Early Detection of Lung Cancer

Biomarkers and CT Scans

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BY
MORTEN HORNEMANN BORG

DISSERTATION SUBMITTED 2022

AALBORG UNIVERSITY
DENMARK

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PREFACE

Historically, survival after receiving a lung cancer diagnosis was poor. A minority of patients were candidates for operation and the majority of the remaining patients received oncological treatment with a survival benefit of a few months (1).

After its introduction at Lillebaelt Hospital Vejle, Denmark, the lung cancer package was implemented nationwide in 2008, with the aim that no patient should experience unnecessary waiting time in relation to examination and treatment of lung cancer. Since then, lung cancer diagnostic work-up has been streamlined and standardized throughout Denmark. Duration of time spent on diagnostic work-up has dropped, while resection rate has increased, and over the last two decades overall survival has improved (2).

Despite these improvements, the task of finding lung cancer at a curative stage is still a challenge. A large proportion of patients diagnosed with early-stage lung cancer have no symptoms. Hence, screening of people at greater risk of developing lung cancer has been proposed (3), and low-dose computed tomography (LDCT) screening has been implemented in the US. Several problematic issues are related to LDCT screening for lung cancer, and a suitable blood-based biomarker with high sensitivity and specificity would be of great benefit.

The overall purpose of this thesis is to evaluate the potential utilization of blood-based biomarkers for detection of early-stage lung cancer and to evaluate the significance of increased use of CT exams on lung cancer stage distribution and management of incidental findings.

This project has been carried out as a collaboration between the Department of Internal Medicine, Lillebaelt Hospital Vejle and Department of Respiratory Medicine, Aalborg University Hospital. I owe thanks to many people for various types of help in the process of this project. First, I would like to thank my supervisors Ulla and Ole for making the thesis possible and for excellent guidance throughout the project.

I would also like to thank my collaborators in Vejle for important input, initiation and recruitment of the DETECT cohort, as well as my external collaborators in Aarhus and Copenhagen.

A special thanks goes to my colleagues in the Invasive Unit at Aalborg University Hospital: Camilla, Helle, Thor and many more, for great input during my PhD. Also, I owe thanks for great discussions during coffee breaks, advice on statistics and interpretation to the Respiratory and Anesthesiology Research Departments at Aalborg University Hospital.

This work could not have been done without the financial support from the Department of Respiratory Medicine at Aalborg University Hospital, Gangstedfonden, Region of Southern Denmark and Lillebaelt Hospital Research Foundation.

Finally, I would like to thank my family, especially my wife Ina, for continuous support and encouragement throughout my PhD-fellowship, and my two sons Oliver and Oscar.

PAPERS

I Performance of the EarlyCDT® Lung test in detection of lung cancer and pulmonary metastases in a high-risk cohort.

Borg M, Wen SWC, Nederby L, Hansen TFH, Jakobsen A, Andersen RF, Weinreich UM, Hilberg O.

Lung Cancer 2021;158: 85-90.

II Assessment of circulating biomarkers for detection of lung cancer in a high-risk cohort.

Borg M, Nederby L, Wen SWC, Hansen TFH, Jakobsen A, Andersen RF, Weinreich UM, Hilberg O.

Submitted to Cancer Biomarkers

III Natural killer cell activity as a biomarker for the diagnosis of lung cancer in high-risk patients.

Borg M, Wen SWC, Hansen TFH, Jakobsen A, Andersen RF, Hilberg O, Weinreich UM, Nederby L.

Journal of International Medical Research 2022;50: 1-10.

IV Increased use of computed tomography: Stage shift towards early-stage lung cancer and management of incidental findings.

Borg M, Hilberg O, Andersen MB, Weinreich UM, Rasmussen TR.

Submitted to Acta Oncologica

ENGLISH SUMMARY

Lung cancer is the leading cause of cancer-related death in Denmark and the world. Survival depends heavily on lung cancer stage at diagnosis. Lung cancer screening is established in the US and discussions are ongoing as to whether to implement similar measures in Europe.

This thesis is divided into two sections: 1) Three sub-studies respectively evaluate the value of the biomarkers "Early-CDT® Lung test", "biomarker panel (CYFRA 21-1, CEA, CA125)" and "natural killer cell activity" in a cohort of patients suspected of having lung cancer; and 2) An examination of the relationship between the number of CT thorax performed in the Danish Regions in the period 2013-2020 and the lung cancer stage distribution over the same period. Furthermore, a clinical audit assessed the referral pattern of stage IA lung cancer and the management of incidental findings in stage IV lung cancer patients.

The DETECT cohort was established at Lillebaelt Hospital Vejle in the period February 2019 to January 2020. All participants were under suspicion of lung cancer and referred for clinical investigation. Before investigation, a blood sample was obtained and analyzed for the specific biomarkers.

Data on CT scan activity was obtained from the Danish Health Data Authority and lung cancer stage distribution was extracted from the Danish Lung Cancer Registry. Journal auditing was performed on stage IA and stage IV patients.

The investigated biomarkers had different lung cancer detection abilities. However, common to all of them were that they were better at detecting late-stage lung cancer, as compared to early-stage lung cancer, and that the detection of early-stage lung cancer was not sufficient for clinical use in a screening program to reduce lung cancer mortality.

The use of CT thorax and percentage of stage IA lung cancer varied significantly among the Danish regions. Journal auditing revealed that 86.8% of stage IA lung cancer were incidental findings. Furthermore, 4.3% of stage IV lung cancer had a CT thorax performed two years before diagnosis with a nodule/infiltrate that most likely developed into stage IV lung cancer, and potentially could have been given curative treatment if follow-up recommendations had been followed.

DANSK RESUME

Lungekræft er den hyppigste cancer-relaterede dødsårsag i Danmark og Verden. Der er markant forskel på overlevelsen for lungekræft afhængig af om sygdommen diagnosticeres i et tidligt eller sent stadie. Lungekræftscreening er indført i USA og det diskuteres i øjeblikket om lignende tiltag skal gennemføres i Europa.

Afhandlingen er opdelt i to typer af studier: 1) Tre substudier evaluerer værdien af biomarkørerne Early-CDT® Lung test, Biomarkør panelet (CYFRA 21-1, CEA, CA125) og natural killer celle aktivitet i en kohorte af patienter under mistanke om lungekræft (DETECT kohorten). 2 Et studie undersøger forholdet mellem antallet af CT-scanninger der udføres i de danske regioner gennem de sidste 8 år og stadiefordelingen af lungekræft i samme periode. Endvidere blev der foretaget auditering for at vurdere henvisningsmønsteret på stadie IA-lungekræft patienter samt håndteringen af tilfældige fund på stadie IV lungekræft patienter.

DETECT kohorten blev etableret ved Sygehus Lillebælt Vejle i perioden februar 2019 til januar 2020. Alle medvirkende var under mistanke for lungekræft og henvist til Lungepakken på Sygehus Lillebælt Vejle. Før vanlig lungekræft udredning blev der taget en blodprøve som blev analyseret for de påtænkte biomarkører.

Data for antallet af CT-scanninger af lungerne og stadieinddeling blev indsamlet fra Sundhedsdatastyrelsen og Dansk Lunge Cancer Register. Der blev foretaget journalopslag vedrørende tidligere CT-scanninger hos stadie III-IV lungekræft patienter.

De undersøgte biomarkører har forskellig evne til at detektere lungekræft. Imidlertid har de til fælles, at de er bedst til at detektere sent stadie af lungekræft og at detektionen af lungekræft i tidligt stadie ikke er sufficient til at biomarkørerne umiddelbart kan indsættes som screeningsforanstaltning for at reducere dødeligheden af lungekræft.

Antallet af CT af brystkassen og procentdelen af stadie IA-lungekræft viste betydelige forskelle imellem regioner. Auditering viste at 86,8% af stadie IA-lungekræft tilfælde var tilfældige fund og at 4,3% af stadie IV lungekræftpatienter havde fået foretaget en CT indenfor to år før diagnose som viste en nodulus/infiltrat som med størst sandsynlighed udviklede sig stadie IV lungekræft og potentielt kunne have modtaget kurativ behandling hvis CT-kontrol forløb ifølge gældende anbefalinger var blevet igangsat.

ABBREVIATIONS

AUC	Area under the curve
CA125	Cancer antigen 125
CEA	Carcinoembryonic antigen
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
ctDNA	Circulating tumor deoxyribonucleic acid
CT	Computed tomography
CYFRA 21-1	Cytokeratin-19 fragment
EBUS	Endobronchial ultrasound
EUS	Endoscopic ultrasound
IASLC	International Association for the Study of Lung Cancer
IFN γ	Interferon γ
IQR	Interquartile range
LDCT	Low-dose computed tomography
NK cells	Natural killer cells
NKA	Natural killer cells activity

RCT	Randomized controlled trial
ROC	Receiver operations characteristic
SD	Standard deviation
pro-SFTPB	Pro-surfactant protein B
USPSTF	United States Preventive Services Task Force

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1. BACKGROUND

1.1 LUNG CANCER EPIDEMIOLOGY

Globally, it is estimated that 1.8 million people die from lung cancer each year (4) and thus, it is by far the leading cause of cancer-related death among both men and women. Known risk factors are tobacco smoking, second-hand cigarette smoke exposure, indoor radon exposure, asbestos, pollution, and diesel exhaust, among others (5). The highest mortality is seen in Western countries. However, as cigarette smoke pollution is increasing in the developing world, the mortality from lung cancer has likewise increased in those countries, as exemplified by the current mortality rates in China (Figure 1).

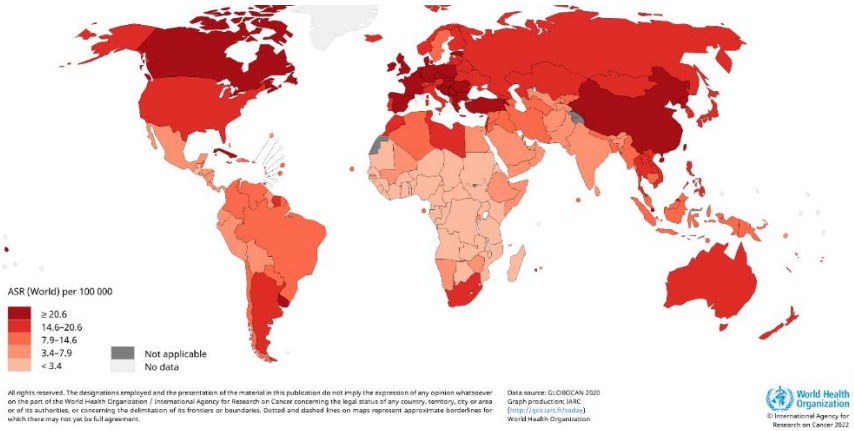


Figure 1. *Estimated age-standardized lung cancer mortality rates worldwide 2020 (Courtey of GLOBOCAN 2020, Graph production IARC (http://gco.iarc.fr/today) World Health Organization).*

In Denmark, approximately 5,000 cases of lung cancer are diagnosed each year, with an overall five-year survival rate of 18.2% - ranging from 56.3% in stage IA lung cancer to 2.2% in stage IVB lung cancer (2).

1.2 SYMPTOMS OF LUNG CANCER

Lung cancer symptoms depend on the stage of the disease. Classical pulmonary symptoms of lung cancer are cough, shortness of breath, hemoptysis, thoracic pain and hoarseness, while constitutional symptoms are predominantly fatigue, weight loss and night sweats (6). In early-stage lung cancer, a large proportion of patients have no symptoms and the finding is often incidental (6). Previous research shows that overall survival proved better in patients without symptoms compared to symptomatic lung cancer patients (7, 8). In stage IV lung cancer patients, symptoms are dominated by constitutional symptoms and metastases-linked symptoms, such as pain from bone metastases or tumor growth in innervated tissue and neurological deficits stemming from brain metastases (Figure 2).

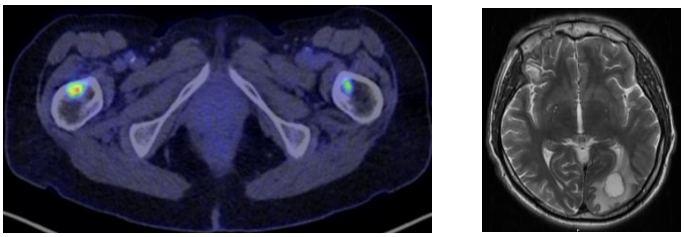


Figure 2. *PET/CT scan showing bone metastases and cerebral MR scan showing a brain metastasis (Courtesy of the Department of Radiology, Aalborg University Hospital, Denmark).*

1.3 DIAGNOSIS AND STAGING

Lung cancer is diagnosed with a tissue sample from either the primary tumor or a local or distant metastasis. Lung cancer staging is conducted using the TNM IASLC 8th edition (9); evaluating tumor size and growth, local lymph node involvement and distant metastasis(es). A sample from the primary lung tumor is most often obtained using a computed tomography (CT)- or fluoroscopy-guided percutaneous needle aspiration biopsy (10). If the location of the lung tumor allows it, the tissue sample will preferably be acquired using ultrasound-guided percutaneous needle aspiration biopsy, bronchoscopy, endobronchial ultrasound (EBUS) or endoscopic ultrasound (EUS), as these procedures carry a smaller risk of iatrogenic pneumothorax and are not associated with radiation (10). The local mediastinal lymph node staging (Figure 3) is most often performed using EBUS and/or EUS, while mediastinoscopy or thoracoscopy might be required in selected cases (11).

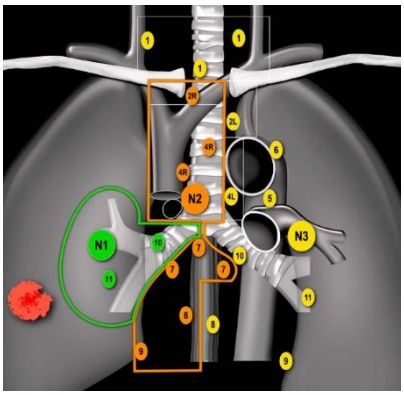


Figure 3. *Mediastinal lymph node staging. Courtesy of Radiology Assistant (radiologyassistant.nl).*

Distant metastases are biopsied depending on their location; liver and subcutaneous metastases will most often be sampled percutaneous using ultrasound, while bone metastases might require CT-guidance. Left adrenal gland will often be biopsied using EUS, while tissue from brain metastases requires open brain surgery and metastasectomy.

1.4 LOW-DOSE COMPUTED TOMOGRAPHY (LDCT) SCREENING

Screening tests for lung cancer began in the 1970s with sputum cytology and chest x-ray. The first randomized controlled trial (RCT), the Mayo Lung Project, enrolled 9,211 males, aged 45 or older who had smoked at least 20 cigarettes a day the last year (12). The screening group received chest x-ray and sputum cytology every four months vs. recommendation of an annual chest x-ray. Despite a larger lung cancer incidence in the screened group, there was no difference in mortality and the trial highlights the importance of overdiagnosis in lung cancer screening, i.e. cancers which do not progress or influence mortality.

The early research on chest LDCT consisted of observational prospective studies (13, 14). The Mayo Clinic enrolled 1,520 participants with a tobacco history of 20 pack years or more in a prospective study, where participants underwent five annual LDCT scans (15). This resulted in 4% of patients diagnosed with lung cancer, while one or more uncalcified nodule(s) of at least 4 mm were seen in 74% of patients. Investigators compared the results with the Mayo Lung Project and found no difference in mortality.

The “Detection And screening of early lung cancer with Novel imaging Technology” (DANTE) trial was the first RCT comparing LDCT screening for lung cancer vs. usual care. It included 2,811 participants, aged 60-75 years, with a smoking history of 20 pack years or more. Even though the study found more lung cancers in the screening group, there was no change in all-cause or lung cancer-specific mortality (16). This led to the largest conducted lung cancer screening RCT – the National Lung Screening Trial (NLST) (17). It enrolled 53,454 participants with at least 30 pack years, comparing biannual LDCT scans vs. annual chest x-ray with a median follow-up of 6.5 years. The relative risk of lung cancer-specific mortality was decreased by 20.3% (95% confidence interval (CI): 6.8%-26.7%) and the number needed to screen to prevent one lung cancer death was 320. Based on these findings, the United States Preventive Services Task Force (USPSTF) in 2013 recommended LDCT screening for people aged 55-80, with a tobacco history of 30 pack years or more, who were currently smoking or had quit smoking within the past 15 years. The age range and pack-year eligibility criteria were changed in 2021 to include ages 50-80 with a tobacco history of 20 pack years or more (3). Since the implementation in the US, a number of RCTs have been conducted in Europe (18-22); the largest was the NELSON study, with 15,789 participants (19), which showed a decrease in lung cancer-specific mortality with a rate ratio of 0.76 (95% CI: 0.61-0.94), although all-cause mortality was not significantly different. However, a recent meta-analysis reported a modest but significant reduction in all-cause mortality (RR = 0.96, 95% CI: 0.92-1.00) (23).

In Europe, there are currently no organized nationwide lung cancer screening programs. The results of the RCTs have raised the question of whether to implement lung cancer screening programs in European countries; however, several issues should be addressed (24, 25). This includes the questions of all-cause mortality vs. lung cancer-specific mortality, overdiagnosis, pulmonary nodule management and capacity of CT scanners and radiologists. Because of these issues, no European country has decided to implement nationwide LDCT screening (26).

1.5 POTENTIAL LUNG CANCER-SPECIFIC BIOMARKERS

New potential lung cancer-specific biomarkers are rapidly being developed (27). The majority are blood-based, although urine metabolites (28), volatile organic compounds in exhaled breath (29) and sputum (30) are also being explored.

Potentially useful blood-based biomarkers include the measurement of microRNA (31, 32), circulating tumor deoxyribonucleic acid (ctDNA) (33, 34), ctDNA mutations (35) or ctDNA methylations (36). These measurements have proved useful in the

detection of lung cancer, and the International Association for the Study of Lung Cancer (IASLC) has suggested that implementation in clinical practice is justified in specific therapeutic settings (37).

The EarlyCDT-Lung test®, developed by OncImmune, is based on the detection of autoantibodies against a panel of tumor-related antigens (38-40). Currently, the seven-panel assay against p53, NY-ESO-1, CAGE, GBU4-5, SOX-2, MAGE A4 and HuD is commercially available. Recent company-sponsored studies have found a sensitivity of 41% and specificity of 91% (41), and it has been postulated that the test detects equally well both early-stage and late-stage disease (42). The Early Detection of Cancer of the Lung Scotland trial (ECLS) randomized participants to an EarlyCDT-Lung test® or usual care. If the EarlyCDT-Lung test® was positive, participants were offered biannual LDCT scans for two years (43). The ECLS enrolled 12,208 participants and a significant stage shift was observed (44). In the intervention arm, 41.1% of diagnosed lung cancers were in stage I-II vs. 26.8% in the control arm. No differences were observed in lung cancer-specific or all-cause mortality and the study does not evaluate the potential incremental contribution of the EarlyCDT-Lung test® to LDCT screening.

Several types of proteins or protein-fragments, measured alone or in a panel, have been proposed as useful lung cancer tumor markers. These include – but are not limited to – cancer antigen 125 (CA125), carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA 21-1), the precursor form of surfactant protein B (Pro-SFTPB), neuron-specific enolase (NE) and squamous cell carcinoma antigen (SCC Ag). Previous studies have provided conflicting results regarding the diagnostic properties of these biomarkers for screening purposes (45-47). Moreover, it has been suggested that some biomarkers could be used as a marker of disseminated lung cancer (48-51). In one study (52), CYFRA 21-1 was found to have an area under the curve (AUC) in a receiver operating characteristic (ROC) curve of 0.85-0.87, while the same biomarker had an AUC of 0.55-0.68 in another study (53). Most of these studies are case-control studies. Some, but not all, are matched on smoking status, and lung cancer stage distribution may differ significantly between studies. The ethnic composition and, possibly, the biological development of lung cancer, differ among the studies and this could potentially explain the discrepancies in study results. In a prominent US study, the biomarkers CA125, CEA, CYFRA 21-1 and Pro-SFTPB were combined with smoking history to develop an integrated risk prediction model (54). This combination resulted in an AUC of 0.83, while a model purely based on smoking had an AUC of 0.73. The authors suggested that a panel of biomarkers could improve lung cancer risk assessment and may be used to define eligibility for LDCT screening.

Natural killer (NK) cells are cytotoxic innate lymphocytes that, without prior sensitization or activation, can kill cancer cells in the body (55). Furthermore, through the secretion of cytokines, such as interferon gamma (IFNγ) and tumor necrosis factor α, they play an important role in the innate and adaptive immune response and directly affect cancer cells (56, 57). A large population-based epidemiological study with

3,625 participants and 11 years of follow-up conducted in the 1980s, found that low NK cells activity (NKA) was associated with increased cancer risk (58). This instigated further interest in the suppression of NK cell effector functions by the presence of tumor cells, the expression of tumor ligands and checkpoint ligands in the tumor microenvironment (59, 60). In recent clinical pilot studies, low NKA has been associated with prostate cancer (61), gastric cancer (62), colorectal cancer (63) and lung cancer (64). However, it remains unclear whether these associations are clinically relevant.

1.6 CT EXAMS AND LUNG CANCER STAGE DISTRIBUTION

Worldwide, the overall number of CT exams is increasing by 4% annually and reached 300 million CT exams per year in 2020 (65). The CT exams performed in a range of Western countries show wide variation ranging from 255 per 1,000 inhabitants in the USA (2021) to 45 per 1,000 inhabitants in Finland (2020) (66). In Denmark, the number of CT exams is relatively high, at 207 per 1,000 inhabitants (2020).

The increased use of CT yields more incidental findings, and it is believed that 31% of CT exams produce an incidental finding (67). These include findings that are not clinically relevant but also potential malignant lesions. It could be expected that, with increased use of CT thorax, more lung cancer cases would be detected at an earlier stage as incidental findings. With high CT activity in a country, it would be expected that more lung cancer cases are detected at an earlier stage as incidental findings. CT-detected incidental tumors are typically smaller and serve as an independent predictor of survival, compared to symptomatic diagnosis or incidental detection on chest x-ray (7, 8, 68, 69). However, this could also be due to detection of indolent tumors that do not influence mortality (70).

Currently, it is not known whether increased use of CT is related to a higher fraction of early-stage lung cancer. It does, however, seem plausible since previous studies have found that the majority of stage I lung cancer cases are incidental findings (6, 7). Furthermore, if the majority of early-stage lung cancer cases are incidental findings, it becomes highly important to ensure management and follow-up of incidental pulmonary findings in accordance with current guidelines (71).

2. AIMS AND HYPOTHESES

2.1 PERFORMANCE OF THE EARLYCDT® LUNG TEST IN DETECTION OF LUNG CANCER AND PULMONARY METASTASES IN A HIGH-RISK COHORT (PAPER I)

Hypothesis: Specific autoantibodies are useful in early detection of lung cancer.

Aim: To investigate the diagnostic value in early detection of lung cancer of specific autoantibodies in the blood.

2.2 ASSESSMENT OF CIRCULATING BIOMARKERS FOR DETECTION OF LUNG CANCER IN A HIGH-RISK COHORT (PAPER II)

Hypothesis: The combination of circulating biomarkers is useful in early detection of lung cancer.

Aim: To explore in detection of lung cancer the diagnostic sensitivity and specificity, individually and combined, of the blood-based biomarkers cancer antigen 125 (CA125), carcinoembryonic antigen (CEA) and cytokeratin-19 fragment (CYFRA 21-1).

2.3 NATURAL KILLER CELL ACTIVITY AS A BIOMARKER FOR THE DIAGNOSIS OF LUNG CANCER IN HIGH-RISK PATIENTS (PAPER III)

Hypothesis: Activation of natural killer cells is useful in detection of lung cancer.

Aim: To investigate the diagnostic value in detection of lung cancer of natural killer cells activation.

2.4 INCREASED USE OF COMPUTED TOMOGRAPHY: STAGE SHIFT TOWARDS EARLY-STAGE LUNG CANCER AND MANAGEMENT OF INCIDENTAL FINDINGS (PAPER IV)

Hypothesis: Increased use of CT thorax lead to an increase in the diagnosis of early-stage lung cancer.

Aim: To assess the potential association between the number of CT thorax performed in Denmark and lung cancer stage distribution, and to evaluate the management of incidental findings.

3. METHODS

3.1 THE DETECT COHORT

The DETECT high-risk cohort was established at Lillebaelt Hospital Vejle – University Hospital of Southern Denmark. In total, 250 participants referred by their general practitioner for suspicion of lung cancer were consecutively enrolled from February 2019 to January 2020. Inclusion and exclusion criteria are summarized in Table 1.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Referred for suspicion of lung cancer	Previous lung cancer diagnosis
Aged 18 and over	Other malignant disease 5 years prior to study enrolment, except non-melanoma skin cancer and carcinoma in-situ cervicis uteri
Written and oral informed consent	Severe comorbidity leading to patients´ incapacity to participate in diagnostic procedures

The inclusion process is shown in Figure 4. At the initial visit of diagnostic work up for suspected lung cancer first lung cancer, a blood sample was obtained for subsequent analysis of potential lung cancer biomarkers. Next, the CT-scan of the thorax and upper abdomen was reviewed. Either suspicion of lung cancer was confirmed, which warranted further investigation, or lung cancer was ruled out and participants served as controls. Lung cancer investigation was conducted with lung biopsy and/or bronchoscopy with EBUS/EUS. Staging was performed using the IASLC 8th edition (9).

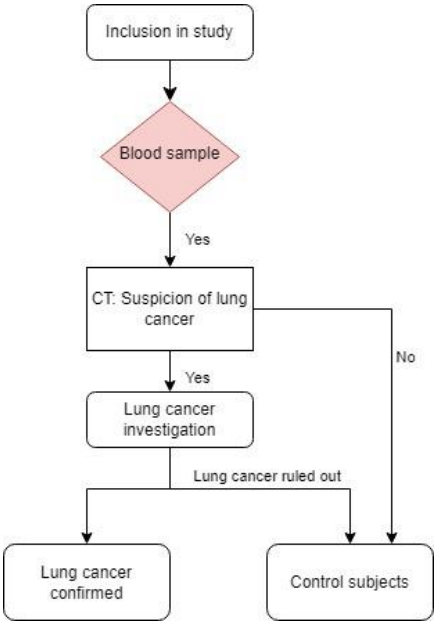


Figure 4. *Flowchart of inclusion and diagnostic work-up of participants*

3.2 BIOCHEMICAL AND MOLECULAR METHODS

3.2.1 Early-CDT® Lung test

Blood samples were collected in BD Vacutainer® Clot Activator Tubes (BD Biosciences, San Jose, CA, USA). After 30 minutes, the samples were centrifuged at 2000xg for 10 minutes and serum was collected and stored at -80°C until use. After thawing, serum samples were analyzed using the EarlyCDT® Lung test (OncImmune Ltd, Nottingham, UK). This enzyme-linked immunosorbent assay (ELISA) detected autoantibodies against the antigens p53, SOX2, CAGE, NY-ESO-1, GBU4-5, MAGE A4 and HuD. The seven-panel assay was performed at the Department of Clinical Biochemistry, Lillebaelt Hospital Vejle – University Hospital of Southern Denmark, Denmark according to the manufacturer's recommendation. The test uses autoantibody-specific cut-off values to evaluate the presence or absence of an antibody in a sample. Results are given as "high level", "moderate level" or "no significant level" for each autoantibody. In an individual participant, if "high level" or "moderate level" were recorded for any autoantibody, the test was treated as positive. If all the autoantibody measurements were "no significant level" in a patient, the test was regarded as a negative result.

3.2.2. Biomarker panel (CYFRA 21-1, CEA, CA125)

Blood samples were collected in BD Vacutainer® Clot Activator Tubes (BD Biosciences, San Jose, CA, USA). After 30 minutes, the samples were centrifuged at 2000xg for 10 minutes and serum was collected and stored at -80°C until use. After thawing, concentrations of CA125, CEA, and CYFRA 21-1 were assessed in the serum using the Human Circulating Cancer Biomarker Magnetic Bead Panel I (Merck, Darmstadt, Germany) on a Bio-Plex 200® analyzer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the manufacturer's recommendations at the Department of Clinical Biochemistry, Lillebaelt Hospital Vejle – University Hospital of Southern Denmark, Denmark. Four samples, two controls included in the kit and two serum samples from patients with lung cancer, all served as quality controls and were analyzed in either duplicates or triplicates in each run. Inter assay variation for CA125, CEA and CYFRA 21-1 were 15%, 13% and 13% respectively, and the intra-assay variations were 12%, 14% and 15%, respectively.

3.2.3. Natural killer cells activity

Natural killer cell activity was measured in whole blood using the NK Vue® assay (NKMAX Co Ltd, Seongnam-si, South Korea) that consists of a special sample tube and an ELISA. The analyses were performed at the Department of Clinical Biochemistry, Lillebaelt Hospital Vejle – University Hospital of Southern Denmark, Denmark and has been described previously (82). Besides Na-heparin as the anticoagulant, the NK Vue® tubes hold an NK cell-stimulating agent, Promoca®. Specifically, 1 mL of whole blood was drawn into NK Vue® tubes and placed at 37°C within 15 minutes of sampling. After 24 hours, the plasma level of IFNγ was measured using the NK Vue® ELISA (NKMAX). Values below the lower limit of quantification (65 pg/mL) were recorded as 32.5 pg/mL. Samples with test results above the assay's upper limit (2000 pg/mL) were diluted at 1:10 and reanalyzed. The in-house intra-assay and inter-assay coefficients of variation of the ELISA were <10% and <12%, respectively, and the lower reference limit was 120 pg/mL measured in-house. A cut-off of 250 pg/mL was used to separate abnormal from normal NKA in accordance with the manufacturer's suggestion (72). It has previously been shown that IFNγ measured in the assay is predominantly secreted from NK cells (73).

3.3 USE OF CT THORAX, LUNG CANCER STAGE DISTRIBUTION AND CLINICAL AUDITING

3.3.1. CT thorax exams and lung cancer stage distribution

The annual use of CT thorax in the period 2013-2020 in the five Danish regions was acquired from the Danish Health Data Authority. Stage distribution of Danish lung cancer patients 2013-2020 was extracted from the Danish Lung Cancer Registry [11].

3.3.2. Clinical audit of lung cancer patients diagnosed in stage IA

A clinical audit of patient journals was performed in a Danish region (74). In the period 2019-2021, all lung cancer patients diagnosed in stage IA were identified from the Danish Lung Cancer Registry [11]. Clinical auditing was performed to investigate whether the reason for referral to the CT thorax was a clinical suspicion of lung cancer or the early-stage lung cancer was an incidental finding. If the CT thorax was performed as a consequence of a chest x-ray, the reason for referral for chest x-ray was noted.

3.3.3 Clinical audit of lung cancer patients diagnosed in stage IV

A clinical audit of patient journals was performed in a Danish region (74). Lung cancer patients diagnosed in stage IV in the period 2019-2021 were identified from the Danish Lung Cancer Registry (11). Clinical auditing was performed as a single-observer study by a respiratory physician subspecialized in lung cancer investigation, to evaluate whether patients had received a CT that showed parts of the lungs within the two years before lung cancer diagnosis. The types of CT scans included were: CT thorax (including CT angiography), CT thorax abdomen pelvis (CT TAP), CT abdomen, showing lower parts of the lungs (including CT urography) and cardiac CT. If patients had a CT scan performed two years before the lung cancer diagnosis, it was noted whether the CT exam was described with a pulmonary nodule/infiltrate and if adequate follow-up with CT was performed. If the CT was not described with pulmonary nodules/infiltrates, the scan was assessed to evaluate, in retrospect, if any pulmonary nodules/infiltrates could be detected at the location where the lung tumor was found. Finally, it was assessed whether any pulmonary nodules/infiltrates seen on the earlier CT scan were likely to be the origin of the later diagnosed stage IV lung cancer.

3.4 STATISTICAL DATA ANALYSES

Normal distributed data are presented as the mean ± standard deviation (SD), non-normal distributed data as the median ± interquartile range (IQR). Categorical data are presented as frequencies.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the Early-CDT® Lung test were computed from the R core package (75).

The logistic regression prediction models of the biomarker panel were computed using K-fold cross-validation technique (5-fold) in R, Caret package (76). ROC curves were computed with assessment of the AUC and with 95% CI.

The Mann-Whitney U test was used to compared continuous variables of NKA. Optimal cut-off value of the ROC curves for NKA were determined using the Youden index. The risk of lung cancer at different cut-off values of NKA was assessed using odds ratio contingency analysis, with significance measured with Fisher’s exact test. Multiple linear regression was performed to evaluate the influence of clinical factors on NKA. Figures of NKA levels were produced using the ggplot2 R package (77).

Correlations between number of CT exams per 1,000 inhabitants and lung cancer stage distribution were calculated using linear regression analysis.

The statistical significance level was set at $p < 0.05$. Data analyses were performed in STATA Statistical Software version 16, StataCorp LLC, College Station, TX, USA and R statistical software version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria (75). Clinical auditing was performed with the use of REDCap (78).

3.5 ETHICAL CONSIDERATIONS

The study setup of the DETECT cohort was observational. Participation had no influence on lung cancer investigation or treatment. The risks involved were low and consisted of temporary soreness and a small hematoma after blood sampling. The study was approved by the Regional Committee on Health Research Ethics in Southern Denmark (ID: S-20180052) and the Danish Data Protection Agency (ID: 18/33058). All participants provided written informed consent to participate. Approval for clinical auditing was acquired from the hospital directors.

4. RESULTS

4.1 THE DETECT COHORT

Among the 250 participants in the DETECT high-risk cohort, 79 (32%) were diagnosed with lung cancer. Lung cancer was ruled out in the remaining 171 participants, and they served as control subjects. Patient characteristics are presented in Table 2. The two groups had a similar sex distribution while patients with lung cancer were slightly older ($p < 0.05$). Among patients with lung cancer, tobacco pack years were significantly higher, compared to control subjects ($p < 0.05$). Similar, control subjects were more often never smokers ($p < 0.01$).

Table 2. Descriptive characteristics of participants

Variable	All participants	Patients with lung cancer	Control subjects
n	250	79	171
Sex (men/women)	128/122	40/39	88/83
Age, years	65 (11)	68 (9)	64 (12)
Tobacco pack years	21 [2-40]	35 [20-48]	15 [0-40]
Current smoker	23%	30%	19%
Former smoker	54%	61%	51%
Never smoker	22%	9%	29%

Presented as percentages (%), mean (standard deviation), or median [interquartile range].

Lung cancer stage distribution in the DETECT cohort is presented in Figure 5. Compared to the Danish Lung Cancer Registry 2003-2021 (79), there are fewer patients in stage IV and proportionally more patients in stage II and III.

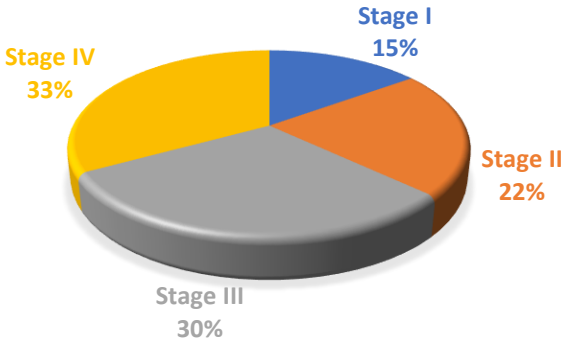


Figure 5. *Diagram of lung cancer stage distribution in the DETECT cohort.*

The histologic differentiation of lung cancer patients in the DETECT cohort is seen in Figure 6. Compared to the Danish Lung Cancer Registry 2003-2021 (79), relatively more patients are diagnosed with adenocarcinoma and fewer with small-cell carcinoma. This is well explained by the fact that the study excluded patients that were incapable of participating in diagnostic procedures, and that histology was obtained from all participants, while a histologic diagnosis is not obtained from 4%-8% of patients in the Danish Lung Cancer Registry.

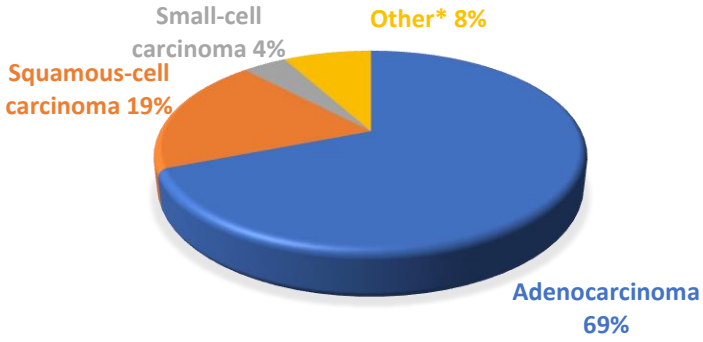


Figure 6. *Histologic differentiation of lung cancer patients in the DETECT cohort. *Primary lung cancers such as carcinoid tumor, low differentiated carcinoma, non-small-cell lung cancer-not otherwise specified.*

4.2 STUDY 1: EARLYCDT® LUNG TEST

The Early-CDT® Lung test was tested in the cohort as a whole, as well as in distinct clinically relevant subgroups (Figure 7) (80). These were based on age groups, sex, tobacco smoking history and lung cancer stage I-II vs. III-IV. Furthermore, a screening group were formed with the participants of aged 55-80 and a minimum of 30 pack years, which were the current USPSTF LDCT screening criteria at the time of the study publication. Four patients with lung cancer were excluded from the analysis due to a missing result of the Early-CDT® Lung test.

Total cohort	Sensitivity; n (95 % CI)	Specificity; n (95 % CI)	PPV	NPV
Lung cancer	0.33; 25/75 (0.23–0.45)	0.88; 150/171 (0.82–0.92)	0.54	0.75
Any malignant tumor	0.31; 27/87 (0.22–0.42)	0.88; 140/159 (0.82–0.93)	0.59	0.70
Smoking history subgroups				
Screening group#	0.37; 13/35 (0.21–0.55)	0.81; 39/48 (0.67–0.91)	0.59	0.64
10+ pack years	0.33; 21/63 (0.22–0.46)	0.86; 80/93 (0.77–0.92)	0.62	0.66
20+ pack years	0.33; 18/54 (0.21–0.47)	0.84; 58/69 (0.73–0.92)	0.62	0.62
30+ pack years	0.34; 15/44 (0.20–0.50)	0.81; 43/53 (0.68–0.91)	0.60	0.60
40+ pack years	0.35; 11/31 (0.19–0.55)	0.76; 31/41 (0.60–0.88)	0.52	0.61
50+ pack years	0.44; 8/18 (0.22–0.69)	0.79; 15/19 (0.54–0.94)	0.67	0.60
Age subgroups				
Age ≤ 60	0.11; 2/18 (0.01–0.35)	0.94; 59/63 (0.85–0.98)	0.33	0.79
Age 61–75	0.31; 11/35 (0.17–0.49)	0.87; 69/79 (0.78–0.94)	0.52	0.74
Age >75	0.55; 12/22 (0.32–0.76)	0.76; 22/29 (0.56–0.90)	0.63	0.69
Lung cancer stage subgroups				
Stage I-II lung cancer	0.21; 6/28 (0.08–0.41)	0.88; 150/171 (0.82–0.92)	0.22	0.87
Stage III-IV lung cancer	0.40; 19/47 (0.26–0.56)	0.88; 150/171 (0.82–0.92)	0.47	0.84
Sex				
Male	0.32; 13/40 (0.19–0.49)	0.86; 76/88 (0.77–0.93)	0.52	0.74
Female	0.34; 12/35 (0.19–0.52)	0.89; 74/83 (0.80–0.95)	0.57	0.76

95 % CI: 95 % confidence interval. #: The screening group consisted of participants aged 55–80 years and with at least 30 tobacco pack years. PPV: Positive predictive value. NPV: Negative predictive value.

Figure 7. *Performance of the EarlyCDT® Lung test (80).*

The Early-CDT® Lung test optimal sensitivity/specificity were 33%/88% in the cohort as a whole. The range in diagnostic properties depended on the clinically relevant factors. In the age groups, sensitivity ranged from 11% (<60 years) to 55% (>75 years). When smoking history was taken into account, sensitivity ranged from 33% (10+ tobacco pack years) to 44% (50+ tobacco pack years). Sensitivity in different lung cancer stages was 21% in stage I-II lung cancer vs. 40% in stage III-IV lung cancer (80).

4.3 STUDY 2: BIOMARKER PANEL (CYFRA 21-1, CEA, CA125)

Concentrations of the biomarkers in patients with lung cancer vs. control subjects are presented in Table 3. They were all significantly higher in patients with lung cancer compared to control subjects.

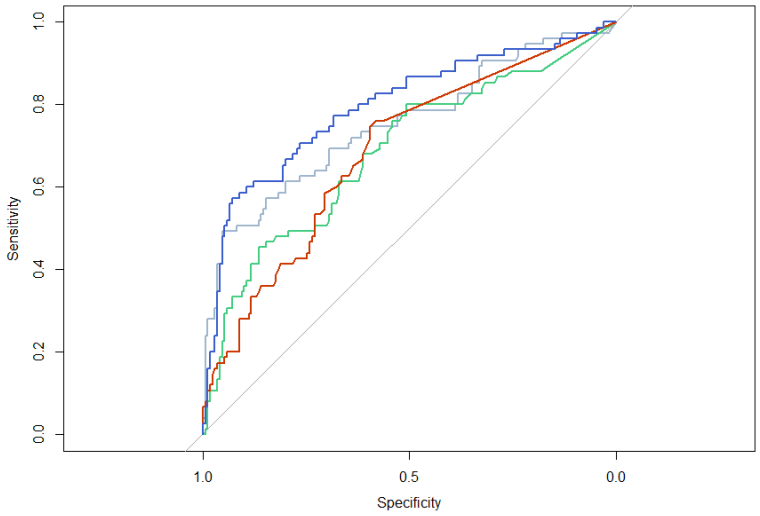
Table 3: Biomarker concentrations

	Patients with lung cancer	Control subjects
CA125	11 kU/L [7 – 32]	6 kU/L [4 – 11]*
CEA	1838 µg/L [664 – 4012]	601 µg/L [366 – 942]*
CYFRA 21-1	1342 ng/L [920 – 3544]	0.1 ng/L [0.1 – 1535]*

*Presented as median [IQR]. CA125: Cancer antigen 125, CEA: Carcinoembryonic antigen, CYFRA 21-1: cytokeratin 19 fragment. *p < 0.05.*

The ability to detect lung cancer in the cohort is presented as ROC curves for each biomarker with the corresponding AUC values. Using the combination of the biomarkers, a cross-validated prediction model was computed (Figure 8). The AUC values are shown in Table 4 (81). The corresponding optimal sensitivity and specificity of the biomarker model were 57% and 93%, respectively. The negative predictive value was 83%. At an overall specificity of 83%, based on the USPSTF criteria (3), sensitivity was 61%.

A



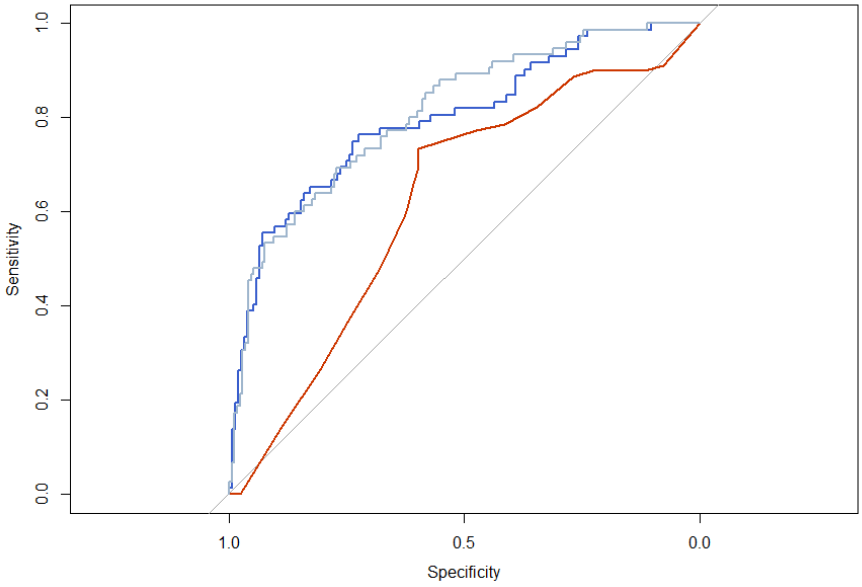
B

	AUC	95% CI
CA125	0.69	0.61-0.76
CEA	0.75	0.68-0.82
CYFRA 21-1	0.69	0.62-0.76
Biomarker model	0.80	0.73-0.86

***Figure 8 - A** Receiver operation characteristic curves* **B:** *Area under the curve of prediction models. CA125, CEA, CYFRA 21-1, combined biomarker model (81). CA125: Cancer antigen 125, CEA: Carcinoembryonic antigen, CYFRA 21-1: cytokeratin 19 fragment, AUC: Area under the curve, 95% CI: 95% Confidence interval.*

To perform a comparison of the biomarker panel model with clinically available information of tobacco pack years and age, three distinct prediction models were computed (81). A model combining the biomarker panel with tobacco pack years, a model combining the biomarker panel with current USPSTF LDCT screening criteria, and a model built solely on USPSTF screening criteria of age and tobacco pack years (Figure 9). The AUC values of the ROC curves are presented in Table 5.

A



B

	AUC	95% CI
Biomarker panel + tobacco pack years	0.80	0.73-0.86
Biomarker panel + screening criteria	0.81	0.75-0.87
USPSTF screening criteria	0.62	0.55-0.70

Figure 9 – A: *Receiver operation characteristic curves* **B:** *Area under the curve of prediction models. Biomarker panel + screening criteria, United States Preventive Services Task Force screening criteria, Biomarker panel + tobacco pack years (81). AUC: Area under the curve; 95% CI: 95% confidence interval.*

Furthermore, the models were evaluated on their abilities to detect stage I-II lung cancer vs. stage III-IV lung cancer (Figure 10) (81). The AUC values of all prediction models are shown in Table 4. It clearly shows that the prediction models are better at detecting late-stage lung cancer. When applying the USPSTF screening criteria, detection of stage I-II vs. stage III-IV were equally low, with AUC values of 0.62 and 0.61, respectively.

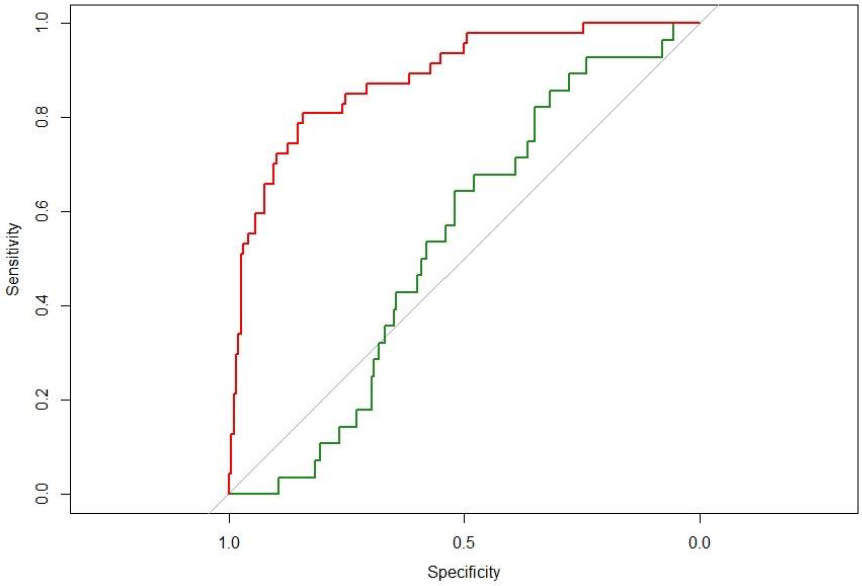


Figure 10. *Receiver operation characteristic curves: Biomarker panel detecting stage I-II, biomarker panel detecting stage III-IV (81).*

Table 4. Area under the curve of prediction models detecting stage I-II and stage III-IV

	Stage I-II	Stage III-IV
Biomarker panel	0.54 [0.44-0.63]	0.88 [0.83-0.94]
Biomarker panel + tobacco pack years	0.66 [0.55-0.76]	0.86 [0.79-0.93]
Biomarker panel + screening criteria	0.67 [0.58-0.76]	0.87 [0.81-0.93]
USPSTF screening criteria	0.62 [0.51-0.72]	0.61 [0.53-0.69]

Detection of stage I-II vs. stage III-IV. Presented as area under the curve with 95% confidence interval. USPSTF: United States Preventive Services Task Force (81).

4.4 STUDY 3: NATURAL KILLER CELLS ACTIVITY

The levels of NKA in control subjects, any-stage lung cancer, early-stage lung cancer (stage I-II) and late-stage lung cancer (stage III-IV) are presented in Figure 11 (82). The level was significantly lower in late-stage lung cancer (median 161 pg/mL, IQR 33-643), compared to both early-stage lung cancer median 753 pg/mL, IQR 172-1957) and control subjects (median 450 pg/mL, IQR 130-1358); ($p<0.01$). No significant difference was seen between controls and early-stage lung cancer.

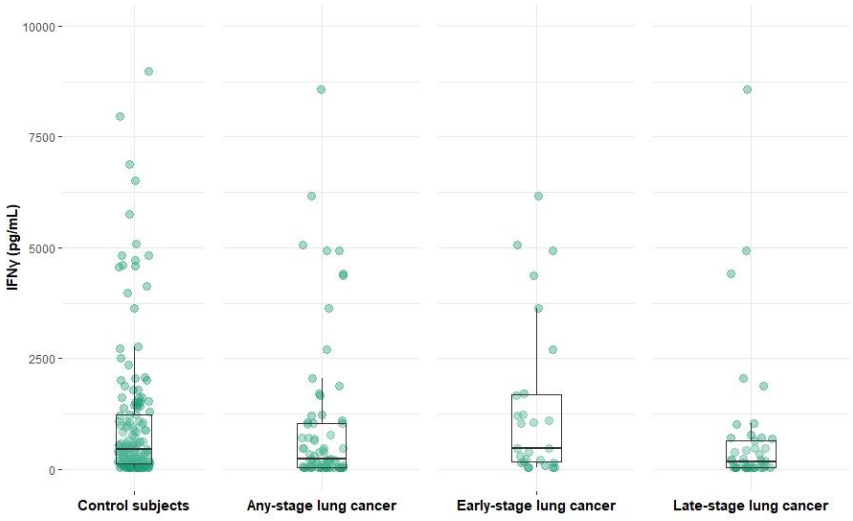


Figure 11. *Natural killer cells activation among groups. IFNγ: Interferon gamma (82).*

The ability of NKA to detect all-stage lung cancer and late-stage lung cancer was assessed using ROC curves (Figure 12) (82). The AUC for detecting any stage of lung cancer was 0.57 (95% CI: 0.49-0.66), while the AUC for detecting late-stage lung cancer was 0.66 (95% CI: 0.57-0.75). For late-stage lung cancer, the AUC corresponds to a sensitivity of 62% and specificity of 64% at the optimal cut-point of 240 pg/mL. At an overall specificity of 83%, based on the USPSTF criteria (3), sensitivity was 38%.

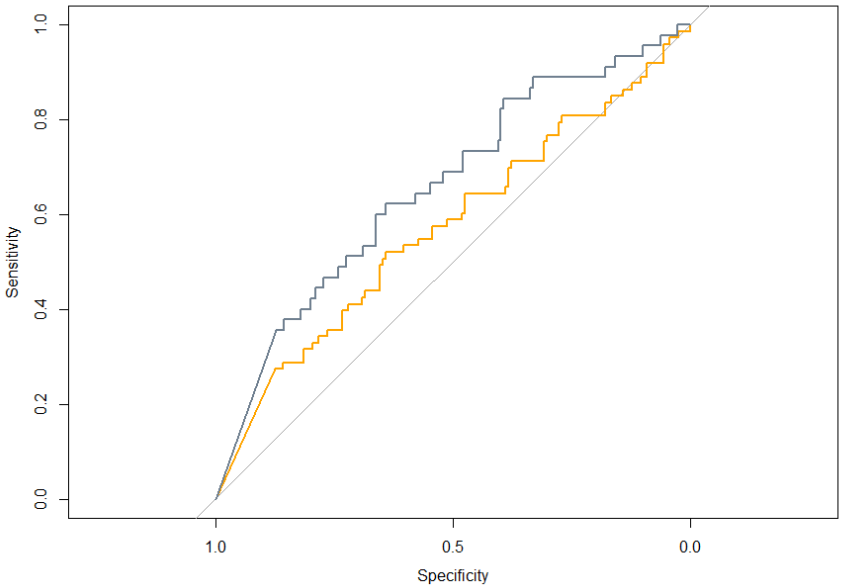


Figure 12. *Receiver operation characteristic curves: Late-stage lung cancer, Any-stage lung cancer (82)*

Next, the importance of clinical factors, such as age, sex and smoking status on NKA was assessed using a multiple linear regression model (82). The results are presented in Table 5 and do not find NKA to be influenced by the above-mentioned factors.

Table 5. Influence of clinical factors on natural killer cells activity

Variable	β-coefficient	p-value
Men	-116.2 [-817.5 – 585.1]	0.74
Ever-smoker (vs. non-smoker)	235.9 [-1093.8 – 622.0]	0.59
Age	11.9 [-19.9 – 43.6]	0.46

Data are presented as the β-coefficient with the 95% confidence interval. Multiple linear regression model (82).

4.5 STUDY 4: INCREASED USE OF COMPUTED TOMOGRAPHY: STAGE SHIFT TOWARDS EARLY-STAGE LUNG CANCER AND MANAGEMENT OF INCIDENTAL FINDINGS

The use of CT thorax increased in all five of the Danish regions in the period 2013-2020, as seen in Figure 13 (74). However, there is some variation among regions, with a difference in number of CT thorax in 2013 of more than 60% (24.3 per 1,000 inhabitants to 38.9 per 1,000 inhabitants, $p < 0.01$). In addition, the increase per year over the eight-year period differed from 1.9 to 3.4 more CTs annually per 1,000 inhabitants ($p < 0.01$). Across the period, the relative difference among regions was reduced, with the Central Denmark Region and North Denmark Region performing almost 60 CT thorax per 1,000 inhabitants and the Capital Region and Region Zealand performing around 45-50 CT thorax per 1,000 inhabitants in 2020.

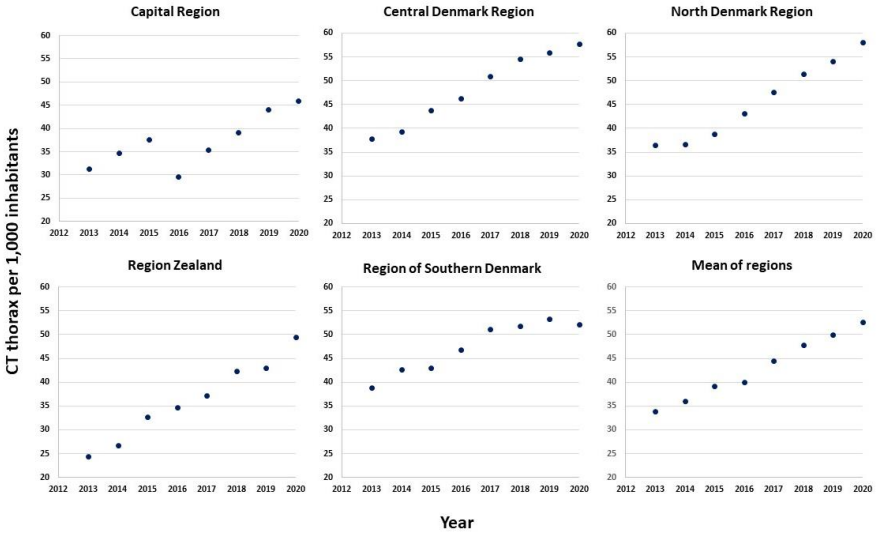


Figure 13. *Development in use of CT thorax in the Danish regions and mean of all regions in the period 2013-2020 (74).*

Figure 14 presents the percentage of stage IA lung cancer cases in the period 2013-2020 (74). In the year 2013, the percentage of stage IA lung cancer ranged from 7.3% to 12.2% ($p < 0.01$). Over the following years, from 2013 to 2020, there were

significant annual increases in the fraction of patients diagnosed in stage IA in the Capital Region, the Central Denmark Region, and the Region of Southern Denmark ($p < 0.01$ for each of these regions). In Region Zealand, only a minor, but still significant, increase in stage IA fraction was seen ($p < 0.05$), while there was no significant change in the North Denmark Region.

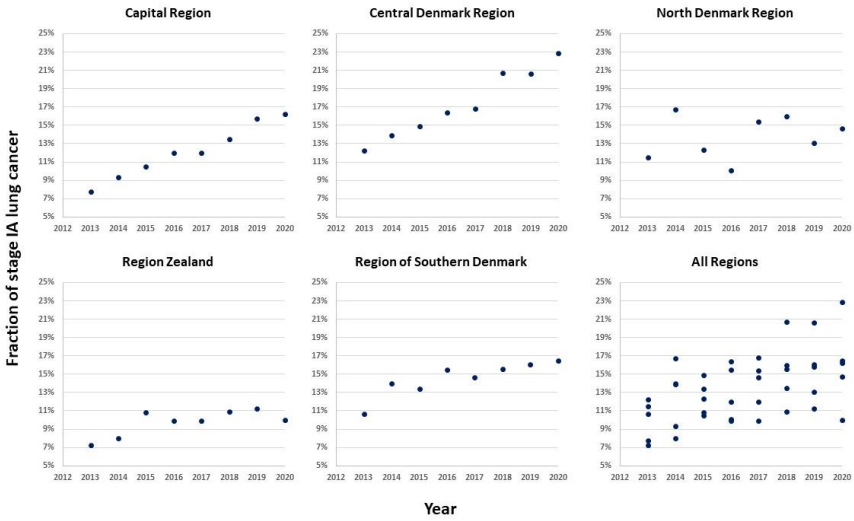


Figure 14. *Percentage of stage IA lung cancer in the Danish regions in the period 2013-2020 (74).*

A correlation analysis was done between the number of CT thorax performed and percentage of stage IA lung cancers (74). The scatter plots are presented in Figure 15. In the Capital Region, Central Denmark Region, and Region of Southern Denmark, the percentage of patients diagnosed in stage IA correlated to approximately the same extent with the number of CT thorax exams per 1,000 inhabitants ($p < 0.05$ for the correlation between number of CT thorax exams and the stage IA fraction, while $p > 0.35$ for difference between these three regions). In Region Zealand, a significant correlation was also seen ($p < 0.05$). However, the correlation was not nearly as marked and there was a significant difference between Region Zealand vs. the Capital Region, the Central Denmark Region, and the Region of Southern Denmark. No significant correlation was seen in the North Denmark Region.

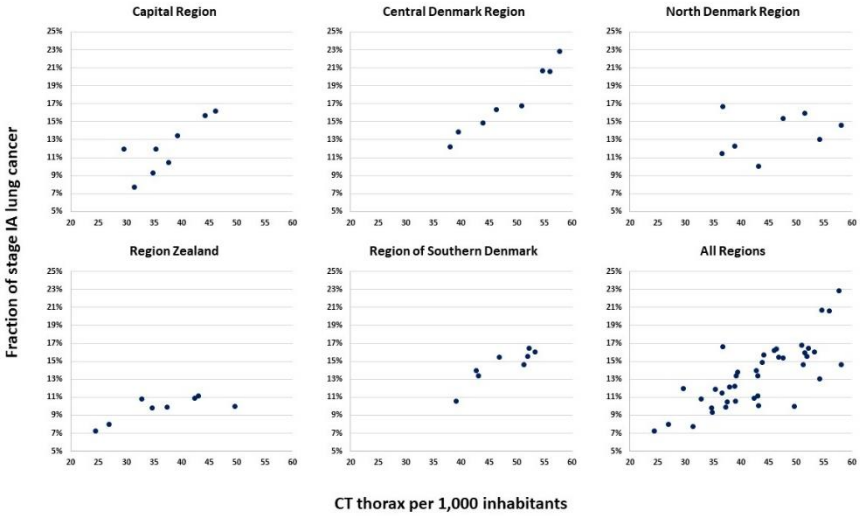


Figure 15. *Correlations between CT thorax and percentage of stage IA lung cancer in the Danish regions in the period 2013-2020 (74).*

Clinical auditing of 273 stage IA lung cancer cases was performed (74). In total, 36/273 (13.2%) were referred for suspicion of lung cancer. Out of these, seven (19.4%) were in stage IA1, 23 (72.2%) in stage IA2 and 6 (16.7%) in stage IA3.

Overall, 237/273 (86.8%) were incidental findings (i.e. the reason for referral was not suspicion of lung cancer) (74). Of these, 38 (16.0%) were in stage IA1, 151 (63.7%) in stage IA2 and 48 (20.3%) in stage IA3. The reasons for referral to CT exam were trauma, cardiology investigation with cardiac CT, suspicion of pulmonary embolism and back or shoulder pain. Alternatively, the early-stage lung cancer was found as a result of staging or follow-up of cancer in the head and neck, colon, prostate, ovaries or hematological cancer.

Next, clinical auditing of 736 stage IV lung cancers was performed (74). The audit results are presented as a flowchart in Figure 16. In total, 81 patients (11%) had a CT performed that showed parts or all of thorax. Of these, a nodule or infiltrate were described by the radiologist in 27 patients; eight patients received CT follow-up while 19 patients did not receive follow-up in accordance with the Fleischner Society pulmonary nodules recommendations (71). The infiltrate/nodule in 23 of these patients was most likely the origin of the subsequently diagnosed stage IV lung cancer; even though four of them received follow-up according to recommendations (71).

The CT exams were not described with a nodule/infiltrate in 54 patients (74). However, retrospectively assessed, nine patients did have a nodule/infiltrate that most likely developed into stage IV lung cancer. In total, 32 patients (4.3%) with stage IV lung cancer had a nodule/infiltrate visible on a previous CT exam, that most likely developed into the subsequently diagnosed stage IV lung cancer.

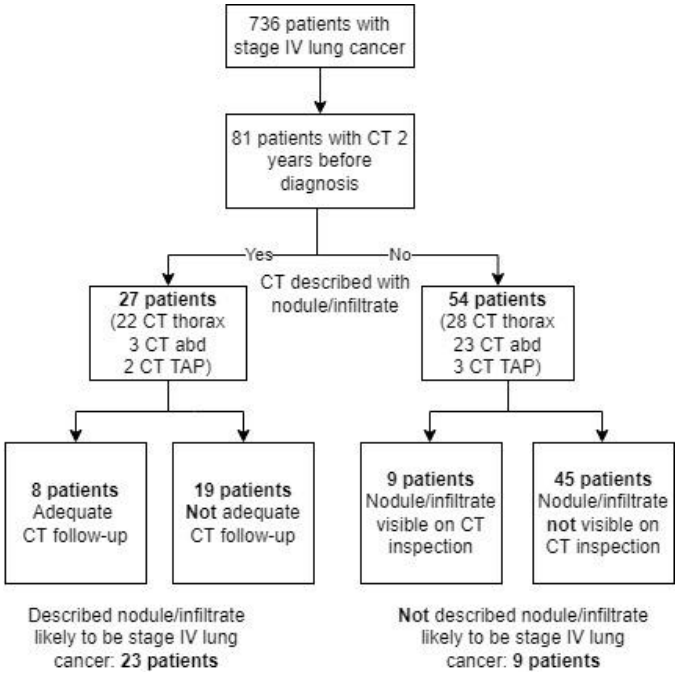


Figure 16. *Flowchart of the clinical auditing results of stage IV lung cancer (74).*

In the 27 patients where a nodule/infiltrate was described by the radiologist, morphology and size of the nodule/infiltrate were recorded to assess whether they actually should receive follow-up according to recommendations by the Fleischner Society (71). As seen in Table 6, the morphology was primarily solid, while no ground glass opacities were seen (74).

Table 6. Morphology of nodules/infiltrates described by the radiologist (74)

Morphology	n
Solid	12
Semi-solid	1
Ground glass opacity	0
Cystic	2
Diffuse infiltrates	1
Central tumor/lymphnode	3

The size of the described nodules/infiltrates are seen in Figure 17. Notably, none of the nodules/infiltrates were below 6 mm in size, and, according to recommendations they should all have received CT follow-up (74).

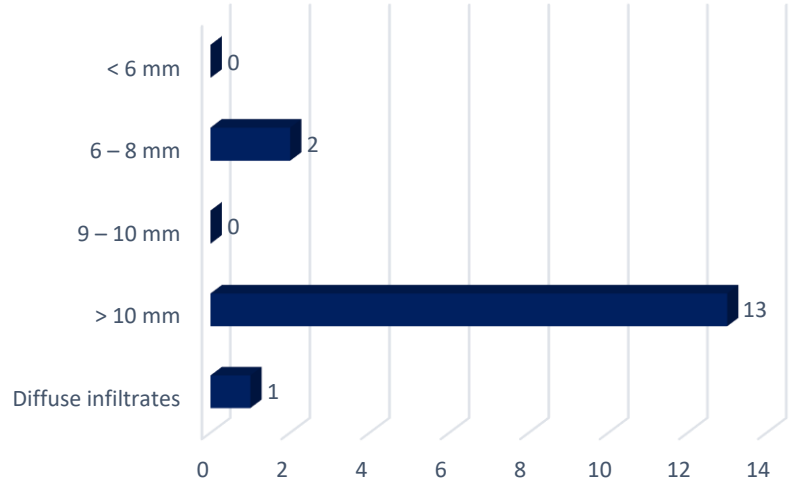


Figure 17. *Size of nodules/infiltrates described by the radiologist*

The following reasons for deviations from follow-up algorithms were identified: The radiologist describes the infiltrate but does not recommend follow-up; the radiologist recommends follow-up but the referring department does not perform CT follow-up; the CT exam is sent for assessment at the regional lung cancer investigation unit, where follow-up is recommended, but the referring department does not perform follow-up; or the referring department uses chest x-ray for follow-up of

pneumonia/diffuse infiltrates. An example from the clinical audit of stage IV lung cancer patients is provided in Figure 18 (74). An infiltrate in the right upper lobe is described as probably infectious by the radiologist and recommendation of follow-up is not given to the clinician. Ten months later, the patient is diagnosed with stage IV lung cancer, small cell carcinoma (T4N2M1c).

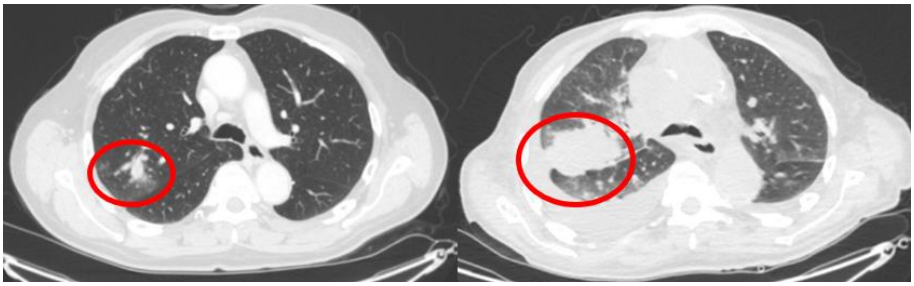


Figure 18. Example of an infiltrate that most likely developed into stage IV lung cancer. The CT exams are 10 months apart and the patient was diagnosed with stage IV lung cancer, small cell carcinoma (T4N2M1c) (74).

5. DISCUSSION

5.1 STUDY 1: EARLYCDT® LUNG TEST

Study 1 evaluates the performance of the EarlyCDT® Lung test antibody assay in the prospective DETECT high-risk cohort (80). The study found an overall sensitivity of 33% (25/75) with a specificity of 88% (150/171). It follows that, if the assay was used in society as a primary screening tool for lung cancer (in a population similar to the DETECT cohort), it would return a false negative result in 67% of the patients that actually had lung cancer. If screening measures were applied in lung cancer, the primary target would be to find patients in a curable stage, preferably stage I-II. However, the EarlyCDT® Lung test would return a false negative result in 79% of patients that had stage I-II lung cancer. Furthermore, evidence from the study suggests that the EarlyCDT® Lung test detects lung cancer less well in patients below 60 years of age and with less smoking history. These patients would often be more likely to cope with thoracic surgery.

Naturally, diagnostic properties like this are not suited for lung cancer screening. The ideal setup for lung cancer screening would be a three-step program. First, patients would be included based on an age- and tobacco pack years cut-off. Next, participants would have annual blood tests taken, analyzed using a biomarker with a very low number of false negative results when detecting lung cancer. Finally, participants with positive biomarker results would have a diagnostic CT thorax performed. Unfortunately, no currently tested biomarker has the potential to fulfill this dream scenario for lung cancer screening.

The EarlyCDT® Lung test has previously been tested in other populations, mainly in collaboration with the developers of the assay. Compared to the current study, distinct differences in results are seen. In a case control study by Chapman et al. (41) comprising 235 lung cancer cases and 266 healthy controls, the overall diagnostic sensitivity was 41% with a specificity of 91%. Information on lung cancer stage distribution in lung cancer cases was not given. However, the authors reported no significant difference in results on lung cancer stage distribution or age.

In a setup similar to the current study, Jett el al. (83) found a sensitivity of 41% (25/61) and specificity of 87%. In the study, the physicians ordered an Early CDT® Lung test in 1613 patients that they suspected of having lung cancer. If the test was positive, the responsible physician was notified. Six-month follow-up then revealed which patients were diagnosed with lung cancer. Overall, 50% of lung cancers were diagnosed in stage I-II, and out of 14 assay-detected NSCLC, 6 were in early-stage. However, the sensitivity of the assay when detecting stage I-II lung cancer is not disclosed in the study. The discrepancies between these two studies (41, 83) and the study 1 are likely to be explained by difference in study setup and stage distribution.

To our knowledge, apart from Study 1, only one other external study has assessed the EarlyCDT® Lung test. In the context of the German Lung Cancer Screening Trial (LUSI) (84), a post-hoc analysis of screening results were performed on blood tests from screened participants. As a natural consequence of the screening trial setup, stage distribution was skewed, with 69.6% of lung cancer cases in stage I. This resulted in a reported sensitivity of 13% with a specificity of 88.9%. Overall, the conclusions of Study 1 – with a large difference in sensitivity of stage I-II vs. stage III-IV (21% vs. 40%) – fits well with the LUSI-related study (84). Hence, these two external studies suggest that the EarlyCDT® Lung test shows insufficient sensitivity for detecting early-stage lung cancer, both as a single biomarker in screening and as a rule-in test as part of LDCT eligibility criteria.

5.2 STUDY 2: BIOMARKER PANEL (CYFRA 21-1, CEA, CA125)

In Study 2, the diagnostic properties of the three blood biomarkers CA125, CEA and CYFRA 21-1 are studied in the DETECT high-risk cohort (81). The biomarker model combined the three biomarkers, which resulted in an AUC value of 0.80. The corresponding sensitivity and specificity were 57% and 93%, respectively. Notably, information on tobacco use did not change the AUC value of the model. If the biomarker assay was used as a screening measure (in a cohort similar to the DETECT cohort), this would mean that 43% of patients with lung cancer would test false negative. When detecting stage I-II lung cancer, the AUC value of the biomarker assay fell remarkably to 0.54, which would mean an even higher fraction of false negative test results. Despite the fact that adding tobacco information improved the AUC value when detecting stage I-II lung cancer, it would still not yield acceptable results as a screening tool.

As previously described, the US has implemented LDCT screening for lung cancer-based eligibility criteria based on age and tobacco pack years (3). If the same criteria were applied on the DETECT cohort, this yielded AUC values of 0.62 and 0.61 for stage I-II and stage III-IV, respectively (81). These results highlight the weakness of the current US screening program and that a large proportion of patients diagnosed with lung cancer would not be eligible for lung cancer screening if a similar LDCT program were introduced in Denmark. A highly sensitive biomarker used as a rule in test, could possibly improve the selection process of a screening program. However, based on the results of Study 2, the currently available biomarkers do not seem to possess the appropriate detection properties. Other efforts to improve the eligibility criteria using clinically available information have been made. In a US context, the PLCOm2012 (85) risk model has shown a higher sensitivity than the NLST screening criteria (86). Apart from age and smoking history, the PLCOm2012 criteria also include race, education level, body mass index, chronic obstructive pulmonary disease

(COPD) yes/no, previous history of lung cancer and family history of lung cancer. In the Nordic countries, the HUNT model has been proposed as an alternative lung cancer risk model (87).

In Study 2, when tested individually, the biomarkers had AUC values of 0.69 (CA125), 0.69 (CYFRA 21-1) and 0.75 (CEA) (81). The individual properties of the biomarkers have been evaluated in previous studies. In a Chinese case control study by Wang et al. (52), the AUC of the biomarkers CA125 and CYFRA 21-1 were higher (CA125: 0.79; CYFRA 21-1: 0.85), while the AUC of CEA was comparable to the current study (CEA: 0.76). The stage distribution was not entirely similar between the two studies (Stage I-II: 37% vs. 44%), but this would normally favor the detection abilities of the assay when tested in the DETECT cohort. However, previous studies have found the Asian and Caucasian lung cancer population to differ (88), with a larger number of non-smoking cases in the Asian (89). This could lead to a difference in tumor development (90) and a distinct tumor micro-environment could explain the variability in blood protein composition (91). Tobacco information was not disclosed in the study (52).

The results from study 2 were comparable to a study by Mazzone et al. (53) (CA125: AUC 0.67; CYFRA 21-1: AUC 0.68; CEA: AUC 0.70). The proportion of stage I-II was a bit higher, at 46%, and tobacco use was prominent with median pack years – around 40. Samples were obtained from biorepositories in the US, and ethnic composition is believed to resemble that of the Study 2. Furthermore, the results from Study 2 were also comparable with those of Hanash at al. (54) who combined the three biomarkers with surfactant protein B (Pro-SFTPB) and tobacco history, reaching an AUC of 0.83, comparable to the results of the current study, with an AUC of 0.80, combining the three biomarkers with tobacco history. When the USPSTF screening criteria were applied, the AUC rose to 0.81.

Often, when assessing lung cancer biomarkers, they will primarily detect disseminated disease, while early disease is more difficult to detect. As mentioned, this is also the case in Study 2. The same issue has been seen in the previous studies (53, 54). This fact has led to a proposal for the biomarkers to be used as a model to detect tumor metastasis (92). However, PET/CT shows high sensitivity in identifying extra-thoracic metastases (93, 94). Hence, in countries where PET/CT is readily available in the lung cancer investigation units, PET/CT will remain the first choice in detection of tumor metastases.

5.3 STUDY 3: NATURAL KILLER CELLS ACTIVITY

Study 3 evaluated whether the activity of NK cells was downregulated in patients with lung cancer from the DETECT high-risk cohort, and whether this was sufficient and robust enough to be used as a diagnostic tool (82). No significant difference was seen when comparing NKA of patients with lung cancer (any stage) with control subjects, and the ROC curve yielded an AUC value of 0.57. However, the difference in NKA between control subjects and stage III-IV lung cancer was highly significant and the AUC value of the ROC curve increased to 0.66. This negative correlation between NKA downregulation and tumor stage found in the current study detecting lung cancer has also been described previously in breast (95) and prostate cancer (96).

In Study 3, the optimal cut-off of NKA was set at 240 pg/mL, yielding a sensitivity/specificity of 52%/64%. Previous studies have shown downregulation of NKA in several cancer types (61-64). Each study has yielded distinct diagnostic properties at an optimal cut-off value of NKA. Depending on the type of cancer investigated and patient cohort composition, optimal cut-offs may vary. However, across previous publications cut-offs are at approximately the same range: Prostate cancer: 200 pg/mL (61); colorectal cancer: 181 pg/mL (63); gastric cancer: 438 pg/mL (62); and lung cancer: 391 pg/mL (64). These cut-off values have provided different diagnostic properties with AUC values of the organ-specific cancers at 0.82 (gastric cancer (62)); 0.73 (colorectal cancer (63)); 0.75 (prostate cancer (61)); and 0.76 (lung cancer (64)). Study 3 found a substantially lower AUC at 0.57 when detecting all-stage lung cancer. This discrepancy is not likely to be explained by stage distribution, as 43% of patients were in stage I-II in the lung cancer study by Choi et al. (64), compared to 37% in Study 3.

Previous work on the impact of clinical factors such as age, sex and smoking on NKA has provided conflicting results (64, 97-99). However, Study 3 finds no evidence to support any impact of age, sex or smoking (82), and hence, the difference in diagnostic properties between the two studies could be due to the designs of the studies with diverging control groups or potentially the different histologic subtypes of lung cancer. In the work by Choi et al. (64), 40.9% of lung cancer cases were squamous cell carcinoma, as compared to 19% in the current study. If NKA was used as a screening measure in a cohort similar to the DETECT cohort, this would result in a false negative rate of 62% at the fixed specificity of 83%, with an even higher rate of false negative results in stage I-II lung cancer cases. Clearly, the assay is not appropriate for lung cancer screening in its current state.

5.4 STUDY 4: INCREASED USE OF COMPUTED TOMOGRAPHY: STAGE SHIFT TOWARDS EARLY-STAGE LUNG CANCER AND MANAGEMENT OF INCIDENTAL FINDINGS

Study 4 evaluated the possible correlations between the increasing use of CT thorax and percentage of stage IA lung cancer, the referral pattern of patients diagnosed with stage IA lung cancer and the management of incidental findings through a clinical audit of stage IV lung cancer patients (74).

Strong correlations between the use of CT thorax and stage IA lung cancer were seen in three of the five Danish regions, while a less marked, but still significant correlation was seen in one region. When information from the clinical audit of stage IA lung cancer patients was added, showing that the vast majority (86.8%) can be characterized as incidental findings, this strongly suggested that increased use of CT results in a higher percentage of early-stage lung cancer.

Both in terms of CT use and percentage of stage IA lung cancer, wide variations were seen in the five Danish regions. The use of CT thorax per 1,000 inhabitants differed by more than 50% in 2013, and the annual increase in CT thorax per 1,000 patients ranged from 1.9 to 3.4 among the regions (74). These regional differences are likely to arise from issues such as variation in patients' demand for CT exams, a potential shortage of radiologists in some regions and, as previously shown in a single-center study, restricted vs. liberal access to CT exams for clinicians (100).

A large difference in percentage of stage IA lung cancer among regions is also seen; ranging from 10.0% to 22.9% in 2020 (74). This could be due, at least in part, to variations in use of CT. However, in one region a significant correlation between CT use and stage IA percentage is not seen. Hence, other reasons could also play a role. The clinical audit of stage IV lung cancer patients aimed to assess the management of incidental findings that could be early-stage lung cancer. It was found that 4.3% of stage IV lung cancer patients had a nodule/infiltrate on an earlier CT and potentially could have been diagnosed in an earlier stage.

The audit provides an excellent learning opportunity to improve management of incidental findings (74). As mentioned above, Study 4 identified a number of scenarios, where deviations from the algorithm were seen. This highlights the importance of firm follow-up of algorithms to secure they have the intended effect, which is early detection of lung cancer.

Out of the nodules/infiltrates that likely developed into stage IV lung cancer, 27% were not mentioned by the radiologist (74). With a mean diameter of 9.8 mm (range 5-24 mm), the sizes were comparable to those seen in a previous study of missed

nodules (101). However, with additional training, the radiologist's ability to detect nodules can be improved significantly (102).

5.5 STRENGTHS AND LIMITATIONS

The study setup of the DETECT high-risk cohort is a feasible approach to obtain blood samples from an at-risk population with both lung cancer cases and control subjects, where lung cancer is ruled out on the basis of a CT scan and, potentially, further diagnostic work-up. The setting is "real-world", and the suspicion of lung cancer relies not only on age and tobacco pack years, but also a suspicion held by the GP, which broadens the strict criteria of age and tobacco history in LDCT screening studies. Naturally, the results cannot be translated directly to an LDCT screening study with only a few percent of lung cancer cases and a large fraction of stage I-II lung cancer cases. However, as the DETECT cohort has a high prevalence of lung cancer cases, the negative predictive value of the biomarkers assessed will be lower than if tested in a screening study cohort with a low prevalence of lung cancer. Furthermore, due to the study setup, control subjects and patients with lung cancer are not matched on clinical factors, such as smoking history or age.

The correlation study on CT thorax and lung cancer stage distribution can only characterize an association, hence, causal effects are uncertain. However, as suggested a causal relationship does seem likely, since these small tumors are practically undetectable without the use of CT and the majority of stage IA lung cancers are incidental findings on CT exams performed for reasons other than suspicion of lung cancer. The clinical audit of stage IV lung cancer patients provides a retrospective assessment of the CT thorax. If the nodule/infiltrate was located at the same place as the primary tumor of the subsequently diagnosed stage IV lung cancer, it was characterized as most likely the origin of the stage IV lung cancer. Of course, a characterization like this comes with some uncertainty.

The audit period of two years before diagnosis was chosen partly due to a legal limit of five years from diagnosis for quality assessment studies and partly because two years is the usual follow-up period for solid pulmonary nodules (74). If the audit period was longer this could have affected the actual number of missed nodules/infiltrates, given that slow growing lung cancers, primarily of subsolid type, often require longer time to progress into stage IV lung cancer. Furthermore, the audit included only lung cancer patients in stage IV. If the clinical audit had included patients in stage II and III, this could also have affected the number of missed nodules/infiltrates.

The clinical audit was performed as a single-observer study and the retrospectively assessment that a nodule/infiltrate most likely was the origin of the later diagnosed stage IV lung cancer, was based on that the nodule/infiltrate was in the approximate same location of the later diagnosed primary tumor of the stage IV lung cancer. Hence, inter-observer assessment variations will probably occur.

6. CONCLUSIONS AND PERSPECTIVES

6.1 CONCLUSIONS

Based on the results obtained, the following conclusions can be drawn.

Study 1: Early-CDT® Lung test

When assessed in the DETECT high-risk cohort, the Early-CDT® Lung test proved to have an overall sensitivity of 33% with a specificity of 88%. When tested in patient subgroups, the assay was best at detecting stage III-IV lung cancer in patients above the age of 75 and with a smoking history of more than 50 tobacco pack years. In this light, the Early-CDT® Lung test is not appropriate as a primary screening tool in detection of early-stage lung cancer.

Study 2: Biomarker panel (CYFRA 21-1, CEA, CA125)

The biomarker panel was tested in the DETECT high-risk cohort. In conjugation with tobacco history, the biomarker panel provided diagnostic properties similar to previous studies, with an AUC value 0.81. However, the biomarker panel was best at detecting stage III-IV lung cancer and, when trying to detect stage I-II lung cancer, the AUC was reduced to 0.66. Hence, the panel is less applicable as an early lung cancer screening tool.

Study 3: Natural killer cells activity

The activity of natural killer cells was evaluated in the DETECT high-risk cohort. It proved that NKA was significantly reduced in patients with lung cancer stage III-IV compared to controls. However, as this was not the case in any-stage or stage I-II lung cancer, NKA does not seem to be suitable for detection of early-stage lung cancer.

Study 4: Increased use of computed tomography: Stage shift towards early-stage lung cancer and management of incidental findings

There are significant regional differences in Denmark regarding use of CT thorax and percentage of stage IA lung cancer. The vast majority of stage IA lung cancers are incidental findings. As much as 4.3% of stage IV lung cancer cases had a previous CT with a nodule/infiltrate that most likely developed into stage IV lung cancer.

6.2 PERSPECTIVES

Overall, based on the results in this thesis, the biomarkers tested in the DETECT high-risk cohort do not possess the necessary properties to be used unassisted as a tool in screening for early-stage lung cancer. However, the tested biomarkers perform better when detecting late-stage lung cancer, and they show potential for use as markers of disseminated disease, treatment effect in disseminated disease or as a surveillance tool for relapse. Future research in the present biomarkers should focus on these areas, possibly in combination with complementary biomarkers.

There is a vast array of potential blood-based lung cancer biomarkers. The biggest challenge is to detect early-stage lung cancer. Beneficially, future research could focus on biomarkers with a high negative predictive value, and let the biomarker be part of an LDCT screening program in high-risk participants with a fixed cut-off, to determine whether patients should undergo LDCT or a new biomarker test in 6 months. In this way, the number of LDCT scans produced by a screening program would be greatly reduced, creating less demand on scanning capacity, radiologists, and respiratory physicians.

The thesis also points out that the number of CT thorax performed in a country or region, in itself, does not ensure early detection of lung cancer. Patients receiving a CT exam could be at risk of developing lung cancer and the necessary resources for careful CT interpretation and proper follow-up regimens in accordance with recommendations should be put in place to minimize the risk of missing an opportunity to diagnose a lung cancer at an early stage.

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