European Cooperation in Science and Technology
- COST -

Secretariat

Brussels, 16 December 2010

COST 4196/10

MEMORANDUM OF UNDERSTANDING

Subject :	Memorandum of Understanding for the implementation of a European Concerted Research Action designated as COST Action BM1005: Gasotransmitters: from basic science to therapeutic applications (ENOG: European Network on Gasotransmitters)

Delegations will find attached the Memorandum of Understanding for COST Action BM1005 as approved by the COST Committee of Senior Officials (CSO) at its 180th meeting on 1 December 2010.

MEMORANDUM OF UNDERSTANDING
For the implementation of a European Concerted Research Action designated as

COST Action BM1005
GASOTRANSMITTERS: FROM BASIC SCIENCE TO THERAPEUTIC APPLICATIONS
(ENOG: EUROPEAN NETWORK ON GASOTRANSMITTERS)

The Parties to this Memorandum of Understanding, declaring their common intention to participate in the concerted Action referred to above and described in the technical Annex to the Memorandum, have reached the following understanding:

1. The Action will be carried out in accordance with the provisions of document COST 4159/10 "Rules and Procedures for Implementing COST Actions", or in any new document amending or replacing it, the contents of which the Parties are fully aware of.

2. The main objective of the Action is to boost the quality, competitiveness and impact of European biomedical research in the field of gasotransmitter molecules and to translate the progress made into potential therapies. The network will pool, categorize and share specific knowledge, skills, expertise and reagents among the ENOG participants; the formation of the Action will also increase the targeted interactions between a multi-disciplinary group of researchers with expertise that ranges from computational and synthetic chemistry to pharmacology, drug design and development.

3. The economic dimension of the activities carried out under the Action has been estimated, on the basis of information available during the planning of the Action, at EUR 48 million in 2010 prices.

4. The Memorandum of Understanding will take effect on being accepted by at least five Parties.

5. The Memorandum of Understanding will remain in force for a period of 4 years, calculated from the date of the first meeting of the Management Committee, unless the duration of the Action is modified according to the provisions of Chapter V of the document referred to in Point 1 above.

A. ABSTRACT AND KEYWORDS

The biological and medical importance of the endogenously generated gaseous transmitter nitric oxide was recognized by the 1998 Nobel Prize for Medicine/Physiology. It is now clear that several "gasotransmitters" exist and this class of mediators also includes carbon monoxide (CO) and hydrogen sulfide (H_2S). Several of the groups leading this research field are based in Europe, thus, the formation of a European network that collates scattered knowledge, resources and expertise on gaseous mediators will encourage the dissemination of innovation, boost collaborations and increase the European competitiveness in this field. In the longer term, we anticipate this concerted effort will accelerate translational research in inflammation, cardiovascular disease and CNS disorders, areas in which the biology of these three gaseous molecules has been implicated. The benefits from the support of such a network are expected in multiple levels: scientific, innovation/technological and societal.

Keywords: nitric oxide, carbon monoxide, hydrogen sulfide, pharmacology, physiology, cardiovascular, inflammation, drug discovery

B. BACKGROUND

B.1 General background

Gasotransmitters are a family of small-molecule signalling mediators which have come to the fore of biomedical research due to their perceived importance in the homeostatic regulation of numerous organ systems (e.g. cardiovascular, gastrointestinal, urogenital, nervous and immune), and the therapeutic potential of drugs that can manipulate these parallel signal transduction pathways.

The importance of this Action is exemplified in the cardiovascular system. Cardiovascular disease (CVD) causes ~1/3 of all deaths in the world (WHO). Of these, ~50% are attributed to coronary heart disease (CHD) and ~28% to stroke. Whilst lipid lowering drugs (e.g. statins), aspirin, early reperfusion therapy and hypertension therapy have substantially reduced the incidence of mortality from CVD (by ~25%). However, significant mortality still remains and is associated with increasing levels of morbidity, inflicting a substantial health burden to society. It is now accepted that one of the initial triggers for CVD, and one that persists throughout the disease course, is a defect in the release of the gasotransmitter NO from the endothelial cells that line the inner surface of all blood vessels; more recently H_2S has also been implicated in the control of blood pressure. Therefore, a more complete understanding of the physiological and pathological roles of gasotransmitters is likely to define new mechanisms which can be manipulated pharmacologically to restore, or replace, the diminished gasotransmitter seen in CVD and consequently have obvious therapeutic use potential.

One of the strengths of this proposal lies in the multi-disciplinary approach**.** The groups assembled to participate in the ENOG network combine expertise from a variety of disciplines including computational chemistry, medicinal chemistry, biochemistry, cell biology, generation of genetic models, drug discovery, as well as in vitro and in vivo pharmacology/physiology and translational studies in healthy volunteers. Thus, this COST Action will jointly apply a molecule-to-man approach to understanding the biology of gasotransmitters, something unattainable by individual groups or small-scale collaborations, but achievable by a pan-European network. Each member of the COST Action possesses funding from country-specific origins to study distinct topics related to gasotransmitter biology.

B.2 Current state of knowledge

The gasotransmitter family consists of endogenously-produced gaseous signalling molecules and includes nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S). All three gasotransmitters have some common properties: they are enzymatically generated by mammalian cells under basal and/or stimulated conditions, freely permeate cell membranes and possess numerous autocrine, paracrine and endocrine effects. NO is synthesized from the amino acid L-arginine, by a two step, O_2-requiring reaction producing L-NG hydroxyarginine as an intermediate and with concomitant citrulline production. Multiple enzyme isoforms have evolved to generate NO; NO is synthesized by the constitutive calmodulin-dependent nitric oxide synthases (NOS; types 1 and 3) or by the inducible NOS2 form that is crucially involved in inflammatory responses. CO is formed by haem oxygenase (HO), which exists in three isoforms i.e. HO-1, HO-2 and HO-3; HO-2 is constitutively expressed while HO-1 is inducible by physiological and pathological stimuli. Our understanding of H_2S-synthesis is not as advanced as the other two gasotransmitters; it is produced in mammalian tissues by the action of both enzymatic and non-enzymatic pathways. In mammalian tissues, H_2S is generated from L-cysteine by at least four separate pathways. Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) appear to be responsible for the majority of the endogenous production of H_2S.

NO, CO and H_2S differ significantly in the signalling mechanisms they employ. NO in mammalian cells is produced through the three NOS isoforms and exerts many of its effects by stimulating soluble guanylyl cyclase. cGMP then alters the activity of a variety of signalling pathways, including protein kinase G, cGMP-gated ion channels and phosphodiesterases. The action of cGMP is terminated by phoshodieasterases. The activities of NOS are regulated at the transcriptional, post-transcriptional and post-translational levels. In addition, NO exerts some of its effects by promoting post-translational modifications of proteins. On the hand CO mainly interacts with heme-containing proteins. H_2S is thought to mediate its effect by activating K_{ATP} channels and by promoting the sulphydration of cysteine residues on proteins. H_2S seems to be less selective in its intracellular targets, since it binds zinc and zinc containing proteins, participates in redox reactions and reduces ATP levels by inhibiting cellular respiration. Gasotransmitters have a wide array of effects on different cells and tissues in the body.

Since a primary focus of our network will be the cardiovascular system it should be emphasized that NO, CO and H_2S exert similar and complementary effects in the vasculature and heart. For example, all of the gasotransmitters are reported to be cardioprotective in ischemia-reperfusion models and exert ionotropic effects. NO, CO and H_2S also all inhibit platelet aggregation, presumably by distinct mechanisms. All three gasotransmitters are vasodilators with their potency depending on the vascular bed and species studied; however, CO and H_2S have been described to constrict some vessels. All of the gases are angiogenic; NO and CO have been implicated in vascular remodelling and atherosclerosis. Low levels of NO are generally protective in the vasculature as they limit leukocyte adhesion to the vessel wall, while high levels, such as those produced by iNOS have been associated with inflammatory responses. The role of H_2S in inflammation is less clear as both pro- and anti-inflammatory properties have been ascribed to this gasotransmitter.

Originally classified as environmental pollutants, gasotransmitters have been radically re-evaluated, starting from the demonstration, 30 years ago in 1980, that NO promotes vasorelaxation, an observation rewarded with the Nobel Prize for Physiology and Medicine in 1998. In fact, the accumulated knowledge on the mechanisms of production, activity and signalling of NO, which is the best-studied gasotransmitter, can serve as a guide to make fast inroads into the biology of CO and H_2S, since many similarities among the three gases seem to exist. Several diseases have been identified where dysfunction of NOS has been detected and/or where modulation of NO levels and signalling can modify the course or severity of the pathology; NO donors have been in clinical use for over a century (e.g. nitroglycerine for angina pectoris and heart failure), while NO gas is clinically used to treat persistent pulmonary hypertension of the newborn. Modifiers of the NO/cGMP pathway are widely used drugs, for example sildenafil (Viagra®) in erectile dysfunction and in pulmonary hypertension. Although considerable progress in NO pharmacology has been achieved, drug discovery in the field of CO and H_2S is still lagging behind. In part this lag is due to the lack of specific and potent reagents with which the biology of both CO and H_2S biology might be analyzed. Testing the efficacy and specificity of novel reagents in multiple systems and by more than one research groups, and having a structured platform for exchanging this information would undoubtedly accelerate evaluation of novel tools and expose any potential problems such as toxicity.

Gasotransmitters have been studied extensively over the past 30 years, particularly from European researchers, with publications in NO, CO and H_2S, in total more than 150,000 in a recent PubMed search. Despite this long history, numerous novel aspects of gasotransmitter research are considered to be at the cutting-edge of research. For example, groups within this COST Action have already shown that dietary nitrate lowers blood pressure and protects against CVD – and discovered that that this bioactivity can partly account for the cardioprotective effects of a diet rich in fruit and vegetables. This seminal work has led to considerable scientific and media interest (http://news.bbc.co.uk/1/hi/7228420.stm) due to its putative societal impact. In addition, members of the consortium have pioneered the work leading to the synthesis of novel CO-releasing molecules that have been tested with promising results in preclinical models of disease and which are expected to enter into clinical trials shortly. Finally, centres that participate in this COST Action have pioneered the experimental use of H_2S and H_2S-releasing molecules in a number of diseases of the vasculature; again, this work has garnered much scientific and general media interest (http://corrieredelmezzogiorno.corriere.it/napoli/notizie/cronaca/2009/17-marzo-2009/solfatara - presenze-decuplicate-weekend-grazie-viagra-naturale--1501094541443.shtml).

The scientific field of gasotransmitter biology is highly competitive because of its therapeutic promise; surprisingly, however, there is no European network currently available that pools together the considerable but fragmented expertise. It is therefore obvious that a COST-based initiative would be a significant boost for therapeutics-oriented European research in two ways. Firstly, by facilitating collaboration it is likely that the pooling of knowledge and expertise on various physiological systems and the combination of complementary angles will result in the identification and in-depth characterization of pathophysiological conditions where CO or H_2S signalling is dysfunctional, Secondly, the combination of know-how of our research consortium will clearly enable joint development of potent and specific gasotransmitter mimics or of selective modifiers of endogenous production. The interdisciplinary team, by exchanging reagents, tools, information, data, expertise and ideas will undoubtedly contribute to the progress of this exciting biological field.

B.3 Reasons for the Action

This COST Action will launch to advance both economical/societal needs and to promote scientific/technological advance. Of note, the most important area of impact is exemplified by cardiovascular disease, which is currently a terrible burden on society in terms of mortality and morbidity and constitutes a huge financial liability, either directly in terms of health costs or indirectly due to lost productivity. The Action comprises expertise in a wide array of disciplines enabling it to combine basic and translational research aimed at patient care and at the development of novel therapeutics. This is in significant degree achieved by including not only world-renowned medicinal chemists and clinicians within the network, but in addition by bringing in expertise from the pharmaceutical industry with a vested interest in the field. In all, this scheme optimises the likelihood of a tangible outcome in terms of advancing therapeutic options in CVD and a number of other diseases where gasotransmitters are of interest (e.g. gastrointestinal, respiratory). We anticipate that the successful implementation of this COST Action will result in sustainable cutting-edge research in the gasotransmitter field within Europe and that the resulting insights will permit a subsequent, larger application within the European Framework Programmes.

The network participants have agreed to directly share and exchange not only laboratory personnel, samples and reagents, but also scientific information via a website, through a newsletter and through the organization of regular network meetings and a final international conference**.** The expected benefits have wide implications: (i) scientific (primary): the advancement of knowledge on the biology of gasotransmitters, with coordinated and intense interaction between the participating groups; (ii) innovation-technological: the acquired knowledge and the novel reagents will facilitate the development of innovative therapeutics-drugs, thus allowing the translation of scientific knowledge into preclinical or (further ahead) clinical therapeutics; (iii) educational*:* the network will be responsible for educating and forming young researchers; (iv) *societal*: in the long run, the translation of scientific advances into novel, more selective and more economical therapies will result in better health management; and finally (v) economical*:* the advancement in knowledge will lead to development of novel therapies, in large part by ENOG participating European companies, boosting enterprise competitiveness and strengthening employment of highly skilled personnel.

The scientific impact of the proposed network will be primarily by bringing an interdisciplinary perspective to the gasotransmitter. In addition, it will foster innovation in the study of gasotransmitters, through the intense integration of technologies, protocols and techniques that are currently scattered in the various research centres throughout Europe.

B.4 Complementarity with other research programmes

This COST Action will be in close collaboration with other existing programmes and networks such as: CORMRA-FP7-SME-2008-1 (on rheumatoid arthritis) and of the project CORMPOI of the Eurostars program within Eureka (on post operative ileus) and as participant in the project NANOFOL-FP7-NMP-2008-Large-2 (on nanodevices with folates).
It is clear that the synergy and harnessing of all these resources across Europe is best achieved through shared funding. In this respect, the COST Action will provide a timely opportunity to capitalise on our unique capabilities to establish a valuable and effective collective.

C. OBJECTIVES AND BENEFITS

C.1 Main/primary objectives

The overall objective of the ENOG Action is to boost the quality, competitiveness and impact of European biomedical research in the field of gasotransmitter molecules and to translate the progress made into potential therapies. This will be achieved by establishing and fostering a broad network of researchers currently working on gasotransmitters in an isolated fashion. The Action will pool, categorize and share specific knowledge, skills, expertise and reagents among the ENOG participants; the formation of the Action will also increase the targeted interactions between a multi-disciplinary group of researchers with expertise that ranges from computational and synthetic chemistry to pharmacology and drug design and development.

C.2 Secondary objectives

The following secondary objectives are an integral and important part of ENOG, and include:

1) Rapid dissemination of specialised knowledge, experience and skills contributed by each participating group, both to other ENOG members as well as to the international community at large, to be achieved by:

   - Publication of joint reviews on gasotransmitters
   - Creation and maintenance of a website, where updates of the ENOG activity will be uploaded
   - Organisation of an international scientific meeting on the chemistry, biochemistry and biology of gasotransmitters

2) Application of knowledge to the development of novel bioactive compounds that have therapeutic potential. We believe that the participation of small medium enterprises (SME) Alfama Ltd, Pharmahungary and Vasopharm, with expertise in developing NO-modifying and CO-releasing molecules will aid greatly in this direction. To this end, we expect that ENOG will result in:

   - Identification of molecules that either release CO and H_2S, or inhibit the release of the above two gases (enough such molecules exist in the field of NO) and molecules that interfere with the biological activity of NO.
   - Preliminary characterization of the above molecules and the comparison of their bioactivity to that of current "golden standards" in vitro and in animal models of disease, and
   - Submission of patent applications by the individual Inventors, covering the above molecules

3) Training of young researchers, to ensure proper continuation of research in the field:

- Encourage the attendance and participation of young researchers in WS, TS and STSM
- Encourage the participation of ENOG members in the scientific committee of MSc or PhD theses of post-graduate students affiliated with other ENOG laboratories

4) Establish a well-coordinated interdisciplinary team of leading scientists that will apply for financial support in relevant Framework Programme calls.

- Identify Framework Programme calls and submit by ENOG members of at least one grant application relating to gasotransmitter chemistry and/or biology by the end of 2012.

C.3 How will the objectives be achieved?

The objectives of this Action will be achieved by networking and bringing together scientists working on complementary fields; this will allow the necessary interactions, exchange of information, dissemination of knowledge and expertise and of course the promotion/intensification of collaborations. The interdisciplinary nature of our network will be re-enforced by: (i) encouraging the participation of ENOG members in more than one WG, (ii) holding joint WG meeting so that every WG will have at least one joint meeting with every other WG and (iii) organize two workshops during which members of all WG will be invited to attend. A number of meetings are held annually or biannually at the European or International level in the fields of NO, CO or H_2S, but there appears to be a fractionation of efforts, as each of these meeting focuses on a narrow aspect of each gasotransmitter. It should also be kept in mind that the vast majority of the meetings are on NO. Given the similarities that exist between gasotransmitters, it is expected that progress in the area of the newer gaseous mediators CO and H_2S will be expedited by utilizing paradigms form the more advanced NO field. In the existing meetings, research topics usually focus on a specific line of research lacking trans- or interdisciplinary approaches. This Action will apply a much-needed interdisciplinary approach to improve the current understanding of the biology of

gasotransmitters and to define future directions of high priority in NO, CO and H_2S research. The ENOG participants will make efforts to attract and involve scientists who work on related areas so that a cross-fertilisation of ideas takes place. To maximise impact, the Action will utilise the available scientific fora, European and International societies etc. (i.e. will link Action-meetings to larger consortia meetings/societies' annual meetings such as the cGMP meeting that is co-organized by one of the ENOG network participants, the British Pharmacological Society meetings, the EUPHAR and IUPHAR congresses).

More specifically, the objectives will be achieved through the following instruments*:*

• Exchange visits between collaborating laboratories for transfer of know-how and technology, especially for -but not limited to- young scientists.
• An annual meeting will be organized by each WG where researchers will present their current research to rapidly disseminate scientific knowledge and stimulate interactions and collaboration.
• Presentations of current research work and results to the biopharmaceutical industry will be organized in parallel with the yearly meetings (a dedicated extra day of the meetings) in order to stimulate greater industrial interest/involvement in the field of gasotransmitters.
• A website will be created that will contain information on gasotransmitter molecules, research results and publications.
• In addition, an electronic newsletter will be published biannually.

C.4 Benefits of the Action

The expected benefits cover the following broad areas: innovative scientific advances in the field of gasotransmitters, collaborative team-building aiming at successful grant applications, training of young scientists, synergy between academia and bio-pharmaceutical industry and finally, increasing awareness, in the scientific community and beyond of the role of NO, CO and H_2S in health and disease.

More specifically, we expect that the ENOG Action will**:**

- Speed up the emergence of a much better understanding of gasotransmitters' basic biology (including new aspects of NO), based on frequent interdisciplinary scientific interactions

- Identify novel pathophysiology areas where NO, CO or H_2S play important roles, to be substantiated by ENOG pre-clinical investigations in vitro and in vivo
- Significantly increase the effectiveness of the research performed by the individual ENOG groups, because of the synergy afforded by this Action
- Generate novel expertise and much-needed (bio)-chemical tools (e.g. antibodies, siRNAs, methods of their use), especially in the newer fields of CO and H_2S.
- Synthesize novel, possibly patentable, experimental molecules, which will significantly advance basic and applied research in the scientific field of gasotransmitters, both in academia and by the participating industrial partners
- Global strengthening of European research in a competitive, high-visibility field, with possible recognition by Framework Programme 7 support, upon successful application
- Disseminate the pooled knowledge within the ENOG team through workshops and conferences, and to all interested researchers in the EU and globally via the maintenance of an ENOG-dedicated website that will be advertised in all meeting attended by members of the Action
- Foster the emergence *of a* "new generation" of researchers, some of who will ultimately contribute high-quality medical advances in the field of gasotransmitter physiology and pathology.

C.5 Target groups/end users

The target groups/end users of the Action are both immediate and indirect:

In the direct targets/beneficiaries are included:

- The Principal Investigators (PIs) and all participating investigators of the ENOG teams
- The young investigators / trainees from the ENOG groups
- The ENOG Industrial partners

Indirectly, thanks to the dissemination of the acquired knowledge and expertise:

- Basic scientists in the Europe or around the world with an interest in gasotransmitter biology, whether they are based in Academia or in the BioPharmaceutical industry
- Medical professionals who, based on the knowledge generated by ENOG, can develop their own hypotheses on new disease areas where manipulation of NO, CO and H_2S levels and function could be of therapeutic potential

D. SCIENTIFIC PROGRAMME

D.1 Scientific focus

The scientific program will include the following topics:

Production:

- Regulation of the expression of gasotransmitter-synthesizing enzymes
- Structure-function analysis, post-translational modification and sub-cellular targeting of enzymes responsible for gasotransmitter synthesis.

Mechanisms of action:

- Molecular mechanisms of action of gasotransmitters.
- Effects of gasotransmitters on cell metabolism and cellular redox status.
- Post translational modifications of cellular proteins by gasotransmitters.
- Gene expression profiling in cells and tissues in response to fluctuations of gasotransmitter levels.

Physiological role:

- Physiological roles of gasotransmitters in the cardiovascular, respiratory, GI, urogenital and immune systems.

- Cross-talk between NO, CO and H_2S at the cellular and organ level.
- Generation and study of genetic models to better understand gasotransmitter biology.

Role in pathology:

- Assessment of endogenous gasotransmitter levels in pathological states.
- Contribution of NO, CO and H_2S to disease pathogenesis.

Drug development:

- Design and synthesis of (i) novel CO and H_2S donors for delivery of the gases to mammalian cells; the action of nitrite as NO donor will also be evaluated as well as (ii) inhibitors that selectively block enzymes that regulate gasotransmitter production.
- Pharmacological evaluation of new agents that modulate gasotransmitter levels in vitro and in preclinical models of human disease.

D.2 Scientific work plan methods and means

A list of examples of research topics to be covered by members in each of the WG of the Action is provided below and is based on their current and planned activities. This list is by no means exhaustive; more areas will be covered both by existing partners, as well as by additional groups that will join the Action after its approval.

WG1: Molecular control of gasotransmitter production and signalling

Apart from the "classical" cGMP-dependent signalling, NO also signals through the specific post-translational modifications of proteins. Although NO-dependent modifications such as S-nitrosylation and tyrosine nitration clearly occur in some circumstances their role in physiological/pathophysiology is unclear. Members of the Action have already generated apo-sGC mice ($sGCb_1^{H105F}$ knock-in mice expressing sGC that lacks heme and is thus insensitive to NO and CO); these mice are the most appropriate model to distinguish between cGMP-mediated effects of NO and cGMP-independent effects of this gasotransmitter *in vivo*. Efforts from members of the Action are expected to shed light on the above-mentioned fundamental issues.

The activity of the endothelial NO synthase (eNOS) is regulated by Ca^{2+}-dependent and – independent processes, particularly by its post-translational modification by phosphorylation[2]. Members of this starting Action have recently identified a novel regulatory tyrosine residue within eNOS (Tyr657) that attenuates enzyme activity; this phosphorylation even is linked to activation of the redox- and insulin-sensitive tyrosine kinase, PYK2. Current work aims to determine the physiological (and pathophysiological) stimuli that affect eNOS Tyr657 phosphorylation and its effects in endothelial cell biology, particularly as a mechanism to limit free radical production as a result of eNOS "uncoupling". To facilitate *in vivo* studies a non-phosphorylatable (Tyr657Phe) mouse is being generated.

Most of H_2S in the body is produced through the action of two pyridoxal-5'-phosphate-binding enzymes CBS and CSE[3]. In many tissues CSE and CBS are co-expressed and their relative contribution to H_2S levels is largely unknown. CBS expression is known to be upregulated by glucocorticoids; little information exists about the regulation of CSE expression. Also, only a handful of studies on the structure-activity relation of CSE and CBS exist (thus precluding rational inhibitor design) and little is known about their subcellular targeting or the post-translational modifications (phosphorylation, acetylation, or protein-protein interactions), which can alter the activity of these two enzymes. The members of ENOG will work on answering some of the pivotal questions posed above. In the end, we hope to derive a much better understanding of the basic function of these two enzymes in order to design selective inhibitors, and thus be able to probe both their activity in physiological and pathophysiological conditions.

CO binds preferentially and with high affinity to transition metals, pointing to heme- and metal-containing proteins as cellular targets of this gas. The demonstration that CO binds to the heme moiety of sGC, suggests that this mechanism of action could possibly explain the vasodilatory effects of CO. However, NO binds GC with an affinity much higher compared to CO, raising doubts that sGC activation by CO has physiologically relevant effects. In addition, although p38-mitogen activated protein kinase (MAPKs) have been demonstrated to mediate the anti-inflammatory effects of CO, MAPKs are unlikely to be direct targets for CO as they lack metal centres. This stresses the need to decipher the direct cellular targets and mechanism(s) by which CO exerts its biological effects; ENOG participants have been engaged in on-going efforts to provide some badly-needed answers.

H_2S is thought to mediate its effect by activating K_{ATP} channels and by promoting the sulphydration of cysteine residues on proteins. The members of the Action have recently demonstrated that H_2S activates MAPK pathways that are important in H_2S-stimulated angiogenesis. In addition, H_2S triggers PI-3K/Akt activation; however the exact mechanism of H_2S-triggered PI-3K activation remains unknown as does the contribution of the PI-3K/Akt axis to vascular cell survival and endothelial differentiation into capillary-like structures. More recently, H_2S was demonstrated to be an endogenous phosphodiesterase inhibitor. The importance of these pathways in vascular and other cells types is currently essentially unexplored. For example, although it has been reported that cGMP partly mediates the vasodilator response to H_2S in rat aorta, it is not known whether it also mediates relaxation of other smooth muscle cell types (tracheal, gastrointestinal). Moreover, the contribution of cyclic nucleotides to the angiogenic actions of H_2S has not been determined. Studies by participants of ENOG are expected to shed light on the above mentioned areas.

All 3 gaseous messengers can relax GI smooth muscle. $sGC_{\alpha1\beta1}$ (vs. $sGC_{\alpha2\beta2}$) plays a major, but not exclusive, role in the relaxant effect of NO. CO also relaxes GI smooth muscle through activation of both $sGC_{\alpha1\beta1}$ and $sGC_{\alpha2\beta2}$ but CO, delivered in a sustained way as CORM-2 or as continuous infusion of CO, additionally acts via a sGC-independent mechanism that remains poorly characterized. H_2S-induced relaxation of GI smooth muscle is independent of activation of K_{ATP} channels but is rather linked to calcium desensitization, and is related to activation of myosin light chain phosphatase; this could be related to the increase in cGMP recently described. The molecular mechanisms of CO- and H_2S-induced relaxations of smooth muscle in different tissues will be an intense area of investigation for the network.

Finally, H_2S and NO have been shown to modulate each others' signalling at various levels, as mentioned above. ENOG members will attempt to characterize the interaction of these two mediators and to determine its biological significance.

WG2: Gasotransmitters in disease

Soluble guanylate cyclase (sGC) is the prototypical receptor for NO, and might also be involved in CO and H_2S signalling. The members of this starting Action have already generated mice with a global deletion of the sGC a_1 subunit (knock-out mice), as well as mice with a floxed allele of the $sGCa_1$, which permits tissue-specific $sGCa_1$ deletion when crossed with CRE-expressing mice. $sGCa_1b_1$ -/- mice are used to delineate the specific contribution of $sGCa_1b_1$ in physiology and disease. $sGCa_1^{-/-}$ mice, available to ENOG members, can be used to delineate the involvement of $sGCa_1b_1$ in CO and H_2S signalling. At this moment further phenotype analysis of these mice under basal and "stressed"/disease conditions is ongoing. Moreover, to better characterize the role of H_2S in physiology and disease, mice with permanent tissue-specific or with drug- (e.g. tetracycline) inducible disruption of CSE and CBS are needed and their generation and subsequent study is already undertaken by ENOG action participants.

Sepsis and related diseases are the main cause of death in Intensive Care Units. The NO/sGC/cGMP axis plays a dual role in shock as it is involved in the generation of cardiovascular collapse, while at the other hand NOS-inhibition aggravates morbidity and mortality. Studies in mice deficient in sGC showed that sGC stimulation has indispensable protective properties in this setting. This observation has led to studies demonstrating that the selective NO-donor nitrite can provide survival benefit in shock in a sGC-dependent way. In anaphylactic shock, eNOS activation has also been demonstrated. This work will be extended by focusing on the potential use of selective stimulation of the NO/sGC/cGMP axis in sepsis; the functional interaction of the NO/sGC/cGMP axis with other known mechanisms operating in sepsis research in anaphylaxis will be centered on the involvement of sGC in the development of shock. The role of the other two gasotransmitters (CO and H_2S) in shock models will also be investigated using the same models, thus allowing comparisons with NO. This approach can also be relevant to the development of novel pharmaceuticals for the treatment of sepsis as both CO-releasing molecules and H_2S donors display interesting anti-inflammatory and anti-bacterial properties.

Endothelial dysfunction is an early, defining event in the development of cardiovascular disease and is characterized by a reduced bioavailability of NO. This defect is directly related to increased vascular oxidative stress as superoxide anions (O_2^-) readily react with NO to form peroxynitrite. Perhaps more importantly, the oxidative depletion of tetrahydrobiopterin (BH_4), a co-factor important in NO generation, causes the so-called "uncoupling" of eNOS, thus leading to a predominant production of O_2^- instead of NO. The uncoupling of eNOS is a major focus in investigation on the onset and development of cardiovascular disease. For this reason, its role will be studied extensively in a number of models of cardiovascular disease.

It has been demonstrated that the mobilization of progenitor cells with vasculogenic potential from the bone marrow depends on eNOS activation, and is impaired in diabetes. Indeed, diabetic mice show an impaired activation of bone marrow eNOS and decreased numbers of CPC. As the latter phenomenon seems to be linked to the phosphorylation of Tyr657 CPC mobilization and function will be assessed in wild-type and Tyr657Phe mice under control conditions and following induction of diabetes. The role of H_2S and CO in CPC mobilization in type II diabetes remains unknown and will also be an area of intense study by the ENOG Action.

WG3: Chemistry and in vitro pharmacology of gasotransmitter-modifying molecules

The members of this Action have already considerable expertise in computer modelling and drug docking simulations. As the crystal structure of CSE has been solved, we will utilize *in silico* approaches to design agents that bind and selectively inhibit CSE. Based on this information, the chemists associated with ENOG will synthesize an array of new molecules and the pharmacologists will subsequently evaluate their pharmacologic properties in isolated enzyme preparations or in vitro cell-based systems. The potency, efficacy and selectivity of these compounds will be determined. For selectivity experiments we will use COS cells transfected with CSE or CBS-expressing plasmids. Those compounds found to be of interest will be used in preliminary cell-based or ex vivo experiments (isolated organ preparations). Lead compound structures will be used as a mould to derive improved molecules. As far as potential H_2S donors go, chemists participating in our network have already synthesized a number of them. These will be initially tested for their

ability to release H_2S in cell-free systems, as well as in a cellular environment. In a parallel and complementary approach, a significant number of plant extracts (>30), known to be rich in sulphur-containing molecules, are available within the ENOG network. For example, H_2S-donor compounds have been isolated from garlic, and are thought to account for its anti-hypertensive properties; it is most likely that similar compounds exist in other members of the Alliaceae family, but also in extracts from the related family of Cruciferae. Synthetic and naturally occurring compounds will be evaluated for their ability to stimulate H_2S signalling pathways, to trigger H_2S-like responses in cells and tissues in vitro and will be pharmacologically characterized and compared to the few H_2S donors currently available. Given the shortcomings of the only two commercially-available H_2S donors (NaHS and Na_2S), we reasonably expect that we will end up identifying and characterizing much more potent H_2S donors, that will greatly help research in this area and can potentially advance to pre-clinical, or even clinical, stages.

This COST Action will pioneer a strategy to deliver CO to organisms by generating and developing novel CO carriers termed CO-releasing molecules (CORMs). These transition metal carbonyls release controlled amounts of CO, are pharmacologically active and elicit vasodilatory, anti-ischemic and anti-inflammatory activities *in vitro* and *ex-vivo*. More than 20 structurally different CORMs are already available to the ENOG partners and can be used along with the only commercially available CORM (CORM-2). It should be noted that the up to now available CORM molecules lacked one or more characteristics that would make them amenable for clinical development (stability in air or plasma, solubility, pharmakokinetics, narrow therapeutic window etc). The recently synthesized CORMs not only exhibit biological activity, but also possess adequate safety, stability, and solubility and PK profiles. These compounds will be pharmacologically characterized for their rate of CO release, their ability to stimulate signalling pathways (for example cGMP generation) and for their ability to relax isolated smooth muscle preparations from different tissues, both vascular and non-vascular (trachea, intestine).

Other participants have already focused their work on known activators of the NO/cGMP pathway. Organic nitrates have long been used in clinical practise, but their mechanism of bioactivation is still under investigation. Recently it was shown that aldehyde dehydrogenase-2 (ALDH-2) contributes to their bio-activation. This work will continue trying to clarify the mechanism and i*n vivo* relevance of NO formation by ALDH2.

WG4: Evaluation of gasotransmitter-modifying agents in animal models of disease

The NO/cGMP pathway has long been a target for therapeutic interventions. Clinically used drugs that are used widely and which exert their therapeutic effects though these pathways include nitrogycerin, sodium nitroprusside, sildenafil, tadalafil and vardenafil, to name a few. Newer agents in this class include sGC stimulators and activators. The first class of agents enhances cGMP formation in a NO-independent, heme-dependent manner, while the latter show preference for oxidized/heme-free sGC which is present in higher amounts in diseased vessels and tissues; sGC activators are more effective in activating oxidized/heme free sGC. Members of the ENOG Action have already ongoing collaborations with Bayer Schering GmbH and Sanofi-Aventis, investigating the use of proprietary, clinically interesting sGC activators and stimulators in several preclinical models of human disease in the areas of cardiovascular, renal, respiratory and inflammatory diseases.

Besides NO-delivering or enhancing drugs compounds which reduce NO production are also discussed to be useful in certain indications such as sepsis or inflammatory processes. Most NO-synthase inhibitors developed so far are arginine analogues. ENOG partner has developed already a tetrahydrobiopterin analog, 4-amino-tetrahydrobiopterin (VAS203), an agent which has the ability to both inhibit the hyperactive NOSs and to attenuate oxidative stress. One important, clinically underserved area of interest is that of brain trauma. Traumatic brain injuries have an incidence rate of one million cases per year in the US, Europe and Japan. Classical therapies of increased cranial pressure rely in large part on physical approaches (for example hyperosmotic therapy, hypothermia etc). Pharmacological treatment options to handle the acute clinical situation are currently non-existent. Cranial trauma triggers a cascade of events such as inflammation and brain edema formation, which in turn induce a rise in intracranial pressure (ICP). One major player in this sequence of events is NO. The proof of principle for VAS203 regarding reduction of ICP has demonstrated in the well-established Controlled Cortical Impact model. The clinical development Phase I program was successfully completed and currently, Phase IIa studies are being carried out. Further research on the mechanisms of involvement of NO, and potentially H_2S and CO, in ICP will be an integral part of the ENOG action.

Other members of the Action will continue to work on NOX, tackling NO-regulated pathways from a different, innovative angle. NOXs are membrane-bound enzymes that generate reactive oxygen species that inactivate NO and contribute to the pathophysiology of a variety of diseases. Initially the effect of NOX inhibitors will be experimentally tested in the treatment of stroke, using standard preclinical models.

CORMs will also be evaluated in a variety of pre-clinical models of cardiovascular disease such as hyperlipidaemia/atherosclerosis and myocardial ischeamia and collaborations among participants of the ENOG Action is expected to yield significant insights concerning new potential applications of CORMs in cardiovascular diseases, and inflammatory-related disorders that could be further extended to lung, the GI tract or pathologies affecting other organs (i.e. skin).

Another line of research that is actively pursued by a participant of the ENOG Action relates to the diagnosis and therapy of cardiovascular and metabolic diseases. It focuses on the development of inhibitors of peroxynitrite and its downstream cellular targets matrix metalloproteinases to treat ischemic heart disease and atherosclerosis. Through interactions with other participants that are involved in clinical trials, these efforts are expected to move at a swift pace shortening the time to enter clinical trials and in time further expanding their potential clinical indications to include, e.g. organ dysfunctions related to the metabolic syndrome.

A novel vantage point that is currently explored by ENOG members is the biology of nitrate and nitrite. The measurement of nitrite (NO_2^-) and nitrate (NO_3^-) in biological samples has been traditionally used as an index of endogenous NO generation. However, nitrite has been shown to also be a source of NO; nitrite reduction increases with decreasing pH and oxygen tension. Members of the network working collaboratively have conclusively shown that nitrite protects against myocardial ischemia/reperfusion injury, an important observation since replicated in numerous other organs. Moreover, it has been demonstrated that this rise in nitrite is associated with a substantial, dose-dependent decrease in blood pressure that is similar whether the nitrate is ingested as a supplement or through the diet. Thus ENOG members will test whether inorganic nitrate through supplement or dietary means may prove useful in the treatment of both hypertension and ischemic disease, using an array of clinically relevant animal models.

E. ORGANISATION

E.1 Coordination and organisation

ENOG Action will be coordinated by a Management Committee (MC), which will be formed during the inaugural meeting and will be composed of up to two representatives from each participating COST country. Thereafter, the MC will be responsible for the scientific and administrative organization of the Action.

The first task of the MC will be to elect a Chair, Vice-Chair and WG-Chairs and to appoint a Web Master, who will be responsible for the website of the network. The MC will also establish a panel to review the Short-term Scientific Mission (STSM) applications. Other tasks of the MC include promoting the interaction between partners, planning and execution of meetings, workshops, conferences, training schools and monitoring the overall progress made by the Action. The MC will decide on all financial issues, launch calls for exchange visits for members and publicise the activities of the action to increase its visibility in Europe and the world and to recruit more members to the network. The MC will draft the scientific and financial reports to COST and guarantee equal participation of all members involved in the Action. Another important aspect of the MC work would be to facilitate the interaction with industrial partners allowing individual investigators to capitalise on their scientific discoveries.

The Management Committee will meet twice annually, with its meetings joined to Working Group Meetings (WG). One of the MC meetings will be scheduled the day before or after of scheduled events such as a WG meetings or Training School in an effort to reduce the expenses.

To facilitate swift and efficient communication and decision-making, the Action will establish a Core Group (GC). This will consist of the Chair and Vice-Chair of the MC, the WG Chairs, the Web Master and the STSM chairperson. Communication within this group will be at frequent (at least once a month) through net-meetings and will guarantee smooth operations and also ensure that the website is kept up to date by the Web Master.

The following instruments will be used within ENOG:

Annual meetings (years 1-4)
Short-term Scientific Missions (open all year)
Training Schools (years 2 and 4)
Workshops (years 1 and 3)
ENOG website (year 1-4)
International Closing Conference (year 4)

The Website will be the single most important constantly functioning tool of communication and outreach of the network and will be updated monthly. The Website will provide information about all the activities of the Action and will create a milieu for researchers involved in the Action to share their progress, questions, achievements and results. The website will have two domains: one that is freely accessible to the public and one that is password-protected; information in this latter domain will only be accessible by COST members. We believe that the website will increase the awareness and the interest of the scientific community for gasotransmitter biology and will help us to recruit new collaborating research teams. The website will also collect information and present links to current activities and achievements in the field of gasotransmitters world-wide. The password-protected space reserved for participating researchers will be also created on the website where all activities, decisions and expenses of MC and WGs will be announced for transparency.

The MC will organize two one-week Training Schools to allow dissemination of know-how and techniques developed within the Action. The participation of early-stage researchers will be highly encouraged so that Early Stage Researchers (ESR) will have a chance to train in advanced aspects of pharmaceutical chemistry, biochemistry, cell biology, generation of genetic models, drug discovery, as well as in vitro and in vivo pharmacology/physiology. Emerging topics will be scheduled as the “hot topics” in the Program of Training Schools (TS). The main function of the workshops will be to bring together members of the different WG, allowing for interdisciplinary interactions. The first WS will be held early on (year 1) so that all experts will participate and update the Action on their current activities, while the second WS will be held during year 3 and its focus will be to make the participants aware in the achievements made in the different WG.

Short Term Scientific Missions (STSMs) are of great importance because they will facilitate interaction between the participating teams and will aid in overcoming the fragmentation currently observed. Moreover, STSM will give further opportunity for participation in the Action of ERS and will aid in helping the ERS get exposure to different lab and scientific cultures and also help them develop new skills. STSMs will be invaluable in initiating collaboration between research teams.

All the Action instruments will be closely monitored and evaluated. After each MC meetings a typed evaluation form containing the aims of the meeting, its program, outcome, conclusions and further suggestions will be generated. Each such memorandum will be placed on the Action website. Monitoring of each WG meetings and TS will be based on the brief typed report addressed to the MC and CG about the aims, program, invited scientists, outcomes, planned STSMs, TS, conclusions and further suggestions. Each WGM will also give the appropriate information about the meeting on the website.

Each STSM mission will be evaluated based on a brief typed report addressed to the MC and CG covering the aims, program, outcomes and conclusions. This report will be written and signed by both participated parts (guest researcher and host group leader). The MC will evaluate each completed action as soon as possible and will reach to conclusions regarding its success

E.2 Working Groups

WG1: Molecular control of gasotransmitter production and signalling
This WG will study how NO, CO and H_2S production is regulated, will identify mechanisms regulating the biological activity of gasotransmitters after they are produced and will determine the signalling pathways activated by gasotransmitters under physiological conditions.

WG2: Gasotransmitters in disease
This WG will study the contribution of altered gasotransmitter production in the pathogenesis and progression of cardiovascular and inflammatory diseases. The opposite approach will also be used by studying how disease states affect gasotransmitter levels.

WG3: Chemistry and in vitro pharmacology of molecules modifying gasotransmitter production
This WG will work on generating new agents (or identify existing natural products and other compounds) that release gasotransmitters or inhibit their production and will characterize them pharmacologically using in vitro systems.

WG4: Evaluation of gasotransmitter-modifying agents in animal models
Newly synthesized and currently used molecules that alter gasotransmitter levels will be characterized in relevant in vivo models of human disease.

All the WGs will organize Work Group Meetings at least once per year to introduce the advancement in knowledge and know-how by lectures, discussions and also by invitation of other experts not involved in this Action coming either from Europe or other parts of the World. To this end, we have already contacted a number of influential researchers in the field of gasotransmitters that reside and work outside the Europe.

E.3 Liaison and interaction with other research programmes

The effectiveness of this Action and dissemination of its results will be strengthened by connection with other ongoing networks that are directly or indirectly connected with cellular signalling and/or cardiovascular diseases. This aspect is regarded to be crucial for the success of the Action

E.4 Gender balance and involvement of early-stage researchers

This COST Action will place special emphasis on securing gender balance in all its activities. The Management Committee will implement a policy to recruit more female scientists through its website and other activities. Attention to the gender aspect will be given in the nomination of the Chair and Vice-Chairs of the MC, and for the chairperson of the WGs and in the examination of the proposals for STSM. Speakers at the meetings, TS and conferences will be selected using similar criteria. The WGs will nominate one researcher that is 35 years old or younger to coordinate the younger scientists of the Action in order to present the ideas and needs of ESR to the MC. A special section of the website will be dedicated to issues of interest to young researchers.

F. TIMETABLE

The duration of the Action will be a full 4 years, with the closing conference taking place at the end of the Action, to allow incorporation of all achievements.

As is evident from the list of participants, while a good-sized ENOG group has already been assembled, we also expect that new groups will apply to join ENOG during its 4-year run. For this reason, we propose an appropriate number of STSMs (>25), to address the anticipated demand.

The deliverables of the Action will be spread over the 4-year period as follows:

- Training schools for young researchers will be held in the middle of Year 2 and Year 4.
- Yearly WG meetings will be held towards the end of each year. In parallel with the yearly meetings, talks to members in the pharmaceutical industry will be organized in order to secure greater industry involvement.
- Closing international conference will be organized at the end of Year 4.
- Website will be created at the beginning of Year 1 and will be updated regularly.
- Electronic Newsletter will be issued biannually.

The scientific activities of ENOG will be carried by:

a) 9 MC meetings (Management Committee Meetings)
b) 16 WG meetings (Work Group Meetings, 4 per Work Group)
c) 2 TS (Training Schools)
d) 2 Workshops (WS)
e) >25 STSM (Short Term Scientific Missions)
f) 1 ENOG Action Closing International Conference

More specifically, the deliverables per year will be as follows:

YEAR 1

Kick off meeting

Month 3 - Website establishment

Month 6 - Open Call for STSMs established; MCM net-meeting; newsletter

Month 9 - Workshop I

Month 12 - WG Meetings & MC meeting; complete STSMs (year 1); yearly meeting; annual progress report; newsletter; publications

YEAR 2

Discussion on training school I inaugurated –working plan developed

Month 6 - Open Call for STSMs established; MCM net-meeting; newsletter

Month 12 - WG Meetings & MC meeting; complete STSMs (year 2); training school 1; yearly meeting; annual progress report; newsletter; publications

YEAR 3

Month 6 - Open call for STSMs established; MCM net-meeting; newsletter

Month 9 - Workshop II

Month 12- WG Meetings & MC meeting; complete STSMs (year 3); yearly meeting; annual progress report; newsletter; publications

YEAR 4

Discussion on training school II inaugurated –working plan developed

Month 6 - Open call for STSMs established; MC net-meeting; newsletter

Month 9 - WG Meetings & MC meeting; international conference; complete STSMs (year4); training school 2; final report; newsletter; publications

Month 12 - Closing International Conference

As far as the scientific deliverables are concerned it is predicted that by the end of Year 4, almost all research targets described in Section D will be fulfilled or considerably progressed.

G. ECONOMIC DIMENSION

The following 12 COST countries have actively participated in the preparation of the Action or otherwise indicated their interest: AT, BE, DE, EL, ES, HU, IT, NL, PT, SE, TR, UK.

On the basis of national estimates, the economic dimension of the activities to be carried out under the Action has been estimated at 48 Million € for the total duration of the Action. This estimate is valid under the assumption that all the countries mentioned above but no other countries will participate in the Action. Any departure from this will change the total cost accordingly.

H. DISSEMINATION PLAN

H.1 Who?

The target audiences of the results of the ENOG Action will include:

- Researchers in Academia and Industry from various disciplines, such as Pharmacology, Synthetic/Pharmaceutical Chemistry, Biochemistry, Cell Biology
- Medical Professionals specializing, e.g., in Cardiovascular diseases, in Respiratory illnesses or in disorders of the CNS

It is important to stress that the above groups are not limited to scientists that have an already declared interest in gasotransmitters. The original publications and reviews generated by ENOG members as well as the information broadcasted on the ENOG website may trigger the interest and involvement of newcomers that could contribute important, novel insights, e.g. a novel method of synthesis of a more stable CO-releasing compound, or up-to-now unrecognized areas of physiology and pathophysiology where gasotransmitters are involved.

The level and location of such target audiences will intentionally be wide and will include*:*

- Members of the Academic scientific community
- Industrial researchers and investors in the industry
- Medical investigators
- Established investigators
- Post-doctoral and post-graduate researchers (M.Sc. or Ph.D. candidates)

- Policy makers
- All citizens interested in biomedical advances

H.2 What?

- A website will be constructed and will be updated regularly with all the latest research results. The website will have a discussion forum where discussion between scientists will be facilitated and encouraged. It will also include the Newsletter.
- A password-protected section of the website will be devoted exclusively to the partners for exchange of confidential information.
- Presentations will be held in international conferences.
- Original papers and reviews will be published in peer reviewed journals.
- A yearly meeting will be organized in order to discuss the latest research findings of the Action.
- Two training schools will be organized that will educate new/young researchers on gasotransmitters.
- Presentations of current research work and results to invited industry professionals will be organized in parallel with the yearly meetings in order to stimulate greater industry involvement.

Finally, for the benefit of all the above groups, an international conference will be organised at the end of the four-year period of the project. It will help bring together gasotransmitter researchers and clinicians from other countries of the world, promote the Action's research results to non-European audiences.

H.3 How?

- A Network Logo will be created and used on all documents and events organized to identify the Action and to help the scientific community become familiar with the existence and the activities of the Action.

To reach these audiences, a variety of methods will be used:

Broad-targeting methods:

- Scientific publications in specialized journals
- State-of-the-art reviews
- Non-technical publications aiming at a general audience, e.g. in Scientific American, or in Science et Vie etc

Specific-target methods**:**

- Hold topic-restricted ENOG conferences and workshops
- Contact with other networks and service organizations, for cross-sectional, complementary interactions (e.g. with EMMA service that focuses on mouse models of disease)
- Contact national educational institutions to include gasotransmitter biology as a topic in their undergraduate or graduate curricula
- Sensitize policy makers at the national level to specifically include or name the area of gasotransmitter biology in a list of "targeted" research funds
- Alert the BioPharmaceutical industry on advances made in the field and on development opportunities, e.g. by invitation to the ENOG conferences and workshops
- Print a flier to be disseminated at universities, research institutes, industry and granting agencies raising awareness to the progress made in the field.