic mice with iron deficiency or haemolytic anaemias. We will also explore the effects of the putative mediators of anaemia of chronic infection, hepcidin and interleukin-6, on EPO production.

The results of these studies will lead to a better understanding of the causes of anaemia of inflammation and provide leads for its improved treatment.

Dr Dirk Hermann¹ (principal applicant)

Prof. Markus Rudin² (co-applicant)



University Hospital Zurich, Switzerland¹ and Swiss Federal Institute of Technology (ETH), Zurich, Switzerland²

Effects of human erythropoietin on brain plasticity and functional recovery following stroke

Clinical studies have recently shown that human erythropoietin (EPO) promotes neurological recovery in stroke patients. This discovery was widely regarded as a breakthrough in stroke research, after many negative trials, in which survival-promoting compounds failed to show efficacy in humans. Indeed, stroke is a highly frequent disorder, which represents the primary cause of long-term disability in Western countries and is a major burden to healthcare systems worldwide. Alleviating long-term consequences of stroke is therefore a priority topic in biomedical sciences.

In this project, we would like to characterise EPO's effects on brain plasticity, as well as on motor and cognitive recovery following ischemic stroke in mice. We would like to show whether EPO's brain plasticity is potentiated by enhanced physical training. This will be studied by housing laboratory mice in so-called enriched environments, and/or by NoGoA deficiency, a condition in which neuronal sprouting is enhanced. Using tract-tracing studies combined with motor-evoked potentials (MEP) and functional MRI, we would like to identify corticospinal and cortical reorganisation processes underlying EPO's brain recovery, thereby establishing this growth factor as a plasticity-promoting therapeutic.

In view that our department has clinical proof-of-concept structures available allowing assessment of rehabilitative therapies in human stroke patients (on behalf of our NCCR project «Neural plasticity and repair»), we hope to provide a basis for future clinical proof-of-concept studies, in which EPO shall be used as add-on treatment to rehabilitation therapies.

3 *Grant awards in Cycle IV*

Prof. Stéphane Picot¹ (principal applicant)

Prof. Ogobara K. Doumbo² (co-applicant)

Dr Abdoulaye Djimde² (co-applicant)

Dr Belco Poudiougou² (co-applicant)



**Claude Bernard University of Lyon, France¹ and
University of Bamako, Mali²**

Randomised trial of erythropoietin to prevent death from cerebral impairment during severe malaria

Cerebral malaria is a complication of this important disease leading to seizure, coma and death within the first 36 hours. Although cerebral malaria shares features with neurological stroke, erythropoietin (EPO) neuroprotective effects have not yet been investigated in this area. We propose a randomised clinical trial to investigate the safety and efficacy of EPO in cerebral malaria patients hospitalised in Bamako, Mali, to reduce the incidence of premature death in children.

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)



Georges Pompidou European Hospital, Paris, France

Study of the characteristics and fate of erythropoietin-producing cells

Anaemia is a well-known consequence of chronic kidney disease (CKD) that is characterised by a lack of red blood cells. It has been shown that CKD-related anaemia is due to a decreased production of erythropoietin (EPO), a glycoprotein that is the primary regulator of red blood cell production, and is indispensable for terminal differentiation of erythroid progenitors. However, the reasons for this inability to produce sufficient quantities of EPO are poorly understood.

While EPO is mostly produced by the liver during embryonic development, the kidney becomes the main source of EPO after birth. In the liver, EPO is produced by hepatocytes located around the central veins and by stellate cells, while in the kidney it is produced by interstitial cells that have characteristics of fibroblastic cells. The aims of this research project are: (1) to precisely define the characteristics of EPO producing cells in the kidney and liver, but also in other organs that can produce low levels of EPO such as the retina, brain or genital tract; (2) to define the fate of liver EPO-producing cells after birth, and to understand why they cannot compensate for decreased renal production of EPO in patients with CKD; (3) to define the fate of renal EPO-producing cells in the case of kidney disease and (4) to understand why interstitial fibrosis is associated with a decreased production of EPO.

To do so, we will generate knock-in mice harbouring an internal ribosomal entry site (IRES) – Cre recombinase transgene or an IRES – enhanced green fluorescent protein (eGFP) transgene in the 3'-untranslated sequence of the endogenous EPO gene. The former mice (EPO-Cre mice) will be bred with Rosa26 reporter mice, in order to induce expression of the *lacZ* gene specifically in cells that express the EPO gene and in their progeny. The latter mice (EPO-eGFP mice) will be used to easily identify EPO-producing cells.

4 *Progress reports of RoFAR award winners*

Dr Nancy C. Andrews

(Cycle I)



Children's Hospital Boston, USA

Hepcidin regulation in the anaemia of chronic disease

The anaemia of inflammation is an acquired condition that affects patients with a variety of disorders including infection, arthritis, inflammatory bowel disease, trauma, organ failure and cancer. It causes a measurable decrease in quality of life and general feelings of well-being. Hepcidin is a central mediator of the anaemia of inflammation. It is a circulating hormone that is induced by inflammation, which blocks the release of iron from macrophages and interrupts intestinal iron absorption. Accordingly, mice over-expressing hepcidin develop severe iron deficiency anaemia.

The first direct evidence supporting the role of hepcidin in the anaemia of inflammation came from our studies of patients with glycogen storage disease (GSD) type 1a¹. GSD1a is an autosomal recessive disorder caused by mutations in the glucose-6-phosphatase gene, resulting in an inability to maintain glucose homeostasis. A subset of the adult GSD1a patient population develops large hepatic adenomas and anaemia with features characteristic of the anaemia of inflammation. Although the cause of these adenomas is unknown, we presented compelling evidence that the anaemia results from inappropriate overexpression of hepcidin in adenoma tissue. When the adenomas are removed, iron homeostasis returns to normal.

It has become clear that hepcidin is also induced in the anaemia of inflammation resulting from other causes. Elegant work from Tom Ganz, Elizabeta Nemeth and their colleagues has shown that induction can be attributed, at least in part, to the inflammatory cytokine interleukin-6 (IL-6). IL-6 treatment stimulates hepcidin expression in isolated hepatocytes and hepatocyte-like cell lines. Administration of IL-6 to human subjects stimulates increased hepcidin production and results in low serum iron (hypoferremia) *in vivo*. Mice lacking IL-6 fail to induce hepcidin and do not become hypoferremic after treatment with endotoxin. Taken together, their observations left little doubt that IL-6 links inflammation to hepcidin production.

However, the mechanism by which IL-6 induces hepcidin expression was not fully understood.

IL-6 signalling is a major regulator of the acute phase response in hepatocytes. Upon an inflammatory stimulus, IL-6 is released and binds to a complex of the IL-6 receptor α and gp130. The IL-6 ligand-receptor interaction results in the activation of Janus kinases (JAKs), which phosphorylate signal transducers and activators of transcription (STAT) proteins, predominantly STAT3. Upon phosphorylation, STAT3 translocates into the nucleus where it regulates transcription of many target genes.

The goals of our study were to determine whether IL-6 acts directly to induce hepcidin expression and to elucidate the downstream mechanism of IL-6 mediated hepcidin induction. We identified an IL-6 responsive element (CE9) centred around -89 bp relative to the hepcidin transcriptional start site, within the hepcidin promoter. We demonstrated that IL-6 regulates hepcidin expression through direct binding of STAT3 to the promoter. Finally, we showed that STAT3 is necessary and sufficient to confer IL-6 responsiveness in a luciferase reporter assay. Our observations not only illuminate IL-6 regulation of hepcidin, but also suggest that, even in the absence of elevated cytokine levels, aberrations in hepatic STAT3 regulation could lead to increased hepcidin and anaemia.

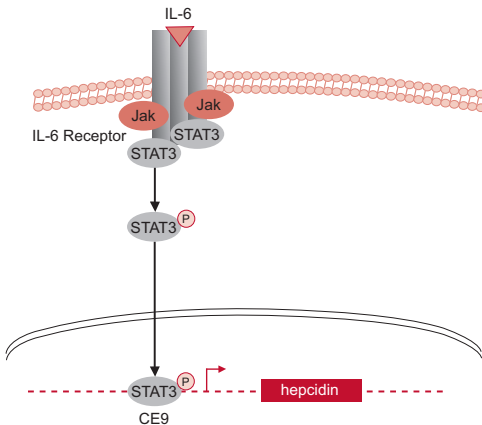


Figure 1. IL-6 induction of hepcidin transcription results from STAT3 binding to the hepcidin promoter. Our interpretation of our results is shown schematically.

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4 *Progress reports of RoFAR award winners*

Dr Andrew McKie (principal applicant)

Dr Robert J. Simpson (co-applicant)

Prof. Robert C. Hider (co-applicant)

(Cycle 1)



King's College, London, UK

Characterisation of a novel intestinal heme transporter

Heme iron is a highly bioavailable form of iron in the diet which is 2–3 times more efficiently absorbed in the proximal intestine than inorganic iron. Heme iron therefore makes an important contribution to body iron stores. HCP1 has recently been identified as a transmembrane transporter for heme iron which is highly expressed in the duodenum¹. It is believed that HCP1 recognises the porphyrin ring and transports the metallo porphyrin ring intact from the diet across the brush border membrane into the enterocyte. Once inside the enterocyte, the heme is broken down by heme oxygenase 1 (HO-1) to yield biliverdin and ferrous iron; the latter, then, is transported across the basolateral membrane via ferroportin.

In the present work we have investigated heme uptake by the duodenal mucosa in mice *in vivo* and *in vitro* using ⁵⁹Fe and ⁵⁵Fe labelled heme respectively, to uncover the role of HCP1 in this process. We found that the duodenal mucosa showed the highest rate of heme uptake compared to the other regions of the intestine (ileum, cecum and colon), which showed very low rates of heme uptake (data not shown). This correlates with the expression of HCP1 being highest in the duodenum.

To investigate whether heme iron absorption was stimulated by hypoxia and iron deficiency, we exposed mice to hypoxia and iron deficient diets and measured ⁵⁵Fe heme uptake in everted duodenal sacs *in vitro*. In mice exposed to chronic hypoxia (0.5 atm) for 3 days, heme uptake was significantly ($p=0.04$) increased (see figure). The addition of HCP1 antibodies to the incubation media reduced the heme uptake in both control ($p=0.02$) and mice subjected to hypoxia ($p=0.03$) (see figure). Control tissues were incubated with preimmune sera which had no effect on heme uptake. Iron deficiency tended to increase duodenal heme iron absorption, but the increase was not significant (data not shown). As with hypoxia, the increase was abolished by addition of the HCP1 antibody indicating that heme uptake was mediated by HCP1. We have repeated these experiments *in vivo* with similar results (data not shown).

Hence we have developed both *in vitro* and *in vivo* methods in mice to measure heme uptake. We have shown that heme uptake is stimulated by hypoxia. The increase in heme uptake was less marked in iron deficiency. However, incubation of tissue with antibodies to HCP1 completely inhibited the increase in heme uptake in both conditions. This provides further evidence that HCP1 is involved in intestinal heme transport.

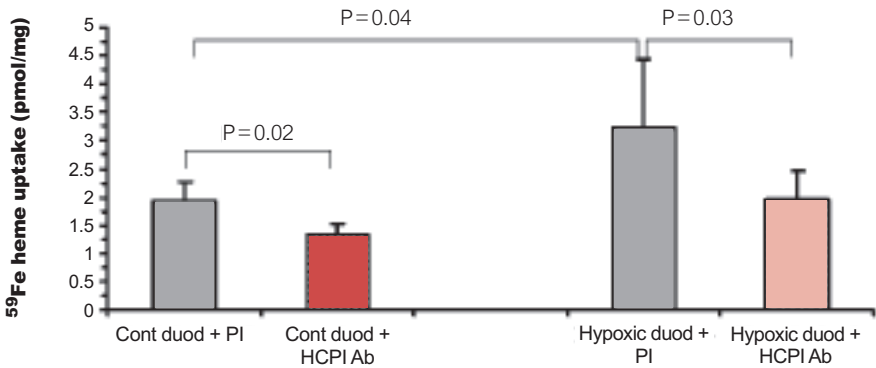


Figure 1. Hypoxia stimulates duodenal ^{59}Fe heme uptake *in vitro*. ^{59}Fe heme (RI Consultants, USA) uptake was measured *in vitro* in everted duodenal sacs at 37°C for 10 minutes using a concentration of $10\mu\text{M}$ heme. Uptake was terminated by rinsing the tissue in ice-cold Hanks solution (Sigma) and then three times in 1% BSA for 1 minute each. After reblotting and weighing, the tissues were solubilised and radioactivity was assayed in the tissue using a twin channel β -counter (LKB Wallac-1209). The results are expressed as pmol/mg wet tissue. PI is preimmune sera and HCP1 Ab is HCP1 antibody.

References

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4 *Progress reports of RoFAR award winners*

Dr Marco Merlano (principal applicant)

Dr Silvana Ungari (co-applicant)

(Cycle I)



S. Croce General Hospital, Cuneo, Italy

In vitro analysis of tumour response to radiation in oxic and hypoxic conditions

The purpose of the presented study is to establish an experimental model and to examine the relationship between hypoxia, EPO/EPOR and EGFR transcription/ expression and their effects on the cellular response to radiation. By identifying the causes due to the negative effects of anaemia on radiotherapy, this may facilitate the development of treatment strategies to improve efficacy and reduce toxicity.

We started establishing 7 human Squamous Cell Carcinoma cell lines from biopsies of HNSCC cancer patients, and we characterised them for the p53 status and the polymorphism in codon 72. All 7 cell line-resulted mutant for p53 and the codon 72 were genotyped. Since p53 is involved in the DNA-damage response following radiation therapy, and knowing that the use of mutant lines may therefore likely produce interpretation biases, we are actually using two wild type p53-bearing HNSCC cell lines: CAL-166, with an elevated EGFR expression and Hep2, of cervical origin and with a low EGFR expression.

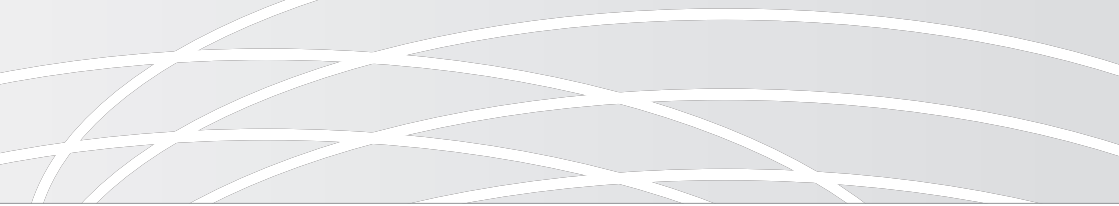
We are performing extended studies to standardise the experimental conditions (time of exposure to hypoxia and time to return of normal culture conditions) and the administration of radiation (fractioned or in a single dose) in order to find the optimal ones in which the effect of the hypoxia on the radio resistance is most pronounced, and where the data obtained are likely to be more effective.

To mimic hypoxic conditions, we are using two different systems for cell culture:

- Campigen Compact (Oxoid): 5% O₂, 10% CO₂, 85% N₂
- Anaerogen (Oxoid): <0.5% O₂, 8%<CO₂<14%

Incubation under oxic and hypoxic conditions is performed as follows:

1. Incubation and irradiation under oxic conditions;
2. Incubation and irradiation under hypoxic and anaerobic conditions;

- 
3. Incubation under either hypoxic or anaerobic conditions, but reoxygenation allowed before irradiation.

Cells are exposed to an X-ray source with a dose escalation from 2–8 Gy.

Cells are then analysed with a classic clonogenic survival assay:

1. Harvesting them immediately after the administration of radiation to test their intrinsic radio resistance;
2. Harvesting them 24 hours after the administration of radiation to test the radio resistance due to their capability of DNA repair;
3. Harvesting them 24, 48, 72, 96 and 120 hours after the administration of radiation to test the radio resistance due to the repopulation of clonogenic cells.

The first round of experiments suggested that clonogenic capability seems not to be influenced by the intrinsic radio resistance caused by hypoxia, but it is likely that accelerated repopulation, in response to cellular depopulation, could be a cause of radio resistance.

Hypoxia induces transcription of the EPO gene via activation of the transcription factor Hypoxia Inducible Factor-1 (HIF-1), the regulatory subunit of HIF-1 that is upregulated under hypoxic conditions and activates hypoxic adaptation pathways. In particular, using Real-Time RT-PCR obtained from the two cell lines, we analysed the basal and hypoxia-stimulated expression of HIF-1 before and after irradiation. We were not able to see an upregulation of the HIF1 alpha mRNA, and we postulate that the lack of correlation between protein and mRNA induction suggests that hypoxia-regulated gene expression may be influenced by post-transcriptional mechanisms. Western blot analysis is in progress to confirm this hypothesis.

4 | *Progress reports of RoFAR award winners*

Dr Peter Mertens

(Cycle I)



University Hospital Aachen, Germany

Mechanisms for erythropoietin resistance in transformed and non-transformed cells

Target genes of YB-1 that are regulated at the transcriptional level under hypoxia and are relevant for inflammation and tumour progression

A novel YB-1 target gene that is regulated under hypoxia and relevant for tumour growth is the epidermal growth factor receptor¹. In collaboration with Sandy Dunn (BC Research Institute for Children's and Women's Health, Vancouver, British Columbia, Canada), tissue arrays from patients with breast cancer were evaluated for YB-1 expression and these data were correlated with herceptin receptor expression. The results indicate that Her-2 receptor is dependent on YB-1 protein expression⁵.

Regulation of hypoxia-responsive genes and evaluation of YB-1 modifications / subcellular shuttling dependent on oxygen sensing

Ongoing projects included the evaluation of YB-1 target genes that are (putatively) relevant in hypoxia-responses, like Smad7 and DNA-polymerase- α . To this extent we have analysed the promoter sequences of both genes and identified YB-1 binding motifs that include a Y-box (inverted CCAAT-box) and inverse repeat sequence. A similar motif is located within the hypoxia-responsive element (HRE) of the EPO gene² (Figure 1). A detailed analysis revealed that YB-1 indeed regulates both genes, Smad7 as well as DNA-polymerase- α , via these elements^{3,4}.

A similar *trans*-regulatory effect was also observed with cells that harbour the HRE in conjunction with a luciferase reporter gene. Under normoxic conditions, the HRE was *trans*-activated about 2-fold by forced YB-1 overexpression, whereas the *trans*-stimulatory effect was enhanced by factor 10 in hypoxic cells (Figure 2). Notably, the *trans*-stimulatory effect that HIF-1 α overexpression has on the HRE

was partially abrogated by forced overexpression of YB-1. These data confirm the hypothesis brought up in the proposal, namely that YB-1 and HIF-1 α interfere with each other in the hypoxia response. From these data it may be derived that YB-1 is a key regulator for hypoxia-dependent gene regulation. Via laser scanning microscopy, we were able to identify a subcellular shift of YB-1 from the cytoplasm to the nucleus under hypoxia: co-localisation of YB-1 and GFP-tagged HIF1 α protein is observed in hypoxic cells. This subcellular co-localisation of both proteins under hypoxia suggests a direct protein interaction that is currently evaluated by co-immunoprecipitation experiments with recombinant HIF1 α and YB-1 proteins. DNA binding analyses revealed that recombinant YB-1 decreases the binding activity of HIF-1 α /ARNT complexes to the HRE oligonucleotide probe. This competitive action of YB-1 on the HIF-1 α /ARNT binding activity may explain the antagonising effect of YB-1 on the HRE-dependent HIF-induced promoter activity. In line with the proposal, we are currently investigating these interactions in detail by DNA binding analyses, e.g. competitor analyses and supershift studies. These findings demonstrate for the first time that the transcription factor YB-1 is part of the transcriptome binding to the HRE.

Setup of model systems to analyse the relevance of YB-1 in hypoxia-response of non-transformed and transformed cells

Different model systems are suited to unravel the role of YB-1 (and its crosstalk with HIF1 α) in hypoxia. To this extent we have initiated the establishment of permanently small interfering RNA expressing cells that target YB-1. As model systems, we have chosen HepG2 and HELA cells. Subsequent experiments will be aimed at testing the hypoxia response of these cells in the hypoxia chamber and to determine the response to erythropoietin incubation.

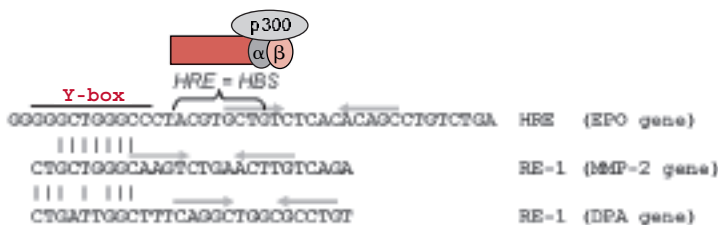


Figure 1. Y-box protein 1 binding elements within the promoters of the erythropoietin (EPO)-, matrix metalloproteinase-2 (MMP-2) and DNA polymerase- α (DPA) genes. The Y-box element as well as the inverted repeat sequence are known binding motifs for YB-1.

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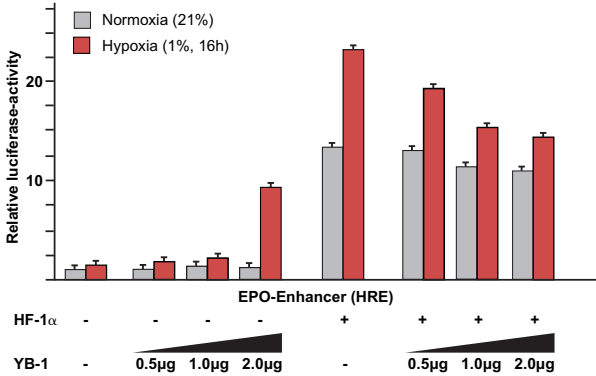


Figure 2. YB-1 trans-activates the HRE under hypoxia and interferes with the HIF1 α -dependent trans-activation of the HRE. YB-1 trans-activates the HRE in a concentration-dependent manner, up to 10-fold, under hypoxia. On the other hand the HIF1 α -dependent trans-activation is diminished with forced overexpression of YB-1.

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Part of this work was published in:

3. En-Nia A *et al.* Serum-induced expression of the DNA-polymerase α gene is mediated by transcription factor YB-1. *J Biol Chem* 2005;280:7702–77012.
4. Dooley S *et al.* Y-box protein-1 is the crucial mediator of antifibrotic interferon-gamma effects. *J Biol Chem* 2006;281(3):1784–95.
5. Wu J *et al.* Disruption of the Y-box binding protein-1 results in suppression of the epidermal growth factor receptor and HER-2. *Cancer Res* 2006;66(9):4872–9.

Dr Chris D. Vulpe (principal applicant)

Dr Ted Holman (co-applicant)

Dr Zhu Zhiwu (co-applicant)

(Cycle I)



University of California, Berkeley, USA

Characterisation of a family of putative mammalian heme chaperones

Multiple eukaryotic proteins require heme as an essential co-factor in order to carry out their functional roles¹. The mechanism of delivery of heme to these proteins is not known². We have identified a heme binding protein in *S. cerevisiae* encoded by *YPL170w* (Dap1p) and four mammalian homologs which could play a role in heme delivery in eukaryotic cells. We have characterised the heme binding of Dap1p and a mouse homolog, mPGRMC1, and determined that they utilise a novel mechanism of heme binding distinct from cytochrome *b*₅ family of proteins to which they are structurally related³. This family of proteins does not contain the conserved histidine ligands of cytochrome *b*₅, but they do have a conserved tyrosine and aspartic acid adjacent to the histidine position in cytochrome *b*₅ (Figure 1). We mutated these residues in Dap1p and found that the mutants still bound heme; there was also minimal change in their structure compared to Dap1p. We therefore mutated other conserved tyrosine residues as potential axial ligands and found one mutant, Y138F, which had no bound heme after purification. These results indicate that Tyr138 is the axial ligand to the heme for Dap1p. Considering that this tyrosine is conserved in this family, it is highly likely that it is the axial ligand for the mammalian homologs as well. We are currently mutating this tyrosine in mPGRMC1 to directly test whether it is the heme binding ligand. We tested the function of the Y138F Dap1p mutant *in vivo* and found that it was unable to complement the observed phenotype in the *dap1Δ* of sensitivity to iron chelators, in contrast to the wild type Dap1p. We similarly assessed if mPGRMC1 could functionally complement for the Dap1p. mPGRMC1 is 57% sequence similar to Dap1p except that mPGRMC1 has a N-terminal extension containing a membrane-spanning domain. As Dap1p is not membrane bound, we expressed a truncated version of mPGRMC1 in yeast which did not contain the N-terminal domain. We found that mPGRMC1 expression was able to rescue the sensitivity of *dap1Δ* to iron chelators, similar to Dap1p itself. These results suggest a remarkable functional conservation of the function of these proteins from yeast to mammals.

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Figure 1. Selected members of the Dap1p family of proteins. mPGRMC1p (accession number NP_058063), Hpr6.6 (accession number NP_006658), Dap1p (accession number Q12091), and the cytochrome b_5 binding motif (pfam00173) using Clustal W v1.82. Conserved amino acid residues that have been mutated in Dap1p are shaded in black and numbers above correspond to the Dap1p sequence. Axial heme ligating histidines from cytochrome b_5 are also highlighted, for comparison. Fully conserved residues are indicated with “*”, strongly conserved amino acids with “:”, and weakly conserved residues with “.”.

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Part of this work was published in:

4. Ghosh K *et al.* Spectroscopic and biochemical characterization of heme binding to yeast Dap1p and mouse PGRMC1p. *Biochemistry*. 2005;44:16729–16736

Prof. Hans Ulrich Bucher¹ (principal applicant)

Dr Joachim Riethmüller² (co-applicant)

(Cycle II)



**University Hospital of Zurich, Switzerland¹ and
University of Tübingen, Germany²**

*Erythropoietin reduces brain, eye and lung damage in very preterm infants:
Proof-of-concept study*

This double blind, randomised clinical trial has been initiated as planned. The following steps have been completed:

1) Safety Pretrial: Three doses of 1500 U/kg (half the planned dose) have been given to six preterm infants in order to rule out severe side effects:

Results Pretrial	EPO-Pretrial	Controls (all Preterm infants 24–31 6/7 born 2004 in Zurich)
number of infants EPO dose	6 3x1500 U/kg	137
Gestational weeks Weight at birth in g	25 6/7–31 5/7 930–1730	24 0/7–31 6/7 490–2480
Patent ductus arteriosus: present	0 0%	36 26%
Sepsis: present (pos. blood culture)	1 17%	13 9%
Cerebral ultrasound abnormal intracranial haemorrhage periventricular echodensities	3 50% 1* 17% 2 33%	65 47% 23 17% 47 34%
ROP (survivors only)	0 0%	23 20%
Bronchopulmonary Dysplasia (survivors only)	0 0%	17 12%
Death	1† 17%	23 17%
Discharged home without severe ROP, severe ICH, PVL or BPD	5 83%	92 67%

* subependymal haemorrhage † cause of death: interstitial pulmonary emphysema

The six infants tolerated the three doses well, and complications were in the range for these high risk patients.

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2) Start with main trial

The first 27 patients have been recruited in Zurich. Their gestational age ranged from 25 to 32 weeks and birth weight ranged from 660 to 1630 g. A total of 157 adverse events and 4 serious adverse events have been recorded. The safety monitoring board made a first interim analysis and allowed the recruitment to be continued.

3) Ancillary pharmacological study (Prof. C. Dame, Berlin): The concentration of EPO in the first 15 samples of umbilical cord blood was analysed. Urine was collected after each dose of EPO in the first 10 infants to measure the loss of EPO by this route.

4) Ancillary study on the effect of EPO on cerebral blood circulation measured by near-infrared spectroscopy. 10 infants receiving EPO and 6 infants receiving placebo were studied so far. Tissue oxygenation index was higher in the placebo group than in the EPO-group. This preliminary result suggests that EPO may affect cerebral perfusion (Figure 1).

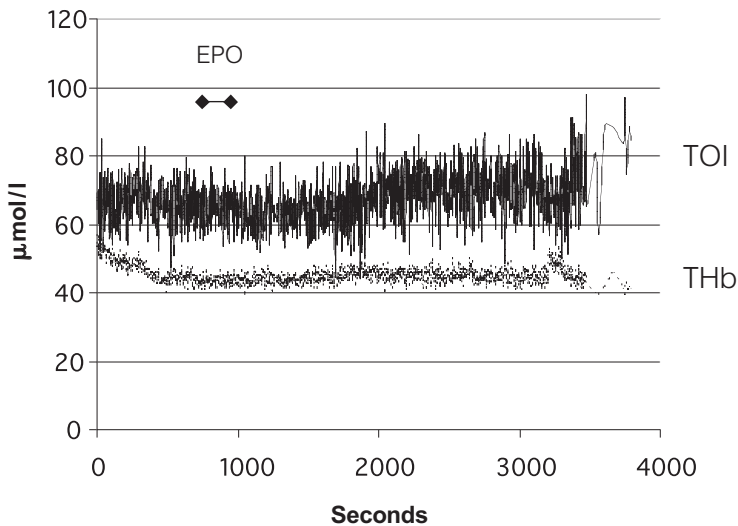


Figure 1. NIRS-recording of cerebral oxygenation (TOI) and perfusion (THb) in an infant before, during and after injection of EPO (◆) intravenously. There is no immediate effect, but a delayed and sustained increase in both, TOI and THb.

Dr Edward Debnam (principal applicant)

Prof. Robert J. Unwin (co-applicant)
(Cycle II)



**Royal Free & University College Medical School,
London, UK**

Is inflammation an important factor in the anaemia of chronic renal failure?

Patients with chronic renal failure (CRF) are commonly treated with erythropoietin (EPO) to correct the associated anaemia; however, they also often require additional iron supplementation. Treatment with systemic iron is usually more effective than oral iron, suggesting impaired intestinal absorption of the metal in CRF. Chronic inflammation is a recognised feature of CRF and is the consequence of many underlying factors including an enhanced incidence of infections (most commonly dialysis related), uraemia, elevated levels of pro-inflammatory cytokines and widespread atherosclerosis.

The hypothesis underlying this research is that CRF-related inflammation per se reduces intestinal iron absorption; this is related to circulating levels of liver-derived hepcidin, a negative regulator of iron absorption. This scenario induces anaemia by suppressing intestinal iron absorption and thus overrides the effect of EPO treatment. To investigate this hypothesis we have induced CRF in rats using the 5/6th nephrectomy model. Animals were later treated with EPO or turpentine, the latter to induce inflammation. *In vivo* uptake studies were used to examine the effect of the different treatments on intestinal iron absorption. Iron absorption was determined by instilling buffer containing ⁵⁹Fe into a cannulated duodenal loop in anaesthetised rats and then removing small samples of blood 5–30 minutes later for measurement of their ⁵⁹Fe activity. After each uptake experiment, samples of liver and duodenal mucosa were stored in order to assess gene (mRNA) expression of liver hepcidin and the known enterocyte iron transporters (DMT1 and Ferroportin) using quantitative RT-PCR. CRF animals had a reduced packed cell volume (PCV) and increased plasma levels of urea and creatinine when compared to sham-operated animals, thus confirming the induction of CRF by 5/6th nephrectomy. Iron absorption was significantly decreased in 5/6 nephrectomised animals (Figure 1). EPO treatment normalised PCV, although iron absorption was only partially restored. These results suggest that changes in iron absorption in the 5/6th nephrectomy model reflect those occurring in

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patients with CRF, demonstrating this to be an appropriate model for investigating the underlying mechanisms of iron homeostasis in CRF.

Although chronic inflammation is not thought to occur in the 5/6th nephrectomy model, iron absorption in these animals was reduced and only partially corrected by EPO (Figure 1); moreover, induction of acute inflammation with turpentine significantly inhibited iron absorption. Preliminary data for expression of liver hepcidin mRNA (Figure 2) suggests that elevated hepcidin levels during CRF reduces intestinal iron absorption, and this will accentuate the effect of reduced EPO secretion on the development of anaemia. EPO treatment failed to completely normalise the rate of intestinal iron absorption and hepcidin expression was only partially restored suggesting that the inflammation of CRF may be a significant underlying factor in the anaemia of CRF. The project is proceeding well and the results that have been generated are in keeping with the proposed hypothesis. No major problems have arisen and the work outlined in the original application should be completed within the time period of the grant.

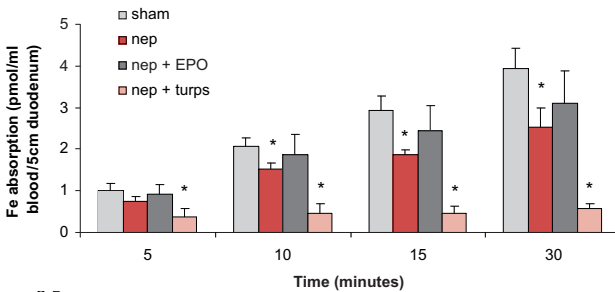


Figure 1. *In vivo* iron absorption by the duodenum of sham, 5/6 nephrectomised, EPO and turpentine treated 5/6 nephrectomised rats. Results are expressed as mean \pm SEM of 6 animals per group. * $P < 0.05$ compared to sham using ANOVA.

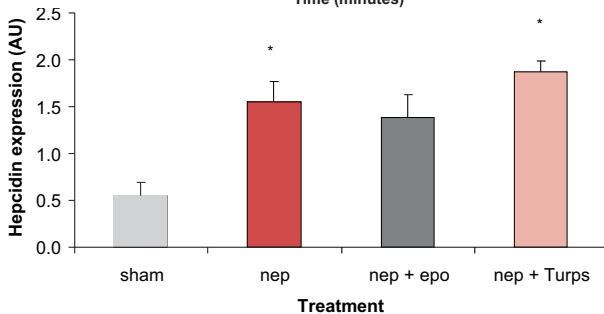


Figure 2. Hepatic expression of hepcidin mRNA in sham-operated, 5/6 nephrectomised, EPO and turpentine treated 5/6 nephrectomised rats. Results are expressed as mean \pm SEM of 4-6 animals per group. * $P < 0.05$ compared to sham using ANOVA.

Part of this work was published in:

1. Solanky N *et al.* Adaptation of iron absorption in the 5/6 nephrectomy animal model reflects the pathological features of chronic renal failure in humans. Accepted for presentation to the UK Physiological Society in July 2006.
2. Marks J *et al.* Is inflammation the key factor in the anaemia of end-stage renal failure (ESRF)? Submitted to the American Society of Nephrology for the annual meeting in November 2006.

Dr Carole Soussain

(Cycle II)



Oregon Health and Science University, Portland (Oregon), USA

Neuroprotective effect of erythropoietin on chemo- and radiotherapy-induced toxicity

Objective 1: To assess the effect of erythropoietin (EPO) on lymphoma cell growth and chemotherapy toxicity *in vitro*.

The activity of EPO was first tested on the dependent growth-factors TF-1 cells line (normal human erythroblasts): EPO sustained growth of the TF-1 cells. EPO did not affect growth of human B-lymphocytes (MC116 cells) (Figure 1). The effect of EPO on radiation and chemotherapy toxicity was tested in MC116 B-lymphocytes. No protection was found. Plans for the upcoming year: We have obtained the new incubator and atmosphere-controlled box for testing the effect of hypoxia on EPO receptor (EPO-R) levels and *in vitro* protective activity against radiation and chemotherapy toxicity.

Objective 2: To determine the neuroprotective activity of EPO *in vitro*.

The activity of EPO against the toxicity of chemotherapy and/or radiotherapy was tested on two *in vitro* models of neuronal cell cultures obtained from differentiation of PC12 and SHSY5Y cells. Viability assays and neurite length measurements were performed. Results were either inconclusive or need confirmation by other methods. Other neuronal *in vitro* models might be needed (Hippocampal cells, neuronal stem cells?). Plans for next year: To test the effect of hypoxia on PC-12 cell growth and EPO-R expression. In order to test EPO neuroprotection using a transfection model, the neurite measurement experiments will be repeated on GFP-transfected PC12 cells for a more accurate method.

Objective 3: To evaluate the effect of EPO on blood-brain barrier (BBB) permeability.

Dr Quentin Smith at the University of Texas evaluated the effect of EPO on the BBB permeability in healthy rats using the diffusion technique. The diffusion of inuline and

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urea was studied after infusion of different doses of EPO. The BBB permeability was not affected by EPO. Plans for next year: To test the effect of EPO on drug delivery in rats with CNS tumors.

Objective 4: To characterise the *in vivo* neuroprotective activity of EPO.

We attempted to develop a model of neurotoxicity in healthy rats treated with chemotherapy and/or radiotherapy (52 rats). Evans blue and fluoresceine dye extravasation experiments were inconclusive and not quantifiable. MR imaging with Gadolinium or Combidex in animals treated with high dose RT did not show leakage. Horse Radish Peroxydase extravasation showed some leakage around vessels. Some toxicity was noted on Hippocampal neuron at pathology but not in a proportion allowing comparative studies. Other methods for evaluating the neurodegeneration are needed. Rat models of B-cell CNS lymphoma (CNSL) were developed, both an intracerebral model and an intraventricular model. 82% of the inoculated rats developed CNS lymphoma. CNSL in the rat brain was assessed by MR imaging and immunohistochemistry¹ (Figure 2). Plans for next year: We will work with Dr Martin Fuss in the Department of Radiation Oncology to assess early hippocampal neurodegeneration in both normal and intracerebral CNSL animals treated with radiation and/or high dose of methotrexate. We will assess the effect of Epo on neurotoxicity in the rat intracerebral CNSL model treated with radiation and/or high dose of methotrexate.

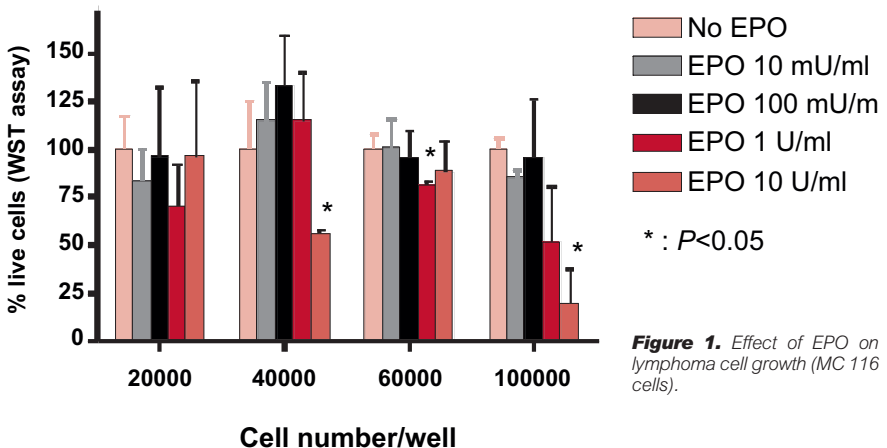


Figure 1. Effect of EPO on lymphoma cell growth (MC 116 cells).

D31 after intracerebral inoculation

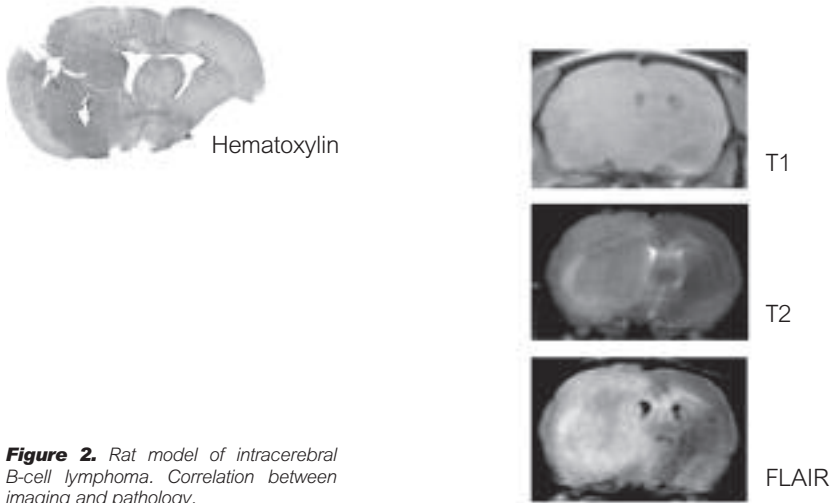


Figure 2. Rat model of intracerebral B-cell lymphoma. Correlation between imaging and pathology.

Part of this work was published in:

1. Soussain C *et al.* Characterization and Magnetic Resonance Imaging of a Rat Model of Human B-Cell Central Nervous System Lymphoma. 2006 *submitted*.

5 *RoFAR Board of Trustees*

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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?

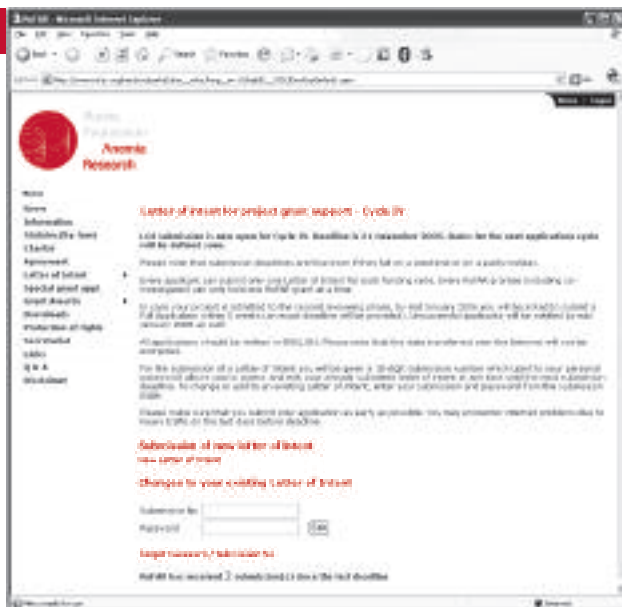
Funds are paid in three instalments over a maximum of 2 years and are dependent on the delivery of an interim and a final report for public use. Additionally, RoFAR must be acknowledged in publications, on posters, etc. Applicants may be asked to attend events organised by RoFAR and present their results.

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.



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).

8 *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.