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2024

Document Version:
Publisher's PDF, also known as Version of record

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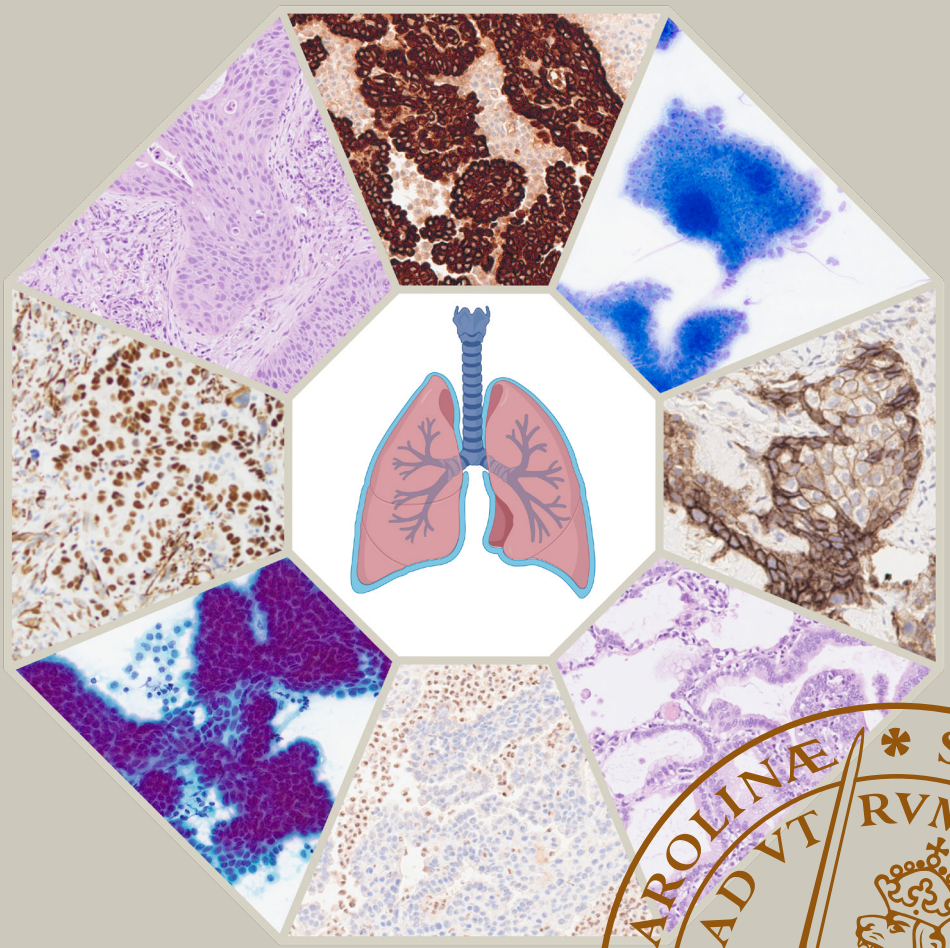

Biomarkers in Mesothelioma and Non-Small Cell Lung Cancer – Investigation of Cytological Specimens with Correlation to Histology

Biomarkers in Mesothelioma and Non-Small Cell Lung Cancer

Investigation of Cytological Specimens with Correlation to Histology

Mohammed S. I. Mansour

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD)
at the Faculty of Medicine at Lund University, Sweden.

To be publicly defended on April 16th, 2024, at 09.00 in the Lecture Hall,
Department of Pathology, Sölvegatan 25B, Lund

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Organization: LUND UNIVERSITY, Division of Pathology, Department of Clinical Sciences Lund, Faculty of Medicine, Lund University, Sweden

Document name: DOCTORAL DISSERTATION **Date of issue** April 16th, 2024

Author(s): Mohammed S. I. Mansour **Sponsoring organization**

Title and subtitle: Biomarkers in mesothelioma and non-small cell lung cancer – Investigation of cytological specimens with correlation to histology

Abstract:

Pleural mesothelioma (PM) and non-small cell lung cancer (NSCLC) are two highly lethal pleuro-pulmonary malignancies where ancillary analyses are needed for accurate diagnosis and (for NSCLC) treatment predictive testing, e.g. PD-L1. Diagnosis and analyses are traditionally based on histological material. However, a significant proportion of patients are diagnosed on cytological specimens, which are rapid and minimally invasive but less standardised regarding handling and fixative.

In Paper I, PD-L1 expression was evaluated immunohistochemically in 61 paired biopsies and cell blocks with PM. The overall percentage agreement (OPA) between histology and cytology was 69%/84%, with a kappa of 0.36/0.08, at the ≥1%/>50% cutoffs. The cyto-histological correlation tended to be higher for epithelioid mesothelioma compared to non-epithelioid mesothelioma at the ≥1% cutoff.

In Paper II, eight diagnostic immunohistochemical biomarkers were investigated in 59 paired biopsies and cell blocks with PM. The cyto-histological OPA for the epithelioid component was for calretinin 93%, CK5 98%, podoplanin 97%, WT1 90%, EMA 86%, desmin 100%, BAP1 91%, and MTAP 72%. Simultaneous loss or simultaneous preservation of both BAP1 and MTAP was observed in 40% and 11% of biopsies, while the corresponding figures were 54% and 8% for the paired cell blocks.

In Paper III, PD-L1 expression was evaluated immunohistochemically in two cohorts of paired biopsies and cytological NSCLC specimens. Using a 3-tier scale, PD-L1 showed concordance in 40/47 (85%) and 66/97 (68%) cases in the two cohorts, with kappa values of 0.77 and 0.49, respectively. In 25 reviewed published studies comprising approximately 1,700 paired cases, the median cyto-histological concordance was 81-85% for a positive PD-L1 staining at the ≥1% cutoff and 89% at the ≥50% cutoff.

In Paper IV, the impact of various clinicopathological and molecular factors on PD-L1 expression was investigated in two NSCLC cohorts of 1131 and 651 unpaired specimens (both 55% PD-L1 positive cases). Lower PD-L1 expression was seen in adenocarcinomas compared to squamous cell carcinomas, *EGFR*-mutated NSCLC compared to *KRAS*/*EGFR* wild-type and *KRAS*-mutated cases (highest expression), and in mucinous *KRAS*-mutated adenocarcinomas compared to non-mucinous.

In Paper V, 24 pleural effusion cell blocks with lung adenocarcinomas were investigated for the effect of four common fixatives on the immunoreactivity of nine diagnostic markers. Differences in staining proportions were seen for TTF-1 clone 8G7G3/1 and EpCAM clone MOC-31, particularly with cases showing negativity in CytoLyt® (33.3% and 83.3% positive, respectively) and PreservCyt® (62.5% and 83.3%), while exhibiting positivity in CytoRich™ Red (76.5% and 94.1%) and formalin (both 95.8%). Weaker staining intensity was seen for all alcohol-based fixatives also for napsin A.

Keywords: BAP1, cytology, fixation, immunocytochemistry, lung cancer, mesothelioma, MTAP, mutation, PD-L1, TTF-1

Classification system and/or index terms (if any) Supplementary bibliographical information

Language English **ISSN and key title:** 1652-8220

ISBN: 978-91-8021-535-0

Recipient's notes **Number of pages**: 211

Price Security classification

Signature Date March 4th, 2024

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Investigation of Cytological Specimens with Correlation to Histology

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Doctoral Dissertation Series 2024:42
ISBN 978-91-8021-535-0 (print)
978-91-8021-569-5 (pdf)
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2024

Preface

If you don't belong, you won't be long.

I write this in Halmstad, Sweden, although I originally came from Palestine. Born and raised in Gaza, I came to Sweden at the age of 19. I have struggled to learn a new language, study in a new country, and take a step into the world of research. Now I have come to the end of the long, serendipitous, and far from straightforward road that led to the completion of this dissertation. I have been able to write this dissertation in the comfort and safety of my office and my own apartment during many global tragedies from the pandemic which still has its fingerprints on our lives, conflicts and wars, frequent earthquakes and storms, and political and economic disasters. I currently feel both stressed and privileged. Stressed because of worrying about my family who are trapped in the madness of the terrible war in the Gaza Strip, and privileged because I can in safety see the fruition of this research journey. As there are two states of my mind, there are also two sides to this thesis, the investigation of two different thoracic malignancies i.e., pleural mesothelioma, and non-small cell lung cancer, in two diagnostic materials i.e., histological, and cytological specimens that are complement for each other.

Mohammed S. I. Mansour

Halmstad, November 9th, 2023

Table of Contents

List of Papers

This thesis is based on the following five original papers and preprints, which are referred to in the text by their Roman numerals as indicated below and the papers are included in full at the end.

Paper I

Mansour MSI, Seidal T, Mager U, Dobra K, Brunnström H, Dejmek A. Higher concordance of PD-L1 expression between biopsies and effusions in epithelioid than in nonepithelioid pleural mesothelioma. *Cancer Cytopathol.* 2021 Jun;129(6):468-478.

Paper II

Mansour MSI, Huseinzade A, Seidal T, Hejny K, Maty A, Taheri-Eilagh F, Mager U, Dejmek A, Dobra K, Brunnström H. Comparison of immunohistochemical mesothelial biomarkers in paired biopsies and effusion cytology cell blocks from pleural mesothelioma. *Cytopathology.* 2023 Sep;34(5):456-465.

Paper III

Mansour MSI, Lindquist KE, Seidal T, Mager U, Mohlin R, Tran L, Hejny K, Holmgren B, Violidaki D, Dobra K, Dejmek A, Planck M, Brunnström H. PD-L1 Testing in Cytological Non-Small Cell Lung Cancer Specimens: A Comparison with Biopsies and Review of the Literature. *Acta Cytol.* 2021;65(6):501-509.

Paper IV

Mansour MSI, Malmros K, Mager U, Ericson Lindquist K, Hejny K, Holmgren B, Seidal T, Dejmek A, Dobra K, Planck M, Brunnström H. PD-L1 Expression in Non-Small Cell Lung Cancer Specimens: Association with Clinicopathological Factors and Molecular Alterations. *Int J Mol Sci.* 2022 Apr 19;23(9):4517.

Paper V

Mansour MSI, Pettersson L, Seidal T, Strömberg U, Mager U, Ali L, Kumbaric S, Hejny K, Taheri-Eilagh F, Mufti J, Nakdali D, Brunnström H. The impact of different fixatives on immunostaining of lung adenocarcinomas in pleural effusion cell blocks. *Manuscript.*

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Additional Publications

The following peer-reviewed papers are not included in this thesis but are of relevance to the field and referred to in the text.

Additional publication I

Mansour MSI, Seidal T, Mager U, Baigi A, Dobra K, Dejmek A. Determination of PD-L1 expression in effusions from mesothelioma by immuno-cytochemical staining. *Cancer Cytopathol.* 2017 Dec;125(12):908-917.

Additional publication II

Mansour MSI, Hejny K, Johansson F, Mufti J, Vidis A, Mager U, Dejmek A, Seidal T, Brunnström H. Factors Influencing Concordance of PD-L1 Expression between Biopsies and Cytological Specimens in Non-Small Cell Lung Cancer. *Diagnostics (Basel).* 2021 Oct 18;11(10):1927.

List of Abbreviations

AC	adenocarcinoma
ALK	anaplastic lymphoma kinase
BAL	bronchoalveolar lavage
BAP1	BRCA1-associated protein 1
BRAF	rapidly accelerated fibrosarcoma oncogene, homolog B
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CI	confidence interval
CK5	cytokeratin 5
CK7	cytokeratin 7
CTLA-4	cytotoxic T cell lymphocyte-associated protein 4
DNA	deoxyribonucleic acid
EBUS	endobronchial ultrasound
EGFR	epidermal growth factor receptor
EMA	epithelial membrane antigen
EpCAM	epithelial cell adhesion molecule
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescent enhanced *in situ* hybridization
FNA	fine needle aspiration
H&E	haematoxylin and eosin
ICC	immunocytochemistry
ICI	immune checkpoint inhibitors
IHC	immunohistochemistry
κ	Cohen's kappa coefficient
KRAS	kirsten rat sarcoma
LBC	liquid-based cytology
MET	mesenchymal-epithelial transition
MGG	May-Grünwald-Giemsa

MPE	malignant pleural effusion
PM	pleural mesothelioma
MTAP	methylthioadenosine phosphorylase
NGS	next-generation sequencing
NPA	negative percentage agreement
NSCLC	non-small cell lung cancer
NTRK	neurotrophic receptor tyrosine kinase
OPA	overall percentage agreement
Pap	Papanicolaou's smear
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PPA	positive percentage agreement
RET	rearranged during transfection protein
RMC	reactive mesothelial cells
ROS1	cytoplasmic c-ros oncogene 1 receptor tyrosine kinase
SCC	squamous cell carcinoma
SCLC	small cell lung cancer
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TMB	tumour mutational burden
TNM	[primary] tumour, [lymph] node, metastasis (staging system)
TP53	transformation-related protein 53
TTF-1	thyroid transcription factor-1
WHO	World Health Organization
Wκ	weighted kappa coefficient
WT1	Wilms tumour 1

Thesis at a Glance

Paper	Study question	Materials & Methods	Results & Conclusions
Pleural Mesothelioma Studies			
I	• Is PD-L1 IHC expression comparable in paired histological and cytological specimens from PM?	• 61 paired pleural biopsies and pleural effusion cell blocks from different PM patients. • PM tumours of different histological subtypes. - Immunohistochemistry. - PD-L1 antibody.	• The OPA between histology and cytology was 69% and 84%, with a kappa of 0.36 and 0.08, at the ≥1% and >50% cutoffs. • The cyto-histological concordance tended to be higher for epithelioid mesothelioma compared to non-epithelioid mesothelioma at the ≥1% cutoff.
II	• Is protein expression of different IHC mesothelioma biomarkers comparable in paired histological and cytological specimens from PM?	• 59 paired pleural biopsies and pleural effusion cell blocks. • PM tumours of different histological subtypes. - Immunohistochemistry. - 8 different antibodies.	• The cyto-histological OPA for the epithelioid component was for calretinin 93%, CK5 98%, podoplanin 97%, WT1 90%, EMA 86%, desmin 100%, BAP1 91%, and MTAP 72%. • The concordance for calretinin, CK5, and WT1 was low (≤45%) for the sarcomatoid component on biopsies compared to cytology. • Simultaneous loss or simultaneous preservation of both BAP1 and MTAP was observed in 40% and 11% of biopsies and in 54% and 8% of the paired cell blocks.
Non-Small Cell Lung Cancer Studies			
III	• Is PD-L1 IHC expression comparable in paired histological and cytological specimens from NSCLC?	• Original study and review of the literature. • Two independent lung cancer cohorts of paired biopsies and cell blocks from different cytological material (47 and 97 cases from Lund and Halmstad, respectively). • NSCLC tumours of different histological subtypes. - Immunohistochemistry. - PD-L1 antibody (two different clones).	• Using a 3-tier scale, PD-L1 showed concordance in 40/47 (85%) and 66/97 (68%) cases in the two cohorts, with kappa values of 0.77 and 0.49, respectively. • The concordance was lower in paired samples from different anatomic sites, and in one cohort, the cytological specimens had a lower PD-L1 score in all discordant cases. • In 25 reviewed published studies comprising approximately 1,700 paired cases, the median (range) cyto-histological concordance was 81-85% (62-100%) for a positive PD-L1 staining at the ≥1% cutoff and 89% (67-100%) at the ≥50% cutoff.
IV	• Is frequency of PD-L1 expression comparable in histological and cytological specimens from NSCLC? • Are there any potential impacts of various clinicopathological and molecular factors on PD-L1 expression? • How frequent is PD-L1 expression in *KRAS* and *EGFR*-mutated NSCLC cases?	• Two independent lung cancer cohorts (1131 and 651 cases) of different histological subtypes. • Unpaired (very few paired cases only) histological - resections and biopsies - and cytological material from different cytological specimens. - Molecular data generated from clinical settings (from targeted NGS, PCR, FISH). - Immunohistochemistry. - PD-L1 antibody (two clones).	• ACs showed lower PD-L1 expression compared to SCC, while no differences were seen for sample types, tumour locations, or between the two cohorts (both 55% PD-L1 positive cases). • *KRAS*-mutated cases showed the highest PD-L1 expression, *EGFR*-mutated the lowest, with *KRAS*/*EGFR* wild-type cases in between. • There were no differences in PD-L1 levels between different prevalent *KRAS* mutations, while mucinous *KRAS*-mutated ACs demonstrated lower expression compared to non-mucinous.
V	• Is expression of ICC biomarkers comparable in cytological cell blocks fixed in formalin and alcohol-based fixative? • Is there a difference in staining properties of the antibodies linked to the fixative or the used antibody clone (for relevant biomarkers)?	• 24 different pleural effusions with lung AC tumour cells. • Matched cell block preparations from the same cases. - Immunocytochemistry. - TTF-1 (clones 8G7G3/1 and SPT24), napsin A, claudin 4, CEA, CK7, and EpCAM (clones BS14, Ber-Ep4, and MOC-31) antibodies.	• Differences in staining proportions were observed for TTF-1 clone 8G7G3/1 and EpCAM clone MOC-31, particularly with cases showing negativity in CytoLyt® (33.3% and 83.3% positive, respectively) and PreservCyt® (62.5% and 83.3%), while exhibiting positivity in CytoRich™ Red (76.5% and 94.1%) and formalin (both 95.8%). • Weaker staining intensity was seen for all alcohol-based fixatives compared to formalin for TTF-1 clone 8G7G3/1, napsin A, and EpCAM clone MOC-31.

Abbreviations: AC = adenocarcinoma; FISH = fluorescence in situ hybridization; ICC = immunocytochemical staining; IHC = immunohistochemical staining; NGS = next generation sequencing; NSCLC = non-small cell lung cancer; PD-L1 = programmed death-ligand 1; OPA = overall percentage agreement; PM = pleural mesothelioma; SCC = squamous cell carcinoma.

Abstract

Diagnosis by aspiration is as reliable as the combined intelligence of the clinician and pathologist makes it.

Fred W. Stewart, 1894-1991

Pleural mesothelioma (PM) and non-small cell lung cancer (NSCLC) are two highly lethal pleuro-pulmonary neoplastic diseases that have a lot in common. Both are rather therapy-resistant malignant tumours, often causing an effusion, both can to a large extent be prevented by the elimination of their main etiological agents, and both conditions have a poor prognosis where early diagnosis provides the best chance for a more favourable treatment response. Also, for both conditions, diagnosis (including subtyping for NSCLC and distinguishing from carcinomas for PM) is morphologically challenging, and a large proportion of patients are diagnosed at an advanced stage, where curative treatment options are no longer feasible. Great progress has been achieved in clinical management for these patients over the past few years, especially for lung cancer, including the introduction of immunotherapy and targeted therapies.

The diagnosis of both PM and NSCLC is traditionally based on histological material. Nevertheless, a significant proportion of patients are diagnosed on cytological specimens which are often the first diagnostic material available. Minimally invasive cytology specimens are an alternative that, like biopsies, require ancillary analyses. Ancillary testing including immunostaining and molecular diagnostics plays a crucial role in evaluating pleuro-pulmonary cytological specimens. These techniques aid not only in tumour subtyping but also in conducting treatment-predictive analyses.

While fixation and processing of tissue samples before immunohistochemistry (IHC) are standardised using formalin-fixed paraffin-embedded (FFPE) tissues, there are multiple fixatives and substantial differences in the handling of cytological specimens for immunocytochemistry (ICC), both in comparison to biopsies and between cytology departments. Cell block cytology preparations are widely used for biomarker analyses thanks to their suitability for immunostaining and molecular testing.

Among biomarkers evaluated in this thesis, programmed death-ligand 1 (PD-L1) is used for treatment prediction in NSCLC. High tumour PD-L1 expression is

linked to a better response to checkpoint inhibitors (ICI) targeting the programmed cell death 1 (PD-1) and its ligand PD-L1 and improved outcomes in NSCLC. Also, part of PM patients shows better response to treatment with ICI, though PD-L1 testing is not used in the clinical setting. The assessment of PD-L1 expression, detected by IHC, to select patients for therapy in NSCLC has mainly been performed on FFPE histological tissue samples. While cytology may be the only available material in the routine clinical setting, testing in clinical trials has mainly been based on biopsies.

The objective of the five original studies included in this thesis was the cyto-histopathological correlation of various diagnostic and predictive biomarkers, mainly PD-L1, in histological and cytological specimens from PM and NSCLC patients. Further, the association between PD-L1 and clinicopathological and molecular alterations in specimens from NSCLC was investigated. Moreover, the impact of different fixatives on the immunohistochemical expression of diagnostic biomarkers was explored.

In Paper I, PD-L1 expression was evaluated immunohistochemically in 61 paired FFPE pleural biopsies and pleural effusion cell blocks from patients with PM. The overall percentage agreement (OPA) between histology and cytology was 69% and 84%, with kappa values of 0.36 and 0.08 at the ≥1% and >50% cutoffs, respectively. The cyto-histological correlation tended to be higher for epithelioid mesothelioma compared to non-epithelioid mesothelioma at a cutoff of ≥1%. Also, at the ≥1% cutoff, PD-L1 positivity was associated with epithelioid subtype in biopsies but not in effusions.

In Paper II, eight diagnostic biomarkers were evaluated immunohistochemically in 59 paired FFPE pleural biopsies and pleural effusion cell blocks from patients with PM. The cyto-histological OPA for the epithelioid component was 93% for calretinin, 98% for CK5, 97% for podoplanin, 90% for WT1, 86% for EMA, 100% for desmin, 91% for BAP1, and 72% for MTAP. The concordance for the sarcomatoid component exhibited in 11 biopsies compared to cytology was low for calretinin, CK5, and WT1 (all ≤ 45%). Simultaneous loss or simultaneous preservation of both BAP1 and MTAP was observed in 40% and 11%, respectively, of the biopsies for epithelioid histology, whereas the corresponding figures were 54% and 8%, respectively, for the paired cell blocks.

In Paper III, PD-L1 expression was evaluated immunohistochemically in two retrospective cohorts of paired biopsies and cytological specimens from NSCLC patients. Using a 3-tier scale, PD-L1 showed concordance in 40/47 (85%) and 66/97 (68%) of the paired NSCLC cases in the two cohorts, with kappa values of 0.77 and 0.49, respectively. In both cohorts, the concordance was lower in paired samples from different anatomic sites. In one cohort, the cytological specimens had a lower PD-L1 score in all discordant cases. In a review of 25 published studies comprising approximately 1,700 paired cases, the median (range) cyto-histological concordance was 81-85% (62-100%) for a positive PD-L1 staining at the ≥1% cutoff and 89% (67-100%) at the ≥50% cutoff.

In Paper IV, the impact of various clinicopathological (sample type, sample site, and histological type) and molecular factors (status of oncogenic drivers) on PD-L1 expression was explored in two cohorts of 1131 and 651 unpaired specimens, respectively. In both cohorts, PD-L1 tested positive in 55% of cases at cutoff value of ≥1%. Adenocarcinomas showed lower PD-L1 expression compared to squamous cell carcinoma ($p < 0.0001$), with no discernible differences observed between sample types, tumour locations, or between the two cohorts (all $p \geq 0.28$). The mutational status showed a significant correlation with PD-L1 expression ($p < 0.0001$), with *KRAS*-mutated cases exhibiting the highest expression, *EGFR*-mutated cases showing the lowest expression, and *KRAS*/*EGFR* wild-type cases falling in between. There were no differences in PD-L1 levels between different prevalent *KRAS* mutations (all $p \geq 0.44$), while mucinous *KRAS*-mutated adenocarcinomas demonstrated lower expression compared to non-mucinous ($p < 0.0001$).

In Paper V, the effect of different common fixatives on the immunoreactivity of pleural effusion cell blocks from 24 malignant pleural effusions from different patients with metastatic lung adenocarcinomas was investigated. From each case, four identical cell blocks were fixed in 10% neutral buffered formalin, PreservCyt®, CytoLyt®, and CytoRich™ Red (only 17 of the cases), respectively. All cell blocks were stained with TTF-1 (clones 8G7G3/1 and SPT24), napsin A, claudin 4, CEA, CK7, and EpCAM (clones BS14, Ber-Ep4, and MOC-31). The fraction and intensity of stained cells were evaluated. Among the investigated biomarkers, significant differences in staining proportions were observed for TTF-1 clone 8G7G3/1 and EpCAM clone MOC-31, particularly with cases showing negativity in CytoLyt® (33.3% and 83.3% positive, respectively) and PreservCyt® (62.5% and 83.3%), while exhibiting positivity in CytoRich™ Red (76.5% and 94.1% positive, respectively) and formalin (both 95.8%). We observed a significantly weaker intensity of staining for all alcohol-based fixatives compared to formalin for TTF-1 clone 8G7G3/1, napsin A, and EpCAM clone MOC-31. Additionally, EpCAM clone Ber-Ep4 exhibited significantly weaker staining intensity only in PreservCyt® compared to formalin.

In summary, a moderate concordance of PD-L1 expression was seen between pleural biopsies and pleural effusion cell blocks from PM patients, particularly noticeable for the epithelioid subtype (Paper I). A good agreement was seen for other IHC stainings, but the somewhat lower concordance found for WT1, EMA and especially for MTAP (Paper II). The lower concordance observed for the sarcomatoid subtype for some biomarkers may suggest biological differences between the two histological subtypes (Paper II). A rather good overall agreement of PD-L1 expression between biopsies and cytology from NSCLC patients, yet there is notable variability between laboratories, highlighting the need for local quality assurance measures (Paper III). The histological and cytological specimens are comparable for PD-L1 evaluation (Paper IV). Given the influence of *KRAS* mutations and the mucinous growth pattern on PD-L1 expression, these factors

warrant further investigation in studies focusing on ICI response (Paper IV). The immunocytochemical expression and concordance with formalin-fixed cell blocks differ depending on the fixative used as well as the antibody and clone employed. This underscores the need to investigate the reliability of each biomarker for non-formalin-fixed cytology (Paper V). Immunostaining on cytology is common practice and has become indispensable for diagnostic and predictive biomarker testing. Furthermore, diagnostic, and predictive immunochemistry on cell blocks is applicable with histology standardised FFPE protocols, but assessment should consider differences between histology and cytology i.e., biological, and methodological aspects including pre-analytical, analytical, and post-analytical factors that may have an impact on the results.

Introduction

Integrity is doing the right thing,
even when no one is watching.

C.S. Lewis, 1898-1963

Historical perspective

The diagnosis of pleuro-pulmonary tumours by cytological methods has a long history. Besides the development of fine-needle aspiration (FNA) cytology, morphological examination of exfoliated cells showed the possibility to diagnose tumours already in the 19th century. Paul Ehrlich introduced the use of air-dried films, published in 1882, [1] which enabled the identification of malignant cells in various serous effusion. In his paper from 1882, he described the features of ovary and breast adenocarcinoma cells in pleural effusions using stained air-dried films, unfortunately without providing any illustrations. The same year Spencer Wells published a textbook containing drawings of adenocarcinoma cells in malignant effusion of gynaecological tumours. [2] During the 1920's MacCarty published a series of studies focusing on malignant cells in exfoliative cytology. [3-6] Cytological material was also used for cellblocks from effusions by Wihman although not primarily in tumour diagnosis. [7, 8] Cytology of effusions was broadly described in a monograph by Papanicaloau in 1954 and in early textbooks on cytology, such as Koss from 1961. More early reports on serous fluids are reviewed by Spriggs and Boddington (1968). [9]

When it comes to airway cytology, sputum samples were historically considered the first diagnostic procedure in patients with suspected lung cancer. A review by Frable and Johnston demonstrated that the exfoliated respiratory cells occurred in sputum already in 1845. [10] Spriggs[11] reviewed that the first description of malignant cells in sputum can be found in A Practical Treatise on the Diseases of the Lungs written by Walshe in 1860, while the first illustration referred to Beale (1861). More early reports are reviewed by Grunze (1960), [12] and Diamantis et al. (2013). [13] In 1886, Mackenzie wrote a whole book on the microscopy of sputum without mentioning lung cancer, maybe because lung cancer was quite uncommon in those days. The first systematic examination of lung cancer in sputum was reported by Hampeln in 1887. He also reported a series in1919 including 25 patients, 13 of

which were diagnosed with lung cancer. In 1934, Dudgeon and colleagues also demonstrated the possibility of identifying malignant cells in sputum using wet-fixed smears. In 1944, in Copenhagen, Wandall published a series of 100 lung cancer cases, 84 of which were correctly diagnosed with histological sub-type, using wet-fixed smears from sputum. [14]

The rigid bronchoscope was introduced by Jackson in 1907, [15] twenty years before Papanicolaou's description of the routine cytological procedure that is still used today. [16] However, the general scepticism regarding cytology prevailed for many years, but gained wider acceptance after the first published monograph on the proving of reliability of Pap-smear by Papanicolaou and Traut in 1943. [17, 18] In the mid-40s bronchoscopy was used for the diagnosis of lung cancer by cytological examination of bronchial secretions. [19]

The use of centrifuged cells was the standard method in Europe and the United States in the early decades of the 20th century, until the introduction of Papanicolaou's wet-fixed smears. As reviewed by Spriggs, [11] the drawings of the initial version of cell blocks using a very concentrated cell deposit were performed by both Zadek (1933), and Merklen *et al*. (1933), without giving proper instructions on how to succeed with this procedure. In 1948, Birge *et al*. performed the first official version of cell block preparation on centrifuged cell pellets from exfoliated neoplastic cells in body fluids, clotted by plasma and thrombin and embedded in paraffin. [20]

By the late-40s and early-50s, the development in respiratory cytology had taken place in parallel with advances in gynaecological cytology which were being pioneered at leading medical centres. [21-23] The first *Atlas of Exfoliative Cytology* including drawings illustrating pleuro-pulmonary neoplastic cells (but without correlation to histology) was performed by Papanicolaou in 1954. [24]

Among many joint efforts during the fifties was the Papanicolaou course at Cornell University, where prominent surgical pathologists began to use the cytological method in successively large numbers. Although the focus was on gynaecological specimens in the beginning, a wider range of cytology specimens was added to the anatomic pathology curriculum by the American Board of Pathology in the mid-sixties.

The first cytopathology textbook covering both gynaecological and non-gynaecological cytopathology based on cytology-histology correlation was written by Leopold Koss in 1961. [25] Koss included side-by-side illustrations of cytology preparations and histological sections. In this book, Koss also demonstrated that cells do not drastically change their appearance except for the subtle differences related to alcohol rather than formalin fixation.

As reviewed by Spriggs, [11] Lebert (1851) is considered the initiator of the idea of tumour puncture, but not as a diagnostic method. However, the first transthoracic FNA was introduced in Sweden by Nordenström in 1965, [26] but it was not until the mid-seventies that the method reached the United States. [27] The transbronchial FNA

procedure for the sampling of lung and mediastinal lesions was not described until 1981 by Wang *et al*. [28]

Sputum as diagnostic material was complemented and successively replaced by bronchial washings and bronchial brushings after the development of the fiber optic bronchoscope in 1968. [29] The interpretation of the specimens obtained with these new types of bronchoscopic washings and brushings could not be easily adapted. [30] Therefore, the progress of pleuro-pulmonary cytopathology followed in the wake of improvements and important advances in imaging modalities, sampling techniques, preparation methods of cytological specimens, microscopic evaluation, and ancillary techniques.

By the early seventies and during the eighties, pleuro-pulmonary cytopathology enjoyed a period of rapid development, particularly FNA which was validated as an alternative to biopsy. In 1974, Saccomanno *et al*. demonstrated that the cytological changes that occur during the development of lung cancer could be reflected in exfoliated cells. [31] Saccomanno's work laid the ground for future studies on the histogenesis of lung cancer in tobacco smokers, [32] and asbestos-exposed individuals. [33]

A report published in 1979 by two iconic pathologists, Juan Rosai and Lauren Ackerman, demonstrating the value of diagnostic cytology, gave it the "seal of approval" from the pathology community, and cytopathology was recognized as a subspecialty of pathology in its own right. [34] Further on, the use of cytology for the detection of lung cancer became a universally applied diagnostic method and one of the most reliable.

In 1989 a qualification in cytopathology was created for the first time in the United States. The same concept of examination was created in the United Kingdom and Australia. In Canada, only in 2012, cytopathology was recognised as an area of focused competency. [35]

Anatomic pathology

Pathology is broadly defined as the scientific study of the causes and effects of diseases. It encompasses the exploration of both the aetiology (cause) and the pathogenesis (mechanisms) of diseases, along with the examination of structural changes and functional manifestations induced by the diseases.

From a broader perspective, pathology refers to the comprehensive study of disease, while anatomic pathology represents a medical laboratory discipline focused on diagnosing diseases through the macroscopic and microscopic examination of histological or cytological specimens. Thanks to recent advancements in molecular biology and the introduction of new techniques, anatomic pathology has progressed in recent decades, now incorporating immunological, molecular, and cytogenetic investigations. [36-41]

The primary application of anatomic pathology is in the diagnosis of cancer, although it is also instrumental in diagnosing a range of non-neoplastic diseases. In recent years, anatomic pathology has evolved beyond being solely a diagnostic speciality to encompass procedures that provide insights into prognosis and the prediction of tumour responses to tailored treatment regimens. [42-47] Thus, anatomic pathology continues to maintain a central role as a branch of medical science that serves as the foundation for all facets of medical care.

Anatomic pathology encompasses numerous subspecialties and subdivisions, including forensic pathology, surgical pathology, neuropathology, hematopathology, histopathology, and cytopathology. The nomenclature of branches and subspecialities in anatomical pathology is based on the origin of samples, the type of specimens used, and the technique employed.

This thesis centres around two primary subdivisions within anatomic pathology: histopathology and cytopathology.

Histopathology

Histopathology, known also as histology, is the study of surgical tissue specimens, including biopsies, resections, whole organs, or the entire body (autopsy). Large specimen is macroscopically assessed by the histopathologist for size, shape, colour, and the presence of abnormalities. The histopathologist chooses noteworthy and pertinent lesions or parts of lesions, which are subsequently fixed, typically in formalin (10% paraformaldehyde). Following fixation, the specimen is embedded in paraffin, cut into exceedingly thin sections, and then placed and mounted onto glass microscope slides prior to staining. Small specimens are processed in toto.

Histopathology is regarded as the gold standard for morphological evaluation, but thanks to modern ancillary techniques, cytopathology alone can be adequate to reach a final diagnosis.

Cytopathology

Cytopathology, known also as cytology, is the study of cells, cell clusters, and tissue micro-fragments. The field of cytopathology is presently well-standardized, and cytological materials are categorized into two major branches, encompassing two main types of specimens based on the cytological method employed: aspiration cytology and exfoliative cytology. Irrespective of acquisition technique, specimens undergo processing before microscopic examination and are subjected to a fixation process using various fixation methods.

Various names are employed to characterize aspiration cytology, with the most notable ones being fine needle aspiration biopsy (FNAB), fine needle aspiration cytology (FNAC), and puncture cytology. All these terms denote the same procedure, which represents a sub-specialty of cytopathology that examines

specimens with aspirated cellular material from organs or lesions, employing a fine needle to establish a diagnosis. This technique has been employed for lesions throughout the body, encompassing two primary areas: palpable lesions and non-palpable lesions. Palpable lesions can be precisely targeted by a clinician or a cytopathologist, whereas non-palpable lesions are typically addressed with the assistance of image analysis techniques such as CT scan-guided, ultrasound-guided, and more recently, endoscopic ultrasound-guided (EUS) and endobronchial ultrasound-guided (EBUS) bronchoscopy with fine needle aspiration.

Exfoliative cytology is a sub-specialty of cytopathology that examines specimens including cells that have exfoliated from superficial or deep serosal or mucosal surfaces. The cells have exfoliated spontaneously or have been mechanically removed, through methods such as swabbing, brushing, or scraping from a surface, or via the instillation of fluid (washing) in hollow organs.

The subdivisions of exfoliate cytology are designated based on the type of specimens. For instance, gynaecological samples i.e., Papanicolaou (Pap) cervical smears, respiratory cytology which includes sputum, bronchial brushing, bronchial washing (bronchoalveolar lavage [BAL]), urinary cytology i.e., voided urine, bladder brushing and washing, and body fluid cytology, also known as serous effusion cytology which includes fluid accumulations in body cavities such as pleural, peritoneal, and pericardial cavities. The respiratory and effusion cytology are commonly used mainly to detect malignancies as well as infections. In this thesis, exfoliative cells found in respiratory and pleural effusions are examined.

Differences between histology and cytology

Histopathology and cytopathology diverge in both biological and methodological aspects.

Histological material comprises tissue sections that enable the assessment of the lesion's architecture, encompassing its appearance, shape, and the arrangement of specific cell types in relation to other cells and stroma within the lesion, as well as in the surrounding tissue.

Cytological specimens allow for the evaluation of only the morphological features of cells, cell clusters, and cell composition.

Biological properties vary between histological and cytological specimens. Cytological specimens frequently include a notable quantity of inflammatory cells, including macrophages, which may be present in smaller numbers in histological specimens. Distinguishing inflammatory cells, especially macrophages, from malignant cells poses a significant diagnostic challenge in cytological material.

Cells in histological materials are situated within the tissue and maintain the same microenvironment, whereas cells in certain cytologies exist in a different environment, which may influence morphological features as well as the expression of various biomarkers. In addition, cytologies, especially effusions, usually contain a diverse range of inflammatory cells, and the yield of malignant cells may be scanty.

Cytology-histology correlation

The cytology-histology correlation (CHC), known also as cyto-histopathological correlation, involves the comparison of cytology interpretations with the histology interpretation of specimens obtained from the same site. CHC serves as a quality indictor for pathology laboratories, functioning as a method for identifying medical errors in accuracy within the diagnostic field [48] and is thereby a vital component in measuring quality within a quality programme. [49] It assesses the disparities between cytology and histology diagnoses in both original and review assessment, as well as discrepancies between different observers. Comparisons between histological biopsies and resections are equally interesting and important, but not covered in the present thesis.

CHC was initially designed to assess cytology performance, but it has equally been proven a valuable resource for evaluating biopsy practices, laboratory processes, [50, 51] as well as studying the type of errors and for quality improvement. [52]

CHC identifies the errors that can be separated by anatomic site and classified as either sampling or interpretive errors. [51, 52] Thus, the CHC may be affected by several pre-examination, examination, and post-examination phases. The pre-examination includes the quality of both cytology and histology specimens related to pre-analytical factors such as transport, fixation methods, and sampling techniques. The examination phase includes the analytical factors such as differences and similarities in processing and preparation technique of specimens. The post-examination is often related to observer reports based on pairs of specimens. The discordant pairs are considered as errors. [48, 53] Errors in diagnosis may lead to incorrect management strategies and can cause delays in reaching a specific diagnosis. [54]

The field of pleuro-pulmonary cytopathology is rapidly evolving, with a growing proportion of personalized medicine-related technique applications performed on cytological specimens. Therefore, the CHC studies play a crucial role in influencing patient management and enhancing the overall quality of treatment.

Until date CHC has been performed mainly in gynaecological cytopathology practice, but a recent survey showed that the CHC practices for non-gynaecological cytopathology have developed in a similar way. [55]

Many studies focused on the understanding of the root cause and addressing the factors that may affect the CHC rates. Some common reasons for the discordance rates may be related to the type of CHC i.e., retrospective vs. prospective, timing and interval of CHC, inadequate history, [51] sampling technique, as well as sampling body site, type of tumour, and type of correlated specimens. [51, 56]

A recent study showed that the discordant rates for CHC of the lung specimens were often due to sampling errors. [52] In addition, the studies indicate that the CHC rates may differ depending on the type of specimens and anatomic sites. [52, 56]

However, there are several aspects that need to be considered prior to the performance of CHC. Adopting binary approaches to interpretation yields the highest concordance rates in pathology, but the differences between histology and cytology may affect the criteria of assessment. The definition of possible influences and parameters of CHC, and determination of how many degrees of variance allowed before a pair correlates, may facilitate the understanding of the obtained CHC rates.

The discordance rates of the pairs may also be due to intra- and interobserver variability, as well as the number of observers. [51] The intra-observer variability means that the assessment performed by the same observer is inconsistent, while inter-observer variability means that the assessment performed by two or more different observers were dissimilar.

The timing and interval of CHC may affect the diagnosis based on cytology specimens. Performing comprehensive CHC at the time of biopsy interpretation, in real time or through concurrent correlation, is optimal for influencing patient clinical management. However, logistical challenges may render this approach impractical, potentially resulting in a failure to capture CHC data for ongoing monitoring purposes. Furthermore, there exists the potential for confirmation bias, wherein an observer interpreting a biopsy as negative might be more inclined to downgrade a cytology preparation with high grade neoplastic cells if only a small number of cells are present, even in the presence of clear diagnostic features. Immediate correlation in real-time offers prompt feedback to the interpreting observer of the biopsy, enabling the healthcare provider to address any discrepancies by either obtaining additional biopsies or preventing unnecessary procedures. In addition, the interval may have a high impact on the concordance rates of CHC, perhaps because the lesion has progressed or regressed as result from an oncologic therapy which also may affect the expression of certain biomarkers. Therefore, the real CHC is based on histological and cytological specimens both obtained before the patient received any oncological treatment as well as obtained at the same time or within reasonable interval that did not affect the set of tumour cells by tumour progression. The interval may vary depending on the type and grade of the tumour.

Advantages and limitations

A revolution in the pathology field has occurred in the last decades, including wide application of immunohistochemistry and molecular analysis. Although tumour tissue is historically the gold standard for cancer diagnostics as well as ancillary analysis, there are considerable innate limitations such as representative, inadequacy, and tumour heterogeneity.

Representativity is one of the basic concepts in both histology and cytology. A representative specimen originates from the lesion to be examined, whereas a non-representative specimen comes from other tissue within or surrounding the lesion. This may be a problem both in histology and cytology, however, often less so in

histology, where the examiner always has a whole-tissue specimen to evaluate. Thus, cytology material, sometimes is non-diagnostic or acellular or even is suboptimal due to multiple factors such as air-drying artifacts, abundant presence of inflammatory cells, or the material contains too many blood elements, mainly erythrocytes, which obscure diagnostic cellular details. However, the general characteristics of malignant cytological specimens are that they are high cellular, e.g., effusions in the body cavities which represent the whole cavity.

Tumour heterogeneity describes the variations among tumour cells both within individual tumours and across different tumours. This concept encompasses differences in morphological and phenotypic profiles, including cellular morphology, gene expression, metabolism, proliferation, and metastatic potential among cancer cells, as well as genotypic differences. [57, 58]

The tumour heterogeneity can be classified into two main types: intertumour and intratumour heterogeneity. Intertumour heterogeneity describes the tumour-by-tumour differences between different patients, where the altered genotype and phenotype are induced by diverse etiological and environmental factors. On the other hand, intratumour heterogeneity refers to differences between cellular populations in a distinct tumour "tumour subpopulations" and describes the cellular diversity that harbouring genomic and biological variations within the same tumour. The intratumour heterogeneity can also refer to differences between distinct tumours within an individual patient, e.g., between primary and metastatic tumours, or between multiple metastatic sites, which is called intersite heterogeneity. The intratumour heterogeneity is gained by tumour cell evolution under diverse microenvironments linked to various aetiologies. [58] A schematic illustration of tumour heterogeneity is showed in Figure 1.

The heterogeneity among tumour cells can be further increased by variations in the tumour microenvironment. The observed intratumour heterogeneity between primary and metastatic tumours within individuals, as well as intertumour heterogeneity observed between different patients with the same tumour type, may probably be, at least partly, explained by the impact of surrounding microenvironment on clonal dominance. [58, 59]

Tumoral heterogeneity also plays a significant role in contributing to varying patient responses to treatment, treatment resistance and failure, and is the basis for precision medicine approaches. [58-60]

Heterogeneity in a tumour can be a major concern for histological cancer diagnosis, especially if the diagnosis has to be based on small specimens, such as biopsies. [57] Cytological techniques often allow sampling from larger parts of the lesion; thus, the cytological specimen may better reflect heterogeneity. This plays an important role, especially in the clinical diagnosis of effusions in the pleural cavities and bronchial washings as these represent the whole cavity and surfaces in hollow of the lung, but it is perhaps less important for bronchial brush specimens taken from a specific location. It is also important to bear in mind that tissue-

biopsies, as well as FNA, usually target the primary tumour, while exfoliative cytology in most cases represents tumour spread, i.e., metastasis.

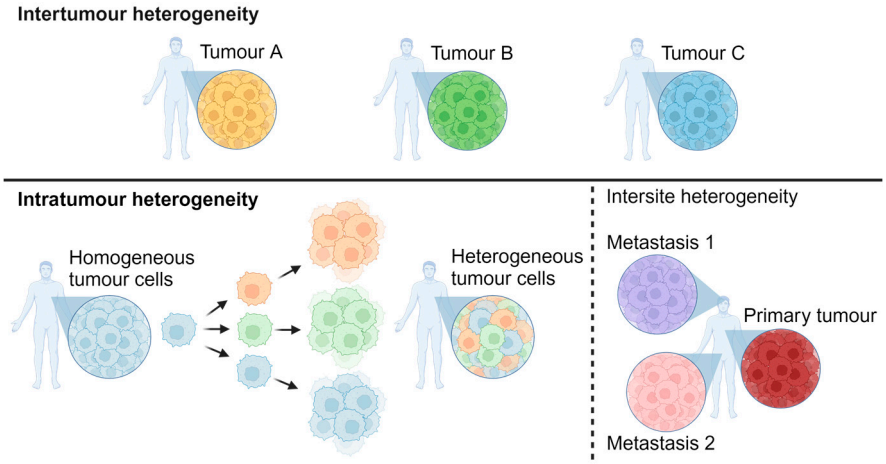

Figure 1. Schematic illustration of the concept of tumour heterogeneity. Intertumour heterogeneity refers to genotypical and phenotypical differences for the same tumour between different patients, while intratumour heterogeneity refers to the differences between tumour cells in a distinct tumour, or between primary and metastatic tumour cells of the same tumour within an individual. Created with BioRender.com

In summary, histology and cytology are two different methods with essential advantages and disadvantages. Cytological material is often the first material received in the laboratory and, thus, cytology is the first diagnostic method used in the diagnosis of PM and NSCLC. Further, cytological material, especially in cases of malignancy, is often abundant and offers enough material for various ancillary techniques. [61] Thus, cytology plays a crucial role in the diagnosis, and work up of malignancy but many novel ancillary applications have mainly been used on histological material. Therefore, histology and cytology may be complementary diagnostic tools, and both have their advantages and limitations.

Advantages of histology

Histology enables the evaluation the architecture of the lesion i.e., the appearance and the position of specific cell types in relation to others within the lesion and in the surrounding tissue. Further, tumour staging, and invasion of tumours can only be assessed on histological specimens (resections), a capability not achievable with cytology. Moreover, prognostic features of many neoplasms are usually evaluated on histological specimens. Additionally, the histological methods are standardized and often quality assured.

Disadvantages of histology

Histology involves higher costs and is more time-consuming than cytology due to its intricate preparation procedures. The sampling procedure is invasive, sometimes requiring anaesthesia, and more traumatic for the patient. This procedure may entail potential complications the sampling in terms of uncontrolled bleeding, local infections, and eventual scars. Some types of histological biopsies such as bronchial biopsies have a higher rate of suboptimal diagnostic yield.

Advantages of cytology

Cytology offers a rapid, cost-effective, safe, and convenient alternative for the patients. Cytology implicates less waiting time, which enables an early diagnosis and in turn early treatment. Sampling of cytological material is essentially non-traumatic, major complications are exceedingly rare, and it almost never leaves scars. Even though histology is frequently deemed the gold standard, cytology can provide a more comprehensive morphological overview of a tumour characteristics compared to a small biopsy. Also, cytology today is used as a screening tool for different programme, such as cervix screening and complement to mammography in breast cancer screening. Cytology examinations are also standard procedure as a follow-up, after establishing the initial diagnosis, which is very common in patients with pleuro-pulmonary malignancies.

Disadvantages of cytology

Cytology sometimes yields a scanty amount of tumour cells, posing challenges in achieving a conclusive diagnosis and securing sufficient material for ancillary techniques. The limited amount of material may arise from either inadequate sampling or inherent properties of the lesion, such as a dense stroma. Further, there may be sampling errors that affect the accuracy and reliability of the sample. Moreover, neither the assessment of the tissue architecture or the evaluation of tumour invasion can be performed by cytology. In addition, the mixture of malignant cells with diverse types and quantities of inflammatory cells, stroma cells and normal cells from surrounding tissue can pose a challenge in selecting the appropriate complementary assays.

Background

The tyrant dies and his rule is over,
the martyr dies and his rule begins.

Søren Kierkegaard, 1813-1855

Cancer is a Latin word that has its roots in the ancient Greek "karkinos" which signifies "crab". The term, commonly attributed to Hippocrates, describes the hardness, the symptomatic pain, and the pattern of swollen blood vessels, resembling a crab crawling in the sand.

Cancer is not a novel phenomenon, and it has existed for a long time in humans, animals, [62, 63] and even plants [64, 65] throughout history, and endeavours to treat human cancer were already undertaken in our ancient past. The earliest known portrayals of cancer in humans are found in ancient Egypt, covering the period between 3200 and 500 BC, as evidenced by findings from mummies and skeletons, [66-68] along with descriptions of various tumour types in the Edwin Smith Papyrus – an ancient medical text. [69]

Cancer is today one of the most lethal forms of disease and is a leading worldwide cause of death. It is a complicated disease that can affect any part of the body with rapid, abnormal, and uncontrolled division of cells, which have also the potential to invade or spread to different organs in the body. The transformation of normal cells into cancer cells is called tumorigenesis, which is a gradual process believed to result from the accumulation of gene mutations. Thus, cancer is a polygenic disorder, rather than being caused by a single mutation. However, cancer is caused by many different aetiological risk factors, including inherited genetic alterations in 5-12% as well as various environmental risk factors. [70]

Cancers constitute a large and heterogeneous group of malignant tumours, although different cancer types at the cellular level share many common features in the process of developing malignant tumours. [71] Therefore, epidemiological rates of cancers, including incidence and mortality, vary by age, gender, and population, as well as the type of tumour. [72] Given the expanding and ageing population, the incidence of cancer is anticipated to rise by 47% from 2020 to 2040, [73] presenting a growing global health concern. Furthermore, the latest data on the global cancer burden is derived from the GLOBOCAN, with estimates of cancer incidence and mortality in 2020, encompassing 185 countries and 36 types of cancer, compiled by

the International Agency for Research on Cancer. Worldwide, the data estimate that there were 19.3 million new cancer cases and nearly 10 million cancer deaths. [73]

In Sweden, according to the statistics from the National Cancer Registry (NCR) and the National Cause of Death Registry, by the National Board of Health and Welfare, for the years 1970 - 2022, the incidence rates of cancer have increased over time, while the mortality rates have decreased. However, the incidence and mortality rates are higher for men than for women. [74]

The formation of different body tissues from undifferentiated cells is called histogenesis. These undifferentiated cells are constituents of the three primary embryonic germ layers: the endoderm, mesoderm, and ectoderm. Cancer is categorized into three main types, depending on the location where it originates, which is according to these three embryonic germ layers.

Malignancies that derive from the mesoderm are called mesodermal or mesenchymal tumours (sarcomas), whereas the malignancies from ectoderm and endoderm are called epidermal, epithelial tumours or carcinomas, which are characterized by their ability to form solid tumours, emerge as the most prevalent form of malignant neoplasm.

Mesothelioma is a malignant tumour that derived from the embryonic mesoderm, while lung cancer arises from the anterior foregut endoderm. [75] Mesothelioma and lung cancer are two malignant tumours that have a lot in common. Both can often be prevented by the elimination of their main etiological agents, and both conditions have a poor prognosis and early diagnosis provides the best chance for a more favourable treatment response. In this thesis, the focus is on variants of these two types of malignancy, i.e., mesothelioma and lung cancer.

Mesothelioma

The mesothelium is a monolayered epithelium-like serosal layer that originates from the lateral plate mesoderm. [75-77] Bichat initially described it almost 200 years ago, but the term was coined later by Minot after his microscopic study of organs. [76, 78]

Mesothelium lines all coelomic cavities i.e., the body's serous cavities and internal organs, which provides protection for the encapsulated organs. [79] Mesothelium envelops the lung, heart, gut, and tunica vaginalis, [78, 80, 81] and is composed of specialised cells known as mesothelial cells. [76, 78] The mesothelium enveloping the lungs, known as the pleura, consists of pleural mesothelial cells. [76]

The serous or serosal membrane including pleura are flat and thin tissue membrane comprising two single-cell layers of mesothelium, both with a layer of loose connective tissue underneath. [82-84] The layer that blankets the internal organ such as the lung is known as the visceral pleura, while the layer that constitutes the inside wall of the cavity and that attached to the chest wall is referred to as the parietal pleura. [85] Between the visceral and parietal layers there is a thin serous space

called the serous body cavity. The pleural space between these layers is called the pleural cavity. The serous cavity normally contains a small amount of fluid, secreted by the two serous membranes, comprising enzymes, immune cells, and blood components. A schematic illustration of the pleura including mesothelial layers and pleural cavity is shown in Figure 2.

The two primary fundamental functions of mesothelial layers in all body cavities that they serve are to secrete lubricants, forming a slippery, non-adhesive, and protective surface that facilitates the movement of underlaying organs and provides a frictionless surface between the layers, and to provide physical and mechanical protection to the underlaying coelomic organ. [78, 86, 87] Nevertheless, mesothelial cells undertake other crucial roles, including the transport of fluid and cells across serosal cavities, antigen presentation, involvement in inflammation and tissue repair, participation in coagulation and fibrinolysis, and facilitation of tumour cell adhesion. [78]

Various pathological conditions increase the production of serous fluid, and an imbalance of fluid formation and removal leads to the development of fluid called an effusion. Effusions in the serous cavities are divided into two main types of fluids i.e., transudates and exudates. Physiologically impaired function such as heart failure, kidney failure, and cirrhosis of the liver that cause changes in hydrostatic or osmotic forces, may lead formation of a transudate. [88] An inflammatory process and tumour cause an increase of the capillary permeability, leading to the formation of exudates. [88] The transudate contains few cells, and the protein content is low, while the exudates are usually cellular, containing inflammatory cells and mesothelial cells shed into the fluid, with a high protein content. Exudates caused by malignant tumours often contain malignant cells. The studies included in this thesis only deal with exudates but according to accepted English nomenclature we consequently use the term effusion. An effusion occurring in the pleural cavity, “thoracic cavity“, is called a pleural effusion. Malignant effusions are a common complication of various types of malignancy, and fluid can be aspirated for both therapeutic and diagnostic purposes.

The malignancy arising from mesothelial cells is termed mesothelioma. [89] Mesothelioma can manifest in various body cavities, including the serosal cavities lining of the pleura, peritoneum, pericardium, and tunica vaginalis testis. [77, 90] Nevertheless, the pericardium and tunica vaginalis testis are rare sites of origin for mesotheliomas. The pleura serves as the predominant site of mesotheliomas, and the mesothelioma growing in the pleural cavity is called pleural mesothelioma (PM), constituting 60-85% of mesothelioma cases, with most of the remainder arising in the peritoneum. [90-94]

A malignant effusion could be the result of a primary serosal tumour in the body cavity, i.e. mesothelioma, or metastases from a tumour in nearby or distant organs. The most common tumours spreading to the serous cavities are adenocarcinomas (AC), a type of cancerous tumour that forms glandular (secretory) cells and can occur in several organs, e.g. lung, breast, pancreas, ovary, prostate, and colon. [95]

PM is one of the primary focuses of this thesis. PM is a rare thoracic malignancy that can develop in both pleural layers. However, it is thought to commonly start in the parietal pleura and subsequently spread to the visceral pleura. [85]

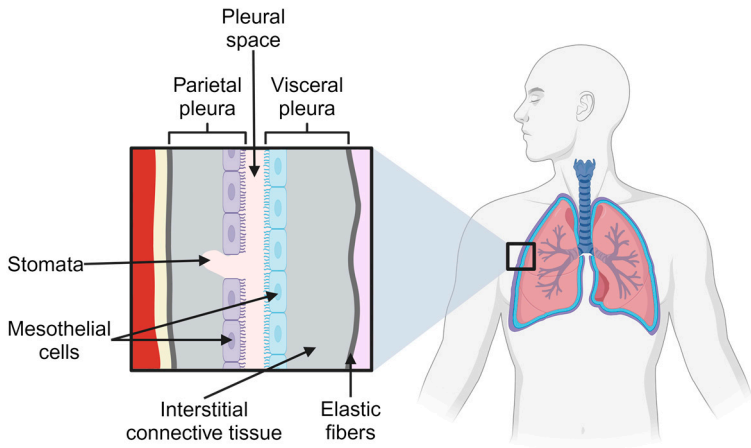

Figure 2. Schematic illustration of the pleura and mesothelial layers. Created with BioRender.com

Classification of mesothelioma

Mesothelial tumours are categorized into benign or preinvasive tumours and mesotheliomas. [96, 97] The benign or preinvasive category comprises adenomatoid tumour, well-differentiated papillary mesothelial tumours, and mesothelioma in situ. Malignant tumours are referred to as mesotheliomas and can be either localized or diffuse. [96, 97]

The localized malignant type refers to a circumscribed mesothelioma mass and is rare and not included in the studies of the thesis, while the diffuse mesotheliomas are diffusely involving the cavity. Both are categorised according to the histological appearance of the tumour and the histological classification exhibits a spectrum of morphologic differentiation.

According to World Health Organization (WHO), diffuse PMs are classified into three main histological subtypes, i.e., epithelioid constitutes 60-80% of cases, sarcomatoid accounts for 10-15%, and biphasic (which is also referred to as mixed of epithelioid and sarcomatoid components) make up 10-30% of the cases. [96-103] Further, each histologic type is classified into sub-variants and some rare unclassified variants constitute a fourth main group, [104] with different therapeutic and prognostic implications. A schematic illustration of the mesothelioma main histological subtypes is shown in Figure 3.

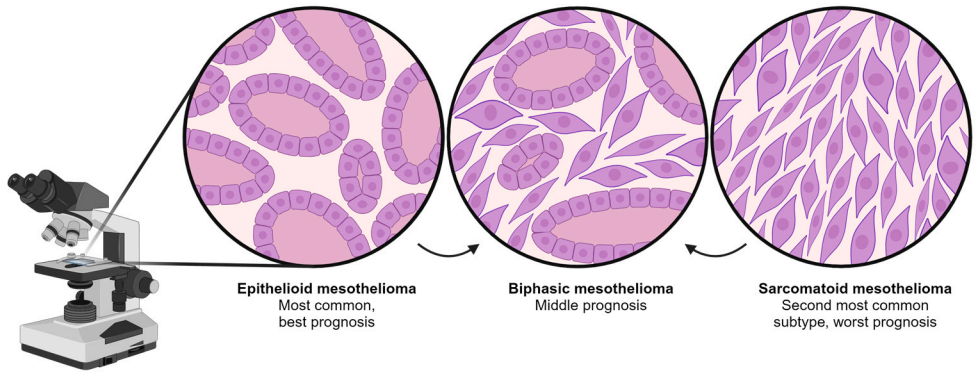

Figure 3. Schematic illustration of the main histological subtypes of mesothelioma. The epithelioid constitutes the majority of cases, sarcomatoid is the second subtype, and the biphasic is mixed of both epithelioid and sarcomatoid components. Created with BioRender.com

Mesothelioma in situ

The majority of malignancies exhibit a pre-invasive stage preceding the onset on invasive cancer, commonly referred to as carcinoma in situ, which means that there is no invasion or spread to adjacent tissue. [105] At this stage, cells harbour multiple genetic alterations, resulting in cellular hyperplasia that can be detected by histology. This stage of disease is clinically significant, as intervention can prevent progression to cancer in many cases. [106] Particularly in the context of PM, where effective therapies are lacking, early diagnosis of pre-malignant pleural lesions which subsequently can ablated or excised offers an important curative treatment option for patients.

For mesothelial tumours, mesothelioma in situ has initially introduced by Whitaker et al as a single layer of small papillary projections of atypical mesothelial cells on a pleural surface. [107, 108] Also, only small case reports have previously suggested the presence of mesothelioma in situ. [109]

Nevertheless, in many years, PM was not considered to have a pre-invasive stage, explained by the lack of conclusive supporting evidence. The challenge with the definition was that these changes were observed against a background of invasive mesothelioma. Consequently, distinguishing whether this represented a genuine non-invasive component or surface spread of an invasive mesothelioma proved difficult. Moreover, the consensus among experts was that mesothelioma in situ cannot be differentiated from a reactive process based on morphology alone.

Recently, a prominent study by Churg et al. definitively proved the existence of mesothelioma in situ, showing a complete loss of BAP1 nuclear immunostaining in surface mesothelial cells. In these cases, there was no evidence to have a diagnosis of pleural pathologies or malignancy, and most patients underwent a biopsy due to repeated effusions of unknown aetiology. Further, there was no local invasion of mesothelial cells, and interestingly a few cases showed cytological atypia or

hyperproliferation in a papillary pattern; in contrast, specimens formed a bland mesothelial cell monolayer on the pleural surface, which would not appear suspicious through the routine evaluation of histological specimens. [110]

However, this concept has been reintroduced as a clinicopathological entity and a recent significant change in the WHO classification is the incorporation of mesothelioma in situ as a distinct diagnostic category. [97, 111] The recent definition pertains to a flat or slightly papillary single layer surface mesothelial proliferation, and the diagnosis requires loss of BAP1 or MTAP by immunohistochemistry, or *CDKN2A* homozygous deletion by FISH. [110, 112] In addition, this occurs in patients with recurrent non-resolving pleural effusions in the context of significant asbestos exposure, with or without pleural plagues, post-radiation, and in patients with familial predisposition. [110, 113]

Epithelioid mesothelioma

Epithelioid mesothelioma constitutes roughly 60-80% of all pleural mesotheliomas and has the best prognosis i.e., the highest life expectancy and more potentially curable treatment options are available.

Epithelioid mesothelioma is morphologically characterised by its resemblance to carcinomas with typically rounded and polygonal epithelioid cells and nuclei, and a tendency to create glandular and papillary cell clusters, cell balls, and rosettes. [114, 115]

The classical histological growth pattern of epithelioid mesothelioma is characterized by bland, uniform cuboidal cells that infiltrate the pleura in a tubulo-papillary growth pattern. This pattern consists of round to oval structures mixed with tumour cells covering fibrovascular cores. Nevertheless, a diverse range of architectural growth patterns may be observed in epithelioid mesothelioma, including trabecular-linear arrangement of interconnected single or dual malignant epithelioid cells, usually one or two layers, micropapillary structures lacking a fibrovascular core, may also be admixed with single cells, solid of continuous sheets, microcystic-sieve-like arrangement of cribriform growth with confluent small acinar spaces, and less commonly, adenomatoid-gland-like structures lined by flat cells which resembles an adenomatoid tumour. [115, 116] Furthermore, tumours may exhibit multiple growth patterns.

The cytological features of epithelioid mesothelioma are defined as well and may also be variable. Apart from the classic bland cuboidal morphology, one may encounter cells with rhabdoid, deciduoid, small cell, clear cell, and signet ring morphologies. [103, 115] In a detailed description, the rhabdoid cell contains a cytoplasmic eosinophilic globule, deciduoid cells with voluminous cytoplasm and may have a range of nuclear features showing pleomorphic features, small cells with hyperchromatism resembling small cell carcinoma, clear cell with clear cytoplasm which may be confused with renal cell carcinoma, and signet ring with intracytoplasmic vacuoles displacing the nucleus to one side.

Nevertheless, case reports on histological variants continue to surface, and the rare types of mesotheliomas such as signet ring and decidual mesothelioma have been reported by Ordóñez *et al*. [117, 118]

Recognizing these growth patterns and cytological features may be important to prevent potential misdiagnosis. Moreover, some of these patterns have been identified as potentially holding prognostic significance. The micropapillary and solid growth patterns have been linked to an unfavourable prognostic significance, as well as rhabdoid cytology. [116, 119, 120]

Sarcomatoid mesothelioma

Sarcomatoid mesothelioma represents the second most common subtype, and has the worst prognosis i.e., shorter life expectancy and fewer treatment options. Sarcomatoid mesothelioma has histologic characteristics that resemble sarcoma type tumours, i.e. the cells may be multi-nucleated, cells and nuclei are elongated or irregular, and the cells grow diffusely or in bundles in stroma.

According to the WHO classification, sarcomatoid mesothelioma is characterized by a proliferation of spindle cells arranged in fascicles or haphazard patterns, invading the adipose tissue or lung parenchyma. [96, 103] Necrosis and atypical mitoses are commonly observed in sarcomatoid mesothelioma.

Sarcomatoid mesothelioma exhibits morphological heterogeneity, and the current WHO classification encompasses variants and cytological features. The identification of distinct sarcomatoid areas is highly beneficial in confirming the diagnosis, as this variant can be prone to be misdiagnosed as benign.

Transitional features exhibit an appearance between epithelioid and sarcomatoid morphology, displaying a sheet-like elongated but plump cell with well-defined cell borders. [121] Studies have demonstrated that transitional features have genomic characteristics similar to the sarcomatoid subtype. [122] Transitional and pleomorphic are two cytological features that were previously categorised as epithelioid patterns. [103] Nonetheless, recent reports indicate that these diagnoses should be reclassified. [122, 123]

Transitional features within biphasic mesothelioma bear negative prognostic significance, as cases with transitional features exhibit a median survival of 6 months compared to 12 months for those without transitional features. [123] Hence, it is crucial not to interpret transitional features as indicative of epithelioid mesothelioma, especially in biphasic tumours.

Biphasic mesothelioma

Biphasic mesotheliomas exhibit a dual composition of both epithelioid and sarcomatoid components and growth patterns. According to the 2021 WHO Classification of Tumours of the Pleura the diagnosis of biphasic mesothelioma can be diagnosed in small biopsies containing both epithelioid and sarcomatoid components, even if one component constitutes less than 10% of the specimen. [97] The clinical pathology report should incorporate the percentage of the sarcomatoid

component due to its significant implications for prognosis and therapeutic decision-making.

Microscopic images from different pleural mesothelioma cases demonstrating the epithelioid and sarcomatoid histological subtypes are shown in Figure 4. The need for a more detailed and clinically valid classification beyond the three current mesothelioma subtypes has been supported by recent genomic data. Further, the recognition of these primary subtypes has an impact on the prognosis and treatment of patients diagnosed with this lethal tumour. [124]

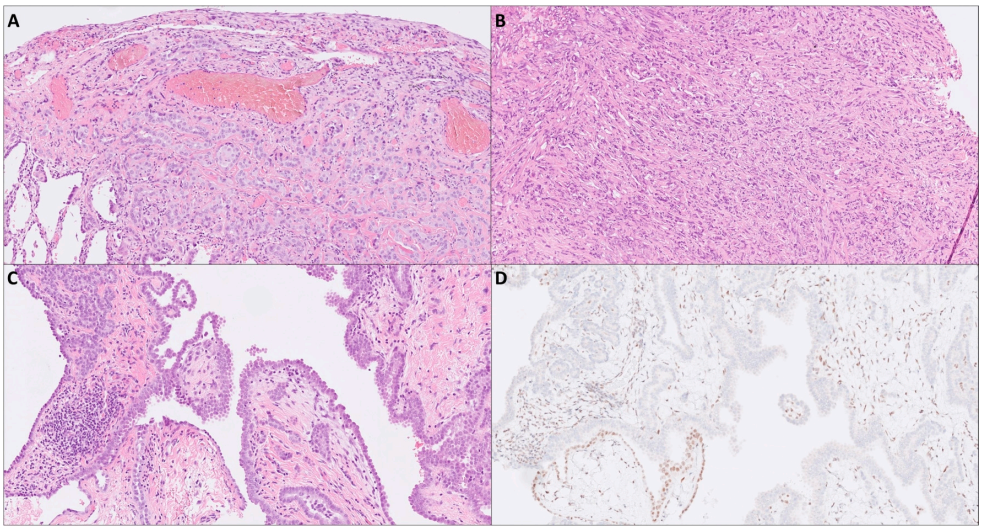

Figure 4. Pleural mesothelioma cases. (A) Pleura with epithelioid mesothelioma with invasion of the lung, H&E staining. (B) Pleura with sarcomatoid mesothelioma, H&E staining. (C) Pleura with epithelioid mesothelioma and benign mesothelial cells, H&E staining. (D) Same case as C stained with BAP1 immunostaining, the epithelioid mesothelioma exhibits loss of BAP1 IHC expression in neoplastic mesothelial cells, while preserved in benign mesothelial cells as well as in stromal and inflammatory cells. Original magnification x25 objective.

Aetiology of mesothelioma

Mesothelioma is primarily associated with the inhalation of asbestos which is a carcinogenic mineral. Thus, the main aetiological agent causing mesothelioma is long-term exposure to asbestos, and prior asbestos exposure is identified in 54-97% of patients, [77, 90, 93, 125-127] but there is no evidence that asbestos exposure is associated with the development of different histologic subtypes of mesothelioma. [128] Nevertheless, the association with asbestos exposure is particularly strong for the pleural site, with 80% of patients reporting a history of asbestos exposure. [94] Moreover, even brief past exposure to asbestos could potentially lead to the development of PM later in life. [129]

Mesothelioma typically exhibits a latency period of 30-40 years between asbestos exposure and the initial clinical manifestations, [130] but in certain instances, it might extend up to 60-70 years. [131-133] Mesotheliomas were linked to certain occupations globally, [125, 126, 134] and in Sweden. [129, 135] PM was classified as an occupational disease in the 1956 by Wagner et al who were the first to discover the link between asbestos and PM. [136]

Asbestos is a naturally occurring silicate mineral that is divided into two primary forms: the serpentine form, which includes chrysotile fibres, and the amphibole form, encompassing crocidolite and amosite. [133]

Various degrees of association were noted based on the type of fibre. The highest risk of developing mesothelioma is associated with exposure to asbestos variants belonging to the amphibole family, while the weakest association was observed when serpentinic chrysotile was used. [133] Mesothelioma among occupationally exposed individuals is primarily attributed to the amphibolic variants i.e., crocidolite and amosite fibres. [133]

The amphibole shape of asbestos is thought to induce chronic irritation, potentially resulting in malignant transformation of the pleura through repeated exposure. [137] Among the various types of amphibolic asbestos, crocidolite is regarded as the most carcinogenic. Nevertheless, in Sweden, the predominant asbestos fibre used was chrysotile, belonging to serpentine type. [138] However, certain industries, like asbestos cement production, used asbestos varieties from both the serpentine and amphibole families. [139]

The widespread use of asbestos occurred for many years during industrialization, as it serves as a form of insulation in various industries. These industries encompass construction, shipbuilding, pipefitting, and car brake assembly.

The increasing incidence of PM in the 1950s spurred research into the clinically significant interaction between asbestos and mesothelial cells. Initial reports documented the migration of inhaled asbestos fibres to the pleura and the capability of mesothelial cells to phagocytose chrysotile asbestos fibres. [140]

A study showed that in cases of asbestos inhalation, the asbestos rapidly migrates the airway epithelium, traversing the alveolar space to reach the surface of the visceral pleura. [141] This research also demonstrated a progressive thickening of the visceral pleura across various time points, extending up to one year after asbestos exposure. Additionally, there was observed recruitment of neutrophils and macrophages of pleural regions. [141] In addition, the study documented a delayed clearance and prolonged persistence of asbestos fibres in the pleura. Once inhaled, asbestos is non-biodegradable, potentially leading to its persistence in the pleura for several decades.

The model proposing the pathogenesis of mesothelioma suggests that prolonged exposure to asbestos induces long-term and chronic inflammation in mesothelial cells. The development of mesothelioma is believed to involve the induction of inflammation caused by pleural irritation from asbestos fibres. Additionally, the production of reactive oxygen species is thought to contribute to mesothelial

hyperplasia and the accumulation of DNA damage, leading to mutations, aneuploidy, and genomic instability. [142, 143] Collectively, these cancer-inducing processes have contributed to PM over several decades. Nevertheless, the exact mechanism by which asbestos induces pleural carcinogenesis remains unclear.

Consequently, there has been bans on asbestos imports into many countries, including Sweden in 1982. [144] Throughout Europe, various countries enacted individual regulations banning asbestos from the 1980s to the early 2000s. In 1991, member states of the European Union (EU) collectively banned five of the six asbestos types (amosite, crocidolite, anthophyllite, tremolite, and actinolite). Furthermore, a ban on the new use of chrysotile came into effect in 2005. [145]

However, the ban was implemented on the use of only 6 of approximately 400 different mineral asbestos fibres. The remaining around 400 mineral fibres, not currently regulated, are freely used, despite many of them being carcinogenic and linked with mesothelioma. [133]

There is no established link between mesothelioma and any occupational exposure beyond asbestos, including factors like air pollution. [146] However, epidemiological evidence indicates that PM might develop due to the interplay of environmental carcinogens, genetic factors, and viral infections. [146]

Mutations mainly in the germline of BRCA1-associated protein 1 (*BAP1*), but also in *CDKN2A*, *NF2*, *LATS2*, *SETD2* and tumour suppressor genes, such as *MLH1* (Lynch syndrome), and *TP53* (Li-Fraumeni syndrome), have been causally associated with mesothelioma, sometimes in conjunction with exposure to asbestos or other carcinogenic fibres, utilising "gene x environment interaction". [133] Furthermore, therapeutic ionising radiation targeted at the chest, typically employed in the treatment of lymphomas, has been causally connected to mesothelioma, particularly among young patients. [133]

In addition, the association to other environmental exposures, including toxicological and carcinogenic fibres such as erionite and talc has also been demonstrated, but studies on simian virus 40 showed controversial results. [133]

Epidemiology of mesothelioma

The incidence of PM started to rise in the 1950s, attributed to asbestos mining and its widespread use in various industries. [85] The incidence rate of mesothelioma varies between countries, [147] with about 30 cases per one million in some developed countries. [148] From 1994 to 2008, a total of 92,253 mesothelioma deaths were reported in 83 countries, with Europe accounting for 54% of these cases. [147]

PM is frequently diagnosed in older patients, given its prolonged disease onset. Indeed, individuals, whether men or women, with comparable asbestos exposure exhibit a similar incidence of PM, [133] but men are more commonly affected than women, [93, 147] with ratio of 3.6:1, [147] which can only be partially explained by male occupational asbestos exposure. [149] Median age at diagnosis of over 60 years, [93] and

mean age at death of 70 years[147] has been reported. The overall survival (OS) ranges from 4 to 14 months. [150, 151]

Nevertheless, the landscape of mesothelioma is evolving, transitioning from predominantly affecting males to increasingly impacting females in substantial numbers. [145] Furthermore, studies indicate that females exhibit significantly better overall survival rates. [133, 152, 153] The suggested reasons for these findings are lower levels and duration of exposure, [154] more favourable clinical characteristics, [155, 156] and the potential protective effect of oestrogen. [157, 158]

In Sweden, mesothelioma affects approximately 12 cases per one million, which annually reflects about 120 cases. [159] Further, a clear difference between the genders for the increased risk of mesothelioma has also been observed in the Swedish population, where women constitute 21% of mesothelioma cases. [135]

Despite being a rare cancer, mesothelioma global rate is still increasing worldwide, [133, 145, 147] and the asbestos ban has still not demonstrated a clear impact on the incidence of PM in many countries; instead, there was an estimated peak in the incidence rate of PM in 2020. [133] Thus, the incidence and mortality continue to increase both in high-resource countries and worldwide. [133] Sweden is no exception, and the same incidence pattern has been observed. [135] Currently, in Sweden, more individuals die from mesothelioma than from workplace accidents. [160]

There are many possible explanations for that such as the long latency period of mesothelioma. Further, the increase in mesothelioma with age is attributed to the aging of the population. Moreover, as regulations currently encompass only 6 out of around 400 fibre types found in nature, under the generic term asbestos, numerous potentially carcinogenic fibres remain unregulated, contributing to ongoing human exposure and the occurrence of mesothelioma. [133] However, a substantial portion of the global population is still exposed to the mining and industrial utilization of this carcinogenic mineral, and asbestos use today is banned in only 55 countries. Additionally, owing to its extensive use in previous decades, numerous buildings still contain asbestos deposits, primarily used for fireproofing and insulation. However, achieving complete remission of the malignancy is not expected until many decades later, primarily due to the ongoing and unabated use of asbestos in some countries. [161]

PMs are universally lethal and aggressive tumours, characterized by a dismal prognosis and extremely poor outcome, [162] with median of 9-12 months. [90] Even with the implementation of modern therapeutic approaches, there have been only modest changes in survival rates observed over time, [89, 163] and 5-year survival of 5%. Nevertheless, the epithelioid subtype tends to have a relatively better prognosis compared to sarcomatoid and biphasic subtypes. [124]

Based on the data from the Surveillance, Epidemiology, and End Results (SEER) database [National Cancer Institute (NCI), the United States], patients diagnosed with epithelioid, biphasic, and sarcomatoid mesotheliomas of the pleura, who underwent surgical treatment, exhibited median survival of 19, 12, and 4 months, respectively. [124]

Certain studies have demonstrated comparable survival rates for biphasic and sarcomatoid mesothelioma, contrasting with the prognosis for epithelioid mesothelioma. [114, 164-167] Sarcomatoid mesothelioma has been correlated with a mere 4-month survival rate in patients who underwent surgical treatment. [124, 168] Mesotheliomas are typically situated in a fibrotic stroma; nonetheless, due to the production of hyaluronic acid, certain tumours may exhibit an abundant myxoid background. This characteristic has been linked to a more favourable prognosis. [169]

Lung cancer

Lung cancer is not a singular entity but rather a spectrum of diverse diseases, representing different heterogeneous tumours with diverse clinicopathological features. [104] Additionally, lung cancer is typically aggressive, initially asymptomatic, and diagnosed at an advanced stage of disease when it cannot be cured. These are some of the reasons why lung cancer tops the list of highly lethal diseases and is the leading aetiology of cancer-related mortality in mankind for people of both genders worldwide and in Sweden. [73, 170, 171]

Classification of lung cancer

Lung cancer has historically been broadly classified into two major groups, namely small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which encompass about 15-20% and 80-85% of all primary lung cancers, respectively. [172-174]

NSCLC is the predominant form, which is histologically subdivided into squamous in 18%, non-squamous (mainly adenocarcinomas) in 78%, and other uncommon subtypes in 4%.[175] The World Health Organization (WHO) has classified NSCLC into two main subtypes, adenocarcinoma (AC) and squamous cell carcinoma (SCC). [176, 177] AC followed by SCC make up the most common subtypes of all lung cancer diagnoses and are the focus of this thesis. According to the Swedish National Lung Cancer Registry, about 56% is AC, 17% SCC and 10% SCLC. [178] The molecular characteristics differ greatly between these subtypes despite certain similarities in histological appearance. [179] A schematic overview of PM and NSCLC neoplasms is shown in Figure 5.

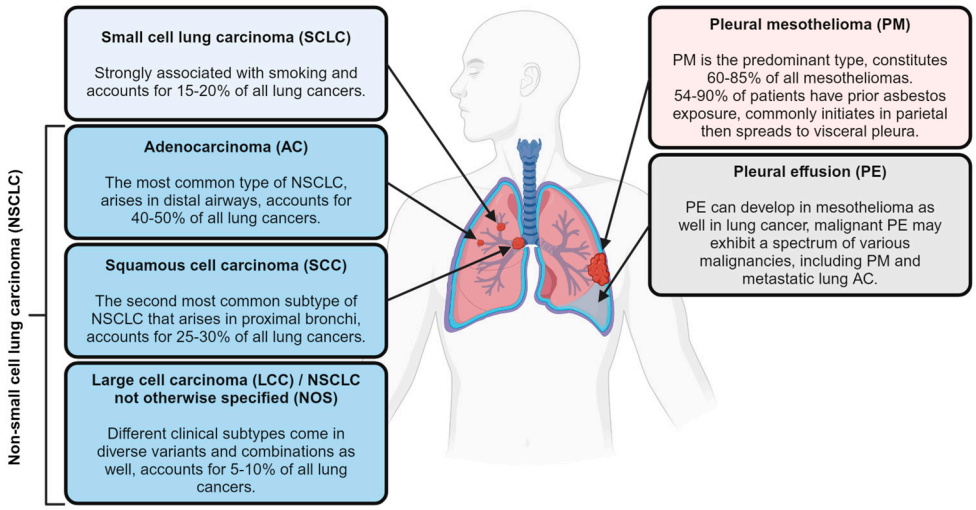

Figure 5. Schematic overview of the pleural mesothelioma and non-small cell lung cancer. This thesis focused on evaluation of different biomarkers in paired histological and cytological specimens from two thoracic malignancies e.g., PM and NSCLC, mainly AC and SCC. Created with BioRender.com

Adenocarcinoma (AC)

AC is the most prevalent subtype, and the most common primary lung cancer, representing roughly 40-50 % of all lung cancers. [104, 173, 174] Locally, AC constitutes around 60% of LC cases in Region Halland (internal statistics). AC is less strongly linked to smoking compared to SCC and SCLC[180] and frequently occurs in female non-smokers.

The tumorigenesis of AC frequently involves genetic alterations in specific driver oncogenes, [181] characterized by activating mutations in oncogenic driver genes, including *KRAS*, *BRAF* and *EGFR*. [172]

AC is a type of carcinoma that develops in glandular tissue and originates from alveolar cells located in the epithelium of the smaller airways (see Figure 5). For classification as AC, tumour cells do not necessarily have to be part of a gland or show glandular formation, as long as they exhibit secretory properties. The term adenocarcinoma derives from “adeno”, signifying “pertaining to a gland”, and “carcinoma”, denoting cancer that arises in epithelial cells, specifically those lining the walls of various organs. Typically, this type is located in the peripheral part of the lung.

AC are morphologically broadly categorized into non-mucinous and mucinous type. AC can occur without invasion, called AC in situ, and the minimally invasive AC is characterised by an invasion area ≤ 5 mm. The non-mucinous invasive AC are subclassified into various subtypes according to distinct growth pattern, with the five most frequent patterns being lepidic, acinar, papillary, micropapillary, and

solid. The majority of invasive ACs exhibit a mixture of these subtypes and the tumours are classified based on the predominant growth pattern, with other tumour components ("subpatterns") noted. The mucinous AC is an uncommon subtype of lung AC, and harbours specific clinicopathological and molecular characteristics. [182]

The subtypes have been shown to correlate with prognosis, and a grading system demonstrated that the lepidic-predominant tumours should be considered low-grade tumours with better prognosis, acinar or papillary-predominant tumours categorised as intermediate, and solid or micropapillary-predominant tumours deemed high-grade tumours with worse prognosis. [183]

Squamous cell carcinoma (SCC)

The frequency of SCC was the most common histological subtype until a few decades ago, but it was surpassed by AC. [184] SCC has markedly decreased, partially attributed to changes in smoking habits and reductions in smoking rates in high-income countries, and modifications in cigarette composition and design such as the introduction of filters, [185] as well as more accurate diagnostic methods using ancillary techniques. Nevertheless, SCC continues to be the predominant subtype in certain countries. [186]

SCC accounts today for about 25-30% of lung cancer cases, [173] originates from non-glandular lining or covering epithelia, and typically arises from cells located in the airway epithelium. The morphological characteristics of SCC encompass keratinisation and the presence of intercellular bridges, which denote connections between adjacent cells. Targetable driver oncogene mutations are infrequent, and SCC frequently exhibits a high overall mutation rate, primarily linked to smoking. [187]

Large-cell carcinoma (LCC)

LCC is defined as a NSCLC tumour devoid of distinctive features associated with other histological types. [103]

LCC used to make up approximately 5-10% of all lung cancer cases. [173] The identification of this condition is possible only through examination of surgical specimens, and its occurrence has diminished since the adoption of the 2015 WHO classification, which imposed limitations on the utilisation of LCC. Nevertheless, in the context of biopsies and cytological specimens, the diagnosis NSCLC not otherwise specified (NOS) is a more frequently employed diagnosis.

It is believed that LCC may signify poorly differentiated AC or SCC, wherein all morphological and immunohistochemical characteristics have been eroded. Although probably accurate for most cases of NSCLC NOS on small specimens, this remains a subject of controversy for true LCC cases. [188]

Other infrequent NSCLC subtypes include adenosquamous carcinoma and sarcomatoid carcinomas and several other rare subtypes.

Small cell lung carcinoma (SCLC)

SCLC is a neuroendocrine tumour, along with the less prevalent large-cell neuroendocrine carcinoma (LCNEC) and carcinoids. SCLC accounts for about 15% of all lung cancers. [189] SCLC is a highly aggressive cancer form associated with a very poor 5-year survival rate of < 7%,[174, 190, 191] closely linked to smoking, and is molecularly characterised by a high mutational burden. [189] As a consequence of the rapid cancer growth and early metastatic dissemination, SCLC usually follows a dynamic course, marked by symptomatic patients at the time of disease. LCNEC is similar to SCLC which is highly correlated with smoking and frequently exhibits clinical aggressiveness. [192] Carcinoids are not linked to smoking, typically exhibit slow growth and have much better prognosis. [193]

Aetiology of lung cancer

Tobacco smoke comprises more than 70 carcinogenic compounds that can promote carcinogenesis through various pathways. [194] Many environmental and lifestyle factors have been associated with the development of lung cancer, of which tobacco smoking is the main etiological cause, and at least 80% of cases are related to smoking alone. [195]

Tobacco does not only elevate the risk for the development of lung cancer but also raises the risk of several other cancer types. [196] In fact, only about 15% of smokers develop lung cancer, and there may be a link to genetic susceptibility. Exposure to environmental tobacco smoke, such as from a person who smokes or exposure in the workplace, has also been shown to increase the risk of developing lung cancer. [195, 197]

There exists a latency period of 10-30 years from the initiation of smoking to the onset of lung cancer, and even after cessation, the risk remains elevated for up to 30 years. [198, 199]

As mentioned above, lung cancer also appears in never-smokers, e.g. due to passive smoke inhalation, [195] where the incidence rate may vary depending on gender. [200, 201] Globally, approximately 85% of lung cancer cases in men, and a comparatively lower proportion in women, are believed to be linked to smoking. [202] In some countries, higher incidence rates among young women compared to young men have been observed, and the difference in tobacco use could not explain the findings. [203] A higher proportion of patients without a history of smoking is found among women than men. [197, 200] These observations may associate with the potential existence of gender-dependent differences in susceptibility or exposure to factors contributing to lung cancer risk, beyond smoking. However, investigations on the impact of hormonal factors, with oestrogen being proposed as a potential carcinogenic factor, indicate the need for additional studies to substantiate these findings. [204]

The environmental exposures to toxins and carcinogenic factors such as radon[195, 205] and other occupational exposure such as metals including asbestos, arsenic, chromium, and nickel, and polycyclic aromatic hydrocarbons, [195, 206] are associated with the risk of lung cancer. In addition, the elevated risk from radon exposure has been shown to be greater among smokers. [205] As for radon, the combination of the extent of smoking and exposure to other risk factors such as asbestos exhibits a synergistic effect for the risk of developing lung cancer. Also, an increased risk of lung cancer has been associated with exposure of indoor and outdoor air pollution such as cooking oil vapours, factory emissions, and exhaust fumes from vehicles. [197, 207]

Elevated risk of lung cancer risk is also associated with exposure to ionizing radiation, notably observed in patients who have a history of Hodgkin lymphoma or breast cancer. [208, 209]

In the carcinogenesis of lung cancer, hereditary factors may contribute, as evidenced by an elevated risk observed in individuals with a family history of the disease. [210] The genetic factors and certain cancer syndromes caused by germline mutations are associated with increased lung cancer risk. [211-215]

Human immunodeficiency virus (HIV) infection, alcohol consumption, and history of pulmonary fibrosis, chronic obstructive pulmonary disease, pneumonia, and tuberculosis have also been defined as risk factors for lung cancer. [216-218]

Human papilloma viruses (HPV) serve as risk factors for various cancer types. [219] While some studies have identified the presence of HPV DNA in lung neoplasms, [220] one study found no evidence for HPV in the aetiology of lung cancer in Swedish non-smokers. [221]

Epidemiology of lung cancer

The incidence rate of lung cancer is globally one of the highest incidences of malignancies in the world. Owing to its elevated incidence and low survival rates, lung cancer stands as the leading global cause of cancer-related deaths. [73] Worldwide, more than 2 million were diagnosed in the year 2020. [73]

The incidence of lung cancer varies between different countries as well as regions, and the fluctuations in incidence over time primarily reflect changes in the smoking patterns within the population. The highest incidence and mortality rates of lung cancer are observed in developed countries. Conversely, developing countries are estimated to exhibit comparatively lower rates of lung cancer. However, the absence of a centralized reporting system in numerous developing countries obscures the true incidence of the disease. [222] The incidence of lung cancer stays infrequent in individuals below the age of 40. [223] Subsequently, it gradually starts to increase, reaching its pinnacle between the ages of 65 and 84 years. Besides, the mortality rate for lung cancer patients is very high due to the change in lifestyle and exposure to environmental risk factors.

According to the World Health Organization (WHO), an expected upward trend in global lung cancer mortality is predicted, mainly due to the escalating of tobacco use on a worldwide scale, notably in Asia. [224] Within the large population of China, a notable rise in lung cancer incidence has been observed, which is anticipated to persist in the upcoming decades. [225]

In the United States, there has been a decrease in age-standardised incidence and deaths from lung cancer over the past few decades. [226] Still, a report for 2018 based on the American population expected that the rate of lung cancer-related deaths is estimated to exceed the combined three next most common cancers, i.e., breast, colon, and prostate cancers. [226]

In Sweden, the incidence of lung cancer in men has decreased in recent decades. However, there has been a notable increase among women, and today lung cancer is the fourth most common malignancy for women and the sixth for men. [199, 227] A total incidence of 4400 newly diagnosed patients annually are seen in Sweden, and the median age at diagnosis is 69 years. [199]

From an international perspective, Sweden has a relatively low incidence, especially when comparing the incidence among men. [199] This is explained by a relatively low smoking frequency in the Swedish population. However, Sweden is no exception, and the same pattern as internationally is also seen, where lung cancer is the most common cause of cancer deaths, with approximately 3600 deaths each year.

The 5-year overall survival rate for NSCLC remains poor, ranging from 68% in early-stage patients to only about 10% in advanced-stage patients. [228] Still, the survival rates have improved in the latest decades, for example in Sweden from about 10% 5-year overall survival in the 1980s to over 25% in the 2020s for the whole population of lung cancer patients. [227] A higher survival rate for women compared to men is observed both internationally and in Sweden. [227, 229]

Preparation procedures of pathological specimen

The processing technique plays a crucial role in both histology and cytology, and may in the extension influence the diagnosis. The handling of tumour tissue is similar worldwide, while the handling of cytological material differs between countries and even between pathology departments within the same county and the same health care unit. There is different material of both histological and cytological specimens, respectively. Histological samples are typically formalin-fixed, paraffin-embedded, and sliced into thin sections, while cytological specimens can undergo various processing methods and fixed in various fixations.

Preparation procedures of histological specimen

The histological tissue specimens were used in four studies included in this thesis (Paper I-IV). Histological tissue material can be in form of small biopsies or resections from major surgery. The most used tissue specimens in our studies were biopsies.

In clinical practice, tumour tissues are typically fixed in formalin and then embedded in paraffin blocks. This process preserves the tissue and facilitates microscopic evaluation of thin tissue sections. The initial step involves immersing the tumour tissue in formalin, which penetrates and stabilises the tissue. Given the hydrophobic nature of the wax used in the ultimate paraffin embedding, the specimen needs to be dehydrated. This process is accomplished by immersing the sample in a series of alcohol solutions, effectively replacing the water. The alcohol needs to be replaced by xylene because alcohol and wax do not fully blend. This process is known as clearing. Subsequently, the specimen can be embedded in paraffin wax.

This procedure guarantees preservation and facilitates the cutting of thin sections with preserved tissue morphology. However, it has the potential to damage DNA, RNA, and proteins in the tissue due to processes such as cross-linking, degradation, and modification. Hence, investigations using nucleic acids obtained from FFPE tissue can be technically challenging. Conversely, nucleic acids extracted from fresh frozen tissue typically maintain high quality. Nevertheless, fresh frozen tissues are typically not collected as part of clinical routine procedures. The general stain of histological tissue is haematoxylin and eosin (H&E) stain, but there are many other histochemical stains that are used for different diagnostic issues (Figure 6).

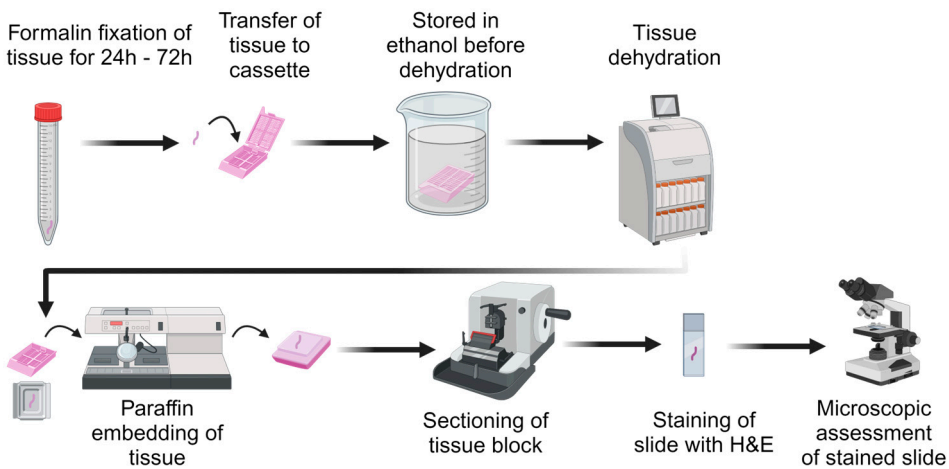

Figure 6. Schematic overview of the preparation procedure of histological material. The same process is performed for all histological specimens, but with differences in fixation and dehydration time depending on type and size of material. Created with BioRender.com

Preparation procedures of cytological specimen

The cytology preparation approach includes a variety of methodologies for diagnosis of cancer, and multiple preparation and fixation methods are available for cytological specimens. The cytological technique employed depends on how the material is submitted to the laboratory. The four traditional primary techniques include conventional air-dried, ethanol- or formalin-fixed smears, cytospins, liquid-based cytology (LBC) preparations, and cell block preparations. [230, 231]

Conventional smears are generated by spreading the cells onto glass slides, and these slides are either air-dried or promptly fixed in ethanol or methanol. The cytospin technique involves centrifugation of fixed or unfixed specimens directly onto glass slides, which are subsequently fixed in the same manner as conventional smears. A significant shift has occurred for the conventional smears, with mainly Pap smears that marked the initiation of the exponential revolution in the field of cytopathology being replaced or complemented with so called LBC. This transition enables more precise interpretation and facilitates molecular testing for e.g. HPV infection. [232] The technique has shown promising results for some type of cytological specimens, [233] producing multiple pathological slides that can be applied to immunocytochemistry (ICC) and molecular analysis. [234] However, unfavourable results have been obtained for non-gynaecological cytology specimens related to low cellularity. [235] Systematic reviews and meta-analyses have demonstrated that LBC may replace smears, but the diagnostic accuracy of LBC depends on different LBC techniques. [236, 237] In LBC, the specimen is transferred to a vial containing a liquid fixative. The material can undergo centrifugation and be processed like conventional smears or cytospin, or the entire cell pellet can be fixed in formalin and embedded in paraffin, similar to histological material.

Both histological and cytological specimens can undergo staining using chemical and immunochemical stains, but the staining protocols vary between histology and cytology. The standard chemical staining method for histology is H&E staining for formalin-fixed tissue, while the standard chemical staining for cytology includes H&E or Pap staining for alcohol-fixed specimens and May-Gründwald-Giemsa (MGG) for air-dried specimens. The staining of alcohol-fixed cytological specimens using either H&E or Pap depends on the question at issue, the type of specimen, and the sampling site.

Cell block processing in cytology

The cell block is a preparation method where the cytological specimen is compacted into a cell pellet and processed as paraffin-embedded blocks in a manner comparable to FFPE histological specimen. [238, 239] Cell blocks have become popular given their similarity to histological sections and already early reports based on refinements of cell block preparation technique using plasma-thrombin contributed remarkably to the acceptance of pleuro-pulmonary cytology. [240] However, cell blocks today play a crucial role in the practice of cytopathology, and from a clinical point of view, they

are the preferred cytology preparations. They serve as a complement to other cytological preparations for morphological diagnosis by improving diagnostic yield and may increase morphological details. [238, 239] The cytology cell blocks provide the same features as when reviewing a section of FFPE histological specimens and facilitate the recognition of the cells lying flat on the slide. Furthermore, cell blocks assume greater importance by yielding tissue sections suitable for further ancillary examinations, including immunohistochemical staining and molecular analyses. [239, 241] In addition, the cytological material in cell blocks is preserved for future utilisation, presenting a substantial advantage in biobanking practices. [241]

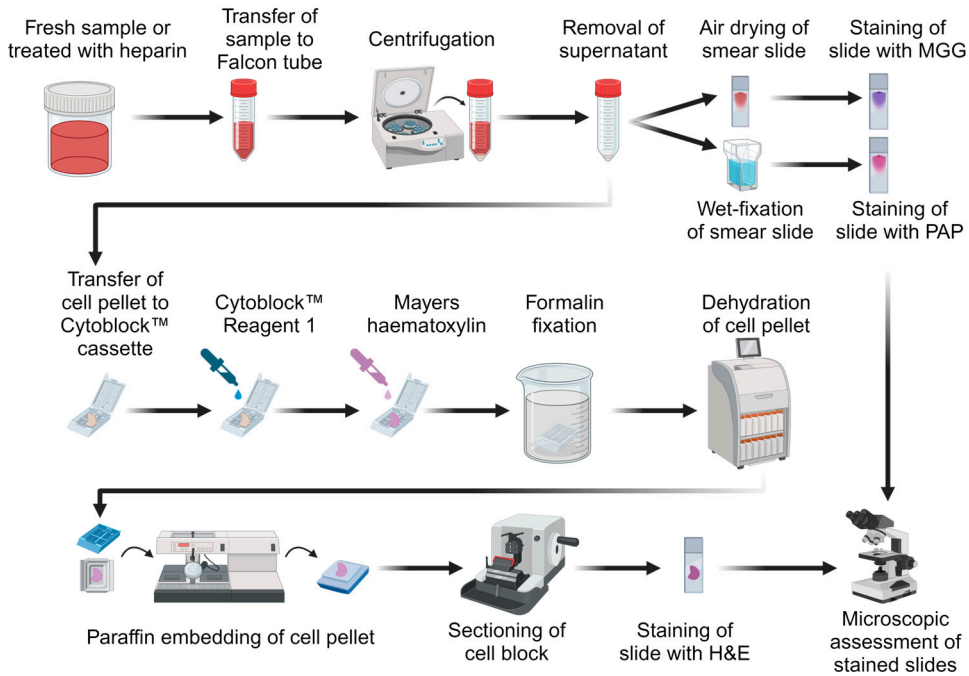

Figure 7. Schematic overview of the preparation procedure of cytological material. This illutration depicts the routine prepartion procedure of pleuro-pulmonary cytology specimens, but the process can be modified depending on the cellularity of the specimen, and cytospin can be used instead of cell block in case of insufficient material. Created with BioRender.com

Cell blocks can be created from any type of cytological sample including scraped off conventional smears and residual material from liquid-based preparations. However, there are substantial differences in the handling of cell blocks, in terms of multiple preparation protocols and fixation methods. The cell block preparation procedures can be manual or automated. The most common cell block preparation techniques include plasma-thrombin, agar-based methods (including HistoGel and Cell-Gel), Cellient™ (used in Paper III and IV), and Shandon™, [238, 239, 241] using different commercially available LBC fixatives such as PreservCyt®, *CytoLyt*®,

and CytoRich™ Red. Moreover, there is a simple sedimentation technique that was used in all Papers included in the thesis. Several reports have discussed and identified the advantages and limitations of each method. [238, 239] A schematic overview of the preparation procedure of cytological specimens including illustration of the cell block preparation using simple sedimentation preparation technique is shown in Figure 7.

Biomarkers

Today's PM and NSCLC diagnostics are going through many changes and the purpose is not only to provide a diagnosis of malignancy, but also for prediction of treatment response by predictive biomarkers. Furthermore, a great need for biomarkers in precision medicine for molecular information and identification of the patient's unique disease risks, which enables guidance for personalised treatment based on the patient's individual needs. [242]

The process of identifying novel biomarkers necessitates screening across a wide array of tumour types, which can prove time-consuming, costly, and technically challenging. The use of tissue samples from various tumour types in a tissue microarray (TMA) format provides a rapid and cost-effective method for biomarker screening. However, this is limited, especially when evaluating cyto-histopathological correlation, where a sufficient amount of tumour cells is required to reach optimal results. The technique and optimization of antibodies are established on histological material, while several reports have demonstrated that the method can also be successfully performed on cytological preparations. [243-245]

Several biomarkers have been identified and integrated into clinical diagnostics over the past few decades. Based on their clinical applications across various disease stages, biomarkers can be categorised into three main groups, i.e., diagnostic, prognostic, and predictive biomarkers. [246]

Diagnostic biomarkers are used to detect, identify, characterise, classify, and monitor or exclude a disease.

Prognostic biomarkers, as defined, relate to the natural history of the disease, reveal how the disease may develop in an individual patient, and determine the risk of a disease outcome, such as death, independent of the type of treatment. They are thus crucial in guiding the clinical decision-making processes. The clinical stage stands out as the most critical prognostic biomarker in both PM and NSCLC, consistently outweighing the impact of other contributing factors.

Predictive biomarkers, also called therapeutic biomarkers, evaluate the probable response to a particular therapy and aim to identify patients who are likely to benefit from the treatment.

Below is a description with brief outlines of immunohistology for all immunohistochemical biomarkers as well as the main use for molecular biomarkers that were used in the studies included in the thesis.

Diagnostic biomarkers

Morphological examination is often not sufficient to determine the type and origin of a tumour. Therefore, ancillary techniques e.g. immunostainings using diagnostic biomarkers, play an important role and are often required for the identification and typing of tumour cells.

The most common diagnostic mesothelial biomarkers were determined in histological and cytological material from PM patients which was the focus of Paper II. Different diagnostic lung AC biomarkers were evaluated in cytology cell block preparations, fixed in different fixatives, which was the focus of Paper V.

Mesothelioma biomarkers

Eight different diagnostic mesothelial biomarkers have been evaluated in paired histology and cytology material from patients with pleural mesothelioma (Paper II). Calretinin, cytokeratin 5 (CK5), podoplanin, and Wilms tumour 1 (WT1) are used to confirm mesothelial origin, while epithelial membrane antigen (EMA), desmin, BRCA1-associated protein (BAP1), and methylthioadenosine phosphorylase (MTAP) are used to differentiate benign from malignant mesothelial cell proliferations. The sensitivity and specificity of those biomarkers vary between studies depending on the antibody clone used, site, and the histological subtype of mesothelioma. [247]

Calretinin

Calretinin is a 29 kDa calcium-binding molecule which is a member of the EF-hand protein group. Calretinin is closely related to S100 and calbindin and is believed to play a role in calcium-dependent intracellular signalling mechanisms that regulate the cell cycle. Calretinin was initially discovered in neurons and characteristically expressed in central and peripheral nervous tissue. Calretinin can also be demonstrated in mesothelium and is expressed on the surface of mesothelial cells. [248] The first study investigating calretinin as a biomarker for mesothelioma was conducted by Doglioni and colleagues in 1996. [249]

Calretinin is used to confirm mesothelial origin, and the immunoreactivity of calretinin is found in both benign and malignant mesothelial cells. Calretinin is extensively utilized in IHC assessments of histological and cytological specimens suspected of PM, encompassing both epithelioid and sarcomatoid subtypes. [116] The ideal staining pattern of calretinin is nuclear, often presenting a “fried egg appearance”, and may lack membranous staining. [250] The interpretation of a cytoplasmic stain alone should be considered negative. [250]

A systematic review including seventeen studies assessed the value of calretinin staining in distinction between epithelioid mesothelioma and lung AC demonstrated an overall diagnostic sensitivity and specificity of 82% and 85%, respectively. [251]

For mesotheliomas in effusions, the sensitivity of calretinin ranges between 81-100%.[250] In AC, weak cytoplasmic staining for calretinin has been reported in 19-57% of cases, while it is typically negative in effusions with ovarian carcinoma. [250] In SCC, the expression of calretinin has been inconsistent in the different reports, ranging between 0-100% of cases. [250]

Cytokeratin 5 (CK5)

Cytokeratins (CK) constitute an extensive family of cytoskeletal proteins, serving as intracytoplasmic intermediate filaments expressed in mesothelial and epithelial tissues, as well as tumours derived from these tissues. CK5 is a basic, type II cytokeratin of weight 58 kDa that is encoded in humans by the *KRT5* gene. CK5 is a basal cytokeratin and a biomarker of squamous differentiation. CK5 is also a biomarker used to confirm mesothelial origin, and the immunoreactivity of CK5 is found in both benign and malignant mesothelial cells. CK5 is useful for distinguishing between mesothelioma and lung AC, [251] and is reported to be expressed in most epithelioid and biphasic mesotheliomas. It is not cross-reactive with CK6. CK5 exhibits as diffuse cytoplasmic staining with perinuclear enhancement.

The majority of studies used the CK5/6 IHC biomarker, and few reports (including our study reported in Paper II of this thesis) used the CK5 IHC biomarker to study mesothelioma. CK5 and CK6 are functionally unrelated but share a similar tissue distribution. They are present in various proportions in many non-keratinizing stratified squamous epithelia, as well as in basal epithelia of many tissues. [252] The expression of CK5 is also associated with the intermediate phenotype of cells undergoing the epithelial-mesenchymal transition.

The positivity rate of lung AC increased from 12.8%, with CK5 alone, to 23.7%, when CK5 and/or CK6 were positive in one study. [253] Further the study showed CK5/6 positivity in 56% of lung AC cases. [253] In contrast, one study demonstrated that AC are never diffusely positive for CK5/6, and only 2% of lung AC may be focally positive in cases with squamous differentiation. [254] An additional study showed CK5/6 reactivity ranging between 7 to 12% depending on whether the lung AC is primary or metastatic. [255] Meanwhile, both SCC and epithelioid mesothelioma have been reported positive in 100% of cases, regardless of whether CK5 alone or CK5/6 were used. [252] Based on the histological review study of mesotheliomas, the frequency may vary depending on the histological subtype and the combined overall expression of CK5/6 is 84%, while only two-thirds of the sarcomatoid subtype is positive. [254] This may, at least in part, explain why other studies showed positivity in only 64% of mesotheliomas. [255]

The frequency of CK5/6 expression for distinguishing between epithelioid mesothelioma from lung AC differs between several studies. In pleural effusions,

CK5/6 immunoreactivity was exhibited in 90% of mesothelioma and 0% of AC. [256] Other studies on serous effusions reported CK5/6 reactivity in 48% of metastatic carcinoma, 10-58% of AC, 43-65% of pulmonary AC, and 87-100% of mesothelioma. [250] More studies indicated that CK5/6 is not useful to distinguish mesothelioma from lung SCC. [250] A systematic review including eight studies evaluated CK5/6 staining in epithelioid mesothelioma and lung AC demonstrated a combined sensitivity and specificity of 83% and 85%, respectively, to identify epithelioid mesothelioma. [251]

Podoplanin

Podoplanin, also known as T1-alpha, aggrus, gp36, E11, and M2A, is a mass 36-43 kDa, type-I transmembrane glycoprotein with extensive O-glycosylation and mucin-like character. Podoplanin is a 162-amino acid encoded by a gene, *1p36.21*, on the short arm of chromosome 1. [257, 258] Podoplanin with platelet aggregation-inducing ability may play a role in cancer cell migration, invasion, metastasis, and malignant progression. [259] Podoplanin was first described by Breiteneder-Geleff and colleagues in 1997. [260]

Podoplanin is recognized by various anti-podoplanin antibodies that have been developed, with a varying degree of characterization. Examples include D2-40 and YM-1, with the monoclonal D2-40 being a widely used antibody clone. [257] Podoplanin is a specific cell surface biomarker selective for lymphatic endothelial cells, and lymphatic differentiation. [250, 257, 258] Podoplanin is also expressed on a range of tumours, and is found in both benign and malignant mesothelial cells. [250, 257-259]

D2-40 immunostaining reveals a robust membranous pattern in mesothelial cells, exhibiting a sensitivity ranging between 83-100% and a specificity of 49-100%.[250] Membranous D2-40 expression is found in 88-96% of epithelioid mesothelioma, 59-83% in biphasic mesothelioma, and 36-77% in sarcomatoid mesothelioma. [257] D2-40 staining in sarcomatoid mesothelioma may manifest as membranous, cytoplasmic, or a combination of both patterns. [257] Prudent interpretation of D2-40 immunoreactivity is essential, because it can also accentuate interspersed lymphatics. Notably, D2-40 is positive in about 20% of sarcomatoid carcinomas, which imposes limitations on its sensitivity in this differential diagnosis. [257] Further, D2-40 demonstrates intense membranous staining in 58% of ovarian AC, while 30% of non-ovarian AC may exhibit a weak and focal membranous reactivity. [250] In addition, D2-40 is positive in about 50% of SCC of the lung, [250, 257] but only in 3% of lung AC. [257]

Studies investigating a pure podoplanin antibody in effusions are limited; however, current findings suggest a specificity comparable to D2-40. Podoplanin demonstrates expression in 94% of mesotheliomas, and 97% of reactive mesothelial cells (RMC), while in only 7% of ovarian AC. In contrast, it is not expressed in lung and breast AC. For the detection of mesotheliomas, these characteristics result in an overall sensitivity and specificity of 94% and 97%, respectively. [250] Further, in the study podoplanin demonstrated strong membranous immunoreactivity in

mesothelioma cells, whereas ovarian AC cells exhibited weak membranous immunoreactivity. [250]

Wilms tumour 1 (WT1)

WT1 is a tumour-suppressor gene involved in the induction of Wilms Tumour, and in the sporadic and familial paediatric kidney tumour. The protein produced by the *WT1* gene is a zinc-finger transcription factor and a tumour-associated antigen. [261] The *WT1* gene is normally expressed in foetal kidney and mesothelium, as well as in a significant number of human neoplasias, including haematological malignancies. [262] In adults, WT1 expression persists in the mesothelium and various other tissues. [263]

While there is no established pan-mesothelial cell biomarker, WT1 is frequently used in studies of embryonic mesothelium. [116, 264] WT1 is expressed in mesenchymal tissues that undergo a transition towards an epithelial phenotype. [264] Thus, WT1 is used to confirm mesothelial origin, and the immunoreactivity of WT1 is found in both benign and malignant mesothelial cells. Therefore, its expression has been suggested as a biomarker for Wilms tumour and mesothelioma.

A systematic review including eight reports investigated the value of WT1 staining in tissues for distinction between epithelioid mesothelioma and lung AC. The combined overall diagnostic sensitivity and specificity were 77% and 96%, respectively. [251]

As WT1 often exhibits cross-reactivity with cytoplasmic proteins in a range of benign and malignant entities, it is crucial to note that only nuclear immunoreactivity of WT1 should be considered diagnostic. [250] Nuclear immunoreactivity of WT1 was observed in over 90% of mesothelioma effusion specimens, in contrast to 20-30% of metastatic adenocarcinomas, particularly those originating from the lungs and breasts. [250] Conversely, WT1 is expressed in 80-90% of ovarian malignancies, making it ineffective in distinguishing between mesothelioma and ovarian tumours. [250] In general, WT1 is not expressed in SCC. [250]

Epithelial membrane antigen (EMA)

Various names have been used for EMA over the last 30 years, including MUC1, CD227, CA15-3, episialin, and many others. [265] EMA is a single-pass type I transmembrane protein with a heavily glycosylated extracellular domain. [265] EMA has a high-molecular weight of >400 kDa[265] and extends up to 200-500 nm from the cell surface. [266]

EMA is normally expressed in glandular or luminal epithelial cells and hematopoietic cells, while being absent in skin epithelium and mesenchymal cells. [267] Particularly, EMA is normally expressed on the apical surface of almost all glandular epithelial cells including respiratory. [265] Overexpression of EMA is frequently observed in malignancies, contributing to tumorigenesis. EMA is highly expressed and aberrantly glycosylated in most carcinomas, especially AC, and haematological cancers. [265, 266]

EMA is not expressed in benign mesothelium, [265] and mesothelial cells develop EMA during the transition to malignancy. EMA is both a mesothelioma and carcinoma biomarker. EMA as a biomarker is used to differentiate benign from malignant mesothelial cell proliferations, as well as being used as a carcinoma biomarker. EMA is positive in the majority of carcinomas, [258] particularly AC in different organs, [268] and commonly used alongside other cytokeratin stains. [258] EMA exhibits cytoplasmic staining in carcinoma cells. [269] In non-carcinomas, EMA is valuable for distinguishing meningioma, certain hematopoietic cancers, and mesothelioma.

For mesothelioma, EMA is primarily located on the cell surface, associated with microvilli, [251] and membranous immunoreactivity is considered positive. [270] Studies have demonstrated that not all mesotheliomas are positive for EMA, [271] and EMA is most useful for the epithelioid subtype. [272] A systematic review demonstrated that the frequency of EMA reactivity in sarcomatoid mesotheliomas was notably lower than that observed in epithelioid mesotheliomas (25% versus 77%, respectively), and EMA had an overall sensitivity and specificity of 74% and 89%, respectively, for distinction of mesotheliomas from benign or reactive pleural lesions. [251] Furthermore, RMC may exhibit membranous staining with EMA, although this is observed in only 3-4% of cases. [273] However, some studies demonstrated that reactive or benign mesothelial cells may exhibit immunoreactivity of EMA in 10-50% of cases. [251] Lastly, a subset of ACs, approximately 14%, may also exhibit a membranous staining pattern. [274]

In effusions, EMA immunoreactivity has a sensitivity of 91-100% and a specificity of 86-100% in distinction of AC from RMCs. [250] Immunoreactivity of EMA is reported in up to 100% of mesotheliomas, and in 9-26% of RMC, [250, 270] and for the diagnosis of mesothelioma, it has a sensitivity and specificity of 100% and 74-91% respectively. The specificity of EMA in diagnosing mesotheliomas is influenced by the clone used. EMA clone E29 from DAKO has been identified to possess higher sensitivity and specificity compared to other clones. [275] This clone was used in Paper II. Given the difference of EMA staining patterns in mesothelioma and carcinoma, EMA can be valuable in the evaluation of effusions for the differential diagnosis between mesothelioma and carcinoma based on the staining patterns. Also in effusion, EMA exhibits a distinctly strong and marked cytoplasmic membrane immunoreactivity in mesotheliomas, whereas it demonstrates a diffuse cytoplasmic staining pattern in carcinomas. [276]

Desmin

Desmin is an intracellular protein and a member of the intermediate filament family. Specifically, desmin is a type III intermediate filament protein, composed of 470 amino acids that has a molecular weight of 52/53 kDa, and is encoded by a single gene, *DES*, located at chromosome 2 (*2q35*). Desmin is the major muscle-specific protein, first purified in 1977, expressed characteristically in skeletal, cardiac, and

smooth muscle cells, and in the neoplasms associated with them. Desmin is essential for proper muscular structure and function. [277, 278]

Desmin exhibits cytoplasmic staining. The anti-desmin antibody is valuable for identifying neoplasms of myogenic origin, and with myogenic differentiation. It has also been described in some non-myogenic tumours, like sporadically reported lung AC, malignant melanoma, Wilms tumour, and gliomas. [251]

Mesothelial cells often exhibit a loss of cytoplasmic desmin expression during the transition to malignancy, [279] particularly in effusions, although the underlying mechanism is not entirely clear. Hence, desmin is a useful biomarker to differentiate reactive (mostly desmin positive) from malignant mesothelial cell proliferations (mostly desmin negative). The studies demonstrated that desmin reactivity is normally exhibited in 84-92% of reactive mesothelial proliferation in effusions, while the immunoreactivity ranges between 0-10% in mesotheliomas. [250, 251, 270, 280, 281] However, in the latter, the expression is often cytoplasmic and dot-like in many cases. [251] Based on three studies, the combined sensitivity and specificity of desmin were both 83% for differentiation the benign pleural diseases from mesotheliomas. [251] It is important to note that a few background RMC can still express desmin in malignant effusion, located among epithelioid mesothelioma cells.

BRCA1-associated protein 1 (BAP1)

BAP1 functions as a nuclear ubiquitin hydrolase, playing roles in diverse cellular processes, notably chromatin remodeling. *BAP1* serves as an important tumour suppressor gene owing to its role as a deubiquitinating enzyme. BAP1 regulates gene expression and cell processes such as DNA repair, chromatin accessibility, and mitochondrial calcium flux to apoptosis through its deubiquitinating activity. [282] Genomic investigations conducted in PM cohorts have revealed that *BAP1* mutations lead to protein truncation, disruption of the nuclear localization signal domain, or heterozygous loss. These alterations result in reduced BAP1 protein levels or loss-of-function. [283] Double-hit inactivation of *BAP1* is a key driver event in approximately half of mesotheliomas. [250] Furthermore, whole-exome sequencing has verified that the development of mesothelioma in situ is linked to somatic mutations in *BAP1*. This indicates that *BAP1* alterations represent an early event in the development of a subset of mesotheliomas. [284]

Somatic mutations within the *BAP1* gene, situated on chromosome *3p21*, manifest in over 50% of PMs, predominantly of the epithelioid subtype. [285] *BAP1* mutations are frequently coupled with concurrent loss of heterozygosity on chromosome *3p21*. [285]

The significance of BAP1 in PM has been underscored by reports of germline *BAP1* mutations inherited in an autosomal dominant pattern. These mutations predispose individuals to a spectrum of cancers, including uveal melanoma and mesothelioma. [282] This finding, along with the prevalent *BAP1* mutations observed in PM, suggests the existence of a *BAP1* cancer syndrome and underscores a significant association between *BAP1* mutation and PM. [91, 133, 282]

BAP1 loss-of-function can be detected by IHC and has become a highly specific method for differentiation of reactive from malignant mesothelial cell proliferations, though its sensitivity is not particularly high. Collectively, substantial evidence suggests that quantifying nuclear BAP1 by IHC can reveal *BAP1* loss-of-function attributable to mutation. This supports the diagnosis of PM, and even signifies a high risk of PM development in certain cases. [286]

The loss of BAP1 IHC expression range between 27-65% in mesotheliomas. [247, 250, 287] *BAP1* mutations are common in epithelioid mesotheliomas, whereas they are infrequently present in sarcomatoid mesotheliomas and provide less help in distinguishing them from benign processes. [288] However, given the prevalence of *BAP1* mutations in epithelioid PM, BAP1 staining provides a potentially valuable method for distinguishing BAP1-negative epithelioid PM from other cancers.

In patients lacking a germline *BAP1* mutation, normal mesothelial cells should exhibit positive BAP1 nuclear expression, which serves to differentiate benign reactive mesothelial cells from malignant. [91] Further, a retained BAP1 nuclear expression in RMC has been demonstrated by several reports. [287]

Methylthioadenosine phosphorylase (MTAP)

The *MTAP* gene, which encodes methylthioadenosine phosphorylase (MTAP), is located approximately 100 kb telomeric adjacent to cyclin-dependent kinase inhibitor 2A (*CDKN2A*, also known as *p16* which encodes the *p16* tumour suppressor) on chromosome *9p21*, and these two genes are commonly co-deleted. [289-291] MTAP is expressed ubiquitously in human cells under normal physiological conditions but is deficient in various types of cancer.

A correlation with the findings of *9p21* FISH and the capability to differentiate malignant from non-neoplastic reactive mesothelial hyperplasia has been demonstrated, and the 9p21 FISH assay has been proved valuable and reliable in diagnosing PM. [292, 293] Nevertheless, FISH has not been adopted for routine clinical diagnosis practices everywhere, and its implementation faces limitations in certain laboratories due to technical challenges and the intricate procedures, and it is relatively costly and less readily available compared to IHC. Hence, many efforts have aimed to identify a reliable IHC biomarker capable of predicting the homozygous deletion of the 9p21 as detected by FISH.

The *9p21* locus encompasses a cluster of genes, including *CDKN2A*, *CDKN2B* (also known as $p15^{INK4B}$), $p14^{ARF}$, and *MTAP*. [294, 295] The most suitable IHC biomarker among the protein products of the genes was *MTAP*. Further, FISH-based and NGS-based studies revealed co-deletion of *MTAP* in 55-91% of PM with *CDKN2A* homozygous deletion. [285, 289-291, 296]

An initial study employing a polyclonal anti-MTAP primary antibody exhibited disappointing specificity of IHC MTAP for diagnosing mesothelioma. [297] However, several studies using a monoclonal anti-MTAP primary antibody and comparing it to FISH have shown that IHC loss of cytoplasmic MTAP expression is a reliable biomarker, with a sensitivity of 37-95% and a high specificity of 96-100% for

detection of *CDKN2A* homozygous deletion and the diagnosis of mesothelioma. [292, 296, 298, 299] The wide range of sensitivity may in part be related to the subtypes of mesothelioma, as the epithelioid mesothelioma exhibits low sensitivity, and sarcomatoid mesothelioma demonstrates high sensitivity, while maintaining high specificity for both subtypes.

IHC loss of cytoplasmic MTAP expression is considered a highly specific gold standard IHC biomarker of diagnosis of mesothelioma and serves as a specific surrogate for *CDKN2A* deletion in mesothelioma. [296, 298, 299] Further studies have demonstrated the reliable applicability of MTAP IHC to cell block preparations. [288, 300]

In-depth investigations have delved into the detailed correlation of MTAP expression with *MTAP* and *CDKN2A* alterations in mesotheliomas, as well as the genomic factors underlying ambiguous MTAP immunostaining patterns. [296] Furthermore, IHC loss of cytoplasmic MTAP expression shows a strong correlation with *MTAP* homozygous deletion, with a sensitivity of 75% and specificity of 95%.[296]

Heterogeneous MTAP immunostaining has been recognised in mesothelioma, [292, 298, 299] but the underlying causes remain unclear. However, sub-clonality of *MTAP* deletion and epigenetic silencing are suggested to play a role and may each contribute to this phenomenon. In mesothelioma, *MTAP* deletion results in decreased gene expression, [285] and *MTAP* deletions have been identified in a subset of tumours exhibiting heterogeneous MTAP immunostaining, indicating potential underlying subclonal *MTAP* deletion. [296] Epigenetic silencing has also been proposed as a factor contributing to *MTAP* silencing and the observed heterogeneous MTAP immunostaining in mesothelioma. [299] The observation of biphasic mesotheliomas with heterogeneous loss of *MTAP* expression in the epithelioid component but diffuse loss of *MTAP* expression in the sarcomatoid component suggests that 9p21 deletion may contribute to the development of sarcomatoid features. [296] *CDKN2A* homozygous deletion may be present in up to 100% of sarcomatoid mesotheliomas, [293] and further studies have demonstrated the utility and high benefit of MTAP IHC in the differential diagnosis of sarcomatoid mesothelial lesions. [292, 299]

Controversially, one study demonstrated that some RMC may exhibit loss of MTAP expression in a small percentage of cells. [292] Thus, MTAP staining results should be interpreted in context with other findings, [298] and only cytoplasmic loss of expression should be considered as true loss of expression. However, the true frequency and intratumoural distribution of heterogeneous *MTAP* expression, as well as the diagnostic significance of heterogeneous MTAP immunostaining, remain unclear.

Carcinoma biomarkers

Six different diagnostic lung AC biomarkers, with multiple antibody clones for two of them, have been evaluated in matched cell block preparations from pleural

malignant effusions with metastatic lung AC. Each case was fixed in different fixatives and the following biomarkers were investigated: two clones of thyroid transcription factor-1 (TTF-1), napsin A, claudin 4, carcinoembryonic antigen (CEA), cytokeratin 7 (CK7), three different clones of epithelial cell adhesion molecule (EpCAM).

Thyroid transcription factor-1 (TTF-1)

TTF-1, also known as NKX2-1, is a 38 kDa member of the NKX2 family of homeodomain transcription factors, which was originally identified in follicular cells of the thyroid. TTF-1 was subsequently identified in pneumocytes, which is required for the morphogenesis of the lungs and the differentiation of the epithelial cells. [301] The gene is situated on chromosome *14q13*.

This biomarker is highly recommended in diagnosing primary lung AC, representing one of the most dependable approaches to differentiate primary lung AC from both primary SCC and metastatic AC. It also exhibits high specificity for thyroid ACs and high-grade neuroendocrine carcinomas. [302]

Several different commercial TTF-1 monoclonal antibody clones are available, e.g., 8G7G3/1 and SPT24. Various clones target distinct epitopes, resulting in diverse IHC staining patterns.

In comparisons between clone SPT24 and 8G7G3/1, findings indicate that SPT24 tends to be more frequently positive in lung SCC and occasionally in lung metastases, [303, 304] whereas clone 8G7G3/1 exhibits a weaker staining intensity and is less frequently positive in lung metastases. [303, 304]

Nevertheless, one study has demonstrated that lung metastases exhibit positivity with both clones to a similar extent. [305]

Nordic immunohistochemical Quality Control (NordiQC), an organization engaged in external technical quality assurance of IHC staining for over 200 pathology departments, including those in Sweden, advocates for a more sensitive biomarker, such as SPT24, in the diagnostic evaluation of pulmonary tumours. [306]

As a result, numerous pathology departments in Sweden opt for clone SPT24 over clone 8G7G3/1, while the latter is recommended in the WHO guidelines for the diagnosis of lung cancer. [103, 307]

One study including 454 cases of primary lung ACs showed that TTF-1 exhibited a sensitivity of 81% and specificity of 90% in detecting lung AC, compared to other biomarkers. [308]

A systematic review including only five studies reported the differential expression of TTF-1 in lung AC and epithelioid mesothelioma, demonstrating that none of the mesotheliomas was positive for TTF-1, while 28% of carcinomas were negative. The sensitivity and specificity of TTF-1 for identifying lung AC were, therefore, 72% and 100%, respectively. [251]

Napsin A

Napsin A is a rather recently discovered aspartic proteinase belonging to the pepsin family, playing a role in the maturation process of surfactant protein B. Its primary presence is observed in the lungs and kidneys. The first study on the expression of napsin A in lung carcinomas was performed by Hirano *et al*, in 2000. [309] Along with TTF-1, napsin A functions as a diagnostic biomarker for lung AC, effectively distinguishing it from lung SCC. [310] Several studies indicate that napsin A is not only more sensitive but also more specific than TTF-1 in the diagnosis of lung AC. [310, 311]

The staining pattern of napsin A in lung ACs is granular and cytoplasmic. In various published studies, the percentage of napsin A positivity in lung ACs has varied, ranging from 58% up to 91% of the cases. [312] A study included 454 cases with primary lung ACs and compared different antibodies showed that napsin A exhibited a sensitivity of 87% and specificity of 95% for detecting lung AC. [308]

Napsin A expression is also evident in renal cell carcinomas, [313] along with clear cell ovarian and endometrial carcinomas. [314, 315] Although, napsin A serves as a valuable biomarker for differentiating primary lung AC from SCC, it has major limitations in distinguishing lung AC from ACs of other organs, and pathologists should exercise caution because napsin A immunoreactivity can lead to an erroneous conclusion of metastasis from renal, thyroid, or endometrial carcinoma as a primary lung AC. [316]

Claudin 4

Claudins are constituents of tight junctions, facilitating cell-to-cell adhesion, and constitute a protein family with 27 highly homologous members. [317] Claudin 4, also known as CLDN4, belongs to this group. Claudin 4 is a key component involved in tight junctions in epithelial cells, including those in the lungs. Claudin 4 is prominently expressed throughout the lung epithelium, and it exhibits high expression in both type I and type II alveolar cells. [318]

Overexpression of claudin 4 has been detected in various cancers, including NSCLC, [319] and correlates with cancer progression. Claudin 4 is regarded as a promising biomarker and potential molecular therapeutic target for several epithelial malignancies. [320] Claudin 4 can also serve as a biomarker for distinguishing mesothelioma from lung cancer. [321]

A few studies have reported claudin 4 expression in various metastatic carcinomas. [250] In tissues, claudin 4 was identified to be expressed in 91% of various types of carcinomas, but it was negative in mesotheliomas. [250] In effusions, claudin 4 exhibits a strong diffuse membranous staining pattern in 84% and a moderate staining pattern in 12% of ACs, while showing no expression in any mesotheliomas. [250] Claudin 4 exhibits a sensitivity ranging between 85-99% and a specificity of 100% in distinguishing carcinomas from mesotheliomas. [250]

Claudin 4 is exceptionally valuable in identifying single tumour cells dispersed within a dense inflammatory reaction, [250] and therefore it may serve as an ideal biomarker for the detection of metastatic epithelial cells in serous effusions.

Carcinoembryonic antigen (CEA)

CEA is an oncofoetal glycoprotein that was initially identified by Gold and Freeman in 1965. [322] While mesothelial cells typically do not express CEA, it is commonly expressed in AC of lung origin. The introduction of diagnostic IHC led to the early exploration of CEA's potential to distinguish between mesothelioma and lung AC. [323]

A systematic review including fifty-one studies evaluated CEA staining in epithelioid mesothelioma and lung AC and demonstrated a diagnostic sensitivity and specificity of 83% and 95%, respectively, to identify lung AC. [251]

For detection of AC in effusions, CEA exhibits a high reported specificity ranging between 90-100%, with variable sensitivity between 43-100%.[250] CEA demonstrates a strong membranous staining pattern. [250]

Early studies used polyclonal CEA antibodies, which may yield false positive results in mesothelioma due to nonspecific cross-reaction The introduction of monoclonal CEA antibodies has enhanced its diagnostic sensitivity and specificity, establishing it as one of the most robust and valuable antibodies in the diagnostic panel tools. Therefore, monoclonal CEA is frequently used in effusions and is generally favoured over polyclonal antibodies to prevent nonspecific staining in background inflammatory cells. However, it is essential to note that carcinomas from different origins and well-differentiated neuroendocrine tumours typically exhibit negativity when assessed with monoclonal CEA antibodies on tissue sections. CEA expression has been reported in 30-70% of SCC. [250]

Cytokeratin 7 (CK7)

CK7, also known as keratin 7, and K7, is similar to other keratins, and serves as a component of intermediate filaments, forming the cytoskeleton of epithelial cells. [324] The protein is encoded by a gene that is a member of the keratin gene family. The genes responsible for encoding the type II cytokeratins are grouped in a region of chromosome *12q12-q13*. This type II cytoskeletal 7 consists of basic low molecular weight of 54 kDa, [325] found in simple non-keratinizing epithelia in a variety of organs and identifies the simple epithelium in most glandular and transitional epithelium, but not in stratified squamous epithelium. [324]

CK7 generally exhibits membranous/cytoplasmic expression in many normal epithelial and epithelial tumours, as well as normal mesothelium. [326] The expression has some variation, ranging from weak and focal to strong and diffuse.

Despite wide distribution, CK7 is useful as part of a panel in determining the origin of metastatic carcinoma, i.e., adenocarcinomas, given its presence in the majority of e.g., lung, ovary, breast, upper gastrointestinal, and many other tissues while being negative in e.g. most colorectal cancers. Mesotheliomas are also characteristically strongly and diffusely positive for CK7. Distinguishing metastatic carcinomas in the lung that express CK7 from a primary lung carcinoma requires an antibody panel.

The IHC workup is contingent upon the patient's history and presenting findings. A significant portion of the literature on CK7 is based on the immunoreactivity patterns of clone OV-TL 12/30 from Dako-Agilent in FFPE tissues.

The vast majority, 97-100%, of primary lung AC are positive for CK7 staining, [254, 326] but the expression can be lower related to the histological growth pattern, [253] or related to whether the lung AC is primary or metastatic. [255] A study included 454 cases with primary lung ACs, compared CK7 expression with other biomarkers showed that CK7 exhibited a sensitivity of 100% and specificity of 80 % for detecting lung AC. [308]

Recently, a study demonstrated CK7-negative primary lung AC and pointed out that the proper identification of primary lung AC can be performed using TTF-1 and napsin A and/or CK7, and management and prognosis of lung AC should not be affected by CK7 status. [327] CK7 expression is exhibited in 44-93% of mesotheliomas. [254, 255, 328]

Epithelial cell adhesion molecule (EpCAM)

The *EPCAM* gene encodes a protein known as epithelial cell adhesion molecule (EpCAM). The *EPCAM* gene has several other names, e.g., *EGP-2*, *TACST-1*, *TACSTD1*, and human epithelial glycoprotein-2. Mutations in this gene have been linked to Lynch syndrome, a disorder elevating the risk of developing various cancers, notably colorectal cancer. [329] Mutations in the *EPCAM* gene can also lead to congenital tufting enteropathy. [330]

EpCAM has first been described in 1979 as a tumour antigen on colorectal carcinoma cells. [331] EpCAM is an antigen associated with different types of carcinomas[332] and belongs to a family comprising at least two type I transmembrane proteins, i.e., two glycoproteins, measuring 34 and 39 kDa. [333] EpCAM has been independently identified by several groups and has therefore been known by various names as epithelial antigen, epithelial specific antigen, epithelial glycoprotein or CD326, and others. The EpCAM protein is found in epithelial cells, which form the linings of body surfaces and cavities. This cell surface glycosylated protein is predominantly expressed on the basolateral membrane of cells in most normal epithelial tissues, excluding those with squamous differentiation. [280, 334] Typical mesothelial cells do not exhibit EpCAM positivity but may express focal reactions when undergoing reactive changes.

EpCAM protein functions as a homotypic calcium-independent cell adhesion molecule and facilitates cell adhesion by promoting their cohesion to one another. [335]

EpCAM expression serves as a biomarker of early malignancy, with increased expression observed in tumour cells. EpCAM expression is detected in the vast majority of ACs across various sites, [332] with rates ranging from 50-100% in different studies. It is also found in neuroendocrine tumours such as small cell carcinoma. EpCAM is found on the surface of ACs and exhibits a membranous staining pattern. [250] Furthermore, mesotheliomas, encompassing epithelioid and

biphasic subtypes, demonstrate EpCAM positivity in 4-26% of cases. Typically, the staining is focal, although occasionally it may be widespread.

EpCAM serves as a valuable aid in the differential diagnosis of malignant engagements within the body cavities. [336] The absence of EpCAM immunoreactivity in most mesotheliomas can be effectively utilized within a suitable panel to distinguish between mesotheliomas and ACs. [333, 337]

In a series of anti-epithelial antibodies, different anti-EpCAM clones may be used in the demonstration of epithelial cell differentiation in scenarios where anti-cytokeratins fail to yield definitive positivity or where a false positive for cytokeratin cannot be ruled out, such as in submesothelial cells. [337]

In paraffin sections, numerous monoclonal antibodies have been developed against EpCAM, many of which have been characterised as tumour-specific molecules on carcinomas.

According to NordiQC, [338] the clones BS14, Ber-Ep4, MOC-31, and VU-1D9 could all be used to yield optimal staining results. The most commonly used antibody for demonstrating EpCAM, clone Ber-Ep4, exhibited the highest proportion of sufficient and optimal results when used with heat-induced epitope retrieval (HIER) in specially formulated buffers such as TRS low pH 6.1 (Dako). Nevertheless, the clones BS14, MOC-31, and VU-1D9 could offer optimal staining results when used with standard HIER buffers. Further, considering performance and for laboratories facing challenges with optimisation of Ber-Ep4 or MOC-31 on the Bond (Leica) or BenchMark (Ventana) platform, both BS14 and VU-1D9 could serve as better alternatives. When utilizing all clones in a laboratory-developed (LD) assay, the use of sensitive 3-step polymer/multimer detection systems yielded the highest proportion of sufficient and optimal results. The Dako ready-to-use (RTU) system GA637 (Omnis), based on monoclonal antibody clone Ber-Ep4, exhibited superiority over all other RTU systems.

Several studies have compared different EpCAM clones in the diagnosis of metastatic carcinomas and distinction of mesotheliomas from NSCLC. [339, 340]

In Paper V, the impact of different fixatives on the immunoreactivity of three different EpCAM clones (BS14, Ber-Ep4, and MOC-31) has been investigated. Detailed data on these three biomarkers focusing on mesothelioma and lung cancer are presented below.

BS14

The expression rates of EpCAM clone BS14 in different tumours including PM and NSCLC are lacking in the literature. However, the only data on BS14 is found on two manufacturer's websites. [341, 342] BS14 clone demonstrates robust and optimal performance across various IHC platforms, utilizing the standard high pH HIER buffer. Furthermore, it is a good alternative providing a broader, flexible, and more adaptable dilution range, which enables adjustments tailored to the specific tissue being stained. This information is proven by assessment according to the NordiQC. [338]

Ber-Ep4

In early studies, Ber-Ep4 demonstrated high sensitivity and specificity for adenocarcinoma, with positive reactivity reported in less than 1% of mesotheliomas.

A systematic review including seventeen studies evaluated Ber-Ep4 staining in epithelioid mesothelioma and lung AC demonstrated an overall diagnostic sensitivity and specificity of 80% and 90%, respectively, for distinguishing between epithelioid mesothelioma and lung AC. [251]

For detecting AC in effusions, Ber-Ep4 exhibits a sensitivity ranging between 76-94%, and specificity ranging between 84-100%.[250, 343] Cytospins are suboptimal for Ber-Ep4 immunostaining, as the cells are typically suspended in a protein-rich fluid, leading to nonspecific reactions. The presence of large, three-dimensional cell groups and crushed, or degenerated and necrotic cells can also contribute to background staining. [231] In a particular study, cytospins showed nonspecific positive Ber-Ep4 staining in 16% of mesothelial cells, along with macrophages and neutrophils. [343] The reported expression of Ber-Ep4 in SCC cases ranged from 87% to 100%.[250]

MOC-31

MOC-31 interacts with epithelial glycoprotein-2 which is an epithelium-associated trans-membranous glycoprotein, derived from a small cell lung cancer cell line. The epitope recognized by MOC-31 is similar to that targeted by the monoclonal antibody to EMA. MOC-31 exhibits a predominantly membranous staining pattern. [250]

A systematic review including seven studies evaluated the ability of MOC-31 staining to distinguish between epithelioid mesothelioma and lung AC in tissues. The diagnostic sensitivity and specificity of MOC-31 were both 93% for identifying lung AC. [251]

In effusions, the sensitivity of MOC-31 for detecting AC ranges from 70% to 100%,[280] and it demonstrates variable specificity in the range of 65% to 100%.[250, 280] MOC-31 may exhibit focal expression in 5% to 35% of mesothelioma cases; however, it is nearly minimal or undetectable in RMC. [250] MOC-31 has also been observed in a subset of SCC of lung, with expression reported in the range of 60% to 97%.[250]

Predictive biomarkers

During the last decades, a number of tumour biomarkers have been discovered and implemented in clinical diagnostics. The diagnostic biomarkers have not lost their importance, but there is a need for prognostic and predictive biomarkers, which predict the natural outcome and response to specific therapies, respectively. Despite the progress in neoadjuvant and adjuvant therapies, including chemotherapy, radiotherapy, hormone therapy, targeted therapy and immunotherapy, there remains a critical need to improve risk stratification and personalise treatment strategies for

cancer patients. Though diverse clinical, histopathological, and genetic factors have been utilized to assess patient prognosis, the quest for more precise and personalized biomarkers persists. These biomarkers are essential for enhancing patient outcomes, guiding treatment decisions, and optimising cancer care.

Currently, genomic studies in mesothelioma are still relatively scarce, [344] and the tumour mutational burden (TMB) in mesothelioma is low, with a lack of genetic targets for contemporary TKI. [344] Hence, it is not recommended to conduct testing for predictive biomarkers of response to non-surgical therapies for PM. [345] In contrast, the molecular landscape of lung cancer is diverse and complex, and several molecular studies performed in NSCLC, especially lung AC, led to a better understanding of the biology of lung cancer, revealing a high TMB, and today there are many predictive biomarkers for NSCLC. Advancements in the detection and treatment of NSCLC have resulted in enhanced survival rates. [172, 174]

There are different types of predictive biomarkers including predictive biomarkers for chemotherapy, targeted therapies, and immunotherapy (Figure 8). The novel biomarker used in three Papers included in the thesis (Papers I, III, and IV), PD-L1, is a recently identified predictive biomarker for immunotherapy with ICIs that offers insight into therapy response across various types of cancer. In addition, *EGFR* and *KRAS* mutations function as predictive biomarkers for targeted therapies and need to be evaluated for treatment with TKIs, playing an important role in the treatment of NSCLC, and is one of the focuses of Paper IV.

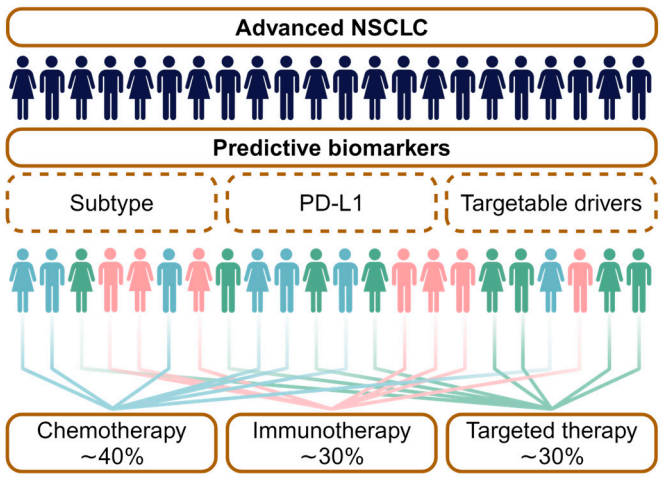

Figure 8. Schematic overview of the main predictive biomarkers used in precision medicine of advanced non-small cell lung cancer patients. PD-L1 = programmed death-ligand 1. Created with BioRender.com

Predictive biomarkers for immunotherapy

Programmed death-ligand 1 (PD-L1)

Immune checkpoint inhibitors (ICIs) have saved many lives and were discovered by James P. Allison and Tasuku Honjo, who later received the 2018 Nobel Prise in Physiology and Medicine. [346] Although the hypothesis of cancer immune surveillance was introduced already at the beginning of the 20th century, the first ICI was not approved for the treatment until 2011. [347] However, Allison discovered an antibody that blocked cytotoxic T-cell lymphocyte-associated protein 4 (CTLA-4) on T-cells, causing the tumour cells to fail to bind, and T-cells to be reactivated and attack the tumour cells. Honjo discovered another receptor on the T-cells, programmed cell death-1 (PD-1), which had a different mechanism. After further studies, anti-PD-L1 antibodies were produced that blocked the receptor so its ligand, programmed death-ligand 1 (PD-L1) on the tumour cells could not bind and reduce the T-cells' activity. This led to the T-cells being activated and able to attack the tumour cells. Today, there are several approved drugs with different points of attack, where nivolumab and pembrolizumab are antibodies against PD-1 that block PD-L1 and PD-L2 from binding to the receptor, while durvalumab targets PD-L1. [347, 348] Three of the five Papers included in this thesis focused on PD-L1.

PD-1 and its ligand PD-L1 play a pivotal role in regulating T-cell activity. PD-1 is a type I transmembrane glycoprotein that is expressed on the surface of activated T-lymphocytes. PD-1 serves as a coinhibitory, i.e., a negative regulatory receptor on T-cells, preventing immune activation. [349]

PD-L1, also known as B7 homolog 1 (B7-H1), serves as a dominant mediator of immunosuppression, functioning as a negative costimulatory molecule. [349] PD-L1 is expressed to a highly variable extent across various types of cells and tissues, including the placenta, pancreatic islet cells, mesenchymal stem cells, certain non-hematopoietic tissues, as well as on antigen-presenting cells (APC) such as macrophages and dendritic cells. [350] PD-L1 expression can also be upregulated in tumour cells and has been observed in numerous malignancies, serving as a mechanism for malignant cells to evade the immune system. [351] This process enables tumour cells to avoid T-cell cytolysis and promote their survival and cancer formation. [352] Upon PD-L1 binding to the PD-1 receptor, it inhibits the proliferation and function of activated lymphocytes, [353] resulting in a condition of T-cell hyporeactivity. [354]

In physiological terms, the PD-1/PD-L1 pathway has evolved to regulate the extent of inflammation at sites expressing the antigen, thereby ensuring the protection of normal tissue from damage. A notable PD-1 protein expression occurs on the surface of all activated cells. When T-cells recognise peptide antigens expressed by the MHC complexes on the target cells, it triggers the production of inflammatory cytokines, thus initiating the inflammatory process. As a result of these cytokines, PD-L1 expression increases in the tissue, which activates the PD-1

protein on the T-cells, ultimately regulating the activity of T-cells and leading to immune tolerance. [347]

PD-L2 is another ligand that competes for interaction with PD-1, but is presented on a limited number of cells, i.e., dendritic cells, mast cells, and macrophages. This ligand has a higher affinity to PD-1 than PD-L1; however, PD-L1 is much more highly expressed in more cells. PD-L1 is the ligand that is used in cancer diagnostics as drugs with antibodies against PD-L1 have been developed and much less is known about PD-L2, but more research is being done on that ligand. [347, 355]

Tumour cells release antigens (Ag) that can be recognised and bind to APCs, initiating the activation of T-cell receptor (TCR) through MHC binding. APCs present the antigens to the inactive T-cells, using their MHC complexes on the surface. On the surface of the T-cells there are TCRs that bind to the MHC complex on the APC surface (further with binding of APC CD80/86 to T-cell CD28), and this interaction leads to the activation of the T-cells into T-helper cells (CD4+) and cytotoxic T-cells (CD8+),[355] which in turn presents PD-1 on their surfaces. PD-L1 expressed on APC has the ability to bind to PD-1 on the activated T-cell, and this interaction leads to inhibition of T-cell activity. In cancer, tumour cells can also serve as APCs. Tumour cells can similarly present PD-L1 on their surfaces and bind to PD-1 membrane-bound receptors on the T-cell and mimic the APC. This causes the tumour cells to send out inhibitory signals and take control of the T-cells, which in turn causes the T-cells to become exhausted and lose their function.

Inflammatory cytokines i.e., interferon-gamma (IFN-γ) is secreted by activated T-cells which in turn proliferate, amplifying the immune response against tumour cells through activation of other immune cells to participate in the antitumour response. [348] CD4+ cells stimulate B lymphocytes to produce antigen-specific antibodies, while CD8+ cells directly eliminate the tumour cells. A minority of CD4+ cells constitute the NK cells and Treg cells.

Production of IFN-γ can also promote the expression of PD-L1 in tumour cells, which may protect tumour cells from cytotoxic cells, inhibiting the immune system. [347, 348]

PD-L1 expression has been observed in several malignancies, including lung cancer and mesothelioma. [356, 357] In NSCLC, PD-L1 expression, evaluated by IHC staining, is used to predict the response to ICIs. [358-361] Various commercial PD-L1 assays are available for different PD-1/PD-L1 inhibitors, and several studies have compared these assays. [362-364] The PD-L1 clones 28-8, 22C3, and SP263 demonstrate similar staining patterns, whereas SP142 exhibits differences from the others (Figure 9). Since ICIs are used in advanced and recurrent NSCLC, biopsies have been the primary sample type for PD-L1 assessment in clinical trials, [358-361] and are considered the gold standard.

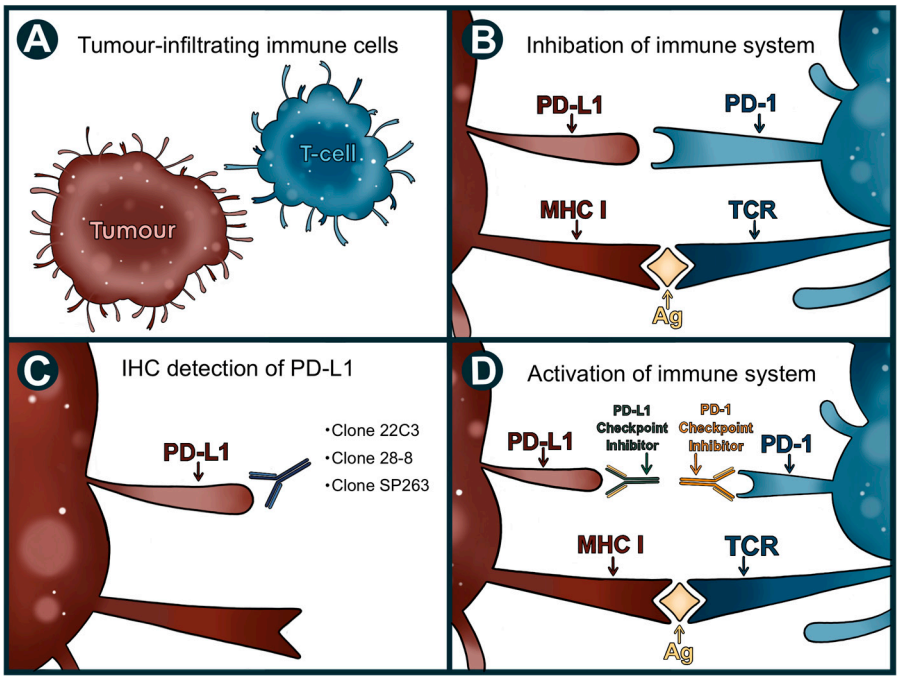

Figure 9. Schematic illustration of the PD-1/PD-L1 binding, immunohistochemical detection of PD-L1, and the principle of immune chechpoint inhibitors against PD-1/PD-L1 pathway. PD-1 = programmed cell death 1, PD-L1 = programmed death-ligand 1. Created by Julia Hansten.

Predictive biomarkers for targeted therapies

Epidermal growth factor receptor (*EGFR*)

The Epidermal growth factor receptor (*EGFR*) gene, commonly known as *HER1* or *ErbB1*, is situated on chromosome *7p11.2*. It is responsible for encoding a 170 kDa glycoprotein. *EGFR* functions as a receptor tyrosine kinase (RTK) and is part of the membrane-bound ErbB/HER family of RTK. [365]

In the early 1960s, Cohen was the first to discover EGF as a protein capable of stimulating the proliferation of epithelial cells. [366] A decade later, Carpenter identified a specific binding receptor for EGF on target cells, [367] which was subsequently termed *EGFR*. In the early 1980s, *EGFR* protein and its corresponding gene were cloned, and *EGFR* characterised as RTK by Ullrich *et al*. [368] The clinical significance of this receptor in NSCLC was revealed concurrently with its discovery. [369] The *EGFR* pathway plays a crucial role in maintaining epithelial tissues, and typically provides a robust signal for epithelial cell proliferation and survival during organogenesis and tissue repair. [365]

EGFR is considered one of the most commonly mutated oncogenes in lung cancer and various other types of cancer. [365] Activating mutations in *EGFR* tyrosine kinase domain is found in 10-40% of NSCLC patients. However, the frequency varies

significantly depending on ethnicity. The prevalence of these mutations ranges from 10-30% in Caucasians, while it increases to 40-60% in Asians. [365, 370] The incidence of these mutations is roughly three times higher in non-smokers compared to smokers, as well as in women compared to men. [365]

In recent decades, *EGFR*-targeted therapies have defined a new era in precision oncology. The discovery of *EGFR* led to a race to develop anti-*EGFR* treatments for lung cancer. In the mid-2000s, researchers discovered the first NSCLC-specific driver mutation in the *EGFR* gene, which ultimately enabled the prediction of response to anti-*EGFR* therapy. [371] Patients who harbour *EGFR* mutations commonly exhibit a notable response to treatment. [365, 370, 371] However, resistance commonly emerges within a few months.

Specifically, the first-generation *EGFR*-TKIs, gefitinib and erlotinib, showed significant survival benefits and dramatic responses to treatment in non-smoking, young Asian women with adenocarcinoma, changing the therapeutic landscape of NSCLC from a solely histology-based approach to testing molecular subtypes based on their genetic alteration variability. [365]

Today, TKIs serve as the standard first-line treatment for patients with advanced NSCLC who classically harbour *Ex19Dels* or *L858R EGFR*-mutations. The TKIs include three generations, first-generation i.e., gefitinib and erlotinib, second-generation i.e., afatinib, and third-generation i.e., osimertinib, which all have demonstrated significant improvements in progression-free survival (PFS). [365]

Clinical trials indicated that patients with *EGFR* mutations have exhibited limited clinical benefits with ICI monotherapy. [370] However, recent reports indicate an improved survival with ICI-based combinations in patients with *EGFR*-mutant advanced NSCLC who have progressed on *EGFR*-TKIs. [370]

Kristen rat sarcoma virus (*KRAS*)

Rat sarcoma virus (*RAS*) GTPase proteins serve as crucial components in cellular proliferation, growth, and differentiation. To date, three different isoforms of *RAS* proteins have been identified: *KRAS*, *NRAS*, and *HRAS*. Mutations in the Kirsten rat sarcoma gene (*KRAS*) have been identified as tumour drivers and are commonly observed in various human malignancies, including NSCLC. *KRAS* mutations are detected in approximately 25-30% of cases NSCLC cases, with the highest frequency observed at codons 12 and 13. [372, 373] These mutations are associated with adenocarcinoma histology, a history of smoking, and Caucasian ethnicity, though variations exist between different *KRAS* mutational subtypes.

The *KRAS* protein serves as an intracellular messenger, binding either guanosine triphosphate (GTP) or guanosine diphosphate (GDP). The protein undergoes a transition from its inactive GDP-bound state to an active GTP-bound state, catalysed by upstream actor enzymes in the signalling cascade, which triggers downstream mitogenic signalling. Substitutions of amino acids at position 12 or 13 cause the protein to become trapped in an active GTP-bound state. [372] Frequently, *KRAS*

mutations co-occur with other molecular alterations, especially *TP53*, *STK11*, and *KEAP1*, which potentially affect treatment effectiveness and patient outcomes. [372]

For a considerable duration, *KRAS* mutations have been deemed challenging to target therapeutically, and considered undruggable, primarily due to their high toxicity profile and the limited specificity of available compounds. Two novel *KRAS* inhibitors i.e., sotorasib and adagrasib, have recently been approved for treating *KRAS*-mutant NSCLC patients with *G12C* mutations. *KRAS*-mutated tumours also show an overall high PD-L1 expression, and these patients are also eligible to be treated with ICIs against PD-1/PD-L1. Therefore, the efficacy of ICIs requires comprehensive evaluation, as responses to anti-PD-1/PD-L1 agents may be significantly influenced by concomitant mutations.

Detection techniques of biomarkers

Assessment of cell morphology through light microscopy of routine-stained slides may not always sufficiently differentiate between normal and abnormal cells or effectively characterise malignant cells. Not only the recognition of malignant cells poses a challenge, but also distinguishing between different types of tumours is difficult. Despite enhancements in routine staining techniques, these techniques often have to be combined with supplementary analyses based on molecular biology findings. [374] Examples of such analyses include immunostaining and in situ hybridization techniques (e.g. FISH and CISH) for selected mutation and amplification analysis. [375] However, studies indicate that molecular changes manifest well before cancer becomes clinically visible, implying that early detection through molecular diagnosis can significantly improve patient survival rates and outcomes. [376, 377]

The choice of technique depends on the characteristics of the biomarkers that are intended to be utilized. Biomarkers can be categorized into three main types based on their characteristics: molecular, cellular, and imaging biomarkers. [246]

The molecular biomarkers are indicators measured using proteomic and genomic techniques. Molecular biomarkers play a crucial role in diagnosing diseases and find applications in prognosis and management. These biomarkers can be measured in different biological samples such as biopsies and various cytological specimens and liquid biopsies (blood/plasma). Molecular biomarkers encompass a broad spectrum of molecules, spanning from small to large entities, including peptides, proteins, metabolites, nucleic acids i.e., DNA and RNA, and various other molecules. Molecular biomarkers may be categorized based on the detected molecules: chemicals, proteins, and genes. [246]

The protein biomarkers are valuable for detecting diverse biological changes and serve as indicators of the progression of inflammation, immunity, or related diseases including cancer. [246] The detection of protein biomarkers typically relies on

established immunoassays including immunostaining, flow cytometry, ELISA, and protein microarrays.

Genetic biomarkers include different genetic variations such as mutations and fusions, which have been most widely utilized as biomarkers for diagnosing disorders over the past few decades. DNA biomarkers represent the biggest category of biomarkers associated with several diseases and conditions. Genetic biomarkers can be identified within the DNA of all nucleated cells extracted from any biological sample, particularly in cancers, as many cancer cells accumulate somatic mutations. [246] Today, cell-free DNA can also be analysed for both diagnostic and predictive purposes, [378] but the liquid biopsies are beyond the scope of this thesis.

In this thesis, both protein and genetic biomarkers were included, and techniques used for the detection of these biomarkers are presented below.

Immunostaining

Immunostaining is based on the recognition of specific antigen-antibody interactions for detection or exclusion of specific antigens, typically proteins, in the tissues or cells, which are located in membranes, cytoplasm, or nucleus, using specific antibodies that bind to the target and can be visualized by chromogen as a (typically) brown colour. [41]

Different cell types contain structures with unique antigenic properties, [379, 380] and immunostaining is used to microscopically visualize important structures and properties of tissues and cells, by revealing and identifying different cellular components, detecting the presence, abundance, and localization of specific elements, and characterising the molecular properties of certain diseased tissues.

The technique has a history spanning over 70 years, but it wasn't until the 1990s that it gained widespread use in diagnostic pathology, [381] and it has been a valuable tool in cancer diagnostics for many decades.

The role of immunostaining in diagnostic pathology has broadened, with its application observed in approximately 11-38% of cases for the diagnosis of carcinoma. [382] Further, immunostaining is currently an established ancillary technique indispensable for both histological and cytological diagnosis, [383, 384] to achieve diagnostic accuracy and precision. [276, 384-389]

Immunostaining serves as a crucial complement to morphology in diagnosing pleuro-pulmonary tumours, [390-392] alongside considerations such as the patient's former cancer history, age, gender, risk factors, and radiological findings. Recently, immunostaining has also been developed to assess prognostic and predictive biomarkers for therapeutic decision-making in many malignancies, including lung tumours. [41, 243, 245]

For a long time, biomarkers for immunostaining have been a part of routine diagnostics using antibodies, either stand-alone or more often within panels of antibodies to affirm or negate a diagnosis, contributing to high sensitivity and specificity in cancer diagnosis, [276, 385, 387] especially for certain differential diagnoses.

[389] Effective utilization of immunostaining requires a comprehensive assessment and the appropriate combination of both positive and negative antibodies. However, the diagnostic of malignancies is based initially on conventional staining i.e. H&E before immunostaining, to distinguish between neoplastic and non-neoplastic alterations, and immunostaining is considered as an ancillary technique. [393]

Immunostaining in histology and cytology

Different terms for immunostaining, also known as immunochemistry, may be used depending on the diagnostic material. Immunostaining is established and routinely performed on FFPE histological material, called immunohistochemistry (IHC). It can also be performed using cytological specimens; the method is then called immunocytochemistry (ICC).

The principles underlying immunostaining for both histological and cytological materials are fundamentally the same. However, there are both biological and methodological aspects that should be considered when a comparison is made between histology and cytology. This is because differences can affect the interpretation of immunostaining which might lead to inaccurate final diagnosis.

The majority of ancillary techniques, including immunostaining, are designed for and mainly performed on FFPE tissue specimens. Nevertheless, many early reports demonstrated the benefits of immunostaining on cytology, [394] and various preparations of cytological specimens for immunostaining are currently used, including air-dried, ethanol- or formalin-fixed smears, cytospins, LBC preparations, and cellblock preparations. [231, 387] The variety of cytological preparation methods may lead to varied results influenced by the specific technique used. [395-397] Hence, it is advisable to evaluate antibodies and validate methods before applying them to cytological specimens. [395] Ideally, employing the same fixation and technique for both immunohistochemistry and immunocytochemistry would offer significant advantages.

In addition, studies emphasize that formalin-fixed tissue is also suitable for other, modern molecular analyses, [398] rendering FFPE the preferred method for ancillary techniques, even in cytological material. However, there are also studies indicating that non-formalin fixation may be superior for molecular analyses. [399, 400]

Immunostaining technique

The IHC process involves various key steps for successful analysis. The process of immunostaining starts with deparaffinization of FFPE specimens, typically by using xylene, and thereon rehydration using graded washes of ethanol to water.

The ability of the antibodies to recognize epitopes of proteins can be affected by the FFPE procedure which can cause cross-linking of proteins and may have led to alterations in the antigens. Therefore, the process of unmasking epitopes that are tied up in cross-links is essential and is typically performed with heat or digestive enzymes. This process is called antigen retrieval.

Antibodies can exist as either monoclonal, binding to a single epitope, or polyclonal, binding to various epitopes on the same antigen. The immunostaining can be performed using different principles of techniques, either direct with one step, indirect with two steps, or indirect with three steps. A schematic illustration of the basic principles of different techniques of immunostaining is presented in Figure 10.

The indirect method of sandwich procedure is more commonly used. This approach offers several advantages e.g., it enhances versatility, allows for the use of the primary antibody at a higher working dilution, and the preparation of the secondary antibody is easily accomplished with a high level of specificity and affinity. [401]

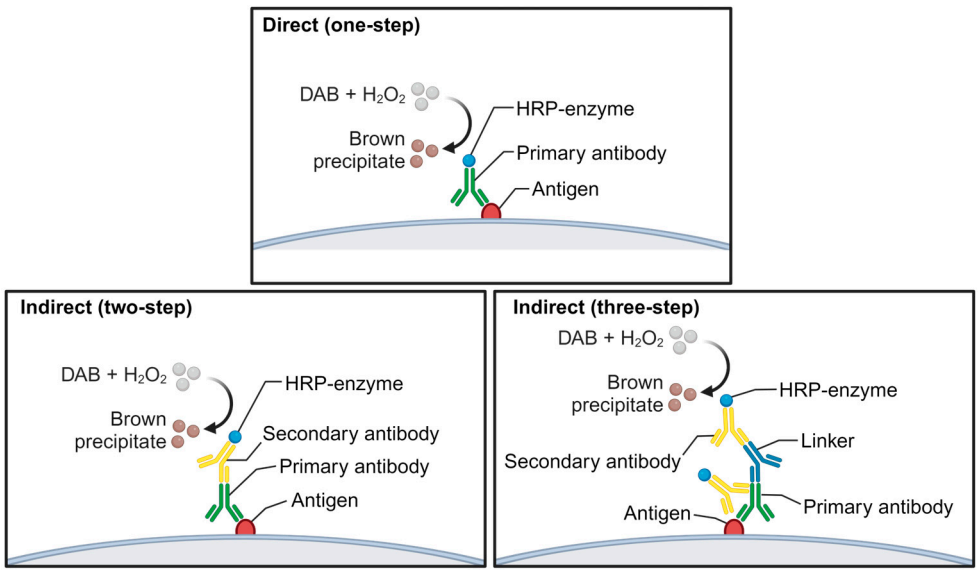

Figure 10. Schematic overview of immunostaining. Illustration of the basic principle of immunohistochemical staining, regardless of different visualisation systems. A primary antibody binds to specific epitopes on the antigen. The secondary antibodies bind to the primary antibodies. A chemical substrate that reacts with the horseradish peroxidase (HRP) enzyme is added, which creates a brown precipitate that can be studied in a microscope. Created with BioRender.com

Immunostaining visualisation systems

The process of immunostaining can be performed either manually or automatically, with the preparation technique adapting to the nature of the material being analysed. Automatic immunostaining is today performed using various IHC staining platforms, and the platforms use different visualisation systems. In the studies included in this thesis, three different IHC platforms were used, Autostainer Link 48, or Dako Omnis (Agilent Technologies, Santa Clara, CA, USA), or BenchMark Ultra (Ventana Medical Systems, Inc., Tucson, AZ, USA).

The Autostainer Link 48 and Dako Omnis use the EnVision™ FLEX or FLEX+ visualisation systems, while the Ventana BenchMark Ultra uses the UltraView or OptiView visualisation systems. Detailed data for all antibodies used in the studies in this thesis, platforms, and visualisation systems are reported in the Material and Methods section (Table II).

EnVision™ visualisation systems

The EnVision™ FLEX visualisation system is an indirect immunostaining technique, where the sections are initially stained with a primary antibody that binds to specific epitopes on the antigen. Peroxidase-Blocking Reagent, consisting of hydrogen peroxide in a buffer solution, is added to block endogenous peroxidase which reduces unspecific signals (it is done either before or after incubation of primary antibody depending on the platform used, e.g., it occurs before binding of primary antibody for Autostainer, while after binding of primary antibody for Omnis). A polymer conjugate is added, comprising multiple secondary antibodies and enzyme molecules called horseradish peroxidase (HRP) that are attached to a dextran backbone. The secondary antibodies are of goat origin and are raised against mouse and rabbit immunoglobulins. The secondary antibodies bind to the primary antibodies. Subsequently, a chemical mixture consisting of hydrogen peroxide (H_2O_2) substrate and 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen, is added, reacting with the HRP enzyme, which generates a brown precipitate that can be evaluated under a light microscope. Lastly, counterstaining with haematoxylin stain is applied to visualise cellular structures.

The EnVision™ FLEX+ visualisation system is essentially the same but includes Linker antibodies (mouse Linker or rabbit Linker depending on the host species of the primary antibody) that bind to the primary antibody and facilitate multiple secondary antibodies to bind to both the Linker and the primary antibody, which in turn strengthens the detected signals. The EnVision™ visualisation system is schematically presented in Figure 11.

UltraView and OptiView visualisation systems

The UltraView visualisation system of the antibody-antigen binding is achieved through an indirect detection method. The sections are first stained with a primary antibody that binds to specific epitopes on the antigen. Peroxidase-Blocking Reagent, consisting of hydrogen peroxide in a buffer solution, is added to block endogenous peroxidase which reduces unspecific signals (e.g., for UltraView is typically before binding of primary antibody). The primary specific antibody is located by an enzyme-labelled secondary antibody attached to HRP multimer conjugate. The complex is subsequently visualized using a H_2O_2 substrate and DAB chromogen that reacts with the HRP enzyme, resulting in a brown precipitate that can be observed by a light microscope. The copper sulphate is used to modify the brown precipitate and thereby amplify the signal. Finally, counterstaining with haematoxylin stain is applied to visualise cellular structures.

The OptiView visualisation system is basically the same, but the primary specific antibody is located by a designated specific secondary antibody (HQ Linker, which is a universal linker contains a cocktail of HQ-labelled antibodies) that is bound by an enzyme-labelled tertiary antibody (HRP multimer conjugate containing a monoclonal anti-HQ-labelled HRP tertiary antibody). This step also strengthens the detected signals. The UltraView and OptiView systems are schematically presented in Figure 11.

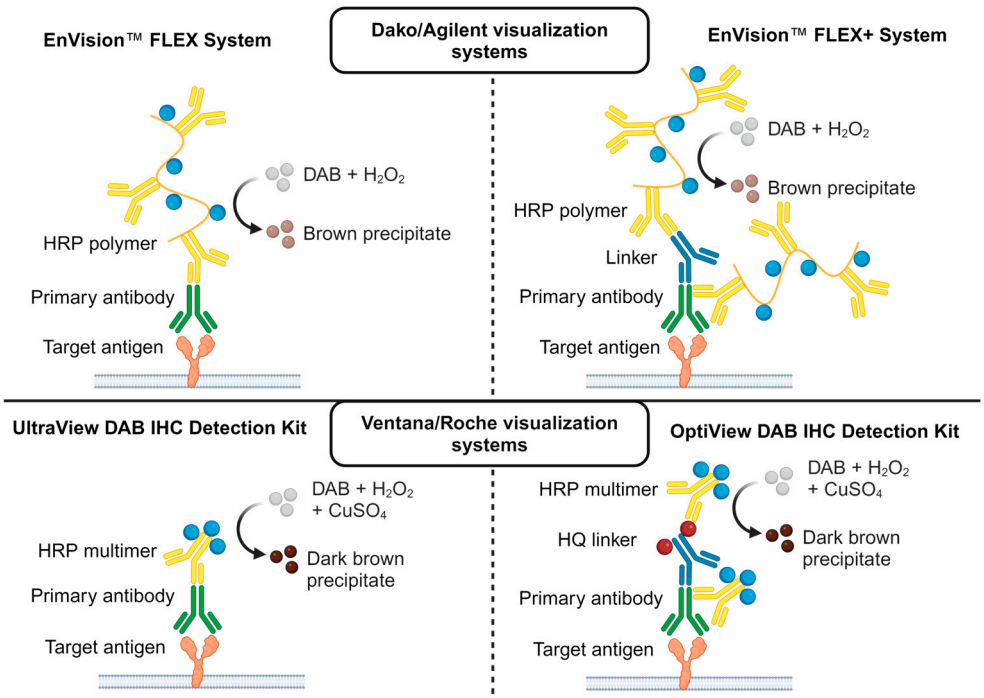

Figure 11. Schematic illustration of EnVision™, UltraView and OptiView visualisation systems. These visualisation systems are established for DAKO/Agilent and VENTANA/Roche immunostaining platforms, which have the same basic principle, with small differences, including the use of linker and different substances. Created with BioRender.com

Factors affecting immunostaining

The immunoreactivity can be exhibiting positive, partially positive, or negative reactivity in the cells of interest, and the pattern of immunoreactivity can depend on biological factors such as tumour heterogeneity and methodological factors (Figure 12). There are several factors that constitute pitfalls, affecting the result of IHC staining. Those encompass various stages throughout tissue handling, the staining process, and the methodology for evaluating the ultimate staining outcome. For instance, correct tissue handling is crucial as both under and over-fixation (over-fixation may be an influencing factor for certain sensitive antibodies but has been

shown to affect FISH and RNA analysis) can impact the intensity of the staining. [402] Moreover, considering that the sensitivity and specificity of the antibody significantly influence the outcomes, it is crucial to properly validate the antibody. In addition, the evaluation of the stained slide or scanned image relies on human eye observation, potentially leading to variations in interpretation of immunostains. A threshold "cut-off" for categorizing specimens as either positively or negatively expressing is frequently established for statistical purposes. However, the process of setting these thresholds for a particular biomarker lacks standardization, leading to discrepancies between studies. The absence of standardization across studies can lead to inconsistent results and challenges in comparing findings from different studies.

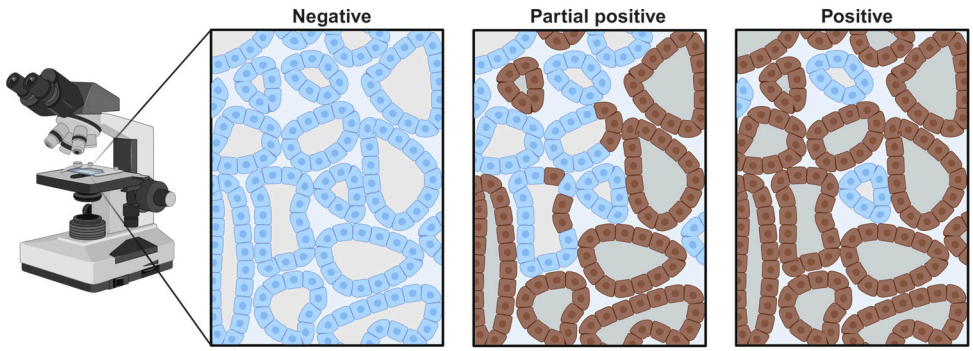

Figure 12. Schematic illustration of the pattern of immunoreactivity. Created with BioRender.com

Factors affecting immunostaining are usually divided into pre-analytical, analytical, and post-analytical factors. A summary including different factors is found in Table I. Additional considerations are that there may be other pitfall factors in studies such as case selection/bias, the proportion of included tumour subtypes, and diagnostic criteria.

Table I. Factors affecting the outcome of IHC stainings.

Type of Factor	Pre-analytical factors	Analytical factors	Post-analytical factors
Indications	Factors relating to the tissue	Factors relating to the analysis "staining"	Factors relating to the evaluation
Type of factors	• Handling artifacts: - laser - freezing • Pre-fixation conditions: - time to fixation (ischemic time) - temperature • The fixation conditions: - tissue type - tissue size - fixation type - medium volume - fixation time • Processing - dehydration - sectioning - drying of slides • Specimen type - cell block vs. cytospin vs. resection vs. biopsy vs. TMA • Specimen age - age of block - age of slide	• Epitope retrieval • Blocking • Antibody conditions: - antibody purity - choice of clone - dilution - time and temperature for primary and secondary antibody - other reagents • Detection system • Platform • Double staining	• Choice of cutoff value • Interpretation of pitfalls • Evaluation of the correct cells • Improper labeling • Age/storage of slide (fading)
Impact on outcomes	• Weak or absent immunoreactivity due to e.g. inadequate fixation and incomplete dehydration • Background staining can be caused by too thick sections of tissue, delayed fixation and necrotic tissue	• Weak or absent immunoreactivity due to e.g. prolonged heating • False negative outcomes • Difficulties in interpretation	• Incorrect diagnosis • Misleading diagnosis

Advantages and disadvantages of immunostaining

Although there may be potential disadvantages associated with immunostaining as mentioned above, it also offers numerous advantages. FFPE tissue sections are commonly utilized in clinical routine, making them easily accessible. Immunostaining is a rapid, cost-effective, and widely recognised method, contributing to its frequent use in research studies. Immunostaining facilitates the visualisation of the target antigen, allowing for quantification and in situ evaluation of the biomarker's cellular localization. Moreover, it is possible to evaluate the expression of the antigen across various types of cells within the tissue section.

Molecular pathology methods

Cancer is a genetic disease driven by genetic alterations. [403, 404] The genetic alterations leading to cancer can either be inherited, result from specific environmental exposures by a carcinogen, or due to errors that arise during cell division by a random mistake. The cell needs an accumulation of genetic alterations over many years to turn healthy cells into cancerous cells. The vast majority of cancers arise spontaneously as a consequence of this process over time. Genetic alterations may occur in oncogenes that may elevate the levels of proteins that e.g. keep the cells growing, or in tumour suppressor genes that reduce the levels of proteins that signal cells to stop growing, or in DNA repair genes that prevent the production of proteins that instruct cells to undergo self-destruction when they are damaged. Genetic alterations that are manifested in gametes are called germline mutations, while the majority of genetic alterations occur in other body cells, which is called somatic mutations.

There are multiple variants of genetic alterations in genes that control the way cells grow and multiply. The genetic alterations can be at the nucleotide level i.e., changes in a single nucleotide or more, or at the chromosomal level i.e., changes in a segment of DNA or more. The point mutations also known as single nucleotide variant (SNV) change a single nucleotide which is replaced by another nucleotide. These alterations can lead to a change in the amino acid sequence of the encoded protein, known as a missense mutation, or an early termination of the protein, referred to as a nonsense mutation. Frameshift mutations involve the addition or deletion of one or a few nucleotides, or more complex of one or a few bases, leading to a shift in the reading frame.

Frequently, mutations that contribute to cancer tend to cluster in “hotspots”, where tumours from various patients exhibit the same recurring mutation. Certain hotspot SNVs may be prevalent, while others are infrequent. For instance, the *BRAF V600E* mutation is present in 40% of all melanomas, whereas the *BRAF L597S* mutation occurs in less than 1% of all melanomas. Approximately 5% of individuals with cancer exhibit a point mutation in the *KRAS* gene.

Chromosomal alterations, also called chromosomal rearrangements, are alterations that modify the structure of the chromosomes. There are four types of chromosomal alterations i.e., deletion, duplication, inversion, and translocation. Gene fusion is a hybrid gene of transcripts derived from two independent genes and can occur as a result of either chromosomal rearrangements or splicing mechanisms that are non-chromosomal rearrangements. [405] These alterations can be at the gene or RNA level and represent a crucial category of somatic alterations in cancer, playing significant roles in the initial steps of tumorigenesis. [406-408] The splicing process can also generate complex RNA patterns within cells. [405]

Genetic tests that are used to identify genetic alterations which drive the growth of cancer may also be regarded as biomarkers. This information is crucial for therapy decision-making and personalized medicine. Molecular biomarker testing is also

called tumour profiling or molecular profiling. Tests for molecular profiling of tumours vary in complexity, ranging from simple to intricate methods. The common widespread technologies used for clinical testing in molecular pathology are real-time polymerase chain reaction (PCR) also known as quantitative PCR (qPCR), Sanger sequencing, multiplex ligation-dependent probe amplification (MLPA), fluorescence in situ hybridization (FISH), and next-generation sequencing (NGS) technologies.

In Paper IV, included in the thesis, the molecular analyses were performed using different techniques including FISH, qPCR, and NGS over time during the study period.

FISH is used to locate genes or specific sequences on one or more chromosomes, with detection performed using fluorescence microscopy. The technique requires unstained specimens with either paraffin-embedded sections or cell smears (usually for UroVysion) and is limited in its ability to detect most types of mutations found in solid tumour neoplasms. Chromosomal rearrangements or fusions can be detected using IHC, but the finding of positivity is then typically confirmed by FISH e.g., *ROS1* and *NTRK* translocations. However, for some fusions such as *ALK* there is approved IHC as a basis for treatment without confirmatory by FISH.

Many mutation detection methods depend on the PCR techniques to amplify the specific region of DNA of interest. Real-time PCR is widely used within many diagnostic fields. Real-time PCR is a well-established method, that enables the quantification of gene expression and verification of differential of genes, using commercially available fluorescence-detecting thermocyclers to amplify specific nucleic acid sequences and simultaneously measure their concentration. The molecular profiling of tumours is typically conducted on DNA extracted from FFPE tissue specimens. The limited size of DNA fragments from tissue blocks constrains the PCR, which forms the basis of most mutation detection methods. Therefore, prior to testing, a pathologist needs to examine the tissue specimen to confirm the presence of the tumour and determine the tumour content in the specimen.

In detail, PCR with fluorescent probes involves the addition of reporter probes for both the wild-type and mutant alleles to the reaction mixture. Required components include template DNA, primers, nucleotides (dNTPs), and thermostable DNA polymerase. [409] After hybridization to the DNA, the polymerase extends the probes in a complementary manner, releasing the reporter molecules for subsequent detection. Successive PCR cycles using the reporter probes lead to amplified signals, enabling the accurate measurement of one or both alleles of interest. Target sequences are both amplified and quantified within the same PCR instrument. Thus, the method provides an advantage of offering qualitative and quantitative analysis, which makes it flexible and adaptable for a wide range of applications. Real-time PCR does not necessitate internal standards for the quantification of DNA or RNA amounts. [410] The primary limitation of the technique is its inability to detect other mutations that may be present in tumour DNA.

NGS is a high-throughput, massively parallel DNA sequencing methodology that has revolutionized the field of molecular diagnostics since its introduction to the

market in 2004. NGS technologies enable targeted, whole exome, and whole genome sequencing, and may analyse both protein-coding and non-coding regions in the human genome. Thus, it has become a powerful tool to perform comprehensive tumour molecular profiling for cancer patients. The mutational profiles have been the basis for personalized medicine in cancer, enabling the genotyping of tumours and the targeting of specific treatments against gene alterations, such as the use of *EGFR* tyrosine kinase inhibitors and recently *KRAS* inhibitors in NSCLC.

There is a wide variety of NGS platforms available, but the two main NGS technologies are Ion Torrent and Illumina with different versions and series of platforms. These techniques have advantages and disadvantages related to their various testing modalities i.e., hybridization capture and amplicon sequencing, and differences in their chemistry, leading to varied sensitivity and specificity. [411]

In Paper IV, two cohorts from NSCLC specimens were analysed with Oncomine™ Focus Assay using Ion Torrent S5™, and with the TruSight Tumor 15 panel (TST15) using Illumina MiSeq™, respectively.

The initial sequencing step for both Ion Torrent and Illumina platforms involves immobilizing each DNA fragment and clonally amplifying it. Clonal amplification is essential to produce a signal of sufficient magnitude for detection. Ion Torrent relies on pH (voltage) change upon nucleotide binding and employs bead and emulsion for clonal amplification, whereas Illumina utilizes fluorescence for nucleotide detection and clonal amplification on a flow cell. The bead or flow cell harbours sequences that hybridize to a part of the adaptor on the DNA fragments. The optimal DNA concentration is crucial to guarantee the binding of only one DNA fragment per bead and to maintain well-spaced distribution of DNA fragments on the flow cell. During the clonal amplification step, a bead or cluster is formed, containing roughly 1000 identical copies of a unique parent DNA molecule that are physically isolated from other molecules. In the Ion Torrent, the beads are subsequently positioned in wells, with one bead per well. Ion Torrent boasts a slightly longer base pair read length, reaching up to 400 bp, whereas Illumina achieves a read length of 300 bp. Ion Torrent exhibits a shorter run time compared to Illumina, but is prone to homopolymer errors, whereas Illumina tends to have errors in GC-rich regions. [411]

Treatment of mesothelioma and lung cancer

In Sweden, the treatment of mesothelioma and lung cancer is carried out according to a national clinical cancer care guideline[174] that closely reflects the international consensus. For both malignancies, the majority of patients are diagnosed at an advanced stage of the disease, requiring systemic medical treatment. However, the treatment of mesothelioma remained unchanged for many years based on

chemotherapy, while the treatment of lung cancer has continuously undergone several changes due to advancements in therapy such as targeted therapy and immunotherapy, also including combination with chemotherapy as neoadjuvant or adjuvant therapies. Briefly, the clinical management of NSCLC patients with advanced stage has been influenced by precision medicine, but with mixed effects for different types of cancer. The advances have been most dramatic for NSCLC and more specifically for lung AC, since lung AC has a growing number of druggable oncogenic drivers. [412] Several additional advances have been made in this field, leading to changes in the therapeutic decision-making for advanced NSCLC. The variation in treatment response and progression of NSCLC among individuals can be attributed to inter-individual variation in genetic makeup, which is called tumour heterogeneity (Figure 1).

The choice of treatment for NSCLC is determined by various factors such as stage of the disease, subtype, and has also evolved with the introduction of several lines of TKIs in patients with *EGFR*, *ALK*, *ROS1*, *KRAS* and other genetic alterations. Similarly, ICIs have dramatically changed the landscape of NSCLC treatment. [413] ICIs are now part of the first-line treatment of NSCLC as monotherapy, combined with chemotherapy, or as adjuvant treatment after definite chemo-radiotherapy in patients with stage III unresectable NSCLC. For metastatic lung cancer the treatment landscape has changed drastically with the introduction of targeted therapy with TKIs and ICI as a complement to chemotherapy. Furthermore, the results of new trials continue to help us understand the role of these novel agents and which patients are more likely to benefit.

Treatment of mesothelioma

Surgery and chemotherapy of PM

Mesothelioma is a highly aggressive neoplasm associated with elevated morbidity and mortality rates. Traditionally, the treatment options for mesothelioma includes surgery, chemotherapy, or a combination of both approaches typically also with radiation therapy. [414]

There are three surgical procedures available for PM including pleurodesis, pleurectomy with decortication, and extrapleural pneumonectomy. [415, 416] To address recurrent pleural effusions, pleurodesis prevents their formation by introducing talc into the thoracic cavity, which effectively seals the space between the pleural layers. [415] Pleurectomy with decortication entails the removal of the pleura along with the excision of visible tumours, while extrapleural pneumonectomy involves the removal of the parietal pleura, the whole lung, and part of pericardium and diaphragm. [415, 416] However, surgery is seldom a treatment option and is only eligible for a limited subset of patients.

PM represents an aggressive tumour and is a chemotherapy-resistant malignancy with a dismal prognosis. [417] Although initial response rates to single-agent

chemotherapy were disappointing, a combination of the platinum compound cisplatin and anti-folate/antimetabolite drug pemetrexed chemotherapies have remained the cornerstone of PM treatment for the past three decades. This doublet chemotherapy has demonstrated an overall survival of 12.1 months in patients compared to the 9.3 months achieved with single-agent cisplatin. [418] Despite the limited efficacy of doublet chemotherapy, the combination of cisplatin and pemetrexed is endorsed by the Swedish national clinical cancer care guidelines as the standard of care for PM patients. Incorporating an antiangiogenic agent into chemotherapy resulted in modest enhancements in survival, both when administrated up front in combination with platinum/anti-folate and in case of relapse. [417]

Targeted therapies and immunotherapy for PM

In the past decade, there has been a gradual elucidation of the genetic and transcriptomic landscape of PM, revealing a low somatic mutation burden, with no difference observed between histological subtypes as reported in several reports. [285, 344] A genomic analysis examined gene expression data from 216 PM tumours, revealed recurrent mutations leading to the loss-of-function of pivotal tumour suppressors. [344] The most common genetic events are somatic copy number alterations, mainly deletions, which are most frequent in *CDKN2A*. [285, 344] Homozygous loss of *CDKN2A* is most frequently observed in sarcomatoid mesotheliomas, followed by biphasic and epithelioid subtypes. The comprehensive genomic analyses revealed that the most common mutated genes include *BAP1*, *NF2*, *TP53*, and *SETD2*. [285, 344] *BAP1* mutations have been reported in several studies, indicating that up to 67% of PM cases harbour a *BAP1* mutation. [282] In general, the genetic landscape of PM is characterised by mutations in tumour suppressor genes, leading to subsequent loss-of-function in tumour suppressor proteins. [344]

Despite the growing focus on PM genomics, there are no potential agents against these targets and only a few studies are being conducted. The protein product encoded by the *MTAP* gene, known as methylthioadenosine phosphorylase (MTAP), plays a crucial role in adenosine monophosphate and methionine salvage pathways. The discussion around therapies exploiting synthetic lethality in *MTAP*-deficient tumours underscores its potential significance. [289] *In vitro* evidence demonstrated that the deficiency of *MTAP* has emerged as a promising target for therapeutic intervention. [419] Recently, the development of novel personalised therapeutics for the treatment of PM has been discussed, including those targeting DNA repair and *EZH2* pathways. Nonetheless, trials have yielded variable outcomes. [417]

The use of immunotherapy, especially those targeting PD-(L)1, has shown important albeit variable efficacy in relapsed PM when used as monotherapy, serving as crucial salvage treatment following first-line chemotherapy. [417] Also, in early clinical trials, the combination of immunotherapy and chemotherapy has

shown modest potential.[420] However, first-line treatment since a few years is double ICI with PD-1 inhibitor nivolumab and the CTLA-4 inhibitor ipilimumab,[421] regardless of PD-L1 expression in the tumour, and upon progression chemotherapy.[174] These findings indicate the efficacy of ICI in treating PM, but a recent systematic review and meta-analysis including 43 studies has demonstrated a slight advantage in OS.[420] Yet, there is no established standard of care for PM, and it is recommended to conduct further randomized controlled trials with consistent criteria and outcomes to steer subsequent therapy in relapsed PM. These trials can also help identify patients with specific characteristics who might benefit from such subsequent therapy.[420]

The field of PM is evolving, emerging treatments offer hope for a largely lethal and challenging malignancy. Despite many developments, there is still lack of understanding of the role of combination and multimodal therapies, the identification of factors driving treatment resistance, and the establishment of predictive biomarkers to enhance patient selection and treatment sequencing. The most well-known reason for variable responses to treatment of PM, regardless of the type of treatment, is histological type. Therefore, the determination of histological subtypes of mesotheliomas holds prognostic significance and plays a pivotal role in determining treatment decisions for patients diagnosed with this lethal disease. The WHO classification of pleural tumours has recently defined the grading and subtyping of epithelioid mesotheliomas.[96] Identifying additional prognostic significance of certain histological subgroups and introducing a grading system for epithelioid mesothelioma may enhance the clinical stratification and management of these patients.[111, 422] Indeed, patients with the epithelioid subtype are potentially eligible for surgical treatment and more likely to derive benefit from surgical treatment in specific circumstances,[168, 345] and have a more favourable OS compared to other subtypes.[150, 423] In addition, double ICI as the new standard first-line treatment for PM has been shown to improve OS, particularly in those with non-epithelioid histology.[417] In contrast, one study showed that dual ICI continued to provide OS benefits compared with chemotherapy, irrespective of histology.[421] Lastly, the concept of mesothelioma in situ has been redefined and may offer the potential for earlier intervention.

Treatment of lung cancer

Surgery and chemotherapy of NSCLC

Apart from the patient's will and general condition, the choice of therapy for NSCLC is affected by stage, histological type, and treatment-predictive alterations.[174] However, the approach of NSCLC is mainly stage-specific, and treatment choices hinge on the clinical stage, which is determined by the primary tumour status, nodal involvement, and distant metastasis according to the TNM system.[96] For patients diagnosed with stage I or II NSCLC, complete surgical resection is

recommended when there are no contraindications. Patients with stage I who are not candidates for surgery should be considered for stereotactic radiotherapy.

Medical intervention including chemotherapy, targeted therapy, and immunotherapy, enhances outcomes across all clinical stages, but different patients show different treatment responses related to different clinicopathological factors and TMB. This is particularly true for ACs which harbouring a high TMB compared to many other malignancies. The clinical use of chemotherapy as a standalone treatment for NSCLC has decreased due to the introduction of targeted therapies and immunotherapy. However, it still holds significance in combined regimens alongside radiotherapy for curative purposes and combined with immunotherapy for palliative care. For lung cancer, the preferred chemotherapy treatment regimens typically involve platinum-based doublets, where either cisplatin or carboplatin is paired primarily with another chemotherapeutic agent that operates through a different mechanism of action. In the treatment of NSCLC, commonly utilized drugs include microtubule inhibitors such as vinorelbine, docetaxel, and paclitaxel, as well as antimetabolites like pemetrexed and gemcitabine. The choice of chemotherapy is affected by histology. While platinum-based combination regimens are used for both histological types, pemetrexed and bevacizumab are not used for SCC due to lack of effect and risk of severe haemorrhage, respectively. [424, 425]

Percutaneous thermal ablation techniques including cryoablation, microwave, and radiofrequency ablation, can used as treatment alternatives for salvage therapy following surgery, radiotherapy, or chemotherapy, as well as for palliative care in advanced NSCLC cases.

Targeted therapies and immunotherapy for NSCLC

Early-stage tumours that are larger or have spread to regional lymph nodes are typically treated with surgery and adjuvant therapy (or sometimes neoadjuvant therapy, if limited involvement of mediastinal lymph nodes). Today, in addition to chemotherapy and radiation therapy, both immunotherapy and tyrosine kinase inhibitors (TKI) for *EGFR* may be used as adjuvant therapy in combination with chemotherapy in case of positive PD-L1 (an indication not existing when the thesis project started) or the occurrence of an *EGFR* mutation, respectively. [426] For locally advanced NSCLC where surgery is not deemed an option, a combination of chemotherapy and radiation therapy is used for curative intent.

In metastasizing NSCLC, the first-hand choice of treatment is typically TKI if a driver molecular alteration (such as *EGFR* mutation or *ALK* translocation) exists. Otherwise, combined chemotherapy and immunotherapy is typically used, but ICI as monotherapy is an option in case of high PD-L1 expression in the tumour. Here, the indication for immunotherapy has changed over time, as upon introduction, a positive or high PD-L1 test was required for ICI in the second and first line, respectively. In addition to molecular treatment-predictive alterations being more common in AC than SCC. [412]

TKIs have been a standard part of NSCLC treatment since 2004, following the approval of the first *EGFR* TKIs for clinical use. Currently, there are TKIs employed in NSCLC treatment which are specifically designed to target *EGFR*, *ALK*, *ROS*1, *RET*, the mesenchymal-epithelial transition kinase (*MET*), rapidly accelerated fibrosarcoma oncogene, homolog B (*BRAF*), *NTRK*, and recently *KRAS* subtype *G12C*.

The earlier trials of immunotherapy involving non-specific activators including interferon and high-dose interleukin 2 treatment, yielded considerable side effects and notably poor outcomes. [427] Yet, in the late 2000s, a trial involving ipilimumab, a groundbreaking cytotoxic T-cell lymphocyte-associated protein 4 (CTLA-4) inhibitory antibody, demonstrated a significant efficacy on patients with stage IV melanoma, [428] essentially heralding the onset of a new era in medical oncology. CTLA-4 serves as a co-inhibitory T cell receptor which competes with the co-stimulatory receptor, cluster of differentiation 28 (CD28), for binding to ligands CD80 and CD86 during antigen presentation. [429] Therefore, CTLA-4 blockade triggers substantial activation of T-cells. CTLA-4 is linked to autoimmune side effects because it is expressed on regulatory and early activated T-cells. [430] Ipilimumab is not currently included alone as a part of the standard treatment for NSCLC. However, it has been used as a component of a dual blockade combination immunotherapy alongside nivolumab (targeting PD-1) in clinical trials, yielding promising results. [431] The research focus subsequently shifted to another immune checkpoint, involving the PD-1 receptor and its ligand, PD-L1, mainly because of side effects related to the therapeutic effect of CTLA-4 blockade. PD-1 shares structural similarities with CTLA-4, and akin to CTLA-4, the interaction between PD-1 and PD-L1 operates as a co-inhibitory mechanism concurrently with antigen presentation. However, unlike CTLA-4, PD-L1 is inducible and exerts negative regulation over the response of T-cells that are actively involved in an effector T-cell response. [432] Upon ligand binding, phosphorylation of a C-terminal immune tyrosine-based inhibitory motif occurs, which in turn recruits Src homology region 2 domain-containing phosphatase 1 (SHP-1) and SHP-2. [433] Additionally, there is an upregulation of ubiquitin ligases Casitas B-cell lymphoma B (CBL-b) and c-CBL, both contributing to T-cell receptor down-modulation through diverse pathways. [434] Given that this process takes place within the tumour itself, the ectopic expression of PD-L1 serves as a tactic of immune evasion frequently utilized by tumour cells. [429] Blockade can target either PD-1 or PD-L1, and drugs for both targets have already received approval for clinical use. Blockade induces a marked increase in T-cell activation during antigen presentation, leading to an intratumoral immune response characterised by reduced systemic side effects, [435] which are linked to better response. [436] Today, immunotherapy employing PD-1/PD-L1 antibodies serves as the primary approach of modern medical treatment of NSCLC lacking druggable driver mutations. A schematic overview of the mechanism of immunotherapy targeting PD-1/PD-L1 is shown in Figure 13.

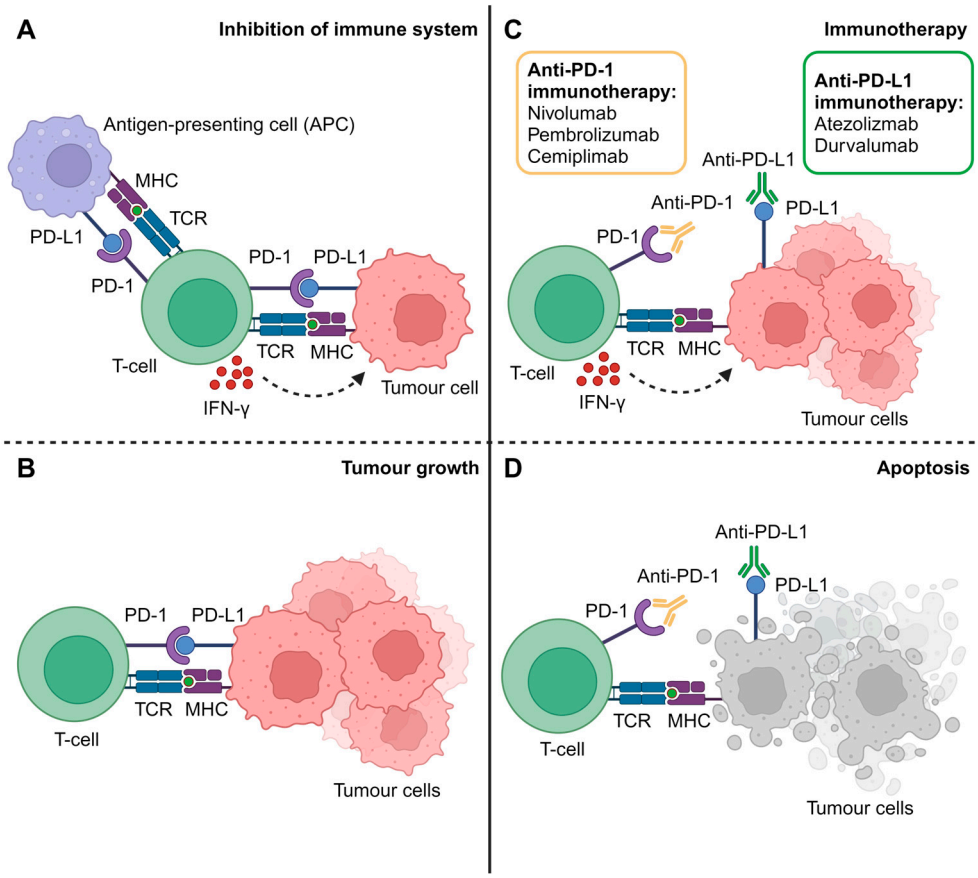

Figure 13. Schematic illustration of mechanism of immunotherapy against PD-1/PD-L1 pathway. The overview also provides some examples of different drugs target either PD-1 or PD-L1. PD-1 = programmed cell death 1, PD-L1 = programmed death-ligand 1. Created with BioRender.com

Rationale

The greatest glory in living lies not in never falling,
but in rising every time we fall.

Nelson Mandela, 1918-2013

This thesis is based on a series of studies, which all are focused on evaluating and improving diagnostics of PM and NSCLC using cytological specimens. The rationale behind the research studies is formed by the following key statements:

i. The majority of patients diagnosed at advanced stages of disease, where surgery is not an option, may benefit from chemotherapy, immunotherapy or targeted therapies, where diagnostic including subtyping and predictive biomarkers guide choice of therapy, all of which require material that preferably can be obtained by less invasive techniques. Cytology enables a quick, less invasive, and cheap investigation. In fact, as much as 80% of PM have effusion cytology specimens as the first indication of disease, and 40% of NSCLC are diagnosed only based on cytological specimens.

ii. Histological diagnosis has been considered as gold standard, and cytological diagnosis has historically been considered more controversial. Variable results in cytological studies reflect the usage of different preparation techniques, fixation methods and the lack of standardization, and cytology of pleuro-pulmonary tumours, including PM and NSCLC, is no exception.

iii. Despite the extensive use of cytology in clinical diagnostic routine, supported by several reports demonstrating that this technique is excellent for ancillary testing, cytology still faces many challenges related to a variety in preparation methods. Systematic comparisons are surprisingly lacking in several clinically relevant areas. Also, scientific evidence for cyto-histopathological correlation is more or less lacking for many ancillary analyses. Discrepancy in outcomes for ancillary analyses including immunostaining on cytologic material compared to biopsy shows that the accuracy of the diagnosis and treatment predictive basis can be influenced by the type of sample.

The role of cytology has changed dramatically, and cancer diagnostics based on cytology specimens has been crucial and is today an indispensable part in a clinical setting. The expanding use of ancillary analyses by clinicians to include not only diagnostic biomarkers for confirmation of diagnosis, but also for predictive biomarkers, which is necessary for therapy decision-making and personalized treatment in precision medicine, is crucial. This development requires more diagnostic material and the histological specimens, which may be small and limited, can in many cases be insufficient. Improvement and standardisation of cytological methods will lead to the full-utilization of the cytology samples, which are often the first and sometimes only specimens available. The current project may contribute to development of cytology to achieve its full potential in clinical practice. Reliable cytological diagnostics and predictive analyses can lead to earlier diagnosis of cancer and hence sometimes earlier start of treatment.

Aims of the Thesis

We cannot solve our problems with the same thinking
we used when we created them.

Albert Einstein, 1879-1955

Overall aim

The overarching objective of my dissertation has been to improve the cytopathological diagnostics of PM and NSCLC. The main strategy was to investigate the staining properties of different IHC biomarkers, particularly PD-L1 but also diagnostic markers in paired histopathological and cytopathological specimens from PM and NSCLC patients. The thesis also focused on the correlation between PD-L1 expression and oncogenic molecular alterations in unpaired histology and cytology specimens from NSCLC patients. Further, the thesis has highlighted the impact of different fixatives on the expression of IHC biomarkers in cytology preparations. In the long term, this thesis has provided important data and highlighted potential research areas concerning biomarkers in the diagnosis of PM and NSCLC in cytology.

Specific aims

The thesis is based on five studies, and the specific and major objectives of each study are presented and listed below, followed by an overview of the studies and papers included in this thesis, see Figure 14.

Paper I

To compare the expression of PD-L1 in paired histological and cytological specimens from PM patients.

Paper II

To compare the expression of eight mesothelial IHC biomarkers, calretinin, CK5, podoplanin, WT1, EMA, desmin, BAP1, and MTAP in paired histological and cytological specimens from PM patients.

Paper III

To compare the expression of PD-L1 in two cohorts of paired histological and cytological specimens from NSCLC patients, based on the standard procedures in southern Sweden. Another aim was to review the current literature on cyto-histological correlation of PD-L1 in NSCLC.

Paper IV

To deepen the understanding of the correlations between PD-L1 expression and various clinicopathological and molecular factors in two NSCLC cohorts.

Paper V

To prospectively explore the impact of different LBC fixatives on the immunoreactivity of nine IHC biomarkers, TTF-1 clones 8G7G3/1 and SPT24, napsin A, claudin 4, CEA, CK7, and EpCAM clones BS14, Ber-Ep4, and MOC-31, in matched cytology cell block preparations made from pleural effusions caused by lung AC.

	Pleural mesothelioma studies		Non-small cell lung cancer studies		
Study number	**Study I**	**Study II**	**Study III**	**Study IV**	**Study V**
Type of tumour	PM patients	PM patients	NSCLC patients	NSCLC patients	Lung AC patients
Number of cases	61 pairs of histological and cytological specimens	59 pairs of histological and cytological specimens	144 pairs of histological and cytological specimens	1782 unpaired histological and cytological specimens	24 quadruple matched cell block preparations made from pleural effusions
Objectives	Comparison of PD-L1 expression in paired histological and cytological specimens	Comparison of expression of eight mesothelial IHC biomarkers in paired histological and cytological specimens	Comparison of PD-L1 expression in two cohorts of paired histological and cytological specimens	Study of the correlations between PD-L1 expression and various clinicopathological and molecular factors	Exploring the impact of different fixatives on immunoreactivity of nine IHC biomarkers
Results	Results presented in Paper I	Results presented in Paper II	Results presented in Paper III	Results presented in Paper IV	Results presented in Paper V

Figure 14. Overview of the studies included in the thesis. Subtypes of the tumours and biomarkers used in Studies I-V and presented in Papers I-V. AC = adenocarcinoma, IHC = immunohistochemistry, NSCLC = non-small cell lung cancer, PD-L1 = programmed cell death ligand 1, PM = pleural mesothelioma.

Material and Methods

If the Olive Trees knew the hands that planted them,
Their Oil would become Tears.

Mahmoud Darwish, 1941-2008

Study design and study populations

The present investigation included different cohorts of PM and NSCLC patients in the various studies included in this thesis.

Paper I and II

Papers I and II in this thesis included 61 and 59, respectively, paired FFPE pleural biopsies and pleural effusion cell block preparations obtained from PM patients. All cases were retrieved from the archives of Skåne University Hospital in Malmö and Lund, and the Halland Hospital in Halmstad, in Southern Sweden. There were no differences in the sampling and preparation methods over time and between the hospitals. Further, the same inclusion criteria were applied for both cohorts included in these studies, and available paired specimens that fulfilled the following criteria were included: (a) the histological specimen was pleural biopsy; (b) the cytological specimen was a cell block from pleural effusion; (c) the biopsy was obtained at the same time as the collection of the effusion or within 12 weeks afterward; (d) both the histology and cytology samples for all cases had been collected before each patient had received any oncological treatment; (e) only cases with >100 evaluable tumour cells in both samples were included. Only one cell block and one biopsy from each patient were stained with antibodies of interest and included in the respective study. Detailed information on mesothelioma cohorts is exhibited in Figure 15.

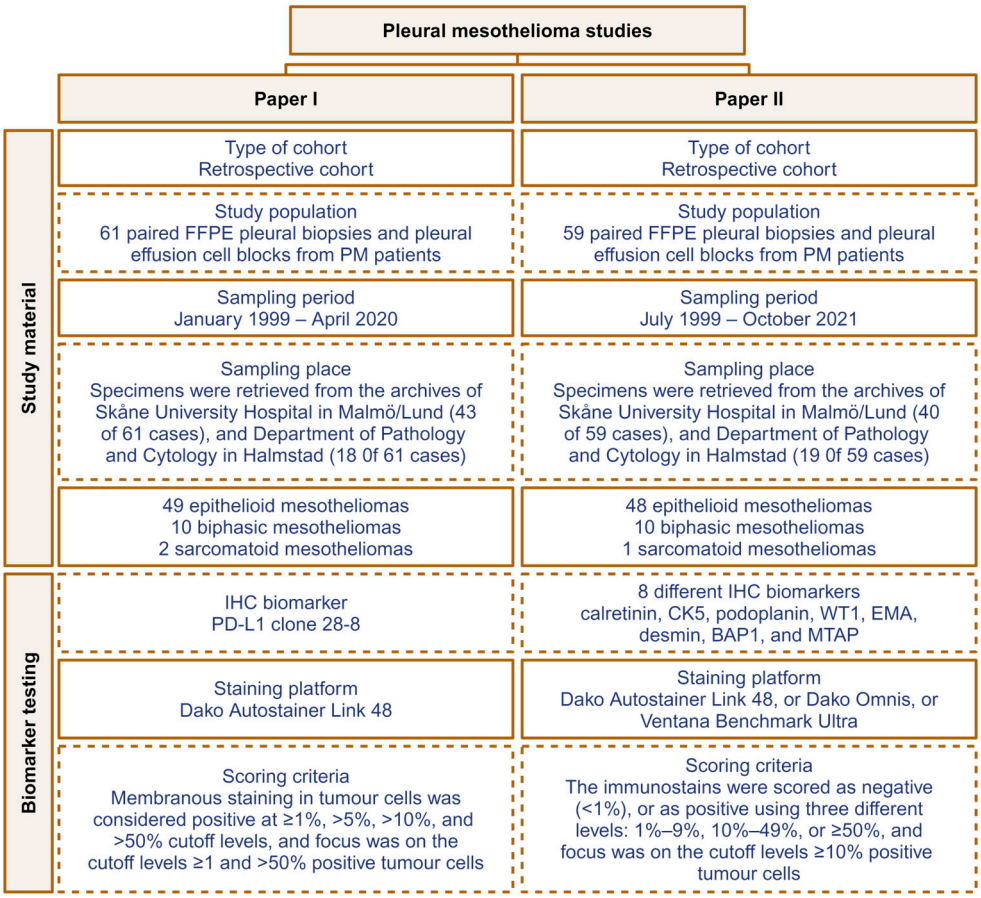

Figure 15. Characteristics of mesothelioma cohorts included in Paper I and II. Differences and similarities of study material, biomarker testing, and scoring criteria for study I and II. BAP1 = BRCA1-associated protein 1, CK5 = Cytokeratin 5, EMA = Epithelial membrane antigen, FFPE = formalin-fixed paraffin-embedded, IHC = immunohistochemistry, MTAP = Methylthioadenosine phosphorylase, PD-L1 = programmed cell death-ligand 1, PM = pleural mesothelioma, WT1 = Wilm's tumour 1.

Paper III

In Paper III, the study material consisted of two NSCLC cohorts from a university-affiliated (Lund) and a regional (Halmstad) pathology department in southern Sweden, including 47 and 97 paired, concurrently sampled, biopsies and cytological cell block specimens, respectively. The same inclusion criteria were applied for both cohorts included in the study, except for the years of inclusion, and only cases where the paired specimens were part of the same diagnostic workup were included (a maximum of 4 weeks between the paired samples). In addition, only cases with >100 evaluable tumour cells in either sample were included. Only one cell block and one biopsy from each patient were stained with PD-L1 and included in the

study, but sometimes the cell block contained material from >1 cytological sample combined into a single cell block. All cases showing different histological types between paired histology and cytology specimens were excluded. For the histology specimens, there were no differences in the sampling and preparation methods over time and between the hospitals. The cytology cell block preparation differs between the cohorts and hospitals using two different cell block preparation methods. In Lund, the cell blocks were prepared using the Cellient™ automated cell block system after alcohol-based fixation, whereas in Halmstad, the cell blocks were prepared by the traditional sedimentation cell block method after formalin fixation. Furthermore, for the Lund cases PD-L1 clone 22C3 was used, while in Halmstad clone 28-8 was used instead. Additional information on the cohorts included in Paper III is shown in Figure 16.

In Paper III, a review of the literature was also accomplished. This review was based on an appraisal and summary of the peer-reviewed, original, English-written articles published up to December 2020 that included a comparison of PD-L1 expression in paired histological and cytological specimens from patients with NSCLC. The search was performed using PubMed with “PD-L1 cytology lung cancer” as the search term.

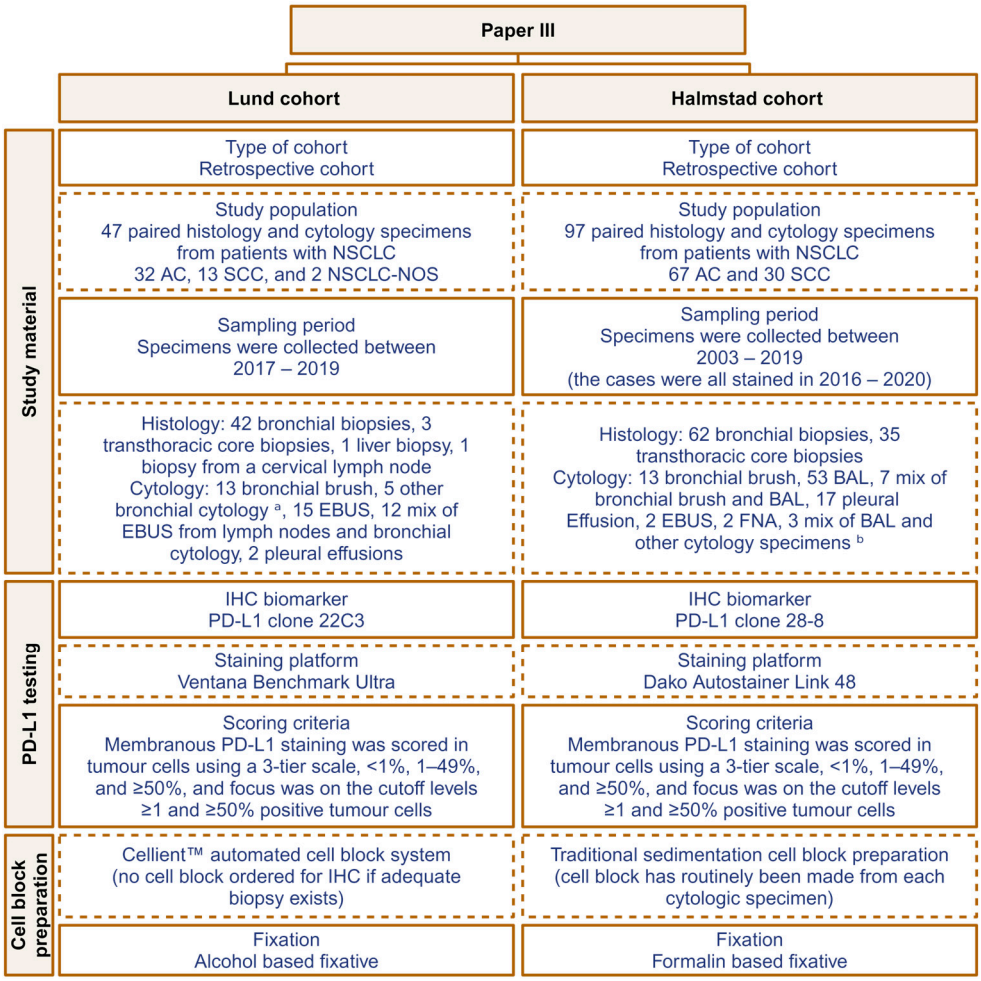

Figure 16. Characteristics of the NSCLC cohorts included in Paper III. Differences and similarities of study material, cell block preparation, PD-L1 testing, and scoring criteria for study III. [a] Other bronchial cytology including suction catheter, BAL, or a mix of any of the two with bronchial brush. [b] both BAL and either pleural effusion, EBUS, or FNA of the lymph node in 1 case each. AC = adenocarcinoma, BAL = bronchoalveolar lavage, EBUS = endobronchial ultrasound-guided lymph node aspirations, FNA = fine-needle aspiration of the lymph node, IHC = immunohistochemistry, NOS = not otherwise specified, NSCLC = non-small cell lung cancer, PD-L1 = programmed cell death-ligand 1, SCC = squamous cell carcinoma.

Paper IV

In Paper IV, the study material consisted of two NSCLC cohorts from a university-affiliated (Lund) and a regional (Halmstad) pathology department in southern Sweden, including 1131 and 651 consecutive, unpaired specimens, respectively. The methods of data collection differ between the cohorts. For the Lund cohort, the cases were collected based on the ordering of molecular analysis, while for the

Halmstad cohort, the cases were collected based on PD-L1 analysis, as in practice possible searches in the database systems differed. Detailed data on the cohorts included in Paper IV showed in Figure 17.

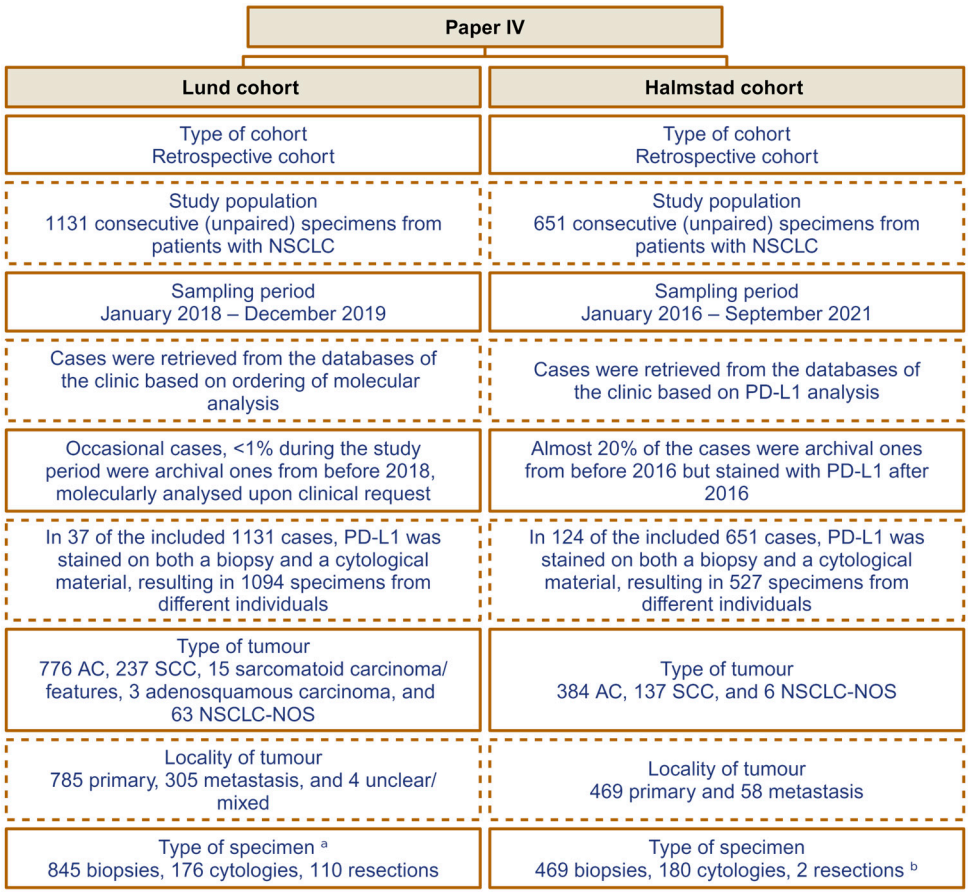

Figure 17. Characteristics of the NSCLC cohorts included in Paper IV. Differences and similarities of study material and collection of data for study IV. [a] For the type of specimen, results from both a biopsy and a cytological specimen were included if both existed for a single patient (for all other parameters, the PD-L1 result from the biopsy was used if both biopsy and cytology existed). [b] Halmstad cases surgically treated in Lund (not included in the Lund cohort). AC = adenocarcinoma, NOS = not otherwise specified, NSCLC = non-small cell lung cancer, PD-L1 = programmed cell death-ligand 1, SCC = squamous cell carcinoma.

Data on the different clinicopathological and molecular features of all cases in both cohorts included in Paper IV were retrieved from the databases of both involved hospitals. Specification of interesting data collected is presented in Figure 18.

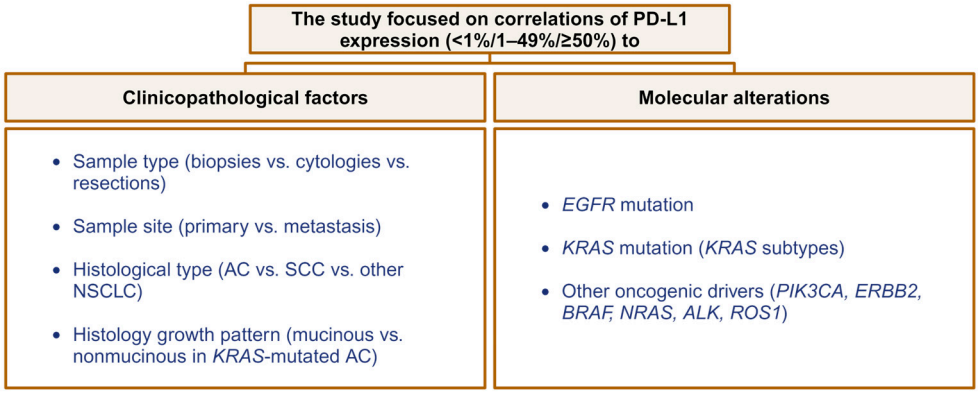

Figure 18. Overview of the data collected in Paper IV. The correlation between PD-L1 and different clinicopathological, and molecular factors explored in study IV. AC = adenocarcinoma, *ALK* = anaplastic lymphoma kinase, *EGFR* = epidermal growth factor receptor, *KRAS* = kirsten rat sarcoma, NSCLC = non-small cell lung cancer, PD-L1 = programmed cell death-ligand 1, *ROS1* = cytoplasmic c-ros oncogene 1 receptor tyrosine kinase, SCC = squamous cell carcinoma.

Paper V

Paper V in this thesis included 24 malignant pleural effusions (MPE) from different patients with lung AC, diagnosed at the Department of Pathology and Cytology, Halland Hospital in Halmstad, in Southern Sweden. Four cell blocks were prepared from each MPE, which were consequently fixed in 4 different fixatives, 10% neutral buffered formalin (NBF), PreservCyt®, CytoLyt®, or CytoRich™ Red, respectively, for a minimum of 24 hours. For 7 of the 24 included cases, only three fixatives were used (CytoRich™ Red not used for the initial cases). There was no difference in the sampling or preparation method over time. All available specimens that fulfilled the following criteria were included: (a) pleural fluid as cytological material; (b) MPE with lung AC; (c) cases with >100 evaluable tumour cells in both cell block preparations were included. All cases showing either different histological types than lung AC, or duplicate samples from the same patient, or samples containing an insufficient number of tumour cells were excluded. Data on the cohort included in Paper V is presented in detail in Figure 19.

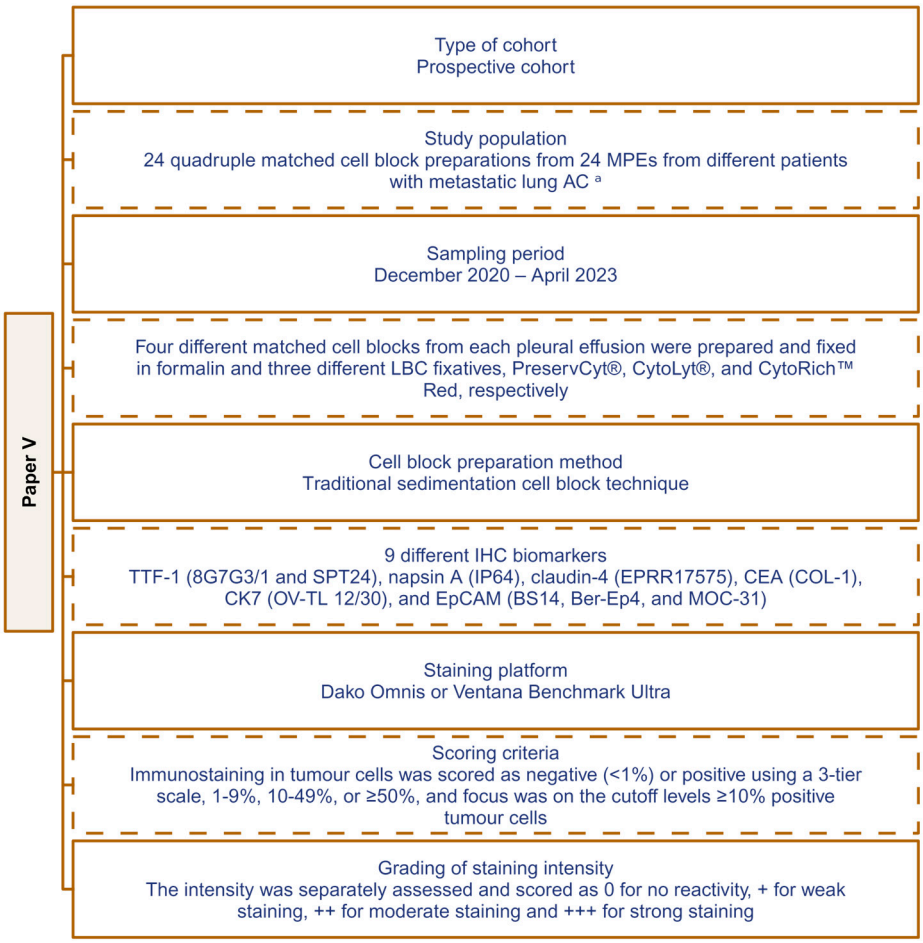

Figure 19. Characteristics of the lung AC cohort included in Paper V. The study material, cell block procedure, and immunocytochemical process used in study V. [a] For 17 cases have only triple cell blocks, fixed in formalin, PreservCyt®, and CytoLyt®, respectively). AC = adenocarcinoma, CEA = carcinoembryonic antigen, CK7 = cytokeratin 7, EpCAM = epithelial cell adhesion molecule, MPE = malignant pleural effusion, TTF-1 = thyroid transcription factor-1.

Handling of specimens

All histological and cytological samples used in all five studies included in this thesis were formalin-fixed paraffin-embedded specimens (exception to cytology cell blocks included in study III and IV, which were fixed in CytoLyt®/PreservCyt® and prepared using Cellient™). There was no difference in handling of histological specimens, whereas the cytological specimens were prepared using two different

cell block preparation techniques, corresponding to two different cohorts from different pathology departments included in two of the studies (Papers III and IV).

Preparation of histological specimens

The histological specimens consisted mainly of either pleural biopsies included in mesothelioma studies (Paper I and II), or bronchial or lung biopsies included in NSCLC studies (Paper III and IV). In addition, a limited number of resected tumours were included in one NSCLC study (Paper IV).

Biopsies were fixed in 10% neutral buffered formalin (NBF) for at least 24 h. Consequently, the biopsies were dehydrated in the tissue processor for 4 hours, followed by paraffin-embedding with a tissue embedding station. Resections were fixed and dehydrated for a longer period depending on the size and type of tissue, followed by the same process as for biopsies. The typical fixation time for lung resections was 48 h. The preparation of histological specimens was consistent over time, and there were no differences in the sampling or preparation methods between cohorts and hospitals.

Preparation of cytological specimens

The cytology cell block preparations in Papers I and II were performed using traditional sedimentation cell block technique (Halmstad cohorts), while in Papers III and IV, the routine procedures of cell block preparation methods varied between the two cohorts from different pathology departments included in these studies. Either traditional sedimentation cell block technique (Halmstad cohorts) or Cellient™ automated cell block system (Lund cohorts) was used. However, Malmö and Lund cases included in mesothelioma studies were prepared using the same method as in Halmstad. In addition, the cell block preparation of pleural effusions differed slightly from other cytology specimens within the same cohort as described below. The cell block preparations in Paper V were also performed by traditional sedimentation cell block technique, but with some conditions.

Sedimentation cell block technique – Halmstad cohorts

All cytology cell block preparations from Halmstad cohorts in Papers I - IV included in this thesis were prepared using the traditional sedimentation cell block technique. The routine procedure for the cytology samples was that the specimen was transferred to the laboratory and fixed in CytoLyt® for a least one hour. EBUS-guided lymph node aspirations were fixated after initially being put in sodium chloride for no more than a few hours (to enable flow cytometry). Centrifugation was performed at 1000 g for 10 minutes within 24 hours, whereafter the supernatant fluid was discarded. After centrifugation, the cell pellet was manually transferred into Shandon Cytoblock cassettes or a plastic netting. Approximately, 2-3 drops of

Cytoblock Reagent 1 (clear fluid) included in the cell block kit were dropped into the centre of the well in the cassette before the cell pellet was placed in the cassette to stabilize and facilitate handling of the pellet. Subsequently, 1–2 drops of Mayer's Haematoxylin were added for colour, and the cassette was directly fixed in 10% neutral buffered formalin (NBF) for at least 6 h up to 24 h. Pleural effusions were received at the laboratory untreated or in Heparin, and were handled differently, as the material was primarily centrifuged and transferred to a cassette which was directly put in NBF. Only a small proportion of bloody pleural effusions may have been rapidly washed in CytoLyt® once before formalin fixation. After fixation, the pellet was later dehydrated in the tissue processor for 8 hours and followed by further processing including paraffin-embedding. The sampling and cell block preparation of specimens was consistent over time.

The cell block preparation procedure included in Paper V was also the traditional sedimentation cell block method, but with determined conditions related to the prospective design of the study. Pleural effusions were received at the pathology department in Halmstad, untreated or in Heparin. In the clinical setting, the formalin-fixed cell blocks were routinely available together with conventional wet-fixed Pap- and air-dried MGG-stained smears. The remaining pleural fluid from patients who were suspected to have pulmonary AC by the physician, and after confirmation of the diagnosis by the cytopathologist, were included in Paper V. Centrifugation was performed within one week, whereafter the supernatant fluid was discarded. The cell pellet from each patient sample was divided into four smaller cell pellets as equal in size as possible, which were thereafter manually transferred to four different Shandon Cytoblock cassettes. The same procedure of cell block processing as mentioned above was used. Lastly, the four cassettes were directly fixed in 10% NBF, PreservCyt®, CytoLyt®, or CytoRich™ Red, respectively, for a minimum of 24 hours. After fixation, the cassettes were removed to a container with 70% alcohol prior to dehydration and before further processing including paraffin-embedding. Cell block preparation of specimens was consistent over time and performed by the same experienced biomedical scientists, in the presence of the author of the thesis. An illustration of the cell block procedure used in Paper V is shown in Figure 20.

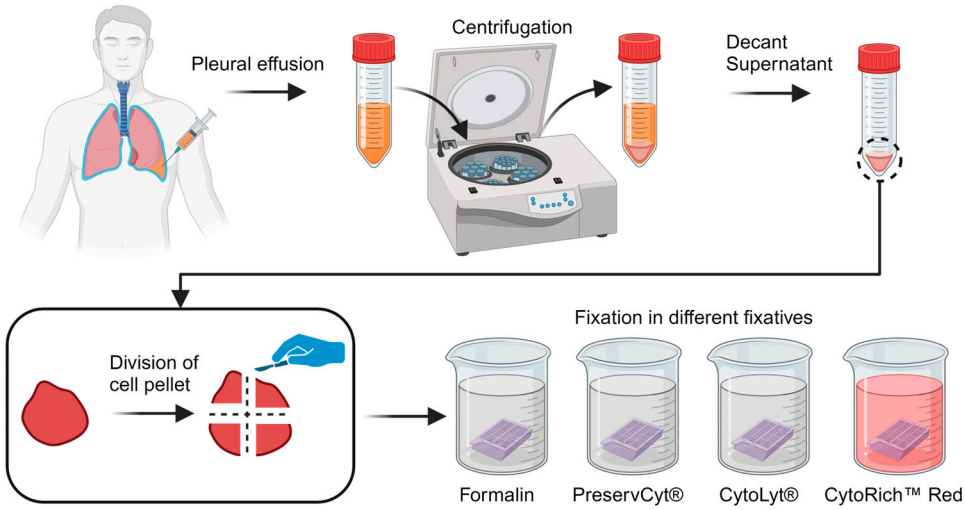

Figure 20. Schematic illustration of the cell block procedure used in Paper V. Sedimentation cell block preparation method of pleural effusion using four different fixation media (formalin and three liquid-based cytology fixatives) for 24 hours. Created with BioRender.com

Cellient™ automated cell block system – Lund cohorts

The cytology cell block preparations from Lund cohorts included in Papers III and IV were prepared using the Cellient™ automated cell block system. The routine procedure was that the cytology specimens were initially fixed in CytoLyt®, before arriving at the pathology department, which usually occurred within a few hours. In the laboratory, the CytoLyt® was replaced by PreservCyt® for further fixation before Cellient™ automated cell block preparation. The exception was pleural effusions where the cells had only been rapidly washed in CytoLyt® once or occasionally twice and then fixed in PreservCyt®. The fixation in PreservCyt® had typically been 1-3 days before further processing and IHC staining (as typically a pathologist first reviewed the initial slides before ordering a cell block). Cell block preparation of specimens was consistent over time, and there was no difference in the sampling or preparation method.

Immunohistochemical staining

All patients included in all the studies of the thesis were diagnosed at either the Department of Pathology and Cytology, the Halland Hospital in Halmstad, or the Department of Pathology, Skåne University Hospital in Malmö, or Lund. In the clinical setting, the final diagnoses were based on all available clinical data and the results of imaging techniques together with morphological diagnoses mainly on

H&E-stained slides of histological specimens, supported by IHC staining. For cytology, the diagnoses were based on conventional wet-fixed PAP or H&E and air-dried MGG-stained smears at all hospitals, as well as FFPE cell blocks at Halland Hospital in Halmstad. The diagnostic immunostains were routinely performed on cell blocks at all hospitals, but sometimes cytospin slides were used at the Skåne University Hospital Malmö. The immunopanels used in the clinical diagnostic situation varied especially with clinical and morphological factors, but also slightly over time and between hospitals.

As part of the research studies, the original diagnostic reports, including histological subtype were retrieved from the databases of all involved departments and from the patients' medical records. Furthermore, all histological slides were reviewed for confirmation of diagnosis and histological subtype in accordance with the World Health Organization classification of thoracic tumours, [96] by at least one experienced cytopathologist together with the thesis author.

From all specimens, an H&E-stained slide was made and examined by the thesis author in unclear cases together with an experienced cytopathologist, when needed, to confirm the presence of malignant cells. If no clearly malignant cells could be identified in H&E-stained slide, the case was not included in the research study in question.

The immunohistochemical stains were performed on FFPE histology and cytology cell block preparations from mesothelioma (Papers I and II), while the cytology cell block preparations in NSCLC cohorts (Papers III - V) were prepared using different cell block preparation techniques, depending on study design and the cohort included, see Figures 15, 16 and 19.

Different in-house multi-tissue control blocks made from various FFPE tissues including both positive and negative controls, depending on the applied antibody, were routinely used on each slide for all included antibodies. The 4-µm-thick specimen sections from the multi-control blocks, biopsies and cell blocks were, for most cases, sectioned at the Halland Hospital in Halmstad, where the majority of specimens were pre-treated on PT Link and stained on an Autostainer Link 48 or automatically pre-treated and stained on a Dako Omnis (Agilent Technologies, Santa Clara, CA, USA), using the EnVision FLEX or FLEX+ visualization system (see Figure 11). Other IHC biomarkers were stained using the automated staining system on a Ventana Benchmark Ultra (Ventana Medical Systems, Inc., Tucson, AZ, USA) using the UltraView or OptiView visualization system at either the Skåne University Hospital in Lund, or the Department of Clinical Pathology and Cancer Diagnostics, Karolinska University Hospital in Huddinge, Stockholm, Sweden.

The specimens were stained for each included IHC biomarker using the same antibody clone, immunostaining protocol, and staining platform for all IHC preparations in the entire respective cohort, with identical staining procedures and without any difference within the same cohort. Furthermore, the same immunostaining procedure for each antibody was used for both histological and cytological specimens. For each cohort stained with multiple biomarkers,

consecutive cut sections from all specimens were stained with the same panel of antibodies for all cases. Immunostainings of the same cohort were performed in batches. An illustration of immunostaining with PD-L1 using EnVision FLEX+ visualization system is shown in Figure 21.

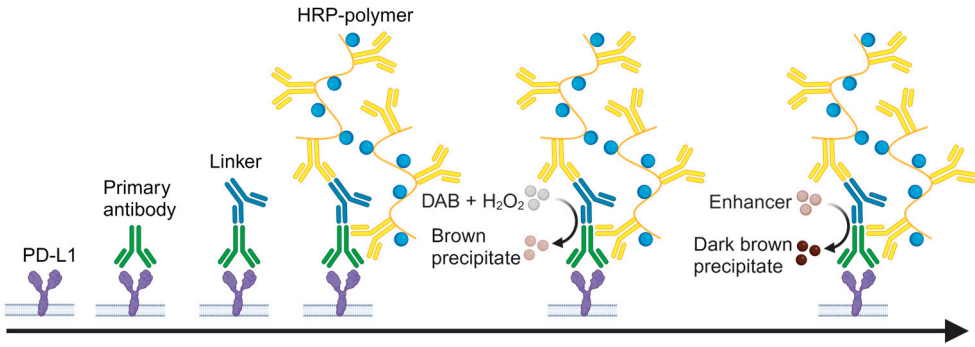

Figure 21. Schematic illustration of the immunohistochemical process of PD-L1 staining. PD-L1 staining reaction using EnVision FLEX+ visualization system. DAB = 3,3'-Diaminobenzidine. Created with BioRender.com

Information on staining procedure including antibodies, clones, platforms, pre-treatment, and control tissues for all IHC biomarkers used in all studies included in the thesis is summarised in detail in Table II.

Table II Characteristics and detailed description of IHC biomarkers used in all studies.

Antibody	Clone	Paper	Vendor	Dilution	Pre-treatment	Staining Pattern		Cutoff level	Visualization system	Staining platform	Control		Article no.
						Localization	Type of cells				Positive control [a]	Negative control	
PD-L1	28-8	I, III and IV	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS Low pH, 20 min, 97°C	Membranous	Positive in mesothelial and NSCLC cells	I: ≥1%, >5%, >10%, and >50%. III and IV: <1%, 1–49%, and ≥50%.	EnVision FLEX+	Autostainer, Dako, Agilent	Tonsil (strong reactivity in crypt epithelium and weak reactivity in histiocytes of germinal centers), placenta, cell line (NCI-H226) [b]	Appendix, cell line (MCF-7) [b], NCR control [c]	SK00521-2
PD-L1	22C3	III and IV	Dako/Agilent Technologies, Glostrup, Denmark	1:40	CC1, 48 min, 99°C	Membranous	Positive in NSCLC cells	<1%, 1–49%, and ≥50%	OptiView	BenchMark ULTRA Ventana, Roche	Tonsil and placenta	n/a	M365329
Calretinin	DAK-Calret 1	II	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS High pH, 20 min, 97°C	Nuclear and cytoplasmic	Positive in mesothelial cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX	Autostainer, Dako, Agilent	Appendix and colon	Tonsil	IR62761-2
CK5	XM26	II	Leica Biosystems/Newcastle Ltd, Newcastle upon Tyne, UK	1:25	TRS High pH, 30 min, 97°C	Cytoplasmic	Positive in mesothelial cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX+	Omnis, Dako, Agilent	Tonsil (epithelium) and prostate	Appendix, placenta	CK5-L-CE
Podoplanin	D2-40	II	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS High pH, 20 min, 97°C	Membranous	Positive in mesothelial cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX	Autostainer, Dako, Agilent	Appendix, tonsil and colon	n/a	IR07261-2
WT1	6F-H2	II	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS High pH, 20 min, 97°C	Nuclear	Positive in mesothelial cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX+	Autostainer, Dako, Agilent	Apenndix, tonsil, kidney and fallopian tube	n/a	IR05561-2
EMA	E29	II	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS High pH, 20 min, 97°C	Membranous	Positive in mesothelioma cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX	Autostainer, Dako, Agilent	Normal breast tissue	n/a	IR62961-2

Antibody	Clone	Paper	Vendor	Dilution	Pre-treatment	Staining Pattern		Cutoff level	Visualization system	Staining platform	Control		Article no.
						Localization	Type of cells				Positive control [a]	Negative control	
Desmin	D33	II	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS High pH, 30 min, 97°C	Cytoplasmic	Negative in mesothelioma cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX+	Omnis, Dako, Agilent	Appendix	Tonsil and liver	IR60661-2
BAP1	C-4	II	Santa Cruz Biotechnology, Dallas, TX	1:100	TRS High pH, 20 min, 97°C	Nuclear	Loss in mesothelioma cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX+	Autostainer, Dako, Agilent	Appendix, tonsil, pancreas, skin and testis	n/a	SC-28383
MTAP	2G4	II	Abnowa, VWR, Stockholm, Sweden	1:10	CC1, 52 min, 95°C	Cytoplasmic	Loss in mesothelioma cells	<1%, 1–9%, 10–49%, and ≥50%	UltraView	BenchMark ULTRA Ventana, Roche	Lung, kidney, lymph node and skin	n/a	ABNOH0000 4507-M01
TTF-1	8G7G3/1	V	Ventana Medical Systems, Inc., Tucson, AZ, USA	RTU	CC1, 64 min, 99°C+A(m)	Nuclear	Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	UltraView	BenchMark ULTRA Ventana, Roche	Thyroid	Tonsil	790-4398
	SPT24		Leica Biosystems/Newcastle Ltd, Newcastle upon Tyne, UK	1:50	CC1, 48 min, 100°C				OptiView				NCL-L-TTF-1
Napsin A	IP64	V	Leica Biosystems/Newcastle Ltd, Newcastle upon Tyne, UK	1:50	TRS High pH, 30 min, 97°C	Granular cytoplasmic	Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX+	Omnis, Dako, Agilent	Kidney (proximal tubules)	Tonsil, colon	NCL-L-Napsin A
Claudin 4	EPRR17575	V	Abcam, Discovery Drive, Cambridge Biomedical Campus, Cambridge, UK	1:100	CC1, 64 min, 100°C O.V-kit	Membranous	Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	OptiView	BenchMark ULTRA Ventana, Roche	Colon, kidney (proximal tubules [weak], collecting ducts)	Tonsil, liver	ab210796
CEA	COL-1	V	BioCare Medical, The Hague, The Netherlands	1:200	TRS Low pH, 30 min, 97°C	Cytoplasmic	Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX+	Omnis, Dako, Agilent	Appendix, tonsil (epithelium)	Liver	CM058C

Antibody	Clone	Paper	Vendor	Dilution	Pre-treatment	Staining Pattern		Cutoff level	Visualization system	Staining platform	Control		Article no.
						Localization	Type of cells				Positive control [a]	Negative control	
CK7	OV-TL 12/30	V	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS High pH, 30 min, 97°C	Cytoplasmic	Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX	Omnis, Dako, Agilent	Liver (bile ducts), tonsil (epithelium [few cells])	Appendix, liver (hepatocytes)	GA61961-2
EpCAM	BS14	V	Nordic Biosite, Täby, Sweden	1:100	CC1, 32min, 100°C P3/4min	Membranous	Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	OptiView	BenchMark ULTRA Ventana, Roche	Appendix, kidney (proximal tubules [weak], collecting ducts), liver (bile ducts)	Tonsil (non-epithelial cells), liver (hepatocytes)	BSH-7402-1
	Ber-Ep4	V	Dako/Agilent Technologies, Glostrup, Denmark	RTU	TRS Low pH, 30 min, 97°C		Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	EnVision FLEX+	Omnis, Dako, Agilent			GA63761-2
	MOC-31	V	Dako/Agilent Technologies, Glostrup, Denmark	1:100	CC1, 48 min, 91°C O.V-kit		Positive in lung AC cells	<1%, 1–9%, 10–49%, and ≥50%	OptiView	BenchMark ULTRA Ventana, Roche			M352501-2

[a] The protein expression varies from low to strong in different control tissues.

[b] A control slide was produced from two cell lines, NCI-H226 (PD-L1–positive) and MCF-7 (PD-L1–negative), included in the antibody kit, was stained for each run.

[c] An additional negative antibody control for every sample slide from an identical slide of each specimen was stained with Negative Control Reagent (NCR), a buffer containing immunoglobulin G antibodies that lack specificity for PD-L1 and works as an isotype control.

Abbreviations: AC = adenocarcinoma; BAP1 = BRCA1-associated protein 1; CC1 = cell conditioning 1; CEA = carcinoembryonic antigen; CK5, 7 = cytokeratin 5, 7; EMA = epithelial membrane antigen; EpCAM = epithelial cell adhesion molecule; IHC = immunohistochemistry; MTAP = methylthioadenosine phosphorylase; n/a = not applicable; NCR = negative control reagent; O.V = OptiView; RTU = ready to use; TRS = target retrieval solution; TTF-1 = thyroid transcription factor 1; WT1 = Wilm's tumour 1.

Immunohistochemical evaluation

All immunostainings included in Papers I-III and V were performed as part of the research studies included in this thesis. Also, a part of immunostainings included in Paper IV were performed as part of the previous study (Paper III).

For all IHC biomarkers, immunoreactivity was assessed using conventional light microscopy. Specimens with sufficient viable tumour cells (>100) were considered adequate, and tumours were judged as positive or negative based on the reactivity of viable malignant cells in the whole specimen slide regardless of intensity. For all evaluations, any reactivity in non-malignant cells, necrotic areas, and immune cells such as lymphocytes or macrophages was disregarded and was not included in the assessment. The evaluation of the immunostainings was supported by correlation with H&E-stained sections and additional diagnostic IHC stainings when performed were available at the time of evaluation of the IHC biomarkers (incl. IHC from the clinical setting not annotated in the present studies). The IHC slides from the clinical setting that were included in the present studies were reassessed regardless of the previous assessment. The same criteria were applied to both histological and cytological specimens, when applicable. The scoring of all immunostainings was performed blindly, independently, and without side-by-side comparison, by at least three different investigators, for most cases primarily by the thesis author, followed by two experienced cytopathologists for confirmation, or three when needed. Cases with discordant reactivity between investigators for each staining or between different specimens from the same patient (i.e., paired histology and cytology specimens or matched cell blocks from the same patient) were reassessed by two investigators together, to achieve consensus without knowledge of the previously reported result, by manual counting of malignant cells for some biomarkers such as PD-L1. Possible reasons for any discrepancy were discussed.

In Paper I, PD-L1 staining in malignant cells was evaluated by the criteria as recommended in an assessment manual from Dako. [437] The positivity of PD-L1 staining was defined as the percentage of well-preserved malignant cells on the entire slide exhibiting positive complete surrounding or linear partial membranous staining. All linear membranous reactivity, regardless of intensity was considered positive. Cytoplasmic reactivity in malignant cells was ignored. The percentages of well-preserved malignant cells, expressing PD-L1 were semi-quantified as negative if viable malignant cells with reactivity were <1%, or positive at different cutoff levels: ≥1%, >5%, >10%, and >50%. See Figure 15.

In Paper II, the positivity of immunoreactivity was defined as both cytoplasmic and nuclear staining for calretinin, cytoplasmic staining for CK5, desmin, and MTAP, and nuclear staining for WT1 and BAP1, while for podoplanin and EMA membranous staining was regarded. Cytoplasmic staining for EMA and BAP1 and nuclear staining for MTAP were disregarded, whereas the cytoplasmic staining for

EMA in sarcomatoid components was included and assessed as positive due to difficulties in the judgment of membranous staining. The immunostains were scored as negative (<1%) or positive using three different levels: 1-9%, 10-49%, or ≥50%. In our analyses, ≥10% positive tumour cells was considered positive (or preserved for BAP1 or MTAP) to reflect a relevant application in the clinical setting. See Figure 15.

In Paper III, the PD-L1 immunostainings were evaluated using the same evaluation criteria applied in Paper I, and in line with assessment manuals. [437, 438] The PD-L1 reactivity was scored as negative, <1%, or positive, 1-49% or ≥50%. See Figure 16.

In Paper IV, PD-L1 staining in malignant cells was evaluated by the same criteria applied in Paper I and III, and as recommended in an assessment manual from Dako. [437, 438] A part of immunostainings was performed as part of Paper III. The rest of the included cases from the clinical setting were evaluated by at least two investigators, and the PD-L1 scores of interest were <1% as negative, or 1-49% and ≥50% as positive, which were retrieved from the databases of the two pathology departments involved. Detailed data on PD-L1 testing for the cohorts included in Paper IV are presented in Figure 22.

In Paper V, the positivity of immunoreactivity was defined as the percentage of well-preserved malignant cells on the entire slide exhibiting membranous staining for claudin 4 and EpCAM stainings, cytoplasmic staining for CEA and CK7, granular cytoplasmic staining for napsin A, and nuclear staining for TTF-1. Cytoplasmic staining for EpCAM was disregarded. The scoring of immunoreactivity was negative (<1%) or positive using three different levels: 1-9%, 10-49%, or ≥50%. In our analyses, ≥10% positive tumour cells was considered positive to reflect a relevant application in the clinical setting. The intensity of immunoreactivity in most target cells in the whole slide was considered, which was also separately assessed and graded as 0 for no reactivity, + for weak staining, ++ for moderate staining, and +++ for strong staining. See Figure 19.

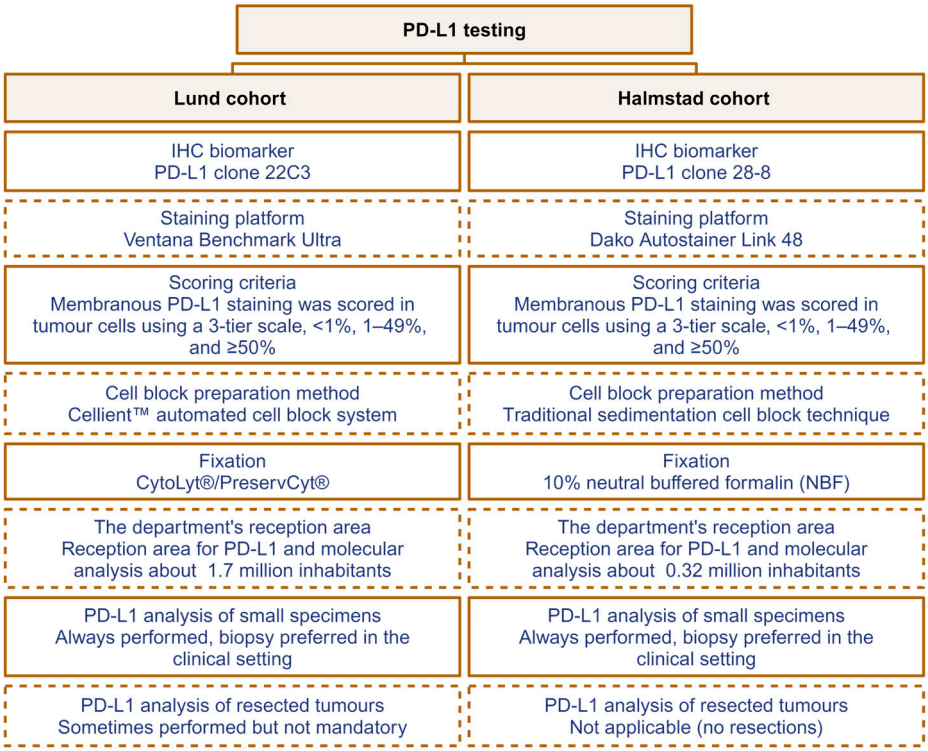

Figure 22. PD-L1 testing in Paper IV. Differences and similarities of the process of PD-L1 testing performed in study IV. IHC = immunohistochemistry, PD-L1 = programmed cell death ligand 1.

Procedures of molecular analysis

The data in Paper IV was mainly based on molecular analyses and PD-L1 status from lung cancer patients. The study included two retrospective cohorts from the Department of Pathology, Skåne University Hospital in Lund, and from the Department of Pathology and Cytology, Halland Hospital in Halmstad, in southern Sweden. The histological FFPE specimens were commonly used for molecular analysis for both cohorts. For cytology, the scraped-off and lysed cells from MGG-stained smears or sections from cell blocks were also used, but in different proportions between the cohorts (in Lund, the first available adequate material is typically selected for NGS, thus quite often cytology). During the study period and the covered time, the molecular analyses varied over time due to the rapid development of molecular diagnostics. The main detection method was NGS, but PCR, FISH, and IHC were also used.

Detailed information on similarities and differences over time in molecular testing between and within the cohorts included in Paper IV including specimen type and detection methods are presented in Figure 23.

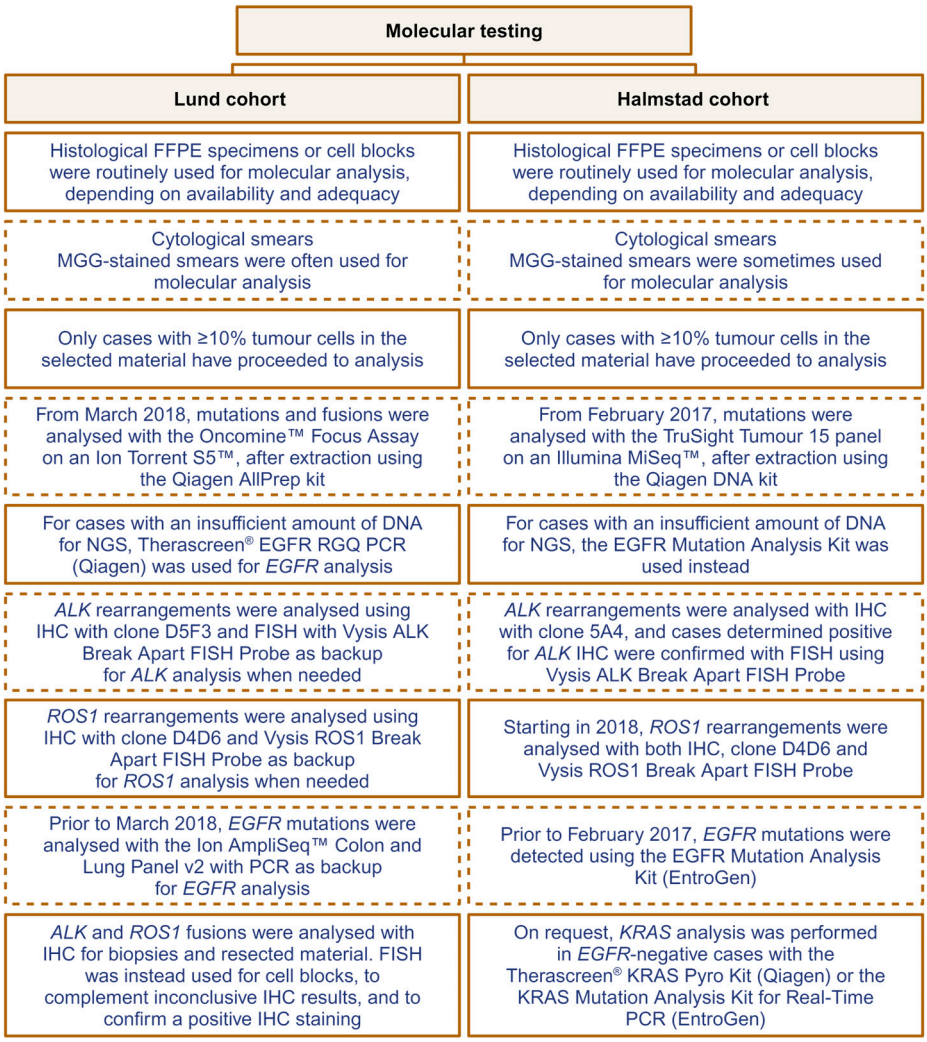

Figure 23. Molecular testing in Paper IV. Differences and similarities of molecular analysis used for the cohorts included in study IV. *ALK* = anaplastic lymphoma kinase, *EGFR* = epidermal growth factor receptor, FFPE = formalin-fixed paraffin-embedded, FISH = fluorescent enhanced *in situ* hybridization, IHC = immunohistochemistry, *KRAS* = kirsten rat sarcoma, PCR = polymerase chain reaction, PD-L1 = programmed cell death-ligand 1. *ROS1* = cytoplasmic c-ros oncogene 1 receptor tyrosine kinase.

Statistical analysis

The main purposes of all the included research studies in this thesis were to investigate the association of various parameters in different groups and compare different biomarkers between groups. All statistical analyses used in all studies included in this thesis are listed in Table III.

Table III. All statistical methods used in different studies included in this thesis.

Statistical method	Type of the test	Characteristics of the test	Purpose	I	II	III	IV	V
Student's *t*-test	Parametric test	For data with normal distribution	To test for differences in the means, i.e. the averages of two groups				X	
Chi-square (χ2) test	Non-parametric test	For large sample sizes	To examine the differences, independence or association between two categorical variables		X		X	
Fisher's exact test	Non-parametric test	For small sample sizes	To determine the association or relationship between two categorical variables	X		X		
Mann-Whitney U test	Non-parametric test	For two independent groups	To compare the distribution of a continuous variable between two groups	X			X	
Wilcoxon signed-rank test	Non-parametric test	For two paired groups	To compare the distribution of a continuous variable between two groups			X		X
McNemar's test	Non-parametric test	For paired nominal data	To determine the differences on a dichotomous (a categorical variables with two categories) dependent variable between two related groups	X	X			X
Kruskal-Wallis test	Non-parametric test	For independent variable on a continuous or ordinal dependent variable	To compare the distribution of a continuous variable between more than two groups				X	
Friedman test	Non-parametric test	For an extension of the paired-data, where the dependent variable being measured is ordinal or continuous data	To determine the difference between the means of three or more groups in which the same subjects show up in each group					X
One-Way ANOVA	Parametric test	For independent groups, with normal distribution data	To test for differences in the means, i.e. the averages of three or more groups				X	
Multivariate regression analysis	Parametric test	For more than one dependent variable and multiple independent variables	To measure the degree to which more than one independent variable (predictors) and more than one dependent variable (responses), are linearly related to each other				X	
Unweighted Cohen's kappa coefficient (κ)	Measure of reliability and rater agreement	For paired observations from same two raters, but are independent	To estimate inter-observer agreement between two raters or two methods for categorical scales	X	X	X		X
Weighted kappa coefficient (Wκ)	Measure of reliability and rater agreement	For ordinal variables, and for multiple raters, i.e., more than two raters	To estimate inter-observer agreement between different raters or methods on the same subjects					X
Overall percentage agreement (OPA)	Measure of overall concordance and agreement	For two or more outcomes, and does not differentiate between the agreement on the positives and agreement on the negatives	To assess agreement between two tests in the absence of a reference standard. For example, the proportion of all cases that are concordant, i.e., cases where the tests (such as biomarker expression) are either both positive or both negative, between two paired tests.	X	X	X		X
Positive percentage agreement (PPA)	Measure of concordance and agreement for positives	Reported with respect to the imperfect reference standard positives	To determine the proportion of comparative/reference method positive results in which the test method result is positive	X	X			X
Negative percentage agreement (NPA)	Measure of concordance and agreement for negatives	Reported with respect to the imperfect reference standard negatives	To determine the proportion of comparative/reference method negative results in which the test method result is negative	X	X			X

In general, studies are often set out to examine a null hypothesis, with the assumption of no difference between the groups. However, the probability of an observed difference may arise purely by chance, meaning that there is no difference between the groups. Thus, the null hypothesis can be confirmed or rejected by statistical calculation of the probability value, also called *p*-value or simply *p*. When the null hypothesis is true, the *p*-value represents the probability of finding a difference between the groups. The null hypothesis is rejected when the probability, i.e., *p*-value, is low enough. The *p*-value threshold is defined and usually albeit arbitrarily, set at 0.05. A *p*-value of 0.05 implies that the probability is 5%. Therefore, in all included studies in this thesis, we used *p*-values to investigate the correlation of parameters in the groups, and all *p*-values were determined using two-sided tests, and outcomes with *p*-values above 0.05 were not considered statistically significant.

Nevertheless, the magnitude or significance of the difference cannot be inferred solely from the p-value, as it is also influenced by the sample size. Also, a statistically significant difference between two large groups may not necessarily be clinically meaningful if the difference is very small. Moreover, a substantial and clinically relevant difference may be challenging to establish as statistically significant if the number of observations is low. A power calculation can be conducted to estimate the necessary number of patients to include in a study to achieve statistically significant results. In the studies featured in this thesis, no power calculations were conducted. This is primarily because the cohorts in each study were predefined and constrained to the material accessible through research biobanks.

The concordance of expression for each biomarker between paired cases and matched preparations was statistically analysed in different ways, including overall percentage agreement (OPA), positive percentage agreement (PPA), negative percentage agreement (NPA), and unweighted Cohen's kappa coefficient (κ) for the pair-wise comparison between two groups, using a 2-tier scale (2X2 table), see Figure 24 A. The agreement of biomarker expression was calculated by weighted kappa coefficient (Wκ) for all matched groups i.e., specimens and preparations, using a 4-tier scale (4X4 table), see Figure 24 B. Examples of illustrating 2X2 and 4X4 tables are exhibited in Figure 24 A and B, respectively.

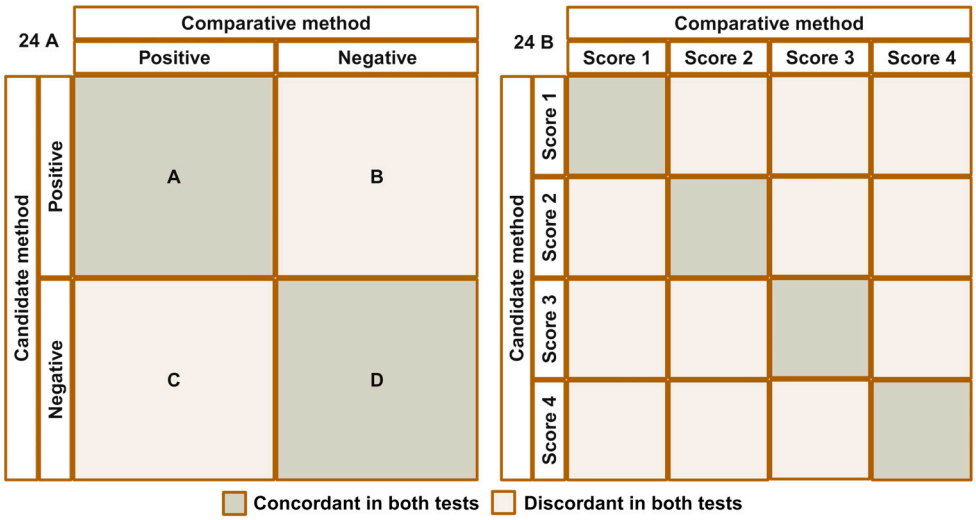

Figure 24 A-B. Illustration of 2X2 table and 4X4 table. A: Illustration of 2X2 table using one cutoff level, resulting in two scores. B: Illustration of 4X4 table using three cutoff levels, resulting in four scores.

In the terminology of Altman, [439] and as shown in the illustration in Figure 24 A.

$$OPA = \frac{\mathrm{a} + \mathrm{d}}{N}$$

$$PPA = \frac{\mathrm{a}}{a + c}$$

$$NPA = \frac{\mathrm{d}}{d + b}$$

The strength of agreement for κ and Wκ is considered poor (<0.2), fair (0.21-0.40), moderate (0.41-0.60), good (0.61-0.80), or excellent (0.81-1.00).

Furthermore, the calculations of all confidence intervals (CIs) in different studies were performed using a modified Wald method with a Wilson score of 95%, according to the website GraphPad Software (https://www.graphpad.com/quickcalcs/). Detailed information on statistical methods used in all Papers are listed in Table III, and the summary of main statistical methods for each present investigation included in different studies of this thesis are presented below.

Paper I

The association between PD-L1 expression and age was evaluated using the Mann-Whitney U test, while the association between PD-L1 expression and gender, and histological subtypes was determined with Fisher's exact test. The agreement of PD-

L1 frequency between histology and cytology was statistically analysed in different ways, including OPA, PPA, NPA, and Cohen's κ for the pair-wise comparison between histology and cytology for PD-L1 expression, using a 2-tier scale (2X2 table). In detail, the concordance of PD-L1 expression for paired histology and cytology was analysed using unweighted κ, and additionally OPA was analysed for the concordant cases (both positives and negatives), PPA for positives separately, and NPA for negatives separately. The McNemar's test was applied to investigate any systematic difference between histology and cytology for discordant cases. The IBM SPSS Statistics for Windows, version 26.0 (IBM, Armonk, NY, USA) software was used for all statistical calculations.

Paper II

The frequency of immunoreactivity for each biomarker was compared between different histological components and cytology using the Chi-square test. The concordance of expression was calculated in different ways, including OPA, PPA, NPA, and Cohen's κ for the pair-wise comparison between histology and cytology for each biomarker, using a 2-tier scale (2X2 table). In detail, the diagnostic concordance of expression for each biomarker in paired histology and cytology was analysed using unweighted κ, and additionally OPA was analysed for the concordant cases (both positives and negatives), PPA for positives separately, NPA for negatives separately, while the McNemar's test was calculated to investigate any systematic difference between histology and cytology for the discordant cases. The MedCalc Statistical Software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium) was used for the determination of *p*-values. The IBM SPSS Statistics for Windows, version 27.0 (IBM, Armonk, NY, USA) software was also used for the production of other data analyses and summary graphs.

Paper III

OPA was used to calculate agreement of PD-L1 expression between histology and cytology, using a three-tier scale. Further, unweighted κ was used to analyse the concordance of PD-L1 between histology and cytology. Wilcoxon signed-rank test was used to analyse the differences in PD-L1 scores between histology and cytology. Fisher's exact test was used to compare the concordance between cohorts. All analyses were performed with MedCalc Statistical Software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium).

Paper IV

Student's *t*-test, chi-square, and, when applicable, one-way ANOVA were performed to investigate correlations between PD-L1 expression and different clinicopathological factors. The same correlations were also reanalysed using Mann-Whitney and Kruskal-Wallis tests. The differences in PD-L1 expression between various clinicopathological parameters in each category was analysed

using multivariate regression analysis. Statistical analyses were produced with MedCalc Statistical Software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium).

Paper V

The frequency of positivity for each biomarker was compared between different fixatives using the Friedman test. Whether the comparison showed any systematic difference between fixatives, the pair-wise comparisons between fixatives were performed using McNemar's test at one cutoff level (2 scores) or the Wilcoxon signed-rank test at three cutoff levels (4 scores). The concordance between biomarker expressions was calculated in different ways, including OPA, PPA, NPA, and Cohen's κ for the pair-wise comparison of the fixatives, using a 2-tier scale (2X2 table), and Wκ for all matched cell block preparations i.e., fixed in different fixatives, using a 4-tier scale (4X4 table). All analyses were performed using IBM SPSS Statistics for Windows, Version 29.0 (IBM, Armonk, NY, USA).

Ethical approval statement

The studies were conducted in adherence to the Declaration of Helsinki and approved by the Regional Ethical Review Board, Southern Health Care Region in Lund, protocol code 2006/399, with addition 2017/708 for all included Papers, and additionally protocol code 2019/04782 for Paper III and IV, and protocol code 2020/00256 for Paper IV.

Results

I'm a great believer in luck,
and I find the harder I work,
the more I have of it.

Thomas Jefferson, 1743-1826

Paper I

Association between PD-L1 expression in PM histology and cytology specimens and patient characteristics

PD-L1 expression was analysed in 61 pairs of pleural biopsies and pleural effusion cell blocks from PM patients. The focus was on membranous staining in tumour cells at ≥1% and >50% cutoff levels. The median age at diagnosis (range) was 72 years (50-90), and the majority of patients, 52/61 (85.2%), were men. The cases were divided according to histological subtype into epithelioid mesothelioma in 49/61 (80.3%), and non-epithelioid mesothelioma in 12/61 (19.7%) cases, which in turn was grouped into biphasic mesothelioma in 10/61 (16.4%), and sarcomatoid mesothelioma in 2/61 (3.3%) cases. Of 61 biopsies, 28 (45.9%) and 7 (11.5%) were PD-L1 positive at ≥1% and >50% cutoffs, respectively. The corresponding figures for effusion cell blocks were 21 of 61 cases (34.4%) and 5 of 61 cases (8.2%) at ≥1% and >50% cutoff levels, respectively. The relationship between PD-L1 expression in histological and cytological specimens and patient characteristics was investigated. PD-L1 positivity showed no difference with respect to age or gender, in either histological or cytological specimens. However, a significant difference in PD-L1 expression was observed in the histological specimens at the ≥1% cutoff level between epithelioid and non-epithelioid mesothelioma, with a higher frequency of positivity in the latter group ($p = 0.049$), see Table IV. Furthermore, the frequency of PD-L1 immunoreactivity was marginally lower in cytological specimens across various cutoff levels.

Table IV. The frequency of PD-L1 positivity in hisology and cytology specimens of pleural mesothelioma.

Parameters	Histology [a]		Cytology	
	≥1% Cutoff level	>50% Cutoff level	≥1% Cutoff level	>50% Cutoff level
Frequency of PD-L1 positivity, n (%)	28/61 (46)	7/61 (12)	21/61 (34)	5/61 (8)
PD-L1 correlation to histology subtype [b] (epithelioid vs. nonepithelioid)	0.049	0.130	0.736	1.00

Abbreviation: PD-L1 = programmed cell death ligand 1.

[a] For biphasic mesothelioma, the positivity of the histological component with the highest score was chosen.

[b] Comparison between PD-L1 reactivity in epithelioid and non-epithelioid subtypes.

Cyto-histological correlation of PD-L1 expression in paired PM specimens

Differences in PD-L1 expression were identified between histological and cytological specimens, either through disparities in positivity between sample types or in the percentages of positive tumour cells when both sample types were positive.

Figure 25 shows an image of a sample illustrating different cell components and proportions of positive cells.

The agreement of PD-L1 immunoreactivity in paired histological and cytological specimens was analysed at ≥1%, >5%, >10%, and >50% cutoff levels. The concordance was calculated for both positives and negatives using OPA, and separately for positives using PPA and negatives using NPA. OPA ranged between 62% and 84%. PPA decreased with increasing cutoff levels, while NPA improved.

At the ≥1% cutoff level, Cohen's κ was 0.36 (CI, 0.13-0.59), indicating fair to moderate agreement statistically. The κ decreased at higher cutoff thresholds. McNamar's test did not yield statistical significance at any cutoff level. Some of the findings are shown in Table V.

The agreement of PD-L1 immunoreactivity in epithelioid and non-epithelioid mesothelioma and corresponding cytology specimens was also analysed at ≥1%, >5%, >10%, and >50% cutoff levels.

At the ≥1% cutoff level, a moderate concordance was observed between histological and cytological specimens for epithelioid ($\kappa = 0.43$), whereas non-epithelioid mesotheliomas showed no significant concordance ($\kappa = 0.08$). The κ values decreased with higher cutoff levels (Table V).

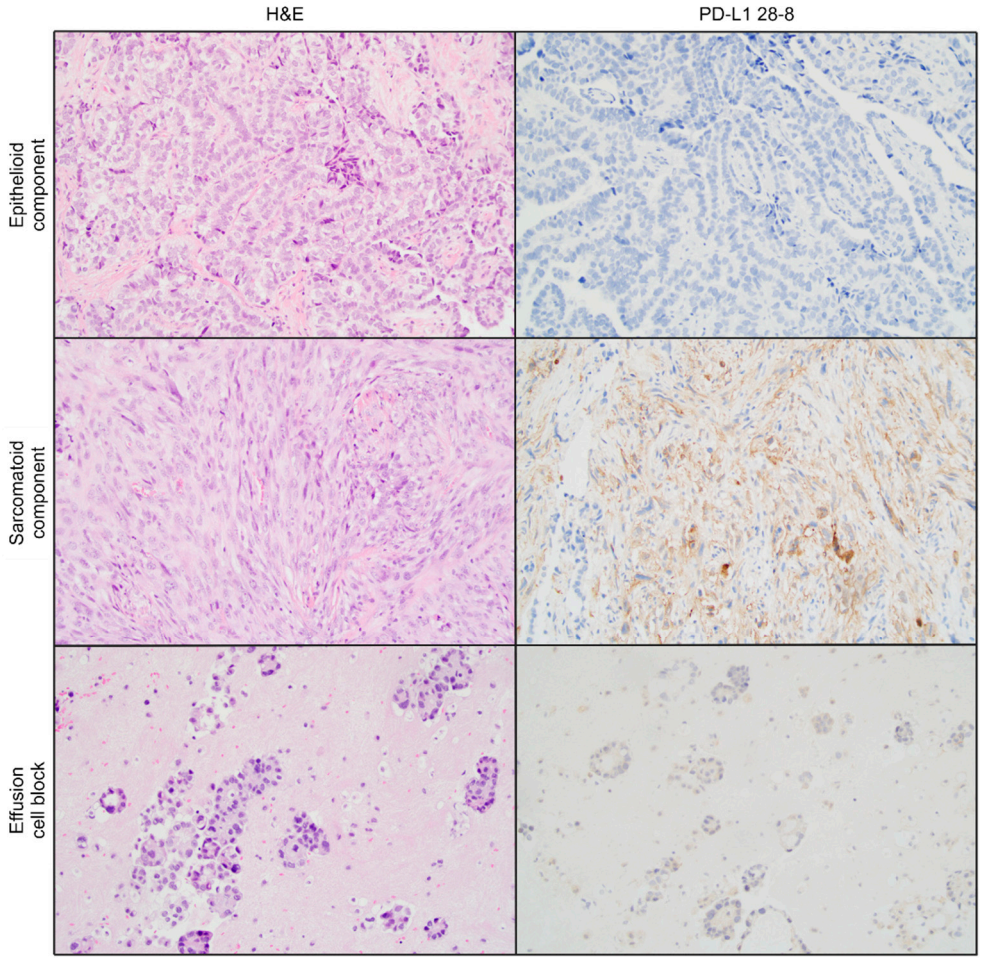

Figure 25. A biphasic mesothelioma with PD-L1 immunoreactivity (clone 28-8) in paired histological and cytological specimens. A single specimen with concordant expression of PD-L1 in epithelioid component of biopsy and cytology effusion cell block (both were PD-L1 negative), while discordant between sarcomatoid component of biopsy and cytology effusion cell block (>50% PD-L1 positive malignant cells vs. negative). H&E = Haematoxylin-eosin staining, PD-L1 = programmed death-ligand 1. Original magnification x20 objective.

Table V. The concordance between paired histology and cytology specimens from pleural mesothelioma.

Parameters	Histology [a] vs. Cytology ≥1% Cutof level	Histology [a] vs. Cytology >50% Cutoff level		
The overall percentage agreement (OPA)	69%		84%	
Cohen's kappa coefficient (κ)	0.36		0.08	
McNemar's test	0.17		0.75	
Parameters	**Histology (epithelioid) vs. Cytology ≥1% Cutoff level**	**Histology (epithelioid) vs. Cytology >50% Cutoff level**	**Histology (nonepithelioid) vs. Cytology ≥1% Cutoff level**	**Histology (nonepithelioid) vs. Cytology >50% Cutoff level**
The overall percentage agreement (OPA)	73%	88%	50%	67%
Cohen's kappa coefficient (κ)	0.43	0.18	0.08	− 0.14
McNemar's test	0.58	1.00	0.22	0.63

Note: Cohen's kappa coefficient (κ) is presented as range -1−1, and McNemar analyses are presented as P-values.
Abbreviation: PD-L1 = programmed cell death ligand 1.
[a] For biphasic mesothelioma, the positivity of the histological component with the highest score was chosen.

Paper II

Patient characteristics and expression status of biomarkers in paired PM specimens

Analysis of 59 patients diagnosed with PM, including paired biopsy and effusion cell block specimens meeting study criteria, was conducted to evaluate the expression of calretinin, CK5, podoplanin, WT1, EMA, desmin, BAP1, and MTAP IHC biomarkers. Eighty-five percent (50 cases) were male. The median age at diagnosis was 72 years, ranging from 51 to 90 years. Among the 59 mesotheliomas examined, 81% (48 cases) exhibited epithelioid morphology, 17% (10 cases) were biphasic, and 2% (1 case) were sarcomatoid. For MTAP, one case was excluded due to no epithelioid component left in the biopsy.

Using a threshold of ≥10% positive tumour cells to define positive staining, the expression rates for traditional IHC biomarkers associated with mesothelial lineage (calretinin, CK5, podoplanin, and WT1) ranged between 93% and 98% in the epithelial component of biopsies and between 92% and 100% in cytological specimens (see Figure 26). No significant disparities in biomarker positivity were observed between histological and cytological specimens. Reduced expression of calretinin, CK5, and WT1 was evident in sarcomatoid components compared to both epithelioid components and cytological specimens (all $p \leq 0.0001$). Additionally, a

significantly lower frequency of podoplanin expression was observed in sarcomatoid components compared to cytological specimens ($p = 0.021$).

Regarding biomarkers utilized to distinguish mesothelioma from benign mesothelial cells, the frequency of EMA positivity was notably lower in histological sarcomatoid components compared to histological epithelioid component and cytological specimens (both $p \leq 0.0001$). Furthermore, the loss of nuclear BAP1 (<10% positive tumour cells) was more prevalent in cytological specimens and histological epithelioid components compared to histological sarcomatoid components, with statistical significance observed only in cytological specimens ($p = 0.039$). However, no difference was noted for the loss of cytoplasmic MTAP and desmin negativity.

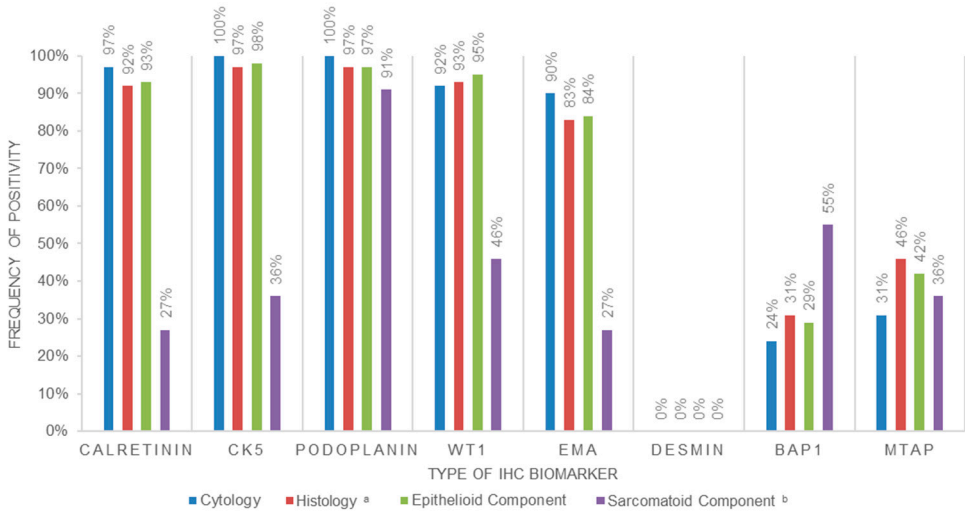

Figure 26. Expression rate of immunohistochemical biomarkers in histology and cytology from mesothelioma patients. Frequency of positive/preserved immunohistochemical reactivity at ≥10% cutoff in different histopathological components and cytology from 59 pleural mesotheliomas. [a] Biphasic mesotheliomas were classified as positive/preserved expression if any component showed positive/preserved expression. [b] Ten biphasic cases and one sarcomatoid case. BAP1 = BRCA1-associated protein 1, CK5 = Cytokeratin 5, EMA = epithelial membrane antigen, MTAP = Methylthioadenosine phosphorylase, WT1 = Wilm's tumour 1.

Cyto-histological correlation of biomarker expression in paired PM specimens

The agreement of protein expression between paired biopsies and cytological specimens for all IHC biomarkers considered, utilizing a threshold of ≥10% positive tumour cells to define positive staining or preserved expression (for BAP1 and MTAP) was calculated by several statistical methods for histological epithelioid components and for histological sarcomatoid components.

Images of a concordant case and a discordant case, illustrating different IHC biomarkers and diagnostic materials, are shown in Figures 27 and 28, respectively.

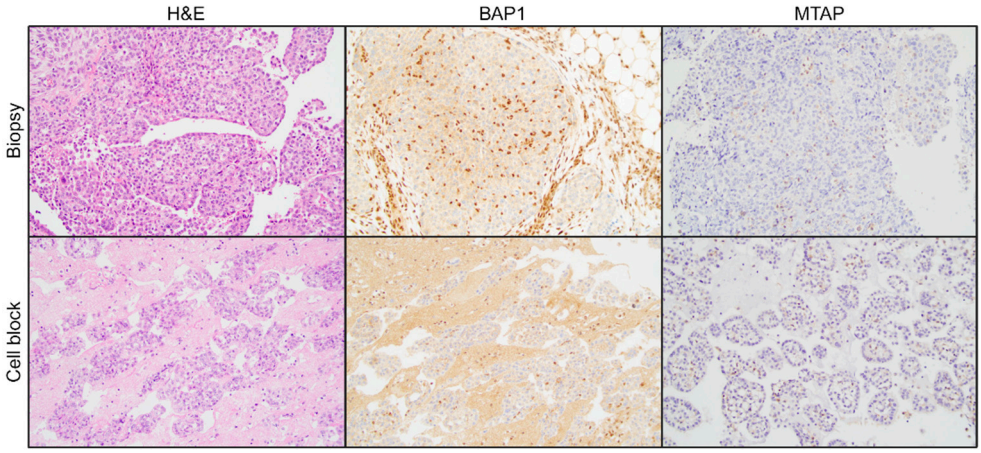

Figure 27. An epithelioid mesothelioma with concordant expression of BAP1 and MTAP in paired pleural biopsy and pleural effusion cell block. Loss of nuclear BAP1 expression and loss of cytoplasmic MTAP expression in paired specimens. H&E = Haematoxylin-eosin staining, BAP1 = BRCA1-associated protein 1, MTAP = methylthioadenosine phosphorylase. Original magnification x20 objective.

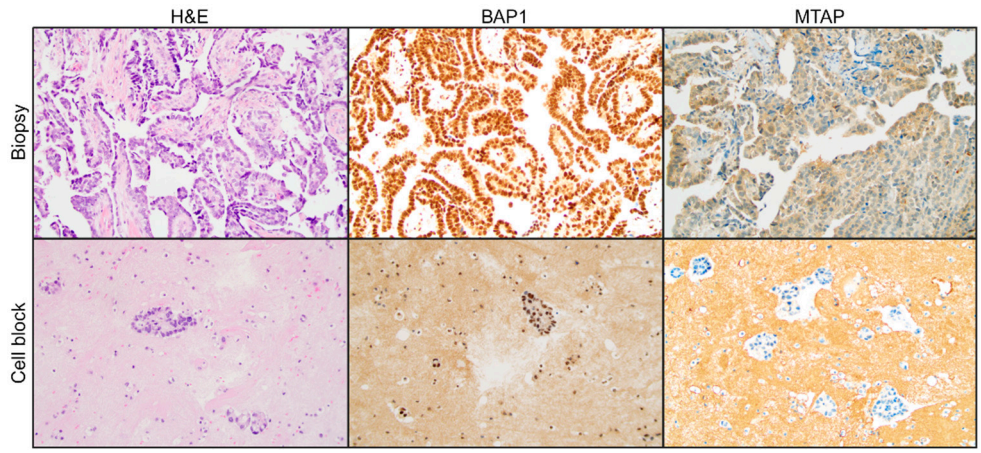

Figure 28. A biphasic mesothelioma with concordant expression of BAP1 and discordant expression of MTAP in paired pleural biopsy and pleural effusion cell block. Preserved nuclear BAP1 expression in ≥50% of malignant cells in epithelioid component of biopsy vs. 10-49% in cell block. Preserved cytoplasmic MTAP expression in ≥50% of malignant cells in epithelioid component of biopsy vs. loss of cytoplasmic MTAP expression in cell block. (The sarcomatoid component of biopsy showed preserved nuclear BAP1 expression in ≥50% of malignant cells and preserved cytoplasmic MTAP expression exhibited in 10-49% of malignant cells.) H&E = Haematoxylin-eosin staining, BAP1 = BRCA1-associated protein 1, MTAP = methylthioadenosine phosphorylase. Original magnification x20 objective.

The concordance of IHC biomarker positivity between paired histological and cytological specimens was analysed through various methods, including OPA, PPA, NPA (using histology as the standard for PPA and NPA), McNemar's test, and Cohen's κ when applicable. The findings obtained when employing ≥10% positive tumour cells as criteria for positive/preserved staining and the OPA is presented in Table VI.

Table VI. The agreement of immunohistochemical reactivity between different histological components and cytology. The concordance of expression for different mesothelial biomarkers in paired specimens from 59 pleural mesotheliomas.

	The overall percentage agreement (OPA) % (95% CI)		
IHC biomarker	**Histology [a] vs. Cytology**	**Epithelioid histology vs. Cytology**	**Sarcomatoid histology vs. Cytology**
Calretinin	92 (81 – 97)	93 (83 – 98)	27 (9 – 57)
CK5	97 (88 – 100)	98 (90 – 100)	36 (15 – 65)
Podoplanin	97 (88 – 100)	97 (88 – 100)	91 (60 – 100)
WT1	88 (77 – 94)	90 (79 – 96)	45 (21 – 72)
EMA	86 (75 – 93)	86 (75 – 93)	64 (35 – 85)
Desmin	100 (93 – 100)	100 (93 – 100)	100 (70 – 100)
BAP1	90 (79 – 96)	91 (81 – 97)	73 (43 – 91)
MTAP	71 (59 – 81)	72 (59 – 82) [b]	73 (43 – 91)
Total of cases, n (%)	59	58	11

Note: Positive/preserved expression defined as ≥10% positive tumour cells.
Abbreviations: BAP1 = BRCA1-associated protein 1; CI = confidence interval; CK5 = Cytokeratin 5; EMA = Epithelial membrane antigen; IHC = immunohistochemistry; MTAP = Methylthioadenosine phosphorylase; WT1 = Wilm's tumour 1.
[a] Biphasic mesotheliomas were classified as positive/preserved expression if any component showed positive/preserved expression.
[b] One case was excluded due to no epithelioid component left in the biopsy.

McNemar's test yielded statistically significant results for calretinin, CK5, and WT1 (all $p \leq 0.04$), suggesting systematic differences between cytological specimens and histological sarcomatoid components. However, no significant differences were observed for the remaining included biomarkers.

Cohen's κ agreement was applicable and employed to calculate concordance for EMA, BAP1, and MTAP. There was good concordance for BAP1 when comparing cytological specimens with the histological epithelioid component, while other comparisons demonstrated moderate concordance. The findings remained consistent for weighted κ when using the four-tier scale for the IHC biomarkers (<1%, 1-9%, 10-49%, ≥50%), and in comparing cytological specimens with histology when adopting the highest score for both components in biphasic cases.

Relationship between BAP1 and MTAP IHC expression in paired PM specimens

The relationship between BAP1 and MTAP immunoreactivity results is reported in Table VII, with specific details provided for different histological components and cytological specimens. As observed, a smaller proportion of epithelioid components on biopsies exhibited loss of both BAP1 and MTAP (<10% positive tumour cells) compared to cytology, accounting for 40% (23 of 57) and 54% (32 of 59), respectively.

Table VII. The relationship between BAP1 and MTAP immunoreactivity. The expression rate of BAP1 and MTAP in different histopathological components and cytology specimens of pleural mesothelioma.

BAP1 and MTAP status	**Cytology** N=59, n (%)	**Histology** [a] N=59, n (%)	**Histology** Epithelioid component [b] N=57, n (%)	**Histology** Sarcomatoid component N=11, n (%)
BAP1 (−) and MTAP (−)	32 (54)	21 (36)	23 (40)	3 (27)
BAP1 (−) and MTAP (+)	13 (22)	20 (34)	18 (32)	2 (18)
BAP1 (+) and MTAP (−)	9 (15)	11 (19)	10 (18)	4 (36)
BAP1 (+) and MTAP (+)	5 (8)	7 (12)	6 (11)	2 (18)
Loss of BAP1 and/or MTAP	54 (92)	52 (88)	51 (89)	9 (81)

Note: Preserved expression defined as ≥10% positive tumour cells.
Abbreviations: BAP1 = BRCA1-associated protein 1; BAP1 (−) = loss of BAP1 expression; BAP1 (+) = expression of BAP1 preserved; MTAP = Methylthioadenosine phosphorylase; MTAP (−) = loss of MTAP expression; MTAP (+) = expression of MTAP preserved.
[a] Biphasic mesotheliomas were classified as preserved expression if any component showed preserved expression.
[b] One case was excluded due to no epithelioid component left in the biopsy.

Paper III

Cyto-histological correlation of PD-L1 expression in paired NSCLC specimens

The concordance of PD-L1 expression in paired biopsies and cytological specimens from NSCLC cases in Lund 2017-2019 and Halmstad 2003-2019 (stained 2016-2020), respectively, was analysed by Cohen's κ and OPA. Forty of 47 cases (from 47 different individuals) exhibited concordance in the Lund cohort, accounting for 85%. All discordant cases exhibited a lower PD-L1 score in cytology, and the disparity in score between biopsies and cytology was statistically significant (Wilcoxon test p = 0.02). The unweighted κ was 0.77 (95% CI: 0.62-0.93). The concordance was 94% (44/47) for the 1% cutoff level (i.e., <1 or ≥1% positive tumour cells) and 89% (42/47) for the 50% cutoff level (Table VIII).

Table VIII. Summary of the results in Paper III. The concordance of PD-L1 expression between paired histology and cytology specimens from 144 non-small cell lung cancer patients in two cohorts.

Parameters	Lund Cohort Histology vs. Cytology		Halmstad Cohort Histology vs. Cytology	
Concordance [a], n (%)	40/47 (85)		66/97 (68)	
Cohen's kappa coefficient (κ)	0.77		0.49	
Parameters	**≥1% Cutoff level**	**≥50% Cutoff level**	**≥1% Cutoff level**	**≥50% Cutoff level**
The overall percentage agreement (OPA) n (%)	44/47 (94)	42/47 (89)	79/97 (81)	82/97 (85)

Note: Unweighted cohen's kappa coefficient (κ) is presented as range -1−1.

Abbreviation: PD-L1 = programmed death-ligand 1.

[a] The concordance represents the simultaneously agreement between histology and cytology, using a 3-tier scale (<1%, 1-49% and ≥50% cutoff levels).

In the Halmstad cohort, 66 of 97 cases (from 97 different individuals) were concordant, representing 68% agreement. The unweighted κ was 0.49 (95% CI: 0.35-0.63). The concordance was 81% (79/97) for the 1% cutoff level (i.e., <1 or ≥1% positive tumour cells) and 85% (82/97) for the 50% cutoff level. There were more instances of lower PD-L1 scores in cytology within the Halmstad cohort, demonstrating a trend that was not statistically significant (Wilcoxon test $p = 0.055$).

The quantity of concordant cases was notably lower in the Halmstad cohort in contrast to the Lund cohort, with a statistically significant difference (Fisher's exact test $p = 0.043$). Examples of a concordant case and a discordant case (using the 3-tier scale <1%/1-49%/≥50%) are shown in Figures 29 and 30, respectively.

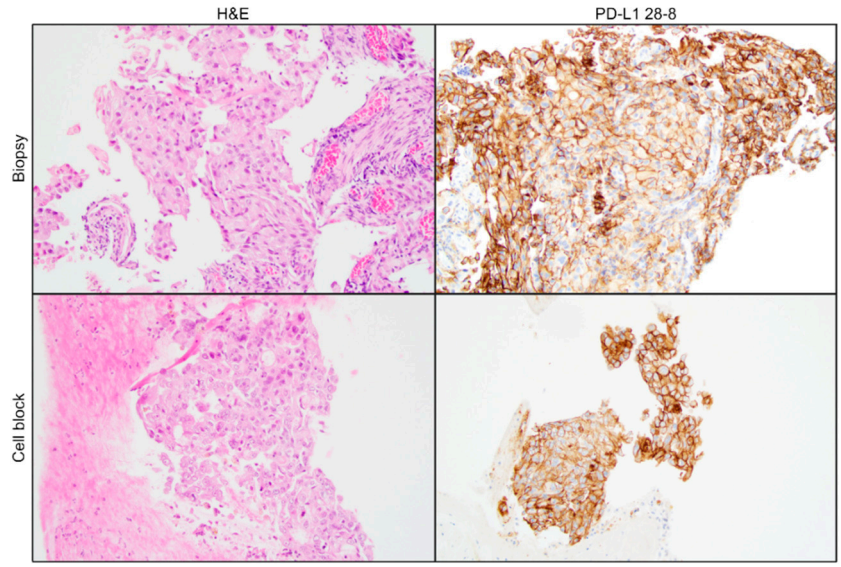

Figure 29. An adenocarcinoma with concordant PD-L1 expression (clone 28-8) between paired bronchial biopsy and BAL cell block. Both have PD-L1 expression in ≥50% of malignant cells. BAL = bronchial lavage, H&E = haematoxylin-eosin staining, PD-L1 = programmed death-ligand 1. Original magnification x20 objective.

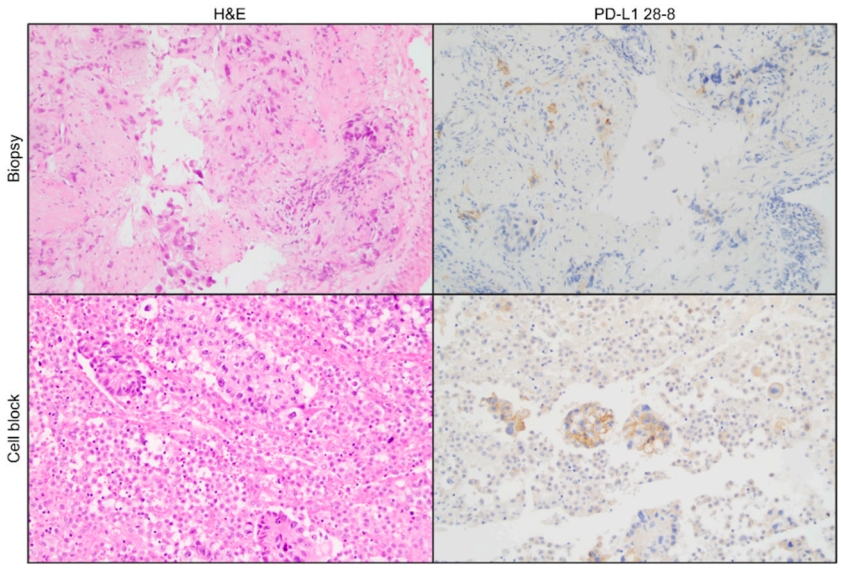

Figure 30. An adenocarcinoma with discordant PD-L1 expression (clone 28-8) between paired bronchial biopsy and pleural effusion cell block. Biopsy exhibited negative PD-L1 expression, <1%, while cell block exhibited 1-49% PD-L1 positive of malignant cells. H&E = haematoxylin-eosin staining, PD-L1 = programmed death-ligand 1. Original magnification x20 objective.

Characteristics of specimens and PD-L1 concordance

In the Lund cohort, out of the 47 cases, 32 were diagnosed as AC (whereof 29 concordant), 13 as SCC (with 9 concordant), and 2 cases as NSCLC not otherwise specified (both concordant). The biopsies comprised bronchial biopsies in 42 cases, transthoracic core biopsies in 3 cases, a liver biopsy in 1 case, and a biopsy from a cervical lymph node in 1 case. The cytological specimens comprised bronchial brush samples in 13 cases, other bronchial cytology including suction catheter, BAL, or a combination of any of the two with bronchial brush in 5 cases, EBUS-guided lymph node aspirations in 15 cases, a combination of EBUS from lymph nodes and bronchial cytology in 12 cases, and 12 pleural effusions in 2 cases.

Out of the 47 samples, 18 cases were from the same site (whereof 16 concordant), 12 were partially from the same site (11 concordant), and 17 were from different sites (13 concordant), respectively. The samples obtained from the same site were all from the primary tumour. The samples partially obtained from the same site comprised biopsies from the primary tumour and mixed cytological specimens, all of which were combinations of bronchial cytology and EBUS from lymph node.

In the Halmstad cohort, out of the 97 cases, 67 were diagnosed as AC (whereof 43 concordant) and 30 cases were identified as SCC (23 concordant). The biopsies consisted of bronchial biopsies in 62 cases and transthoracic core biopsies in 35 cases. The cytological specimens included bronchial brush samples in 13 cases, BAL in 53 cases, a combination of both bronchial brush and BAL in 7 cases, pleural effusion in 17 cases, EBUS-guided lymph node aspirations in 2 cases, FNA of the lymph node in 2 cases, and a combination of BAL with either pleural effusion, EBUS, or FNA of the lymph node in 1 case each.

Out of the 97 samples, 73 cases were from the same site (whereof 54 concordant), 3 were partially from the same site (all concordant), and 21 were from different sites (9 concordant), respectively. The samples partially obtained from the same site comprised biopsies from the primary tumour and mixed cytological specimens, including a combination of BAL with either pleural effusion, EBUS, or FNA of the lymph node. The paraffin blocks had been stored for at least 3 years before PD-L1 staining in 36 of the cases (whereof 25 concordant) and for a duration shorter than 3 years in the remaining 61 cases (with 41 concordant).

Review of the literature

Based on 25 published studies, the mean, median, and range for PD-L1 concordance in studies on paired cytology/histology cases were 83-85%, 81-85%, and 62-100%, respectively, at a cutoff level of 1% for positive PD-L1 staining. At a cutoff level of 50%, the figures were 87-89%, 89%, and 67-100%, respectively.[440-464] The intervals provided (here and below) reflect that some studies presented separate data for more than one cytological preparation, PD-L1 assay, or histological specimen type. The numbers remained consistent when the data from the present study were incorporated. Further details on the reviewed studies are presented in Table IX.

Combining all paired cytology/histology cases from the studies (including cases from the present study) yielded a concordance of 82-83% (1,465/1,785-1,488/1,792 cases) at a cutoff level of 1% for positive PD-L1 staining and 88-89% (1,398/1,587-1,408/1,580 cases) at a cutoff level of 59%.[440-464] Excluding studies involving resections, 6 studies (including the present one) remained, reporting a concordance in paired cytology/biopsy cases of 81-86% (228/283-248/290 cases) at a cutoff level of 1% for positive PD-L1 staining and 86-90% (249/290-254/283 cases) at a cutoff level of 50%.[443, 445, 448, 451, 461]

In studies where cytology was fixed in formalin, specifically excluding studies with non-formalin or mixed formalin/non-formalin fixation, the concordance with paired histological specimens was 81-82% (333-336/410 cases) at a cutoff level of 1% and 88-89% (316-317/358) at a cutoff level of 50%, based on 7 studies. [440, 441, 450, 452, 460, 462, 464] If instead, only including studies with cytology fixed in non-formalin fixatives, excluding mixed formalin/non-formalin fixation, the concordance with paired histological specimens was 79-81% (500-512/633 cases) at a cutoff level of 1% and 88-89% (554-565/633 cases) at a cutoff level of 50%, based on 9 studies including the Lund cohort from the present study. [443, 447, 448, 451, 454, 457, 458, 464]

The agreement in PD-L1 expression in studies utilizing only cytological cell blocks compared to histology was 82-84% (1,181/1,436-1,217/1,443 cases) at a cutoff level of 1% and 88-89% (1,085/1,238-1,091/1,231 cases) at a cutoff level of 50%, based on 23 studies including the present. [440-446, 449-453, 455-464] Correspondingly, the concordance for cytological smears compared to histology was 79-80% (327-329/413 cases) at a cutoff level of 1% and 93% (383-384/413 cases) at a cutoff level of 50%, based on 5 studies. [445, 447, 448, 451, 454]

Table IX. Summary of the data of review literature in Paper III. A literature review of 25 published studies conducted until December 2020 including cyto-histological correlation of PD-L1 expression in NSCLC patients.

			Concordance			
			≥1% Cutoff level		≥50% Cutoff level	
Type of included study	Number of studies	Number of included cases	Mean % (range)	Median % (range)	Mean % (range)	Median% (range)
All published studies all type of cytology preparations vs. all type of histology specimens	25	about 1,700	83-85 (62-100)	81-85 (62-100)	87-89 (67-100)	89 (67-100)
All studies including the present study	26	1,580-1,792	82-83	—	88-89	—
Only studies with biopsies cytology vs. biopsy	6	283-290	81-86	—	86-90	—
Only studies with formalin formalin fixed cytology vs. histology	7	358-410	81-82	—	88-89	—
Only studies with nonformalin nonformalin fixed cytology vs. histology	9	565-633	79-81	—	88-89	—
Only studies with cytology cell blocks cytological cell block vs. histology	23	1,231-1,443	82-84	—	88-89	—
Only studies with cytology smears cytological smears vs. histology	5	384-413	79-80	—	93	—

Note: The reason for the intervals is that some studies presented separate data for >1 cytological preparation, PD-L1 assay, or histological specimen type.
Abbreviations: NSCLC = non-small cell lung cancer; PD-L1 = programmed death-ligand 1.

Paper IV

Collection and characteristics of the cases

During the study period, molecular analysis was requested for 1275 NSCLC cases in Lund. PD-L1 analysis was conducted in 1094 of these cases, which comprised the Lund cohort of study IV. In 37 of the included cases, PD-L1 staining was performed on both biopsy and cytological material, resulting in a total of 1131 specimens. The Halmstad cohort comprised 527 NSCLC cases with PD-L1 analysis, of which 124 cases had paired biopsy and cytology, totalling 651 specimens.

The frequency of PD-L1 positivity was 55% in both cohorts at a cutoff level of ≥1%. Additional details concerning PD-L1 expression using a three-tier scale (<1%,

≥1-49%, and ≥50%) and its correlation with sample type, tumour locality, histological type, and oncogenic drivers were compiled. Not all cases underwent analysis for all mutation/fusions due to limited material and discrepancies in panels and routines between the two pathology departments (e.g., concerning *TP53*) and changes in methodologies over time (e.g., affecting gene fusion testing).

Correlation between PD-L1 expression status and clinicopathological and molecular features

From the two cohorts, a total of 1381 specimens were used for statistical analyses, including 90 cytological samples from patients who had a biopsy analysed for PD-L1. These specimens had complete data on sample type, tumour location, histological diagnosis, and mutation status. The findings of correlations are shown in Table X.

Using different statistical methods including Student's *t*-test, Analysis of variance (ANOVA), and Chi2 test, AC demonstrated significantly lower PD-L1 scores compared to SCC and "other NSCLC", whereas no significant difference was observed between SCC and "other NSCLC" (see Table X). Additionally, the Lund cohort exhibited slightly higher PD-L1 scores compared to the Halmstad cohort, with statistical significance observed but at a minimal level. No significant differences were found in comparisons between sample types and tumour locations.

In cases with *EGFR* mutations, PD-L1 scores were significantly lower compared to *EGFR* wild-types (Student's *t*-test, $p < 0.0001$). Conversely, *KRAS* and *PIK3CA* mutations were associated with elevated PD-L1 expression compared to *KRAS* wild-types ($p < 0.0001$) and *PIK3CA* wild-types ($p = 0.006$), respectively. In cases with *ERBB2*, *BRAF*, or *NRAS* mutations, as well as *ALK* or *ROS1* fusions, there were no significant differences in PD-L1 scores compared to those negative for these alterations in corresponding separate analyses (all $p = 0.12$-0.58). Out of the 73 cases with *PIK3CA* mutations, 5 (7%) exhibited a concurrent *EGFR* mutation and 27 (37%) had a *KRAS* mutation. The cases were categorised into *EGFR*-mutated, *KRAS*-mutated, and *EGFR*/*KRAS* wild-type groups for subsequent analysis. With this categorisation, cases harbouring *EGFR* mutations exhibited significantly lower PD-L1 scores compared to cases with *KRAS* mutations and cases lacking either mutation. Conversely, *KRAS*-mutated cases exhibited significantly higher PD-L1 scores than those devoid of *EGFR*/*KRAS* mutations (see Table X).

As evident in Table X, the significant differences observed between histological types (with lower PD-L1 scores for AC compared to SCC) and mutational status (*EGFR* mutation vs. *KRAS* mutation vs. *EGFR*/*KRAS* negative) persisted in the multiple regression analysis. However, no significant differences were noted between cohorts, sample types, or tumour locations. The significances persisted when resections and "other NSCLC" than AC and SCC were excluded from the analysis. When the cohorts were analysed independently, the significances persisted for the Lund cohort. In the Halmstad cohort, only mutational status, not histological type, was

significantly correlated with PD-L1 expression. In the Lund cohort, there was merely a nonsignificant trend for lower PD-L1 in cytology than biopsies (p = 0.08).

Table X. Summary of the results in Paper IV. The correlations of different clinicopathological and molecular factors to PD-L1 expression from 1381 lung cancer specimens with complete data.

		Statistical analysis *P*-value		
Characteristics	**Parameters**	**Student's *t*-test**	**Chi-square (χ2)**	**Multiple regression (coefficient)**
Cohort	Lund vs. Halmstad	0.010	0.12	0.81 (-0.012)
Sample type	Biopsy vs. Cytology vs. Resection	0.75 [a]	0.88	0.28 (-0.041)
	Biopsy vs. Cytology	0.61	—	—
	Cytology vs. Resection	0.81	—	—
	Biopsy vs. Resection	0.53	—	—
Locality of tumour	Primary vs. Metastasis	0.98	0.30	0.37 (0.049)
Diagnosis	AC vs. SCC vs. Other NSCLC	<0.001 [a]	<0.0001	<0.0001 (0.17)
	AC vs. SCC	0.0003	—	—
	SCC vs. Other NSCLC	0.61	—	—
	AC vs. Other NSCLC	0.0062	—	—
Molecular profile	*EGFR*-mutation vs. *KRAS*-mutation vs. *EGFR*/*KRAS* wild-type	<0.001 [a]	<0.0001	<0.0001 (0.22)
	EGFR-mutation vs. *KRAS*-mutation	<0.0001	—	—
	KRAS-mutation vs. *EGFR*/*KRAS* wild-type	0.0020	—	—
	EGFR-mutation vs. *EGFR*/*KRAS* wild-type	<0.0001	—	—

Note: The calculations represent correlations between different clinicopathological and molecular factors, and PD-L1 expression using a 3-tier scale (<1%, 1-49% and ≥50% cutoff levels).
Abbreviations: AC = adenocarcinoma; *EGFR* = epidermal growth factor receptor; *KRAS* = kirsten rat sarcoma; NSCLC = non-small cell lung cancer; PD-L1 = programmed death-ligand 1; SCC = Squamous cell carcinoma.
[a] One-way analysis of variance (ANOVA).

PD-L1 expression status in EGFR-mutated lung carcinomas

Out of the 163 cases harbouring *EGFR* mutations in the two cohorts, 74 (45%) presented a deletion in exon 19, whereas 66 (40%) had an L858R mutation. The PD-L1 expression was comparable for the two types, with 39% and 38% at a cutoff level of ≥1% of tumour cells, and 9% and 12% at a cutoff level of ≥50%, respectively (Student's *t*-test, p = 0.91). All four *EGFR*-mutated SCC cases tested positive for PD-L1, with three of them showing PD-L1 expression of ≥50%.

There were 14 *EGFR*-mutated cases sampled after treatment for purpose of investigating resistance mutations or confirming metastasis (while nearly all small specimens analysed for PD-L1 expression in the cohorts were obtained as part of the investigational work-up, i.e., prior to any treatment). In 71% of these cases ≥1%

of the tumour cells tested positive for PD-L1, while in 14% of the cases ≥50% of the tumour cells were positive for PD-L1. This percentage was significantly higher compared to those with sampling before treatment (p = 0.034).

PD-L1 expression status in KRAS-mutated adenocarcinomas

In the two cohorts, 403 cases of AC harboured a *KRAS* mutation, accounting for 35% of all 1160 AC cases in the cohorts, or 42% of the 959 AC cases analysed for *KRAS*. The predominant *KRAS* mutations included *p.G12C* in 38%, *p.G12V* in 18%, *p.G12D* in 15%, and *p.G12A* in 8% of the *KRAS*-mutated AC. Among the 403 AC cases with a *KRAS* mutation, a morphological mucinous growth pattern was observed in 62 (15%) cases.

PD-L1 expression was observed in 247 out of 403 (61%) *KRAS*-mutated AC at a cutoff of ≥1%. More data utilizing the three-tier scale (<1%, ≥1-49%, and ≥50%) was analysed and compiled. Based on a Student's *t*-test, no significant difference was found in PD-L1 levels among the four most prevalent *KRAS* mutations (p = 0.44-0.92). Additionally, the ANOVA analysis yielded nonsignificant results (p = 0.88). In contrast, there was a significantly lower PD-L1 expression level in mucinous *KRAS*-mutated AC compared to non-mucinous cases (Student's *t*-test, p < 0.0001) (see Figure 31).

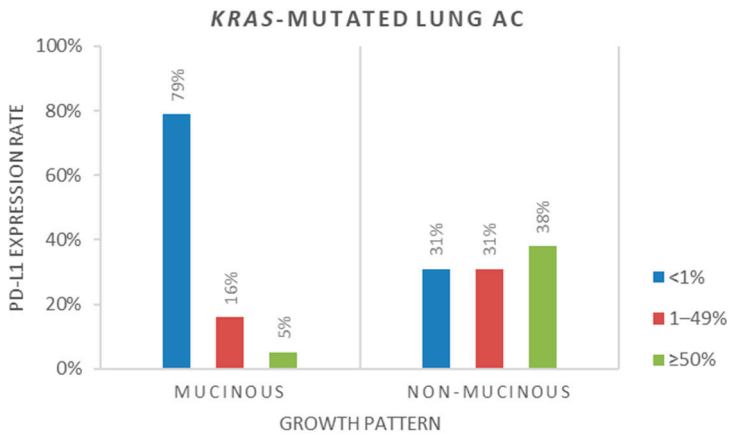

Figure 31. PD-L1 expression rate in *KRAS*-mutated lung adenocarcinomas. Differences in PD-L1 expression according to mucinous and non-mucinous growth pattern. AC = adenocarcinoma, *KRAS* = kirsten rat sarcoma, PD-L1 = programmed death-ligand 1.

Paper V

Characteristics of the cases and expression status of biomarkers in matched cell blocks

During the observed study period, a total of 77 consecutive malignant pleural effusions were collected, with 60 sourced from distinct patients. Twenty-five out of the 60 patients were excluded because their final diagnosis revealed a malignancy other than lung AC. Among the 35 remaining cases, 24 had adequate material in all cell blocks. In 7 cases, three cell blocks each were fixed in formalin, PreservCyt®, and CytoLyt® respectively. For the remaining 17 cases, there were four cell blocks each, fixed also in CytoRich™ Red.

All cell blocks were subsequently examined for the expression of TTF-1 (clones 8G7G3/1 and SPT24), napsin A, claudin 4, CEA, CK7, and EpCAM (clones BS14, Ber-Ep4, and MOC-31). Among the cases examined, 7 (29%) were male and 17 (71%) were female, with a median age at diagnosis of 73 years, range (43-85).

The status of the examined ICC biomarkers was assessed at a cutoff of ≥10% positive tumour cells, regardless of intensity, to define positive staining (Figure 32). The positive expression frequencies for all biomarkers (with the expectation that the selected ones would test positive in all or most cases of lung ACs) ranged from 87.5% to 100% for formalin-fixed cell blocks.

PreservCyt® and particularly CytoLyt®-fixed cell blocks demonstrated lower scores for TTF-1 clone 8G7G3/1 compared to formalin-fixed cell blocks (62.5% and 33.3%, respectively, versus 87.5%). These differences were statistically significant (Friedman's test, $p < 0.001$; McNemar's test, $p = 0.031$ and $p < 0.001$, respectively). The rate of positivity frequency for TTF-1 clone 8G7G3/1 was somewhat lower for CytoRich™ Red (76.5%) compared to formalin, although not significantly. Moreover, the TTF-1 clone 8G7G3/1 for CytoLyt®-fixed cell block exhibited significantly lower scores compared to PreservCyt® and CytoRich™ Red (both $p = 0.016$).

The rate of positivity frequency for napsin A and EpCAM clone Ber-Ep4 was slightly and nonsignificantly lower only in PreservCyt® compared to formalin and other fixatives. For EpCAM clone MOC-31, both PreservCyt® and CytoLyt® were linked to slightly and nonsignificantly lower scores. However, the expression rate was consistent across all fixatives for the other included biomarkers (Figure 32).

Concordance of biomarker expression between matched cell blocks

The concordance of positivity for the ICC biomarker between matched cell blocks, fixed in different fixatives was analysed using various methods including OPA, PPA, NPA, McNemar's test, and Cohen's κ when applicable. The calculations were based on a threshold of ≥ 10% positive tumour cells to define positive staining, with formalin as the standard for the PPA and NPA calculations. Unweighted Cohen's κ was also calculated, applicable to TTF-1, napsin A, and EpCAM clone MOC-31

(for the other biomarkers, the frequency of positivity and concordance was too high for any calculation).

When compared with formalin, the lowest Cohen's κ and OPA values were observed for PreservCyt® and CytoLyt® for TTF-1 clone 8G7G3/1, with κ of 0.385 and 0.133, and OPA of 75% and 46%, respectively. Similarly, for EpCAM clone MOC-31, κ was 0.357 and OPA was 88% for both fixatives. Higher corresponding figures for CytoRich™ Red compared with formalin were found, with κ of 0.821 and OPA of 94% for TTF-1 clone 8G7G3/1 and, κ of 1.0 and OPA of 100% for EpCAM clone MOC-31. However, differences and overlaps in positivity for TTF-1 clone 8G7G3/1 and EpCAM clone MOC-31 among all fixatives were found.

For napsin A and EpCAM clone Ber-Ep4, two and one cases, respectively, tested negative in cell blocks fixed with PreservCyt® but not in the other cell blocks (for napsin A, there was a CytoRich™ Red cell block for only one of the cases), resulting to a slight decrease in OPA and, for napsin A, κ value. Results from OPA analyses using ≥10% positive tumour cells to define positive staining are available in Table XI.

Further comparisons were conducted using pairwise Wκ and Wilcoxon signed-rank tests for all antibodies and fixatives, employing the four-tier scale (<1%, 1-9%, 10-49%, and ≥50%). PreservCyt®, CytoLyt®, and CytoRich™ Red yielded significantly lower scores compared to formalin for both TTF-1 clone 8G7G3/1 and EpCAM clone MOC-31 (Wilcoxon signed-rank test, $p = 0.046$ for CytoRich™ Red and $p < 0.001$ for the others). The scores demonstrated significant discrepancies, with PreservCyt® and CytoLyt®, showing significantly lower scores compared to CytoRich™ Red. Specifically, CytoLyt® exhibited lower scores compared to PreservCyt® only for TTF-1 clone 8G7G3/1 (Wilcoxon signed-rank test, all $p \leq 0.03$).

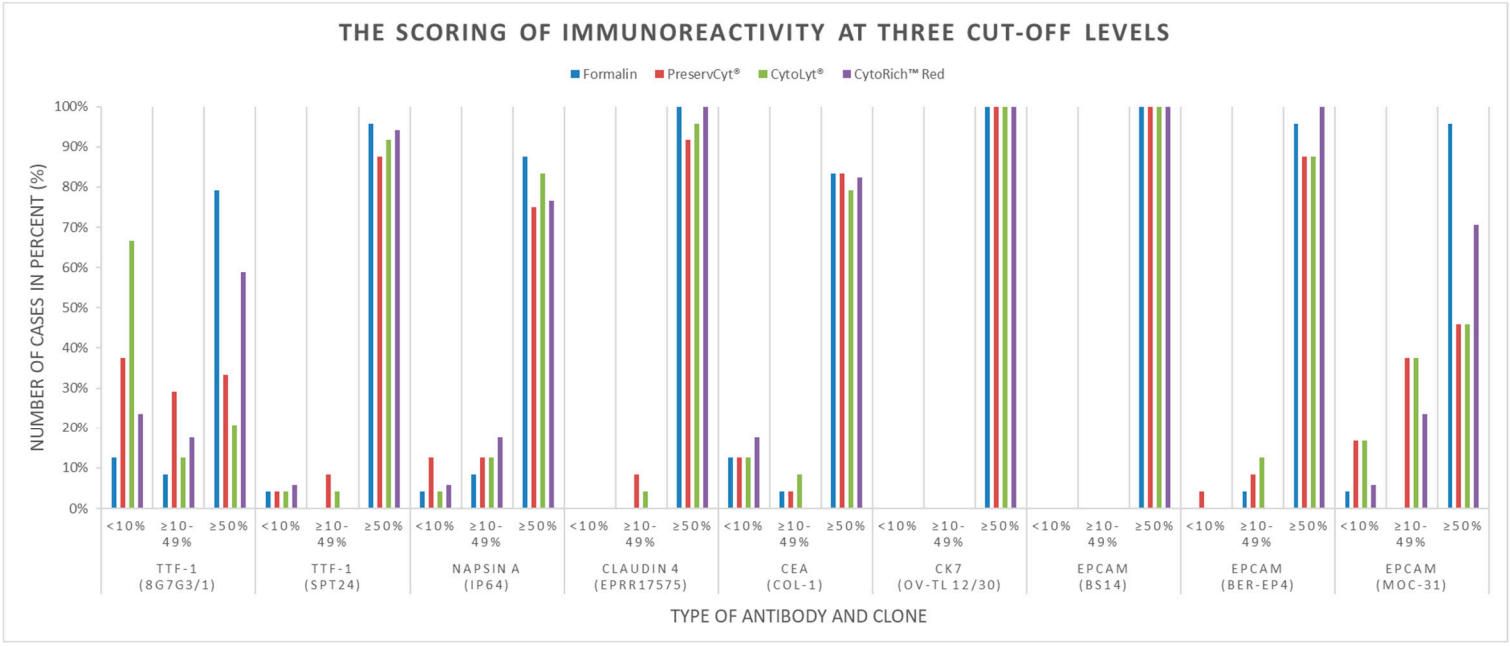

Figure 32. The status of biomarker expression at different cutoff levels. Four matched cell block preparations fixed in different fixatives. CEA = carcinoembryonic antigen, CI = confidence interval, CK7 = cytokeratin 7, EpCAM = epithelial cell adhesion molecule, TTF-1 = thyroid transcription factor 1.

Table XI. The agreement of immunohistochemical reactivity between matched cell block preparations. The concordance of expression for different lung adenocarcinoma biomarkers in matched cell blocks from pleural effusion specimens.

IHC biomarker antibody (clone)	The overall percentage agreement (OPA) % (95% CI)		
	Formalin vs. PreservCyt®	**Formalin vs. CytoLyt®**	**Formalin vs. CytoRich™ Red**
TTF-1 (8G7G3/1)	75 (58 – 87)	46 (28 – 65)	94 (71 – 100)
TTF-1 (SPT24)	100 (84 – 100)	100 (84 – 100)	100 (78 – 100)
Napsin A (IP64)	92 (73 – 99)	100 (84 – 100)	100 (78 – 100)
Claudin 4 (EPRR17575)	100 (84 – 100)	100 (84 – 100)	100 (78 – 100)
CEA (COL-1)	100 (84 – 100)	100 (84 – 100)	100 (78 – 100)
CK7 (OV-TL 12/30)	100 (84 – 100)	100 (84 – 100)	100 (78 – 100)
EpCAM (BS14)	100 (84 – 100)	100 (84 – 100)	100 (78 – 100)
EpCAM (Ber-Ep4)	96 (78 – 100)	100 (84 – 100)	100 (78 – 100)
EpCAM (MOC-31)	88 (68 – 96)	88 (68 – 96)	100 (78 – 100)
Total of cases, n (%)	**24**	**24**	**17**

Note: Positive expression defined as ≥10% positive tumour cells.
Abbreviations: CEA = carcinoembryonic antigen; CI = confidence interval; CK7 = cytokeratin 7; EpCAM = epithelial cell adhesion molecule; TTF-1 = thyroid transcription factor 1.

Additionally, significant differences in expression rates for TTF-1 clone 8G7G3/1 between fixatives persisted when applying the widely used cutoff of ≥1% positive tumour cells as the criterion for defining positive staining. In four out of the 24 cases (17%), <1% of positive tumour cells were observed in PreservCyt® and CytoLyt®, whereas they exhibited ≥1% in formalin. Conversely, the corresponding number was only one out of 17 cases (6%) for CytoRich™ Red.

Figures 33 and 34 show images illustrating differences in immunostaining with TTF-1 and EpCAM clones in matched cell block preparations fixed in four fixatives.

Intensity of biomarker expression between matched cell blocks

The distribution of intensity for the examined ICC biomarkers based on the fixative of cell block was evaluated. Significant differences in intensity were observed for TTF-1 clone 8G7G3/1, napsin A, and EpCAM clones Ber-Ep4 and MOC-31 (Friedman's tests, all $p \leq 0.01$). For all these biomarkers, the strongest intensity was observed with formalin, whereas there was some variability among biomarkers in terms of which LBC fixative exhibited the lowest staining intensity. For TTF-1

clone 8G7G3/1, napsin A, and EpCAM clone MOC-31, PreservCyt®, CytoLyt®, and CytoRich™ Red all exhibited significantly weaker staining compared to formalin (Wilcoxon signed-rank test, $p < 0.001$ to 0.034). For EpCAM clone Ber-Ep4, only PreservCyt® demonstrated significantly lower intensity than formalin ($p = 0.02$). Moreover, both PreservCyt® and CytoLyt® exhibited significantly weaker intensity than CytoRich™ Red for TTF-1 Clone 8G7G3/1 and EpCAM clone MOC-31 (both $p \leq 0.001$ and $p \leq 0.013$, respectively). When considering all 24 cases (excluding CytoRich™ Red), significantly reduced intensity compared to formalin was also observed for TTF-1 clone SPT24 with PreservCyt® and for claudin 4 with CytoLyt® (Wilcoxon signed-rank test, $p = 0.034$ and $p = 0.024$, respectively).

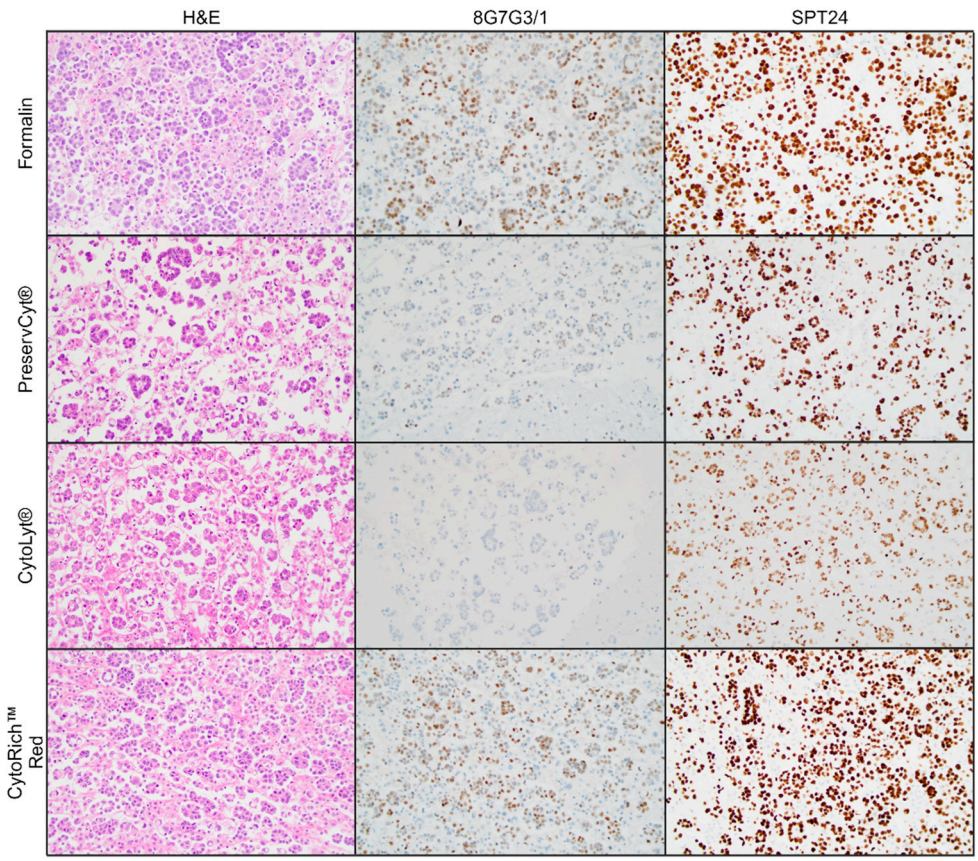

Figure 33. Four cell block preparations with lung adenocarcinoma from a single malignant pleural effusion fixed in four different fixatives, i.e., formalin, PreservCyt®, CytoLyt®, and CytoRich™ Red, respectively. All cell blocks were stained with haematoxylin-eosin (H&E) and immunocytochemical (ICC) stains with two different TTF-1 clones, i.e., 8G7G3/1 and SPT24, respectively. Original magnification x20 objective.

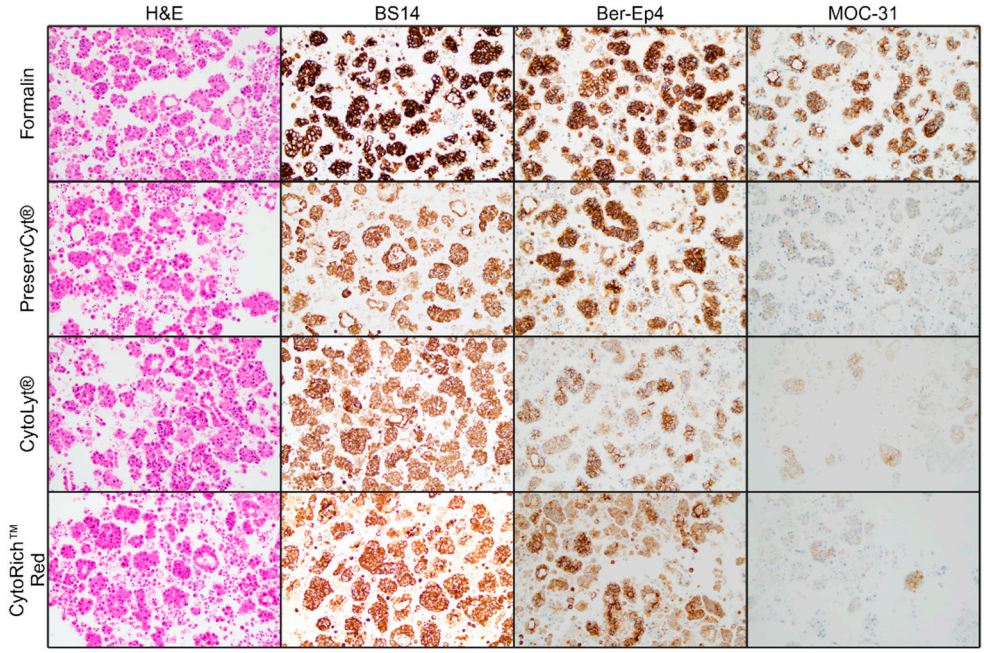

Figure 34. Four cell block preparations with lung adenocarcinoma from a single malignant pleural effusion fixed in four different fixatives, i.e., formalin, PreservCyt®, CytoLyt®, and CytoRich™ Red, respectively. All cell blocks were stained with haematoxylin-eosin (H&E) and immunocytochemical (ICC) stains with three different EpCAM clones, i.e., BS14, Ber-Ep4, and MOC-31, respectively. Original magnification x20 objective.

General Discussion

A lion chased me up a tree,
and I greatly enjoyed the view from the top.

Confucius 551-479 BCE

Clinical trials have predominantly relied on biopsy-based testing, but cytology often represents the sole accessible material in the routine clinical setting. Therefore, cytology remains pivotal in diagnosing thoracic malignancies, including PM and NSCLC, that is, despite the limitations mentioned in the introduction of this thesis. Nevertheless, the indications have changed over time, including greater use of different cytological specimens, both for primary diagnosis and staging purposes. Furthermore, the emergence of genomic medicine and the growing adoption of personalised molecularly targeted therapies and immunotherapies in clinical practice necessitates more use of ancillary techniques including complex diagnostic techniques and targeted sequencing panels, such as immunostaining and NGS.

Research has shown that cytology samples are well suited to provide the necessary information and can be a surrogate method. Optimizing sample procurement and utilization requires a close working relationship between cytopathologists and clinicians, [465] as well as the improvement of diagnostic and predictive cytology techniques. As cytological material is handled in different ways it is crucial to maintain a high quality of cytological diagnostics across laboratories preferably by ensuring a strong correlation with biopsies. It is not only important for confident diagnoses established on cytology as diagnostic material but also for the accurate utilization of cytology for ancillary methods. Therefore, the scope of all included Papers in this thesis aimed to improve diagnostic and predictive cytology focused on specimens from PM and NSCLC.

Our investigations relied on cytology-histology correlation, based on the most commonly used diagnostic materials i.e., biopsies and effusion cytology (Paper I and II) in the diagnosis of PM, or biopsies and different lung cytologies (Paper III) in the diagnosis of NSCLC. Also, the predictive biomarker PD-L1 was evaluated in unpaired cytology and histology samples (Paper IV). Given the wide range of cytology material preparation and fixation methods, cytology-cytology correlation was investigated using the same preparation technique, i.e., cell blocks, but different fixatives of the same cytology material for evaluation of immunostainings (Paper V).

In Paper I, we evaluated the PD-L1 expression in paired biopsies and effusion cytology cell blocks from PM patients. The PD-1/PD-L1 pathway assumes a pivotal role in facilitating tumour immune evasion. PD-L1 expression has been detected across various malignant tumour types, prompting the use of IHC assessment to identify patients eligible for immune checkpoint inhibitor (ICI) therapy.

Most studies investigating PD-L1 expression have focused on histological samples, particularly those derived from NSCLC tissue specimens. [362] Numerous studies have investigated the comparison of PD-L1 reactivity between histological and cytological materials from NSCLC, [466] which we have also performed in Paper III. Diverse cytological samples have been encompassed, with only a limited number of studies offering findings from paired histology and pleural effusions. In NSCLC studies, the concordance between histological and cytological samples has been reported diversly, including as overall positive or negative agreement, Kappa (κ) agreement statistics, or correlation coefficients. Consequently, comparing results across different studies proves challenging. In a systematic review comprising 142 studies, the OPA reached 88.3% at the ≥1% cutoff level between histological and cytological specimens across 9 studies fulfilling the inclusion criteria. [466] In Paper III, as discussed below, we observed an agreement of 85% (κ = 0.77) and 68% (κ = 0.49) in our two cohorts, respectively. We also reviewed up-to-date literature and based on 25 published studies including about 1,700 paired histology/cytology cases, the median concordance was 81-85% at cutoff 1% for a positive PD-L1 staining.

In studies conducted on histological mesothelioma specimens, the positivity rates for PD-L1 varied from 11% to 72% at the ≥1% cutoff level. [247] Studies conducted on mesothelioma effusions are scarce.

In our previous study (not included in the thesis) encompassing 74 mesothelioma effusions, of which 61 met the inclusion criteria, PD-L1 positivity was observed in 38% and 10% of cases at the ≥1% and >50% cutoff levels, respectively. The report suggested that the PD-L1 expression in cytology is similar to previously reported studies on histological specimens. [467] Beyond our investigation, we found only 2 additional studies, collectively involving 8 mesothelioma cases. [441, 468] A study conducted on diverse cytological samples comprised 3 pleural effusions, one of which was mesothelioma. [441] Khanna et al. reported another 7 mesothelioma effusions with corresponding biopsies. [468]

Among our histological samples (Paper I), we identified PD-L1 positivity in 28 out of 61 cases (46%) at the ≥1% cutoff level, and in 7 out of 61 cases (12%) at the >50% (Table IV). The reported positivity range in previous studies conducted on histological mesothelioma specimens varies widely. [247] In a recent study utilizing the same antibody clone (28-8 pharmDx) and deemed most comparable to ours, a positivity rate of 23% (25 out of 112) was reported in all pleural mesotheliomas. Specifically, positivity rates of 16% (9 out of 56), 30% (14 out of 47), and 22% (2 out of 9) were observed in epithelioid, biphasic, and sarcomatoid mesotheliomas, respectively. [469]

In our study, the frequency of PD-L1 positive samples in epithelioid and non-epithelioid mesotheliomas differed for the histological specimens at the ≥1% cutoff level. Epithelioid mesotheliomas showed positivity in 19 out of 49 cases (39%), while non-epithelioid mesotheliomas showed positivity in 9 out of 12 cases (75%). This aligns with previous studies indicating higher positivity rates in sarcomatoid mesotheliomas. [356, 470] The positivity rates observed in the cytological specimens (21 out of 61 [34%] at the ≥1% cutoff and 5 out of 61 [8%] at the >50% cutoff) remained consistent with the findings from our previous study.

We presented the concordance between histological and cytological specimens in terms of OPA, PPA, NPA, Cohen's κ, and McNemar's probability.

Lacking studies comparing effusions and biopsies from mesotheliomas, we juxtaposed our findings with those reported in analogous NSCLC studies. A review of studies comparing histological and different types of cytological specimens has notably revealed a high degree of concordance. [466] However, only three of the included studies focused partially on effusions. One of the studies, utilizing antibody clone 22C3, examined 30 effusions and 40 bronchial wash samples with NSCLC, revealing very high agreement across the total material (~90%). This agreement was reported in terms of OPA, PPA, and NPA at both the ≥1% and ≥50% cutoff levels. [444] Our findings in PM mirrored those at the ≥1% cutoff level but exhibited lower agreement at the ≥50% cutoff level. Another study on NSCLC reported fair to moderate concordance ($\kappa = 0.39$) across 15 effusions and their corresponding histological specimens, [449] which is in line with our results. As indicated in Table IV, the frequency of positivity was marginally lower in cytological specimens compared with histological specimens in our study. The κ values at various cutoff levels suggested that in cases of highly positive samples, histology and cytology may exhibit disagreement and could be complementary (but correlation to outcome – prognosis or treatment response – would be of interest for such investigation). McNemar's test revealed no significant evidence of systematic discrepancies between histological and cytological specimens (Table V). The agreement declined at higher cutoff levels, potentially attributed to the relatively small sample size of this study and the limited number of positive cases at higher cutoff levels, thereby increasing the impact of random variation. Nevertheless, it suggested that both the chosen cutoff level and the type of material used exert an influence on the outcome.

The concordance between histological and cytological specimens at the ≥1% cutoff level varied between epithelioid and non-epithelioid mesotheliomas. In epithelioid mesotheliomas, the concordance was moderate ($\kappa = 0.43$), whereas in non-epithelioid mesotheliomas, there was no concordance ($\kappa = 0.08$) (Table V). The κ values notably decreased for higher cutoff thresholds. Varied proportions of histological subtypes of mesotheliomas in the study comparing histological specimens with effusions impact the results. While sarcomatoid mesothelioma gives rise to effusions, it seldom sheds tumour cells into the effusion. In cases where tumour cells are identified in the effusion, they frequently have adopted epithelial features. Hence, the mesenchymal component tends to be present in a small

proportion, while the epithelial component typically dominates in cytological specimens.

A conceivable rationale for the distinct concordances observed for epithelioid and non-epithelioid mesotheliomas in our study is the absence of the sarcomatoid tumour component that tends to be more frequently positive, and which is not found in the cytological material. Thus, the malignant cells present in effusions from epithelioid mesotheliomas may offer a more comprehensive representation of the entire tumour. A recent study demonstrated that the overall survival and proportion of long-term survival were notably better for mesothelioma patients diagnosed by cytology than by histology, suggesting that malignant cells in effusions may represent a different population. [471]

The presence of macrophages and benign mesothelial cells in the mix may cause interpretation problems, given their morphological resemblance to tumour cells and the expression of PD-L1 in some macrophages. Cellular specimens with a predominance of malignant cells generally presented no interpretation issues. However, distinguishing between tumour cells and immune cells proved challenging in certain cases. This challenge was even greater, particularly in instances where the tumour cells were few, compared to the inflammatory background. The evaluation of the cytological specimens may also be hampered by low overall cellularity together with an admixture of tumour cells dispersed among macrophages. Hence, in this study, a routine H&E-stained slide, along with relevant immunostainings for calretinin and CK5 or CK5/6, were available for most of the cases. These served as additional aids to identify the malignant cells, facilitating the assessment of PD-L1.

The malignant cells shed into effusions may constitute a distinct subpopulation, possibly not indicative of the entire tumour. This discrepancy could elucidate variations in the frequency of PD-L1 reactivity observed between biopsies and effusions. Some studies based on histological specimens indicate that there is an association between PD-L1 reactivity and histological tumour subtype of mesothelioma, with the expression being higher in sarcomatoid histology. [356, 470]

The low occurrence of sarcomatoid mesotheliomas (2/61) and biphasic mesotheliomas (10/61) in our study can be attributed to our fundamental inclusion criterion, which required effusions containing diagnostic malignant cells.

The rationale underlying the assessment of PD-L1 activity in tumour tissue is based on the assumption that PD-L1 positivity could predict the response to treatment with PD-1 or PD-L1 inhibitors. While several studies have indeed demonstrated a correlation between such response and PD-L1 expression, some patients with low levels of PD-L1 expression in their mesothelioma tumours have also shown good response to treatment. [472] Currently, there is no consensus on whether tumour PD-L1 expression reliably predicts outcomes[473] and mesotheliomas are not tested for PD-L1 in the clinical setting.

In many of our cases, significant differences were observed in the fraction of positive cells between biopsies and effusions. However, the design of Paper I

mitigates disparate outcomes stemming from methodological variations, including using identical preparation methods for both material types and adhering rigorously to the positivity criteria recommended by the manufacturer. [437] Further, all samples were obtained at the time of diagnosis, prior to the administration of any oncological treatment to the patients. Certain studies suggest that chemotherapy might influence PD-L1 expression in cancer cells, potentially biasing the apparent outcomes. [474] Detailed information on the strengths and limitations of Paper I is presented in Table XII.

In Paper II, we examined multiple diagnostic IHC biomarkers in 59 pleural mesotheliomas using paired biopsies and FFPE pleural effusion cell blocks. As discussed above, effusion cytology predominantly comprises epithelioid mesothelioma cells. As anticipated, we observed a notably high concordance between cytology and the histological epithelioid component, particularly regarding the expression of desmin, CK5, and podoplanin, followed by calretinin, BAP1, WT1, and EMA. Nevertheless, there were more cases with discrepancies observed for MTAP as well as for several biomarkers when comparing cytology with the histological sarcomatoid component.

While numerous studies have explored diagnostic IHC biomarkers in either cytological or histological material, few have incorporated paired specimens. Five studies, each comprising 6-30 paired cases, consistently reported 100% concordance for BAP1 and MTAP between histology and cytology, [288, 475-478] whereas in a larger study encompassing 57 paired cases, a κ agreement value of 0.85 was reported between surgical and cytological specimens for BAP1. [479] However, the applied cutoff levels varied, with thresholds ranging from 50% to 100% negative tumour cells defining loss of expression, thereby making comparisons between studies challenging.

Several investigations have reported partial loss of BAP1 and MTAP in certain mesotheliomas. [296, 299, 475, 478, 480] Moreover, a wide range of frequencies of loss has been reported for mesotheliomas, 10-90% for BAP1 and 33-76% for MTAP. [300, 478, 481-485] In two studies, loss of both BAP1 and MTAP, as well as preserved expression of both biomarkers, was observed in 21-35% and 20-21% of mesotheliomas, respectively. [476, 486] In our material, the loss of expression of both biomarkers was more prevalent, while preserved expression of both biomarkers was less common, particularly in cytology (Table VII). In our view, defining loss of expression and exploring cytological-histological concordance, especially for MTAP, warrants further attention.

One study reported a 71% concordance rate for desmin in paired histological and cytological specimens, considering a positive staining as ≥1% positive tumour cells. [475] If we were to apply the same cutoff to our material, the concordance would be 93% instead of 100%, as there are some cases with less than 10% positive cells in cytology. We cannot rule out the possibility that the focal desmin positivity observed in our cytological specimens may represent RMC, as these cells are very difficult to distinguish from mesothelioma cells. Dual staining with desmin/EMA

could prove valuable for accurately differentiating between mesothelioma and RMC. [487] Nonetheless, focal desmin expression has been reported in 5-10% of mesotheliomas in histological specimens. [281, 475, 488] Hence, our findings may also represent genuine expression.

To the best of our knowledge, there is a lack of data on paired histological and cytological specimens in the literature for most of the biomarkers investigated in our study. In unpaired cases, the reported frequencies of positivity in epithelioid mesotheliomas are as follows: 87-100% for calretinin, 69-100% for CK5 or CK5/6, 66-100% for podoplanin, 72-99% for WT1, and 80-96% for EMA. [281, 489-496] Lower frequencies have been reported for sarcomatoid mesotheliomas. This aligns with our findings (Figure 26).

In our study, the frequency of EMA positivity differed between histological and cytological specimens, with epithelioid histology specimens being positive in 84% and cytology specimens in 90% of the cases (Figure 26). However, we noted more frequent focal reactivity of EMA in biopsies compared to cytology.

For several of the investigated biomarkers, positive IHC expression was observed in the epithelioid component of both biopsies and cytology specimens, while the sarcomatoid component of biopsies was negative in some biphasic cases in our material. Furthermore, the agreement was lower between cytology and the histological sarcomatoid component compared to the histological epithelioid component for most biomarkers (Table VI). This observation may reflect the biological differences of sarcomatoid mesothelioma and the fact that effusion cytology predominantly contains epithelioid mesothelioma cells. Thus, the single pure sarcomatoid case (as per histology) in our material was likely biphasic but lacked an epithelioid component represented in the biopsy. Considering the inclusion criteria of our study, which required the presence of mesothelioma cells in the pleural effusion, the limited number of sarcomatoid cases was unsurprising. Fine needle aspirations may offer superior cytological material for investigating cytological-histological concordance in sarcomatoid cases, but this aspect falls beyond the scope of our study.

The main strengths of Paper II comprise the utilization of paired histological and cytological specimens from uncommon malignancy like PM, along with the evaluation of multiple IHC biomarkers within the same cohort. A list of strengths and limitations of Paper II is shown in Table XII.

In Paper III, the paired cytology/biopsy NSCLC cases indicate that PD-L1 expression is lower in cytological specimens, with significance observed in one cohort and a trend noted in the other of our 2 cohorts. Indeed, the PD-L1 score was lower in the cytological specimen for all discordant cases in the Lund cohort. It is worth noting that the concordance was relatively limited in the Halmstad cohort, with a kappa value of 0.49. While discordant expression was more prevalent in paired samples from distinct sites (e.g., biopsy from the primary tumour and cytology from pleura or lymph nodes) with a concordance of 58% (22 out of 38 cases) in our combined cohorts, discordant cases were also noted in paired samples

from the same site, with a concordance of 77% (70 out of 91 cases). Concordance was comparable for both ACs and SCCs, as well as for new and archival blocks.

An important distinction between, as well as within, our two cohorts was the duration in alcohol-based fixative (usually 1-3 days vs. <24 h, with some solely undergoing formalin fixation) and the utilization of Cellient™ versus manual cell blocks. While the fixation and processing of biopsies remain essentially consistent across all pathology departments, there are notable differences in the handling of cytological specimens for IHC. Aside from formalin, cells may be fixed in various ethanol- or methanol-containing solutions (e.g., CytoLyt®, PreservCyt®, CytoRich™ Red, CytoRich™ Blue, CytoRich™ Clear and Novaprep®), while different techniques including direct smears, Cytospin™ preparations, or cell blocks (e.g., plasma-thrombin, agar, Cellient™, and Shandon™) may be used, [497] which is also evidenced by the published studies reviewed in our study.

Alcohol fixation of tissue has been suggested to impact PD-L1 staining negatively, [498] and some studies, such as our Lund cohort, have reported a lower PD-L1 score for alcohol-fixed cytology in all discordant paired cytology/histology cases. [447, 499] Moreover, Koomen et al. [464] clearly demonstrated that alcohol-fixed Cellient™ cell blocks yield lower concordance with histology compared to formalin-fixed agar-based cell blocks. However, the compiled literature data in Paper III did not reveal any obvious difference between formalin and non-formalin fixation. Additionally, Lou et al. [462] demonstrated a very good concordance for formalin-fixed cell blocks, with or without prefixation with CytoLyt®, while a perfect concordance was observed in the study by Gosney et al. [500] with EBUS cytology fixed in formalin versus alcohol. Fixation in, for example, CytoLyt®, is very rapid, and it has been suggested that fixation for less than 1 hour does not affect IHC staining (personal communication). This area thus merits future investigation.

Furthermore, specimen types differed between our cohorts, with the Lund cohort comprising more bronchial biopsies, brushes, and EBUS-guided lymph node aspirations, whereas the Halmstad cohort contained more transthoracic core biopsies, BAL, and pleural effusions. Both cohorts, but especially the Lund cohort, contained cell blocks mixed from different cytological samples to enhance tumour cell content. This complicates the analysis of sample types but reflects the real-world diagnostic situation in our setting. Grosu et al. [452] and Zou et al. [459] demonstrated a higher concordance for PD-L1 in pleural effusions compared to matched histological samples (87-97% at 1% cutoff) than Yoshimura et al. [501] and Jug et al. [460] did for EBUS-guided samples (70-84%). Such a trend was not observed when comparing our cohorts but may be obscured by other differences with greater impact. There were too few pleural effusions in the Lund cohort and too few EBUS-guided aspirations in the Halmstad cohort for comparisons of these two sample types within each cohort. However, in an incremental study not included in the thesis, we have further investigated the potential impact of different types of cytological specimens, different types of biopsies, histological tumour type, and specimen cell

content on the concordance of PD-L1 expression between biopsies and cytology in the Halmstad cohort, with an extended number of cases. [502]

Interestingly, in the extended Halmstad cohort, the cyto-histological PD-L1 concordance was lower for bronchial brushes and pleural effusions compared to BAL specimens, while there was no obvious difference between bronchial and transthoracic core biopsies, or histological tumour type. Additionally, a high number of tumour cells (>500) in biopsies was associated with better concordance at the ≥50% cutoff. [502] However, our cohorts lack sufficient cases for adequate multivariable analyses, and ideally, future studies may include multiple samples from the same patient for further analysis of the potential impact of sample type on PD-L1 expression (ideally with correlation to treatment outcomes).

Nevertheless, the overall concordance of PD-L1 expression between histology and cytology was rather good based on our results on our results and previously published studies. In the literature, discordant cases typically exhibit either a higher score in histology or in cytology, [441, 444, 445, 457, 503] which is expected owing to the heterogeneity of PD-L1 expression. [504-509] However, the range of concordance was quite broad, spanning from 62% to 100% at a cutoff of 1% and from 67% to 100% at a cutoff of 50%, as based on 25 publications. Moreover, several studies indicate a positive PD-L1 expression (≥1%) in less than 40% of NSCLC cases when using cytology, [443, 444, 446, 454, 456, 463, 510, 511] a figure notably lower than what has been reported in both early treatment studies and large studies with real-life data, where PD-L1 expression ≥1% has been reported in 53-86%[512] and 56-63%[513, 514] of NSCLC cases, respectively, with a rate of 61% for cytology. [513] Moreover, for instance, the large study by Kuempers et al. [454] demonstrated a high concordance (93%) at the 50% cutoff for a positive staining. However, there were still no cases with >50% in both cytology (mainly imprints from resections) and the paired resected tumours, raising questions about the applicability of the results. We believe it would be reasonable for any department that utilizes cytology for PD-L1 assessment in NSCLC to (a) examine the proportion of PD-L1-positive cases in 50-100 nonselective cytological specimens, and (b) assess the concordance between biopsies and cytological specimens in 20-30 paired cases. A laboratory with less than 55% PD-L1-positive cases and/or a concordance rate below 85% should consider investigating possible causes and exploring potential improvements.

Biopsies currently serve as the standard for PD-L1 testing, and positivity in histological samples, at varying threshold levels, has been a prerequisite for the use of some anti-PD-1/PD-L1 immunotherapy (but with changes in the role of testing and treatment indications over time since the start of our research studies). However, there is no complete concordance between positivity in histology samples and therapy response, and based on histology, PD-L1 is not regarded as an optimal predictor of immunotherapy response. [358-361, 515-518] Additionally, there is a variation in positive PD-L1 expression between studies for biopsies as well which is also evident from our review of the literature, but also observed in treatment studies. [512]

Hence, it could be argued that the predictive value of PD-L1 in cytology (and ideally the concordance with biopsies), and whether basing therapy decisions on PD-L1 positivity in cytology samples would be an improvement compared to histology or not, or whether the methods would be complementary should be assessed in a population of patients undergoing immunotherapy and can only be determined by investigating paired samples together with therapy response, which probably requires a multicentre study. Currently, such studies are lacking. Further, the identification of accurate predictive biomarkers beyond PD-L1 expression remains essential to select the most appropriate candidates for ICI therapy.

PD-L1 expression in immune cells, which is pertinent to a different PD-L1 assay (SP142), was not the focus of the present study. The representation of tumour-infiltrating immune cells in small samples has been discussed, and the interobserver concordance for PD-L1 has been demonstrated to be low for immune cells. [363, 364] In our Halmstad cohort, we observed that more than 1% positive lymphocytes were present in 7 (7%) out of 94 cytological specimens, and more than 1% positive macrophages were detected in 60% (65%) out of 93 cytological specimens. However, it cannot be investigated in cytological samples if lymphocytes are tumour-infiltrating or originally present far from the tumour.

In Paper III, we did not restrict the material to cases with sampling from the same site to eliminate another potentially confounding factor. However, data for samples from both the same and different sites are presented. All cases in each cohort were stained with the same biomarker, either 28-8 or 22C3, respectively. As very similar staining patterns have been reported for these two PD-L1 clones, [362-364] the staining is likely comparable. The Halmstad cohort included some old archival cases (associated with lower PD-L1 expression and not recommended[363, 519]), but this was approximately equal for the cell blocks and biopsies, which likely mitigates the impact on the results. In the Lund cohort, there was a slight selection bias for PD-L1-positive cases since a cytological cell block was not ordered as frequently in the clinical setting when a biopsy was PD-L1 negative. However, this likely does not significantly impact the results. The inclusion of cell blocks with mixed material from different sites limits the possibility of fully evaluating any potential impact of the sampling site on PD-L1. It is worth noting that the interobserver discrepancy was 17% (3/18) for these cell blocks, compared to 8% (10/126) for the remaining cases. Additional strengths and weaknesses of Paper III are listed in Table XII.

In recent decades, there has been a paradigm shift in the treatment of NSCLC with the introduction of targeted therapy and immunotherapy. This breakthrough has led to an increasing number of treatment-guiding biomarkers, including oncogenic driver alterations and PD-L1 expression. While targeting the PD-1/PD-L1 pathway leads to improved survival, not all patients respond well to anti-PD-1/PD-L1 immunotherapy. [348, 520-522] The optimal role and potential enhancement of PD-L1 testing, along with other mechanisms affecting responsiveness, have not yet been fully clarified. Here, additional insights from large studies with real-world data may be a way forward.

Given the results in Paper III, with good and moderate PD-L1 concordance, respectively, across two independent cohorts, with all discordant cases exhibiting lower expression in alcohol-fixed cytology in the former, and a lower concordance for pleural effusions compared to bronchial cytology, we continued with Paper IV. Here, our aim was to delve deeper into this aspect using larger (unpaired) cohorts, while also examining potential correlations of PD-L1 with molecular alterations and relevant pathological characteristics.

In our cohorts, 55% of the cases tested positive for PD-L1 (≥1% positive tumour cells), aligning with other large real-world studies predominantly from Europe and North America, which reported a frequency ranging from 52% to 63%.[513, 514, 523, 524] Zheng et al. reported a lower level of PD-L1 expression (43%) in a larger study from China, [525] while Wang et al. reported a frequency as high as 71% from Canada. [526]

In our study, we observed only a very slight and non-significantly lower frequency of PD-L1 positivity for cytological samples compared to biopsies, and for metastases compared to primary tumours. There are limited large-scale studies addressing these factors, but the study by Evans et al. found a higher PD-L1 expression in cytology, [513] while Wang et al. found no difference. [526] This suggests that PD-L1 expression in cytology is generally comparable to that in biopsies, but given the variation in handling and fixation of cytology, the matter should be further investigated. Both Evans et al. and Zheng et al. reported a higher PD-L1 expression in metastases, [513, 525] and in biopsies compared to resections (the latter was consistent with our results), but slightly higher expression in resections has also been reported. [523]

In our current study, we observed lower PD-L1 expression in AC compared to SCC, primarily driven by the Lund cohort, even when considering mutational status in multivariate analysis (Table X). Lower PD-L1 expression in AC has also been demonstrated in two large studies from China and North America, [524, 525] while no difference or higher expression in AC has been reported in two other large studies, [527, 528] and in systematic reviews. [529, 530]

Based on our findings and the large studies in the literature discussed above, drawing firm conclusions about sample type, sample site, and histological type is challenging. However, there appears to be no consistent evidence supporting significant differences in PD-L1 levels between biopsies and cytological specimens. In contrast, robust evidence suggests lower PD-L1 expression in *EGFR*-mutated AC compared to *EGFR* wild-type tumours, as supported by several studies. [513, 523-525, 527, 531, 532] However, some reports indicate a nonsignificant difference in this regard. [526] Reports have consistently indicated lower TMB and a reduced number of tumour-infiltrating lymphocytes (TILs) in *EGFR*-mutated NSCLC, alongside a poor response to ICI. [533-535] The possible efficacy of ICI in *EGFR*-mutated cases remains under discussion. [536] Increased PD-L1 expression has been observed post-targeted therapy, particularly in cases demonstrating therapy resistance. [537]

Consistent with our findings, previous studies have reported higher PD-L1 expression in cases with *KRAS* mutations, [527, 528, 531, 532] although statistical significance was not always reached. [526] Studies with comprehensive data on PD-L1

expression and both *EGFR* and *KRAS* status (especially after excluding *EGFR*-mutated cases which typically exhibit low PD-L1 expression, from the *KRAS* wild-type group) are relatively uncommon in the literature. Upon analysing AC, we observed no disparity in PD-L1 levels concerning prevalent *KRAS* mutations. Furthermore, when categorizing cases based on mutations, no discrepancy in PD-L1 levels was evident between *KRAS* mutations associated with high (*G12A*, *G12C*, *G13D*, *Q61L*) or low (*G12D*, *G12R*, *G12V*) Raf affinity. [538] Additionally, we found a significant difference between mucinous and non-mucinous *KRAS*-mutated AC (Figure 31). This is of great interest as one retrospective study demonstrated that immunotherapy of mucinous ACs exhibited a favourable therapeutic response and correlated to improved overall survival. [539] This may indicate that the predictive value of PD-L1 is lower in mucinous AC, but more studies on ICI response correlated to both the status of most common mutations and growth patterns are required.

The retrospective design of Paper IV and the inclusion of a proportion of older archival cases within one of the cohorts should be acknowledged. This may have potentially contributed to a higher frequency of AC cases in the group with a cytological diagnosis, although it is unlikely that this has significantly influenced the main results. More strengths and limitations of Paper IV are listed in Table XII.

In Paper V, we assessed multiple diagnostic biomarkers in 24 malignant pleural effusions (MPE) from metastatic lung AC with four matched cell block preparations. These were fixed in formalin, PreservCyt®, CytoLyt®, and CytoRich™ Red, respectively. We observed apparent discordance in staining properties between fixatives for TTF-1 clone 8G7G3/1 and EpCAM clone MOC-31. Additionally, significant differences in staining intensity were noted for TTF-1 clone SPT24, EpCAM clone Ber-Ep4, napsin A, and claudin 4. However, the biomarkers EpCAM clone BS14, CEA, and CK7 were not significantly affected. We particularly observed differences in staining proportion between PreservCyt® and CytoLyt® compared to formalin, while we noted a higher concordance between formalin and CytoRich™ Red.

The biomarkers and fixatives investigated were selected based on their clinical relevance and common usage. The included biomarkers are typically positive in most lung AC and are utilized to differentiate lung AC from lung SCC (TTF-1 and napsin A), mesothelioma (TTF-1, napsin A, EpCAM, CEA, and claudin 4), and AC of non-pulmonary origin (TTF-1, napsin A, and CK7). [116, 307] We included CytoLyt® due to the common practice of transporting cytological specimens in CytoLyt® and occasionally storing them in the medium overnight before preparation. This practice depends on the distance between the place of sampling and the laboratory.

In a study conducted by van Hemel & Suurmeijer, 30 different diagnostic IHC biomarkers were individually assessed in 3-8 PreservCyt®-fixed Cellient™ cell blocks across various malignancies, and they were compared with paired histology. [540] In their case series, TTF-1 clone 8G7G3/1, EpCAM clones Ber-Ep4 and MOC-31, CK7, and CEA showed concordance for all cases. However, negative ICC

staining was observed in some cases for calretinin, CD3, CD56, melan-A, and S100, and in all cases for WT1. Kawahara et al. observed impaired staining for TTF-1 clone 8G7G3/1 and reduced staining for napsin A in cell blocks with pleural lung AC fixed in CytoRich™ Blue and 95% ethanol, compared to CytoRich™ Red and formalin. However, no significant difference was noted for CK7. [541]

More extensive study materials have been presented regarding PD-L1. Koomen et al. demonstrated notably lower PD-L1 scores for 34 CytoLyt®/PreservCyt®-fixed Cellient™ cell blocks with lung cancer compared to paired histology (κ = 0.28-0.49 depending on the PD-L1 antibody clone), a trend not observed for formalin-fixed agar-based cell blocks. [464] The main conclusions were further substantiated in a follow-up Dutch nationwide interlaboratory comparison[542] and were consistent with a study conducted by our group (with a κ of 0.77). The study involved 47 CytoLyt®/PreservCyt®-fixed Cellient™ cell blocks with lung cancer alongside paired histology. Nevertheless, PD-L1 and other treatment-predictive ICC biomarkers[243, 245] were not included in the scope of the current study.

As evidenced by our findings and the literature cited, there is no generalized rule of thumb for ICC. Essentially, unless using formalin fixation, each antibody (or even antibody clone, particularly for some biomarkers) should undergo validation for every used fixation method. In the clinical setting, various potential solutions may exist for diagnostic analyses, including the selection of fixation method, choice of antibody clone, or selection of biomarker. According to our findings, biomarkers such as EpCAM clone BS14, CEA, or claudin 4 could be considered as carcinoma biomarkers (instead of EpCAM clone MOC-31) for pleural cytology, particularly if PreservCyt® is the preferred fixation method in the laboratory. However, such solutions may not be applicable to all biomarkers, especially treatment-predictive biomarkers, which may not be interchangeable or replaceable.

Our findings underscore the clinical relevance and emphasize the need to investigate the reliability of each ICC biomarker for the various fixatives used within individual pathology departments. Immunoreactivity should be interpreted cautiously in alcohol-fixed cell blocks, as some cases may occasionally exhibit false negative staining.

The interpretation of the findings of Paper V should take into account both its strengths and limitations. The main limitation is the restricted number of cases, especially for CytoRich™ Red, as this fixative was not utilized for the initial cases. Consequently, there is especially a risk that subtle differences in the influence of fixatives on ICC results may not have been identified. Detailed data on several strengths and limitations of Paper V is identified in Table XII.

In general, the disparities observed for frequencies of expression of all IHC biomarkers, especially predictive biomarker PD-L1, in unpaired histology and cytology specimens (Paper IV), or in cytology-histology correlation (Paper I, II, and III) and cytology-cytology correlation (Paper V), but also differences between published studies, regardless the tumour type, could be attributed to biological and methodological factors which are discussed below. Beyond PD-L1 studies, the

comparison studies of IHC biomarkers between histology and cytology are scarce, therefore the discussion regarding factors is focused on PD-L1.

The biological factors include i.e., tumour microenvironment, and intratumour and intertumour heterogeneity (Figure 1), while the methodological factors generally include differences in preparation and fixation methods, antibody clones, staining platforms, interobserver variability, the utilization of different sample types, and variations in cutoff values and evaluation criteria.

For PD-L1, the selection of PD-L1 assay, platform, and cutoff for positive staining may also impact on PD-L1 results regardless of specimen type. While factors such as fixation and preparation methods, specimen cell content, sampling site, heterogenous expression, and interobserver agreement may vary between histology and cytology specimens. [363, 543, 544] Moreover, there is a mixture of both histological (biopsies, resections, and tissue microarrays) and cytological specimens (FNA, BAL, bronchial brush, and pleural effusions, etc.) in various studies, which may hold significance as well. Additional pre-analytical, analytical, and post-analytical factors that may influence immunostaining are discussed below and listed in detail in Table I.

The microenvironment plays an important role in tumour biology, and immunological traits can vary among primary tumours, metastatic tumours, circulating tumour cells, and effusions, with potential heterogeneity within the same tumour manifestation. [545]

From a biological perspective, the cells in certain cytological materials are positioned outside their usual natural microenvironment. This is particularly evident in e.g., pleural effusions, where the microenvironment of cells in effusions differs from those fixed in solid tissue. Malignant cells in effusions exist within a fluid environment containing essential nutrients, thereby maintaining the viability of the cells, whereas tumour cells in biopsies proliferate within a solid stroma. Furthermore, malignant effusions frequently comprise a blend of malignant cells (primary or metastatic), benign mesothelial cells, and various inflammatory cells, and harbour various soluble substances secreted by tumour or benign mesothelial cells. Moreover, the interaction between inflammatory cells and tumour cells dispersed in the fluid can have effects on the cells and potentially induce phenotypic changes, and it is known that malignant cells in serous effusions may undergo changes in their phenotype. [546-548] Our findings in Paper I and II indicated that the biopsies were primarily composed of tumour cells, whereas the effusions exhibited varying proportions of lymphocytes, macrophages, and tumour cells. The potential interaction between tumour cells and immune cells in effusions could influence the expression of PD-L1 and also other biomarkers; however, this aspect was beyond the scope of the current studies.

The process of sampling cytological material can influence the evaluation of the PD-L1 expression levels, and tumour sampling may disrupt its topography which means that the sampling is a mixture of tumour cells, immune cells, and tissue fragments. [549] However, the tissue structure is preserved in histological material.

Variability stemming from heterogeneity in malignant tumours can also be related to sampling errors in both histology and cytology, particularly when dealing with small samples. [550] Two studies have shown that the variations in scoring between histological and cytological specimens appear to be partially attributable to sampling from heterogeneous tumours in histological material and the insufficient material available in the cytological specimens. [441, 449] The analysis of malignant effusions may also face challenges due to low overall cellularity, with tumour cells often being scarce compared to the inflammatory background cells. Conversely, small histological samples may incur damage or lack tumour cells, whereas cells in effusions typically remain well-preserved and contain malignant cells that may have exfoliated from the entire tumour area. Therefore, an effusion may offer a more representative sample compared to a small biopsy. The variability in histology could be attributed to the narrowly localized sampling, which might not adequately represent the entire tumour. The impact of local sampling is not as pronounced in effusions, which are well mixed and thus to some extent may represent the entirety of the exposed tumour mass, but it may also be that only a small part of pleural invasive tumour is represented. The "age" of the effusion could potentially act as a confounding factor if various cell types are shed at different rates and pronounced cell degeneration is sometimes seen in pleural effusion samples. However, the most probable cause of the differences lies in the biological characteristics of the lesions and their propensity for shedding.

The biological aspects of malignant cells in FNA from lymph node and EBUS material are that they are aspirates of cells in their natural environment but represent metastatic tumours. Other cytological material such as bronchial washings and brushes used in diagnosis of lung cancer face the same challenge due to low overall cellularity as for effusions but represent primary tumour. However, cells in bronchial washings and brushes may resemble cells in effusions that exfoliate from superficial or deep serosal or mucosal surfaces.

Numerous biological factors that could potentially influence PD-L1 staining results have also been explored. The expression of PD-L1 may vary between the primary tumour and metastases, exhibiting either gain or loss of expression in the metastatic sites. [551-555] A prolonged time interval between samplings has been reported to correlate with increased intertumour discrepancy, suggesting a change in PD-L1 expression over time. [551] Reports suggest that the metastatic site does not influence PD-L1 expression, [556] which is in line with our findings in Paper IV.

The impact of intratumour heterogeneity has also been investigated. For instance, the concordance of PD-L1 expression between paired biopsies and resected tumours has been reported to range from 63% to 92% for <1% versus ≥1% positive tumour cells. [504-508]

From a methodological perspective, differences in fixation and preparation methods between biopsies and cytology are recognised to potentially influence the results of IHC. [542, 557, 558] Short fixation times, certain decalcification procedures, older paraffin blocks, and long storage time of slides before staining have been

associated with reduced staining of tumour cells. [498, 519, 559-561] On the contrary, delayed, or prolonged formalin fixation has not demonstrated any significant effect on PD-L1 staining. [561, 562]

However, the criteria of assessment should be based on differences between histology and cytology. Therefore, for PD-L1, it is demanded to modify the standard PD-L1 interpretation criteria with respect to cytoplasmic and globular staining patterns based on various fixatives and different processing methods, [563] and it is likely the same for other biomarkers.

In summary, although there remain some limitations in making an initial diagnosis of PM and NSCLC merely based on cytological material, these constraints are shrinking day by day. The role of cytology as an initial diagnostic tool is now considered standard procedure, reflecting advancements in the field, and more research is needed to demonstrate the predictive utility of cytology. Therefore, the comparison of histology and cytology is necessary to utilize the cytological methods we currently use in the laboratories, and only through further studies can cytology achieve its full potential in clinical settings.

Consideration of specific analysis methods are described in the Background and Materials & methods sections, suggestions for further and continued studies in the Future perspective section, and the strengths and limitations of all included Papers are partially described in the Discussion section but summarised in Table XII.

Tabel XII: Summary of strengths and limitations of the studies in the thesis.

Partial thesis	Strengths	Limitations
Paper I	• One type of tumour at the same tumour stage. • The preparation, fixation and staining methods were consistent for both types of material. • Only one PD-L1 antibody clone. • Only cases with > 100 evaluable tumour cells were included. • The paired samples were from the same diagnostic workup before oncological treatment. • The scoring of PD-L1 expression at different cutoff levels. • Reporting of the concordance by different ways.	• The retrospective study design. • The cohort contained some old archival cases. • The limited number of cases, especially biphasic/sarcomatoid cases. • The study performed without correlation to treatment response and survival rates.
Paper II	• One type of tumour at the same tumour stage. • The preparation, fixation and staining methods were consistent for both types of material. • Evaluation of multiple IHC biomarkers in the same cohort. • Only cases with > 100 evaluable tumour cells were included. • The paired samples were from the same diagnostic workup before oncological treatment. • Reporting of the concordance by different ways.	• The retrospective study design. • The cohort contained some old archival cases. • The limited number of cases, especially biphasic/sarcomatoid cases. • The study did not include two recently reported biomarkers, namely HEG1 and EZH2.
Paper III	• Only biopsies (not resections) as histological material. • The same PD-L1 antibody clone was used in each cohort. • Only cases with > 100 evaluable tumour cells were included. • The paired samples were from the same diagnostic workup before oncological treatment.	• The retrospective study design. • The Halmstad cohort contained some old archival cases • Cases with sampling from different anatomic sites. • Uneven distribution of different included cytological samples in both cohorts. • The study performed without correlation to treatment response and survival rates.
Paper IV	• The study included two large cohorts. • Investigation of PD-L1 expression in different subtypes of *KRAS* mutations. • Reporting of PD-L1 expression and both *EGFR* and *KRAS* status. • Correlation of PD-L1 expression with mucinous growth pattern in *KRAS*-mutated adenocarcinomas.	• The retrospective study design. • The Halmstad cohort contained some old archival cases. • The next-generation sequencing panels used during the studied time did not cover some interesting targets, such as *STK11*, *KEAP1*, and *SMARCA4*. • The study performed without correlation to treatment response and survival rates.
Paper V	• The prospective study design encompassing cases from a limited period. • One type of tumour at the same tumour stage. • The inclusion of matched and identically handled cytological specimens from the same patients. • The same preparation and staining methods for all matched cell blocks. • Investigation of multiple fixatives. • Evaluation of multiple ICC biomarkers in the same cohort. • Only cases with > 100 evaluable tumour cells were included. • A uniform evaluation protocol for all cases was used. • Reporting of the concordance in different ways.	• The limited number of cases. • Correlation with paired histological specimens was not performed. • The study did not include other relevant fixatives, such as CytoRich™ Blue, CytoRich™ Clear, Novaprep®, and TACAS™ Ruby, or mixes or sequential fixation.

Conclusions

Generosity is giving more than you can, and pride is taking less than you need.

Khalil Gibran, 1883-1931

Overall conclusion

The main conclusion based on the findings of the Papers in this thesis is that there was generally a notable consistency in the IHC staining patterns of various diagnostic and predictive biomarkers observed by cyto-histopathological concordance in paired specimens from pleural mesothelioma and non-small cell lung cancer. Nevertheless, a relatively diminished agreement was observed for some biomarkers, underscoring the need for additional exploration and local quality assurance. The rate of cyto-histopathological correlation may depend on biological as well as methodological differences, given the fact that there is no standardization of preparation techniques for cytology. Furthermore, the concordance rate of expression for some IHC biomarkers in matched cytological specimens differs depending on the used fixation method and, at least for some markers, different clones of antibodies.

Specific conclusions

The thesis consists of five studies, published in five respective Papers. The specific conclusions of each study are summarized and listed below:

Paper I

- A moderate concordance of PD-L1 expression between paired FFPE biopsies and pleural effusion cell blocks was seen from patients with pleural mesothelioma.
- There was higher PD-L1 concordance for epithelioid mesotheliomas than non-epithelioid mesotheliomas, which indicates biological differences between histology and cytology.

Paper II

- Generally, a high concordance for IHC staining was seen between paired FFPE biopsies and pleural effusion cell blocks in cases of pleural mesothelioma. However, the somewhat lower agreement for WT1, EMA and particularly MTAP calls for further investigation and local quality assurance and awareness in the clinical situation.
- The lower concordance for the sarcomatoid subtype for calretinin, CK5, and WT1 may indicate biological differences between the two types of specimens.

Paper III

- The overall concordance of PD-L1 expression between biopsies and cytology cellblocks from NSCLC patients was rather good, but with significant variation between laboratories.
- Local quality assurance at each laboratory is important as fixatives and preparation techniques for cytology may probably affect PD-L1 expression.
- The variation in concordance strengthens the consensus approach to use histological specimens for PD-L1 evaluation, when possible, but cytology when this is the only available material.

Paper IV

- Histological and cytological specimens from NSCLC seem comparable for PD-L1 evaluation.
- PD-L1 expression was lower in:
    - AC compared to SCC.
    - *EGFR*-mutated compared to *EGFR*/*KRAS* wildtype and *KRAS*-mutated NSCLC.
    - Mucinous compared to non-mucinous *KRAS*-mutated AC.

Paper V

- Difference in staining proportion was seen for TTF-1 clone 8G7G3/1 and EpCAM clone MOC-31 with especially cases negative in CytoLyt® and PreservCyt®, while being comparable between CytoRich™ Red and formalin.
- Weaker intensity was seen for all alcohol-based fixatives compared to formalin for TTF-1 clone 8G7G3/1, napsin A, and EpCAM clone MOC-31, while claudin 4 and EpCAM clone Ber-Ep4 was significantly weaker only in CytoLyt® and PreservCyt®.
- Immunocytochemical expression, and concordance with formalin-fixed CBs, differ depending on the used fixative as well as the antibody and clone, warranting investigation of the reliability of each biomarker for non-formalin-fixed cytology.

Future Perspectives

The youth will not get tired
their goal is your independence.

Ibrahim Tuqan, 1905-1941

Cytology has long been the dark art that came into the light, and the growing importance of proper cytology specimen sampling, sample collection, handling, and preparation became increasingly appreciated and more widely performed. Further, several significant developments have occurred in recent decades, such as the use of cytology as a mainstream diagnostic tool in different screening programs, advances in specimen sampling, and the development of standardised international reporting systems. All the latter allowed cytology to be used in many clinical settings. However, despite many successes, cytology still faces challenges and there are many areas of development.

The conclusions of our studies point toward two main development areas for future research:

i. systematic investigation of ancillary analysis, inclusive diagnostic, and predictive biomarkers, on paired histology and cytology specimens, with correlation to treatment response and patient outcomes; and
ii. examination of pre-analytical, analytical, and post-analytical factors on different cytological preparation techniques, using prospective and systematic studies.

Cytology as diagnostic material

The high frequency of diagnosis of pleuro-pulmonary malignancies including pleural mesothelioma and non-small cell lung cancer based on cytological specimen stresses cytology as a valuable diagnostic tool. However, the main limitations of cytology are due to problems with sample collection and poor diagnostic yields, and the biggest challenge is what can be done on limited material. Therefore, the choice of preparation technique is crucial. Hence, standardization of the handling of cytological specimens and the creation of suitable protocols for different cytological preparation methods are needed.

From a cyto-histopathological correlation perspective, histology and cytology may be complementary diagnostic procedures, with both advantages and

limitations. However, cytological materials are often the first samples analyzed when cancer is suspected. Reliable cytological diagnosis can allow faster initiation of proper treatment and the therapeutic response to tailored treatment can be predicted. Cytology is a useful method that provides quick results at a lower cost compared to other diagnostic methods. Cytological methods must therefore continue to be further developed and evaluated for novel cancer biomarkers; thereby the diagnostic certainty can be improved, and several other, more expensive examinations can be avoided. This is therefore an important area of research that many laboratories strive to invest in.

In the case of a predictive biomarker such as PD-L1, the cytology could accelerate the diagnosis and provide a faster onset of treatment, by identifying and selecting those patients who express PD-L1 at an early stage of the cancer evolution and thereby can be expected to contribute most benefit from treatment with immunotherapy. The use of cytology means that the method would save the patient and healthcare the need for biopsy, which is often a more extensive procedure than the cytology.

Given to the plausible rationale mentioned above, the current and novel biomarkers, especially predictive biomarkers, need to be investigated clinically on cytological material.

Cytology and ancillary analysis

The role of cytology has changed dramatically, and cancer diagnostics based on cytology specimens has been a crucial and unavoidable part in the clinical setting, particularly when essential expansion of ancillary analysis to include not only diagnostic biomarkers but also predictive biomarkers in precision medicine. [243-245, 564] Ancillary analyses, including immunohistochemistry and molecular testing, are typically established on histological specimens, and improvement in cytology methods leads to the full utilization of the cytology samples, which is often the first and only specimen available.

Although several reports have demonstrated many successes that cytology can be used as a surrogate for ancillary testing, cytology still faces many challenges related to the variety in preparation and fixation methods and there are many areas of development. Therefore, ancillary analyses need to be evaluated on different cytological preparation techniques, utilizing different fixatives to reach the most optimal outcomes.

Several ancillary analyses have been investigated on cytological material, exploring different pre-analytical, analytical, and post-analytical factors. Nevertheless, most of these studies were performed on either animals or cell lines or are retrospective studies, without correlation to clinical reports. The significant outcomes and obvious conclusions can solely be reached by systematic investigations of the biomarkers in paired cytological and histological specimens as well as matched cytology preparations through multicentre, prospective, and

systematically designed studies, with correlation to clinical reports. This kind of systematic research is needed.

Cytology and technological advances

There is a major challenge in the integration of cytology and molecular techniques, and the future of cytology will include technological advances such as greater uses of Telecytology, the involvement of artificial intelligence, and increased use of digital systems for reporting, education, and training which facilitates standardization of outcome reports, and increase credibility of cytology.

The full integration of cytology and confident cytological diagnoses will increase the reliability of cytology as a diagnostic material among physicians, including pulmonologists and oncologists, by being able to perform patient management on one sample, leading to an early diagnosis, which in turn leads to early treatment and better outcomes.

Suggestions for further studies on mesothelioma and lung cancer

Here at the end of this thesis, I echo our plea that the findings from the included Papers in this thesis shed light on the important scientific and clinical relevance of cytology-histology correlation for PM and NSCLC diagnostic and predictive biomarkers. Nevertheless, the results from these studies cannot be generalized to other cancer types and biomarkers, but they could give indications that can also contribute to a greater understanding of how the different biological and methodological factors can affect the outcomes of various ancillary analyses. The issue of "histology" versus "cytology" approaches to immunostaining and molecular testing for tumour diagnosis and prediction of treatment response needs further research that could therefore focus on improving study design sophisticatedly and validating these conclusions in larger and diverse patient cohorts to enable the clinical application of cytology diagnosis. Some specific proposals for certain research projects as incremental or in-depth studies of our studies included in this thesis are presented below.

In Paper I and III, we explored the PD-L1 concordance in paired histology and cytology samples from PM and NSCLC patients, respectively. The predictive value and the manner in which this correlates with therapy response, and the potential enhancement of therapy decisions by relying on PD-L1 positivity in cytology specimens, instead of histology, or the possibility of these materials being complementary can only be determined by thorough and comprehensive investigations involving paired specimens collected prior to therapy with correlation to therapy response. Furthermore, it is of interest to evaluate the current and novel predictive IHC biomarkers other than PD-L1 in paired histological and different cytological specimens (including different cytology preparation techniques such as cytospin and LBC preparations) from PM and lung cancer patients including both SCLC and NSCLC.

In Paper II, we investigated the most common diagnostic biomarkers, but we did not include two recently reported biomarkers, HEG1 and EZH2, [287, 300, 489, 565] which should be of interest to explore in paired specimens in future investigations. Further, investigation of the SPOCK1 antibody as a diagnostic and prognostic IHC biomarker in paired histology and cytology specimens from mesothelioma patients and correlating the expression to overall survival would be interesting. Moreover, the next step would be to investigate whether the same findings from immunostaining of mesothelioma biomarkers on cell block could be obtained using different preparation techniques such as cytospin preparations, compared to corresponding histological specimens. In the case of PM, which is far less common than NSCLC, it would probably require a multicentre study.

In Paper III, we have also compared the PD-L1 expression in two cohorts of cell blocks from NSCLC patients. The cell blocks have two different fixation methods, i.e., formalin and CytoLyt®/PreservCyt®. Given that there is no consensus, based on the results from our investigation and the literature review performed, on the extent to which alcohol-based fixatives can affect the PD-L1 expression, this area merits future investigation. Prospective studies could further validate that issue and include a large sample size to identify and detect additional factors, which we started with through a prospective and systematic investigation as presented in Paper V. Currently, we are working on including more lung AC cases and investigate expression of different PD-L1 clones on the same material.

In Paper IV, our findings suggest elevated PD-L1 levels in *EGFR*-mutated cases with sampling after or during treatment. However, conclusive evidence would require a larger study encompassing cases assessed for PD-L1 expression both before and after treatment. Interestingly, in our study, we observed a significant distinction between mucinous and non-mucinous *KRAS*-mutated AC, a finding that, to our knowledge, has not been previously documented in the literature. In a retrospective analysis of mucinous ACs, the administration of immunotherapy correlated with improved overall survival rates. [539] The data on *KRAS* mutations and PD-L1 status was unavailable for all cases in our study. However, given the favourable therapeutic response observed in that study and the low frequency of PD-L1 expression in our data, additional research is warranted to determine whether PD-L1 serves as a less predictive biomarker in mucinous compared to non-mucinous ACs. Moreover, studies on ICI response should include reporting on both *KRAS* mutation status and mucinous growth patterns for a comprehensive understanding of their predictive value. Furthermore, we lacked information regarding overall survival, which patients received treatment with immunotherapy, and their response to therapy. In addition to its predictive role, high PD-L1 expression has been inconsistently associated with a worse prognosis. [527, 529, 566] A comprehensive correlation between PD-L1 and different molecular alterations, and treatment response, including the potential prognostic and predictive value, could be assessed in our cohorts in the future, considering longer follow-up times and taking into account stage and treatment modalities. Moreover, the NGS panels used

during the study period did not encompass certain interesting targets, including *STK11*, *KEAP1*, and *SMARCA4*.[357] Additionally, numerous molecular alterations are relatively uncommon, thereby limiting our conclusions to prevalent types.

In Paper V, we were able to investigate the impact of different fixatives on immunostaining with carcinoma biomarkers on matched cell blocks from lung AC patients. Given the differences between histology and cytology, and although the formalin-fixed cellblocks are assumed to be comparable to biopsies, a correlation of our findings with paired histological specimens would be valuable. Moreover, we did not include other relevant fixatives, such as CytoRich™ Blue, CytoRich™ Clear, Novaprep®, and TACAS™ Ruby, or variations in fixation methods like mixtures or sequential fixation (e.g., formalin before, after, or mixed with an alcohol-based fixative). This was not addressed in the present study and hence this type of research is needed to further increase the understanding to what extent the various fixatives can affect immunostaining. Additionally, we did not assess the potential influence of different fixation durations, fixation delay (cold ischemia time), or varying time from sectioning to staining as pre-analytical factors, although all these factors remained consistent for all fixatives across each case. We aim to explore various fixation times, mixed/sequential fixatives, and additional biomarkers in future studies. This will involve a larger number of cases and paired biopsies to yield further clinically relevant insights.

Popular Scientific Summary

Appreciate everything around you,
before moments turn into memories.

Lung cancer is the leading cause of cancer-related death in the world. It is a both rather common and aggressive malignant tumour, and the chance to cure has essentially been limited to early-detected tumours that can be surgically treated. Mesothelioma is also an aggressive and lethal malignancy, which arises from the cells lining the lung surface and adjacent chest wall, called the pleura. In comparison, it is a quite rare malignancy. Both lung cancer and mesothelioma are strongly linked to exposure in the form of smoking and asbestos, respectively. Also, the diagnosis may be challenging for both tumours.

Malignant tumours are normally diagnosed using a microscope in a pathology department. The investigation of tissue pieces and cell smears is called histology and cytology, respectively. For lung cancer and mesothelioma, both histological biopsies and cytological specimens such as pleural effusions (fluid in the pleural space) are common samples from the clinical investigation. To enable the investigation of tissues and cells in the microscope, the material needs to be treated with a fixative and stained. While the handling and fixation of biopsies are standardised over the world (with the use of formalin as fixative), cytological specimens are handled and fixed differently (often with alcohol-based fixative) between and also often within pathology departments.

Based on the appearance of tumour cells in the microscope (called morphology), it is often difficult and not seldom impossible to distinguish lung cancer from mesothelioma and also to distinguish different types of lung cancer from each other. This is of great importance since treatment differs a lot between these entities. For example, the most common type of lung cancer called adenocarcinoma, in contrast with the second most common type squamous cell carcinoma, quite often displays mutations in the tumour cells that are associated with the development of cancer, and that may be specifically targeted with drugs called targeted therapy.

Immunotherapy is another group of drugs that stimulate the immune system to attack the tumour cells, or counter the tumour's blocking of the immune system, with the same result. Immunotherapy can be used for both lung adenocarcinoma and squamous cell carcinoma and also mesothelioma, but for lung cancer, it has been

shown that the effect of these drugs is better if the tumour cells express a protein called PD-L1.

PD-L1, as well as other proteins expressed by the tumour cells that may be of aid to distinguish different types of lung cancer and mesothelioma from each other, is detected in the microscope after the tumour cells or tissues have been stained with immunohistochemical staining. In this thesis, such analyses have been investigated, often with a cytological specimen and a biopsy from the same patient to detect any differences in the result of the stainings due to sample type or fixative used for the cytology.

In the first Paper, staining of PD-L1 was compared in cytological pleural effusions and biopsies from the same patients in 61 mesotheliomas. The agreement in the result between cytology and histology was rather good, 69% or 84% depending on the threshold used to call the PD-L1 staining positive (i.e., the protein counting as significantly present). Mesotheliomas can have different appearances in the microscope, and the type called epithelioid showed a higher concordance between cytology and histology than the sarcomatoid type, which probably relates to the tumour's biology since the sarcomatoid cells do not tend to spread in the pleural space.

In the second Paper, eight different immunohistochemical markers were investigated in 59 paired pleural effusions and biopsies. The markers were selected based on their common use to either separate mesothelioma from e.g. lung cancer or distinguish mesothelioma from the normal mesothelial cells that are present in the pleura. The agreement in the result between cytology and histology was ≥90% for most markers, but a new marker called MTAP, which may be lost in mesotheliomas but preserved in normal mesothelial cells, only had an agreement of 72%. Also, both MTAP and a similar marker called BAP1 were less often positive in cytology than in biopsies.

In the third Paper, staining of PD-L1 was compared in cytological specimens and biopsies from the same patients in two cohorts of 47 and 97 lung cancers from Lund and Halmstad, respectively. The agreement in results between cytology and biopsies was higher for the Lund cohort (85% vs. 68%), but in all cases where there was a disagreement in the Lund cohort, the PD-L1 staining was weaker in the cytological sample. In this paper, a comprehensive literature review was also performed on the cyto-histological agreement of PD-L1 staining.

In the fourth Paper, the results of PD-L1 staining from the clinical setting were investigated in a different material based on 1131 and 651 consecutive lung cancer cases from Lund and Halmstad, respectively. Here, there was no significant difference in PD-L1 expression between cytology and biopsies, but PD-L1 was lower in adenocarcinomas compared to squamous cell carcinomas and in lung cancers with a mutation in the gene *EGFR* compared to a mutation in the gene *KRAS*. Also, in *KRAS*-mutated adenocarcinomas, PD-L1 was lower in cells with abundant mucin cell inclusions seen in the microscope.

In the fifth and final Paper, four cytological materials were produced from each patient of 24 with lung adenocarcinoma spread to the pleura. The four materials were fixed differently, with either formalin or an alcohol-based fixative. Nine different immunohistochemical markers commonly used for diagnosing lung adenocarcinoma (including variants of two of the markers) were investigated. For some markers, the result was exactly the same regardless of the fixative used, while for others, and more for some variants than others, there was a great difference with lower expression in some or all of the alcohol-based fixatives.

Cytology is a rapid method that is often less difficult for the patient. The studies of the thesis have foremost highlighted the importance of quality assurance and some vital pitfalls, and to some extent how to avoid them, in diagnostics of lung cancer and mesothelioma.

Populärvetenskaplig Sammanfattning (Summary in Swedish)

Bättre att veta att man gör fel än tro att man gör rätt.

Lungcancer är den vanligaste orsaken till cancerrelaterad död i världen. Det är en både ganska vanlig och aggressiv elakartad tumör, och chansen att bota har i huvudsak begränsats till tidigt upptäckta tumörer som kan behandlas kirurgiskt. Mesoteliom, lungsäckscancer, är också en aggressiv tumör med hög dödlighet, som utgår från cellerna i den dubbelskiktade hinna som omger lungytan och intilliggande bröstvägg, kallad lungsäcken. I jämförelse med lungcancer är det en sällsynt malignitet. Lungcancer är ofta relaterad till rökning, medan mesoteliom är starkt relaterad till exponering för asbest.

Elakartade tumörer diagnostiseras vanligtvis med hjälp av ett mikroskop på en patologavdelning. Mikroskopisk bedömning av vävnadsprover och cellutstryk kallas histopatologi respektive cytologi. För utredning av lungcancer och mesoteliom används både histologiska biopsier och cytologiska prover såsom pleuravätskor (vätska i lungsäcken), vilka är vanliga förekommande prover vid klinisk diagnostik. För att möjliggöra undersökning av vävnader och celler i mikroskopet behöver materialet fixeras och färgas. Hantering och fixering av biopsier är standardiserad över hela världen (med användning av formalin som fixering), medan hantering och fixering av cytologiska prover är olika (ofta med alkoholbaserat fixativ) mellan och även ofta inom patologavdelningar.

Diagnosen kan vara utmanande för båda tumörerna och utifrån utseende av tumörceller i mikroskopet är det ofta svårt, och inte sällan omöjligt, att skilja lungcancer från mesoteliom och även att skilja olika typer av lungcancer från varandra. Detta är av stor betydelse eftersom behandlingen skiljer sig mycket mellan olika tumörtyper. Till exempel visar den vanligaste typen av lungcancer, som kallas adenokarcinom, i motsats till den näst vanligaste typen, skivepitelcancer, i en del fall mutationer i tumörcellerna som är associerade med utvecklingen av cancer och som kan ge möjlighet till specifik så kallad målriktad terapi.

Immunterapi är en annan typ av behandling, som stimulerar immunsystemet att attackera tumörcellerna, eller motverka tumörens blockering av immunsystemet, med samma resultat. Immunterapi kan användas för både lungadenokarcinom och skivepitelcancer och även mesoteliom, men för lungcancer har det visat sig att

effekten av dessa läkemedel blir bättre om tumörcellerna uttrycker ett protein som kallas PD-L1.

PD-L1, liksom andra proteiner som uttrycks av tumörcellerna som kan vara till hjälp för att skilja olika typer av lungcancer och mesoteliom från varandra, detekteras i mikroskopet efter att tumörcellerna eller vävnaderna har färgats med immunhistokemisk färgning. I denna avhandling har sådana analyser undersökts, ofta med ett cytologiskt prov och en biopsi från samma patient för att upptäcka eventuella skillnader på grund av provtyp eller fixativ som används för cytologin.

I det första delarbetet jämfördes färgning för PD-L1 i pleuravätska (cytologiskt prov) och histologiska biopsier från samma patienter i 61 fall av mesoteliom. Överensstämmelse i resultaten mellan cytologi och histologi sågs i 69 % till 84 % beroende på vilket tröskelvärde för positivitet som användes för att kalla PD-L1-färgningen positiv (dvs. proteinet räknas som signifikant förekommande). Mesoteliom kan ha olika utseende i mikroskopet, och den typ som kallas epitelioid visade en högre överensstämmelse mellan cytologi och histologi än den typ som kallas sarkomatoid.

I det andra delarbetet undersöktes åtta olika immunhistokemiska biomarkörer i 59 parade pleuravätskor och biopsier. Biomarkörerna valdes baserat på deras vanliga användning för att dels separera mesoteliom från till exempel lungcancer och dels för att skilja tumörceller från normala mesotelceller som kan förekomma i lungsäcken. Överensstämmelsen i resultatet mellan cytologi och histologi var ≥ 90 % för de flesta biomarkörer, men en ny biomarkör kallad MTAP, som kan gå förlorad i mesoteliom men bevaras i normala mesotelceller, hade lägre överensstämmelse, 72%. Dessutom var både MTAP och en liknande biomarkör kallad BAP1 mindre ofta positiv i cytologi än i biopsier.

I det tredje delarbetet jämfördes färgning för PD-L1 i cytologiska prover och biopsier från samma patienter i två kohorter med 47 respektive 97 lungcancerfall från Lund och Halmstad. Överensstämmelsen i resultat mellan cytologi och biopsier var högre för Lund-kohorten (85 % vs. 68 %), men i samtliga fall där det fanns en diskrepans i Lund-kohorten var PD-L1-färgningen svagare i det cytologiska provet. I detta arbete utfördes också en omfattande litteraturgenomgång av den cyto-histologiska överensstämmelsen vid PD-L1-färgning.

I det fjärde delarbetet undersöktes resultaten av PD-L1-färgning i ett annat material baserat på 1131 och 651 konsekutiva lungcancerfall från Lund respektive Halmstad. Här fanns ingen signifikant skillnad i PD-L1-uttryck mellan cytologi och biopsier, men PD-L1 var lägre vid adenokarcinom jämfört med skivepitelcancer och vid lungcancer med en mutation i *EGFR*-genen jämfört med en mutation i *KRAS*-genen. I *KRAS*-muterade adenokarcinom var PD-L1 lägre i tumörer med riklig slembildning, något som ses i mikroskopet.

I det femte och sista delarbetet producerades fyra cytologiska material från varje patient i 24 fall av lungadenokarcinom med spridning till lungsäcken. De fyra cellmaterialen fixerades på olika sätt, med antingen formalin eller ett alkoholbaserat fixeringsmedel. Nio olika immunhistokemiska biomarkörer som vanligtvis används

för att diagnostisera lungadenokarcinom (inklusive varianter/kloner av två av biomarkörerna) undersöktes. För vissa biomarkörer var resultatet exakt detsamma oavsett vilket fixativ som användes, medan det för andra, och mer för vissa varianter/kloner (undertyper av en biomarkör) än andra, var stor skillnad med lägre uttryck i vissa eller alla de alkoholbaserade fixativen.

Cytologi är en snabb metod som ofta är enklare och mer skonsam för patienter jämfört med biopsier. Studierna i avhandlingen har framför allt belyst vikten av kvalitetssäkring och några viktiga fallgropar, och i viss mån hur man undviker dem, vid diagnostik av lungcancer och mesoteliom.

Acknowledgements

Whoever does not thank the people,
does not thank Allah.

Prophet Muhammad, 570-632

This thesis would not be a reality without the support from several collaborations and assistance from a tremendous number of people; supervisors, mentors, collaborators, colleagues, friends, and family who have been involved directly or indirectly in the making of this dissertation. If you are looking for your name without finding it, please know that any omissions are due to the stress that comes with writing a thesis under exceptional circumstances, not a lack of gratitude. However, I would like to acknowledge and extend my sincere gratitude especially to the following persons for their support, help and kindness during my journey as a PhD student:

First and foremost, all mesothelioma and lung cancer patients, who have participated in my research projects and chose to donate the residual or their archival biological material. Your generous consent laid the groundwork of this thesis, and without you I would not have been able to carry out this research. Despite your illness and the struggling period in your lives, you have chosen to contribute to improving cancer diagnostics for the benefit of current and future patients.

Hans Brunnström, my main supervisor. My most heartfelt thanks to you for accepting me as a PhD student in your group, giving me the enviable opportunity to work with projects of my choosing, supporting me during this research journey with your extensive knowledge in thoracic pathology, answering all my questions with great enthusiasm, positive attitude, and endless patience, and for the last-minute help. You supervise me with a careful balance between guidance and giving me great opportunities to scientific freedom in designing the studies, which was crucial for me to become an independent researcher. Your ability to stay focused among manifold ventures and remain constructive is something I can only hope to emulate. You are a source of inspiration. I consider myself lucky to have you as my PhD main supervisor and I look forward to continuing with further research projects with you.

HANS, I WILL ALWAYS BE THANKFUL TO YOU!

Katalin Dobra, my co-supervisor. Sincere thanks for your great support and invaluable advice and comments in writing some of the Papers, sharing one of your many inspiring projects with me, interesting discussions, and sharing your vast knowledge about mesothelioma during those occasions we had to evaluate the immunostaining slides together, provided interesting research opportunities, and for always having a new perspective on research projects.

Maria Planck, my co-supervisor. Sincere thanks for your words of encouragement which were invaluable during difficult and stressful times during my period as a PhD student, providing guidance and inspiration, contributing with your extensive expertise in oncology and for your comments and advice on the oncological part of included studies in this thesis. Your invaluable input was much appreciated, and I really enjoyed the opportunity to work with you.

KATALIN AND MARIA, THANK YOU FOR EVERYTHING!

Annika Dejmek, supervisor, and mentor. Sincere gratitude from the depth of my heart for believing in me as a researcher, for all your support and encouragement during this scientific adventure throughout the years, your constructive comments and regular pep-talks, inspiring discussions, your amazingly comprehensible explanations, cheerfully sharing your wide knowledge in diagnosis of mesothelioma with me, for always challenging me to think one step further, and for reminding me of what is truly important and what it is all about in the end. Without your critical analysis and input, the studies in this thesis would not have been carried out in such a professional manner. A special thanks also to your husband, Petr Dejmek for his invaluable support with illustrations and sound advice about statistics-related matters.

Tomas Seidal, supervisor, and mentor. Sincere gratitude for supporting, encouraging, challenging, and guiding me throughout it all, for working with me to reach the finish line, and for answering my many pathology-related questions. You truly are an encyclopaedia of pathology. Thank you also for the time we spent discussing research, proofreading of many students' thesis that we supervised together, and for the great flexibility to accommodate my other endeavours. You never said no to me when I needed to discuss anything related to research or personal matters. Because of you, I never felt alone in the department.

ANNIKA AND TOMAS, I consider you as my family and hope that you do the same. I AM GRATEFUL TO YOU FOR EVERYTHING!

All my co-authors. Many thanks for your input, feedback, ideas, essential work, contribution, collaboration, and a great teamwork.

Sincere thanks to the former and current heads of the Department of Pathology and Cytology at Halland Hospital in Halmstad, Sweden, Tomas Seidal, Åsa Fred, Monica Rolling, Sofia Axelsson, and Sara Runestam for giving me the opportunity to combine clinical work with research, encouraging me to perform clinically relevant studies, support in all administrative, and computer-related issues.

All past and present staff and colleagues at the Department of Pathology and Cytology at Halland Hospital in Halmstad, Sweden. thanks for helping with big and small issues, especially the Cytology-laboratory Unit and Immuno-laboratory Unit. I am sincerely grateful to everyone who helped me along the way. Particular thanks are due to Ante Vidis, Lana Ali and Sana Kumbaric who helped out with collection of malignant effusions, cellblock preparation and practical support, Kim Hejny and Frida Kronqvist for the technical advice and assistance with the immunohistochemistry, Gentijane Kastrati for archival- and administrative assistance, and Benjamin Holmgren for support with illustrations and technical issues.

All other colleagues and collaborators in Division of Respiratory and Internal Medicine at the Department of Clinical Medicine, Halland Hospital in Halmstad, Sweden. My sincere thanks especially to Ulrich Mager for our project collaborations and great teamwork.

All past and present staff and collaborators at the Department of Research and Development in Halland (FoU-Halland). My gratitude for all of you for support, excellent administrative work, help with statistical issues, and for always doing your best for the PhD students by creating a thriving research environment in Halland county. In particular, I would like to thank Hanna Svensson, Stefan Lönn, Amir Baigi, Anders Holmén, Ulf Strömberg and Louise Pettersson.

All members in my big family. Firstly, **my parents**, for raising me with love and care and always supporting and believing in me during my whole life. You are my role model in many aspects. I could not have done this without you in my corner and your prayers. Secondly, **my sisters and brothers**, for always being there for me with your encouragement and support in all my more or less crazy endeavours. Last but definitely not least, **my own family** "the love of my life", SAID, HAMZA, SELMA, ZEINELDIN and SARA for making my everyday life full of joy and surprises, and making me smile and laugh.

MY PARENTS, SIBLINGS AND FAMILY. I am lucky to have you in my life and the words "thank you" are silly and woefully insufficient in this context. I LOVE YOU ALL♡!

The research presented in this thesis was supported with grants from the Research and Development by Halland county and Skåne county, the Sparbank Foundation in Varberg, Southern Sweden, the Swedish Governmental Funding of Medical Training and Clinical Research within the national health services (ALF), and the Swedish Cancer Society. My thanks are also directed to Swedish Society of Clinical Cytology and Swedish Society of Cytotechnologists and Cytologists for funding my conference travels.

THANKS FOR ALL OF YOU FOREVER!

Mohammed S. I. Mansour

Halmstad, February 29th, 2024

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Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

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FACULTY OF MEDICINE

Division of Pathology
Department of Clinical Sciences, Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2024:42
ISBN 978-91-8021-535-0
ISSN 1652-8220