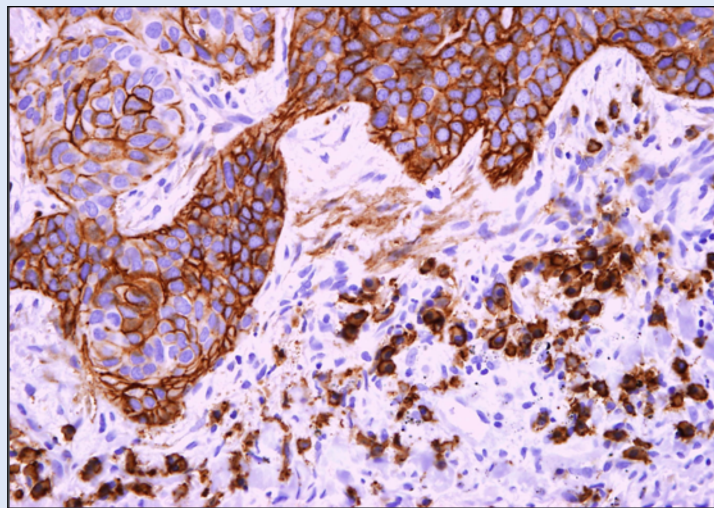
THE PROGNOSTIC SIGNIFICANCE OF THE INNATE AND ADAPTIVE IMMUNE SYSTEMS IN NON-SMALL CELL LUNG CARCINOMA



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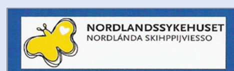
Dissertation for the degree philosophies doctor (PhD)
University of Tromsø



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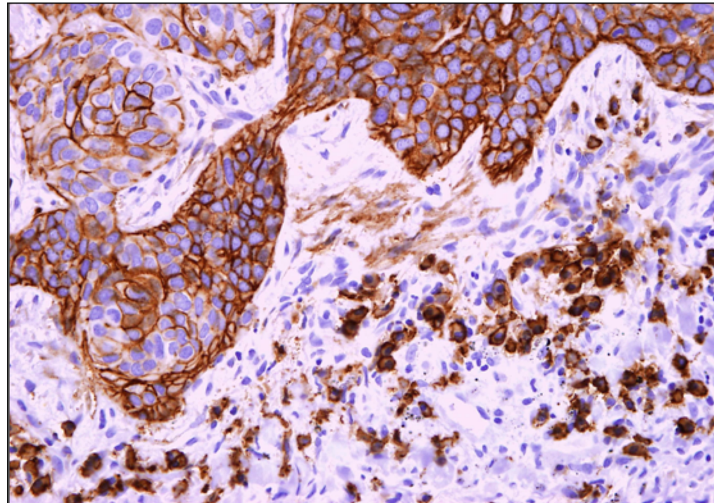

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'My parents, sister, brother and all my teachers in medicine and pathology '.

ABBREVIATIONS

AAH: Atypical Adenomatous Hyperplasia.
ADCC = Antibody Dependant Cell Cytotoxicity.
BAC = Bronchioalveolar Carcinoma.
COX = Cyclo-Oxygenase.
CSF-R1 = Colony Stimulating Factor-Receptor 1.
DC = Dendritic Cells.
DSS = Disease Specific Survival.
FNA = Fine Needle Aspiration.
HLA = Histocompatibility Antigens.
IHC = Immunohistochemistry.
IFN = Interferon.
IL = Interleukin.
M-CSF = Macrophage-Colony Stimulating Factor.
MDSC = Myeloid Derived Suppressor Cells.
MHC = Major Histocompatibility Cmplex
NK = Natural Killer.
NSCLC = Non-Small Cell Lung Carcinoma.
SCLC = Small Cell Lung Carcinoma.
TGF = Transforming Growth Factor.
TAM = Tumor Associated Macrophages.
TIL = Tumor Infiltrating Lymphocytes.
TMA = Tissue Microarry.
TNF = Tumor Necrosis Factor.
Treg cells = T regulatory cells.
VEGF = Vascular Endothelial Growth Factor.

LIST OF PAPERS

1. **Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT.** Prognostic Impact of Epithelial and Stromal Lymphocyte Infiltration in Non-Small Cell Lung Cancer. Clin Cancer Res. 2008 Aug 15;14(16):5220-7.

2. **Al-Shibli K, Al-Saad S, Donnem T, Persson M, Bremnes RM, Busund LT.** The Prognostic Value of Intraepithelial and Stromal Innate Immune System Cells in Non-Small Cell Lung Carcinoma. Histopathology. 2009 Sep;55(3):301-12.

3. **Al-Shibli K, Al-Saad S, Andersen S., Donnem T, Bremnes RM, Busund LT.** The Prognostic Value of Intraepithelial and Stromal T cells and Plasma cells in Non-Small Cell Lung Carcinoma. Accepted in APMIS 2010.

1. INTRODUCTION.

1.1. Lung cancer

1.1.1 Lung cancer incidence and clinical presentation

Cancer is a major public health problem worldwide and lung cancer is the leading cause of cancer death in the western world, including Norway (Figure 1). In some countries, lung cancer accounts for more deaths than prostate cancer, breast cancer, and colorectal cancer combined.[1] There are two main categories of lung cancer: Non small cell lung carcinoma (NSCLC) (80%) and small cell lung cancer (SCLC) (20%).[2]

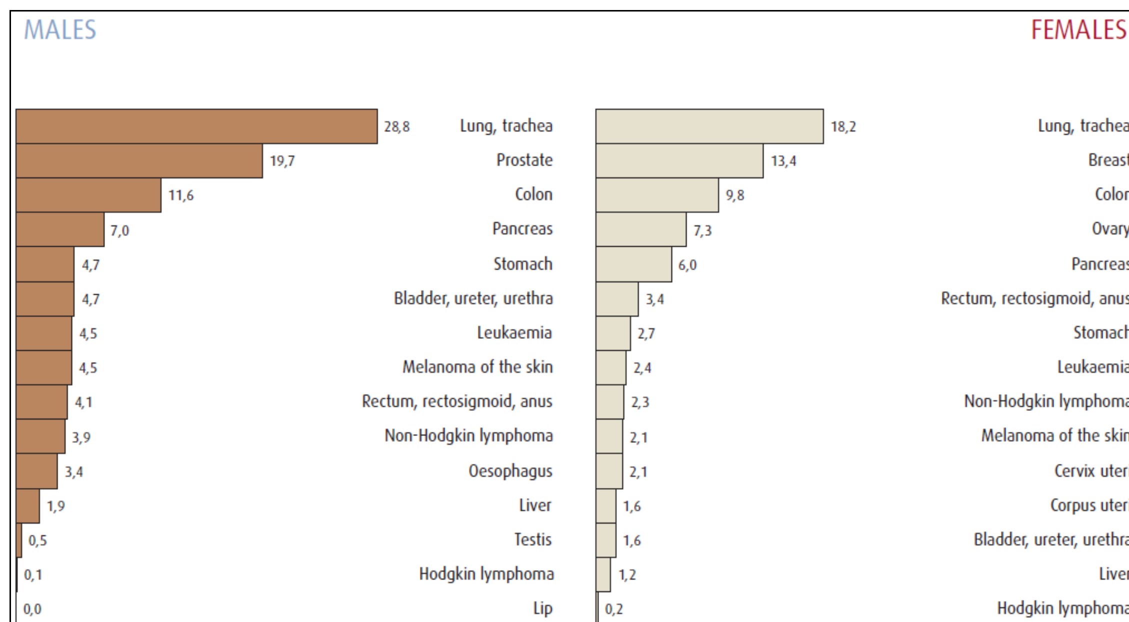

Figure 1: *Age standardised mortality rates in Norway 2007 for selected cancers (source: Statistics Norway), (from www.kreftregisteret.no).*

Lung carcinomas are insidious lesions that more often than not have spread to be unresectable before they produce symptoms. In few instances, chronic cough with sputum call attention to still localized, resectable tumors. When hoarseness, chest pain, superior vena cava syndrome, pericardial or pleural effusion, atelactasis or pneumonitis appear, the prognosis is grim. Often, the tumor presents with symptoms related to metastatic spread to the brain, liver or other organs. Although metastasis to the adrenal is common, adrenal insufficiency is

uncommon. Clinical manifestations due to paraneoplastic syndrome can also be seen (see table in appendix).

At present, surgery remains the primary therapy modality for solid tumors, including NSCLC. However, despite the advancement in molecular knowledge and the introduction of multiple new therapeutic lung cancer agents, the dismal 5-years survival rate (<15%) remains relatively unaltered (Figure 2), with a median survival still less than a year. The lack of advancement reflects the limited available knowledge about factors, which promote oncogenic transformation and proliferation and progression of NSCLC cells.

Nearly 75% of NSCLC patients have unresectable advanced disease with lymph nodes and/or visceral metastases at the time of diagnosis. Even among patients treated for stage I-III NSCLC and considered postoperatively tumour-free, about 65% will relapse within two years after surgery and subsequently die of metastatic spread.[1]

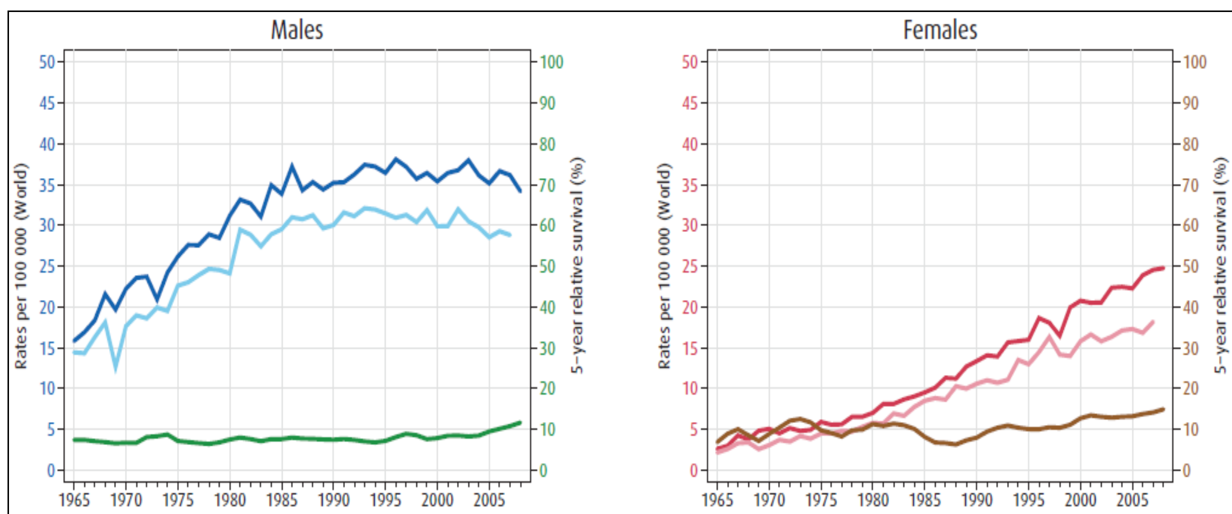

Figure 2: *Incidence (dark blue/red), mortality rate (light blue/pink) and 5-year survival (green/brown) proportions of lung carcinoma in Norway (source www.kreftregisteret.no).*

1.1.2. Lung cancer etiology

There is overwhelming evidences that tobacco smoking is the major cause of lung cancer, and the geographical and temporal patterns of lung cancer today reflect tobacco consumption 2-3 decades back. In fact, 80-90% of lung cancers are attributed to carcinogens

from cigarette smoking, and tobacco smoke contain 4800 chemical components, of which 20 are oncogenes.[3] However, epidemiological studies have shown that no more than 11% of smokers will develop this disease, suggesting additional environmental and ⁄ or genetic determinants. Two groups of enzymes exhibit a genetic polymorphism that could play a role in lung cancer incidence: P450 enzymes, encoded by CYP family genes, and glutathione S transferase (GST). The former is responsible for activation of tobacco carcinogen intermediate metabolites, whereas the latter is able to detoxify them.[4] Recently, a nicotinic acetylcholine receptor gene variant (acetyl choline receptors 3 and 5) at 15q24 has been identified and is suspected of being responsible for a higher risk of lung cancer and/or nicotine addiction.[4] Examples of other chemicals associated with lung cancer are in Table 1.

Table 1: *Some agents and exposure circumstances associated with lung carcinoma (modified from http://monographs.iarc.fr).*

Agent	**Use/industry**
Arsenic	Glass
Asbestos	Insulation, textiles
Radon	Mining
Nickel	Catalyst, alloy
Chromium compounds	Metal plating, pigments

1.1.3. Pulmonary preinvasive lesions

Three pulmonary pre-neoplastic lesions are identified to date[5]: 1) Squamous bronchial dysplasia and carcinoma in situ (CIS), preceding bronchial squamous cell carcinoma and basaloid carcinoma, 2) atypical adenomatous hyperplasia (AAH) representing the preneoplastic condition for a subset of adenocarcinoma, namely bronchioalveolar carcinoma (BAC), and 3) diffuse idiopathic pulmonary neuroendocrine cell hyperplasia, a proposed precursor of carcinoid tumors. No morphological epithelial lesion has been identified to date as a precursor for SCLC.

1.1.4. Molecular genetics of lung cancer

Concomitant to morphological changes from normal epithelium to preneoplastic to neoplastic lesions, multi-step accumulation of 10–20 genetic alterations occurs at the genomic level, leading to initiation, development and maintenance of lung cancer.

For squamous carcinoma, allelic losses (loss of heterozygosity) at multiple 3p chromosome sites are the earliest change, followed by loss of heterozygosity at 9p21. Later changes include alterations at 8p21-23, 13q14, and 17p13.[6] RAR (retinoic acid receptor) loss occurs in 40 % at an early stage (mild dysplasia); whereas P53 mutation and vascular endothelial growth factor (VEGF) overexpression occurs later on. Inactivation of p16 was demonstrated in 75 % of in situ carcinoma adjacent to invasive squamous cell carcinoma of the lung often associated with VEGF, cyclin D1 and E overexpression.[5]

Although the cell of origin for most adenocarcinomas remains unknown, peripheral adenocarcinomas arise from Clara cells or type 2 pneumocytes, and for a subset of these adenocarcinomas, they are preceded by AAH. Two different molecular pathways have been detected in lung adenocarcinoma pathogenesis (Figure 3): smoking-associated activation of RAS signalling, and non-smoking-associated activation of epidermal growth factor receptor (EGFR) signalling. KRAS codon 12 mutations are reported in 15% to 39% of AAH lesions, and p53 mutation has been demonstrated with increasing frequency in the progression from AAH through BAC to invasive adenocarcinoma.[6] EGFR mutation stimulate PI3K/AKT cycle leading to carcinogenesis. Finally, there is no molecular or genetic marker to distinguish diffuse idiopathic pulmonary neuroendocrine cell hyperplasia from reactive neuroendocrine proliferation, but allelic imbalance at 11q13, where the tumor suppressor gene MEN1 is located, is observed in up to 50% of typical carcinoids and 50–70% of atypical carcinoids.

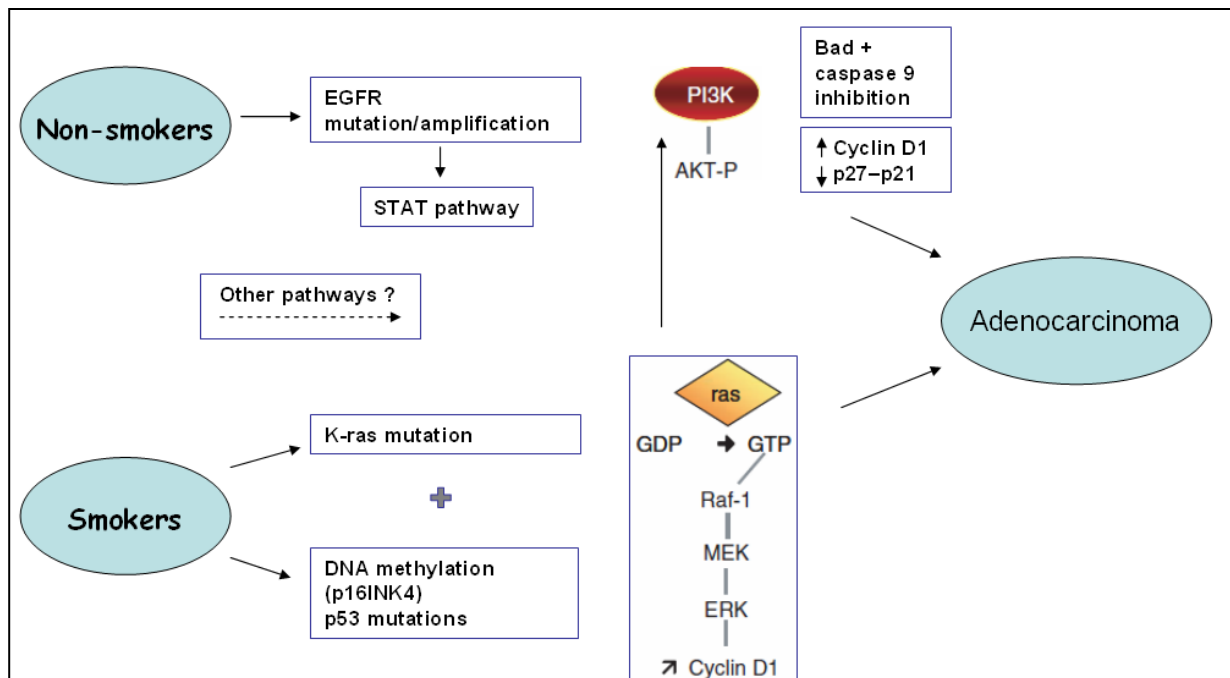

Figure 3. *Two main pathways proposed for the development of peripheral adenocarcinoma of the lung.*

1.1.5. Lung cancer staging

All patients with suspected lung cancer undergo a chest x-ray and a CT of the chest including the upper abdomen with the adrenal glands. Tissue biopsies and/or brush cytology are usually obtained by bronchoscopy, but for peripheral tumors CT guided fine needle aspiration (FNA) cytology and biopsy are often performed. In patients with enlarged mediastinal glands, mediastinoscopy or open surgery were regularly done to confirm or to rule out N2-status. Today, positron emission tomography (PET), transoesophageal or endobronchial ultrasound as well as FNA via bronchoscopy have been added to the staging procedures. These procedures are vital for a correct staging, treatment and prognosis. Clinical TNM (cTNM) is based on clinical examination of the patients while pathological TNM (pTNM) is based on examination of the surgical specimen. The novel TNM lung cancer classification has defined new tumor size cut-off values, (T1a, $\leq$ 2.0 cm; T1b, > 2.0 cm $\leq$ 3.0cm; T2a, > 3.0 cm $\leq$ 5.0 cm; T2b, > 5.0cm $\leq$ 7.0 cm; T3, > 7.0 cm).[7] In addition, the M factor is also modified in the updated TNM classification (Table 2).[7] Furthermore, the use of

special stain for elastic fibres is recommended in assessing pleural invasion[8]

Table 2: *Modification of the M factor in the new, 7th edition staging system*

M factor characteristics	6th edn	7th edn
Malignant pleural or pericardial effusion, pleural nodules	T4	M1a
Contralateral lung additional nodules of similar histology	M1	M1a
Distant metastases	M1	M1b

TNM stage estimates the postoperative outcome as well as rational for adjuvant therapy. Despite the prognostic power of this staging system, determining the outcome for patients with NSCLC has, however, remained inaccurate. The detection of micrometastasis in lymph nodes or in other locations might improve staging accuracy. However, it is uncertain if this offers a prognostic marker for recurrence. Hence, micrometastasis detection is not included in the routine staging process of NSCLC and using immunohistochemistry with cytokeratin is not a routine test in examining the lymph nodes from NSCLC patients.

1.1.6. Histopathology of NSCLC (Table 3 and Figure 4)

Other than the distinction between NSCLC and SCLC, there has historically been limited interest in further histological subtyping of NSCLC, and the staging system was considered quite adequate. This perception has changed dramatically during the past decade with the evolution of novel therapies. For example, treatment with EGFR tyrosine kinase inhibitors is more efficacious in patients who were never smokers, females or had adenocarcinoma histology.[9] Similarly, the new multitargeted antifolate agent, Pemetrexed (Alimta) give best effect in adenocarcinoma.[10] Therefore, how much longer the term NSCLC will be accepted as the bottom line on the pathological reports to the oncologists is uncertain.

Adenocarcinoma is the most histologically heterogeneous form of lung cancer (Table 3), both between cases and within individual tumors, and this has implications for pathologists reporting on this tumor type. Small biopsy samples may not be representative of the whole tumor or allow appreciation of much tumor architecture, and the specificity for preoperative diagnosis of adenocarcinoma is less than 50%.[11] The vast majority of pulmonary adenocarcinomas are heterogeneous tumors showing a mixture of patterns that probably have different biological behaviour and therefore potential therapeutic implications for the patient.[12] The value of subclassifying lung adenocarcinoma is still relatively uncertain, and our understanding of this area of tumor pathology is still evolving. A consistent, reproducible, biologically meaningful classification and pathological diagnosis of lung adenocarcinoma will facilitate comparison between case databases from different centres, be of great importance in epidemiological studies, and ensure that the patient receives the most appropriate treatment.[12] In the future, oncologists may require more than a simple diagnosis of 'adenocarcinoma' before proceeding with patient management. BAC is the only subtype of adenocarcinoma that has been classified separately in our study, as it has a well-documented prognostic significance. The WHO definition used to include a case in BAC category as ‘neoplastic cells growing along pre-existing alveolar structure without evidence of stromal, vascular or pleural invasion’.[13]

Squamous cell carcinoma is seen as sheets or islands of large polygonal malignant cells containing keratin (individual cells or keratin pearls) and/or intercellular bridges; adjacent bronchial dysplasia or carcinoma in situ are common. Subtypes include: Papillary, clear cell, small cell and basaloid.[2] No subtyping was used in our study.

Table 3: *WHO classification of lung adenocarcinoma (2004). Adopted from reference (adopted from ref.* [12]*).*

Types
Adenocarcinoma, mixed subtype
Acinar adenocarcinoma
Papillary adenocarcinoma
Bronchioloalveolar carcinoma
Non-mucinous
Mucinous
Mixed non-mucinous and mucinous or indeterminate
Solid adenocarcinoma with mucin production
Variants
Fetal adenocarcinoma
Mucinous ('colloid') carcinoma
Mucinous cystadenocarcinoma
Signet ring adenocarcinoma
Clear cell adenocarcinoma

Large cell (anaplastic) carcinoma may be undifferentiated squamous cell or adenocarcinomas. It is a diagnosis by exclusion and hence cannot be diagnosed, with certainty, on small biopsies. Microscopically, it is seen as large polygonal and anaplastic cells growing in solid nests without obvious squamous or glandular differentiation; vesicular nuclei, prominent nucleoli, moderately abundant cytoplasm and well defined cell borders. No morphological features of neuroendocrine architecture are seen.[13]

1.1.7. Potential for new prognostic factors and therapeutic approach in NSCLC

To date the only relevant prognostic factor for including patients in therapeutic trials remains the TNM classification.[14] Improving the survival rate for lung cancer patients requires the comprehension of all molecular events leading to lung cancer development including the significance of the immune system. The cancer research community anticipates

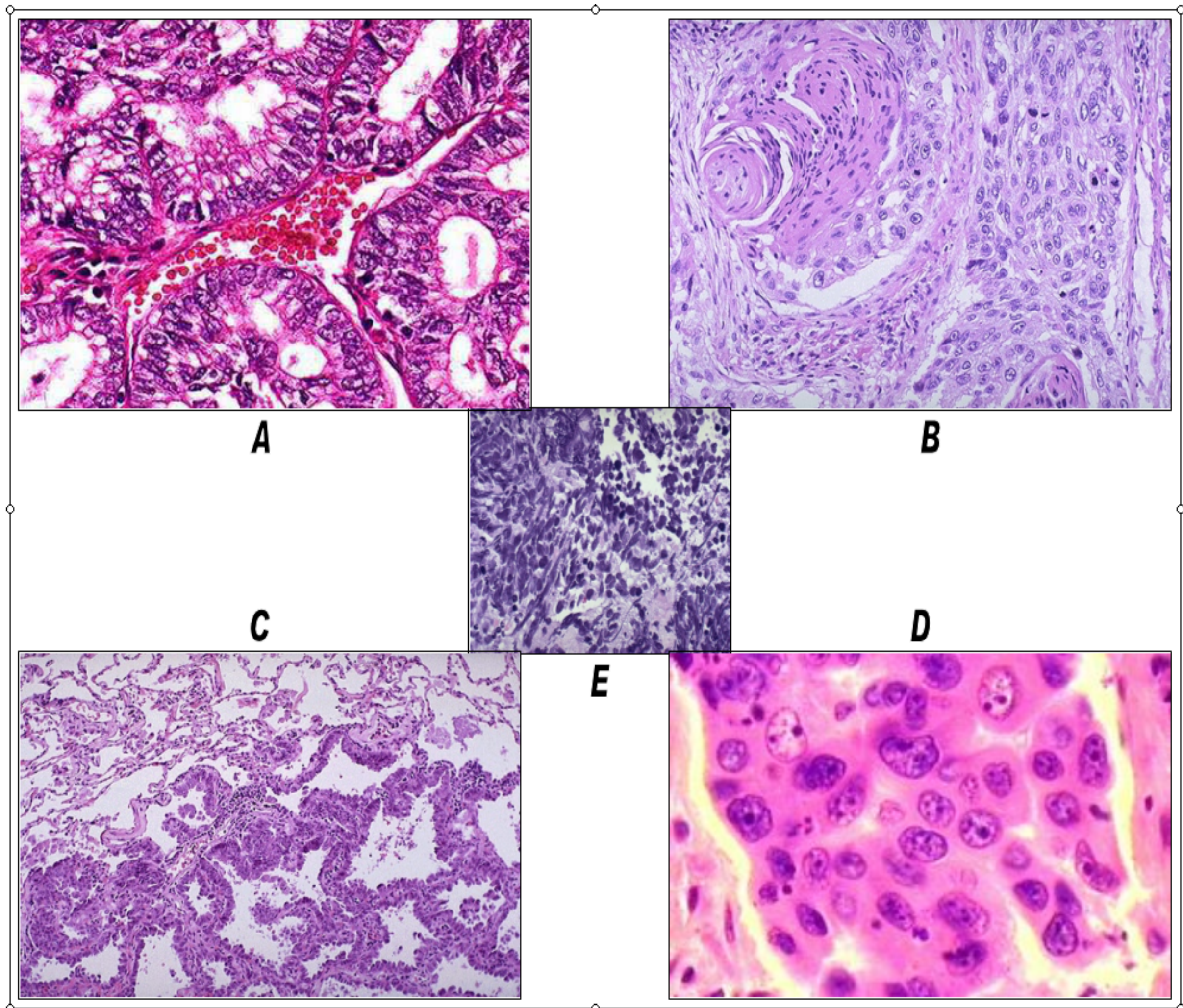

Figure 4: *Major histological types of lung carcinoma. Adenocarcinoma (A), squamous cell carcinoma (B), bronchioalveolar carcinoma (C), large cell anaplastic carcinoma (D) and small cell carcinoma (E).*

that an improved understanding of the genetic and epigenetic mechanisms driving the tumor may provide new tools for clinicians to stratify patients for optimizing therapy or assessing the prognosis within the same subgroup of tumors or at the individual level. Improved understanding of NSCLC biology has recently led to the development of new therapeutic agents (e.g. EGFR' and VEGF inhibitors). However, the challenge is still to identify the proper combination of agents for those novel therapies to enhance efficacy, as well as to increase the accuracy of prognosis prediction.

Increased accuracy of prediction of relapse/metastases might be obtained by non-

tumoral variables, as both primary tumor and metastasis develop within a tumor microenvironment which includes the inflammatory cells and mediators.[15] Hence studying tumor microenvironment including the inflammatory component is paramount.

1.2. The immune system

Our immune system is composed of two distinct compartments; the innate and the adaptive immune system. Each compartment has developed advanced communication networks, which enable rapid and effective responses to tissue injury.

1.2.1. The innate immune system

The uniqueness of this system is the inherent ability to rapid response when tissue injury occurs. Consisting of dendritic cells (DC), natural killer (NK) cells, macrophages, leukocytes (neutrophils, basophiles, and eosinophils), mast cells as well as soluble factors, the innate immune system is the first line of defence against foreign pathogens (e.g. virally infected cells or transformed cells). In the acute response, DCs, macrophages and mast cells are the primary effectors, as they are posted in the tissue and supervise its environment. Macrophages and mast cells immediately release soluble mediators such as cytokines, chemokines, matrix metalloproteinases (MMPs), oxygen species and bioactive mediators (histamine) inducing mobilization and infiltration of additional leukocytes, angiogenesis and remodelling of the damaged tissue.[16, 17]

1.2.2. The adaptive immune system

The innate immunity response leads to activation of the more sophisticated adaptive immune system. Primary adaptive immune response requires direct interactions with mature antigen-presenting cells as well as a proinflammatory environment. The adaptive immune

system is composed of B and T lymphocytes and antibodies. The two major T lymphocyte subsets are CD4+ helper and CD8+ cytotoxic T lymphocytes. These lymphocytes are specialized cells by expression of somatically generated, diverse antigen-specific receptors allowing a flexible and broader repertoire of responses than the innate immune cells.[18, 19]

Primary adaptive responses are slower than the innate responses since clonal expansion due to recognition of foreign antigens is required to obtain adequate antigen-specific B and T lymphocytes to fight the infection.[20, 21] However, during the primary adaptive immune response subsets of lymphocytes differentiates to long-lasting memory cells with a heightened state of immune reactivity at subsequent exposures of the same antigen.

1.2.3. Combined innate and adaptive immune responses (Figure 5)

The innate immune system regulates adaptive immune responses by production of cytokines, interactions between DCs and lymphocytes, and activation of the complement system. The adaptive immune system modulates the innate immune responses by cytokine and antibody production.[22]

Being a key player in the interphase between innate and adaptive immunity, DCs take up foreign antigens, migrate to lymphoid organs and present these antigens to adaptive immune cells. NK cells also participate in the cellular crosstalk between the two systems through interacting bidirectionally with DCs, promoting DC maturation and eliminating immature DCs, thus reciprocally regulating activation of NK cells.[23-25]

Through interaction of all cellular components, the key role of the immune system is to maintain tissue homeostasis. But it is, however, also implicated in the pathogenesis of several chronic diseases, also cancer.[26]

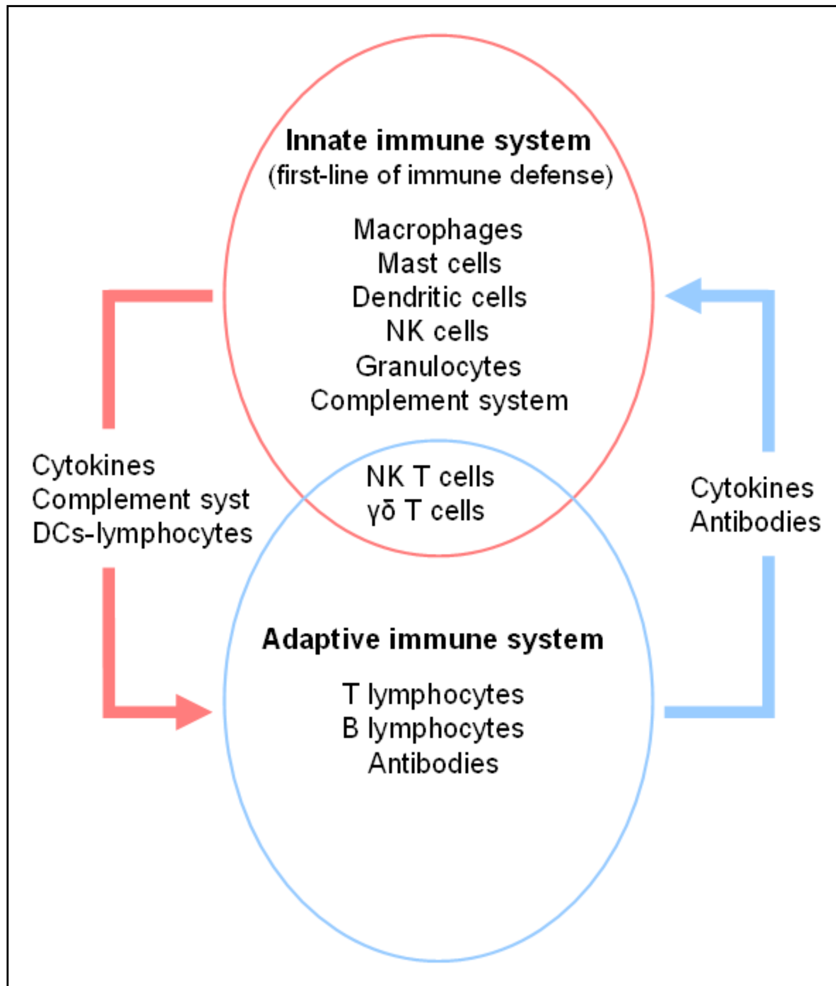

Figure 5: *Schematic presentation of the interplay between innate and adaptive immunity. NK T cells and γδ T cells play their roles in the crossroad between the innate and adaptive immune system. The crosstalk between these systems is mediated by complex interactions between cells of both immune subsets and their soluble factors. The innate immune system, i.e. the first line of immune defense, regulates adaptive immune responses by the production of cytokines, interactions between dendritic cells and lymphocytes and activation of the complement system. The adaptive immune system modulates innate immune responses by cytokine and antibody production. Adapted from de Visser et al.*[22]

1.3. Tumour Immunity

Malignant transformation is a complex process that results in the expression of proteins that are seen as non-self by the immune system. The fact that cancers exist suggest that the immune surveillance is imperfect. The fact that some tumors escape these lines of defence does not, however, preclude the possibility that others may have been aborted.[27] A

strong argument for the existence of immunosurveillance with respect to transformed or malignant cells, is the increased frequency of cancers in immunodeficient hosts (e.g. AIDS patients).[28]

The predominant host cells recruited to and/or activated in the vicinity of the tumor are immune cells, fibroblasts and endothelial cells. Furthermore, there is an abundant collection of growth factors, pro-angiogenic mediators, cytokines, chemokines and components of the extra cellular matrix. There are increasing evidence indicating that many of the processes occurring in the tumor microenvironment are hijacked by the malignant tumor to facilitate its progression.[29] However, the results of several studies indicated that the immune system could be harnessed and directed as an anticancer therapy. This may be a reality if we gain a better understanding of its mechanisms of action in malignancy.[30]

Cancers are often infiltrated by inflammatory cells (T and B lymphocytes, NK cells, DCs, macrophages, neutrophils, eosinophils and mast cells), which are variably scattered within the tumor and loaded with different inflammatory mediators.[15] Tumor infiltrating lymphocytes (TIL) are mostly T cells[30], and cytotoxic T lymphocytes are capable of specific lysis of tumor cells. Tumor-specific CD4+ T cells can be isolated from various human solid tumors.[31] Furthermore, selective expansion of tumor-specific CD8+ cells in a neoplasm is a probable indication of an ongoing immune response in various tumors, including lung carcinoma.[32, 33]

1.3.1. Inflammatory cells and cancer prognosis

The presence of different tumor infiltrating inflammatory cells has been correlated to a lower tumor stage and a better survival in a variety of human cancers like colorectal carcinoma[34], squamous cell carcinoma of the esophagus[35] and ovarian cancer[36]. In contrast, no such correlation was found in other types cancer like hepatocellular carcinoma[37], and an

association with shorter survival was reported in renal cell carcinoma.[38] In many studies, distinction could not be made with respect to inflammatory cell localization, which may have caused the lack of significant results, as inflammatory cells in different tumor compartments (stromal vs. intraepithelial) may have different functions and roles in this respect. It was only after such a categorization was done, that survival effect could be associated with in situ immunological processes in some studies.[39] In NSCLC, the published results are contradictory. While, some studies reported that a high number of some types inflammatory cells was associated with a better survival[39, 40], others found no such correlation.[41]

1.3.2. Antitumor effector mechanisms

Antitumor effectors are mediated by many mechanisms (Figure 6):

1. Cytotoxic T lymphocytes as evidenced by the presence of major histocompatibility complex (MHC)-restricted CD8+ cells within human tumors.[30]

2. Natural killer cells that can destroy tumor cells without prior sensitization. After activation by IL-2, NK cells can lyses a wide range of human tumors even if they appear not to be immunogenic for T cells. CD8+ T lymphocytes and NK cells seem to provide complementary antitumor mechanisms. Tumors that fail to express MHC class I antigens cannot be recognized by CD8+ T-cells, but NK cells, which will lose the inhibition mediated by the recognition of normal autologous class I molecules, can trigger them.[42]

3. Humoral mechanisms mediated by tumor-specific antibodies produced by B lymphocytes, mediate tumor killing by activation of the complement system as well as antibody-dependant cellular cytotoxicity (ADCC).[30]

4. Cytotoxic molecules secreted by immune cells; e.g. cytokines like TNF-α secreted by macrophage; perforin and granzyme secreted by NK and T cells.[43]

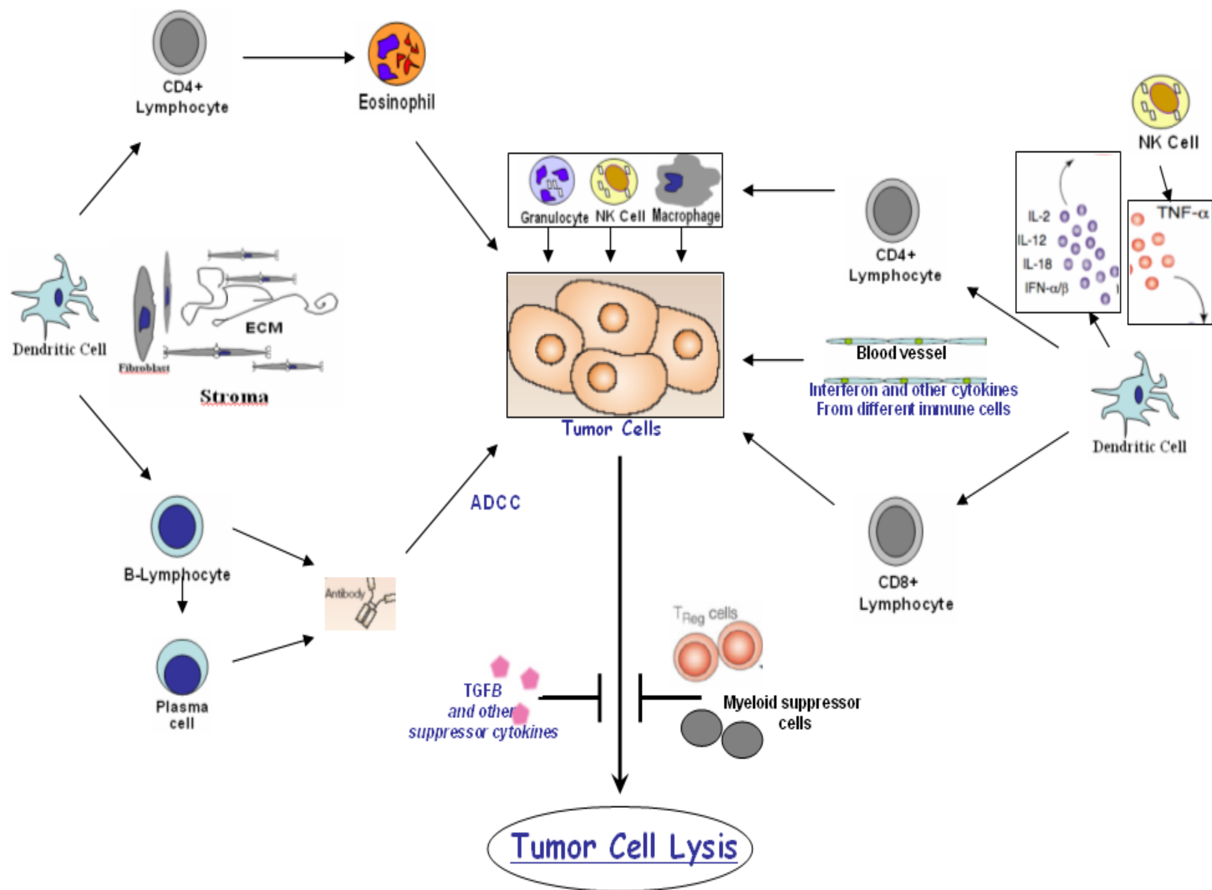

Figure 6: *Cellular effectors of antitumor immunity and some cytokines that modulate antitumor activities. DC present tumor antigen to cytotoxic T cells (CD8+) which can kill tumor cells directly by releasing cytotoxic granules (e.g. perforin and granzyme). The survival and persistence of CD8+ memory cells are regulated by CD4+ helper T cells. Both CD8+ and CD4+ secrete IFN-γ which up-regulate MHC class I and further sensitize cancer cells to CD8+ cells, promote recruitment of NK cells, granulocytes and macrophages and interfere with angiogenesis. Dendritic cells can also activate IL-5 secreting CD4+ T-helper 2 lymphocytes, which recruit eosinophils and stimulate the humoral immune system leading to antibody production by B-lymphocytes and plasma cells. Antibodies against tumor antigens can mediate antibody-mediated cell cytotoxicity (ADCC) by macrophages and NK cells.*

1.3.3. Cancers and immunosurveillance

Cancer can evade the immune system by several escape mechanisms (reviewed by Marincola et al [44]):

1. Selective outgrowth of antigen-negative variants during tumor progression. Strongly immunogenic subclones may be eliminated.

2. Loss or reduced expression of HLA. Cytotoxic CD8+ and CD4+ T cells can recognize cell-bound antigens only in association with HLA class I and II, respectively. However, this loss interrupts the inhibitory signals of NK cells and allowing their activation with lyses of target cells.

3. Lack of co-stimulatory molecules that is required for T cell sensitization. This may render T cells anergic or cause them to undergo apoptosis.

4. Immunosuppression by tumor growth factors like TGF-β, which inhibit NK cells, NK-T cells and cytotoxic T cells, and IL-10 which can affect DCs function.[29]

5. The tumor-induced immune response includes T regulatory (Treg) and myeloid derived suppressor cells (MDSC), which suppress tumor immunity.

6. Tumor cell may inhibit the T cell-receptor-induced surface expression of Fas-ligand on effector cells. Normally, Fas-ligand, cross links Fas receptor (CD95) on target cells, initiating caspase activation and leading to apoptosis.[45]

1.3.4. The pro-tumorigenic role of the immune system

The immune system has a paradoxical role during cancer development. Generally, chronic activation of various types of innate immune cells might contribute to tumor development/progression. Whereas, cells of the adaptive immune system carry out surveillance and can eradicate various tumors.[29, 46]

In tissues, monocytes migrate to the site of injury guided by chemotactic factors, and once activated; tumor associated macrophages (TAMs) are the main source of growth factors and cytokines which facilitate tumor growth, angiogenesis, cell motility and invasion (Figure 7).[40] This antagonizes the anti-tumorigenic effect of macrophages and the net result may be pro-tumorigenic.[41] In fact, an experimental study has demonstrated that interaction between lung cancer cells and macrophages promotes the invasiveness and matrix-degrading activity of cancer cells.[47] Macrophages are polarized into M1 (antitumorigenic) and M2 (pro-tumorigenic). Differentiation of M1 TAMs is induced by IFN-γ and TNF-α, whereas M2 TAMs activation may be induced by signals derived from Treg cells or cancer cells themselves (M-CSF, IL-10 and TGF-β).[48]

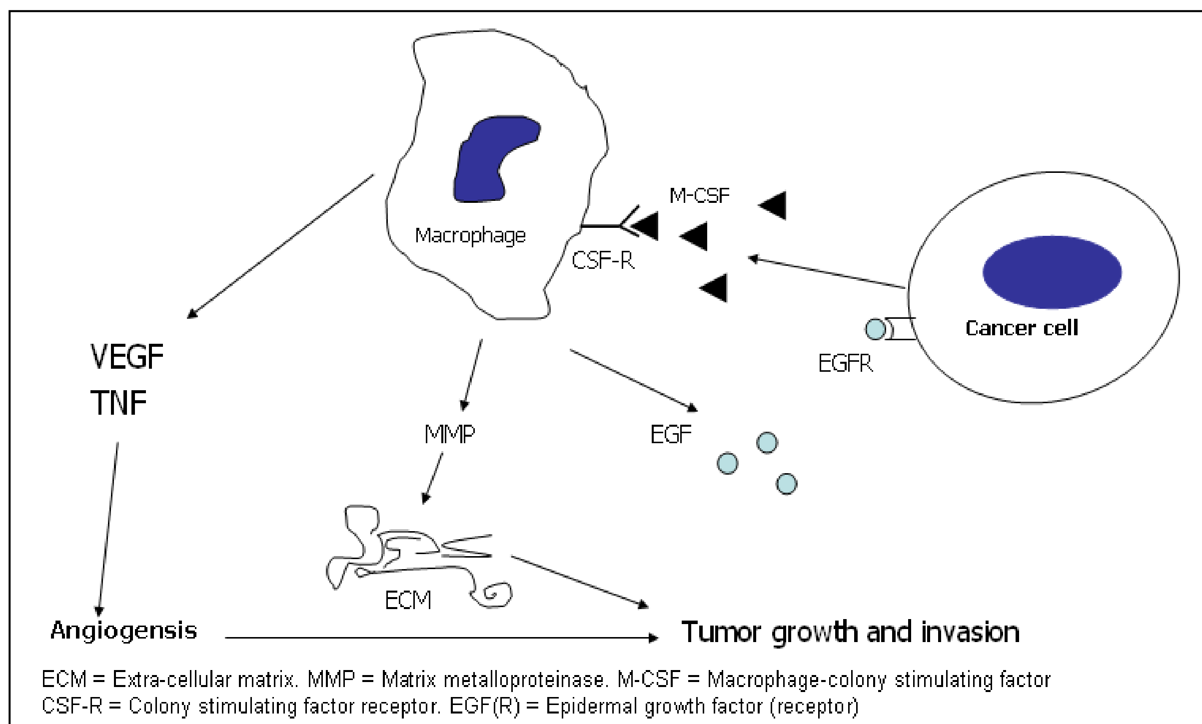

Figure 7. *The role of macrophages, M-CSF and CSF-1R in tumor growth and invasion. Macrophages stimulated by M-CSF can stimulate the growth of cancer cells directly, as well as via stimulating angiogenesis. Angiogenesis and matrix degradation facilitate invasion and metastasis.*

Similarly, CD4+ cells include, in addition to the T helper cells, Treg cells which are CD4+, CD25+ and Forkhead Box Protein P3-psitive (FOXP3+) cells and which suppress the effecter functions of cytotoxic T cells.[49] Furthermore, a subset of innate immune cells

(MDSC, which are CD11b+ cells) may accumulate in tumors and lymphoid tissue. MDSC are known to induce T lymphocyte dysfunction by direct cell-cell contact as well as production of immunosuppressive mediators.[50]

Perhaps the most compelling clinical evidence for a causative link between chronic inflammation and cancer comes from studies reporting that inhibiting chronic inflammation in patients with pre-malignant disease has preventive effect. Long-term usage of anti-inflammatory drugs (as COX2 inhibitors) significantly reduce the risk of some cancers.[51]

Finally, B lymphocytes were found to be required for establishing chronic inflammatory states that promote de novo carcinogenesis via recruiting innate immune cells.[52]

1.3.5. Immunotherapy for Tumours

The facts that cancer is more common in immuno-compromized patients as well as the significantly better survival in cases with higher number of immune cells in the cancer tissues indicate that directed immunotherapy may be a way to battle against malignant cells. This may be accomplished by:

1. Adoptive cellular therapy. Incubation of lymphocytes harvested from surgically resected tumor masses with IL-2 and IL-7[53] generates activated lymphocytes with potent antitumor activity in vitro (Figure 8).

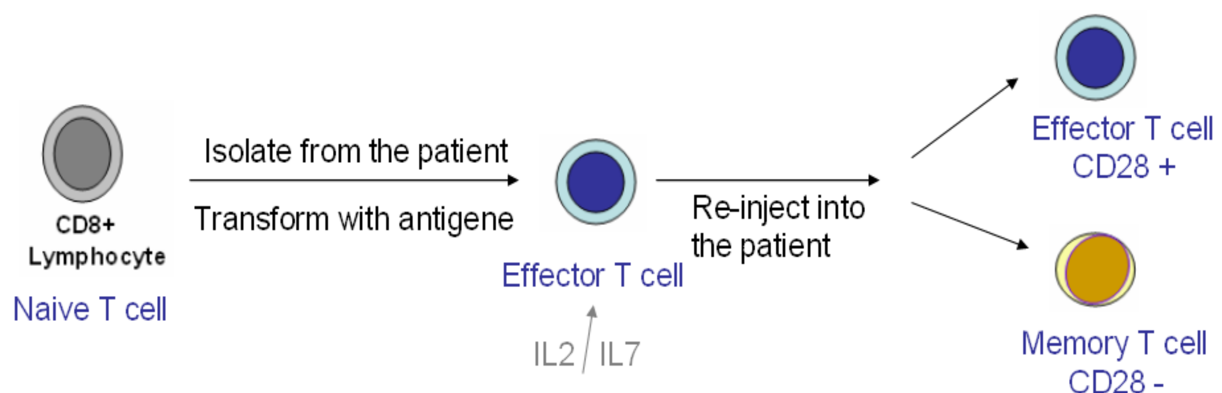

Figure 8: *Modification of CD8+ T cells with anti-tumor activity.*

2. Cytokine therapy, e.g. IFN-γ is directly cytotoxic, it also activates NK cells and increase expression of HLA on tumor cells.

3. Antibody-based therapy. Monoclonal antibodies against certain B-cell lymphoma are already in use. However, antibodies against tumor-associated antigens have not proven efficacious in other types cancer.

4. Tumor vaccination utilizing DCs.[54]

5. Disrupting tumor-mediated mechanisms hindering host immunity could be a novel approach to tumor immunotherapy. Treg depletion and blockade of differentiation improve endogenous anti-tumor immunity and the efficacy of active immunotherapy in animal models for cancer.[49] Some chemotherapeutic agents, like sunitinib, decreased the number of MDSC and Treg in tumor-bearing animals and reduced expression of IL-10, TGF-β, and Foxp3.[55]

1.4. Tumor compartments

Until recent years, the principal focus in cancer research has mostly been the malignant cell itself. Today, it is hypothesized that understanding the nature of the tumor environment is equally important for future cancer therapy as understanding the cancer genetics. Cancers are not simply collections of autonomous malignant cells, but also composed of multiple cell types such as fibroblasts, innate and adaptive immune cells, endothelial cells, and specialized mesenchymal cells. These different cell types in the stromal environment can be recruited by malignant cells in order to support tumor growth and facilitate metastatic dissemination. The historical lack of research interest in the tumor microenvironment has led to a significant discrepancy between the vast knowledge about cancer cell biology and a limited understanding of the tumor microenvironment's role as a whole.

1.4.1. Definitions

Each tumor is composed of two compartments:

1. The cancer cells (epithelial cells in case of carcinoma).

2. Tumor stroma: A peritumoral specialized connective tissue consists of: 1) The non-malignant cells of the tumor; activated fibroblasts, specialized mesenchymal cell types distinctive to each tissue environment, innate and adaptive immune cells, and the vasculature with endothelial cells and pericytes, as well as 2) the extracellular matrix (ECM). ECM consists of structural proteins (collagen, elastin), specialized proteins (fibrillin, fibronectin, elastin) and proteoglycans.[56]

Tumor stroma plays a central role in tumor development, as it provides growth factors, blood supply, and exctracellular matrix and removes waste and dead cells. On the other hand, a variety of antitumor mechanisms acts through the tumor stroma.[56] The expansion of the tumor stroma with an enhanced number of fibroblasts and increased deposition of ECM is termed tumor desmoplasia. Tumor stroma can be divided topographically into:

1. The stroma between tumor cells/clusters.

2. The stroma at the advancing edge of the tumor.

However, in most cases of NSCLC (especially in adenocarcinoma), cancer cells proliferate along the alveolar walls of the lung in the periphery of the tumor, and does not have stroma at the invasive margin[39]. Hence, such topographical stromal distinction was not done in our study.

1.4.2. The role of tumor stroma in the interaction between tumor and immune system

It has become clear that analysing the tumor microenvironment is of crucial importance to better understand cancer.[56] Various cytokines contribute to tumour rejection

mainly by their action on tumor stroma (e.g. by attacking angiogenesis, Figure. 9), whereas killing mechanisms mediated by molecules, such as perforin or Fas ligand, act on the epithelial cells as well as the stroma.[56] IL-4 secreted by CD4+ and CD8+ T cells helps in tumor rejection mainly by acting on the tumor associated fibroblasts in the tumor microenvironment converting them to a phenotype that is not able to participate in angiogenesis.[57]

IFN-γ counteract tumor-induced angiogenesis by acting on the endothelial cells.[58] Finally, TNF-α suppress tumor growth by acting directly on endothelial cells, or indirectly by acting on non-bone-marrow derived cells inhibiting the release of pro-angiogenic factors.[56]

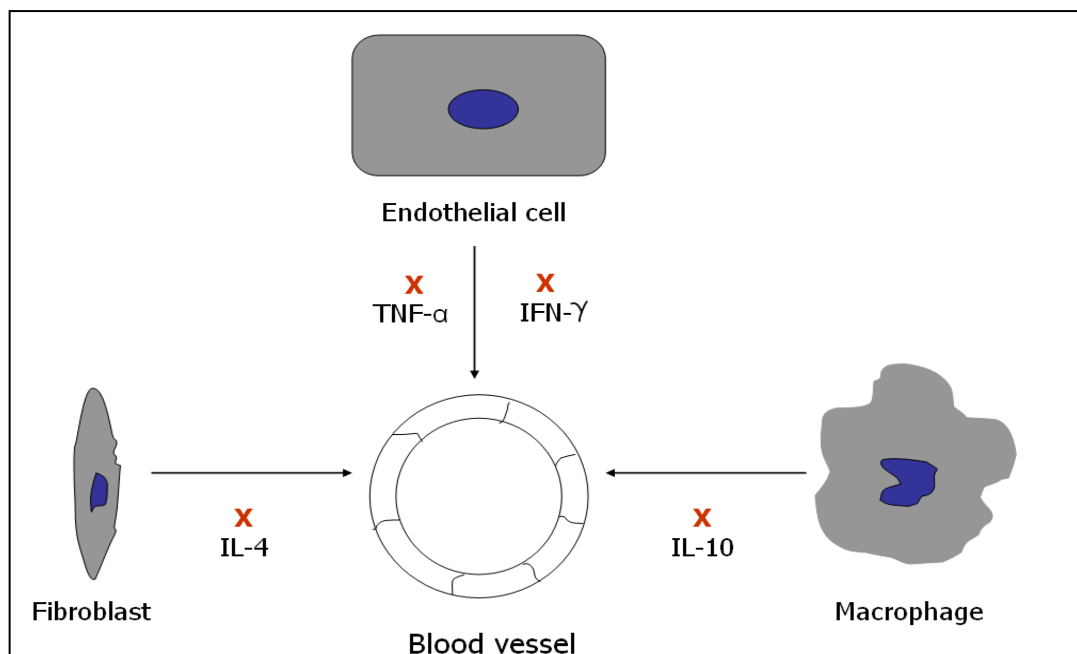

Figure 9: *Different cytokines mediate their anti-tumour effect by attacking angiogenesis in the tumor stroma. IL4 acts mostly on tumor-associated fibroblasts, IL-10 on macrophages and TNFα and IFN-γ on endothelial cells (x=inhibition), modified from Blankenstein et al).*[56]

1.5. Tissue microarray

Tissue microarrays (TMAs) may be used for large-scale investigation of the biologic and prognostic value of molecular marker families. TMAs allow rapid visualization of molecular targets in hundreds of tissue specimens on a single slide, either at DNA, RNA or protein level.[59] The technique may facilitate rapid translation of molecular discoveries to

clinical applications. However, it is a population-level research tool and is not intended for making clinical diagnosis for individual cases.[60] Table 4 summarizes some of the advantages and disadvantages of TMA.

Table 4. *Advantages and disadvantages with TMA technology.*

Advantages	**Disadvantages**
- Time saving. - Cost effective. - Large number of cases can be analyzed. - Consecutive sections can be taken. - Permits the whole staining procedure to be done as one experiment.	- Low representativity in heterogeneous tissue. - Not suitable for diagnosis.

The history of TMAs is relatively short and one of the first premature multicore blocks was used in 1986, when Battifora described a method of embedding 100 or more different tissue samples in a normal sized paraffin block. During the last decade has this high-throughput technique been commonly used.[60] One of the first large scale TMA studies on NSCLC was published by Bremnes et al. in 2002.[61]

TMAs, in contrast to routine hematoxylin and eosin–stained sections, provide a far more efficient workflow. With TMAs, the diagnostic skills of the pathologist occurs only once and up front avoiding the bias of post hoc analysis that can occur with conventional whole section analysis.[62] Routine hematoxylin and eosin–stained sections of tumors in the cohort are analyzed, and areas of tumor are circled by the pathologist (Figure 10). Thus, the pathologist‘s diagnostic skills are used in a prospective manner.[62]

However, TMAs are only as good as the cohorts from which they are created. Because TMA technology facilitates the development of large cohorts, researchers often use archival tissue from greater time spans, increasing the chance that variations in tissue processing techniques over time can confound results.[62] Although a common concern is whether the small core samples used in TMA analysis give meaningful information on large tumor

specimens, up to 95% correlation has been demonstrated when comparing tumor cell assessment in duplicate 0.6mm cores versus the whole slide.[60] To our knowledge, we are the first using TMA technique to study variables such as immunological cell markers, in NSCLC tumor cells and tumor related stroma.

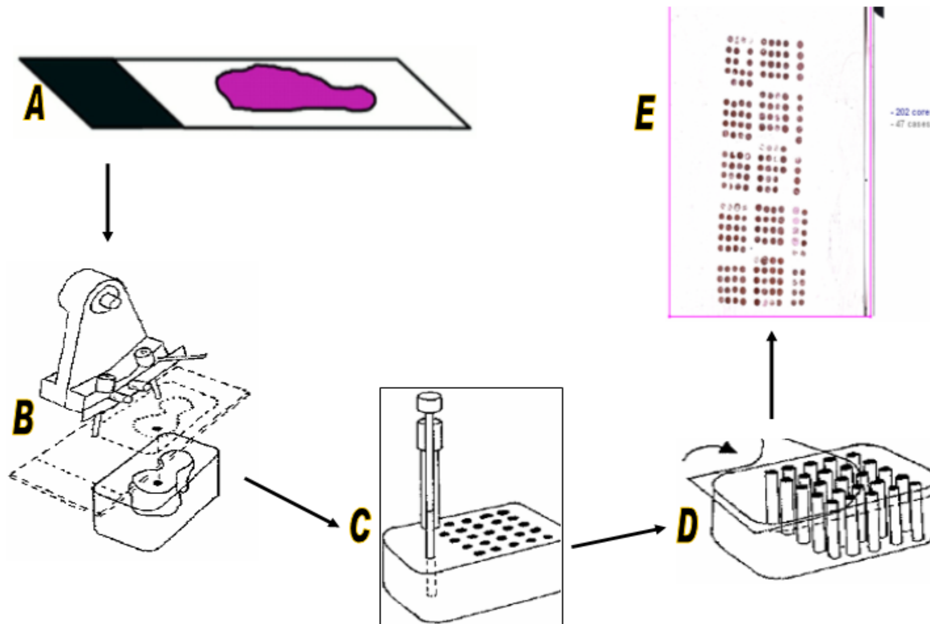

Figure 10: *The principle of tissue microarray. A: The hematoxylin-eosin slides from each tumor were studied and the desired areas marked. B: A tissue core biopsy was punched from the preselected region of neoplastic cells or tumor related stroma (0.6 mm in diameter). C: The cylindrical samples were extruded directly into the recipient block at defined array coordinates. D: Multiple 5-µm sections were cut and E: Immunohistochemical staining with selected antibodies to be studied. E.g. slide containing 202 cores, representing 47 patients.*

2. AIMS OF THE STUDY

The aims of this study are:

1. To assess the role of different immune cell types in NSCLC and their relation to survival as well as to other clinicopathological variables.

2. To study separately the meaning, if any, of the stromal and intraepithelial immune cells as different compartments of the same tumor.

3. MATERIALS AND METHODS

3.1. Patients and tissue samples

371 patients diagnosed with NSCLC pathological stage I-III at the University Hospital of Northern Norway and the Nordland Central Hospital during 1990 through 2004 were considered for this study. Normal lung tissue was secured from normal paraffin embedded lung areas, but also from normal lung tissue obtained during autopsy. Demographic, clinical, treatment, and outcome data have been collected from medical records. An anonymised database was established. Survival data were updated in early 2009. 36 patients were excluded from the study due to: (i) Radiotherapy or chemotherapy prior to surgery (n = 10); (ii) Other malignancy within five years prior to NSCLC diagnosis (n = 13); (iii) Adequate paraffin-embedded fixed tissue blocks not available (n = 13). Thus, 335 patients were included in this study (Figure 11).

The median follow-up of survivors was 86 months (range 48-216). Two pathologists (Samer Al-Saad and Khalid Al-Shibli), blinded to any pathological or clinical information, reviewed all the cases; the diagnosis of carcinoma, histological type, vascular invasion and pathological stage were confirmed before including any case in the study. Nodal metastasis was assessed using the haematoxylin-eosin slides without using immunohistochemistry with cytokeratin for detecting micrometastasis. The tumors were staged according to the International Union Against Cancer's TNM classification[63], stage IA and IB patients were grouped together as stage I, and stage IIA and IIB patients were grouped as stage II. Histological classification was done according to the World Health Organization guidelines.[2] The National Data Inspection Board and The Regional Committee for Research Ethics in Northern Norway approved this study.

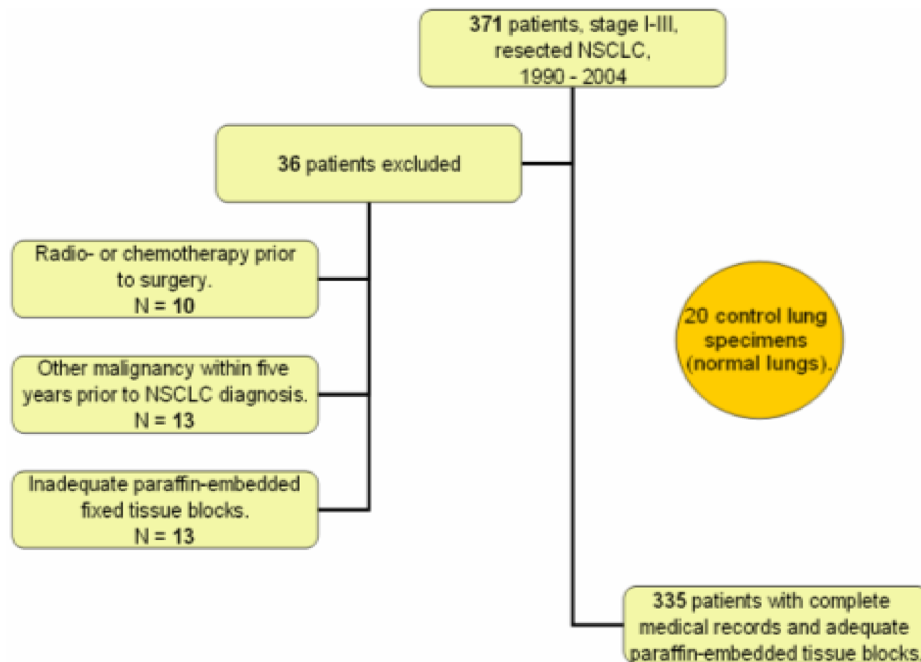

Figure 11: *The patients included in the study.*

3.2. Microarray construction

TMA were constructed by choosing the most representative paraffin block(s) for each case. Studies suggest that punching multiple 0.6mm cores from different regions captures the heterogeneity of the tumors more accurately than a single 2-4mm core.[60] Hence, we chose to use four 0.6mm cores [two areas of viable invasive carcinoma tissue (neoplastic epithelium) and two from the surrounding tumor stroma] that were selected to be as representative as possible, after reviewing all the original sections of the tumor and taking the fact of heterogeneity in consideration. In addition, all the surface areas of the four cores were assessed. The TMAs were obtained using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD), consisting of thin-walled stainless steel biopsy needles and stylets to biopsy the donor block and transfer the needle content and empty it into the recipient block. We used 0.6 mm diameter stylet to sample two separate pre-defined neoplastic epithelial areas and two stromal areas. To include the entire 1340 plus the control cores, eight tissue array blocks were constructed. Multiple 5-µm sections were cut with a Micron microtome

(HM355S) and stained by specific antibodies for immunohistochemistry analyses.

3.3. Immunohistochemistry

Sections were de-parafinized with xyline and re-hydrated with ethanol. Antigen retrieval was performed by placing the specimens in 0.01M citrate buffer at PH 6.0 and exposed to two-repeated microwave heating of 10 minutes at 450W. The slides were then transferred to the Ventana Benchmark, XT automated slide stainer (Ventana Medical System, France). Tissue sections were incubated with primary mouse monoclonal antibodies recognizing CD1a, CD3, CD4, CD8, CD20, CD34, CD56, CD68, CD117, CD138, M-CSF, and CSF-1R. The details of the antibodies and their incubation periods are shown in Table 5.

As secondary antibodies biotinylated goat anti-mouse IgG and mouse anti-rabbit IgM, both 200µg/ml, were used. The kit DAKO EnVision+ System-HRP (DAB) kit was used as endogen peroxidase blocking. The DAB kit was used to visualize the antigens by application of liquid diaminobenzidine and substrate-chromogen, yielding a brown reaction product at the site of the target antigen. Finally, all slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including negative staining controls, all staining was performed in a single experiment. As negative staining controls, the primary antibodies were replaced with the primary antibody diluents.

3.4. Scoring of immunohistochemistry

By light microscopy, the tissue sections were scored for the degree of infiltration of different inflammatory cells. The percentages of these cells, compared to the total amount of nucleated cells in the epithelial and stromal compartments were assessed. The percentage of cells, and not a number, was used to make data more reproducible independent of the slide thickness. The CD138+ and CD4+ cells were scored as high if represent >5% of the

Antibody	Production lab.	Antibody Clon	Lot number	Dilution**	Antibody Incubation Time (minutes)
CD 1a	Ventana	JMP	457650	VMS	32
CD 3	Ventana	PS1	504194	VMS	24
CD 4	Novacastra	1F6	116057	1/5	20
CD 8	Ventana	1A5	492779	VMS	32
CD 20	Ventana	L26	4198946	VMS	16
CD 34	Ventana	QBEnd/10	508686	VMS	32
CD 56	Ventana	123C3.D5	21036	VMS	16
CD 68	Ventana	KP-1	508662	VMS	16
CD117	Ventana	Anti-C-kit (9.7)	506649	VMS	32
CD138	Ventana	B-A38	20057	VMS	32
CSF-1R	Santa Cruz*	H-300	K0404	1:25	16
M-CSF	Santa Cruz*	H-300	K0304	1:5	28

Table 5: *The antibodies used in the study.*

*Santa Cruz Biotechnology.

**Ventana antibodies are ready diluted from the manufacturer.

nucleated cells in the epithelial compartment or >25% in the stromal compartment, and low otherwise. Both CD20+ and CD1a+ cells were scored as low if absent or representing <1% of the nucleated cells and high otherwise in both epithelial and stromal compartments. DCs were identified by their brown membranous immunoreactivity as well as their cytoplasmic flame-like extensions. The same scoring (<1% of the nucleated cells) was applied to intraepithelial CD68+ cells, whereas CD68+ cells in the stroma were more abundant and scored as low if they represented <25% of total nucleated cells and high otherwise. Both CD56+ and CD117+ cells were present in the epithelial compartment in only seven cases and in the stromal compartment in few cases, and when present they were very sparse and their highest concentration did not exceed 1%. Hence, they were scored as absent or present in both the stromal and epithelial cores. CD3+ cells were abundant in the stroma and were scored as high if they represented >50% of nucleated cells in the stroma, whereas <1% in the epithelium was

scored as low and high otherwise. For CD8+ cells the cut-off point was ≤5% and ≤50% for epithelial and stromal components, respectively.

CD138 is known to stain epithelial cells themselves and the staining intensity in the epithelial compartment was scored as 0 = negative; 1 = weak; 2 = intermediate; and 3 = strong. A similar scoring was used for epithelial M-CSF and CSF-1R. High expression in tumor epithelial cells was defined as score >1.0 for CD138 and ≥1.5 for both M-CSF and CSF-1R. Stromal M-CSF and CSF-1R expressions were calculated by adding intensity score (as above) to density score [scored by measuring the ratio of positive cells compared to the surface area of the extracellular matrix, and was categorized accordingly as: 1 = low density (<25% cell/matrix ratio); 2 = intermediate density (25-50%) and 3 = high density (>50%)] before categorizing into low and high expression. High expression in the stroma was defined as >3.5 and >3 for M-CSF and CSF-1R, respectively.

The interstitial tissue of the non-neoplastic normal controls showed few (1-5%) CD68+ CD3+, CD4+ and CD8+ cells; and very sparse (0-1%) CD117+, CD138+, CD56+ and CD1a+ cells. There was no significant infiltration of the normal bronchial columnar epithelium by any type inflammatory cells. The bronchial columnar epithelium showed moderate membranous and cytoplasmic positivity for CD138. Almost all pneumocytes in the control sections were weakly positive for M-CSF, whereas 30% were weakly positive for CSF-1R. Almost all alveolar macrophages showed moderate to severe positive staining for both M-CSF and CSF-1R.

MVD was assessed using CD34 immunohistochemistry. Any stained endothelial cell or endothelial cell cluster was considered as a single countable microvessel. Stromal MVD was scored as 0, negative; 1, 1-10 vessels per core; 2, 11-20 vessel per core; 3, >20 vessel per core. A mean score for duplicate cores from each patient was calculated, and high stromal MVD was defined as a mean score ≥3.[64]

All samples were anonymized and independently scored by two pathologists (Samer Al-Saad and Khalid Al-Shibli). In case of disagreement, the slides were re-examined and the observers reached a consensus. When assessing one marker in a given core, both observers were blinded regarding the scores of other markers as well as to the patient's outcome. The inter-observer scoring agreement between the two pathologists was tested on the current material in a previous report.[64] The mean correlation coefficient (r) was 0.95 (range 0.93-0.98).

3.4.1 Cut-off points

Variation in methods including differences in tissue preparation, antigen retrieval, and assessment of positive staining makes it difficult to standardize cut-off values. Many studies use the median as cut-off value, but the obvious disadvantage with this approach is missing biological interesting mechanisms. For instance, this may be the case where only the minority or the majority of the patients had a high expression level linked to a certain biological effect. In our binary cut-off points for the biomarkers, the cut-off was determined for each variable so that the two resulting subgroups were the most different according to disease specific survival (DSS). The main drawback with this approach is the danger of false positive results, and especially borderline significant results in the analyses must be interpreted carefully. Arbitrary cut-off points at 1%, 5%, 25%, or 50% for each cell/compartment according to the degree of cell densities were used, as these percentages are easy to follow and reproduce in daily practice.

3.4.2. Controls and limitations

Both reagent and tissue controls were used. Of all components used for immunohistochemistry, the primary antibody is the most critical. Occasionally other reagents

may need to be replaced. As reagent control, diluent without primary antibody was used.

The limitation of this study is that a single marker identifies each immune cell type. CD68 is not specific for macrophages and has been found in CD1a-positive DCs as well.[65] Although most tumor-infiltrating CD8+ T cells are cytotoxic T cells, some CD8+ T cells are regulatory T cells in cancers including NSCLC.[66] In addition to different T cells, CD4 can be expressed by immature DCs but decrease during maturation.[67] Therefore, it is possible that some of the cells identified by used markers are not the named immune cells.

3.5. Statistical analysis

All statistical analyses were performed using the statistical package SPSS. The Chi-square test and Fishers Exact test were used to examine the association between the density of the inflammatory cell infiltrates and clinicopathological parameters. Univariate analysis was performed by using the Kaplan-Meier method, and statistical significances between survival curves were assessed by the log-rank test. DSS was determined from the date of surgery to the time of lung cancer death. To assess the independent value of different pre-treatment variables on survival, multivariate analysis was carried out using the Cox proportional hazards model. Only variables with a significant P value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.10, respectively. The significance level was set at $P < 0.05$.

4. MAIN RESULTS

The patients' age ranged between 28 and 85 years (median was 67 years), and 75% were males. The NSCLC tumors comprised 191 squamous cell carcinomas, 95 adenocarcinomas, 31 large-cell carcinomas and 18 BACs. Due to nodal metastasis and/or non-radical surgical margins, 59 (18%) patients received postoperative radiotherapy. There

were 232 lymph node negative cases (N0), and 103 cases with regional lymph node metastases (76 N1, 27 N2).

Performance status, pathological stage, T-status, N-status, differentiation, surgical procedure used, vascular infiltration, and postoperative radiotherapy were all significant indicators for disease free survival in univariate analyses. Tumor stage (P = 0.002) and nodal status (P < 0.0001) were the strongest prognostic factors.

Infiltration of different types inflammatory cells were seen in both the intraepithelial and stromal compartments. T and B-lymphocytes were the most common inflammatory cell types, followed by macrophages. Whereas NK cells (CD56+) and mast cells (CD117+) were the rarest. Tumor infiltrating lymphocytes were observed in both the epithelial and stromal compartments, and they were generally more abundant in the stroma.

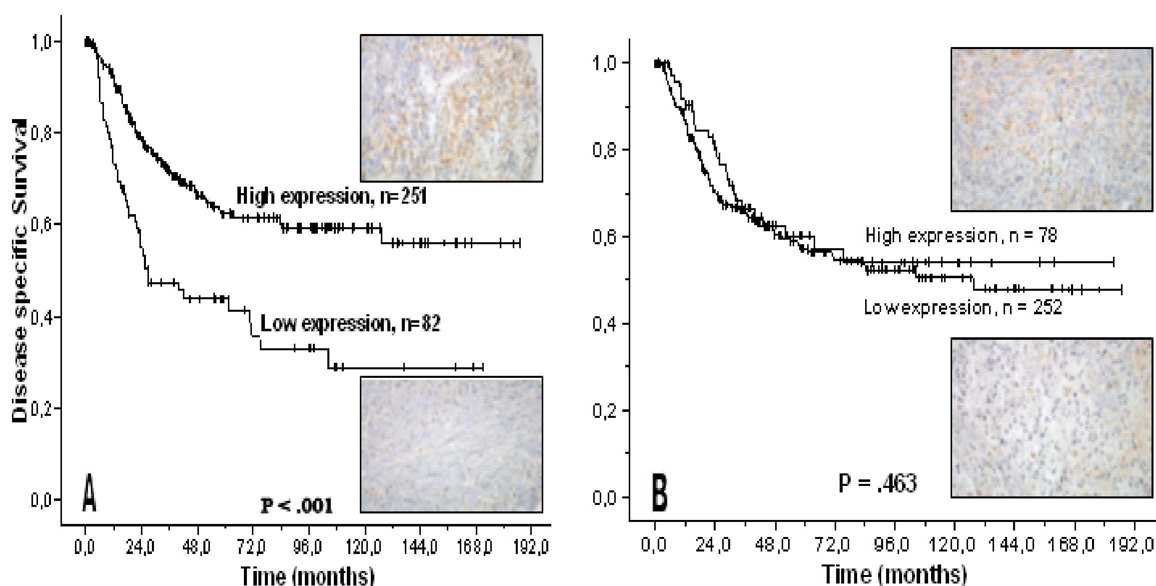

Figure 12: *Kaplan-Meier survival curves with microscopic pictures comparing high and low CD4+ cells in the stromal (A) and epithelial (B) compartments.*

4.1. Paper I

In this study, the aim was to assess the significance of CD4+ and CD8+ T cells, as well as B-lymphocytes (CD20+) in NSCLC intraepithelial and stromal compartments. In univariate analyses, increasing numbers of stromal CD4+ (P < 0.001, Figure 12), stromal

CD20+ (P < 0.001) and stromal CD8+ (P = 0.002, Figure 13) lymphocytes correlated significantly with an improved DSS. The main findings in this study were related to the tumor stromal compartment, where a high number of stromal CD4+ (P = 0.002) and stromal CD8+ (P = 0.043) lymphocytes were independent prognostic factors for DSS. Stromal B-lymphocytes had a positive prognostic effect only in univariate analysis. Furthermore, a high level of stromal CD8+ lymphocytes was associated with a lower incidence of angiolymphatic invasion (P = 0.032).

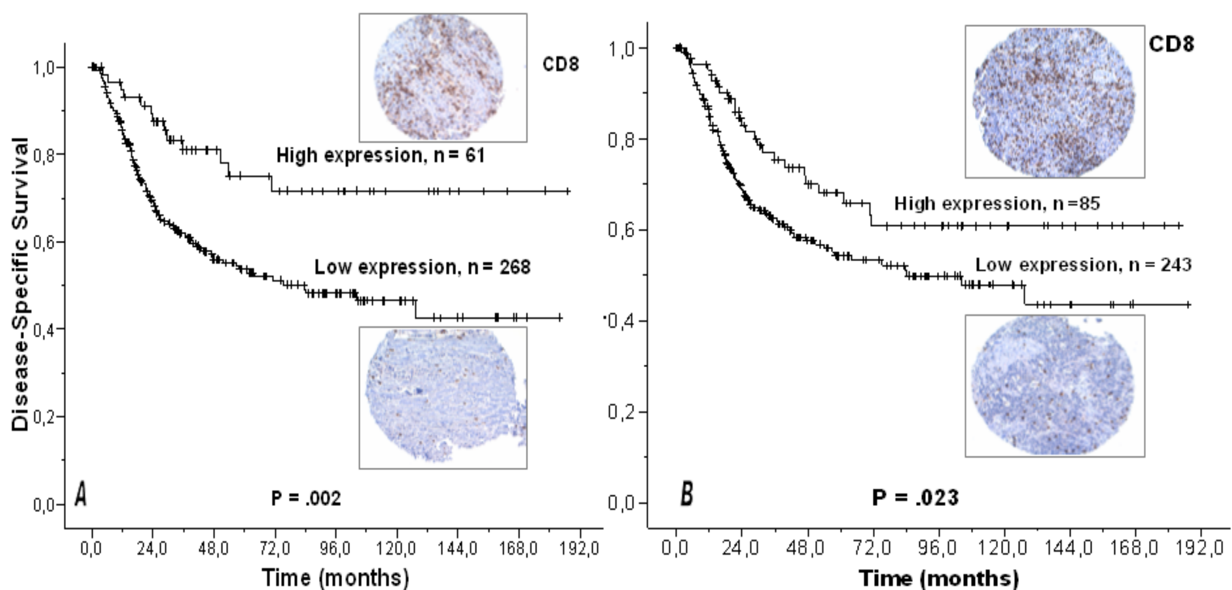

Figure 13: *Kaplan-Meier survival curves with microscopic pictures comparing high and low CD8+ cells in the stromal (A) and epithelial (B) compartments.*

Epithelial lymphocytes showed a significant antitumor effect, but only in the univariate analyses. A large number of epithelial CD8+ lymphocytes had a significantly better DSS than those with low CD8+ cell numbers (P = 0.023). This was observed, however, only in patients without lymph node metastasis (N0) (P = 0.018), whereas patients with lymph node metastasis (N1 and N2) did not showed a significant tendency for better DSS (N1 P = 0.583, N2 P = 0.760). Epithelial CD20+ lymphocytes showed a similar significance (P = 0.023) that was limited to squamous carcinoma (P = 0.030). Epithelial CD4+ cells showed no significant correlation with DSS, and analysis of the cases in subgroups failed to show a

significant prognostic association.

4.2. Paper 2

In this paper, we assessed the role of the innate immune system cells in NSCLC. Tumor infiltrating CD56+ cells were very sparse in both compartments. The 37 cases in which CD56+ cells were present in the stroma, showed a significantly better DSS than those in which NK cells were absent (P = 0.014). No significant association with other clinicopathological variables was noted. For the epithelial compartment, only seven cases have CD56+ cells, an inadequate number for statistical analysis. Macrophages (CD68+) were observed in the epithelial and stromal compartments, and they were generally more abundant in the stroma. In neither compartment did the CD68+ cells show any significant correlation with DSS (epithelial, P = 0.13; stromal, P = 0.11, Figure 14).

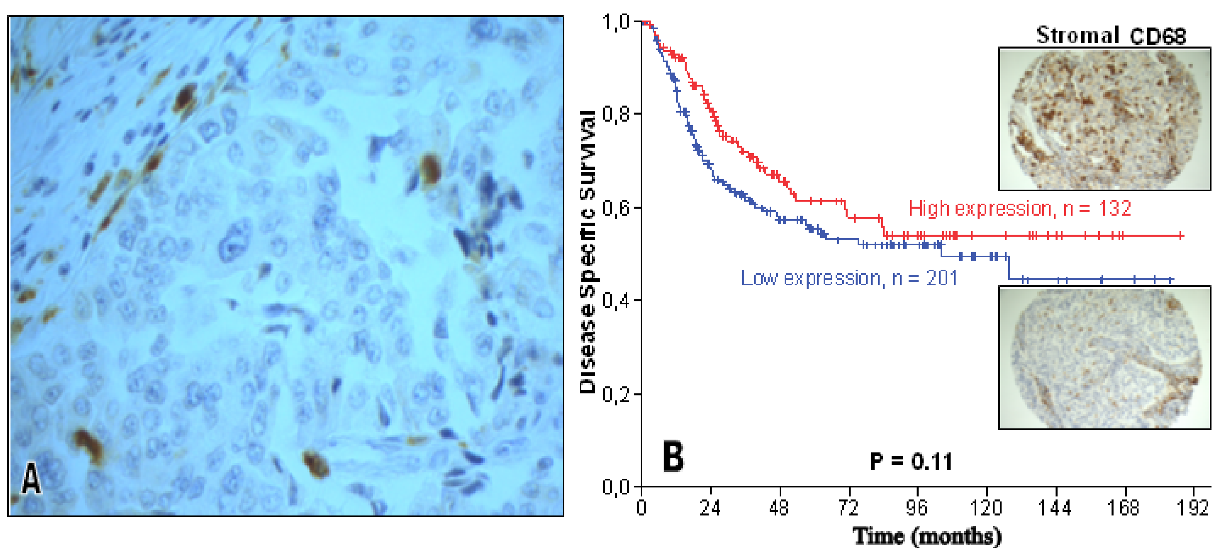

Figure 14 : *Macrophages stained with CD68 in both tissue compartments (A, x400). Kaplan-Meier survival curve comparing high and low stromal CD68+ cell count (B).*

Regarding CD1a+ cells, they were more abundant in the tumor stroma of females than males (high expression; 33% males and 56% females, P < 0.001), and in well differentiated than in less differentiated tumors (high expression; 31%, 37% and 60% for low, moderate and highly differentiated tumors, respectively, P = 0.001). The group of patients with a high stromal CD1a+ cell count had a significantly better DSS than those with a low one (P =

0.011). This was not seen for epithelial CD1a (P = 0.65). Furthermore, patients without lymph node metastases showed a significantly higher stromal CD1a count than those with nodal metastasis (high expression; 45% for N0, 25% for N1 and 19% for N2, P = 0.001). No such associations were noted for epithelial CD1a+ cells.

M-CSF and CSF-1R showed high staining score in the epithelium in 63% and 42% of the cases, respectively (Figure 15). No significant association with DSS was noted (P = 0.37 and 0.83, respectively). The same applies for stromal M-CSF and CSF-1R (P = 0.82 and 0.71, respectively). Neither did subgroup analysis show any significant association with DSS.

In multivariate analyses, high number of stromal CD56+ cells was an independent prognostic factors for DSS (HR 2.3, CI 1.1-5.0, P = 0.031).

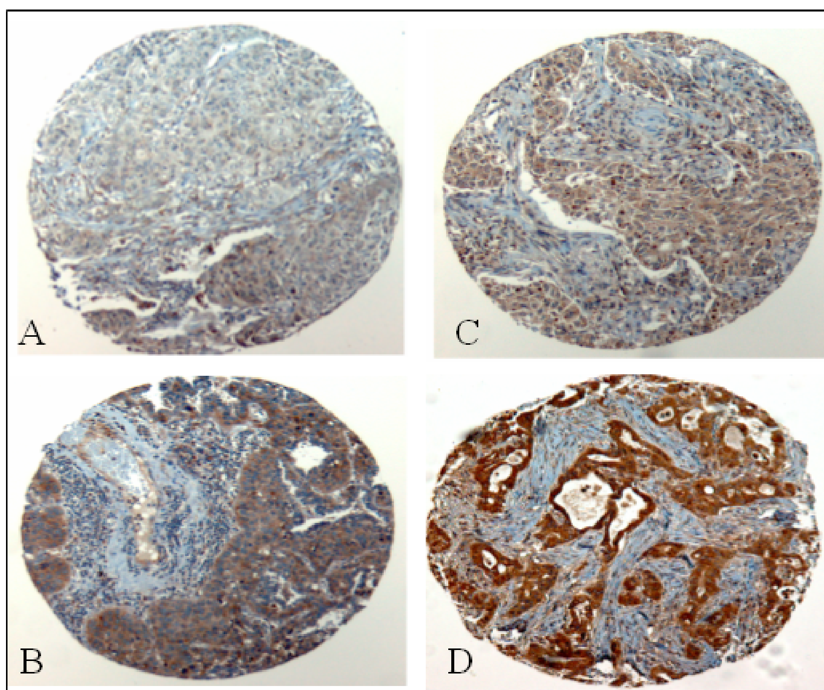

Figure 15. *Epithelial CSF-1R low (A), high (B); M-CSF low (C) and high score (D). X200.*

4.3. Paper 3

Tumor infiltrating CD3+ cells were seen in both compartments. Patients with high stromal CD3+ cells had a significantly better DSS than those with a low count (P = 0.001) (Figure 16). No significant association with other clinicopathological variables was noted. For

the epithelial compartment, high CD3+ cells was also associated with a better DSS (P = 0.004).

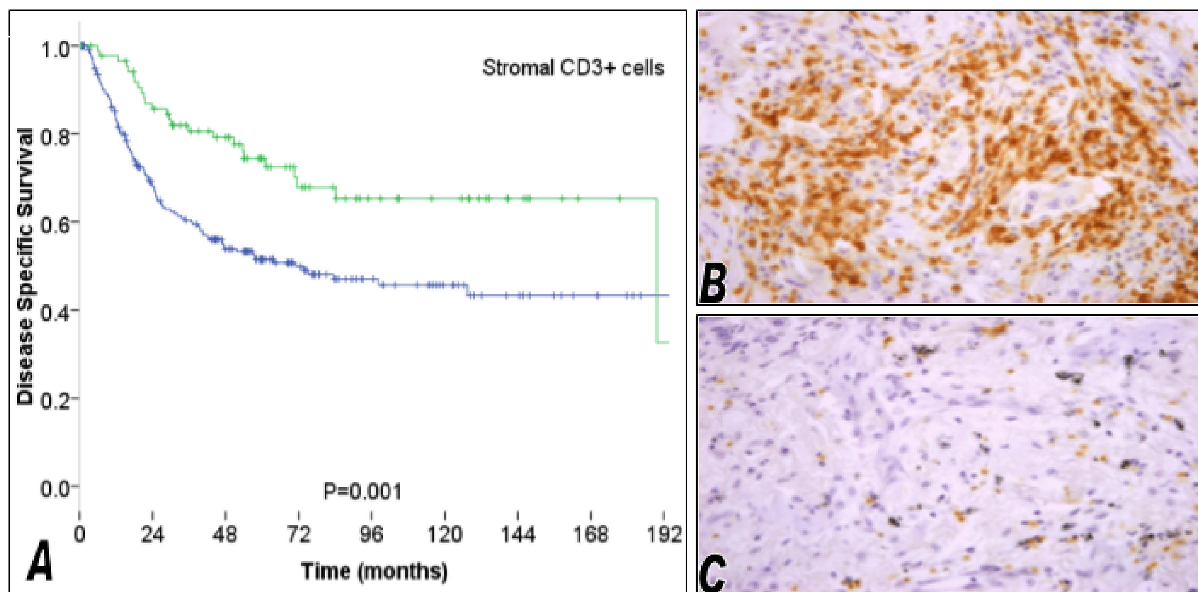

Figure 16: *Kaplan-Meier survival curve (A) with microscopic pictures comparing high (B) and low (C) CD3+ cells in the stromal compartment, x400.*

CD138+ cells were more abundant in the stroma than in the epithelium. In the epithelial compartment CD138+ cells showed no significant correlation with DSS (P = 0.85). Sub-analyses according to histological type, gender, stage and differentiation did not reveal significant prognostic associations. Similarly, high stromal CD138+ cells was associated with a tendency for a better DSS but did not reach significance (P = 0.12). However sub-analyses of CD138+ cells according to gender revealed a significant better DSS in males (P = 0.029), and sub-analyses according to T and N status showed a positive prognostic effect in advanced stage (T3, P = 0.046; N2, P = 0.029 and stage III, P = 0.034).

With respect to cancer epithelial cell staining, CD138 showed a significantly higher positivity in squamous cell carcinoma compared to other histological types (squamous cell carcinoma, 82%; adenocarcinoma, 49%; bronchioalveolar carcinoma, 50%; large cell anaplastic carcinoma, 63%; P<0.001). However, CD138 was not associated with DSS in any histological type.

CD117+ cells in the stroma showed no significant association with DSS, or with any of the clinicopathological variables studied. There was a weak correlation between MVD, assessed by CD34 (Figure 17) and CD117+ cells (R=0.226, P = 0.001).

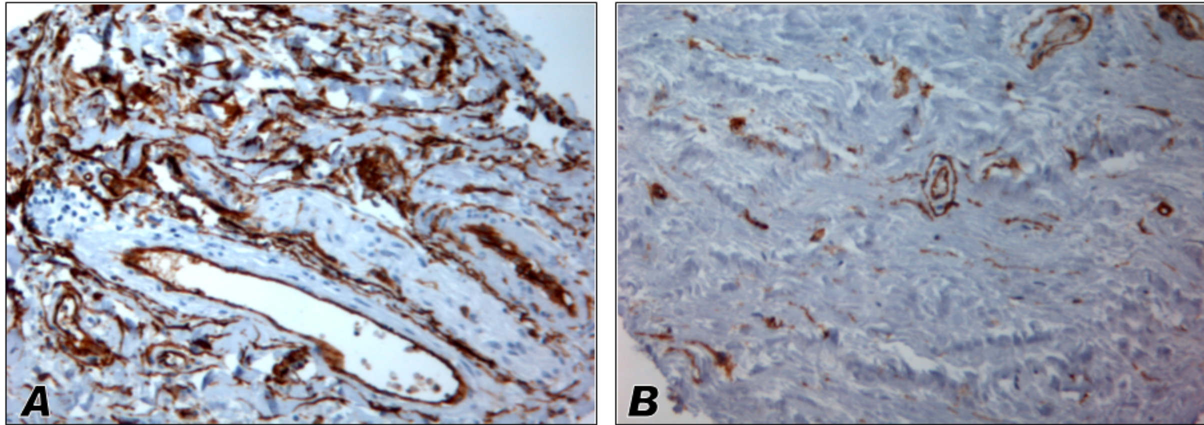

Figure 17. *Stromal staining with CD34 showing high (A) and low (B) MVD score, x400*

In the multivariate analysis a limited number of stromal CD3+ cells was an independent negative prognostic factor for DSS (HR 1.925, CI 1.21-3.04, P = 0.005).

5. DISCUSSION

Approximately one-third of NSCLC patients are diagnosed with early disease and for those patients surgery is the most effective treatment with an intent-to-cure. Following surgery, distant recurrence is the most common form of relapse and eventual cause of death as demonstrated by Subotic et al[68] in their prospective study in which they found locoregional, distant or both types of relapse in 26%, 70% and 4% patients respectively. Hence, there is a need for new prognostic indicators and therapeutic agents. Our study suggests that intratumoral lymphocytes modify tumor stroma or tumor epithelium (or both) in ways that attenuate tumor progression. Most of the beneficial effect of TILs was related to the stromal compartment, where CD8+, CD4+, CD3+ and CD56+ cells were independent positive prognostic factors in our NSCLC cohort. This highlights the importance of the tumor microenvironment, and may point to the fact that using simple immunological parameters on

the routine histological materials may single out those individuals who are more likely to benefit from e.g. adjuvant treatment. To our knowledge, this project is the first to analyse a wide spectrum of immune cells in both tumor compartments of a large number of resected NSCLC tumors.

Two important questions must be addressed prior to introduction of a prognostic marker in clinical practice: 1) Does the marker have an effect independent of the TNM stage? and 2) is there a reliable measurement method available for this factor in routine practice?[15] The investigated immune components appear to satisfy both questions. In our research subjects, the primary tumors were surgically removed. The prognostic impact associated with the different lymphocytes may indicate an ability by systemic effectors to recognize and kill circulating cancer cells in the peripheral blood, pleura, bone marrow and lymph nodes, which may lead to elimination of cancer cells or to a state of equilibrium.

5.1. Chronic inflammation and cancer

Near 150 years ago, Virchow postulated that inflammation is a predisposing factor for tumorigenesis. This hypothesis was based on his observation that cancerous tissue often arose at sites of chronic inflammation, and that inflammatory cells were present in the resected tumors.[69] In contrast, Burnet[31] proposed in 1970 the hypothesis that the immune system has the ability to spontaneously identify and eliminate cancer cells, and will consequently protect against tumor development; the concept of immunological surveillance. When tissue homeostasis is chronically perturbed, interactions between innate and adaptive immune cells can be disturbed. While innate immune cells form the first line of immune defence and regulate activation of adaptive immune responses, this role can be reversed during chronic inflammation.[29] In the latter situation, adaptive immune responses can cause T lymphocyte dysfunction instead of activation.[70] The inability to properly engage and/or disengage the

innate or adaptive immune system can result in excessive tissue remodelling, loss of tissue architecture due to destruction, protein and DNA alterations due to oxidative stress and increased risk of cancer development.[29]

So, host immunity protect against cancer. But according to recent research, subsets of chronically activated immune cells may promote tumor growth and/or facilitate survival of cancer cells.[29] This raises several questions about the underlying mechanisms for these tumor-promoting effects, and whether they can be blocked while maintaining or enhancing the antitumor immune responses.

The connection between inflammation and cancer were characterized along two pathways by Mantovani et al[71] (Figure 18): 1) an extrinsic pathway, driven by chronic inflammatory/infectious conditions which increase cancer risk (e.g., chronic inflammatory disease) and 2) an intrinsic pathway, driven by genetic alterations (oncogenes) that cause inflammation and subsequently neoplasia. These two pathways converge, resulting in the activation of transcription factors, mainly nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α) in tumor cells. These factors again coordinate the production of inflammatory mediators as cytokines and chemokines, which recruit and activate various leukocytes, mainly cells of the myelomonocytic lineage. The cytokines activate the same key transcription factors in inflammatory cells as well as in other stromal cells and cancer cells, resulting in an elevated production of inflammatory mediators and establishment of a cancer-related inflammation.

5.2. Different immune cells and NSCLC

5.2.1. CD4+ cells and NSCLC

CD4 is a nonpolymorphous glycoprotein belonging to immunoglobulin superfamily. It is a marker of T helper lymphocytes, which play a crucial role in initiating and maintaining

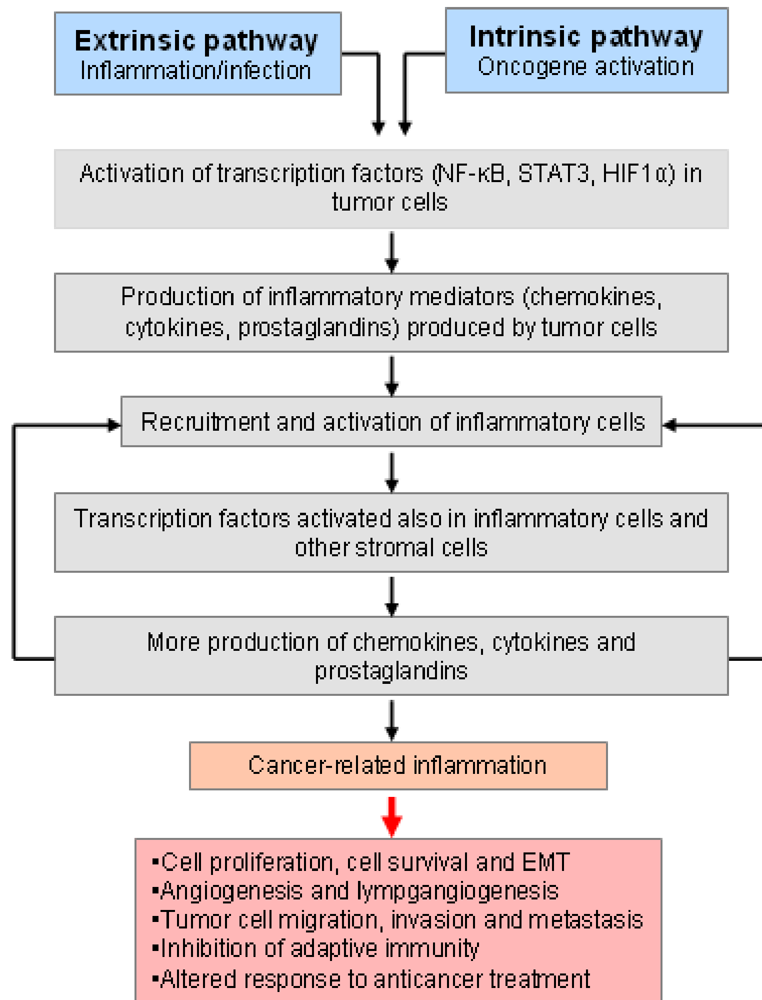

Figure 18. *Pathways connecting inflammation and cancer. Chronic inflammatory or infectious conditions, enhancing the risk for cancer development, constitutes the extrinsic pathway. The intrinsic pathway is activated by genetic events (oncogene mutation, inactivation of suppressor genes, amplifications) which cause neoplasia. The pathways merge and results in the activation of transcription factors. Adapted from Mantovani et al*[71]. *Abbreviations: NF-κB, Nuclear factor-κB; STAT3, signal transducer and activator of transcription 3; HIF1α, hypoxia-inducible factor 1α.*

anticancer immune response. In the absence of CD4+ cells, cytotoxic lymphocytes can become lethargic or even be deleted. In fact, CD4+ helper cells are needed to imprint CD8+ cells with the ability to develop into long lasting memory cells. In addition, CD4+ cells can recruit other immune cells and can directly kill tumor cells. Our results highlight the independent beneficial effects of CD4+ T cells in NSCLC, mediated via the tumor stroma. Similar conclusion has been found in other studies involving NSCLC[39] as well as other

cancers, including lymphomas.[72] The favorable prognostic effect was augmented in the group of patients having a simultaneously high CD8+ cells, confirming the co-operation between CD4+ and CD8+ cells and the most optimal antitumor effect in case of concurrent high CD4+ and CD8+ cells as noted earlier by Hiraoka.et al.[73] However, CD4+ cells are heterogeneous and in addition to the effector cells, regulatory cells also found. The regulatory cells are CD4+, CD25+ and FOXP3+ and may mediate immune tolerance and eradication of T effector cells. Our results show that the net effect of CD4+ cells is a beneficial one in NSCLC. In fact, even Treg cells were found, paradoxically, to be associated with a better loco-regional control in head and neck carcinoma[74]; whereas, high ratio of Treg over Th2 cells resulted in a significantly shortened disease-free survival in Hodgkin lymphoma.[75]

5.2.2. CD8+ cells and NSCLC

CD8 is a cell surface glycoprotein and a member of immunoglobulin superfamily; located at 2p12. It seems that despite HLA-haplotype loss, a vigorous antitumor immune response mediated by CD8+ cells can be present in NSCLC.[45] The presence of numerous CD8+ cells has been associated with epithelial damage resulting in the clinical manifestations of diseases like lymphocytic colitis and Celiac disease. A similar CD8+ cell kill is beneficial in association with different types of malignant tumors[34, 76, 77], including NSCLC.[45]. It is thought that tumor rejection by CD8+ T-cell effectors is primarily mediated by direct killing preceded by IFN-γ mediated inhibition of tumor-induced angiogenesis.[58] Anyway, it seems that the beneficial effect of CD8+ and CD4+ cells is mediated primarily via the tumor stroma, corroborating the findings by Fassnacht et al[78] who targeted tumor stromal antigens to stimulate cytotoxic CD8+ responses and reduce the ability of tumors to evade immune elimination.

5.2.3. CD20+ cells and NSCLC

CD20 is a phosphoprotein initially expressed on B cells after CD19/CD10 expression and before CD21/CD22 and surface immunoglobulin expression; retained on mature B cells until plasma cell development.

We postulate that patients' humoral immunity may play a role in long-term survival. This notion is based on the observed survival in patients who were found to have high B-lymphocytes in the stroma, although this was not an independent prognostic factor. There are many possible mechanisms that may explain why the presence of peritumoral B cells is involved with prolonged survival. Possibly, this immune response helps to locally contain some tumors, thereby reducing the true incidence of occult micrometastases.[79] If occult metastases are present, such immunity might prolong survival by limiting further tumor dissemination. In addition, the presence of B cells may mirror the host's overall immunity. Those able to mount an immune response may preselectively be in better overall condition. Finally, antibodies produced by B cells are important for the killing of tumor cells by NK cells and other inflammatory cells through ADCC.

5.2.4. CD1a+ cells and NSCLC

DCs are the most potent antigen presenting cells, and represent a heterogeneous group of cells that express different markers. We used CD1a which is a common DC marker in the daily routine. CD1a is a transmembrane glycoprotein, which is structurally related to the MHC proteins and form heterodimers with beta-2-microglobulin. Although mostly expressed on immature DCs, mature DCs also express this marker.[80, 81] Other DC markers are not specific: The CD83 molecule has also been found in other cells including B cells; while CD208, originally considered a specific marker of mature DCs, is also expressed in normal lung and lung adenocarcinoma epithelial cells.[82, 83] In our cases CD1a does not show any

staining in the tumor epithelial cells. Hence, CD1a appears to be a reasonable marker for visualizing DCs.

The positive prognostic impact of stromal DCs in our cohort is consistent with several previous studies reporting of their prognostic significance in different neoplasms, as colorectal[84], hepatocellular[85], breast carcinoma[80] as well as NSCLC.[86] In addition to their key function as antigen presenting cells, DCs may themselves contribute to cancer cell killing by direct cytotoxicity or cytokine production (so-called killer DCs and IFN-producing killer DCs).[86-88] Many tumors, however, inhibit the maturation and function of DCs by secreting mediators like IL6 and VEGF. In this context, anti-VEGF treatment may be a promising therapy capable of restoring DC function in addition to suppressing angiogenesis.[86] In addition, many tumors (like colon carcinoma) secret COX2 which leads to increased prostaglandin which acts on EP2 and 4 receptors on DC cells affecting their function (Figure 19).[84] Finally, following culture with human lung carcinoma cells, DCs showed decreased production of TNF-α and IL-12, and were converted to TGF-β-producing DCs, which were poor at eliciting the activation of naive CD4+ T cells and sustaining their proliferation and differentiation into Th1, IFN-γ + effector cells.[89]

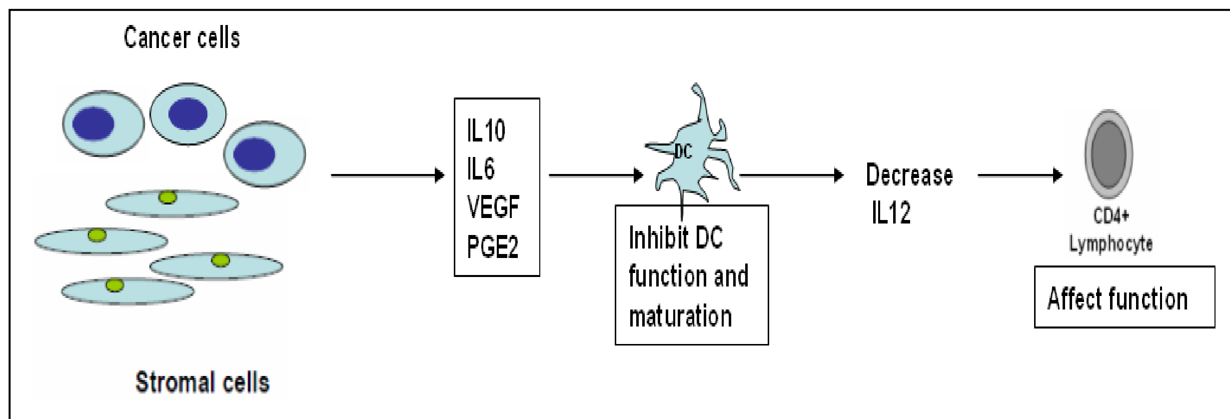

Figure 19: *Cancer can evade the anti-tumorigenic effect of DC. Cancer epithelial and/or stromal cells can produce IL-10, IL-6, VEGF and prostaglandins that can inhibit DCs maturation and function (e.g. decrease IL-12 and TNF-α, and increase TGF-β production), resulting in an abnormal CD4+ helper cells differentiation.*

5.2.5. CD56+ cells and NSCLC

CD56 is the most commonly used surface marker to identify NK cells. It presents on near all NK cells as well as T memory cells and neuronal tissue. NK cells kill malignant cells in two ways: 1) Granule mediated cytotoxicity (perforin, granzyme and TNF-related granules) and 2) secreting cytokines (e.g. IFN-γ, IL5, M-CSF, GM-CSF) and chemokines (e.g. CCL1 and CCL3)[42]. NK cell activation is regulated by a balance of signals that are generated from activating and inhibitory receptors. Every NK cell expresses at least one inhibitory receptor that recognizes a self-MHC class I molecule. So, normal cells that express MHC class I molecules are protected from self-NK cells, but transformed or infected cells that have down-regulated MHC class I expression are attacked.[90] Although NK cells in our study were relatively sparse, their presence in the stroma was an independent prognostic marker for a better DSS. A similar finding has previously been noted in a study on squamous cell carcinoma[91] and adenocarcinoma[92] of the lung, as well as in other malignancies as colorectal[93] and gastric carcinomas.[94] Moreover, the presence of a higher number of NK cells in normal regional lymph nodes was associated with a lower incidence of disease recurrence in gastric carcinoma.[95] The fact that there is a high level of loss of MHC class I in NSCLC[96], increases the significance of NK cells in tumor immunity, as loss of MHC class I will remove the inhibitory effect of NK cells and stimulate their tumor killing capability. Carrega et al[97] assessed NK cells in NSCLC and found that their cytolytic activity is lower than that of their peripheral blood counterparts, whereas no difference was observed regarding cytokine production. They also found that NK cells were localized in the stroma.

5.2.6. CD68+ cells, MCSF and CSF-1R in NSCLC

The role of M-CSF, a major regulator of the mononuclear phagocytic lineage, and its receptor CSF-1R in tumor progression has been extensively studied in several types of cancer.

Expression of these have correlated with poor prognosis especially in breast[98], endometrial[99] and prostate[100] carcinomas. Although CSF produced by lung cancer cells might promote tumor progression by autocrine and paracrine mechanisms through stimulating enhanced production of extracellular matrix degrading proteinases by the tumor cells or by prolonging tumor cell survival[101], the role of M-CSF and its receptor at tissue level is not well studied in NSCLC. M-CSF secreted from the tumor acts to divert antitumor macrophage responses and suppresses the differentiation of mature tumor-antigen-presenting DCs.[98] Although M-CSF and its receptor showed high epithelial staining in 63% and 42% of our NSCLC cases, there was no significant association with survival.

The macrophage constitute a major component of the inflammatory infiltrate in tumors.[69] TAMs may enhance cancer progression through activation to M2 macrophages which facilitate matrix remodelling, angiogenesis and stimulation of tumour growth and motility through the synthesis of growth and chemotactic factors.[48] Interestingly, these functions are also normally found in wound healing and inflammation. This supports the notion that tumors are 'wounds that never heal' and suggests that chronic inflammation through persistent infection or by other means might be an important co-factor in the genesis and promotion of malignancy.[102] Meanwhile, macrophages have also an anti-tumorigenic action mediated by classically activated M1 macrophages that produce effector molecules such as reactive oxygen intermediates, reactive nitrogen intermediates, and TNF-α, to limit tumor growth.[48] Heterogeneity of macrophages may account for part of the controversies with respect to the prognostic role of TAMs, and may explain the lack of significant survival effect in our study cohort.

5.2.7. CD3+ cells and NSCLC

In paper 1, we demonstrated the independent prognostic significance of CD4+ and

CD8+ cells in NSCLC. CD3 stains both these cell groups and, in addition, a subset of NK cells (NK-T cells) that are CD3+ and CD56+, as well as CD4-, CD8- T cells (with gamma/delta T cell receptor). The aim in assessing CD3+ cells was to confirm the prognostic effect of CD4+ and CD8+ cells, and to see if such an effect is maintained taking into consideration other cell types represented by the pan T marker CD3. NK-T cells kill malignant cells without the need for previous sensitization and in an MHC-unrestricted manner[29]. High numbers of CD3+ cells have been associated with increased apoptosis in NSCLC patients.[103] Kataki et al[41] concluded that the CD3+ cells in lung carcinoma result from recruitment of circulating precursors rather than from local replication, which may be important when considering the possible therapeutic relevance of these cells. Our results, specifically the independent significance of immune cells in the stromal component, highlight the significance of the stromal compartment in cancer survival and progression. T cells utilize cytokines to inhibit tumor stroma formation and cytotoxic molecules to kill epithelial and stromal cells that cross-present antigens.[56] Any possibilities to modulate stromal lymphocyte cytotoxicity in the close vicinity of cancer cells would be of substantial clinical interest.

5.2.8. CD117+ cells and NSCLC

CD117 is the receptor for kit protein, a 145 kD tyrosine kinase growth factor receptor protein important for development and survival of mast cells, haematopoietic stem cells, melanocytes, germ cells and interstitial cells of Cajal. Apart from mast cells, the other above-mentioned cell types are unlikely to be observed in NSCLC tissue. As a consequence, we used CD117 as a marker of mast cells in our cohort. We observed only sparse number of mast cells, solely in the stromal compartments.

Mast cells are found in various solid tumors and can enhance angiogenesis via multiple interacting factors[104]. In a previous NSCLC study, the density of mast cells correlated

with MVD, but not survival[105]. However, mast cells can express natural cytotoxicity against tumor cells and produce cytokines with anti-tumor activity, like TNF-α. This can explain the finding by Tomita et al[106] where a higher density of mast cells correlated with improved DSS in 90 adenocarcinoma patients. In a study on gastric carcinoma, Yano et al[107] found, in contrast, accumulation of mast cells in the stroma to correlate with increased vascularization, angiolymphatic invasion, metastases, and poor DSS. For this reason, mast cells have been regarded as a double-edged sword in cancer immunity. This may explain the lack of prognostic impact by mast cells in our study cohort. Corroborating our data, two previous studies[40, 108] reported a neutral prognostic effect of mast cell density in stage I-IV NSCLC tumors. The lack of a strong association between MVD and mast cell density in our cohort may be related to the different mast cell phenotypes, as only the tryptase+ mast cell is closely associated with increased angiogenesis[109]. CD117 do not differentiate between these different types of mast cells.

Previous studies have reported a negative prognostic impact of high MVD in NSCLC.[110] To our knowledge, this is the first TMA study to evaluate the effect of MVD in NSCLC. There was no prognostic impact of MVD assessed in tumor or stromal cores. This may be explained by the TMA technique's unsuitability for evaluating MVD.[64]

5.2.9. CD138+ cells and NSCLC

CD138+ is a member of the syndecan family which regulates cell-cell and cell-extracellular matrix adhesion, cell migration and growth factor activity. It is expressed mainly in plasma cells, normal epithelial cells, transiently in condensing mesenchyme during embryogenesis as well as some stromal cells.[111] CD138 stains epithelial, in particular squamous cells. Loss of CD138 expression in epithelial cells is associated with tumor aggressiveness in head and neck carcinoma;[112] and reduced CD138 expression has been

observed in invasive squamous cell carcinoma when compared to carcinoma in situ.[113] Shah et al[114] demonstrated reduced survival with loss of epithelial CD138 expression in 63 NSCLC cases. No such association could be demonstrated in our cohort, in consistency with the study by Toyoshima et al[115] on 97 NSCLC cases.

CD138+ cells infiltrating both the stromal and epithelial compartments were also analyzed, neither tumor compartments had significant survival effect, though the stromal density showed a trend for a better prognosis. In a study assessing CD79a+ cells (stains B lymphocytes as well as plasma cells) no significant prognostic effect was noted in either tumor compartment[116]. CD138 stains, in addition to plasma cells, some stromal cells (fibroblast), which enhance tumor angiogenesis and hence tumor growth and progression.[111] However, in stage III NSCLC survival was significantly better in patients with a higher number of stromal CD138+ cells. This may indicates a crucial role of plasma cells and antibody mediated immunity in advanced disease, and needs to be re-examined by a larger series of stage III as well as stage IV disease.

6. CONCLUSION

The results of our study suggest that:

1. NSCLC patients respond towards their tumors by cellular immunity and in patients with stage I-IIIa NSCLC, different types immune cells infiltrate the tumor epithelial and stromal compartments.
2. As surgically treated patients with similar histology and stage can have a significantly different prognosis, prognostic effects can be identified by assessing the status of the tumor infiltrating inflammatory cells in the stromal and epithelial tumor compartments.
3. High number of stromal NK cells and T lymphocytes (assessed by CD4, CD8 and CD3) are independent prognostic marker predicting good survival. This highlights the significance of

the tumor microenvironment as a prognostic indicator and a possible target for therapy.

7. FUTURE PERSPECTIVES

1. A more accurate insight in the mechanism of action of inflammatory cells as well as their interaction with the tumor microenvironment may lead to a useful adjuvant immunotherapy in NSCLC.

2. Prospective studies are essential before any implementation of TIL count in clinical decision making in NSCLC. This may pave the way for new therapeutic concepts that augment the protective effect of anti-tumoral immune responses.

3. Assessing tumor immune status may be also of great importance to be considered in designing clinical trials of emerging therapies, because those patients who are predicted to do well anyway (due to a favorable immunological infiltrate) may not gain significant benefit from trial therapies, this will create a bias which diminishes the benefit from the trial.[40]

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PAPER I

PAPER II

PAPER III

APPENDIX

Signs and symptoms of lung carcinoma. Approximately 5-20% of cases are clinically occult. Modified, from T.V. Colby et al. {391}

Systemic symptoms Weight loss, loss of appetite, malaise, fever **Local /direct effects** From endobronchial growth and/or invasion of adjacent structures including chest wall and vertebral column Cough, dyspnoea, wheeze, stridor, haemoptysis Chest pain/back pain Obstructive pneumonia (+/- cavitation) Pleural effusion **Extension to mediastinal structures** Nerve entrapment : recurrent laryngeal nerve (hoarseness), phrenic nerve (diaphragmatic paralysis), sympathetic system (Horner syndrome), brachial plexopathy from "superior sulcus" tumours Vena cava obstruction: superior vena cava syndrome Pericardium: effusion, tamponade Myocardium: arrythmia, heart failure Oesophagus: dysphagia, bronchoesophageal fistula Mediastinal lymph nodes: pleural effusion **Metastatic disease** Direct effects related to the organ(s) involved **Paraneoplastic syndromes** Dermatomyositis/polymyositis Clubbing Hypertrophic pulmonary osteoarthropath Encephalopathy Peripheral neuropathies Myasthenic syndromes (including Lambert-Eaton) Transverse myelitis Progressive multifocal leukoencephalopathy	**Endocrine syndromes** Parathormone-like substance: hypercalcemia Inappropriate antidiuretic hormone: hyponatremia ACTH: Cushing syndrome, hyperpigmentation Serotonin: carcinoid syndrome Gonadotropins: gynecomastia Melanocyte-stimulating hormone: increased pigmentation Hypoglycemia, hyperglycemia Hypercalcitonemia Elevated growth hormone Prolactinemia Hypersecretion of vasoactive intestinal polypeptide (VIP): diarrhea **Hematologic/coagulation defects** Disseminated intravascular coagulation Recurrent venous thromboses Nonbacterial thrombotic (marantic) endocarditis Anemia Dysproteinemia Granulocytosis Eosinophilia Hypoalbuminemia Leukoerythroblastosis Marrow plasmacytosis Thrombocytopenia **Miscellaneous (very rare)** Henoch-Schönlein purpura Glomerulonephritis, Nephrotic syndrome Hypouricemia, Hyperamylasemia Amyloidosis Lactic acidosis Systemic lupus erythematosus

Imaging techniques in lung cancer staging.
From T.V. Colby et al. {391}.

Technique	Uses
Conventional radiographs	Primary detection/characterization of parenchymal tumour Assessment of main bronchi/tracheal involvement Detection of chest wall invasion Assessment of hilar and mediastinal invasion/adenopathy Detection of obstructive atelectasis/pneumonitis Detection of pleural effusion
CT	Assessment of main bronchi/tracheal involvement Detection of chest wall invasion Assessment of hilar and mediastinal invasion/adenopathy Detection of liver, adrenal, brain metastases
MRI	Detection of chest wall invasion (particularly superior sulcus [tumours]) Detection of mediastinal or spinal canal invasion Assessment of hilar and mediastinal adenopathy in patients with equivocal CT examinations or contraindications to intravenous contrast media Characterization of isolated adrenal masses
Ultrasound	Detection of pleural effusion/guidance for thoracentesis Guidance for biopsy of peripheral lung or mediastinal mass
Gallium-67 scan	Detection of hilar and mediastinal adenopathy Detection of distal metastases
Pulmonary angiography	Evaluation of central pulmonary artery invasion

WHO histological classification of tumours of the lung

Tumour	Code
Malignant epithelial tumours	
Squamous cell carcinoma	8070/3
Papillary	8052/3
Clear cell	8084/3
Small cell	8073/3
Basaloid	8083/3
Small cell carcinoma	8041/3
Combined small cell carcinoma	8045/3
Adenocarcinoma	8140/3
Adenocarcinoma, mixed subtype	8255/3
Acinar adenocarcinoma	8550/3
Papillary adenocarcinoma	8260/3
Bronchioloalveolar carcinoma	8250/3
Nonmucinous	8252/3
Mucinous	8253/3
Mixed nonmucinous and mucinous or indeterminate	8254/3
Solid adenocarcinoma with mucin production	8230/3
Fetal adenocarcinoma	8333/3
Mucinous (“colloid”) carcinoma	8480/3
Mucinous cystadenocarcinoma	8470/3
Signet ring adenocarcinoma	8490/3
Clear cell adenocarcinoma	8310/3
Large cell carcinoma	8012/3
Large cell neuroendocrine carcinoma	8013/3
Combined large cell neuroendocrine carcinoma	8013/3
Basaloid carcinoma	8123/3
Lymphoepithelioma-like carcinoma	8082/3
Clear cell carcinoma	8310/3
Large cell carcinoma with rhabdoid phenotype	8014/3
Adenosquamous carcinoma	8560/3
Sarcomatoid carcinoma	8033/3
Pleomorphic carcinoma	8022/3
Spindle cell carcinoma	8032/3
Giant cell carcinoma	8031/3
Carcinosarcoma	8980/3
Pulmonary blastoma	8972/3
Carcinoid tumour	8240/3
Typical carcinoid	8240/3
Atypical carcinoid	8249/3
Salivary gland tumours	
Mucoepidermoid carcinoma	8430/3
Adenoid cystic carcinoma	8200/3
Epithelial-myoepithelial carcinoma	8562/3
Preinvasive lesions	
Squamous carcinoma *in situ*	8070/2
Atypical adenomatous hyperplasia	
Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia	

Tumour	Code
Mesenchymal tumours	
Epithelioid haemangioendothelioma	9133/1
Angiosarcoma	9120/3
Pleuropulmonary blastoma	8973/3
Chondroma	9220/0
Congenial peribronchial myofibroblastic tumour	8827/1
Diffuse pulmonary lymphangiomatosis	
Inflammatory myofibroblastic tumour	8825/1
Lymphangioleiomyomatosis	9174/1
Synovial sarcoma	9040/3
Monophasic	9041/3
Biphasic	9043/3
Pulmonary artery sarcoma	8800/3
Pulmonary vein sarcoma	8800/3
Benign epithelial tumours	
Papillomas	
Squamous cell papilloma	8052/0
Exophytic	8052/0
Inverted	8053/0
Glandular papilloma	8260/0
Mixed squamous cell and glandular papilloma	8560/0
Adenomas	
Alveolar adenoma	8251/0
Papillary adenoma	8260/0
Adenomas of the salivary gland type	
Mucous gland adenoma	8140/0
Pleomorphic adenoma	8940/0
Others	
Mucinous cystadenoma	8470/0
Lymphoproliferative tumours	
Marginal zone B-cell lymphoma of the MALT type	9699/3
Diffuse large B-cell lymphoma	9680/3
Lymphomatoid granulomatosis	9766/1
Langerhans cell histiocytosis	9751/1
Miscellaneous tumours	
Harmatoma	
Sclerosing hemangioma	8832/0
Clear cell tumour	8005/0
Germ cell tumours	
Teratoma, mature	9080/0
Immature	9080/3
Other germ cell tumours	
Intrapulmonary thymoma	8580/1
Melanoma	8720/3
Metastatic tumours	

[1] Morphology code of the International Classification of Diseases for Oncology (ICD-O) {6} and the Systematized Nomenclature of Medicine (http://snomed.org). Behaviour is coded /0 for benign tumours, /3 for malignant tumours, and /1 for borderline or uncertain behaviour.

TNM classification of the lung

TNM classification of carcinomas of the lung {738,2045}

T – Primary Tumour

- TX Primary tumour cannot be assessed, or tumour proven by the pres ence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy
- T0 No evidence of primary tumour
- Tis Carcinoma in situ

- T1 Tumour 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus, i.e., not in the main bronchus (1)
- T2 Tumour with any of the following features of size or extent:
  - More than 3 cm in greatest dimension
  - Involves main bronchus, 2 cm or more distal to the carina
  - Invades visceral pleura
  - Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung
- T3 Tumour of any size that directly invades any of the following: chest wall (including superior sulcus tumours), diaphragm, mediastinal pleura, parietal pericardium; or tumour in the main bronchus less than 2 cm distal to the carina1 but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung
- T4 Tumour of any size that invades any of the following: mediastinum, heart, great vessels, trachea, oesophagus, vertebral body, carina; separate tumour nodule(s) in the same lobe; tumour with malignant pleural effusion (2)

Notes: 1. The uncommon superficial spreading tumour of any size with its invasive component limited to the bronchial wall, which may extend proximal to the main bronchus, is also classified as T1.

2. Most pleural effusions with lung cancer are due to tumour. In a few patients, however, multiple cytopathological examinations of pleural fluid are negative for tumour, and the fluid is non-bloody and is not an exudate. Where these elements and clinical judgment dictate that the effusion is not related to the tumour, the effusion should be excluded as a staging element and the patient should be classified as T1, T2, or T3.

N – Regional Lymph Nodes[#]

- NX Regional lymph nodes cannot be assessed
- N0 No regional lymph node metastasis
- N1 Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
- N2 Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
- N3 Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)

M – Distant Metastasis

- MX Distant metastasis cannot be assessed
- M0 No distant metastasis
- M1 Distant metastasis, includes separate tumour nodule(s) in a different lobe (ipsilateral or contralateral)

Stage Grouping

Occult carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T1	N1	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T1, T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	Any T	N3	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1

A help desk for specific questions about the TNM classification is available at http://www.uicc.org/tnm/

[#]The regional lymph nodes are the intrathoracic, scalene, and supraclavicular nodes.