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The Pulmonary Neuroendocrine System

Physiological, pathological and tumourigenic aspects

Thesis for the degree of philosophiae doctor

Trondheim, August 2007

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Science and Technology
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NTNU
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Lungenes nevroendokrine system

- betydning ved fysiologiske og patologiske tilstander

Nevroendokrine (NE) celler er en benevnelse på spesialiserte celler som finnes diffust utbredt i flere organ i kroppen og som har evnen til å produsere og skille ut hormon-liknende substanser. I lungene oppfattes ansamlinger av disse cellene som sanseorgan som monitorerer oksygen-nivået, og de spiller sannsynligvis en viktig rolle for lungenes utvikling, regulering av lungesirkulasjon og luftstrøm, samt immunrespons.

Hovedmålet med avhandlingen har vært å se på ulike sider ved lungenes NE system ved fysiologiske og patologiske tilstander, med fire delarbeider som hver for seg belyser ulike aspekt ved dette.

I det første arbeidet ble den generelle NE markøren kromogranin A (CgA) målt i blodprøver fra personer som deltok i Helseundersøkelsen i Nord-Trøndelag (HUNT 1995-97). Resultatene viste at mannlige deltakere med dårlig lungefunksjon hadde høyere nivå av CgA enn deltakere med normal lungefunksjon, som et uttrykk for NE aktivering.

Det andre arbeidet omhandler et 72 ukers eksponerings-forsøk med inhalasjon av karbon monoksid (CO) hos rotter gitt i konsentrasjoner som tilsvarer blod-verdier hos stor-røykere. Bortsett fra forstørret hjerte, ble det ikke funnet andre røyke-relaterte skader på hjerte/kar-systemet eller lungene. CO hadde ingen effekt på svulstforekomst eller forandringer i antall NE celler.

I det tredje arbeidet ble ulike NE markører undersøkt med immunhistokjemiske, immunelektronmikroskopiske og biokjemiske metoder hos pasienter med ikke-småcellet lungecancer. Hovedfunnet her var et større antall svulster positive for NE markører enn tidligere beskrevet når signalforsterkende teknikker ble brukt ved immunhistokjemi. Dette kan ha betydning for forståelsen av svulstenes biologi, og kan være uttrykk for at lungenes NE celler er opphavsceller for flere slike svulster enn tidligere antatt.

Det siste delarbeidet belyser sekresjon av substanser fra lungenes NE system ved hypoksi i en isolert, ventilert og sirkulert rottelunge-modell. Ved lave oksygennivå falt konsentrasjonen av proteinet bombesin i buffer sirkulert gjennom lungekretsløpet. I tillegg ble det funnet øket antall immunmerkede celler med calcitonin gene-related peptide, noe som tyder på redusert cellulær utskillelse ved eksponering for hypoksi. Resultatene viser at hypoksi er assosiert med raske forandringer i lungenes NE system for å opprettholde en balansert ventilasjon og sirkulasjon.

Samlet gir arbeidene økt kunnskap om det nevroendokrine system ved ulike sykdoms-prosesser som luftveisobstruksjon, inhalasjon av gasser som CO, i svulstutvikling og ved fysiologiske prosesser som hypoksi.

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Veiledere: Helge L. Waldum og Sigurd L. Steinshamn

Ovennevnte avhandling er funnet verdig til å forsvares offentlig for graden Philosophiae Doctor (PhD) i klinisk medisin. Disputas finner sted i Auditoriet, Kvinne-Barn-Senteret, St. Olavs Hospital onsdag, 22.08.07, kl. 12.15

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Trondheim, March 2007

Sveinung Sørhaug

Abbreviations

APUD	amine precursor uptake and decarboxylation
BLPs	bombesin-like peptides
BONT	Bronchial Obstruction in Nord-Trøndelag
CgA	chromogranin A
CGRP	calcitonin gene-related peptide
CO	carbon monoxide
COHb	carboxyhaemoglobin
COPD	chronic obstructive pulmonary disease
DCV	dense core vesicle
DIPNECH	diffuse idiopathic pulmonary neuroendocrine cell hyperplasia
DNES	the diffuse neuroendocrine system
EM	electron microscopy
GRP	gastrin releasing peptide
H&E	haematoxylin and eosin
HO	heme oxygenase
HP	helicobacter pylori
HUNT	Nord-Trøndelag Health Study
IASLC	International Association for the Study of Lung Cancer
IEM	immunoelectron microscopy
IH	intermittent hypoxia
IHC	immunohistochemical
LCNEC	large cell neuroendocrine carcinoma
NCAM	neural cell adhesion molecule
NE	neuroendocrine
NEB	neuroepithelial body
NO	nitric oxide
NSCLC	non-small cell lung cancer
NSCLC-ND	non-small cell lung cancer with neuroendocrine differentiation
NSE	neuron-specific enolase
PBF	phosphate-buffered formaldehyde
PNEC	pulmonary neuroendocrine cells
Ppa	pulmonary artery pressure
ppm	parts per million
Ppv	pulmonary venous pressure
RIA	radioimmunoassay
SCLC	small cell lung cancer
SYN	synaptophysin
WHO	World Health Organisation

1. List of papers

This thesis, which is based on the following papers, referred to by roman numerals in the text, is presented to the Faculty of Medicine, the Norwegian University of Science and Technology, for the Doctoral Degree Ph.D. in Clinical Medicine.

Paper I

Sveinung Sørhaug, Arnulf Langhammer, Helge L. Waldum, Kristian Hveem and Sigurd Steinshamn.
Increased serum levels of chromogranin A in male smokers with airway obstruction.
European Respiratory Journal 2006; 28: 542-548.

Paper II

Sveinung Sørhaug, Sigurd Steinshamn, Odd G. Nilsen and Helge L. Waldum.
Chronic inhalation of carbon monoxide: Effects on the respiratory and cardiovascular system at doses corresponding to tobacco smoking. *Toxicology* 2006; 228: 280-290.

Paper III

Sveinung Sørhaug, Sigurd Steinshamn, Rune Haaverstad, Ivar S. Nordrum, Tom C. Martinsen and Helge L. Waldum.
Expression of neuroendocrine markers in non-small cell lung cancer. A biochemical, immunohistochemical and ultrastructural study. *Acta Pathologica, Microbiologica et Immunologica Scandinavica, (APMIS)* 2007; 115: 152-163.

Paper IV

Sveinung Sørhaug, Sigurd Steinshamn, Bjørn Munkvold and Helge L. Waldum.
Effects of intermittent alveolar hypoxia on the release of neuroendocrine products in isolated rat lung. *Submitted 2007.*

2. Summary

Paper I

Increased serum levels of chromogranin A in male smokers with airway obstruction.

The neuroendocrine (NE) system may play an important role in smoking-induced airway diseases. The peptide chromogranin A (CgA), which is a general NE marker, was evaluated in sera from three study groups selected from the bronchial obstruction study (BONT) of the large cross-sectional Nord-Trøndelag Health Study (HUNT). The study groups included never-smokers with normal lung function, smokers with normal lung function and smokers with airway obstruction. The results showed that male smokers with airway obstruction had significant higher serum CgA than both smokers without airway obstruction and never-smokers with normal lung function. The elevated serum levels of CgA correlated with the degree of airway obstruction. Moreover, presence of respiratory symptoms and chronic bronchitis among male smokers were associated with increased serum CgA levels. Women had CgA levels similar to male smokers independent of smoking status and lung function. Elevated serum CgA levels in subjects with airway obstruction and respiratory symptoms may represent NE activation in inflammatory or remodelling processes in the lung.

Paper II

Chronic inhalation of carbon monoxide: Effects on the respiratory and cardiovascular system at doses corresponding to tobacco smoking.

Long-term effects of low doses of carbon monoxide (CO), as in the gaseous component of tobacco smoke, are not well known. In paper II, the effects of chronic inhalation of CO on the respiratory and cardiovascular system at doses corresponding to tobacco smoking and its effect on tumourigenesis and pulmonary NE cells were evaluated in rats. In the cardiovascular system, only cardiac hypertrophy was observed. No signs of atherosclerosis were found. In the lungs, no signs of pathology similar to that associated with cigarette smoking were observed, and no differences in number of pulmonary NE cells were found between the exposure groups. In addition, no exposure related carcinogenic effects were observed. The results in paper II suggest that low dose CO

exposure is probably not responsible for the respiratory pathology associated with tobacco smoking, but may contribute to smoking-related cardiac pathology.

Paper III

Expression of neuroendocrine markers in non-small cell lung cancer. A biochemical, immunohistochemical and ultrastructural study.

NE differentiation is reported in some cases of non-small cell lung cancer (NSCLC). In paper III, 20 cases of NSCLC were examined using immunohistochemical (IHC) methods with signal amplification technique and immunoelectron microscopy (IEM). In addition, circulating levels of the NE markers CgA and neuron-specific enolase (NSE) were measured. The results revealed that for some NE markers, a higher number of immunoreactive tumours than previously reported were identified with the use of a signal amplification technique. Furthermore, labelling of CgA in secretory granules using IEM was not found to be as sensitive as IHC methods in detecting NE features in NSCLC. Finally, no association between circulating levels of NE markers and IHC reactivity was observed. Knowing the expression of different NE markers may improve our understanding of the tumour biology and represent an important diagnostic tool for future targeted therapy of cancer.

Paper IV

Effects of intermittent alveolar hypoxia on the release of neuroendocrine products in isolated rat lung.

Alveolar hypoxia is associated with several reactions in the lung, and the pulmonary NE system may play an important role in the homeostatic control. In paper IV, the effects of acute intermittent alveolar hypoxia on the release of NE products in isolated buffer-perfused and ventilated rat lungs were examined. Perfusate collected from isolated rat lungs ventilated intermittently with hypoxic and normoxic gas was analysed for the bioactive NE products bombesin-like-peptides (BLPs) and serotonin. In lungs ventilated with intermittent hypoxia (IH), perfusate levels of BLPs decreased compared to lungs ventilated with normoxic gas only. No difference was observed in perfusate levels of serotonin between the two groups. At the end of the study, immunohistochemical evaluation of the lungs revealed significantly increased numbers of pulmonary NE cells

immunoreactive to calcitonin gene-related peptide (CGRP) in IH ventilated lungs, indicating diminished release of the neuropeptide during hypoxia. No difference was observed in the immunoreactivity for CgA between the groups. Together, these results suggest that intermittent periods of hypoxia are associated with a rapid physiological response in the pulmonary NE system probably in order to maintain a well balanced ventilation and perfusion relationship in the lung.

3. Introduction

3.1. The diffuse NE system - general aspects

In most animal species, NE cells are found in several organs and systems, especially in the epithelial surfaces. They are scattered among other cells either as single cells or clusters of cells. Although these cells are not well defined anatomical entities or organs, they share several important functional and morphological properties. Collectively, they are named as "the diffuse neuroendocrine (or endocrine) system" (DNES) (Montuenga *et al.* 2003). In the nineteenth century, Heidenhain (1870) and Kultschitzky (1896) first described these cells as "clear cells" with basal granules in the epithelium of stomach and intestine. Some decades later, Feyrter (1938) reported the presence of pale cells (helle Zellen) scattered distributed in many organs. It is now well accepted that NE cells are found diffusely spread in both the gastrointestinal and respiratory epithelium. In addition, these cells are seen in the urogenital tract, skin (Merkel cells) and thyroid glands (C cells).

NE cells share some specific functional and morphological properties. They are endocrine in the way that they synthesise and release bioactive peptides and amines that have effects on other target cells via the blood (endocrine). In addition, their secretory products can act directly on neighbouring cells (paracrine) or its own cell (autocrine). They are also ascribed neurosecretory properties as they possess several common regulatory factors (neurotransmitters) with neurons. Furthermore, some NE cells, like pulmonary NE cells, seem to have a rich innervation (Lauweryns *et al.* 1985). Another important property of the NE cells is the uptake of amino acids and transformation of these into bioactive amines by decarboxylation, which clarify the previous acronym APUD (Amine Precursor Uptake and Decarboxylation) of these cells (Pearse & Polak 1971). Morphologically, NE cells are identified by their contents of peptides, visualised by IHC methods or ultrastructural findings of electron dense granules (dense core granules, DCG) using electron microscopy (EM).

3.2. The pulmonary NE cell

3.2.1. Terminology and origin

Since Feyrter reported the presence of some cells without affinity for haematoxylin and eosin in the airway epithelium in 1938, the existence of these NE cells in the lungs have been a subject of increasing interest (Montuenga *et al.* 2003). Previously, these cells have been named "clear cells", APUD-cells or Kultschitzky-cells. The present terminology, pulmonary NE cells (PNEC), includes cells with NE phenotypes in the respiratory epithelium (Van Lommel *et al.* 1999). These cells are found either as single cells among other airway epithelial cells or as aggregates of PNEC, called neuroepithelial bodies (NEBs)(Lauweryns & Peuskens 1972). NE cells in respiratory-like systems are found in most of the species investigated, such as amphibians, reptiles, birds, mammals and even in gill filaments of fish (for review, see Van Lommel *et al.* 1999).

The embryological origin of the PNEC has been a subject of debates. In the past, suggestion of a neuroectoderm (neural crest) origin of PNEC has been made based on the similarities in chemical, functional and morphological qualities with neural derived cells. This hypothesis has been supported by findings of expression of neural cell adhesion molecule (NCAM) in NEBs, which is a membrane-bound protein expressed by cells of neuroectoderm origin (Ito *et al.* 1995). Another argument for this hypothesis is the necessity of a critical transcription factor (achaete-scute homologue-1) for neuronal development in the formation of mouse PNEC (Borges *et al.* 1997). However, evidence for an endodermal origin has also been found. Ito *et al*. reported formation of NEBs in cultures of foetal airway epithelium without neural tissue or mesenchyme, indicating that they were derived from airway epithelium (1997). Futhermore, the airway epithelium, like the upper gastrointestinal tract with its NE cells, are developed from the primitive foregut. However, this topic has not been fully clarified.

3.2.2. Localisation and morphology

In humans, single PNEC are found scattered in the respiratory epithelium from the nose to the bronchioalveolar region, while NEBs are usually found in the intrapulmonary

airways. PNEC are often triangular in shape, with the main cytoplasmic contents located near the basal membrane. The apical portion is narrowed and may reach the airway lumen. NEBs are often located at airways bifurcations or bronchioalveolar junctions, occupying strategic positions for sensing of air contents. They consist of clusters of PNEC forming an intraepithelial “organ”, which is innervated at its basal part and with protruding microvilli into the airway lumen at its apical part (Montuenga *et al.* 2003) (Figure 1). Most of the luminal surface are covered with adjacent cells like Clara cells or type 1 or 2 pneumocytes (Ito 1999).

3.2.3. Markers and quantification

The ability to produce and store peptides and amines are utilized to identify PNEC/NEBs in the lung (Figure 1). Using IHC methods with antibodies against peptide products like *calcitonin gene-related peptide (CGRP)*, *gastrin releasing peptide (GRP, mammalian bombesin)* and *calcitonin* separates PNEC easily from other epithelial cells (Scheuermann 1997). In addition, general NE markers as *neuron-specific enolase (NSE), chromogranin A (CgA), synaptophysin (SYN)* and *neural cell adhesion molecule (NCAM)* are also used for visualisation of NE cells in the lung, as well as the main amine in the vesicles - *serotonin (5-hydroxytryptamine, 5-HT)* (Lauweryns *et al.* 1987; Gosney *et al.* 1988). Even though some similarities exist, important differences in the specificity of the markers are observed between different species.

Owing to the rarity and scattered distribution of the PNEC and NEBs in the lung, the quantification of NE cells in the respiratory system has been a challenge. Different methods of quantification, like counting all NE cells in serial paraffin sections or in a whole-mount preparation (Peake *et al.* 2000) have revealed different results that are difficult to compare. However, in most studies and species the number of PNEC is found at its maximum around the time of birth, and thereafter declining (Redick & Hung 1984). It seems that NEBs are most frequent in animals with immature lungs at birth, such as rodents. In adult humans, the reported number of NE cells among airway epithelial cells has been varying from 1 – 12.5 PNEC / cm basement membrane, or up to 0.5 % of all the epithelial cells (Boers *et al.* 1996). In a recent study by Weichselbaum *et al*, the area densities of PNEC in normal human respiratory epithelium

were reported ranging from $65/mm^2$ to $250/mm^2$, using confocal microscopy of whole-mounts preparations (2005).

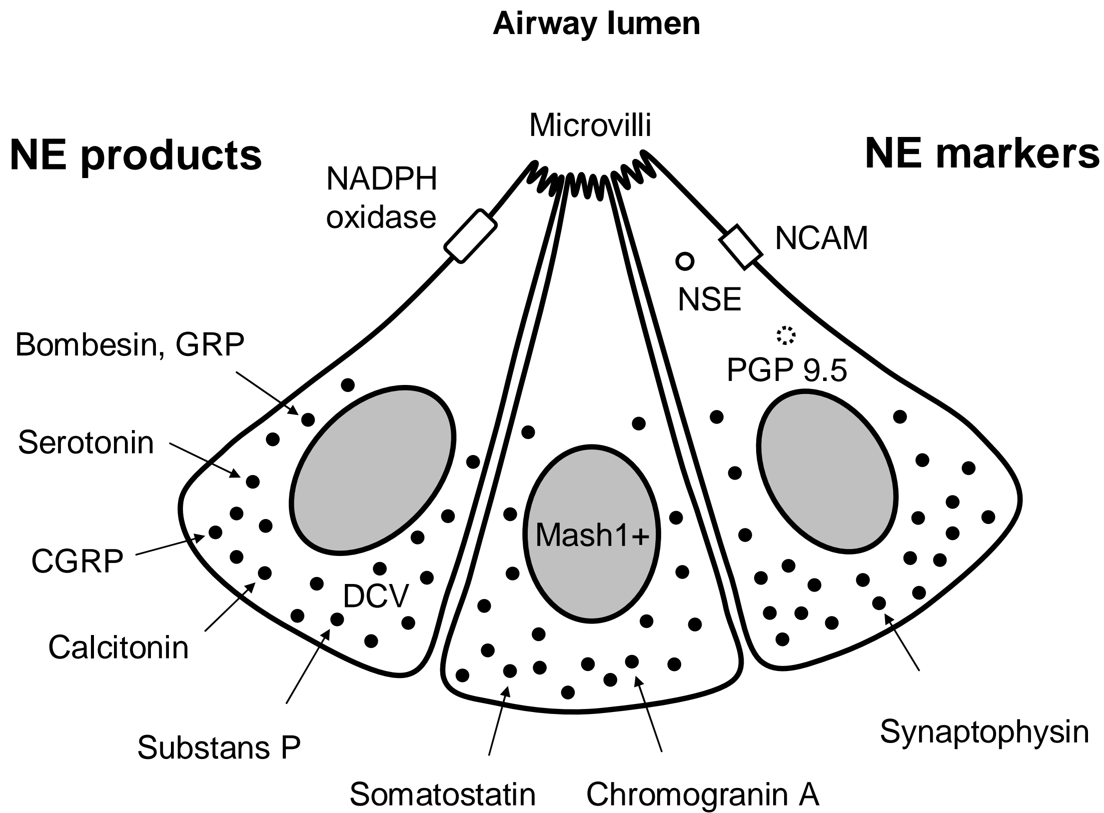

Figure 1. Diagram of pulmonary neuroendocrine (NE) cells (PNEC) forming a neuroepithelial body (NEB) with some of their secretory products, membrane proteins and general NE markers. GRP: gastrin releasing peptide; CGRP: calcitonin gene-related peptide; NSE: neuron-specific enolase; PGP 9.5: protein gene product 9.5; NCAM: neural cell adhesion molecule; DCV: dense core vesicles; Mash1+: positive mammalian achaete-scute 1 complex (neuronal transcriptional factor).

3.3. Functions of the pulmonary NE system

3.3.1. Airway oxygen sensors

NEBs are thought to be specialized airway oxygen (O_2) sensors (Figure 2). They are in close contact with nerve fibres, and were previously thought to be mainly afferently innervated from the vagus nerve. However, recent studies have shown that the innervation may be more complex, including spinal sensory and intrinsic bronchial nerve fibres connecting individual NEBs and PNECs (Adriaensen *et al.* 2003; Pan *et al.* 2004). Products secreted from NEBs may therefore act as neurotransmitters, which induce both stimuli to the central nervous system and accommodation of local regulatory axon reflexes.

Airway hypoxia is a powerful stimulus to NEBs and leads to exocytosis of DCV containing peptides and serotonin (Cutz *et al.* 1993) (Figure 2). In a study by Youngson *et al.*, voltage-activated potassium, sodium and calcium membrane currents in rabbit NEB culture exposed to hypoxia were detected using the patch clamp technique (1993). Hypoxia led to reduced outward potassium current and a subsequent membrane depolarisation. The precise mechanism of O_2 sensing is not fully known, but a membrane-bound O_2-sensing enzyme complex, such as NADPH oxidase, is thought to be the potential receptor (Fu *et al.* 2000). In the postulated model for oxygen sensing, hypoxia affects NADPH oxidase via reduced O_2 concentration, which leads to reduced production of reactive O_2 derivates. This further induces closure of voltage-gated potassium channels, which in turn leads to membrane depolarisation and opening of calcium channels. The influx of calcium finally triggers release of stored substances from secretory vesicles (for review see Cutz & Jackson 1999).

The impact of the ability of pulmonary NE cells to sense the alveolar contents of O_2 and react upon hypoxia is not fully known in adults, but in the neonatal lung this system seems essential (Bolle *et al.* 2000). At birth the O_2-sensing cells of the carotid body, which are activated by blood hypoxemia, have a low chemo-sensitivity, and may react incomplete in the rapid homeostatic responses needed. NEBs, which are abundant at the

time of birth, may therefore be important in sensing hypoxia and maintaining respiratory control at that time.

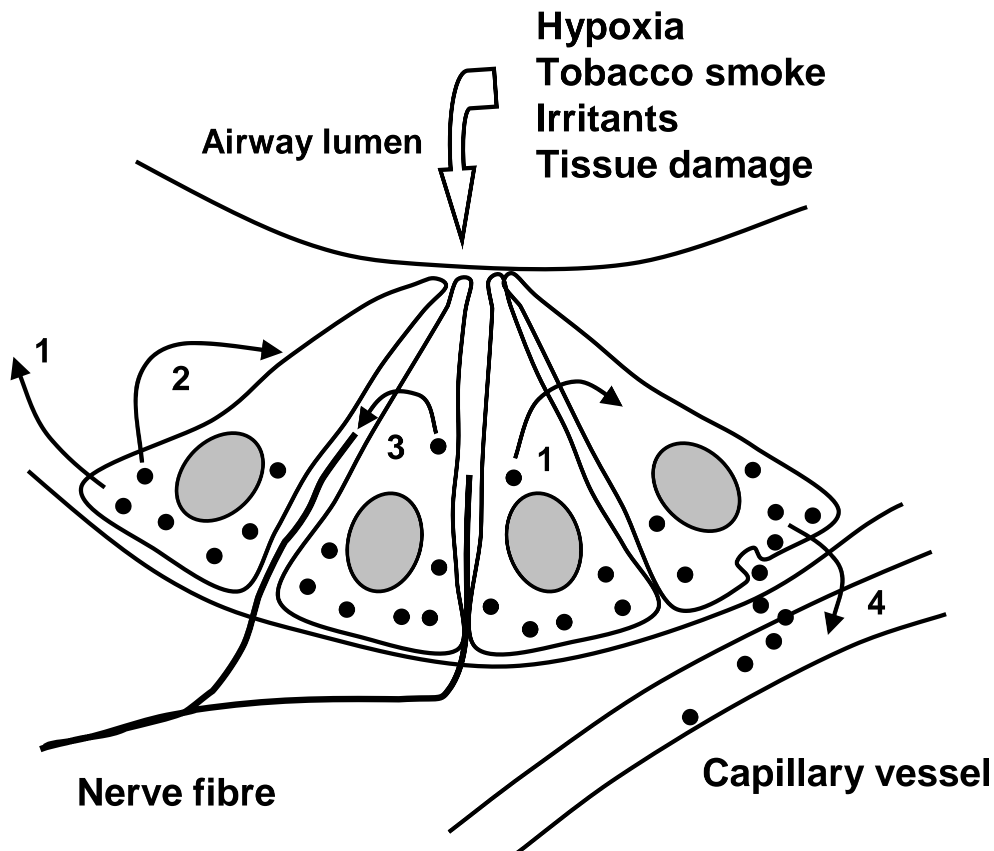

Figure 2. Schematic presentation of a neuroepithelial body (NEB) with some of its functions. NEBs are thought as "receptors" sensing different gases or substances in the airways. As a response, NE products are released and may act in a 1) paracrine, 2) autocrine, 3) neurocrine or an 4) endocrine way.

3.3.2. Regulation of lung development

The high number of pulmonary NE cells in the late foetal and neonatal period is thought to be related to the development of the lung. During pulmonary organogenesis, PNEC are the first cell type to become mature. They are differentiated in a centrifugal pattern, from the central airways and subsequently into the peripheral airways (Sorokin *et al.* 1993). It is postulated that the peptides secreted from PNECs have a paracrine

stimulatory effect on surrounding epithelial cells responsible for lung maturation (Hoyt *et al.* 1993). BLPs and CGRP seem to have mitogenic and maturing effects. They serve as growth factors for several pulmonary cell types and stimulate airway branching and differentiation of cells (Emanuel *et al.* 1999).

In addition, it is known that mechanical stretch is important for lung growth and differentiation in the foetal period. In a recent study by Pan *et al*., PNEC/NEBs are proposed to have mechanoreceptor properties in addition to chemoreceptor qualities (2006). They found that mechanical strain was an important stimulus for release of serotonin from foetal PNEC via mechanosensitive channels. The release of serotonin was independent of potassium-mediated exocytosis, which is the predominant way of hypoxia-induced secretion of serotonin. Taken together, this illustrates the important role of the pulmonary NE system in lung development.

3.3.3. Regulation of pulmonary blood flow

It has been known for decades that alveolar hypoxia induces pulmonary vasoconstriction (hypoxic pulmonary vasoconstriction, HPV) (von Euler 1946). This physiological response is important for optimal oxygenation of the blood. In insufficiently ventilated parts of the lung, the vasoconstriction results in re-distribution of blood to better ventilated regions. The basic mechanisms of the HPV are not completely understood, but the complex reaction seems to involve multiple mediators from different cell types, including PNEC/NEBs (Dumas *et al.* 1999). Several products of pulmonary NE cells have vasoactive properties. Serotonin, which is secreted from the PNECs exposed to hypoxia, is a strong pulmonary vasoconstrictor (Fu *et al.* 2002). Furthermore, CGRP, which is a pulmonary vasodilator, is tonically secreted in normoxic conditions. During hypoxia, the release of CGRP is reduced and may result in a vasoconstriction (Helset *et al.* 1995).

3.3.4. Regulation of bronchial tonus

Several studies support the view that NE cells of the lung could have a regulatory role of the bronchomotor tonus of the airways. The neuropeptide CGRP is found to constrict airway smooth muscle cells in cultures (Palmer *et al.* 1987). Furthermore, hypoxia is

associated with reduced CGRP release from the pulmonary NE cells (Roncalli *et al.* 1993; Helset *et al.* 1995) and may result in a physiological bronchodilatation. In addition, studies on guinea pig tracheal preparations have shown that the spontaneous tonus in these preparations is partly controlled by NE cells of the airways (Skogvall *et al.* 1999).

3.3.5. Immunomodulatory effects

Some studies have suggested a role of PNECs/NEBs in the immunological responses of the airways. Secreted peptides may modulate the inflammatory reaction in diseases like asthma and chronic obstructive lung disease (COPD). Sensitisation with ovalbumine stimulates PNECs to produce and store secretory substances that are released when exposed to antigens (Bousbaa *et al.* 1994; Tsukiji *et al.* 2004). The secretory products may have both pro- and anti-inflammatory effects. One example is CGRP, that has chemotactic effects on eosinophils in the airways (Bellibas 1996), and in addition inhibit edema-promoting actions of inflammatory mediators (Raud *et al.* 1991).

3.4. PNEC and non-malignant respiratory diseases

Morphological changes of the pulmonary NE system are found in many non-malignant conditions (Table 1). The hyperplastic alterations include both increased number of single PNECs/NEBs and the cell density of each NEB. Most often these changes are seen in lung diseases involving inflammatory or fibrotic processes. In conditions like asthma (Stanislawski *et al.* 1981), chronic bronchitis and emphysema (Gosney *et al.* 1989), bronchiectasis (Gould *et al.* 1983; Pilmane *et al.* 1995), cystic fibrosis (Dovey *et al.* 1989) and eosinophilic granuloma (Aguayo *et al.* 1990) the number of immunoreactive NE cells is increased compared to normal lungs.

The important question whether the hyperplasia of PNEC/NEBs is a primary or secondary occurrence has so far not been clarified (Aguayo 1994b). Many of these diseases are chronic diseases characterised by persistent inflammation leading to structural changes and destruction of normal lung tissue. By time this eventually leads

to chronic hypoxia, and it is postulated that hypoxia may be the major stimulating factor for NE cell proliferation. In addition, many of the chronic lung diseases are caused by long-term cigarette smoking which also may lead to hyperplasia of NE cells.

Furthermore, in many pulmonary diseases with a damaged lung parenchyma, NEBs may play an important role as growth regulators. They secrete peptides that are thought to be involved in the repairing process, and may stimulate differentiation of primitive epithelial cells.

Other non-inflammatory conditions like pulmonary hypertension are associated with PNEC hyperplasia (Heath *et al.* 1990). This could in fact be a consequence of chronic alveolar hypoxia. However, it has also been proposed that NE peptides or amines such as serotonin may have bioactive effects on the pulmonary vasculature, representing a primary cause for vascular remodelling (Marcos *et al.* 2004).

Table 1 Pulmonary conditions and exposures associated with hyperplasia of pulmonary NE cells

Human conditions	**Experimental animal models**
Asthma	Acute and chronic hypoxia
Chronic bronchitis, emphysema	Hyperoxia
Eosinophilic granuloma	Tobacco smoke
Bronchiectasis	Nitrosamines
Cystic fibrosis	Naphthalene
Tobacco smoking	Ozone
Pulmonary hypertension	Asbestos
Bronchopulmonary dysplasia	Silica
Sudden infant death syndrome	Diaphragmatic hernia
Congenital diaphragmatic hernia	
Diffuse idiopathic NE cell hyperplasia	
Tumours	

Sources: (Van Lommel *et al.* 1999; Van Lommel 2001; Linnoila 2006)

The fact that PNEC seem to be important for lung development, has initiated several studies looking for changes in the pulmonary NE system in paediatric lung disorders. In bronchopulmonary dysplasia, a condition secondary to mechanical ventilation and high O_2 levels in infants, high number of PNEC/NEBs is reported in addition to other tissue damages (Johnson *et al.* 1982). Other paediatric conditions associated with hyperplasia of pulmonary NE cells compared to age-matched controls are sudden infant death syndrome (Cutz *et al.* 1997), congenital pneumonias (Saad *et al.* 2003) and congenital diaphragmatic hernia (Asabe *et al.* 1999). The reasons for these alterations are not fully known, but may be related to hypoxia, tissue injury and inflammation.

3.5. Lung cancer

Lung cancer is currently one of the most common neoplasms worldwide and is the most frequent cause of cancer death in men. The most important etiological agent of lung cancer is tobacco smoke, accounting for approximately 85-90% of all cases. Other etiological factors include exposure to asbestos, nickel, chromium, polycyclic aromatic hydrocarbons, radon and presents of pulmonary fibrosis or genetic susceptibility (Albert 2004).

3.5.1. Preinvasive lesions

Carcinogenesis of lung cancer is thought as a multistep process involving transformation of normal bronchial epithelium through a continuous spectrum of preneoplastic lesions into invasive carcinoma (for review see Kerr 2001). Along with the morphological changes, increasing molecular and genetic abnormalities occur. This is best recognised for *squamous dysplasia* and subsequent *carcinoma in situ*, which is observed prior to development of invasive squamous cell carcinoma. Two other preinvasive lesions have recently been classified by WHO (Travis *et al.* 1999). *Atypical adenomatous hyperplasia* may be a precursor for adenocarcinoma and *diffuse idiopathic neuroendocrine cell hyperplasia (DIPNECH)* is considered as a precursor for tumorlets and carcinoids (see section 3.5.7). However, lung cancer is often histological

heterogenic and transition and dedifferentiation of the tumour may complicate the finding of a single cell type as the cell of origin.

3.5.2. Classification

The histological diagnosis of lung cancer is based primarily on light microscopy, supported by IHC and EM. Microscopic findings are classified according to the accepted WHO/IASLC (World Health Organisation/International Association for the Study of Lung Cancer) Histological Classification of Lung and Pleural Tumours (Travis *et al.* 1999). The four most common histological types of lung cancer are *squamous cell carcinoma*, *adenocarcinoma*, *small cell carcinoma (SCLC)* and *large cell carcinoma* (for review see Travis 2002). However, many of the lung tumours display a heterogenic picture with a mixture of histological types. In such cases, the portion that is most highly differentiated defines the specific diagnosis, except for tumours that contain features of SCLC, which are classified as SCLC. However, the currently most clinical relevant classification is the distinction between SCLC and the other sub-types, collectively named non-small cell lung carcinoma (NSCLC). These two types of tumours have major differences in presentation, progression and response to therapy.

3.5.3. Squamous cell carcinoma

Squamous cell carcinoma accounts for approximately 30 % of all lung carcinomas (Travis *et al.* 1995). The overall incidence of this histological type is decreasing in North America and Europe, but in some countries like Norway it is raising rapidly among women (Devesa *et al.* 2005). Most of these are central tumours, originates in a segmental or lobar bronchus and often grow intraluminally. Large tumours often present with a central necrosis which leads to cavitation. Histopathological features include intracellular bridging, squamous pearl formation and individual cell keratinisation.

3.5.4. Adenocarcinoma

Approximately 30-35 % of lung carcinomas are *adenocarcinomas*, and the trend is increasing in both gender the latest decades (Devesa *et al.* 2005). They often present as peripheral tumours. Most adenocarcinomas are histologically heterogeneous, and may grow in an acinar/glandular or papillary pattern. *Bronchioalveolar carcinoma (BAC)* is

a subtype of adenocarcinoma characterised by a growth pattern along the alveolar septa but without invasive growth (Beasley *et al.* 2005).

3.5.5. Large cell carcinoma

Large cell carcinoma accounts for about 9 % of all lung carcinomas (Travis *et al.* 1995). The histological diagnosis of large cell carcinoma is applied to tumours that do not have the typical pattern of SCLC and show no squamous or glandular differentiation by light microscopy (Travis *et al.* 1999). They often have large cells with abundant cytoplasm and large nuclei with prominent nucleoli. Several subgroups of large cell carcinoma are recognised by the WHO/IASLC classification, including the clinical important *large cell neuroendocrine carcinoma (LCNEC)* (see section 3.5.9).

3.5.6. Small cell carcinoma

Approximately 20 % of all lung carcinomas are *SCLC*. According to a recent published multinational study this type of lung cancer is slowly decreasing in most counties both in North America and Europe among men (Devesa *et al.* 2005). In women, however, the incidence is increasing especially in Norway and the Netherlands. SCLC is often situated as a central perihilar mass, with infiltration of submucosa and peribronchial tissue (Albert 2004). The histological appearance is characterised by small round or fusiform cells with scanty cytoplasm and finely granular nuclear chromatin with absence of nucleoli. In addition, the mitotic rates are high (> 10 mitoses/2 mm^2). A combination of SCLC with other histological types is seen in up to 28 % of SCLC, and is classified as *combined SCLC* (Nicholson *et al.* 2002).

3.5.7. Pulmonary NE tumours

The WHO/IASLC classification incorporates several different lesions into the term *NE proliferations and neoplasms* as summarised in table 2 (Travis *et al.* 1999). These lesions show NE features like NE growth pattern, express positive NE markers and possess DCV in the cytoplasm.

Hyperplasia of NE cells, as described in section 3.4, is often seen as secondary lesions in conditions with inflammation, hypoxia, and exposure to toxic or irritating substances.

When increasing number of PNEC/NEBs is detected in the airway epithelium without known causes, the term *diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH)* is used (Aguayo *et al.* 1992b). The findings of small aggregates of NE cells named *tumourlets* (< 5mm in diameter) or *carcinoids* (> 5mm) in DIPNECH, propose this hyperplasia as a potential precursor for carcinoid tumours (Kerr 2001; Adams *et al.* 2006). DIPNECH is previously considered as a rare disease, most often seen in non-smoking females without other known lung disease. However, a recent retrospective histological study by Davies *et al.*, concludes that DIPNECH occurs more commonly than previously thought and may be associated by impaired lung function and atypical carcinoids (Davies *et al.* 2006). Fortunately, the condition is considered as an indolent lesion as the majority of the cases remained stable for many years.

Table 2 WHO Classification of Pulmonary Neuroendocrine Lesions

NE cell hyperplasia and tumourlets	
	NE hyperplasia
	Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia
	Tumourlets
Tumours with NE morphology	
	Typical carcinoid
	Atypical carcinoid
	Large cell NE carcinoma
	Small cell carcinoma
Non-small cell carcinoma with NE differentiation	

3.5.8. Carcinoid tumours

Carcinoid tumours account for 1-2 % of all invasive lung carcinomas (Travis *et al.* 1995). They are often found in younger patients without a smoking history and may be associated with paraneoplastic syndromes. The histological pattern consists of cells with finely granular cytoplasm and nuclei with a finely granular chromatin. They are characterised by an organoid or rosette-like growth pattern. *Typical and atypical carcinoids* are distinguished by the number of mitoses (< 2 mitosis/ mm^2 versus 2-10 mitosis/ mm^2) (Travis *et al.* 1999).

3.5.9. Large cell NE carcinoma

In the latest classification by WHO/ IASLC the term *large cell NE carcinoma* has become a separate entity (Travis *et al.* 1999). This sub-group of large cell carcinoma is recognised by the NE morphology (organoid pattern) and positive NE markers confirmed by immunohistochemistry. Mitotic counts are high and necrosis is common. Whether this recently classified type of malignancy should be regarded as a separate clinical entity, and treated like other highly malignant NE carcinomas such as SCLC, has not been clarified yet (Harada *et al.* 2002; Fernandez & Battafarano 2006).

3.5.10. Non-small cell carcinoma with NE differentiation

It is known that some NSCLC with no obvious histological signs of NE features (organoid/palisade -like growth pattern) show IHC and ultrastructural characteristics of NE differentiation (Linnoila *et al.* 1988; Baldi *et al.* 2000). These are collectively referred to as *NSCLC with NE differentiation (NSCLC-ND)*, but are not formally classified as a separate entity as its clinical and prognostic significance has been questioned (Carnaghi *et al.* 2001; Ionescu *et al.* 2007). The portion of NE differentiated tumours has previously been regarded as low (< 20 %) among NSCLC (Baldi *et al.* 2000). However, this largely depends on the sensitivity of the methods used for detection of NE markers. Fresvig *et al.* have previously shown that a higher percentage of squamous cell carcinoma of the lung has IHC signs of NE differentiation (10 of 29) (Fresvig *et al.* 2001), especially when techniques for increasing the sensitivity of IHC staining like the tyramide signal amplification (TSA) method was used.

3.6. Chromogranin A

The human CgA is a single-chain, acidic, water-soluble glycoprotein consisting of 439 amino acids. In the 1960s, CgA was originally discovered in chromaffin granules of the adrenal medulla (Banks & Helle 1965; Blaschko *et al.* 1967). It is now considered as a secretory protein found in DCV of several endocrine and NE cells where it is co-released with other peptide hormones from the secretory granules (Nobels *et al.* 1998). CgA has been found in the adrenal medulla, in nerves and throughout the diffuse NE

system, including the anterior pituitary gland, the thyroid and parathyroid glands, islet cells of the pancreas, NE cells of the bronchial tree and GI tract and Merkel cells of the skin (Feldman & Eiden 2003).

The function of CgA has mainly been linked to its presence in the secretory granules. CgA binds calcium and aggregates in its presence in the acidic granule interior. It is therefore proposed that CgA is important for secretory granulogenesis, sorting of peptides and granule maturation (Feldman & Eiden 2003). In addition, CgA is considered as a prohormone that is intra -or extracellularly degraded in an organ-specific process, generating several bioactive peptides exerting its effects on other organs. Some examples of CgA-derived peptides are; pancreastatin, which is able to inhibit insulin secretion in the pancreas; parastatin, which inhibits parathormone secretion in the parathyroid gland and vasostatins with vasoactive properties (Taupenot *et al.* 2003).

The serum concentration of CgA is the sum of all CgA secreted from endocrine or NE tissue. Elevated levels may therefore reflect hyperplasia of NE cells, an increased secretion of NE peptides or a decreased elimination of CgA from the body as seen in renal failure. In patients with NE neoplasia elevated levels of circulating CgA are detected (Syversen *et al.* 2004), and there exists a strong correlation between the level of CgA and the volume of the NE tumour (Hsiao *et al.* 1990).

3.7. Carbon monoxide

Carbon monoxide (CO) is a colourless, odourless gas produced by incomplete combustion of carbon-containing materials. Its main environmental sources include vehicles, industrial processes, and other fuel combustion sources. Indoor sources may be gas-, oil-, and wood-burning stoves or heaters (WHO 1999). In addition, CO is a product of cigarette smoking, and the greatest source of individual exposure to CO is probably tobacco smoke. CO constitutes about 5% of total effluent of the vapour phase of mainstream smoke. And the concentration of CO in the smoke inhaled into the lung

has been estimated to 400 parts per million (ppm). (Goldsmith & Landaw 1968; WHO 1999).

CO is also endogenously produced in human tissues, through degradation of haemoglobin to bile pigments. Heme is degraded to biliverdin by the enzyme heme oxygenase (HO), with the release of iron and CO. Like nitric oxide (NO), CO activates guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP), which in turn can result in smooth muscle relaxation and vasodilatation. Another important property of CO, like NO, is that these are molecules small enough to easily pass across the plasma membrane, without binding to receptors or transport-proteins. This enables them to act directly on the intracellular target molecule. Therefore, CO is regarded as a cellular signal molecule in normal physiology, and may act as a neurotransmitter, vasodilator, bronchodilator and inhibitor of platelet function (for review see (Sethi 2005; Kim *et al.* 2006). In addition, in small concentrations, it may exert a protective role in a wide variety of diseases, with its anti-inflammatory and anti-proliferative effects (Ryter & Otterbein 2004).

In high concentrations, CO is a poisonous gas, resulting in a severe hypoxic condition with cerebral dysfunction and cardio-respiratory failure. The gas is rapidly absorbed in the lungs, and bound to the oxygen-binding site of haemoglobin forming carboxyhaemoglobin (COHb). CO binds to haemoglobin about 240 times the affinity of oxygen, and in addition causes a left shift in the oxyhaemoglobin dissociation curve. These effects lead to both reduced oxygen transport and release of oxygen to the tissues (WHO 1999).

Although the effects of acute severe exposure of CO are well known, the effects of prolonged low level CO exposure are unclear. Some of the effects may be related to the formation of COHb and hypoxia, which are shown in some animal studies leading to cardiac hypertrophy, increased haemoglobin and haematocrit (Stupfel & Bouley 1970; Turner *et al.* 1979). However, the results are conflicting, and no information exists of long-term effects of CO inhalation on the pulmonary morphology and tumourigenesis.

4. Aims of the study

The main purpose of this thesis was to evaluate the possible roles and regulatory functions of the pulmonary neuroendocrine (NE) system in physiological and pathophysiological conditions and in tumourigenesis. In order to meet this purpose several distinct aims were defined.

1. (Paper I) To examine the relationship between the serum levels of the general NE marker chromogranin A in humans and
   a. smoking habits
   b. lung function
   c. respiratory symptoms.

2. (Paper II) To examine the long-term effects of inhaled carbon monoxide in rats at doses corresponding to tobacco smoking on the
   a. respiratory system
   b. cardiovascular system
   c. tumourigenic processes
   d. the pulmonary NE cells.

3. (Paper III) To examine the expression of different NE markers in surgically treated non-small cell lung cancer using
   a. biochemical analyses of patient sera and plasma
   b. immunohistochemical methods with signal amplification techniques
   c. immunoelectron microscopy methods.

4. (Paper IV) To examine the effects of acute intermittent alveolar hypoxia in an isolated buffer-perfused and ventilated rat lung model
   a. on the release of NE products in the pulmonary circulation
   b. using immunohistochemical methods for detecting changes in the immunoreactivity of the pulmonary NE cells

5. Methodological considerations

In the present thesis several different methods and procedures are used which are described in details in each paper. Some general methodological considerations are given below.

5.1. Study populations

5.1.1. Human studies

In paper I all subjects were selected from a sub-study of the Nord-Trøndelag Health Study (the HUNT study II). The HUNT study is a cross-sectional survey conducted in 1995-1997 in the Norwegian county of Nord-Trøndelag representing 71% of the adults (> 20 years). The sub-study BONT (the Bronchial Obstruction in Nord-Trøndelag) included a 5 % random sample (n = 2 791) of the total study population of the HUNT study (n = 65 225) and those reporting asthma or asthma-related symptoms (n = 8 150) (Langhammer *et al.* 2001). From the BONT study 3 groups were randomly selected for further serological analysis: 1) never-smokers with normal lung function (n = 1 649), 2) ever-smokers with normal lung function (n = 879), and 3) ever-smokers with obstructive spirometric values (n = 359). Among these groups random samples of Helicobacter Pylori (HP) negative subjects (151, 138 and 116) were further analysed for CgA (for details see figure 1 in paper I). The selection of HP negative subjects was done to reduce a possible gastric source of CgA as a previous study has shown a relationship between infection with HP and hyperplasia of NE cells in the gastric mucosa with increased levels of circulating CgA (Sanduleanu *et al.* 2001).
The study subjects (n = 20) in paper III were all recruited from the Department of Pulmonary Medicine, St. Olavs Hospital, Trondheim. They had a histological confirmed NSCLC, Stage I-IIIA (Mountain 1997), and were all treated with a surgically resection of the tumour. In addition, one subject with a typical carcinoid was included as a positive control. Written informed consent was given prior to the surgery. Both studies were approved by the Regional Committee for Ethics in Medical Research and the Norwegian Data Inspectorate.

5.1.2. Animal studies

All animal studies (papers II and IV) were performed with Wistar rats. In paper II, female rats were used as they are previously well characterised in a long-term exposure study in our laboratory (Waldum *et al.* 1996). In paper IV, male Wistar rats were used. This strain and sex has been described in previously published studies of isolated rat lung models (Hauge 1968; Helset *et al.* 1995). The studies were approved by the Animal Welfare Committee of the St. Olavs Hospital, Trondheim, the Norwegian Council for Animal Research, Oslo, and conformed to the "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes"

5.2. Anaesthesia of animals

In papers II and IV, all animals were anaesthetised with a subcutaneously injected mixture of fentanyl 12.5 µg/ml, midazolam 1.25 mg/ml and haloperiodol 0.83 mg/ml at doses of 0.4 ml/100g rat weight. This mixture is a local preparation at the Dept. of Laboratory Animals, St. Olavs Hospital and gives a deep sedation with a good analgesia without respiratory depression. In addition, peripheral perfusion is preserved allowing peripheral venous blood sampling.

5.3. Light microscopy

All tissue specimens for histological and immunohistochemical examination were fixed in 4% phosphate-buffered formaldehyde (PBF) for 24 hours, dehydrated in 80 % alcohol before embedded in paraffin. Haematoxylin and eosin (H&E) staining was used for routine histological evaluation, as described in details in papers II, III and IV. In paper II, a commercial Elastica von Gieson staining kit (Merck KGaA, Darmstadt, Germany) were used to evaluate the number of muscularized pulmonary arteries as described by Keegan et al. (Keegan *et al.* 2001) and in details in paper II.

5.3.1. Immunohistochemistry

In papers II, III and IV, IHC methods are used to visualise different cellular proteins. Immunohistochemistry is a method used to detect molecules in the cells or tissues.

Using specific antibodies in combination with different detection reagents, the antigen-antibody reaction can be visualised by its in situ localisation in a tissue slide. Commonly used detection reagents include fluorescent dyes (for fluorescent microscopy), enzymes (for light microscopy) and colloidal gold spheres (for EM).
The primary antibodies used in the present thesis are both polyclonal and monoclonal. Polyclonal antisera contain several different antibodies directed towards different epitopes on the antigen. The immunoreaction of the polyclonal antibodies is therefore more sensitive than monoclonal antibodies, but has an increased risk of non-specific immunoreaction (background staining). On the other hand, monoclonal antibodies are more specific but have a lower sensitivity. Therefore, in the present study, monoclonal antibodies were used when possible.
All immunohistochemistry in the present study was done using the two-step EnVision-system (DakoCytomation, Glostrup, Denmark). EnVision is a staining technique in which the primary antibody is followed by a detection reagent that consists of a dextran backbone with a large number of peroxidase molecules and secondary antibodies coupled. The EnVision-system has been reported to be a very sensitive method that allows high dilutions of the primary antibodies without loss of specificity and with low non-specific background staining (Sabattini *et al.* 1998).

5.3.2. Tyramide signal amplification technique

To further increase the sensitivity of immunohistochemistry, TSA technique was used in paper III. This method was first described by Adams (1992) and makes it possible to increase the sensitivity up to 1000-fold for several antibodies. By adding biotinylated tyramine (tyramide) additional binding sites for peroxidase are created, before the reaction is visualised by attaching signal molecules (chromogens) to streptavidin.

5.3.3. NE markers

Several antibodies towards different NE cell contents are used to visualise NE features and cells. The expression of different markers depends on the species examined. In our animal studies (papers II and IV) a polyclonal antibody towards CGRP was preferred to visualise PNEC and NEBs. CGRP is a secretory protein in NE cells and neurons, and is highly expressed in rats (Avadhanam *et al.* 1997). In the human study (paper III),

antibodies against CgA, SYN, NSE and NCAM were used. These are among the most common NE markers used in human pulmonary immunohistochemistry. NSE is considered as a sensitive NE marker, but with a low specificity (Carlei & Polak 1984; Brambilla *et al.* 1992). CgA, which is one of the major matrix components of the NE granules, is regarded as a specific NE marker and is easily detected in all human NE cells (Nobels *et al.* 1998). Furthermore, NCAM, a membrane attached molecule, is expressed in most NE cells and NE tumours (Jin *et al.* 1991; Lantuejoul *et al.* 1998).

5.4. Electron microscopy

In papers II and III, EM was used for ultrastructural analyses. All tissue samples were cut into 1mm^3 blocks, immediately immersed in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide for 60 min, before the samples were dehydrated in a graded series of ethanol and propylene oxide and embedded in epoxy resin LX 112 (Ladd Research Industries, Willinton, VT, USA). The samples were further sliced in ultra-thin sections (70-90nm, RMC MTX Ultramicrotom, Boecklerand, Germany) and mounted on grids before being contrasted with uranyl acetate and lead citrate. For conventional transmission EM copper grids were used (paper II). Nickel grids were used for IEM to prevent chemical precipitations. The grids were further examined in a JEOL 1011 transmission EM (Tokyo, Japan).

5.4.1. Immunoelectron microscopy

Like immunohistochemistry, the reason for using IEM is to localise molecules in the cells, but at an ultrastructural level. Immunolabelling is performed either before (pre-embedding) or after (post-embedding) the embedding of the tissue. The latter was done in the present study (described in details in paper III). Some of the advantages of the post-embedding technique are that the ultrastructure is well preserved, the method is relatively easy to perform, and it is a reliable technique for localising intracytoplasmic antigens (Merighi *et al.* 1992). Using primary antibodies towards sub-cellular structures or molecules, which are further attached to secondary antibodies conjugated with an electron dense particle, specific structures are easily detected and distinguished from

normal cellular contents. The most common detection reagent used in IEM is colloidal gold spheres (5-50nm in diameter). Labelling efficiency of the gold probe seems to be inversely proportional to the diameter of the gold particle, and for single procedures 10nm gold probes are therefore recommended, as used in paper III (Merighi *et al.* 1992).

Before labelling with the primary antibody, retrieval of antigens of the epoxy embedded specimens must be performed. In paper III, antigen retrieval was achieved by placing the grids in an alkaline solution, (ph 10, Target Retrieval Solution, TRS, Dako Corporation, Carpinteria, CA, USA) and heating in an autoclave at 140°C for 15 min. In a study by Fossmark *et al*. at our laboratory, the CgA labelling efficacy after antigen retrieval in an alkaline solution was higher in an autoclave at 135°C compared to a microwave at 100°C for NE vesicles without deterioration of the ultrastructure (2005a).

5.5. CO exposure

5.5.1. Exposure chambers

For experimental inhalation studies, well designed exposure chambers are essential. The chambers with its equipments should be able to give a constant concentration of the gas in the chamber, allow observation of the animals and measurement of the exposed environment, and, for safety reasons, leakage of the gas to the ambient air should be avoided. In addition, sufficient area and easy access to the animals should be provided, to facilitate the cleaning and feeding. To meet these requirements, three stainless steel and glass chambers were used for gas exposure in the CO inhalation study (figure 4). The chambers, each 650 l, were designed as a cube with a conical top and bottom, as described in a previous exposure study from our laboratory (Waldum *et al.* 1996). A mixture of hospital medical quality air and CO (AGA, Oslo, Norway) was continuously circulated through two of the chambers and created a constant CO concentration of 200 ppm. Pure hospital medical quality air was circulated through the control chamber, at equal rate to the two CO exposed chambers. The CO concentration, chamber temperature and humidity were monitored daily.

Figure 4. Stainless steel and glass chambers used for the chronic CO inhalation study (paper II). A mixture of CO and air was continuously circulated through the exposure chambers (from the left, 1st and 2nd chamber) creating a CO concentration of 200 ppm. Only pure air was circulated through the chamber containing the control animals (3rd chamber).

5.5.2. CO exposure protocol

The animals in the CO inhalation group were exposed to CO for 20 hours a day, five days a week (Monday to Friday) for 72 weeks. During the weekends they were only exposed to pure air for practical reasons. The animals had only access to food when not exposed to CO, to avoid any gas contamination of the food, and fulfil the criteria of a pure inhalation study. All animals were taken directly from the exposure chambers before sacrificing or blood sampling.

5.6. Isolated perfused and ventilated rat lung

Isolated animal organ models are well suited for exposure studies on endocrine or NE systems. In our laboratory, we have previously developed and used an isolated vascularly perfused rat stomach model for studies on the endocrine function of the stomach (Kleveland *et al.* 1986; Sandvik *et al.* 1989). Given the knowledge that the respiratory organs also show endocrine or NE properties, the purpose of paper IV was to investigate the effects of intermittent hypoxia (IH) on the release of NE products from the rat lung. To exclude any systemic origin of the bioactive substances, the experiments were performed on isolated buffer-perfused and ventilated rat lungs. This model allows full control over the humoral factors released in the pulmonary circulation since the vascular perfusate is not recirculated. In addition, the isolated lung model is a viable organ with intact metabolic function for several hours (Baker *et al.* 1999). The present isolated perfused and ventilated rat lung model is developed in our laboratory but methodologically based on previously described isolated rat lungs by Hauge and Bjertnæs (1968; 1977). The model is described in detail in paper IV, and only some general considerations are discussed below.

5.6.1. Isolated lung preparation

After the rats were deeply anaesthetised, a tracheostoma was made and the animals were connected to a rodent ventilator. Thereafter, the lungs were exposed via a medial sternotomy. To prevent thrombi formation in the lungs, 350 IU Heparin (LEO, Copenhagen, Denmark) was injected into the right ventricle before the lungs were removed from the thorax. The ventilation was then stopped and the trachea-lung-heart preparation was dissected free from the chest. During this procedure special care was taken not to touch the fragile lungs. Only morphologically undamaged lungs without leakage were used. The inflow cannula with two additional outlets (one for pressure monitoring and the other serving as an air trap) was then placed into the pulmonary artery and ligated. Through a cut in the left ventricle, the outflow cannula (with one additional outlet for pressure monitoring) was placed in the left atrium and secured with a band around the ventricles. The preparation was mounted in a special designed humidified water-jacketed perspex chamber (36-37 ºC) suspended by the air-tap tube of

the pulmonary artery cannula, and connected to the ventilator and the tubes for in- and outflow and pressure monitoring. This allowed the lungs to expand freely during the ventilation.

5.6.2. Perfusion, ventilation and measurement

The lungs were perfused through the pulmonary artery with a pre-heated (38 ºC) Krebs-Ringer-albumin buffer (paper IV) in single-pass perfusion using a peristaltic perfusion pump (Ismatec IPC, Glattbrugg, Switzerland). Bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used as a buffer-colloid to prevent pulmonary oedema. A positive pressure ventilator (Harvard Rodent Ventilator Model 683, Massachusetts, US) was used for ventilation with either a normoxic gas-mixture (21% O_2, 5% CO_2, 74% N_2, AGA, Norway) or a hypoxic gas-mixture (2% O_2, 5% CO_2, 93% N_2, AGA, Norway) with a tidal volume of 2 ml, a respiratory frequency of 80/min and an end-expiratory pressure of 2 cm H_20 to avoid collapse of the lungs. The pressure in the pulmonary artery (Ppa) and left atrium (pulmonary venous pressure, venous outlet pressure, Ppv) were continuously recorded by pressure transducers (Marquette Tramscope, Marquette electronics inc, Milwaukee, WI, USA) that were connected to tubes from the inlet and outlet cannulas. The Ppv was adjusted and kept constant at minus 1 mmHg during the experiment. Since the perfusion flow and the Ppv were kept constant, changes in the pulmonary vascular tonus were reflected as changes in Ppa.

5.6.3. Experimental protocol

After 15 min equilibration on normoxic gas, the hypoxic exposed lungs were alternately ventilated with the hypoxic and normoxic gas-mixture for cycles of 5 min duration. The control lungs were ventilated with normoxic gas only. During the experiment samples of the outflow perfusate were repeatedly collected.

5.7. Measurements and analyses

5.7.1. Animal and organ weights

In paper II, the animal weights were measured every 4th week. During the exposure period, all rats from the same cage were weighed together, and the weights were reported as means. At the end of the study, each animal and the reported organ were weighed separately.

5.7.2. Spirometry

In paper I, spirometric measurements from the BONT study were used for classifying subjects according to lung function. Flow/volume spirometry was recorded by HUNT staff using pneumotachographs according to the recommendations of the American Thoracic Society (ATS 1995), as described in detail by Langhammer *et al*. (2001). The predicted forced expiratory volume in one second (FEV1%) was calculated using prediction equations estimated for the population of Nord-Trøndelag (Langhammer *et al.* 2001).

5.7.3. Immunoassays for Helicobacter Pylori and NSE

In paper I, a commercial enzyme immunoassay (Pyloriset EIA-IgG, Orion Diagnostica, Espoo, Finland) was used for detection of immunoglobulin G antibodies to Helicobacter Pylori in serum. The analyses were done at Levanger Hospital, The Nord-Trøndelag Hospital Trust, and titer values >300 were scored as positive.

In paper III, an electrochemiluminescence immunoassay (ECLIA) method with reagents from Roche Diagnostics GmbH (Mannheim, Germany) was used for measurement of serum NSE. These tests were performed at the Department of Biochemical Medicine, St. Olavs Hospital, Trondheim.

5.7.4. Radioimmunoassays

Circulating CgA in papers I and III were analysed at the Department of Biochemical Medicine, St. Olavs Hospital, Trondheim, using a commercial radioimmunoassay (RIA)

method with reagents from EuroDiagnostica, Malmø, Sweden. This method is based on polyclonal antibodies raised in rabbits against a purified fragment containing amino acid sequence 116-439 of the CgA molecule, and has been shown to detect both intact CgA and fragments of CgA (Stridsberg *et al.* 1993; Stridsberg *et al.* 1995).

In paper IV, perfusate levels of BLPs and serotonin were analysed at the Department of Cancer Research and Molecular Medicine (Laboratory of Basal Physiology) by competition binding assays using commercially available RIA kits. BLPs immunoreactivity was measured using a Bombesin RIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA), where the antibody has a 100% cross-reactivity with bombesin, 50% with porcine gastrin releasing peptide (GRP) and < 0.01% with substance P and vasoactive intestinal peptide (VIP). The lower limit of detection was 5.8 pg/tube. Levels of serotonin were determined using a Serotonin-RIA kit (DLD Diagnostica GmbH, Hamburg, Germany) with a 100% antibody cross-reactivity for N-Acetylserotonin, and a lower limit of detection of 2 ng/ml in liquor. The samples were assayed in duplicate and calculated mean values were used.

5.7.5. Quantification of NE cells

In the animal studies (papers II and IV), quantification of pulmonary NE cells was done using antibodies to the secretory peptide CGRP, which is highly expressed in PNECs and NEBs in rat (McBride *et al.* 1990). In addition, antibodies to CgA were used in paper IV. Both PNECs and NEBs were identified as distinct positive immunoreactive cells with a stained cytoplasm located within all levels of the respiratory tree down to the respiratory bronchioles. The size of the NEBs was reported as number of immunoreactive cells with a visible nucleus. In addition, single PNEC in the airways were counted. Total number of NE cells/NEBs was divided by the total area of the section, which was calculated from photos of the lung sections using iTEM Analysis (Soft imaging system GmbH, Münster, Germany) software.

5.7.6. Statistical analyses

All data were analysed using the statistical package for social sciences (SPSS, version 13.0, Chicago, IL, USA) and GraphPad Prism Software (version 4.01, San Diego, CA

USA). The data are presented as means ± standard deviation (SD) or standard error of the mean (SEM). The non-normal distributed data for serum CgA (papers I and III) and NSE (paper III) are presented as medians with interquartile range. A two-tailed p-value < 0.05 was considered statistically significant.

For continuous normally distributed data Student`s t-test and analyses of variance (ANOVA) with Bonferroni`s post hoc test were used for comparisons between two or multiple groups, respectively. The non-parametric Mann-Whitney U test was used for comparisons between two groups of non-normally distributed data. In paper I, the variable serum CgA was transformed to log-CgA to achieve a normal distribution of data, before analyses were performed stratified by sex. Differences between proportions were analysed using the Chi-squared test and Fisher`s exact test. The Spearman rank correlation test was used to test the correlation between non-normally distributed variables such as serum CgA and plasma NSE in paper III.

A multiple linear regression model was used in paper I to assess the impact of the independent variables age, pack-years, FEV1%, presence of respiratory symptoms and serum creatinine on the dependent variable log-CgA. Analyses were done separately for each sex. The assumptions for linear regression analyses, such as normally distributed residuals, constant variability of the independent variables and a linear relation between the independent and dependent variable, were met for this model.

6. Results and discussion

6.1. The pulmonary NE system and respiratory pathophysiology

6.1.1. Serum levels of CgA in smoking-induced airway diseases

Based on morphological studies, it has been proposed that the NE system may play an important role in the pathogenesis of smoking-induced airway diseases (Aguayo 1994b). In addition, some papers have also reported increased urinary levels of NE peptides in smokers or smoking-related airway obstruction (Aguayo *et al.* 1989; Aguayo *et al.* 1992a; Meloni *et al.* 1998). In paper I, we report for the first time circulating levels of the general NE marker CgA according to the smoking habits, lung function and respiratory symptoms. Among the selected subjects from the HUNT study, we observed significantly higher levels of serum CgA in male smokers with airway obstruction than in smokers with normal lung function and in never-smokers. In addition, respiratory symptoms were associated with elevated CgA levels in male smokers. Among females, these differences were not significant. Using multiple linear regression analysis, age, lung function and serum creatinine were statistically significant predictors of CgA in males, accounting for 25% of the variability of CgA.

In paper I, all data were stratified by sex. This revealed a different pattern of CgA levels between the gender according to smoking habits, lung function and respiratory symptoms. However, results after including the interaction terms ((FEV1% x sex) and (pack-years x sex)) in a non-stratified multiple linear regression model did not support the thought of an interaction of sex on the serum CgA. One possible explanation for the apparently sex difference in the analyses may be the small number of females in some of the study groups.

Another finding in paper I was the increasing levels of CgA with decreasing lung function and elevated levels of CgA in smokers with respiratory symptoms and chronic bronchitis. This indicates that increased CgA levels observed in paper I may be related to lung disease and inflammation, and not to pharmacological or toxic effects of nicotine or cigarette smoke alone.

Based on the results of paper I, it may be suggested that the pulmonary NE system plays a role in some airway diseases. This paper does not allow us to confirm the origin of elevated circulating levels of CgA. As discussed in paper I, the higher circulating CgA levels in smokers with airway obstruction or in subjects with respiratory symptoms and chronic bronchitis may reflect either a local secretion from the lungs or a general NE activation. Hypothetically, PNEC/NEBs hyperplasia associated with airway inflammation may increase the serum levels either as increased number of NE cells (constitutive pathway) or as increased release from pulmonary NE cells (regulatory pathway). Increased levels of NE peptides, such as BLPs have previously been found in bronchioalveolar lavage fluid from smokers compared with non-smokers (Aguayo *et al.* 1989). However, severe airway obstruction like COPD, which is considered a systemic disease (Wouters 2002), may also lead to a general NE activation. A comparable situation may be patients with chronic heart failure, where elevated circulating levels of CgA are reported (Ceconi *et al.* 2002).

In conclusion, elevated serum CgA levels in subjects with airway obstruction and respiratory symptoms may represent NE activation in inflammatory or remodelling processes in the respiratory organs.

6.1.2. Effects of chronic inhalation of CO on the respiratory and cardiovascular system

Chronic CO inhalation, at exposure levels comparable to heavy smokers, may have important effects on health. However, few long-term exposure studies have been published (Stupfel & Bouley 1970; Turner *et al.* 1979), and no information exists of chronic CO inhalation and effects on the respiratory morphology and pulmonary NE cells. In paper II, the effects of long-term CO exposure were evaluated in rats, with particular emphasis on morphological findings in the respiratory organs and the cardiovascular system.

The results from the exposure study show that chronic inhalation of CO does not appear to induce morphological changes in the lung of rats. The respiratory pathology usually associated with cigarette smoking, such as emphysema, inflammation or remodelling of

the parenchyma, were absent in the present study (paper II). The same conclusion was made by Hugod in a short-term study of adult rabbits exposed to 200 ppm for 6 weeks (1980). However, another study by Penney *et al.* has reported an increase in lung weight in rats exposed to 250-1300 ppm for 7.5 weeks, which was not explained by specific morphological changes (1988). This finding was not confirmed in our study.

Experimental exposure studies of different gases have previously shown morphological changes in the pulmonary NE system. An increase in the number of PNEC and NEBs is reported in response to chronic high concentration of oxygen, ozone and non-filtered urban ambient air (Schuller *et al.* 1988; Ito *et al.* 1989; Ito *et al.* 1994). Furthermore, some studies have linked cigarette smoking to changes in the pulmonary NE system. Components of tobacco smoke are reported to have trophic effects on pulmonary NE cells (Novak *et al.* 2000). However, even though NEBs may exhibit a potential binding site for CO through the NADPH-oxidase, no difference in number of NEBs was observed between the groups in the present study (paper II). Chronic CO inhalation did not affect the pulmonary NE cells in a way detected by current morphological evaluation. We may therefore conclude that other components of the tobacco smoke than CO are responsible for changes in the pulmonary NE system.

On the other hand, recent studies have reported several favourable effects of low-dose CO inhalation. CO may have cytoprotective effects in acute lung injury and lung fibrosis (Otterbein *et al.* 1999; Sato *et al.* 2001). In addition, CO inhalation seems to decrease airway hyperresponsiveness in mice models (Ameredes *et al.* 2003). Whether these effects of CO on pathological conditions are mediated via changes in the pulmonary NE cells are not known. Our study was not designed to investigate possible protective effects of CO on pathological processes.

Some previous studies have proposed a link between CO exposure and cardiovascular diseases (Astrup *et al.* 1967; Stern *et al.* 1988; Burnett *et al.* 1997; Melin *et al.* 2005). However, the evidences are inconsistent. In the present study (paper II), CO exposure for 72 weeks did not lead to any morphological changes in the cardiovascular system, except for cardiac hypertrophy. Histological examination of the myocardium did not

reveal any signs of scarring, which could indicate previous myocardial infarction. In the aorta and femoral artery, no signs of atherosclerosis were observed in CO exposed rats. The mechanisms for cardiac hypertrophy in CO exposed animals, which has been reported by several authors (Penney *et al.* 1984; Clubb *et al.* 1986; Loennechen *et al.* 1999), are not completely known. One hypothesis is that ventricular hypertrophy results from an increase in volume overload due to blood volume and viscosity enhancement and increased ventricular preload. However, it may also be proposed that CO may have some direct effects on the myocardium. In a sub-study of paper II (Bye *et al.*, submittet 2007), cellular analyses showed both longer and wider cardiomyocytes in the CO exposed animals. In addition, several regulatory proteins associated with pathological cardiac hypertrophy were up-regulated suggesting intrinsic effects of CO on the myocardium. This is also supported by the fact that CO, like NO, is regarded as a cellular signal molecule (Kim *et al.* 2006).

6.2. The pulmonary NE system and tumourigenesis

6.2.1. Chronic inhalation of CO and tumourigenesis

Inhalation of cigarette smoke is the main etiological agent of lung cancer (Hutt *et al.* 2005). Experimental studies have shown that several compounds of tobacco smoke, like polycyclic aromatic hydrocarbons and nitrosamines are associated with cancer in the respiratory organs (Hecht 2002). However, little is known about the effects of long-term CO exposure on induced or spontaneous tumourigenesis. In the present study (paper II), we could not detect any carcinogenic effects of inhalation of CO at doses corresponding to tobacco smoking with an exposure time of three quarters of the rats life expectancy. No difference of the overall tumour prevalence was detected between the groups. Only one lung tumour (an adenocarcinoma) was observed. Even though this tumour was observed in the CO exposed group, the finding could be accidental. In addition, one papillary NE hyperplasia was observed in each of the study groups. Another study by Ito *et al.* reported bronchial papillomas with NE differentiation in rats exposed to polluted ambient air for 18 months (1989). However, this study did not include a control group for comparison and CO level in the ambient air was not measured. Furthermore,

in the present study (paper II), no increased number of PNEC or NEBs was observed. Therefore, our findings of papillary NE hyperplasia may represent a normal spontaneous occurrence in aged rats. Taken together, the present exposure study did not support any idea of a tumourigenetic effect of CO in rats.

6.2.2. NE markers in non-small cell lung cancer

In paper III, we report the expression of NE markers in 20 cases of NSCLC using biochemical, IHC and ultrastructural methods. The results of IHC evaluation of the NE markers NSE, SYN, CgA and NCAM showed a wide variation in the immunoreactivity. The proportion of immunoreactivity ranged from only 5 % with CgA to 50 % with NSE using conventional methods. Adding the tyramide signal amplification technique, the number of immunoreactive cases increased significantly for CgA and SYN. With the use of immunoelectron microscope only one of 15 representative tumours showed ultrastructural immunolabelling for CgA in cytoplasmic vesicles.

These findings illustrate some of the problems in assessing the differentiation of a tumour. The formation of a tumour involves several steps from a genetically disturbed cell into uncontrolled growth of cells (Alberts 2002). During these steps the cell of origin often looses its characteristics and may be difficult to recognise in clinically presented tumours. The ability to detect the general NE marker CgA for instance, is directly related to the number of secretory vesicles in the cytoplasm. Tumours with small number of vesicles may therefore show no immunoreactivity for CgA using conventional IHC methods. However, amplification of the IHC signals may increase the sensitivity, as shown in the present study (paper III) with the use of tyramide signal amplification technique. Even though no "gold standard definition" of NE differentiation exists, ultrastructural finding of CgA labelled vesicles in the tumour cells represents a strong hallmark for NE differentiation. In the present study (paper III) only one of five IHC positive CgA tumours showed immunogold labelling of the DCV, which may be explained by sampling errors in ultrastructural analyses. In a comparable study on SCLC, which is a well defined NE tumour, Dardick *et al.* reported only three immunogold labelled tumours of 15 CgA IHC positive tumours (1996). Taken together,

ultrastructural evaluation with IEM does not seem to increase the sensitivity of NE differentiation compared to sensitive IHC techniques.

It has been argued that looking for NE differentiation in NSCLC is only of academic interest since the clinical significance of NE differentiation is disputable. The current opinion is that the finding of NE features in NSCLC does not influence prognosis or response to treatment (Hiroshima *et al.* 2002; Pelosi *et al.* 2003; Howe *et al.* 2005; Ionescu *et al.* 2007). However, paper III illustrates some important aspects regarding the role of pulmonary NE cells in carcinogenesis and tumour classification for future therapeutic modalities.

Even though lung cancer is one of the most common neoplasms, the exact cell of origin of the different histological types of lung cancer is not well understood. As described in section 3.5.1, some preneoplastic changes are suggested, which is mainly based on histological findings associated with resected carcinomas of the lung. In addition, accumulations of genetic abnormalities have been found in correlation with increasing morphological changes (Hirano *et al.* 1994; Greenberg *et al.* 2002). However, in smokers, who in particular are at risk, often several different preneoplastic changes are seen at the same time at different locations (lung "field cancerisation") (Greenberg *et al.* 2002).

The finding of NE markers in non-NE tumours (paper III) may suggest that the NE cells of the lung are the cellular origin of the NE differentiated lung carcinomas. NE cells are multipotent cells with the ability to divide, and may differentiate into many types of cells (Sunday & Willett 1992). Classical NE tumours of the lung have been, according to their NE features with positive NE markers and DCV, proposed to originate from NE cells in the bronchial mucosa (Kerr 2001). The findings in the present study of NE features of NSCLC (paper III) may suggest an origin from the same cells. It can be hypothesised that NSCLC with NE characteristics rather are de-differentiated NE lesions than tumours with NE differentiation. Furthermore, in gastric carcinogenesis, some studies have proposed that the NE enterochromaffin like cell (ECL cell) in the stomach may be the origin of gastric adenocarcinomas (Waldum *et al.* 1998). Both

human observations and experimental animal studies have suggested the principle of NE cell de-differentiation in tumourigenesis (Qvigstad *et al.* 1999; Fossmark *et al.* 2004; Fossmark *et al.* 2005b). On the other hand, lung cancers often show a heterogeneous histology with a mixture of subtypes, including both SCLC and NSCLC (Brereton *et al.* 1978). This may also suggest that some lung carcinomas may be derived from a common endodermal stem cell with potential of multidirectional differentiation (Brambilla *et al.* 2000).

Finally, knowledge of the expression of various NE markers in NSCLC may have implications for future therapy. Increasingly experimental and clinical use of molecular targeted therapy with drugs targeting important molecules involved in different steps in the neoplastic transformation of cells may necessitate further sub-classification using various markers (Ho *et al.* 2005; Janson 2005; Maione *et al.* 2006). This may give additional information concerning prognosis and response to new therapeutics. In the future, lung cancer treatment may be individually adjusted according to a set of markers including different NE markers.

6.3. The pulmonary NE system and the physiological hypoxic response

In order to maintain a well balanced ventilation and perfusion in the lung, different homeostatic reactions to low oxygen levels are observed in the respiratory organs (von Euler 1946). It is proposed that several systems and cells are involved in the hypoxic response of the airways and pulmonary vasculature, including the pulmonary NE system (Gosney 1994; Dumas *et al.* 1999; Jain & Sznajder 2005). In paper IV, we have evaluated the effects of intermittent alveolar hypoxia on the pulmonary NE system. Using an isolated buffer-perfused and ventilated rat lung model, release of the NE products BLPs and serotonin into the pulmonary circulation during IH was examined. The findings revealed that during the first periods of IH, levels of BLPs in the perfusate gradually decreased. Even though a lot of knowledge exists about the functions of bombesin and BLPs (Willey *et al.* 1984; Sunday *et al.* 1990; Lemaire 1991), little is known about the effects of alveolar hypoxia on the pulmonary release of BLPs. In the

respiratory system, immunoreactivity for BLPs is found mainly in the pulmonary NE cells (Aguayo *et al.* 1990) and in some GRP containing nerve fibres (Uddman *et al.* 1984). Levels of BLPs in the pulmonary circulation may therefore correspond to changes in the pulmonary NE system.

Given the proposed effects of NEBs to sense the alveolar oxygen contents (Cutz & Jackson 1999), the finding of decreased levels of BLPs during intermittent hypoxia illustrates some important aspects of the function of the pulmonary NE system. Even though alveolar hypoxia is associated with membrane depolarisation and release of vesicle contents from the pulmonary NE cells (Cutz *et al.* 2003), this reaction may be modified by local actions. By autocrine, paracrine or neurocrine feedback mechanisms the NE cells may adjust the release of products to suit the appropriate physiological response. In this regard, reduced levels of BLPs, which among other functions act as bronchoconstrictors (Impicciatore & Bertaccini 1973), may results in a bronchodilatation in order to maintain adequate ventilation. This is also supported by another study by Helset *et al.* showing decreased release of the vasodilator CGRP in the perfusate of blood-perfused rat lungs ventilated with hypoxic gas (1995).

In paper IV, we did not find any association between the release of serotonin and IH. During the experiment the levels of serotonin detected in the perfusate varied considerably at different periods in both groups. In an experimental study of dogs, Yemen *et al.* reported increased levels of serotonin in blood-samples from the left ventricle during hypoxic ventilation (2003). In addition, a recent in vitro study has shown release of serotonin from intact rabbit NEB cells during hypoxia (Fu *et al.* 2002). The results from paper IV did not confirm these findings. However, this may have some methodological explanations. Additional sources of serotonin may have masked the hypoxic serotonin response from the pulmonary NE cells, like serotonin stored in platelets (Omenn & Smith 1978) and pulmonary mast cells (Aldenborg *et al.* 1993) in addition to neurotransmitter-release from neurons.

Increased number of pulmonary NE cells has been described in association with hypoxia in both experimental animal studies and human pathologic reports (Keith &

Will 1981; Johnson *et al.* 1982; Gosney *et al.* 1989; Aguayo *et al.* 1990; Aguayo 1993, 1994a). This has most often been ascribed to hyperplasia of NEBs/PNEC, either as a primary pathological event or as a secondary response to low levels of oxygen (Aguayo 1994a). However, changes in the intercellular level of bioactive substances and thereby the immunoreactivity for antibodies may occur rapidly. In paper IV, we report an increase in number of CGRP immunoreactive NEBs in lungs ventilated for only 40 min with IH compared to normoxic ventilated lungs. This finding is supported by other studies showing increased CGRP immunoreactive pulmonary NE cells in rats exposed to ambient hypoxia for 4 hours (Roncalli *et al.* 1993), and an observation of decreased levels of CGRP in blood from isolated perfused rat lungs ventilated with intermittent hypoxic gas for 5 min (Helset *et al.* 1995). Together, this support the idea that hypoxia leads to decreasing release and thereby an up-concentration of CGRP in the NEBs, rendering more cells detectable with IHC methods.

Finally, using antibodies to the general NE marker CgA, only a few NEBs/PNEC were detected in the present study. In contrast to the expression of CGRP, no difference in the number of CgA immunoreactive pulmonary NE cells between the IH ventilated lungs and the controls was observed. Again, this suggests that the hypoxic response of the pulmonary NE cells may be complex and involves specific reactions for the actual NE product.

In conclusion, the results from paper IV indicate a rapid response to intermittent alveolar hypoxia on the release of some NE products in isolated buffer-perfused rat lungs. This further suggests that the pulmonary NE system may play a role in order to maintain a well balanced ventilation and perfusion relationship in the lung.

7. Main conclusions

1. Serum levels of CgA
   - are increased in male smokers with airway obstruction compared to non-smokers and smokers with normal lung function
   - are correlated to the degree of airway obstruction in men
   - are associated with the presence of respiratory symptoms and chronic bronchitis

2. Chronic inhalation of CO in rats at levels corresponding to tobacco smoking
   - induces right and left ventricular hypertrophy
   - does not lead to increased atherosclerosis
   - is not associated with tobacco smoking related pathology of the respiratory system
   - has no impact on the morphology of pulmonary NE cells
   - has no tumourigenic effects

3. Evaluation of NE markers in NSCLC demonstrated that
   - using sensitive IHC methods, like the tyramide signal amplification technique, a greater proportion of NE differentiated tumours was detected
   - IEM methods with immunogold-labelling of CgA were not as sensitive for detection of NE features as IHC techniques with signal amplification
   - levels of circulating CgA or NSE did not correlated to positive IHC findings

4. Evaluation of NE products in isolated perfused and ventilated rat lung revealed
   - a decreased release of BLPs in perfusate from lungs intermittently ventilated with hypoxic gas compared to normoxic controls
   - a release of serotonin in lung perfusate independent of hypoxic or normoxic ventilation
   - an increase in CGRP immunoreactive NE cells in hypoxic ventilated lungs
   - no difference in number of CgA immunoreactive NE cells between hypoxic and normoxic ventilated lungs

Together, the findings presented in this thesis have elucidated some important aspects of the pulmonary NE system in man and rodents. The thesis suggests that the NE system of the lung may play a role in pathological conditions like inflammatory or remodelling processes in the respiratory organs, in the tumourigenic process of the lung and in physiological adaptations such as for instance hypoxia. In addition, the results indicate that environmental substances such as CO do not have any impact on the pulmonary NE cells. However, the basic mechanisms behind the changes in the NE cells in different conditions are still not known. Further studies are needed, especially on the role of PNEC/NEBs in inflammatory lung diseases and pulmonary carcinogenesis.

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Paper I

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Paper II

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Chronic inhalation of carbon monoxide: Effects on the respiratory and cardiovascular system at doses corresponding to tobacco smoking

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Abstract

Carbon monoxide (CO) is a dangerous poison in high concentrations, but the long-term effects of low doses of CO, as in the gaseous component of tobacco smoke, are not well known. The aims of our study were to evaluate the long-term effects of inhaled CO on the respiratory and cardiovascular system at doses corresponding to tobacco smoking and its effect on tumourigenesis and pulmonary neuroendocrine (NE) cells. Female Wistar rats were exposed to either CO (200 ppm) for 20 h/day ($n = 51$) or air ($n = 26$) for 72 weeks. Carboxyhaemoglobin was $14.7 \pm 0.3\%$ in CO exposed animals and $0.3 \pm 0.1\%$ in controls. In the lungs, no signs of pathology similar to that associated with cigarette smoking were observed, and no differences in number of pulmonary NE cells were observed between the groups. Chronic CO inhalation induced a 20% weight increase of the right ventricle ($p = 0.001$) and a 14% weight increase of the left ventricle and interventricular septum ($p < 0.001$). Histological examination of the myocardium did not reveal any signs of scarring. In the aorta and femoral artery, no signs of atherosclerosis were observed in CO exposed rats. No exposure related carcinogenic effects were observed. Spontaneous tumours were identified in 29% of CO exposed animals and in 28% of the controls. Our results suggest that low dose CO exposure is probably not responsible for the respiratory pathology associated with tobacco smoking. The effects on the cardiovascular system seem to involve myocardial hypertrophy, but not atherogenesis.
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Keywords: Carbon monoxide; Cardiac hypertrophy; Lung; Pulmonary neuroendocrine cells; Tobacco smoke; Tumourigenesis

Abbreviations: CGRP, calcitonin gene related peptide; CO, carbon monoxide; COHb, carboxyhaemoglobin; COPD, chronic obstructive lung disease; EM, electron microscope; Hb, haemoglobin; HO, heme oxygenase; IHC, immunhistochemistry; LV, left ventricle; LV + S, left ventricle + interventricular septum; NE, neuroendocrine; NEB, neuroepithelial body; PBF, phosphate-buffered formalaldehyde; PNEC, pulmonary neuroendocrine cells; ppm, parts per million; RV, right ventricle; S.E.M., standard error of mean; TBS, Tris-buffered saline

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1. Introduction

Cigarette smoking is one of the most important etiologic factors of diseases in the respiratory and cardiovascular system. The mechanism of the detrimental effects of cigarette smoke involves several mediators and pathways. Cigarette smoke is composed of hundreds of chemicals, including tar with its many carcinogens, nicotine, free radicals and gaseous compounds, such as carbon monoxide (CO). The gas component of cigarette smoke contains 4.5% CO, and the CO concentration of inhaled cigarette smoke may reach as high as 500 parts per million (ppm) (WHO, 1999). Smoking increases carboxyhaemoglobin (COHb) levels from 1 to 2% in non-smokers up to 15% in heavy smokers (Omaye, 2002). Some studies have also reported elevated COHb levels in non-smokers exposed to environmental tobacco smoke (Scherer et al., 1990).

CO is considered a toxic chemical at high concentrations, leading to a severe hypoxic condition by displacing oxygen from haemoglobin (Hb), leftward shift of the oxyhaemoglobin dissociation curve, and binding to intracellular enzymes. However, several reports indicate that even low levels of chronic CO exposure may have important effects on health. Epidemiological studies have shown that ambient CO levels correlate with onset of heart diseases, increased mortality rates, and hospital admission for cardiovascular diseases (Stern et al., 1988; Kleinman et al., 1989; Burnett et al., 1997). In addition, recent animal studies have shown that inhalation of CO at doses corresponding to tobacco smoking worsens cardiac failure both in rats with experimental myocardial infarction and pre-existing hyperthrophic cardiomyopathies (Melin et al., 2005; Mirza et al., 2005). Furthermore, CO exposure has been suggested as an important etiological factor for atherosclerosis (Astrup et al., 1970; Kleinman et al., 1989). However, these findings have been questioned by other experimental studies, which did not show any association between CO exposure and atherosclerotic diseases (Weir and Fabiano, 1982; Penn et al., 1992).

Effects of acute high dose CO exposure on the respiratory system are well known, including pulmonary cell damage, endothelial and alveolar swelling and oedema (Niden and Schulz, 1965). Conversely, little epidemiological and experimental information is available on the pulmonary effects of long-term low dose CO exposure. Tobacco smoke is the main source of CO exposure in the general population. Therefore, many respiratory effects of CO may be confounded by the effects of tobacco smoke, which includes chronic obstructive pulmonary disease (COPD) and lung cancer.

An association between cigarette smoking and hyperplasia of a subgroup of airway epithelial cells called pulmonary neuroendocrine cells (PNEC), has been postulated by some authors (Gosney et al., 1989; Aguayo, 1994). These cells, which belong to the diffuse neuroendocrine (NE) system, are located among other epithelial cells in the airways, either as solitary PNEC or as aggregates of NE cells known as neuroepithelial bodies (NEBs). The function of the pulmonary NE system is not completely known, but it may be important in control of growth and development of the foetal lung. In addition, it may contribute to regulation of ventilation and circulation in the postnatal and adult lung (for review, see Van Lommel, 2001). NEBs have a rich innervation, and are hypothecated to be specialised chemoreceptors, responsible for detecting the alveolar oxygen levels (Cutz and Jackson, 1999). It has been proposed that CO, through binding to the oxygen receptor, may interact with the pulmonary NE system (Haddad, 2002).

To our knowledge, only a few experimental long-term studies with low levels of CO exposure have been published (Stupfel and Bouley, 1970; Armitage et al., 1976; Turner et al., 1979). However, the results are conflicting, and no information exists of long-term effects of CO inhalation on the pulmonary morphology and tumourigenesis. Therefore, a 72 weeks experiment was performed on female rats to study inhaled CO exposure at levels comparable to heavy smokers. The main aims of the study were to investigate the effects of chronic CO exposure *in vivo*, with particular emphasis on the respiratory and cardiovascular system, including pulmonary NE cells and a possible effect on tumourigenesis.

2. Materials and methods

2.1. Animals

Outbred 6–8 weeks old female Wistar rats (Harlan Netherlands B.V., The Netherlands) with an initial weight of 169 ± 4.5 g (mean ± S.E.M.) were exposed to either CO (n = 51) or air (n = 26). The animals were caged in groups of six or seven. They were fed a pellet rodent diet (RM1, Special Diets Services, Essex, England) available 4 h a day (8:00 a.m. to 12:00 noon), 5 days a week and with free access to food through the weekends. Tap water was provided *ad libitum*. Light was controlled in a 12:12-h light–dark cycle. Bedding was changed two times a week. The rats were weighed monthly. The study was approved by the Norwegian Council for Animal Research and conformed to the “European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes”.

2.2. CO exposure

Four animal steel cages were placed in each of three 650 l stainless steel and glass chambers designed as a cube with a conical top and bottom, as described in a previous study (Waldum et al., 1994). The positions of the cages in the chambers were changed systematically. A mixture of hospital medical quality air and CO (AGA, Oslo, Norway) was continuously circulated through two of the chambers. An airflow of 165 l/min and a CO flow of 0.03 l/min created a CO concentration of 200 ppm and about 15 air changes/h in the inhalation chambers. Pure hospital medical quality air was circulated through the control chamber, at equal rate to the two CO exposed chambers. The CO concentration was monitored daily by a gas monitor (NEOTOX XL single-gas monitor, Neotronics, UK) and no statistical differences were measured between the two CO exposure cambers (202 ± 1.7 ppm versus 199 ± 1.6 ppm). No CO was detected in the control chamber. The animals were CO exposed for 20 h a day (12:00 noon to 8:00 a.m. the following day), 5 days a week (Monday to Friday) for 72 weeks. Temperature in the three chambers (two CO exposure and one control) was 23.0 ± 0.1, 22.8 ± 0.1 and 22.9 ± 0.2 °C and with a relative humidity of 71.6 ± 1.0, 71.3 ± 1.1 and 64.1 ± 1.0%, respectively.

2.3. Animal procedures and tissue preparation

Before start of exposure, some animals from the CO exposure group ($n = 8$) and controls ($n = 8$) were anaesthetised with a subcutaneously injected mixture of fentanyl 12.5 μg/ml, midazolam 1.25 mg/ml and haloperiodol 0.83 mg/ml (0.4 ml/100 g rat weight), before collecting blood from the saphenous vein. After 2 weeks of exposure, two CO exposed rats and one control rat were sacrificed after anaesthesia, and blood collected by puncture of the abdominal aorta for determination of COHb levels. The animals were taken directly from the exposure chambers before the sacrifice.

After 3 months exposure, eight rats from each chamber ($n = 24$) were anaesthetised and blood sampled from the saphenous vein. At 6 months exposure, two animals from each chamber were anaesthetised, sacrificed and examined for pathology. Throughout the exposure period, animals exhibiting signs of illness were removed from the chambers, anaesthetised, sacrificed and examined.

At the end of the study (72 weeks of exposure) the remaining animals (CO exposed; $n = 42$, control; $n = 22$) were anaesthetised, and killed with blood-drawing from the abdominal aorta. Blood samples were collected in heparin-coated tubes and placed on ice until analysed. COHb and Hb levels were measured in an ABL SYSTEM 625 spectrophotometer (Diamond Diagnostics, USA) within 3 h of sampling. The animals were examined for macroscopic pathology of the brain, lungs, heart, thoracic aorta, femoral artery, gastrointestinal (GI) tract, liver, spleen, kidneys, ovaries and urinary bladder. In addition, weight of the lungs, stomach, liver, spleen, kidneys, ovaries and urinary bladder were measured. The hearts were incised at the level of the valves, and the left ventricle (LV) together with the interventricular septum (LV + S) was dissected free from the right ventricle (RV) and weighed. Each ventricle was sectioned coronarily, fixed in 4% phosphate-buffered formalaldehyde (PBF), and dehydrated in 80% ethanol, before embedding in paraffin for histological analyses. In addition, tissue samples from the thoracic aorta and femoral artery were collected and fixed in 4% PBF. The lungs were dissected free from the heart, greater vessels, and oesophagus, and weighed. The left lung was carefully filled intrabronchially with 1.5 ml 4% PBF and immersed in 4% PBF overnight before dehydration in 80% ethanol. Thereafter, the lung was sectioned into four slices from the hilus, perpendicular to the main bronchus, and embedded in paraffin. In addition, blocks of 1 mm^3 lung tissue from some animals were fixed in 2.5% glutaraldehyde for electron microscopy (EM).

2.4. Histopathologic evaluation

Paraffin embedded tissue was cut in 4 μm thick sections, mounted on slides (Super Frost® Plus, Braunschweig, Germany) and stained with regular haematoxylin and eosin (H&E). The tumours were classified according to the most comparable human terminology, based on H&E sections. Sections were examined for visible microscopic pathology, described in Table 1, and the degree of inflammation was calculated as number of sectioned airways with an adjacent lymphoid follicle divided by the total number of airways of the section. In addition, four lung sections from each study group were stained using the Elastica van Gieson staining kit (Merck KGaA, Darmstadt, Germany) for evaluation of pulmonary hypertension, as described by Keegan et al. (2001). Pulmonary arteries (25–100 μm external diameter) associated with an airway were counted and considered muscularized, if possessing a distinct double-elastic lamina, visible for at least half of the vessel circumference in a cross section.

2.5. Immunohistochemistry

Lung sections for immunohistochemistry (IHC) were dewaxed with xylene, rinsed in graded alcohol, re-hydrated in water and immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigen retrieval was achieved by heating the sections in 10 mM Tris–EDTA (pH 9.0) in a commercial microwave oven at 160 W for 15 min. For visualisation of pulmonary NE cells, sections were incubated with polyclonal anti-calcitonin gene related peptide (CGRP) (diluted 1:12,000, L-8198, Sigma–Aldrich, St. Louis, MO, USA) for 60 min at room temperature. The antibodies were diluted in Tris-buffered saline (TBS, pH 7.4) with 0.025% Tween 20 (DakoCytomation, Glostrup, Denmark) and 1% bovine serum albumin (BSA, Sigma, St. Louis, MS). Between each step, the sections were washed in TBS with 0.05% Tween 20. The immunoreactivity was visualised with an Envision-HRP kit (K5007, DakoCytomation, Glostrup, Denmark) and DAB+ (K4065, DakoCytomation, Carpinteria, CA, USA). All sections were finally counterstained with haematoxylin for 6 s.

Table 1
Morphological signs of respiratory and cardiovascular pathology

Emphysema	Enlargement of the alveolar spaces or destruction of alveolar septal tissue
Inflammation	Accumulation of inflammatory cells in airways, alveoli or in the parenchyma Increased fraction of airways associated with lymphoid follicles
Bronchial/peribronchial thickening	Increased epithelium layer, bronchial muscle hypertrophy or submucosal gland enlargement
Fibrosis	Increased collagen deposit (Elastica van Gieson staining)
Pulmonary hypertension	Fibrotic thickening of the lamina interna and hypertrophy of the muscular lamina media of the pulmonary arteries (H&E staining) Increased muscularized small arteries with a double elastic lamina (Elastica van Gieson staining)
Atherosclerotic lesion	Accumulation of foam macrophages, proliferation of myointimal cells, fibrosis, inflammation or destruction of the lamina elastica interna in systemic arteries
Myocardial scarring	Fibrovascular granulation tissue and fibrosis

PNEC and NEBs were identified as clear positive immunoreactive cells with a stained cytoplasm located within all levels of the respiratory tree down to the respiratory bronchioles. The locations of the NEBs were classified into alveolobronchiolar (aNEBs) (located in respiratory bronchioles or alveoli) and bronchiolar/bronchial (bNEBs), and the size reported as number of immunoreactive cells with a visible nucleus. In addition, single PNEC in the airways were counted. Total number of NE cells/NEBs was divided by the total area of the section. The area was calculated from photos of the lung sections using iTEM Analysis (Soft Imaging System GmbH, Münster, Germany) software.

2.6. Electron microscopy

Lung tissue from two CO exposed and two control animals were immersed in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide for 60 min, before the samples were dehydrated in a graded series of ethanol and propylene oxide and embedded in epoxy resin LX 112 (Ladd Research Industries, Willinton, VT, USA). The samples were further sliced in ultra-thin sections (70 nm, RMC MTX Ultramicrotom, Boecklerand) and mounted on copper grids, before being contrasted with uranyl acetate and lead citrate. The grids were examined in a JEOL 1011 (Tokyo, Japan) transmission electron microscope. The thickness of the fused basal membrane of the air-blood barrier was measured at 12 locations along the alveolar wall of each animal using iTEM Analysis (Soft Imaging System GmbH, Münster, Germany) software.

2.7. Statistical analysis

Data are presented as means ± standard error of mean (S.E.M.). Differences between groups of normally distributed data were analysed using Student's *t*-test and ANOVA for multiple comparison. The χ^2-test was used to compare differences between proportions. Statistical significance was set at $p < 0.05$ (two-sided). All data were analysed using the statistical package for social sciences (SPSS, version 13.0, Chicago, IL, USA).

3. Results

3.1. Effects of CO on animal and organ weights

There was not observed any difference in the weight gain between CO exposed and control animals during the study period, as shown in Fig. 1. At the end of the study, the CO exposed group had a mean body weight of 275 ± 4 g compared to 270 ± 6 g in the control group ($p = 0.544$). Specific organ weights are provided in Table 2, showing that the only difference between the CO exposed and control groups was seen on cardiac weights (described in Section 3.4.1).

3.2. Effects of CO on COHb and Hb

During the study, levels of COHb and Hb were measured in the animals (Table 3). In CO exposed animals,

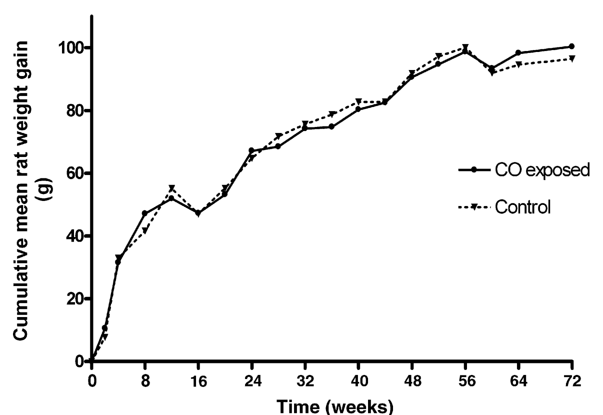

Fig. 1. No difference was observed in animal weight gain during the 72 weeks exposure period between CO exposed and control rats. The data are presented as cumulative mean rat weight gain corrected for decreasing number of animals with time.

Table 2
Organ weights

Organ	CO exposed	Control
Lung (mg)	1416 ± 26.6	1387 ± 29.8
RV (mg)	134 ± 3.2*	112 ± 5.2
LV (mg)	642 ± 12.4*	561 ± 14.7
RV/BW (mg/g)	0.48 ± 0.01*	0.42 ± 0.02
LV/BW (mg/g)	2.33 ± 0.03*	2.09 ± 0.04
RV/LV	0.21 ± 0.01	0.20 ± 0.01
Stomach (mg)	1900 ± 21.4	1890 ± 52.2
Liver (g)	7.05 ± 0.16	6.57 ± 0.22
Kidneys (mg)	1667 ± 25.6	1622 ± 34.3
Urinary bladder (mg)	90 ± 2.8	86 ± 4.1
Ovaries (mg)	121 ± 4.5	121 ± 6.4
Spleen (mg)	691 ± 75.0	557 ± 23.1
No. of animals	43	23

Data are presented as: no., numbers and means ± S.E.M. RV: right ventricle; LV: left ventricle; BW: body weight. * $p \leq 0.001$ vs. control group.

COHb levels ranged from 11.0 to 14.7%. COHb levels in control animals were <0.3%. Similarly, Hb levels were increased in the CO exposed animals compared to control animals from 12 weeks exposure time and throughout the study.

3.3. *Effects of CO on the respiratory system*

3.3.1. *Morphology*

H&E stained sections of the lungs did not reveal any of the morphological signs of smoking associated pathology summarised in Table 1. No distinct morphological differences between the CO exposed and control animals were observed. The fraction of airways with an associate lymphoid follicle was not statistically different between the CO exposed and control group (14.0 ± 1.6% versus 15.1 ± 1.7%, $p = 0.664$) (Fig. 3D). Furthermore, no ultrastructural differences in alveolar epithelial cells or alveolar septas between the two groups were observed with the use of EM (Fig. 2). The thickness of the fused basal laminas of the alveolar epithelial and endothelial cells of the blood-air barrier did not differ significantly between CO exposed and control animals (89.21 ± 2.6 nm versus 85.22 ± 2.2 nm, $p = 0.252$).

3.3.2. *Pulmonary neuroendocrine cells*

Chronic CO exposure was not associated with any significant morphological changes in the pulmonary NE system. The number of single PNEC immunoreactive for CGRP (Fig. 3A) in the airway epithelium was slightly higher in CO exposed animals compared to control animals, but without a statistically significant difference (1.9 ± 0.2 cells/cm^2 versus 1.7 ± 0.3 cells/cm^2, $p = 0.579$). Similarly, no statistically significant difference was found between the CO exposed group and control group regarding pulmonary NEBs (Fig. 3B), although the number of NEBs located both in the alveolobronchiolar (1.7 ± 0.3 cells/cm^2 versus 1.8 ± 0.6 cells/cm^2, $p = 0.837$) and bronchial (1.9 ± 0.1 cells/cm^2 versus 2.1 ± 0.3 cells/cm^2, $p = 0.530$) area were fewer in the CO exposed group than in the control group. Interestingly, the number of alveolobronchial NEBs was nearly equal to the number of NEBs located in the airway epithelium, independent of the exposure group. No difference between CO exposed and control animals was seen regarding the size of the aNEBs (5.6 ± 0.6 cells versus 5.2 ± 0.7 cells, $p = 0.696$) or bNEBs (7.0 ± 0.5 cells versus 7.1 ± 0.5 cells, $p = 0.902$).

3.4. *Effects of CO on the cardiovascular system*

3.4.1. *Cardiac weights*

Chronic CO exposure for 72 weeks induced a 20% ($p = 0.001$) increase in RV weight and a 14% ($p < 0.001$) increase in LV + S weight compared to control animals (Table 2). The same trend was seen after only 6 months of exposure, where analysis of some animals showed an increase in LV + S weight in the CO exposed versus the

Table 3
Blood levels of carboxyhaemoglobin (COHb) and haemoglobin (Hb)

Time (weeks)	COHb (%)				Hb (g/dl)			
	CO exposed	*n*	Control	*n*	CO exposed	*n*	Control	*n*
0	–		–		13.7 ± 0.2	8	13.2 ± 0.2	8
2	11.0 ± 1.0	2	0.1	1	–		–	
12	–		–		14.5 ± 0.6*	16	13.4 ± 0.3	8
24	12.6 ± 0.7	4	0.1 ± 0.1	2	15.0 ± 0.9	4	11.8 ± 0.2	2
72	14.7 ± 0.3*	43	0.3 ± 0.1	22	14.7 ± 0.1*	43	13.1 ± 0.2	22

Data are presented as means ± S.E.M. * $p < 0.02$ vs. control group.

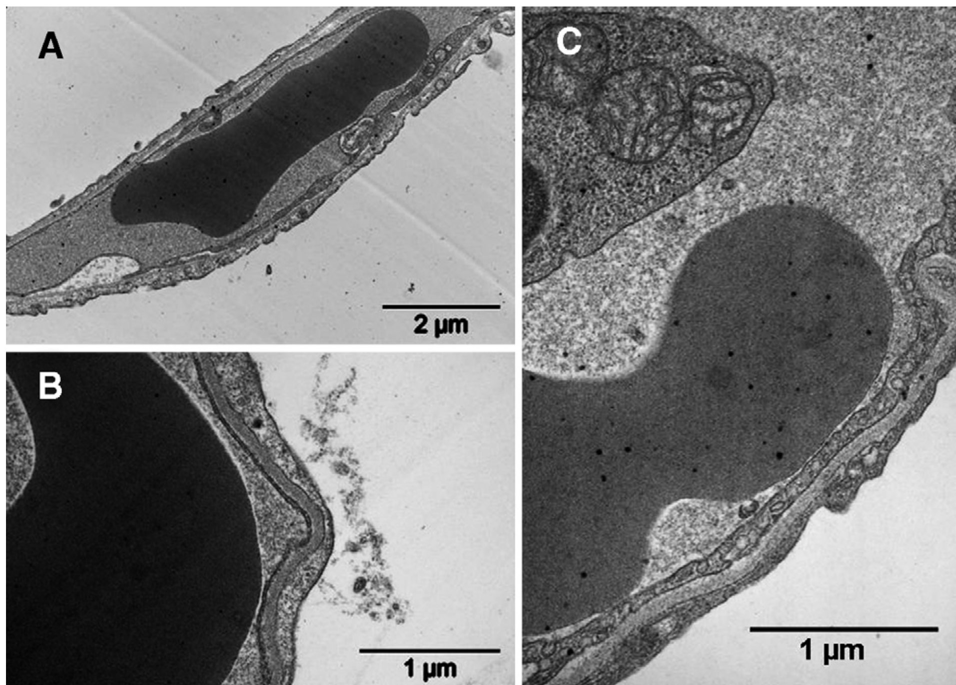

Fig. 2. Electron micrograph of the alveolar wall showing no differences between CO exposed and control rats. (A) Capillary vessel with an electron dense erythrocyte surrounded by the alveolar lumen from a CO exposed rat. (B and C) The gaseous diffusion barrier between blood and the alveolar air at higher magnification from a CO exposed and a control rat, respectively. The barrier consists of a thin cytoplasmic layer of the endothelial cell and the pneumocyte with a fused basal membrane between.

control group (556 ± 33 mg versus 510 ± 14 mg, $n = 4$ versus 2, respectively).

3.4.2. Cardiac morphology

No macroscopic differences of the heart were observed between the study groups. Furthermore, no histopathologic changes like oedema, inflammation, or signs of scarring were observed in H&E stained sections of the right and left ventricular wall.

3.4.3. Pulmonary arteries

H&E stained sections of the lung showed no structural signs of hypertension in the arteries. In addition, no differences in proportion of small muscularized arteries to non-muscularized arteries were observed between CO exposed and control animals ($47.9 \pm 2.3\%$ versus $47.6 \pm 1.9\%$, $p = 0.936$) in Elastica van Gieson stained sections.

3.4.4. Systemic arteries

H&E stained cross sections of the femoral artery and three sections of the distal part of the thoracic aorta showed no signs of atherosclerotic lesions in the CO exposed group. In the control group, only one of 15 examined animals showed a plaque-like lesion in the femoral artery with thickening of the lamina interna. No abnormality was observed in the thoracic aorta in the control group.

3.5. Tumourigenesis

The location and type of tumours observed are summarised in Table 4. In both groups, tumours of the anterior pituitary gland were the most frequent neoplasia, observed in 12 and 16% of the CO exposed and control animals, respectively. These tumours were all classified as benign adenomas, with a NE morphology without atypia and with a low rate of mitosis. One of the tumours measured 1 cm in diameter, causing compression of normal cerebral tissue and neurological symptoms with gait disturbance. Tumours of the mammary gland were only observed in the CO exposed group. Only one of the tumours (an adenocarcinoma of the uterus) was associated with metastasis. In the lungs, no macroscopic tumours were seen. Microscopic examination revealed one small tumour, exhibiting adenocarcinoma characteristics, in the CO exposed group. Using immunohistochemistry one NE hyperplastic lesion was found both in the CO exposed and control group (Fig. 3C). This hyperplasia of NE cells into small tumour-like lesions <0.5 cm

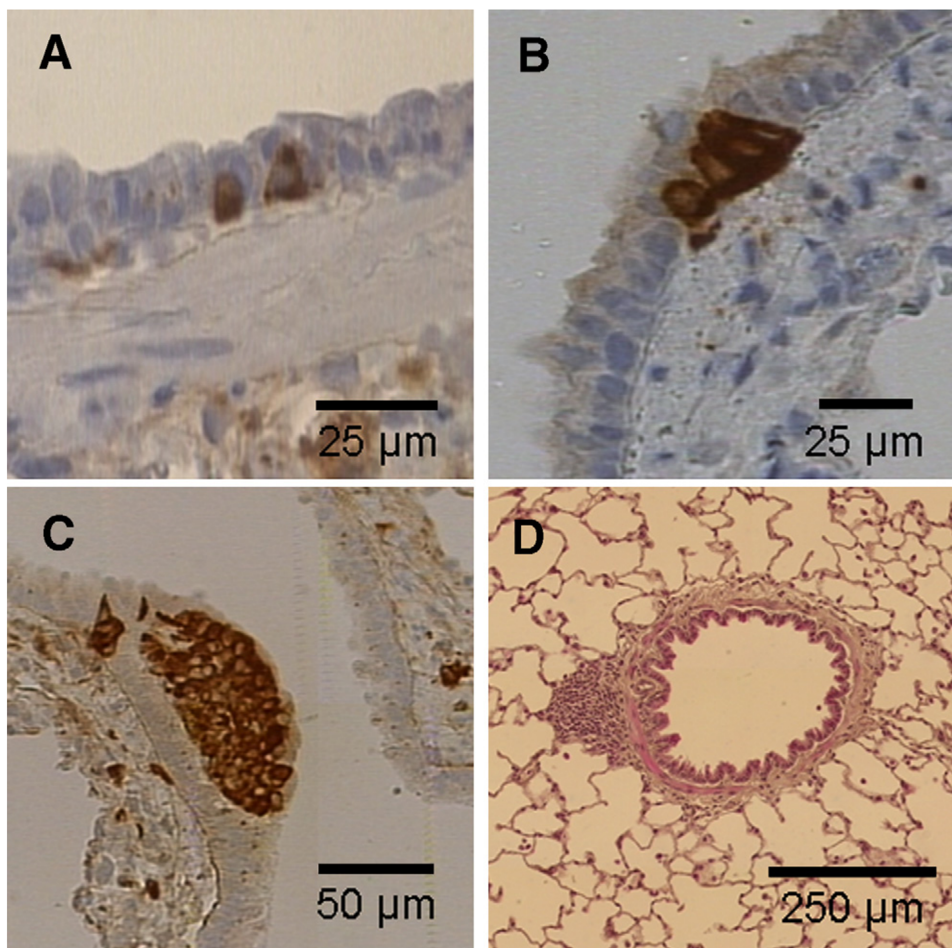

Fig. 3. Histological findings in the lungs. Immunohistochemical reactivity for CGRP visualising two single PNEC (A) and a NEB (B) among epithelial cells in a bronchus from a CO exposed rat. (C) NE hyperplastic lesion immunoreactive for CGRP in an airway from a control rat. (D) Heamatoxylin and eosin stained section from a CO exposed rat showing normal alveolar parenchyma and a bronchiole associated with a lymphoid follicle.

in diameter seems to resemble the human parallel named tumourlets, except their papillary appearance in the rats. The proportion of total number of animals with tumours did not differ significantly between the groups (29% versus 28%, $p = 0.959$). Only one of the animals (CO exposed) died spontaneously during the exposure period, suffering from leukaemia. In addition, two CO exposed rats were sacrificed after 15 and 17 months, respectively, and one control rat after 17 months, because of signs of illness (Table 5). Examination of the animals revealed a large mammary tumour, an adenocarcinoma of the ovary and a pituitary adenoma, respectively.

4. Discussion

The present work reports an inhalation study of CO on rats, at doses comparable to tobacco smoking with an exposure time of three quarters of their life expectancy. Except for cardiac hypertrophy, no structural changes in the respiratory and cardiovascular system were observed. Furthermore, no tumourigenic effect of CO exposure was seen. To our knowledge, few long-term experimental studies of low level CO exposure have been published (Stupfel and Bouley, 1970; Armitage et al., 1976; Turner et al., 1979). Compared to previous studies, with a CO concentration $\leq$250 ppm and an exposure time >12 months, this study adds important information regarding the morphology of the respiratory system, including pulmonary NE cells in chronic CO exposed animals.

Chronic CO exposure does not appear to induce morphological changes in the lungs. The respiratory pathology usually associated with cigarette smoking was absent in the current study. To our knowledge, no detailed information of the respiratory morphology from

Table 4
Number of animals with tumours

Tumours (site and type)	CO exposed ($n = 49$)	Control ($n = 25$)
Pituitary gland		
Adenoma	6 (12%)	4 (16%)
Mammary gland		
Fibroadenoma	3	0
Ovary		
Adenocarcinoma	1	0
Haemangioma	0	1
Uterus		
Leiomyoma	1	0
Salivary gland		
Adenocarcinoma	0	1
Haematopoietic system		
Leukaemia	1	0
Liver		
Metastasis from adenocarcinoma of the uterus	1	0
Lung		
Adenocarcinoma	1	0
Tumourlets/NE hyperplasia	1	1
Total number of animals with tumours	14 (29%)	7 (28%)

Data are presented as *n*, numbers and % of each group.

long-term CO exposure exists. In a short-term, study by Hugod (1980), no morphological changes were observed in the lungs of adult rabbits exposed to 200 ppm for up to 6 weeks. Another study of CO exposure to rats (250–1300 ppm) for 7.5 weeks showed an increase in lung weight, which was not due to increase in blood volume in the lung, oedema or fibrosis (Penney et al., 1988). However, our study did not confirm this finding, even after near life-time exposure.

Changes in the pulmonary NE system with hyperplasia of the PNEC/NEBs have been described in susceptible smokers and diseases associated with tobacco smoking (Gosney et al., 1989; Aguayo, 1993). The mechanism for this response is unknown, but one proposed hypothesis is that components of tobacco smoke may stimulate NE cells to proliferate (Novak et al., 1999). In addition, experimental studies have shown an increase in number of PNEC/NEBs in response to chronic high concentration of gases like oxygen and ozone or non-filtrated urban ambient air (Schuller et al., 1988; Ito et al., 1989, 1994). It has been proposed that NEBs are acting as an oxygen sensor through a membrane bound heme-protein (Youngson et al., 1993; Cutz and Jackson, 1999). The oxygen molecule is bound to the heme-containing membrane receptor (NADPH-oxidase), where decreasing oxygen levels lead to depolarisation and secretion of bioactive substances (Kemp et al., 2002). Secreted peptides or amines may further act as auto or paracrine mediators, like CGRP which has a growth stimulating effect.

Similar to the binding of CO to the heme of haemoglobin, it is postulated that CO may bind to the NADPH-oxidase (Haddad, 2002). To our knowledge, no experimental studies have been published, reporting the effects of CO exposure on the pulmonary NE system. The immunohistological results of the present study exhibit a trend of increased single PNEC and a lower number of NEBs in the CO exposed group, although changes were not statistically significant. We conclude that chronic CO inhalation does not significantly affect the pulmonary NE system in a way detectable by current morphological evaluation.

The carcinogenic effect of tobacco smoke is well known. Recent epidemiological studies have shown an association between cigarette smoking and cancers of the lung, urinary tract, aerodigestive tract, larynx, pancreas, stomach, liver, kidney, uterine cervix and myeloid

Table 5
Number of withdrawal rats during the exposure period

Time (weeks)	CO exposed			Control		
	Withdrawal (planned)	Withdrawal (illness)	Remaining animals	Withdrawal (planned)	Withdrawal (illness)	Remaining animals
0			51			26
2	2		49	1		25
20		1	48			25
24	4		44	2		23
60		1	43			23
68		1	42		1	22
72			42			22

Data are presented as numbers.

leukaemia (Mauderly et al., 2004; Sasco et al., 2004). In our study, we found a tumour at the time of sacrifice in nearly 1/3 of the animals, independent of CO exposure. This rate of spontaneous tumours is similar to that reported in elderly rats from previous studies and indicate that long-term inhalation of CO does not have tumourigenic effects (Stupfel and Bouley, 1970; Kroes et al., 1981; Waldum et al., 1994).

In recent years, several studies have focused on the possible beneficial physiological and cytoprotective effects of low dose CO. CO is produced endogenously in human tissues through breakdown of heme by heme oxygenase (HO), releasing iron and CO (Omaye, 2002). During conditions like hypoxia or inflammation, increased levels of HO, and thereby CO, have been found. CO is regarded as a cellular signal molecule in normal physiology, and may act as a neurotransmitter, vasodilator, bronchodilator and inhibitor of platelet function (for review, see Sethi, 2005; Kim et al., 2006). In addition, it may exert a protective role in a wide variety of diseases, with its anti-inflammatory and anti-proliferative effects (Ryter and Otterbein, 2004). Some recent in vivo studies have shown the cytoprotective effects of low-dose CO in acute lung injury, lung fibrosis and vascular transplants (Otterbein et al., 1999; Sato et al., 2001; Zhou et al., 2005). Our study was not designed to investigate possible protective effects on pathological processes. However, for future therapeutic considerations, the present work may support the safety of low dose CO inhalation on the respiratory system.

Current evidence for a role of CO in cardiovascular diseases is inconsistent. Some previous studies have linked CO exposure to the process of atherosclerosis and coronary artery diseases. Astrup et al. (1967) found cholesterol and lipid deposits in the aortic wall of rabbits, induced by several months of CO exposure at COHb levels of 20–33%. In addition, in vitro studies of blood platelets exposed to CO at concentrations of 50–100 ppm, displayed an increase in released nitric oxide-derived oxidants, which may damage the vascular endothelium (Thom and Ischiropoulos, 1997). However, only a few experimental studies have investigated long-term CO exposure covering most of the animals' lifetime. A study by Stupfel and Bouley (1970) reported no pathological changes in the aorta of rats exposed to 50 ppm CO for up to 2 years. In contrast to this, an exposure study of white Carneau pigeons, fed a high cholesterol diet for 52 weeks, resulted in enhanced coronary artery lesion development in those pigeons exposed to 150 ppm CO (Turner et al., 1979). The present study, with near life-long exposure and COHb level equal to heavy smokers, revealed no significant pathology indicating atherosclerosis in the aorta or the systemic femoral artery. It must be noted that, unlike some previous studies, the rats in our study were fed a standard rodent pellet diet not enriched with lipids or cholesterol for only 4 h a day, 5 days a week, with free access during weekends. This may have delayed the onset of the atherosclerotic process, but is probably a more representative physiologically model than a lipid-rich diet. Altogether, our findings support the hypothesis that chronic CO inhalation has no atherogenic effect in animals fed normal diets.

The finding of increased cardiac weight in long-term CO exposed animals is consistent with previous studies, even with a shorter exposure time than used in our study (Penney et al., 1982, 1984; Clubb et al., 1986). In addition, a recent study of experimental myocardial infarction in rats showed an increase in infarct size and worsening of heart failure, but also remodelling of the ventricles with myocardial hypertrophy of the opposite wall (Mirza et al., 2005). However, inconsistent data exists concerning the relationship between right and left ventricular hypertrophy (Penney et al., 1984; Loennechen et al., 1998). The present study shows that the weight of both ventricles increased, since the ratio RV/(LV + S) was similar in CO exposed and control animals. The reasons for CO-induced cardiac hypertrophy are not completely known. It appears that cardiac hypertrophy develops in spite of the CO-induced lowering of systemic blood pressure and thereby lowering LV afterload (Penney and Formolo, 1993). One hypothesis is that ventricular hypertrophy results from an increase in volume overload due to blood volume and viscosity enhancement and increased ventricular preload. However, mechanisms other than increased work load or haemodynamic effects may by postulated. Our findings, indicating a complete absence of pulmonary arterial hypertension and hypertrophy of both ventricles, support the idea of a possible intrinsic effect on the myocardium mediated by CO. At present, the exact pathways involved are unknown.

The current findings of an apparent safety of CO inhalation at doses of 200 ppm regarding to tumourigenesis, the respiratory organs and cardiovascular system, except cardiac hypertrophy, may be applied to only healthy adult rats. Other studies have shown that effects of CO on pathological conditions in rats may include both detrimental effects, like increased infarct size (Mirza et al., 2005), but also favourable effects like cytoprotection in acute lung disease (Otterbein et al., 1999). Furthermore, studies have described several effects on developing rats (Stockard-Sullivan et al., 2003). In addition, even though some physiological mechanisms are conserved, there may be species dif-

ferences. In some recent studies, the anti-inflammatory effects of inhaled CO observed in a mouse model, could not be detected in a comparable human model (Mayr et al., 2005). This shows that the findings have to be interpreted with caution when applied to humans.

In conclusion, long-term CO inhalation in rats at levels corresponding to tobacco smoking, induces right and left ventricular hypertrophy, but not increased atherosclerosis. CO exposure is not associated with tobacco smoking related pathology of the respiratory system, and has no impact on the morphology of pulmonary NE cells or tumourigenesis.

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128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.

139.Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140.Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.
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141.Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142.Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143.Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144.Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145.Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146.Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147.Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148.Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149.Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150.Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151.Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152.Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153.Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154.Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155.Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156.Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
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158.Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160.Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161.Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162.Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163.Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164.Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165.Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
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168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
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183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
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186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
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192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM

198.Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199.Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200.Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES
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201.Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202.Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203.Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204.Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205.Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206.Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207.Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208.Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209.Pål Klepstad: MORPHINE FOR CANCER PAIN
210.Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211.Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212.Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213.Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214.Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215.Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS
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216.Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217.Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218.Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219.Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220.Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221.Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222.Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223.Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL

224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
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250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS

251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
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255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
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267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
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273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS

280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
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283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OG PATHOLOGICAL GAMBLING IN NORWAY
288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
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291. Anne Engum: DEPRESSION AND ANXIETY – THEIR RELATIONS TO THYROID DYSFUNCTION AND DIABETES IN A LARGE EPIDEMIOLOGICAL STUDY
292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
295. Gunilla Klensmeden Fosse: MENTAL HEALTH OF PSYCHIATRIC OUTPATIENTS BULLIED IN CHILDHOOD
296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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298. Haakon R. Skogseth: INVASIVE PROPERTIES OF CANCER – A TREATMENT TARGET ? IN VITRO STUDIES IN HUMAN PROSTATE CANCER CELL LINES
299. Janniche Hammer: GLUTAMATE METABOLISM AND CYCLING IN MESIAL TEMPORAL LOBE EPILEPSY
300. May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
302. Siri Malm: LEFT VENTRICULAR SYSTOLIC FUNCTION AND MYOCARDIAL PERFUSION ASSESSED BY CONTRAST ECHOCARDIOGRAPHY
303. Valentina Maria do Rosario Cabral Iversen: MENTAL HEALTH AND PSYCHOLOGICAL ADAPTATION OF CLINICAL AND NON-CLINICAL MIGRANT GROUPS
304. Lasse Løvstakken: SIGNAL PROCESSING IN DIAGNOSTIC ULTRASOUND: ALGORITHMS FOR REAL-TIME ESTIMATION AND VISUALIZATION OF BLOOD FLOW VELOCITY

305.Elisabeth Olstad: GLUTAMATE AND GABA: MAJOR PLAYERS IN NEURONAL METABOLISM
306.Lilian Leistad: THE ROLE OF CYTOKINES AND PHOSPHOLIPASE A_2s IN ARTICULAR CARTILAGE CHONDROCYTES IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS
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