UiT
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Faculty of Health Sciences,
Departments of Medical Biology and Clinical Medicine

Immune Infiltration and Clinical Outcome in Non-Small Cell Lung Cancer

Mehrdad Rakaee

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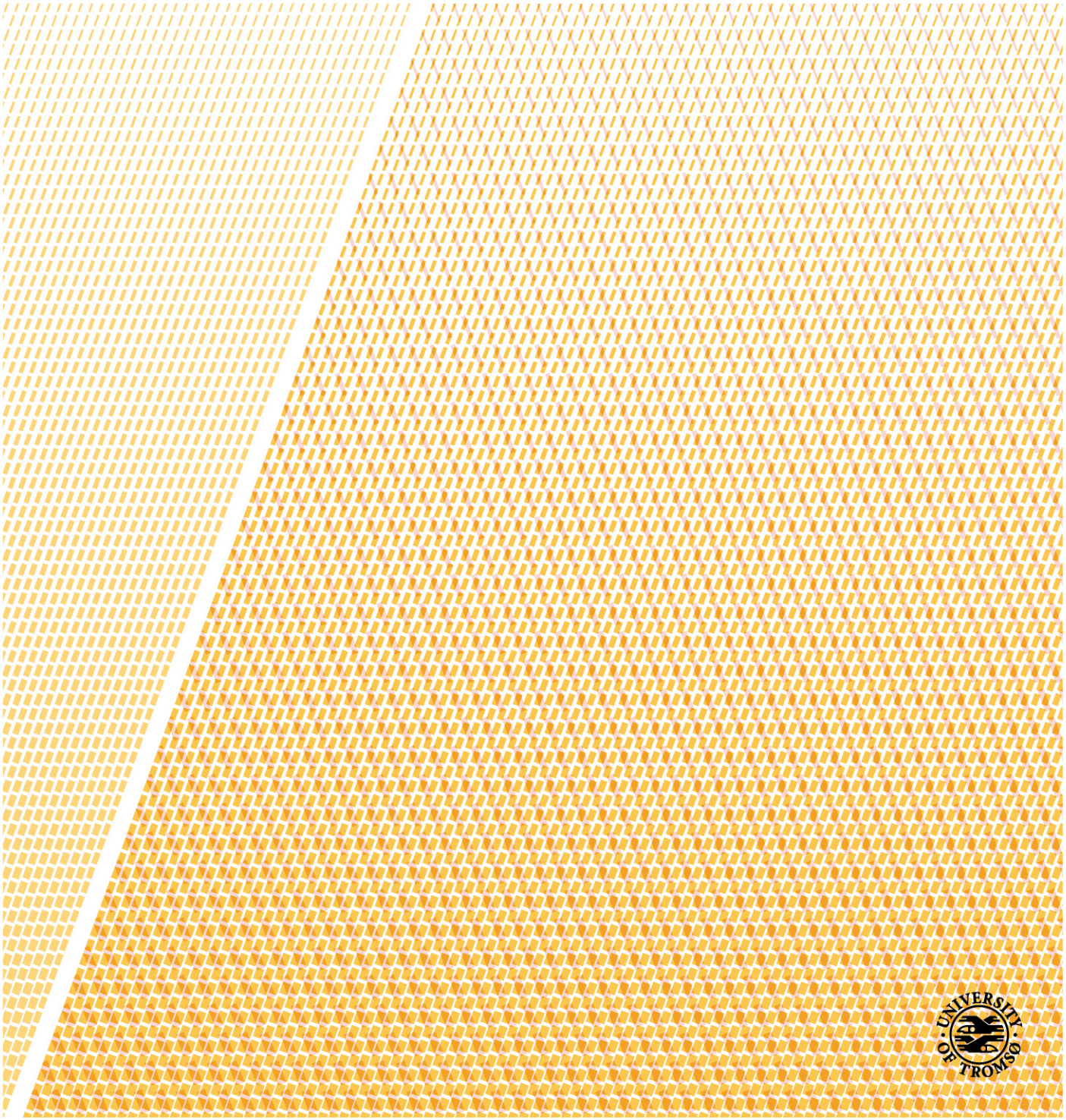

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Preface

Lung cancer is the cancer responsible for the most deaths worldwide. Treatment is multimodal and based on information specific to the tumor (histology, stage, biology and genetics aberrations) and patient-related factors. During the last decade, the arrival of novel targeted therapies and immunotherapy, has led to a paradigm shift in the management of lung cancer. In this regard, assessment of tumor immunology is of great interest to researchers and clinicians.

The immune system undoubtedly plays an important role in the progression and development of cancer. Although it is just a snap-shot picture, it is established that the local immune status, at the time of resection, can provide important prognostic information and influence the clinical management and survival of cancer patients. Currently, the most prominent examples, where immune cell assessment are clinically relevant, are colorectal and breast cancer.

The immune infiltrate comprises adaptive and innate immune cell subsets in which lymphocytes, macrophages and neutrophils are the major populations orchestrating tumor immunity. Advancing the understanding of immune infiltration has significant potential for the development of clinical prognostic and predictive immune markers for patients with NSCLC. The ultimate goal in studying the *in situ* immunity of NSCLC is to apply this information for optimization of immunomodulation and immunotherapy.

The present study was designed to study tumor-infiltrating lymphocytes, macrophages and neutrophils, which seems to represent a potential powerful prognostic instrument for NSCLC. In addition, this thesis emphasizes the importance of the choice of methodology for reliable identification of relevant immune biomarkers.

List of papers

- **Paper I**

  Rakaee M, Busund L-T, Paulsen E-E, Richardsen E, Al-Saad S, Andersen S, Donnem T, Bremnes RM, Kilvaer TK. Prognostic effect of intratumoral neutrophils across histological subtypes of non- small cell lung cancer. *Oncotarget*. 2016;7(44):72184–96.

- **Paper II**

  Rakaee M, Kilvaer TK, Dalen SM, Richardsen E, Paulsen E-E, Hald SM, Al-Saad S, Andersen S, Donnem T, Bremnes RM, Busund L-T. Evaluation of tumor-infiltrating lymphocytes using routine H&E slides predicts patient survival in resected non-small cell lung cancer. *Hum Pathol*. 2018 Jun 6; 79: 188–98.

- **Paper III**

  Rakaee M, Busund L-T, Jamaly S, Richardsen E, Paulsen E-E, Al-Saad S, Andersen S, Donnem T, Bremnes RM, Kilvaer TK. Prognostic value of macrophage phenotypes in non-small cell lung cancer assessed by multiplex immunohistochemistry. *Submitted*.

Abbreviations

ADC	adenocarcinoma
ALK	anaplastic lymphoma kinase
AP	alkaline phosphatase
ASCO	American Society of Clinical Oncology
BAC	bronchioloalveolar carcinoma
BCRs	B-cell receptors
BRAF	B-Raf proto-oncogene
CCL	chemokine (C-C motif) ligand
CE-IVD	European conformity *in vitro* diagnosis
CI	confidence interval
CIS	carcinoma *in situ*
CRC	colorectal cancer
CSF1R	macrophage colony-stimulating factor receptor
CT	computed tomography
ctDNA	circulating tumor DNA
CXCL	C-C-motif ligand
CXCR	C-X-C motif receptor
DAB	3,3'-Diaminobenzidine
DCs	dendritic cells
DFS	disease-free survival
DSS	disease-specific survival
EBUS-NA	endobronchial ultrasound needle aspiration
ECOG	Eastern Cooperative Oncology Group
EGFR	epidermal growth factor receptor
EUS-NA	endoscopic ultrasound needle aspiration
FFPE	formalin fixed paraffin embedded
FISH	fluorescence *in situ* hybridization
GM-CSF	granulocyte-macrophage colony-stimulating factor
H&E	hematoxylin and eosin
HGF	hepatocyte growth factor
HR	hazard ratio
HRP	horseradish peroxidase
IFN-γ	Interferon gamma
IHC	immunohistochemistry
IL	interleukin
IVD	*in vitro* diagnostic
LCC	Large cell carcinoma
LN+	metastatic lymph node
LPBC	lymphocyte-predominant breast cancer
MCSF	macrophage colony stimulating factor 1
MDSCs	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MIA	minimally invasive adenocarcinoma
mIHC	multiplex immunohistochemistry
MMP	matrix metallopeptidase
MRI	magnetic resonance imaging
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCN	National Comprehensive Cancer Network
NETs	neutrophil extracellular traps
NGS	next-generation sequencing
NK	Natural killer
NSCLC	non-small cell lung cancer
OS	overall survival
PD-L1	programmed death ligand-1
PDGF	platelet-derived growth factor

PET-CT	positron emission-tomography-CT
PFS	progression-free survival
PRRs	pattern recognition receptors
pStage	pathological stage
ROS	reactive oxygen species
ROS1	ROS proto-oncogene 1
RUO	research use only
SCC	squamous cell carcinoma
SCLC	small-cell lung cancer
TAMs	tumor-associated macrophages
TANs	tumor-associated neutrophils
TBNA	transbronchial needle aspiration
TCRs	T-cell receptors
TGF-β	transforming growth factor beta
Th	T helper
TILs	tumor-infiltrating lymphocytes
TIME	tumor immune microenvironment
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TLSs	tertiary lymphoid structures
TMA	tissue microarray
TME	tumor microenvironment
TNF-α	tumor necrosis factor-α
TNM	Tumor, node, metastasis
TNM-I	TNM-Immunoscore®
Tregs	regulatory T cells
TTF1	thyroid transcription factor-1
TTNA	transthoracic needle aspiration
UICC	Union for International Cancer Control
VATS	video-assisted thoracoscopic surgery
VEGF	vascular endothelial growth factor
WHO	World Health Organization

1 Introduction

1.1 Lung cancer

1.1.1 Epidemiology

Global: Lung cancer is the leading cause for cancer-related deaths worldwide. Approximately 2.1 million persons will be diagnosed with lung cancer in 2018, accounting for almost 12% of all cancer patients [1]. In men, lung cancer is both the most common cancer and cause of cancer-specific mortality. In women, lung cancer is the fourth most common cancer and the second highest cause of cancer-specific mortality [2]. The incidence of lung cancer is mainly driven by exposure to cigarette smoking. In the 1920s and1960s, the incidence started to raise for men and women, respectively, elucidating the earlier uptake of smoking in males. Initially smoking was adapted throughout society, regardless of socioeconomic status. However, equivocal evidence of the link between smoking and lung cancer, and other smoking related diseases, has led to a socioeconomic gap where those with higher education and income are more likely not to start or to cease smoking [3]. Currently, developed countries have 5-7 folds higher incidence of lung cancer compared to developing countries. However, declining smoking rates in the western world is already started to reflect in lung cancer incidence and, as of now Central and Eastern Europe along with Eastern Asia has the highest incidence rate in males, while North America and Europe have the highest incidence rates in females [4]. In the coming decades the incidence of lung cancer will likely fall in developed countries and rise in developing countries.

Norway: The latest report of cancer statistics by the Norwegian National Cancer Registry [5] shows that lung cancer is the second most common cancer in men (after prostate cancer) and the third most common in women (after breast and colon cancer; **Figure 1**). In total, lung cancer has the highest mortality rate and constituted 19.8% of all cancer related deaths in 2016. For women, the incidence of lung cancer was 9% higher in the period of 2012-2016 compared to 2007-2011, exhibiting a consistent increase since the 1950s. In men, the incidence of lung cancer showed a further 6% decline, consistent with a leveling off during the last two decades [5]. Interestingly, overall survival of lung cancer patients has improved over the last 17 years [6].

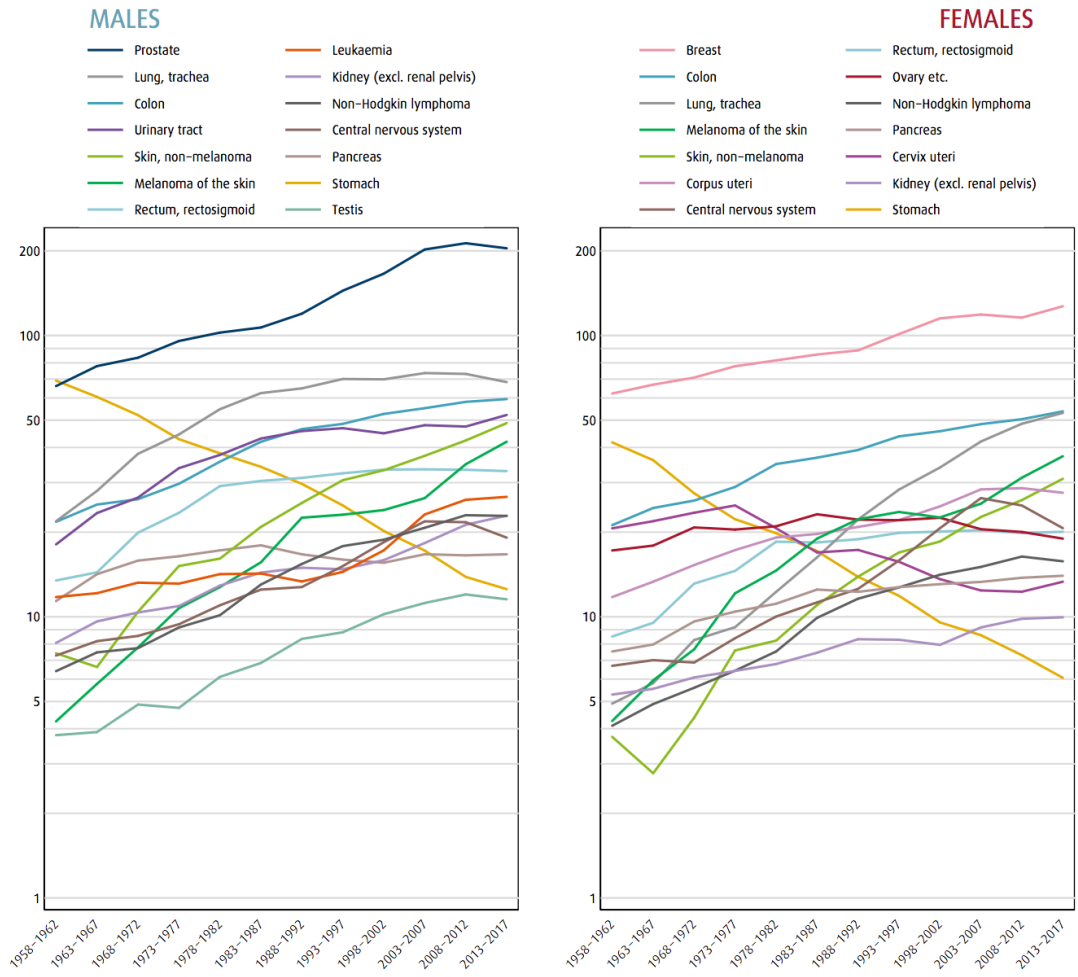

Figure 1: Incidence trends for selected cancers in Norway from 1958 to 2017 (age-standardized; adopted from Norwegian National Cancer Registry, Cancer in Norway 2017) [5].

1.1.2 Risk factors

Among all cancers, the most well-known environmental factor causing cancer is tobacco consumption. Tobacco exposure from smoking cigarettes, cigars and pipes is considered the primary cause of 87% of lung-cancer cases [7]. In the early 20th century, lung cancer was a rare disease. However, the incidence and mortality rates increased sharply when smoking became epidemic [8]. Duration and amount of consumed tobacco is a powerful determinant of lung cancer risk. Besides lung cancer, cigarette smoking is also an important cause of cancer in head & neck, pancreas, bladder, stomach, liver and kidney [9].

Cigarette smoke contain about 60 carcinogenic substances, and their carcinogenicity have been proven by several animal models and experimental studies [10]. Passive smoking, called second-hand smoke, is also associated with a greater risk of lung cancer. A lifelong exposure

to second-hand smoke (smokers at home or workplace), 16-30% increases the life-time risk of lung cancer for nonsmokers [9].

Long-term occupational or residential exposure to ionizing radiation/radon or chemical compounds, including asbestos, chromium, silica, polycyclic aromatic hydrocarbons and diesel exhaust, are considered risk factors [11]. Lung cancer in non-smokers is more common in females and in East Asia, and has been associated with environmental exposures including passive smoking, pollution, occupational carcinogens and inherited genetic susceptibility [12]. For instance, indoor air pollution (burning coal for cooking and heating) and exposure to cooking oil vapors, are considered risk factors, particularly in Asian women [13].

In addition to environmental factors, age and inheritable factors are associated to lung cancer. Incidence is higher in the older population, with a median age of 70 for both smokers and non-smokers [14]. Polymorphisms and variations in chromosomal region 15q24-25.1 [15], DNA repair genes [16] and epidermal growth factor receptor (EGFR) T790M gene [17] increases the risk of lung cancer.

1.1.3 Histological classification

In general, lung cancer can be classified into two pathologically distinct main groups: non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). The current histological and immunohistochemical criteria for the classification of lung cancer is based on the World Health Organization (WHO) guidelines, 4th version, announced in 2015 (**Appendix 1**) [18]. In brief comparison to the 2004 WHO classification, the following major points were highlighted in latest edition: use of immunohistochemistry for classification; molecular tests for managing advanced stages; reclassification of adenocarcinoma (ADC) and squamous cell carcinoma (SCC) subgroups [18,19].

SCLC is a highly aggressive neuroendocrine malignancy comprising nearly 15% of lung cancer cases. Initially, SCLC was believed to originate from the lymphatic system due to its morphological resemblance to lymphoma [20]. SCLCs typically derive from peribronchial tissues. Clinically, when compared to NSCLC, SCLC generally present more aggressive behavior, high initial response to chemotherapy, and an earlier development of distant

metastases [21]. In difficult cases and small biopsies, immunohistochemical staining is applied to differentiate SCLC from NSCLC. Almost 90% of SCLCs are positive for thyroid transcription factor-1 (TTF-1) and neuroendocrine markers such as CD56, chromogranin-A and synaptophysin. Staining for cytokeratins and epithelial membrane markers are used to differentiate SCLCs from lymphoma and subsets of small round cell tumors [22].

The majority, approximately 85%, of lung cancer patients are histologically classified as NSCLC. NSCLC can be further subclassified by pathological characteristics into two major groups: ADC and SCC. For NSCLC patients in advanced stages, therapeutic decisions are heavily dependent on histological subtype and molecular properties [23].

SCCs are characterized by squamous differentiation with intercellular bridges, individual cell keratinization and squamous pearl formation [24]. They arise from the bronchial epithelium of the proximal airways and are thought to progress through a series of preinvasive neoplastic lesions from squamous metaplasia, to squamous dysplasia (mild, moderate and severe) and finally into carcinoma *in situ* (CIS) [18]. SCC is largely associated with a history of smoking and chronic inflammation [25]. Classically, SCC was the most common subtype of NSCLC, but during the recent decades a shift towards ADC has been observed. This alteration is believed due to changes in the carcinogenic substances and the introduction of cigarette filters [26]. The routine tests for differentiation between SCC and ADC are: p40 and p63 [27].

ADCs are histologically characterized by the presence of glandular differentiation and/or mucin production. They are thought to arise from the alveolar or bronchial epithelium (pneumocytes or club cells) of distal airways and mostly arise in the peripheral parts of the lung [28]. The 2015 WHO classification further categorize invasive adenocarcinoma based on the dominant growth pattern into solid, papillary, micropapillary, acinar and lepidic subtypes. Recent reclassification has unified terminology and diagnostic criteria, and consequently the terms bronchioloalveolar carcinoma (BAC) and mixed adenocarcinoma are obsolete. In addition, the term minimally invasive adenocarcinoma (MIA) was recommended to define small lepidic tumors ($\leq$ 3cm) with an invasive component $\leq$ 5mm [18,29].

Large cell carcinoma (LCC), which constitute < 5% of lung cancers, is a less common subtype of NSCLC [30]. LCCs do not exhibit squamous or glandular morphology. Although LCC and SCLC exhibit some similarities, such as positivity for neural markers, they can be

distinctly separated. Immunohistochemical markers for LCC identification are TTF-1 and/or cytokertain-1, -5, -10, -14 and -20. Almost 50% of LCCs express TTF-1, while they to a lesser extent express epithelial cytokeratin markers [31]. As the diagnosis of LCC is based on ruling out ADC, SCC and SCLC, diagnostic accuracy is significantly improved when resected specimens are available instead of biopsies [18,28].

1.1.4 Diagnosis and staging

Most lung cancer patients are diagnosed in advance stages of their disease. Probably because clinical signs are varied or missing and patients can be asymptomatic for a long time. Typical symptoms of a primary tumor in the chest is cough, dyspnea and/or hemoptysis. The clinically suspected lung cancer patient will undergo radiology with thoracic x-ray and/or computed tomography (CT) scan. CT is more sensitive and specific than x-ray which have relatively poor resolution and accuracy. Other imaging tools, such as magnetic resonance imaging (MRI) or positron emission-tomography-CT (PET-CT), can be utilized in addition to CT or plain x-ray [32,33].

Although imaging studies are noninvasive and provide valuable information, tissue evaluation remains the gold standard for a confirmatory diagnosis. A broad range of techniques are available for tissue sampling and staging purposes, including conventional or navigational bronchoscopy, endobronchial ultrasound needle aspiration (EBUS-NA), endoscopic ultrasound needle aspiration (EUS-NA), combined EBUS/EUS, transthoracic needle aspiration (TTNA), transbronchial needle aspiration (TBNA) and sputum cytology [34].

Following clinical assessment, imaging studies and tissue diagnosis, malignant lung tumors are clinically staged according to the updated 8th edition of the Union for International Cancer Control (UICC) TNM classification (**Appendix 2**) [35]. The purpose of TNM staging is to provide a description of the extent of cancer at the time of diagnosis based on information of three parameters: Size and growth pattern of the primary tumor (T), involvement of regional lymph nodes (N) and distal metastasis (M). The TNM model has been designed based on the experience and clinical outcomes of groups of previous patients with similar stage. TNM staging serve as a prognostic indicator and assists clinicians in treatment decisions. The main differences between the two latest TNM models (7th vs 8th version) are: 1) T category have

been subdivided further by size; 2) N category, no change; 3) M category, distinguishes single *versus* multiple extrathoracic metastasis [35,36].
Notably, there are some important prognostic features, such as vascular infiltration and surgical margins, that are not included in the TNM classification model and needs to be considered separately [37].

1.1.5 Molecular diagnosis

Molecular testing of genetic alterations has become a valuable approach to guide therapeutic-specific decision-making in advanced NSCLC. The diagnostic molecular tests detect three classes of genomic alterations: mutations, translocations and amplifications [38]. The rapid development and availability of next-generation sequencing (NGS) platforms, has significantly changed the molecular diagnostic practice. NGS enables simultaneous assessment of several target genes in a single test with high sensitivity and specificity.

In NSCLC, the first discovery of targetable oncogenic aberrations was mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR) in 2004 [39]. Currently, EGFR mutation analyses are well-established and are the most widely used predictive molecular markers in NSCLC. The most frequent hotspots, where EGFR alterations occur, are deletions in exon 19 (45% of EGFR positive patients) and missense mutation at exon 21 codon 858 (40% of EGFR positive patients). Almost 75% of patients harboring EGFR alterations experience tumor regression and improved survival by the use of TK inhibitors (TKI) like erlotinib, gefitinib and afatinib [40].

Other predictive molecular biomarkers in this context are anaplastic lymphoma kinase (ALK) and ROS proto-oncogene 1 (ROS1). Approximately 2-7% of all NSCLC patients have translocations in encoding-genes of ALK. Patients carrying ALK translocations are EGFR-TKI resistant, while their clinical characteristics are the same as EGFR-mutated patients [41]. Translocation of the ROS1 gene occur in 1–2 % of NSCLC patients. Patients positive for translocations in ALK or ROS1 benefit from targeted therapies such as alectinib and crizotinib [42].

With the advent of immunotherapy, the expression status of programmed death ligand-1 (PD-L1) in tumor and immune cells have become important tests to select patients for immune-check point inhibitors in NSCLC.

Briefly, according to the latest (May.2017) National Comprehensive Cancer Network (NCCN) and American Society of Clinical Oncology (ASCO) guidelines, the following molecular tests should be performed for all ADC NSCLCs, regardless of their clinical characteristics: 1) EGFR mutation analysis (at least for exon 19 deletion and exon 21 point mutation); 2) ALK translocation analysis by immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH) or NGS; 3) ROS1 translocation analysis by FISH or NGS; 4) B-Raf proto-oncogene (BRAF) V600E mutation analysis; and 5) PD-L1 expression analysis by IHC [43,44] (**Figure 2**). However, currently there is not any approved *in vitro* diagnostic test for ROS1 and BRAF, hence the clinicians should use well-validated assays to study ROS1 and BRAF.

Broader NGS panels can detect a range of alterations in tumors related to either oncogenic or tumor suppressor genes including p53, KRAS, MET, ERBB2, RET, STK11, FGFR1 and others. This additional molecular data on patients' samples could be beneficial for clinical decisions. For instance assessing tumor mutation load, derived either from large targeted or whole exome NGS panels, may predict response to immunotherapy [45].
However, these large DNA and RNA panels are not implemented in the routine clinical setting and their use should be limited to clinical trials [46].

In the near future, circulating tumor DNA (ctDNA) may provide a noninvasive and easy test for cancer diagnosis, prognosis and treatment-guidance. When fully established, the use of ctDNA for molecular evaluation (e.g., EGFR mutation) can be a potential alternative to rebiopsy for patients with inadequate tissue [43].

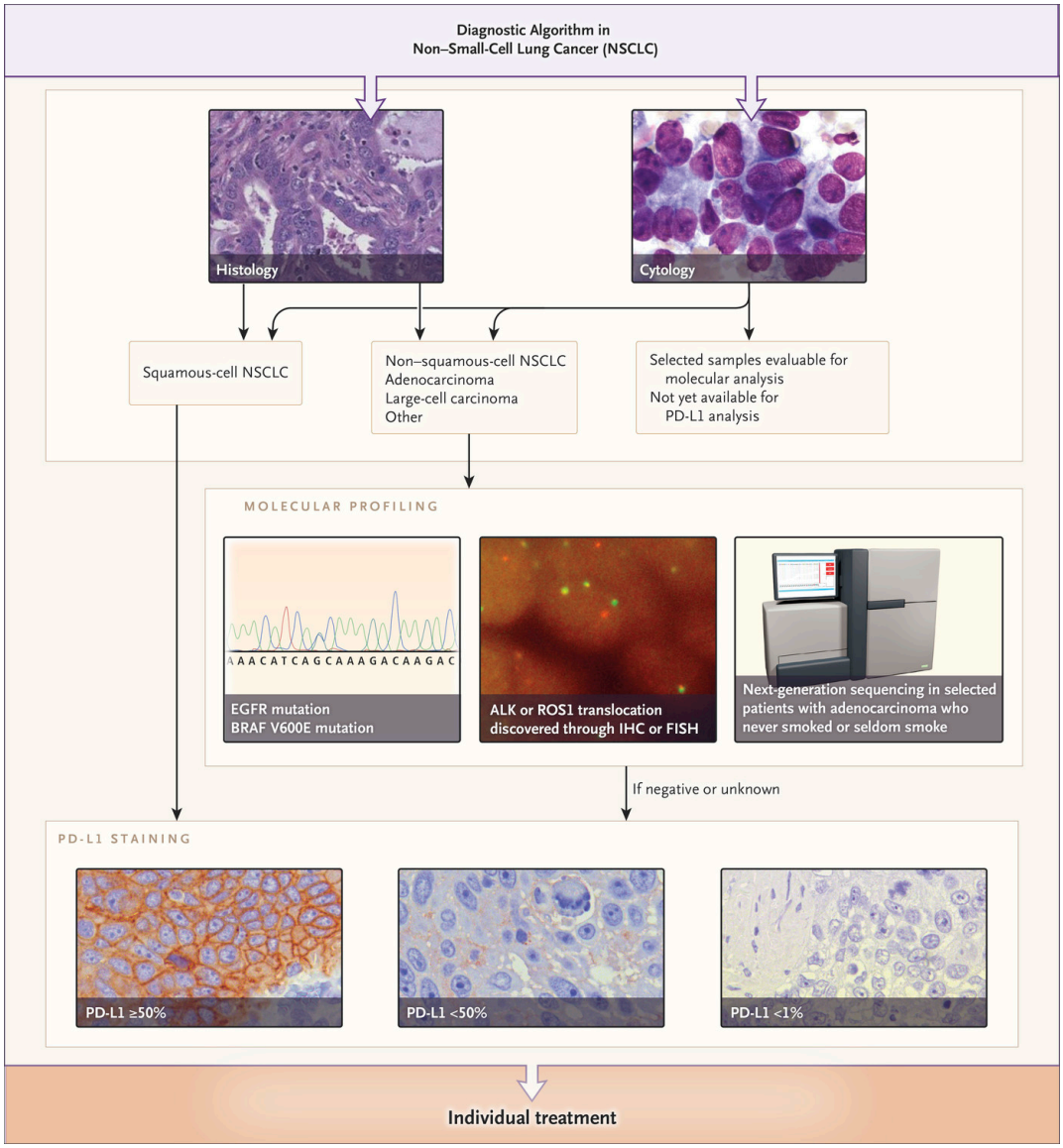

Figure 2. Diagnostic Algorithm for NSCLC
The upper portion of the algorithm shows the morphological classification of NSCLC based on histological (hematoxylin and eosin) and cytological (Giemsa) evaluation. The middle portion of the algorithm shows the molecular analysis for the key treatable oncogenic alterations: EGFR and BRAF V600E mutations and ALK and ROS1 translocations, as well as additional molecular analyses in selected patients. The lower portion of the algorithm shows the assessment of programmed death ligand 1 (PD-L1) expression by means of immunohistochemical staining (Reproduced with permission from [44] Copyright Massachusetts Medical Society).

1.1.6 Treatment and prognosis of NSCLC

Several factors, such as clinical staging, histological classification, molecular tumor features, Eastern Cooperative Oncology Group (ECOG) or Karnofsky performance status, age and so forth are influencing the choice of treatment for lung cancer patients. The optimal management of NCSCL requires careful evaluation of these factors in order to maximize the safety and efficiency of treatment.

Surgical resection is the standard of care for NSCLC stage I and II. Expected 5-year survival is 73-90% for pathological stage I and 56-65% for stage II. Risk assessment of patients eligibility for surgical resection is conducted in accordance with recommended guidelines [35,47]. Different types of surgical approaches are available according to size and localization of the tumor: pneumonectomy, lobectomy, segmentectomy or wedge resection. Lobectomy with systematic lymphadenectomy has been the conventional standard procedure and accounts for the majority of surgical cases (60-70%). In stage I, minimally invasive lobectomy such as video-assisted thoracoscopic surgery (VATS) lobectomy may be preferred to thoracotomy with respect to combined outcome and patient´s quality of life [48]. No survival benefit has been shown for adjuvant chemotherapy in stage I [49]. For stage I patients deemed not to be candidates for lobectomy or segmentectomy, stereotactic body radiation therapy (SBRT) or surgical wedge resection may be considered [50]. In this group, SBRT may improve 3 year survival rate from 25-35% to approximately 50%, with low rates of local failure, and moderate treatment toxicity [51]. Platinum-based adjuvant chemotherapy is recommended for patients (ECOG:0-1) with completely resected pathological stage IIA and IIB (N1) [50] .

Stage IIIA NSCLC is the most challenging group for clinicians as the optimal treatment for this group remains unknown. If feasible, surgery should be performed. Expected 5-year survival in this group is 41% [35,52]. For patients not eligible for surgery, the standard of care is concurrent platinum-based chemoradiotherapy with a curative purpose for patients (N2 and N3; ECOG:0-1). The optimal radiation dose for concurrently treated patients is typically 60 to 70 Gy [52]. In patients with completely resected NSCLC, adjuvant chemotherapy is recommended in T1-2, N1-2, M0 tumors, and tumors >4 cm. Adjuvant chemotherapy confers more benefits in stage IIIA. The 5-year survival of patients who had surgery plus adjuvant chemotherapy versus single modality-surgery were 39% vs 26% [53]. In addition, adjuvant

radiotherapy is a proper choice to reduce the incidence of local recurrence in patients with occult pathological N2 or R1 resection, but it is still unclear whether it improves survival [52].

Stage IIIB-IV patients are generally managed without surgery. In this patient group, the individual patient's performance status has major impact on treatment selection and survival. Median survival is 12-19 and 6-11 months for clinical stage IIIB and IV respectively [35,46]. Classically, a doublet chemotherapeutic regime of platinum-based compounds (carboplatin or cisplatin) and third-generation chemotherapy agents (paclitaxel, docetaxel, gemcitabine, pemetrexed or vinorelbine) is the standard of care. Alternatively, the platinum-based doublet may be combined with angiogenesis inhibitors (bevacizumab), which leads to a modest improvement of both overall- and progression free survival (PFS) in non-SCC NSCLC patients [54,55].

Recent evidence supports that the choice of first line therapy for advanced NSCLC should be based on molecular profiling. Utilizing this approach, many patients will be selected for targeted therapies or immunotherapy [37]. In patients with sensitizing EGFR mutations, first-line therapy with an EGFR TKI is recommended due to prolonged response rates, progression free survival and favorable toxicity profiles compared to standard platinum-based chemotherapy. In patients with ALK or ROS1 translocations, receiving ALK inhibitors (such as crizotinib, ceritinib or alectinib), showed superior response rate, progression-free survival (PFS), and quality of life when compared to standard chemotherapy [46].

Currently, there are two clinically FDA-approved immune check-point inhibitors for lung cancer: anti-PD1(nivolumab) and anti-PDL1(pembrolizumab). Nivolumab is recommended as subsequent therapy in patients with metastatic non-SCC or SCC NSCLCs (>1% PDL1 expression), who has progressed during or after first line chemotherapy. For ALK/ROS1^{-} patients (>50% PDL1 expression), pembrolizumab is recommended as first line treatment [56,57].

1.2 Tumor immunity

1.2.1 Tumor immune microenvironment

A tumor is a product of developing interactions between various cell types both within the tumor and the surrounding tumor microenvironment (TME) or stroma. The tumor stroma consists of extracellular matrix and various cell types such as immune cells, fibroblasts, endothelial cells, pericytes, adipocytes and others [58]. In recent years, in the dawn of immunotherapy, assessment of the tumor immune microenvironment (TIME) has become an interesting biological and clinical consideration. The cellular components of TIME consist of lymphocytes (T and B cells) responsible for adaptive immunity, myeloid cells (macrophages, dendritic cells, neutrophils and mast cells) that participate in both the innate and adaptive immunity and other stromal cells [59]. Communication between these cells are either by juxtacrine or paracrine mechanisms involving inflammatory cytokine networks. The localization, density, functional orientation as well as expression of immune derived-mediators and modulators of TIME have principal roles in directing tumor-associated inflammation toward tumor development or regression. In addition to the regulatory role of tumor-derived cytokines, chemokines, and growth factors, the mutational profile (particularly encoding-genes that create neoantigens) of the cancer cells can impact on the subset and amount of immune infiltration in stroma [60].

1.2.2 Innate and adaptive response

Innate response: The immune system protects the body from disease through two interrelated arms, the innate (natural) and adaptive immunity. Innate immunity involves a large number of different cell populations mainly derived from the myeloid lineage. These include, but are not limited to, monocytes, macrophages, granulocytes (neutrophils, eosinophils and basophils), dendritic cells, and natural killer cells (from the lymphoid lineage). Innate immune cells generally arise from hematopoietic stem cells in the bone marrow. The fist-line defense against pathogens relies on the activation of these cells. They recognize pathogens (or stress-associated and damage-associated molecular patterns) through different classes of pattern recognition receptors (PRRs). After recognition, the innate cells react against perturbing pathogens through general processes such as phagocytosis, complement cascade activation, and induction of inflammation. The inflammation process mainly occur due to the release of

soluble inflammatory mediators (cytokines, extracellular matrix remodeling enzymes and ROS) and bioactive mediators (e.g., histamine) by activated cells, which induce recruitment and infiltration of additional immune cells into damaged tissue [61].

Neutrophils, and to some extent eosinophils, are first recruited to the site of acute inflammation. They eliminate pathogens directly by releasing toxins and through phagocytosis. The second wave of cells are monocytes, which differentiate to macrophages within the tissue. Activated macrophages are an important source of cytokines and growth factors profoundly affecting tissue structure. Mast cells can also release pro-inflammatory effectors such as cytokines, proteases, and histamine. In acute inflammation, both mast cells and macrophages affect epithelial and vascular endothelial cell function, important for elimination of pathogen and initiation of tissue repair processes. Basophils (subclass of granulocytes) are functionally close to mast cells, and their primary role is to secrete histamine which induces inflammation by increasing blood flow to the inflamed site [62].

Natural killer (NK) cells are important lymphoid-derived components of the innate immune system. NK cells defend the host from pathogens by direct cytotoxic attack on their targets or by producing a large array of mediators (importantly IFN-γ). The released inflammatory mediators contribute to initiation of the antigen-specific immune response. NK cells also participate in cellular crosstalk between innate and adaptive immune cells through bidirectional interaction with dendritic cells (DCs) [63].

DCs are part of the antigen-presenting cells system that initiate and modulate the adaptive immunity. These act as sentinel cells that basically monitor the microenvironment for danger signals (damage-associated molecular patterns). Activation of DCs depend on the local proinflammatory effectors milieu and pathogenic antigens. DCs undergo a maturation phase after capturing the foreign pathogen and migrating to lymphoid organs, where they present antigen peptides in association with major histocompatibility complex (MHC) to naive CD8 (by MHC class I) and CD4 (by MHC class II) positive T-cells [64].

Adaptive response*:* Acute activation of innate immunity sets the stage for induction of the more tailored adaptive immune system. Adaptive immunity is a specific response to a particular antigen mainly driven by two leukocyte subsets, B and T cells. This defense system functions explicitly by a somatic rearrangement process in lymphoblasts, to produce a huge

number of antigen receptors such as T-cell receptors (TCRs) and/or immunoglobulin-based B cell receptors (BCRs). When T-cells are activated, they initiate the adaptive immunity in three ways: 1) direct attack on antigen-bearing cells by cytotoxic T lymphocytes (CD8+ T-cells), 2) stimulation of B lymphocytes to generate specific antibodies against the antigens, and 3) boosting the innate response and thereby inducing inflammation at the site of antigen engagement [62,65].

B-cells constitute a subpopulation of lymphocytes which express various cell surface immunoglobulin receptors recognizing specific antigenic epitopes. The majority of B-cells reside within lymphoid follicles, where they face and interact with T-cell-specific antigens bound to follicular DCs, proliferate, and either differentiate into plasma cells or memory B-cells [66]. After activation, selected B- and T-lymphocytes undergo clonal expansion after presentation and recognition of foreign particles, to obtain sufficient antigen-specific B- and T-cells for eliminating pathogens. Hence, the responsiveness of the primary adaptive immunity is slower than the innate system. However, during primary adaptive responses a subpopulation of lymphocytes differentiate into memory T- or B-cells, resulting in more robust responses after subsequent recurrence of the same antigen. Together, the innate and adaptive immunity cooperate during host defense to eliminate pathogens and restore tissue homeostasis. When expressed inappropriately or subjected to long-term involvement (chronic inflammation such as viral hepatitis infections), immune cells can give rise to autoimmune diseases or cancer, respectively [67].

1.2.3 Cancer immunoediting

The inverse tumor-promoting and tumor-inhibiting effects of the immune system, have resulted in the hypothesis of cancer immunoediting. The theory of cancer immunoediting underlines that extrinsic immune mechanisms may either prevent tumor progression or promote tumor growth by inhibition of host antitumoral immune responses. Cancer immunoediting (also named ´ the three E's`) relies on three steps: elimination (previously known as cancer immunosurveillance), equilibrium (persistence) and immune escape (progression) [68].

Elimination: The elimination of malignant tumors may occur at an early stage. Such a process consists of four phases: (i) Tumor antigens are recognized by innate cells which partially

remove the tumor cells. ECM remodeling due to tumor progression, induce pro-inflammatory signals resulting in recruitment of additional innate immune cells (including macrophages, DCs, NK cells, natural killer- and γδ-T lymphocytes) to the tumor site [69]. (ii) Interferon Is and γ limits tumor growth via initiation of interferon-dependent processes with antiproliferative, antiangiogenic and proapoptotic effects [69,70]. (iii) Recruited DCs activate after exposure to cytokines or interaction with NK cells. Then, the activated (mature) DCs migrate to lymph nodes, where they promote activation of T-helper (CD4+) cells and tumor antigen-specific CD8+ T-cells. (iv) In the last step, in order to complete elimination of tumor, the activated CD4+ and CD8+ T-cells of the adaptive system contribute in killing the antigen-specific tumor cells via direct and indirect (ex. IFN-γ-dependent) mechanisms [68].

Equilibrium: In Equilibrium, the immune system holds the residential cancer cells in a state of functional dormancy which is clinically undetectable. Equilibrium is the longest of the three phases and may last several years in humans [68]. Compared to elimination and escape, less detail is available about equilibrium as it is difficult to model this state of immunity in animals. Specific components of the adaptive immune system, including CD8+ and CD4+ T-cells (and not innate cellular components), are thought to be responsible for keeping the occult tumor cells in equilibrium. At this point there is probably a balance between antitumoral (e.g., INF- γ and interlukin-12) and protumoral cytokines (e.g., interleukin 10 and 23) [71].

Escape: At this step, nascent tumors are fully immunoedited and the immune control fails to restrict their progression. Hence, the cancer become clinically apparent. The tumor cell escape process can occur through diverse mechanisms such as: (i) absence or reduced immune recognition due to loss of tumor-antigenicity or MHC expression, (ii) deficiency in apoptotic signaling pathways and activation of anti-apoptotic signals including overexpression of STAT3 and BCL2, or (iii) development of an immunosuppressive milieu through the effect of immunosuppressive mediators (e.g., IL-10, TGF-β and VEGF) and immune cells (e.g., (myeloid-derived suppressor cells and regulatory T-cells) or immunoregulatory molecules (e.g., IDO, LAG-3, PD-1/PD-L1 and Tim-3/ galectin-9) [68,72].

1.2.4 Lymphocytes

Further support for cancer immunoediting can be found in reports correlating the quantity of tumor-infiltrating lymphocytes (TILs) with favorable clinical outcome [73]. These findings imply that TILs are effective at postponing tumor development. However, it is important to consider that different TILs have distinct functions in the TME. Cytotoxic CD8+ TILs, are capable of killing cancer cells directly [65]. CD8+ regulatory T cells (Tregs) possess an immunoinhibitory function and are able to maintain immune homeostasis via CXCL4 [74]. However, their role in cancer is poorly understood. CD4+ TILs are a heterogeneous class of cytokine secreting lymphocytes, comprising several distinct subpopulations. For cancer, the Th1, Th2, Th17, and Treg CD4+ TILs are deemed the most important [75]. Th1 cells produce IFN-γ and IL-2 which mediates activation of CD8+ TILs. Th2 cells produce a broader range of cytokines (e.g., IL-4, IL-5, IL-9, IL-10, IL-13, IL-25) and limit CD8+ TIL proliferation. In terms of antitumoral responses, Th2 activation is less effective than Th1 activation (**Table 1**). Th17 cells secrete IL-17 and mediate induction of many organ-specific autoimmune diseases. CD4+ Tregs secrete IL-10 and TGF-β, which maintains self-tolerance through the suppression of effector TILs [76,77]. Overall, immune infiltration of various adaptive lymphocyte subsets has been associated with improved prognosis in many different cancers [73,78].

Table 1. Innate and adaptive immune cells involved in regulating tumor growth in human	
Stimulate Cancer growth	Inhibit cancer growth
Innate Immune cells	
Neutrophils	Dendritic Cells*
Macrophages (M2)	Macrophages (M1)
Myeloid derived suppressor cells	
Adaptive immune cells	
TH2 $CD4^+$ T cell	Cytotoxic $CD8^+$ T cell
$CD4^+$ T regulatory cell	TH1 $CD4^+$ T cell
B lymphocytes*	TH17 $CD4^+$ T cell
Abbreviation: Th, T helper *Have been associated with both stimulation and inhibition. Reproduced with permission from American Society of Clinical Oncology [77].	

In NSCLC, extensive stromal infiltration by CD8+ or CD3+ TILs is strongly associated with patient survival [79–81]. No conclusive results has been achieved on the prognostic impact of CD4+ TILs [82]. However, among the CD4+ TIL subsets, Th1 cells have been associated with improved survival [83], while Th2 cells were associated with tumor progression [84].

High levels of Th17 CD4+ TILs have been associated with lymphangiogenesis and a poor clinical outcome [85]. Notably, CD45RO+ T-cells are other subclasses of TILs, considered as memory T lymphocytes. Overexpression of CD45RO+ TILs has been associated with improved outcomes in various cancers as well as NSCLC [86]. FOXP3 is a key regulatory transcription factor for the development and function of Tregs. High infiltration of FOXP3+Tregs has been correlated with poor survival in NSCLC [87,88]. In contrast to T-cells, the precise prognostic impact of B-cells and plasma cells are currently not well defined and remains controversial [80]. Until now, the most robust prognostic TIL marker in NSCLC, is CD8 [89].

Immunohistochemistry is the optimal method to evaluate TIL subsets. Nevertheless, several studies have investigated total TIL levels using standard hematoxylin and eosin (H&E) staining and found strong prognostic and predictive impact [60,90]. Initially, assessment of TIL levels in breast cancer H&E slides was reported as a powerful predictor of response to neoadjuvant chemotherapy [91]. Since then, several studies in various cancers have evaluated H&E TILs [60]. In 2014, Salgado *et al* (in collaboration with a panel of international pathology experts) recommended a standardized guideline for evaluating H&E TILs in breast cancer [92]. A general update of this guideline, for many solid tumors, was proposed by Hendry et al [93]. In NSCLC, a couple of studies have evaluated TILs in H&E routine slides with various scoring models [94–98]. However, no consensus for the evaluation of TILs in H&E for NSCLC have been reached. The original breast cancer H&E TILs assessment guideline is an attractive choice for adaptation, but requires comprehensive validation for other types of cancer, including NSCLC.

1.2.5 Neutrophils

In addition to TILs, the cellular composition of the TME contains various types of immune cells including neutrophils, macrophages, mast cells, DCs, and NK cells. Tumor-associated neutrophils (TANs) constitute a significant portion of the TME and are the most prevalent immune cell type found in lung cancer [99,100]. In humans, it is still unclear whether the presence of TANs stimulate or suppress tumor growth. Based on studies in murine models, it has been proposed that TANs polarize into either a N1 antitumoral or N2 protumoral phenotype [101]. TANs are recruited to target sites by local overexpression of chemokine receptors including CXCR1 and CXCR2. Tumor cells and associated mesenchymal cells

express various ligands (e.g., CXCL1, CXCL2 and CXCL5) that accelerates recruitment of TANs [102].

N2—pro-tumor role: In various cancer models, TANs were found to facilitate cancer cell extravasation via a number of direct and indirect mechanisms. Neutrophils and other inflammatory cells capable of remodeling ECM have long been considered mediators of cancer cell invasion and metastasis through surface expression of selectins and integrins (adhesion receptors) or production of neutrophil extracellular traps (NETs) [103,104]. TANs release granules containing neutrophil elastase, matrix metalloproteinase-8 (MMP8), MMP9 and proinflammatory cytokines, which degrade ECM and facilitate tumor progression [104]. MMP-9, in contribution with CXCL8, activates VEGF-A and FGF2 and initiates angiogenesis [105]. Hepatocyte growth factor (HGF) is a pleotropic cytokine with angiogenic attributes. TANs may produce HGF following exposure to local pro-inflammatory mediators, which promote invasion and metastasis of tumor cells [106]. ROS (generated by NADPH oxidase of phagolysosomes), produced by neutrophils is a powerful defense against pathogens. In malignancy, however, ROS may exert both genotoxic effects by initiating tumor proliferation and DNA damage, or conversely, cytotoxic effects mediating tumor suppression [107].

N1—anti-tumor role: In addition to the broad literature on their protumoral impact, there is also evidence of antitumoral activity mediated by TANs. In the original study [101], which proposed distinct N1 and N2 TAN subsets, N1 TANs were described as cells generating a broad specter of cytokines, which were highly cytotoxic to tumor cells. Depletion of the N1 antitumor TANs decreased CD8+ T-cell activity and led to increased tumor burden [101]. In addition, TANs may exert antitumor activity by directly killing tumor cells, or by producing factors leading to recruitment and activation of innate and adaptive immune cells. In early stage cancer, TANs induce T-cell responses and release proinflammatory cytokines (e.g., TNF-α, IL-6 and IL-8), which enhance the antitumoral activity [108] (**Figure 3**).

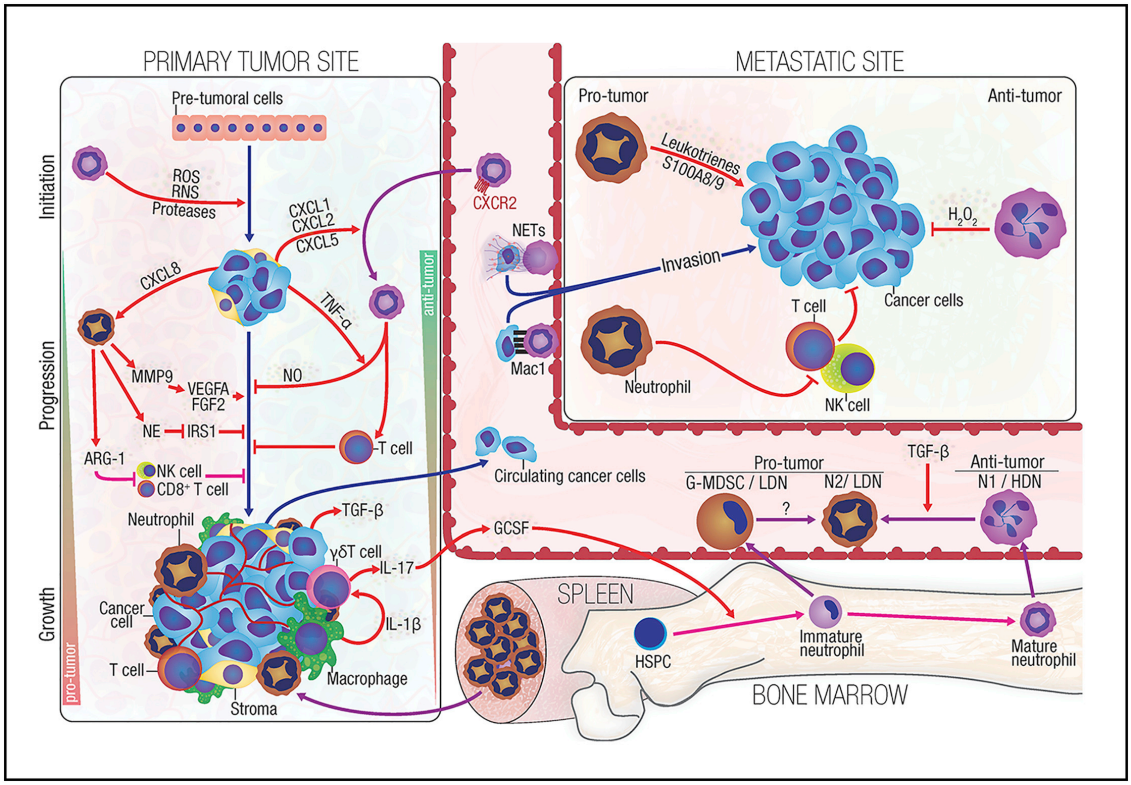

Figure 3: Neutrophils in cancer
Neutrophils influence the tumor environment and cancer progression through multiple mechanisms. At the primary tumor site (left box), activated neutrophils can induce genetic damage or signaling in pretumoral cells through reactive oxygen species (ROS), reactive nitrogen species (RNS), and proteases, thereby promoting tumorigenesis (Initiation). In primary tumors, neutrophils can prevent tumor progression by activating cytotoxic immunity or nitric oxide (NO) production. As the tumor progresses, neutrophils become predominantly protumorigenic: transfer of elastase (NE) activates proliferation within tumor cells; arginase-1 (ARG1) suppresses CD8+ T cell and NK cell responses; and release of MMP9 activates VEGF-A and FGF2 to support angiogenesis. As the tumor grows, cancer cells and the supporting stroma produce tumor-supporting factors: macrophages release IL-1β that induces IL-17 production by intratumoral γδ T cells, resulting in G-CSF-dependent expansion and recruitment of protumoral neutrophils from the bone marrow or the spleen; TGF-β programs immune competent neutrophils (N1) toward an immunosuppressive (N2) state. Neutrophils also influence tumor metastasis in negative and positive ways (right box). Production of hydrogen peroxide (H2O2) is toxic for metastatic cells. In contrast, capture of circulating cancer cells through neutrophil-derived Mac-1 or NETs favors their entry into tissues; and inhibition of natural killer (NK) and T cell responses supports the survival of metastatic cells, whose proliferation is additionally favored by neutrophil-derived leukotrienes. Reproduced with permission from Elsevier [102].

The prognostic significance of the innate immune cells is controversial and appears best studied within the context of individual tumor types. For TANs, high tissue infiltration was associated with a poor prognosis in kidney, esophagus and head & neck cancers [109–111], and with a good prognosis in gastric and colorectal cancers [112,113]. In NSCLC, previous studies have failed to reveal significant associations between TAN and patient survival [114,115]. The reasons behind varying outcomes are not clear, but may be related to the type of cancer, stage and histology. Until now, there are no specific markers available for differentiation between N1 and N2 phenotypes in human tissues. In published studies, CD66b

(alternative names: carcinoembryonic antigen-related cell adhesion molecule-8 or CEACAM8, NCA-95 and CD67), which is localized in human neutrophil and eosinophil granules, is the most widely used marker to label pan-TANs [116,117].

1.2.6 Macrophages

Macrophages are a heterogeneous population of mononuclear phagocytic leukocytes. Their functions are specialized for the anatomical location in which they reside. Tissue macrophages derive from two main sources: Yolk sac and bone marrow progenitor cells. Dependent on tissue type, some tissues are populated by yolk sac-derived macrophages like Langerhans cells and microglia in the skin and brain, while, other tissues are populated by macrophages from bone marrow (circulating monocytes) [118]. Most macrophages involved in pathogenic responses, especially cancer, appear to originate from circulating monocytes [119]. The macrophages involved in cancer-initiated inflammatory responses are often named tumor-associated macrophages (TAMs). TAMs are a significant component of the myeloid-derived infiltrate in tumor stroma, and studies of TAMs formed the basis for the models proposing that inflammatory infiltrates are involved in tumor development. Similar to TANs, a binary phenotype with distinct divergent functions were defined for TAMs: M1 antitumoral and M2 protumoral. TAM differentiation, growth, and chemotaxis is regulated by local cytokines and growth factors such as GM-CSF, MCSF, IL-3, CCL-2 etc [120].

M1—anti-tumor role: Th1 cytokines (IFN-γ and TNF-α) are the key players when macrophages polarize into the M1 phenotype. M1 macrophages typically: (I) overexpress proinflammatory cytokines such as IL-12, IL-23 and TNF; (ii) express MHC class II and costimulatory molecules such as those in the B7 family; (iii) express CXCL9 and CXCL10 to amplify Th1 responses; (iv) underexpress IL-10. Functionally, M1 macrophages contribute to the host defense mechanisms through activation of NADPH and production of ROS. This process is mainly regulated by the sustained production of IFNγ secreted by Th1 cells. As described for TANs, ROS produced by TAMs may lead to both progression and regression of tumors. The M1 phenotype is vital to the initial antitumoral defense and their activation is partly regulated by the anti-inflammatory activity of M2 subpopulations—to protect against tissue damage driven by M1 cytokines and mediator products [121,122].

M2—pro-tumor role: Similar to the M1—Th1 axis, M2 polarization is significantly influenced by Th2 cytokines. M2 macrophages can be further subclassified into M2a, M2b, M2c, and M2d according to different environmental signals. M2 TAMs produce high levels of IL-10 and express scavenger receptors, mannose receptors, IL-1 decoy receptor and hyaluronan receptor LYVE-1 [123]. In general, M2 TAMs promote tumor growth and dissemination through ECM remodeling, angiogenesis, immunoregulation and immunosuppression [124]. M2 TAMs, typically present at hypoxic areas in tumor stroma and induce proangiogenic factors such as VEGFs and PDGFs via overexpression of HIF-1α. M2s are the major source of enzymes and proteases (e.g., MMPs, plasmin, osteonectin, and cathepsins) that regulate the degradation of surrounding ECM, thereby allowing tumor cells to spread and metastasize [125,126]. Different direct and indirect mechanisms allow M2 TAMs to inhibit anti-tumor Th1-mediated adaptive immunity. In direct (cell-to-cell) mechanisms, M1 TAM´s surface receptors/ligands interact with their counterpart´s inhibitory receptors/ligands of target immune effector cells. For example, M2 TAMs possess the ligand for PD-1 and CTLA-4 immune checkpoints that upon activation inhibit the cytotoxic activity of CD8+ T-cells, NK and NKT cells. Through indirect signaling, IL-10 secretion by TAMs may suppress the cytotoxic activity of CD8+T-cells and induce the regulatory activity of Tregs [122].

In addition, macrophages may polarize into an 'M2-like' phenotype, which shares, but do not express all the signature properties of M2 macrophages. Antigen-antibody complexes, together with TGF-β and IL-10 can induce macrophage differentiation into the M2-like subsets which have shared features of IL-4/IL-13 activated cells, such as overexpression of mannose receptors, IL-10 and angiogenic markers. Different *in vivo* and transcriptome studies, in both normal and cancerous tissue, have confirmed different scenarios for M2 polarization [124,127] (**Figure 4**).

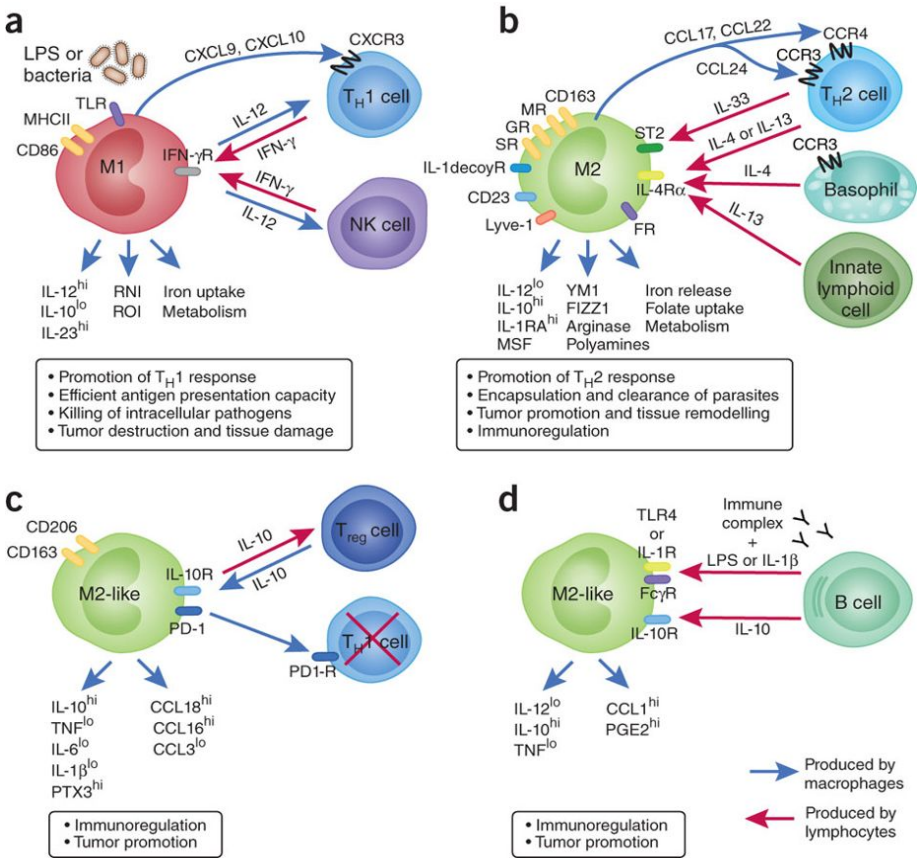

Figure 4: The orchestration of macrophage activation and polarization by lymphoid cells
(a) M1-polarized macrophages and their crosstalk with TH1 and NK cells. (b) M2 polarization of macrophages driven by TH2 cells, basophils and innate lymphoid cells through their secretion of IL-4, IL-13 or IL-33. (c) M2-like macrophages polarized by interaction with Treg cells. (d) M2-like polarization of macrophages by interaction with B cells through antibody-mediated FcγR activation or cytokines. FR, folate receptor; GR, galactose receptor; IFN-γR, IFN-γ receptor; IL-1decoyR, IL-1 decoy receptor; MHCII, major histocompatibility complex class II; MP, macrophage; MR, mannose receptor; SR, scavenging receptor; ST2, receptor; PGE2, prostaglandin E2; PTX3, pentraxin 3; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate. Reproduced with permission from Springer Nature [124].

In accordance to the protumor and antitumor mechanistic properties of TAMs, discrepant results exist concerning their prognostic significance. In some human tumors, an increased frequency of TAMs have been associated with poor prognosis, as shown in breast, head and neck, ovarian, gastric and bladder carcinomas, while in others, such as colorectal carcinoma, TAMs seem to convey a favorable prognosis [128]. In NSCLC, the prognostic impact of TAMs is still a matter of controversy [129].

1.2.7 Tumor immune profile

For most cancers, state-of-the-art prediction of clinical outcome is achieved by utilizing the TNM classification. However, outcome may vary among patients within the same TNM stage. Hence there is room for additional prognostic information to be considered beyond the TNM grading system [36,130]. The tumor immune contexture (defined as type, density, and location of immune cells) has demonstrated impact on clinical outcome [60].
In colorectal cancer (CRC), a large body of evidence has revealed a tight correlation between the immune contexture status and patient survival [131,132]. Evaluation of CD8, CD3, CD45RO and granzyme B positive immune cells in different tumor compartments (invasive margin and tumor core), has added to the TNM staging system for CRC and given the name TNM-Immunoscore® (TNM-I). The TNM-I classifier is an easy model applicable for use in a routine practice through scoring the quantity (scoring range: I0-I4) of established immune markers in specified tumor areas. E.g., a low quantity of markers in both invasive margin and tumor cores scores "I0" whereas a high quantity of markers in both areas scores "I4" [133,134].

In breast cancer, data obtained from large-scale studies have revealed the prognostic and potential predictive effect of TILs for both HER2 positive and triple negative patients. The clinical benefit of evaluating TIL in breast cancer is linked to models predicting the usefulness of pre-and post-operative chemotherapy and immunotherapy [135]. As discussed in **Section 1.2.4**, recommendations for an assessment of TILs have been proposed by an international breast cancer TILs working group, which endorses H&E staining and morphological evaluation of TILs for their proposed immunoscore [90,92].

In NSCLC, similar models have been proposed as complements to the TNM classification [89]. Various immune cell populations such as CD8, CD45RO, PD1, PD-L1, CTLA-4 and LAG-3, have been explored [79,86,136–138]. Until now, the most promising candidates are CD8 and CD45RO (SCC subgroup) [79,86,89]. In this context, there is clearly a potential to explore further immune-related markers for establishing a prognostically conclusive immune panel for implementation in a NSCLC TNM-I model.

2 Aim of thesis

The general aim of this thesis was to explore the *in situ* presence of the most prevalent immune cell subsets (neutrophil, macrophages and lymphocytes) in NSCLC. And further assess their association with disease progression.

Specific aims:

I. To explore associations between tumor-associated neutrophils and clinical outcome in histological subtypes of NSCLC.

II. To validate H&E TILs assessment guidelines, originally proposed for breast cancer, in order to study its prognostic relevance in NSCLC whole-tissue section slides.

III. To apply a reliable technical approach for identification of tumor-associated macrophage phenotypes in NSCLC, and to analyze their relationship with survival and other adaptive/innate immune infiltrates.

3 Materials and methods

3.1 Patient cohort

Primary tumor: Formalin fixed paraffin embedded (FFPE) blocks were collected from consecutive stage I-III NSCLC patients who underwent radical resection at the University Hospital of North Norway (Tromsø) or the Nordland Central Hospital (Bodø) from 1990 to 2010. Of a total of 633 primary tumor samples, 536 (in **study I**), 537 (in **study II**) and 553 (in **study III**) were included in the analyses. An overview of the of the cohorts is given in **Table 2**.

Exclusion criteria were as follows: i) Patients having malignancies other than lung cancer within five years of diagnosis (n=39, not including superficial skin cancer) as they are likely to either have received treatment that may alter the host immune response, experience relapses or harbor mutations making them susceptible to cancer, all of which may obscure statistical analyses; ii) Patients who received neoadjuvant chemo- and/or radiotherapy (n=15) as neoadjuvant treatment may alter the local host immune reaction via modifications in stroma immune cell composition; iii) Patients with in-adequate tissue in FFPE blocks (n=26); iv) Patients with H&E samples of poor quality (n=16 in **study II**).

Lymph nodes: From the 633 surgically resected primary tumor samples, 172 patients were diagnosed with lymph node metastasis (LN+). Of 172 patients, 143 had adequate tissue for expression analysis and this LN+ cohort was included in **study I** and **III**. The details of both primary tumor and LN+ cohorts has been previously described [137–139].

Table 2: A glance overview of the cohorts and applied methods					
	Primary tumor cohort			**LN+ cohort**	
	Study I	**Study II**	**Study III**	**Study I**	**Study III**
Tumor type	NSCLC	NSCLC	NSCLC	NSCLC	NSCLC
Sample type	FFPE	FFPE	FFPE	FFPE	FFPE
Cohort size (original/after exclusion)	(633/536)	(633/537)	(633/553)	(172/142)	(172/143)
Histological classification	WHO 2004	WHO 2015	WHO 2015	WHO 2004	WHO 2015
SCC	289	298	307	74	78
ADC	201	232	239	58	65
Other	46	7	7	10	-
TNM staging	UICC 7th edition	UICC 8th edition	UICC 8th edition	UICC 7th edition	UICC 8th edition
pStage					
I	256	226	232	-	-
II	194	181	185	70	59
III	86	130	136	72	84
Methods	TMA mIHC	Whole tissue H&E	TMA mIHC	TMA mIHC	TMA mIHC
Clinical endpoints	DSS, DFS, OS	DSS, DFS, OS	DSS	DSS	DSS

Abbreviations: NSCLC, non-small cell lung cancer; LN+, node metastases; FFPE, formalin fixed paraffin embedded; WHO, world health organization; SCC, squamous cell carcinoma; ADC, adenocarcinoma; UICC, The Union for International Cancer Control; pStage, pathological stage; TMA, tissue microarray; mIHC, multiplex immunohistochemistry; H&E, hematoxylin and eosin; DSS, disease-specific survival; DFS, disease-free survival; OS, overall survival.

Pros and cons: Significant strengths of the cohort is the large number of patients, the lack of patient selection, and, since all patients were recruited from two local hospitals, the reliable clinical data. Major considerations include the study's retrospective nature and the long inclusion period.

Retrospective studies are cost-benefit and time-saving compared to prospective studies, but have more potential for bias and cofounder effects. Besides, there may be lack of homogenous data and standardized follow-up. Other drawbacks include limited access to further demographic data about patients' lifestyle and concomitant diseases.

The patients in this study were included over a period of 20 years. During this period

guidelines for diagnosis and treatment (especially after implementation of adjuvant therapy in 2005) of lung cancer have changed. To partly compensate for this, the cohort has been updated according to the latest guidelines for TNM- and histological-classifications. Updating the cohort will lead to a few patients changing overall stage. In some cases, patients will change to a stage where the treatment they originally received might not be considered appropriate according to current treatment strategies. This is exemplified by 21 patients being classified as stage IIIB after reclassification from the 7th to the 8th edition of the UICC guidelines. Moreover, improvements in imaging techniques during the inclusion period are significant. For example, many of the patients with occult N2 nodes included in the cohort would likely have been discovered by PET imaging and deemed not to be candidates for surgical resection.

3.2 Clinical data

The demographic and clinical data were retrieved from medical journals by an oncologist. In all three studies, the records included follow-up data until October 2013. The median follow-up of survivors was 86 (range 34–267) months. In **study I**, the TNM staging was conducted according to the 7th edition of UICC guidelines [36] and the histological classification was in accordance to 2004 WHO guidelines [19]. However, in **study II** and **III**, the patients were restaged and the tissue specimens reclassified based on the latest UICC (2016) and WHO (2015) guidelines [18,35]. Notably, after histological transition from the 2004 to 2015 version, previously excluded patients histologically classified as bronchioloalveolar carcinoma (BAC), were re-classified and re-included in the ADC subgroup of the cohort. The major difference in TNM staging after the transition from the 7th to the 8th version, was that 21 patients were staged as IIIB. The reporting of clinicopathological variables, survival data and biomarker expressions was conducted in accordance with the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines [140]. One major limitation with our database was lack of molecular alteration information for patients such as EGFR, ALK, KRAS and ROS1.

Clinical endpoints: Disease-specific survival (DSS), disease-free survival (DFS) and overall survival (OS) are the clinical endpoints measured in our cohort. DSS was calculated from time of surgical treatment to lung cancer death. DFS was defined from time of surgery to first

relapse. OS defined the time between surgery and death of any cause. The time of death was retrieved from death certificates.

The primary endpoint was DSS. DSS may be considered more sophisticated compared to other endpoints. As death by other causes is disregarded, DSS may potentially provide better data about the underlying biology. However, differentiating endpoints is difficult and requires comprehensive information about the cause of death. In the current cohort, the official cause of death was cross-referenced to the information available in the patient's journal both in the regional and local hospitals and in cases with missing information, the patient's general physician was contacted. These steps ensure high confidence in the clinical endpoints. Nevertheless, the cause of death was interpreted subjectively and potential differences in interrater variability was not tested. Therefore, it is possible that some few patients may change endpoints if a full revision, by separate investigators, was performed. Because of this latter argument, many researchers consider OS a more robust endpoint. In the studies included in the present thesis, OS and DFS were used as supplementary endpoints in **study I** and **II**.

3.3 Ethics

The initial database and cohort (involving 335 patients) was approved by the Regional Committee for Medical and Health Research Ethics (REK Nord) and the Norwegian data protection authority (DPA). In the latest ethical reapproval for the updated cohort with Id.no: 2011/2503, the informed consent from patients was considered. Since this project was a retrospective study and most of the patients were deceased, the need for patients' consent was waived by the boards of DPA and REK Nord. All patient's personal data were anonymized prior to database entry.

3.4 Tissue microarray

Tissue microarray (TMA) is a cost-effective and time-saving technique suitable for large-scale tissue-based studies and beneficial in order to preserve the tissue. A broad range of techniques, to evaluate DNA, RNA and/or proteins, utilizing immunohistochemistry and *in situ* hybridization principles, are compatible with TMAs. The staining or probing variability is greatly decreased when using TMAs, as large numbers of tissue samples are processed

simultaneously in consistent experimental conditions [141,142]. Each TMA block consist of several cylindrical cores sampled from different FFPE tumor/tissue blocks, arrayed in a single recipient paraffin block. The area of interest in each donor block has been marked by a pathologist, on the corresponding H&E slides. This area is then punched and transferred to a recipient block. Depending on the purpose of use, various tissues/compartments can be selected for transfer into recipient TMA blocks, such as tumor epithelial, normal- or tumor-associated stroma, normal tissue, invasive margins and etc. The diameter of cores may vary from 0.6 to 1mm and the depth is normally 3mm [142,143].

The common concern regarding the use of TMAs is whether the small core samples arranged in recipient TMA blocks represent the whole heterogenous "face" of the tumor, especially when the donor specimen is rather large. For biomarker evaluations, a significant number of cores from donor blocks is essential for statistical considerations, primarily to reduce the bias associated to tumor heterogeneity. For the studies presented in this thesis, four to five cores from each patient were transferred to the recipient blocks, consisting of two cores from tumor epithelial, two from tumor-associated stroma, and one from normal alveolar tissue (if present). Besides, in both **study I** and **III**, a random comparison of 20 patients was performed between TMA cores and paired whole tissue slides, in which a correlation >95 % was observed. However, an interesting recent study in breast cancer evaluated the number of TMA cores required for a reliable assessment of lymphocytic infiltration. This study found that four cores represents a good trade-off between performance and the amount of tissue required. However, six cores were needed to achieve consistent prognostic value in the HER2 subgroup [144].

Other issues with TMAs include that they are not validated for routine clinical use and that some cores may detach and be missing from the TMA slides during the IHC or ISH procedure due to loss of paraffin elasticity, old FFPE blocks, antigen retrieval or washing steps in the protocol.

3.5 Immunostaining

3.5.1 Immunohistochemistry

Immunostaining is commonly utilized to detect diagnostic, prognostic and predictive biomarkers. Among different immunostaining approaches, IHC offers a broad range of research and diagnostic applications for detection and visualization of proteins in tissues through labelling of antibodies, either direct or indirect antigen binding. In the clinic, IHC is an important ancillary tool in order to differentiate histology and diagnose different cancer types. In research, IHC is an essential technique for biomarker discovery. Identifying novel molecular or immunotherapeutic targets and developing personalized therapy has emerged a successful approach to improve patient's outcome and care. IHC is a well-established and affordable technique, appropriate to complement TMA in order to deliver rapid and high throughput assessment in large-scale studies. Its ability to be applied for fresh frozen or formalin fixed specimens makes it highly practical, as specimens of these natures are the mainstay of most surgical pathology centers worldwide. It is a remarkably sensitive and specific approach when the proper antibodies and reagents are thoroughly chosen following prior validation on known tissue or staining controls [145].

While IHC is a powerful laboratory technique, it has certain limitations related to technical reproducibility. The technical issues are mostly derived from the preanalytical and analytical phases. Preanalytical variables are variation on tissue processing, fixation methods, and storage time prior to the analytical phase [146]. It is known that prolonged fixation in formalin may decrease the immunoreactivity of antibody/antigen in FFPE sections [147]. Due to the retrospective nature of this thesis, the findings may have been affected by the variables related to the preanalytical phases, particularly with respect to uncertainty regarding fixation time. However, the recent developments in antigen retrieval steps (within the analytical phase) has improved the unmasking of antigen significantly; even in samples with longer exposure to tissue fixatives [148]. Fixation duration varies depending on tissue type and size. The penetration time for formalin is longer in large resections and highly cellular dense tissues. Potential differences may occur when central versus peripheral parts of the tissue undergo such analyses, due to quicker fixation of the area of tumor at the periphery [149]. In the presented analyses, the quality of all patient specimens was verified and re-checked by two experienced pulmonary pathologists, and poorly qualified tissue was excluded.

Storage time may be an issue for IHC. However, in a study of antigenicity, up to 68 years of archiving FFPE blocks did not significantly impact IHC staining quality for most markers [150]. However, prolonged storing of sectioned slides impacts staining immunoreactivity. Due to this, all IHC procedures for the studies (**study I** and **II**) in this thesis were performed in fresh cut sections. Moreover, no significant differences in staining intensities and distributions were observed, when comparing patient samples collected before and after year 2000 in the present cohort.

However, there has been a surge of novel technologies for IHC, including automation, multiplexing, and digital pathology; which may overcome some of IHC's inherent drawbacks. The development of automated platforms for tissue processing, fixation and performing IHC staining have directed laboratories closer to the goal of standardization and have improved technical reproducibility. The current IHC-autostainers vary significantly in their design and capabilities. Regarding analytical variables, utilizing autostainers provides uniform standardized microenvironments which results in higher intra- and inter-laboratory consistency and reproducibility. Besides labor saving, less hands, and reduction in manual variations, the main advantage of autostainers is providing stable temperature condition for antigen unmasking and enzymatic reaction [151]. The IHC assays included in this thesis were performed with one of the most advanced available platforms: Discovery-Ultra (Ventana, Roche). Discovery-Ultra is an open-system with high flexibility to run double IHC/ISH and chromogenic/ fluorescence multiplexing. The disadvantage of automated IHC-systems is that it requires skilled histotechnologists for troubleshooting and optimization.

3.5.2 Multiplexed-IHC

To better understand the complex expression pattern of biomarkers, IHC applications are shifting from single- towards multi-antigen detection. Multiplex IHC (mIHC) provides greater insights into tumors as well as helping to conserve tissue via simultaneous visualization of a larger number of markers. Chromogen-based mIHC is preferable when less than three markers are of interest. Fluorescence-based mIHC is more practical when assessing a large panel of markers [152]. Staining is either simultaneous (cocktail) or sequential. In simultaneous IHC, the antibodies are used to label antigens in one staining process. The cocktail method is useful for double labeling in which the two primary antibodies should be from different host species. Sequential IHC involves several iterations of labeling single

antigens, each with a different label or secondary antibody type (e.g., IgM vs IgG) until all desired antigens are visualized [153]. In our automated setting, the sequential strategy was employed (**Figure 5**).

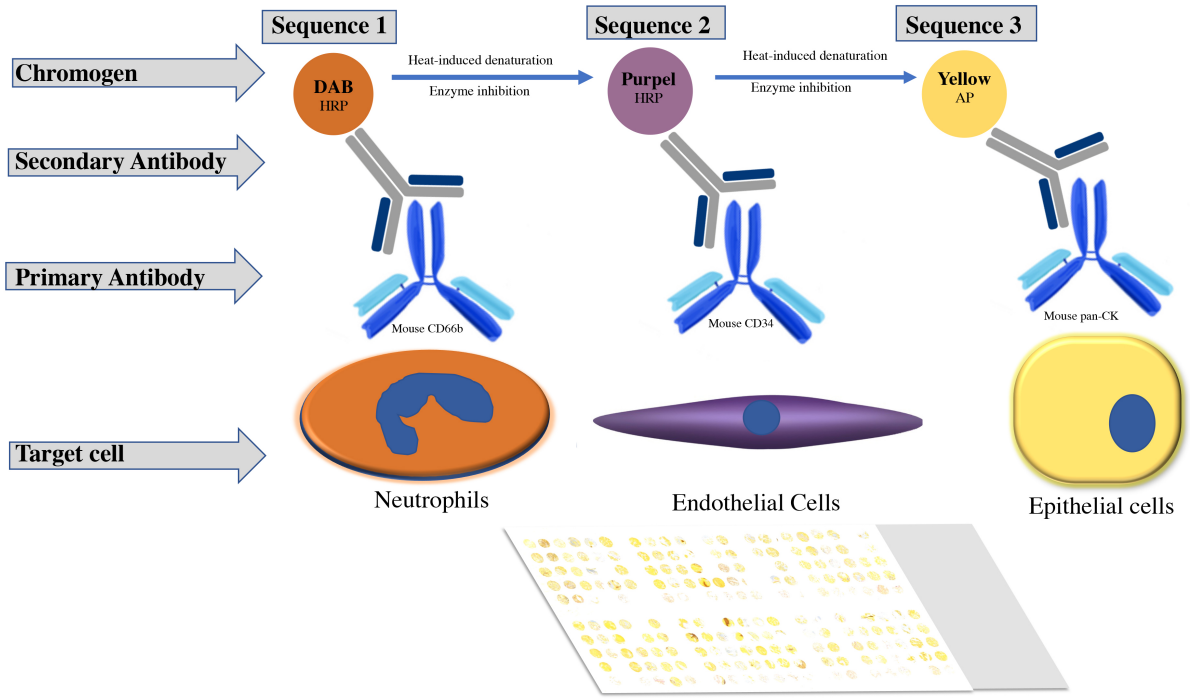

Figure 5. Sequential Multiplex IHC.
Schematic model to represent the principal of 3-plex staining performed in **paper I**. Brown, purple and yellow chromogens were loaded to identify target cells. Between each sequence, two different approaches were used in order to denature the excess antibodies including: heat-induced denaturation and enzyme inhibitors.

Drawbacks of mIHC include antibody cross-reactivity and chromogen overlap [154]. Cross reaction may occur between incomplete eluted antibodies and newly applied antibodies in each staining sequence. To overcome this hinder, enzymatic inhibition and heat-induced denaturation between each sequence (prior to loading the 2nd or 3rd primary antibody) were applied. Both the duration of enzymatic inhibition and the temperature were validated and verified in single-IHC to ensure that the structural integrity of the antigen was retained.

The most common reporter enzymes for chromogenic labelling are: Horseradish peroxidase (HRP) and alkaline phosphatase (AP). Enzymes can be conjugated with different chromogens to produce a colored precipitate at the site of the antigen/antibody reaction. Cross-reactivity can be avoided or reduced by using separate HRP and AP conjugates [155]. Therefore, wherever mIHC was applied in this thesis, different conjugated chromogens were utilized (**paper I**: HRP—HRP—AP; **paper III**: HRP—AP).

Chromogens: Chromogens are molecules that allow detection of a target using enzyme-based precipitation reactions. For microscopical evaluation of mIHC, the chromogen combination is critical because visual contrast is the key requirement [154].

mIHC works best for the visualization of different tissue elements without colocalization. However, visualizing colocalized elements in a single cellular compartment, although challenging, is possible. Light shades overshadowed by darker shades or heavier dyes (e.g., DAB) can obstruct the visualization of colocalized or cellularly labelled antigens [156]. Thus, for visual assessment, careful selection of chromogens is required. In the history of IHC, many different chromogen combinations have been proposed for multiplexing. However, only a few have proven suitable for manual observation of mixed color at the sites of colocalization using bright-filed microscopy [157]. In recent years, the development of new and improved chromogen- and fluorescent-based detection systems have significantly expanded the application of immunolabelling. For instance, translucent chromogens, in conjunction with other chromogens, allows for detection of signal colocalization, via formation of a tertiary color. In **study I** and **III**, yellow was used as a landmark marker to stain the epithelial compartment without interfering with other chromogens. In **study III**, in order to visual assessment of colocalized signals, yellow in combination with teal created tertiary green color, which was easily distinguishable even with unaided eyes. Notably, the majority of previous studies based on chromogenic-IHC was performed using DAB as chromogen. In our setting, DAB in combination with any other chromogens (e.g., purple, teal, yellow, green, blue or red), was not a reliable choice for assessing colocalized markers, because the dominant brown color significantly obstructed the other dyes.

However, similar to the necessity of technical adjustments (on dilution, temperature, antigen unmasking) in IHC protocols, chromogens have their own sensitivity and efficiency characteristics, which needs to be tuned rigorously (e.g., incubation time and temperature).

3.5.3 Antibodies

Antibodies have a broad range of applications in research, diagnostic and therapeutics. Two classes of antibodies are available: polyclonal and monoclonal. Polyclonal antibodies are produced from repeated immunization of various species (e.g., mouse, goat, donkey, sheep, chicken and so on). They are a heterogenous mixture of antibodies against various isoforms of

the target proteins. Compared to monoclonal antibodies, they have higher affinity, but lower specificity. Polyclonal antibodies in general suffer from a lack of reproducibility due to batch-to-batch inconsistency. Monoclonal antibodies derive mainly from rabbit and mouse. Compared to polyclonal antibodies, they exhibit higher specificity and homogenous affinity to antigen. Hence, cross reactivity with other antigens will be significantly reduced with monoclonal antibodies. However, in the case of close similarity in shape or in amino acid sequence of the targeted peptides, cross reactivity may occur [158]. In addition, monoclonal antibodies are relatively complicated and expensive to produce and vulnerable to epitope-loss due to unmasking or fixation treatments. This issue can be offset by pooling two or more monoclonal antibodies to the same antigen [159]. Overall, monoclonal antibodies have proved effective reagents in terms of specificity for routine diagnostic practice.

Successful IHC assays are highly dependent on the selection of proper antibodies. An important principle for validation is antibody reproducibility. Almost all the primary antibodies used in this thesis are commercially available and FDA approved for *in vitro* diagnostic (IVD) assays. The only exceptions were the antibodies for CD66b and CD204. However, these were extensively cited in the literature. **Table 3** is an overview of the applied primary antibodies. Even though IVD antibodies are highly reproducible and require minimal optimization in single IHC, there are some issues that needs to be considered in the mIHC setting such as: The antibodies should be 1) oriented in proper disposition within staining cycles, 2) paired with fitting chromogens with regards to either cellular compartmentalization or disposition, and 3) validated in the target tissue and in controls.

Table 3: List of primary antibodies

Antibody	supplier	Cat.no	Clone	Host	Reagent status
CD66b	BD.Bioscience	# 555723	G10F5	mouse	RUO
CD34	Roche	#790-2927	QBEnd/10	mouse	CE-IVD
Pan-keratin	Roche	# 760-2135	AE1/AE3/PCK26	mouse	CE-IVD
CD68	Roche	#790-2931	KP-1	mouse	CE-IVD
CD163	Roche	# 760-4437	MRQ-26	mouse	CE-IVD
HLA-DR	Dako	M074601-2	TAL.1B5	mouse	CE-IVD
CD204	TransGenic	#KT022	SRA-E5	mouse	RUO

Abbreviations: CE-IVD, European conformity *in vitro* diagnosis; RUO, research use only

3.5.4 Staining controls

Multiple immunolabeling requires stringent controls and careful combination of enzymes and chromogens to achieve the best color discrimination (contrast) of the IHC reaction. Although the primary antibodies applied in this thesis have been extensively validated by vendors, it is always recommended to control the specificity and sensitivity of antibodies using both positive and negative controls [159].

Tissue controls: In each staining run, a TMA slide containing multiple samples of normal and tumor tissue was included to verify the quality of staining across different tissue types. The multi-TMA control involved selections of normal (skin, breast, liver, pancreas, colon, tonsil, ventricle, kidney, prostate, lung and brain) and malignant tissues (melanoma, basal cell carcinoma, ductal/lobular carcinoma, hepatocellular carcinoma, colon/prostate ADC, sarcoma, lung ADC/SCC, glioma and glioblastoma). The TMA control is carried out the same way as test samples. In **study I** and **III**, the key parameters in the characterizing of our antibodies was whether the antibody captures its cognate cellular antigen. In **study I**, the neutrophils were effortlessly identified in tissue controls, due to their multilobulated nucleus characteristic. In contrast, macrophages exhibit complex morphology and have the additional drawback of false positive staining with many antibodies. For this reason, macrophages were labelled with more than one marker. The documentation of the exact cellular and subcellular locations for immunoreactivity of macrophage markers is listed in **paper III**, **Table S3**.

Processing controls: Assessing the expression of vimentin (positive in endothelial and mesenchymal cells) is recommended as a measure of internal quality control in immunoreactivity [160]. The level of its expression may verify the quality of antigen preservation and the uniformity of tissue fixation in FFPE samples. The vimentin expression in stroma was evaluated in our initial cohort (with 335 patients). In addition, both for the purpose of validation and categorization (stroma *vs* tumor), pan-keratin was integrated in the mIHC protocol (**paper I** and **III**). Homogenous epithelial staining intensity was observed for pan-CK in almost all TMA sides.

Negative controls: Omitting the primary antibody is a conventional negative control for the secondary antibody and detection kits. The alternative negative control is utilizing isotype

match control, which is same class and type of immunoglobulins from which the primary antibody is derived. With this tool it is possible to distinguish if there is unspecific binding to tissue depending on the Fc domain of the immunoglobulin, and not on the idiotype [161]. In each mIHC sequence, both methods (isotype and no-antibody) were tested to check for cross-reactivity between the secondary antibody and the detection reagents as well as with other sequences.

3.6 Histological assessment

3.6.1 H&E slides

In **study II**, TILs were defined as lymphocyte and/or plasma cell infiltration and assessed in whole tissue H&E slides by manual light microscopy. A team of experienced pulmonary pathologist designed a scoring scheme based on the recommendations for TIL scoring in breast cancer by Salgado *et al.* [92]. TILs were scored as the percentage of tumor stroma containing mononuclear immune cells using a four-tiered ordinal scale: 0=0-5 %, 1=6-25%, 2=26-50% and 3>50%. The scores from multiple areas (at least five high power fields) were averaged for the final count. The definition of the tumor-associated stromal region was crucial. For clarification, **Figure 6** exemplifies the definition of tumor-associated stroma in H&E slides. Interestingly, different stromal configurations were observed depending on the level of immune infiltration (low to high). In tissues with low TIL levels, stroma appeared fibroblastic without the presence of TLSs, while tissues with high TIL levels were rich in the number of TLSs. In breast cancer, this latter group of patients (>50% of stroma occupied with TILs) are called lymphocyte-predominant breast cancer (LPBC) [91,92].

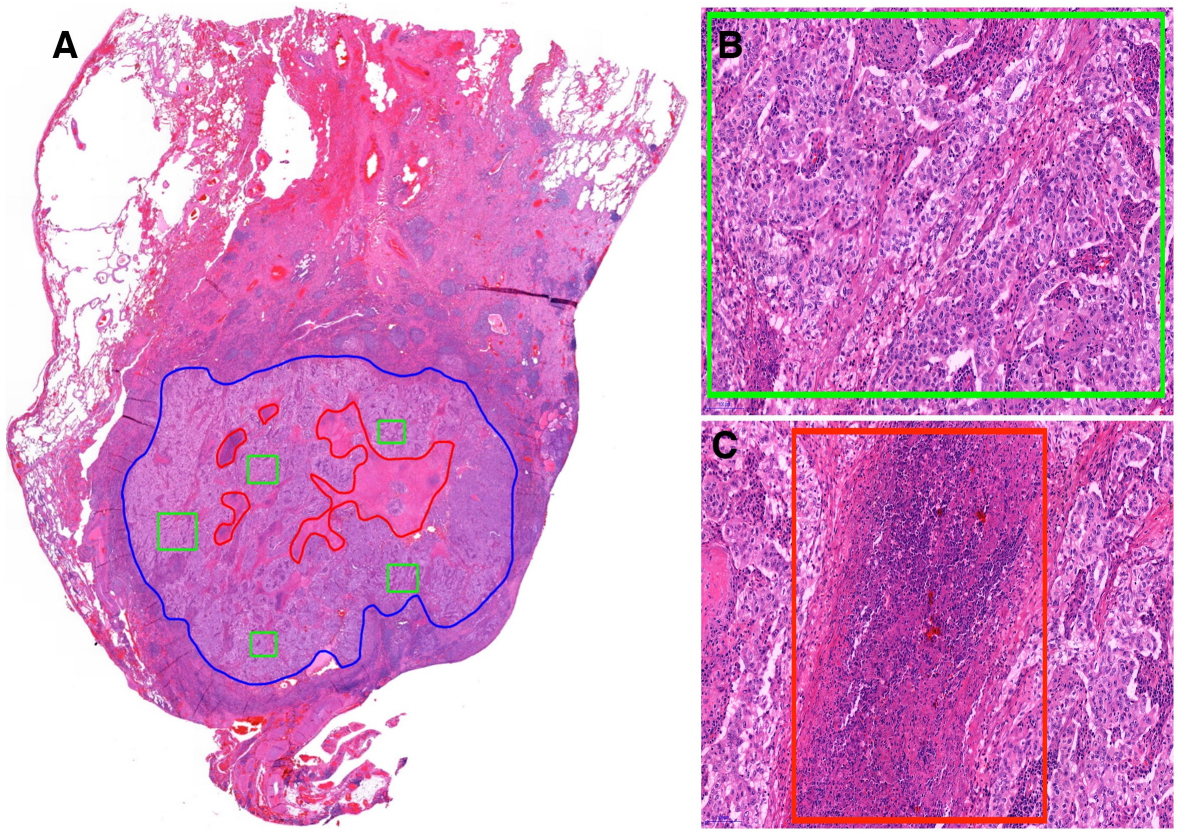

Figure 6: The stromal areas included for TILs assessment.
A) Tumor border were marked with blue color and the stromal areas (green rectangular) within this region were considered for scoring (at least 5 HPF). The normal area outside the tumor and the necrotic areas (red marked) were excluded (magnification x5); B) x20 magnification of stroma area; C) x20 magnification of necrotic area within the tumor cores.

In order to maximize the reproducibility of the scoring model and minimize the inter-observer variations, the following parameters were not included in the scoring scheme: granulocytes of any type, TLSs, necrotic areas, tumor borders (invasive margins) and normal alveolar areas. **Table 4** is a side-by-side comparison between the original scoring criteria for TILs in breast cancer and the adjustments made for scoring TILs in NSCLC, showing the degree of adherence to the original guideline.

Table 4: Comparing TILs assessment guideline in breast cancer and the criteria executed in study II.	
Original guideline	**Adopted criteria in study II**
TILs should be reported for the stromal compartment and in percentage (=% stromal TILs).	Accordingly, TILs were scored in stromal compartment.
TILs should be evaluated within the borders of the invasive tumor.	Inter-tumoral stroma included in our assessment (see details in **Figure 6**).
Exclude TILs outside of the tumor border and around DCIS and normal lobules.	Normal alveolar area was excluded.
Exclude TILs in tumor zones with crush artifacts, necrosis, regressive hyalinization as well as in the previous core biopsy site.	Necrotic area and regions having crush artifact were excluded.
All mononuclear cells, including lymphocytes and plasma cells, should be scored, but PMN leukocytes are excluded.	Lymphocytes and plasma cells considered as TILs and tumor-associated granulocytes were excluded.
One section (4–5 µm, magnification ×200–400) per patient is currently considered to be sufficient.	1-2 section per patient were used for analysis with x200 magnification, and if needed x400.
A full assessment of average TILs in the tumor area by the pathologist should be used. Do not focus on hotspots.	Average score of at least five area were used as final score.
Scoring scale according to original methodology firstly described by Denkert et al. [91] Continuous per 10%.	A four-tiered scale designed by four experienced lung cancer pathologists: 0=0-5 %, 1=6-25%, 2=26-50% and 3>50%.
No formal recommendation for a clinically relevant TIL threshold(s) was given. LPBC can be used as a descriptive term for tumors that contain more lymphocytes than tumor cells. However, the thresholds vary between 50% and 60% stromal lymphocytes.	Multi-level cutoff was used.
Additional parameters, including TLS in the peritumoral region, TILs at the invasive edge or intratumoral TILs can still be included for research purposes to further determine and/or confirm their potential clinical relevance.	TLS, intraepithelial and invasive margin TILs were not included in our assessment.
Abbreviations: TILs, tumor-infiltrated lymphocytes; LPBC, lymphocyte-predominant breast cancer; TLS, tertiary lymphoid structures. Partly reproduced with permission from Oxford University Press [92].	

3.6.2 IHC analysis

In **study I** and **III**, a semiquantitative scoring model was applied for visual assessment of the neutrophil and macrophage infiltration in different compartments. In primary tumors, intratumoral and stromal areas were scored separately, while in the LN+ cohort only intratumoral areas were scored. In LN+, the reason for exclusion of stroma was: 1) high abundance of immune cells, 2) difficulty to differentiate between normal- and tumor-associated stroma regions.

In stroma, the overall percentage of positive neutrophils or macrophages among nucleated cells were scored as follows: 0 (0–5%), 1 (6–25%), 2 (26–50%) and 3 (>50%). In the intratumoral compartments (**paper I**), due to the lower presence of target cells in tumor compared to stroma, the following four-tiered scale was applied: 0 ≤ 1, 1 = 1-5, 2 = 6-15, 3 >15. Notably, macrophages were less prone to infiltrate into the tumor area. Hence a three-tiered scale was applied: 0 (no cells), 1 (1–5), 2 (≥6). LN+ cohorts were scored similar to the intratumoral scoring models.

Even though manual scoring is practical, it is based on subjective visual perception. The semi-quantitative nature and low reproducibility of manual scoring is often criticized. To minimize the subjectivity of our assessments, the following available options were adhered to: excluding areas with high risk of error (false positive/negative count), and analyzing the inter-observer variability.

Excluded areas: For both neutrophils and macrophages, necrotic and pre-necrotic areas presented highly infiltrated zones and were ignored to avoid false positive counts. Moreover, areas where neutrophils resided together with their released granules (due to neutrophil autolysis), and areas with neutrophil aggregates (**Figure 7**) were ignored.

In addition, it is known that the systemic inflammatory response caused by cancer is associated with alterations in circulating leukocytes, specifically with signs of neutrophilia and lymphocytopenia in advanced NSCLC [162,163]. With this understanding and in order to avoid the potential bias of circulating/intravascular neutrophils, neutrophils clearly located within vessels were excluded from scoring.

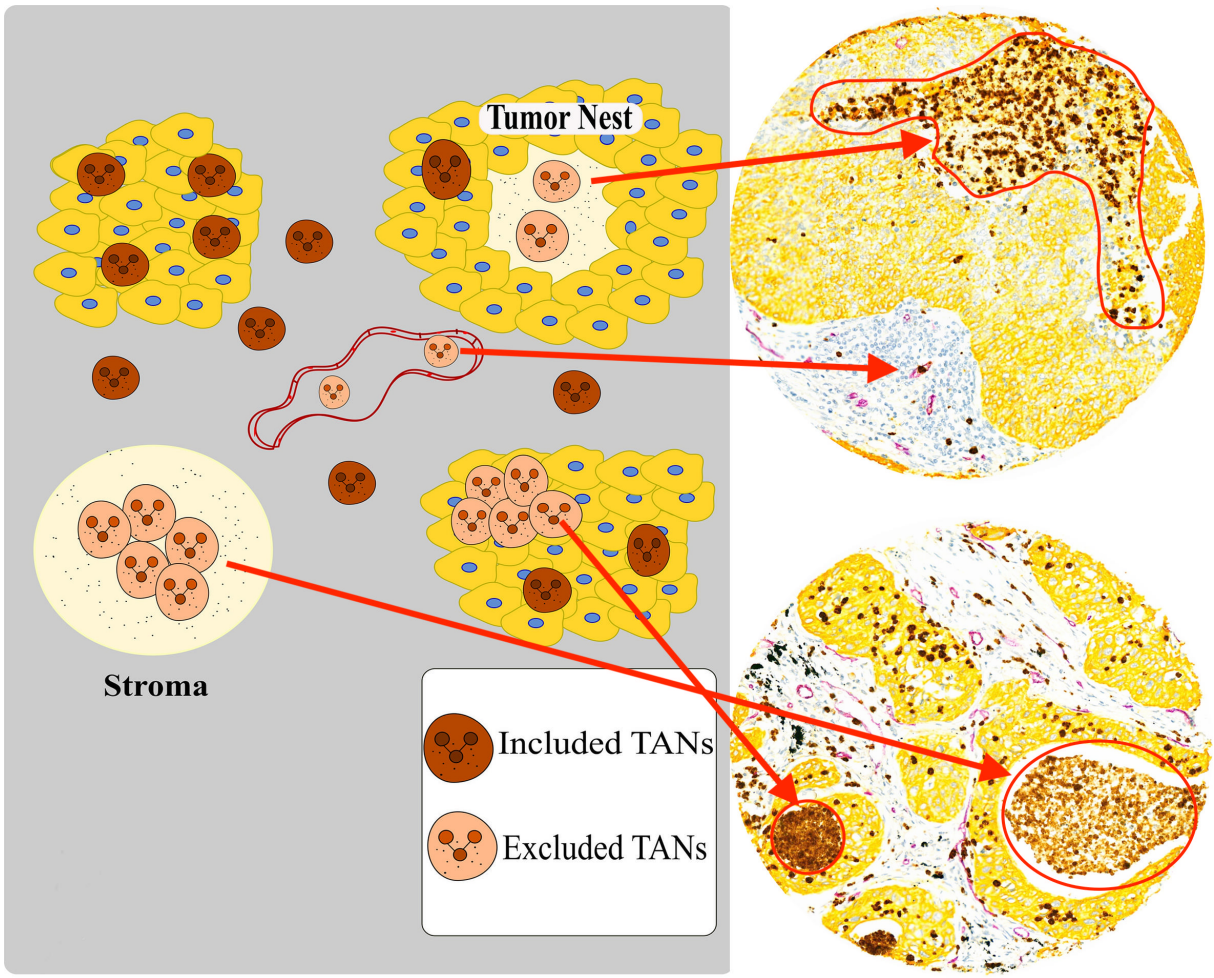

Figure 7: Excluded areas for scoring in **study I**.
Representative CD66b (brown)+CD34 (purple)+pan-CK (yellow) mIHC stained TMA slides scanned by a pathology digital imaging system (Pannoramic P250 Flash III whole-slide scanner; 3DHistech, Hungary). Red arrows from the schematic model (adopted from **paper I** with modification) indicating the areas excluded in scoring.

Inter-observer variability: Two observers, blinded to patient clinical data, scored all stained slides. In general, there was a strong agreement between scorers with regard to inter-observer correlation coefficient. Interestingly, high interobserver kappa values were observed in **study I** and **III** wherein mIHC was implemented for the purpose of accurate differentiation of positive cells. This may indicate that the technical approaches chosen for these studies, led to reduced interobserver variation. In the manual scoring setting for various markers, it is common to carry out reassessments if there is a two-score disagreement or more between the observers. In such a case the slides go for reassessment to reach a consensus. In **study I** and **III**, there was almost no disagreement between observers further highlighting the advantage of mIHC in manual scoring.

Recently, digital image analysis has started to compensate for the variability of human perception. Current imaging technologies are capable of measuring characteristics that are extensively beyond the reach of human visual subjective observations. In addition, the information that could be uncovered through detection of multiple biomarkers by mIHC, is critically important in developing a fully quantitative model to evaluate IHC.

3.7 Cutoff identification

Biomarkers are direct or indirect measures of biological processes. Even though biological processes are inherently continuous, it is often necessary to use a cut-off value to apply a biomarker in prognostication or treatment-decision-making. The cut-off value will typically stratify the patients into two or more groups based on a risk-profile. The alternative would be to use a continuous scale as this would be a true reflection of the biological process. Although a tempting approach, the shear complexity of using continuous scales and the number of patients needed to conduct meaningful trials is for the moment precluding its use. In the field of biomarker discovery, no standard tool (similar to Allerd or IR score) to convert a metric or ordinal variable into a discrete variable exist. Thus, the chosen cut-off(s) are likely to be error-prone.

The number and values of the cut-off may be based on previous results (confirmatory studies), clinical or physiological data, mean or median values, tertiles, quartiles or quintiles, greatest separation of groups (lowest P-value) or other approaches. All these approaches have limitations related to risk of type I and II errors. Provided an exploratory design, it is up to the investigator to choose the cut-off strategy. In some of the studies included in this thesis (**paper I** and **III**) mean cut-offs were used. Mean cut-offs are resistant to type I errors, but may be prone to type II errors if the effect of the biomarker is only evident in either strong positive or negative cases. In addition, a mean cut-off may be easier to reproduce compared to for instance an optimal cutoff. In **study III** (for CD68), an optimal cut-off strategy was applied. Although, caution is advised when using optimal cut-offs [164], this strategy is commonly used in explorative studies [165,166]. Optimal cut-off strategies increase the chance of type I errors and reduces the chance of type II errors. Subgroup analyses, for which many explorative studies are underpowered, should be considered with caution as the chance of type I errors increases as number of patients in each group dwindles. In addition,

reproducibility might prove to be an issue, especially when optimal cut-offs are applied to semi-quantitative scores.

Of the studies included in this thesis, **study II** may have the most robust form of cut-off as the original scores is used without further modification. This scoring model seems to be easily reproducible and reliable for potential translation into the clinic

3.8 Statistical analysis

All statistical analyses were performed using two programs: IBM SPSS (v23-25) and Rstudio (v3.2.2; packages: survival, gridExtra, car, Hmisc, irr and ggplot2). The association of TAN, TIL and TAM levels and different clinicopathological parameters was evaluated using chi square and Fisher's exact tests. Interobserver reliability was calculated using a two-way random-effects model with an absolute agreement definition and Cohen's kappa coefficient with equal weighting. A non-parametric test (Mann–Whitney U) was used since the patients were not normally distributed across pathological stages (**paper III**). Spearman correlation coefficient was used to examine the associations between protein/protein expressions and when needed Bonferroni correction was applied. Kaplan-Meier estimates were plotted for DSS, OS and DFS. Log rank test was used to test the statistical significance of the difference between low/high and stepwise increased groups. A multivariable cox model, with estimated hazard ratios (HR) and 95% confidence interval (CI), was used to model associations between survival and known prognostic variables (age, pathological stage, ECOG score, gender, vascular invasion and differentiation). Stepwise backward conditional selection using 0.10 and 0.05 as entry-and exit-points was used to select variables for the final models. Two-tailed probability values <0.05 were considered to be statistically significant.

4 Main results

4.1 Paper I

The clinical impact of tissue-based neutrophils is still unclear in NSCLC. Using mIHC, CD66b+ TANs were evaluated in primary tumors and paired metastatic lymph nodes. The positive cells were manually scored in the tumor and stroma compartments, based on criteria described in **Section 3.6.2**. In short, pre-/necrotic areas and intravascular neutrophils were ignored. CD66b showed membranous and cytoplasmic localization on neutrophils.

Correlation: In the overall cohort, intratumoral TANs were positively associated with increasing tumor stage ($P = 0.011$) and pathological stage IIB ($P = 0.002$). In SCC, TANs were negatively associated with nodal stage ($P = 0.032$). In the overall cohort and SCC subgroup, stromal TANs were associated with weight loss ($P = 0.044$, $P = 0.031$; respectively). No associations between clinicopathological variables and TANs were detected in the ADC or LN+ groups. Correlation analyses were performed between TANs and 104 tumor molecular markers previously evaluated in this cohort. Concisely, there was a significant correlation between TANs and innate immune-related markers including: CD68, CSF1R and MCSF.

Survival analysis: For stromal TANs, no significant impact on patient outcome was observed in the overall cohort and histology subgroups. For intratumoral TANs, when stratified according histology, high level in the SCC subgroup was an independent positive prognosticator for DSS (univariate $P = 0.038$, multivariable $P = 0.021$, HR 0.59, CI 0.38-0.92). In contrast, we found a significantly shorter DSS for primary tumors with high level of TANs in the ADC group (univariate $P = 0.032$; multivariable $P = 0.020$, HR 1.7, CI 1.1-2.65). Consistently, in LN+ ADC histology, high level of intratumoral TANs was associated with poor prognosis (univariate $P = 0.003$; multivariable $P = 0.004$, HR 2.87, CI 1.39-5.91). In primary tumors when DFS and OS were explored, similar effects were observed.

4.2 Paper II

Normally, TILs are ignored during the routine diagnostic process on H&E slides of resected tumors. However, accumulating data show that *in situ* TILs, even in routine H&E slides, can provide pivotal prognostic and predictive information. Currently, there is a guideline available for evaluation of TILs in H&E slide for breast cancer. Hence, we aimed to validate the compatibility of this guideline´s criteria for use on NSCLC whole tissue H&E slides. The following areas and cells were excluded from assessment: Intraepithelial TILs, tumor-associated granulocytes, TLSs, invasive margins, normal and necrotic regions.

Correlation: In the overall cohort, higher level of TILs was statistically associated with a favorable ECOG performance status.

Survival analysis: In the overall cohort, high TIL levels was associated with better outcome for DSS, DFS and OS (P=0.008, P=0.006 and P=0.036 respectively). In the SCC subgroup, high TIL levels was a positive prognostic factor for DSS (P=0.047) and a positive trend was observed for OS and DFS (P=0.058, P=0.054). In the ADC subgroup, high TIL levels conveyed no survival benefits for any clinical endpoints. Multivariable models adjusted for known prognostic factors including pathological stage, histological differentiation, vascular invasion, gender and age were conducted. Elevated TIL levels were independent positive predictors of OS (P=0.006, HR 0.51, CI 0.32-0.82,), DSS (P<0.001, HR 0.3, CI 0.15-0.6) and DFS (P<0.001, HR 0.34, CI 0.19-0.64). When patients were stratified according to TNM stage, TIL levels was a nearly significant positive prognostic indicator in stage II (DSS, P=0.057) and III (DSS, P=0.082) and a notable positive trend was detected in stage I (DSS, P=0.51).

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4.3 Paper III

The significance of TAMs in NSCLC has been the subject of conflicting reports. The present study was designed to set up a reliable IHC-based method in order to phenotype, and to evaluate the clinical significance of TAMs in NSCLC. As TAMs are thought to differentiate mainly into two anti-tumoral M1 and pro-tumoral M2 subsets, the study aimed to phenotype TAMs using mIHC with the following marker combinations: HLA-DR/CD68 (M1), CD163/CD68(M2), CD204/CD68(M2) and CD68/CK (pan-TAM).

Correlation: Stromal M1 was associated with T-stage and ECOG status and $CD204^+$M2 was associated with patient age. In the intratumoral compartment (both for primary tumors and in the LN+ cohort), M1 was associated to ECOG status. Correlation analysis was performed between TAMs and innate/adaptive immune-associated markers, previously evaluated in this cohort. In both stromal and intratumoral compartments, moderate to strong correlations were observed between TAMs (both M1 and M2) and CD3, CD8, CD4 and CD45RO positive immune cells. When TAMs distribution was assessed across TNM stages, levels of stromal $CD163^+$M2, $CD204^+$M2, and CD68 infiltration did not differ significantly, but notably declined for M1 from pathological stage I to III.

Survival analysis: In multivariable models, stromal M1 (HR 0.73; CI 0.5-0.97; P=0.03), $CD204^+$M2 (HR 0.7; CI 0.5-0.94; P=0.02) and CD68 (HR 0.69; CI 0.5-0.94; P=0.02) and intratumoral M1 (HR 0.7; CI 0.5-0.99; P=0.04), $CD204^+$M2 (HR 0.6; CI 0.4-0.8; P=0.004) and CD68 (HR 0.73; CI 0.5-0.99; P=0.04) were independent favorable prognostic indicators of increased DSS. In the LN+ cohort, high intratumoral M1 level was an independent favorable indicator of DSS (HR 0.38; CI 0.2-0.7; P=0.001).
When stratified according to histology, high levels of stromal $CD163^+$M2 (P <0.001) and $CD204^+$M2 (P= 0.005) and both stromal and intratumoral M1 (P <0.001, P=0.016) subsets, were associated with increased DSS in the SCC subgroup, while high levels of stromal CD68 TAMs was a predictor of increased DSS (P= 0.039) in the ADC subgroup.

5 Discussion

5.1 Tumor-associated neutrophils

As seen in **paper I**, our results show that intratumoral TANs have diverging prognostic impact in the ADC and SCC subgroups of NSCLC. This may explain why no prognostic impact was observed in the overall cohort.

A recent meta-analysis showed that high *in situ* neutrophil infiltration is associated with unfavorable outcome in solid tumors [167], however the role of neutrophils in NSCLC is still unclear. Overall, the presence of neutrophils are associated with unfavorable outcome in bronchioloalveolar, esophagus, renal cell, and head & neck cancers [106,109–111], but with a favorable outcome in colorectal and gastric malignancies [112,113]. In a previous study in NSCLC, the ratio of $CD66b^+$ TAN/$CD8^+$ T-cells was associated with increased risk of progression and shorter OS [115], while another study found no association between neutrophils and clinical outcome [168].

The data on the biological activity of TANs are majorly derived from experimental model studies, rather than studies in human. The findings from animal models underscore the influence of the TGF-β pathways on TAN recruitment and activation of CD8+ TILs [169]. For instance, in a mouse model study, they found that TGF- β signaling is pivotal to differentiate PMN subset of MSDCs, and to induce a distinct N2 TAN phenotype with tumor-promoting properties. Also, abrogation of TGF- β signaling differentiated TANs from a N2 to N1 phenotype with anti-tumoral effect. Further, N1 subset depletion reduced $CD8^+$ T-cells function and promoted tumor growth [101]. In fact, N1 TAN mediated $CD8^+$ T cell activation, is postulated as the main mechanism for tumor-inhibition responses exerted by these cells. Despite the many differences between tumor-bearing mice and humans, the same may apply to tumors in humans. Macrophages and neutrophils derive from the same progenitor, thus the complexity of macrophage differentiation in humans is also likely for neutrophils [170]. In this study, we observed strong associations between TANs and $CD68^+$ TAMs, and expression of CSF1R and MCSF.

Human TANs may distinctively influence tumor immunity depending on tumor stage and histology. In early stage NSCLC, TANs are not mainly immunosuppressive; instead they

stimulate T cell-mediated immune reaction by producing co-stimulatory molecules that increase the proliferation of $CD4^+$ and $CD8^+$ TILs [171,172]. The mechanisms by which human TANs influence immunity is poorly understood. Moreover, it is unclear whether *in vitro* data from murine studies, supporting the notion of N1 /N2 TANs, can be applied to humans. TANs are influenced by a wide range of signaling molecules that differ between and within different stages of the same histological subtype. Thus, the prognostic significance of TANs may vary, not only according to histological subtype, but also due other tumor intrinsic properties such as oxygenation, nutrient supply, etc. Adding complexity, the predominant NSCLC histological phenotypes (SCC vs ADC) are often regarded as different entities, with distinct genomic and morphological profiles, growth patterns and sensitivity to treatment [173].

5.2 Tumor-infiltrating lymphocytes

In this study, the guideline for assessing TILs using standard H&E slides in breast cancer were validated for use in NSCLC. The results show that increasing levels of stromal TILs is a strong independent prognostic factor for DSS, OS and DFS in NSCLC patients. While others have explored overall TILs on H&E in lung cancer before, this is the first large study to incorporate well-defined scoring methods for reliable evaluation of TILs in NSCLC patients using routine H&E slides.

The prognostic significance of TILs in primary NSCLC has been investigated in numerous studies [174]. However, only a few of these studies utilized routine H&E staining (rather than IHC) to assess lymphocyte infiltration [81,94–98]. High TIL density was associated with increased survival in three of the studies on pathological stage I [96,97] or III [98]. A large Italian-cohort study (including 1290 patients) found survival benefit of high TILs infiltration in the SCC histology (549 patients), while no survival-association observed in their whole cohort [95]. In a large TMA-based study, high level of CD8+ TILs was correlated with improved survival, while TILs evaluated with H&E did not show a significant difference [81]. In the current study, we found an association between stromal TILs and all clinical endpoints in the whole cohort, consistent with the findings of another recent large NSCLC study by Brambilla and coworkers [94]. In addition, TILs in the SCC patient subgroup, were found to be significantly associated with a better prognosis.

There is strong support for the prognostic significance of TIL in NSCLC, but there is no standardized method for assessing TILs in whole tissue H&E slides. To design a consistent approach to assess TILs in this setting (see **Section 3.6.1**), three histological components were excluded:

1. *Intratumoral TILs*. Intratumoral TILs have prognostic and predictive associations in breast cancer patients [91], but in the setting of NSCLC it was difficult to differentiate between tissue lymphocytes and apoptotic tumor epithelial cells. For this reason, intraepithelial TILs were considered unscorable. A recent study observed low interobserver agreement among scorers, and poor reproducibility for quantifying TILs in the intraepithelial compartments [175].

2. *TILs within the invasive margins*. TILs within the invasive margins in CRC have an important prognostic role [176,177]. One study on liver metastases of CRC found that a high CD8+ T-cell density in invasive margins was associated with improved prognosis and response to chemotherapy; the investigators defined the invasive margin as an area of 500 μm on each side of the edges between tumor epithelium cells and normal tissue [177]. However, due to the highly unstable growth patterns of some NSCLCs, the invasive margin was not considered in order to increase the reproducibility of the results.

3. *Tertiary lymphoid structure (TLS)*: TLSs may have a critical antitumoral role by inducing systemic and local T-cell immune responses and are observed in many solid tumors, including lung cancer [178]. In NSCLC, the density of TLS-localized mature dendritic cells has been associated with increased survival [83]. TLS have similar structures to lymph nodes, comprising T-cell-DC clusters, follicular B cells, and high endothelial venules. In the original guideline, TLS were recommended to be exclude or evaluated as a separate research parameter (**Table 4** in **Section 3.6.1**).

Subgroup analyses according to pathological stages revealed high density of TILs to be near-significant prognostic factor for patients with stage II and III. A trend for this relationship was also noted in stage I tumors. Two previous NSCLC studies found that high TIL density is also associated with improved overall survival in stage I disease [95,96]. We found no statistically significant prognostic impact in stage I patients, which is consistent with previous reports

on other early stage cancers involving esophageal, colorectal and breast [179–181]. TIL levels may therefore have a stronger prognostic role in NSCLC with more aggressive phenotypes. These results should be interpreted with caution. Further studies powered for subgroup analyses would be valuable, especially in patients with stage I NSCLC.

Although rational, the present study is the first to demonstrate that a stepwise increase of TIL levels in tumor stroma leads to a stepwise increase in patient prognosis. Feng *et al.* [98] did not detect any prognostic potential of TILs using a four-step method. The detailed scoring system proposed in this study should be easy to use and will likely reduce inter-observer variation and enhance detection of the prognostic potential of TILs in NSCLC. Even though the assessment of TILs would be simple, inexpensive, and easy to introduce into routine practice, it is still a semi-quantitative measurement and may be further refined using digital cell-counting systems.

5.3 Tumor-associated macrophages

Many studies have evaluated the prognostic potential of TAMs in NSCLC, but the significance is debatable and would also benefit from further investigation. To the best of our knowledge, this is the first large-scale study to investigate the prognostic significance of *in situ* TAMs in NSCLC. Independent positive associations between high levels of HLA-DR$^+$M1, CD204$^+$M2 and pan-CD68$^+$ TAMs and DSS, were found in both tumor stroma and in the intratumoral compartments.

Traditionally analyses of TAMs have been based on CD68 expression alone [182]. In a previous study of 335 patients from our group, using single-color IHC a positive trend between high CD68$^+$TAM levels in both stromal and intratumoral compartments was noted [183]. The current study used a larger number of patients and co-stained samples with pan-CK. In multivariable analyses, we found a statistically significant relation between high levels of CD68$^+$ TAMs and a favorable prognosis. **Table S7** (**paper III**) summarizes previous studies on the prognostic impact of TAMs in NSCLC. Two of these studies, by Kim [184] and Eerola *et al.* [185] also showed improved outcomes associated with high intratumoral densities of CD68$^+$TAMs. However, some studies found negative [186–188], none [189–191] or diverging [192,193] associations. This inconsistency may relate to methodologic difference

and CD68 antibody specificity. As interpretation of IHC stains varies considerably, the reproducibility of CD68 scoring is also variable. Some of this variability may relate to expression of the marker in tumor cells and other immune cell infiltrates [194]. We found CD68 positivity in the tumor cells of 23% of our patient sample.

Non-specific staining may overestimate TAM density, but the use of pan-CK to differentiate between epithelial and non-epithelial cells improves accuracy when detecting intratumoral $CD68^{+}$TAMs. Some studies use digital analysis to quantify TAMs [114,195]. For the detection of TAMs, digital analyses may be biased more compared to using visual microscopy due to the wide variation in the size of macrophages (5–30 μm) in the lung tissue [196]. In the future, detection of TAMs using digital pathology will likely rely on a combination of artificial intelligence or computer vision, that depend on huge annotated datasets of TAM morphology and antibody panels designed especially for this purpose.

Currently there is no consensus on the most accurate methods for identifying and differentiating tissue-based macrophage subsets in solid tumors. Recent studies use multiple antibodies to identify macrophages and to characterize TAM subsets [197]. The most common markers for M2 identification when co-staining with CD68, or using a single IHC assay, are CD163, CD204 and CD206 [198]. For identifying the M1 subset, the best choice of antibodies is undecided. Some studies used HLA-DR [195,199–201], but this is expressed on the membranes of antigen-presenting cells, including macrophages, monocytes, dendritic cells, B cells, activated T cells [202] and tumor cells [203]. In NSCLC, only two studies used mIHC to analyze TAM subsets. The others used single-IHC against M2 antigens (e.g., CD204 or CD163) (see **Table S7** in **Paper III**). Intratumoral subpopulations, including M1-like and M2-like TAMs, were found to predict superior outcomes in NSCLC patients [199]. We also found a survival benefit in relation to high M1 or M2 phenotype levels in both tumor islets and stroma. In one study, only the intratumoral M1 subset (not M2) was found to have independent prognostic significance [195]. However, investigators in both of these studies [195,199] were unable to identify a statistically significant association between stromal TAM subsets and survival.

There is a higher proportion of immune cells in tumor stroma than in intratumoral tissue, where some subsets of immune cells are positive for the markers studied here, together with TAMs. Additionally, IHC-based analysis of TAM subsets in stroma requires a reliable

method to account for co-localized macrophage markers. For this reason, we carried out several experiments to characterize the macrophage subsets. Due to the challenges of mIHC, (discussed in **Section 3.5.2**), we tested different chromogens and enzymatic reactions to determine the most appropriate color combination for visual assessment of co-localized areas. Using translucent chromogens enabled us to reliably label co-localized antigens of interest on TAMs. We found that the commonly used DAB/red dual-chromogen set was unreliable because of the dominant brown color.

We had two novel findings in the study. First, the level of intratumoral M1 subset in metastatic lymph nodes was found to be an independent positive predictor of prognosis. This is in line with its prognostic role in primary tumors. There was no significant correlation between TAM subsets in lymph nodes and those in primary tumor tissue, possibly because of the heterogeneity of macrophages in the tissues [204]. Second, stromal infiltration of M1 significantly dropped from stage I to stage III. This is in support of the finding that macrophage phenotypes change from proinflammatory to immunosuppressive states during the disease course [205]. It also supports the findings of an animal study of advanced stage hepatocellular carcinoma in which a high M1-like phenotype found in the early stage changed to a low M1-like phenotype [206]. Some of the complexity of macrophage expression relates to this temporal plasticity during tumor development.

From a biological perspective, M1 and M2 macrophage subsets are expected to associate inversely with tumor-inhibiting or tumor-promoting effects, respectively. However, studies on NSCLC, CRC and gastric carcinomas (including the current study) observed that infiltration of both M1 and M2 subtypes were positively associated with clinical outcome [199,207,208]. Different inferences can be made regarding the survival benefits of M2 TAM infiltration. Further research might reveal mutual interactions between M1 and M2 TAMs in NSCLC [199]. In CRC, the M1 antitumoral activity may dominate over the M2 protumoral activity since the two subtypes co-exist, thus leading to improved outcomes [207]. Further, in the unique intestinal environment that comprises various microorganisms, macrophages may require this functional alteration to maintain gut-tissue homeostasis [207]. The prognostic influence of TAMs may relate to lymphocytic infiltration; this is based on observations of high levels of both TILs and $CD163^{+}$M2 in gastric cancer [208]. In our study, the moderate to strong correlation between M1 and M2 with lymphocytic infiltration of CD3, CD8 and CD4 cells implies that both phenotypes are involved in effective recruitment of lymphocytes,

operating with T-helper and cytotoxic cells to induce an antitumoral response [124]. Interestingly, a recent study found a close relation between the quantity of $CD206^+$ M2-like TAMs and "bystander" $CD8^+$ TILs in lung tumor stroma of TAM-depleted mice [209]. They also found that TAMs have prolonged interactions with $CD8^+$ TILs in the stroma, limiting their entry into cancer islets and thus interrupting their antitumoral activity [209].

Taken together, the distribution of macrophage phenotypes clearly differs between different tissues and within specific tissues, in terms of polarization, disease stage and environmental signals. This degree of macrophage plasticity limits understanding of the role of M1 and M2 subtypes in the distinct protumoral and antitumoral activities of tumors. The existing nomenclature based on macrophage function probably has little relevance in the complex microenvironment of tumors [122,210].

6 Concluding remarks and outlook

Numerous studies have detected prognostic and predictive markers, both for cancer in general and for NSCLC in particular. However, only a few have entered clinical studies and even fewer to clinical practice. This is in stark contrast to the need of tools to select, or to spare, patients for an ever-increasing arsenal of treatment options. Immune cell infiltration to the malignant environment is a typical feature of many solid neoplasms. Over the past decade, an extensive body of evidence have demonstrated a fundamental interaction between inflammatory and cancer cells. In NSCLC, our group found that patients with high levels of CD8+T-lymphocytes in the malignant environment, exhibited better disease-specific and overall survival when compared to patients with low levels [79]. This work was followed by a recommendation to include IHC-based immune profiling in routine practice as NSCLC TNM-Immune cells score classifier [89]. The studies in this thesis were conducted to further the understanding of the immune contexture, and to search for novel immune markers in NSCLC.

Neutrophils, lymphocytes and monocytes are the most abundant subsets of leukocytes in blood and tumor tissues. While the positive prognostic impact of adaptive immune cells are widely investigated in most cancers, the prognostic impact of innate immune cells are suffering from discrepant reports in different cancers, including NSCLC. In paper I, we identified intratumoral neutrophils to be both an independent positive and negative predictor of prognosis in the SCC and ADC subgroup of NSCLC patients, respectively. However, we were not able to provide a definite answer why the prognostic influence diverged in these two main subgroups. When tested in pathological subgroups, we proposed the level of CD66b+ neutrophils as an appropriate candidate marker for a NSCLC-ADC TNM-I, along with other proposed markers such as CD8+ and CD45RO+ T-lymphocytes. However, these preliminary results require validation in larger cohort of NSCLC-ADC patients.

In paper III, a technical strategy for the assessment of colocalized markers in chromogenic IHC-based assays was described. Moreover, we demonstrated that the high infiltration of macrophages into the primary tumors of NSCLC patients (HLA-DR$^+$M1, CD204$^+$M2 and pan-CD68 macrophages) are independent determinants of better clinical outcome. In metastatic lymph nodes, high level of HLA-DR+M1 phenotype was an independent favorable prognosticator. We also observed there is a phenotypic shift from high to low levels of HLA-DR$^+$M1 macrophages during the development of disease across pathological stage I to III.

These results are in contrast to preclinical studies that supports the theory of distinct M1 anti-tumor and M2 pro-tumor macrophages. While the reason behind the positive survival-association of both M1 and M2 macrophages in NSCLC remains elusive, the significant correlation between these two phenotypes and adaptive immune cells clearly warrants further attention.

In paper II, we found that stromal TIL levels, evaluated in routine H&E slides, are predictors of favorable prognosis in NSCLC patients. This finding confirms a previous study by Brambilla *et al*. [94]. However, in contrast to Brambilla, we found that a four-tiered score provided prognostic information in a step-wise manner likely reflect the underlying biology. The study was conducted in accordance to the original guideline for TILs assessment in breast cancer with some minor adjustments for use in NSCLC. We concluded that H&E TILs is a promising candidate for a NSCLC TNM-I. H&E TILs are especially attractive because they are evaluated in the same H&E slides already used for histopathological reporting and thus is easily integrated in current clinical practice.

Multiple hurdles must be crossed prior to the execution of a novel prognostic or predictive marker in a clinical setting. In order to ensure marker performance, independent prospective validations, in different patient cohorts, are needed. Notably, our research team is conducting a prospective multi-cohort Scandinavian study (NCT03299478) in order to validate the prognostic and predictive benefit of a NSCLC TNM-I in resected samples. However, the majority of lung cancer patients are diagnosed in advanced stages. For most of these patients surgical resection is not an option and is likely to worsen an already bleak prognosis. Future research on the immune infiltration in lung cancer should emphasize its use on bioptic materials. In a clinical setting, successful assessment of immune status in biopsies may help select patients for immunotherapy, and could be applicable both for patients in an advanced and in a neoadjuvant setting.

In summary, this thesis provides a comprehensive evaluation of the major innate and adaptive immune cell populations (neutrophils, lymphocytes and macrophages) and their association with clinical outcome in NSCLC. The novel data on the prognostic value and distribution of these markers, will hopefully contribute to an enhanced understanding and aid in the identification of immunopanels for prognostication and therapeutic intervention in NSCLC.

7 References

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8 Appendix

8.1 Appendix 1

The 2015 WHO classification of lung tumors.
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TABLE 1. 2015 WHO Classification of Lung Tumors[a,b,c]

Histologic Type and Subtypes	ICDO Code
Epithelial tumors	
Adenocarcinoma	8140/3
Lepidic adenocarcinoma[e]	8250/3[d]
Acinar adenocarcinoma	8551/3[d]
Papillary adenocarcinoma	8260/3
Micropapillary adenocarcinoma[e]	8265/3
Solid adenocarcinoma	8230/3
Invasive mucinous adenocarcinoma[e]	8253/3[d]
Mixed invasive mucinous and nonmucinous adenocarcinoma	8254/3[d]
Colloid adenocarcinoma	8480/3
Fetal adenocarcinoma	8333/3
Enteric adenocarcinoma[e]	8144/3
Minimally invasive adenocarcinoma[e]	
Nonmucinous	8256/3[d]
Mucinous	8257/3[d]
Preinvasive lesions	
Atypical adenomatous hyperplasia	8250/0[d]
Adenocarcinoma in situ[e]	
Nonmucinous	8250/2[d]
Mucinous	8253/2[d]
Squamous cell carcinoma	8070/3
Keratinizing squamous cell carcinoma[e]	8071/3
Nonkeratinizing squamous cell carcinoma[e]	8072/3
Basaloid squamous cell carcinoma[e]	8083/3
Preinvasive lesion	
Squamous cell carcinoma in situ	8070/2
Neuroendocrine tumors	
Small cell carcinoma	8041/3
Combined small cell carcinoma	8045/3
Large cell neuroendocrine carcinoma	8013/3
Combined large cell neuroendocrine carcinoma	8013/3
Carcinoid tumors	
Typical carcinoid tumor	8240/3
Atypical carcinoid tumor	8249/3
Preinvasive lesion	
Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia	8040/0[d]
Large cell carcinoma	8012/3
Adenosquamous carcinoma	8560/3
Sarcomatoid carcinomas	
Pleomorphic carcinoma	8022/3
Spindle cell carcinoma	8032/3
Giant cell carcinoma	8031/3
Carcinosarcoma	8980/3
Pulmonary blastoma	8972/3
Other and Unclassified carcinomas	
Lymphoepithelioma-like carcinoma	8082/3
NUT carcinoma[e]	8023/3[d]
Salivary gland-type tumors	
Mucoepidermoid carcinoma	8430/3
Adenoid cystic carcinoma	8200/3
Epithelial-myoepithelial carcinoma	8562/3
Pleomorphic adenoma	8940/0
Papillomas	
Squamous cell papilloma	8052/0
Exophytic	8052/0
Inverted	8053/0
Glandular papilloma	8260/0
Mixed squamous and glandular papilloma	8560/0
Adenomas	
Sclerosing pneumocytoma[e]	8832/0
Alveolar adenoma	8251/0
Papillary adenoma	8260/0
Mucinous cystadenoma	8470/0
Mucous gland adenoma	8480/0
Mesenchymal tumors	
Pulmonary hamartoma	8992/0[d]
Chondroma	9220/0
PEComatous tumors[e]	
Lymphangioleiomyomatosis	9174/1
PEComa, benign[e]	8714/0
Clear cell tumor	8005/0
PEComa, malignant[e]	8714/3
Congenital peribronchial myofibroblastic tumor	8827/1
Diffuse pulmonary lymphangiomatosis	
Inflammatory myofibroblastic tumor	8825/1
Epithelioid hemangioendothelioma	9133/3
Pleuropulmonary blastoma	8973/3
Synovial sarcoma	9040/3
Pulmonary artery intimal sarcoma	9137/3
Pulmonary myxoid sarcoma with *EWSR1–CREB1* translocation[e]	8842/3[d]
Myoepithelial tumors[e]	
Myoepithelioma	8982/0
Myoepithelial carcinoma	8982/3
Lymphohistiocytic tumors	
Extranodal marginal zone lymphomas of mucosa-associated Lymphoid tissue (MALT lymphoma)	9699/3
Diffuse large cell lymphoma	9680/3
Lymphomatoid granulomatosis	9766/1
Intravascular large B cell lymphoma[e]	9712/3
Pulmonary Langerhans cell histiocytosis	9751/1
Erdheim–Chester disease	9750/1
Tumors of ectopic origin	
Germ cell tumors	
Teratoma, mature	9080/0
Teratoma, immature	9080/1
Intrapulmonary thymoma	8580/3
Melanoma	8270/3
Meningioma, NOS	9530/0
Metastatic tumors	

[a]The morphology codes are from the ICDO.[2] Behavior is coded /0 for benign tumors, /1 for unspecified, borderline or uncertain behavior, /2 for carcinoma in situ and grade III intraepithelial neoplasia, and /3 for malignant tumors.
[b]The classification is modified from the previous WHO classification[3] taking into account changes in our understanding of these lesions.
[c]This table is reproduced from the 2015 WHO Classification by Travis et al.[1]
[d]These new codes were approved by the International Agency on Cancer Research/WHO Committee for ICDO.
[e]New terms changed or entities added since 2004 WHO Classification.[3]
LCNEC, large cell neuroendocrine carcinoma, WHO, World Health Organization; ICDO International Classification of Diseases for Oncology.

8.2 Appendix 2

The 2016 (8th edition) of TNM classification for lung cancer.

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T, N, and M descriptors

T (Primary Tumor)		Label
T0	No primary tumor	
Tis	Carcinoma in situ (Squamous or Adenocarcinoma)	Tis
T1	Tumor ≤3 cm,	
T1a(mi)	Minimally Invasive Adenocarcinoma	T1a*(mi)*
T1a	Superficial spreading tumor in central airways[a]	T1a *SS*
T1a	Tumor ≤1 cm	T1a *≤1*
T1b	Tumor >1 but ≤2 cm	T1b *>1-2*
T1c	Tumor >2 but ≤3 cm	T1c *>2-3*
T2	Tumor >3 but ≤5 cm or tumor involving:	
	visceral pleura[b],	T2 *Visc Pl*
	main bronchus (not carina), atelectasis to hilum[b]	T2 *Centr*
T2a	Tumor >3 but ≤4 cm	T2a *>3-4*
T2b	Tumor >4 but ≤5 cm	T2b *>4-5*
T3	Tumor >5 but ≤7 cm	T3 *>5--7*
	or invading chest wall, pericardium, phrenic nerve	T3 *Inv*
	or separate tumor nodule(s) in the same lobe	T3 *Satell*
T4	Tumor >7 cm	T4 *>7*
	or tumor invading: mediastinum, diaphragm, heart, great vessels, recurrent laryngeal nerve, carina, trachea, esophagus, spine;	T4 *Inv*
	or tumor nodule(s) in a different ipsilateral lobe	T4 *Ipsi Nod*
N (Regional Lymph Nodes)		
N0	No regional node metastasis	
N1	Metastasis in ipsilateral pulmonary or hilar nodes	
N2	Metastasis in ipsilateral mediastinal/subcarinal nodes	
N3	Metastasis in contralateral mediastinal/hilar, or supraclavicular nodes	
M (Distant Metastasis)		
M0	No distant metastasis	
M1a	Malignant pleural/pericardial effusion[c] or pleural /pericardial nodules	M1a *Pl Dissem*
	or separate tumor nodule(s) in a contralateral lobe;	M1a *Contr Nod*
M1b	Single extrathoracic metastasis	M1b *Single*
M1c	Multiple extrathoracic metastases (1 or >1 organ)	M1c *Multi*

TX, NX:T or N status not able to be assessed

[a]superficial spreading tumor of any size but confined to the tracheal or bronchial wall

[b]such tumors are classified as T2a if > 3 ≤4 cm , T2b if > 4 ≤5 cm

[c]Pleural effsuions are excluded that are cytologically negative, non-bloody, transudctive, clinically judged not to be due to cancer

Stage groupings for the eighth edition of the TNM classification for lung cancer

T/M	Label	N0	N1	N2	N3
T1	T1a *≤1*	IA1	IIB	IIIA	IIIB
	T1b *>1-2*	IA2	IIB	IIIA	IIIB
	T1c *>2-3*	IA3	IIB	IIIA	IIIB
T2	T2a *Cent, Yisc Pl*	IB	IIB	IIIA	IIIB
	T2a *>3-4*	IB	IIB	IIIA	IIIB
	T2b *>4-5*	IIA	IIB	IIIA	IIIB
T3	T3 *>5-7*	IIB	IIIA	IIIB	IIIC
	T3 *Inv*	IIB	IIIA	IIIB	IIIC
	T3 *Satell*	IIB	IIIA	IIIB	IIIC
T4	T4 *>7*	IIIA	IIIA	IIIB	IIIC
	T4 *Inv*	IIIA	IIIA	IIIB	IIIC
	T4 *Ipsi Nod*	IIIA	IIIA	IIIB	IIIC
M1	M1a *Contr Nod*	IVA	IVA	IVA	IVA
	M1a *Pl Dissem*	IVA	IVA	IVA	IVA
	M1b *Single*	IVA	IVA	IVA	IVA
	M1c *Multi*	IVB	IVB	IVB	IVB

Research Paper

Prognostic effect of intratumoral neutrophils across histological subtypes of non-small cell lung cancer

Mehrdad Rakaee[1], Lill-Tove Busund[1,2], Erna-Elise Paulsen[3,4], Elin Richardsen[1,2], Samer Al-Saad[1,2], Sigve Andersen[3,4], Tom Donnem[3,4], Roy M. Bremnes[3,4], Thomas K. Kilvaer[3,4]

[1]Department of Medical Biology, University of Tromsø, The Arctic University of Norway, N-9037 Tromso, Norway

[2]Department of Clinical Pathology, University Hospital of North Norway, N-9038 Tromso, Norway

[3]Department of Clinical Medicine, University of Tromsø, The Arctic University of Norway, N-9037 Tromso, Norway

[4]Department of Oncology, University Hospital of North Norway, N-9038 Tromso, Norway

Correspondence to: *Mehrdad Rakaee,* ***email:*** *Mehrdad.r.khanehkenari@uit.no*

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ABSTRACT

Recent data indicate that tumor-associated neutrophils (TANs) serve a dual role in tumor progression and regression. CD66b is a neutrophil marker and has been associated with patient outcome in various cancers. However, its clinical impact in non-small cell lung cancer (NSCLC) remains controversial. 536 NSCLC patients, of which 172 harbored lymph node metastases, were included in this study. Tissue microarrays were constructed and multiplexed immunohistochemistry of CD66b, CD34 and pan-keratin was performed to evaluate the localization and quantity of CD66b$^+$ TANs. High intratumoral CD66b$^+$ TANs density in squamous cell carcinoma (SCC) subgroup was an independent positive prognosticator for disease-specific survival (*P* = 0.038). In contrast, high intratumoral TANs density was an independent negative prognostic factor in the adenocarcinoma (ADC) subgroup (*P*= 0.032). Likewise, in ADC patients with lymph node metastases, high level of intratumoral TANs was associated with poor prognosis (*P* = 0.003). Stromal CD66b$^+$ TANs were not associated with outcome of NSCLC patients. In conclusion, CD66b$^+$ TANs show diverging prognostic effect in NSCLC patients according to histological subgroups. The presence of CD66b$^+$ TANs could prove pivotal for development of an immunoscore in ADC NSCLC patients.

INTRODUCTION

Lung cancer is the leading cause for cancer-related deaths worldwide [1]. Even after complete surgical resections, the prognosis of NSCLC patients remain poor due to locoregional relapses and/or metastases [2]. Huge efforts are being invested in identifying new prognostic and predictive molecular markers in order to improve treatment stratification and overall survival (OS).

Tumor infiltrating leukocytes (TILs) positively affect NSCLC patient outcomes [3]. Of these, a significant proportion constitute tumor-associated neutrophils (TANs) [4]. It has been hypothesized that TANs polarize into either a N1 antitumoral or N2 protumoral phenotype in response to cancer epithelial- and stromal-derived signals [5, 6]. The protumoral functions of the N2 phenotype include increased angiogenesis [8], tumor cell proliferation [9], extracellular matrix remodelling, lymphangiogenesis and inhibition of the anti-tumoral immune response [10]. The anti-tumoral activity of the N1 phenotype comprises immune-surveillance including cytotoxicity towards cancer cells. The cellular cytotoxicity leads to recruitment and activation of cells related to both the innate and adaptive immune systems [11, 12].

CD66b is an established marker of TANs [13], stored in neutrophil granules and constitutively expressed by human neutrophils [14]. In contrast to the cells of adaptive immune system, the prognostic role of CD66b$^+$ TANs has been associated with unfavorable outcome for a number of malignancies [15–18], whereas improved survival has been reported for gastric and colorectal carcinoma [19, 20]. In NSCLC, two previous studies

failed to reveal significant associations between TANs and patients outcome [21, 22], neither of these studies evaluated histological subtypes.

Differential outcome of CD66b$^+$ TAN presence according to histological subtypes could be expected since squamous cell carcinoma (SCC) and adenocarcinoma (ADC) are recognized as different diseases regarding biology, treatment stratification and efficacy [23]. This harmonizes with our previous studies on the immune contexture in SCC vs ADC NSCLC patients [24].

Herein, we 1) investigate the prognostic role of CD66b$^+$ TANs in primary tumors of NSCLC patients stratified into SCC and ADC subgroups, 2) assess the prognostic effect of intraepithelial CD66b$^+$ TANs in metastatic lymph nodes from N+ patients and 3) correlate the presence of CD66b$^+$ TANs with 104 tumor molecular markers previously evaluated in this same cohort.

RESULTS

Patient characteristics

A retrospectively registered cohort of 536 patients with NSCLC, of which 172 had LN metastases at the time of diagnosis, was investigated for CD66b$^+$ TAN density. Detailed information of the cohort has previously been published [24, 25]. The average age of the patients at the time of surgery was 67 years old (range, 28-85), and 68% of the patients were men. According to histology, primary tumors were divided into the following histotypes: 289 squamous cell carcinomas (SCC), 201 adenocarcinomas (ADC) and 46 large-cell carcinomas (LCC). Histological features in the LN+ cohort were: 91 (53%) patients SCC, 68 (40%) patients ADC and 13 (8%) patients undifferentiated carcinomas (NOS). During the period 2005-2010, 43 (20%) patients received adjuvant chemotherapy. 76 (14%) patients received adjuvant radiotherapy.

Interobserver variability

The intraclass correlation coefficients (ICCs) and Cohen's Kappa for the CD66b scores were as follows: Intratumoral primary tumor (ICC = 0.94, $P < 0.001$, Kappa = 0.79, $P < 0.001$), stromal (ICC = 0.93, $P < 0.001$, Kappa = 0.75, $P < 0.001$) and intratumoral lymph node metastases (ICC = 0.95, $P < 0.001$, Kappa = 0.80, $P < 0.001$).

Expression and association of CD66b$^+$ TANs with histopathological variables

CD66b was expressed on the membrane and in the cytoplasm of TANs, while multiplex IHC allowed effortless distinction between intratumoral, stromal and intra-vascular CD66$^+$ TANs (Figure 1). Associations between CD66b$^+$ TAN density and clinicopathological variables in the overall cohort (primary tumor, LN+) and within histological subtypes are presented in Table 1. Intratumoral CD66b$^+$ TANs in the primary tumor were positively associated with increasing tStage ($P = 0.011$) and pStage IIB ($P = 0.002$) in the whole cohort, and was negatively associated with nStage ($P = 0.032$) in the SCC subgroup. Stromal CD66b$^+$ cells were associated with weight loss in the whole cohort ($P = 0.044$) and in the SCC subgroup ($P = 0.031$). No associations between clinicopathological variables and the presence of CD66b$^+$ TANs were observed in the ADC-group or in the N+ subgroups (Table 1).

Survival analyses

Tables 2 and 3 and Figure 2 summarize the intratumoral presence of CD66b$^+$ cells in primary tumors and lymph node metastases and their role on diseases-specific survival (DSS) in univariable and multivariable analyses. No significant effects on survival were observed in the overall cohort. In the SCC subgroup, the presence of intratumoral CD66b$^+$ cells was an independent positive prognostic factor [univariate $P = 0.038$ (Figure 2B), multivariable $P = 0.021$, HR 0.59 (0.38-0.92)], while in the ADC subgroup, the presence of intratumoral CD66b$^+$ cells was an independent adverse prognostic factor [univariate $P = 0.032$ (Figure 2C), multivariable $P = 0.020$, HR 1.7 (1.1-2.65)]. In patients with nodal metastases (Figure 2G–2I), the presence of intratumoral CD66b$^+$ cells was an independent adverse prognostic factor for the ADC subgroup [univariate $P = 0.003$ (Figure 2I), multivariable $P = 0.004$, HR = 2.87 (1.39-5.91)]. Results were largely similar when assessing disease-free survival (DFS) and OS (Supplementary Figures S1 and S2). The density of CD66$^+$ neutrophils in the stroma was not significantly associated with survival (Figure 2D–2F).

CD66b$^+$ TANs and treatment interactions

The adjuvant chemotherapy treatment-TAN interaction was not significant for OS, DFS and DSS in either overall cohort or ADC and SCC subgroups (data not shown).

Further, for patients who received adjuvant radiotherapy, there was a tendency towards increased survival differences between patients with high vs low CD66b density. Although, similar tendencies were seen for patients who did not receive radiotherapy, and subgroup analysis should be interpreted with caution because of the small sample size [SCC: n=41; ADC: n=19] (data not shown).

Presence of CD66b$^+$ neutrophils and their correlations with prognostic markers

Table 4 summarizes the significant correlations between the presence of CD66b$^+$ cells and prognostic

Table 1: Correlations between clinicopathological variables and A) Intratumoral CD66b$^+$ cells in resected primary tumors of NSCLC patients in the overall cohort and stratified into the SCC and ADC subgroups (chi-square test and fisher exact test, N = 536, 289 and 201 respectively); B) Intratumoral CD66b$^+$ cells in lymph-node tissue from N+ NSCLC patients in the overall cohort and stratified into the SCC and ADC subgroups (chi-square test and fisher exact test, N = 172, 91 and 68 respectively)

	A									B							
	All			SCC			ADC			All			SCC			ADC		
	Low	High	P	Low	High	P	Low	High	P	Low	High	P	Low	High	P	Low	High	P
Age			0.535			0.223			0.023			1.000			0.284			0.367
≤65	129	85		65	38		54	39		41	17		22	6		18	10	
>65	187	108		96	79		71	24		42	17		18	11		17	5	
Gender			0.221			0.158			0.522			0.130			0.706			0.069
Female	92	67		35	35		48	28		28	6		8	2		17	3	
Male	224	126		126	82		77	35		55	28		32	15		18	12	
ECOG			0.092			0.189			0.394			0.407			0.432			1.000
0	185	113		88	65		75	42		48	15		22	6		20	9	
1	115	61		65	40		43	16		28	15		15	9		11	5	
2	16	19		8	12		7	5		7	4		3	2		4	1	
Smoking			0.376			1.000			0.497			0.931			0.299			0.175
Never	12	3		4	2		7	1		3	1		2	0		1	1	
Previous	200	124		101	74		77	40		49	21		25	8		20	12	
Present	104	66		54	41		41	22		31	12		13	9		14	2	
Weight loss			0.061			0.183			1.000			0.195			0.058			0.654
<10%	291	167		147	100		116	58		76	28		38	13		30	14	
≥10%	25	25		14	17		9	5		7	6		2	4		5	1	
Surgical procedure			1.000			0.276			0.867			0.703			1.000			0.746
Wedge/ Lobectomy	229	140		103	83		100	49		44	16		16	7		24	9	
Pulmonectomy	87	53		58	34		25	14		39	18		24	10		11	6	
Margins			0.357			0.031			1.000			0.131			0.428			0.152
Free	286	180		138	110		118	60		75	27		35	13		33	12	
Not free	30	13		23	7		7	3		8	7		5	4		2	3	
tStage			0.011			0.119			0.431			0.064			0.495			0.371
IA	48	22		23	13		23	9		6	1		3	1		3	0	
IB	60	30		27	19		27	10		15	1		5	0		7	1	
IIA	120	58		57	30		50	23		31	13		15	6		15	7	
IIB	43	30		29	22		10	7		12	6		7	5		4	1	
III	42	50		23	32		14	13		15	13		7	5		6	6	
IV	3	3		2	1		1	1		4	0		3	0				
nStage			0.072			0.032			0.607			0.584			0.315			1.000
0	204	143		101	90		81	45										
1	76	36		46	23		27	10		55	25		30	15		21	9	
2	36	14		14	4		17	8		28	9		10	2		14	6	

(*Continued*)

	A									B							
	All			SCC			ADC			All			SCC			ADC		
	Low	High	P	Low	High	P	Low	High	P	Low	High	P	Low	High	P	Low	High	P
pStage			0.002			0.061			0.070			0.229			0.386			0.548
IA	81	47		36	30		39	17										
IB	70	44		32	23		28	16										
IIA	82	33		50	25		26	7		36	10		19	6		15	4	
IIB	30	41		19	27		8	12		6	5		4	4		2	1	
IIIA	53	28		24	12		24	11		41	19		17	7		18	10	
Histology			0.096									0.908						
SCC	161	117								40	17							
ADC	125	63								35	15							
NOS	30	13								8	2							
Differentiation			0.674			0.851			0.083			0.655			0.794			0.236
Poor	132	84		58	40		44	31		41	20		15	7		18	11	
Moderate	143	89		87	63		56	26		37	13		22	10		15	3	
Well	41	20		16	14		25	6		5	1		3	0		2	1	
Vascular infiltration			0.493			0.218			1.000			0.686			0.752			1.000
No	253	161		124	98		106	54		58	25		27	13		27	11	
Yes	61	32		37	19		17	9		25	8		13	4		8	3	

Abbreviations: SCC, squamous cell carcinoma; ADC, adenocarcinoma; ECOG, Eastern Cooperative Oncology Group; NOS, not otherwise specified

Table 2: A) Intratumoral CD66b$^+$ cells in the primary tumors of resected NSCLC patients as predictors of disease-specific survival in the overall cohort and stratified into SCC and ADC subgroups (univariate analyses, log-rank test, N = 536, 289 and 201 respectively). B) Intratumoral CD66b$^+$ cells in lymph-node tissue from N+ NSCLC patients as predictors of disease-specific survival in the overall cohort and stratified into SCC and ADC subgroups (univariate analyses, log-rank test, N = 172, 91 and 68 respectively)

	All					SCC					ADC				
A	N(%)	5 Year	Median	HR (95%CI)	P	N(%)	5 Year	Median	HR (95%CI)	P	N(%)	5 Year	Median	HR (95%CI)	P
CD66b Primary tumor					0.540					0.038					0.032
≤5%	316(59)	56	104	1		161(56)	62	235	1		125(62)	50	71	1	
>5%	193(36)	60	NA	0.92 (0.69-1.21)		117(40)	70	NA	0.64 (0.43-0.96)		63(31)	43	47	1.57 (1-2.46)	
missing	27(5)					11(4)					13(6)				
B															
CD66b LN+					0.075					0.688					0.003
≤5%	83(48)	33	30	1		40(44)	45	35	1		35(51)	23	30	1	
>5%	34(20)	19	16	1.57 (0.9-2.74)		17(19)	40	19	1.17 (0.52-2.61)		15(22)	0	8	2.71 (1.1-6.65)	
missing	55(32)					34(37)					18(26)				

Abbreviations: SCC, squamous cell carcinoma; ADC, adenocarcinoma; LN+, metastatic lymph nodes

markers previously studied in this cohort. Supplementary Table S1 includes all markers involved in the correlation analysis (n=104). After corrections for multiple testing, intratumoral CD66b$^+$ cells in the whole cohort, were correlated with stromal CD66b$^+$ (r = 0.76), CSF1R (r = 0.23), MCSF (r = 0.28), MCT1 (r = 0.23) and MCT4 (r = 0.22) and intratumoral CD68$^+$ cells (r = 0.23). Stromal CD66b$^+$ cells were correlated with intratumoral CD66b$^+$ (r = 0.76) and stromal CSF1R (r = 0.25), CD68$^+$ cells (r = 0.23), MCSF (r = 0.25) and MCT1 (r = 0.26). In the SCC subgroup, intratumoral CD66b$^+$ cells were correlated with stromal expression of CD66b$^+$ (r = 0.80) and MCSF (r = 0.32), while stromal CD66b$^+$ cells were correlated with intratumoral CD66b$^+$ (r = 0.80), CD68$^+$ cells (r = 0.28), MCT4 (r = 0.27) and stromal CSF1R (r = 0.28), stromal CD68$^+$ cells (r = 0.27), MCSF (r = 0.32), FOXO1A (r = 0.28) and MCT1 (r = 0.30). In the ADC subgroup intratumoral CD66b$^+$ cells correlated only with stromal CD66b$^+$ cells (r = 0.66).

DISCUSSION

In our large cohort of unselected stage I-IIIA NSCLC patients, we demonstrate that the presence of intratumoral CD66b$^+$ neutrophils mediate opposing independent prognostic significance in the ADC versus SCC subtype. Moreover, this prognostic significance goes undetected when the role of TANs is investigated in the whole NSCLC cohort, and not according to histology. Lymph nodes from LN+ patients of the same cohort, when evaluated as a validation cohort, revealed similar prognostic harmony with their corresponding primary tumors.

CD66b is recognized as a TAN marker in several studies, and a recent meta-analysis reported the presence of CD66b$^+$ TANs to be a significant unfavorable prognosticator in solid malignant tumors [26]. In NSCLC, the role of CD66b$^+$ TANs remains controversial. Ilie et al. [22] found the CD66b$^+$ TAN/CD8$^+$ T cell ratio to predict recurrence and poor OS. In contrast Carus et al. [21] did not

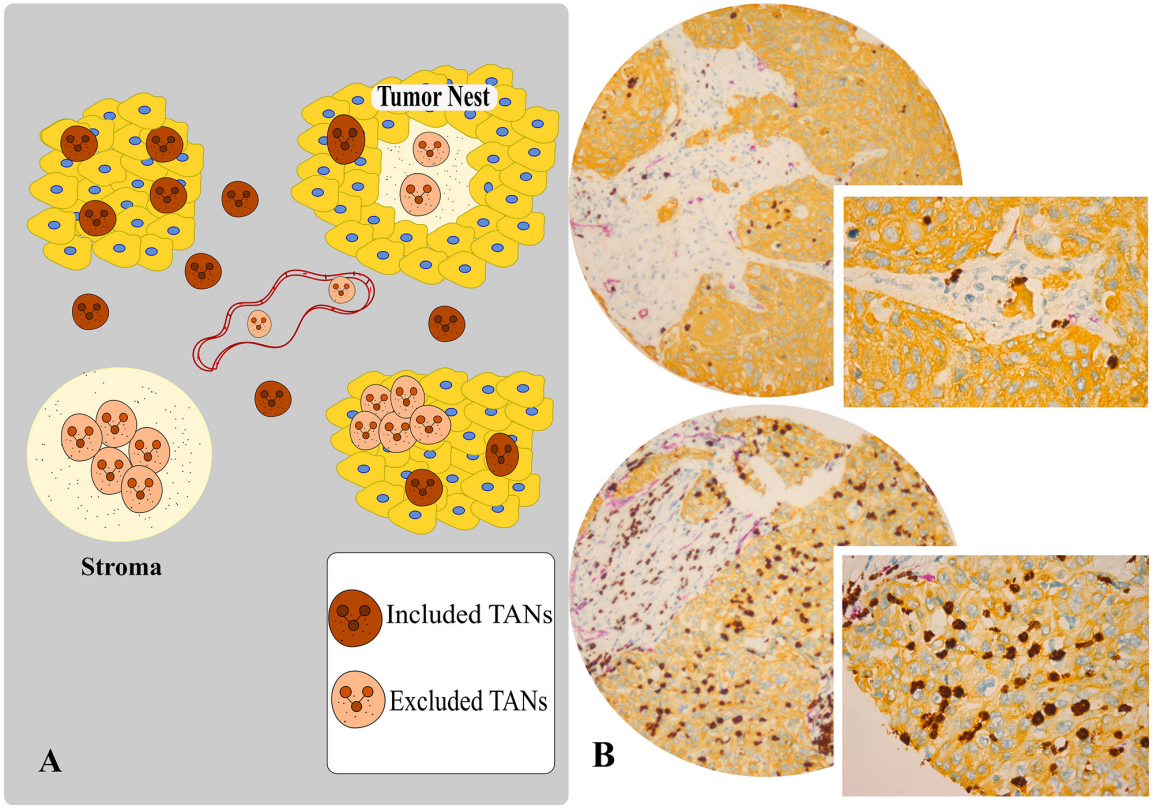

Figure 1: A. Scoring assessment guideline. Scoring of intratumoral (tumor nest) and stromal CD66b$^+$ neutrophils was conducted utilizing the following exclusion- and inclusion-criteria. In intratumoral assessment, we have included only unaggregated CD66b$^+$ cells completely surrounded by tumor epithelial cells. Stromal assessment only regarded extravascular CD66b$^+$ cells. The excluded areas consisted of intratumoral and stromal aggregates of neutrophils, intravascular neutrophils and CD66b$^+$ cells with granular background in stroma. Especially in SCC tissue, the central tumor zone often had dense granular CD66$^+$ structures with some CD66b$^+$ cells, considered a pre- or necrotic area, which were excluded from scoring. **B.** Multiplexed IHC analysis of TANs with CD66b/CD34/pan-CK panel, high versus low intratumoral densities. Brown, purple and yellow substrates were applied to visualize CD66b, CD34 and pan-CK respectively (magnification 10x, 20x).

Table 3: Multivariable models summarizing significant independent prognostic factors in A) The SCC and ADC subgroups of the total cohort (Cox regression analyses, N = 289 and 201 respectively) and B) The ADC subgroup of the N+ NSCLC patients (Cox regression analyses, N = 68)

A	Overall cohort of NSCLC patients	SCC		ADC		B	NSCLC patients with N+ cohort	ADC	
		HR (95% CI)	P	HR (95% CI)	P			HR (95% CI)	P
Intratumoral CD66b									
≤5%		1.000		1.000				1.000	
>5%		0.59 (0.38-0.92)	**0.021**	1.7 (1.1-2.65)	**0.020**			2.87 (1.39-5.91)	**0.004**
pStage									
IA		1.000		1.000					
IB		1.17 (0.51-2.71)	0.715	1.93 (1.02-3.64)	**0.043**				
IIA		2.09 (1.05-4.17)	**0.036**	3.07 (1.58-5.97)	**<0.001**				
IIB		4.43 (2.19-8.94)	**<0.001**	2.51 (1.18-5.35)	**0.017**				
IIIA		7.98 (3.97-16.03)	**<0.001**	4.62 (2.38-8.97)	**<0.001**				
Different									
Poor				1.000					
Moderate				0.91 (0.59-1.42)	0.682				
Well				0.44 (0.22-0.89)	**0.022**				
ECOG									
0				1.000					
1				1.76 (1.13-2.74)	**0.012**				
2				2.93 (1.28-6.7)	**0.011**				

Abbreviations: SCC, squamous cell carcinoma; ADC, adenocarcinoma; ECOG, Eastern Cooperative Oncology Group

detect an association between $CD66b^+$ TANs and survival. Nevertheless, studies in renal cell [15], head and neck [16], bronchioloalveolar [17] and oesophageal carcinoma [18] have demonstrated association between the presence of intratumoral $CD66b^+$ neutrophils and poor prognosis, while in gastric and colorectal carcinoma high levels of $CD66b^+$ neutrophils indicated a favorable prognosis [19, 20].

The current knowledge of TAN function is based on studies in tumor-bearing animal models, not in humans. Deletion or alteration of TGF-β signaling within the tumor lead to reduced tumor progression through the activation of $CD8^+$ T cells and recruitment of myeloid-derived suppressor cells (MDSCs) [27]. In a previous *in vivo* study, TGF- β signaling observed in tumor-bearing mice exerted effects on polymorphonuclear lineages of MDSCs and induced a distinct N2 TAN subtype, which promoted cancer development. Furthermore, abrogation of TGF- β signaling polarized TANs from

the protumoral N2 to the antitumoral N1 phenotype. Depletion of antitumoral N1 TANs following TGF-β blockade reduced $CD8^+$ T cells activation and promoted tumor growth [5]. In a mouse model, N1 TAN mediated activation of $CD8^+$ T cells has been determined the main mechanism responsible for mediating an antitumoral response. It is tempting to infer that the same mechanisms are present in humans, but as with M1/M2 tissue macrophage differentiation, there are broad differences between tumor-bearing mice and humans. As macrophages and neutrophils ascend from a common progenitor, the complexity observed in human macrophage differentiation should be expected for neutrophils as well [28]. Not surprisingly, we observe

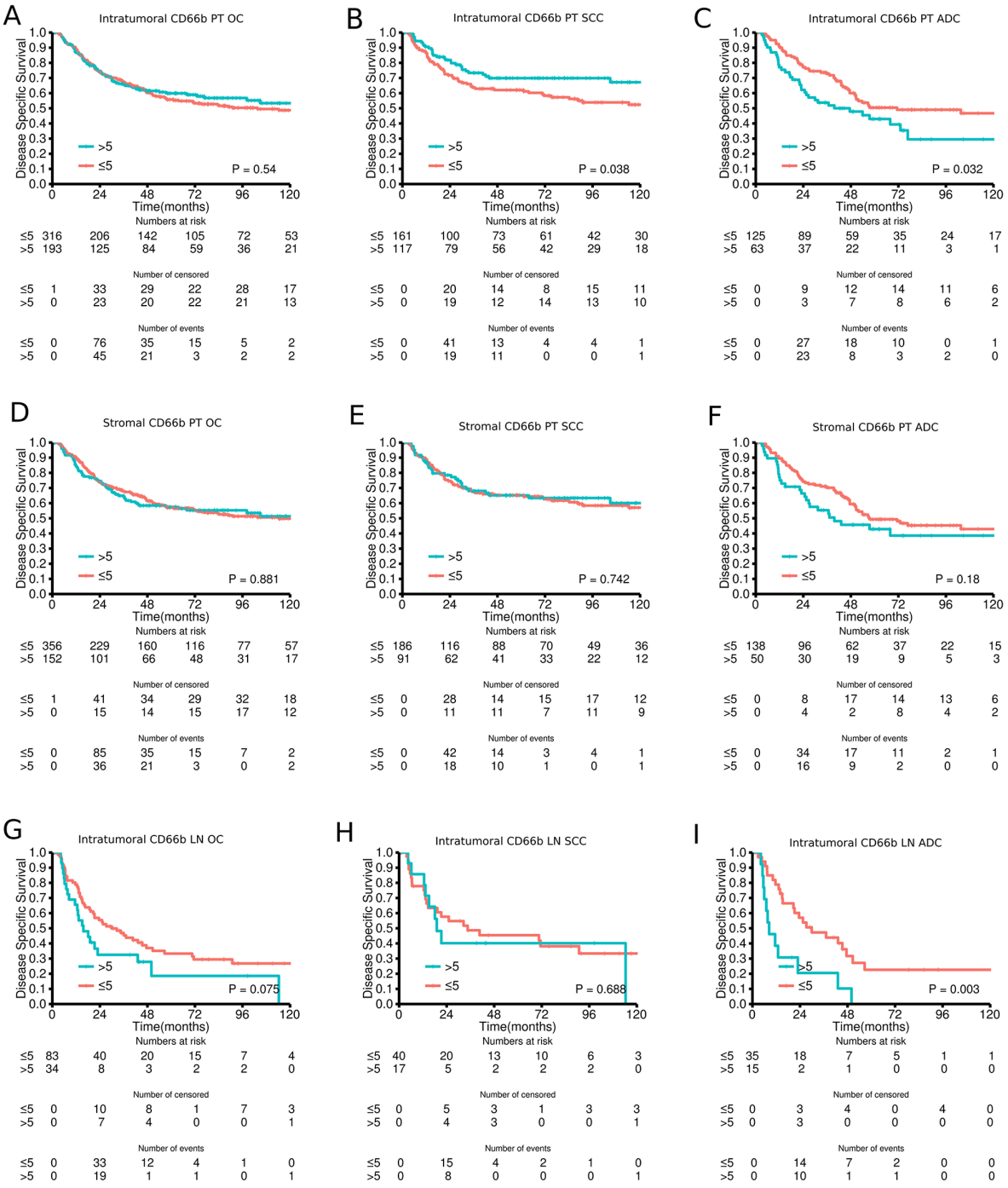

Figure 2: Disease-specific survival curves for A. Intratumoral CD66b in the overall cohort (OC) of primary tumors (PT); **B.** Intratumoral CD66b in squamous cell carcinomas (SCC) of PT; **C.** Intratumoral CD66b in adenocarcinomas (ADC) of PT; **D.** Stromal CD66b in the overall cohort of PT; **E.** Stromal CD66b in SCC of PT; **F.** Stromal CD66b in ADC of PT; **G.** Intratumoral CD66b in the overall cohort of LN+; **H.** Intratumoral CD66b in SCC of LN+; **I.** Intratumoral CD66b in ADC of LN+

Table 4: Significant Spearman rank-correlations with *R*-values > 0.20 between intratumoral and stromal CD66b$^+$ TANs and tumor-associated markers in samples from NSCLC in the total cohort and in subgroups according to histology (Total cohort = 326, SCC = 191; ADC = 95)

	ALL		SCC		ADC			
	Correlations with markers expressed in intratumoral cells							
	Tumor	Stroma	Tumor	Stroma	Tumor	Stroma		
CAIX						0.23*	ANG	T
CD34					-0.27**	-0.27**	ANG	T
D240					0.21*		ANG	T
DLL4	-0.20***					-0.24*	ANG	T
FGFR1					-0.23*		ANG	T
Glut1					0.28**		ANG	T
NOTCH1					0.25*		ANG	T
NOTCH4			-0.22**				ANG	T
PHD-3						-0.33***	ANG	T
Bad-cyt			-0.24***		-0.21*	-0.22*	EMT	T
Bad-Nuc			-0.24**				EMT	T
Her3			-0.21**				EMT	T
Ki67					0.22*		EMT	T
pHer2				-0.22**			EMT	T
pi3K			-0.21***				EMT	T
CD66b		0.76#		0.80#		0.66#	IMM	T
CD68	0.23#		0.28***	0.28#			IMM	T
MCT1						0.22*	MET	T
MCT4		0.22#	0.22**	0.27#			MET	T
PGC1-α						-0.23*	MET	T
	Correlations with markers expressed in tumor stroma							
	Tumor	Stroma	Tumor	Stroma	Tumor	Stroma		
Ang2					0.21*		ANG	S
D240					0.24*		ANG	S
DLL4				0.21**			ANG	S
miR21		0.21***				0.23*	ANG	S
NOTCH4				0.21**			ANG	S
PDGF-A			0.22**	0.23**	0.23*		ANG	S
VEGF-A			0.22**				ANG	S
VEGF-D				0.21**	0.21*		ANG	S
cAkt				0.24***			EMT	S
ERK3				0.20**			EMT	S
IGF1				0.21**			EMT	S
NfκB				0.23**			EMT	S

(*Continued*)

	ALL		SCC		ADC			
	Correlations with markers expressed in intratumoral cells							
	Tumor	Stroma	Tumor	Stroma	Tumor	Stroma		
PAR6			0.22**				EMT	S
CD138				0.21**			IMM	S
CD1a			0.20**				IMM	S
CD66b	0.76#		0.80#		0.66#		IMM	S
CSF1R	0.23#	0.25#	0.26***	0.28#	0.23*	0.21*	IMM	S
CXCL16				0.25***			IMM	S
FOXP3				0.21**			IMM	S
CD68		0.23#	0.23**	0.27#			IMM	S
MCSF	0.28#	0.25#	0.32#	0.32#			IMM	S
FOXO1A				0.28#			IMM	S
LDH5				0.24***			MET	S
MCT1	0.23#	0.26#	0.23**	0.30#	0.28**	0.24*	MET	S
MCT4	0.22#	0.20***	0.20**		0.23*	0.27**	MET	S

Abbreviations: CD, cluster of differentiation; SCC, squamous cell carcinoma; ADC, adenocarcinoma;ANG, angiogenesis; EMT, epithelial-mesenchymal transition; MET, metastasis; IMM; immunology; CAIX, Anti-Carbonic Anhydrase IX;DLL4, Delta ligand 4; FGFR1, Fibroblast Growth Factor Receptor 1; GLUT1, Glucose transporter 1; PHD, prolyl hydroxylase-domain; BAD, Bcl2 Associated Death Promoter; Cyt, in cytoplasm; Nuc, in nucleus; Her, human epidermal growth factor receptor; MCT, monocarboxylate transporter; PGC1, Peroxisome proliferative activated receptor gamma coactivator 1; Ang, angiogenin; miR, micro RNA; PDGF, Platelet-derived growth factor; VEGF, Vascular endothelial growth factor; IGF1, Insulin like growth factor 1; PAR6, Partitioning defective 6; CXCL16, C-X-C motif ligand; FOX, forkhead box; MCSF, Macrophage Colony Stimulating Factor 1; CSF1R, Colony stimulating factor 1 receptor; LDH, lactate dehydrogenase.

*significant at $p > 0.05$,

**significant at $p > 0.01$,

***significant at $p > 0.001$,

significant after Bonferroni correction for multiple tests

a close correlation between $CD66b^+$ TANs and $CD68^+$ (pan-macrophage marker) expression in our cohort (Table 4) and the MDSC associated colony-stimulating factor-1 receptor, indicating a close relationship between TANs and macrophages in NSCLC.

The idea that human TANs may differentially affect the tumor-host immune activity based on stage and histological subtype of cancer is intriguing. In early stage NSCLC, TANs have been shown not to be mainly immunosuppressive, but would rather stimulate T cell-mediated immunity through the production of co-stimulatory molecules enhancing proliferation of $CD4^+$and $CD8^+$ T cells [29, 30]. The role of human TANs in perturbing immunity is poorly defined mechanistically, and gaps remain in understanding TANs plasticity and the switch between pro- and antitumoral effects *in vitro*. Further, little is known of the *in vivo* properties of TANs and whether *in vitro* data from studies in mice can be extrapolated to humans. Moreover, a plethora of signaling molecules, differing between and even within different stages of the same histological subtypes, are available to influence TANs. This is a plausible explanation for why the prognostic significance of $CD66b^+$ TANs diverging according to histological subtypes. This is supported by the two predominant NSCLC histological phenotypes, SCC and ADC, displaying distinct differences in genomic and stromal heterogeneity, association to smoking, growth pattern and sensitivity to treatment [23], and are by many regarded as different entities.

Tumor infiltrating immune cells have a pivotal contribution in cancer progression and critically influences the clinical outcome of patients depending on density and localization of various immune cell subsets [31]. The analysis of the immune contexture in NSCLC has revealed supplementary prognostic

and predictive data which may be combined with the standard pathological TNM classification to form a TNM-Immunoscore (TNM-I) [32]. Recently, our group have reported stromal $CD8^+$ and $CD45RO^+$ TILs to positively associate with survival, hence being good candidate markers for a TNM-I in NSCLC [24, 33]. The effect of TILs in NSCLC is best documented in the SCC subgroup, while the data presented herein indicate that $CD66b^+$ TANs to be a candidate in TNM-I for the ADC subgroup. Supplementary Figure S3 shows how incorporation of $CD66^+$ TAN status could improve the prognostic properties of the established TNM staging system for NSCLC ADC patients. However, these data are preliminary and need to be confirmed in larger NSCLC ADC cohorts.

In conclusion, we observed that intratumoral $CD66b^+$ TANs is both an independent positive and negative prognosticator in the SCC and ADC subgroup of NSCLC patients, respectively. While $CD8^+$/$CD3^+$/$CD45RO^+$ TILs seem to be pivotal for the establishment of a TNM-I for NSCLC SCC patients, $CD66b^+$ TANs may prove an appropriate choice for ADC patients.

MATERIALS AND METHODS

Patients and ethical clearance

An unselected population of 536 patients with surgically resected stage I-IIIA NSCLC from the University Hospital of North-Norway and Nordland Central Hospital from 1990-2010 were included in this study. Of 536 patients, 509 were involved in survival analysis, while the remaining cases are highlighted as missing due to poor tissue quality and unscorable cores.

For the N+ patients, the total number (n=172) constitutes all patients in the cohort with N+ disease. Of these 55 were either missing due to bad or missing cores or due the fact that LN tissue was not available in the archival tissue. Both study populations are described previously by our group [24, 25].

The study was approved by the Regional Committee for Medical and Health Research Ethics (Northern Norway, UNN: protocol ID: 2011/2503). Data collection and storing of the clinical database were approved by the National Data Inspection Board. The study instructions for biomarker expression, clinicopathological features and survival data is reported according to the REMARK guidelines [34].

Tissue micro-array construction and Immunohistochemistry

All tissue samples were reviewed by two experienced pathologists (ER.LTB). The most representative areas was marked on the hemotoxylin and eosin (H&E) slide and sampled for tissue micro-array (TMA) blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA). The methodology has previously been explained [35]. Briefly, four 0.6 mm cores were sampled for each patient: two from central tumor epithelium and two from tumor stroma.

Multiplexed Immunohistochemistry

Triple IHC staining was carried out sequentially using the Discovery-Ultra automated immunostainer (Ventana Medical Systems, Tucson, AZ). Deparaffinization and on-board antigen retrieval were performed for 32 minutes at approximately 100°C with CC1 reagent, which is an EDTA-based proprietary Ventana solution (pH 8.0–8.5). CD66b mouse monoclonal antibody (#555723, clone:G10F5, BD Biosciences, dilution 1/400) was applied and incubated for 32 min and amplified for 4 min, followed by an ultraWash step to wash off excess antibody. Antibody denaturation for 8 minutes at 90°C was performed to ensure that the first primary antibody was completely inactivated before applying the second antibody. The pre-diluted CD34 mouse monoclonal antibody (#790-2927, Ventana, Clone: QBEnd/10) was then applied as a second primary antibody and was incubated for 32 minutes, and then washed, followed by denaturation. In the last sequence pre-diluted mouse monoclonal anti-pan keratin (CK) (#760-2135, Ventana, Clone: AE1/AE3/PCK26) was applied with 16 min incubation. The primary antibodies CD66b, CD34 and pan CK were visualized using Ventana DAB, Purple, Yellow detection kits respectively with 32 min incubation time for DAB, 16 min for purple and 1 hour for yellow chromogens. Finally, the slides were counterstained with hematoxylin and bluing reagent.

All triple stained sections were compared with the corresponding single stained section slide. Three different controls for our staining method were applied: 1) A blank control by omission of the primary antibody in every sequence of staining, 2) control staining of the sections with an isotype-matched control antibody without the primary antibody and 3) multiple organ TMAs as positive and negative tissue controls to verify the specificity of the staining for every staining procedure

Scoring of immunohistochemistry

Two pathologists (LTB, ER) established a semi-quantitative score. Pan-keratin identified normal and malignant epithelium, while CD34 differentiated intra- and extra-vascular $CD66b^+$ TANs.

The TMA slides were scored by two observers (MR, EEP) for intratumoral (primary tumors and lymph nodes) and stromal (primary tumors) $CD66b^+$ TANs. A four-tiered scale with the following levels: $0 \leq 1$, 1 = 1-5, 2 = 6-15, 3 >15 for both the tumor epithelial and

stromal compartments was used. Intravascular $CD66b^+$ TANs, necrotic and pre-necrotic areas were disregarded. The method used for pathological evaluation of $CD66b^+$ neutrophil density in tumor and stromal compartments is presented in Figure 1A. Representative images of triple staining with low and high densities are shown in Figure 1B.

For statistical analysis, dichotomization was done and high presence defined as >5 $CD66b^+$ neutrophils (score 2 or 3).

TMA Validation

Whole-tissue section slides of 20 patients were evaluated for tumor heterogeneity with corresponding TMA cores. The selected cases was from different histological and pathological stages with following detail: 10 specimen of ADC (5 tStage I, 5 tStage III), 10 specimen of SCC (5 tStage I, 5 tStage III). The applied staining procedure for whole-tissue section was the same as for TMA slides. Heterogeneity between paired TMA core and whole tissue was low and a significant concordance was observed for TAN density intratumorally (paired *T*-test correlation= 0.91; $p = <0.001$).

Statistical methods

All data analyses were conducted in RStudio with R version 3.2.2 utilizing the packages survival, gridExtra, car, Hmisc, irr and ggplot2.

IHC scores were compared for interobserver reliability using a two-way random effects model with an absolute agreement definition and Cohen's kappa-statistics with equal weights. The intraclass correlation coefficient and Cohen's kappa were obtained from these results.

The Chi-square and Fischer's Exact tests were used to examine the association between marker expression and clinicopathological variables. Spearman`s rank-correlation was used to examine the associations between marker expressions. Due to the large number variables analyzed in the correlation analyses, Bonferroni corrections were conducted for these analyses.

Univariate survival analyses, were done using the Kaplan-Meier method and statistical difference between survival curves assessed by the log-rank test. DSS, DFS and OS were used as end-points. Multivariable analysis, using the Cox proportional hazards model, was carried out to assess the independent value of pretreatment variables in the presence of other variables. Only variables with $P < 0.25$ from the univariate analyses or otherwise deemed important were explored in multivariable analyses. The significance level used for survival analyses was $P < 0.05$.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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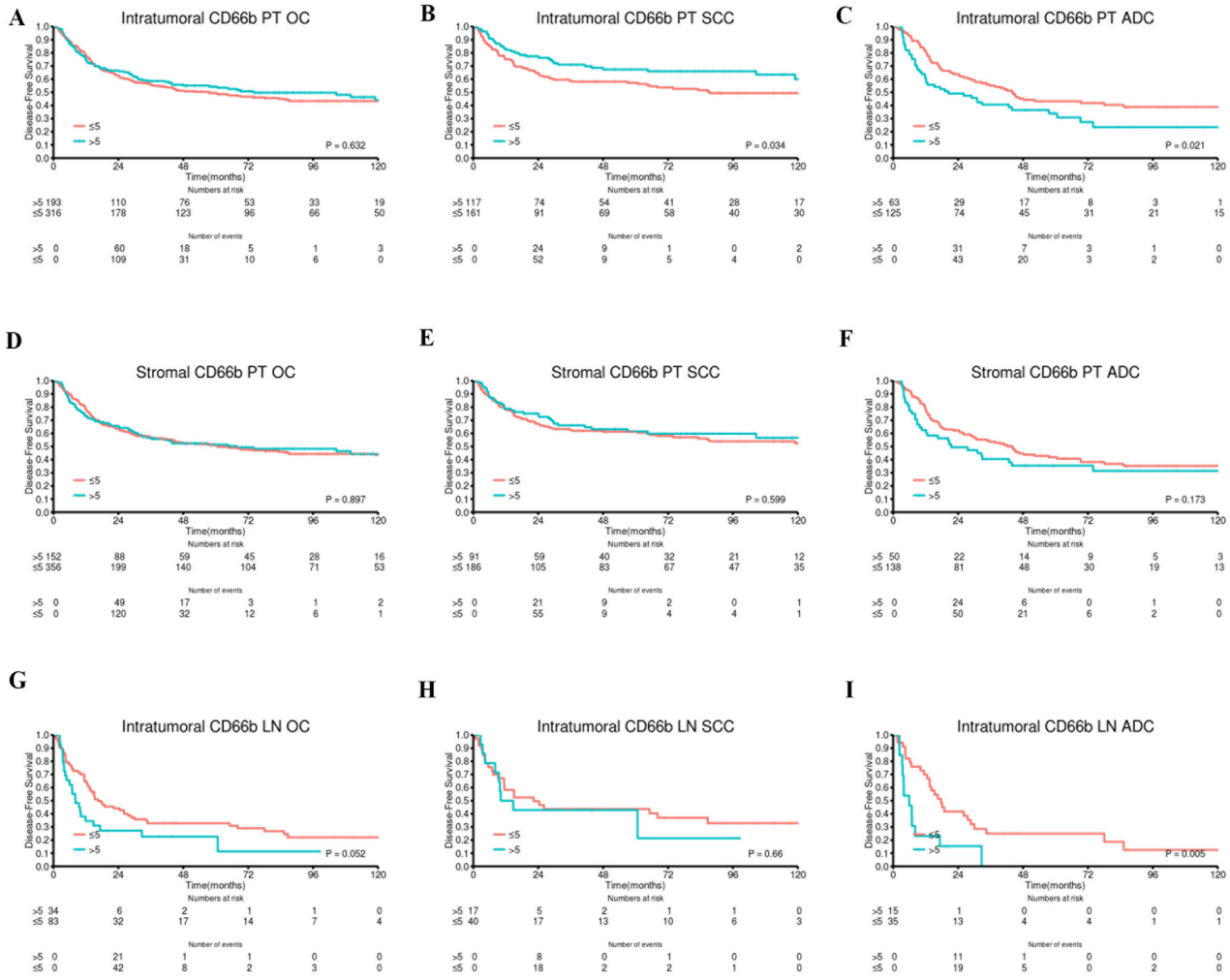
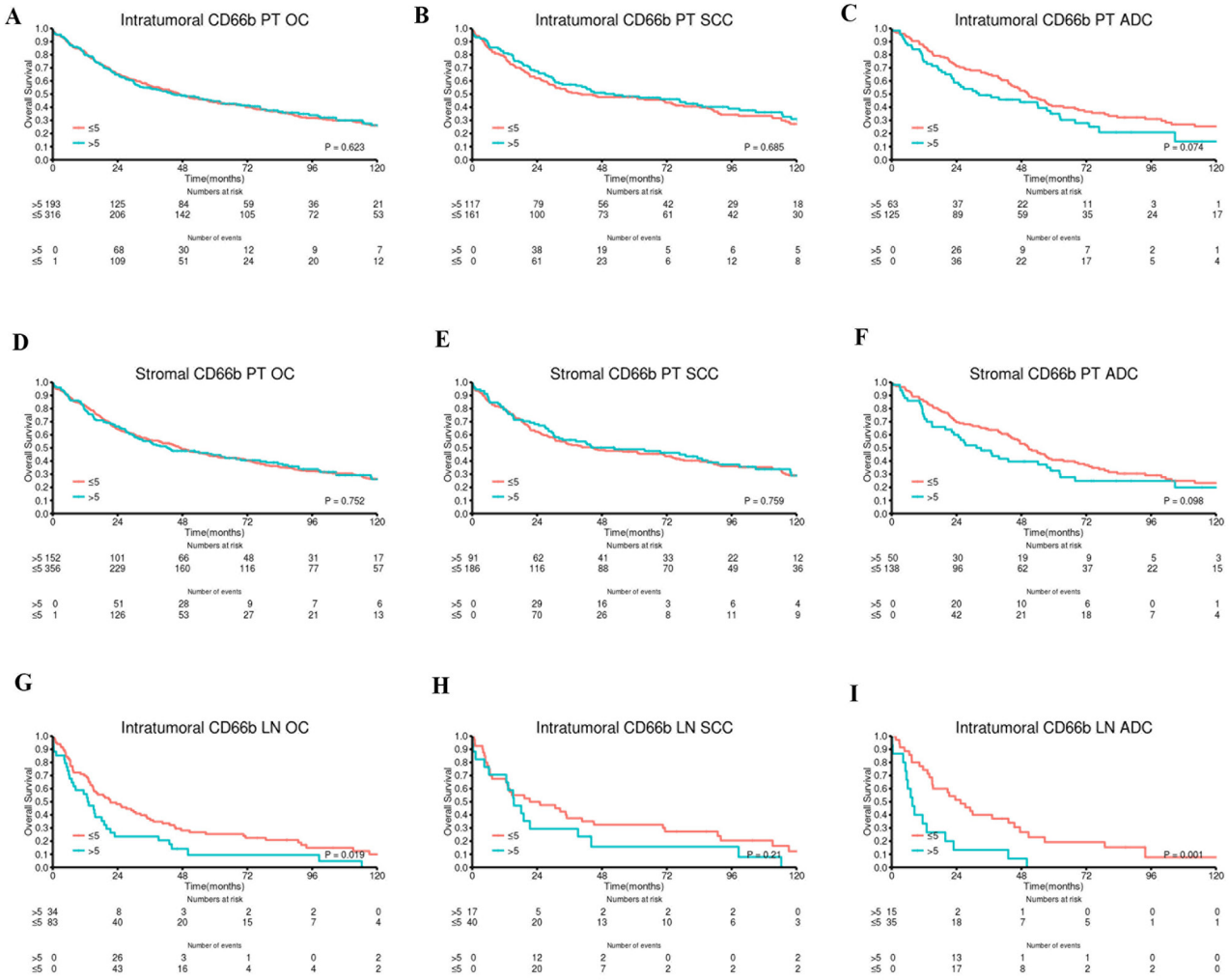
Prognostic effect of intratumoral neutrophils across histological subtypes of non-small cell lung cancer

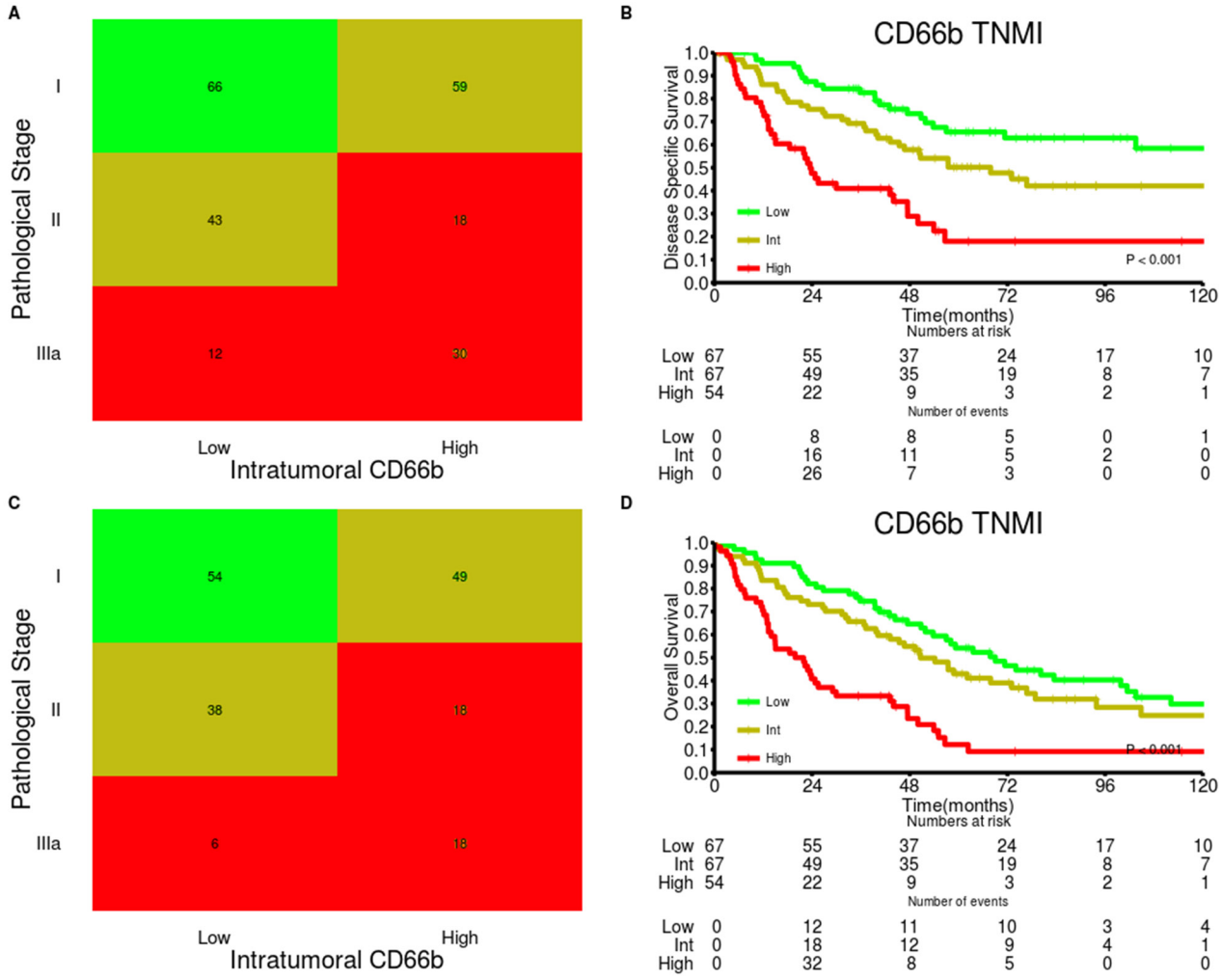
SUPPLEMENTARY FIGURES AND TABLE



Supplementary Figure S1: Disease-free survival curves for A. Intratumoral CD66b in the overall cohort (OC) of primary tumors (PT); **B.** Intratumoral CD66b in squamous cell carcinomas (SCC) of PT; **C.** Intratumoral CD66b in adenocarcinomas (ADC) of PT; **D.** Stromal CD66b in the overall cohort of PT; **E.** Stromal CD66b in SCC of PT; **F.** Stromal CD66b in ADC of PT; **G.** Intratumoral CD66b in the overall cohort of LN+; **H.** Intratumoral CD66b in SCC of LN+; **I.** Intratumoral CD66b in ADC of LN+



Supplementary Figure S2: Overall survival curves for A. Intratumoral CD66b in the overall cohort (OC) of primary tumors (PT); **B.** Intratumoral CD66b in squamous cell carcinomas (SCC) of PT; **C.** Intratumoral CD66b in adenocarcinomas (ADC) of PT; **D.** Stromal CD66b in the overall cohort of PT; **E.** Stromal CD66b in SCC of PT; **F.** Stromal CD66b in ADC of PT; **G.** Intratumoral CD66b in the overall cohort of LN+; **H.** Intratumoral CD66b in SCC of LN+; **I.** Intratumoral CD66b in ADC of LN+.



Supplementary Figure S3: CD66b TNM-I in ADC of NSCLC through DSS **A, B.** and OS **C, D.** endpoints. A, C) Distribution of ADC patients with similar survival in each pathological stages, and B, D) CD66b Immunoscore. (good prognosis: light green; intermediate prognosis: moss green; poor prognosis: red).

Supplementary Table S1: List of 104 tumor-associated markers (99 proteins and 5 microRNAs) investigated in our cohort.

See Supplementary File 1

Supplemental Table 1: List of 104 tumor-associated markers (99 proteins and 5 microRNAs) investigated in our cohort

	Tumor	**Stroma**	**Abbreviation/alternative name**
1	VEGF-A	VEGF-A	vascular endothelial growth factor A
2	VEGF-C	VEGF-C	vascular endothelial growth factor C
3	VEGF-D	VEGF-D	vascular endothelial growth factor D
4	VEGFR1	VEGFR1	vascular endothelial growth factor receptor 1
5	VEGFR2	VEGFR2	vascular endothelial growth factor receptor 2
6	VEGFR3	VEGFR3	vascular endothelial growth factor receptor 3
7	PDGF-A	PDGF-A	platelet-derived growth factor A
8	PDGF-B	PDGF-B	platelet-derived growth factor B
9	PDGF-C	PDGF-C	platelet-derived growth factor C
10	PDGF-D	PDGF-D	platelet-derived growth factor D
11	PDGFR-α	PDGFR-α	platelet-derived growth factor receptor alpha
12	PDGFR-β	PDGFR-β	platelet-derived growth factor receptor beta
13	FGF2	FGF2	fibroblast growth factor 2
14	FGFR1	FGFR1	fibroblast growth factor receptor 1
15	Notch1		neurogenic locus notch homolog protein 1
16	Notch4	Notch4	neurogenic locus notch homolog protein 4
17	Jag 1	Jag 1	jagged 1
18	DLL4	DLL4	delta ligand 4
19	SKB2		SHK1 binding protein
20	ME2		malic enzyme 2
21	HIF1	HIF1	hypoxia inducible factor 1
22	HIF2	HIF2	hypoxia inducible factor 2
23	GLUT1		glucose transporter 1
24	LDH5	LDH5	lactate dehydrogenase A
25	CAIX	CAIX	carbonate dehydratase IX
26	Ang1	Ang1	angiopoietin 1
27	Ang2	Ang2	angiopoietin 2
28	Ang4	Ang4	angiopoietin 4
29	Tie2	Tie2	angiopoietin 1 receptor
30	PHD1		prolyl hydroxylase domain containing protein 1
31	PHD2		prolyl hydroxylase domain containing protein 2
32	PHD3		prolyl hydroxylase domain containing protein 3
33	FIH		factor inhibiting HIF-1
34	MMP2	MMP2	matrix metalloproteinase 2
35	MMP7		matrix metalloproteinase 7
36	MMP9	MMP9	matrix metalloproteinase 9
37	IntA5B1		Integrin alpha 5 beta 1
38	MET-k		hepatocyte growth factor receptor
39	MET-g		phospho-Met (Tyr1234/1235)
40	MCT1	MCT1	monocarboxylate transporter 1

41	MCT2	MCT2	monocarboxylate transporter 2
42	MCT3	MCT3	monocarboxylate transporter 3
43	MCT4	MCT4	monocarboxylate transporter 4
44	PGC1-α		peroxisome proliferator activated receptor gamma coactivator 1
45		agma	alpha smooth muscle Actin
46	COL4A3	COL4A3	collagen type IV alpha 3
47	End		endostain
48	TSP1		thrombospondin 1
49	D240	D240	podoplanin
50	CD34	CD34	
51		CD31	
52	CD4	CD4	
53	CD45	CD45	
54	CD8	CD8	
55	CSF1R	CSF1R	colony stimulating factor 1 receptor
56	MCSF	MCSF	macrophage colony-stimulating factor
57	CD68	CD68	
58	CD20	CD20	
59	CD56	CD56	
60	CD1A	CD1A	
61	CD3	CD3	
62	CD138	CD138	
63		CD117	
64	CXCL16	CXCL16	chemokine CXC motif ligand 16
65	CXCR6	CXCR6	chemokine CXC motif receptor 6
66	FOXP3	FOXP3	forkhead box protein P3
67	tAkt	tAkt	phospho-Akt (Thr308)
68	sAkt	sAkt	phospho-Akt (Ser473)
69	bAkt	bAkt	Akt2
70	cAkt	cAkt	Akt3
71	pi3k	pi3k	phosphatidylinositol 3 kinase
72	PTEN	PTEN	phosphatase and tensin homolog
73	pHer1		phospho- human epidermal growth factor receptor 1
74	pHer2		phospho- human epidermal growth factor receptor 2
75	pHer3		phospho- human epidermal growth factor receptor 3
76	Her4		phospho- human epidermal growth factor receptor 4
77	NfKB	NfKB	nuclear factor kappa-B
78	Vimentin	Vimentin	
79	Par6	Par6	partitioning-defective protein 6
80	APKc		protein kinase C zeta type
81	E-cadherin		epithelial calcium dependant adhesion protein
82	Fascin		
83	TGF-β		transforming growth factor beta
84	Bad		Bcl-2 associated death promotor

85		Masson	masson's trichrome
86	COX-2	COX-2	cyclooxygenase-2
87	IGF1	IGF1	insulin like growth factor 1
88	IGFBP2	IGFBP2	insulin like growth factor binding protein 2
89	Dicer		double-strand-specific ribonuclease
90	Drosha		double stranded RNA specific endoribonuclease 3
91	ERK3_N		extracellular signal-regulated kinase(nucleus)
92	ERK3_C		extracellular signal-regulated kinase(cytoplasm)
93		ERK3	extracellular signal-regulated kinase
94	p27		cyclin-dependent kinase inhibitor 1B
95	p21		cyclin-dependent kinase inhibitor 1A
96	p16		cyclin-dependent kinase inhibitor 2A
97	FOXO1A	FOXO1A	forkhead box protein O1A
98	pFOXO1A	pFOXO1A	Phospho- forkhead box protein O1A (phospho-S256)
99	Ki67	Ki67	
100	miR182b		micro RNA 182b
101	miR21	miR21	micro RNA 21
102	miR210	miR210	micro RNA 210
103	miR126		micro RNA 126
104	miR155	miR155	micro RNA 155

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Original contribution

Evaluation of tumor-infiltrating lymphocytes using routine H&E slides predicts patient survival in resected non–small cell lung cancer☆,☆☆

Mehrdad Rakaee MSc[a,*], Thomas K. Kilvaer MD, PhD[b,c], Stig Manfred Dalen MD[d], Elin Richardsen MD, PhD[a,d], Erna-Elise Paulsen MD, PhD[b,c], Sigurd M. Hald MD, PhD[c], Samer Al-Saad MD, PhD[a,d], Sigve Andersen MD, PhD[b,c], Tom Donnem MD, PhD[b,c], Roy M. Bremnes MD, PhD[b,c], Lill-Tove Busund MD, PhD[a,d]

[a]*Department of Medical Biology, UiT The Arctic University of Norway, Tromsø, Norway, 9019*
[b]*Department of Oncology, University Hospital of North Norway, Tromsø, Norway, 9019*
[c]*Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway, 9019*
[d]*Department of Clinical Pathology, University Hospital of North Norway, Tromsø, Norway, 9019*

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Prognosis;
Lung cancer;
Immunoscore

Summary The presence of tumor-infiltrating lymphocytes (TILs) positively impacts the outcome of non–small cell lung cancer (NSCLC) patients. Most previous studies have assessed TILs using different immunohistochemical assays. The purpose of this study was to develop and validate a histopathological scoring model for the assessment of TILs in whole-tissue hematoxylin and eosin (H&E)–stained section slides of NSCLC patients and to evaluate the model in an immunoscore setting. Therefore, TIL was evaluated manually on H&E slides from 537 surgical specimens of primary resected stage I-III NSCLC patients. Using stromal TIL score as a stepwise discrete variable, increasing survival was seen with rising TIL level: disease-specific survival (DSS; $P = .008$), overall survival ($P = .036$) and disease-free survival ($P = .006$). Subgroup analysis revealed that high stromal TILs level was associated with superior DSS ($P = .047$) in patients with squamous cell carcinoma, but not in patients with adenocarcinoma. Multivariable analysis confirmed that high TIL levels independently predict improved prognosis for all endpoints in the overall cohort. In conclusion, high stromal TIL level is an independent favorable prognostic factor in stage I-III NSCLC patients. The comprehensive histological evaluation conducted in this study may be helpful in streamlining TIL quantification for routine clinical use in a future NSCLC immunoscore setting.

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* Corresponding author at: Translational Cancer Research Group, Department of Medical Biology, UiT The Arctic University of Norway, 9019 Tromsø, Norway.
E-mail addresses: Mehrdad.r.khanehkenari@uit.no (M. Rakaee), kilvaer@gmail.com (T. K. Kilvaer), Stig.Manfred.dalen@unn.no (S. M. Dalen), elin.richardsen@unn.no (E. Richardsen), epa014@post.uit.no (E. -E. Paulsen), sigurd.hald@uit.no (S. M. Hald), samer.al-saad@unn.no (S. Al-Saad), sigve.andersen@uit.no (S. Andersen), tom.donnem@uit.no (T. Donnem), roy.bremnes@uit.no (R. M. Bremnes), lill.tove.busund@unn.no (L. -T. Busund).

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1. Introduction

The characteristics of the tumor microenvironment (TME), in particular immune cells, has been a topic of considerable interest in non–small cell lung cancer (NSCLC). Different immune profiles have been proposed as prognostic and predictive factors for NSCLC patients [1,2]. With the advent of immunotherapy, assessment of the tumor immune-contexture has become an even more important clinical consideration [3].

Tumor-infiltrating lymphocytes (TILs) constitute the predominant immune cell populations in the TME. TILs belong to both the adaptive and innate arms of the immune system. In NSCLC, commonly detected TIL subsets associated with a positive clinical outcome, and previously reported by our group and others, are CD3+, CD4+, CD8+, CD20+, CD45RO+ [4-6]. Intriguingly, the presence of TILs expressing immune-checkpoint regulators PD-1, PD-L1, CTLA-4 and LAG-3 were also associated with improved survival [7-9].

The TNM classification system divides solid tumors into strictly localized or more advanced disease (stages I-IV). Although the TNM classification, combined with histological and genetic features of the tumor, provide valuable prognostic information, a considerable variation in prognosis is reported within the same TNM stage [10]. Recently, assessments of the immune contexture in solid tumors has supplemented reliable prognostic and predictive data to the TNM classification [11-13]. Our group has previously proposed potential candidate immune cell markers to establish a TNM immunoscore (TNM-I) for NSCLC [5-7,14].

Most studies evaluating TILs in lung cancer have applied immunohistochemistry (IHC) to differentiate TIL subsets and to assess their density, distribution and localization. A standardized methodology to assess TILs using routine hematoxylin and eosin (H&E) is an attractive alternative to IHC as it easily integrates into the workflow of pathology laboratories without the need of extra staining protocols. Ease of use may prove H&E TILs as a valuable marker when establishing an immunoscore for NSCLC patients. Only a limited number of lung cancer studies have explored TILs in whole-tissue H&E–stained section (WT-HE) slides [15-19]. A few of these studies used a similar scoring scheme for the evaluation of TILs [15,19]. However, no consensus has been reached on a standard quantification of TILs in lung cancer WT-HE slides.

This study aims to validate a comprehensive pathological assessment of TILs, originally proposed for breast cancer [20,21], for use on NSCLC tissues. Stromal TILs in WT-HE slides from 537 surgically resected stage I-III patients were assessed and confirmatory evidence of the prognostic value of TILs for these patients is provided. Further, the potential of stromal TILs in the setting of a NSCLC TNM-I is explored.

2. Materials and methods

2.1. Patient cohort

Clinical and pathological data were obtained through a detailed retrospective review of the medical records of 633 consecutive patients with NSCLC who had undergone radical resection between 1990 and 2010. Out of 633 patients, 96 were excluded from the study due to: neoadjuvant radio-chemotherapy (n = 15), other malignancy within 5 years before NSCLC diagnosis (n = 39), inadequate tissue in paraffin-embedded formalin fixed blocks (n = 26) and de-colored staining or poor tissue quality (n = 16). Thus, 537 patients with complete demographic and clinicopathological data were eligible for this study.

Most of the patients diagnosed at clinical stage II-III received adjuvant chemotherapy. After postoperative recurrence, eligible patients underwent cytotoxic and targeted therapies. A few patients received radiotherapy and/or resections due to recurrent disease.

The median follow-up of survivors was 86 months (range 34–267 months). Follow up data was last updated October 1, 2013.

The tumors have been restaged in accordance with the 8th edition of the TNM classification [22], published by the Union for International Cancer Control (UICC) and patient samples have been histologically reclassified based on the 4th edition of the 2015 World Health Organization (WHO) Classification of Lung Tumors [23]. REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines were followed in this study [24]. Further information regarding cohort has previously been published [9].

The study was approved by the Norwegian Data Protection Authority and the Regional Committee for Medical and Health Research Ethics (protocol ID, 2016/714).

2.2. Histological evaluation

Histological analysis of TILs was performed on WT-HE slides. TILs were assessed by two observers (S.M.D, M.R), who were blinded to each other, clinical data and patient outcome, using a four-tiered scale designed by four experienced lung cancer pathologists (L.T.B, E.R, S.A.S, S.M.D). The percentage of tumor stroma containing mononuclear immune cells (including lymphocytes and plasma cells) was classified as: 0 = 0–5%, 1 = 6%–25%, 2 = 26%–50% and 3 >50%. To obtain an estimate of mean infiltrative area, TILs were assessed in multiple stromal regions and not only in hot spots. In case of disagreement of 2 or more scale units, slides were reevaluated until the observers reached a consensus. Supplementary Fig. S1 is a schematic representation of which areas were selected for assessment. The histomorphology of the different groups according to the scoring scheme were as follows: (0) The stroma has completely loose or dense fibroblastic appearance with no/very rare immune cells; (1) Loose and

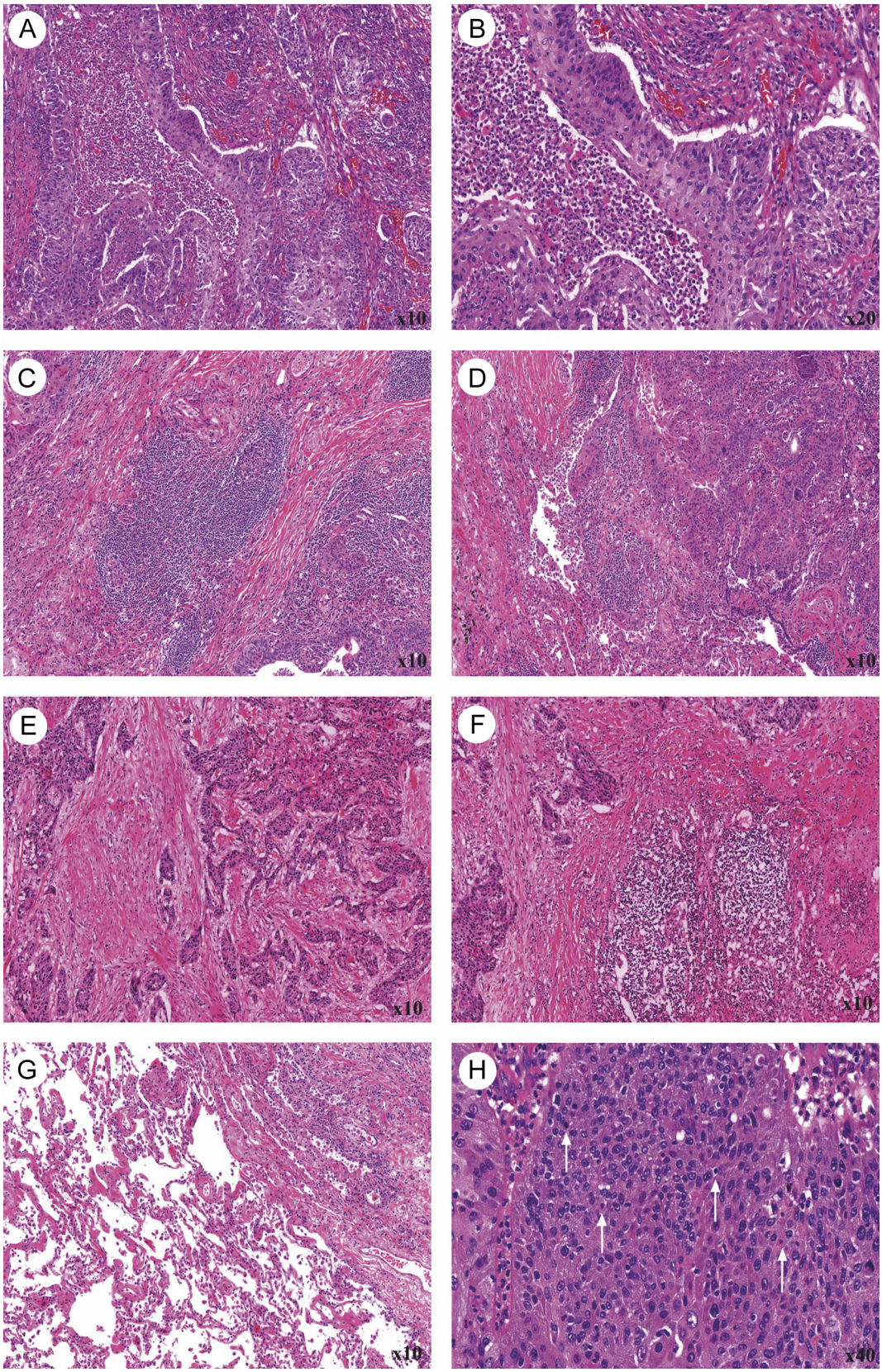

Fig. 1 Standard H&E–stained sections of NSCLC. A and B, Islands of necrotic squamous cells with high presence of neutrophils. C, Tertiary lymphoid structure at the tumor border. D, Lymphoid aggregates without germinal center. E and F, Both images from a single patient specimen represents irregular tumor border: (E) high fibrotic tumor edge, (F) inflammatory cell reaction in tumor edge, (G) adjacent normal lung, (H) TILs and apoptotic cells (white arrows) within neoplastic epithelial cells, (×10, ×20, ×40 magnifications).

Table 1 Clinicopathological variables as predictors of disease-specific survival in NSCLC patients

	Total (%)	5-Year (%)	Median (mo)	HR (95%CI)	*P*
Age					.630
<65	227 (42)	57	134	1	
≥65	310 (58)	58	144	0.94 (0.72–1.22)	
Gender					.040 [a]
Female	173 (32)	63	152	1	
Male	364 (68)	55	133	1.35 (1.03–1.79)	
Weight loss					1.000 [a]
<10%	483 (90)	58	138	1	
>10%	53 (10)	59	148	1 (0.62–1.6)	
Missing	1 (0)				
Smoking					.030 [a]
Never	19 (4)	50	117	1	
Present	343 (64)	62	146	0.64 (0.31–1.35)	
Previous	175 (33)	50	126	0.9 (0.42–1.93)	
ECOG-PS					.020 [a]
0	316 (59)	62	143	1	
1	184 (34)	52	106	1.47 (1.1–1.96)	
2	37 (7)	51	126	1.47 (0.78–2.76)	
Histology					.020 [a]
SCC	298 (55)	63	108	1	
ADC	232 (43)	51	95	1.26 (0.97–1.65)	
LCC	3 (1)	100	170	0 (0–0)	
ASC	3 (1)	50	92	1.74 (0.15–20.22)	
NOS	1 (0)	0	11	10.43 (0.03–4186.78)	
Tstage					<.001 [a]
T1a	14 (3)	93	186	1	
T1b	70 (13)	80	179	1.33 (0.6–2.96)	
T1c	92 (17)	63	134	3.21 (1.46–7.07)	
T2a	132 (25)	57	125	3.79 (1.75–8.2)	
T2b	72 (13)	47	107	4.84 (2.13–11)	
T3	100 (19)	56	130	3.68 (1.67–8.13)	
T4	57 (11)	26	71	8.81 (3.63–21.38)	
Nstage					<.001 [a]
N0	365 (68)	69	156	1	
N1	117 (22)	37	83	2.74 (1.92–3.93)	
N2	55 (10)	20	52	4.25 (2.45–7.36)	
Pstage					<.001 [a]
IA1	9 (2)	89	153	1	
IA2	61 (11)	83	168	0.67 (0.25–1.77)	
IA3	71 (13)	70	136	1.61 (0.61–4.26)	
IB	85 (16)	69	136	1.52 (0.58–3.98)	
IIA	45 (8)	59	118	2.1 (0.76–5.83)	
IIB	136 (25)	59	112	2.3 (0.89–5.96)	
IIIA	109 (20)	28	65	5.15 (1.93–13.72)	
IIIB	21 (4)	0	20	10.03 (2.54–39.54)	
Differentiation [b]					<.001 [a]
Poor	230 (43)	48	120	1	
Moderate	228 (42)	62	137	0.68 (0.51–0.91)	
Well	79 (15)	73	190	0.38 (0.26–0.56)	
Vascular invasion					<.001 [a]
No	442 (82)	62	131	1	
Yes	92 (17)	36	89	1.95 (1.31–2.89)	
Missing	3 (1)				

Abbreviations: ECOG-PS, Eastern Cooperative Oncology Group performance status; ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; ASC, adenosquamous carcinoma; NOS, not otherwise specified; Nstage, nodal stage; Pstage, pathological stage; Tstage, tumor stage.

[a] Statistically significant

[b] Differentiation data is based on the 2004 World Health Organization (WHO) Classification of Lung Tumors.

Table 2 Correlations between clinicopathological variables and TILs in resected tumor tissue from NSCLC patients

	Tumor-infiltrating lymphocytes				
	0–5%	6%–25%	26%–50%	>50%	*P*
Total	52	270	167	48	
Age					.456
<65	22	121	62	19	
≥65	30	149	105	29	
Gender					.099
Female	13	78	65	17	
Male	39	192	102	31	
Weight loss					.648
<10%	46	239	153	45	
>10%	6	30	14	3	
Missing		1			
Smoking					.315
Never	3	11	5	0	
Present	32	168	105	38	
Previous	17	91	57	10	
ECOG-PS					.005 [a]
0	23	150	106	37	
1	23	96	55	10	
2	6	24	6	1	
Histology					.59
SCC	27	142	97	32	
ADC	25	124	68	15	
LCC	0	1	1	1	
ASC	0	2	1	0	
NOS	0	1	0	0	
Tstage					.080
T1a	0	8	1	5	
T1b	8	32	23	7	
T1c	8	39	40	5	
T2a	15	70	35	12	
T2b	6	33	26	7	
T3	12	52	29	7	
T4	3	36	13	5	
Nstage					.599
N0	31	189	113	32	
N1	15	53	40	9	
N2	6	28	14	7	
Pstage					.380
IA1	0	5	1	3	
IA2	6	27	22	6	
IA3	5	34	29	3	
IB	7	51	20	7	
IIA	4	18	17	6	
IIB	18	65	42	11	
IIIA	10	56	32	11	
IIIB	2	14	4	1	
Differentiation [b]					.225
Poor	26	108	71	25	
Moderate	21	117	69	21	
Well	5	45	27	2	
Vascular invasion					.337
No	45	226	135	36	
Yes	6	44	30	12	
Missing	1		2		

Abbreviations: ECOG-PS = Eastern Cooperative Oncology Group performance status, ADC = adenocarcinoma, SCC = squamous cell carcinoma, LCC = large-cell carcinoma, ASC = adenosquamous carcinoma, NOS = not otherwise specified, Nstage = nodal stage, Pstage = pathological stage, Tstage = tumor stage.

[a] Statistically significant.

[b] Differentiation data is based on the 2004 World Health Organization (WHO) Classification of Lung Tumors.

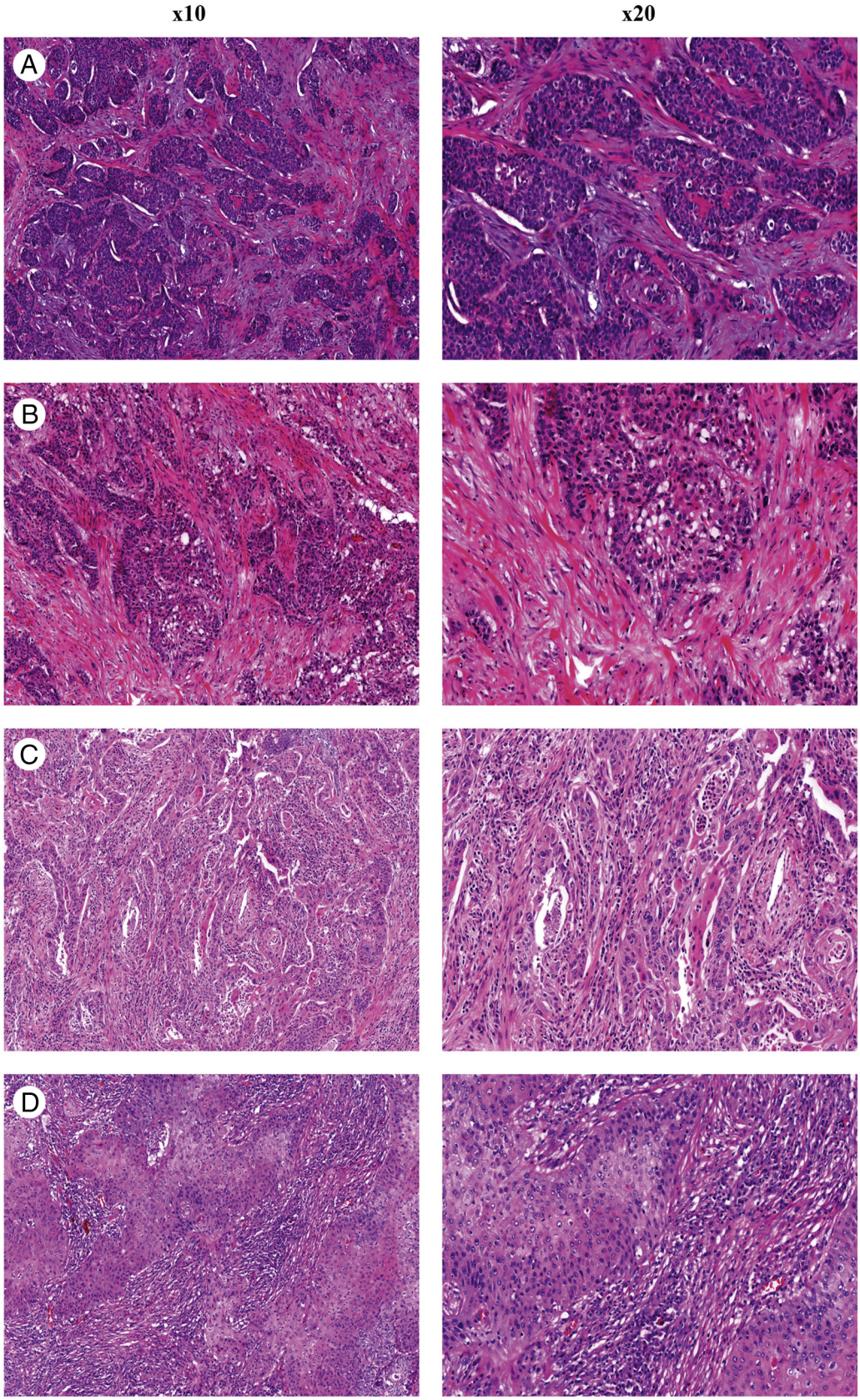

Fig. 2 Low to high level of stromal TILs in NSCLC on hematoxylin and eosin–stained sections. Infiltration scale (A) 0: 0-5%, (B) 1: 6%-25%, (C) 2:26%-50% (D) 3: >50% (×10 and ×20 magnification, left and right column)

Table 3 TILs evaluated in whole tissue H&E–stained slides from lung cancer patients as predictors of OS, DSS and DFS

	OS					DSS					DFS				
	N (%)	5-Year (%)	Median (mo)	HR(95%CI)	*P*	N (%)	5-Year (%)	Median (mo)	HR(95%CI)	*P*	N (%)	5-Year (%)	Median (mo)	HR(95%CI)	*P*
TILs					.036 [a]					.008 [a]					.006 [a]
0–5%	52 (9)	34	60	1		52 (9)	42	106	1		52 (9)	33	77	1	
6%–25%	270 (49)	41	73	0.82 (0.56–1.18)		270 (49)	54	131	0.71 (0.43–1.17)		270 (49)	48	116	0.66 (0.41–1.05)	
26%–50%	167 (30)	50	85	0.68 (0.47–1)		167 (30)	63	140	0.6 (0.36–1.01)		167 (30)	56	121	0.58 (0.36–0.95)	
>50%	48 (9)	59	96	0.56 (0.36–0.89)		48 (9)	77	179	0.34 (0.18–0.62)		48 (9)	73	155	0.37 (0.21–0.65)	

Abbreviations: OS = overall survival, DSS = disease-specific survival, DFS = disease-free survival, HR = hazard ratio, CI = confidence interval.
[a] Statistically significant.

scattered pattern of TILs within the stroma; (2) TILs are more often localized adjacent to neoplastic epithelial cells, and in some stromal areas TILs form patchy lymphocytic aggregates; (3) Highly dense infiltration of immune cells into the stroma. In breast cancer this group has been termed lymphocyte-predominant breast cancer or LPBC [20,25].

The stromal areas and parameters excluded from assessment were as follows: (1) Tumor associated stromal granulocytes (TAGs): Neutrophils and eosinophils were excluded (Fig. 1A and B). (2) Tertiary lymphoid structures (TLS): Follicular lymphoid aggregates with a central germinal center located in the stroma adjacent to tumor epithelium were considered as TLS and excluded (Fig. 1C). In addition, distal lymphoid aggregates without germinal center situated at the invasive margin were ignored to assess (Fig. 1F). (3) Necrosis: Intact tumor and intervening stromal tissue was evaluated in full-tissue disregarding areas with crushing artifacts or necrosis (Fig. 1A). (4) Tumor border: TILs outside of the juxtatumoral stromal area, such as the invasive margin, were ignored (Fig. 1E and F). (5) Normal lung tissue: TILs located in normal lung tissue adjacent to the tumor were ineligible to count (Fig. 1G).

2.3. Statistical procedures

Statistical analyses were performed using the R project (version 3.2.2) for statistical computing. The associations between TILs presence and clinicopathological markers were compared using either Chi-squared or Fisher's exact tests whenever appropriate. For univariate analyses, the Kaplan–Meier method was used to estimate survival curves and the log-rank test was used to compare groups. Disease-specific survival (DSS), disease-free survival (DFS) and overall survival (OS) were defined as the time from surgery to lung cancer death, to first lung cancer relapse, and to death of any cause, respectively. Multivariate analyses were conducted using Cox-regression models. All clinicopathological variables with $P < .25$ from the univariate analyses were entered into the multivariate Cox regression analysis. The final multivariable models were selected through a supervised manual approach. The significance level used was $P < .05$.

3. Results

3.1. Clinicopathological characteristics

The 537 tumor specimens comprised 298 squamous cell carcinomas (SCC), 232 adenocarcinomas (ADC), 3 large cell carcinomas (LCC) and 3 adenosquamous carcinoma (ASC) and 1 carcinoma NOS. The ADC patients were further subtyped as follows: solid (n = 89), acinary (n = 80), papillary (n = 42), micropapillary (n = 17) and mucinous lepidic (n = 4). Distribution of patients according to the UICC 8th TNM staging were: I (n = 226), II (n = 181),

Table 4 Multivariable models summarizing significant independent prognostic factors for OS, DSS and DFS

	OS		DSS		DFS	
	HR(95%CI)	*P*	HR(95%CI)	*P*	HR(95%CI)	*P*
TILs						
0–5%	1		1		1	
6%–25%	0.76 (0.54–1.07)	.114	0.69 (0.45–1.07)	.096	0.66 (0.44–0.98)	.041 [a]
26%–50%	0.65 (0.45–0.93)	.019 [a]	0.63 (0.39–1)	.050 [a]	0.62 (0.4–0.94)	.026 [a]
>50%	0.51 (0.32–0.82)	.006 [a]	0.3 (0.15–0.6)	<.001 [a]	0.34 (0.19–0.64)	<.001 [a]
Age	1.03 (1.02–1.04)	<.001 [a]				
Gender						
Female	1		1			
Male	1.36 (1.08–1.71)	.008 [a]	1.31 (0.97–1.77)	.076		
Pstage						
IA1	1		1		1	
IA2	0.74 (0.29–1.93)	.541	0.58 (0.12–2.7)	.487	0.96 (0.22–4.25)	.958
IA3	1.08 (0.43–2.73)	.867	1.17 (0.27–4.99)	.835	1.7 (0.4–7.16)	.468
IB	1.22 (0.49–3.04)	.676	1.08 (0.26–4.6)	.913	1.7 (0.41–7.09)	.469
IIA	1.61 (0.63–4.14)	.319	1.54 (0.35–6.73)	.566	2.05 (0.48–8.79)	.335
IIB	1.23 (0.5–3.06)	.648	1.6 (0.39–6.62)	.515	2.18 (0.53–8.97)	.278
IIIA	2.62 (1.06–6.48)	.038 [a]	3.84 (0.93–15.84)	.063	4.55 (1.11–18.7)	.036 [a]
IIIB	5.16 (1.89–14.03)	.001 [a]	6.49 (1.46–28.81)	.014 [a]	8.12 (1.84–35.84)	.006 [a]
Differentiation [b]						
Poor			1		1	
Moderate			0.88 (0.66–1.18)	.396	0.87 (0.67–1.13)	.31
Well			0.55 (0.33–0.9)	.020 [a]	0.48 (0.3–0.76)	.002 [a]
Vascular invasion						
No	1		1		1	
Yes	1.69 (1.29–2.2)	<.001 [a]	1.72 (1.23–2.4)	.001 [a]	1.41 (1.03–1.92)	.030 [a]

Abbreviations: OS = overall survival, DSS = disease-specific survival, DFS = disease-free survival, HR = hazard ratio, CI = confidence interval, Pstage = pathological stage.

[a] Statistically significant.

[b] Differentiation data is based on the 2004 World Health Organization (WHO) Classification of Lung Tumors.

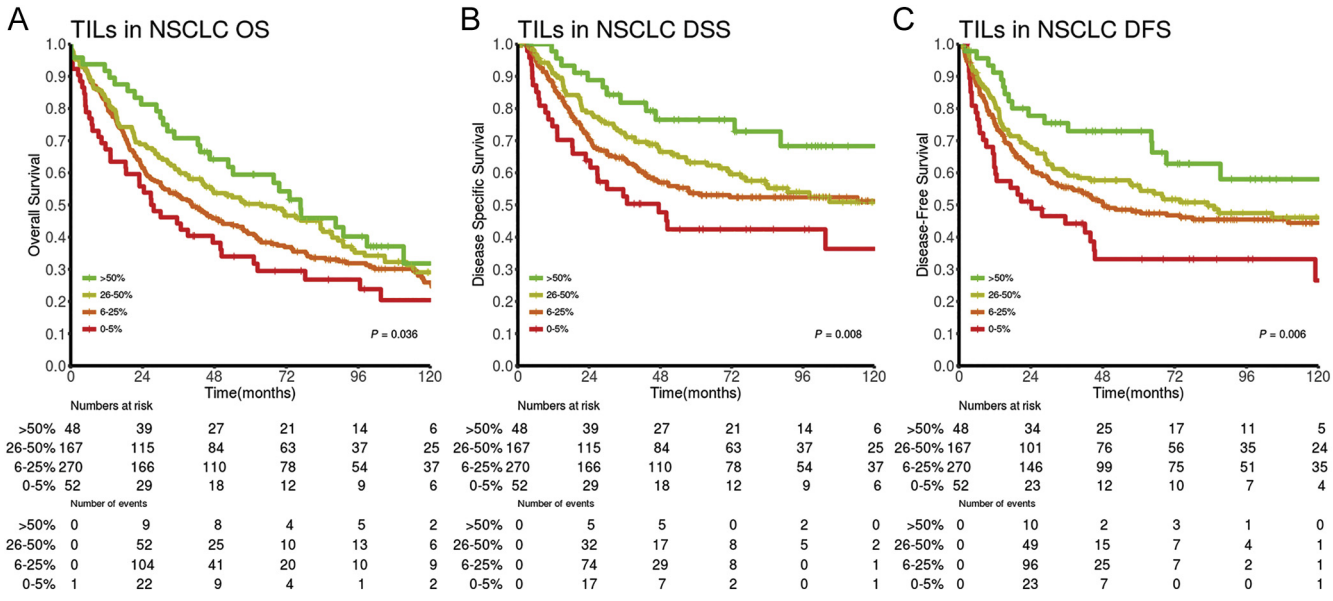

Fig. 3 Survival curves for the overall cohort according to stromal TIL levels. (A) overall survival; (B) disease-specific survival; (C) disease-free survival.

III (n = 130) (Table 1). For further analyses the cohort was divided in four groups based on TIL level. There were no major differences in clinicopathological characteristics between patients with high to minimal TIL levels in the overall cohort (Table 2).

3.2. Interobserver validity

Scores from the observers were compared using a two-way random effects model measuring absolute agreement of values. The intraclass correlation coefficients (ICC) showed an excellent agreement (ICC = 0.84, $P < .001$) between the observers (S.M.D, M.R).

3.3. TILs distribution

Lymphocyte infiltration was found within the tumors, around the tumor (stromal) and at the tumor edges. Based on the assessment criteria summarized in Supplementary Table S1, the cohort was classified into four semi-quantitative categories according to TIL levels: score 0 (9%, 52 of 537), score 1 (49%, 270 of 537), score 2 (30%, 167 of 537), and score 3 (9%, 48 of 537). Example images of representative stromal TIL scores are provided in Fig. 2A-D.

3.4. Survival analysis

The results of the univariate analyses are presented in Table 3 and Fig. 3. In the overall cohort, high TIL level was associated with improved DSS ($P = .008$), DFS ($P = .006$) and OS ($P = .036$). Five-year survival rates for high, moderate, mild and minimal TILs score in the overall cohort were as follows: OS: 59%, 50%, 41% and 34%; DSS: 77%, 63%, 54% and 42%; DFS: 73%, 56%, 48% and 33%, respectively.

Subgroup analyses showed that high TILs score was a positive prognostic factor for DSS ($P = .047$) and with a positive trend observed for OS (P = .058) and DFS ($P = .054$) in the SCC subgroup (Supplementary Fig. S2, A-C). No significant association with survival was found for ADC patients (Supplementary Fig. S2, D-F).

Table 4 summarizes the multivariable models for OS, DSS and DFS in the overall cohort. High TIL levels were an independent positive predictor of OS (HR 0.51 95% CI 0.32–0.82, $P = .006$), DSS (HR 0.3 95% CI 0.15–0.6, $P < .001$) and DFS (HR 0.34 95% CI 0.19–0.64, $P < .001$). In addition to TILs level, pathological stage, histological differentiation, vascular invasion, age and gender were significant independent indicators for almost all endpoints.

4. Discussion

Our group, and others, have previously demonstrated that the presence of different TIL subsets (CD3, CD4, CD8, CD20 and CD45RO) are associated with a marked survival advantage, independent of tumor stage, in NSCLC patients [4-6]. The present study examined the prognostic relevance of TILs in whole-tissue hematoxylin and eosin-stained (WT-HE) slides of NSCLC surgical specimens, utilizing and validating a structured and reproducible evaluation method previously proposed for breast cancer patients. The findings indicate that an increasing stromal TIL levels is an independent positive prognosticator associated with a significantly lower risk of progression, lower overall mortality and an improved DSS in NSCLC patients. To the best of our knowledge, this is the first large study to incorporate a well-defined scoring instruction for reliable evaluation of TILs on WT-HE slides in patients with lung cancer.

The prognostic influence of in situ immune cell infiltrates in primary NSCLC was described decades ago, often in the context of small cell carcinoma [26]. However, only a few studies have used standard H&E stained slides instead of IHC to evaluate lymphocyte infiltration in resected lung tissue [15-19,27]. Survival benefit associated with high TIL density was observed in three of the studies, in which patients cohort were limited either to pathological stage I [17,18] or III [19]. Ruffini et al showed that TILs are more frequent in neuroendocrine tumors and positively associated with outcome in the SCC subgroup, but not in the overall cohort [16]. In a large TMA-based study, the presence of CD8+ cells was correlated with an improved survival while TILs evaluated by H&E failed to reach statistical significance [27]. In the present study, stromal TILs were associated with DSS, OS and DFS ($P = .008$, $P = .03$, $P = .006$ respectively) in the overall cohort. This is in concordance with a large and recent NSCLC study by Brambilla et al [15]. After stratification by histotype, a significant positive prognostic impact of TILs was observed in the SCC subgroup (DSS: $P = .047$).

Despite solid evidence supporting the prognostic impact of TIL density in NSCLC, a standardized scheme for WT-HE TIL assessment has not been established. In this study, the recommendation for TIL evaluation in breast cancer [20] was adapted for use on lung cancer tissue. To design a consistent framework for assessing TILs, the following three components were excluded: TILs within intratumoral areas, invasive margins, and TLS. (A) Intratumoral TILs: In breast cancer, Denkert et al [25] demonstrated that intratumoral TILs had both a prognostic and predictive role. In lung cancer tissue, distinguishing lymphocytes and apoptotic tumor epithelial cells proved challenging in some cases (Fig. 1H). Consequently, intraepithelial TILs were considered unscorable. (B) TILs within the invasive margins: In colorectal cancer (CRC), the importance on the prognostic role of TILs within the invasive margins has been highlighted [28,29]. For example, in hepatic metastases of CRC, high density of CD8+ TILs in the invasive margin predicts a better prognosis and response to chemotherapy. In this study, the invasive margin was defined as an area of 500 μm on each side of the edges between tumor epithelium cells and normal tissue [29]. In our assessment, we were not able to define a unique margin size for all patients. The invasive margin around the tumor nest, for each NSCLC patient,

had a highly unstable pattern (Fig. 1E and F). Moreover, in some specimens, small islands of neoplastic epithelium were observed, making it hard or impossible to have a consistent definition of the invasive margin. Due to these observations, evaluation of TILs at the invasive margin may have low reproducibility and should be excluded in the context of lung cancer. (C) TLS: It has been hypothesized that TLS has a critical anti-tumoral role by induction of a systemic and local T-cell immune response, and TLS have been observed in almost all solid tumors including NSCLC [30]. In lung cancer, the density of mature dendritic cells within TLS was associated with a favorable outcome [31]. Architecturally, TLS are very similar to lymph nodes composed of T-cell–DC clusters, follicular B cells and specialized vessels known as high endothelial venules. Due to the profound presence of immune cells in TLSs and to avoid false positive count, TLS were not included in this setting.

In subgroup analysis according to TNM stage, TIL levels was a near significant prognostic factor for stage II and III patients and a trend could be observed for stage I patients (Supplementary Fig. S3). However, previous studies have observed that a high density of TILs also predicts extended overall survival at earlier stages of disease. Both Ruffini and Horne et al (1290 and 273 patients, respectively) indicated a survival advantage associated with TIL levels in NSCLC stage I [16,17]. In our study, the positive prognostic effect was not statistically significant in stage I for all endpoints, consistent with previous reports in early-stage breast, esophageal and colorectal cancers [32-34]. It appears that TIL level may have a stronger prognostic role for patients with a more aggressive NSCLC phenotype. Nevertheless, these results should be interpreted cautiously and a study powered for subgroup analyses might shed more light on our data, especially for stage I patients. Moreover, as we have previously proposed for stromal CD8 [5], intratumoral CD45RO [6] and the combination of stromal PD-L1 and intratumoral PD-1 [7] in NSCLC, which predicted outcome independently of other variables and within each pathological stage, WT-HE TILs appear to be a promising candidate marker to supplement NSCLC TNM-I with respect to prognosis and stratification for adjuvant therapy. Further, our research group is presently conducting a prospective Scandinavian study (NCT03299478) to validate a NSCLC TNM-I as a prognostic tool in the post-surgical treatment setting. Evaluation of WT-HE TILs may be integrated into this trial.

The novelty of the present study is primarily the demonstration that increasing TIL levels in the tumor stroma were associated with prognostic information. All the statistical analyses were conducted with a four-category discrete cutoff value. In contrast, Feng et al [19] were not able to detect any prognostic impact of TILs after implementation of a four-step method. We believe that an easy-to-use grading scheme, as suggested in this study, will reduce confusion and avert interobserver reproducibility problems, and provide enhanced prognostic accuracy for TILs in NSCLC. Although the evaluation of TILs is lucid, promising, inexpensive, and can be easily introduced into routine histopathology reporting, it remains a semi-quantitative marker and requires further validation utilizing a computer-based cell counter.

5. Conclusions

This study identifies a stepwise increase in stromal TIL levels, assessed on WT-HE slides, to be an independent, favorable prognostic factor in a cohort of resectable stage I-III NSCLC patients. When deciding which TIL identifier to include in a NSCLC TNM-I, the exhaustive morphological TIL assessment using WT-HE slides proposed in this study, may be a favorable choice due to ease of implementation and low cost.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.05.017.

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Figure S1: Schematic model showing NSCLC tissue with the area used for evaluation of TILs. The most advanced edge of the tumor was considered as tumor border or invasive margin and excluded from assessment.

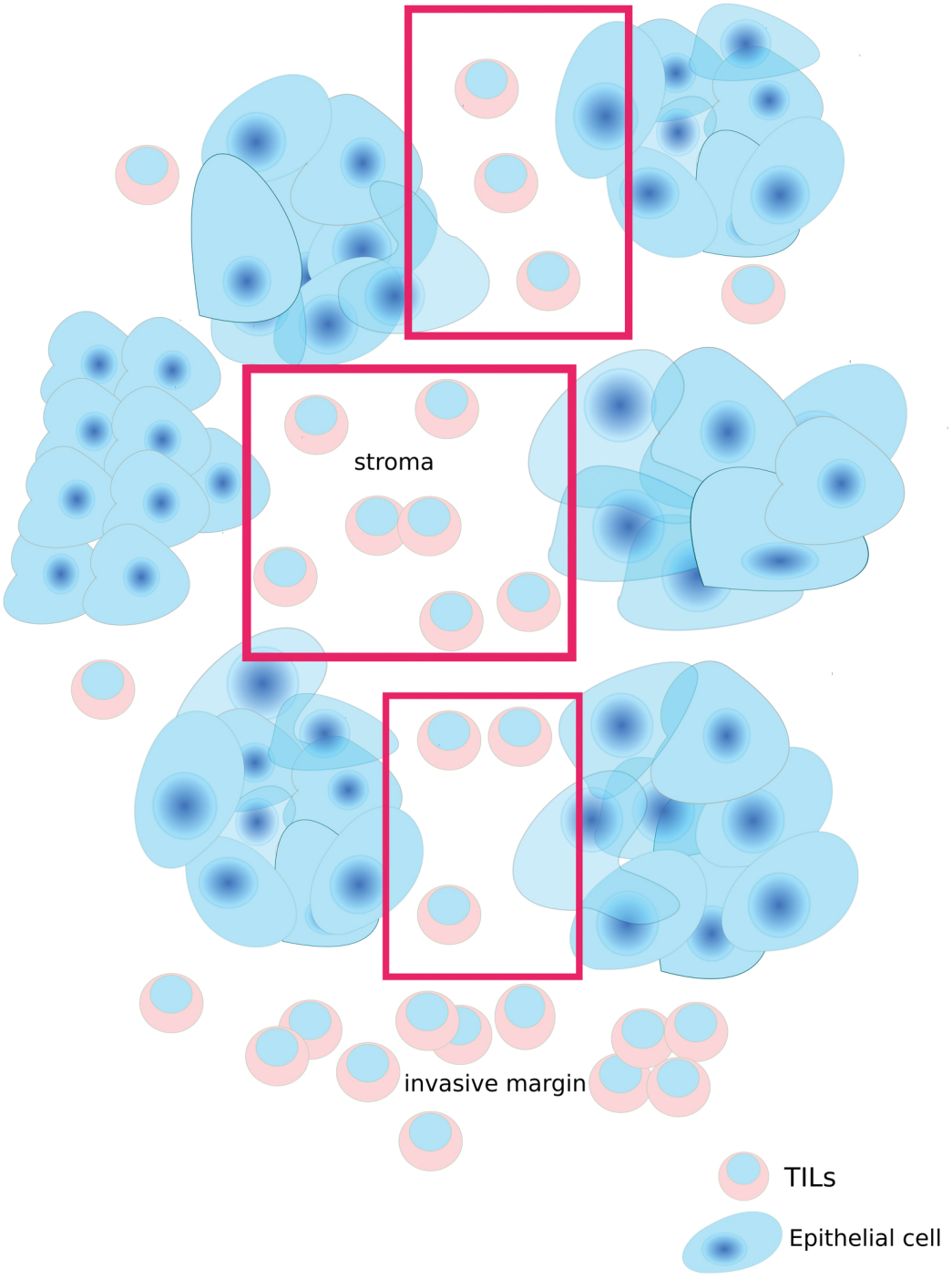

Figure S2: Survival curves according to TIL levels representing OS, DSS and DFS in the SCC (A-C) and ADC (D-F) subgroups, respectively.

Abbreviations: OS, overall survival; DSS, disease-specific survival; DFS, disease-free survival; TIL, tumor-infiltrating lymphocyte

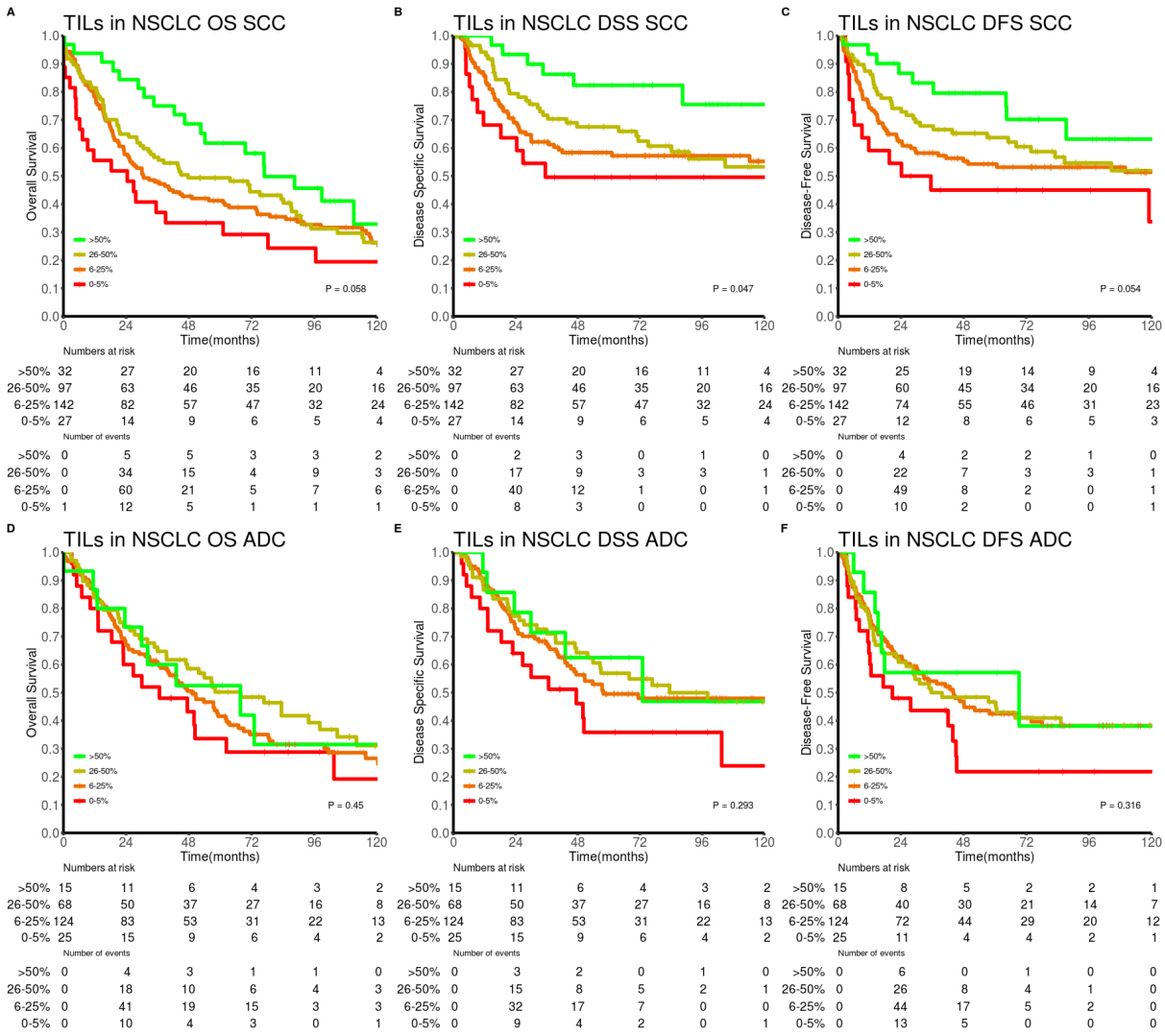

Figure S3: Survival curves for OS (A-C), DSS (E-G) and DFS (I-J) and distribution of patients with similar D) OS; H) DSS; L) DFS, according to pathological stage and TIL levels in the overall cohort (good prognosis: light green; intermediate prognosis: dark green; poor prognosis: orange; very poor prognosis: red). These illustrations highlight how TIL levels may be used to calibrate the current TNM staging system.

Abbreviations: OS, overall survival; DSS, disease-specific survival; DFS, disease-free survival; TIL, tumor-infiltrating lymphocyte

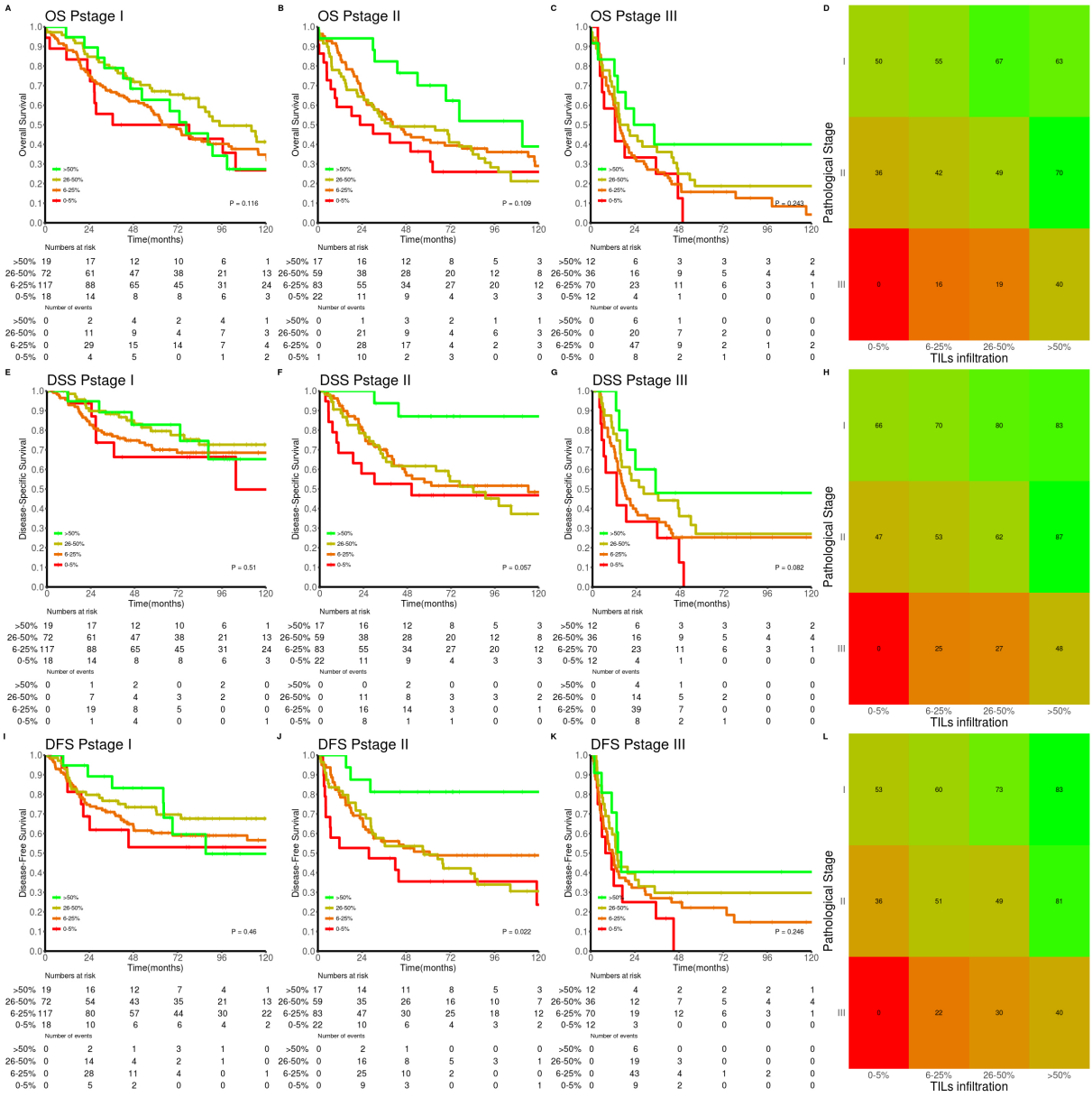

Table S1: Scoring assessment criteria; A) Scoring scale and morphological characteristics of TILs in tumor stroma, B) Parameters excluded in the evaluation.

A		
Score	**TIL %**	**Stroma configuration**
0	0-5%	very rare immune cells, almost no cell
1	6-25%	loose, scattered immune infiltration
2	26-50%	patchy aggregates of mononuclear inflammatory cells
3	>50%	severe diffuse, dense, more lymphocytes than tumor cells

B	
Area	**Cells**
Intra-epithelial Necrotic and pre-necrotic Distal stroma Normal lung Invasive margin Crushing artifact	TAG: TAN, TAE TAM Adjacent/distal follicular lymphoid aggregates: TLS

Abbreviations: TILs=tumor-infiltrating lymphocytes, TAG=tumor-associated granulocytes, TAN= tumor-associated neutrophils, TAE= tumor associated-eosinophils, TAM= Tumor-associated macrophages, TLS= tertiary lymphoid structure

Title page

Prognostic value of macrophage phenotypes in resectable non-small cell lung cancer assessed by multiplex immunohistochemistry

Mehrdad Rakaee, MSc[1]; Lill-Tove Rasmussen Busund, MD, PhD[1,2]; Simin Jamaly, PhD[3]; Erna-Elise Paulsen, MD, PhD[3,4]; Elin Richardsen, MD, PhD[1,2]; Sigve Andersen, MD, PhD[3,4]; Samer Al-Saad, MD, PhD[1,2]; Roy M. Bremnes, MD, PhD[3,4]; Tom Donnem, MD, PhD[3,4]; Thomas K. Kilvaer, MD, PhD[3,4]

[1]Department of Medical Biology, UiT The Arctic University of Norway, Tromsø, Norway, 9019
[2]Department of Clinical Pathology, University Hospital of North Norway, Tromsø, Norway, 9019
[3]Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway, 9019
[4]Department of Oncology, University Hospital of North Norway, Tromsø, Norway, 9019

Corresponding author:
Mehrdad Rakaee
Translational Cancer Research Group
Department of Medical Biology
UiT The Arctic University of Norway
9019 Tromso, Norway
Telephone: +47 77625237
Fax: +47 77672704
E-mail: Mehrdad.r.khanehkenari@uit.no

Running title:
TAM phenotypes in NSCLC

Abstract

Macrophages are important inflammatory cells that regulate innate and adaptive immunity in cancer. Tumor-associated macrophages (TAMs) are thought to differentiate into two main phenotypes, pro-inflammatory M1 and pro-tumorigenic M2. Currently, the prognostic impact of TAMs and their M1 and M2 phenotypes is unclear in non-small cell cancer (NSCLC). The present study was set up to evaluate an approach for identifying common M1 and M2 macrophage markers and explore their clinical significance in NSCLC. Using multiplex chromogenic immunohistochemistry, tissue micro-arrays of 553 primary tumors and 143 paired metastatic lymph nodes of NSCLC specimens were stained to detect various putative macrophage phenotypes: M1 (HLA-DR/CD68), M2 (CD163/CD68), M2 (CD204/CD68) and pan-macrophage (CD68/CK). Correlation analyses were performed to examine the relationship between TAMs and adaptive/innate immune infiltrates. Greater frequency of stromal HLA-DR$^+$/CD68$^+$M1 subpopulation was statistically associated with lower T stage and more favorable ECOG performance status in primary tumors. HLA-DR$^+$/CD68$^+$M1 TAM level significantly decreased from pathological stage I to III. In a compartment-specific correlation analysis, moderate to strong correlations were observed between both TAM subsets (M1 and M2) with CD3, CD8, CD4 and CD45RO positive immune cells. Survival analyses, in both stromal and intra-tumoral compartments, revealed that high levels of HLA-DR$^+$/CD68$^+$M1 (stroma, HR=0.73, *P*=0.03; intra-tumor, HR=0.7, *P*=0.04), CD204$^+$M2 (stroma, HR=0.7, *P*=0.02; intra-tumor, HR=0.6, *P*=0.004) and CD68 (stroma, HR=0.69, *P*=0.02; intra-tumor, HR=0.73, *P*=0.04) infiltration were independently associated with improved NSCLC-specific survival. In lymph nodes, the intra-tumoral level of HLA-DR$^+$/CD68$^+$M1 was an independent positive prognostic indicator (Cox model, HR=0.38, *P*=0.001). In conclusion, high levels of M1, CD204$^+$M2 and CD68 macrophages are independent prognosticators of prolonged survival in NSCLC.

Introduction

In addition to intrinsic mechanisms within neoplastic cancer cells, cancer development depends on complex crosstalk between the tumor and the host's innate and adaptive immune systems [1]. Assessment of the tumor-immune contexture may provide information on the prognostic and predictive value of immune-related biomarkers, and improve understanding of tumor behavior [2,3]. Current knowledge suggests that the composition of the immune response influences the development and prognosis of non-small cell lung cancer (NSCLC) [4]. More recently, immune profiling of NSCLCs has provided prognostic data able to supplement the current TNM classification, producing a TNM-Immune-cell score (TNM-I) model [5]. In search for other immunological markers which could potentially contribute to a NSCLC TNM-I, *in situ* macrophages, known as tumor-associated macrophages (TAMs), are of great interest.

Macrophages constitute a heterogeneous and ubiquitous population of innate myeloid-derived cells, with pivotal roles in phagocytosis, inflammation and tissue repair in both normal homeostasis and disease [6]. In malignancy, TAMs interact with tumor cells to produce a rich source of cytokines, growth factors and proteases that shape the tumor microenvironment [7]. TAMs mainly originate from bone marrow (monocytic precursors), and differentiate according to tumor-derived signals[8]. It is proposed that TAMs polarize into one of two major lineages: M1 (classically activated) and M2 (alternatively activated) [9]. M1 macrophages secrete pro-inflammatory cytokines, largely express MHC class II (such as HLA-DR), and are thought to exhibit anti-tumoral functions through stimulation of T-cell mediated anti-tumor immunity [10]. M2 macrophages are often identified by the expression of CD163 (hemoglobin-scavenger receptor) or CD204 (macrophage-scavenger receptor-1), and are thought to contribute in tumor progression through increased metastatic ability, angiogenesis, immunosuppression via inhibition of the anti-tumoral immunity of both M1 and T-helper (Th1) cells, and by attracting activating regulatory T-cells and Th2 cells [9,11].

The prognostic impact of TAMs is inconsistent for different types of cancer. In a meta-analysis of different solid tumors, the presence of TAMs was associated with unfavorable outcomes in breast, head and neck, ovarian, gastric and bladder carcinomas, and with favorable outcomes in colorectal carcinoma (CRC) [12]. In NSCLC, the prognostic relevance of TAMs is still under debate [13]. Contradictory reports in NSCLC may relate to choice of

marker, low statistical power, homogeneous cohorts (using a particular tumor stage), and wide variation in the used method to assess patterns of macrophage infiltration [14].
The most common marker used to identify TAMs is the pan-macrophage CD68 antibody. However, CD68 is not exclusively expressed by TAMs; and other tumor tissue components (such as malignant epithelial and stromal cells) may express CD68 on their surface to some extent [15]. Moreover, single labeling of macrophages based on CD68 does not distinguish between M1 and M2 subsets. Recent studies attempt to use two or three different macrophage-associated markers to phenotype M1 and M2 and assess their effector functions [16]. Measuring TAMs using multiplex chromogenic immunohistochemistry (IHC) provide subset detail and may have higher detection accuracy, but this is limited to the use of appropriate chromogens for visualizing co-localized markers. The use of translucent chromogens produces color changes at sites of co-localization, allowing easy and reliable identification within the boundaries set by the sensitivity and specificity of the primary antibodies [17].

Due to previous contradictory findings and their wide methodological variation in NSCLC [13], the current study was conducted to profile tissue-based macrophages according to widely accepted M1 (HLA-DR) and M2 (CD163 and CD204) markers in combination with the pan-macrophage marker CD68. TAMs infiltration and association to prognosis was evaluated, in tissues from 553 resected NSCLC specimens and 143 matched lymph nodes, both in cancer cell islets and associated-stroma.

Materials and methods

Study cohort

The study population (previously described in [18,19]) is a consecutive series of 633 stage I-III NSCLC patients operated at University Hospital of North Norway and Nordland Hospital between 1990 and 2010. Of 633 potential cases, 553 were eligible for inclusion and 80 were excluded due to: neoadjuvant therapy before surgical resection (n=15), inadequate tissue in FFPE blocks (n=26), and presence of other malignancies before NSCLC diagnosis (n=39). Of the 553 eligible cases, 172 were diagnosed as LN+, of which 143 (N1, n=97; N2, n=47) had available tissue for assessment. Clinicopathological data were retrieved from clinical records and histopathology reports. The records included follow-up data until October 2013. The median follow-up was 86 months (34-267 months). All tumor specimens were restaged and reclassified by two lung pathologists according to the latest UICC and WHO guidelines [20,21]. The collection and reporting of clinicopathological variables, survival information and marker expression data was conducted according to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines [22]. The study was approved by the Norwegian data protection authority and regional committee for health research ethics (Reference no.2016/714).

Tissue microarray

The tissue microarray (TMA) methodology has been described in detail [23]. Briefly, full-faced tissue section slides were evaluated and the most representative areas were marked on H&E slides. From each patient's FFPE block, four or five representative core punches of 0.6 mm in diameter were transferred from donor to TMA recipient blocks, including two cores from tumor epithelium, two cores from tumor stroma, and one core from the normal alveolar area. TMAs were constructed using a manual MTA-1 tissue arrayer (Estigen, Estonia).

Multiplexed-IHC

The TMA blocks were sectioned at a thickness of 4 μm and baked overnight at 37 °C. The slides were processed using the Ventana Discovery-Ultra platform (Roche, Tucson, USA). The following mouse primary monoclonal antibodies were used for immunostaining: CD68 (clone: KP-1, #790-2931; Ventana), CD163 (clone: MRQ-26, #760-4437; Ventana), CD204

(clone: SRA-E5, #KT022; Transgenic), HLA-DR (clone: TAL.1B5, #M074601-2; Dako) and pan-cytokeratin (CK, clone: AE1/AE3/PCK26, #760-2135; Ventana). CD68, CD163, HLA-DR and pan-CK have clinical applications for *in vitro* diagnostic (IVD) assays. The staining protocol steps are detailed in Table S1. According to applied enzymatic reaction for each staining sequence, the corresponding secondary antibody was loaded: UltraMap anti-mouse (#760-4312, Ventana) and OmniMap anti-mouse (#760-4310, Ventana) for AP and HRP reactions respectively. All the detection kits were from Ventana (#760-124: DAB; #760-247: teal; #760-239: yellow; #760-229: purple). To inactivate the first primary antibody before loading the second primary antibody, enzymatic inhibition, using discovery inhibitor (#760-4840, 12 min,) as well as temperature-induced denaturation (8 min at 90 °C), was applied. All double-stained slides were compared with their corresponding single-stained slide. Three different methods were used for staining quality control: no primary antibody control for each sequence; isotype-matched control; and multi-tumor and normal TMAs.

Evaluation of immunostaining

All slides were digitized using a Pannoramic 250 Flash II scanner (3DHistech, Budapest, Hungary) with a maximum resolution of x40, and viewed using Pannoramic viewer 1.15.4 (3DHistech) and QuPath v.0.12 (Queen's University Belfast, Northern Ireland) software. The CD68 antibody was co-stained with HLA-DR to label M1, and with CD163 or CD204 to label M2. For pan-macrophage assessment, CD68 was co-stained with pan-CK.

The digitized slides were scored independently by two observers (M.R and S.J) for macrophage infiltration in different compartments: (a) tumor stroma (in the primary tumor); and (b) the intra-tumoral area (in both primary tumors and metastatic lymph nodes). Necrotic areas were ignored. In tumor stroma, the percentage of macrophages in the total number of nucleated cells was scored using the following scale: 0 (0–5%), 1 (6–25%), 2 (26–50%) and 3 (>50%). In the intra-tumoral area of both the primary tumor and metastatic lymph nodes, the total number of infiltrating macrophages was scored as follows: 0 (no positive cells), 1 (1–5 positive cells), 2 (≥6 positive cells). If there were more than two disagreements on scores, slides were reassessed to reach a consensus. A mean value of the marker scores was obtained for each patient.

Finally, the stromal M1, $CD204^{+}$M2 and $CD163^{+}$M2 scores were dichotomized into high and low groups using mean as cut-off values. For intra-tumoral infiltration and stromal CD68,

optimal cut-offs (minimal *P*-value) were used for dichotomization. The applied cut-off values are listed in Table S2.B.

Statistical analysis

The statistical analyses were performed using SPSS (Mac OS, version 25) and R (version 3.5.1). Interobserver reliability was calculated using a two-way random-effects model with an absolute agreement definition and Cohen's kappa coefficient with equal weighting. Mann–Whitney U tests were used to examine the association between distribution of different macrophage phenotypes across pathological stages. Correlations were explored between macrophage infiltration and clinicopathological variables (Chi-squared test) and between variables (Spearman's rho coefficient). Survival analysis was estimated by the Kaplan–Meier method, and the log-rank test was used to compare survival between the groups. Disease-specific survival (DSS) was calculated from the date of surgery to the date of NSCLC death. Multivariable Cox regression analyses were performed to identify independent predictors of survival. Stepwise backward conditional selection using 0.10 and 0.05 as entry-and exit-points was used to select variables for the final models. *P* values of < 0.05 were considered statistically significant.

Results

Reliable assessment of macrophage phenotypes

The study evaluated the presence and expression patterns of macrophage subpopulations co-expressing HLA-DR$^+$/CD68$^+$ (M1), CD163$^+$/CD68$^+$ (M2) and CD204$^+$/CD68$^+$ (M2). To find the most appropriate chromogen for cellular colocalization, different dye combinations (DAB, purple, red, yellow, and teal) were tested. HLA-DR, CD163 and CD204 in teal (HRP) and CD68 in yellow chromogen (AP), were the best for manual double-antigen visualizing. In this assay, two over-lapping signals on macrophages appear with a tertiary (green) color, making spatial assessment of the two markers considerably easier (Figure 1A-F). In order to improve differentiation of CD68$^+$ TAMs in tumor islets, pan-CK as an epithelial landmark marker, was co-stained with CD68 (Figure 1G-H).

Figure 2A-B represents the correlation matrix between TAM subsets and immune-related markers previously studied in this cohort. There was a strong correlation between stromal CD163$^+$M2 and CD204$^+$M2 (r=0.92), and moderate correlations between stromal M1 and CD204$^+$M2 or CD163$^+$M2 (r=0.46 and r=0.42, respectively). In the tumoral areas, strong correlation was also observed between CD163$^+$M2 and CD204$^+$M2 (r=0.91), and moderate correlations between M1 and CD204$^+$M2 (r=0.51) or CD163$^+$M2 (r=0.50).

To validate the specificity of TAM subset staining, a single TMA slide consisting of tumor samples from 54 patients were stained in multiplexed-IHC and compared in the combinations of HLA-DR/CD204/CD68 and HLA-DR/CD163/CD68, and the proportion of macrophages coexpressing both M1 and M2 markers were evaluated. By an absolute count of shared-phenotypic positive cells, the majority of TAMs showed a unique phenotypic expression, either M1 or M2, with few macrophages positive for both differentiating markers: HLA-DR$^+$/CD204$^+$/CD68$^+$: median (range) 3.1% (0–10.26%); HLA-DR$^+$/CD163$^{+/}$CD68$^+$: 2.7% (0–11.42%) (Figure 3A-B).
The intraclass correlation coefficients and Kappa values for the macrophage scores are listed in Table S2.A. There was substantial interobserver agreement between the two scorers, with greater consensus for the stroma compartment than the tumor compartment.

To further validate the TMA results, full-faced section slides of 20 (SCC, n=10; ADC, n=10; random selection) patients were evaluated. Heterogeneity between paired sections (full-face

tissue versus TMA cores) from the same patient was very low, and a significant concordance was observed for different macrophage subsets (Table S2. C).

Expression pattern of macrophage markers

The expression patterns of the used markers were fully evaluated in different tumor tissue cell types by two expert pulmonary pathologists (Table S3). As previously reported [15], and confirmed in this assessment, none of the applied antibodies were exclusively expressed on macrophages and can be expressed to some extend by other inflammatory and immune cells. Among these markers, CD68 and HLA-DR had broad immune cell and tissue expression, while CD204 and CD163 were restricted to particular macrophages. In addition, CD68 and HLA-DR were expressed on cancer cells in 23% (n=125) and 51% (n=281) of patients in the cohort, respectively (as illustrated in Figure. 1B). In positive cases, the intensity of CD68 protein expression in the cancer cells was homogenous while varied highly for HLA-DR. The M2-like phenotype was the dominant subset of TAMs in almost all necrotic areas (Figure 3C-D). All the explored antibodies displayed membranous and diffuse cytoplasmic localization on macrophages. CD163 and CD204 antigens had slightly higher cell membrane expression than HLA-DR or CD68.

Macrophage distribution and correlation

High stromal M1 was statistically associated with lower T stage and more favorable ECOG performance status in primary tumors. $CD204^{+}$M2 were closely correlated with patients age. (Table S4). No consistent associations (except between M1 and ECOG) were found between the level of macrophage subsets and clinicopathological variables in the intra-tumoral compartment of primary tumors or metastatic lymph nodes (Table S5).

In the stromal areas, moderate to strong correlations were observed between TAM subsets with CD3 (M1 r=0.47; $CD163^{+}$M2 r=0.39; $CD204^{+}$M2 r=0.38), CD8 (M1 r=0.38; $CD163^{+}$M2 r=0.31; $CD204^{+}$M2 r=0.30), CD4 (M1 r=0.48; $CD163^{+}$M2 r=0.41; $CD204^{+}$M2 r=0.43), and CD45RO (M1 r=0.29; $CD163^{+}$M2 r=0.31; $CD204^{+}$M2 r=0.3) positive immune cells (Figure 2.A). In the tumor area, similar correlations were observed between TAM subsets and T-cell markers (Figure 2.B).

Macrophage distribution was evaluated across TNM stages I, II and III. For pathological stages I to III, levels of stromal CD204$^+$M2, CD163$^+$M2 and pan-CD68 infiltration did not differ significantly, but notably decreased for M1 macrophages (Figure 2.C).

Macrophage and survival: univariate analysis

In the overall cohort, high levels of both intra-tumoral and stromal M1 (P = 0.021 and P = 0.003), CD204$^+$M2 (P = 0.004 and P = 0.013) and pan-CD68 (P = 0.01 and P = 0.006) macrophages were significantly associated with longer DSS (Figure 4; Table 1). For CD163$^+$M2 TAMs, a positive trend was seen for high infiltration in the stromal and intra-tumoral compartments.

In the SCC subgroup (n=307), high levels of stromal CD163$^+$M2 (P <0.001) and CD204$^+$M2 (P= 0.005) and both stromal and intra-tumoral M1 (P <0.001, P=0.016) macrophage infiltration, were associated with improved DSS (Figure S1, Table.S6).

In the ADC subgroup (n=239), high levels of stromal CD68-positive macrophages was associated with longer DSS (P= 0.039) (Figure S2, Table.S6).

In the metastatic lymph nodes, the presence of intra-tumoral M1 macrophages was a significant positive prognostic factor (P= 0.002) (Table 1).

Multivariate survival analysis

To test the prognostic significance of macrophage infiltration when adjusted for known prognostic factors, Cox proportional hazard models were used. In the overall cohort, stromal M1 (HR 0.73; CI 0.5-0.97; P=0.03), CD204$^+$M2 (HR 0.7; CI 0.5-0.94; P=0.02) and CD68 (HR 0.69; CI 0.5-0.94; P=0.02) were associated with significantly longer DSS independent of pStage, vascular invasion, ECOG performance status, and gender. Consistent with findings in stroma, intra-tumoral M1 (HR 0.7; CI 0.5-0.99; P=0.04), CD204$^+$M2 (HR 0.6; CI 0.4-0.8; P=0.004) and CD68 (HR 0.73; CI 0.5-0.99; P=0.04) were independent positive prognostic factors for DSS (Table 2). In metastatic lymph nodes, high intra-tumoral M1 infiltration was an independent positive predictor of DSS (HR 0.38; CI 0.2-0.7; P=0.001).

Discussion

The study describes a multiplex IHC assay for simultaneous identification of co-localized markers in macrophage phenotyping. To our knowledge, this is the first large study to investigate the clinical significance of *in situ* TAMs in stage I-III NSCLC using a chromogen-based IHC approach. The study reveals independent positive associations between the levels of HLA-DR$^+$M1, CD204$^+$M2 and pan-CD68$^+$ TAMs with disease-specific survival in both stromal and intra-tumoral compartments. Our findings also indicate that the presence of intra-tumoral HLA-DR$^+$M1 macrophages in metastatic lymph nodes is a predictor of improved survival.

The traditional approach of TAM analysis is based solely on CD68 expression [24]. Our previous study, involving 335 patients, showed a positive trend between high numbers of CD68$^+$TAMs and clinical outcome in both stromal and intra-tumoral compartments by single-color IHC [25]. In the current study, using a larger sample size and co-staining with pan-CK, CD68$^+$ TAMs showed statistical significance with multivariable analyses. Table S7 summarizes previous studies assessing the prognostic impact of TAMs in NSCLC. In line with the present study, Kim and Eerola *et al* [26,27] showed superior outcome with high intra-tumoral CD68$^+$TAMs. In contrast, other investigators found negative [28–30], none [31–33] or diverging [34,35] associations of CD68$^+$TAM density with patient outcome. These inconsistencies may partly be explained by two major issues, namely CD68 antibody specificity and methodological variation. Evidently, the subjectivity of IHC-stain interpretation can remarkably influence the reproducibility of CD68 scoring. Part of the variability in CD68$^+$TAM scoring may be caused by expression of this marker in tumor cells and other infiltrated immune cells [15]; in this study, tumor cells were positive for CD68 in 23% of the cohort. Non-specific staining may overestimate the level of TAMs and consequently affect the results. The use of pan-CK to differentiate between epithelial and non-epithelial cells probably increases the detection accuracy of intra-tumoral CD68 macrophages. Digital pathology has been used to quantify TAMs in some studies [36,37]. Antibody specificity may bias these studies more than visual microscopic evaluation due to the wide range of macrophage size distribution (5-30 μm) in lung tissue [38]. At the very least, detection of macrophages using morphological attributes in digital pathology requires highly specific algorithms relying on huge annotated datasets for the shape of TAMs.

Currently, there is no consensus on the identification and differentiation of tissue-based macrophage subsets in solid tumors. Recent publications advocate the use of multiple antibodies both to identify macrophages and to characterize TAM subpopulations [39]. When co-staining with CD68 (clone:KP1; IVD antibody) or even in single IHC assays, the most commonly used markers for M2 identification have been CD163 (clone: MRQ-26; IVD antibody), CD204 (clone: SRA-E5, widely used) and CD206 (used mainly for flow cytometry)[16]. For M1, there is less agreement about the best choice of antibodies, however several studies have used HLA-DR (clone: TAL.1B5; IVD antibody) for M1 identification [36,40–42]. HLA-DR is expressed on the membrane of antigen-presenting cells such as macrophages, monocytes, dendritic cells, B cells and activated T cells [43]. Tumor cell expression of HLA-DR has also been reported [44]. In NSCLC, only two studies employed double-IHC staining for analyzing different subsets of TAMs, while the majority used single-IHC staining against M2 antigens (CD204 or CD163) (Table S7). Ohri *et al* reported that intra-tumoral subpopulations, including M1- and M2-like TAMs, were predictors of superior outcome in NSCLC [40]. Similarly, we observed a survival advantage related to high M1 or M2 phenotypes in tumor islet as well as in stromal compartments. Ma *et al* found only intra-tumoral M1 (not M2) to be an independent prognostic indicator [36]. However, both Ma and Ohri *et al* were unable to identify any statistically significant associations between stromal TAM subsets and survival [36,40].

Biologically, the M1 and M2 subpopulations of macrophages are expected to associate with inverse anti-tumoral or pro-tumoral functions, respectively. However, we and other researchers (studying NSCLC, CRC and gastric carcinomas) have observed that both M1 and M2 subtype infiltration were positively associated with the patient's clinical outcome [40,45,46]. Different inferences were made in these studies for the survival benefits of M2 TAM infiltration. In NSCLC, Ohri *et al* suggested that further research might reveal mutual interactions between M1 and M2 TAMs [40]. Edin *et al* anticipated that due to co-presence of M1 and M2 in tumor tissue of CRC, the M1 anti-tumoral attribute may dominate over the M2 pro-tumoral functionality, leading to improved outcome. They also suggested that the intestinal environment is unique, comprising various microorganisms whereby macrophages require functional alteration in order to maintain local tissue homeostasis [45]. In gastric cancer, Kim *et al* speculated that the prognostic aspects of TAM may be largely oriented in relation to

lymphocytic infiltration, as concomitantly high levels of tumor-infiltrating lymphocytes (TILs) and $CD163^{+}$M2 were observed in their population [46]. In our study, the moderate to strong correlation between M1 or M2 and lymphocytic infiltration of CD3, CD8 and CD4 cells may imply that both macrophage phenotypes are involved in effective recruitment of lymphocytes and co-operate with T-helper/cytotoxic cells to induce anti-tumoral immune response [47]. Interestingly, in a recent lung cancer study, Peranzoni *et al* indicated a close relationship between the quantity of $CD206^{+}$ M2-like TAMs and "bystander" $CD8^{+}$ TILs in stroma [48]. Further, using a TAM-depleted murine model, they found that TAMs engage in prolonged interaction with $CD8^{+}$ TILs in stroma, limiting their entry into cancer islets and thereby interrupting their antitumor activity [48]. Taken together, macrophage phenotype clearly differs from tissue to tissue or within a single tissue in relation to their steps of polarization, disease stages and environmental signals. It also appears that, due to the high plasticity of macrophages, such a definition of M1 and M2 subpopulations and their involvement in distinct pro-tumoral and anti-tumoral activities of tumor is limiting; and such established nomenclature based on function probably bears no relevance in the complex tumor microenvironment [49,50].

Tumor stroma consists of a higher proportion of immune cells than intratumoral compartment, in which some immune cell subsets are positive for the markers studied here, together with TAMs (Table S3). Consequently, IHC-based analysis of TAM subsets in tumor stroma requires a reliable technical method that accounts for macrophage markers being co-localized in this context. With this understanding, a set of experiments to characterize macrophage subsets were conducted. In multiplexed chromogenic-IHC, the choice of chromogen or substrate is not important when protein biomarkers are expressed in different cell types. However, evaluating target proteins is more challenging when these are expressed in a single cellular compartment. In this situation, there is a risk of misinterpretation due to the overlap of chromogens and obstruction of one dye with another. By using translucent chromogens, we were able to reliably label co-localized antigens of interest on TAMs. When they are mixed, they can create a unique color, making it relatively easy to identify cells co-expressing the markers. The common dual-chromogen set used by researchers is conventional DAB/red, but in our experiment, this failed to be reliable because the dominant brown color significantly obstructed the red.

A novel finding in this study was the significant prognostic relevance of the M1 phenotype in resected metastatic lymph nodes—the level of intra-tumoral M1 infiltration was a very strong positive predictor of DSS in multivariable analysis, which is in line with its prognostic contribution in primary tumors. We did not find a significant correlation between TAM subsets in lymph nodes compared with primary tumor tissue (data not shown), which may relate to the heterogeneity of macrophages in these tissues [51]. Moreover, in pathological subgroups, stromal infiltration of M1 significantly dropped from stage I to stage III, which supports the previous concept about transition of macrophage phenotypes from proinflammatory to immunosuppressive states during the course of disease [52]. In further support, an animal study on hepatocellular carcinoma showed a shift from a high M1-like phenotype in the early stage to a low M1-like phenotype in the advanced stage [53]. Part of the complexity of macrophage expression can be linked to this temporal plasticity during tumor development.

In conclusion, this study demonstrates that high levels of either stromal or intra-tumoral pan-CD68, HLA-DR$^+$M1 and CD204$^+$M2 macrophages infiltration are independent determinants of favorable clinical outcome in stage I-III NSCLC patients. In addition, high levels of HLA-DR$^+$M1 macrophages in locoregional nodal metastases is an independent positive prognostic marker. From a technical aspect, the current observations support the use of translucent chromogens as a more practical choice for assessing co-localized TAM biomarkers in brightfield multiplex IHC.

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Figure 1: Multiplexed protein detection using translucent IHC-chromogens for TAM phenotyping in NSCLC. Compartment-specific infiltration of different TAM phenotypes in primary tumor: [A, B] HLA-DR^{+}(teal)/CD68^{+} (yellow) M1subset, [B] an example of HLA-DR tumor epithelial positive case in which the labelled M1 macrophages are easily distinguishable; [C, D] CD163^{+} (Teal)/CD68^{+} (yellow) M2 subset; [E, F] CD204^{+}(Teal)/CD68^{+} (yellow) M2 subset, all the co-localized markers appeared in a tertiary green color. [G, H] CD68^{+} (brown)/pan-CK (yellow). (magnification 15x)

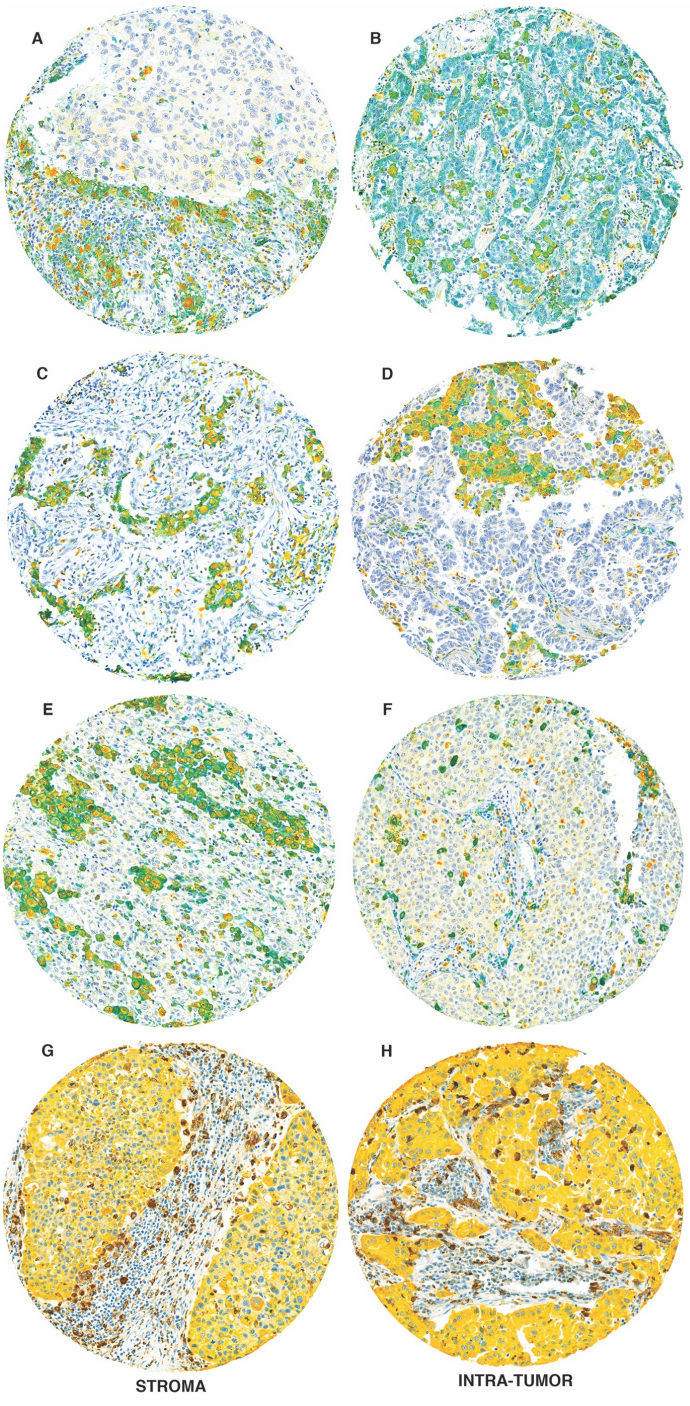

Figure 2: Spearman's rank correlation and Mann-Whitney U test on TAM phenotypes. [A, B] Correlation matrix between different stromal [A] and tumoral [B] TAM subsets and immune-related markers. [C] Dot- and Box-plots of various stromal (S, left column) and tumoral (T, right column) TAM subset distributions across pathological stages I-III in NSCLC. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

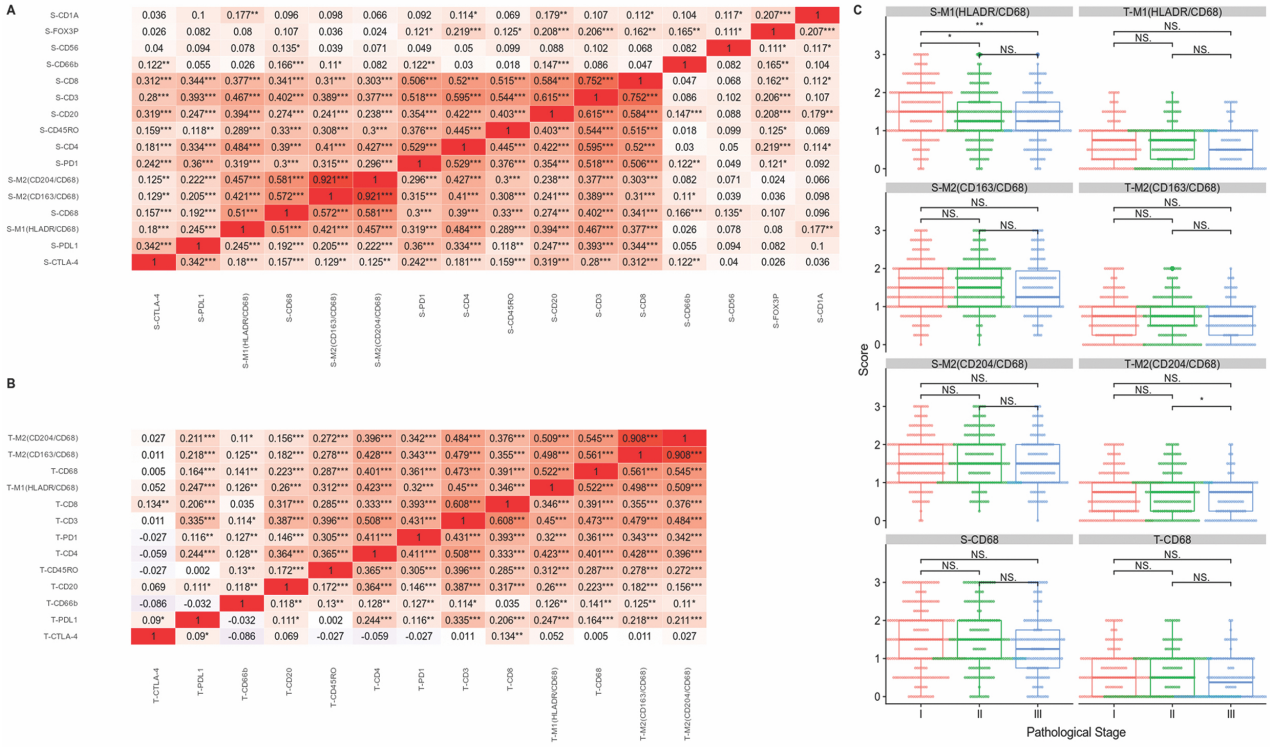

Figure 3: Multiplex IHC for validation of TAM subset (M1 *vs* M2) staining specificity. [A] 3-plexed IHC of M1 and CD163^{+}M2 marker: HLA-DR^{+} (teal)/CD163^{+} (purple)/CD68^{+} (yellow). Distinct phenotypic expression of the markers, M1 (green arrow), CD163^{+}M2 (red arrow), shared M1^{+}/ CD163^{+}M2 (black arrow) phenotype.
[B] 3-plexed IHC of M1 and CD204^{+}M2 marker: HLA-DR^{+} (teal)/CD204^{+} (purple)/CD68^{+} (yellow). Distinct phenotypic expression of the markers, M1 (green arrow), CD204^{+}M2 (red arrow), shared M1^{+}/ CD204^{+}M2 (black arrow) phenotype. [C, D] TAM phenotyping on consecutive TMA sections, demonstrating the dominant level of CD163^{+}M2 over M1 in necrotic areas of same core. [C] HLA-DR^{+} (Teal)/CD68^{+} (yellow) M2, [D] CD163^{+} (teal)/CD68^{+} (yellow) M1, the co-localized markers appeared in a tertiary green color

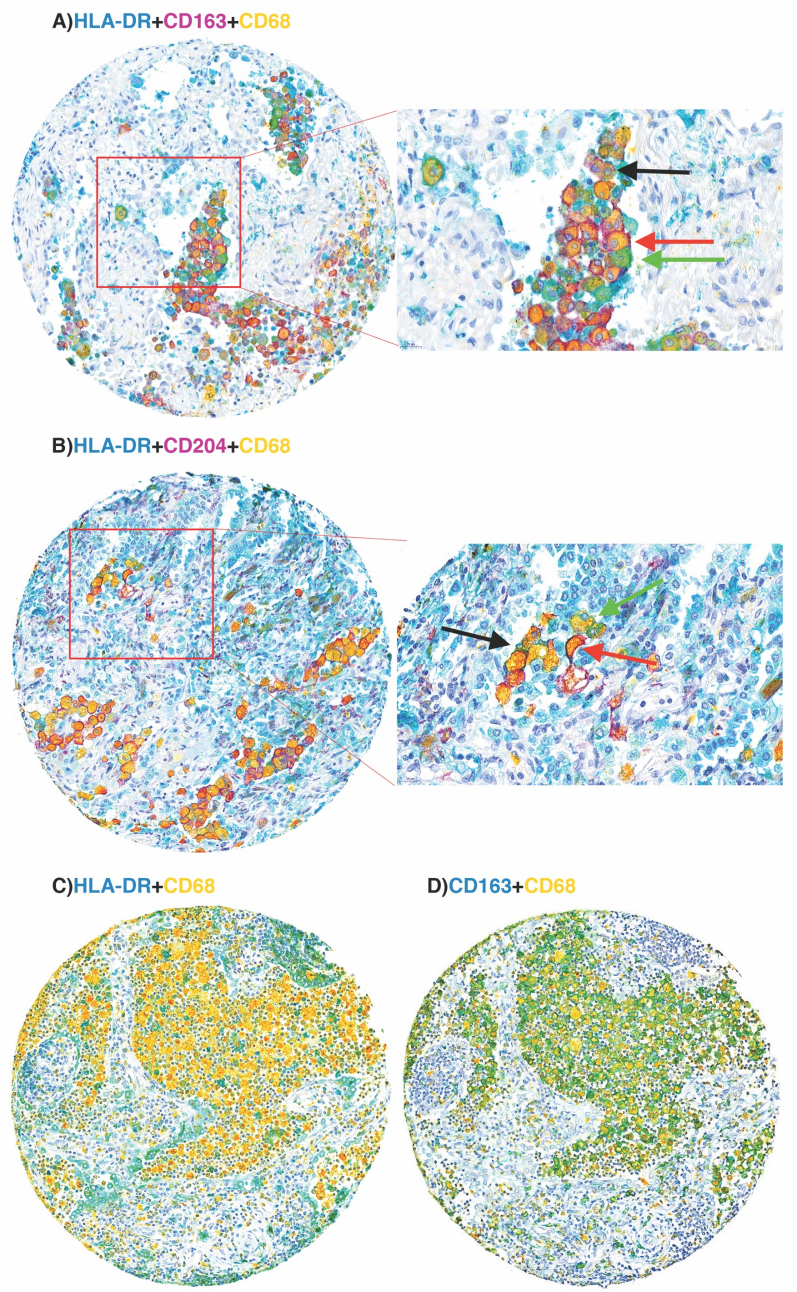

Figure 4: Disease-specific survival curves according to stromal and intratumoral TAM subset levels in primary tumor of NSCLC.

Intratumoral [A] HLA-DR^{+}M1; [B] CD163^{+}M2; [C] CD204^{+}M2; [D] pan-CD68.

Stromal [E] HLA-DR^{+}M1; [F] CD163^{+}M2; [G] CD204^{+}M2; [H] pan-CD68.

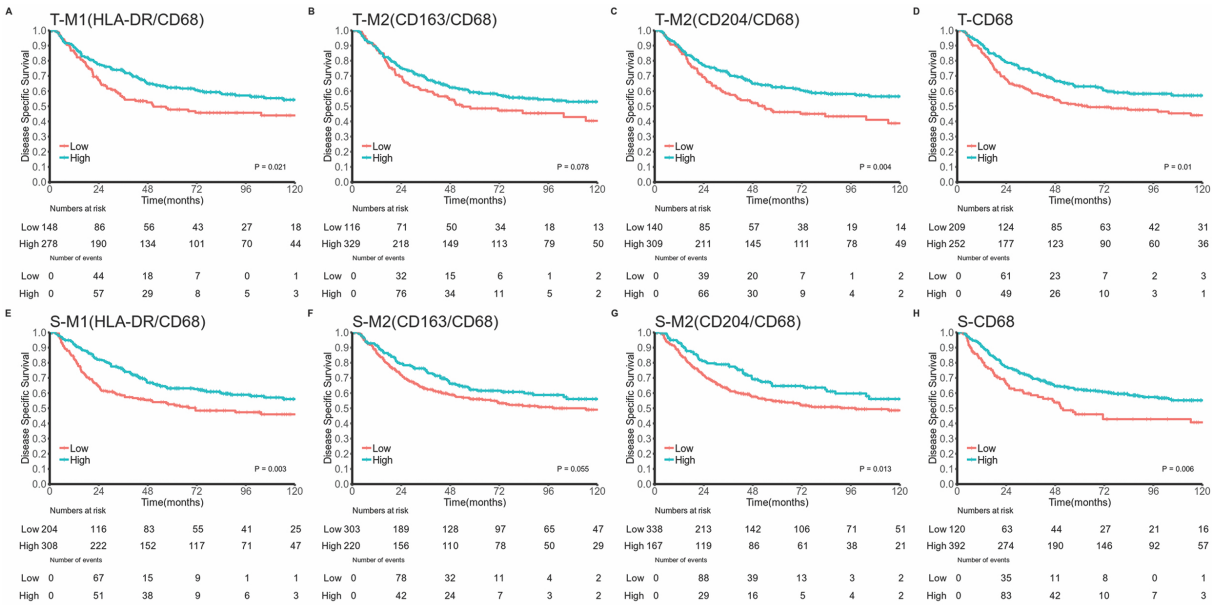

Table 1: Prognostic impact of stromal and intra-tumoral macrophage phenotypes in primary and metastatic lymph nodes of NSCLC patients

	Stroma					**Intra-tumor**				
	N(%)	DSS (%)	Median (month)	HR (95% CI)	*P*	N (%)	DSS (%)	Median (month)	HR(95% CI)	*P*
Primary Tumor										
M1 (HLA-DR$^+$/CD68$^+$)					**0.003**					**0.021**
Low	204(37)	52	71	1		148(27)	48	51	1	
High	308(56)	63	189	0.65(0.5-0.87)		278(50)	62	189	0.7(0.51-0.94)	
Missing	41(7)					127(23)				
M2 (CD163$^+$/CD68$^+$)					0.055					0.078
Low	303(55)	56	98	1		116(21)	48	54	1	
High	220(40)	62	189	0.76(0.57-1.01)		329(59)	59	235	0.75(0.5-1.03)	
Missing	30(5)					108(20)				
M2 (CD204$^+$/CD68$^+$)					**0.013**					**0.004**
Low	338(61)	55	98	1		140(25)	46	54	1	
High	167(30)	65	N.A	0.68(0.41-0.92)		309(56)	62	235	0.65(0.48-0.87)	
Missing	48(9)					104(19)				
CD68$^+$					**0.006**					**0.01**
Low	120(22)	46	51	1		209(38)	51	64	1	
High	392(71)	62	189	0.6(0.47-0.88)		252(46)	63	235	0.68(0.52-0.91)	
Missing	41(7)					92(16)				
LN+										
M1 (HLA-DR$^+$/CD68$^+$)										**0.002**
Low						28(19)	10	15	1	
High						62(43)	35	56	0.41(0.23-0.72)	
Missing						54(38)				

Abbreviations: NSCLC: non-small cell lung cancer, DSS: disease-specific survival, HR: hazard ratio, CI: confidence interval.

Table 2: Multivariable Cox models for disease specific survival of A) various stromal and intra-tumoral macrophage phenotypes in primary tumor and B) metastatic lymph nodes

Parameter	Stroma		Intra-tumor	
	HR (95% CI)	*P*	HR (95% CI)	*P*
A				
Model 1 M1(HLA-DR$^+$/CD68$^+$) Low vs high	0.73(0.5-0.97)	**0.03**	0.7(0.5-0.99)	**0.04**
Pstage				
I	1		1	
II	1.6(1.1-2.3)	**0.01**	0.2(0.16-0.35)	**<0.001**
III	4.1(2.8-5.7)	**<0.001**	0.3(0.2-0.5)	**<0.001**
Vascular invasion No vs yes	1.8(1.3-2.5)	**0.001**	0.5(0.3-0.8)	**0.002**
ECOG				
0	1		1	
1	1.4(1.05-1.9)	**0.02**	0.5(0.3-1.1)	0.09
2	1.4(0.8-2.5)	0.28	0.9(0.5-1.7)	0.8
Gender Female vs male	1.4(1.03-1.9)	**0.03**	0.7(0.5-1.01)	0.06
Model 2 M2 (CD163$^+$/CD68$^+$) Low vs high	0.76(0.57-1.1)	0.053	0.7(0.5-1.03)	0.08
Pstage				
I	1		1	
II	1.6(1.1-2.3)	**0.007**	0.25(0.17-0.36)	**<0.001**
III	3.8(2.7-5.4)	**<0.001**	0.4(0.2-0.5)	**<0.001**
Vascular invasion No vs yes	1.9(1.3-2.6)	**<0.001**	0.6(0.4-0.8)	**0.002**
ECOG				
0	1		1	
1	1.5(1.09-1.9)	**0.009**	0.6(0.3-1.1)	0.1
2	1.5(0.8-2.6)	0.17	0.9(0.4-1.6)	0.7
Gender Female vs male	1.4(1.04-1.9)	**0.03**	0.7(0-4-0.9)	**0.02**
Model 3 M2 (CD204$^+$/CD68$^+$) Low vs high	0.7(0.5-0.94)	**0.02**	0.6(0.4-0.8)	**0.004**
Pstage				
I	1		1	
II	1.6(1.2-2.3)	**0.005**	1.4(1.03-2.1)	**0.03**
III	3.7(2.6-5.3)	**<0.001**	3.6(2.5-5.2)	**<0.001**
Vascular invasion No vs yes	1.8(1.3-2.5)	**0.001**	1.7(1.2-2.5)	**0.002**
ECOG				
0	1		1	
1	1.4(1.04-1.8)	**0.02**	1.4(1.03-1.9)	**0.03**

2	1.4(0.8-2.5)	0.25	1.5(0.8-2.7)	0.1
Gender Female vs male	1.4(1.04-1.9)	**0.02**	1.3(0.9-1.9)	0.058
Model 4 $CD68^+$ Low vs high	0.69(0.5-0.94)	**0.02**	0.73(0.5-0.99)	**0.04**
Pstage				
I	1		1	
II	1.6(1.1-2.26)	**0.01**	1.5(1.05-2.2)	**0.02**
III	3.7(2.6-5.3)	**<0.001**	3.6(2.5-5.2)	**<0.001**
Vascular invasion No vs yes	1.8(1.2-2.5)	**0.001**	1.8(1.3-2.6)	**<0.001**
ECOG				
0	1		1	
1	1.4(1.05-1.8)	**0.02**	1.4(1.03-1.9)	**0.03**
2	1.4(0.8-2.6)	0.2	1.5(0.8-2.8)	0.1
Gender Female vs male	1.3(0.9-1.8)	**0.053**	1.4(1.06-2.02)	**0.01**
B				
M1 ($HLA\text{-}DR^+/CD68^+$) Low vs high			0.38(0.2-0.7)	**0.001**
T stage				
1			1	
2			1.7(0.7-3.9)	0.18
3			1.7(0.7-4.2)	0.2
4			2.6(0.9-7.1)	0.06
N stage (N1 vs N2)			1.7(0.9-3.1)	0.07

Abbreviations: HR: hazard ratio, CI: confidence interval, ECOG: Eastern Cooperative Oncology Group

Figure S1: Disease-specific survival curves of TAM subset levels in squamous cell carcinoma (SCC) subgroup. Intratumoral [A] HLA-DR^{+}M1; [B] CD163^{+}M2; [C] CD204^{+}M2; [D] pan-CD68. Stromal [E] HLA-DR^{+}M1; [F] CD163^{+}M2; [G] CD204^{+}M2; [H] pan-CD68.

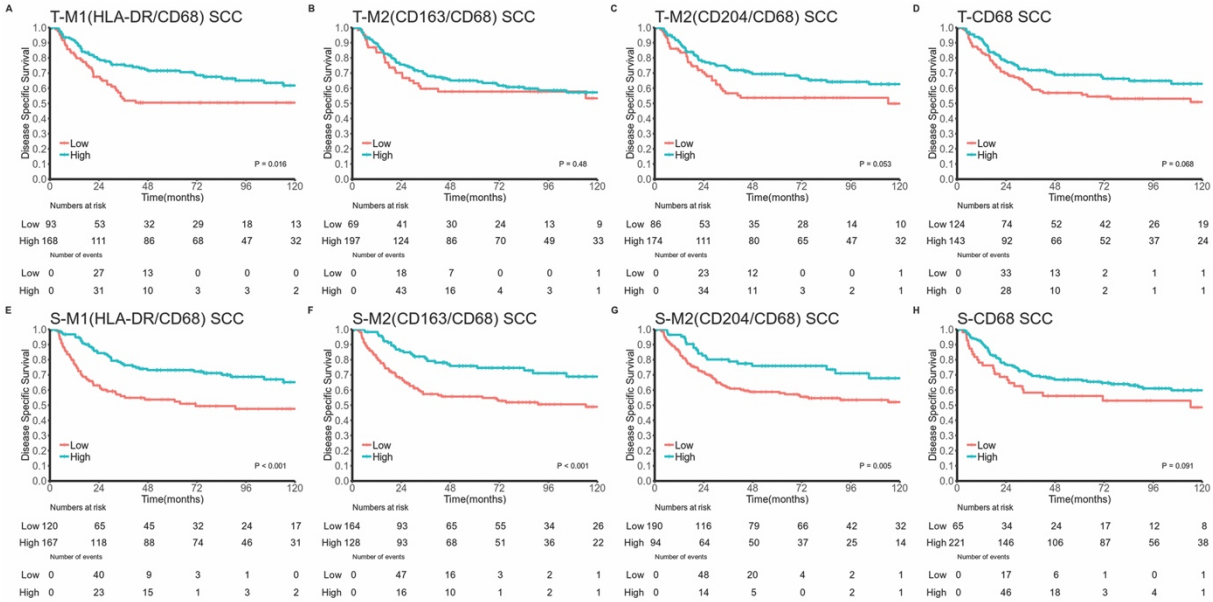

Figure S2: Disease-specific survival curves of TAM subset levels in adenocarcinoma (ADC) subgroup. Intratumoral [A] HLA-DR^{+}M1; [B] CD163^{+}M2; [C] CD204^{+}M2; [D] pan-CD68. Stromal [E] HLA-DR^{+}M1; [F] CD163^{+}M2; [G] CD204^{+}M2; [H] pan-CD68.

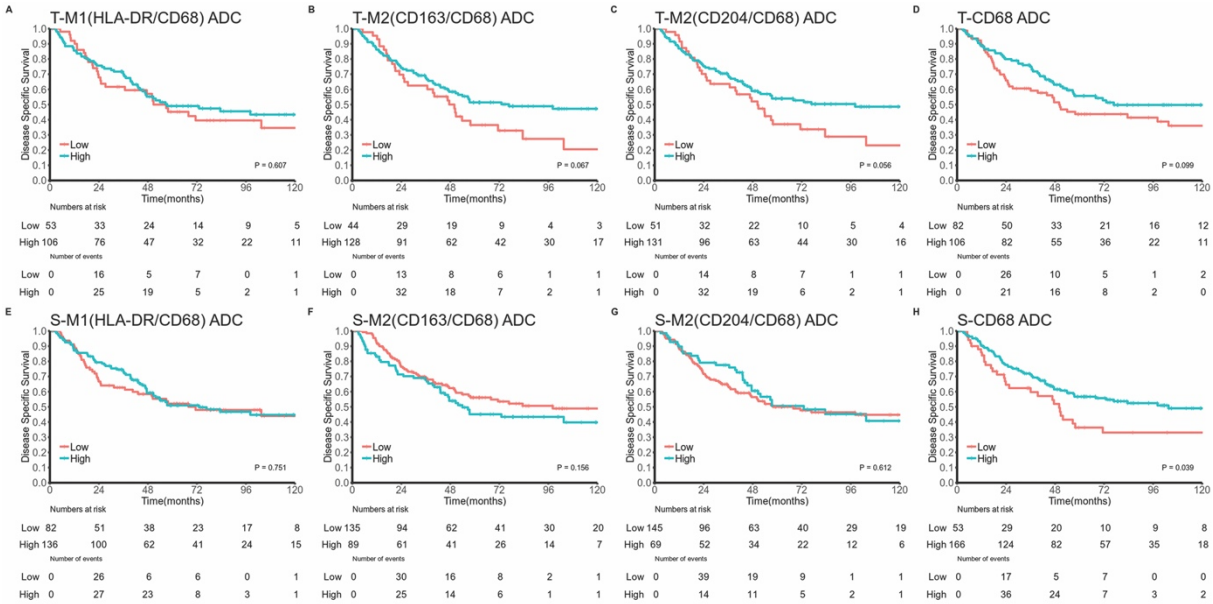

Table S1: The detailed protocol of the sequential multiplexed-IHC for profiling TAMs.

	1st sequence					**2nd sequence**				
	Deparaffinization Cycle (Time)	Pretreatment (Time)	Antibody Dilution (Time)	Chromogen (Time)	Substrate	Antibody Dilution (Time)	Chromogen (Time)	Substrate	Counterstain (Hematoxylin)	Post Counterstain (bluing reagent)
HLADR/CD68	3 (12 min)	CC1 (32 min)	1/150 (32 min)	Teal (H2O2:32 min) (Act:16 min)	HRP	Prediluted (32 min)	Yellow (44 min)	AP	4 min	NA
CD163/CD68	3 (12 min)	CC1 (32 min)	Prediluted (28 min)	Teal (H2O2:32 min) (Act:16 min)	HRP	Prediluted (16 min)	Yellow (44 min)	AP	4 min	NA
CD204/CD68	3 (12 min)	CC1 (32 min)	1/50 (32 min)	Teal (H2O2:32 min) (Act:16 min)	HRP	Prediluted (16 min)	Yellow (44 min)	AP	4 min	NA
CD68/CK	3 (12 min)	CC1 (32 min)	Prediluted (24 min)	DAB (default)	HRP	Prediluted (16 min)	Yellow (60 min)	AP	28 min	4 min

Abbreviations: CD: cluster of differentiation, HLA-DR: human leucocyte antigen-DR isotype, CK: cytokeratin, CC1: cell conditioning 1, Act: activator, HRP: horseradish peroxidase, AP: alkaline phosphatase, NA: not applied.

Table S2: A) Intraclass correlation and Cohen's kappa analysis between scorers for assessed markers in both intratumoral and stromal compartments of primary tumor and metastatic lymph nodes**, B)** the used cut points in the statistical analysis for dichotomization, **C)** paired sample correlation between full-faced tissue section and TMA core scores for 20 cases (*t*-test). For intratumoral compartment of the full-faced section slides, an average number of five fields were used.

	A	Marker localization	ICC	*P*-value	Kappa	*P*-value	**B**	Cut point	**C**	*r*	*P*-value
Primary tumor											
M1(HLA-DR$^+$/CD68$^+$)		intra-tumor	0.86	< 0.001	0.48	< 0.001		0.25		0.84	0.004
M1(HLA-DR$^+$/CD68$^+$)		stroma	0.93	< 0.001	0.53	< 0.001		1		0.86	0.003
M2(CD163$^+$/CD68$^+$)		intra-tumor	0.81	< 0.001	0.33	< 0.001		0.25		0.82	0.01
M2(CD163$^+$/CD68$^+$)		stroma	0.89	< 0.001	0.32	< 0.001		1.5		0.91	< 0.001
M2(CD204$^+$/CD68$^+$)		intra-tumor	0.81	< 0.001	0.35	< 0.001		0.25		0.78	0.02
M2(CD204$^+$/CD68$^+$)		stroma	0.88	< 0.001	0.49	< 0.001		1.75		0.86	0.01
CD68		intra-tumor	0.95	< 0.001	0.8	< 0.001		0.25		0.82	0.01
CD68		stroma	0.97	< 0.001	0.74	< 0.001		0.75		0.84	0.004
Metastatic Lymph nodes											
M1(HLA-DR$^+$/CD68$^+$)		intra-tumor	0.86	< 0.001	0.44	< 0.001		0.25		N.E	N.E

Abbreviation: ICC: intraclass correlation coefficient, N.E: not evaluated

Table S3: Expression pattern of macrophages markers among various tissue cellular compositions assessed in random 50 NSCLC patients (single-IHC staining)

	CD68 (pan)	HLA-DR (M1)	CD163(M2)	CD204 (M2)
TAMs	+++	+++	+++	++
TILs	-/+	++	-/+	-
TANs	-/+	-	-	-
Dendritic Cells*	+	++	-/+	-/+
Plasma Cells	-	-	-/+	-
Fibrocyte/ fibroblast	-/+	-/+	-	-
Endothelial Cells	-	-	-	-
Type 1 Pneumocyte	-	-/+	-	-
Type 2 Pneumocyte	-/+	-/+	-	-
Cancer cells	-/+	++	-/+	-

*Dendritic cell expression assessed in lymphoid aggregates area.
Rarely (-/+), lowly (+), moderately (++), highly (+++) expressed.

Abbreviations: TAMs: tumor-associated macrophages, TILs: tumor-infiltrated lymphocytes, TANs: tumor-associated neutrophils

Table S4: Associations between stromal macrophage phenotypes and clinic-pathological variables in overall cohort of resected primary NSCLC

	M1			CD163 M2			CD204 M2			CD68		
	low	high	*P*	low	high	*P*	low	high	*P*	low	high	*P*
Total	204	308		303	220		338	167		120	392	
Age			0.6			0.2			**0.02**			0.1
≤ 65	91	131		136	87		158	60		44	176	
>65	113	177		167	133		180	107		76	216	
Sex			0.6			0.3			0.4			0.2
Female	65	105		94	78		107	59		35	138	
Male	139	203		209	142		231	108		85	254	
Weight loss			0.07			0.7			0.5			0.8
<10%	177	282		270	199		301	152		107	351	
≥10%	27	25		32	21		36	15		13	40	
Missing		1		1			1				1	
Smoker			0.4			0.4			0.9			0.1
Never	4	12		8	9		11	6		2	15	
Current	129	198		198	133		214	106		73	255	
Former	71	98		97	78		113	55		45	122	
ECOG			**0.02**			0.8			0.2			0.6
0	107	194		177	130		190	104		66	233	
1	77	99		102	76		119	55		44	133	
2	20	15		24	14		29	8		10	26	
Histology			0.52			0.6			0.4			0.8
SCC	120	167		164	128		190	94		65	221	
ADC	82	136		135	89		145	69		53	166	
Other	2	5		4	3		3	4		2	5	
Tstage			**0.004**			0.2			0.5			0.5
T1	63	128		124	73		131	59		34	135	
T2	68	111		103	80		115	61		45	145	
T3	49	40		45	44		54	33		26	70	
T4	24	29		31	23		38	14		15	42	
Nstage			0.4			0.5			0.8			0.7
N0	135	215		205	154		232	118		170	184	
N1	44	66		64	48		72	35		49	59	
N2	25	27		34	18		34	14		26	24	
Pstage			0.08			0.1			0.2			0.1
I	75	135		131	84		141	68		50	163	
II	67	105		92	86		107	64		34	140	
III	62	68		80	50		90	35		36	89	
Vascular invasion			0.5			0.6			0.09			0.6
No	165	255		251	177		285	131		96	322	
Yes	38	51		51	41		51	35		23	68	
Missing	1	2		1	2		2	1		1	2	

Abbreviations: ECOG:Eastern Cooperative Oncology Group performance status, ADC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large-cell carcinoma, ASC: adenosquamous carcinoma, NOS: not otherwise specified, Nstage: nodal stage, Pstage: pathological stage, Tstage: tumor stage.

Table S5: Associations between intratumoral macrophage phenotypes and clinic-pathological variables in overall cohort of resected primary NSCLC tumor and paired metastatic lymph node

	Primary Tumor												LN+		
	M1			CD163 M2			CD204 M2			CD68			M1		
	low	high	*P*	low	high	*P*	low	high	*P*	low	high	*P*	Low	High	*P*
Total	148	278		116	329		140	309		209	252		28	62	
Age			0.8			0.9			0.8			0.9			0.6
≤ 65	63	121		49	140		61	132		89	109		15	30	
>65	85	157		67	189		79	177		120	143		13	32	
Sex			0.1			0.1			0.3			0.6			0.2
Female	40	93		42	94		50	95		66	85		6	20	
Male	108	185		74	235		90	214		143	167		22	42	
Weight loss			0.9			0.3			0.1			0.2			0.3
<10%	132	248		107	291		130	272		192	221		23	56	
≥10%	16	29		9	37		10	36		17	30		5	6	
Missing		1			1			1			1				
Smoker			0.3			0.6			0.4			0.3			0.5
Never	2	10		3	9		2	11		9	5		0	2	
Current	96	172		68	209		87	194		129	163		19	37	
Former	50	96		45	111		51	104		71	84		9	23	
ECOG			0.1			**0.03**			0.1			0.09			0.7
0	73	167		55	198		71	187		110	158		17	33	
1	63	92		48	111		56	102		82	77		9	23	
2	12	19		13	20		13	20		17	17		2	6	
Histology			0.8			0.5			0.4			0.8			0.2
SCC	93	168		69	197		86	174		124	143		15	30	
ADC	53	106		44	128		51	131		82	106		12	32	
Other	2	4		3	4		3	4		3	3		1	0	
Tstage			0.1			0.4			0.6			0.053			0.3
T1	38	98		39	100		51	98		71	82		3	14	
T2	60	96		38	129		46	119		65	104		11	25	
T3	33	49		27	58		27	55		48	37		7	15	
T4	17	35		12	42		16	37		25	29		7	8	
Nstage			0.2			0.9			0.1			0.3			0.5
N0	95	199		81	227		97	216		144	171				
N1	35	58		25	74		26	72		41	61		21	42	
N2	18	21		10	28		17	21		24	20		7	20	
Pstage			0.2			0.8			0.3			0.5			0.3
I	55	116		46	131		59	127		82	106				
II	49	102		40	121		43	115		70	89		9	27	
III	44	60		30	77		38	67		57	57		19	35	
Vascular invasion			0.6			0.6			0.9			0.3			0.1
No	119	219		94	264		113	250		173	198		17	49	
Yes	28	58		20	64		26	58		35	52		10	13	
Missing	1	1		2	1		1	1		1	2		1		

Abbreviations: ECOG: Eastern Cooperative Oncology Group performance status, ADC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large-cell carcinoma, ASC: adenosquamous carcinoma, NOS: not otherwise specified, Nstage: nodal stage, Pstage: pathological stage, Tstage: tumor stage.

Table S6: Subgroup analysis on prognostic value of stromal and intra-tumoral macrophage phenotypes in NSCLC patients

Variable	**Stroma**										**Intra-tumor**									
	Squamous cell carcinoma					Adenocarcinoma					Squamous cell carcinoma					Adenocarcinoma				
	N (%)	DSS (%)	Median (month)	HR(95% CI)	*P*	N(%)	DSS(%)	Median (month)	HR(95% CI)	*P*	N (%)	DSS (%)	Median (month)	HR(95% CI)	*P*	N(%)	DSS(%)	Median (month)	HR(95% CI)	*P*
M1 (HLA-DR$^+$/CD68$^+$)					**<0.001**					0.75					**0.016**					0.6
Low	120(39)	54	71	1		82(34)	52	71	1		93(30)	50	N.A	1		53(22)	45	51	1	
High	167(54)	73	235	0.48(0.32-0.71)		136(57)	51	73	0.94(0.63-1.4)		168(55)	71	235	0.6(0.4-0.91)		106(44)	49	57	0.8(0.56-1.4)	
Missing	20(7)					21(9)					46(15)					80(34)				
M2 (CD163$^+$/CD68$^+$)					**<0.001**					0.15					0.5					0.06
Low	164(53)	56	114	1		135(57)	56	98	1		69(23)	58	N.A	1		44(18)	36	50	1	
High	128(42)	76	N.A	0.44(0.28-0.68)		89(37)	45	54	1.3(0.9-1.9)		197(64)	65	235	0.8(0.54-1.3)		128(54)	51	76	0.6(0.42-1.03)	
Missing	15(6)					15(6)					41(13)					67(28)				
M2 (CD204$^+$/CD68$^+$)					**0.005**					0.61					0.053					0.056
Low	190(62)	59	235	1		145(61)	50	68	1		86(28)	54	114	1		51(21)	37	50	1	
High	94(31)	76	N.A	0.51(0.3-0.8)		69(29)	51	73	0.9(0.59-1.3)		174(57)	70	235	0.6(0.43-1.01)		131(55)	54	98	0.65(0.42-1.01)	
Missing	23(7)					25(10)					47(15)					57(24)				
CD68$^+$					0.09					**0.039**					0.06					0.09
Low	65(21)	56	114	1		53(22)	36	50	1		124(40)	57	N.A	1		82(34)	44	50	1	
High	221(72)	67	235	0.68(0.43-1.07)		166(70)	57	103	0.63(0.4-0.98)		143(47)	69	235	0.68(0.46-1.03)		106(45)	56	77	0.7(0.47-1.06)	
Missing	21(7)					20(8)					40(13)					51(21)				

Abbreviations: DSS: disease-specific survival, HR: hazard ratio, CI: confidence interval.

Table S7: Published data on prognostic effect of tumor-associated macrophages by chromogenic-IHC in NSCLC

(Author/ year)	Cohort size	Sample type	Tumor histology	Tumor stage	IHC	Marker(s)	Subset	Antibody clone	Cut-off	Assessed area	Prognostic impact (PI) for high infiltration
(Li *et al*, 2018)	297	WT	ADC Other	I-IV	SS	1-CD68	Pan	PG-M1	median	stroma	CD68 and CD204 independent negative PI (Overall cohort and ADC subgroup)
						2-CD204	M2	SRA-E5			
										tumor	NS
(Pei *et al*, 2014)	417	TMA	ADC, SCC	I-IIIA	SS	CD68	Pan	KP-1	zero	ND	NS
(Carus *et al*, 2013)	335	WT	ADC, SCC	I-IIIA	SS	CD163	M2	EDHu-1	median	ND	NS
(Ito *et al*, 2012)	304	WT	ADC	I	SS	CD204	M2	SRA-E5	median	stroma	Negative PI for PFS NS in multivariable analysis
(Hirayama *et al*, 2012)	208	WT	SCC	I-III	SS	CD204	M2	SRA-E5	median	stroma	Independent negative PI
(Ma *et al*, 2010)	100	WT	ADC, SCC	I-IV	DS	1-CD68/ HLA-DR	M1	KP1/ LN3	median	tumor	M1: independent positive PI M2: NS
						2-CD68/ CD163	M2	KP1/ 10D6			
										stroma	NS
	170	WT	ADC	I-III	SS	1-CD204	M2	SRA-E5	median	stroma	CD204: negative PI

(Ohtaki *et al*, 2010)						2-CD68	Pan	KP1			CD68: NS
(Dai *et al*, 2010)	99	WT	SCC, ADC	I-IV	SS	CD68	Pan	KP1	median	tumor	Independent positive PI
										stroma	Independent negative PI
(Al-Shibli *et al*, 2009)	335	TMA	SCC, ADC	I-IIIA	SS	CD68	Pan	KP1	optimal	tumor	NS
										stroma	NS
(Ohri *et al*, 2009)	40	WT	SCC, ADC	I-IV	DS	1-CD68/ HLA-DR	M1	PGM1/ TAL.1B5	median	tumor	All M1 and M2 markers: positive PI CD68/MRP8-14: positive independent PI
						2-CD68/ iNOS	M1	PGM1/ 2D2-B2			
						3-CD68/ CD163	M2	PGM1/ 10D6			
						4-CD68/ MRP8-14	M1	PGM1/ 27E10			
						5-CD68/ VEGF	M2	PGM1/ 14-124			
										stroma	NS
(Kim *et al*, 2008)	144	TMA	SCC, ADC	I-IV	SS	CD68	Pan	M0876	median	tumor	Independent positive PI

										stroma	NS
(Kawai *et al*, 2008)	199	WT	SCC, ADC, other	IV	SS	CD68	Pan	KP1	median	tumor	NS
										stroma	Negative PI
(Kojima *et al*, 2005)	129	WT	ADC	I-III	SS	CD68	Pan	PGM1	mean	stroma	NS
(Welsh *et al*, 2005)	162	WT	SCC, ADC, other	I-IV	SS	CD68	Pan	PGM1	median	tumor	Independent positive PI
										stroma	Independent negative PI
(Chen *et al*, 2003)	35	WT	SCC, ADC	I-III	SS	CD68	Pan	KP1	median	ND	Negative PI
(Takanami *et al*, 1999)	113	WT	ADC	I-IV	SS	CD68	Pan	KP1	optimal	ND	Independent negative PI
(Eerola *et al*, 1999)	38	WT	LCC	I-III	SS	CD68	Pan	PGM-1	median	tumor	Positive PI

Abbreviations: TMA: tissue microarray, WT: whole tissue, SCC: squamous cell carcinoma, ADC: Adenocarcinoma, LCC: large cell carcinoma; IHC: immunohistochemistry, SS: single staining, DS: double staining, ND: not differentiated, NS: not significance, CD: cluster of differentiation, HLA-DR: human leukocyte antigen- DR isotype, iNOS: Inducible nitric oxide synthase, MRP8-14: myeloid-related proteins 8-14, VEGF: Vascular endothelial growth factor

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