NATUR VÅRDS VERKET

Miljöforskningsnämnden
Ansökan om projektbidrag inom Naturvårdsverkets forskningsprogram

Forskningsprogram
SNAP ☐ **REPROSAFE** ☐ **FLIPP** Inriktning: Ekonomiska styrmedel ☐
Inriktning: Informationssystem och indikatorer IPP ☐

Projekttitel (svensk): **Experimentella studier av gen-miljö interaktioner vid riskbedömning av hälsoeffekter av luftföroreningar**

Projekttitel (engelsk): **Experimental investigations of gene-environment interactions in risk assessment of health effects of air pollution**

Huvudsökande

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Medsökande

Efternamn, förnamn, tjänst, organisation, institution: **Kjell Larsson, prof; Åke Ryrfeldt, prof; Per Gerde, docent; Maria Kumlin, docent; Lena Palmberg, docent; Lena Låstbom, Med.Dr.; Josephine Hjoberg, PhD**.
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Sammanfattning på **svenska** strukturerad enligt följande: 1) Projektets betydelse för programmet 2) Miljörelevans och förväntad betydelse för miljöpolitiken 3) Mål och hypotes 4) Metodik och genomförande 5) Kommunikationsinsatser i relation till programmet:

1/ Betydelse: Projektet syftar till att utnyttja befintlig fysiologisk och experimentell kompetens för att utreda biologiska effekter och verkningsmekanismer för luftföroreningar. De föreslagna modellstudierna av enskilda faktorer och deras samverkan samt relation till känsliga grupper (genetiskt eller livstilsmässigt, t.ex. rökare) bearbetar många av forskningsprogrammets prioriterade områden och kan skapa kunskap som påverkar riskbedömning.
2/ Miljörelevansen är direkt och resultaten har potential att skapa underlag för miljöpolitiken.
3/ Mål och hypotes: Att karakterisera mekanismerna för biologiska effekter av akut och kronisk exponering för fina och ultrafina partiklar samt gaser (ozon och NO_2) enskilt, och i samverkan, samt ifall dessa luftföroreningar påverkar allergiutveckling, luftvägsinflammation samt blodproppsbildning. Hypotesen är att luftföroreningarnas skadliga effekter beror på fysiologiska och immunologiska försvarsreaktioner, speciellt då sådana förmedlade av mastceller, epitelceller och den medfödda immuniteten.
4/ Metodik och genomförande: Befintlig *in vivo* mus modell utnyttjas för akuta och kroniska inhalationsexponeringar samt registrering av förändringar i lungfunktion, luftvägsinflammation, genutryck samt hjärt-kärlfunktion. Tekniskt avancerad egen utvecklad teknik används för generering av fina aerosoler av diesel partiklar och andra agens. Genetiskt modifierade djurstammar används för att undersöka mekanismer samt identifiera egenskaper som medför ökad känslighet för luftföroreningar. Ytterligare djupanalys genomförs i isolerade lungmodeller från olika djurslag samt cell och vävnadsmodeller från människa och djur. En projektledare har ansvar för genomförandet och integrationen av kompetens från forskarlagets åtta seniora medlemmar, samt handleder den doktorand som förväntas bearbeta projektet.
5/ Kommunikationsinsatser: Rapporter till Naturvårdsverket och vetenskapliga tidskrifter med internationell spridning samt vid konferenser och expertmöten.

	År 2004	År 2005
Summa sökta medel per år i kr:	**1467450**	**1367281**

Miljöforskningsnämnden
Ansökan om projektbidrag inom Naturvårdsverkets forskningsprogram

Sökta projektmedel fördelade på kostnadsslag	År 2004 (kr)	År 2005 (kr)
Personalkostnad inkl. soc. avgifter (3% löneuppräkning år 2005) Laboratorieassistent (månadslön 21500 kronor x1.54x12.16) Doktorand (månadslön 20000 kronor x 1.54x12.16)	402618 374528	414696 385764
Övriga omkostn exkl moms (förbrukningsmtrl, analyser, resor etc) Förbrukningsmaterial Resor Apparatur (exponeringskammare, anslutningar, mm)	122854 20000 80000	129000 21000 40000
Delsumma av ovanstående poster:	1000000	990460
Förvaltningspåslag:35 % + högskolemoms 8.7%	467450	376821
Totalsumma per år: (införs sid. 1):	**1467450**	**1367281**

*) Specificera namn, tjänst **) Specificera

Samtliga övriga miljörelaterade projekt för vilka de sökande har beviljats anslag eller söker anslag för 2004-2006. OBS Även EU-finansiering.

Projekttitel och huvudman	Finansiär	Tidsperiod	Sökt kr	Beviljat kr
Metabolisk aktivering av inhalerade carcinogener vid höga och låga doser-underskattas lungcancerrisken? Per Gerde	FAS	2004-2005		650000 per år
Aerosolgenerering för mikrodosimetri/Per Gerde	VR Medicin	2004-2005	1391000	
Försöksdjursallergi i yrkeslivet/Kjell Larsson	FAS	2004		1300000
Studier av organiskt damm i svinstallar /Kjell Larsson	Stiftelsen Lantbruks-hälsan	2004 2005	437590 154510	

Miljörelaterade projekt för vilka sökande har beviljats anslag för 2000-2003
OBS Även EU-finansiering

Projekttitel	Finansiär	Tidsperiod	Beviljat Kr
Metabolisk aktivering av inhalerade carcinogener vid höga och låga doser-underskattas lungcancerrisken? Per Gerde	FAS	2003	650000
Försöksdjursallergi i yrkeslivet/Kjell Larsson	FAS	2002-2003	1300000 per år
Hälsoeffekter gasexponering i svinstallar.. /Lena Palmberg	Stifelsen Lantbrukshälsan	2002-2003	totalt 500000
Studier av organiskt damm i svinstallar /Kjell Larsson	Stifelsen Lantbrukshälsan	2003	225000

Datum och sökandes underskrift, vilken samtidigt ger Naturvårdsverket tillåtelse att publicera sökandes namn på sin webbplats:	Datum och underskrift av prefekt eller motsvarande med namnförtydligande:
Den 15 oktober 2003 Sven-Erik Dahlén	Den 15 oktober 2003 Magnus Ingelman-Sundberg

Ansökan skall bestå av detta formulär jämte högst sex sidor lång projektbeskrivning på **engelska** (strukturerad som den svenska sammanfattningen samt en redovisning av kunskapsläget). Referenser till egna publikationer ges med sifferhänvisning till CV. Andra referenser ges i löpande text. Sökandes och eventuell medsökandes CV får omfatta högst två sidor. Inga bilagor kommer att beaktas vid bedömningen. Ansökan (max 10 A4-sidor, 12 punkters teckenstorlek) skall inlämnas i **original + 15 kopior samt elektroniskt** till ansok@naturvardsverket.se. Häfta ihop ansökan och använd hålat papper. Ansökan skall ha inkommit senast den 15 oktober 2003 till Naturvårdsverket, Forskningssekretariatet, 106 48 STOCKHOLM.

Bilaga 1 ansökan till Miljöforskningsnämnden 2003:
Forskningsprogram SNAP

EXPERIMENTAL INVESTIGATIONS OF GENE-ENVIRONMENT INTERACTIONS IN RISK ASSESSMENT OF HEALTH EFFECTS OF AIR POLLUTION

Principal Investigator: **Professor Sven-Erik Dahlén**

Co-applicants: **Dr.Med.Sci. Lena Låstbom (Project co-ordinator)**
Professor Kjell Larsson
Professor Åke Ryrfeldt
Docent Per Gerde
Docent Maria Kumlin
Docent Lena Palmberg
Ph.D. Josephine Hjoberg

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The National Institute of Environmental Medicine
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Background information and State of the Art

Epidemiological studies have demonstrated an association between elevated levels of air pollution and various health outcomes, including mortality, exacerbation of asthma, chronic obstructive pulmonary disease (COPD), respiratory tract infections, ischemic heart disease and stroke (Pope, Bates, and Raizenne 1995; Areskoug et al. 2000,Camner et al. 1997; Morgan, Reger, and Tucker 1997; Nyberg et al. 2000). Despite such compelling evidence from epidemiology, the knowledge about the mechanisms behind the alleged health risks is limited. Although the acute airway effects of particular matter and volatile components of air pollution have been studied to some extent in human volunteers (Sydbom et al., 2002), there is a definite paucity of information about the mechanisms involved in the chronic effects of different kinds of air pollution on the pulmonary and cardiovascular systems. Furthermore, the relevance of many previous attempts to investigate the mechanisms involved in chronic exposures of experimental animals have been questioned because of the excessive exposures used to examine for example the effects of diesel exhaust (Sydbom et al., 2002). There is thus an urgent need to better understand the mechanisms of relevant levels of air pollutants, alone and in combinations, with respect to acute or chronic effects, and on different outcomes such as pulmonary and cardiovascular end-points. Such information is required to guide risk assessments and the development of preventive strategies.

Parallel to the increase in air pollution, there has also been a rapid increase in the global incidence of allergic diseases such as asthma and rhinitis in the last two decades. Observations in Japan have suggested that children living close to roads with heavy traffic are more likely to develop allergies (D'Amato, Liccardi and D'Amato,1998). Epidemiological data support the theory that atopic children may constitute a group of individuals that run a heightened risk of developing negative health effects following exposure to airborne particles (e.g. Boezen et al., 1999). The existence of such sensitive subgroups amongst the general population deserve particular attention for risk assessment. Allergy or asthma with increased bronchial responsiveness are two examples of diseases where genetic traits and environmental influences interact to produce a phenotype that may predispose susceptibility to adverse health effects of air pollution. Other potential risk groups include smokers and workers exposed to aggressive fumes in industries or as firefighters, for example.

There is in fact early experimental data to support that individuals with increased risk of response to air pollution may be identified. For example, it is known that humans display considerable variability in their responsiveness to inhaled ozone. In an experimental study of ozone, it was demonstrated that a locus on chromosome 17 encoding the gene for the pro-inflammatory cytokine tumour necrosis factor alpha (TNF_{α}) in fact determined if ozone led to an inflammatory response in the mice (Kleeberger et al., 1997), and subsequent work defined the mast cell as one central component in the reaction to ozone (Kleeberger, Longphre and Tankersley, 1999).

The investigators behind this application have recently joined forces and established a laboratory for translational lung research. The methods range from basic cell biology and molecular biology, over *in vitro* and *in vivo* animal experimentation into experimentations on human volunteers and patients with asthma and COPD. This application is submitted with the intention to obtain funding to allow us to build on our current expertise and apply reliable models for scientific investigations to define the health effects of air pollution. There is also a strong tradition of inhalation toxicology at the Division, as the legacy of the late Professor Per Camner.

Relevance of proposed research for SNAP programme

First of all, as discussed in the background section, there is an unmet research need to find plausible mechanisms behind a number of potentially alarming associations that are indicated by epidemiological observations of air pollution exposures. We believe that the project we offer has great potential to provide new facts on these matters.

With regard to the priorities of the Swedish Environmental Protection Agency(SEPA), we also believe that the project meets a number of the key criteria listed as important in the evaluation of applications.

Thus, the project will clearly provide new important evidence for quantitative risk assessment, and there is no doubt that the data can be incorporated in the final report.

Furthermore, members of the current research team have collaboration with Professors Thomas Sandström in Umeå and Stephen Holgate in Southampton (Member of WHO expert committee on Air Quality Control and Chairman of similar body in the UK). Support to the project will accordingly enhance the proposed mechanistic research and facilitate collaboration between Swedish and European research groups, another priority of SEPA.

The focus of the project is on the development of models that with better precision can explain different biological effects of environmental pollutants, with primary aim at health effects. This also seems to be in line with the priorities of the SNAP research programme.

The project will address the issues of gene-environment interactions by the subjecting genetically defined animals and cells to relevant air pollution exposures, and, again, with the aim to establish mechanistic explanations and exposure-effect relations.

The involvement of a PhD student in the project is necessary to obtain sufficient operational power, but has a long-term value in itself as it will increase the future potential for air pollution research in Sweden.

Relevance for environmental control and policies

The indications of detrimental health effects obtained in epidemiological studies requires mechanistic explanations in order to establish cause-effect relations. Such information will greatly enhance risk assessments and thus improve the ability of government agencies and political bodies to make evidence-based decisions. In particular, the current view that there is a linear relation, without null-effect, between exposures to air pollutants and health effects, may be challenged by experimental evidence from robust models.

Aims and hypotheses

The main aim of the project is to use existing models to investigate the mode of action of different air pollution components, alone and in combination. Particular focus will be on the use of genetically modified mice to study effects of chronic *in vivo* exposures. This is not an end in itself, but the means to assess long-term health effects of exposures on a compressed time scale. As indicated in the next section, we have methods to study effects in mice on several levels, intact animal, isolated tissues and cells and whole isolated lungs. Findings in the acute and chronic challenges of the mice will then be tested in other animal models and in human cells such as airway epithelium, mast cells and macrophages. The latter cells have prominent roles in the host defence reactions towards noxious agents.

The overall mechanistic hypothesis of the project is that most, if not all, effects of air pollution components may be explained by interactions between the air pollution and endogenous host defence mechanisms. In particular, activation of mast cells and epithelial defence mechanisms as a part of the innate immune response are considered to have a pivotal role in the reactions to air pollution that ultimately translate into adverse health effects. The latter hypothesis is supported by observations in mice suggesting that Toll-like receptors and endogenous nitric oxide have definite roles in the adverse reactions to inhaled ozone (Kleeberger et al., 2000).

Another key hypothesis is that two different air pollution components generally have additive damaging effects (eg. ultrafine ($PM_{0.1}$) particles plus ozone, fine (PM_1) particles plus NO_2, DEP plus NO_2, etc). This is actually an important area to investigate in detail, as current risk assessments generally build on one factor at a time, and have limited bearing on the effects of mixed natural exposures. With regard to interactions, it is hoped further along the project to study whether or not tobacco smoking mice (there are such models available) are more sensitive to air pollution challenges. An interaction between noise and air pollution could also in the future be assessed in a well defined mouse model. An immediate interest that can be approached directly in the existing mouse model, is to characterise the effects of air pollution components on allergic sensitisation and bronchial responsiveness.

It should be recognised that the project team also conducts human experimentation, alone and in collaboration with others. Together, these interfaces provide the option for swift transfer of discoveries in the animal models and cell culture studies to validation in humans.

Workplan, operational structure and methodologies

The applicants propose to form a project team aiming at the elucidation of the mechanism involved in different effects of air pollution and its components. The PI and Dr. Lena Låstbom will be responsible to cause an integrated and directed action, and a newly recruited PhD student will be instrumental for the progress of the project. The essentials of the available models and the proposed adoptions to air pollution research are given below.

In vivo mouse model. Dr Hjoberg has as the first investigator at KI established a reliable mice model for assessment of airway inflammation and physiology. Following allergen (OVA, ovalbumin) sensitization, repeated inhalations in awake animals is used to create a state of bronchial hyperresponssiveness to the cholinergic agonist methacholine (MCh). The changes in lung physiology are measured with a new direct and exact method of measuring in vivo airway physiology in smaller animals, made possible by combining the technique of forced oscillation and a ventilator for small animals (the Flexivent system by Scireq Inc., Canada). This method is superior to commonly used but potentially misleading body plethysmograph methods for assessing airway function in small animals *in vivo*. The degree of airway inflammation is assessed by histological and molecular biology examinations of lung tissue, total and differential cell counts of broncho-alveolar lavage (BAL), and measurements of inflammatory cytokines and other mediators in BAL. The hypotheses in the project are tested in appropriate protocols, using genetically modified mouse strains (gene deletions or transgenically overexpressed traits) as well as standard pharmacologic interventions in order to define mechanisms.

The focus of the *in vivo* experiments in mice is to study chronic and subacute effects of air pollution components, and their interactions. For this purpose, the exposure chambers currently used for allergic sensitization of the animals can with small modifications be used to expose animals to air pollution components (Ozone, NO_2, DEP, etc). With regard to DEP and other predominantly particular challenges, it is proposed to take advantage of the unique DustGun aerosol delivery system (see below). Another priority is to develop a new system for chronic exposures of animals during several months, in order to study long term effects on airway inflammation and remodelling. There are such systems available, for example at the Lovelace Respiratory Research Institute in USA that Dr Gerde has collaboration with. In the future we also hope to incorporate high throughput gene expression microarrays (Affymetrix GeneChips® or similar) and proteomics to assess changes in gene expression after exposures to air pollutants. With regard to end-points, it is also planned to measure heart rate and blood pressure and to adopt a method recently developed in hamsters for *in vivo* studies of thrombosis in the mouse (Nemmar et al., 2003). These developments may permit simultaneous assessment of pulmonary and cardiovascular effects of inhaled air pollutants.

<u>*Ex vivo* and *in vitro* mouse models.</u> For certain mechanistic questions, the effects of exposures directly at the airway and vascular smooth muscle level may be analysed by the use of the isolated ventilated perfused lung (see below) and tracheal ring preparations put into tissue bath chambers. These models often provide the possibility to test a larger number of possible interactions, than does the *in vivo* model.

<u>The isolated ventilated and perfused lung model</u> (IVPL) has been developed at the Department (Ryrfeldt et al 1990). These preparations from guinea-pigs and rats are well characterised and particularly suitable to evaluate acute irritating and toxic effects on the lung. The models maintain some of the advantages of the *in vivo* situation, thus the whole organ is intact, the cells have their right neighbours, one may study the direct effect of the lung, there are several exposure routes e.g. inhalation, instillation, bolus injections and constant perfusion. At the same time, the problems associated with for example cardiovascular and neural reflexes can largely be eliminated. The isolated perfused lung model has previously been used for studies of for examples SO_2, NO_2, chemicals causing occupational induced asthma, such as terpenes and isocyanates. We have also recently characterised the mediators of mast cell driven allergen-induced bronchoconstriction in the guinea-pig lung (Sundström et al., 2003). More recently we have also exposed the isolated lung to particles using the DustGun aerosol generator (see below). The aerosol generator in combination with the isolated lung gives us unique possibilities to study were in the lung different particles are deposited, what the actual dose to the epithelium is and how metabolism and interaction with endogenous mechanisms determine toxicity. As indicated above, the isolated lung model is currently being adopted for use also in mice. In addition to the opportunities provided by the use of genetically modified animals, there is a large body of knowledge with

regard to immunological mechanisms and hence also tools in the form of many specific antibodies for mice that are absent in rats and guinea pigs. Many reactions that damage the lung have an immunological component.

Exposure Methods. For short-duration exposures we will use the DustGun aerosol generator, which produces short bursts of micron-size particles at concentrations up to several g/m^3. It is particularly useful for studying acute effects of resuspended dry powder inhalants. Collected diesel exhaust particles can be resuspended at a concentration of 3 g/m^3 and a mass median aerodynamic diameter (MMAD) of 0.5 µm. The particle size distribution is determined for all generated aerosols using a cascade impactor, and the specific surface area of studied particulates is measured using the BET method. Recently the aerosol system has been combined with the IVPL model in rats (Gerde et al. 2003). For the first time the IVPL can be exposed by inhalation to respirable aerosols in high concentrations. Used in conjunction with the IVPL the DustGun system will be used to study a number of dosimetry issues that also will guide the development of chronic models. Another important use of the combined aerosol generator/IVPL system is to study airway reactivity of particulate air pollutants. Following short duration exposures to inhaled aerosols, changes in airway resistance and compliance can be detected immediately. It is also feasible and planned to develop a connection between the holding chamber of the aerosol generator and the Flexivent system for assessment of pulmonary mechanics in mice (see above). Our capability to conduct chronic inhalation exposures is being expanded. A continuous generator version of the DustGun system under development, and we plan to include a commercially available cigarette smoke generator in our standard methods.

Primary cultures of human bronchial airway epithelium cells (PBEC) have been established according to methods learned from Dr Pieter Hiemstra at Leiden University Medical Centre. The cells have been isolated from bronchi obtained at pulmonary lobectomy and treated with proteases. The cells are kept in liquid nitrogen until used for experimentation. The cells are grown in a serum-free complete keratinocyte medium including several growth factors and conventional antibiotica. Functional responses and molecular mechanisms can be studied both acutely and in short term exposures (24-48h).

Human Cord Blood derived Mast Cells (CBMC) are grown from umbilical cord stem cells. Mononuclear cells are separated from heparinized blood by Ficoll-Paque gradient centrifugation. The cells are grown in the presence of recombinant human stem cells factor (rhSCF) and rh IL-6. After about ten weeks in culture, more than 96% of the cells have matured into the mast cell phenotype, as assessed by tryptase staining. Functional responses and molecular mechanisms can be studied both acutely and in short term exposures (24-48h).

Exploitation and communication of research findings

The research team normally publishes findings in international peer-reviewed scientific journals. This is obviously the best way to obtain scientific impact, and the strategy we envisage for findings in the project we hope to obtain funding for. Incidentally, the PI was the senior author on a review paper on health effects of diesel exhaust (Sydbom et al., 2002) that in fact was the third most quoted paper in the European Respiratory Journal in 2002.

In addition, SEPA will get easy first access to findings and we are willing to participate in future expert panels addressing health effects of air pollution and associated issues. Furthermore, the investigators normally get a number of invitations to present at scientific meetings as well as to contribute to public appearances in media and focus groups. Such channels are important for the dissemination of findings to the public and to opinion leaders and politicians.

Concluding remarks

With considerable dedication, the project team proposes to use a number of established models to study the acute and chronic health effects of air pollution in animal models and human cells. The support is required to allocate staff and a PhD student to the project, and to build on existing expertise to develop sensitive and improved methods for chronic exposures. We are keen to embark on these studies and believe that our previous experiences and collective competence represent unique success factors that have great potential to lead to new understanding that will aid risk assessments.

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