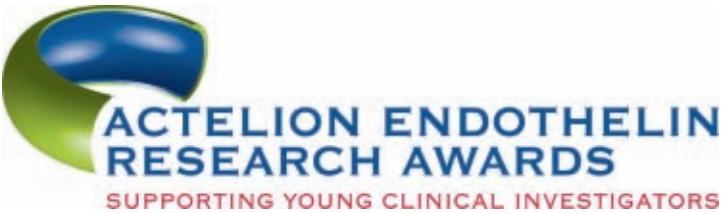
The New Generation in Endothelin Clinical Research

AERA Projects and Progress

2009



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Foreword

Prof. Robert Moots
Chairman of the AERA Steering Committee

Dear colleagues,

We are delighted to announce that in 2008 the Actelion Endothelin Research Awards (AERA) programme, a mentored and global grant-based programme targeting young investigators at universities and research institutes, awarded three new endothelin-related research projects proposed by clinicians in Austria, Germany and Sweden. As in the first two competition cycles in 2006 and 2007 the quality of the 2008 applications was quite impressive, with many innovative and scientifically excellent projects.

The AERA Steering Committee is proud that with AERA's support, previous grant winners have been able to publish their project outcomes in peer-reviewed journals, including *Diabetes Care, Journal of Heart and Lung Transplantation* and *Investigative Ophthalmology & Visual Science,* as well as present at several national and international meetings.

AERA has continued its commitment to young scientists conducting endothelin research by launching the fourth competition cycle in January 2009. As in previous cycles, applications will be assessed by an independent board of renowned experts in endothelin research, the AERA Steering Committee, and the award winners will be publicly announced in autumn of this year.

On behalf of the AERA Steering Committee, I would like to express our gratitude to Actelion for their generous gift to the endothelin research community and enduring commitment to advancing cutting-edge clinical science.

With kind regards,

Robert J. Moots

Supporting young investigators

The Actelion Endothelin Research Awards (AERA) is a **grant-based** programme aimed at encouraging **clinical endothelin research** and targeted at **young investigators**. The AERA is funded by Actelion Pharmaceuticals Ltd and is part of a global programme committed to endothelin research.

Global support to clinical research

Only **12-month mentored clinical projects with patient involvement** will be evaluated and considered for funding. Investigator-initiated clinical trials for young researchers at universities and research institutes are supported in **all countries except the US**. Please visit the AERA website (www.endothelin.org) for further eligibility criteria.

The AERA is an independent programme

Applications are evaluated by the **AERA Steering Committee (SC)**, an independent board of renowned experts who also decide which projects should be awarded the grants and provide strategic guidance to the AERA programme.

How to submit

Applications are **submitted electronically** as Letter of Intent applications. The timelines, the submission procedure and the submission forms are available on the AERA website (www.endothelin.org). Submitted applications are reviewed by the SC, who invites selected applicants to submit a full proposal. Award winners are announced publicly.

Review cycles completed: 3

in 2006, 2007 and 2008

Applications submitted: 123

Cardiovascular disease: 79%
Pulmonology: 57%
(with some overlaps between areas)
Rheumatology: 22%
Others: 28%

Applications submitted from 19 countries:
Australia, Austria, Belarus, Belgium, China, France, Germany, Greece, Hong Kong, Italy, Mexico, Romania, Spain, Sweden, Switzerland, Taiwan, The Netherlands, Turkey, UK

Awards distributed: 12

Overview of awarded AERA grants

AERA 2006 – Awarded July 2006

Dr Marina Anderson	University Hospital Aintree, Liverpool, UK
Dr Gerhard Garhöfer	Medical University of Vienna, Austria
Dr Adrian Gonon	Karolinska University Hospital, Stockholm, Sweden
Dr Eugene Kotlyar	St. Vincent's Hospital, Sydney, Australia
Dr John Wort	Royal Brompton Hospital, London, UK
Dr Ling Zheng	Monash Medical School, Melbourne, Australia

AERA 2007 – Awarded July 2007

Dr Christopher Adlbrecht	Medical University of Vienna, Austria
Dr Sandrine Launois-Rollinat	University Joseph Fourier / CHU, Grenoble, France
Dr David Montani	Hôpital Antoine Béclère, Clamart, France

AERA 2008 – Awarded September 2008

Dr Judith Maria Löffler-Ragg	Innsbruck Medical University, Austria
Dr Gabriela Riemekasten	Charité University Hospital, Berlin, Germany
Dr Magnus Settergren	Karolinska University Hospital, Stockholm, Sweden

The interplay of vascular function, low grade inflammation and innate immunity in the disease process of Raynaud's phenomenon and systemic sclerosis

Effects of non-specific endothelin-1 receptor blockade on ocular blood flow in patients with glaucoma

The importance of endothelin-1 for vascular complications in patients with diabetes

Non-invasive assessment of airway markers in the diagnosis of pulmonary hypertension

The effect of a dual receptor antagonist on platelet function in patients with pulmonary arterial hypertension

Endothelin-1 as a mediator of fibrogenesis in the development of bronchiolitis obliterans syndrome post lung transplant

Active endothelin in pulmonary embolism

Cardiovascular consequences of obstructive sleep apnoea: role of endothelin and preventive effects of a dual endothelin receptor antagonist

Endothelin receptor antagonism and dendritic cell function in pulmonary arterial hypertension

Drug target analysis and interactions of the endothelin system in adult pulmonary Langerhans' cell histiocytosis

Evaluation of differential ET_A and ET_B expression on phenotype and function of peripheral blood mononuclear cells

The importance of endothelin-1 for myocardial microvascular dysfunction in patients with type 2 diabetes

Dr Judith Maria Löffler-Ragg
Innsbruck Medical University, Austria

Dr Elvira Stacher (co-investigator)

Drug target analysis and interactions of the endothelin system in adult pulmonary Langerhans' cell histiocytosis

Adult pulmonary Langerhans' cell histiocytosis (PLCH) is a smoking-related interstitial lung disease characterised by focal Langerhans' cell (LC) granuloma destroying distal bronchioles.

Clinical manifestations range from spontaneously regressing lesions to fatal disorders such as respiratory failure and/or pulmonary hypertension. There is no proven therapy for PLCH and improved understanding of underlying disease mechanism could help in the development of specific therapeutic strategies.

In a retrospective immunohistochemical analysis of lung biopsies of 25 PLCH patients we want to investigate the expression of potential drug targets such as endothelin receptor A and B, phosphodiesterase type 1 and 5, platelet derived growth factor receptor-beta, stem cell factor, c-Abl, vascular growth factor receptors KDR, flt-1 and the stem cell surface marker AC133 (indicating neoangiogenesis). In an *in vitro* study, possible relations of the endothelin system to phenotype and function of LCs and to the development of inflammation, fibrosis and proliferation of endothelial cells observed in PLCH will be analysed. The constitutive expression of endothelin-1 (ET-1) and endothelin receptors will be investigated using murine LCs. After ET-1-stimulation and further inhibition with a selective or dual endothelin receptor antagonist (ERA), alterations in the endothelin system will be studied. With supernatants from stimulated, unstimulated and ERA-treated LCs the proliferation capacity of pulmonary arterial endothelial cells, pulmonary smooth muscle cells and pulmonary fibroblasts will be determined. Specific ET-1 and ERA-induced proteins will be assessed by proteome analysis of treated LCs. This study may identify active signalling pathways in PLCH and provide data for clinical trials offering individualised targeted therapy to modulate disease course.

Dr Gabriela Riemekasten
Charité University Hospital, Berlin, Germany

Mike Oliver Becker (co-investigator)
Angela Kill (co-investigator)

Evaluation of differential ET_A and ET_B expression on phenotype and function of peripheral blood mononuclear cells

Systemic sclerosis (SSc) is a devastating multi-organ disease characterised by a triad of autoimmunity, vasculopathy and tissue fibrosis. Recent studies indicate an importance of endothelin (ET) receptor activation in the pathogenesis of SSc either by the natural ligand endothelin-1 or by stimulatory anti-receptor antibodies. Their effects on inflammation and vasculopathy are mediated by their specific endothelin A/B receptors expressed on various cell types including vascular cells and immune cells. At present, there are only few data concerning the interaction of the immune system and the cardiovascular system and the impact of endothelin receptor activation on the initiation and perpetuation of the inflammatory process.

We hypothesise that endothelin receptor activation in cells of the adaptive and innate immune system can induce inflammatory processes that contribute to the pathogenesis of SSc. Endothelin receptor activation might provide an important link between the vasculopathy and autoimmunity present in SSc leading to tissue fibrosis. In the present project, we will analyse the ET_A and ET_B receptor expression in different peripheral blood mononuclear cells derived from SSc patients versus healthy volunteers by flow cytometry and realtime PCR. The phenotype of the endothelin receptor positive cells will be identified. We will correlate the expression of the different receptors on different cell types with clinical data of the SSc patients. The functional consequences of the receptor activation and the stimulation by ET-1 or the anti-receptor antibodies will by studied by the analysis of the cytokine and chemokine expression via flow cytometry, ELISA and bioplex assays.

Dr Magnus Settergren
Karolinska University Hospital, Stockholm, Sweden

Dr Reidar Winter (co-investigator)

The importance of endothelin-1 for myocardial microvascular dysfunction in patients with type 2 diabetes

Background

Patients with type 2 diabetes are known to have coronary microvascular dysfunction. This is associated with an unfavourable prognosis and may also play a role in the pathogenesis of diabetic cardiomyopathy. The mechanism behind impaired microvascular function in patients with diabetes remains unclear. We have previously shown that increased endothelin-1 (ET-1) signalling contributes to peripheral microvascular dysfunction in patients with type 2 diabetes, and we therefore hypothesise that ET-1 is of importance for the development of coronary microvascular dysfunction.

Methods

Twelve patients with type 2 diabetes and coronary flow reserve (CFR) <2.5 will be included in the study. By using transthoracic echocardiography, CFR is assessed by the determination of blood flow in the mid-portion of the left anterior descending artery by pulsed wave Doppler before and during intravenous infusion of adenosine. CFR is defined as the ratio of maximum to baseline coronary flow. Left ventricular diastolic function will be determined by pulsed–Doppler of the mitral valve inflow and tissue Doppler imaging. Peripheral microvascular endothelial function will be assessed by peripheral arterial tonometry using the Endo-PAT2000.

Study Protocol

Each patient will be randomised to three different treatment protocols using a 3-way cross-over design. Baseline determination of CFR, diastolic function and PAT will be performed as described above. On one occasion an infusion of the selective ET_A receptor antagonist BQ123 (5 nmol/kg/min) will be given. On another occasion BQ123 (5 nmol/kg/min) and the ET_B receptor antagonist BQ788 (4 nmol/kg/min) will be given, and on the third occasion 0.9 % saline will be given. All infusions are given for 30 minutes and CFR, diastolic function and peripheral endothelial function are re-assessed 60 minutes after the start of the infusions.

Significance

The study will give important new insight into the role of ET-1 in the pathophysiology of coronary microvascular dysfunction in patients with diabetes. A positive outcome of the study may result in a new therapeutic strategy using ET receptor antagonists to improve microvascular flow in this patient group.

Dr Christopher Adlbrecht
Medical University of Vienna, Austria

Active endothelin in pulmonary embolism

FINAL REPORT

Background

Chronic thromboembolic pulmonary hypertension (CTEPH) can occur after acute pulmonary embolism. Surgical pulmonary endarterectomy (PEA) is the treatment of choice for CTEPH. Recently, medical treatment with endothelin (ET) receptor antagonists (ERA) has been demonstrated to effectively decrease pulmonary vascular resistance. However, the pathophysiological basis for the ERA action is not entirely clear.

We hypothesised that CTEPH-thrombus-associated ET mediates vasoconstriction in the distal vascular bed, impairing pulmonary capillary flow and furthering adverse remodelling.

Aim

The aim of the present study was to investigate the role of thrombus-borne ET in CTEPH.

Methods

19 surgical thrombus samples from patients with CTEPH undergoing PEA were homogenised and subjected to *in vitro* vasoconstriction experiments on porcine artery rings evaluating dual and selective ET_A receptor antagonism. Thrombus ET synthesis was analysed in an animal model of CTEPH.

Results

CTEPH thrombi exerted significant vasoconstrictive properties in 17 of 19 (90 %) samples and injection of thrombus homogenate into arterial ring segments (final concentration 1 µg/mL) led to an average constriction of 16.1 % (range 10.6 – 30.0 %) of the maximum constrictive capacity.

Dual ET receptor blockade inhibited thrombus-induced constriction by 30.4 % (range 18.6 – 62.9 %) of the crossover-self control. Selective ET_A receptor blockade with BQ123 inhibited thrombus-induced constriction by 51.5 % (range 48.2 – 53.4 %) of the crossover-self control (Figure 1).

Vasoconstriction by *in vitro* formed control thrombi was not susceptible to inhibition with dual ET receptor antagonism: 97.2 % (range 93.1 – 101.3 %).

Immunohistological evaluation revealed co-localisation of ET-1 positivity (Figure 2, panel A) with $CD68^+$ macrophages (Figure 2, panel B) within the CTEPH thrombi (Figure 2, panels C and D: Negative control).

Conclusion

This is the first study analysing the ET content of surgical thrombus specimens of patients with CTEPH undergoing PEA. ELISA-determined immunoreactive ET concentrations were 83.2 fmol/mL thrombus (32.8 – 142.9). Homogenates of CTEPH thrombi induced significant arterial vasoconstriction that was inhibited by pre-incubation with an ET receptor antagonist, indicating that thrombus-borne ET is biologically active.

Our data suggest that ET shedding by proximal thrombi causes vasoconstriction and may further microvascular remodelling in distal vascular territories.

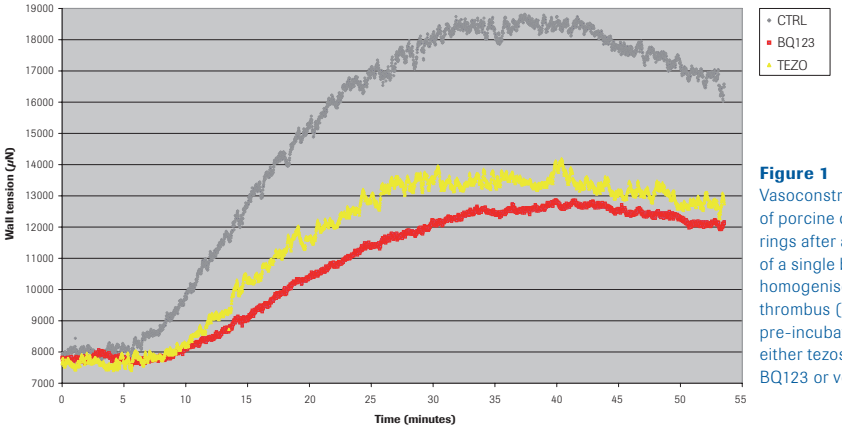

Figure 1
Vasoconstrictive response of porcine coronary artery rings after administration of a single bolus of homogenised CTEPH thrombus (130 µg/ml, t=0), pre-incubated with either tezosentan (TEZO), BQ123 or vehicle (CTRL).

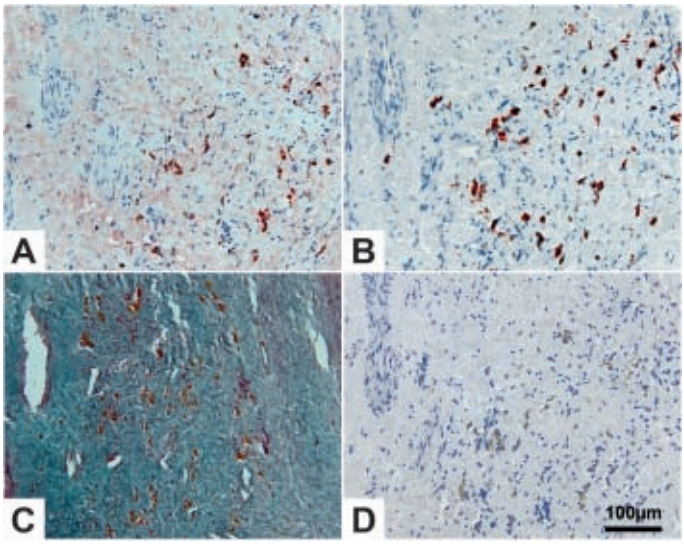

Figure 2
Representative immunohistochemistry of a CTEPH thrombus using anti-ET-1 (panel A), anti-CD68^{+} (panel B) and trichrome stain (panels C and D).

Age (years)	64 (50 – 66)
Mean pulmonary arterial pressure (mmHg)	45 (41 – 50)
Pulmonary vascular resistance (dyne·s·cm^{-5})	642 (419 – 760)
Cardiac index (L·min^{-1}·m^{-2})	2.3 (2.1 – 2.7)
Six-minute-walk distance (meters)	350 (223 – 450)
NYHA class (%)	
NYHA II	17
NYHA III	39
NYHA IV	44

Table 1
Baseline characteristics at inclusion.

All values are displayed as median (25th – 75th percentile)
NYHA = New York Heart Association

Dr Marina Anderson
University Hospital Aintree, Liverpool, UK

The interplay of vascular function, low grade inflammation and innate immunity in the disease process of Raynaud's phenomenon and systemic sclerosis

Our aim is to develop novel, integrated measures of disease activity and severity for use in systemic sclerosis (SSc), whilst elucidating and characterising the link between measures of low-grade inflammation, innate immune response and vasculopathy of this disease. We aim to apply these new measures in clinical studies of therapeutic agents, starting with the study of endothelin receptor antagonism.

We have studied a cohort of patients with SSc, primary Raynaud's phenomenon (RP), undifferentiated connective tissue disease (UCTD) and healthy age and sex-matched control subjects.

1. The innate immune system
NK cells phenotype and function

We determined the production of Th1/Th2 cytokines by natural killer (NK) cells in an *in vitro* stimulation assay.

	IL-6	IFN-γ	IL-2
UCTD	high	high	high
lcSSc	normal	normal	normal
dcSSc	normal	normal	low

Table 1. Production of IL-6, IFN-γ and IL-2 by NK cells.

IL-6, IFN-γ and IL-2 production distinguish between the different phenotypic presentations of autoimmune Raynaud's phenomenon (RP) (Table 1). In addition to decreased levels of the pro-inflammatory IL-2 in the supernatants of cultured NK cells, there were decreased levels of anti-inflammatory IL-10 in patients with diffuse cutaneous SSc (dcSSc). We observed marked variability in the levels of other cytokines tested within control and patient groups with no specific correlations to disease type.

Neutrophil phenotype and function

Proteomics

Building on our pilot proteomic work, we have shown that a number of proteins, including actin related peptide and actin capping protein (both integral to control of actin polymerisation), are expressed in higher concentrations in SSc neutrophils than in controls.[1,2] These protein changes are similar to those found in neutrophils activated by bacterial products or the inflammatory cytokine TNF.

In addition, a higher percentage of neutrophils from SSc patients have focal increases in the polymerised form of actin (F-actin) at the plasma membrane. After stimulation with fMLP, patient neutrophils have significantly higher increases in total F-actin content compared with controls.

Apoptosis

Compared with healthy neutrophils, significantly more apoptosis was observed in patient neutrophils after 2 and 5 hours, although this effect was no longer apparent after 20 hours, due to the cells reaching an apoptosis plateau. However, when control neutrophils were treated with sera from patients with RP, limited cutaneous SSc (lcSSc) and dcSSc, apoptosis of these healthy neutrophils was increased at 5 hours compared with the treatment with healthy serum, but again this effect was not apparent after 20 hours. Apoptosis of neutrophils from lcSSc patients was not significantly altered when treated with sera from healthy controls or patients with UCTD, lcSSc or dcSSc, although after 5 hours of treatment with disease sera, neutrophil apoptosis was comparable to that of neutrophils forced into apoptosis with the protein synthesis inhibitor, cycloheximide.[3] Therefore, neutrophils from SSc patients became apoptotic much earlier than those from healthy controls, but, importantly, were not prone to necrosis. Sera obtained from patients with UCTD, lcSSc or dcSSc caused increased apoptosis of healthy neutrophils when compared with healthy sera.

2. Peripheral venous blood measures of inflammatory response and vascular activation

We established that low grade inflammation, as measured by high sensitivity C-reactive protein (hsCRP), is associated with severe disease manifestations of SSc including pulmonary artery hypertension, digital ulceration / amputation and pulmonary fibrosis.[4] Characterising and correlating hsCRP and the panel of other measures of inflammatory response (IL-6) and vascular activation (von Willebrand factor, endothelial cell adhesion molecules, vascular endothelial growth factor and endothelin-1) with the innate immunity and vascular physiological function results are therefore of major interest in the work of this project.

3. Vascular physiological function

Macrovascular function, microvascular morphology and function have been examined and the data are currently being analysed. These will be correlated with results from the studies of the innate immune system.

In summary, our results support the hypothesis that the innate immune system is important in the pathogenesis of UCTD and SSc, with neutrophils from SSc patients being activated and altered in function to healthy neutrophils, and NK Th1/Th2 cytokine production distinguishing between groups. The patient serum contains a mediator that may be responsible for the change in neutrophil function, and future projects will determine which mediator is responsible, as well as to verify any other modifications in neutrophil function. Furthermore, the NK cell results suggest loss of function that may evolve with disease progression and phenotype, and a postulated course of events is outlined in Figure 1.

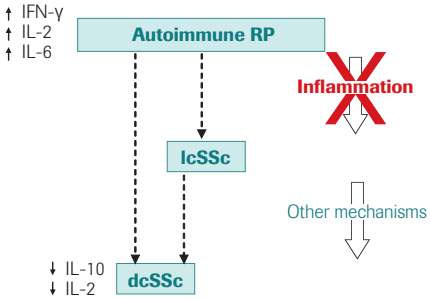

Figure 1. Evolution of Th1 / Th2 cytokine production from NK cells in Raynaud's phenomenon (RP) and systemic sclerosis (SSc). lcSSc = limited cutaneous SSc, dcSSc = diffuse cutaneous SSc

Following achievement of these initial aims, our long-term goal is to use the above measures to go on to test the hypothesis that ET-1 receptor antagonism concomitantly modulates innate immunity, low-grade inflammation and vascular activation and function in SSc and pre-SSc.

References:

1. Barnes T, *et al.* Proteomic studies suggest that neutrophils are activated in systemic sclerosis. *Arthritis & Rheumatism.* 2006; 54:S733.
2. Barnes T, *et al.* Neutrophil cytoskeletal abnormalities in systemic sclerosis. Poster presentation, British Society for Rheumatology Annual Scientific Meeting, Liverpool, April 2008.
3. Naylor EJ, *et al.* Effect of sera from patients with systemic sclerosis and Raynaud's phenomenon on neutrophil apoptosis. Poster presentation at the same meeting as in reference 2.
4. Sadik HY, *et al.* High sensitivity C reactive protein as a disease marker in systemic sclerosis. American College of Rheumatology Annual Scientific Meeting, Boston, November 2007.

Dr Adrian Gonon
Karolinska University Hospital, Stockholm, Sweden

Dr Magnus Settergren (co-investigator)
Dr Alexey Shemyakin (co-investigator)

The importance of endothelin-1 for vascular complications in patients with diabetes

FINAL REPORT

The first study within the project has been completed and published.[1] The objective of that study was to investigate if endothelin (ET) receptor blockade (selective ET_A and dual $ET_A + ET_B$) improves insulin sensitivity in patients with insulin resistance and coronary artery disease. Seven patients (age 58±2) with insulin resistance and coronary artery disease completed three hyperinsulinemic-euglycemic clamp protocols: a control clamp (saline infusion), during ET_A receptor blockade (BQ123) and during combined ET_A (BQ123) and ET_B receptor blockade (BQ788).

Main Results

Total body glucose uptake (M) differed between the clamp protocols with the highest value in the BQ123 + BQ788 clamp ($p<0.05$). The M-value corrected by insulin was higher in the BQ123+BQ788 than in the control clamp ($p<0.01$) or the BQ123 clamp ($p<0.05$) (Figure 1). There was no difference between the control clamp and the BQ123 clamp. Mean arterial pressure did not change during the control clamp, whereas it decreased during both the

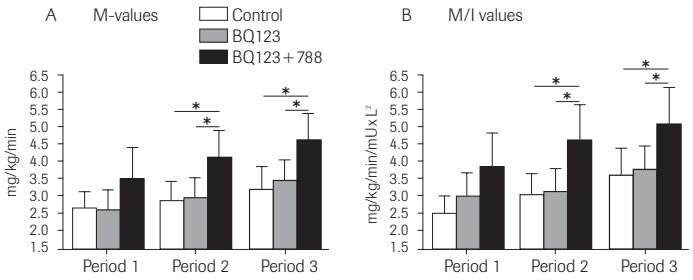

Figure 1
Effect of selective ET_A (BQ123) and combined ET_A/ET_B receptor blockade (BQ123 and BQ788) on (A) total body glucose uptake (M-value) and (B) insulin sensitivity (M/I value) in patients with insulin resistance and coronary artery disease. Combined receptor blockade improves both glucose uptake and insulin sensitivity in comparison with saline (control) and selective ET_A receptor blockade.

BQ123 ($p<0.01$) and BQ123+BQ788 ($p<0.05$) clamps. Renal blood flow increased and renal vascular resistance decreased in the BQ123 + BQ788 clamp ($p<0.05$) but not in the BQ123 clamp. There was no change in splanchnic blood flow in either clamp.

These data demonstrate that dual ET_A+ET_B receptor blockade enhances insulin sensitivity in patients with insulin resistance and coronary artery disease indicating an important role for endogenous endothelin-1 (ET-1) in the regulation of glucose metabolism in this patient group.

In a second study we evaluated the effect of ET receptor blockade on endothelial function and glucose uptake in skeletal muscle in patients with insulin resistance and in isolated skeletal muscle cells. Eleven male subjects (age 61 ± 3) with insulin resistance (total body glucose uptake <6 mg/kg/min and/or HOMA index >2.5) participated in 3 protocols: (1) control protocol: saline infusion followed by insulin infusion (0.05 mU/kg/min), (2) dual ET-blockade protocol: infusion of the ET_A receptor antagonist BQ123 (10 nmol/min) and the ET_B receptor antagonist BQ788 (10 nmol/min) for 45 min, followed by co-infusion of the antagonists and insulin for 1 hour, (3) ET-1 protocol: insulin infusion alone for 45 min, followed by co-infusion with ET-1 (20 pmol/min) for 1 hour. All infusions were given into the brachial artery at a rate of 1 ml/min. Forearm blood flow was measured with venous-occlusion plethysmography. Endothelium-dependent vasodilatation and endothelium-independent vasodilatation were determined by intra-arterial administration of acetylcholine and sodium nitroprusside, respectively. Forearm glucose uptake (FGU) was calculated from the arterio-venous plasma glucose concentration difference and plasma flow.

Insulin infusion increased FGU in all protocols ($p<0.001$). Dual ET receptor blockade increased forearm blood flow from 26.5 ± 2.6 to 33.8 ± 3.8 ml/min/1000ml ($p<0.05$) and FGU from 5.1 ± 1.0 to 8.3 ± 1.2 µmol/min/1000 ml ($p<0.05$) after 45 min of infusion. Co-administration of insulin further increased FGU to 15.7 ± 1.8 µmol/min/1000ml ($p<0.001$).

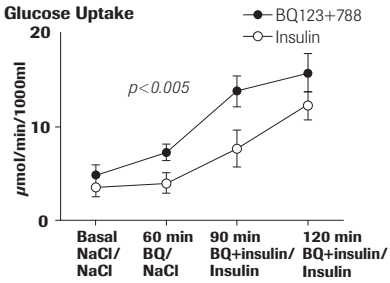

Figure 2
Effect of dual ET receptor blockade by BQ123 and BQ788 on skeletal muscle glucose uptake in the forearm of patients with insulin resistance. Dual BQ significantly increased glucose uptake in comparison with insulin.

The total glucose uptake by ET receptor blockade and insulin was significantly greater than that induced by insulin only (Figure 2). ET-1 decreased basal forearm blood flow by 35% but did not affect insulin-stimulated FGU. Insulin enhanced endothelium-dependent and endothelium-independent vasodilatation *per se* ($p<0.05$) as well as during ET blockade ($p<0.05$), but this effect was abolished by co-infusion of ET-1.

The study demonstrates that dual ET_A/ET_B receptor blockade enhances basal and insulin-induced skeletal muscle glucose uptake in subjects with insulin resistance, suggesting that endogenous ET-1 contributes to skeletal muscle insulin resistance. Insulin improves endothelial and smooth muscle cell function in subjects with insulin resistance. This effect is not further improved by ET receptor blockade.

The third study is the randomised study with oral treatment with the dual endothelin receptor antagonist/placebo on microvascular and macrovascular function in patients with type 2 diabetes and microvascular complications. The third study started in December 2007 and had not yet been completed at the time of this report.

References

1. Ahlborg G, Shemyakin A, Böhm F, Gonon A and Pernow J. Dual endothelin receptor blockade acutely improves insulin sensitivity in obese patients with insulin resistance and coronary artery disease. *Diabetes Care* 2007; 30:591-6.

Project data have been presented at the following scientific meeting

Pernow J, Gonon A, Shemyakin A and Settergren M. The pathophysiological significance of endothelial dysfunction in cardiovascular disease and diabetes. Swedish Cardiovascular Spring Meeting 2007, Gothenburg, April 2007.

Dr Sandrine Launois-Rollinat
University Joseph Fourier / CHU, Grenoble, France

Dr Jean-Philippe Baguet (co-investigator)
Prof. Diane Godin-Ribuot (co-investigator)

*Cardiovascular consequences of obstructive sleep apnoea: role of endothelin and preventive effects of a dual endothelin receptor antagonist **

Obstructive sleep apnoea (OSA) has become a major public health issue because of its cardiovascular consequences (hypertension, coronary syndromes and stroke), which appear to be triggered by nocturnal intermittent hypoxia (IH). Standard treatment by nocturnal application of continuous positive airway pressure (CPAP) reduces cardiovascular morbidity and mortality but is rejected by approximately 20 % of OSA patients. Identifying biomarkers of OSA-related cardiovascular risk and evaluating alternative treatment strategies will improve management of patients who cannot use CPAP. Endothelin-1 (ET-1) is a potent vasoconstrictor produced by endothelial cells under hypoxia. Its role in endothelial dysfunction, atherosclerosis and inflammation is well-established. In animals exposed to chronic IH as well as in OSA patients plasma ET-1 levels are increased and in patients they return to normal following CPAP treatment. A dual endothelin receptor antagonist currently indicated in pulmonary hypertension, lowers systemic blood pressure, improves endothelial function and decreases atherosclerosis in animals and humans.

The aim of our project is two-fold: (1) to evaluate the role of ET-1 in the cardiovascular risk in OSA and (2) to examine the effect of the above-mentioned dual endothelin receptor antagonist on the cardiovascular alterations in OSA patients. In the first study, we hypothesised that OSA patients with a recent history of cardiovascular events would have higher plasma ET-1 levels than matched OSA patients with no such history and matched non-OSA patients with a similar cardiovascular history (Table 1). However, we found no difference in circulating ET-1 levels in these groups of patients, although patients with severe OSA had higher ET-1 plasma concentration than those with milder OSA or than healthy individuals (Figure 1). Although our study does not confirm that ET-1 levels could be used as a marker of cardiovascular risk in OSA patients, the role of the ET-1 system in the pathogenesis of OSA cardiovascular consequences still needs to be

** Note: The use of bosentan in this project falls outside of the approved indications.*

further investigated in apnoeic patients. In the second study, we are currently evaluating the effects of a dual endothelin receptor antagonist administration on blood pressure, endothelial function and inflammation markers in hypertensive OSA patients.

In summary, this project is testing the hypothesis that endothelin is involved in the physiopathology of OSA and will help clarify its role as well as its potential use as a therapeutic target in the clinical management of OSA patients.

	OSA-CVE *n=20* (1 female)	**CVE** *n=12* (2 females)	**OSA** *n=32* (6 females)	**C** *n=16* (6 females)
Age (years)	53.8 ± 8.8	52.5 ± 9.1	51.2 ± 8.9	45.8 ± 7.9
BMI (kg/m^2)	25.7 ± 3.5	24.8 ± 2.7	25.6 ± 2.7	23.9 ± 3.3
RDI (per hour)	38.1 ± 18.1	9.4 ± 4.2	42.1 ± 16.2	7.4 ± 4.4
T90 (%TST)	5.19 ± 13.5	1 ± 2.5	3.7 ± 6.4	0.04 ± 0.1

Table 1
Main characteristics of the four subject groups. The four groups were: OSA patients with a recent cardiovascular event (OSA-CVE group; CVE is defined as stroke or acute coronary syndrome), patients without OSA but recent CVE (CVE group), OSA patients with no history of CVE (OSA group) and a control group of healthy subjects (C group). Age and body mass index (BMI) were not different in the four groups. BMI and T90 (percentage of sleep with hemoglobin saturation under 90%) were not significantly different between the two OSA groups and between CVE and control subjects. BMI and T90 were significantly different ($p<0.05$) between the two OSA groups and CVE and between the two OSA groups and control subjects. Data are presented as mean ± SD. RDI = respiratory disturbance index; TST = total sleep time

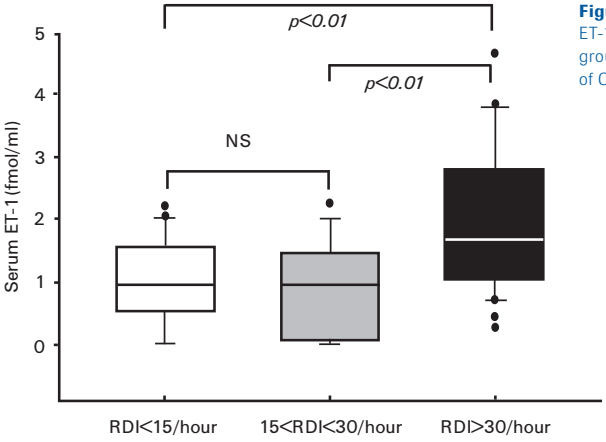

Figure 1
ET-1 concentration in subjects grouped according to the severity of OSA

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Dr Peter Dorfmuller (co-investigator)
Frédéric Perros (co-investigator)
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Endothelin receptor antagonism and dendritic cell function in pulmonary arterial hypertension

The aim of this study is to demonstrate *in vivo* that there is a differential distribution in myeloid dendritic cell (mDC) and plasmacytoid DC (pDC) subpopulations in the circulating DCs between patients with pulmonary arterial hypertension (PAH) and controls and that DCs and their precursors release endothelins. The second aim is to show *in vivo* that endothelin receptor blockade with a dual endothelin receptor antagonist decreases the expression of immune-stimulating molecules by DCs. We also hypothesised that endothelin-1 (ET-1) may contribute to the DC-induced proliferation of smooth muscle cells and may be a chemotactic factor for DCs.

Blood samples

Blood is obtained from PAH patients and controls after written informed consent in the Pulmonary Hypertension Centre, Antoine Béclère Hospital. The French Ethics Committee authorised this at the beginning of the recruitment. At the present time, blood samples have been obtained from 20 PAH patients and 15 controls to evaluate circulating DCs and their distribution as mDC or pDC.

Circulating dendritic cells

Isolation of mononuclear cells from peripheral blood: peripheral blood mononuclear cells (PBMCs) are obtained by gradient separation of fresh whole blood using cellular preparation tubes or Ficoll-Histopaque. mDCs and pDCs are analysed by four-colour flow cytometry. The Blood Dendritic Cell Enumeration Kit is used in whole blood. Briefly, 300 µl of whole blood is incubated for 10 minutes in the light with a pre-mixed antibody cocktail for DC detection or for isotype control, and a dead cells discrimination dye. The pre-mixed antibody cocktails for DC detection are composed of the following antibodies: anti-BDCA-1 PE, anti-BDCA-2 FITC, anti-BDCA-3 APC, anti-CD14 PE-Cy5, anti-CD19 PE-Cy5. From each tube, 800,000 events are acquired and analysed on a FACScalibur flow cytometer using

CellQuest Pro software. Evaluation of distribution of mDCs and pDCs was effective and more samples are needed to have complete results.

Dendritic cell isolation

PBMCs will be isolated from buffy coats from the blood with Miltenyi's kits, following the manufacturer instructions. Isolations of pDC, mDC and monocytes from these samples are in progress.

Cell cultures

Cell cultures from pulmonary smooth muscle cells are obtained by enzymatic digestion of rings of control- and PAH-pulmonary arteries. Cell culture from monocyte-derived DCs (Mo-DCs) are isolated, monocytes are cultured for 7 days with IL-4 and GM-CSF to generate immature Mo-DCs and then cultured for additional 3 days with TNF-α for maturation. The procedures for these cell cultures are currently in place in our laboratory.

Measurement of ET-1, big ET-1, ET-2 and ET-3

ELISA kits for ET-1, big ET-1, ET-2 and ET-3 are currently being tested to measure levels of endothelin in the supernatants of cell culture from Mo-DCs and monocytes.

Dr S. John Wort
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Dr Simon Davidson (co-investigator)
Dr Michael Gatzoulis (co-investigator)
Dr Mark Griffiths (co-investigator)
Dr John Park (co-investigator)

The effect of a dual endothelin receptor antagonist on platelet function in patients with pulmonary arterial hypertension *

In situ thrombosis is commonly seen in post-mortem specimens from patients with pulmonary arterial hypertension (PAH), and mounting evidence suggests that activated platelets are involved in the pathogenesis of this disease. Although anti-coagulation with warfarin is a standard therapy in the management of PAH, anti-platelet agents are not. It is hypothesised that platelets play a role in endothelial cell activation. However, there is conflicting *in vitro* evidence for the role of ET-1 and endothelin receptor antagonists in platelet activation. We, therefore, set out to assess the effect of a dual endothelial receptor antagonist on platelet function in patients with idiopathic PAH and PAH associated with other causes.

Data from healthy volunteers

We have performed preliminary *in vitro* investigations on healthy human subjects. We have assessed the effect of the addition of a dual endothelin receptor antagonist (1 μM) on whole blood impedance platelet aggregometry. Collagen, a potent platelet stimulator, was selected as the agonist for these experiments. Whole blood was used to retain the larger more active platelets, often lost when isolating platelets for other forms of platelet analysis. In addition, red and white blood cells are also important in supplying ADP and ATP to platelets during aggregation. This medium is therefore more physiological. The results of the impedance aggregation can be seen in Figure 1. The concentration of collagen used was 2 μg/mL. The final concentration of the dual endothelin receptor antagonist was 1 μM. As shown in Figure 1, the impedance aggregation (collagen) in healthy volunteers was 15.38 ± 5.3 ohms, and in healthy volunteers receiving the dual endothelin receptor antagonist 3.75 ± 4.4 ohms. Results are expressed as mean $\pm$ SD, $p \leq 0.001$ ($n = 10$).

Patient data

As per protocol, blood was taken from patients before administration of the dual endothelin receptor antagonist, 4 hours after administration, 1 month after chronic dosing and after

3 additional months of therapy. Whole blood was tested with collagen (2 mg/ml) as the platelet aggregation stimulus.

The antagonist-naïve sample was spiked with 1 µM of the antagonist to study the *in vitro* response. At all stages, plasma is separated and stored at –20 °C for future ELISA analysis (as per protocol). Of the 4 patients studied, one has idiopathic PAH and the other three Eisenmenger complex. Results of the impedance aggregation can be found in Figure 2. Results are expressed as mean ± SD. As sample numbers are small, no statistical analysis has been performed. As in healthy volunteers, the *in vitro* spike of the antagonist had a profound anti-aggregatory effect on platelets stimulated with collagen. This response appeared to persist *in vivo* at day 1, although there was more variation in response. It is unclear at present whether this anti-platelet effect is maintained at one month ($n = 3$).

We believe that these preliminary data support the concept that the dual endothelin receptor antagonist used in our study has an anti-platelet aggregation effect both *in vitro* and *in vivo*. As patient numbers are low for the *in vivo* data, we have not been able to perform statistical analysis. We are particularly interested whether patients with sub-groups of PAH will have different responses to the antagonist. By the end of the study we will have looked at patients with idiopathic PAH, Eisenmenger complex and PAH associated with interstitial lung disease.

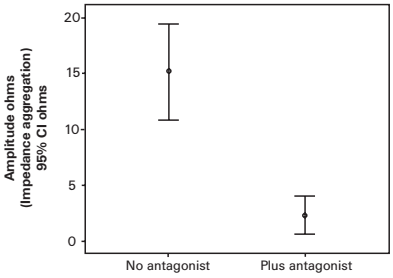

Figure 1
Healthy volunteers ± dual endothelin receptor antagonist (1 µM) – Impedance aggregation (collagen 2 mg/ml). $n=10$.

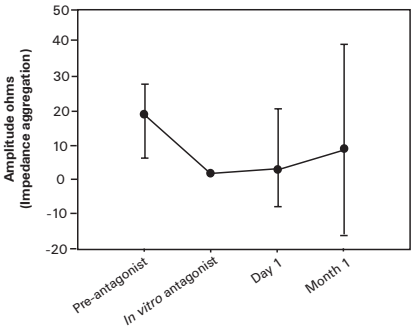

Figure 2
Patient data. Dual endothelin receptor antagonist (1 µM) – Impedance aggregation (collagen 2 mg/ml). $n=3–4$.

* *Note: The use of bosentan in this project falls outside of the approved indications.*
** *Please note that this is a re-print of Dr. Wort's progress report from the 2008 AERA activity report. A final report is still pending.*

Prof. Robert J. Moots
Liverpool, UK (Chairman)

Robert J. Moots is Professor of Rheumatology and Head of the Division of Inflammation at the University of Liverpool, U K. After qualifying in medicine in London, he trained in immunology at the University of Oxford, earning his PhD, and continued his research on immunology of autoimmune diseases at Harvard Medical School, returning to Liverpool in 1997. In 2002 he became Professor of Rheumatology in Liverpool, the youngest appointment to such a post in the UK. His research interests lie in clinical and basic science aspects of inflammatory rheumatic diseases, particularly innate cellular immunity in rheumatoid arthritis and systemic sclerosis. He runs a drug discovery programme, has published extensively in both clinical and laboratory science, has won national and international prizes for research, is active in many medical and scientific societies, and is an active international speaker. He has recently become Editor of the journal *Rheumatology*.

Prof. Damien Bonnet
Paris, France

Damien Bonnet is Professor of Paediatric and Congenital Cardiology and the Director of the Reference Centre for Complex Cardiac Malformations at the Necker Hospital for Sick Children, René Descartes University, Paris, France. Professor Bonnet's main interests include the mechanisms and molecular basis of congenital heart disease (CHD), prenatal diagnosis of CHD, non-invasive vascular physiology in children, and pulmonary hypertension in children and in patients with CHD.

Prof. Christian Kähler
Innsbruck, Austria

Christian Kähler is Director of Pneumology at the University Hospital for Internal Medicine, Innsbruck, Austria, and head of one of the three University Specialised Centres for Pulmonary Hypertension in Austria. Professor Kähler has spent much of his professional career in Innsbruck where he has worked in the fields of clinical/experimental research in pneumology. His main topics of research are pulmonary hypertension, stem cell biology and neurogenic inflammation. He has over 80 publications in peer-reviewed journals and besides other scientific prizes his team was awarded the ERS Clinical Allergy and Immunology Award in 2003. Professor Kähler is a member of the Austrian Society of Pneumology (ASP), Secretary of the assembly group "mechanisms of lung injury and repair" of the European Respiratory Society (ERS) and member of the American Thoracic Society (ATS).

Dr Alessandra Manes
Bologna, Italy

Alessandra Manes received her medical degree with the academic honour *cum laude,* in 1996. She earned her PhD, also graduating *cum laude* at the Medical Faculty, University of Bologna (UoB), Italy in 2003.

She is a staff member of the Institute of Cardiology of the UoB and serves as the coordinator of the Pulmonary Hypertension Centre. Dr Manes teaches at the international master's degree programme on pulmonary vascular diseases at the UoB. She has authored more than 200 scientific publications on pulmonary hypertension and heart failure, and has received the "François Brenot Award" of the European Respiratory Society in 1999 as "Promising Young Investigator in Pulmonary Vascular Sciences".

Dr John Pernow
Stockholm, Sweden

John Pernow, MD, PhD, FESC is Chief of the Cardiology Unit, Department of Medicine at Karolinska Institutet and Senior Consultant in the Department of Cardiology at Karolinska University Hospital, Stockholm. He has authored over 100 original publications and 40 reviews in the field of cardiovascular pharmacology and endothelial function.

Prof. Thomas Unger
Berlin, Germany

Thomas Unger serves as the Chair of Pharmacology, Director of the Institute of Pharmacology, and Director of the Center for Cardiovascular Research at the Charité Berlin. He is also the Chairman of the German Institute for High Blood Pressure Research in Heidelberg. From 2001 until 2006 he was Director of the Institute of Pharmacology and Toxicology Charité Berlin and between 1994 and 2001 the Director of the Institute of Pharmacology at the University of Kiel, Germany. Until 1994, Professor Unger held professorships in pharmacology and hypertension research at the University of Heidelberg. He is a member of several national and international scientific societies, and has authored more than 700 scientific publications. He has also been a member of diverse editorial boards of major scientific journals. Professor Unger has received several awards in recognition of his scientific achievements.

Dr Frank van den Hoogen
Nijmegen, The Netherlands

Frank van den Hoogen is Director of the Rheumatology Centre of Sint Maartenskliniek, Nijmegen, the Netherlands. He graduated with his PhD in Internal Medicine in 1994. The subject of his thesis was systemic sclerosis: effects of treatment with methotrexate.

He is also head of the Scleroderma Unit Nijmegen. Since 1987 he has initiated and has been involved in many studies in the areas of etiopathogenesis, molecular biology, innovative therapies (including biologics and stem cell transplantation), social impact and follow-up of patients with systemic rheumatic autoimmune diseases. Dr van den Hoogen's scientific work has resulted in more than 150 papers in peer-reviewed journals.

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