UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTMENT OF BIOTECHNOLOGY

Ph.D. Thesis

ANALYSIS OF IMMUNOREGULATORY BIOMARKERS IN NON-SMALL CELL LUNG CANCER

MARTA USÓ MARCO

SUPERVISORS:

DR. CARLOS CAMPS HERRERO

DR. ELOISA JANTUS LEWINTRE

DR. RAFAEL SIRERA PÉREZ

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CARLOS CAMPS HERRERO, Dr. por la Universidad de Valencia, Profesor Titular del Departamento de Medicina de la Universidad de Valencia y Jefe del Servicio de Oncología del Hospital General Universitario de Valencia.

CERTIFICA:

Que Dña. Marta Usó Marco, Licenciada en Biotecnología, ha realizado bajo mi dirección la Tesis Doctoral que lleva por título **"Analysis of Immunoregulatory Biomarkers in Non-Small Cell Lung Cancer".** Dicha tesis reúne todos los requisitos necesarios para su juicio y calificación.

En Valencia, 13 de Febrero de 2015.

Prof. Carlos Camps Herrero

D.N.I: 22517012N

FUNDACIÓ INVESTIGACIÓ HOSPITAL GENERAL UNIVERSITARI VALÈNCIA

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ELOISA JANTUS LEWINTRE, Dra. por la Universidad de Valencia, Jefa del Laboratorio de Oncología Molecular de la Fundación del Hospital General Universitario de Valencia y Profesora Asociada del Departamento de Biotecnología de la Universidad Politécnica de Valencia.

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En Valencia, 13 de Febrero de 2015.

Dra. Eloisa Jantus Lewintre

D.N.I: 24474835Y

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DE VALÈNCIA

RAFAEL SIRERA PÉREZ, Dr. por la Universidad de Valencia y profesor titular del Departamento de Biotecnología de la Universidad Politécnica de Valencia.

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Dr. Rafael Sirera Pérez
D.N.I: 22695276A

“Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.”

Marie Curie

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Resumen

El cáncer de pulmón es una de las principales causas de muerte relacionada con cáncer en el mundo, siendo el tercer tipo de cáncer más común. El cáncer de pulmón no microcítico (CPNM) representa casi el 85% de todos los cánceres de pulmón y la supervivencia a los 5 años va desde el 50% en estadios IA hasta el 15% en estadios IIIA. Hasta el momento, no se han descubierto biomarcadores capaces de predecir la progresión de la enfermedad en pacientes tanto en estadios resecables como en estadios avanzados, por lo que existe una clara necesidad de realizar estudios centrados en la búsqueda de biomarcadores pronósticos y diagnósticos en los diferentes tipos de muestra disponibles, como por ejemplo sangre, tejido fresco y tejido parafinado.

El campo de la inmunología tumoral ha cambiado en la última década y actualmente se sabe que el sistema inmune juega un papel clave en cáncer. Las células inmunes que infiltran el tumor son un componente más del microambiente tumoral. Pese a que son potencialmente capaces de eliminar los antígenos tumorales, estas células no pueden evitar la formación y progresión tumoral. Esto es debido a que el tumor adquiere diversos mecanismos de regulación del microambiente tumoral con el objetivo de escapar del ataque del sistema inmune, como por ejemplo liberación de factores que impiden el correcto funcionamiento de los mecanismos de reacción inmune, modulación de vías co-estimuladoras y reclutamiento y activación de células inmunoreguladoras como las células T reguladoras, las células mieloides supresoras y los macrófagos asociados a tumores. El estudio de marcadores relacionados con la respuesta inmune y concretamente con los procesos de inmunoregulación puede proporcionarnos información pronóstica y predictiva relevante sobre los pacientes con cáncer.

Por todo ello, el principal objetivo de esta tesis doctoral es analizar la presencia de marcadores relacionados con la inmunoregulación y evaluar su posible correlación con las variables clínico-patológicas y pronósticas en pacientes con CPNM mediante el uso de técnicas fiables y aplicables en la práctica clínica como la PCR cuantitativa y la inmunohistoquímica. Así mismo, esto nos permitirá conocer en mayor profundidad las características inmunológicas del microambiente tumoral en pacientes con CPNM.

Summary

Lung cancer is the leading cause of cancer-related death worldwide, and is the third most common cancer type; it can be classified into two subgroups based on histology: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). The 5-year survival still remains poor and despite the existence of several distinct tumour phenotypes, therapeutic decisions are mainly based on clinical features such as stage or performance status. This highlights the need for new diagnostic and prognostic biomarkers in different types of samples (such as blood, fresh-frozen tissue or formalin-fixed, paraffin-embedded [FFPE] samples).

The field of tumour immunology has changed in the last decade, and it is now accepted that the immune system plays a pivotal role in cancer. Although the immune cells that infiltrate the tumour microenvironment are potentially capable of eliminating tumour cells, they cannot prevent tumour development and progression. Tumours acquire mechanisms to regulate their immune microenvironment such as the release of a series of factors to subvert normal reaction mechanisms, the modulation of co-stimulatory pathways, also known as immune checkpoints, and the induction and attraction of suppressor cells (myeloid-derived suppressor cells, tumour-associated macrophages, and regulatory T cells). The potential effect of the patient's immune system on clinical outcome is important for the identification of prognostic markers as well as markers that predict treatment responses. The study of immune-related markers, especially those implicated in immunoregulatory processes, could provide valuable prognostic information that could help in many applications in future clinical practice.

Thus, the objective of this thesis is to characterise cancer immunoregulation biomarkers and to evaluate the possible correlation between these biomarkers and clinicopathological and prognostic variables in patients with NSCLC by the use of well-tested and accurate techniques such as quantitative PCR and immunohistochemistry. Furthermore, this study will provide information about the immunological features of the tumour microenvironment in NSCLCs.

Resum

El càncer de pulmó és una de les principals causes de mort relacionades amb càncer al món, sent a més a més el tercer tipus de càncer més comú. El càncer de pulmó no microcític (CPNM) representa el 85% de tots els casos de càncer de pulmó aproximadament i la supervivència als 5 anys continua sent molt baixa. Fins el moment, no s'han descobert biomarcadors capaços de predir la progressió de la malaltia tant en pacients en estadis inicials com en estadis avançats. Per aquest motiu, existeix una clara necessitat de realitzar estudis centrats en la recerca de biomarcadors pronòstics i predictius en els diferents tipus de mostres disponibles, com per exemple sang, teixit fresc i teixit parafinat.

El camp de la immunologia tumoural ha canviat en l'última dècada i actualment se sap que el sistema immune exerceix un paper clau en el càncer. Les cèl·lules immunològiques que infiltren el tumour són un component més del microambient tumoural. Malgrat que aquestes cèl·lules són potencialment capaces d'eliminar el antígens tumourals, s'ha evidenciat que no poden previndre la formació i progressió tumoural. Una de les raons per les quals s'observa aquest fenomen és que el tumour adquireix diversos mecanismes de regulació del microambient tumoural. Aquests mecanismes es basen en l'alliberació de factors que impedeixen el correcte funcionament del sistema immune, la modulació de vies coestimuladores i el reclutament i activació de cèl·lules immunoreguladores com poden ser les cèl·lules T reguladores, les cèl·lules mieloides supressores i els macròfags associats a tumour. L'estudi de marcadors relacionats amb la resposta immune i més concretament amb els processos d' immunoregulació pot proporcionar informació pronòstica i predictiva rellevant sobre els pacients amb càncer.

Per tot això, el principal objectiu d'aquesta tesi doctoral és analitzar la presència de marcadors relacionats amb la immunoregulació i avaluar la seva possible correlació amb les variables clinicopatològiques i pronòstiques de pacients amb CPNM mitjançant l'ús de tècniques fiables i aplicables a la pràctica clínica com són la PCR quantitativa i la immunohistoquímica. Així mateix, aquestes anàlisis ens permetran conèixer amb major profunditat les característiques immunològiques del microambient tumoural de pacients amb CPNM.

ABBREVIATIONS

ADC: Adenocarcinoma

ALK: Anaplastic lymphoma kinase

APC: Antigen presenting cell

AUC: Area under the curve

BAC: Bronchioloalveolar carcinoma

CCL2: Chemokine (C-C motif) ligand 2

CCL22: Chemokine (C-C motif) ligand 22

CCL5: Chemokine (C-C motif) ligand 5

CI: confidence interval

CLEC4C: C-type lectin domain family 4, member C

CSF1R: Colony stimulating factor 1 receptor

CSF3R: Colony stimulating factor 3 receptor (granulocyte)

CT: Computed tomography

CTL: Cytotoxic T lymphocytes

CTLA4: Cytotoxic T-lymphocyte-associated protein 4

CXCL12: Chemokine (C-X-C motif) ligand 12

CXCR4: Chemokine (C-X-C motif) receptor 4

DC: Dendritic cells

DSS: Disease specific survival

EGFR: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial-mesenchymal transition

FOXP3: Forkhead box P3

GITR: Gluco-corticoid-induced TNF receptor family-related

HE: Haematoxylin and eosin staining

HPF: High power fields

IDO: Indoleamine 2, 3-dioxygenase

IFN γ: Interferon γ

IHC: Immunohistochemistry

IL10: Interleukin 10

IL12B: Interleukin 12B

IL13: Interleukin 13

IL23A: Interleukin 23A

IL2RA: Interleukin 2 receptor alpha

IL4: Interleukin 4

IL4RA: Interleukin 4 receptor alpha

IL7RA: Interleukin 7 receptor alpha

IL8: Interleukin 8

ITGAM: Integrin, alpha M (complement component 3 receptor 3 subunit)

ITGB2: Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)

LAG3: Lymphocyte-activation gene 3

LCC: Large cell carcinoma

LCM: Laser capture microdissection

LGALS1: Lectin, galactoside-binding, soluble, 1

LGALS2: Lectin, galactoside-binding, soluble, 2

LN: Lymph nodes

MDSC: Myeloid-derived suppressor cell

MHC: Major histocompatibility complex

MMP2: Matrix metallopeptidase 2

NA: Not available

NF: Nuclease-free

NGS: Next generation sequencing

NK: Natural killer

NRP1: Neuropilin 1

NS: Not specified

NSCLC: Non-small cell lung carcinoma

OS: Overall survival

PD1: Programmed cell death 1

PDGF: Platelet-derived growth factor

PDL1: Programmed cell death 1 ligand

PET: Positron emission tomography

PFS: Progression free survival

PGE2: Prostaglandin E2

PS: Performance status

RB: Retinoblastoma-associated

RECIST: Response evaluation criteria for solid tumours

RFP: Recurrence-free probability

RFS: Recurrence-free survival

ROC: Receiver operating characteristic

RTqPCR: Quantitative real time PCR

SCC: Squamous cell carcinoma

SCLC: Small cell lung carcinoma

SDF1: Stromal cell-derived factor 1

TAM: Tumour-associated macrophages

TCR: T cell antigen-specific receptor

TGFβ: Transforming growth factor β

THBD: Thrombomodulin

TIL: Tumour-infiltrating lymphocyte

TKI: Tyrosine-kinase inhibitors

TLS: Tertiary lymphoid structures

TNF: Tumour necrosis factor

TNM: Tumour/Nodules/Metastasis

Treg: Regulatory T cells

VEGF: Vascular endothelial growth factor

WT: Wild type

INDEX

I. INTRODUCTION

1. CANCER

1.1. THE CANCER CONCEPT

Cancer is a term that describes a large number of pathologies which have the uncontrolled growth and the spread of abnormal cells in common. The carcinogenesis process implies dynamic changes in the genome which lead to the transformation of cells which are consequently released from the homeostatic mechanisms that control normal proliferation, as well as from their normal interactions with the microenvironment (Hanahan and Weinberg, 2000). In addition to cancer cells, tumours exhibit another dimension of complexity: they contain a repertoire of recruited cells that contribute to the acquisition of hallmark traits by creating the ‘‘tumour microenvironment” in which neoplastic epithelial cells constitute a compartment that is distinct from the cells forming the tumour-associated stroma. Both tumour parenchyma and stroma contain distinct cell types that enable tumour growth and progression, such as immune cells, cancer stem cells, pericytes, endothelial cells, and cancer-associated fibroblasts (Hanahan and Weinberg, 2011).

1.2. THE MOLECULAR BIOLOGY OF CANCER

In 2000, Hanahan and Weinberg proposed that there are six essential characteristics, known as the hallmarks of cancer, for the development of cancer disease (Hanahan and Weinberg, 2000). Some years later, in 2011, they incorporated four new hallmarks (Figure 1), and pointed out the importance of the microenvironment in the cancer process (Hanahan and Weinberg, 2011). Hence, the proposed hallmarks are as follows:

1. **Self-sufficiency in growth signals.** In normal conditions, cells require mitogenic growth signals to move from a quiescent state into a proliferative state. By the union of signalling molecules (such as soluble growth factors or extracellular matrix components) to transmembrane receptors, growth signals are transmitted into cells. In the absence of these signals, normal cells are not capable of growing. However, tumoural cells grow even when these interactions do not take place because they can generate their own intrinsic growth signals.
2. **Evading growth suppressors.** In normal conditions, cells receive antiproliferative signals in order to maintain their quiescence, many of which depend on the actions of tumour suppressor genes. For instance, RB (retinoblastoma-associated) protein

integrates signals from diverse extracellular and intracellular sources, making it a cell-cycle progression gatekeeper. Hence, when the RB pathway is disrupted, cells become insensitive to inhibitory growth signals and inappropriate replication continues.

3. **Apoptosis evasion.** Programmed cell death by apoptosis is a natural barrier to cancer development. However, different strategies can be used by tumoural cells to escape from these mechanisms. One of the most common strategies is the loss of the proapoptotic regulator TP53, which induces apoptosis when DNA damage is detected.
4. **Enabling replicative immortality.** The number of cell divisions that can occur during a lifetime in mammals is limited by an intrinsic cell program, which is known as the Hayflick limit. Once cells have achieved this limit, they stop growing and start senescing (Hayflick, 1997), which results from the loss of the protective function of telomeres. It has been shown that in neoplastic cells, telomeres maintain their length due to higher activity of the telomerase enzyme (Shay and Bacchetti, 1997).
5. **Inducing angiogenesis.** Like normal tissues, tumours require nutrients and oxygen, and need to evacuate metabolic wastes. Hence, the generation of new vasculature from the pre-existing one is essential for tumour growth (Folkman, 2003). This process is known as angiogenesis and it is regulated by the equilibrium between inducer and inhibitor factors. Tumours have the capacity to activate the angiogenesis process by stimulating inducer factors.
6. **Invasion and metastasis.** Neoplastic cells can escape from primary tumour masses and invade adjacent tissues or distant sites. The success of this process depends on the other five characteristics and on complex changes in the physiological relationship between cells and their microenvironment.
7. **Genome instability and mutations.** Defects affecting components of the DNA-maintenance machinery lead to the accumulation of a large number of alterations in neoplastic cells, which are related to the aforementioned characteristics. The different steps involved in tumour progression are a succession of clonal expansions produced by the accumulation of mutations that generate selectively advantageous neoplastic cells.

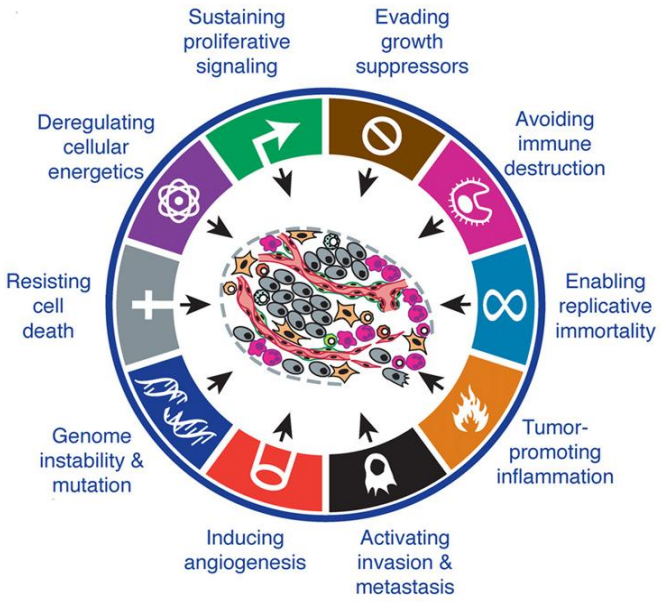


Figure 1. The ten hallmarks of cancer. This illustration represents the capabilities of cancer cells as proposed by Hanahan and Weinberg. One example is the ability of cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. Also of great importance is the inflammation produced by innate immune cells. Reproduced from Hanahan and Weinberg, 2011.

8. **Inflammation.** Tumours are densely infiltrated by immune cells which were initially thought to be acting against the tumour. However, it has now become clear that inflammation can contribute to tumourigenesis and tumour progression by supplying different molecules to the tumour microenvironment such as growth factors, angiogenic factors, and extracellular matrix-modifying enzymes. Inflammation seems to play a pivotal role in the earliest stages of cancer because inflammatory cells release potentially mutagenic chemicals that induce genetic mutations in the neoplastic cells.
9. **Reprogramming energy metabolism.** During the neoplastic process, changes in energy metabolism are produced in order to avoid apoptosis and to maintain and stimulate the growth and division of neoplastic cells.
10. **Avoiding immune destruction.** It has been proven that neoplastic cells have developed different strategies in order to avoid being detected by the immune system. For instance, inflammatory cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which are actively immunosuppressive, are recruited to the tumour environment, where they suppress the action of cytotoxic lymphocytes against tumour cells.

2. LUNG CANCER

2.1. EPIDEMIOLOGY

Currently, cancer is one of the principal causes of death in developed countries, making it an important health care problem. In 2008, 3.2 million new cancer cases were diagnosed and there were more than 1.7 million deaths related to this disease in Europe (Ferlay et al., 2010). Among the different types of cancer, lung cancer is the most frequently diagnosed and is the leading cause of cancer-related death worldwide, with more than 1.5 million new cases diagnosed per year (Jemal et al., 2011). In Europe, lung cancer is the third most common cancer and causes the highest mortality. Moreover, gender analysis showed that lung cancer is the most frequent cancer among men and the third among women (Ferlay et al., 2010). Trends in lung cancer mortality in Spain are similar to those observed in the EU; 20,000 new lung cancer cases are diagnosed each year, which represents 18.4% of all tumours diagnosed in men (18,000 cases) and 3.2% in women (2,000 cases; information obtained from the Spanish Lung Cancer Group website). Starting in the nineties, age-specific death rates in males decreased for each age group under 85 years old. However, a statistically significant annual increase of 6.3% in truncated mortality rates has been observed in women since 1992 (Cayuela et al., 2008).

2.2. RISK FACTORS

Tobacco smoking (cigarettes, pipes, and cigars) is the principal cause of lung cancer due to the toxic compounds present in its smoke and is responsible for 85-90% of these tumours (Freedman et al., 2008). The carcinogens present in cigarette smoke lead to the accumulation of a large number of mutations: it has been observed that in lung cancer an average of 200 mutations are accumulated per tumour, which is far more than the 25-50 mutations that have been observed in other frequent tumours such as breast and prostate cancer. Interestingly, the average number of somatic mutations that appeared in smokers is ten times higher than the number of mutations found in non-smoker lung cancer patients as shown in Figure 2 (Vogelstein et al., 2013).

Furthermore, the risk of developing lung cancer increases depending on the intensity and duration of smoke exposure (Jemal et al., 2008), and this risk starts to decrease two or three years after smokers stop smoking (Ebbert et al., 2003; Peto et al., 2000). An increased

risk (approximately 30%) of developing lung cancer in passive smokers has also been observed (Bilello et al., 2002; Hirayama, 2000). Since not all smokers develop lung cancer, the existence of genetic variations related to susceptibility and predisposition has been postulated (Gorlova et al., 2006; Matakidou et al., 2005).

Other factors that have been associated with lung cancer development are environmental or occupational carcinogens such as radon gas, arsenic, asbestos, and polycyclic hydrocarbons (Fraumeni, Jr., 1982;Tyczynski et al., 2003). Additionally diet type, alcohol consumption, and other diseases such as diffuse cystic fibrosis are also considered to be risk factors (Bilello et al., 2002; Hubbard et al., 2000).

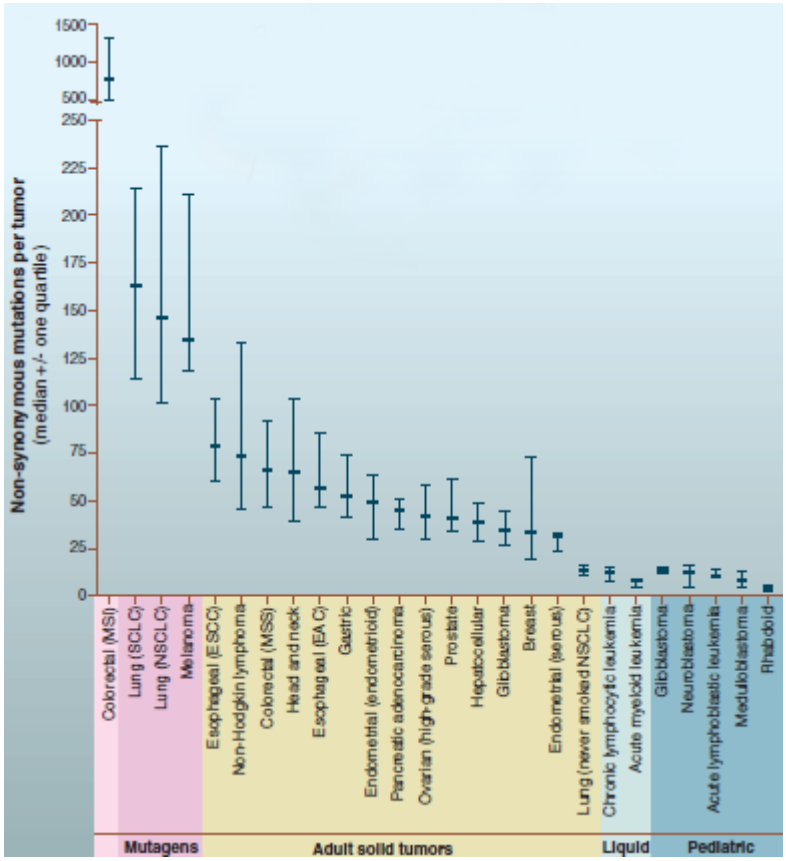


Figure 2. The average number of somatic mutations found in representative human cancers as detected by genome-wide sequencing studies. In lung cancer, the average number of somatic mutations that appeared in smokers is ten times higher than the number of mutations found in non-smokers. Reproduced from Vogelstein et al., 2013.

2.3. PATHOLOGY

The most commonly used pathological classification was published in 2002 and is based on histological and pathological techniques. Lung cancer is classified into four histological subtypes: small cell lung carcinoma (SCLC: 15% of cases), squamous cell carcinoma (SCC: 30%), adenocarcinoma (ADC: 35-40%), and large cell carcinoma (LCC: 10%), the last three subtypes are grouped into a greater entity: non-small cell lung carcinoma (NSCLC), which represents 80-85% of all lung cancer cases (Travis, 2002). NSCLC generally originates in the bronchial epithelium and is usually a slow-growing tumour with low sensitivity to chemotherapy and radiotherapy. Its three subtypes are discussed in more detail here:

1. SCC originates in the squamous epithelium of the lungs or bronchi and displays certain morphological features such as intercellular bridging, squamous pearl formation, and individual cell keratinisation. They usually occur in the central portion of the lung or in one of the main airway branches and can form cavities in the lung if they grow to a large size. SCCs are closely associated with tobacco smoking.
2. ADC originates in broncho-alveolar cells and is histologically heterogeneous. They are usually found in the peripheral areas of the lung and therefore are more likely to be surgically resected. In 2011 Travis et al. published a review that classified ADC histology into different subtypes: *lepidic, acinar*, *papillary*, and *solid* patterns; the *micropapillary* category was later added as a new histological subtype. Variants include invasive mucinous ADC, colloid, foetal, and enteric ADC (Travis et al., 2011).
3. LCC is the least frequent subtype. The prognosis is similar to that for ADCs but the tumours are usually more necrotic. This subtype has a neuroendocrine origin and they are usually found in the periphery of the lung. Their clinical evolution is aggressive due to their rapid growth and their capacity to generate metastasis (Travis, 2002).

2.4. DIAGNOSIS AND PROGNOSIS

Lung cancer is mainly diagnosed because patients develop symptomatic manifestations such as pain, haemoptysis, dyspnoea, and weight loss, or it is identified in a routine chest X-ray. A substantial number of patients with lung cancer show tumour spread at the time of diagnosis, and 40% of patients with NSCLC have distant metastases at presentation

(Morgensztern et al., 2010). Although most patients are initially diagnosed by chest radiograph, a range of technologies are now available for further staging, such as low-dose chest computed tomography (CT) or fluorodeoxyglucose (18F-FDG)-positron emission tomography (PET), which might play a major role in the prediction and assessment of future treatment responses (Cuaron et al., 2012). Moreover, bronchoscopic techniques and endoscopic ultrasound (EUS) or endobronchial ultrasound (EBUS) are now available for biopsying the tumour. Since routine screening for lung cancer is still a very expensive option, there is increasing interest in the development of risk models or biomarkers for predicting lung cancer (for a review see Jantus-Lewintre et al., 2012).

Prognostic assessment is an important factor to take into consideration when selecting an appropriate treatment regimen. The variables that have been associated with prognosis so far can be grouped into different categories: a) tumour-related, such as primary site, cell type and disease extension; b) patient-related, such as performance status, comorbidity, and sex; and c) environmental factors, such as nutrition (Goldstraw et al., 2011). The anatomical extent of disease, as described by the TNM (Tumour/Nodules/Metastasis) classification shown in Table 1, is one of the most important prognostic factors in lung cancer (Shepherd et al., 2007). The seventh edition of the TNM classification, published in 2009, included some changes affecting the organisation of the different stages (Detterbeck et al., 2009). Although the TNM classification provides pivotal prognostic information (Figure 3), histological confirmation (including molecular characterisation of the tumour) is also needed in order to make adequate decisions regarding patient treatment (Goldstraw et al., 2007).

Table 1. The T, N, and M descriptors in the seventh edition of the TNM classification for lung cancer. T refers to the primary tumour, N refers to the regional lymph nodes, and M refers to distant metastases. Reproduced from Goldstraw et al. 2011

T—primary tumour

TX: Primary tumour cannot be assessed, or tumour proven by the presence of malignant cells in sputum or bronchial washings, but not visualised 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 (ie, not in the main bronchus)

T1a: Tumour 2 cm or less in greatest dimension*

T1b: Tumour more than 2 cm but not more than 3 cm in greatest dimension

T2: Tumour more than 3 cm but not more than 7 cm; or tumour with any of the following features†:

- 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

T2a: Tumour more than 3 cm but not more than 5 cm in greatest dimension

T2b: Tumour more than 5 cm but not more than 7 cm in greatest dimension

T3: Tumour more than 7 cm or one that directly invades any of the following: chest wall (including superior sulcus tumours), diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium; or tumour in the main bronchus less than 2 cm distal to the carina* but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung or separate tumour nodule(s) in the same lobe as the primary.

T4: Tumour of any size that invades any of the following: mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, oesophagus, vertebral body, carina; separate tumour nodule(s) in a different ipsilateral lobe to that of the primary.

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

M0: No distant metastasis

M1: Distant metastasis

M1a: Separate tumour nodule(s) in a contralateral lobe; tumour with pleural nodules or malignant pleural or pericardial effusion‡

M1b: Distant metastasis

The resultant stage groupings are:

Occult carcinoma: TX, N0, M0

Stage 0: TisN0M0

Stage IA: T1a,bN0M0

Stage IB: T2aN0M0

Stage IIA: T2bN0M0; T1a,bN1M0; T2aN1M0

Stage IIB: T2bN1M0; T3N0M0

Stage IIIA: T1a,b, T2a,b, N2M0; T3N1, N2M0; T4N0, N1M0

Stage IIIB: T4N2M0; any T N3M0

Stage IV: Any T any NM1

Reproduced from reference 42, by permission of Blackwell Publishing. *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 T1a. †T2 tumours with these features are classified T2a if 5 cm or less or if size cannot be determined, and T2b if greater than 5 cm but not larger than 7 cms. ‡Most pleural (pericardial) effusions with lung cancer are due to tumour; in a few patients, however, multiple microscopical examinations of pleural (pericardial) 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 M0.

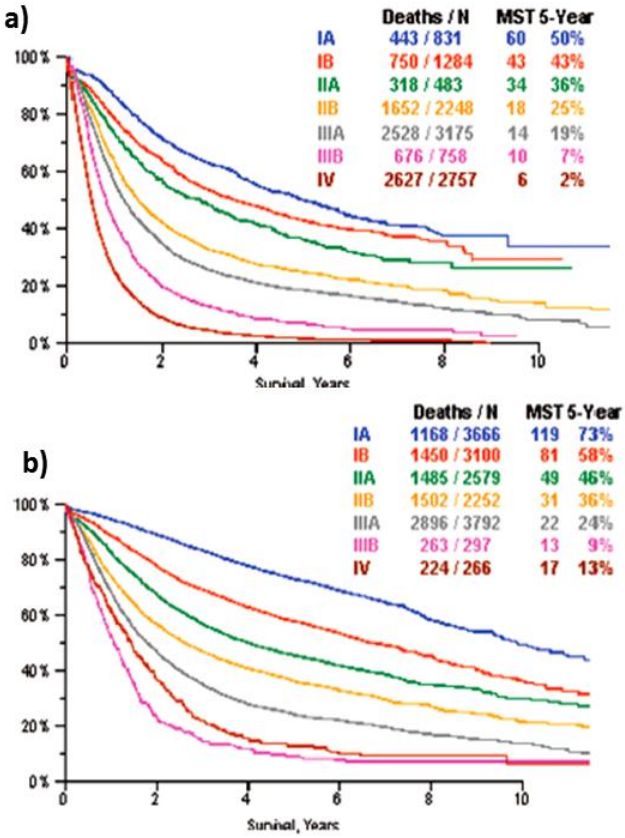


Figure 3. Prognostic information regarding TNM classification. The overall survival is expressed as median survival time and 5-year survival of NSCLC patients, classified by a) clinical stage and b) pathological state, according to the sixth edition of the TNM classification. Reproduced from Goldstraw et al., 2007.

2.5. TREATMENT

Lung cancer treatment essentially depends on anatomopathological classification, tumour stage, and performance status (PS). Surgery is still the standard treatment for early-stage patients with a good PS, that accounts for 20-30% of diagnosed NSCLC patients (Myrdal et al., 2001). However, stereotactic body radiotherapy (SBRT) has emerged as an alternative treatment for stage I-II patients with a borderline medical indication for surgery (Robinson et al., 2013). It has also been demonstrated that adjuvant chemotherapy after surgery with platinum salts in combination with vinorelbine, etoposide, or docetaxel increases patient survival rates (Felip et al., 2010; Molina et al., 2008).

In 2002, Carney pointed out that a treatment-efficacy plateau had been reached for NSCLC which could not be further improved with conventional chemotherapeutic drugs (Carney, 2002). However, since then, the overall survival (OS) has improved to a median of 12 months and it is even longer in clinical trials due to the introduction of new drugs and patient

selection. This selection is based on recognising different histological lung cancer subtypes and driver mutations that determine the biology of the tumour and predict drug efficacy (Scagliotti et al., 2008). A meta-analysis of 16 randomised trials confirmed that the most efficient treatment for the advanced stages of lung cancer was chemotherapy, which significantly increased patient OS (Burdett et al., 2008). Currently, standard chemotherapy is the combination of a third-generation cytotoxic agent such as gemcitabine, vinorelbine, or paclitaxel, with a platinum compound. Furthermore, recent data has shown that histology is an important factor to consider when selecting a specific treatment because of safety and/or efficacy reasons: for instance, the pemetrexed label restricts its use to non-SCCs (Langer et al., 2010). Radiotherapy also has an important role in the curative and palliative treatment of NSCLC patients. Although it can be indicated in patients with a good PS and non-resected disease, palliative intention is still its main use (Goldstraw et al., 2011).

In recent years specific anti-target therapies have emerged, which have increased NSCLC patient survival and decreased the toxicity that can be produced by conventional chemotherapy. Good examples are tyrosine-kinase inhibitors (TKIs) such as gefitinib and erlotinib, which are specific treatments for patients bearing mutations or deletions in the epidermal growth factor receptor (EGFR) gene. These mutations have been observed more frequently in women, in patients who have never smoked, and in those with ADC (Rosell et al., 2009). Another example of personalised treatment that has improved objective responses and survival in combination with chemotherapy in patients with a non-SSC histology is bevacizumab, a recombinant, humanised, monoclonal vascular endothelial growth factor (VEGF) antibody (Soria et al., 2013).

Recently, treatment with crizotinib, a small-molecule TKI that is highly specific for the inhibition of mesenchymal epithelial transition factor proto-oncogene, receptor tyrosine kinase (cMET), anaplastic lymphoma kinase (ALK), and reactive oxygen species proto-oncogene 1, receptor tyrosine kinase (ROS1) has been approved in several countries. The best studied of these targets is the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (*EML4-ALK*). This 5'fusion is a potent oncogenic rearrangement that activates the downstream function of *ALK*. Two methods are currently available for clinical selection of patients with this oncogene. The standard diagnostic tool is a break-apart fluorescence in-situ hybridisation assay (FISH) and the alternative method is immunohistochemistry (IHC) with anti-ALK monoclonal antibodies (Peters et al., 2013).

Over the last few decades, cancer immunotherapy has become a very attractive option for treating several cancer types. It refers to a number of approaches intended to activate the immune system in order to induce objective responses and disease stabilisation. Among the different possible options, blocking immune checkpoint molecules with monoclonal antibodies has emerged as a promising strategy. The CTLA4 (cytotoxic T-lymphocyte-associated antigen 4, also known as CD152) blocking antibody ipilimumab (BMS, Princeton, NJ) has already been approved by the federal drugs agency (FDA) for the treatment of patients with metastatic melanoma. In lung cancer, it is thought that infiltrating T cells might be partially responsive to CTLA4 blockade, and thus ipilimumab is currently being evaluated in a randomised phase III trial for the treatment of advanced NSCLC in combination with chemotherapy. Another monoclonal antibody, nivolumab, that blocks the interaction between the immune checkpoint molecule programmed cell death 1 (PD1) and its ligand (PDL1), is also being evaluated in NSCLC patients. On the basis of the promising preliminary activity seen in a large phase-Ib trial, more nivolumab clinical trials were recently initiated in patients with NSCLC (Drake et al., 2014).

Regarding the treatment of SCLC patients, early concurrent thoracic radiotherapy and prophylactic cranial irradiation should be considered for patients with or without metastases whose disease does not progress after induction chemotherapy and radiotherapy. A combination of chemotherapy, generally based on platinum, plus etoposide or irinotecan, is still the first-line standard treatment for metastatic disease. It is, however, noteworthy that despite high initial response rates, most patients eventually relapse, which then leaves few remaining treatment options, with the exception of topotecan (van Meerbeeck et al., 2011).

2.6. LUNG CANCER MOLECULAR CLASSIFICATION

Over recent decades, substantial advances have been made in the understanding of the molecular and cellular mechanisms driving lung cancer initiation, maintenance, and progression. These advances have led to the discovery of several novel drug targets and the development of new treatment strategies. NSCLC is one of the most genomically diverse tumours, and therefore there are a variety of molecularly defined subsets of patients characterised by specific sets of driver mutations (Figure 4). Among these driver mutations, some are potentially targetable molecules such as EGFR and ALK in ADC patients or PI3K in both histologies (Figure 5).

EGFR alterations are implicated in many types of cancer, including lung cancer. Overexpression or aberrant activation of this gene is present in approximately 60% of cases (Hirsch et al., 2003). *EGFR* encodes a transmembrane tyrosine kinase with an extracellular binding domain and an intracellular component including a tyrosine kinase domain. Binding to its ligand leads to receptor homo- or heterodimerization with other members of the EGFR family and activation of the tyrosine kinase domain (Scagliotti et al., 2004). In NSCLC, *EGFR* mutations have been observed in the first four exons of the intracellular tyrosine kinase domain, mainly (approximately 45%) in frame deletions in exon 19. The next commonest *EGFR* mutations are missense mutations, particularly L858R, a single nucleotide point mutation in exon 21 that leads to a single amino acid change from leucine to arginine at codon 858 accounting for about 40% of cases. Less common mutations, including in-frame duplications or insertions in exon 20 (representing 5-10% of cases), have also been identified (Kosaka et al., 2004; Okabe et al., 2007). In 2004, it was demonstrated that somatic mutations in the *EGFR* gene (exon 19 and 21) were correlated with sensitivity to TKI agents, whilst insertions in exon 20 (or the T790M mutation) produced resistance to this specific treatment (Lynch et al., 2004; Paez et al., 2004).

KRAS (Kirsten rat sarcoma viral oncogene homolog) is part of the *RAS* family of proto-oncogenes, which in humans comprises *KRAS, NRAS,* and *HRAS* and encodes a G-protein with a critical role in controlling signal transduction pathways. These pathways regulate cell proliferation, differentiation, and survival. KRAS plays a critical role in downstream signal transduction induced by several growth factor receptors including EGFR. *KRAS*-activating mutations in codons 12 and 13 are the most frequent oncogenic alteration identified in lung ADCs, occurring in about 25-40% of cases (Downward, 2003; Karnoub and Weinberg, 2008). However, to date, none of these mutations have been correlated with prognosis or chemotherapy response in NSCLC patients (Camps et al., 2011). Interestingly, the *EGFR* and *KRAS* mutations are mutually exclusive in NSCLC patients.

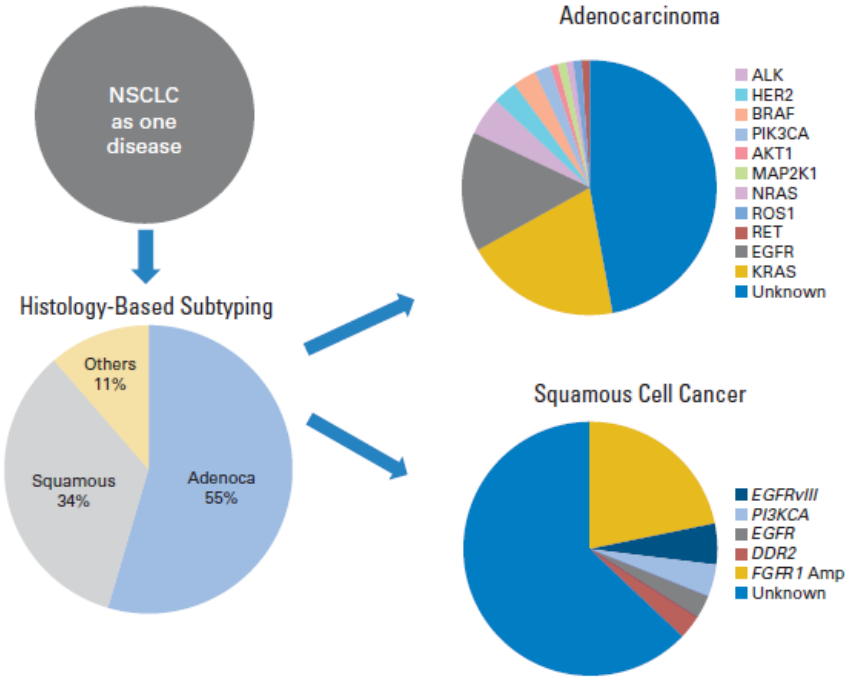


Figure 4. Evolution of NSCLC subtyping from histological to molecular classification. EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; MAP2K1, mitogen-activated protein kinase 1. Reproduced from Li et al., 2013.

ALK rearrangements, which result in fusions of the intracellular kinase domain with the amino terminal end of EML4, are also very important in NSCLC. The rearrangement occurs in chromosome 2p (intron 13 of *EML4* is fused to intron 19 of *ALK)* and causes a constitutive oligomerization that produces a mitogenic signal and therefore a malignant transformation (Soda et al., 2007). More recently, different partner genes have been identified in a small subset of *ALK* rearrangements (less than 1% of cases) including *KIF5B* (kinesin family member 5b), *TFG* (TRK-fused gene) and *KLC-1* (kinesin light chain 1). *ALK* rearrangements have been observed in 4% of ADC NSCLC patients, and are mutually exclusive to *EGFR* and *KRAS* mutations (Peters et al., 2013). ALK inhibition with the TKI crizotinib produces profound responses. However, drug resistance eventually develops, and there is evidence for the formation of secondary ALK point mutations and EGFR signalling activation implicated in some cases (Choi et al., 2010).

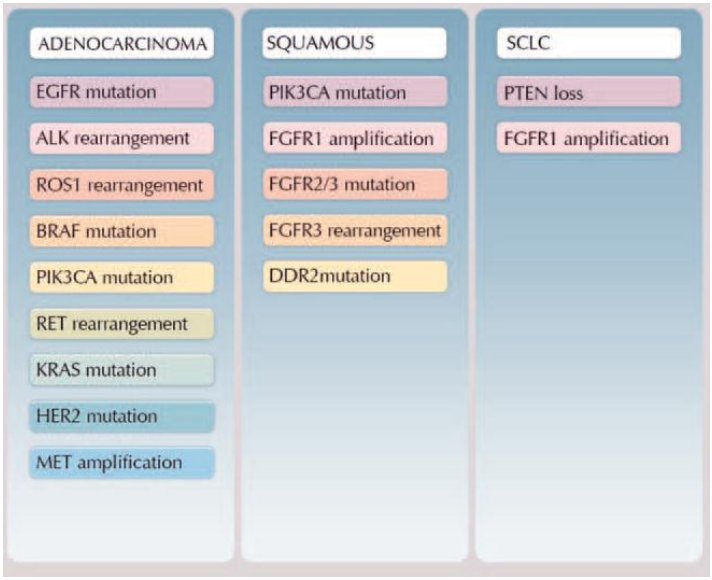


Figure 5. Potential targetable oncogenes by histological subtype. *EGFR*, epidermal growth factor receptor; *ALK*, anaplastic lymphoma kinase; *DDR2*, discoidin domain receptor tyrosine kinase 2; *FGFR1*, fibroblast growth factor receptor 1; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *MET*, MET proto-oncogene; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; *PTEN*, phosphatase and tensin homology deleted on chromosome 10; *RET*, ret proto-oncogene; SCLC, small-cell lung cancer. Reproduced from Morgensztern et al., 2015.

PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) signalling plays important roles in metabolism, growth, survival, and motility. Both copy-number gains and mutations in *PIK3CA* have been identified in lung cancer. *PIK3CA* copy-number gains occur in approximately 20% of lung cancers, with a higher frequency in SCCs. Preclinical data has suggested that cancers harbouring activating mutations in PIK3-catalytic subunit alpha *(PIK3CA)* may be among the most sensitive to single-agent PI3K pathway inhibitors (Morgensztern et al., 2015). Other druggable targets, also mainly in lung ADCs, include *ROS1* and *RET* (*ret proto-oncogene*) translocations and *HER2,* B-Raf proto-oncogene, serine threonine kinase (*BRAF*), *PIK3CA*, and catenin (cadherin-associated protein), beta 1 (*CTNNB1*) mutations (Li et al., 2013). Although *KRAS* mutations are not yet a druggable target, patients carrying mutations in this gene can be treated with synthetic lethal approaches such as a combination of mitogen-activated protein kinase (MEK) and PIK3CA or AKT1 inhibitors (Yang et al., 2010a). In SCC, fibroblast growth factor receptor 1 (FGFR1) and discoidin domain receptor tyrosine kinase 2 (DDR2) have been reported as potential biomarkers but only a few clinical trials are ongoing, and diagnostic methods still need to be standardised (Drilon et al., 2012). Genetic assessment by PCR and next generation sequencing (NGS) platforms are being evaluated in order to identify new driver alterations, and also for developing new clinical tools. Molecular classification of lung

cancer holds great promise for the advancement and personalisation of cancer treatments, with the goal of maximising efficacy and minimising toxicity.

Apart from the driver mutations mentioned above, it is known that all lung cancers carry high numbers of somatic mutations: high levels of inter- and intra-chromosomal rearrangements and copy-number alterations compared with other tumour types (Figure 6). In fact, lung cancers are among the most mutated types of cancer (Alexandrov et al., 2013). The diversity and complexity of the somatic mutational processes underlying carcinogenesis in human beings is now being revealed by identifying mutational patterns buried within cancer genomes. This diversity of mutation makes it difficult to treat all patients with targeted therapies. However, cancer-cell mutations that do not directly contribute to cancer initiation and progression (passenger mutations) generate new antigens which play a key part in tumour immunity. This fact is a great opportunity for therapies based on potentiating the body's own immune system to help fight cancer, also known as immunotherapies.

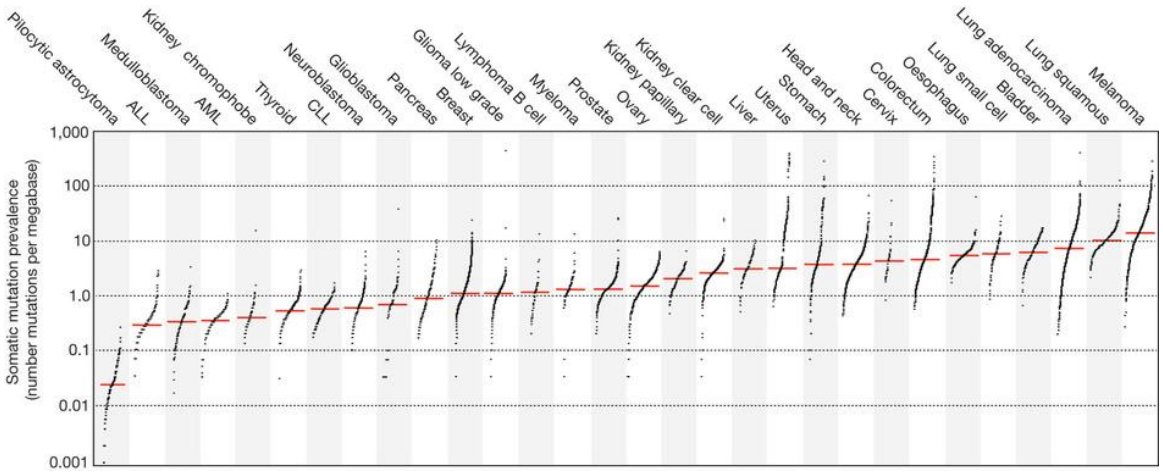


Figure 6. The prevalence of somatic mutations across human cancer types. Every dot represents a sample whereas the red horizontal lines are the median numbers of mutations in the respective cancer types. Lung cancer subtypes are among the most mutated human cancer types. Reproduced from Alexandrov, 2013.

3. THE IMMUNE SYSTEM AND CANCER

The main functions of the mammalian immune system are to control tissue homeostasis, to protect against invading or infectious pathogens, and to eliminate damaged cells. It is divided into two major components, innate and adaptive immunity, both of which produce an effective immune response through a variety of cells and soluble mediators. The innate immune system, which represents the first line of resistance, is composed of macrophages, neutrophils, dendritic cells (DCs), mast cells, eosinophils, basophils, natural killer (NK) cells, and NK T cells. Macrophages and mast cells release soluble mediators such as cytokines, chemokines, matrix metalloproteinases, and reactive oxygen species (ROS), and also attract additional leukocytes (de Visser et al., 2006). NK cells play a major role in the rejection of tumour cells or virus infected cells, whilst NK T cells are able to detect the presence of pathogens and induce DC maturation, functional activation, migration, and antigen presentation (Vivier and Anfossi, 2004). The adaptive immune system comprises B and T cells that further promote activation of innate immunity and support the expansion and production of tumour-specific T cells and antibodies. There are two main T cell subtypes: T helper cells (Th or CD4) and cytotoxic T cells (CTL or CD8). These cells express antigen-specific receptors that allow a flexible and broad number of responses (Bremnes et al., 2011).

The immune system plays three important roles in preventing tumours: it protects the host from virus-induced tumours by eliminating virus infections; it resolves the elimination of pathogens in a prompt manner avoiding the establishment of an inflammatory environment; and finally, the immune system eliminates tumour cells which express tumour-specific antigens in a process known as immunosurveillance (Vesely et al., 2011). In fact, early research showed that the presence of intratumoural T cells is correlated with improved clinical outcomes in advanced ovarian carcinomas (Zhang et al., 2003), which was also observed in other types of tumours such as NSCLCs (Al-Shibli et al., 2008). Among these intratumoural T cells, $CD8^+$ cells are the most important source of anti-tumour activity in vivo. Major histocompatibility complex (MHC) class I and B7 are two indispensable signals for activating $CD8^+$ T cells. After stimulating and interacting with $CD4^+$ T cells, $CD8^+$ T cells convert into cytotoxic lymphocytes (Russell and Ley, 2002). However, when the immune system cannot completely eliminate cancer cells, a state of equilibrium is developed whereby the tumour does not progress or further metastasise. In this case cancer cells that can resist, avoid, or suppress the antitumour immune response are selected for, leading to tumour escape and progressive tumour growth (Finn, 2012).

Taking all this evidence together, it has become increasingly clear that cancer cells can not only avoid immunosurveillance but also exploit native immune mechanisms to their own benefit. In fact, three capabilities or characteristics have been defined as essential features of cancer: the ability to thrive in a chronically inflamed microenvironment, the ability to evade immune recognition, and the ability to regulate and suppress immune activity. The relative strength of each of these abilities may vary from one kind of tumour to another (Cavallo et al., 2011).

Even in the absence of external inflammatory stimuli, signals driven by oncogenes can activate intrinsic pro-inflammatory pathways that accelerate the carcinogenesis process (Mantovani et al., 2008). The most important inflammatory pathways that are involved in inflammation-induced carcinogenesis converge at the level of transcription factor signal transducers. Some examples are activator of transcription 3 (STAT3) and nuclear factor-κB (NFκB). By using these pathways, tumours modulate the inflammatory environment by secreting soluble growth factors and chemoattractants which stimulate inflammatory-suppressive cells to counteract anticancer T cell responses (Elinav et al., 2013). As a result of this inflammation several types of immune cells accumulate in the tumour microenvironment (de Visser et al., 2006) in addition to the cancer cells and their surrounding stroma (the latter consisting of fibroblasts, endothelial cells, pericytes, and mesenchymal cells) which are shown in Figure 7. It is the expression of immune mediators and modulators, as well as the abundance and the activation state of different kinds of cells in the tumour microenvironment that define which direction the balance is tipped in, and therefore whether tumour-promoting inflammation or antitumour immunity will ensue (Lin and Karin, 2007).

Another characteristic of cancer cells is that they can avoid immune recognition. Because of the increasing instability of their genomes, tumours are able to produce different immunogenic clones, and the poorly immunogenic ones escape from immunosurveillance (Dunn et al., 2004). One of the mechanisms by which tumour cells escape is the loss or alteration of MHC glycoproteins on their cell membrane. Escape can also be facilitated by the subversion of cell physiology as a consequence of the overexpression of oncogenes, or/and alterations of antigenic peptide-processing machinery (Norell et al., 2006). These processes disturb direct T cell tumour antigen recognition and prevent direct priming of an immune response against the tumour (Cavallo et al., 2011). Finally, tumour cells can regulate immune system activity in order to suppress its response against them and to modulate the different immune system mechanisms to their own benefit.

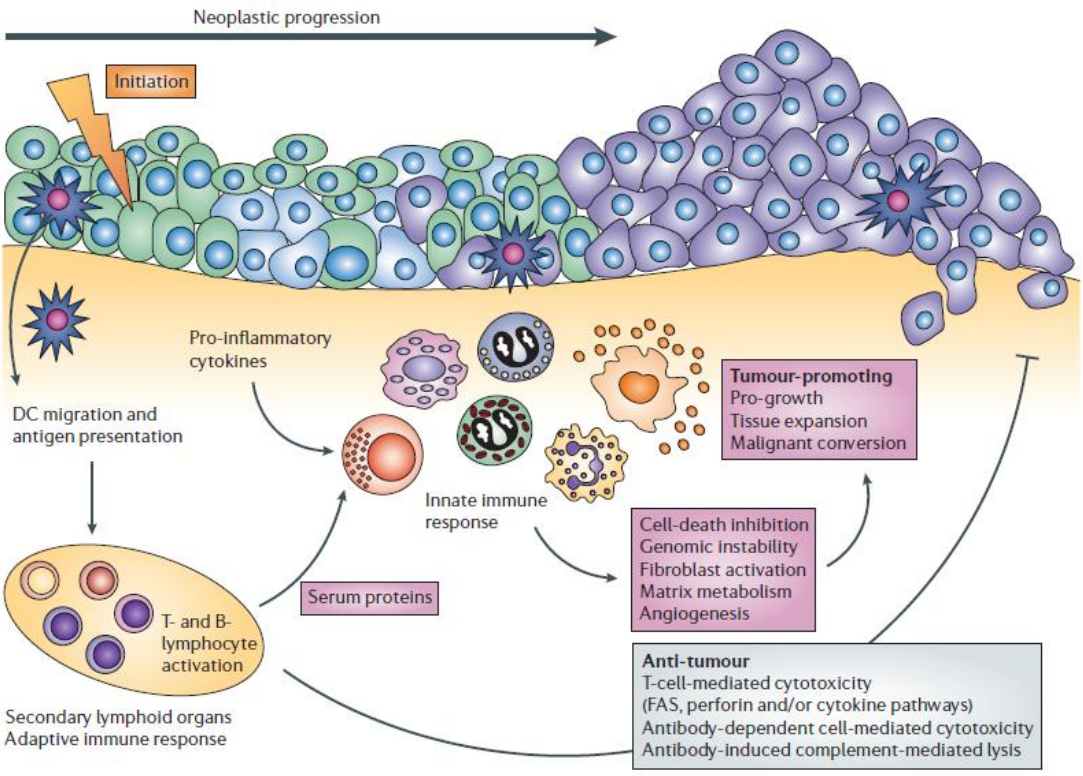


Figure 7. Representation of innate and adaptive immune-cell accumulation in the tumour microenvironment. Antigens that are present in early neoplastic tissues are transported to lymphoid organs by dendritic cells (DCs) that activate adaptive immune responses resulting in both tumour-promoting and antitumour effects. Activation of B cells and humoural immune responses results in chronic activation of innate immune cells in neoplastic tissues. Activated innate immune cells, such as mast cells, granulocytes and macrophages, promote tumour development by the release of potent pro-survival soluble molecules. Inflammatory cells positively influence tissue remodelling and development of the angiogenic vasculature by production of pro-angiogenic mediators and extracellular proteases. In contrast, activation of adaptive immunity also enhances antitumour responses through T-cell-mediated toxicity (by induction of TNF receptor superfamily member 6 [FAS], perforin and/or cytokine pathways) in addition to antibody-dependent cell-mediated cytotoxicity and antibody-induced complement-mediated lysis. Reproduced from de Visser et al., 2006.

3.1. CANCER IMMUNOREGULATION

Tumours acquire mechanisms to regulate their immune microenvironment which include: the release of a series of factors to subvert normal reaction mechanisms, the modulation of co-stimulatory pathways (also known as immunological checkpoints), and the induction and attraction of suppressor cells such as MDSCs, tumour-associated macrophages (TAMs), DCs, and Tregs.

3.1.1. IMMUNOSUPPRESSIVE FACTORS

Immunosuppressive mediators (such as cytokines) released by tumour cells may function to suppress DCs, indirectly inhibiting T-cell penetration into the tumour bed or

directly suppressing effector T-cell activation while also enhancing the function of Treg cells. **TGFβ** (transforming growth factor β) is one of the most important immunosuppressive cytokines produced by tumour cells and infiltrating leukocytes in the tumour microenvironment. It has been proven that TGFβ can promote cancer metastasis by enhancing tumour cell invasion and inhibiting the function of immune cells (Massague, 2008): TGFβ can suppress or alter the activation, maturation, and differentiation of both innate and adaptive immune cells, including NK cells, DCs, macrophages, neutrophils, and $CD4^+$ and $CD8^+$ T cells. Moreover, TGFβ has an important role in the differentiation and induction of natural and induced Tregs, which contributes creating an immuno-tolerant environment (Li et al., 2006). However, it is important to note that TGFβ plays a dual role in cancer; this was first shown in a set of skin cancer experiments performed in mice which demonstrated that TGFβ expression targeted keratinocytes to inhibit benign tumour outgrowth, whereas later it enhances the progression of malignancy and promotes benign papillomas towards malignancy (Cui et al., 1996). It was later demonstrated that TGFβ suppresses tumour initiation and early development by inhibiting cell cycle progression, inducing apoptosis, and suppressing the expression of growth factors, cytokines, and chemokines (Yang et al., 2010b).

Another function of TGFβ is the upregulation of **IL10** (interleukin 10), which in turn enhances TGFβ expression in a positive feedback circuit. IL10 inhibits antigen presentation, MHC class II expression, and the upregulation of the co-stimulatory molecules CD80 and CD86 (Mosser and Zhang, 2008). IL10 suppresses the inflammatory cytokines IL1, IL6, IL12, and tumour necrosis factor alpha (TNFα). IL10 seems to act on DCs and macrophages and protects tumour cells from CTL-mediated cytotoxicity (Kurte et al., 2004); however, some evidence indicates that IL10 may also possess immune stimulating properties. In fact, overexpression of IL10 in the tumour microenvironment synergises with other cytokines to promote tumour rejection instead of inducing immunosuppression (Lopez et al., 2005).

Tumours are also capable of expressing immunosuppressive factors other than cytokines; **Galectins** are important immunosuppressive molecules released in the tumour microenvironment. Current research indicates that galectins have important roles in cancer: they contribute to neoplastic transformation, tumour cell survival, angiogenesis, and tumour metastasis. Moreover, they can modulate the immune and inflammatory responses and might have a key role helping tumours to escape immune surveillance. The most extensively studied galectin in the context of immune response regulation is galectin-1, encoded by the gene LGALS1 (lectin galactoside binding soluble 1). Galectin-1 inhibits full T-cell activation, induces

the growth arrest and apoptosis of activated T cells, and suppresses the secretion of proinflammatory cytokines (Liu and Rabinovich, 2005). Galectins-2 and -9 also seem to play a role in tumour immunoregulation (Ito et al., 2012). In fact, Galectin-2 can induce T-cell apoptosis and control the secretion of lymphotoxin-α by macrophages (Liu and Rabinovich, 2005).

Indoleamine 2,3-dioxygenase (IDO), the key metabolic enzyme implicated in tryptophan catabolism, also effects immune response suppression. It produces a decrease in tryptophan availability and generates tryptophan metabolites which have negative effects on T lymphocytes. IDO activity seems to favour a regulatory phenotype in $CD4^+$ T cells. On the other hand, tryptophan catabolism by IDO can act as a negative modulator of tumour growth because of the inhibitory effect of interferon gamma (IFNγ) on cancer cell proliferation mediated by tryptophan deprivation (Godin-Ethier et al., 2011). However, IDOs role and mechanism of action in immune evasion and tumour growth are still poorly understood in human cancer. Other factors released by tumour cells that can affect the immune response are prostaglandin E2 (PGE2), gangliosides, stromal cell-derived factor 1 (SDF1 or CXCL12), and VEGF (Banerjee et al., 2013).

3.1.2. IMMUNE CHECKPOINTS

T cell activation requires at least two signals to become fully activated. The first occurs after engagement of the T cell antigen-specific receptor (TCR) by MHC, and the second by subsequent engagement of co-stimulatory molecules, the T cell co-stimulator CD28 being the most potent. It has been demonstrated that tumour cells may upregulate surface ligands which act as negative co-stimulatory signals for T cells. Undoubtedly, one of the best-studied regulatory signals is **CTLA4** which is induced in T cells at the time of their initial antigen response. Naïve and memory T cells express high levels of cell surface CD28 but do not express CTLA4: it is sequestered in intracellular vesicles and is transported to the cell surface after the TCR is triggered by encountering antigen (Figure 8a). The stronger the stimulation through the TCR (and CD28), the greater the amount of CTLA4 deposited on the T cell surface (Pardoll, 2012). CTLA4 counteracts the activity of the CD28 T cell co-stimulatory receptor and binds two identical ligands: CD80 (also known as B7.1) and CD86 (also known as B7.2; Carreno et al., 2000). Moreover, CTLA4 is constitutively expressed on the surface of Tregs (Jain et al., 2010). Therefore, CTLA4 functions as a signal dampener to maintain consistent levels of T cell activation. Tumour cells and antigen presenting cells (APCs) bind CTLA4 which circumvents

anticancer T cell activity. Allison and colleagues were pioneers in demonstrating that blocking CTLA4 signalling may enhance antitumour responses (Leach et al., 1996). The initial studies demonstrated significant anti-tumour responses without overt immune toxicities when mice bearing partially immunogenic tumours were treated with CTLA4 antibodies as single agents. The blockade of CTLA4 physiological functioning in T cells is currently used as a therapeutic approach in a variety of human malignancies, particularly in advanced melanoma, with the aim of promoting the activation and expansion of antitumour immune cells (Pardoll, 2012).

Another immune checkpoint receptor, **PD1** is emerging as an additional promising target; the major role of PD1 is regulation of inflammatory responses in peripheral tissues caused by effector T cell antigen recognition. Inflammatory signals in the tissues induce the expression of PD1 ligands, which downregulate T cell activity and thus limit collateral tissue damage (Figure 8b). Tumour cells are able to express PDL1 and, by doing so, they stop anticancer T cell activity (Pardoll, 2012). This receptor is expressed on a large proportion of tumour-infiltrating lymphocytes (TILs), including Tregs, whereas PDL1 is commonly upregulated on the cell surface of many different human tumours (Zou and Chen, 2008). In addition to tumour cells, PDL1 is commonly expressed on myeloid cells in the tumour microenvironment. Encouragingly, blocking antibodies against PD1 or PDL1 have been demonstrated to have substantial clinical effect in patients with metastatic melanoma, renal cell carcinoma, NSCLC, and other tumours. Moreover, preliminary findings raise the possibility that PD1 blockade might be less toxic than ipilimumab, although more detailed testing is still required (Topalian et al., 2012).

Other immune checkpoints that are currently being explored are lymphocyte activation gene 3 (LAG3; also known as CD223), 2B4 (also known as CD244), B and T lymphocyte attenuator (BTLA; also known as CD272), T cell membrane protein 3 (TIM3; also known as HAVcr2), adenosine A2a receptor (A2aR), and the family of killer inhibitory receptors. These molecules have all been associated with lymphocyte activity inhibition, and in some cases, the induction of lymphocyte anergy (Pardoll, 2012).

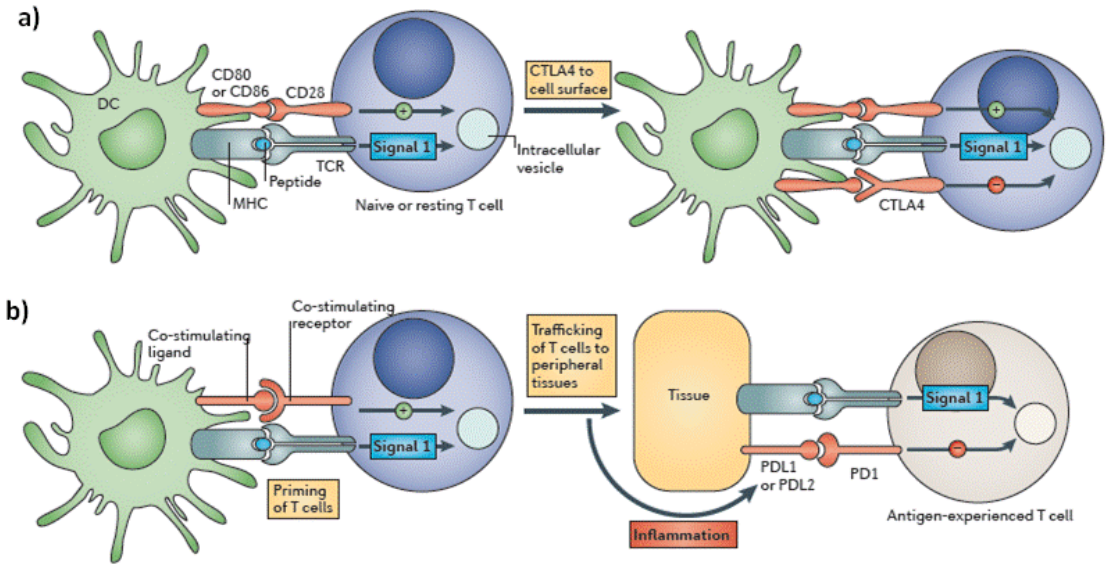


Figure 8. Immune checkpoint mechanisms of producing T cell anergy. a) Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4, also known as CD152) mechanisms of action in T cell response and; b) programmed cell death 1 (PD1) pathway activity on the surface of T cells. Reproduced from Pardoll, 2012.

3.2. IMMUNOREGULATORY CELLS

The contribution of stroma cells in the immunescape, which has been intensively studied over the last decade, is represented by its rapid recruitment, expansion, and activation of various lymphoid and myeloid origin immunosuppressive cells in the tumour microenvironment including Tregs, tumour-associated M2 macrophages, and MDSCs.

3.2.1. REGULATORY T CELLS

Tregs are vital for keeping the immune system in check, helping to avoid immune-mediated pathologies and unrestricted expansion of effector T cell populations. Tregs have been the focus of extensive research over the past few years, which have revealed diverse roles for these cells in numerous pathologies, including autoimmunity, allergy, microbial infection, and cancer. Defects in Treg cell function are an important factor in the development of autoimmunity or in the failure to control immunopathology, whereas overactive Treg cell function may contribute to the suppression of tumour immunity.

In 1995, a subset of $CD4^+$ T cells which constitutively express high amounts of IL2 receptor α-chain (CD25), and which were highly enriched in suppressor activity, were identified and termed regulatory T cells (Sakaguchi et al., 1995). CD25 was intended to be used as a Treg marker because it allowed their identification for functional analysis following their

isolation from non-immune animals; however, it soon became clear that its utility was limited because all activated T cells present CD25 upregulation.

The biology of Tregs was better understood after the identification and study of mutations in the X-chromosome encoded transcription factor FOXP3 (forkhead box P3) in mice and in human IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome patients. Mice and humans with a loss-of-function mutation in the *FOXP3* gene are afflicted with a fatal, early-onset, T cell-dependent, lymphoproliferative, immune-mediated disorder. Interestingly, the disease only affects hemizygous mutant males, because in females random X-chromosome inactivation ensures that some T cells express a wild-type *FOXP3* allele (Bennett et al., 2001; Brunkow et al., 2001). After this discovery, different laboratories assessed the stable expression of FOXP3 in mouse $CD25^{+}CD4^{+}$ Tregs, but not in naïve $CD25^{-}$ $CD4^{+}$ T cells or inactivated $CD4^{+}$ T cells (Hori et al., 2003).

Initially, it was thought that Tregs were a unique entity that originate in the thymus, but this assumption changed with the revelation that naïve $CD4^{+}$ T cells can be differentiated to become $FOXP3^{+}$ T cells. It is now accepted that the Treg population comprises various subsets (Figure 9), those derived from the thymus (or natural Tregs) and those produced in the periphery (or induced Tregs). Interestingly, TGFβR signalling appears to be required for most, if not all, FOXP3 induction in peripheral $CD4^{+}$ T cells (Selvaraj and Geiger, 2007).

a) TRANSCRIPTIONAL CONTROL OF REGULATORY T CELLS: FOXP3

Extensive studies in mice and humans have revealed the critical importance of FOXP3 as a master regulator of Treg development and function. The *FOXP3* gene, which is highly conserved, is located on the X chromosome at Xp11.23 and is subject to X chromosome inactivation. The gene contains 11 coding and 3 non-coding exons (Bennett et al., 2001). FOXP3 directly or indirectly controls hundreds of genes (approximately 700) and directly binds to about 10% of them, acting as an activator or suppressor of their expression (Zheng et al., 2007).

FOXP3 dimerises with nuclear factor of activated T cells (NF-AT) leading to suppression of IL2, IL4, and IFNγ expression, while inducing CD25, CTLA4, and glucocorticoid-induced TNF receptor family-related gene/protein (GITR), among others (Wu et al., 2006). Analysis of the transcriptional signatures and functional characteristics of cells expressing either null or functional FOXP3 reporter alleles further highlighted some of the features of Tregs: high CD25 and CTLA4 expression and diminished IL7Ra and immune response-promoting cytokine

expression are conferred upon these precursor cells prior to FOXP3 expression, most likely by TCR and cytokine signalling, but FOXP3 exaggerates this pre-existing pattern and makes it permanent (Zheng et al., 2007). Although FOXP3 is presently considered the most reliable (intracellular) phenotypic marker for Tregs, major concerns arose when it became evident that FOXP3 expression could be transiently induced in $CD4^+$ and $CD8^+$ effector T cells upon stimulation, albeit at lower levels (Roncador et al., 2005).

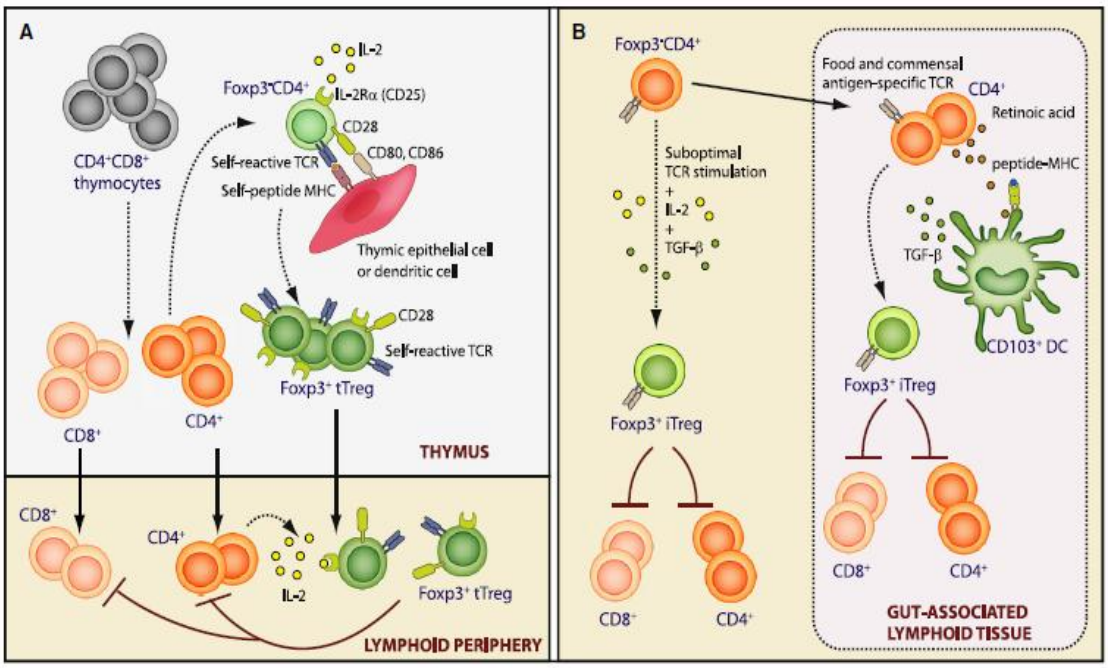


Figure 9. Differentiation of thymic and induced Tregs. Most $FOXP3^+$ thymic Tregs (tTregs) differentiate from $FOXP3^-$ $CD4^+$ thymocytes. The process of tTreg cell differentiation requires strong T cell receptor (TCR) stimulation by the self-peptide-MHC complexes presented by thymic epithelial cells (TECs) or dendritic cells (DCs); CD28 signalling induced by the CD80 and CD86 ligands expressed on antigen-presenting cells; and high-affinity IL2 receptor, and other γc cytokine receptor signalling. $Foxp3^+$ Tregs can also be induced from peripheral naive $CD4^+$ T cells (iTregs). In this case, the conditions favouring the peripheral induction of FOXP3 include: chronic low-dose antigen stimulation under tolerating conditions, suboptimal co-stimulation, and the presence of the immunomodulatory cytokine TGFβ, which plays a very important role in this process. Additionally, IL2 and the vitamin A metabolite retinoic acid (RA) facilitate the induction of FOXP3 in peripheral naïve $CD4^+$ T cells. Reproduced from Josefowicz and Rudensky, 2009.

Although FOXP3 plays a key role in Treg cell development and function, other molecular mechanisms are also necessary. Genome-wide comparison of DNA methylation status in conventional T cells and Tregs demonstrated that the presence of Treg-specific DNA hypomethylation in the genes associated with Treg function is also essential, more specifically, a pattern conserved in a non-coding region in the *FOXP3* locus, known as CNS2 (Ohkura et al., 2012). Furthermore, proteomic analysis in Treg cells indicates that FOXP3 forms complexes with other factors, including Helios, X-box binding protein 1 (Xbp1), Eos, and GATA-binding

factor 1 (GATA1), to activate the expression of most of the genes associated with the Treg-cell signature (Fu et al., 2012). Altogether, these findings suggest that the generation of functional Tregs requires more than just FOXP3 expression. However, stable expression of this transcription factor is critical in Treg development (Josefowicz and Rudensky, 2009).

b) MOLECULAR MECHANISMS OF REGULATORY T CELL SUPPRESSION

Defining the mechanisms of Treg function is clearly of crucial importance in order to understand their pivotal role in the immune system. From a functional perspective, the various potential suppression mechanisms of Treg cells can be grouped into four basic modes of action (Vignali et al., 2008): 1) suppression by the release of inhibitory cytokines such as IL10, TGFβ, and IL35; 2) induction of cytolysis through the expression of granzyme A and B, which can kill either responder T cells or APCs; 3) suppression by metabolic disruption produced by the high levels of IL2R expression on Treg cells that deprive effector T cells of IL2, and inhibits their proliferation; 4) suppression by targeting DCs, via expression of CTLA4 which binds CD80 and/or CD86 on DCs, thus inhibiting their maturation and proper function. Tregs could also condition DCs to express IDO, which induces the catabolism of tryptophan into pro-apoptotic metabolites, resulting in the suppression of effector T cells. Finally, Tregs can also express LAG3 which binds to MHC class II molecules, suppressing DC maturation as well as their immunostimulatory capacity.

c) REGULATORY T CELLS IN CANCER

Shortly after the publication of evidence for the existence of $CD4^{+}CD25^{high}$ Tregs in the peripheral blood of healthy individuals (Baecher-Allan et al., 2001), Carl June's group reported the presence of these cells in patients with epithelial malignancies, in particular ovarian and NSCLCs. They observed increased levels of $CD4^{+}CD25^{high}$ Tregs in the circulation and also within the tumour infiltrating lymphocytes. Upon identification of FOXP3 as a more reliable Treg marker, it was demonstrated that $CD4^{+}CD25^{high}FOXP3^{high}$ Tregs were present in the peripheral blood, malignant ascites, tumoural tissue, and draining lymph nodes (LNs) of ovarian cancer patients. They proposed that the accumulation of these cells was due to the presence of the chemokine CCL22, which is secreted by ovarian cancer cells and TAMs, and which binds to the CCR4 expressed on Tregs (Curiel et al., 2004).

Different mechanisms driving Treg cell accumulation within tumours have been described. First, it was suggested that Tregs have an enhanced capacity for infiltrating the tumour by migration from lymphoid and non-lymphoid organs, mediated by the coordinated

action of chemokines secreted by the tumour and their cognate receptors expressed on T cells, such as CCL22/CCR4, CXCL12/CXCR4, and CCL5/CCR5 (Oleinika et al., 2013). A second mechanism could be through expansion of Tregs within the tumour due to the presence of IL2 released by effector T cells (Quezada et al., 2011). A third mechanism is the de novo conversion of $FOXP3^-$ T cells into Tregs due to tumour-cell derived TGFβ (Chen et al., 2003).

The presence of $FOXP3^+$ lymphocytes has been associated with a poor prognosis in cohorts of patients affected by different types of tumours, including ovarian (Curiel et al., 2004), breast (Bates et al., 2006), NSCLC (Petersen et al., 2006), hepatocellular (Gao et al., 2007), renal (Griffiths et al., 2007), pancreatic (Hiraoka et al., 2006b), and gastric cancer (Perrone et al., 2008). In line with these results, it has been shown that the clinical response to chemotherapies is often associated with a reduction in Tregs and the recruitment of intratumoural $CD8^+$ T cells in breast cancer (Ladoire et al., 2008). On the contrary, it has been reported that intratumoural Treg infiltration in head and neck cancer tumours was correlated with better locoregional control and a good prognosis (Badoual et al., 2006; Bron et al., 2013); the latter was also observed in colorectal carcinoma (Salama et al., 2009) and bladder cancer (Winerdal et al., 2011). Various factors might explain these discrepancies: 1) the fact that although Tregs can potentially suppress antitumour immunity and therefore promote cancer progression, they also have the ability to dampen inflammation thereby reducing cancer progression (Banerjee et al., 2013); 2) the functional heterogeneity displayed by intratumoural Tregs depends on the factors they released and their activation markers (Kryczek et al., 2011); 3) the accumulation of Tregs may reflect the overall level of tumour infiltration by immune cells, including effector T cells; 4) the imperfect markers used to phenotype suppressive cells or technical differences; and 5) the fact that tumours have different phenotypes, grow in different organs, and are associated with unique host factors and, therefore, have unique microenvironments (Fridman et al., 2012). Since the relative contribution of different Treg populations in tumour immunity and in blocking tumour-associated inflammation is still unclear, a key question that arises out of recent work is whether Tregs directly influence cancer development and progression, or if they are merely a prognostic feature of malignancy (Banerjee et al., 2013).

3.2.2. MYELOID-DERIVED SUPPRESSOR CELLS

Myeloid cells are the most abundant haematopoietic cells in the human body and have diverse functions. The three groups of terminally differentiated myeloid cells are macrophages, DCs, and granulocytes, which are essential for the normal functions of the innate and adaptive immune systems. However, in cancer, myeloid cell differentiation is diverted from its normal pathway towards the differentiation of pathological MDSCs. These are immature myeloid cells that fail to complete their differentiation under the chronic inflammatory conditions that are typical in the tumour microenvironment. These cells impair immunosuppressive functions that allow them to efficiently inhibit T-cell mediated anti-tumour reactivity (Ostrand-Rosenberg and Sinha, 2009).

In mice, MDSCs express the Gr1 and CD11b surface markers; the human counterparts of these markers are CD97 and ITGAM respectively. They consist of two major subsets: granulocytic $CD11b^{+}Ly6G^{+}Ly6C^{low}$ (G-MDSCs) and monocytic $CD11b^{+}Ly6G^{+/-}Ly6C^{high}$ cells (M-MDSCs) which may differ in their immunosuppressive mechanisms. However, the human counterpart situation is much more complicated: The same two subsets can be distinguished as $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$ or $CD11b^{+}CD14^{-}CD15^{+}$ for granulocytic cells and $CD14^{+}HLA\text{-}DR^{-/low}$ or $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}$ for monocytic cells (Gabrilovich and Nagaraj, 2009). MDSCs derived from bone marrow haematopoietic precursors due to alteration of myelopoiesis by chronic inflammatory mediators such as the STAT family of factors (STAT3, STAT6, and STAT1), NF-κB, and S100 calcium-binding protein A8. These factors induce strong activation of inducible nitric oxide synthase (iNOS) and arginase (ARG)-1, the upregulated production of TGFβ, and the expression of cyclin D1, MYC, and survivin (Sonda et al., 2011).

a) THE MOLECULAR MECHANISMS OF MYELOID-DERIVED SUPPRESSOR CELL SUPPRESSION

The immunosuppression mechanisms used by MDSCs in tumours are: 1) production of nitric oxide (NO) and ROS that produce T cell apoptosis; 2) nitration of chemokines and TCRs, thus blocking T cell migration and tumour cell killing; 3) induction of the expression of TGFβ1 on cell membranes, stimulating anergy of immune effector cells; 4) deprivation of arginine and cysteine which are needed for multiple T cell functions; 5) reduction of T cell migration to the lymph nodes via the downregulation of L-selectin; and 6) downregulation of TCR ζ-chain expression, thus disabling the transmission of activation signals from the cell membrane by T cells (Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009). Furthermore, it has

also been observed that MDSCs produce NO that is accumulated in the tumour microenvironment and stimulates the development of tumour cell chemo resistance by inactivation of the caspase cascade (Sebens et al., 2007). Thus, MDSCs play a critical role in the development of an immunosuppressive tumour microenvironment.

b) MDSCS AND CANCER

It has been reported that chronic inflammatory mediators strongly stimulate MDSC expansion, migration into tumour lesions, and their immunosuppressive response. These mediators include IL1β, IL4, IL5, IL6, IL10, IL13, TNFα, IFNγ, VEGF, TGFβ, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), CCL2, CCL4, CCL5, CXCL1, CXCL8, CXCL12, cyclooxygenase-2 (prostaglandin synthase-2; COX2), and PGE2 (Gabrilovich et al., 2012). The pattern of mediators involved in MDSC recruitment to the tumour seems to be dependent on the tumour type, and is likely specific for the particular MDSC subset (Figure 10). It has been reported that the recruitment of the monocytic subset occurs via an interaction between CCL2 and its receptors CCR2, CCR4, and CCR5. However, migration of the granulocytic subset has been observed to be regulated by enhanced CXCR2 ligand production in the tumour milieu (Lesokhin et al., 2012).

While some aberrant myeloid populations can be detected in virtually every cancer type that has been studied, the heterogeneity of MDSCs in human malignancies is striking. To characterise them, most researchers agree on general myeloid markers such as CD33 and CD11b, but the human MDSC phenotypes described range from $CD34^+$ or $Lin^-DR^-CD33^+$ myeloid precursors to $CD15^+$ granulocyte-like cells and cells resembling monocytes [$CD14^+$ human leukocyte antigen-DR [HLA-DR]$^{-/low}$] (Poschke et al., 2011). It has been observed that levels of peripherally circulating $CD11b^+CD33^+HLA^-DR^-Lin1^{-/low}$ MDSCs correlate with the clinical stage in breast cancer and gastrointestinal malignancies. Moreover, therapy with cytotoxic agents can further increase the burden of circulating MDSCs, indicating that these cells might play a role in treatment failure (Diaz-Montero et al., 2009). Furthermore, in patients with gastrointestinal malignancies $CD15^+$MDSC levels correlated with elevated IL6 plasma levels whereas the $CD15^-$ MDSC subset revealed a strong correlation with IL10 (Mundy-Bosse et al., 2011). Similarly, elevated levels of $CD11b^+CD33^+HLA^-DR^-Lin1^{-/low}$ MDSCs may represent an independent prognostic factor in pancreatic, oesophageal, and gastric cancers, which also correlates with increased Treg numbers, and an increased risk of death (Gabitass et al., 2011).

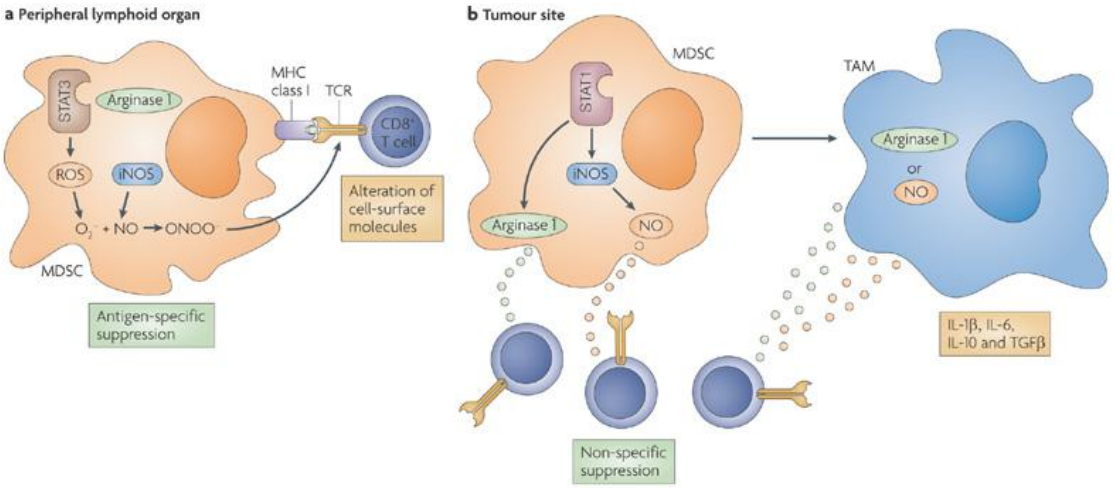


Figure 10. Mechanisms of MDSC-mediated immune suppression in peripheral lymphoid organs and at the site of a tumour. a) In peripheral lymphoid organs, MDSCs produce high levels of reactive oxygen species (ROS), including peroxynitrite (ONOO-), and upregulate signal transducer and activator of transcription 3 (STAT3) activity. This is associated with a moderate increase in arginase 1 (ARG-1) activity and low levels of nitric oxide (NO) production. MDSCs can take up, process, and present antigens to antigen-specific $CD8^+$ T cells, which causes these T cells to become unresponsive to antigen-specific stimulation. b) In contrast, MDSCs that migrate to the tumour site upregulate STAT1 activity, and produce high levels of inducible nitric oxide synthase (iNOS), NO, and arginase 1; this is associated with low levels of ROS. The high levels of ARG-1 and NO that are released by MDSCs inhibit $CD8^+$ T-cell function in a non-specific manner. MDSCs at the tumour site can also differentiate into tumour-associated macrophages (TAMs). Reproduced from Gabrilovich and Nagaraj, 2009.

3.2.3. TUMOUR-ASSOCIATED MACROPHAGES

Macrophages display phenotypic plasticity, and therefore, they are able to perform and participate in a diverse range of functions such as inflammation, tissue remodelling, antimicrobial activity, immunoregulation, and tumour promotion, depending on the signals they receive from their microenvironment. Macrophages have two main phenotypes: classical activation that is promoted by microbial stimuli like lipopolysaccharides (LPS) and Th1 cytokines including IFNγ; and the alternative activation drive by Th2 cytokines such as IL4 and IL13. It has also been proposed that macrophages could be classified into M1 or M2, corresponding to their Th1 and Th2 responses. A polarised macrophage phenotype that has received a great deal of attention in the literature is the tumour-associated macrophages which are often considered to be synonymous with M2 macrophages (Figure 11; Lawrence and Natoli, 2011).

Different mechanisms have been postulated for the differentiation of blood monocytes into TAMS, including the abundant release of colony stimulating factor 1 (CSF1) by tumours and their surrounding stroma. IL34, a ligand for CSF1R, was recently shown to be expressed by breast carcinoma epithelial cells following cytotoxic therapy and was also implicated in TAM

recruitment (DeNardo et al., 2011). Early studies linked the origin of TAMs to "tumour-derived chemotactic factors" (later characterised as CCL2), which recruit these cells to tumour tissues. In fact, studies using overexpression of CCL2 and transgenic modulation of CSF1 (using CSF1R knockouts or siRNA) indicated a functional relationship between macrophage infiltration and tumour progression (Aharinejad et al., 2007). Other chemokines, such as CCL3, CCL4, CCL5, CCL7, CCL8, CXCL12, VEGF, platelet-derived growth factor (PDGF), and IL10, are also reported to promote macrophage recruitment to the tumour microenvironment (Murdoch et al., 2004).

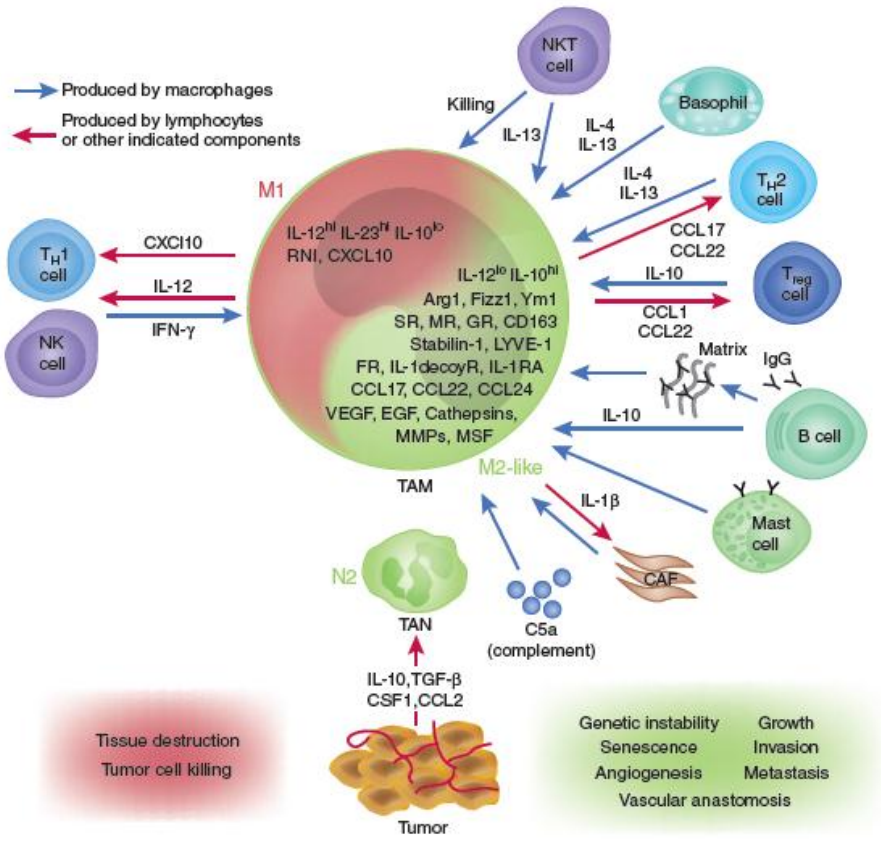


Figure 11. The yin-yang of myelomonocytic cells in tumour progression. Myelomonocytic cells can have either beneficial or pathological roles in cancer depending on the cellular and tissue environment. Red, M1 polarisation; green, M2 or M2-like polarisation; red and green shading, functional outputs for M1 and M2 macrophages, respectively; black lettering in cells, salient features of M1 and M2 macrophages; arrows, crosstalk between macrophage and lymphoid cells. TAM, tumour associated macrophage. Reproduced from Biswas et al., 2010.

a) THE MOLECULAR MECHANISMS OF TUMOUR-ASSOCIATED MACROPHAGES

Since the formal demonstration in the 1970s, by isolation and transplantation, that TAMs are bona fide host cells rather than cancer cells in disguise, considerable progress has been made in defining their function and significance in tumours. It has been demonstrated that macrophages promote cancer-related inflammation through the release of inflammatory mediators like TNF, IL6, and IL1β and they are believed to mediate DNA damage, oncogenic transformation, and cancer-cell survival. They also facilitate angiogenesis, invasion, and metastasis by the expression of a variety of pro-angiogenic factors such as VEGFA, epidermal growth factor (EGF), and CXCL8. Furthermore, TAMs also seem to interact with cancer stem cells (Biswas et al., 2013).

Interestingly, TAMs display a pivotal role in immunoregulation. Early on, some studies demonstrated that macrophages from tumour-bearing animals often had an immunosuppressive phenotype. For example, macrophages from different murine tumours (such as mammary carcinoma, chemically induced fibrosarcoma, ovarian carcinoma, and Lewis lung carcinoma) expressed reduced levels of the pro-inflammatory cytokine IL12, while displaying elevated expression of the anti-inflammatory cytokine IL10 (Torroella-Kouri et al., 2005). It has also been suggested that the $IL12^{low}/IL10^{high}$ characteristic of TAMs, together with the upregulation of TGFβ and CCL22, alter T-cell response in favour of the tumour. Various Th2 cytokines like IL4, IL13, and IL10 polarise TAMs into a M2-like phenotype, as for example, in the mammary carcinoma model (DeNardo et al., 2011). It is also interesting that IL10 derived from tumour-infiltrating Tregs may trigger PDL1 activation on TAMs which favours the inhibition of tumour-specific T-cell immunity (Kuang et al., 2009).

b) TUMOUR-ASSOCIATED MACROPHAGES: CLINICAL VALUE IN CANCER

Some evidence from epidemiological studies support the role of TAMs in human cancer, showing that an increased number of TAMs correlated with poor prognosis in many cancers such breast, bladder, and prostate, but not in others like gastric and colon cancer. Most of these studies were performed using macrophage markers like CD68 and CD163 in human sections (Biswas et al., 2013). A macrophage-specific gene signature and the presence of $CD68^{+}$ TAMs have also been reported to predict treatment outcome in classic Hodgkin's Lymphoma (Steidl et al., 2010). A recent meta-analysis summarised the current prognostic value of the presence of macrophages, which was associated with decreased survival in

endometrial, prostate, urothelial bladder, ovary, gastric, and oral carcinomas. Interestingly, TAM infiltration was associated with improved OS in colorectal cancer patients (Zhang et al., 2012).

In lung cancer a significant portion of the available evidence points to a positive correlation between TAM infiltration and a good prognosis. In a study in patients with resected NSCLCs, an increased tumour islet $CD68^+$ macrophage density and tumour islet/stromal macrophage ratio were significant independent predictors of increased survival (Welsh et al., 2005). On the other hand, substantial evidence correlating TAMs with poor prognosis in human lung cancer also exists. For instance, Takanami et al. found significantly lower 5-year survival rates in ADC patients with high $CD68^+$ TAM densities (Takanami et al., 1999). This work was furthered by Chen et al. who found that $CD68^+$ TAM density correlated positively with tumour IL8 mRNA expression and intratumour microvessel counts, but negatively with prognosis in patients with NSCLC (Chen et al., 2005).

4. BIOMARKERS

According to the National Cancer Institute (NCI), a biomarker is “a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease”. There are a tremendous variety of biomarkers, which can include proteins, nucleic acids, antibodies, and peptides, among other categories. Furthermore, a biomarker can be a set of alterations, such as gene expression profiles, proteomics, and metabolomic signatures. There are different types of biomarkers: 1) diagnostic biomarkers, which can be used to screen or detect otherwise healthy patients for malignancy; 2) prognostic biomarkers, which determine the outcome of the disease independently of treatment; and 3) predictive biomarkers which determine which treatment is likely to be most effective or, in other words the likelihood of response to a specific treatment.

Traditionally, the clinicopathological characteristics of a tumour have been used to assess prognosis. The search for a prognostic and predictive biomarker has to take two key points into consideration: the strength of evidence to support its use and the depth of information provided by a biomarker which adds to what is already known about the disease based on clinical parameters. For instance, there are a number of gene expression signatures, which have been developed in breast cancer that can be used to estimate prognosis for an individual patient (Van't Veer et al., 2005). On the other hand, an example of predictive

biomarkers is the determination of KRAS mutations in colorectal cancer, which correlates to poor responses to EGFR TKI treatments (Allegra et al., 2009).

Over the last decade, a large amount of research has been performed in order to discover and validate biomarkers in NSCLC. Some results from these studies have been adopted in current clinical practice, although a plethora of potential biomarkers could have not yet been validated. Among these validated biomarkers, is the activation EGFR mutations, which have become predictive biomarkers for TKI therapy (Paez et al., 2004), or the presence of ALK translocations, which are predictive biomarkers for ALK inhibitor therapy (Peters et al., 2013). Apart from these, there are several potential biomarkers still being researched which have been proposed as possible tools for the future management of NSCLC, especially early-stage NSCLC. The tumour suppressor gene *p53*, which is frequently altered in NSCLC patients, is a well-established poor-prognosis biomarker in many tumours, although its prognostic role in NSCLC is still controversial (Graziano et al., 2010). The prognostic significance of KRAS also remains controversial due to discrepancies in the results reported so far. Some studies seem to show that *KRAS* mutations, especially at codon 12, are associated with worse progression free survival (PFS) and OS (Vega et al., 1996), while others claim that the presence or absence of mutations in codon 12 of KRAS do not confer a survival disadvantage (Shepherd et al., 2013). Other individual biomarkers that have been suggested as prognostic or predictive biomarkers in NSCLC are Her2, excision repair cross-complementation group 1 (ERCC1), ribonucleotide reductase M1 (RRM1), and breast cancer 1 (BRCA; Burotto et al., 2014).

However, not only individual markers but also gene signatures have been postulated as useful biomarkers in NSCLC. Several authors have attempted to identify prognostic gene signatures based on gene expression levels by using several platforms such as microarrays and quantitative real time PCR (RTqPCR). For instance, Chen et al. identified a five-gene signature associated with an increased risk of death (Chen et al., 2007) and, more recently, Kratz et al. reported a 14-gene prognostic signature using RTqPCR in a large cohort of non-squamous NSCLCs (Kratz et al., 2012). Our group has recently reported a gene expression signature comprising the combination of three angiogenic factors (VEGFA/VEGFB/VEGFD) which is associated with a worse outcome and which was an independent prognostic biomarker in resectable NSCLCs (Sanmartin et al., 2014).

4.1. IMMUNE SYSTEM-RELATED BIOMARKERS IN LUNG CANCER

In NSCLC several attempts have been made to evaluate the prognostic and predictive role of immune microenvironment markers. Furthermore, they have acquired special importance due to the increasingly important role of immunotherapeutic approaches in the treatment of solid tumours.

Previous studies have demonstrated the prognostic importance of tumour infiltrating cells and their location. On one hand, it has been observed that the presence of antitumour cells such as $CD4^+$ and $CD8^+$ T lymphocytes and M1 macrophages predict prolonged patient survival (Al-Shibli et al., 2008). On the other, the presence of immunoregulatory cells such as Tregs, MDSCs, and TAMs has generally been associated with poor clinical outcome, although there are discrepancies between the different studies. For instance, patients with tumours infiltrated by three or more Tregs in 10 high-power fields (HPF) have significantly worse recurrence-free survival (RFS), especially in stages I and II of the disease (Shimizu et al., 2010). Regulatory signals through molecules like CTLA4 and PD1/PDL1 are also of great importance; in fact, CTLA4 overexpression has been reported to be an independent favourable prognostic factor (Salvi et al., 2012). As for PDL1, Velcheti et al. have recently demonstrated that the expression of PDL1 was associated with better OS independently of other factors (Velcheti et al., 2014).

Altogether, the data obtained so far indicates that immune system-related biomarkers, and more specifically those involved in immunoregulation, may become useful in the development of the next generation of prognostic and predictive biomarkers in NSCLC. Therefore it is important to continue searching for, studying, and validating new immunoregulation biomarkers using feasible techniques such as RTqPCR and immunohistochemistry (IHC), before these biomarkers can become useful oncological clinical tools.

II. OBJECTIVES

NSCLC is a heterogeneous and etiopathologically complex disease. Five-year survival remains poor, mainly because these tumours may have already developed regional or distant metastases at the time of diagnosis. Moreover, these patients have strikingly different outcomes which highlight the importance of establishing new prognostic biomarkers that allow better patient stratification. Given that we are now in the era of precision oncology, the objective of this thesis is to find new immunoregulatory biomarkers that could provide relevant information on the immunological features of the tumour microenvironment and their possible clinical translation in NSCLC.

The specific aims of this study are:

1. To analyse biomarkers related to immunoregulation by performing RTqPCR on tumour samples from resected NSCLCs.
2. To determine the presence of infiltrating $CD4^+$, $CD8^+$, and $FOXP3^+$ lymphocytes in the tumour and stroma by immunohistochemistry.
3. To analyse biomarkers related to immunoregulation by performing RTqPCR on blood samples from advanced NSCLCs and to determine their diagnostic and prognostic value.
4. To integrate the gene expression analysis with data about the levels of infiltrating immune cells to gain new insights into their role in the tumour microenvironment.
5. To find new immune-related profiles and signatures and to evaluate their possible roles as biomarkers for better NSCLC patient stratification.

III. MATERIALS & METHODS

1. PATIENTS AND SAMPLES

1.1. PATIENTS INCLUDED IN THE STUDY

1.1.1. EARLY STAGE NON-SMALL CELL LUNG CARCINOMA PATIENTS

In this study 178 patients from *Consorcio Hospital General Universitario de Valencia* and 20 from *Hospital Clínico Universitario de Valencia* with NSCLC were included between 2004 and 2013. Patients that met the eligibility criteria had resected non-pre-treated stage I to IIIA cancer (according to the American Joint Committee on Cancer staging manual) with a histological diagnosis of NSCLC. The study was conducted in accordance with the Declaration of Helsinki, and the institutional ethical review board approved the protocol.

1.1.2. ADVANCED STAGE NON-SMALL CELL LUNG CARCINOMA PATIENTS

Forty-nine samples from patients enrolled in a multicentre study coordinated by the Spanish Lung Cancer Group were retrospectively analysed. The clinical study was carried out between February 2003 and January 2005. Eligibility criteria were: the diagnosis of stage IIIB disease with pleural effusion or stage IV NSCLC. Patients were treated with cisplatin (75 mg/m^2) and docetaxel (75 mg/m^2) on day 1 every 3 weeks and then the objective responses were evaluated using the Response Evaluation Criteria for Solid Tumour (RECIST) criteria (Therasse et al., 2000). All individuals provided informed consent. The study was conducted in accordance with the Declaration of Helsinki and the institutional ethical review board approved the protocol.

1.1.3. CONTROL GROUP

The control group consisted of 54 anonymous, aged- and gender-matched, healthy volunteers without any acute or chronic inflammatory conditions. Control samples were taken at the same time points as the patient samples at the *Consorcio Hospital General Universitario de Valencia.*

1.2. SAMPLES

A total of 178 pairs of patient tumour and adjacent normal lung tissue specimens were obtained at the time of surgery and immediately separated by a pathologist. Tissue samples

were preserved in RNALater® (Applied Biosystems, USA) to avoid RNA degradation, and were fresh-frozen at -80° C until their analysis. Furthermore, 102 formalin-fixed, paraffin-embedded (FFPE) samples from the same cohort of patients were provided by the Pathology Service at the *Consorcio Hospital General Universitario de Valencia* as well as 20 FFPE samples from the Pathology Service at the *Hospital Clínico Universitario de Valencia*. A pathology report was available for all the samples, enabling their characterisation. We included the different NSCLC histology subtypes in this study.

Peripheral blood samples (2.5 ml) from 49 advanced stage NSCLC patients and 54 controls were obtained in PAXgene™ Blood RNA (PreAnalytiX, Switzerland) tubes and were stored at -80° C until they were used. These specialised tubes contain an additive that stabilises the gene transcription profile by reducing RNA degradation and minimising gene induction. Samples from patients enrolled in this multicentre study were obtained on two occasions: before the treatment, and after three cycles of chemotherapy with docetaxel and cisplatin.

2. HISTOPATHOLOGICAL METHODS

The histopathological study was performed using lung tumour tissue fixed in 10% formaldehyde and embedded in paraffin. Each FFPE sample was cut with different section thicknesses using a Leica RM 2135 Microtome: a) 5 µm sections each for non-treated slides for haematoxylin and eosin staining (HE) and for Dako charged slides (Code K8020, Dako, Canada) for IHC; and b) 10 µm sections for membrane slides (PEN-Membrane 2.0 µm; Leica, Germany) for microdissection.

2.1. HAEMATOXYLIN AND EOSIN STAINING

HE staining is the most common staining technique used in histology. Haematoxylin stains the nucleus of the cell blue and the eosin stains the cytoplasm pink. HE staining was used to assess the morphology of the tissue studied, which enabled for further analysis steps as described in the sections below. HE was performed on 5 µm sections for non-treated slides and 10 µm sections in membrane-slides. Briefly, deparaffinization was performed by incubating the slides at 60° C for 20 minutes and then submerging in xylene. The slides were hydrated with ethanol (absolute/96°/80°/70°) and rinsed with water. They were then stained with haematoxylin, decolourised with acid chloride, and immersed in lithium carbonate. The slides were counterstained with eosin, dehydrated with ethanol (96°/absolute) and cleared

with xylene. Finally, the 5µm sections were mounted with a cover using DPX Mountant for histology (Sigma, USA), whereas the 10µm sections were left uncovered.

2.2. LASER-CAPTURE MICRODISSECTION

Laser-capture microdissection (LCM) was performed in order to separately collect tumour and stroma tissue areas from FFPE tumour specimens. For this purpose, a Leica Laser Microdissection System LMD6500 was used with the support of a Hitachi HV-D20 camera. This system uses a laser to isolate a specific region in the tissue sample and transfers the area of interest into devices by gravity (Figure 12). Five micrometre HE sections were used to assess the morphology of the tissue, and to visually select the different areas (tumour vs. stroma) to be microdissected with the help of the computer. These areas were then translated onto the 10 µm membrane-slide sections where they were microdissected. An area of at least $1 \cdot 10^7$ μm^2 was microdissected from the tumour and stroma compartments and collected on the lids of separate 0.2 ml tubes containing 30 µl of Digestion Buffer from the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Qiagen, Germany).

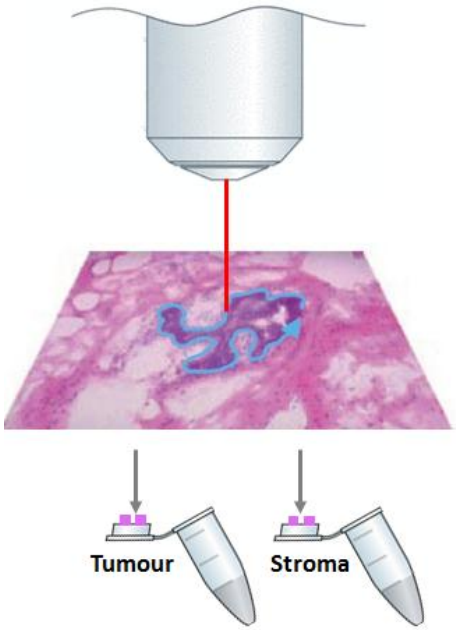


Figure 12. Representation of the laser capture microdissection and the recollection of the dissected areas.

2.3. IMMUNOHISTOCHEMISTRY

IHC is a method that allows the location of proteins (antigens) in tissue sections to be identified by the union of specific antibodies. The antibody-antigen interaction is then visualised by a chromogenic reaction, in which an enzyme conjugated to the antibody cleaves the substrate in order to produce a coloured and insoluble precipitate that can be visualised using a microscope. Depending on the system used and the type of tissue samples, the IHC technique requires specific optimisation.

The pivotal reagent common to all IHC techniques is the antibody, which can be divided according to clonality: antibodies are either polyclonal or monoclonal. Polyclonal antibodies are directed against one antigen, but may bind different epitopes. This type of antibody is generated by different B-cell clones in an animal; hence they have slightly different specificities and affinities. On the other hand, monoclonal antibodies are homogeneous populations of immunoglobulins which are directed against one epitope. They are generated by a single B-cell clone from one animal and are therefore immunochemically similar. Polyclonal antibodies are more robust in antigen binding because they can identify several epitopes of the antigen and consequently false negative results are infrequent. However, monoclonal antibodies are more specific, and false positive results are less frequent.

In this study, the expression of three proteins (FOXP3, CD4, and CD8) was evaluated. For this purpose, we used a Dako Autostainer Link 48, which is a compact, bench-top, open system that allows decoupled pre-treatment, and in which up to 48 IHC slides can be run at the same time. The Dako EnVision™ FLEX detection system was used, which consists of a dextran backbone to which a large number of horseradish peroxidase (HRP) molecules and secondary antibody molecules have been coupled. This system allows the detection of mouse monoclonal antibodies and rabbit polyclonal antibodies.

2.3.1. IMMUNOHISTOCHEMISTRY PROTOCOL

One 5µm section on Dako charged slides was analysed for each sample and antibody. The protocol, which is explained below, was performed with three different antibodies (detailed in Table 2). First, the sections were dried at 60° C for 30 minutes prior to IHC and loaded into the PT Link instrument where the antigen retrieval/dewaxing process took place. The protocol comprised the following steps: temperature increase to 97° C over a period of 10 minutes; incubation at 97° C for 20 minutes with high pH EnVision™ FLEX Target Retrieval Solution (Dako); cooling for an additional 10 minutes; and, finally, section transfer to an

Autostainer Link 48 instrument. The immunostaining was done with Dako FLEX Ready to-Use format for CD4 and CD8, and with a primary antibody diluted at 1:300 with Dako Antibody Diluent (Dako) for FOXP3. The protocol was the following: incubation with peroxidase blocking reagent for 10 minutes; incubation with the primary antibody for 20-30 minutes, incubation in the detection system for 20 minutes, and finally incubation with the chromogen (3,3'-diaminobenzidine, DAB) for 10 minutes. At the end of the run, the slides were flooded with distilled water and then manually counterstained with haematoxylin, dehydrated, and coverslipped.

Table 2. Characteristics of the antibodies and conditions used to detect each protein.

Antigen	Antibody type	Company and clone	Incubation time
FOXP3	Monoclonal Mouse	Abcam, 236A/E7	30 minutes
CD4	Monoclonal Mouse	Dako, 4B12	20 minutes
CD8	Monoclonal Mouse	Dako, C8/144B	20 minutes

2.3.2. IMMUNOHISTOCHEMISTRY CONTROLS

As in many other biological experiments, IHC requires positive and negative controls to assess the experimental quality. Each run should include at least a positive and a negative control. The positive control is usually a section of tissue which expresses the antigen, which is fixed and processed in a similar manner to the samples analysed. The negative control refers to two possible options: a section treated in the same way as the samples analysed but omitting the primary antibody, or a section of a tissue that it is known not to express the antigen.

In this study we included two controls in each run. Human normal tonsil tissue was used as a positive control for the three antibodies, as recommended on the data sheets. Controls were fixed and processed in the same way as the rest of the samples. As a negative control, one of the samples included in the run was duplicated, and one, but not the other, was incubated with the primary antibody.

2.3.3. MICROSCOPE EVALUATION AND SCORING

The slides were independently examined by one experienced pathologist and one investigator, both blinded to the case. Before initiating the scoring, controls were reviewed for quality assurance; the compartments for scoring, as well as the semiquantitative scale to be used, were defined. First, each sample was semiquantitatively scored for the degree of

immune-cell infiltration into the tumour nest and tumour-associated stroma (0 = negative, 1 = weak, 2 = intermediate, and 3 = strong).

To evaluate FOXP3 immunostaining, the percentage of nuclear-stained lymphocytes present in tumour and stroma was defined and graded on a scale of 0-3 according to the percentage of positive lymphocyte cells: 0 = no staining, 1 = less than 10% positive, 2 = 10-33% positive, 3 = 33% or more positive. Furthermore, in 10 tumour and stroma area high power fields (HPFs; magnification = X400) the absolute number of $FOXP3^+$ lymphocytes was determined and then averaged. $CD4^+$ and $CD8^+$ lymphocytes were also counted in 10 HPFs for both tumour and stroma areas and were then averaged.

3. MOLECULAR BIOLOGY METHODS

RNA was isolated from fresh-frozen tissue, samples isolated from FFPE slides, and peripheral blood in order to further perform gene expression analyses. Additionally, genomic DNA was also isolated to assess the most common mutations in lung cancer patients.

3.1. NUCLEIC ACID ISOLATION

3.1.1. RNA AND DNA ISOLATION FROM FRESH-FROZEN TISSUE SAMPLES

RNA and DNA were isolated from 178 tumoural and normal tissue samples using Tri Reagent® (Invitrogen, USA) according to the manufacturer's instructions. Briefly, a piece of 10-20 mg of tissue was dissected and 1 ml of Tri Reagent® was added. Samples were homogenised using TissueLyser (Qiagen) and chloroform was added in order to separate the aqueous phase containing the RNA. Isopropanol was used to precipitate the nucleic acids and ethanol was used for washing. Messenger RNA was redissolved in nuclease free (NF) water and stored at -80° C until further analysis. The DNA interphase was recollected in absolute ethanol and was washed first with buffer (10% ethanol/0.1 M sodium citrate) and then with 75% ethanol. It was redissolved in NF water and stored at -80° C until further analysis.

RNA and DNA quantification was performed using a nano-spectrophotometer (Nano Drop 2000C, Thermo Fisher Scientific, USA). RNA integrity and size was assessed using an Agilent RNA 600 Nano Bioanalyzer with a microfluidics-based platform (Agilent Technologies, USA).

3.1.2. RNA ISOLATION FROM MICRODISSECTED FORMALIN-FIXED PARAFFIN-EMBEDDED SAMPLES

RNA was isolated from the tumour and stroma areas microdissected from FFPE sections using the RecoverAll™ Total Nucleic Acid Isolation Kit (Qiagen) following the manufacturer's recommendations. Briefly, samples were incubated with the digestion buffer for 3-4 hours at 50° C and then for 15 minutes at 80° C. RNA was purified using ethanol and the buffers provided with a glass fibre filter column, followed by DNase treatment for 30 minutes at room temperature. RNA was eluted into NF water that had been previously heated to 50° C. Finally, the elution was incubated at 70° C for 20 minutes in order to revert the cross-linking produced by the formaldehyde. As described above, RNA quantification was performed by nano-spectrophotometry and the samples were stored at -80° C until further analysis.

3.1.3. RNA ISOLATION FROM PERIPHERAL BLOOD

Peripheral blood was collected in PAXgene™ Blood RNA (PreAnalytiX) tubes. RNA isolation from these samples was carried out using the PAXgene™ Blood RNA kit following the manufacturer's recommendations. Briefly, the purification began with a centrifugation step to pellet the nucleic acids in the tube. The pellet was then washed and redissolved in NF water followed by proteinase k incubation. Ethanol was added to adjust the binding conditions, and the lysate was applied to a PAXgene RNA spin column. This column was centrifuged so the RNA could selectively bind to a silica membrane and allowing efficient subsequent washing. The membrane was also treated with DNase I to remove any bound DNA, and finally the RNA was eluted into a buffer and heat-denatured. Again, the RNA was quantified using nano-spectrophotometry and stored at -80° C until further analysis.

3.2. DETECTION OF EGFR MUTATIONS

The theraScreen® EGFR RGQ PCR (Qiagen) kit was used to analyse the *EGFR* mutations. This kit allows 29 somatic mutations in the *EGFR* gene to be detected in exons 18-21 (Table 3) by RTqPCR combined with ARMS® and Scorpions® technology. This method is highly selective and, depending on the total amount of DNA present, enables detection of a low percentage of the mutant gene in a background of wild-type genomic DNA.

Table 3. EGFR mutations detected using theraScreen kit.

EXON	MUTATION
19	19 deletions*
20	T790M
21	L858R
21	L861Q
18	G719X (G719S, G719A and G719C)*
20	S768I
20	3 insertions *

* The kit does not distinguish between them.

Each reaction was performed in 96-well plates with a reaction volume of 12.5 µl comprising 9.75 µl reaction mix (primers, probes, dNTPs and reaction buffer containing Cl_2Mg), 0.25 µl Taq DNA Polymerase, and 2.5 µl DNA (2-10 ng/µl) isolated from fresh-frozen lung cancer specimens. A positive and negative control (provided by the manufacturer) was included in each run. The reaction was performed using a LightCycler 480 II (Roche, Switzerland) thermocycler following the conditions in Table 4.

Table 4. TheraScreen® EGFR RGQ PCR cycling parameters.

Cycles	Time	Temperature
1	15 minutes	95° C
40	30 seconds	95° C
	60 seconds	60° C

3.3. DETECTION OF KRAS MUTATIONS

The analysis of KRAS mutations was carried out using a theraScreen® KRAS Pyro® kit (Qiagen). This kit is used for quantitative detection of mutations in codons 12, 13, and 61 of the human *KRAS* gene by pyrosequencing. Codons 12/13 and codon 61 were amplified by PCR using 5 µl of template DNA (10 ng of genomic DNA), 12.5 µl of PyroMark® PCR Master Mix 2x, 2.5 µl of Coral Load Concentrate 10x, 4 µl of NF water, and 1 µl of KRAS 12/13 or 61 PCR primers. The reactions took place in a MasterCycler® thermocycler (Eppendorf, Germany) following the conditions described in Table 5.

The amplicons were immobilised on Streptavidin Sepharose® High Performance beads to prepare the single-stranded DNA and anneal the sequencing primers to it using a PyroMark Q24 plate and a vacuum workstation. PyroMark Gold Q24 reagents (enzyme mixture, substrate mixture, and nucleotides) were then prepared and loaded into a cartridge so they could be dispensed during the sequencing process. Finally, the plate and the cartridge were loaded into the PyroMark Q24 System and the sequencing process was started. The sequences were

analysed using software provided by the manufacturer. In each run, two controls were included: unmethylated control DNA which worked as a positive control for PCR and sequencing reactions, and a negative control (without template DNA).

Table 5. TheraScreen® KRAS Pyro® PCR cycling parameters.

Phases	Time	Temperature
Initial activation step	15 minutes	95° C
3-step cycling:		
Denaturation	20 seconds	95° C
Annealing	30 seconds	53° C
Extension	20 seconds	72° C
Number of cycles	42	
Final extension	5 minutes	72° C

This kit allowed the most frequent mutations at codons 12, 13, and 61 to be detected. The nucleotide dispensation order for sequencing codons 12/13 was *TACGACTCAGATCGTAG,* and for codon 61 it was *GCTCAGTCAGACT.* The analysis sequence for codons 12/13 was *GNTGRCGTAGGC*, which allowed the most frequent mutations in codon 12, nucleotide 35 (second position) to be detected. To analyse if the mutation was present in nucleotide 34 (first position), the analysis sequence was changed into *NGTGRCGTAGGC.* In the case of exon 61, the analysis sequence was *CTCDTGACCTG*, which represents the most frequent mutation in this codon, detected in nucleotide 183 (third position). To analyse if the mutation was present in nucleotide 182 (second position) the analysis sequence was changed into *CTCTHGACCTG*, and to analyse if it was present in nucleotide 181 (first position), the sequence was *CTCTTSACCTG.* The expected histograms for each sequence are represented in Figure 13.

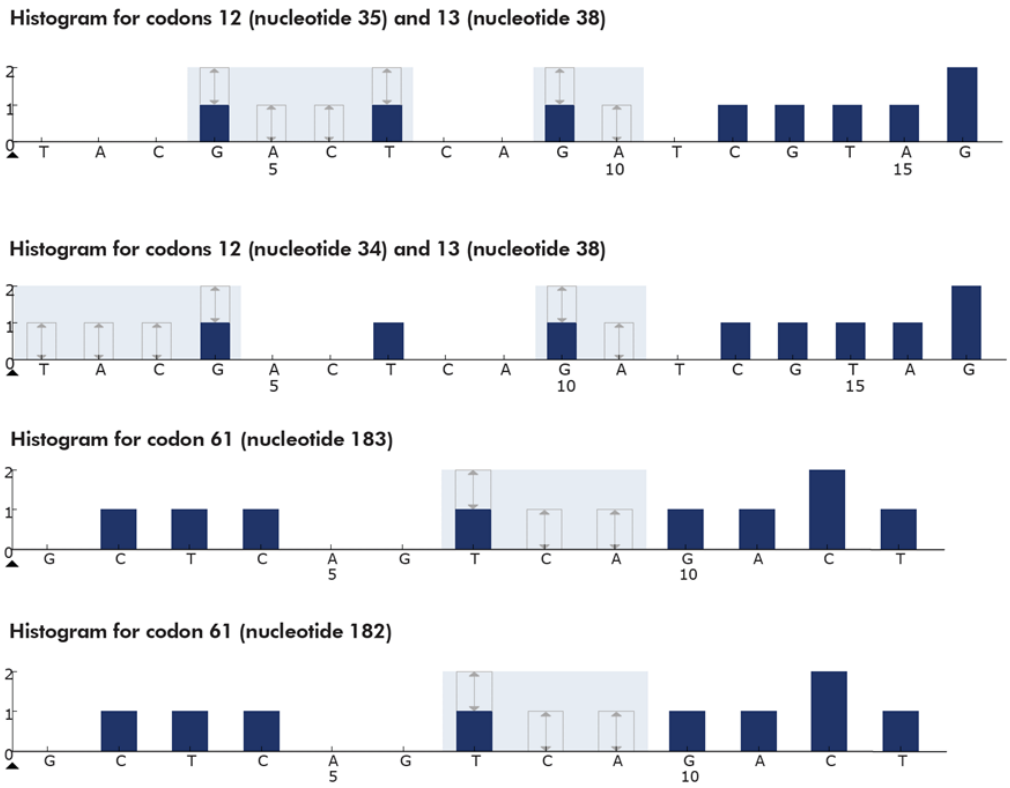


Figure 13. Representation of the histograms for the codons and nucleotides according to the sequences for analysis.

3.4. REVERSE TRANSCRIPTION

Reverse transcription was performed in order to transform RNA into complementary DNA (cDNA), which was required for the subsequent analyses, by using a High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems). The total quantity of RNA included in each reaction depended on the initial samples and isolated RNA: from fresh-frozen tissue the RNA input was of 1000 ng, from microdissected samples it was 100 ng, and from peripheral blood it was 500 ng. Each reaction comprised 2 µl of reverse transcription (RT) buffer, 0.8 µl of dNTP mix, 2 µl of RT random primers, 1µl MultiScribe™ Reverse Transcriptase, 1µl RNase inhibitor, and RNA, made up to a total volume of 20 µl with NF water. The reactions took place in a MasterCycler® thermocycler (Eppendorf) following the conditions described in Table 6 and the resulting cDNA was stored at -80° C until further analysis.

Table 6. Cycling program for reverse transcription reaction.

Phase	Time	Temperature
1	10 minutes	25° C
2	2 hours	37° C
2	5 seconds	85° C

3.5. PREAMPLIFICATION

Preamplification was performed using a TaqMan® PreAmp Master Mix Kit to increase the quantity of specific cDNA targets. This was necessary due to the low initial quantity of RNA obtained from microdissected samples and the large number of genes to be analysed. This kit enables multiplex preamplification of up to 100 targets at a time and provides unbiased amplification. First, a TaqMan assay pool was prepared by mixing 20x TaqMan® Gene Expression Assay mix for the genes of interest which were then diluted with 1x Tris-EDTA (TE) buffer so that each assay was at a final concentration of 0.2x. Each reaction comprised: 20µl of Master Mix PreAmp 2x, 10 µl of assay pool, and 10 µl of sample. Ten preamplification cycles were performed and the reaction took place in the MasterCycler® thermocycler (Eppendorf) following the cycling conditions described in Table 7. After preamplification, samples were diluted 1:5 with 1x TE buffer.

The uniformity of the preamplification process was assessed in order to check whether all the amplicons were amplified uniformly without bias, using preamplified human reference cDNA (Clontech®, USA) preamplified and at a concentration of 0.3 ng/µl. A conventional RTqPCR run was carried out and used for relative quantification to determine their ΔΔCt values between 0.3 ng/µl of cDNA and the preamplified cDNA as follows:

1. ΔCT (cDNA) = avg. CT (Target X) – avg. CT (Uniformity ref. gene)

2. ΔCT (Preamp) = avg. CT (Target X) – avg. CT (Uniformity ref. gene)

3. ΔΔCT = ΔCT (Preamp) – ΔCT (cDNA)

A ΔΔCT value close to zero indicates adequate preamplification uniformity. Typically, 90% of targets produce ΔΔCT values within ± 1.5 of zero.

Table 7. Cycling program for preamplification reaction.

Phases	Time	Temperature
Enzyme activation	10 minutes	95° C
Preamplification PCR (10 cycles)		
Denature	15 seconds	95° C
Anneal/Extend	4 minutes	60° C

3.6. QUANTITATIVE REAL TIME PCR

Target gene quantification was performed by RTqPCR using hydrolysis probes labelled with a reporter dye linked to the 5' end of the probe (TaqMan®, Applied Biosystems). This system also includes a non-fluorescent quencher (NFQ) at the 3' end of the probe and a Minor Grove Binder (MGB) attached to the NFQ which increases the melting temperature (Tm) without increasing the length of the probe (Figure 14).

In this study we analysed a total of 46 genes that were selected according to their relevance to the biology of tumour immunoregulation (Table 8). The relevance of these genes was established from a PubMed database search, which revealed published information demonstrating or suggesting a role for these genes in normal immune reaction mechanisms against tumours, co-stimulatory pathway modulation (also known as immune checkpoints), induction and attraction of suppressor cells (such as MDSCs, TAMs, and other APCs), and Tregs.

Gene expression levels were assessed using TaqMan® Gene Expression Assays (Applied Biosystems) which are described in Table 8. Different endogenous gene controls (Table 9) were tested in fresh-frozen, microdissected, and peripheral blood samples in order to evaluate the best internal control for each case using GeNorm software. This software automatically calculates the gene-stability measurement 'M' for all control genes and allows the worst-scoring housekeeping genes to be eliminated (Vandesompele et al., 2002).

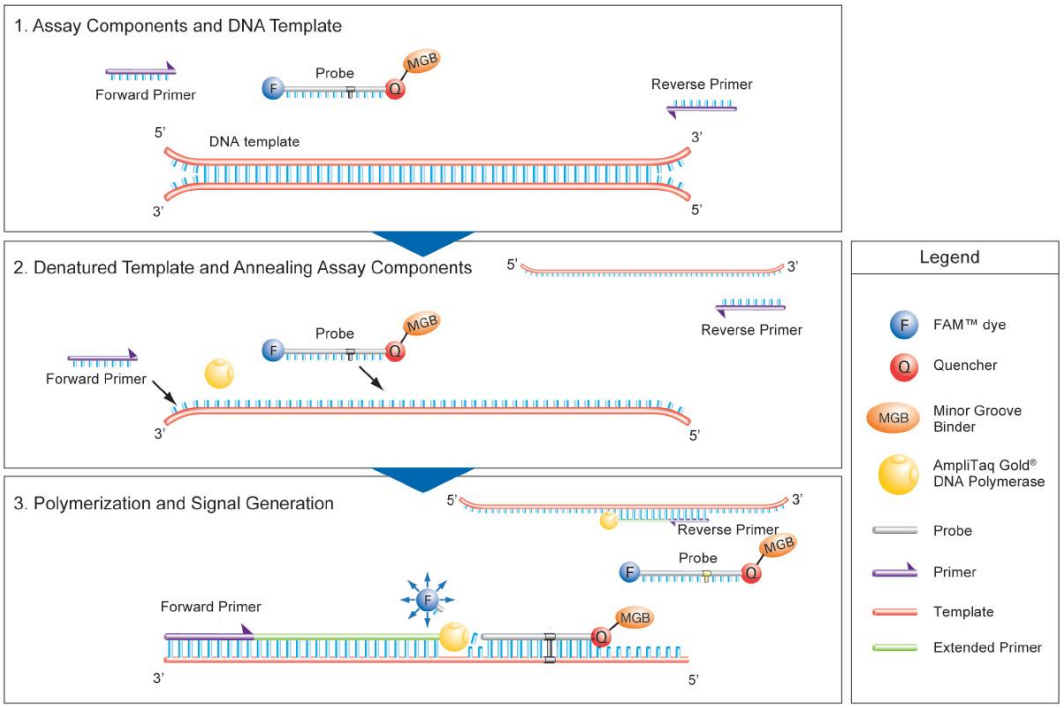


Figure 14. TaqMan® qPCR reaction steps (figure reproduced from Life Technologies).

Table 8. Genes analysed in this study, their description and TaqMan® assays used for RTqPCR.

Gen	Description	Assay	Amplicon length
CCL2	Chemokine ligand 2	Hs00234140_m1	101
CCL22	Chemokine ligand 22	Hs99999075_m1	65
CCL5	Chemokine ligand 5	Hs00174575_m1	63
CD1C	CD1c molecule	Hs00233509_m1	71
CD209	CD209 molecule	Hs00253550_m1	83
CD33	CD33 molecule	Hs01076284_m1	79
CD34	CD34 molecule	Hs00156373_m1	63
CD4	CD4 molecule	Hs00181217_m1	60
CD40	CD40 molecule	Hs00386850_m1	64
CD40LG	CD40 ligand	Hs00163934_m1	81
CD44	CD44 molecule	Hs01075861_m1	70
CD80	CD80 molecule	Hs99999103_m1	70
CD86	CD86 molecule	Hs01567025_m1	74
CD8	CD8A molecule	Hs00233520_m1	58
CD97 (TM7LN1)	C97 molecule	Hs00173542_m1	72
CLEC4C	C-type lectin domain family 4, member C	Hs01092462_m1	72
CSF1R (CD115)	Colony stimulating factor 1 receptor	Hs00911250_m1	74
CSF3R (CD114)	Colony stimulating factor 3 receptor (granulocyte)	Hs01114427_m1	83
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	Hs01011591_m1	79
CXCL12 (SDF-1)	Chemokine ligand 12	Hs00171022_m1	77
CXCR4	Chemokine receptor 4	Hs00237052_m1	78
FOXP3	Forkhead box P3	Hs00203958_m1	64
IDO1	Indolamine-1	Hs00984148_m1	66
IFNG	Interferon gamma	Hs00989291_m1	73
IL10	Interleukin 10	Hs00961622_m1	74
IL12B	Interleukin 12B (p40)	Hs01011518_m1	72
IL13	Interleukin 13	Hs99999038_m1	68
IL23A	Interleukin 23A	Hs00413259_m1	53
IL2RA (CD25)	Interleukin 2 receptor alpha	Hs00166229_m1	67
IL4	Interleukin 4	Hs00174122_m1	70
IL4R	Interleukin 4 receptor alpha	Hs00166237_m1	70
IL7RA (CD127)	Interleukin 7 receptor alpha	Hs00233682_m1	68
IL8 (CXCL8)	Interleukin 8	Hs99999034_m1	81
ITGAM (CD11b)	Integrin, alpha M (complement component 3 receptor 3 subunit)	Hs00355885_m1	60
ITGB2 (CD18)	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	Hs00164957_m1	76
LAG3	Lymphocyte-activation gene 3	Hs00158563_m1	60
LGALS1 (Galectina-1)	Lectin, galactoside-binding, soluble, 1	Hs00355202_m1	63
LGALS2 (Galectina-2)	Lectin, galactoside-binding, soluble, 2	Hs00197810_m1	73
MMP2	Matrix metallopeptidase 2	Hs01548727_m1	65
NRP1	Neuropilin 1	Hs00826125_m1	60
PD1 (PDC1)	Programmed cell death 1	Hs01550088_m1	127

PDL1 (CD274)	Programmed cell death 1 ligand	Hs01125301_m1	89
TGFB1	Transforming growth factor, beta 1	Hs00171257_m1	63
THBD	Thrombomodulin	Hs00264920_s1	91
TNF	Tumour necrosis factor	Hs00174128_m1	80
TNFRSF18 (GITR)	Tumour necrosis factor receptor superfamily, member 18	Hs00188346_m1	76

Table 9. Endogenous gene TaqMan® assays used to normalisation the results.

Gen	UniGene	Description	Assay	Amplicon length
ACTB	Hs.520640	Actin, beta	Hs01060665_g1	63
GAPDH	Hs.544577	Glyceraldehyde-3-phosphate dehydrogenase	Hs03929097_g1	58
GUSB	Hs.255230	Glucuronidase, beta	Hs01558067_m1	71
HPRT1	Hs.412707	Hypoxanthine phosphoribosyltransferase 1	Hs01003267_m1	72
CDKN1B	Hs.238990	Cyclin-dependent kinase inhibitor	Hs00153277_m1	71

Each reaction was performed twice in 384-well plates with a final volume of 5 µl comprising: 2.5 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems), 1.25 of NF water, 0.25 µl of TaqMan® Gene Expression Assay mix (Applied Biosystems), and 1 µl of cDNA. Non-template controls (NTCs) were included in each run, as well as positive reference controls: the NCI-H23 cell line from NSCLC (ATCC Number: CRL-5800TM) and T lymphocyte Jurkat cells (ATCC Number: TIB-152TM), in addition to a commercially available reference cDNA (Clontech). The reactions took place in a 7900HT Fast Real-Time PCR thermocycler system (Applied Biosystems) following the cycling conditions described in Table 10.

The efficiency of each TaqMan® assay was evaluated by carrying out serial dilutions (50 ng/µl, 5 ng/µl, 0.5 ng/µl, 0.05 ng/µl, and 0.005 ng/µl) using the cDNA as a template. The efficiency was calculated by using the following equation: $E = 10^{-1/slope}$ and the results indicated that almost all the assays used were adequately efficient (Supplementary Table 1). However, the efficiency of IL4 and CLEC4C could not be assessed.

Table 10. Cycling program for RTqPCR.

	Step	Time	Temperature
Pre-PCR	UNG incubation	2 min	50° C
	Taq activation	10 min	95° C
PCR (40 cycles)	Denature	15 sec	95° C
	Anneal/Extend	1min	60° C

The step at 50° C is required for optimal UNG enzyme activity. The step at 95° C is required to activate the AmpliTaq Gold enzyme.

Relative gene expression levels were expressed as the ratio of target gene expression to reference gene expression by using the Pfaffl formula (Pfaffl, 2001). Relative quantification determines the changes in steady-state mRNA levels of a target gene across multiple samples and expresses it relative to the levels of control RNA. The expression is normalised against a reference gene, which is often a housekeeping gene.

3.7. DATA ANALYSIS

Non-supervised hierarchical analysis was carried out with Cluster software (version 3.0) and visualised with Tree View software version 1.0.6 which can be found at http://rana.lbl.gov/EisenSoftware.htm (Eisen et al., 1998). All analyses were carried out on normalised and log2-transformed dataset values. Uncentered correlation was used as the similarity metric and average linkage was used as the clustering method.

First, we evaluated if the variables followed a normal distribution by using the Kolmogorov-Smirnov test. Because the variables did not follow a normal distribution, statistical analyses were conducted by nonparametric tests. Continuous variables were compared using non-parametric Mann Whitney U and Kruskall Wallis tests. Spearman's rank was used to test for correlations between continuous variables, and associations between dichotomised variables were evaluated using the Chi-square test. In order to represent gene expression data in a heat-map we used Genesis software [Institute for Genomics and Bioinformatics, Gratz, Austria] (Sturn et al., 2002).

The performance of each parameter (i.e. its ability to predict the disease vs. control status) was evaluated using a receiver operating characteristic (ROC) curve, and the area under the curve (AUC) was measured. Differences between ROC curves were estimated according to De Long methods (DeLong et al., 1988). Confidence intervals for the AUC were calculated according to Hanley and McNeil (Hanley and McNeil, 1982). The best cut-off value was selected using the Youden index.

OS and PFS were calculated from the date of surgery to the end point of the study or to the last recorded follow-up and patient progression was assessed following the Response Evaluation Criteria in Solid Tumours (RECIST) criteria (Therasse et al., 2000). The survival analysis was performed using a univariate Cox regression method using clinicopathological variables and dichotomised gene expression markers. Survival curves were created using the

Kaplan-Meier method, and the statistical significance between survival curves was assessed using the log-rank test.

Combined markers were also analysed in order to find expression profiles with a prognostic value. The combinations were performed according to the biological functions of the genes analysed or the processes in which their products are involved. The markers were combined in order to interrogate their possible prognostic value, even though these markers did not significantly independently correlate with survival rates.

Furthermore, we also calculated the gene expression scores based on multi-gene signatures, which can provide more accurate predictions than a model, using single genes. For this purpose, we constructed a gene expression score using a method previously reported by Lossos et al. (Lossos et al., 2004). All the genes analysed were included, and expression values were introduced as continuous variables. First, univariate COX regression analysis was performed to identify which genes were moderately associated with OS, which were those that had a |Z-score| higher than 1.5 ($p < 0.13$). Z-scores are defined as the regression coefficient (b) obtained from univariate Cox regression, divided by their standard error. The selected genes were included in a multivariate model and regression coefficients from this model were multiplied by the gene expression values and summed to build the expression score. For multivariate regression models, missing values for genes were replaced with the average values (Schetter et al., 2009).

Finally, to assess the independent value of the tested biomarkers, a Cox proportional hazard model for multivariate analyses was used. All significant variables (both biomarker and clinicopathological markers) from the univariate analyses were entered into the multivariate analyses in a forward stepwise Cox regression analysis.

A $p < 0.05$ was considered statistically significant for all analyses. The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 (Chicago, IL). Finally, GraphPad Prism v5.0 (GraphPad Software Inc.) was used in order to build some of the graphics presented here.

IV. RESULTS & DISCUSSION

STUDY I: BIOMARKERS IN RESECTED NON-SMALL CELL LUNG CARCINOMAS

A) IMMUNOREGULATION GENE EXPRESSION BIOMARKERS

1. ANALYSIS IN FRESH-FROZEN SAMPLES

The immunoregulatory biomarkers expressed in NSCLC patient biopsies, based on mRNA expression levels, were analysed in the tumours and compared with normal lung tissue both obtained from surgeries.

1.1. PATIENT CHARACTERISTICS

This retrospective study included 178 patients with NSCLCs who underwent resection at *Consorcio Hospital General Universitario de Valencia*. Fresh frozen tumour and adjacent normal lung tissue specimens were used. The most relevant demographic and clinicopathological characteristics of the cohort are shown in Table 11. The median patient age was 65 years [range: 26-85], 86.5% were male, and 47.2% had SCCs. Moreover, 59% of the patients were diagnosed at stage I of the disease and 66.3% presented a PS of 0.

Table 11. Clinicopathological characteristics of the patients included in the study.

Characteristics	N	%
Age at surgery (median, range):	**65 [26-85]**	
Gender		
Male	154	86.5
Female	24	13.5
Stage		
I	105	59
II	35	19.7
IIIA	38	21.3
Histology		
SCC	84	47.2
ADC	74	41.6
Others	20	11.2
Performance Status		
0	118	66.3
1-2	60	33.7
Differentiation grade		
Poor	43	24.2
Moderate	77	43.3
Well	31	17.4
NS	27	15.2
Smoking Status		
Current	86	48.3
Former	72	40.4
Never	20	11.3

ADC, adenocarcinoma; SCC, squamous cell carcinoma; NS, not specified.

1.1.1. *EGFR* AND *KRAS* MUTATIONAL ANALYSIS

KRAS mutational analysis was performed in 173 patients; 151 (87.3%) were wild type and 22 (12.7%) presented one of the possible mutations. Most of the patients with mutations were male (77.3%) and had ADC histology (77.3%; Table 12). This is similar to other study cohorts, which have also reported that the percentages of mutations in the *KRAS* gene were higher in men and in ADC patients (Schmid et al., 2009; Tam et al., 2006). Moreover, the most frequent mutation was 12ASP, which was present in 36.4% of our cases (Table 13).

EGFR mutational analysis was performed in 64 patients; 54 (84.4%) were wild type and 10 (15.6%) presented a mutation. Most of the patients with these mutations were women (70%), with an ADC histology (90%), who had never been smokers (60%; Table 12). These percentages were also in agreement with previously reported data, including multicentric studies (Molina et al., 2008; Rosell et al., 2009). In our cohort, exon 21 was the most frequently mutated (60%), in particular the mutation L858R stood out; this is a single nucleotide point mutation leading to a single amino acid change from leucine to arginine in codon 858 (Table 13).

Table 12. Frequency of *EGFR* and *KRAS* mutations according to the patient characteristics.

	EGFR		KRAS	
Characteristics	**Analysed patients N (%)**	**Mutated patients N (%)**	**Analysed patients N (%)**	**Mutated patients N (%)**
Total	64	10	173	22
Gender				
Male	49 (76.6%)	3 (30%)	149 (86.1%)	17 (77.3%)
Female	15 (23.4%)	7 (70%)	24 (13.2%)	5 (22.7%)
Stage				
I	36 (56.3%)	7 (70%)	101 (58.4%)	16 (72.7%)
II	17 (26.6%)	1 (10%)	35 (20.2%)	5 (22.7%)
IIIA	11 (17.2%)	2 (20%)	37 (21.4%)	1 (4.5%)
Histology				
SCC	9 (14.1%)	1 (10%)	80 (46.2%)	3 (13.6%)
ADC	44 (68.6%)	9 (90%)	73 (42.2%)	17 (77.3%)
Others	11 (17.1%)	-	20 (11.6%)	2 (9.1%)
Age (years)				
≤ 65	37 (57.8%)	5 (50%)	93 (53.8%)	11 (50%)
> 65	27 (42.2%)	5 (50%)	80 (46.2%)	11 (50%)
Smoking Status				
Current	29 (45.3%)	2 (20%)	85 (49.1%)	8 (36.4%)
Former	21 (32.8%)	2 (20%)	68 (39.3%)	7 (31.8%)
Never	14 (21.9%)	6 (60%)	20 (11.6%)	7 (31.8%)

ADC, adenocarcinoma; SCC, squamous cell carcinoma; EGFR, epidermal growth factor receptor.

Table 13. Frequency of the different mutations detected for EGFR and KRAS.

Type of mutation	N	%
EGFR		
Del. 19	1	10
Exon 20	3	30
Exon 21	6	60
KRAS		
12ASP	8	36.4
12CYS	6	27.3
12VAL	7	31.8
12SER	1	4.5

EGFR, epidermal growth factor receptor.

1.2. GENE EXPRESSION ANALYSIS

1.2.1. RNA QUANTIFICATION AND QUALITY ASSESSMENT

For relative gene expression determination 178 samples from tumour and adjacent normal lung tissues were analysed. RNA was isolated from small pieces of tissue (approximately 10-20 mg). An optimal RNA concentration was obtained from all the samples; the median for normal adjacent tissues was 385 [76.8 - 3642] ng/µl and was 1340 [92-5779] ng/µl for tumour tissues.

The quality and integrity of the RNA extracted was evaluated by capillary electrophoresis which is widely used for detecting the presence or absence of RNA degradation products. Depending on the entire electrophoretic trace of the RNA sample this method provides an RNA integrity number (RIN) which is calculated by a software algorithm. All the samples presented an adequate quality and integrity because their RIN was superior to 7, the 18S and 28S peaks were clear and defined, and there was low molecular-weight noise (Figure 15).

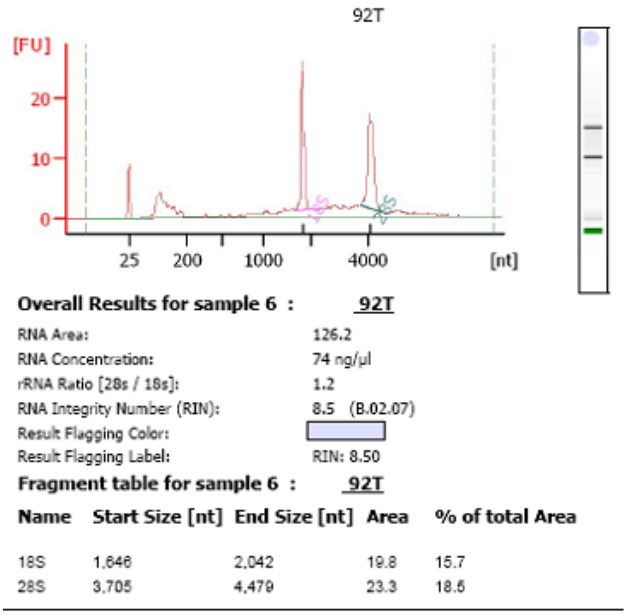


Figure 15. Example of an electropherogram provided by Agilent software after a capillary electrophoresis run. This figure represents a good-quality tumour sample electropherogram in which the 18S and 28S peaks are clear and defined, there is low molecular-weight noise, and the RNA integrity number (RIN) is over 7.

1.2.2. RELATIVE IMMUNOREGULATORY GENE MESSENGER RNA EXPRESSION

Relative gene expression analysis of genes of relevance to tumour immunoregulation was performed in this part of the study. The 20 genes were: CCL2, CCL22, CD1C, CD127, CD209, CD25, CD4, CD8, CLEC4, CTLA4, FOXP3, IDO1, IL10, IL23A, LGALS1, LGALS2, NRP1, PD1, PDL1, and TGFB1. Relative gene expression levels were based on the expression of the 20 target genes analysed in tumour tissues versus their expression in normal adjacent tissue; these were normalised against endogenous genes and were calculated using the Pfaffl mathematical formula.

The expression of five endogenous genes (ACTB, GAPDH, GUSB, HPRT1, and CDKN1B) was tested in a subset comprising approximately 30% of the samples in order to establish the best internal control. For this purpose, we used GeNorm software (see materials and methods), which indicated that the most stable option was the ACTB, GUSB, and CDKN1B combination. Following the procedure proposed by Vandesompele et al., a normalisation factor based on the expression of these three endogenous genes was calculated using the geometric mean (Vandesompele et al., 2002).

A gene was considered to be overexpressed in tumour samples compared to normal tissue when the median of the relative gene expression or fold-change was above 2, and it was considered to be downregulated when the fold-change was below 0.5. Using this criteria, we found that FOXP3 (3.87X) and CD25 (2.66X) were overexpressed, whilst CD1C (0.42X), CD127 (0.40X), PDL1 (0.38X), and CCL2 (0.25X) were downregulated in the tumour. To better visualise these results, they were log2-normalised and their mean was represented (Figure 16).

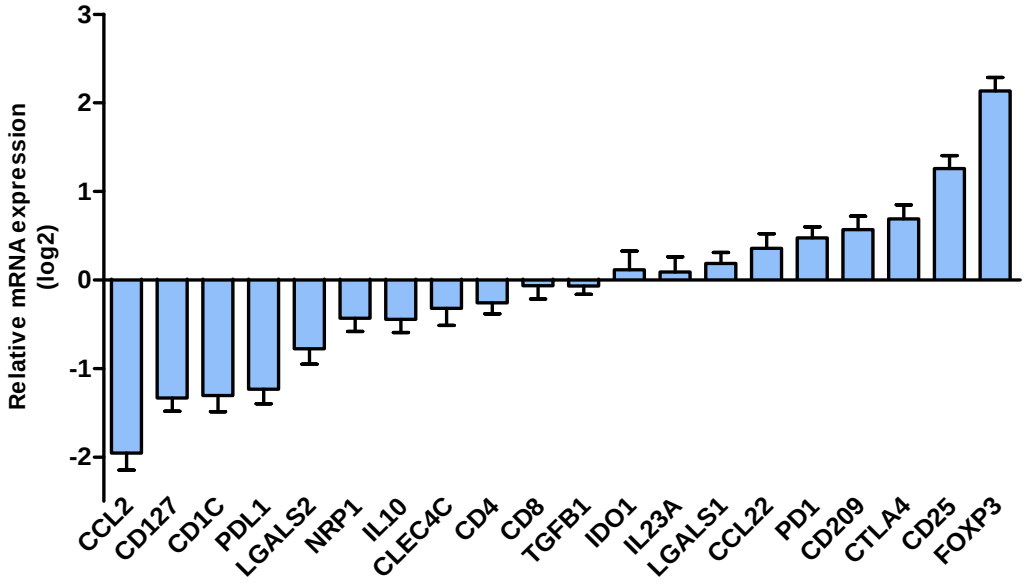


Figure 16. Relative mRNA expression levels of the 20 genes analysed. In this graphic the log2-tranformed relative gene expression mean is represented for each gene. The results represented are the mean ± SEM.

FOXP3 was found to be the most overexpressed gene. FOXP3 is a master Treg regulator and is one of the most reliable markers for the identification of these cells. However, FOXP3 expression is also transiently induced in $CD4^+$ and $CD8^+$ T cells upon stimulation (Roncador et al., 2005) and interestingly, its expression in tumour cells has been reported in different types of cancers (Triulzi et al., 2013). The overexpression of the non-selective biomarker CD25 in tumour compared to normal tissue could indicate strong infiltration by different types of immune cells. CD25 is the alpha chain of the IL2 receptor, which becomes rapidly expressed upon activation of conventional T cells, Tregs, and NK cells, as well as B cells and myeloid precursors (Becknell and Caligiuri, 2005).

On the other hand, some genes were found to be downregulated. One of these genes was CCL2, which encodes a chemokine that recruits and activates monocytes during inflammatory responses and which has been reported to induce prostate cancer cell

proliferation (Zhang et al., 2010). Other genes found to be downregulated were PDL1, which binds to PD1 and has a role in suppressing T cells (Zou and Chen, 2008); CD127, that stands for the interleukin-7 receptor-α and is expressed on various cell types, including naïve and memory T cells (Gregory et al., 2007); and CD1C, which is expressed on a subset of myeloid DCs (Dzionek et al., 2000).

1.2.3. UNSUPERVISED CLUSTERING ANALYSIS

Unsupervised hierarchical clustering analysis was used to group patients and genes based on the similarity of their expression patterns. Patients were classified into a cluster tree with two major subgroups: Cluster I (n = 78) and Cluster II (n = 76). Patients in Cluster I had lower expression levels of most of the genes analysed, whilst Cluster II comprised patients with higher gene expression levels (Figure 17).

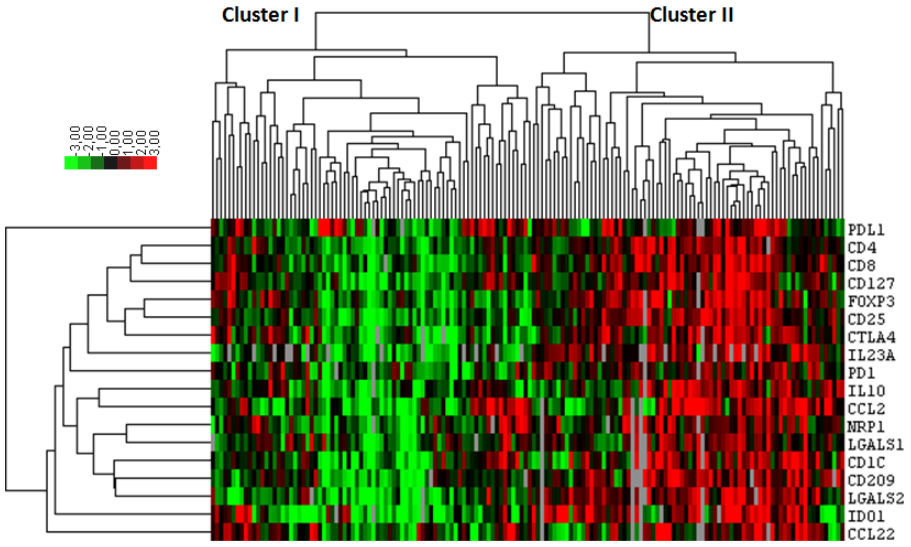


Figure 17. Hierarchical clustering based on gene expression. Patients in the original cohort were clustered into a hierarchical tree based on gene expression. The clustering analysis separated patients into two distinct groups. Red indicates high expression and green indicates low expression levels.

Genes were also grouped into two clusters: the first was mainly composed of genes related to conventional and regulatory T cells (CD4, CD8, CD127, FOXP3, CD25, and CTLA4) and the second comprising genes involved in different immunoregulatory processes (IL10, CCL2, NRP1, LGALS1, LGALS2, CD1C, and CD209). Three genes (PDL1, CCL22, and IDO1) did not fit into these two clusters so we decided to run a second clustering without them. In this new tree the patients, as well as the genes, were classified into two main clusters. In this case, in Cluster I (n = 70) most of the genes were downregulated, and in Cluster II (n = 84) most of the genes were overexpressed (Figure 18).

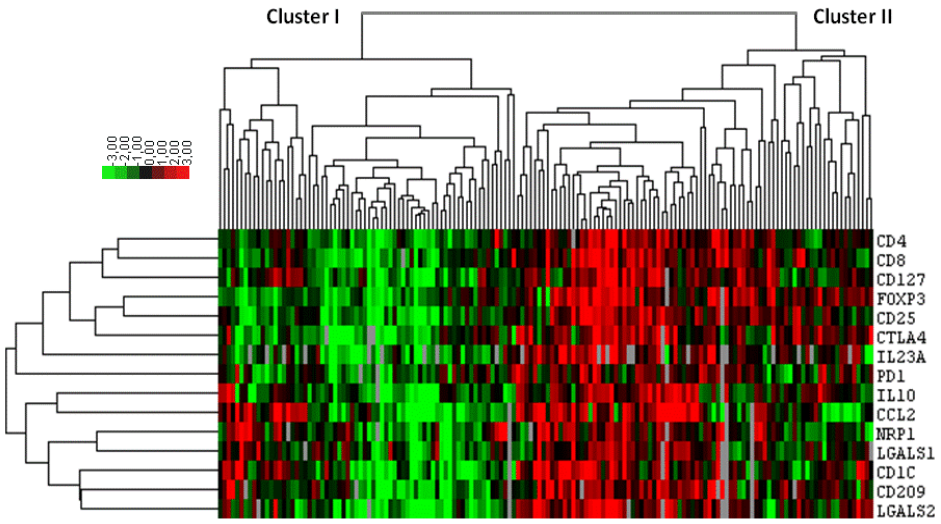


Figure 18. Hierarchical cluster based on selected gene expression. Patients in the original cohort were clustered into a hierarchical tree based on the expression of 15 genes. The clustering separated the patients into two distinct groups. Red indicates high expression and green indicates low expression levels.

Hierarchical clustering is a powerful bioinformatics method, which analyses multiple factors in order to classify tumours by their similarities. The strategy of unsupervised hierarchical patient classification according to the expression patterns of genes related to tumour immunology was previously reported for other types of tumours such as hepatocellular carcinoma (Gao et al., 2011) as well as in lung ADCs (Seike et al., 2007). In this study, a 15-cytokine gene expression profile in noncancerous lung tissue and corresponding lung tumour tissues from 80 lung ADC patients was analysed. These data were used to construct a classification algorithm based on the analysis of noncancerous and tumour tissues to predict the prognosis of lung ADC patients with stage I disease. Thus, the principal objective of this clustering analysis was to find groups of patients with specific characteristics, like for instance, those with specific histology or survival groups based on the mRNA levels detected in the tumour microenvironment.

1.3. CORRELATION OF BIOMARKERS WITH CLINICOPATHOLOGICAL VARIABLES

Non-parametric tests such as the Mann-Whitney U, Kruskal Wallis, and Chi-square tests were carried out in order to investigate the association of the immunoregulatory markers with clinicopathological characteristics.

The Mann-Whitney U test revealed significant differences between histology (SCC vs. non-squamous), and TGFB1 (p = 0.020) and NRP1 (p = 0.022) expression (Figure 19a). TGFB1 expression was higher in patients with SCC histology and, in the case of NRP1, the expression was higher in non-squamous patients. The first observation seems to be in line with a previous study reporting that TGFB1 plays a very important role in SCC carcinoma because it induces angiogenesis, inflammation, and epithelial-mesenchymal transition (EMT; Han and Wang, 2011). To the best of our knowledge no association between NRP1 and non-squamous histology has previously been reported.

Moreover, higher levels of PDL1 (p = 0.018) and LGALS2 (p = 0.023; Figure 19b) were found to be associated with the absence of lymph node involvement, whilst higher levels of FOXP3 (p = 0.045) and PD1 (p = 0.029) correlated with tumour size. These correlations could be explained by the fact that larger tumours attract a greater number of immune cells including cells expressing PD1, such as T lymphocytes and, in the case of FOXP3, Tregs. An association between *KRAS* status and FOXP3 (p = 0.007), IL10 (p = 0.030), PD1 (p = 0.028), PDL1 (p = 0.036), and IL23A (p = 0.029) was also observed, and the expression of these genes was higher in patients with a wild type (WT) *KRAS* phenotype.

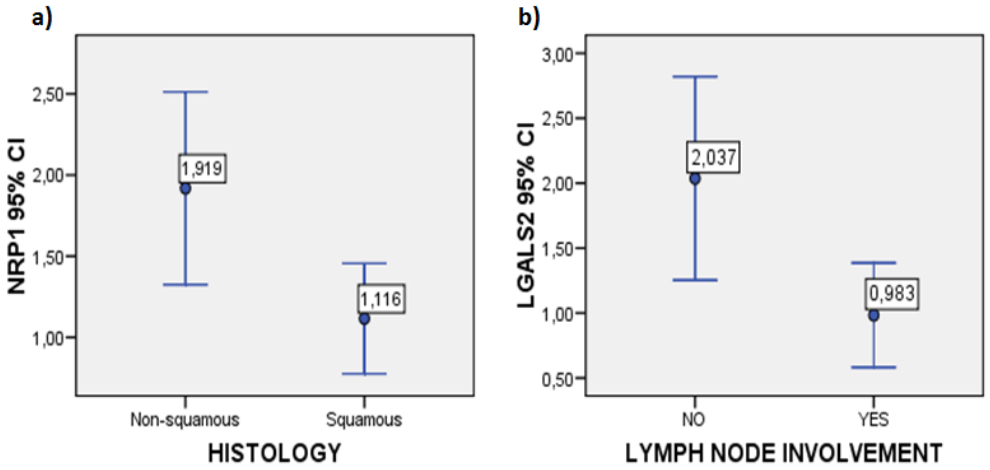


Figure 19. These figures represent two examples of correlation between clinicopathological variables and gene expression markers. a) Representation of NRP1 expression according to histology and, b) representation of LGALS2 according to lymph node involvement. Error bars represent the 95% confidence interval (CI) of the mean.

Gene expression levels were then dichotomised according to their medians into high and low expression levels, thus converting them into qualitative variables. Correlations between them and clinicopathological variables were then assessed using the Chi-square test. We found that LGALS2 correlates with lymph node involvement (p = 0.006; Figure 20a) and

that tumour size correlated with FOXP3 (p = 0.042), but in addition it also correlated with CD8 (p = 0.036) and CCL2 (p = 0.005; Figure 20b). Moreover, CD4 (p = 0.006), FOXP3 (p = 0.04), PD1 (p = 0.038), and IL23A (p = 0.006) correlated with *KRAS* status (Figure 20c).

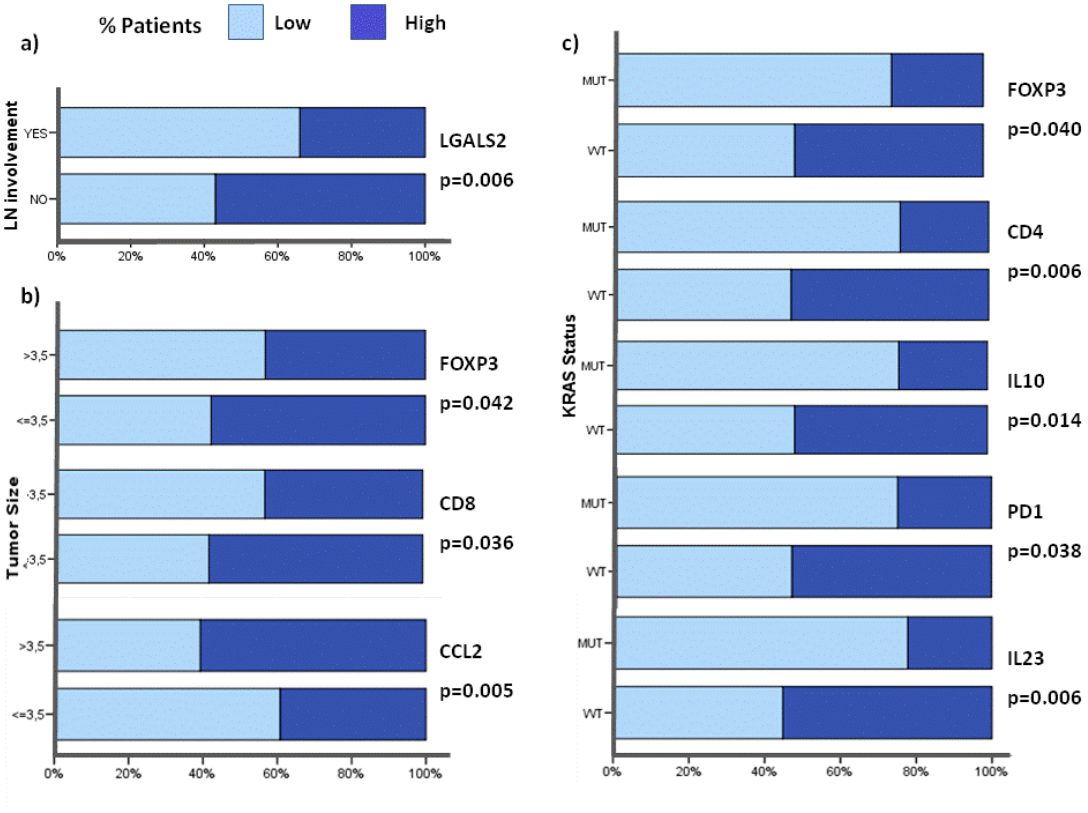


Figure 20. Histograms showing the associations between clinicopathological variables and gene expression markers. Statistically significant associations of dichotomised gene expression values with a) lymph node involvement, b) tumour size and c) *KRAS* status. Histograms show the percentage of patients. The *p*-value was obtained using the Chi-square test.

Chi-square test showed that there were significantly more patients with stage I (p = 0.017), absence of LN involvement (p = 0.016), and wild type KRAS phenotype (p = 0.011) in Cluster II than in Cluster I. These correlations may indicate that patients in Cluster II, which comprises patients expressing higher levels of tumour immune-related genes compared to normal tissue, presented a less aggressive type of NSCLC.

1.4. BIOMARKER SURVIVAL ANALYSIS

1.4.1. CLINICOPATHOLOGICAL VARIABLES

Of the 178 resected NSCLC patients included in this part of the study, 80 (45%) relapsed and 76 (42.7%) died during the follow-up. The median follow-up was of 81.23 months [Range: 1-113]. The prognostic value of the different clinicopathological variables was assessed using the univariate Cox regression method for OS and PFS, and are shown in Table 14 along with the hazard ratios and *p*-value for each variable.

Table 14. Results from survival analysis based on clinicopathological variables for the entire cohort.

	OS			PFS		
Variable	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	***p*-value**
Gender						
Male vs. Female	2.499	0.909-6.875	0.076	1.638	0.791-3.391	0.184
Age (years)						
>65 vs. ≤ 65	1.290	0.792-2.100	0.065	1.207	0.792-1.841	0.382
Stage						
II/IIIA vs. I	1.081	0.662-1.768	0.755	1.258	0.824-1.920	0.288
Histology						
ADC vs. SCC vs. Others	1.038	0.702-1.536	0.850	1.141	0.813-1.601	0.446
Tumour size						
>3.5 cm vs. ≤ 3.5 cm	1.503	0.910-2.484	0.111	1.478	0.962-2.271	0.075
LN involvement						
Yes vs. No	1.502	0.892-2.529	0.126	1.635	1.046-2.556	0.031*
PS						
1/2 vs. 0	1.405	0.829-2.382	0.206	1.457	0.924-2.296	0.105
Differentiation grade						
Poor vs. Well/Moderate	0.943	0.546-1.756	0.979	1.009	0.614-1.659	0.972
Smoking status						
Former/Current vs. Never	1.835	0.667-5.048	0.240	0.844	0.352-2.024	0.353
EGFR						
Wild type vs. Mutated	0.477	0.144-1.576	0.225	2.499	0.909-6.875	0.704
KRAS						
Mutated vs. Wild type	1.970	1.024-3.790	0.042*	1.886	1.038-3.429	0.037*

CI, confidence interval; EGFR; epidermal growth factor receptor; HR, hazard ratio; LN, lymph node; OS, overall survival; PFS, progression free survival; PS, performance status. The results were obtained using the univariate Cox regression method.*$p < 0.05$.

Significant results obtained from the univariate COX regression method were also analysed using the Kaplan-Meier method (long-rank) in order to obtain the survival plots. This univariate analysis of clinicopathological variables showed that patients with the *KRAS* mutation had a worse OS ($p = 0.038$) and a shorter PFS ($p = 0.034$; Figure 21), which agrees with previously published results (Meng et al., 2013). Also, as previously reported (Suzuki et

al., 2013), LN involvement was significantly associated with PFS (p = 0.029). No other clinicopathological variables appeared to have any prognostic value.

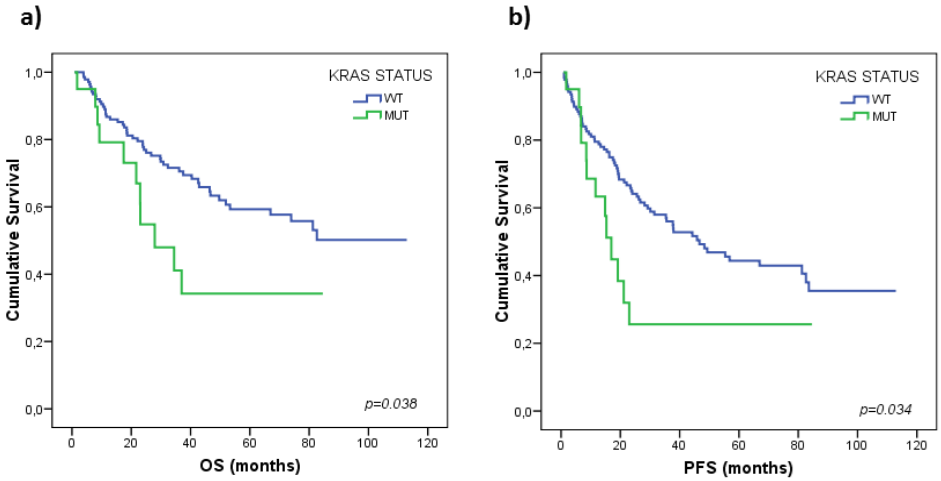


Figure 21. Kaplan-Meier plots according to KRAS status. a) Analysis for OS, and b) PFS**.** Blue line represents KRAS wild type patients, whilst green line represents mutated patients. *P*-values from the Kaplan-Meier test.

ANALYSIS ACCORDING TO HISTOLOGY

The prognostic value of clinicopathological variables was also assessed according to histology. The ADC subgroup comprised 78 patients (including ADCs and adenosquamous histology): of these, 33 (42.3%) relapsed and 33 (42.3%) died. In the univariate analysis, only LN involvement was associated with PFS (p = 0.038; Table 15). The SCC subgroup comprised 84 patients; 40 (47.6%) relapsed and 36 (42.9%) died. In contrast to the findings in ADC patients, no significant association was found between the clinicopathological variables and OS or PFS in SCC patients (data not shown).

Table 15. Results from survival analysis based on clinicopathological variables for ADC patients.

	OS			PFS		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Gender						
Male vs. Female	3.215	0.963-10.728	0.058	1.956	0.860 -4.449	0.110
Age (years)						
>65 vs. ≤ 65	1.619	0.741-3.537	0.227	1.205	0.636-2.284	0.567
Stage						
II/IIIA vs. I	0.967	0.430-2.174	0.935	1.265	0.653-2.451	0.487
Tumour size						
>3.5 cm vs. ≤ 3.5 cm	1.925	0.872-4.248	0.105	1.812	0.948-3.464	0.072
LN involvement						
Yes vs. No	1.786	0.707-4.511	0.220	2.428	1.153-5.113	0.020*
PS						
1/2 vs. 0	1.364	0.541-3.442	0.511	1.698	0.816-3.534	0.157
EGFR						
Wild type vs. Mutated	0.339	0.079-1.461	0.147	20.819	0.309-2.168	0.688
KRAS						
Mutated vs. Wild type	2.146	0.947-4.864	0.067	1.976	0.971-4.020	0.060

CI, confidence interval; HR, hazard ratio; LN, lymph node; OS, overall survival; PFS, progression free survival; PS, performance status; EGFR; epidermal growth factor receptor. The results were obtained using the univariate Cox regression method. *$p < 0.05$.

1.4.2. HIERARCHICAL CLUSTERS

We then carried out univariate Kaplan-Meier survival analysis using the two major clusters obtained from the second unsupervised clustering (where some genes were excluded). This showed that patients in Cluster II had better OS (not reached (NR) vs. 46.6 months, $p = 0.040$) and longer PFS (81.2 vs. 26.2 months, $p = 0.027$) than patients in Cluster I (Figure 22a-b). We also analysed their prognostic value according to histology, and observed that ADC patients classified in Cluster II had a significantly better OS (NR vs. 42.9 months, $p = 0.034$) and PFS (81.2 vs. 17.8 months, $p = 0.005$) than patients in Cluster I.

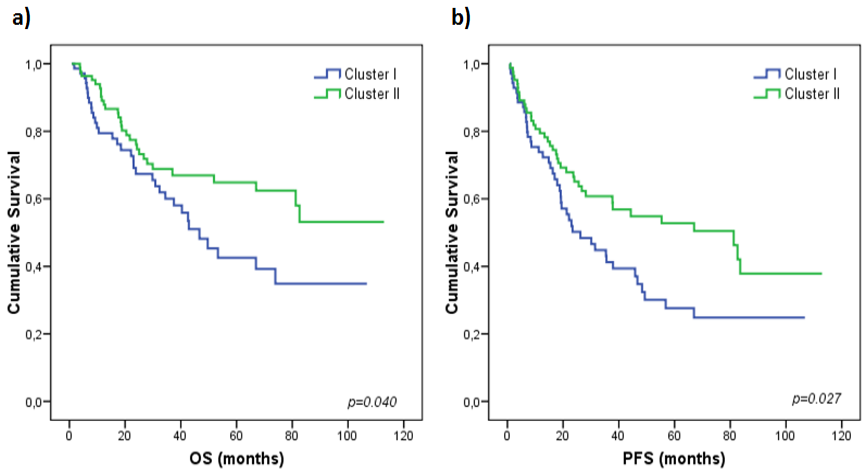


Figure 22. Kaplan-Meier plots for OS and PFS according to the clustering classification of patients. a) OS and, b) PFS. Blue line represents patients classified in Cluster I, whilst green line represents patients in Cluster II. *P*-values were calculated using the Kaplan-Meier test.

Molecular profiling based on mRNA microarrays or RTqPCR data has been extensively studied in lung tumour tissues. Some of these studies found significant associations between specific clusters of patients and their survival or metastasis (Beer et al., 2002; Bhattacharjee et al., 2001). Hierarchical clustering of immune-related genes performed in different types of cancer have demonstrated that clusters with an over-representation of genes related to cellular immunity are, in general, associated with a better outcome (Fehlker et al., 2014; Seike et al., 2007). In our study, unsupervised clustering analysis of immune-related genes in NSCLC samples indicated that the group of patients with the highest expression levels of immune-related genes had better outcomes than the other group, although most of these genes are involved in immunoregulatory processes. This might point towards a beneficial impact of an active immune response within the tumour microenvironment of NSCLCs.

This is not the first study to observe that patients with higher expression of genes related to immunoregulation have better survival rates. In fact, in breast cancer, a molecular signature (obtained from microarray data analysis) which was associated with relapse-free patients had a higher representation of genes involved in B cell development and antigen presentation, but also of genes involved in T-cell apoptosis, CTLA4 signalling, or activation of IL23R, which are all pathways involved in the negative regulation of effector T cells (Ascierto et al., 2012). In a recent study, the expression of immunosuppressive factors such as PD1, PDL1, CTLA4, and FOXP3 measured in 481 breast tumours, were highly significant predictors of therapy response and improved outcome (Denkert et al., 2014).

Although it is of interest that the unsupervised cluster analysis of immune-related genes was able to identify a group of patients with a better prognosis, hierarchical clustering can only be applied retrospectively and cannot be used to predict a patient's future outcome. Therefore, next we investigated the prognostic value of genes analysed individually or as small groups.

1.4.3. INDIVIDUAL BIOMARKERS

The prognostic value of the different immune biomarkers was assessed using the univariate Cox regression method for OS and PFS. Gene expression levels were dichotomised according to their median. Results obtained in the univariate analysis are shown in Table 16.

Table 16. Results from survival analysis based on gene expression biomarkers in the entire cohort.

	OS			PFS		
Variable	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	***p*-value**
FOXP3 *High vs. Low*	0.728	0.443-1.198	0.212	0.607	0.392-0.940	0.025*
CD4 *High vs. Low*	0.670	0.406-1.106	0.117	0.641	0.414-0.992	0.046*
CD8 *High vs. Low*	0.750	0.456-1.234	0.258	0.693	0.447-1.075	0.101
CD25 *High vs. Low*	0.584	0.353-0.967	0.036*	0.702	0.450-1.083	0.110
CD127 *High vs. Low*	0.700	0.425-1.153	0.161	0.543	0.349-0.844	0.007*
CTLA4 *High vs. Low*	0.639	0.385-1.060	0.083	0.655	0.421-1.018	0.060
IL10 *High vs. Low*	0.674	0.409-1.110	0.121	0.847	0.549-1.308	0.455
TGFB1 *High vs. Low*	1.177	0.715-1.937	0.521	1.096	0.711-1.690	0.677
PD1 *High vs. Low*	0.657	0.399-1.082	0.099	0.731	0.473-1.129	0.157
PDL1 *High vs. Low*	0.92	0.564-1.517	0.757	0.882	0.570-1.365	0.573
NRP1 *High vs. Low*	1.058	0.634-1.764	0.830	1.057	0.678-1.649	0.806
IL23A *High vs. Low*	0.390	0.215-0.708	0.002*	0.480	0.294-0.783	0.003*
CCL2 *High vs. Low*	0.801	0.483-1.329	0.391	0.794	0.512-1.232	0.304
CCL22 *High vs. Low*	0.957	0.574-1.595	0.867	0.966	0.619-1.507	0.879
CD1C *High vs. Low*	0.755	0.453-1.258	0.281	0.924	0.593-1.438	0.725
CD209 *High vs. Low*	0.684	0.409-1.144	0.148	0.931	0.598-1.448	0.750

IDO1						
High vs. Low	0.800	0.479-1.336	0.394	0.824	0.526-1.291	0.399
LGALS1						
High vs. Low	1.072	0.642-1.789	0.791	1.173	0.752-1.831	0.481
LGALS2						
High vs. Low	0.495	0.293-0.837	0.009*	0.485	0.306-0.768	0.002*
CLEC4						
High vs. Low	0.684	0.386-1.212	0.194	0.745	0.439-1.264	0.275

Gene expression levels dichotomised as high and low according to their medians. The results were obtained using the univariate Cox regression method. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; *$p < 0.05$.

Univariate Cox regression analysis found that high levels of IL23A were associated with OS [HR, 0.390; 95% CI, 0.215-0.708; $p = 0.002$] and PFS [HR, 0.480; 95% CI, 0.294-0.783; $p = 0.003$]. Kaplan-Meier analysis was carried out in order to obtain the survival plots and it indicated that patients with high levels of IL23A did not reach the survival median for OS ($p = 0.001$), and that the median PFS was higher than for the other group (81.2 vs. 23.4 months, $p = 0.003$; Figure 23a-b). IL23 is a heterodimeric cytokine composed of the p40 subunit, expressed by IL12 gene, and the p19 subunits, expressed by IL23A gene (Oppmann et al., 2000). In T cells, IL23 induces activation of STAT family members, but it is predominantly produced by activated myeloid dendritic cells and by type 1 macrophages (Parham et al., 2002). This cytokine is now considered the master switch in several T cell-mediated inflammatory disorders, but the antitumour activity of IL23 is controversial. On the one hand, it has been shown that pro-inflammatory cytokines, including IL17A, IL6, and IL23 can impair $CD8^+$ T cell-mediated immune surveillance and promote tumour neovascularisation (Langowski et al., 2006). But on the other hand, other groups have reported that IL23 exerts antitumour activity by stimulating T cells and natural killer (NK) cells (Kaiga et al., 2007). Its prognostic value was studied in ovarian cancer, and an improved OS was observed in patients with high p19 mRNA expression (expressed by the IL23A gene; Wolf et al., 2010). In lung cancer, a recent study in NSCLC tumour samples and cell lines reported that gemcitabine, a chemotherapy drug indicated for first-line treatment of NSCLC, induced IL23A expression and that it was found to induce NSCLC cell line proliferation. However, they failed to correlate IL23A expression with NSCLC patient prognosis (Baird et al., 2013).

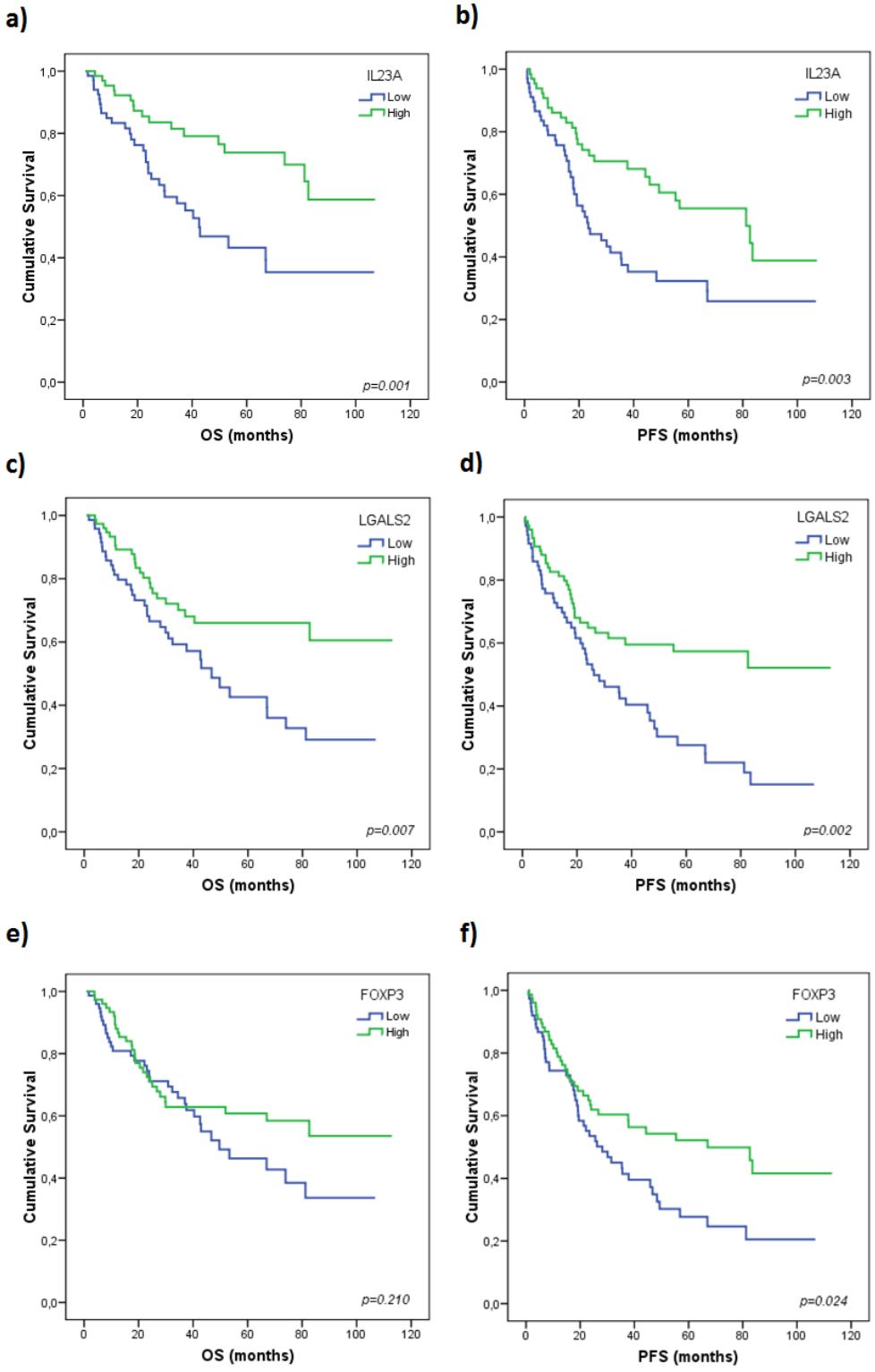
Another gene that correlated with better OS [HR, 0.495; 95% CI, 0.293-0.837; $p = 0.009$] and longer PFS [HR, 0.485; 95% CI, 0.306-0.768; $p = 0.002$] was LGALS2, which encodes galectin-2. The median survival for patients with high levels of LGALS2 was, again, not reached at the end of the study for either OS or PFS (Figure 23c-d). In contrast to galectin-1 and galectin-3, relatively few studies have examined the expression of galectin-2 in animals and

human tumours. Galectins are members of a highly conserved family of β-galactoside-binding lectins, which have a broad variety of functions including immune function regulation. The most extensively-studied galectin function is their regulation of apoptosis. Furthermore, galectin-1 functions as a soluble mediator used by tumour cells to evade the immune response (Liu and Rabinovich, 2005). Galectin-2 can induce T-cell apoptosis and control the secretion of lymphotoxin-α by macrophages (Ozaki et al., 2004). Similar to our findings that lower LGALS2 expression is associated with a worse outcome, in gastric cancer, it has been reported that decreased galectin-2 expression is associated with LN involvement and advanced clinical stage (Jung et al., 2012).

Furthermore, higher levels of FOXP3 were correlated with longer PFS [HR, 0.607; 95% CI, 0.392-0.940; p = 0.025] and, as it can be observed in the Kaplan-Meier plot, higher levels of FOXP3 were associated with a better prognosis (Figure 23e-f). This association seems to be discordant with previous work in which the presence of $FOXP3^+$ cells was associated with a poor prognosis in NSCLC (Petersen et al., 2006; Suzuki et al., 2013). However, our result could be explained by the fact that we are detecting mRNA expression levels from the tumour microenvironment, and therefore we do not know their origin. In fact, cancer cells positive for FOXP3 have been detected in several distinct types of cancer (Triulzi et al., 2013) and in breast cancer, its presence was associated with better OS (Ladoire et al., 2012). In lung cancer, a piece of work published in 2012 reported that FOXP3 expression in NSCLC tumour cells attenuated the unfavourable prognostic influence of the tumour-infiltrating Tregs also detected by IHC (Tao et al., 2012). Since we may be detecting FOXP3 mRNA produced by both Tregs and tumour cells, and the role of FOXP3 expression in the later is still unknown, this result should be interpreted with care.

Finally, high expression levels of CD4 [HR, 0.641; 95% CI, 0.414-0.992; p = 0.046] (Figure 23g-h) and CD127 [HR, 0.543; 95% CI, 0.349-0.844; p = 0.007] (Figure 23i-j) were also correlated with longer PFS. High expression levels of CD127, which codes for interleukin-7 receptor-α and is expressed on various cell types including naïve and memory T cells (Gregory et al., 2007), could indicate a high T cell infiltration into the tumour microenvironment, and therefore, could be a surrogate marker for an active anti-tumour response in this subgroup of patients. These results do not agree with a previous publication in which high IL7-R expression in tumours was associated with a worse outcome (Suzuki et al., 2013). This discrepancy could be explained by different reasons: 1) In the other study IL-7R expression was detected as positive IHC staining in tumour cells, whereas in our study we detected mRNA expression in

the tumour microenvironment, which could be enriched in mRNA from T cells as well as tumour cells; and 2) They only included stage I lung ADC patients, whereas we included patients with different histologies and stages.



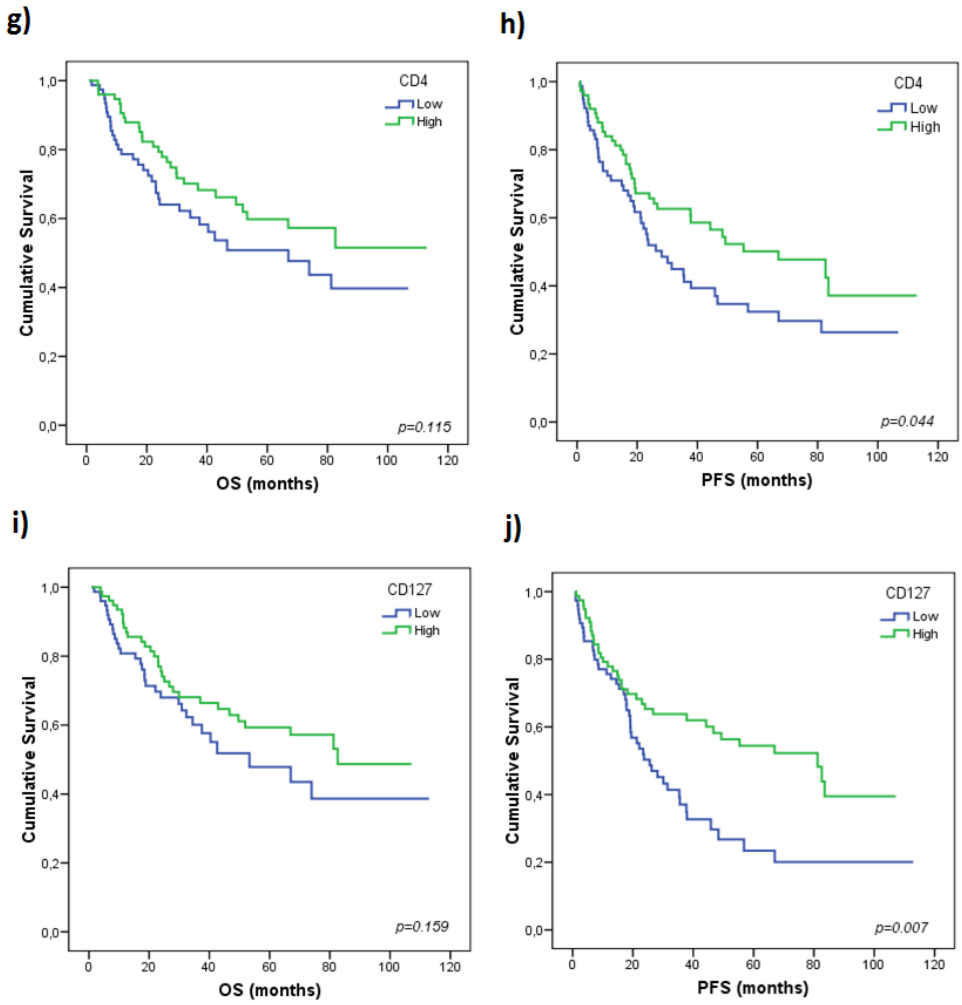


Figure 23. Kaplan-Meier plots for OS and PFS according to gene expression levels. a-b) IL23A; c-d) LGALS2; e-f) FOXP3; g-h) CD4, and i-j) CD127. Gene expression levels were dichotomised according to the median. Blue line represents patients with low levels of expression, whilst green line represent patients with high levels of expression. *P*-values were calculated using the Kaplan-Meier test.

ANALYSIS ACCORDING TO HISTOLOGY

NSCLC is one of the most genomically diverse tumours, and there are a variety of molecularly-defined subsets of patients. Different driver mutations have been identified in SCC and ADC histologies, which have led to the assumption that they are molecularly different diseases. For this reason, survival analysis was also performed according to the patient histology. Results from univariate COX regression analysis for ADCs are provided in Table 17. However, SCC results are not included because we did not find any significant correlation for this subgroup of patients.

Table 17. Results from survival analysis based on gene expression biomarkers for ADC patients.

	OS			PFS		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
FOXP3						
High vs. Low	0.609	0.268-1.383	0.236	0.510	0.257-1.008	0.053
CD4						
High vs. Low	0.848	0.385-1.866	0.682	0.760	0.366-1.363	0.300
CD8						
High vs. Low	0.735	0.333-1.21	0.445	0.674	0.344-1.318	0.249
CD25						
High vs. Low	0.419	0.180-979	0.045*	0.449	0.226-0.891	0.022*
CD127						
High vs. Low	0.859	0.387-1.907	0.709	0.574	0.293-1.127	0.107
CTLA4						
High vs. Low	0.336	0.143-0.790	0.012*	0.349	0.174-0.701	0.003*
IL10						
High vs. Low	0.414	0.182-0.941	0.035*	0.477	0.242-0.940	0.033*
TGFB1						
High vs. Low	1.056	0.478-2.335	0.893	1.107	0.570-2.152	0.763
PD1						
High vs. Low	0.575	0.248-1.335	0.198	0.489	0.242-0.988	0.046*
PDL1						
High vs. Low	0.698	0.308-1.582	0.389	0.672	0.332-1.360	0.269
NRP1						
High vs. Low	1.189	0.520-2.721	0.682	1.308	0.654-2.616	0.447
IL23A						
High vs. Low	0.281	0.091-0.866	0.027*	0.361	0.158-0.824	0.016*
CCL2						
High vs. Low	0.614	0.279-1.35	0.226	0.541	0.280-1.043	0.067
CCL22						
High vs. Low	0.943	0.428-2.076	0.884	0.910	0.467-1.774	0.783
CD1C						
High vs. Low	0.571	0.256-1.273	0.171	0.575	0.293-1.126	0.106
CD209						
High vs. Low	0.518	0.231-1.161	0.110	0.651	0.332-1.276	0.211
IDO1						
High vs. Low	0.446	0.196-1.016	0.055	0.539	0.269-1.077	0.080
LGALS1						
High vs. Low	0.864	0.642-1.789	0.716	0.9193	0.472-1.791	0.805
LGALS2						
High vs. Low	0.234	0.092-0.595	0.002*	0.348	0.168-0.721	0.005*
CLEC4						
High vs. Low	0.526	0.207-1.336	0.177	0.558	0.234-1.330	0.188

The results were obtained using the univariate Cox regression method. Gene expression levels were dichotomised as high and low according to their median. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; *$p < 0.05$.

The univariate COX regression model performed with ADC patients (including ADCs and adenosquamous histologies), showed the same association between high IL23A and LGALS2 and better prognosis that were found in the entire cohort. CD25 was also associated with better OS [HR, 0.419; 95% CI, 0.180-979; p, 0.045] and longer PFS [HR, 0.449; 95% CI, 0.226-891; p, 0.022]. Kaplan-Meier plots represented in Figure 24a and b, showed the survival differences between the two groups of patients. Few studies have focused on the expression of CD25 in NSCLC, and similar to other immune-related markers, these were performed using IHC methodologies. One example is a study published in 2012 in which double staining for CD3/CD8 and CD4/CD25 was performed in lung cancer patients from all stages. The authors observed that high numbers of stromal T-lymphocytes (both sets of staining) had a positive prognostic influence in NSCLC patients and were also an independent prognostic factor in ADCs (Kayser et al., 2012).

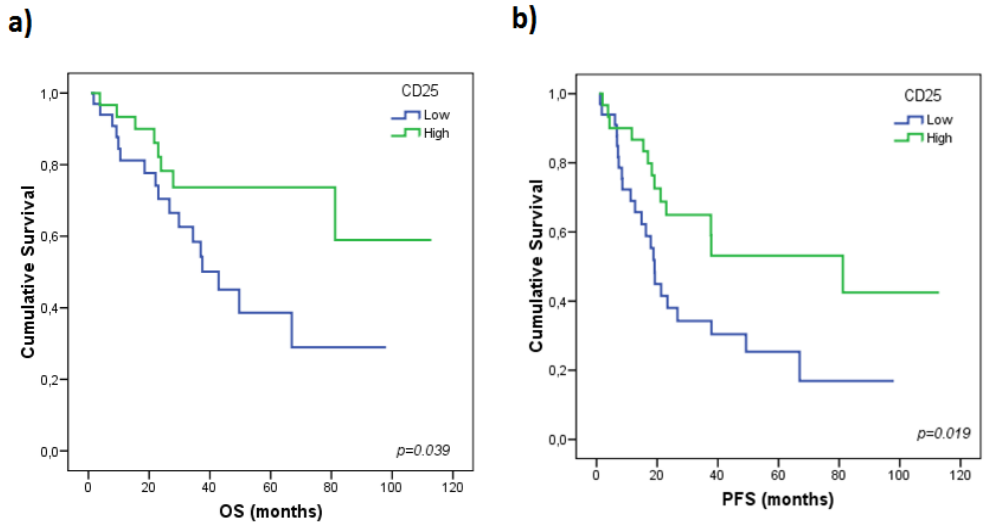
CTLA4 also correlated with OS [HR, 0.336; 95% CI, 0.143-0.790; p, 0.012] and PFS [HR, 0.349; 95% CI 0.174-0.701; p = 0.003] in this subset of patients. In this case, Kaplan-Meier plots revealed that for the group of patients with high CTLA4 expression levels the median OS was not reached, and for PFS it was 81.2 vs. 18.2 months (Figure 24c-d). This result is in agreement with previous work in NSCLCs (resected I-III) reporting that although CTLA4 did not show a statistically significant prognostic value, patients with higher CTLA4 expression levels experienced a 40% reduction in death risk, and a higher frequency of CTLA4 overexpression was found in non-squamous NSCLCs (Salvi et al., 2012). Similarly, in B-cell chronic lymphocytic leukaemia (CLL) cells patients with high CTLA4 mRNA levels were associated with a good clinical outcome (Joshi et al., 2007). The favourable effect of CTLA4 might appear in contrast to the notion that this receptor presents immunosuppressive characteristics, but could be explained by the fact that CTLA4 also mediates negative signals into tumour cells. Indeed, it has been observed that CTLA4 expression in early disseminated NSCLC cells (microscopic disease) might interact with B7 ligands, producing the inhibition of lung cancer cell proliferation (Contardi et al., 2005).

Furthermore, we found that high levels of IL10 correlated with a higher OS [HR, 0.414; 95% CI, 0.182-0.941; p = 0.035] and PFS [HR, 0.477; 95% CI, 0.242-0.940; p = 0.033]. Patients with high IL10 expression levels had a median of survival for OS of 81.2 vs. 37 months (p = 0.030, Kaplan-Meier test) and for PFS the median was 49.3 vs. 18.8 months (p = 0.029, Kaplan-Meier test; Figure 24e-f). IL10 is an anti-inflammatory, immunosuppressive cytokine with a double role in cancer. Its release by cancer and immune cells may result in the suppression of

cell mediated immunity, allowing tumours to proliferate, but this immunosuppression may also inhibit angiogenesis and therefore prevent cancer progression (Mocellin et al., 2005).

In NSCLCs, two older pieces of work report contradictory results: on the one hand increased IL10 production was associated with a worse prognosis (Hatanaka et al., 2000), while on the other hand, the lack of this cytokine also correlated with a worse outcome (Soria et al., 2003). Since then, other studies have tried to elucidate the role of IL10 in NSCLC. For instance, it has been reported that IL10 expression by TAMs, but not by tumour cells, may play a role in the progression and prognosis of NSCLC (Zeni et al., 2007). Moreover, $CD8^+/IL10^+$ cells accumulated in the tumour stroma of patients with early stage NSCLC correlate with longer OS (Miotto et al., 2010).

In a recent meta-analysis of cohort studies, the prognostic effect of different circulating inflammatory factors was evaluated and it was observed that IL10, among others, was not significantly associated with OS (Liao et al., 2014). Putting all of this data together, and given that IL10 can act as both an immune-stimulator and -inhibitor, the balance of these biological activities seems to be highly context-dependent. According to our results, in resected ADC patients the presence of high IL10 mRNA expression is a protective characteristic of the tumour microenvironment.



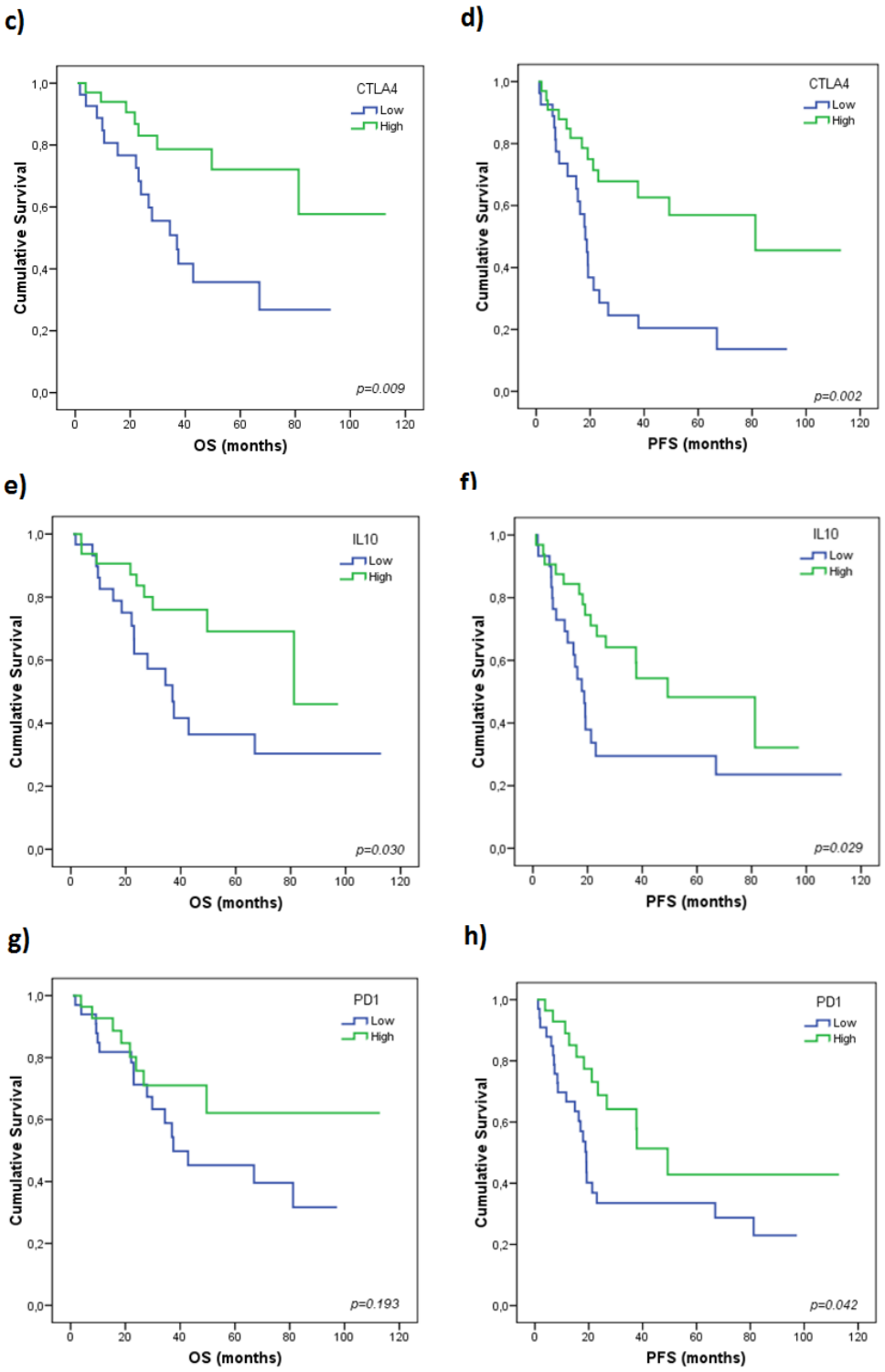


Figure 24. Kaplan-Meier plots for OS and PFS according to gene expression levels in ADCs. a-b) CD25; c-d) CTLA4; and e-f) IL10. Gene expression levels were dichotomised according to the median. Blue line represents patients with low levels of expression, whilst green line represents patients with high levels. *P*-values calculated using the Kaplan-Meier test.

1.4.4. EXPRESSION PROGNOSTIC SCORE

We also decided to create a gene expression score based on a multi-gene signature, which can provide more accurate predictions than a model using single genes. For this purpose, we followed the steps described in the data analysis section of the materials and methods section. Based on univariate Cox regression analysis, expression of IL23, IL10, CCL2, PD1, and CTLA4 were moderately associated with mortality (|Z-score| >1.5; Figure 25).

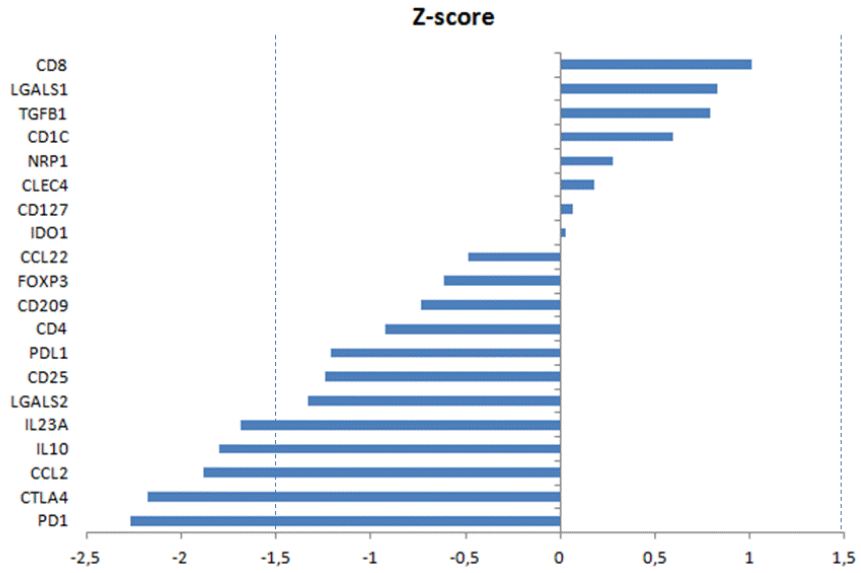


Figure 25. Univariate analysis of the expression of 20 immunoregulatory genes for OS. The genes are ranked based on their predictive power (univariate Z-score). Dashed lines indicate |Z-score|= 1.5. This criterion was used to select genes to include in the multivariate Cox regression model used to calculate the expression score.

These genes were selected to construct the prognostic signature by introducing them into the multivariate model; the results are detailed in Table 18. Absolute regression coefficients from multivariate analysis were used to calculate the expression prognostic score.

Table 18. Results from the multivariate model with genes included in the expression score.

Variable	Regression coefficient	SE	*p*-value	HR	95% CI
IL23A	-0.016	0.067	0.804	0.984	0.862-1.122
IL10	-0.077	0.129	0.550	0.926	0.719-1.192
CCL2	-0.154	0.138	0.262	0.856	0.653-1.123
PD1	-0.132	0.094	0.159	0.876	0.729-1.053
CTLA4	-0.173	0.064	0.006	0.840	0.742-0.952

CI, confidence interval; HR, hazard ratio; SE, standard error.

The formula to calculate the signature was the following:

(IL23A x 0.016) + (IL10 x 0.077) + (CCL2 x 0.154) + (PD1 x 0.132) + (CTLA4 x 0.173)

Kaplan–Meier log-rank analysis showed that patients with a high expression score have longer OS (NR vs. 42.9 months, p = 0.007) and longer PFS (82.6 vs. 23.4 months p = 0.011; Figure 26a-b). Furthermore, to evaluate the potential use of the score as a biomarker, we did a stratified analysis by TNM-staging and histology. We observed that when we stratified patients according to histology, the association between the ADC patient score and the prognosis was even stronger (OS: NR vs. 18.2 months, p = 0.001 and PFS: NR vs. 37 months, p = 0.001).

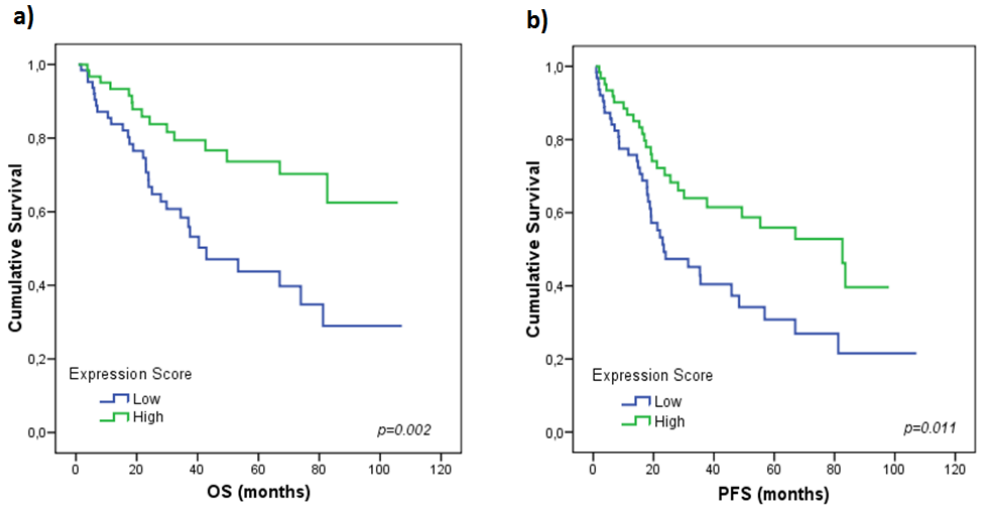


Figure 26. Kaplan-Meier plots for OS and PFS according to the gene expression score. a) OS and, b) PFS. The score was divided as low and high according to its median. Blue line represents patients with low levels of expression, whilst green line represents patients with high expression scores. *P*-values were calculated using the Kaplan-Meier test.

We identified a prognostic score, based on the expression of five immunosuppressive genes, which is associated with OS and PFS in NSCLC. Patients with a high expression score and therefore high expression levels of these genes had increased survival, which was even stronger in patients with ADC histology. This expression score comprised two immune checkpoint-related genes (CTLA4 and PD1), IL10 and IL23A (their association with survival is described above), and CCL2.

CCL2 is a chemokine responsible for the recruitment of monocytes during inflammatory responses, and it has been implicated in the development of multiple inflammatory disorders (Zhang et al., 2010). The correlation between CCL2 and NSCLC

prognosis was recently assessed by IHC in a study analysing the expression of this chemokine in 134 stage I-IV patients. The authors concluded that the presence of CCL2 in NSCLCs predicted improved prognosis relative to its absence (Zhang et al., 2013). However, the prognostic role of CCL2 in cancer is controversial due to the existence of studies pointing out that its expression is associated with angiogenesis and tumour progression (Salcedo et al., 2000); other work, e.g. in colorectal cancer, claim the contrary (Watanabe et al., 2008). It has been reported that CCL2 regulates the infiltration of inflammatory cells in tumour tissue, elevates the cytotoxic activity of monocytes and NK cells, and mediates the activity of macrophages. However, the interaction between macrophages and tumour cells is complicated because of the existence of two phenotypes: M1 and M2 macrophages. M1 macrophages are activated by IL2 and IFNγ and produce antitumour activity (Deshmane et al., 2009), whereas M2 macrophages promote tumour progression by stimulating angiogenesis through cytokines and proteases (Vande, I et al., 2006).

Prognostic scores based on gene expression levels have been previously described in the literature. For instance, Endoh et al. assessed the prognostic value of 48 candidate genes previously identified in two studies using ADC patients. They identified an eight-gene prognostic score that could separate patients with different prognoses (Endoh et al., 2004). Similarly, Lau et al. found a three-gene classifier that stratified a cohort of 147 early-stage NSCLC patients with significantly different prognoses (Lau et al., 2007). Although several prognostic scores or classifiers have been reported for NSCLC so far, very few genes have been observed to overlap between any of these classifiers.

1.4.5. IMMUNE CHECKPOINT SCORE

Because two of the genes composing the gene expression score described above were immune checkpoint genes, we decided to study if a score composed only by the expression of CTLA4 and PD1 would also have a prognostic value. Furthermore, a prognostic score based on fewer genes is preferred both in terms of time and cost. Following the method described in the previous section we created a multivariate model including these two genes. Absolute regression coefficients from this analysis (Table 19) were used to calculate the immune checkpoint score:

(PD1 x 0.116) + (CTLA4 x 0.058)

Table 19. Results from the multivariate analysis for OS based on PD1 and CTLA4 expression.

Variable	Regression Coefficient	SE	*p*-value	HR	95% CI
PD1	-0.116	0.075	0.121	0.890	0.769-1.031
CTLA4	-0.058	0.035	0.102	0.944	0.881-1.012

CI, confidence interval; HR, hazard ratio; SE, standard error

Kaplan- Meier analysis showed that patients with a high immune checkpoint score had longer OS (NR vs. 40.4 months, $p = 0.008$) and longer PFS (82.6 vs. 23 months, $p = 0.009$; Figure 27a-b). To evaluate the potential use of the score as a biomarker, we did a stratified analysis by TNM staging and histology. We found that for ADC patients, the association between a high immune checkpoint score and prognosis was stronger than for the entire cohort of patients (OS: NR vs. 34.4 months, $p = 0.002$ and PFS: NR vs. 16.2 months, $p < 0.001$). *P*-values obtained for OS and PFS were similar to the ones obtained using the five-gene expression score, which indicates that, in this case, there were no benefits to including a larger number of genes in the score, and therefore the immune checkpoint score is more practical.

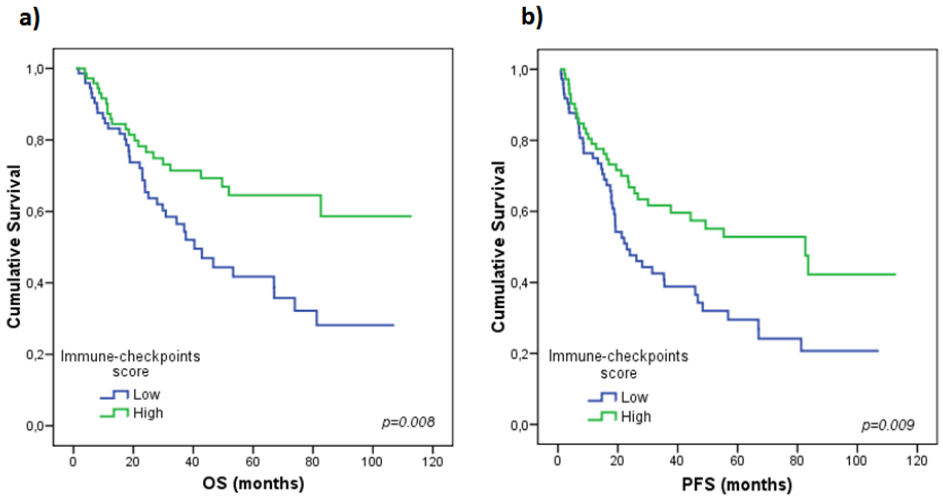


Figure 27. Kaplan-Meier plots for OS and PFS according to the immune checkpoint expression score. The score was divided as low and high according to its median. Blue line represents patients with low levels of expression, whilst green line represent patients with high scores. *P*-values were calculated using the Kaplan-Meier test.

The immune checkpoint score encompasses the expression of two genes, CTLA4 and PD1, which have become of great interest in the last few years. This is because researchers have demonstrated the importance of how the immune checkpoint blockade leads to robust antitumour effects in patients with metastatic melanoma, NSCLC, and other tumour types. As previously mentioned, CTLA4 overexpression is more common in ADC and appears to be an independent prognostic factor in NSCLC (Salvi et al., 2012). In a similar way to CTLA4, PD1 is

also an immune checkpoint receptor with immunosuppressive properties. However, in contrast to CTLA4, PD1 is activated during the effector stages of T cell activation, interaction with its ligand (PDL1) occurs primarily in peripheral tissues instead of LNs, and importantly, it can be expressed in tumour tissue as well as in immune cells (Ott et al., 2013).

PD1 expression in cancer has still not been thoroughly studied. In 2013, PD1 expression on $CD4^+$ and $CD8^+$ T cells obtained from peripheral blood mononuclear cells (PBMCs), normal gastric mucosa, and gastric cancer tissue cells was evaluated by multicolour flow cytometry, which showed that its expression in gastric cancer patients was significantly higher than that of normal controls (Saito et al., 2013). In breast cancer, another study demonstrated that the presence of $PD1^+$ TILs was associated with a poor prognosis (Muenst et al., 2013). To the best of our knowledge, so far only one piece of work has assessed the expression of PD1 in NSCLC tissues; the authors analysed the expression of PD1 and PDL1 in a cohort of 125 NSCLC patients in order to evaluate if they were differently expressed according to the presence or absence of EGFR mutations, ALK translocation, or KRAS mutations. Although they observed that the sensitivity to treatment was higher and the OS was longer in patients treated with EGFT TKIs when PDL1 expression was higher, no differences were observed for PD1 (D'Incecco et al., 2014). As for PDL1, recent work has analysed its expression in two large NSCLC patient cohorts, observing that high expression of PDL1 protein or mRNA was associated with a better outcome (Velcheti et al., 2014). However, we failed to obtain this correlation in our data when the prognostic value of the markers was analysed individually. This discrepancy could be explained by methodological differences: we performed RTqPCR whereas Velcheti et al. used IHC and in situ hybridisation.

Our results indicate that the combination of these immune checkpoints, but not their individual expression, has a strong prognostic value in resected NSCLC. Although we found that CTLA4 correlated with survival in ADC patients, the *p*-values obtained for this subgroup of patients were more strongly correlated when CTLA4 was combined with PD1. This information could be very useful in NSCLC management, especially because many new and promising immune checkpoint blockade therapies are now becoming available.

1.4.6. MULTIVARIATE ANALYSIS

In order to select the best prognostic biomarker obtained from fresh frozen tissue in resected NSCLCs, we interrogated all the biomarkers that were significantly associated with prognosis ($p < 0.05$) and included them in a multivariate model for OS and PFS. In the OS

multivariate model the following variables were included: *KRAS* status, cluster classification, individual biomarkers such as CD25, IL23A, LGALS2, the expression score, and the immune checkpoint score. The variables included in the PFS model were: LN involvement, *KRAS* status, cluster classification, individual biomarkers such as FOXP3, CD4, CD127, IL23, LGALS2, the expression score, and the immune checkpoint score. Results obtained from this multivariate analysis indicated that *KRAS* status and the immune checkpoint score were independent biomarkers for both OS and PFS, and in the later, CD127 expression was also identified as an independent biomarker (Table 20).

Table 20. Multivariate Cox regression model results, including all the significant variables.

	OS			PFS		
Variables	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
KRAS status						
Mutated vs. WT	2.984	1.338-6.659	0.008	3.807	1.764-8.214	0.001
Immune checkpoint score						
High vs. low	0.308	0.156-0.609	0.001	0.527	0.298-0.933	0.028
CD127						
High vs. low	-	-	-	0.513	0.292-0.901	0.020

CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; WT, wild type.

Multivariate analysis was also performed with significant results for ADC patients; the following variables were included in the model for OS: CD25, CTLA4, IL10, IL23A, LGALS2, cluster classification, the expression score, and the immune checkpoint score. In this case, only the immune checkpoint score proved to be an independent biomarker. For PFS, we included the same variables plus LN involvement and PD1. Again the immune checkpoint score was an independent prognostic factor as well as LN involvement (Table 21). Our results indicate that the immune checkpoint score is an independent biomarker for both OS and PFS, and moreover, its prognostic value proved to be stronger for OS than factors such as *KRAS* status in the entire cohort, or LN involvement in ADC patients.

Table 21. Results from a multivariate Cox regression model including all the significant results for ADC patients.

	OS			PFS		
Variables	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Immune checkpoint score						
High vs. low	0.131	0.038-0.451	0.001	0.156	0.063-0.388	<0.001
LN involvement						
Yes vs. No	-	-	-	3.896	1.539-9.858	0.004

CI, confidence interval; HR, hazard ratio; LN, lymph node; OS, overall survival; PFS, progression free survival.

Expression scores are well established methods for separating patients into prognostic groups. In this study, we used RTqPCR, the gold standard method for gene expression quantification, because of its high sensitivity and specificity. Other advantages of this technology are that it requires a low RNA input, it is less time consuming than other methods, and it is robust and flexible. Thus, RTqPCR is clinically applicable for detecting patient subgroups with specific prognostic characteristics. This is of great importance because current clinicopathological staging methods have limited success in predicting patient survival and great outcome uncertainty for same-stage NSCLC cancers remains: we still cannot predict which patients will be cured and which ones will suffer recurrence or death after surgical resection. It is remarkable that we have found an independent prognostic biomarker, defined as the immune checkpoint score, which identifies a subset of NSCLC patients with a better outcome. Two recent publications in the *Nature* journal indicated that the expression of immune checkpoints, PD1 and PDL1, in infiltrating immune cells was correlated with better responses to immune checkpoint blockade treatment. The findings suggest that the presence of these biomarkers might indicate that these tumours have already been recognised by the immune system, and therefore they are key predictors of clinical treatment responses (Herbst et al., 2014; Tumeh et al., 2014). If these predictive markers are a reflection of a pre-existing immune recognition, and taking the theory that predictive markers are also likely to be of prognostic value into account (Angell and Galon, 2013), our results suggest that immune checkpoint marker expression may also be of future value as a new prognostic NSCLC biomarker. They may also have some therapeutic value for managing NSCLC via emerging targeted immunotherapies, especially immune checkpoint blockade-based therapies, and so further studies to asses both of these uses should be conducted in order to better understand these processes.

In summary, in this part of the study we identified some biomarkers based on the expression of immune-related genes, especially immunoregulatory cells and processes. Unsupervised hierarchical analysis revealed that patients were grouped according to their gene expression levels and patient clusters with higher expression of these biomarkers were associated with better outcomes. Some of these biomarkers presented a significant association with survival when individually analysed or combined into expression scores. In fact, one score comprising two immune checkpoint-related genes was found to be an independent prognostic factor. Interestingly, the prognostic value of most of these biomarkers was amplified in ADC patients.

2. ANALYSIS IN FORMALIN-FIXED PARAFFIN EMBEDDED SAMPLES

In this part of the study a set of immunoregulatory biomarkers was assessed in the different compartments of the tumour microenvironment: the tumour itself and the adjacent stroma. It is important to assess the specific location of a given marker in the tumour microenvironment because its prognostic value may be different or even opposite depending on where it is expressed.

2.1. PATIENT CHARACTERISTICS

For this purpose FFPE samples from 122 patients (102 patients included in the previous section and 20 more from another hospital) were studied. The most relevant demographic and clinicopathological characteristics are shown in Table 22.

Table 22. The clinicopathological characteristics of patients included in the FFPE-analysis.

Characteristics	N	%
Age at surgery (median, range)	65 [26-85]	
Gender		
Male	104	85.4
Female	18	14.6
Stage		
I	72	59
II	26	21.3
IIIA	24	19.7
Histology		
SCC	58	47.5
ADC	51	41.8
Others	13	10.7
Performance Status		
0	70	57.4
1-2	35	28.7
NS	17	13.9
Differentiation grade		
Poor	29	23.8
Moderate	48	39.3
Well	28	23
NS	17	13.9
Smoking Status		
Current	59	48.4
Former	46	37.7
Never	17	13.9
EGFR		
Wild type	43	35.2
Mutated	9	7.4
NS	70	57.4
KRAS		
Wild type	83	68
Mutated	14	11.5
NS	25	20.5

ADC, adenocarcinoma; SCC, squamous; NS, not specified; EGFR, epidermal growth factor receptor.

2.2. GENE EXPRESSION ANALYSIS

2.2.1. LASER CAPTURE MICRODISSECTION AND RNA ISOLATION

Laser capture microdissection was carried out in order to separately obtain tumour and stroma areas from FFPE samples. First, we selected specific areas of at least 10^7 μm^2 on 5 µm HE stained sections and then we performed the microdissection on 10 µm membrane-slide sections. Figure 28 shows two examples of how the areas were selected for the different NSCLC histologies.

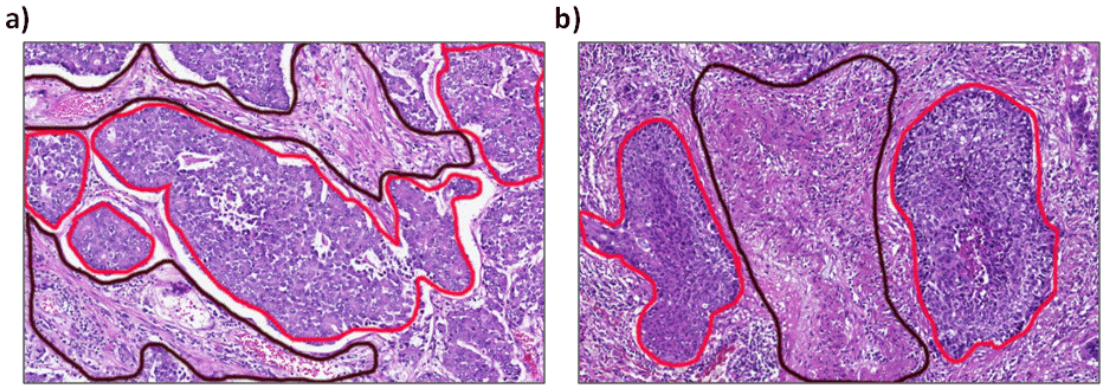


Figure 28. Examples of areas selected for laser capture microdissection. The figure represents areas selected for microdissection, areas of tumour cells are highlighted in red and areas composed of adjacent stroma are highlighted in black. a) Adenocarcinoma tumour and b) squamous cell tumour.

RNA was isolated from microdissected areas of at least 10^7 μm^2 from the tumour and stroma compartments. The median RNA concentration for tumour areas was of 46.8 [range: 8–194.7] ng/µl and for stroma areas it was of 38 [range: 4.8-202.5] ng/µl. The RNA quantity obtained in 10 of the 122 cases included was not enough for further analysis. The quality of RNA isolated was assessed by capillary electrophoresis. In the majority of the samples the RIN was under 7, which was expected due to the nature of the fixation process, however this did not produce an adverse impact on the subsequent gene expression analysis.

2.2.2. ASSESSMENT OF PRE-AMPLIFICATION UNIFORMITY

We then checked whether all amplicons were uniformly amplified during the pre-amplification process. We assessed the expression of a human reference cDNA (preamplified and at a concentration of 0.3 ng/µl) and the Cts obtained were included in the equation described in the material and methods section. Genes with ΔΔCt values within ±1.5 were

considered to be uniformly amplified (Table 23). As recommended by the manufacturer, we used CDKN1B as the endogenous reference gene.

Table 23. Results of the pre-amplification uniformity test for the gene expression assays.

GENES	ΔΔCt	GENES	ΔΔCt	GENES	ΔΔCt
CCL2	1.399	**CD114**	-0.617	**IL7RA**	-0.920
CCL22	0.953	**CLEC4C**	0.036	**IL8**	-1.367
CCL5	-0.263	**CSF1R**	1.173	**ITGAM**	0.101
CD1C	0.953	**CSF3R**	-0.617	**ITGB2**	-0.675
CD209	1.276	**CTLA4**	-1.163	**LAG3**	-1.279
CD34	0.077	**CXCL12**	1.393	**LGALS1**	0.262
CD4	-0.444	**CXCR4**	-0.088	**LGALS2**	-1.414
CD40	0.503	**FOXP3**	-0.165	**MMP2**	-0.222
CD40LG	-0.723	**IDO1**	-0.805	**NRP1**	0.273
CD44	-0.634	**IL10**	1.475	**TGFB1**	-0.199
CD80	-1.423	**IL13**	-0.167	**THBD**	1.010
CD86	1.232	**IL23A**	0.539	**TNF**	0.750
CD8	-0.300	**IL2RA**	0.040	**GITR**	0.779
CD97	0.064	**IL4RA**	1.043	-	-

A ΔΔCT values close to zero indicate pre-amplification uniformity. 90% of targets typically produce ΔΔCT values within ±1.5. CDKN1B was used as a reference gene.

All the genes for which uniformity could be assessed proved to be correctly amplified. However, the uniformity of CD33, INFG, IL12B, and IL4 could not be evaluated because the threshold cycles for the 0.3 ng/µl template were undetermined.

2.2.3. RELATIVE MESSENGER RNA EXPRESSION OF IMMUNOREGULATORY GENES IN TUMOUR AND ADJACENT STROMA COMPARTMENTS

Forty-four genes relevant to tumour immunoregulation were assessed by relative gene expression analysis in 122 tumour and stroma area samples that were microdissected from FFPE samples obtained from resected NSCLC patients. All the analysed genes could be amplified using the selected primers/probes except IL13 and IL4. In addition, the CD33, INFG, CLEC4 and IL12B genes could not be studied further because more than 50% of the samples had undetermined values. Thus, a total of 38 genes were included in this analysis.

The expression of five endogenous genes (ACTB, GAPDH, GUSB, HPRT1, and CDKN1B) was tested in a subset comprising approximately 30% of the samples in order to establish the best internal control using geNorm software, with the GAPDH and CDKN1B being the best combination. Consequently, a normalisation factor was calculated based on the expression of these two endogenous genes using the geometric mean (Vandesompele et al., 2002). Furthermore, relative expression of the genes analysed in the tumour and stroma was normalised against a pre-amplified reference cDNA.

A gene was considered to be overexpressed in the tumour or stroma compartment compared to the reference cDNA when the median of the relative gene expression or fold-change was above 2 and, it was considered to be downregulated when the fold-change was below 0.5. We observed strong overexpression of five genes CD25 (19.46X, 11.59X), FOXP3 (4.96X, 4.08X), CTLA4 (2.89X, 3.02X), CD80 (4.62X, 2.87X), and TGFB1 (3.24X, 2.117X) in both the tumour and stroma compartments, respectively, and one gene, MMP2 (3.25X), was overexpressed only in the stroma. On the other hand, we found downregulation of eleven genes: LGALS2 (0.04X, 0.01X), ITGB2 (0.46X, 0.27X), IL4R (0.22X, 0.17X), CD97 (0.23X, 0.15X), CD40 (0.16X, 0.13X), CD34 (0.28X, 0.11X), CCL2 (0.10X, 0.04X), CXCL12 (0.25, 0.07X), THDB (0.24X, 0.09X), CD1C (0.35X, 0.34X), and TNF (0.39X, 0.27X) in both tumour and stroma respectively. Moreover, six genes were downregulated only in the tumour compartment: LGALS1 (0.39X), IL10 (0.39X), CD40LG (0.41X), CD209 (0.31X), ITGAM (0.27X), and CSF1R (0.37X) (Figure 29).

The continuous cross-talk between cancer cells and tumour microenvironment-associated cells has a significant role in tumour carcinogenesis and tumour progression. The tumour microenvironment is a crucial source of angiogenic cytokines, proteases and other factors that are important for maintaining intercellular communication. The immune cells that can be present in both tumour and stroma compartments are also of great importance. Gene expression analysis of immunoregulatory markers indicated that genes related to the presence of T cells and other immunoregulatory cells were overexpressed in both tumour and stroma locations. The overexpression of the Treg-related genes FOXP3, TGFB1, and CTLA4 was also observed. MMP2 was overexpressed in the stroma but not the tumour compartment which could be due to its role in degrading extracellular matrix so that the tumour can metastasise. On the other hand, we found that other genes such as the chemokines CCL2 and CXCL12, which are related to MDSCs and TAMs, were downregulated in the tumour and stroma

compartment. Some genes were downregulated only in the tumour, which might indicate that the infiltration of certain types of immune cells may be concentrated in the stroma compartment instead of the tumour nest.

We then analysed if there were significant differences between the gene expression levels in tumour versus stroma. We found significant differences for several genes (see Table 24) and in most of the cases the expression was higher in stroma than in tumour areas. This information could indicate differences in the immune infiltrate present in each compartment and more importantly, it justifies gene expression analysis at the compartment level because biomarkers may have diverging prognostic impacts depending on whether they are expressed in tumour or in stroma locations.

The role of stromal cells is becoming increasingly understood, and it is probable that they also contribute to the development of the inflammatory phenotype. Although they are typically associated with wound healing through the deposition of extracellular matrix, fibroblasts have important roles in both immune modulation and angiogenesis (Gajewski et al., 2013). The evidence suggests that in general the stroma and the microenvironment activate homeostatic tissue repair mechanisms that include cellular and molecular events traditionally considered to pertain to either angiogenic or immunosuppressive mechanisms (Motz and Coukos, 2011). Moreover, molecular profiling of stromal cells from a variety of different human tumour specimens has yielded information of prognostic value, further highlighting the critical role that the tumour microenvironment plays in directing tumour development.

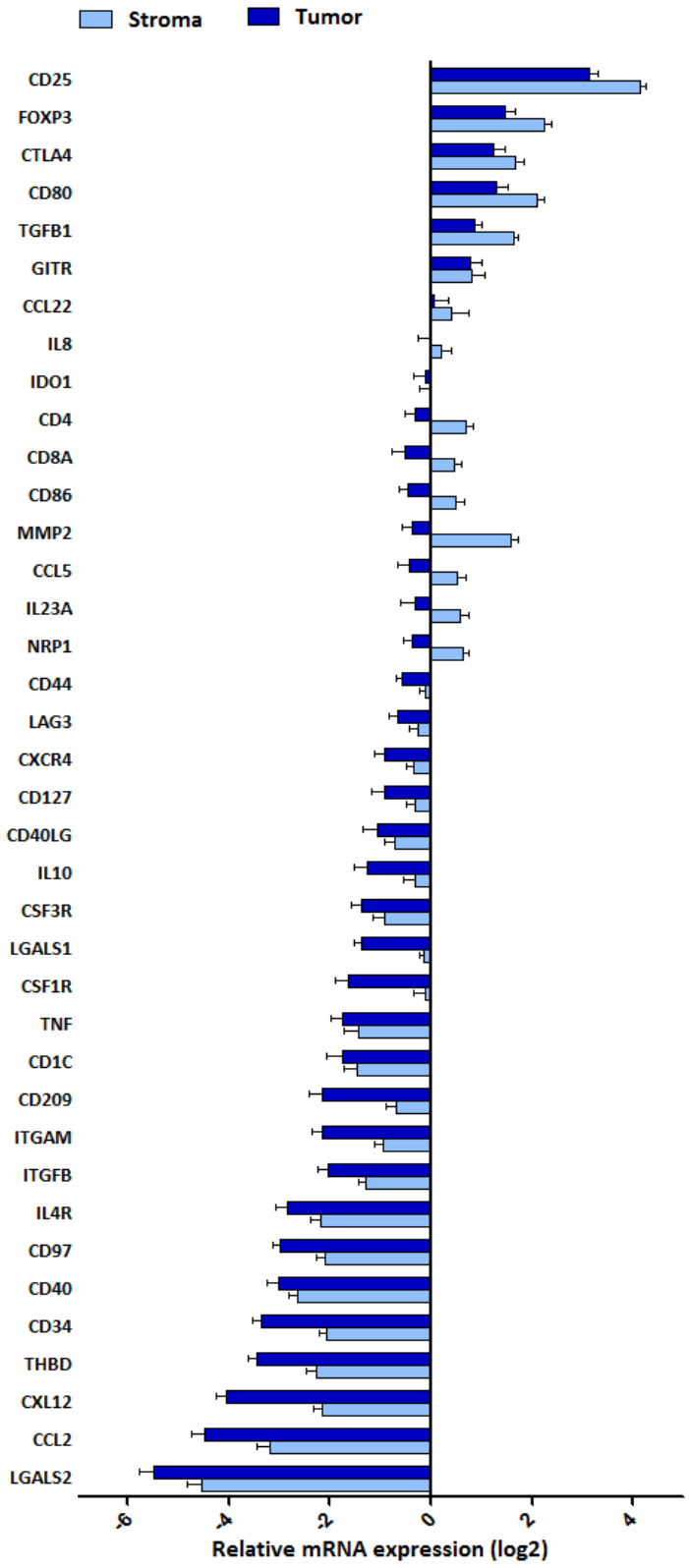


Figure 29. Relative mRNA expression of the genes analysed in the tumour and stroma compartments. In this graphic the relative gene expression is represented as the mean of the log2-normalised data. Results represent the mean ± SEM.

Table 24. Relative expression levels of genes with significant differences between tumour and stroma.

GENE	TUMOUR	STROMA	*P*-value	GENE	TUMOUR	STROMA	*P*-value
CCL2	0.046	0.104	<0.001	**CD97**	0.153	0.234	<0.001
CCL5	0.888	1.459	<0.001	**CXCR4**	0.618	0.738	0.003
CSF3R	0.536	0.688	0.020	**CXL12**	0.070	0.255	<0.001
CSF1R	0.376	1.029	<0.001	**FOXP3**	4.081	4.965	0.007
CD127	0.555	0.942	0.001	**IL10**	0.397	0.887	0.002
ITGAM	0.276	0.636	<0.001	**IL23A**	0.861	1.619	0.013
CD209	0.315	0.782	<0.001	**IL4R**	0.174	0.229	0.012
CD25	11.593	19.464	<0.001	**ITGFB2**	0.275	0.462	<0.001
CD34	0.117	0.281	<0.001	**LAG3**	0.668	0.806	0.012
CD4	1.075	1.791	<0.001	**LGALS1**	0.392	0.944	<0.001
CD44	0.771	1.014	0.009	**MMP2**	0.956	3.250	<0.001
CD80	2.875	4.625	<0.001	**TGFB1**	2.117	3.244	<0.001
CD86	0.960	1.697	<0.001	**THBD**	0.094	0.244	<0.001
CD8	1.011	1.470	0.002	**NRP1**	0.854	1.663	<0.001

Tumour and stroma were compared for each gene using a pair-wise Wilcoxon test.

2.2.4. UNSUPERVISED CLUSTERING ANALYSIS

Unsupervised hierarchical clustering analysis was performed in order to classify patients and genes based on the similarity of their expression pattern. Before starting, some genes were excluded from the analysis due to similar expression among patients or because they did not pass the filter (expression data in more than 80% of the samples). We ran a hierarchical analysis with gene expression data from both the tumour and stroma compartment to test if the samples clustered according to their compartment. Although the clusters were enriched in samples from the same compartment, they did not completely separate (data not included).

We then separately performed the hierarchical analyses with expression data from the tumour and stroma compartments. Using gene expression data from the stroma compartment, patients were classified into two major subgroups: Cluster I (n = 32) and Cluster II (n = 49). Patients in Cluster I had lower expression levels for most of the genes, whilst patients in Cluster II presented higher levels of expression in general, although there were subgroups with variable expression (Figure 30).

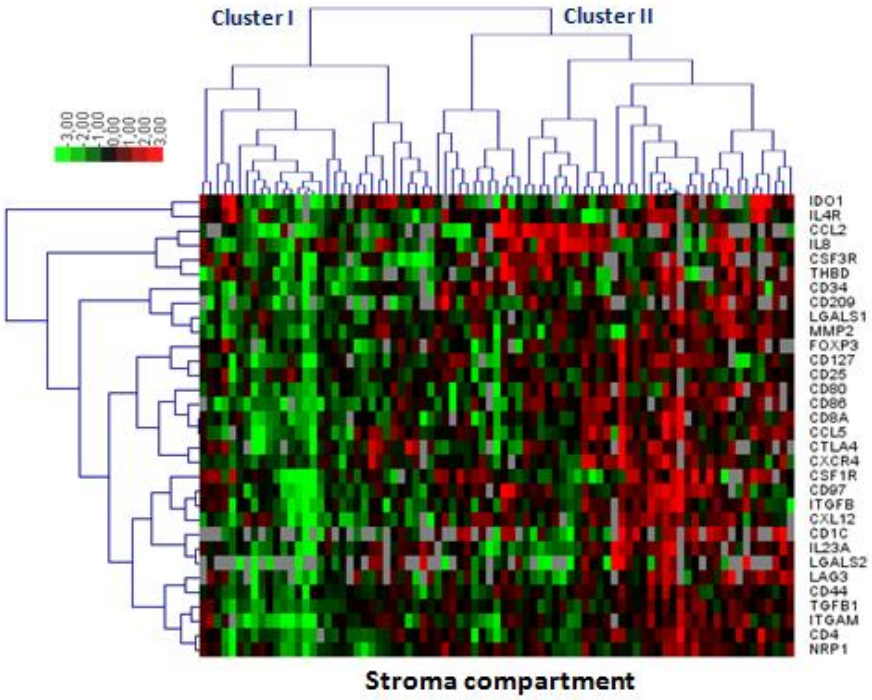


Figure 30. Hierarchical cluster based on gene expression data from the stroma compartment. Patients in the original cohort were clustered into a tree hierarchy based on the expression of 32 genes. Red indicates high expression and green indicates low expression.

Hierarchical analysis performed with gene expression data from the tumour compartment also revealed two major subgroups: Cluster I (n = 51) and Cluster II (n = 39). Cluster II presented homogeneously lower gene expression levels (except from one specific region in the heat map). The expression pattern in Cluster I was more heterogeneous (although there was a tendency towards higher expression levels). Some gene expression patterns could be observed, for instance genes related to inflammation such as THBD, IL8, MMP2, and CSFR3 were grouped into the same cluster, as well as genes related to Tregs such as CD127, CD25, FOXP3, and CD4 (Figure 31).

Gene expression analysis according to the compartment has been previously reported in other studies where sample microdissection and RNA isolation were also considered to be critical steps in order to obtain reliable results. For instance, using this approach a gene expression profile with elevated expression of genes related to extracellular matrix remodelling was identified exclusively in epithelial cells in breast cancer (Vargas et al., 2012). In colorectal cancer, a differential expression pattern with several MMPs and angiogenic cytokines in tumour cells compared to adjacent tumour stroma was observed. Moreover, cluster analysis showed that the expression of MMPs and angiogenic cytokines in stromal cells was tumour-site-dependent (Kahlert et al., 2014). The fact that we observed differential

patterns of expression in tumour and stroma cells points towards the differences between these two compartments in the tumour microenvironment.

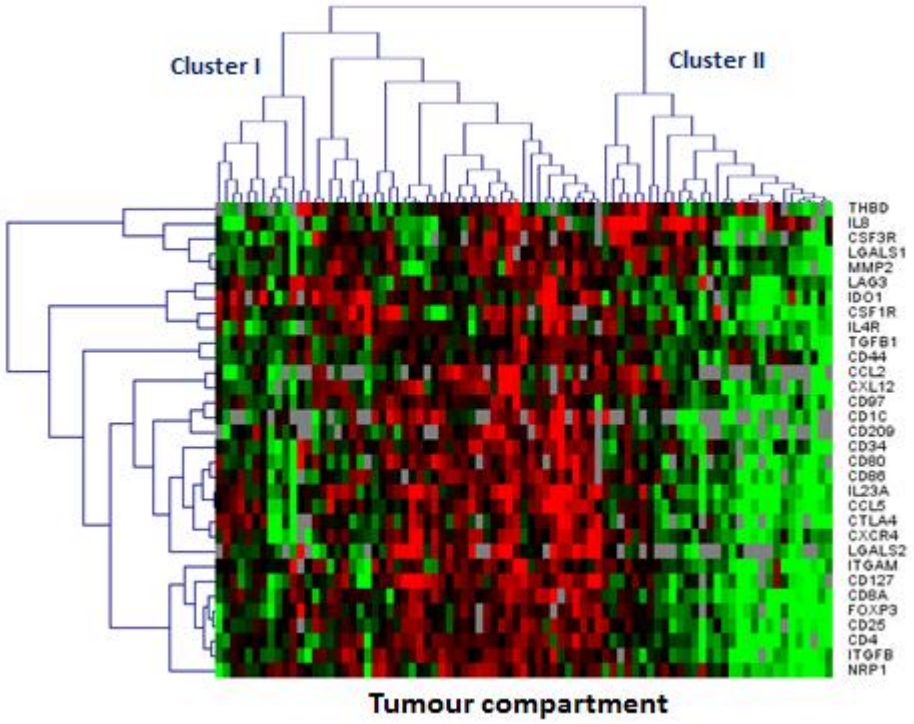


Figure 31. Hierarchical cluster based on gene expression data from the tumour compartment. Patients in the original cohort were clustered into a tree hierarchy based on expression of 32 genes. Red indicates high expression and green indicates low expression.

2.3. CORRELATION OF BIOMARKERS WITH CLINICOPATHOLOGICAL VARIABLES

The Mann-Whitney U test revealed that there were several significant associations between the expression of different genes in tumour or/and stroma compartments and clinicopathological variables such histology, stage, and mutational status (see Table 25). The expression of several genes, such as TNFα, CD97, and NRP1 was associated with histology, and their expression levels were higher in ADC tumours. The stage of the disease was correlated with the expression of IL10, ITGAM, and CD115 in the tumour compartment and with the expression of CD40 and LGALS2 in the stroma compartment; in all cases the expression was higher in patients with stage I vs. II/III cancer. Turning to the mutational status, *KRAS* correlated with CSF1R and IL4: in both cases patients with mutated versions of this gene presented higher expression levels of CSF1R and IL4. Patients with a mutated *EGFR* gene showed reduced IL8 expression compared to the wild type group, whereas CD40LG expression was higher in the *EGFR* mutated group.

Chi-square tests showed that when analysing the tumour compartment, there were significantly more ADC patients in Cluster I and more SCC patients in Cluster II (p = 0.007) and, moreover, there were more patients with larger tumours (larger than 3.5 cm) in Cluster II (p = 0.001). These results agree with observations from individual correlations where patients with higher gene expression levels were grouped into Cluster I, which also contained significantly more ADC patients. Thus, ADC patients presented higher expression rates of immunoregulatory genes in the stroma and the tumour compartments. Although, to the best of our knowledge, this is the first study to assess expression differences in immune-related genes (especially immunoregulatory genes) between lung ADC and SCC, previous work has shown that ADC patients presented higher Treg infiltration rates than SCC patients (Black et al., 2013).

Table 25. Significant correlations between gene expression values and clinicopathological variables.

Gene		Variable		*p*-value
		Histology		
		SCC	ADC	
CD8	T	0.624	1.299	0.044
	S	1.094	1.648	0.008
CD4	T	0.742	1.406	0.024
	S	1.335	2.034	0.008
LAG3	T	0.545	0.843	0.008
	S	0.626	1.163	0.008
CD97	T	0.080	0.221	<0.001
	S	0.176	0.282	0.014
CD80	T	1.740	3.499	0.029
	S	3.571	5.732	0.037
IL23A	T	0.592	1.225	0.019
	S	1.205	2.003	0.021
NRP1	T	0.377	1.749	<0.001
	S	1.135	2.130	<0.001
ITGB2	**T**	0.102	0.482	<0.001
CSF1R	**T**	0.256	0.550	0.032
IL4R	**T**	0.079	0.252	0.001
CCL5	**T**	0.555	0.938	0.018
CD34	**T**	0.081	0.153	0.014
LGALS1	**T**	0.303	0.474	0.014
MMP2	**T**	0.559	1.212	0.018
CD86	**T**	0.575	1.059	0.021
CSFR3	**T**	0.267	0.633	0.002
IL10	**S**	0.627	1.261	0.014
TNF	**S**	0.172	0.474	<0.001
CD1C	**S**	0.199	0.631	0.004
CSF3R	**S**	0.461	0.834	0.003
IL23A	**S**	1.205	2.003	0.021
CD40	**S**	0.128	0.227	0.029
CXCR4	**S**	0.606	0.945	0.020

Gene		Variable		*p*-value
		Stage		
		I	II/IIIA	
IL10	**T**	0.631	0.262	0.027
ITGAM	**T**	0.360	0.242	0.032
CSF1R	**T**	0.476	0.234	0.041
CD40	**S**	0.240	0.123	0.013
LGALS2	**S**	0.052	0.037	0.035
		KRAS status		
		WT	Mutated	
CSF1R	**T**	0.354	0.941	0.018
IL4R	**T**	0.148	0.389	0.022
		EGFR status		
		WT	Mutated	
IL8	**S**	1.289	0.368	0.025
CD40LG	**S**	0.449	1.867	0.026

ADC, adenocarcinoma; SCC, squamous cell carcinoma;
T, tumour; S, stroma; WT, wild type.

2.4. BIOMARKER SURVIVAL ANALYSIS

2.4.1. CLINICOPATHOLOGICAL VARIABLES

Of the 122 resected NSCLC patients included in this part of the study, 68 (55.7%) relapsed and 67 (54.9%) died. The median follow-up was of 53.3 months [range: 1-113]. The results obtained in the univariate analysis using the Cox regression method are shown in Table 26. The only variable that was associated with prognosis in this set of patients was the tumour size (p = 0.025); patients with tumours more than 3.5 cm had a shorter PFS.

Table 26. Results from survival analysis based on clinicopathological variables.

	OS			PFS		
Variable	**HR**	**[95% CI]**	***p*-value**	**HR**	**[95% CI]**	***p*-value**
Gender *Male vs. Female*	3.558	0.864-14.656	0.079	2.157	0.782-5.952	0.138
Age (years) *>65 vs. ≤ 65*	1.707	0.970-3.003	0.064	1.217	0.730-2.028	0.452
Stage *II/IIIA vs. I*	0.785	0.447-1.379	0.400	1.048	0.634-1.734	0.854
Histology *ADC vs. SCC vs. Others*	0.806	0.515-1.262	0.346	1.061	0.709-1.586	0.773
Tumour size *>3.5 cm vs. ≤ 3.5 cm*	1.218	0.694-2.135	0.492	1.806	1.079-3.024	0.025*
LN involvement *Yes vs. No*	1.224	0.669-2.239	0.512	1.289	0.743-2.237	0.366
PS *1/2 vs. 0*	1.291	0.687-2.424	0.427	1.469	0.838-2.577	0.179
Differentiation grade *Poor vs. Well/Moderate*	0.991	0.521-1.885	0.979	1.042	0.585-1.856	0.888
Smoking status *Former/Current vs. Never*	1.427	0.513-3.975	0.496	1.314	0.564-3.058	0.527
EGFR *Wild type vs. Mutated*	0.426	0.097-1.864	0.257	1.019	0.379-2.744	0.970
KRAS *Mutated vs. Wild type*	1.914	0.744-4.926	0.178	1.901	0.800-4.518	0.146

CI, confidence interval; EGFR; epidermal growth factor receptor; HR, hazard ratio; LN, lymph node; OS, overall survival; PFS, progression free survival; PS, performance status; *p < 0.05.

2.4.2. HIERARCHICAL CLUSTERS

Survival analysis carried out with cluster groups obtained from the gene expression data showed that patients in stromal Cluster I had a worse PFS than in Cluster II (17.4 vs. 44.3 months, p = 0.006; Figure 32b). With regard to the tumoural clustering, patients in Cluster II had a worse OS than patients in Cluster I (34.4 vs. 70.4 months, p = 0.005) as well as a shorter

PFS (19.1 vs. 32.5 months, $p = 0.010$; Figure 32c-d). As we previously observed when analysing clusters obtained from fresh-frozen samples, groups of patients with higher immune-related gene expression had better outcomes.

Here we found an association between stromal clusters and PFS, but tumoural clusters were also associated with PFS and OS. Other studies have previously reported the prognostic value of expression signatures according to specific compartments in the tumour microenvironment. For instance, in a study carried out in breast cancer using microdissection and microarray technologies, a new stroma-derived prognostic predictor that stratified disease outcome, independently of standard clinical prognostic factors, was identified. Interestingly, the presence of immune cells within the stroma was diminished in individuals in the poor-outcome cluster. Instead, stroma from individuals in the poor-outcome cluster showed increased hypoxic and angiogenic response markers and a decrease in the expression of chemokines that stimulate NK cell migration and mediate pro-survival signals in T lymphocytes (Finak et al., 2008). Another group analysed expression signatures in the tumour and stroma compartment of SCC cells from NSCLC patients, and observed that many markers related to longer survival were predominantly expressed in the stroma, particularly MHC-II complex genes (Bendrat et al., 2012).

These results provide insight into the importance of immunoregulatory markers in NSCLC prognosis, and indicate that tumour and stroma are independent entities inside the tumour microenvironment, with different characteristics due to the differences in immune cell infiltration. Cluster analysis has provided a global vision of the impact of immunoregulatory markers on prognosis, however these results must also be dissected in order to assess which of these markers have a prognostic role when individually analysed.

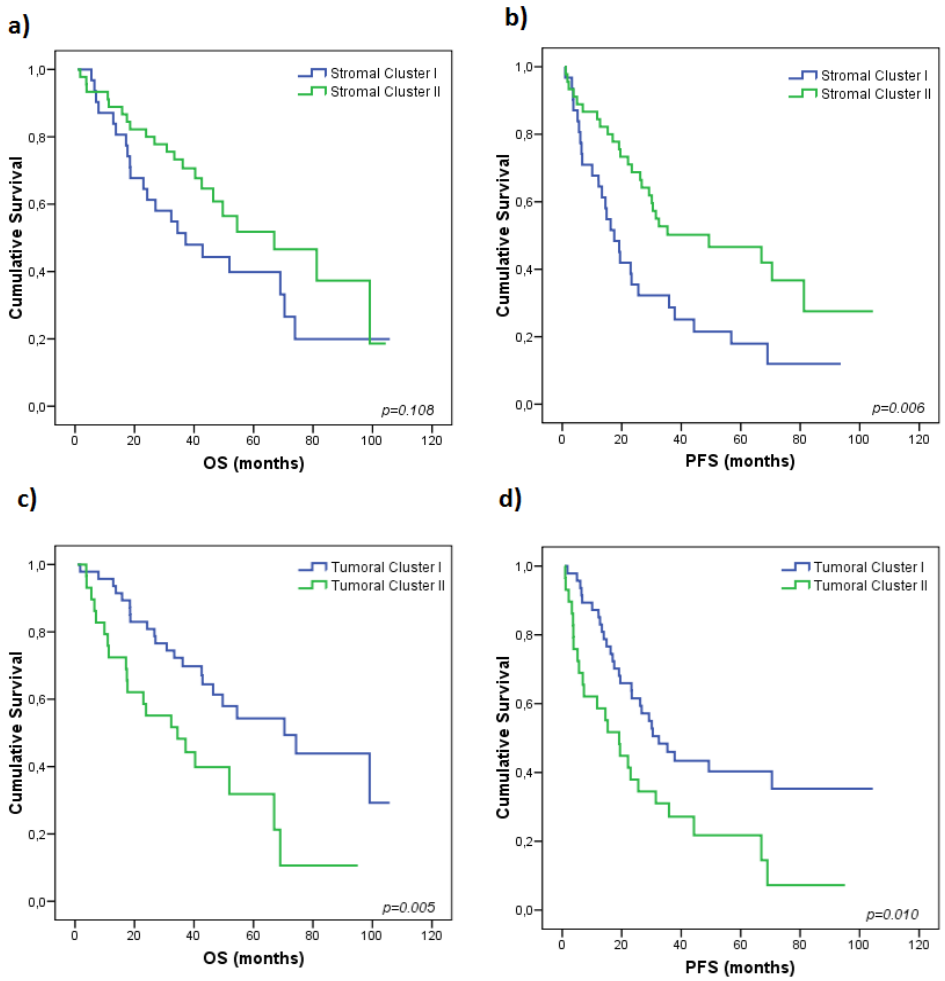


Figure 32. Kaplan-Meier plots for OS and PFS according to clustering classification. a-b) Clusters obtained from stroma gene expression data and, c-d) clusters obtained from tumoural data. Blue line represents patients classified in Cluster I and green line represent patients in Cluster II. *P*-values were obtained using the Kaplan-Meier test.

2.4.3. INDIVIDUAL BIOMARKERS

The prognostic impact of the analysed genes that were involved in tumour immunoregulation was assessed both for their expression in tumour and in adjacent stroma by univariate COX regression analysis. For this purpose the gene expression levels were dichotomised according to the median of each one. The results obtained from these univariate analyses are shown in Tables 27 and 28.

Table 27. Results from the survival analysis based on gene expression biomarkers in stroma areas.

STROMA						
	OS			PFS		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
FOXP3						
High vs. Low	0.772	0.413-1.441	0.416	0.869	0.498-1.515	0.620
CD4						
High vs. Low	0.942	0.536-1.655	0.835	1.235	0.744-2.050	0.415
CD8						
High vs. Low	0.521	0.285-0.954	0.035*	0.728	0.430-1.234	0.239
CD25						
High vs. Low	1.565	0.864-2.837	0.139	1.612	0.944-2.754	0.080
CD127						
High vs. Low	0.822	0.448-1.508	0.526	0.936	0.545-1.607	0.810
TLA4						
High vs. Low	0.873	0.454-1.681	0.686	0.579	0.321-1.042	0.068
NRP1						
High vs. Low	1.170	0.667-2.053	0.584	1.038	0.624-1.726	0.885
LAG3						
High vs. Low	0.828	0.424-1.617	0.581	1.004	0.562-1.796	0.989
GITR						
High vs. Low	0.616	0.195-1.943	0.408	0.620	0.256-1.505	0.291
TGFB1						
High vs. Low	0.720	0.404-1.283	0.265	0.889	0.531-1.489	0.655
IL10						
High vs. Low	0.949	0.374-2.411	0.913	0.756	0.345-1.652	0.483
LGALS1						
High vs. Low	1.320	0.748-2.332	0.338	1.091	0.654-1.820	0.738
LGALS2						
High vs. Low	1.485	0.634-3.478	0.362	0.891	0.444-1.788	0.745
IDO1						
High vs. Low	0.824	0.435-1.558	0.551	0.827	0.470-1.458	0.512
ITGAM						
High vs. Low	1.441	0.792-2.622	0.232	1.313	0.770-2.241	0.317
ITGB2						
High vs. Low	1.162	0.652-2.069	0.611	1.038	0.613-1.756	0.891
CD97						
High vs. Low	0.841	0.459-1.539	0.573	0.958	0.560-1.640	0.876
IL4R						
High vs. Low	0.928	0.505-1.704	0.810	0.966	0.560-1.665	0.900
CD209						
High vs. Low	1.031	0.508-2.094	0.933	0.831	0.442-1.560	0.564
CD1C						
High vs. Low	0.794	0.337-1.871	0.597	1.232	0.603-2.519	0.567
CD86						
High vs. Low	0.610	0.320-1.160	0.132	0.794	0.451-1.399	0.424
CD80						
High vs. Low	0.545	0.279-1.067	0.077	0.698	0.393-1.238	0.218
CD34						
High vs. Low	0.764	0.416-1.402	0.384	0.655	0.375-1.144	0.137
CD40						
High vs. Low	0.976	0.420-2.267	0.954	0.898	0.448-1.803	0.763
CD40LG						
High vs. Low	1.068	0.447-2.552	0.883	1.175	0.571-2.415	0.661

TNF						
High vs. Low	0.396	0.110-1.431	0.158	0.922	0.331-2.568	0.877
IL8						
High vs. Low	1.335	0.736-2.421	0.341	1.274	0.745-2.179	0.376
MMP2						
High vs. Low	0.925	0.526-1.629	0.788	1.069	0.642-1.781	0.798
THBD						
High vs. Low	0.876	0.460-1.669	0.687	1.074	0.605-1.907	0.808
CSF1R						
High vs. Low	1.304	0.717-2.371	0.384	1.076	0.630-1.836	0.789
CSF3R						
High vs. Low	0.692	0.361-1.324	0.266	0.588	0.330-1.049	0.072
IL23A						
High vs. Low	0.950	0.527-1.714	0.866	0.838	0.491-1.430	0.518
CCL2						
High vs. Low	0.954	0.476-1.911	0.895	0.963	0.523-1.775	0.904
CCL22						
High vs. Low	1.473	0.629-3.450	0.372	1.296	0.638-2.631	0.473
CCL5						
High vs. Low	0.471	0.259-0.858	0.014*	0.700	0.415-1.181	0.181
CXCL12						
High vs. Low	0.668	0.369-1.210	0.183	0.652	0.384-1.107	0.113
CXCR4						
High vs. Low	0.819	0.455-1.474	0.505	0.855	0.501-1.460	0.567
CD44						
High vs. Low	0.955	0.545-1.672	0.871	1.150	0.691-1.913	0.591

Gene expression levels dichotomised as high and low according to their median. The results were obtained using a univariate Cox regression method. CI, confidence interval, HR, hazard ratio; OS, overall survival; PFS, progression free survival; *$p < 0.05$.

Univariate Cox regression analysis showed significant associations between CD4 and CD8 expression levels and survival in NSCLCs. In particular, we observed that CD4 expression in the tumour compartment was correlated with OS [HR, 0.491; 95% CI, 0.269-0.896; $p = 0.021$] and PFS [HR, 0.585; 95% CI, 0.347-0.986; $p = 0.044$]. Kaplan-Meier analysis performed to obtain survival plots indicated that patients with high CD4 expression had a better OS (81.2 vs. 42.9 months, $p = 0.018$) and longer PFS (37.8 vs. 23 months, p 0.042; Figure 33a-b). CD8 was also significantly correlated with OS [HR, 0.340; 95% CI, 0.182-0.636; $p = 0.001$] and PFS [HR, 0.417; 95% CI, 0.243-0.716; $p = 0.002$]. In fact, the Kaplan-Meier test revealed that high levels of CD8 expression were associated with better survival (OS: 81.2 vs. 37.2 months, $p < 0.001$ and PFS: 81.2 vs. 19.4 months, $p = 0.001$; Figure 33c-d). In the stroma compartment no significant results were obtained for CD4 gene expression, and only for OS but not PFS for CD8 [HR, 0.521; 95% CI, 0.285-0.954; $p = 0.035$].

Table 28. Results from the survival analysis based on gene expression biomarkers in tumour areas.

TUMOUR						
	OS			PFS		
Variable	**HR**	**[95% CI]**	***p*-value**	**HR**	**[95% CI]**	***p*-value**
FOXP3						
High vs. Low	0.407	0.214-0.773	0.006*	0.521	0.298-0.911	0.022*
CD4						
High vs. Low	0.491	0.269-0.896	0.021*	0.585	0.347-0.986	0.044*
CD8						
High vs. Low	0.340	0.182-0.636	0.001*	0.417	0.243-0.716	0.002*
CD25						
High vs. Low	0.792	0.433-1.447	0.447	0.772	0.448-1.331	0.352
CD127						
High vs. Low	0.592	0.320-1.096	0.095	0.626	0.362-1.083	0.094
CTLA4						
High vs. Low	0.811	0.431-1.525	0.515	0.825	0.470-1.450	0.504
NRP1						
High vs. Low	0.757	0.420-1.362	0.353	0.912	0.539-1.543	0.731
LAG3						
High vs. Low	0.513	0.284-0.924	0.026*	0.649	0.382-1.102	0.109
GITR						
High vs. Low	0.859	0.318-2.320	0.765	0.736	0.337-1.607	0.442
TGFB1						
High vs. Low	0.534	0.298-0.957	0.035*	0.703	0.422-1.171	0.176
IL10						
High vs. Low	0.887	0.307-2.561	0.824	1.176	0.489-2.829	0.718
LGALS1						
High vs. Low	1.019	0.576-1.803	0.948	1.263	0.754-2.113	0.375
LGALS2						
High vs. Low	0.872	0.438-1.735	0.695	1.013	0.548-1.873	0.967
IDO1						
High vs. Low	0.573	0.313-1.052	0.072	0.598	0.348-1.027	0.062
ITGAM						
High vs. Low	0.941	0.519-1.706	0.842	0.715	0.416-1.228	0.224
ITGB2						
High vs. Low	1.162	0.652-2.069	0.611	0.647	0.376-1.115	0.117
CD97						
High vs. Low	0.659	0.367-1.183	0.163	0.772	0.456-1.307	0.335
IL4R						
High vs. Low	1.293	0.702-2.382	0.410	1.267	0.730-2.200	0.401
CD209						
High vs. Low	0.346	0.167-0.713	0.004*	0.302	0.157-0.581	0.000*
CD1C						
High vs. Low	0.527	0.223-1.249	0.146	0.738	0.364-1.497	0.400
CD86						
High vs. Low	0.739	0.401-1.364	0.334	0.791	0.456-1.371	0.403
CD80						
High vs. Low	0.478	0.243-0.941	0.033*	0.811	0.455-1.443	0.475
CD34						
High vs. Low	0.712	0.395-1.285	0.260	1.002	0.592-1.697	0.993
CD40						
High vs. Low	0.479	0.206-1.112	0.087	0.551	0.271-1.122	0.101
CD40LG						
High vs. Low	0.426	0.165-1.101	0.078	0.498	0.223-1.111	0.088

TNF *High vs. Low*	0.522	0.178-1.533	0.237	0.596	0.242-1.465	0.259
IL8 *High vs. Low*	1.012	0.556-1.844	0.968	0.624	0.363-1.072	0.088
MMP2 High *vs. Low*	0.485	0.262-0.896	0.021*	0.594	0.350-1.007	0.053
THBD *High vs. Low*	1.175	0.607-2.276	0.632	1.570	0.871-2.829	0.134
CSF1R *High vs. Low*	0.782	0.437-1.398	0.407	0.827	0.489-1.399	0.478
CSF3R *High vs. Low*	0.878	0.463-1.663	0.689	0.820	0.465-1.443	0.490
IL23A *High vs. Low*	0.514	0.283-0.934	0.029*	0.634	0.374-1.074	0.090
CCL2 *High vs. Low*	0.852	0.415-1.749	0.662	0.963	0.523-1.775	0.904
CCL22 *High vs. Low*	0.316	0.131-0.760	0.010*	1.296	0.638-2.631	0.473
CCL5 *High vs. Low*	0.639	0.350-1.169	0.146	0.622	0.361-1.071	0.087
CXCL12 *High vs. Low*	0.753	0.410-1.385	0.362	0.881	0.511-1.517	0.647
CXCR4 *High vs. Low*	0.920	0.513-1.650	0.781	0.976	0.575-1.656	0.928
CD44 *High vs. Low*	0.663	0.374-1.174	0.159	0.784	0.472-1.301	0.346

Gene expression levels dichotomised as high and low according to their median. The results were obtained using a univariate Cox regression method. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; *$p < 0.05$.

CD4 is a glycoprotein found on the surface of immune cells such as T helper cells, monocytes, macrophages, and DCs. The CD4 T helper lymphocyte is responsible for orchestrating two different but overlapping cytokine patterns that influence other effector cells and in turn shape the pattern of the inflammatory response. CD4 has been widely assessed in the tumour microenvironment by IHC, but little has been published regarding its expression at the mRNA level. Contradictory results have been reported regarding the presence of $CD4^+$ cells in different types of cancer. Some publications associate their presence with a better prognosis in pancreas adenocarcinoma and oesophageal squamous carcinoma (Cho et al., 2003; Fukunaga et al., 2004), whereas in others tumours , like in renal cell cancer, the presence of $CD4^+$ cells was correlated with shorter survival (Bromwich et al., 2003). In lung tumours, multivariate analyses show a low risk of death for SCC patients with a relatively high percentage of $CD4^+$ lymphocytes (da Costa et al., 2012). Other authors have reported that the presence of stromal $CD4^+$ lymphocytes correlates with improved survival (Al-Shibli et al., 2008; Hald et al., 2013; Wakabayashi et al., 2003). However, in our study mRNA expression levels of CD4 in stroma were not associated with prognosis.

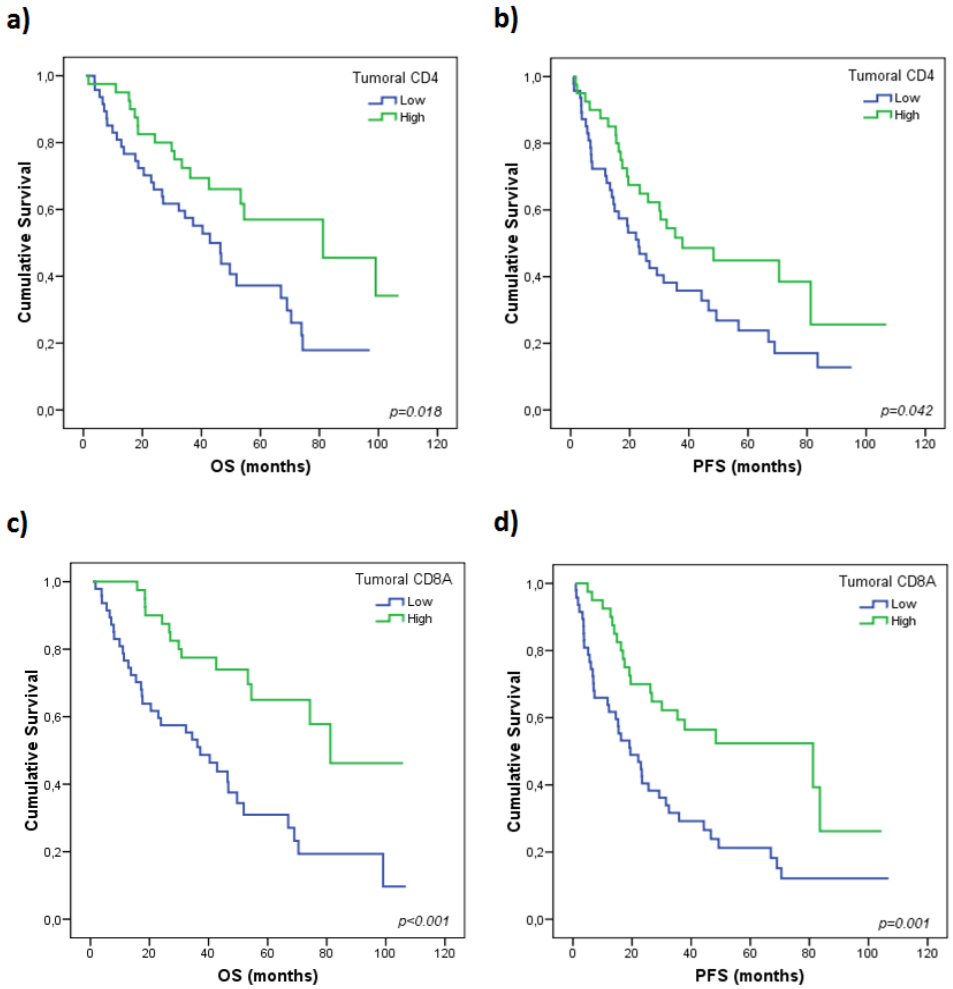


Figure 33. Kaplan-Meier plots for OS and PFS according to gene expression markers in the tumour compartment. a-b) Tumoural CD4 and c-d) tumoural CD8. Gene expression levels were dichotomised according to their medians. The blue line represents patients with low expression levels, whilst the green line represents patients with high expression levels. *P*-values were obtained using the Kaplan-Meier test.

CD8 is a transmembrane glycoprotein that serves as a co-receptor for the TCR, which is predominantly expressed on the surface of cytotoxic T cells, but can also be found on NK cells, cortical thymocytes, and DCs. The presence of $CD8^+$ cells in tumour specimens has been associated with a good prognosis in virtually every tumour in which it has been analysed (Fridman et al., 2012). Ruffini et al. showed that $CD8^+$ cells in NSCLCs were associated with prolonged survival, but only in a subset of SCCs (Ruffini et al., 2009). However, in our study this correlation was observed in the entire cohort, including ADC patients. Other studies have also reported that high $CD8^+$ cell infiltration in stroma was a favourable prognostic factor in NSCLC (Al-Shibli et al., 2008; Hiraoka et al., 2006a).

Another marker with a prognostic value when analysed only within the tumour nest was FOXP3. Univariate COX regression analysis showed that it correlates with both OS [HR, 0.407; 95% CI, 0.214-0.773; p = 0.006] and PFS [HR, 0.521; 95% CI, 0.298-0.911; p = 0.022]. Patients with high levels of FOXP3 had a better OS (NR vs. 37.2 months, p = 0.005, Kaplan-Meier log rank), and longer PFS (35.3 vs. 22.1 months, p = 0.020, Kaplan-Meier log rank). Interestingly, FOXP3 gene expression in stroma was not associated with prognosis (Figure 34a-b). This result is in line with our observations, described in the previous section, that higher levels of FOXP3 in the whole tumour were correlated with a better prognosis, although these results might seem controversial because FOXP3 is a specific Treg marker. As previously discussed, we analysed the mRNA expression markers present in the whole tumour microenvironment and therefore it is not possible to know which specific cells express this transcription factor. Therefore in this section we decided to evaluate the prognostic value of FOXP3 taking its location into consideration. The fact that FOXP3 was found to correlate with a better outcome in the tumour but not in the stroma compartment reinforces the theory that tumour cells may also express FOXP3.

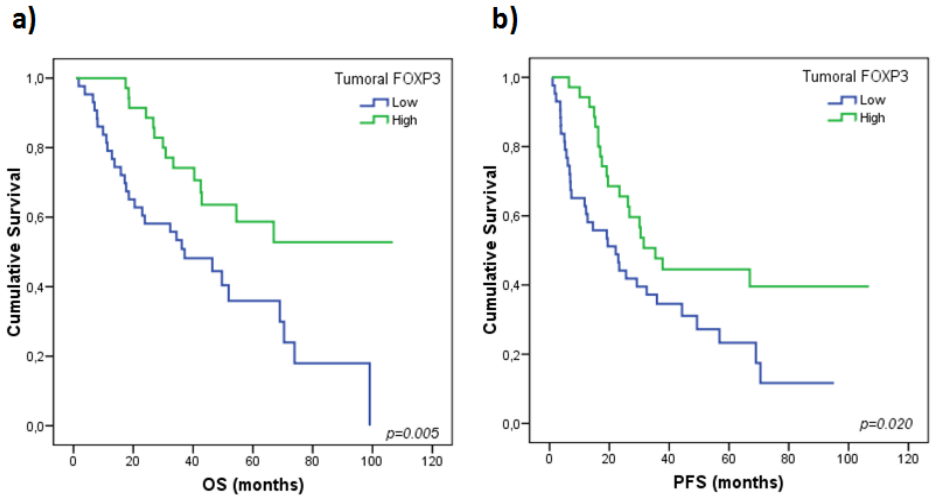


Figure 34. Kaplan-Meier plots for OS and PFS according to FOXP3 gene marker expression in the tumour compartment. a) OS and, b) PFS. Gene expression levels were dichotomised according to their median. Blue line represents patients with low levels of expression, whilst the green line represents patients with high levels of expression. *P*-values were calculated using the Kaplan-Meier test.

Other less-studied immunoregulatory genes were also associated with survival in our patient cohort. For instance, CCL5 expression levels in the stroma compartment were associated with patient survival [HR, 0.471; 95% CI, 0.259-0.858; p = 0.014]. Patients with high levels of CCL5 expression levels had a longer OS (70.4 vs. 37.2 months, p = 0.012), as shown in the Kaplan-Meier plot (Figure 35a). C-C chemokine ligand 5 (CCL5), also known as Regulated

upon Activation, Normal T-cell Expressed, and Secreted (RANTES), is expressed by T lymphocytes, macrophages and platelets, and plays an active role in recruiting a variety of leukocytes into inflammatory sites including T cells, macrophages, eosinophils, and basophils (Aldinucci and Colombatti, 2014). In collaboration with certain cytokines that are released by T cells such as IL2 and IFNγ, CCL5 induces the activation and proliferation of particular NK cells to generate C-C chemokine-activated killer cells (Soria and Ben-Baruch, 2008). CCL5 production is relevant to the induction of proper immune responses against tumours but it is also associated with cancer progression and metastasis (Hanahan and Coussens, 2012).

A number of solid tumours express CCL5 and/or CCR5, but the involvement of this pair of genes in cancer progression and development has only been studied in depth in some malignancies. CCL5 expression by breast tumour cells is a valuable prognostic factor for detection of stage II breast cancer in patients at risk for disease progression (Yaal-Hahoshen et al., 2006). In lung cancer, CCL5 mRNA expression by tumour cells in patients with stage I lung adenocarcinoma was associated with improved survival (Moran et al., 2002). The authors pointed out that the reasons for the different prognostic values obtained from one tumour type to another remain unknown and therefore may be worthy of future study. A recent publication showed that low plasma levels of CCL5 at the time of diagnosis (in patients treated with EGFR-TKIs) were significantly associated with long-term survival. These results suggest that the network of pro-inflammatory cytokines was affected by EGFR-TKI treatment and that the patient outcome may be influenced by the status of plasma pro-inflammatory cytokines at diagnosis (Umekawa et al., 2013).

In the tumour nest, more markers proved to have a prognostic value than in the stroma compartment. For instance, we found an association between gene expression levels and OS for LAG3 [HR, 0.513; 95% CI, 0.284-0.924; p = 0.026], and patients that had high expression levels of this marker had better outcomes (69 vs. 36.2 months, p = 0.023 Kaplan-Meier test; Figure 36a). LAG3 was cloned over 20 years ago as a CD4 homologue, but its function as an immune checkpoint was not defined until 2005 when it was shown to have a role in enhancing the function of Treg cells. LAG3 is one of various immune-checkpoint receptors that are coordinately upregulated on both Treg and anergic T cells, and PD1 and LAG3 are commonly co-expressed on anergic or exhausted T cells (Pardoll, 2012). LAG3 blockade, alone and in combination with nivolumab, is currently being explored in early-phase investigations (Creelan, 2014). To the best of our knowledge, the prognostic value of LAG3 has not yet been assessed in NSCLCs or in any other tumour types. Therefore our results are the

first to indicate the positive prognostic value of this immune checkpoint, which may have a role in cancer that is similar to PD1. However, further studies are still required to validate the prognostic role of LAG3 and to evaluate if LAG3 blockade is of any clinical use for immunotherapy.

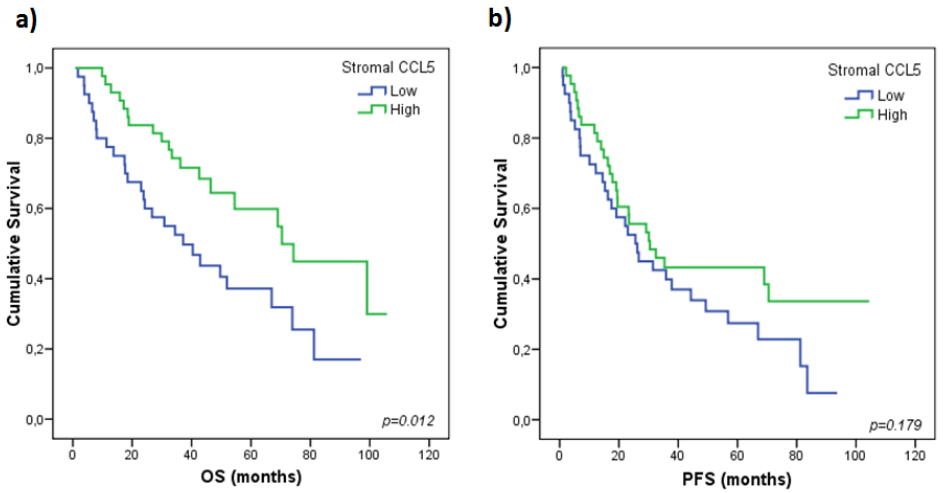


Figure 35. Kaplan-Meier plots for OS and PFS according to CCL5 gene expression in the stroma compartment. a) OS and, b) PFS. Gene expression levels were dichotomised according to their medians. The blue line represents patients with low levels of expression, whilst the green line represent patients with high levels of expression. *P*-values were calculated using the Kaplan-Meier test.

Another marker which was significantly associated with OS when expressed in the tumour compartment was TGFB1 [HR, 0.534; 95% CI, 0.298-0.957; p = 0.035]. The group of patients with higher TGFB1 expression levels had a better OS (46.6 vs. 74.3 months, p = 0.032 Kaplan-Meier test; 36b). The TGFB1 signalling pathway plays a critical and dual role in the progression of human cancer: during the early phase of tumour progression, TGFB1 acts as a tumour suppressor, but it promotes processes that support tumour progression such as tumour cell invasion, dissemination, and immune evasion. Consequently, the functional outcome of the TGFB1 response is strongly dependent on the context of the cell, tissue, cancer type, etc. (Meulmeester and Ten, 2011). Most of the studies reporting TGFB1 expression assessed its expression using two different approaches: IHC or enzyme-linked immunosorbent assay (ELISA). Using the first approach Valkov et al. observed that high TGFB1 expression was an independent negative prognostic factor for disease-specific survival (DSS) in soft tissue sarcomas (Valkov et al., 2011). Other work using the second approach showed that serum TGFB1 levels were elevated in breast cancer patients and that this had a favourable prognostic value (Ciftci et al., 2014). In NSCLC, recent work assessed TGFB1 expression using IHC in 105 samples and reported that patients with positive TGFB1 and negative Eps15 homology domain

1 (EHD1) expression had much longer survival times than patients with other combinations (Gao et al., 2014). However, other studies have reported the opposite prognosis for high TGFB1 expression levels (Zhao et al., 2010).

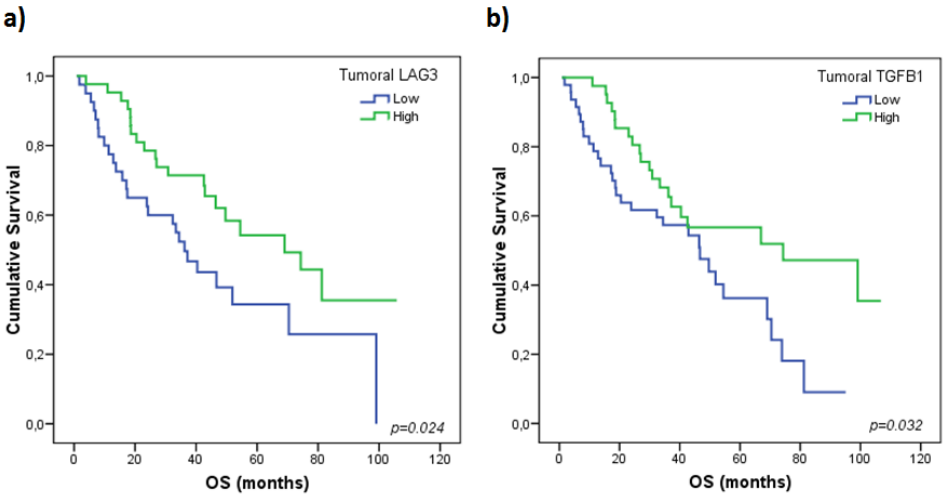


Figure 36. Kaplan-Meier plots for OS according to gene expression levels in the tumour compartment. a) LAG3 and b) TGFB1. Gene expression levels were dichotomised according to their medians. The blue line represents patients with low levels of expression, whilst the green line represents patients with high levels of expression. *P*-values were calculated using the Kaplan-Meier test.

Expression of CD209 in the tumour compartment was also associated with a better OS [HR, 0.346; 95% CI, 0.167-0.713; $p = 0.004$] and longer PFS [HR, 0.302; 95% CI, 0.157-0.581; $p < 0.001$], and Kaplan-Meier plots (Figure 37a-b) indicated that patients with high expression levels of CD209 had a better OS (34.4 vs. 81.2 months, $p = 0.003$) and PFS (16.2 vs. 81.2 months, $p < 0.001$). This is the first study to explore the prognostic value of CD209, which is also known as DG-SIGN. DC-SIGN is a type II transmembrane C-type lectin receptor that is mostly expressed on myeloid DCs, but can also be expressed in macrophages. DCs have often been denominated master immune response regulators and several groups have proposed vaccination strategies aimed at targeting antigens to DCs for the treatment of cancer (van et al., 2013). In a recent publication, the expression of soluble DC-SIGN (sDC-SIGN) and its receptor was evaluated by ELISA in the serum of patients with colon cancer, and by IHC in cancer tissue, showing that high levels of sDC-SIGN were indicative of much longer survival times (Jiang et al., 2014). Although in this publication it was the soluble form that had a prognostic value, our observations are in line with these results. In summary, it seems that CD209 expression may indicate the presence of DCs which might have a positive impact on the tumour microenvironment. Therefore follow-up studies with larger cohorts should be carried out in order to validate the prognostic value of this very promising immune-marker.

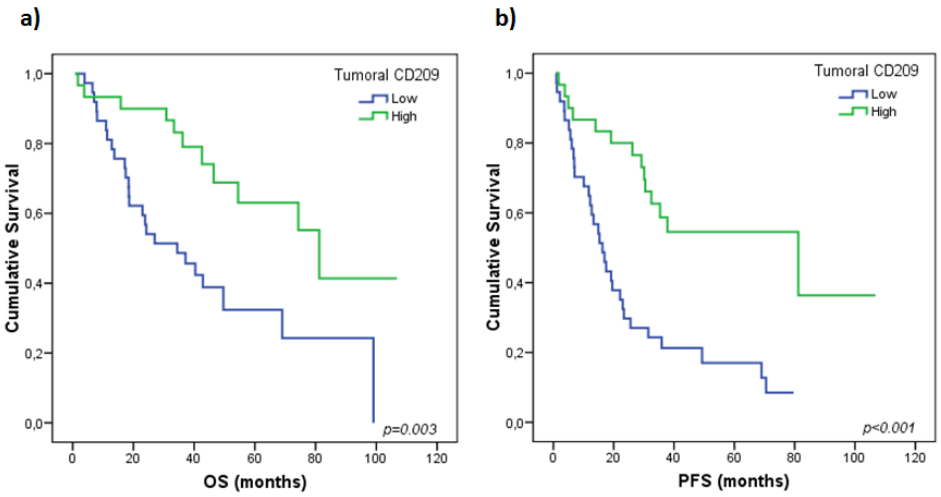


Figure 37. Kaplan-Meier plots for OS and PFS according to CD209 gene expression levels in the tumour compartment. a) OS and, b) PFS. Gene expression levels were dichotomised according to their medians. The blue line represents patients with low levels of expression, whilst the green line represent patients with high levels of expression. *P*-values were calculated using the Kaplan-Meier test.

Univariate analysis was also performed in ADC and SCC patients separately. The number of patients was quite low but some of the statistically significant results obtained in the entire cohort were also observed. In ADC patients, we found that tumoural expression levels of CD4, CD8, and FOXP3, but not the rest of the markers, were associated with survival. In SCC patients CD8 expression in the stroma and CD209 expression in the tumour were correlated with OS and PFS (Supplementary Table 2).

2.4.4. COMBINED BIOMARKERS

In this part of the study combined biomarkers were assessed in order to evaluate the prognostic value of some of the biomarkers together. Only the combinations that were associated with survival have been included. First we analysed the CD25 and CD127 combination. Although FOXP3 is a specific marker of regulatory T cells, these can also be defined as T cells expressing high levels of CD25 and low levels of CD127 (Liu et al., 2006). Therefore, we decided to evaluate the prognostic role of this combination in both the tumour and stroma compartments. To do this we calculated the ratio of CD25 to CD127 for both sample sets, which showed that patients with a high ratio level in the stroma compartment (patients with high CD25 expression and low CD127 expression) had a worse OS [HR, 2.089; 95% CI, 1.093-3.991; p = 0.026] and shorter PFS [HR, 1.882; 95% CI, 1.066-3.322; p = 0.029]; the differences in the median survival rates for OS were 42.9 vs. 81.2 months (p = 0.023, Kaplan-Meier test) and for PFS it was 19.1 vs. 35 months (p = 0.029, Kaplan-Meier test; Table 29; Figure 38).

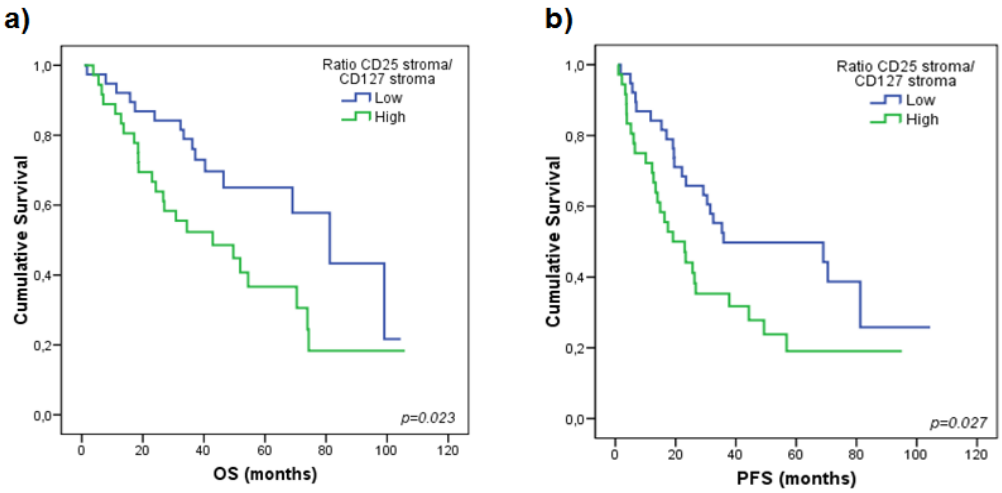


Figure 38. Kaplan-Meier plots for OS and PFS according to the combination of CD25 and CD127 in the stroma compartment. a) OS and, b) PFS. Gene expression levels were dichotomised according to their medians. The green line represents patients with high levels of the ratio, whilst the blue line represents patients with low levels. *P*-values were calculated using the Kaplan-Meier test.

This combination of markers has been previously used to isolate Tregs from the T cell population (Yu et al., 2012); therefore, our results might indicate that patients with a Treg phenotype in the stroma compartment have worse survival rates than the other groups of patients. However, since the expression of these markers was assessed at the mRNA level, their specific cell origin remains unknown. No previous studies have evaluated the CD25 and CD127 combination at the mRNA level; however the frequency of $CD4^{+}$ $CD25^{high}$ $CD127^{low}$ cells assessed by flow cytometry in patients with gastric cancer suggested that these cells are present at higher frequencies in patients with advanced stages of the disease (Shen et al., 2009).

We also decided to further study the prognostic value of conventional T cell markers such as CD4 (a T helper cell marker), and CD8 (a T cytotoxic cell marker) in combination with FOXP3, the most specific Treg marker so far found. To do this we calculated new variables based on the ratio of these markers. From the different combinations that were tested by univariate Cox regression analysis, we found that the ratio between FOXP3 expression assessed in the stroma compartment, and the expression of CD4 and CD8 in the tumour nest, had a prognostic value. In fact, patients with high FOXP3 expression levels in the stroma and low CD4 levels in the tumour, or high FOXP3 levels in stroma and low CD8 levels in the tumour, had worse survival rates. In particular, patients with a high Foxp3 stroma/CD4 tumour ratio had a worse OS [HR, 2.341; 95% CI, 1.179-4.649; p = 0.015], with a median survival of 46.6 vs. 81.2 months (p = 0.012, Kaplan-Meier test) and a worse PFS [HR, 2.070; 95% CI, 1.149-3.730; p

= 0.015], with a median survival of 19.4 vs. 37.8 months (p = 0.013, Kaplan-Meier test; Figure 39a-b). As for the FOXP3 stroma/CD8 tumour ratio, patients with a high ratio had a worse OS [HR, 2.141; 95% CI, 1.083-4.232; p = 0.029] and PFS [HR, 1.830; 95% CI, 1.012-3.310; p = 0.046]. Kaplan-Meier plots (Figure 39c-d) indicated that the median survival was shorter than in the low ratio group for both OS (46.4 vs. 74.3 months, p = 0.025) and PFS (23 vs. 37.8, months p = 0.042; Table 29). Although, we cannot be certain about the origin of any of these markers due to the nature of the methodology we used, we can say that the proportion of certain immune mRNA markers in the different locations of the tumour microenvironment have a prognostic value. To the best of our knowledge, no similar studies have been reported so far, and any attempt to evaluate the proportion of immune cells has always been made by IHC.

The ratio between FOXP3 expression in the stroma to its expression in the tumour was also evaluated. The rationale for doing this analysis was that high FOXP3 expression in the tumour compartment alone was correlated with better survival rates, but patients with high expression levels of the same marker in the stroma in comparison to the expression of CD4 and CD8 in the tumour had shorter survival rates. Thus, to further analyse this matter, we calculated a new ratio (FOXP3 stroma/FOXP3 tumour ratio), observing that patients with high levels of FOXP3 in stroma and low levels of FOXP3 in tumour had a worse OS [HR, 3.150; 95% CI, 1.464-6.777; p = 0.003], with a median survival of 42.9 months vs. NR (p = 0.002, Kaplan-Meier test) and a shorter PFS [HR, 2.962; 95% CI, 1.545-5.679; p = 0.001] with a median survival of 19.4 months vs. NR (p = 0.001 Kaplan-Meier test; Figure 39e-f; Table 29).

Table 29. Results from univariate Cox regression analysis of combined biomarkers.

	OS			PFS		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Ratio CD25 stroma/ CD127 stroma						
High vs. low	2.089	1.093-3.991	0.026*	1.882	1.066-3.322	0.029*
Ratio FOXP3 stroma/ CD4 tumour						
High vs. Low	2.341	1.179-4.649	0.015*	2.070	1.149-3.730	0.015*
Ratio FOXP3 stroma/ CD8 tumour						
High vs. Low	2.141	1.083-4.232	0.029*	1.830	1.012-3.310	0.046*
Ratio FOXP3 stroma/ FOXP3 tumour						
High vs. Low	3.150	1.464-6.777	0.003*	2.962	1.545-5.679	0.001*

Gene expression levels dichotomised as high and low according to their medians. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; *$p < 0.05$.

From these results we infered that the prognostic value of FOXP3 expression depends on its location. When we analysed FOXP3 expression in the stroma compartment alone no association with survival was observed. However, if we take what is happening in the tumour at the same time into account, the picture changes and a subgroup of patients with high expression levels of FOXP3 in the stroma but low expression levels in the tumour associated with worse survival emerge. The reasons behind this phenomenon remain unknown, but one could hypothesise that FOXP3 expressed by other cells, such as tumour cells for instance (Tao et al., 2012), may attenuate the negative prognostic effect of FOXP3 expression by Tregs in the stroma compartment. In order to obtain further insight into this problem and to elucidate what is happening in the tumour microenvironment in NSCLCs, the presence of Tregs in situ was assessed by IHC and its prognostic consequences were analysed.

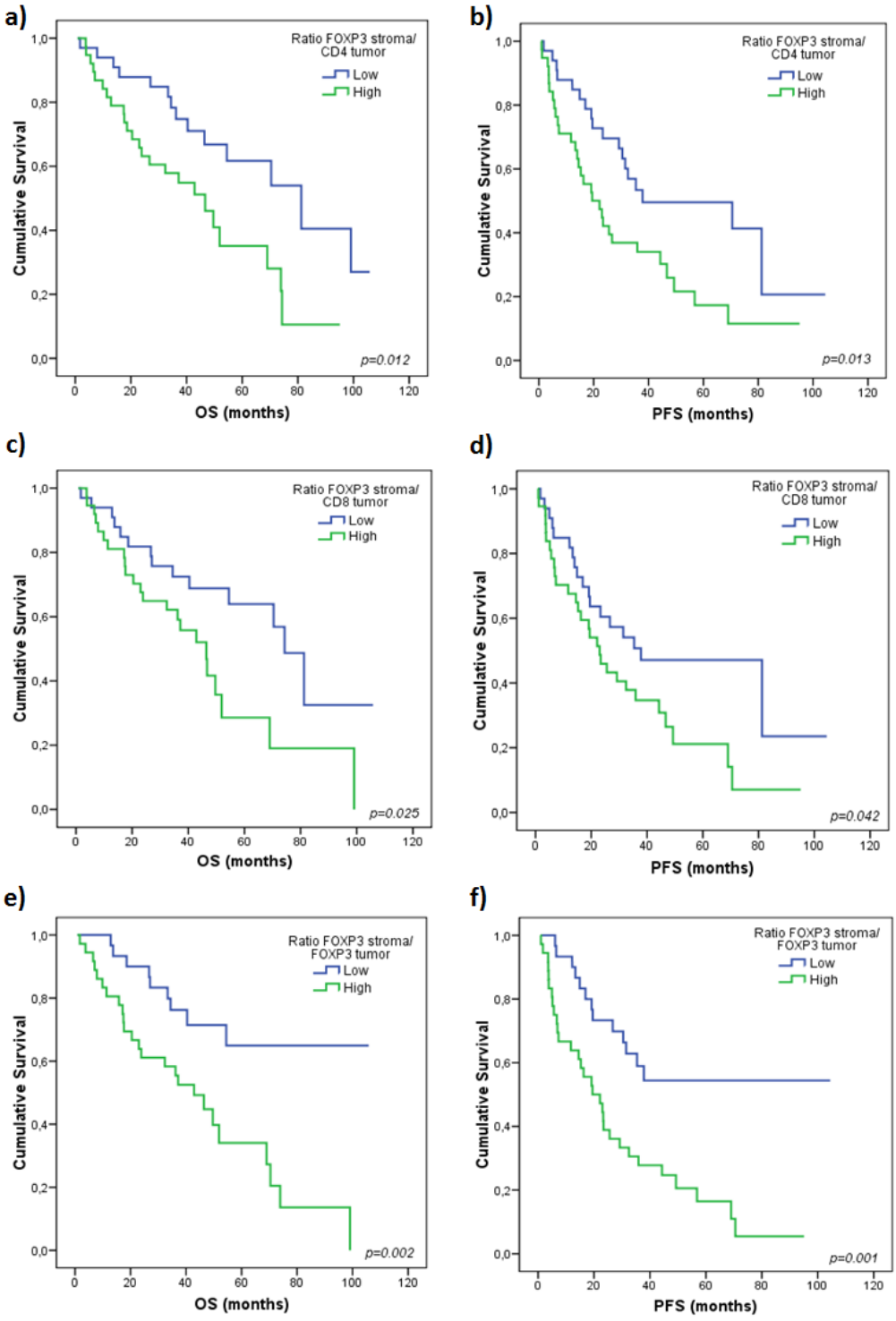


Figure 39. Kaplan-Meier plots for OS and PFS according the ratios calculated. a-b) FOXP3 stroma/ CD4 tumour ratio, c-d) FOXP3 stroma/CD8 tumour ratio, and e-f) FOXP3 stroma/FOXP3 tumour ratio. Gene expression levels were dichotomised according to their medians. The blue line represents patients with low levels, whilst the green line represents patients with high levels. *P*-values were calculated using the Kaplan-Meier test.

2.4.5. GENE EXPRESSION SCORE

Gene expression levels obtained in this part of the study were also used to find a score associated with patient survival. As described in a previous section, and following the criteria from Lossos et al. (Lossos et al., 2004), genes were selected to comprise the expression score according to their Z-scores. Expression levels of CD80, CXCL12, and CCL22 were selected for construction of the stroma score, whilst expression of CD25, CD4, TGFB1, CD44, CD1C, and THBD were used to construct the tumour score (Figure 40).

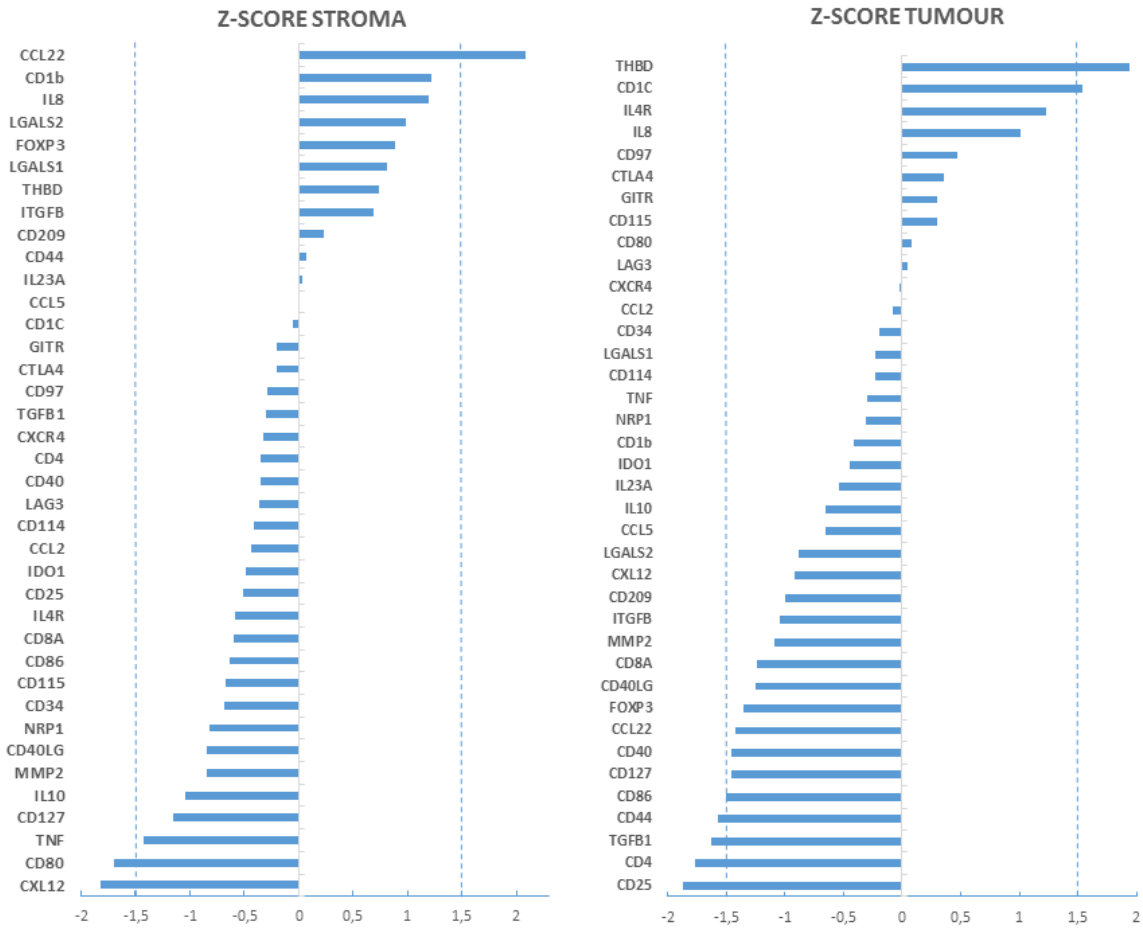


Figure 40. Univariate analysis of 37 genes analysed in order to assess their association with the overall survival (OS). The genes are ranked based on their predictive power (univariate Z-score). Dashed lines indicate |Z-score| = 1.5. Blue bars represent the Z-scores. This criterion was used for selecting genes to include in the multivariate Cox regression model used to calculate the expression score.

These genes were selected to construct the prognostic signatures by introducing them into multivariate models; the results from these are detailed in Table 30 and Table 31. Positive regression coefficients from multivariate analysis indicate that the genes are associated with

longer survival, whilst genes with negative regression coefficients are associated with shorter survival. The expression scores were classified as high or low according to their median.

Table 30. Results from the multivariate model with genes included in the stromal expression score.

Variable	Regression coefficient	SE	*p*-value	HR	95% CI
CD80	-0.034	0.036	0.339	0.966	0.900-1.037
CXCL12	-1.176	0.651	0.070	0.308	0.086-1.103
CCL22	0.030	0.013	0.024	1.030	1.004-1.058

CI, confidence interval; HR, hazard ratio; SE, standard error.

Table 31. Results from the multivariate model with genes included in the tumoural expression score.

Variable	Regression coefficient	SE	*p*-value	HR	95% CI
CD25	-0.027	0.016	0.085	0.973	0.944-1.004
CD4	-0.197	0.169	0.242	0.821	0.590-1.143
TGFB1	-0.112	0.140	0.424	0.894	0.679-1.177
CD44	-0.575	0.267	0.031	0.562	0.333-0.948
CD1C	0.246	0.083	0.003	1.280	1.088-1.504
THBD	2.205	0.782	0.005	9.072	1.959-42.015

CI, confidence interval; HR, hazard ratio; SE, standard error.

Therefore, the formula used for the stroma score was:

(CD80 x -0.034) + (CXCL12 x-1.176) + (CCL22 x 0.030)

The formula used for the tumour score was:

(CD25 x -0.027) + (CD44 x -0.575) + (CD1C x 0.246) + (THBD x 2.205) + (CD4 x -0.197) + (TGFB1 x -0.112)

The prognostic impact of tumour and stroma expression scores were then evaluated, observing that: a) the stroma-expression score did not correlate with survival, and b) a high tumour-expression score significantly correlated with worse OS (33.4 vs. 99 months, $p = 0.001$) as well as with shorter PFS, although this latter association was weaker (19.4 vs. 35.4 months, $p = 0.026$; Figure 41a-b). Because a high tumour-expression score was correlated with worse survival, it should be considered as a risk score.

The association between the tumour-risk score (TRS) and the clinicopathological variables was assessed, and a correlation between this score and tumour size (≤ 3.5 and > 3.6 cm) was observed (p = 0.006, Chi-square test). Patients with larger tumour sizes were more likely to be classified as having a high expression-TRS. When stratified according to the tumour size, the tumour-expression score was associated with OS in patients with large tumours (23.8 vs. NR months, p = 0.001).

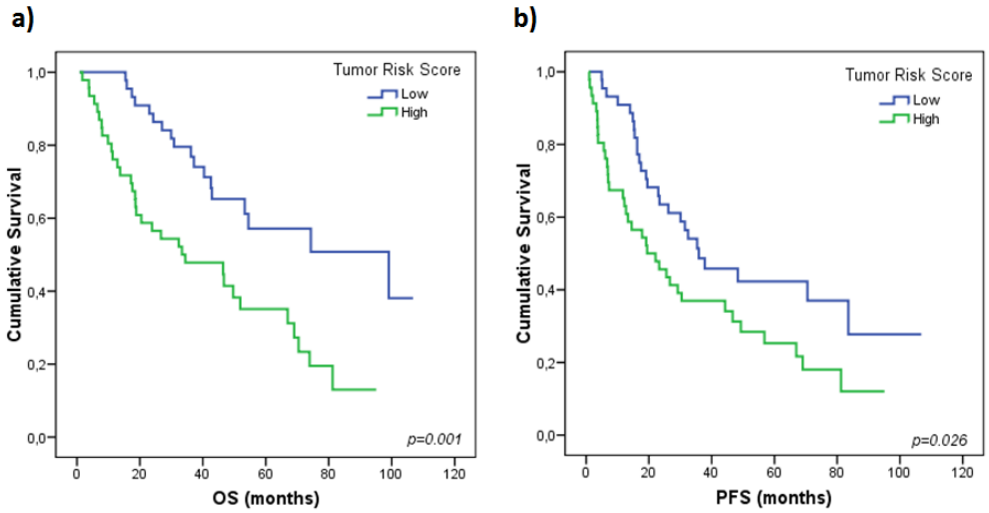


Figure 41. Kaplan-Meier plots according to the tumour risk score (TRS). a) OS and, b) PFS. The score was divided into low and high according to its median. The blue line represents patients with low levels of expression, whilst the green line represents patients with high score levels. *P*-value calculated using the Kaplan-Meier test.

There was no correlation between survival and the stroma expression score, which comprised the expression of genes related to immune cell trafficking and antigen presentation. This lack of correlation is in line with results observed in individual gene expression survival analysis in the stroma compartment, with the exception of CD8 and CCL5. However, unsupervised hierarchical analysis, based on gene expression data from the stroma compartment, revealed two clusters with different prognostic values. The fact that the combination of a larger number of genes was related to the prognosis of patients in the stroma compartment, but very few significant associations were observed when a small group of genes or single genes were analysed, might be an indication of the complexity of the stroma, which is composed of a variety of cells, apart from infiltrating immune cells.

On the other hand, the tumour risk score comprised the expression of six genes (CD25, CD44, CD1C, THBD, CD4, and TGFB1), some of which are related to the presence of immune cells such as lymphocytes and DCs, involved in immunoregulation and inflammation. In

particular, CD25 is expressed in a variety of immune cells including activated CD4 T cells, as well as in Tregs; CD44 is implicated in migration after T cell activation and is related to memory T cells (Zoller, 2011); CD1C is an APC marker but also marks some types of MDSCs; CD4 is a T helper cell marker and TGFB1 is an immunosuppressive factor. The last component of the score was THBD, which was given the biggest weight in the equation. This gene encodes a transmembrane glycoprotein, known as thrombomodulin, which has potent anticoagulant activity and also has anti-inflammatory functions mediated via a variety of molecular mechanisms. Thrombomodulin is implicated in the production of activated protein C and in the suppression of thrombin, a potent inflammatory reaction stimulator which regulates the proliferation and activation of lymphocytes and monocytes (Li et al., 2012). Moreover, thrombomodulin inhibits neutrophil and monocyte adhesion to endothelium as well as complement activation. However, insufficient information is currently available regarding its role in cancer (Conway, 2012).

The expression of these genes is likely to be correlated with the immune-state of the tumour nest and it might provide information regarding the cross-talk that takes place between the tumour cells and immune cells present there. Our results indicate the presence of markers related to immune cells that may produce a response against tumour cells, as well as the possible presence of immunosuppressive factors and immunoregulatory cells. Although future research is needed, it is possible that this prognostic score, or a similar inflammatory or immune-related biomarker, may be able to identify patients who are more or less likely to respond to immunotherapy.

Multiple gene expression-based scores that are predictive for survival outcomes have been identified in the last few years. Here, we used a predictive model based on the multivariate analysis first reported by Lossos et al. (Lossos et al., 2004) and slightly modified by Schetter et al. (Schetter et al., 2009). In the latter study, the authors reported an expression pattern of inflammatory-related genes in the tumour and paired non-cancerous tissues that was an independent prognostic marker for colon adenocarcinoma patients, and which indicated the importance that inflammatory cells, and consequently immune cells, have in the cancer prognosis.

2.4.6. MULTIVARIATE ANALYSIS

In this section multivariate analysis was performed with biomarkers that were significantly associated with prognosis ($p < 0.05$). To construct OS and PFS multivariate models, we introduced the following variables: individual gene expression values correlated with survival in both the tumour and stroma compartments (detailed in the section 2.4.3), the significant combinations (section 2.4.4), expression clusters, and tumour risk score. In the PFS model the tumour size was also included. The results for OS indicated that the expression of CD8 in the stromal compartment was an independent prognostic biomarker, whereas for PFS the resulting independent biomarkers were the expression of CD8 in the tumour and the ratio between the expression of CD25 and CD127 in the stroma compartment (Table 32).

Table 32. Results from multivariate Cox regression model including all the significant factors from this part of the study.

	OS			PFS		
Variables	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Stromal CD8 expression *High vs. Low*	0.171	0.048-0.610	0.007	-	-	-
Tumoural CD8 expression *High vs. Low*	-	-	-	0.219	0.098-0.490	<0.001
Ratio CD25 stroma/ CD127 stroma *High vs. Low*	-	-	-	2.601	1.220-5.547	0.013

CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival.

The main conclusion that can be drawn from these results is that CD8 expression plays a pivotal role in the tumour microenvironment in NSCLCs. The objective here was to obtain surrogate markers of the tumour microenvironment immunity and to assess their prognostic value. High expression of CD8 mRNA may indicate the presence of high numbers of cytotoxic T cells, which have an important role in antitumour immunity, as demonstrated in a number of different cancer types, like for instance, breast cancer (Ali et al., 2014). CD8 T cells can circumvent many of the barriers inherent in cancer-induced stroma, while optimizing T-cell specificity and producing antitumour effects (Kawai et al., 2008).

The other biomarker that was found to be an independent prognostic factor for PFS was the CD25/CD127 ratio in the stroma compartment. It was observed that patients with high ratio levels, and therefore high CD25 expression, and low CD127 expression in the stroma had worse survival rates. As previously mentioned, this combined biomarker has also been used to analyse Treg infiltration in some studies. Because this phenotype is correlated with worse survival, and Tregs have been previously associated with this outcome (Shimizu et al., 2010),

one might think that the stroma of these patients could be populated with this subset of immune cells and that their presence there would have a prognostic value. However, this association was not observed in the tumour compartment. Therefore it would be interesting to reassess the presence of Tregs in the stroma compartment in situ in order to corroborate these findings.

Altogether, the results detailed in this part of the study indicate the importance of the immune system in the tumour microenvironment and how the expression of immune-related genes has a different prognostic impact depending on its location. To further validate these results and to gain greater insight into the immune picture in the NSCLC microenvironment, the presence of three different types of immune cells was assessed by IHC, as detailed in the following section of this thesis.

B) TUMOUR MICROENVIRONMENT INFILTRATING IMMUNE CELLS

1. IHC ANALYSIS OF CD4, CD8, AND FOXP3

IHC analysis was performed in 84 FFPE tumour samples in order to detect the presence of certain tumour-infiltrating immune cells in the tumour and the tumour-associated stroma in NSCLCs. We assessed three protein markers, each of them characteristic of one type of immune cell: CD4 (helper T cells), CD8 (cytotoxic T cells), and FOXP3 (regulatory T cells). The system used for this analysis was a Dako Autostainer Link 48 combined with a Dako EnVision™ FLEX detection system. A negative and a positive control were included in each run. The positive control for the three antibodies was human normal tonsil tissue (recommended on the data sheet); all the preparations were referred back to these controls. In tonsil tissue samples, positive CD4 and CD8 staining was observed in the cytoplasm of lymphocytes, and in the nucleus for FOXP3 staining (Figure 42). We did not observe any positive staining in the negative controls included.

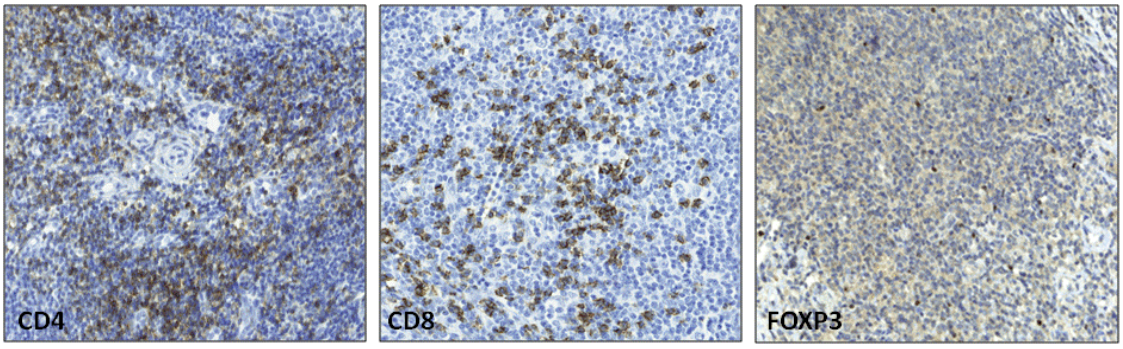


Figure 42. Positive cells for CD4, CD8 and FOXP3 in normal human tonsil samples. The figures represent positive staining for CD4 and CD8 in lymphocyte cytoplasm and in the nucleus for FOXP3. Original magnification, X200.

2. DETECTION OF INFILTRATING IMMUNE CELL MARKERS

Under HPF (X400) magnification each sample was semiquantitatively scored for the degree of immune cell infiltration into the tumour nest and tumour-associated stroma. Three types of immune cells were studied: $CD4^+$, $CD8^+$, and $FOXP3^+$ cells. $CD4^+$ cells were detected in all the assessable samples, although in 13 (15.5%) of the 84 samples, positive cells were only found in the stroma location in which there were significantly more positive TILs (pair-wise Wilcoxon test, $p < 0.001$; Figure 45a). The number of $CD4^+$ cells ranged from 1 to 76 (median: 18.8, mean: 20.2) per HPF in the stroma location and from 0 to 21 (median: 1.8, mean: 3.5) per HPF within the tumour. Patients were classified with low or high $CD4^+$ cell infiltration in the

tumour or stroma location according to the median calculated in each case. Examples of IHC images representing different $CD4^+$ cell scores in the tumour and stroma are shown in Figure 43.

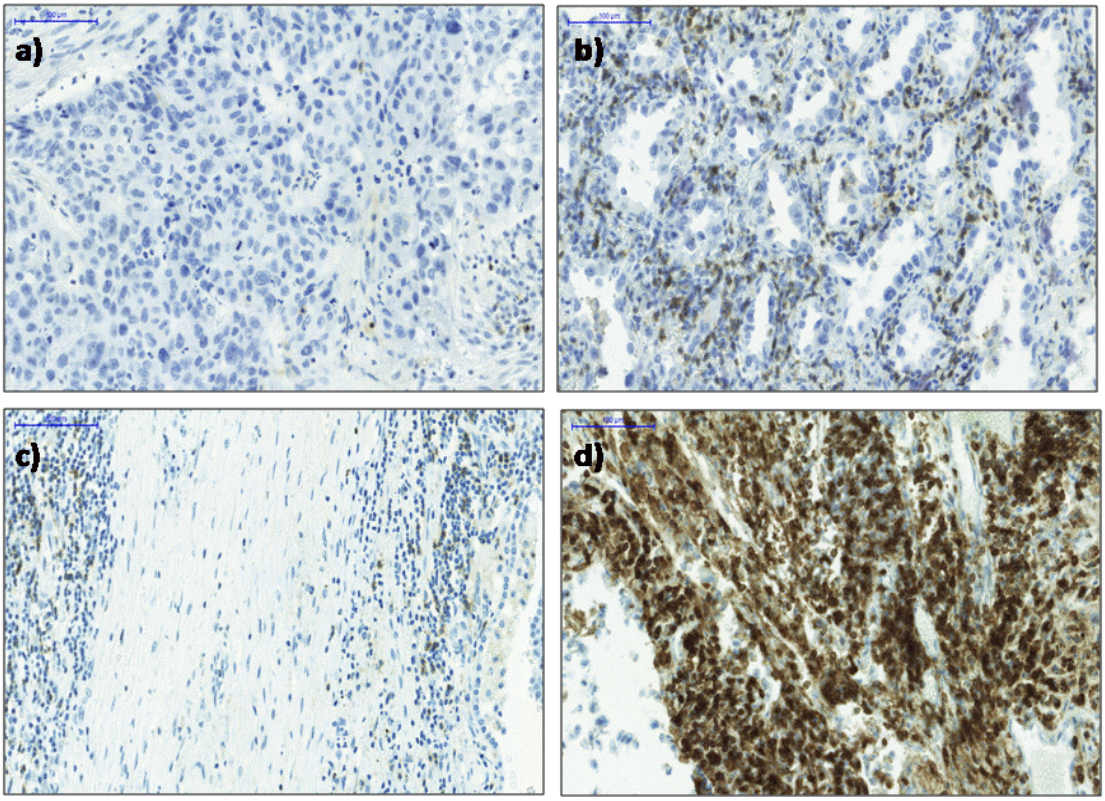


Figure 43. Representative immunohistochemical CD4 staining. Microscopic immunohistochemical images of NSCLC samples with $CD4^+$ cell staining. a) Tumour area with low $CD4^+$ cell infiltration, b) tumour area with high $CD4^+$ cell infiltration, c) stroma area with low $CD4^+$ cell infiltration and, d) stroma area with high $CD4^+$ cell infiltration. Original magnification X200.

$CD8^+$ cells were also detected in all the assessable samples, and were present throughout tumours, with a tendency towards a stromal location (pair-wise Wilcoxon test, $p < 0.001$; Figure 45b). The number of $CD8^+$ cells ranged from 3 to 73 (median: 29.8, mean: 29) per HPF in the stroma location and from 1 to 82 (median: 5.6, mean: 8.5) per HPF within the tumour. Patients were classified as having low or high $CD8^+$ cell infiltration in the tumour and stroma location according to the median calculated in each case. Examples of IHC images representing different $CD8^+$ cell infiltration scores in tumour and stroma locations are shown in Figure 44.

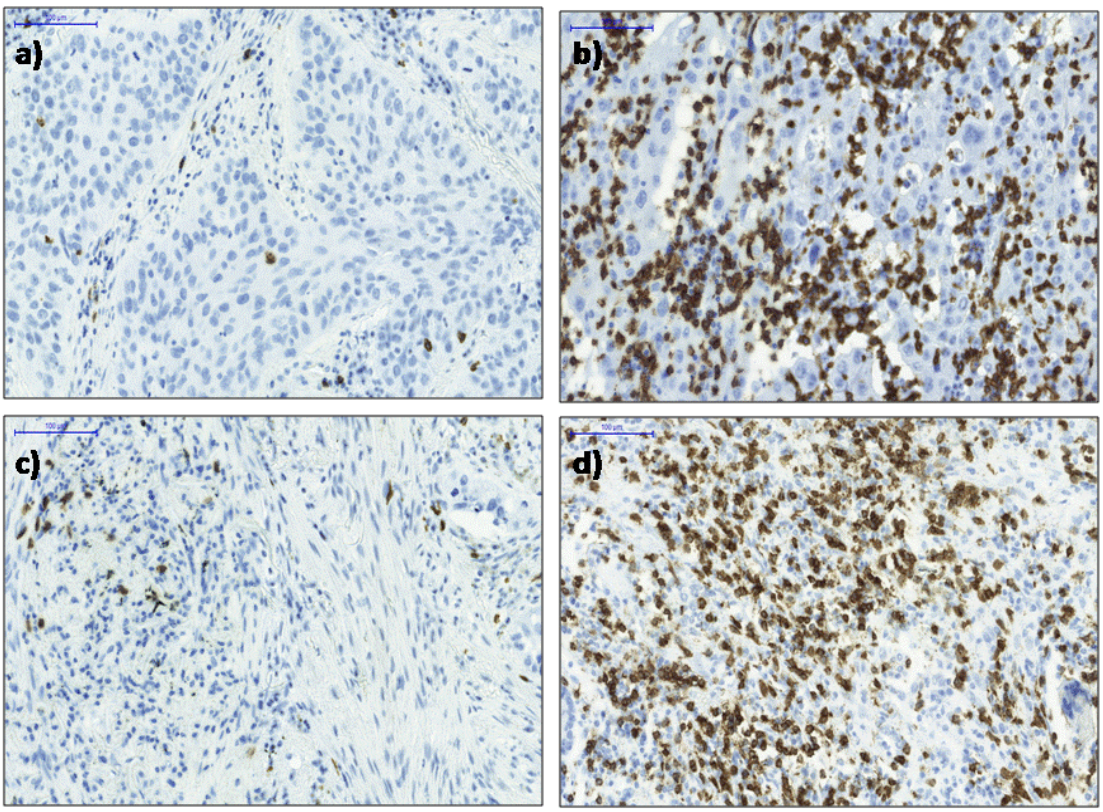


Figure 44. Representative immunohistochemical CD8 staining. Microscopic immunohistochemical images of NSCLC samples with $CD8^+$ staining cells. a) Tumour area with low $CD8^+$ cell infiltration, b) tumour area with high $CD8^+$ cell infiltration, c) stroma area low high $CD8^+$ cell infiltration and d) stroma area with high $CD8^+$ cell infiltration. Original magnification, X200

The presence of $FOXP3^+$ cells was evaluated by two different approaches. First, the percentage of infiltrating lymphocytes with nuclear-staining in both the tumour and stroma compartments was defined and graded as: no staining, less than 10% positive lymphocytes, 10-33%, and more than 33% of the total number of lymphocytes present in these areas. $FOXP3^+$ cells were detected in 80 (95.2%) of the 84 assessable samples, although in 8/80 (10%) of the samples, positive cells were only found in the stromal location, and in one sample (1.8%), positive cells were only found in the tumour compartment. Most of the cells in the samples, in both the stroma (60.8%) and tumour compartment (85.7%), were less than 10% FOXP3 positive (detailed information is provided in Table 33). This percentage was established as a cut-off value for the dichotomisation of $FOXP3^+$ cells into low and high percentages.

Furthermore, the total number of $FOXP3^+$ cells per HPF was also assessed. Again, there were significantly more positive TILs (pair-wise Wilcoxon test, $p < 0.001$; Figure 45c) in the stroma than in the tumour compartment. The number of $FOXP3^+$ cells ranged from 0 to 45 (median: 11.6, mean: 13.7) per HPF in the stroma location and from 0 to 15 (median: 1, mean:

1.6) per HPF within the tumour. Patients were classified as having low or high FOXP3$^+$ cell infiltration in tumour or stroma location according to the median calculated in each case. Examples of IHC images representing different FOXP3$^+$ cell scores in the tumour and stromal locations are shown in Figure 46.

Table 33. Percentages of FOXP3$^+$ cells in either the stroma or the tumour compartment.

	Stroma		Tumour	
	N	%	N	%
No staining	5	6.0	12	14.3
< 10%	46	54.8	60	71.4
10-33%	32	38.1	11	13.1
>33%	1	1.2	1	1.2

The percentage of infiltrating cells with nuclear FOXP3$^+$ staining in either the tumour or the stroma compartments was defined and graded as no staining, less than 10% positive cells, 10-33%, and more than 33% positive cells from the total number of cells present in these areas.

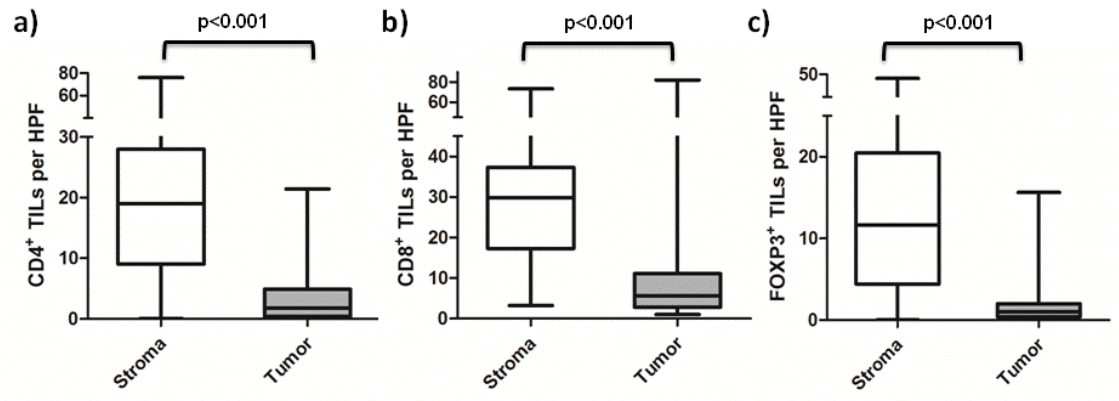


Figure 45. Box plots representing the levels of positive cells in the stroma and tumour compartments. a) CD4$^+$ cells per HPF in either the stroma or the tumour, b) CD8$^+$ cells per HPF in either the stroma or the tumour and c) FOXP3$^+$ cells per HPF in either the stroma or the tumour.

As we wanted to study the presence of Treg cells in the tumour microenvironment, we focused only on the expression of FOXP3 in lymphocytes. However, the number of articles reporting the expression of this transcription factor in other types of cells, especially epithelial malignant cells, has increased exponentially in recent years. In fact, it has been suggested that FOXP3 is expressed in carcinoma cells in all cancer types except in ovarian carcinoma (Triulzi et al., 2013). For instance, FOXP3 expression was detected immunohistochemically in the tumour cells of 24/39 patients with pancreatic carcinoma; subcellular FOXP3 staining in these patients was mostly cytoplasmic in some patients, but predominantly nuclear in others (Hinz et al., 2007). In breast cancer carcinomas, FOXP3 staining was localised predominantly in the

cytoplasm, although both cytoplasmic and nuclear staining was present in some specimens and a few showed only nuclear staining (Ladoire et al., 2011; Merlo et al., 2009). A study by Tao et al. (Tao et al., 2012) on NSCLC specimens revealed tumour cell FOXP3 expression in 31% of patients, and in another immunohistochemical analysis on NSCLC tissues, FOXP3 staining was consistently nuclear and was stronger in tumour cells than in adjacent normal bronchial epithelium (Dimitrakopoulos et al., 2011).

Although we did observe what seemed to be positive tumour cell staining, this is not reported here for various reasons: 1) the aim of this part of the study was to analyse the presence of $FOXP3^+$ cells which have an immunosuppressive role (Tregs); 2) FOXP3 expression in tumour cells needs to be further investigated and validated, and the role of its expression in these cells is still unknown; and 3) the possible tumour staining in our set of samples needs to be further evaluated in order to totally discard any possible background effects. Thus, the study of FOXP3 positive staining in tumour cells is beyond the objectives of this study.

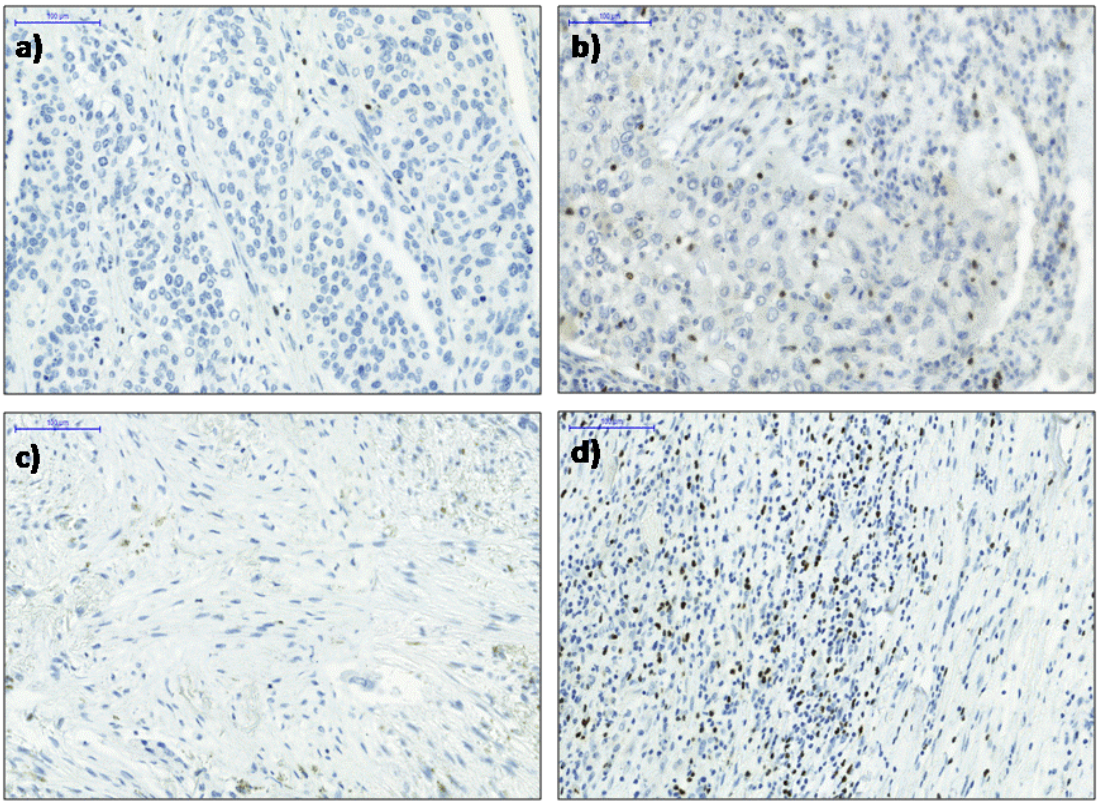


Figure 46. Representative immunohistochemical FOXP3 staining. Microscopic immunohistochemical images of NSCLC samples with $FOXP3^+$ staining cells. a) Tumour area with low $FOXP3^+$ cell infiltration, b) tumour area with high $FOXP3^+$ cell infiltration, c) stroma area low $FOXP3^+$ cell infiltration and d) stroma area with high $FOXP3^+$ cell infiltration. Original magnification, X200.

3. CORRELATION BETWEEN INFILTRATING IMMUNE CELLS AND CLINICOPATHOLOGICAL VARIABLES

Correlations between the presence $CD4^+$, $CD8^+$, and $FOXP3^+$ cells infiltrating the tumour and stroma compartments and clinicopathological variables were assessed using the Chi-square test. We observed a correlation between *EGFR* status and the presence $CD4^+$ cells (p = 0.047; Figure 47a). In particular, patients with mutated *EGFR* presented high levels of $CD4^+$ cells in the stroma; this could be explained by the fact that somatic mutations in the *EGFR* gene produce new peptides that are not recognized as self-antigens by the immune system and therefore $CD4^+$ T helper cells might be recruited in order to produce an immune response. In this regard, the detection of IgG responses to EGFR-derived peptides in a recent study suggested that the presence of $CD4^+$ cells may be a promising method for prognostication of NSCLC patients receiving gefitinib (Azuma et al., 2014).

Furthermore, smaller tumours (less than 3.5 cm) were correlated with the presence of higher numbers of tumour-stroma infiltrated $CD8^+$ cells (p = 0.047; Figure 47b), suggesting that these cells are recruited in the early stages of tumour development, when the immune response is more robust, in order to stop tumour growth. A higher number of $CD4^+$ cells in the stroma was also observed in patients with an ADC histology (p = 0.03; Figure 47c). This is a tendency which has already been observed in previous analyses performed in this study: higher expression of genes related to immune response and immunoregulation were more frequently observed in ADC patients. In addition, a higher percentage of $FOXP3^+$ cell infiltration in the stroma compartment was associated with a worse PS (p = 0.005), which could be indicative of more aggressive disease (Figure 47d).

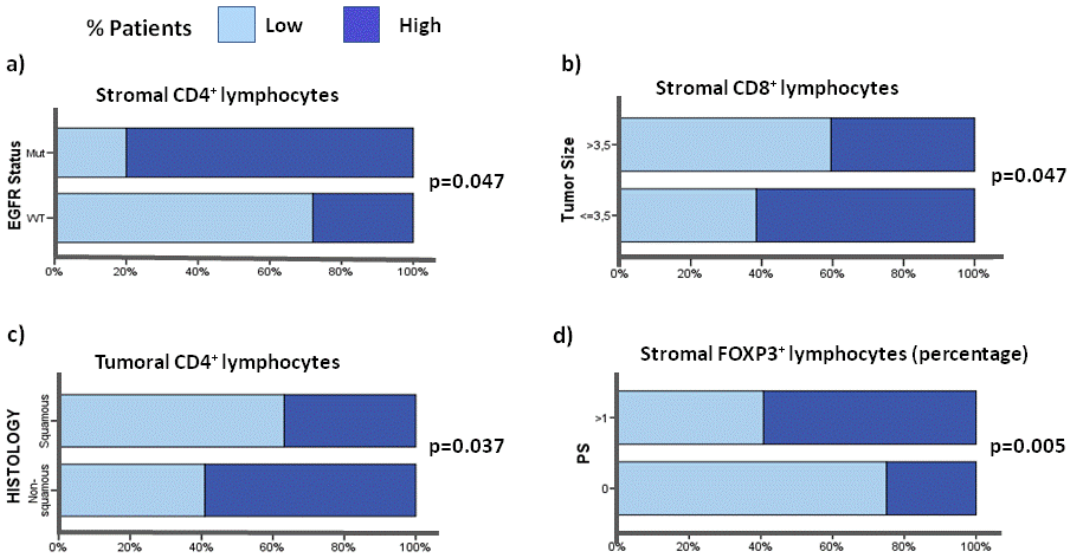


Figure 47. Histograms showing the association between clinicopathological variables and immune cell infiltration. Statistically significant associations with a) EGFR status, b) tumour size, c) histology and d) PS. Histograms show the percentage of patients. *P*-values were calculated using the chi-square test.

4. IMMUNE CELL INFILTRATION SURVIVAL ANALYSIS

4.1. THE PROGNOSTIC ROLE OF INDIVIDUAL IMMUNE CELLS

The density of immune cells detected by IHC in the tumour nest and tumour-associated stroma was independently assessed for its ability to predict patient survival. The results, detailed in Table 34, showed that the presence of T helper lymphocytes ($CD4^+$ cells) alone was not associated with prognosis either in tumour or stroma compartments. This result is in concordance with previous work, in which the presence of $CD4^+$ cells did not correlate with NSCLC survival (Hernandez-Prieto et al., 2014; Liu et al., 2012). However, other studies reported that the presence of these cells in the NSCLC tumour stroma was correlated with better prognosis (Al-Shibli et al., 2008; Wakabayashi et al., 2003).

However, it should be considered that $CD4^+$ T cells from a heterogeneous population of immune cells with different phenotypes and different functions in the tumour microenvironment. Th1 $CD4^+$ T cells are a subtype of immune cells with antitumour properties, whereas other types of $CD4^+$ T cells such as Th2, Th17, and Tregs are suspected to stimulate cancer growth, although this functional distinction has to be placed into the context of each tumour type (Fridman et al., 2011). Therefore, the fact that we detected subpopulations of

immune cells with opposing action upon the tumour may explain the lack of prognostic significance in our set of patients.

Table 34. Survival analysis results based on individual immune cell infiltration.

	OS			PFS		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Tumoural CD4$^+$ cells						
High vs. Low	0.908	0.499-1.653	0.753	0.931	0.531-1.632	0.803
Stromal CD4$^+$ cells						
High vs. Low	0.655	0.359-1.193	0.166	0.750	0.428-1.315	0.315
Tumoural CD8$^+$ cells						
High vs. Low	0.493	0.267-0.910	0.024*	0.525	0.295-0.936	0.029*
Stromal CD8$^+$ cells						
High vs. Low	0.774	0.426-1.408	0.402	0.698	0.396-1.232	0.215
Tumoural FOXP3$^+$ cells						
High vs. Low	1.088	0.458-2.585	0.849	1.253	0.559-2.806	0.584
Stromal FOXP3$^+$ cells						
High vs. Low	1.929	1.058-3.518	0.032*	1.608	0.907-2.850	0.104

Patients were classified as having low or high positive cell infiltration in the tumour or stroma locations according to the medians calculated in each case. The results were obtained using a univariate Cox regression method. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival.

In contrast, the presence of T cytotoxic lymphocytes (CD8$^+$ cells) in the tumour nest was significantly associated with better OS [HR, 0.493; 95% CI, 0.267-0.910; p = 0.024] and PFS [HR, 0.525; 95% CI, 0.295-0.936; p = 0.029], which was also reflected in the Kaplan-Meier plots for OS (73.9 vs. 40.4, months, p = 0.021) and PFS (56.8 vs. 23 months, p = 0.026; Figure 48a-b). Among the tumour infiltrating immune cells, CD8$^+$ cytotoxic lymphocytes form the effector arm of adaptive immunity and are thought to have protective roles against tumours, including lung cancer (Suzuki et al., 2011). Their prognostic role has been studied in several types of tumour and, in general, their presence is correlated with better disease outcomes. For instance, in breast cancer a large (n = 12,439 patients), recently published, multicentre study showed that intratumoural and stromal CD8$^+$ lymphocytes were independently associated with a reduced risk of death from cancer (Ali et al., 2014). In a similar analysis in NSCLC, Al-Shibli et al. assessed the presence of CD8$^+$ cells, among other immune cells, by performing tissue microarrays on 335 resected stage I to IIIA NSCLCs. They observed that high CD8$^+$ lymphocyte infiltration was associated with better survival in both tumoural and stromal areas (Al-Shibli et al., 2008).

In previous studies, in which the influence of the tumour compartment was not evaluated, a correlation between the presence of CD8$^+$ lymphocytes and tumour cell

apoptosis, as well as a vigorous antitumour immune response in NSCLCs was observed (Tormanen-Napankangas et al., 2001; Verdegaal et al., 2007). The fact that we found an association with survival only when we analysed the presence of $CD8^+$ cells in the tumor compartment could be explained by previous finding suggesting that antitumour cytokines contributing to tumour suppression act mainly in the stroma, whist on tumour cells this antitumour activity is more often mediated by molecules such as perforin or Fas ligand, which are expressed by $CD8^+$ cells (Blankenstein, 2005).

We also observed that patients with a higher percentage of $FOXP3^+$ cells in the stroma compartment had worse OS [HR, 1.929; 95% CI, 1.058-3.518; p = 0.032], with a median survival of 37.2 vs. 68 months (p = 0.029, Kaplan-Meier test) in this group of patients (Figure 48c). $FOXP3^+$ cells or Tregs are a specific subset of the T cell repertoire which have been demonstrated to be involved in decreasing the antitumour response (deLeeuw et al., 2012). Previous studies have assessed the prognostic value of Tregs in tumour samples from NSCLC patients; Shimizu et al. demonstrated that patients with NSCLC (stages I-IIIB) containing three or more Tregs infiltrating the tumour in 10 HPFs had significantly worse RFS, and among patients with node-negative NSCLC, this was an independent prognostic factor (Shimizu et al., 2010). An increased Treg count was also found to be associated with worse OS and RFS in another study with I-IIIA stage NSCLC, in which it was also observed that positive tumour cell staining attenuated the prognostic value of Treg infiltration (Tao et al., 2012).

In a recent study, the clinical impact of the tumour microenvironment was evaluated in stage I lung adenocarcinoma (n = 956). Using tissue microarrays and IHC, Suzuki et al. investigated eight types of tumour-infiltrating cells in the tumour nest and tumour-associated stroma, observing that a high density of stromal $FOXP3^+$ cells was associated with a shorter RFP (Suzuki et al., 2013). This result, which is in line with our observations, emphasizes the importance of assessing the location of TILs within the tumour microenvironment. In fact, the importance of immune cells in the tumour stroma in NSCLC has been shown in a study with patients with stages I-IIA of the disease; this work demonstrated the presence of tertiary de novo lymphoid structures in the tumour microenvironment, a structure they termed the tumour-induced bronchus-associated lymphoid tissue (Ti-BALT). Moreover, the presence of mature dendritic cells in the Ti-BALT correlated with prolonged OS (Dieu-Nosjean et al., 2008). In breast cancer, a study reported that the presence of Tregs within lymphoid infiltrates surrounding the tumour, but not within the tumour itself, was associated to a higher risk of relapse and death. The authors elegantly provided evidence that this clinical observation could

result from the selective recruitment of Tregs within lymphoid infiltrates of breast tumours where they are locally activated (likely through tumour-associated antigen recognition) leading to their in situ proliferation and prevention of conventional T cell activation (Gobert et al., 2009). We hypothesise that this explanation could be extrapolated to lung tumours, in which the importance of lymphoid structures has already been reported, and the prognostic value of Tregs located in the stroma has been observed in our results and in previous studies.

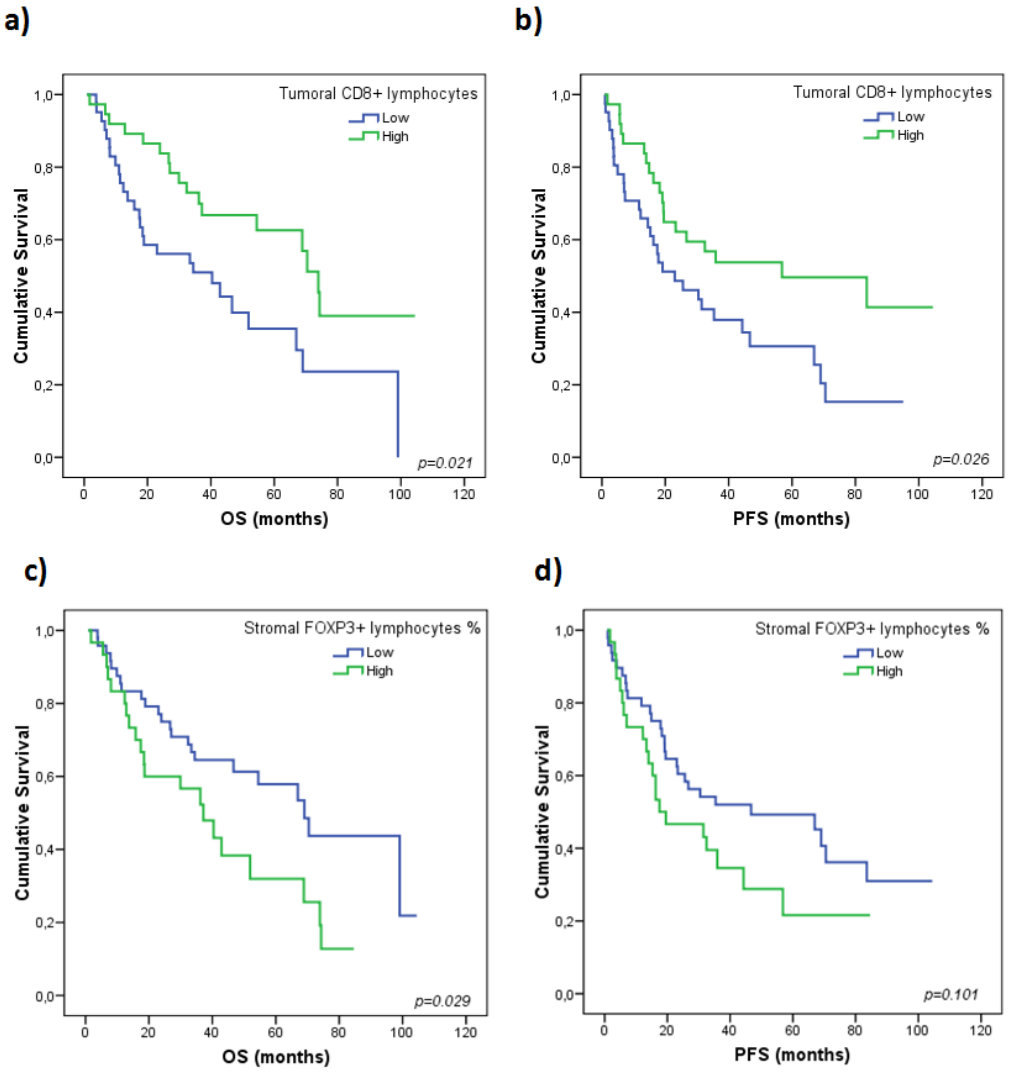


Figure 48. Kaplan-Meier plots for OS and PFS according the density of immune cells infiltrating the tumour microenvironment. a-b) Tumoural $CD8^+$ cells and c-d) stromal $FOXP3^+$ lymohocytes. Densities were dichotomized according to the median expression values. The blue line represents patients with low infiltratinon, whilst the green line represents patients with high infiltration. *P*-values were calculated using the Kaplan-Meier test.

4.2. PROGNOSTIC ROLE OF THE COMBINATION OF IMMUNE CELLS

Since the survival analysis carried out in the previous section showed a prognostic value for the proportion of conventional T lymphocytes (defined by the CD4 and CD8 markers) and regulatory T cells (defined by FOXP3 expression) at the mRNA level, we decided to corroborate these results by analysing the proportion of these types of immune cells in situ. Univariate survival analysis showed that patients with high $FOXP3^+$ cell infiltration in the stroma and low $CD4^+$ cell infiltration in the tumour presented worse OS [HR, 2.385; 95% CI, 1.095-5.194; p = 0.029], with a survival median of 17.4 vs. 66.9 months (p = 0.024, Kaplan-Meier test; Figure 49a-b). The other variable studied compared cytotoxic T cells with Tregs, indicating that patients with high $FOXP3^+$ cell infiltration in the stroma and low $CD8^+$ cell infiltration in the tumour also presented worse OS [HR, 2.391; 95% CI, 1.195-4.787; p = 0.014] and shorter PFS [HR, 2.046; 95% CI, 1.036-4.043; p = 0.039]. Kaplan-Meier plots showed that the survival median for OS was 17.4 vs. 68.8 months (p = 0.011) and for PFS it was 15.3 vs. 35.9 months (p = 0.035; Figure 49c-d).

Table 35. Survival analysis results based on the proportion of immune cell infiltration.

	OS			PFS		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
$FOXP3^+$ stroma/ $CD4^+$ tumour *↑$FOXP3^+$ stroma/↓$CD4^+$ tumour vs. Other combinations*	2.385	1.095-5.194	0.029	1.936	0.897-4.177	0.092
$FOXP3^+$ stroma/ $CD8^+$ tumour *↑$FOXP3^+$ stroma/↓$CD8^+$ tumour vs. Other combinations*	2.391	1.195-4.787	0.014	2.046	1.036-4.043	0.039

The results shown in the table are for the combination specified vs. other combinations. The results were obtained using a univariate Cox regression method. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival.

In addition to type, density, and location, we have demonstrated that the relative proportion of pro- and antitumour immune cells is also an important parameter to study when analysing tumour-infiltrating cells. In this case, the proportion of helper and cytotoxic cells, to regulatory T cells in different tumour microenvironment locations had a prognostic value in resected NSCLCs. The first study to investigate the correlation between $FOXP3^+$ Treg cells, tumour infiltrating lymphocytes, and lung cancer survival was carried out by Petersen et al. (Petersen et al., 2006). They observed that patients with stage I NSCLC who had a higher proportion of tumour Tregs relative to TILS (defined as $CD3^+$ T cells) had a significantly higher risk of recurrence. They pointed out that since Tregs exert their effect by inhibiting antitumour

T cells, they should always be considered in the context of total TILs. In another important piece of work, tumours containing high levels of stromal FOXP3 and low levels of stromal CD3 were considered to be high-risk and this parameter was found to be a strong predictor of disease recurrence (Suzuki et al., 2013).

It should be taken into consideration that previous work has assessed $CD3^+$ cells, i.e. every T cell, without making any distinction between helper and cytotoxic T cells. To the best of our knowledge, ours is the first study reporting the prognostic value of the combination of Tregs and conventional T cells, and which differentiates the type of lymphocytes which infiltrate the tumour in resected NSCLCs. In advanced NSCLC, the prognostic and predictive value of different types of infiltrating immune cells has also been previously investigated: the results indicated that the ratio of $FOXP3^+/CD8^+$ TILs was an independent predictor of poor response to platinum-based chemotherapy (Liu et al., 2012). However, neither TIL subtypes alone, nor their ratio was correlated with OS.

Over the last decade, the analysis of large libraries of annotated human tumours has allowed researchers to identify prognostic markers based on immune cell infiltration. In this regard, colorectal cancer has been extensively studied and represents the paradigm of these findings. It has been shown that not only the overall density of memory $CD45RO^+/CD8^+$ T cells was important, but also the location of the immune microenvironment (infiltration inside the tumour core and at its invasive margin). These observations have led to the definition of an "immunoscore", based on quantifying the density of infiltrating T cells using two marker combinations (CD8/CD45RO or CD3/CD8) in these two regions of the tumour microenvironment to define a score ranging from 0 to 4, which predicts the PFS and OS in colorectal cancers up to stage III with a high degree of significance (Galon et al., 2006; Galon et al., 2012; Pages et al., 2009). The clinical application of the immunoscore is currently being validated in a multicentre international study. It should be noted that the immunoscore is defined by the infiltration of cells that have been traditionally linked to antitumour properties, and therefore, to a better outcome. In contrast, we have demonstrated that analysing different types of immune cells, such as cytotoxic and regulatory T cells, in combination could also provide good information regarding patient outcome.

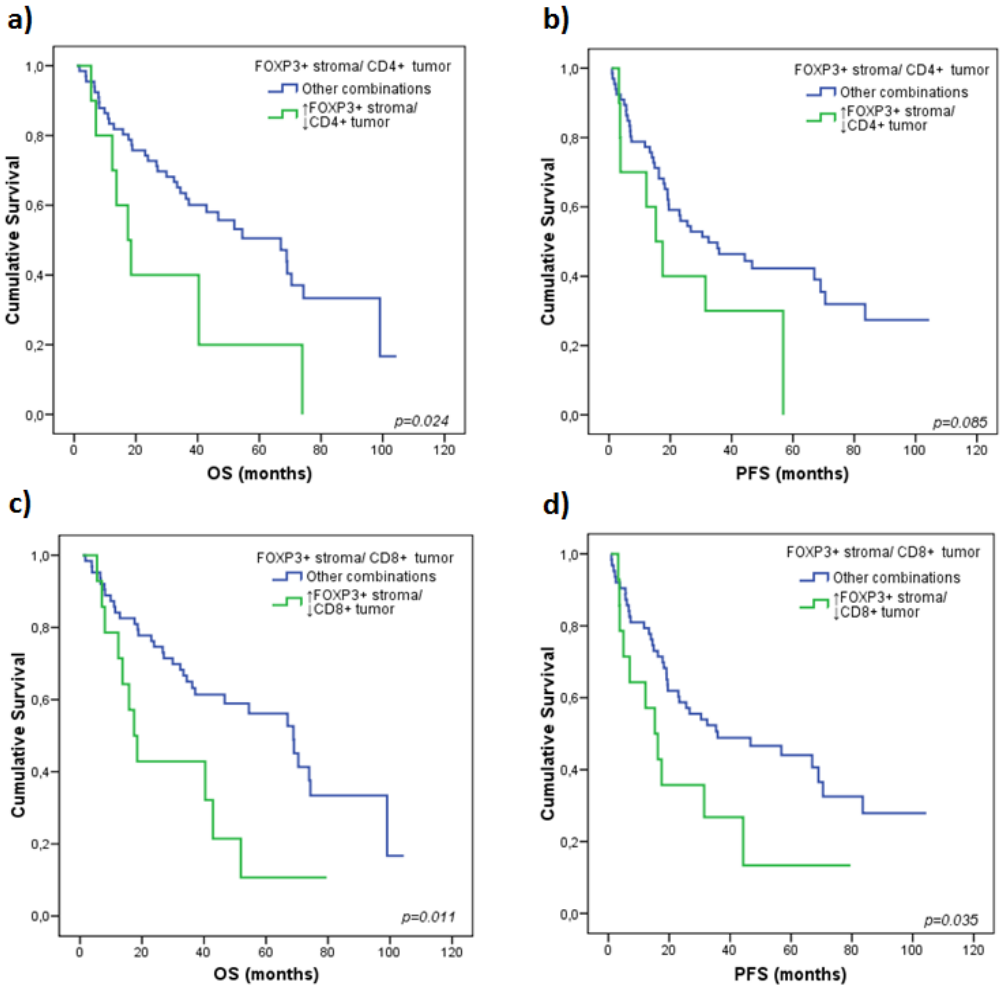


Figure 49. Kaplan-Meier plots for OS and PFS according to the proportion of FOXP3$^+$ cells in the stroma to CD4$^+$ and CD8$^+$ cells in the tumour. a-b) Combination of FOXP3$^+$ cells in the stroma/CD4$^+$ cells in the tumour, and c-d) FOXP3$^+$ cells in the stroma/CD8$^+$ cells in the tumour. The green line represents patients with the combination of interest, whilst the blue line represents patients with other combinations. *P*-values were calculated using the Kaplan-Meier test.

4.3. MULTIVARIATE ANALYSIS

In this part of the study we performed multivariate analyses with the biomarkers described in the previous sections, in this case positive immune cells identified by IHC, which were significantly associated with survival. Moreover, we also included the clinicopathological variables that presented prognostic value in this subset of patients. Thus, the OS multivariate model was composed of tumoural CD8$^+$ cells, stromal FOXP3$^+$ cells, FOXP3$^+$ stroma/CD4$^+$ tumour, and FOXP3$^+$ stroma/CD8$^+$ tumour expression, whereas the PFS model was composed of tumoural CD8$^+$ cells and FOXP3$^+$ stroma/CD8$^+$ tumour expression. Gender, LN involvement, and PS were correlated with survival in the univariate analysis, and so they were also included in both multivariate models. The results, which are detailed in Table 36, indicated that the

presence of $CD8^+$ cells in the tumour compartment was an independent prognostic biomarker for OS and PFS. Moreover, the presence of $FOXP3^+$ cells in the stroma compartment was also found to be an independent prognostic factor for OS.

Table 36. Results from the multivariate Cox regression model including all the significant results from this part of the study.

	OS			PFS		
Variables	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Tumoural $CD8^+$ cells						
High vs Low	0.386	0.175-0.850	0.018	0.305	0.137-0.680	0.004
Stromal $FOXP3^+$ cells						
High vs Low	2.203	1.109-4.379	0.024	-	-	-

CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival.

Infiltrating immune cells have been shown to have prognostic value in several solid malignancies, including colorectal, ovarian, and breast cancer; Galon et al. have advocated the use of three important parameters of tumour infiltrating lymphocytes (type, density, and location) to predict clinical outcomes in patients. Results from these analyses suggest that the prognostic value of this immunophenotype may be more powerful than traditional staging (Galon et al., 2012). In this part of the study, we investigated three infiltrating immune cell markers and found that $CD8^+$ cells in the tumour compartment and $FOXP3^+$ cells in the stroma compartment were independent prognostic factors. Indeed, this immune pattern remained the only significant criterion over any other clinicopathological variables for PFS and OS.

The positive prognostic value of $CD8^+$ cell infiltration in the tumour compartment may indicate an ongoing immune response against the tumour. Presuming that this infiltrate includes tumour-antigen specific T cells that have been activated spontaneously in response to the growing tumour, perhaps through immune system surveillance mechanisms (Bui and Schreiber, 2007), these cells would be attempting to control the tumour, and would thus create a favourable clinical outcome. However, how a subset of patients can generate a $CD8^+$ T cell response against tumour-associated antigens, apparently in the absence of pathogen involvement, remains an enigma. Two hypotheses have been postulated: one suggests the likely participation of stress-associated or damage-associated molecular patterns that may trigger innate immune activation and provide a bridge toward adaptive immunity. The other hypothesis explains the presence of T cells in the tumour microenvironment because of the generation of tertiary lymphoid structures (TLSs). As their presence is also associated with the accumulation of $CD8^+$ effector T cells, it is conceivable that spontaneous priming of an

antitumour T cell response leads to chronic inflammation in the tumour microenvironment that subsequently generates signals for TLS generation (Gajewski et al., 2013).

The other independent prognostic factor was the presence of $FOXP3^+$ cells in the stroma compartment. FOXP3 is a Treg marker: a subset of lymphocytes that, as previously explained, is known to suppress the host immune response. In patients with lung cancer, Tregs are thought to play protumour roles in all histological subtypes (Petersen et al., 2006). Interestingly, our results indicated that FOXP3 in the stroma (and not in the tumour nest) was associated with survival, emphasising the importance of assessing the location of immune cells within the tumour microenvironment. In addition, these findings have significant implications for devising potential immunomodulatory therapies. For instance, in patients with high $FOXP3^+$ cells infiltrating the stroma compartment an intervention that decreases FOXP3 and that increases other positive immune cells, such as $CD8^+$ cells, would likely be beneficial. Beyond their cytostatic characteristics, some conventional chemotherapeutic agents were found to affect the adaptive immune system, resulting in the inhibition of Tregs function or viability, and thereby enhancing antitumour immunity. Interestingly, cyclophosphamide has been shown to modulate the tumour immune microenvironment by depleting Tregs (Le and Jaffee, 2012). Paclitaxel, a taxane-based chemotherapeutic agent that is currently the most widely used in the clinic, was found to specifically impair cytokine production and viability in $FOXP3^+$ Treg cells but not in $FOXP3^-$ $CD4^+$ effector T cells (Zhu et al., 2011). Ipilimumab, an antibody that is specific for CTLA4, blocks an important inhibitory signal for activated T cells, thereby bolstering T cell responses and decreasing Treg cell-dependent immune suppression (Peggs et al., 2009). Studies that aim to deplete Treg cells in patients with cancer are hampered by the fact that Treg cells do not express an exclusive surface molecule that can be targeted. Therefore novel strategies are needed that permit specific Treg cell targeting and therefore elimination of their suppressive function.

C) CORRELATION BETWEEN IMMUNE CELL INFILTRATION AND IMMUNOREGULATORY GENE EXPRESSION

1. CORRELATION BETWEEN THE CD4+, CD8+, AND FOXP3+ CELL COUNT AND THE GENE EXPRESSION LEVELS OF THESE MARKERS

1.1. GENE EXPRESSION DATA FROM FRESH-FROZEN SAMPLES

In this section we wanted to correlate the expression of CD4, CD8, and FOXP3 at the protein level (detected as positive cells by IHC) with their mRNA expression levels (assessed by RTqPCR). In order to better analyse these correlations, we compared gene expression levels without normalising to normal tissue, so that only tumour tissue expression was considered. This step was made because it allows comparison of the total number of positive cells with the absolute expression of the genes in the tumour, and not in the tumour compared to normal tissue. As for the positive cell count, because they were originally evaluated in tumour and stroma compartments separately, an average of tumoural and stromal cell counts was calculated for each patient. A Spearman test (R statistic) revealed a correlation between mRNA expression levels and the positive cells count for CD4 (p = 0.013, R2 = 0.326), CD8 (p = 0.007, R2 = 0.345), and FOXP3 (p = 0.003, R2 = 0.374; Figure 50). Although these are positive results, because they indicate that mRNA expression could be a good surrogate marker for actual immune cells, they do not confirm that these proportions are equivalent at the protein level, which may be due to possible effects from post-transcriptional and post-translational modifications.

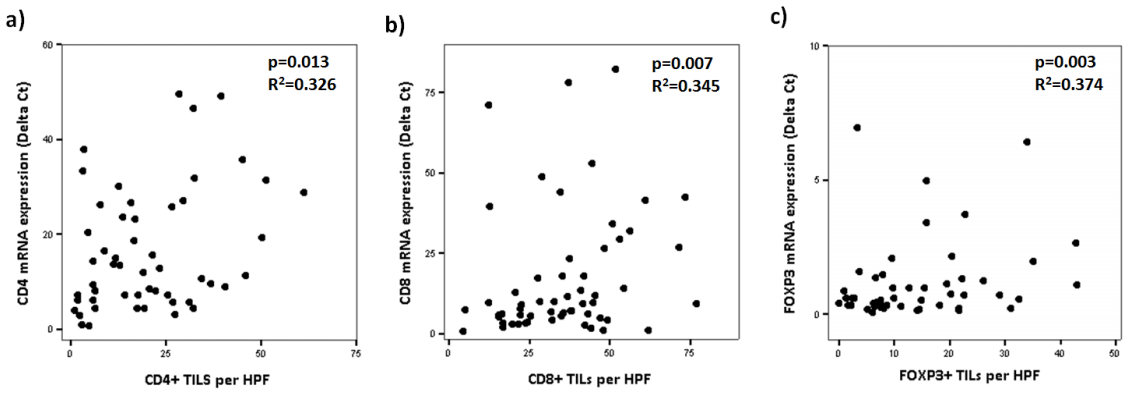


Figure 50. Correlation between mRNA expression levels and the number of immune cells positive by immunohistochemistry in the tumour microenvironment. a) CD4, b) CD8, and c) FOXP3. R represents the Spearman correlation coefficient.

1.2. GENE EXPRESSION DATA FROM FORMALIN-FIXED PARAFFIN-EMBEDDED SAMPLES

The correlation between mRNA expression levels and positive-cell count was also evaluated independently in tumour and stroma compartments using the data obtained from FFPE microdissected samples. In order to better analyse this correlation we compared gene expression levels without normalising to human reference cDNA, as justified in the previous section. In this case, we only observed a correlation between the presence of $CD8^+$ cells and CD8 gene expression levels in the stroma compartment (p = 0.029, R^2 = 0.261) and the tumour nest (p = 0.008, R^2 = 0.311; Figure 51).

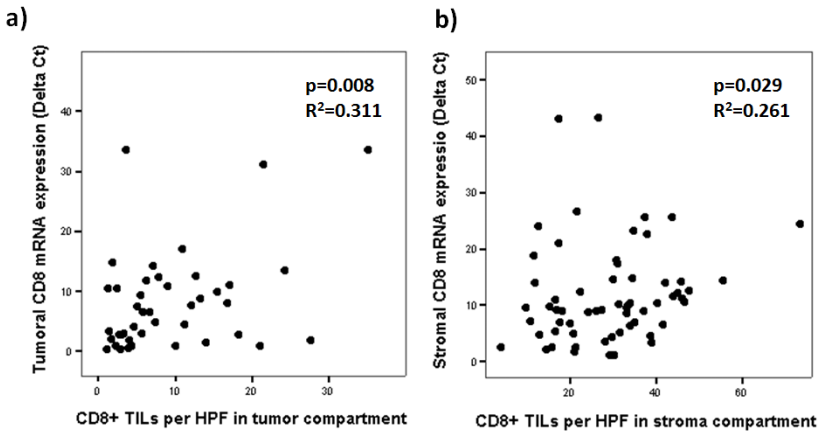


Figure 51. Correlation between mRNA expression levels and the number of or immune cells positive by immunohistochemistry for CD8 in the tumour microenvironment. a) In the tumour compartment and, b) in the stroma compartment. R represents the Spearman correlation coefficient.

The lack of FOXP3 correlation can help us to understand some of the results we obtained in previous sections. High FOXP3 expression in the tumour compartment was associated with better survival but no correlation was observed for the stroma compartment when the prognostic value was analysed at the mRNA level. We hypothesised that the positive prognostic value might be due to the fact that not only Tregs express FOXP3 in the tumour compartment. Therefore, this lack of correlation, together with the fact that high $FOXP3^+$ cell infiltration in the stroma compartment had a negative prognostic impact, reinforces our hypothesis that FOXP3 mRNA is not exclusively expressed by Tregs in the tumour microenvironment and that other cells, presumably tumour cells, as already suggested (Tao et al., 2012), could also be a source of FOXP3 mRNA.

2. THE IMMUNE CONTEXTURE CHARACTERISATION ASSOCIATED WITH THE PRESENCE OF IMMUNE CELLS

Immune cell infiltrates are heterogeneous between tumour types, and are very diverse from patient to patient. In fact, as we have demonstrated in previous sections, the differences in density, location, and proportion of pro- and antitumour immune cells between different individuals with NSCLC affects their clinical outcome. Once the prognostic value of these tumour-infiltrating immune cells has been established other questions arise. For instance, it would be interesting to better understand the key elements involved in shaping the immune microenvironment in which these immune cells are present, or in other words, to further explore the immune contexture of these microenvironments.

According to the authors who first proposed the "immunoscore", the immune contexture is defined as the type, density, location and functional orientation of the immune cells within distinct tumour regions (Angell and Galon, 2013). The immune contexture represents the complexity of the immune parameters within the tumour microenvironment and is associated with patient survival (Galon et al., 2013). The functional orientation of the immune contexture is characterised by immune signatures that might reflect the functional characteristics or the molecular phenotypes of the immune cell infiltrate. In order to analyse these functional characteristics, gene expression patterns related to different immune processes and the presence of different immune cells were studied in specific groups of patients. Therefore, in this part of the study, we investigated whether there are significant differences in gene expression patterns according to $CD4^+$, $CD8^+$, and $FOXP3^+$ cell infiltration.

2.1. CORRELATION BETWEEN INFILTRATING IMMUNE CELLS AND EXPRESSION PATTERNS IN FRESH-FROZEN SAMPLES

Firstly, the correlation of specific sets of genes analysed at the mRNA level in fresh-frozen samples, and the level of infiltration of three types of immune cells, was assessed in the whole NSCLC tumour microenvironment. Although a Mann-Whitney U test revealed no association between the expression patterns and $CD4^+$ cell infiltration (Figure 52a) individual expression of CCL22 (p = 0.020) and PDL1 (p = 0.022) did positively correlate. Correlation with CCL22 could indicate the active recruitment of naïve and memory T cells, including Tregs, from blood into the tumour microenvironment (Fridman et al., 2012), and correlation with PDL1

might mean that when the $CD4^+$ cell density increases, PDL1 expression also increases in the tumour microenvironment in order to suppress lymphocyte function (Pardoll, 2012).

Patients with higher $CD8^+$ cell infiltration in the tumour microenvironment presented higher gene expression for gene groups related to TAMs, APCs, and immune cell trafficking processes (Figure 52b). Moreover, individual analysis showed that $CD8^+$ cell infiltration was associated with TGFB1 (p = 0.039), PDL1 (p = 0.018), CCL22 (p = 0.001), and IDO1 (p = 0.036). In this case, a high density of $CD8^+$ cells in the tumour microenvironment was correlated with an expression pattern that indicates an active immune response, in which immune cells, especially APCs, might be present. On the other hand, correlation with individual genes might also point towards the likelihood that the tumour is trying to disarm the immune response by releasing immunosuppressive factors and chemokines that have previously been associated with the recruitment of immunoregulatory cells, especially Tregs (Curiel et al., 2004).

Finally, the presence of $FOXP3^+$ cells in the tumour microenvironment was significantly associated with three of the four groups of genes analysed: genes related to Tregs, immune checkpoints, immunosuppressive factors, and TAMs, APCs, and immune cell trafficking processes (Figure 52c). Individual analysis showed that infiltration of these lymphocytes was positively associated with expression levels of CD25 (p = 0.001), CD127 (p = 0.002), TGFB1 (p = 0.030), PDL1 (p = 0.003), CCL2 (p = 0.025), CD1C (p = 0.022), and IL23A (p = 0.041). A heat map displaying gene patterns according to the density of $FOXP3^+$ cells in the tumour microenvironment is shown in Figure 53; high levels of $FOXP3^+$ cell infiltration in the tumour microenvironment were associated with upregulation of the cluster referring to the Treg profile, as well as with individual genes related to Tregs. This positive correlation was also observed in the case of the immune checkpoint group because some of these markers are also expressed by Tregs (Pardoll, 2012).

Furthermore, correlation with the presence of other immune cells and trafficking processes was also observed. Although this correlation was observed in tumours with high levels of $CD8^+$ cell infiltration, the polarisation of these cells could be different, thus facilitating a more immunosuppressive microenvironment. Thus a greater number of genes must be studied in order to better understand the processes that are taking place, especially within different locations within the tumour. Therefore, we carried out the same analysis with data obtained from FFPE samples in which a larger number of genes were independently analysed in the tumour and stroma compartments.

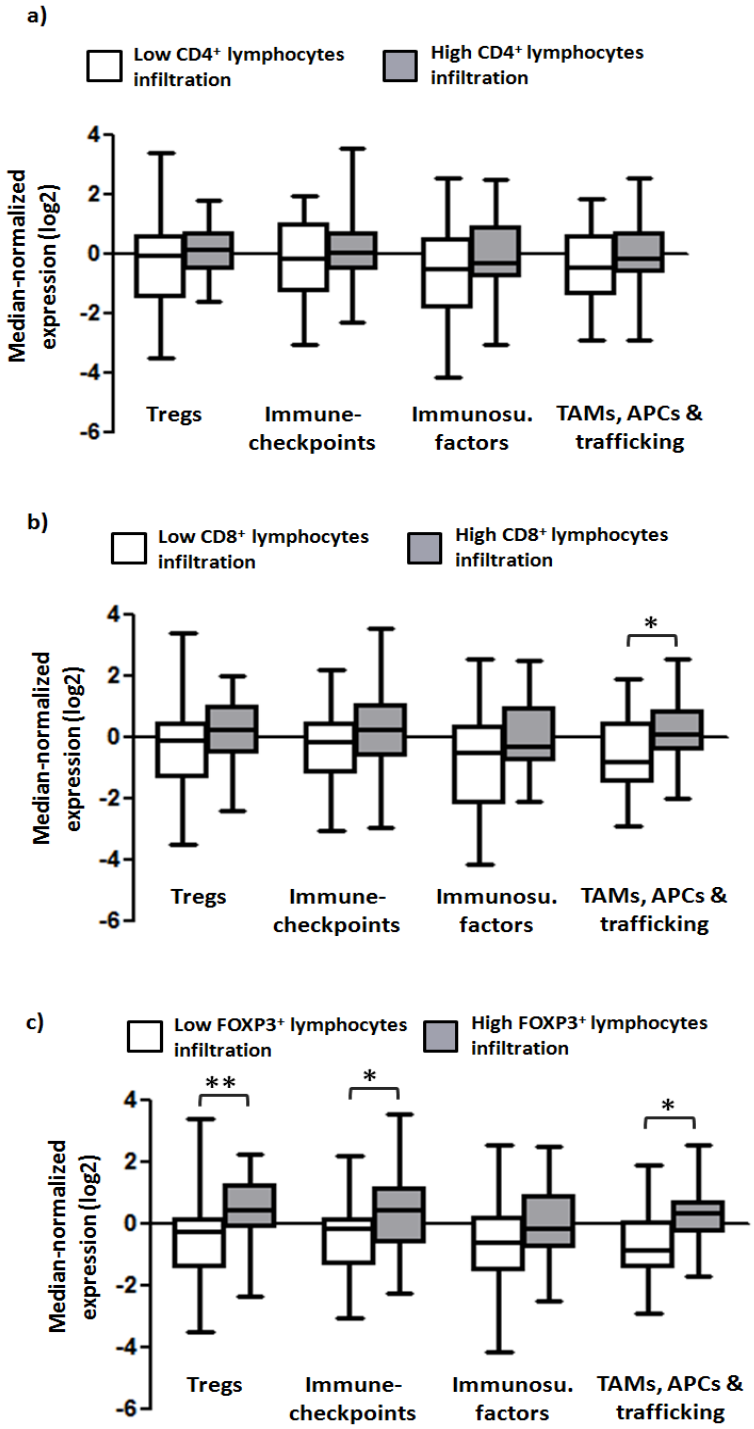


Figure 52. Gene expression patterns analysed in fresh-frozen samples according to a) $CD4^+$, b) $CD8^+$ and c) $FOXP3^+$ cells in the tumour microenvironment. Expression patterns of genes related to regulatory T cells (Tregs), immune checkpoints, immunosuppressive factors, TAMs, and APCs and immune cell trafficking processes according to the level of immune cell infiltration. The relative gene expression is represented as the log2-median of the expressions obtained for each gene. In the figures, the asterisks represent Mann-Whitney U significance test levels as follows: *p = 0.05-0.01 and **p < 0.01.

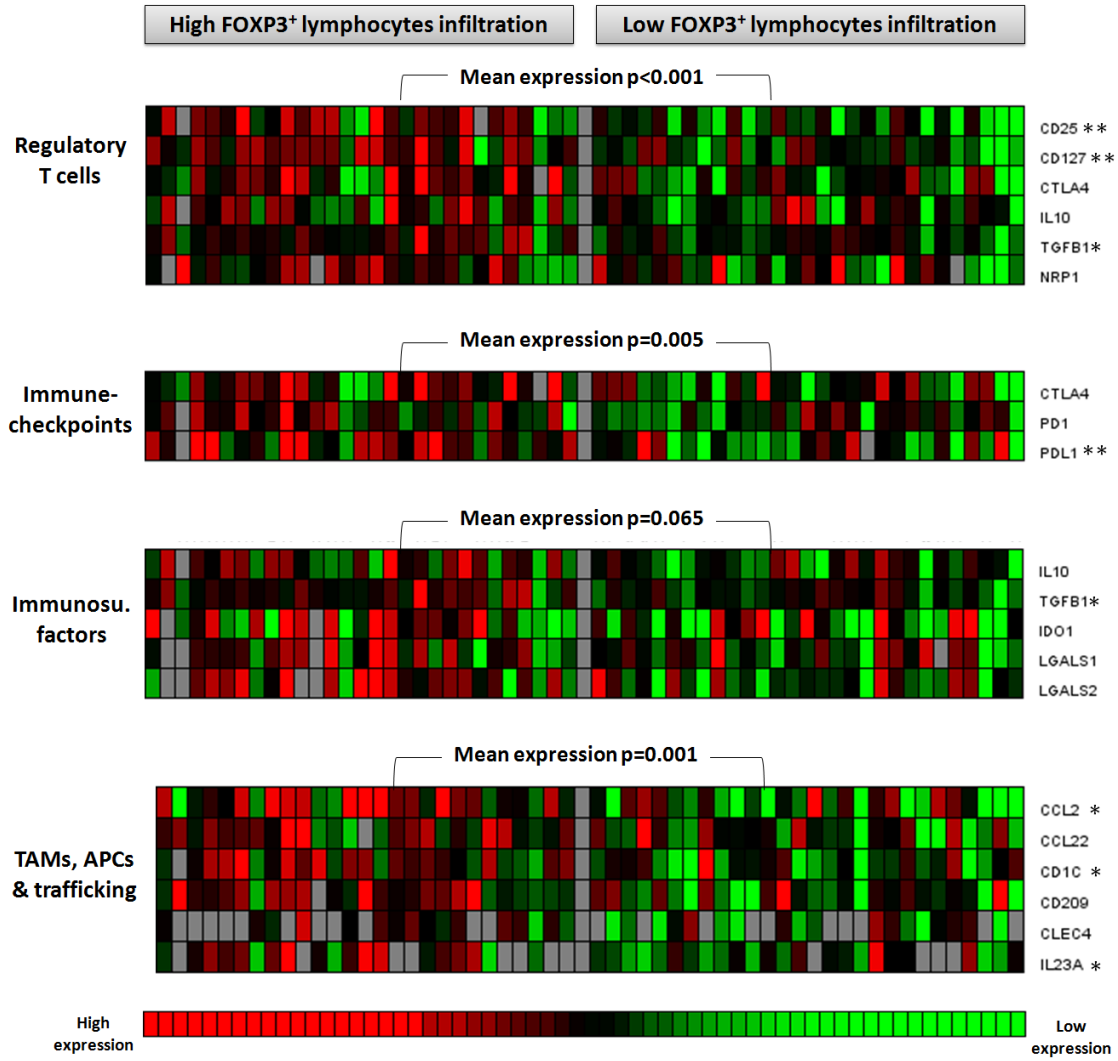


Figure 53. Heat map representation of the expression pattern of genes analysed in fresh-frozen samples according to the presence of FOXP3$^+$ cells in the tumour microenvironment. Expression patterns of genes related to regulatory T cells (Tregs), immune checkpoints, immunosuppressive factors, TAMs, APCs, and immune cell trafficking processes according to the level of immune cell infiltration. Patients are plotted according to their level of FOXP3$^+$ cell infiltration per HPF, and thus are divided into patients with high and low levels of infiltration. Gene expression is represented as the log2-median of the expression obtained for each gene and the asterisks represent Mann-Whitney U significance test levels as follows: *p = 0.05-0.01, ** $p < 0.01$.

2.2. CORRELATION BETWEEN INFILTRATING IMMUNE CELLS AND EXPRESSION PATTERNS IN FORMALIN-FIXED PARAFFIN-EMBEDDED SAMPLES

In this part of the study we investigated if there were significant differences in the expression patterns of specific sets of genes analysed at the mRNA level and the infiltration of the three types of immune cells assessed in the tumour and stroma locations. A Mann-Whitney U test revealed that patients with high levels of $CD4^+$ cells in the tumour compartment expressed higher levels of genes linked to Tregs, MDSCs, TAMs, and other APCs, whereas no correlation was found in the stroma compartment (Figure 54a-b). Thus, high $CD4^+$ cell infiltration might indicate the possible infiltration of immunosuppressive cells, among other immune cells, into the tumour microenvironment. However, no changes in the expression patterns were linked to the presence of these cells in the stroma compartment. Moreover, the immune contexture characterised by high levels of $CD4^+$ cell infiltration did not have a prognostic impact.

Of special interest is the analysis of expression pattern differences between tumours with high and low numbers of $CD8^+$ cells, due to the positive prognostic value that infiltration of these cells into the tumour compartment presented. Tumours with higher levels of $CD8^+$ cell infiltration expressed significantly higher levels of genes related to immunosuppressive factors, chemokines and their receptors, MDSCs and TAMs, and other APCs (Figure 54c-d). Moreover, significant positive correlations were observed between higher levels of $CD8^+$ cell infiltration and the expression of certain individual genes such as IDO1 ($p < 0.001$), CCL5 ($p = 0.005$), CD209 ($p = 0.031$), CD86 ($p < 0.001$), and IL23A ($p = 0.008$; Figure 55). Interestingly, IL8 was inversely correlated with high levels of $CD8^+$ cell infiltration ($p = 0.015$). We also found a significant correlation between the expression of genes related to chemokines and their receptors in the stroma compartment.

The microenvironment characterised by high $CD8^+$ cell infiltration seems to fit a T cell-inflamed phenotype, consisting of infiltrating T cells, chemokines and their receptors, and the possible presence of APCs which are required to activate $CD8^+$ cytotoxic cells by presenting tumour antigens to them. If these were the only types of cells acting in the tumour microenvironment the immune system would likely have a higher chance of completely eliminating tumour cells. However, as it can be interpreted from these gene expression patterns, other processes also take place. In fact, tumours with high levels of $CD8^+$ cell

infiltration express higher levels of markers related to immunosuppressive factors. One of these factors is IDO1, a key metabolic enzyme implicated in tryptophan catabolism. Its immune suppression functions are due to decreased tryptophan availability and the generation of tryptophan metabolites, culminating in multipronged negative effects on the T lymphocytes in proximity to IDO-expressing cells, notably on proliferation, function, and survival (Godin-Ethier et al., 2011). Interestingly, mechanistic studies in mice revealed that upregulated expression of IDO and PDL1 depended on the presence of $CD8^+$ T cells within the tumour microenvironment (Spranger et al., 2013).

Moreover, a higher MDSC expression pattern was also observed, indicating the possible presence of these cells when there is high $CD8^+$ cell infiltration. MDSCs have been implicated in indirectly altering anti-tumour immune responses by contributing to the tumour microenvironment suppressive network. However, it is still unclear which subset of MDSCs might be responsible for T cell suppression, or what the specific nature of MDSC suppression is. In fact, it has been observed that MDSCs intricately influence different $CD8^+$ T-cell activation events in vitro, whereby some parameters are suppressed while others are stimulated (Schouppe et al., 2013).

Also of interest are the positive correlations observed between $CD8^+$ cell infiltration and genes such as CD209 and IL23A. In previous sections, it has been observed that high expression of these markers was associated with longer survival, which might indirectly point to the presence of high numbers of $CD8^+$ cells, and indeed, a positive effect on the prognosis. The only inverse correlation observed in this study was with IL8 expression. This is a proinflammatory CXC chemokine associated with the promotion of neutrophil chemotaxis and degranulation. The expression of IL-8 receptors on cancer cells, endothelial cells, neutrophils, and tumour-associated macrophages suggests that the IL8 secretion by cancer cells may have a profound effect on the tumour microenvironment. IL8 receptor activation on endothelial cells is known to promote an angiogenic response, inducing the proliferation, survival, and migration of vascular endothelial cells (Waugh and Wilson, 2008). Thus, IL8 expression might be associated with a more aggressive type of immune contexture in which antitumour immunity is less important and processes like angiogenesis might play a more active role.

Finally, the presence of $FOXP3^+$ cells did not correlate with any expression patterns either in the tumour or in the stroma compartment (Figure 54e-f). Individual analysis showed that only the CTLA4 gene was positively correlated with high levels of $FOXP3^+$ cell infiltration in the stroma compartment.

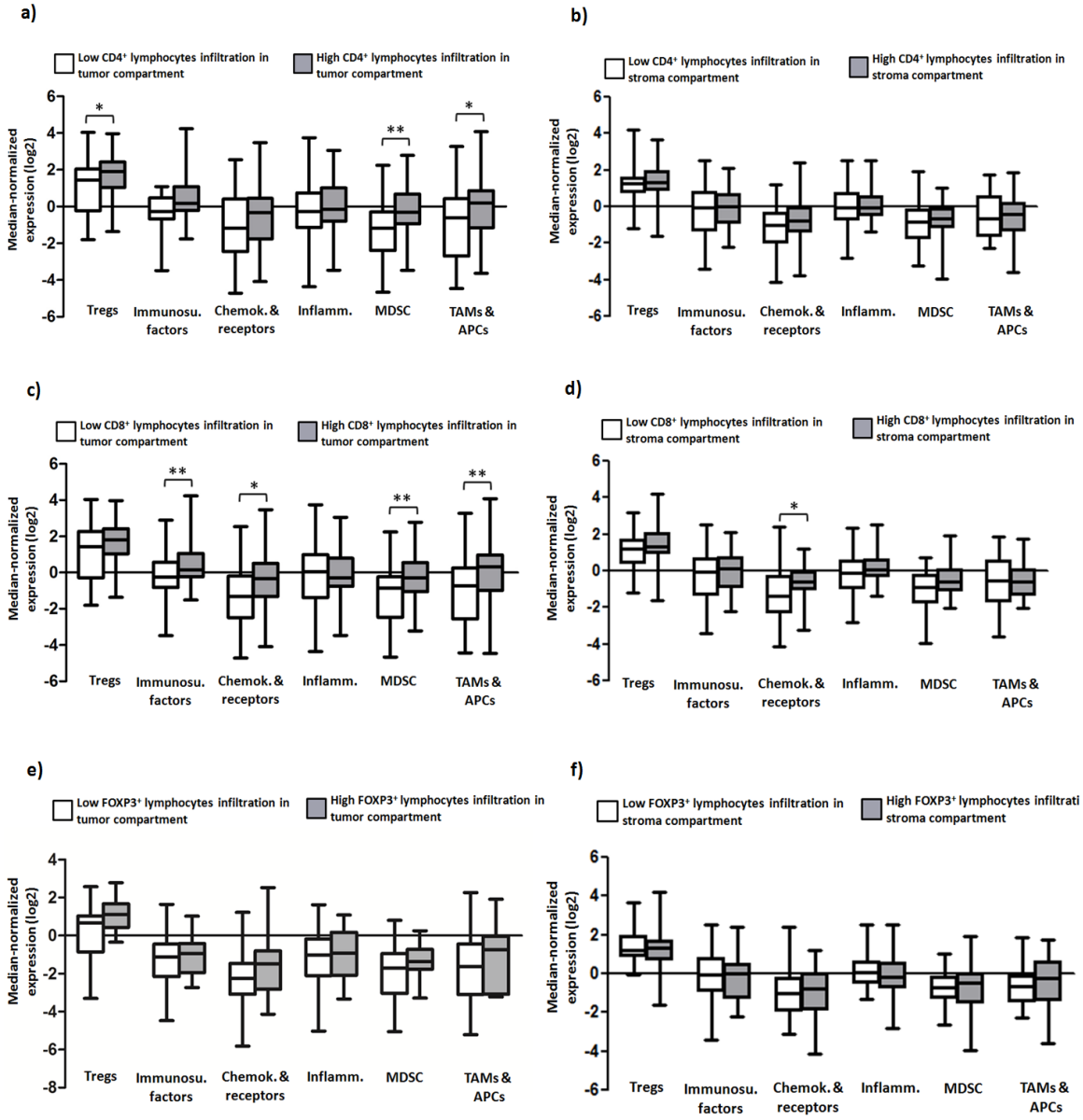


Figure 54. Gene expression patterns in tumour and stroma locations according to a) tumoural $CD4^+$ cell infiltration, b) stromal $CD4^+$ cell infiltration, c) tumoural $CD8^+$ cell infiltration, d) stromal $CD8^+$ cell infiltration, e) tumoural $FOXP3^+$ cell infiltration, and f) stromal $FOXP3^+$ cell infiltration. Gene expression patterns represent regulatory T cells (Tregs), immunosuppressive factors, chemokines and their receptors, inflammation processes, myeloid-derived suppressor cells (MDSCs), tumour associated macrophages (TAMs), and other antigen presenting cells (APCs) according to the immune cell infiltration level. The relative gene expression level is represented as the log2-median of the expressions obtained for each gene. In the figures, the asterisks represent Mann-Whitney U significance test levels as follows: $*p = 0.05\text{-}0.01$, and $**p < 0.01$.

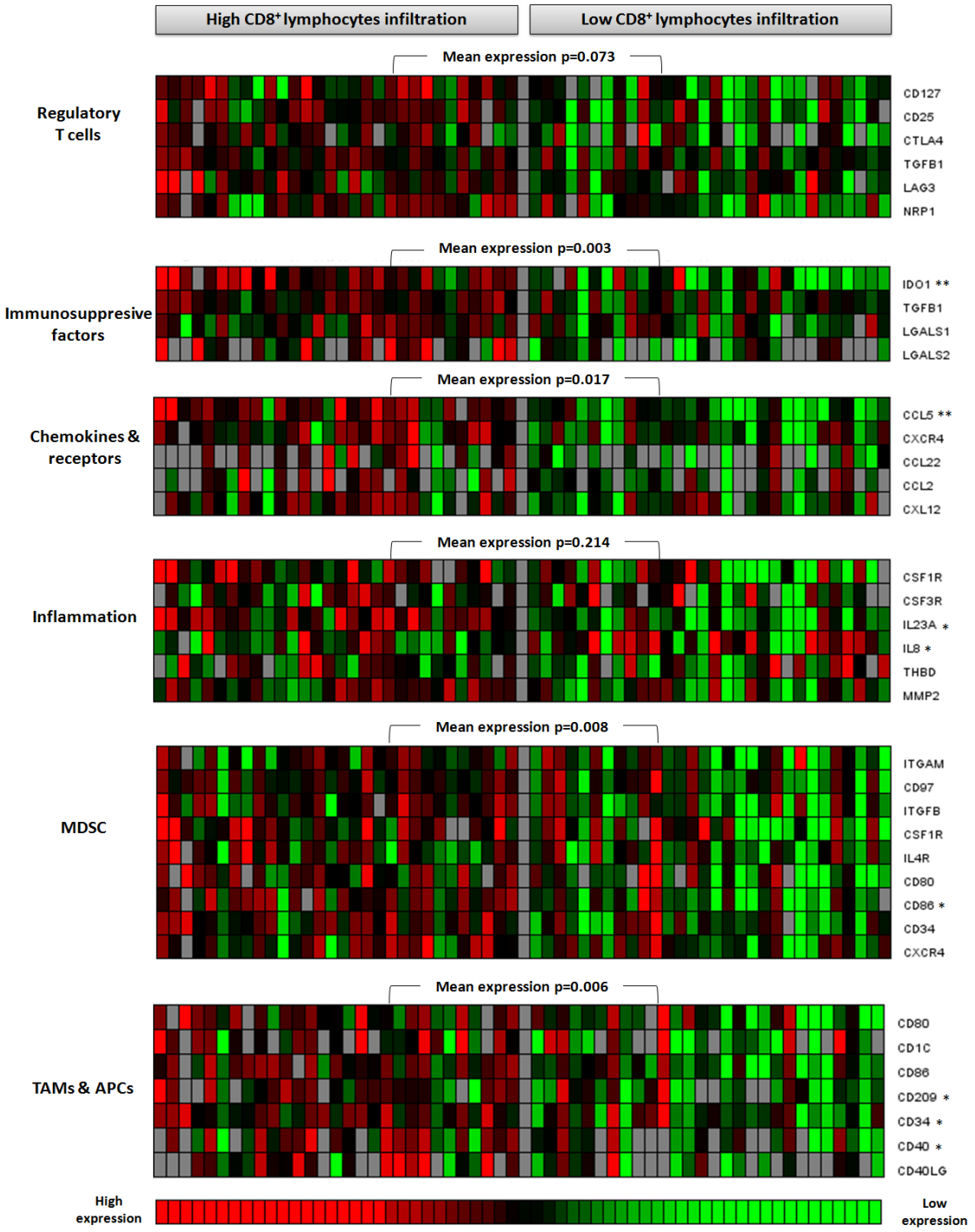


Figure 55. Heat map representation of patterns of genes related to immunoregulation according to CD8$^+$ cell infiltration in the tumour nest. Gene expression patterns represent regulatory T cells (Tregs), immunosuppressive factors, chemokines and their receptors, inflammation processes, myeloid-derived suppressor cells (MDSC), tumour associated macrophages (TAMs), and other antigen presenting cells (APCs) according to the level of immune cell infiltration. Patients are plotted according to their CD8$^+$ cell infiltration levels in each HPF, and thus, are divided into patients with high and low levels of infiltration. Gene expression is represented as the log2-median of the expressions obtained for each gene and the asterisks represent Mann-Whitney U significance test levels as follows: *p = 0.05-0.01, ** p < 0.01.

According to these results, we postulated the existence of two major tumour microenvironment phenotypes. One major subset presents a T cell-inflamed phenotype consisting of infiltrating T cells, which reflects innate immune cell activation, and in which immunoregulation processes are activated in order to resist immune attack. The other major phenotype lacks this T-cell inflamed phenotype and resists immune attack through immune system exclusion or ignorance. These differences may be due to the antigens presented in the tumour. On one hand, some tumour antigens are shared by different types of tumours and were identified as possible therapeutic targets, however, many of these shared antigens are also expressed at some level by self-tissues, which can lead to immunological tolerance. On the other hand, neoantigens generated by point mutations in normal genes, which are unique to individual tumours, can result in much more potent antitumour immune response which tries to control the tumour via immune effector cells, thus creating a more favourable clinical outcome (Gajewski et al., 2013)

It is remarkable that even with the activation of this immunosuppressive network, the immune contexture dominated by high levels of $CD8^+$ cell infiltration is associated with longer survival. Therefore, our results indicate that patients with a tumour microenvironment in which the immune system is able to recognise tumour antigens and activate a potent immune response, are more likely to have better outcomes, and that the activation of the immunosuppressive network is a consequence of this immune response. In fact, the group of patients with low levels of $CD8^+$ cell infiltration presented lower levels of immunosuppressive cell and process pattern expression, and it was associated with worse survival. The positive correlation of immunosuppressive markers with improved outcome and improved therapy responses has also been described in other studies (Denkert et al., 2014; West et al., 2013).

Although the presence of $FOXP3^+$ cells in the stroma compartment was correlated with worse OS, we did not observe any differences in the expression pattern analysed. However, it is known that these cells play an important role in tumour progression. Interestingly, the negative prognostic value of the presence of $FOXP3^+$ cells in the stroma compartment was increased in the subgroup of patients with low levels of $CD8^+$ cell infiltration in the tumour compartment. This observation might indicate that there may be crosstalk between these two types of immune cells.

3. CORRELATION BETWEEN INFILTRATING IMMUNE CELLS AND IMMUNOREGULATION GENE EXPRESSION SIGNATURES

In this section we assessed if there was any correlation between the type, density, and location of immune cells and gene expression signatures of prognostic value. The term "expression signature" refers to a group of genes whose expression pattern is uniquely characteristic of a biological phenotype. Thus, clusters and expression scores with prognostic value were included in this analysis. Among the gene expression signatures obtained from fresh-frozen samples, we observed a significant correlation with the immune checkpoint score and $CD8^+$ cell infiltration (Figure 56). Patients with a high immune checkpoint score (those with longer survival medians), had a higher number of $CD8^+$ cells in their tumour microenvironment (p = 0.030). High immune checkpoint scores might reflect high levels of $CD8^+$ cell infiltration and therefore high levels of antitumour activity, which could explain the association with longer survival. In addition, the immune checkpoint score was identified as an independent prognostic factor which indicated its possible utility in clinical practice.

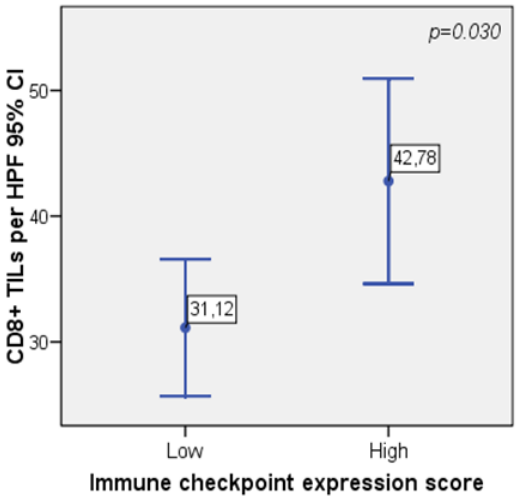


Figure 56. Correlation between the immune checkpoint expression score obtained in fresh-frozen samples and $CD8^+$ cell infiltration levels in the tumour microenvironment. Error bars represent the 95% CI of the mean.

As for the expression signatures obtained from FFPE samples, and independently obtained from tumour and stroma locations, we observed two correlations, both in the tumour compartment. Firstly, patients grouped in tumoural Cluster I had significantly a higher levels of $CD8^+$ cell infiltration (p = 0.001; Figure 57a). This cluster comprised a group of patients with a more heterogeneous gene expression pattern compared with patients in Cluster II (who had low levels of $CD8^+$ cell infiltration), although there was a tendency for higher gene expression levels. It is also important that the group of patients, who had significantly better survival, also had high levels of $CD8^+$ immune cell infiltration.

Finally, we also found an association between the tumour risk score and tumoural $CD8^+$ cell infiltration: patients with a high tumour risk score presented less $CD8^+$ cell infiltration (p = 0.004; Figure 57b). The tumour risk score was composed of the expression of 6 genes, some of which had a negative regression coefficient (indicating their protective value) while others had a positive regression coefficient, indicating that they were associated with an increased risk. Among these 6 genes the one with the strongest effect on the score was THBD. Because THBD plays an anti-inflammatory role, the high tumour risk score might indicate that this patient subgroup has a phenotype lacking T cell-inflamed properties. Therefore their tumours might be ignored by their immune systems and are likely to be more aggressive than tumours with a low tumour risk score. No correlation was observed between these expression signatures and $CD4^+$ or $FOXP3^+$ cell infiltration.

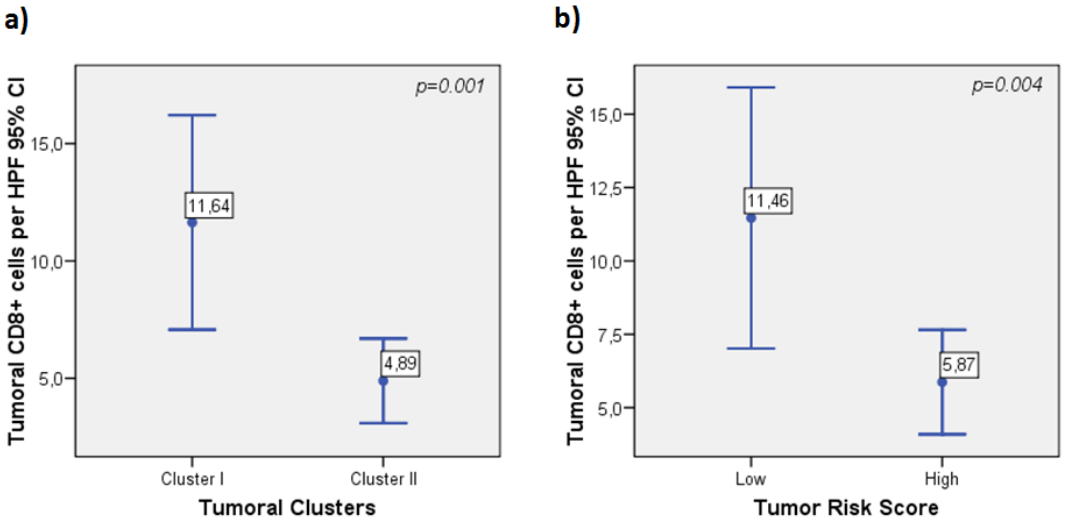


Figure 57. Correlation between the expression signatures obtained from FFPE samples and $CD8^+$ cell infiltration. a) Correlation with the tumoural clusters and, b) correlation with the tumour risk score. Error bars represent the 95% CI of the mean.

D) INTEGRATION OF RESULTS FROM STUDY I

Study I focused on the analysis of prognostic biomarkers related to immunoregulatory processes in patients with resected NSCLCs (stages I-IIIA). Although most of these patients are treated with curative intent, the associated survival is less than optimal with the 5-year survival ranging from 50% for stage IA to 15% for stage IIIA (Burotto et al., 2014). Currently, there are still shortfalls in the approach used for selecting patient adjuvant therapies based on the surgical stage alone. Thus, the great challenge is to identify patients at the greatest risk of recurrence and their potential response to specific treatments, avoiding any unnecessary chemotherapy-associated toxicities. Therefore, it is essential to discover new biomarkers that could help physicians in the management of the disease. Over the last decade, the field of tumour immunology has changed, and it is now accepted that the immune system plays a pivotal role in cancer. The potential effect of the patient's immune system on clinical outcome is important not only for the identification of prognostic markers, but also markers that predict treatment-response. Thus, the study of immune-related markers, especially those implicated in immunoregulatory processes, could provide valuable prognostic information about resected NSCLCs that could help in future clinical practice.

In this study, the prognostic value of immune-related markers was analysed in patients with resected NSCLCs using different approaches. In the first part of the study, the aim was to identify molecular biomarkers by RTqPCR. This technology is considered to be the gold standard in gene expression quantification and some of its major advantages are its speed, sensitivity, and the low amount of RNA that it requires. The analysis of gene expression biomarkers was first assessed in fresh-frozen tumour and normal lung tissue samples. An introductory analysis indicated the existence of different groups of patients according to their expression pattern, and the group of patients who expressed high levels of immune-related and immunoregulatory genes survived longer. Individual survival analysis also revealed associations between markers like IL23A, FOXP3, and LGALS2 with better outcomes. Furthermore, a score composed of the expression of immune checkpoint related genes was constructed following a mathematical model. A high immune checkpoint score was associated with longer OS and PFS, and was demonstrated to be an independent prognostic biomarker, along with *KRAS* status. Because immune checkpoint blockade demonstrated promising clinical results in NSCLC patients, due to the activation of antitumour immunity (Creelan, 2014), the fact that high immune checkpoint scores were correlated with better survival seemed

paradoxical. However, previous studies have also demonstrated that the expression of individual immune checkpoint genes was associated with better outcomes (Salvi et al., 2012; Velcheti et al., 2014).

In the light of these results, and because of increasing evidence suggesting that biomarkers can present distinct prognostic values depending on where they are expressed, in a second approach, gene expression levels were assessed separately in the tumour nest and the adjacent stroma. For this purpose, microdissected FFPE samples were used to better understand the prognostic value of immunoregulatory markers, by analysing a larger number of genes. Although fresh-frozen samples were of higher quality than FFPE samples, the latter are easy to obtain and allowed us to work with the tumour and stroma compartments separately. Unsupervised clustering analysis using these gene expression data indicated the existence of groups of patients with distinct outcomes. Patients with over-representation of immunoregulation genes in both tumour and stroma compartments had longer survival. Moreover, individual survival analysis indicated that several markers had a positive prognostic value when present in the tumour compartment (CD4, CD8, FOXP3, LAG3, and CD209 among others) but only two genes (CD8 and CCL5) were correlated with survival when expressed in the stroma compartment. Finally, an expression prognostic score, comprising the expression of six genes (CD25, CD4, TGFB1, CD44, CD1C, and THBD) in the tumour compartment, allowed the identification of a subgroup of patients with an increased risk of progression and death.

Taking the results from both approaches into account, we observed that clustering analysis in both types of samples indicated that, in general, the groups of patients expressing higher amounts of immunoregulatory genes had better outcomes. However, individual analysis of the markers yielded different results. For instance, in fresh-frozen samples markers such as IL23A and LGALS2 had a prognostic value that was not corroborated when their expression was assessed in the tumour and stroma compartments separately. On the other hand, the prognostic value of FOXP3, the most specific marker of Tregs, was observed in both types of samples. In particular, high levels of FOXP3 expression were associated with longer survival when present in the whole tumour samples and in the tumour nest, but not in the stroma compartment. This positive prognostic value differs from previous studies, suggesting that the presence of Tregs is associated with worse outcomes due to their inhibition of the antitumour immune response (Petersen et al., 2006). Thus, we hypothesised that Tregs might not be the only cells that express FOXP3 in the tumour microenvironment. Other authors have observed that tumour cells can express FOXP3, and that this expression attenuates the

negative prognostic value of the presence of high numbers of Tregs (Tao et al., 2012). Therefore, the expression of FOXP3 by tumour cells and not by Tregs could be responsible for the positive prognostic value of this marker in the whole tumour and in the tumour nest.

We also observed that conventional T cell markers, such as CD4 and CD8, had a positive prognostic value when analysed in the tumour, and in the case of CD8, also in the stroma compartment. Furthermore, combining these biomarkers with FOXP3 allowed a group of patients with comparatively worse survival to be identified, as defined by high levels of FOXP3 in the stroma and low levels of CD4 or CD8 in the tumour. Although the expression of FOXP3 in the stroma was not correlated with survival, the combination of high CD25 expression levels and low CD127 levels, which are also characteristics of the Treg phenotype, was associated with worse PFS in this compartment. This combination, along with CD8 expression in tumour nest, were identified as independent prognostic factors for PFS, whereas only the expression of CD8 in the stroma was a significant prognostic factor for OS.

To further validate these results and to gain a greater insight into the immune picture in the NSCLC microenvironment, the presence of $CD4^+$, $CD8^+$, and $FOXP3^+$ positive cells was assessed by IHC in the same FFPE samples in the third part of the study. Survival analysis revealed that $CD8^+$ cell infiltration in the tumour compartment was associated with longer survival, whereas $FOXP3^+$ cell infiltration in the stroma was associated with the opposite outcome. However, $FOXP3^+$ cells infiltrating the tumour nest were not associated with any prognostic factor, which differs from its positive prognostic value when analysed at the mRNA level. This might indicate that Tregs are not the only cells that express FOXP3 in the tumour microenvironment; the negative prognostic role of $FOXP3^+$ cell infiltration in the stroma compartment agrees with the prognostic value of the combined biomarker, defined by high CD25 and low CD127 expression levels in the stroma compartment, which may indicate that this combined biomarker could possibly be used as a surrogate marker for the presence of Tregs.

Furthermore, the negative prognostic value of the combined biomarkers (indicating high levels of FOXP3 in the stroma and low levels of CD4 or CD8 in the tumour) was corroborated in situ with the results from IHC analysis. $CD8^+$ cell infiltration in the tumour compartment remained an independent prognostic factor for OS and PFS; moreover, $FOXP3^+$ cell infiltration was also found to be an independent predictor for OS. Therefore, it seems that the strongest prognostic value lies in the presence of CD8 markers present at both the mRNA and protein levels. The presence of $FOXP3^+$ cells could also be used in order to distinguish a

subtype of patients with worse survival and who could benefit from adjuvant chemotherapy, especially with drugs such as cyclophosphamide that can modulate or deplete Tregs (Le and Jaffee, 2012).

In order to further explore the immune contexture in resected NSCLC patients, we combined the results obtained by the different approaches. The objective was to evaluate if there were differences between immunoregulatory expression patterns in patients with high and low infiltration of $CD4^+$, $CD8^+$, or $FOXP3^+$ cells. Gene expression patterns from fresh-frozen samples indicated that patients with higher $CD8^+$ cell infiltration in the tumour microenvironment presented higher expression of genes related to APCs and the immune response but also higher expression of immunoregulatory markers. In contrast, high levels of $FOXP3^+$ cell infiltration in the tumour microenvironment were associated with the upregulation of the clusters of Tregs, and with immune cell trafficking and immune checkpoint markers. As for the results obtained from tumour and stroma FFPE samples, we observed that high levels of $CD8^+$ cell infiltration in the tumour nest were associated with a T cell-inflamed phenotype characterised by higher expression of genes related to innate immune response as well as immunosuppressive pathways. However, no significant differences were observed in patients with high and low levels of $FOXP3^+$ cell infiltration. Therefore, our results indicate that patients with high levels of $CD8^+$ cell infiltration (those with better outcomes) seem to present active immune responses, which could be activated by immune system recognition of tumour antigens, whilst also presenting feedback activation of immunosuppressive pathways. Importantly, the positive correlation of immunosuppressive markers and improved outcome has also been observed in other types of tumour (Denkert et al., 2014).

Finally, the correlation between the different expression signatures with prognostic value and the level of immune cell infiltration was assessed. Of note, the immune checkpoint score calculated from fresh-frozen sample data was positively correlated with $CD8^+$ cell infiltration, whereas the tumour risk score, calculated from FFPE sample data, presented a negative correlation. Thus, the immune checkpoint score may reflect a favourable immune context in which the immune system recognises the tumour, whereas the opposite situation may be occurring in the tumours of patients with high risk scores. This score, whose main component (THDB) has anti-inflammatory properties, might reflect a detrimental immune context characterised by the lack of T-cell inflammation that leads to an immune response.

In summary, in this study several prognostic immune-related biomarkers were identified in resected NSCLCs. Our results indicated that, in general, patients with high

expression of immunoregulatory markers presented better outcomes. Furthermore, immune contexture analysis revealed that tumours with high levels of cytotoxic T cell infiltration, whose presence was correlated with longer survival, expressed high levels of immune response markers as well as immunoregulation markers. Taken together, our results indicate the existence of two possible immune-scenarios in NSCLCs. In the first, the tumour is recognised by the immune system and a T-cell response is activated, which in turn activates immunoregulatory pathways. In this case patients had better outcomes. In the second scenario, which is associated with worse outcomes, the immune system does not recognise the tumour and there is no immune response activation, therefore immunoregulatory pathway activation is not required. These results provide new insight into the tumour immunity field in NSCLC, and could be useful in the future development of prognostic and therapeutic tools.

STUDY II. BIOMARKERS IN ADVANCED NON-SMALL CELL LUNG CARCINOMA

A) ANALYSIS OF GENE EXPRESSION BIOMARKERS IN BLOOD SAMPLES

In this study the analysis of immunoregulatory biomarkers was assessed in order to evaluate the viability of detecting mRNA markers in the peripheral blood of patients with advanced NSCLC. Determining the presence of biomarkers in peripheral blood has some advantages over using patient tumour biopsies: the collection of the sample is minimally invasive, it allows patients to be monitored during the disease, and more importantly, it permits biomarkers to be determined in every single patient even if there is no surgical or biopsy specimens available from the tumour. The latter is of great importance in patients with a diagnosis of advanced NSCLC because their tumours are not resected as part of the treatment, thus no tumour tissue sample is available (Hanash et al., 2011). Furthermore, the information acquired from a single biopsy provides a spatially and temporally limited snap-shot of a tumour and might fail to reflect its heterogeneity. A liquid biopsy, or blood sample, can provide information about the genetic landscape of all the cancerous lesions present (primary and metastases) as well as offering the opportunity to systematically track genomic evolution (Crowley et al., 2013).

The following describes our first approach to assessing immunoregulatory biomarkers in peripheral blood by determining the expression of 11 genes related to immune response, Tregs, and MDSCs. We aimed to obtain initial insights into possible changes in tumour immunoregulation process markers in patient blood samples, to correlate these results with clinicopathological variables, and to evaluate their prognostic, diagnostic, and predictive value in advanced NSCLC.

1. PATIENT CHARACTERISTICS AND SAMPLES ANALYSED

This retrospective study included 49 patients with advanced NSCLC enrolled in a multicentre study coordinated by the Spanish Lung Cancer Group. Peripheral blood samples (2.5 ml) were collected and stored in PAXgene™. RNA was extracted from blood samples obtained at two time points: before treatment and after three cycles of chemotherapy with

docetaxel and cisplatin. The most relevant demographic and clinicopathological characteristics of the cohort are shown in Table 37. The median patient age was 57.8 [37-75] years, 89.8 % were male and 55.2 % had ADC. Moreover, 85.7% of the patients were diagnosed at stage IV of the disease and 51% had a PS of 1. Our control group comprised 54 anonymous, age- and gender-matched, healthy volunteers without any acute or chronic inflammatory conditions. From a total number 102 samples analysed, 49 were obtained pre-treatment, 25 post-treatment, and 54 were from healthy donors.

Table 37. Clinicopathological characteristics of the advanced NSCLC patients included in this study.

Characteristics	N	%
Age at surgery (median, range)	**57.8 [37-75]**	
Gender		
Male	44	89.8
Female	5	10.2
Stage		
IIIB	7	14.3
IV	42	85.7
Histology		
ADC	27	55.2
SCC	11	22.4
Others	11	22.4
Performance Status		
0	24	49
1	25	51
Response[a]		
PR	11	22.4
SD	13	26.5
PD	23	46.9
NA	2	4.1

ADC, adenocarcinoma; SCC, squamous cell carcinoma; PR, partial response; SD, stable disease; PD, progressive disease; NA, not available. [a] Tumour response was evaluated according to the Response Evaluation Criteria for Solid Tumours [RECIST] (Therasse et al., 2000).

RNA was isolated from 2.5 ml peripheral blood samples, and an optimal RNA concentration was obtained for all samples: the median concentration obtained was 66.7 ng/µl (Range: 20.1-342.0). The quality and integrity of the RNA extracted was confirmed by capillary electrophoresis, with all the samples showing representative 18S and 28S RNA peaks in the electropherogram, and a RIN higher than 7.

2. RELATIVE GENE EXPRESSION

In this study we evaluated the expression levels of different immunoregulatory markers, mainly related to two types of immunoregulatory cells: Tregs (FOXP3, CD25, CD4, CD8, CD127, and TGFB1), and MDSCs (CD97, CSFR1, IL4R, ITGAM, and ITGB2). The expression of three endogenous genes (ACTB, GAPDH, and GUSB) was tested in a subset of approximately 30% of the samples in order to establish, using geNorm software, the best internal control, which was the combination of GAPDH and ACTB. Furthermore, relative expression was normalised against a reference sample, which in this case was the Jurkat cell line. In order to better visualise gene expression results, they are represented as the log2-median of expression. As shown in Figure 58, all the genes showed higher expression in samples than in the control cell line; ITGAM was the most abundantly expressed gene and TGBF1 had the lowest expression.

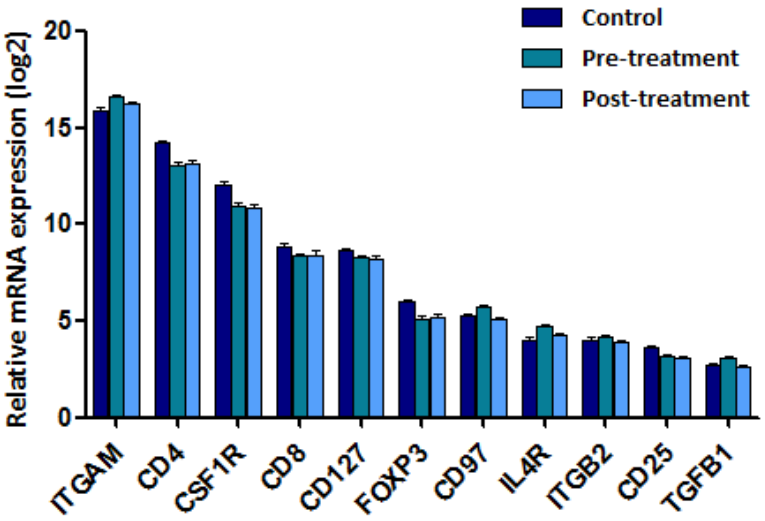


Figure 58. Relative expression of the genes analysed in peripheral blood samples. The relative gene expression (fold-change) is represented as the log2-median of the expressions obtained for each gene in three samples: controls vs. advanced NSCLC patients before and after treatment with three cycles of chemotherapy with docetaxel and cisplatin.

ITGAM is the subunit of the heterodimeric integrin alpha-M beta-2 (αMβ2) molecule, also known as macrophage-1 antigen (Mac-1) or complement receptor 3A (CR3A). ITGAM is expressed on the surface of many leukocytes involved in the innate immune system, including monocytes, granulocytes, macrophages, and NKs. It mediates inflammation by regulating leukocyte adhesion and migration (Solovjov et al., 2005). Moreover, ITGAM is expressed in every MDSC type and is frequently used as a marker to identify and isolate these cells

(Gabrilovich et al., 2012). Because it is expressed in several types of immune cells that can be found in peripheral blood, we expected its expression levels to be high, as for other markers also present on the surface of different immune cells, such a $CD4^+$ and $CSFR1^+$ cells. In contrast, TGFB1 showed the lowest expression in blood. This factor is released by immunosuppressive cells or tumour cells in order to reduce inflammation or to suppress tumour cell immune responses (Flavell et al., 2010). A Wilcoxon pair-wise test revealed significant differences in the expression of some of the markers before and after the treatment. These markers were CD97 ($p = 0.005$), IL4R ($p = 0.020$), ITGB2 ($p = 0.045$), and TGFB1 ($p < 0.001$), and in all the cases their expression decreased after the treatment. These could indicate that chemotherapy based on docetaxel and cisplatin reduced the levels of circulating MDSC mRNA markers, as well as immunosuppressive factors like TGFB1.

B) ANALYSIS OF THE DIAGNOSTIC, PROGNOSTIC, AND PREDICTIVE VALUE OF IMMUNOREGULATORY BIOMARKERS

1. THE DIAGNOSTIC VALUE OF THE GENES ANALYSED

A Mann-Whitney U test revealed that there were significant differences in the gene expression levels in advanced NSCLC patients in comparison to healthy donors. Significant increases in gene expression levels were observed for CD4, CSF1R, CD8, CD127, FOXP3, and CD25 in controls in comparison to patients. On the other hand, ITGAM, CD97, and TGFB1 showed significantly higher expression in the patient samples (Table 38).

Table 38. Expression levels of the analysed genes in patients with advanced NSCLC or healthy donors.

	Genes	CD4	CD8	CD25	CD97	CD127
Controls	**Median**	2,0427.3	471.755	12.549	39.283	405.758
	Minimum	4,688.6	83.847	3.591	2.312	161.441
	Maximum	4,1354.1	1,745.4	37.908	145.245	940.219
Patients	**Median**	9,237.7	334.346	8.808	51.585	289.747
	Minimum	1,532.6	66.970	2.644	30.276	55.423
	Maximum	3,0004.3	2,097.6	44.937	108.417	1,378.1
	***p*-value**	<0.0001	0.020	0.005	0.002	0.022
	Genes	**CSF1R**	**FOXP3**	**IL4R**	**ITGAM**	**TGFB1**
Controls	**Median**	4,522.7	65.033	16.591	6,5700.4	6.755
	Minimum	378.833	16.607	0.844	6101.445	0.806
	Maximum	1,2815.7	168.226	51.633	27,3741	12.159
Patients	**Median**	1,973.7	38.679	22.985	10,5587	8.239
	Minimum	349.823	9.753	11.333	5,4950.6	5.483
	Maximum	8181.007	120.539	78.758	17,8420	15.232
	***p*-value**	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Because most of the genes analysed presented differences in gene expression levels between advanced NSCLC patients and controls, we decided to investigate their possible role as diagnostic biomarkers. For this purpose, we used the receiver operating characteristic (ROC) curve and area under the curve (AUC) metrics. The curves represent the relationship between the sensitivity and specificity of each biomarker. Statistically significant predictive models for distinguishing patients from controls could be constructed using TGFB1, ITGAM, IL4R, and CD97 which yielded AUCs of 0.770 (95% CI, 0.675-0.862), 0.816 (95% CI, 0.730-0.901), 0.704 (95% CI, 0.604-0.804), and 0.681 (95% CI, 0.578-0.784) respectively (Figure 59), and significant differences were found between them (p < 0.001, Chi-square test). Using the ROC curve

coordinates, the Youden Index was calculated (sensitivity + specificity) for each biomarker and cut-off values that better distinguished between patients and controls were selected (Table 39). ITGAM gene expression levels were the most suitable biomarker for discriminating patients and controls, with a sensitivity of 85.71 % and a sensitivity of 71.69 %.

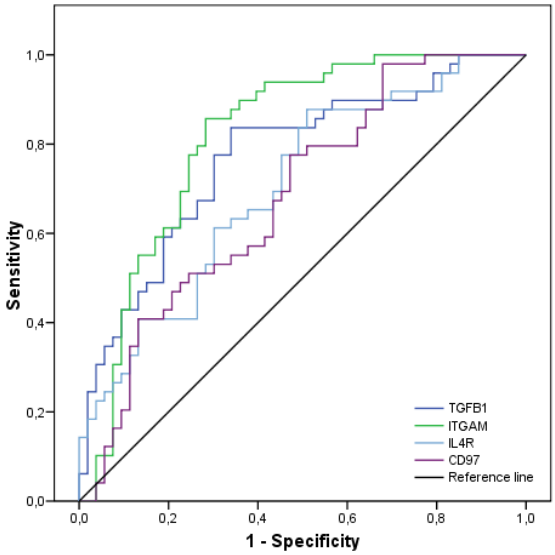


Figure 59. ROC curves representing the diagnostic value of the biomarkers. ROC curves represent the sensitivity and 1- specificity obtained when all the cut-off values for the markers are considered.

The only marker studied that was sufficiently sensitive and specific to make it a candidate diagnostic biomarker was ITGAM, which is expressed by different immune cells such as macrophages, neutrophils, and other leukocytes. Although it is a marker used to identify MDSCs, which are immunosuppressive cells of interest in this study, ITGAM expression cannot be linked to this subtype of cells because of the nature of the methodology we used. However, this was not the only MDSC marker with higher expression in advanced NSCLC patients than in controls. In fact, other genes that are used along with ITGAM to identify MDSCs, such as CD97 and IL4R, were also expressed in higher amounts in these patients than in controls.

Table 39. Cut-off values that better distinguish between patients and controls.

Genes	Cut-off value	Sensitivity	Specificity	Youden Index
TGFB1	7.143	83.673	66.038	1.497
ITGAM	7,6281.9	85.714	71.698	1.574
IL4R	15.705	87.755	49.057	1.368
CD97	40.017	77.551	52.830	1.304

The table represents the gene expression cut-off values for each biomarker, with higher sensitivity and specificity selected using the Youden Index.

Haematopoietic abnormalities in tumour-bearing hosts, originally described as "neutrophilia" and characterised by the abundance of less mature cells, have long been observed. Mechanistically, this is associated with the tumour secretion of cytokines and chemokines that induces myeloid cell proliferation, cell accumulation, and tumour infiltration. Furthermore, not only do tumours secrete growth factors that induce myelopoiesis and chemokines that mobilise MDSCs, but they may also limit their maturation and differentiation, thereby contributing to their accumulation (Talmadge and Gabrilovich, 2013). Therefore, the presence of MDSCs could be one of the main reasons for the detected increase in ITGAM and other markers related to these immune cells in the peripheral blood of patients with advanced NSCLC.

These results suggest that there are expression differences in some of the biomarkers analysed in advanced NSCLC patients vs. healthy controls, and that these changes are measurable using our methodological approach. The changes observed indicated a decrease in markers such as CD4, CD8, and CD25 in NSCLC patients, and an increase in markers related to MDSC. In fact, the expression of ITGAM allowed advanced NSCLC patients to be discriminated from controls with adequate sensitivity (85.7%) and specificity (71.6%), suggesting that these biomarkers have a diagnostic value which warrants further study in order to validate them.

2. CORRELATION WITH CLINICOPATHOLOGICAL VARIABLES

We observed significantly higher levels of CD97 and ITGAM in patients with a PS of 1 (p = 0.006 and p = 0.001, respectively; Figure 60) and higher levels of CSF1R and FOXP3 in patients with a PS of 0. Furthermore, male patients presented significantly higher levels of IL4R (p = 0.006) and ITGAM (p = 0.027; Figure 61). There was no significant association between gene expression levels and clinical characteristics such as tumour stage, histology, metastatic location, age, or treatment response.

The presence of higher levels of expression of genes such as ITGAM and CD97 in patients with a worse PS at diagnosis might indicate that these patients have a more aggressive disease mediated by the increase of immunosuppressive cells, in this case MDSCs which expressed high levels of these markers (Gabrilovich and Nagaraj, 2009). To the best of our knowledge, this is the first time that higher expression of markers related to MDSCs (or other immune cells expressing ITGAM and IL4R) has been reported in male patients than in female patients.

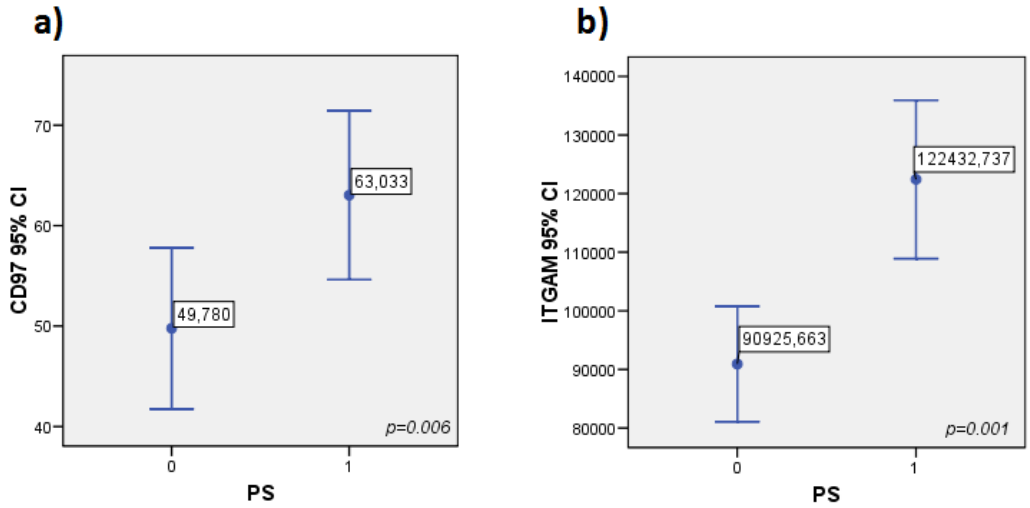


Figure 60. Some examples of the correlation between performance status (PS) and immune-marker gene expression. a) CD97 and b) ITGAM. Error bars represent the 95% CI of the mean.

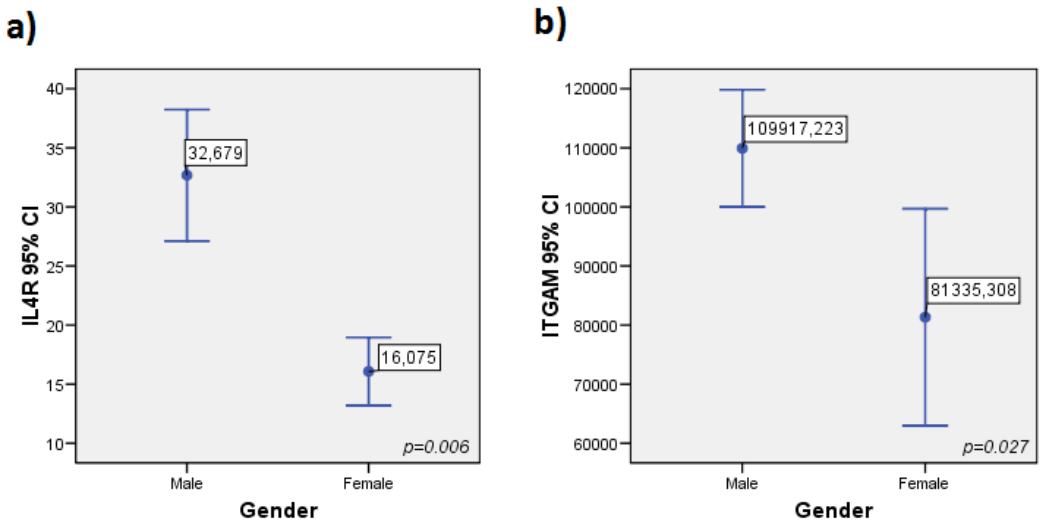


Figure 61. Correlation between gender and immune-marker gene expression. a) IL4R and b) ITGAM. Error bars represent the 95% CI of the mean.

As for clinical responses to the treatment, 11 patients (22.4 %) presented a PR, 13 patients (26.5 %) had SD, and 23 patients (46.9%) had PD. Although a Wilcoxon pair-wise test revealed significant differences in the expression of some of the genes before and after the treatment, we did not observe any correlation between gene expression levels and responses to treatment. This rendered assessment of their possible role as predictive response-biomarkers pointless.

3. SURVIVAL ANALYSIS

Of the 49 patients with advanced NSCLC included in this part of the study, 46 (94%) relapsed and a total of 42 (85.7%) died. The median follow-up was of 9.63 months [range: 1.6-39.9]. The prognostic value of the different clinicopathological variables and immune biomarkers was assessed using univariate a Cox regression for OS and PFS. The gene expression levels of the biomarkers analysed were dichotomised according to their individual medians. The results obtained in the univariate analysis by Cox regression are shown in Table 40, and also show the hazard ratios and *p*-value for each marker.

Table 40. Results obtained from survival analysis according to clinicopathological variables.

	OS			PFS		
Variable	**HR**	**[95% CI]**	***p*-value**	**HR**	**[95% CI]**	***p*-value**
Gender						
Male vs. Female	0.729	0.259-2.056	0.551	0.459	0.163-1.291	0.140
Age						
≤ 60 vs. > 60	0.513	0.277-0.951	0.034*	0.706	0.394-1.265	0.242
Stage						
III vs. IV	0.694	0.303-1.591	0.388	0.907	0.397-2.070	0.816
Histology						
ADC vs. SCC vs. Others	0.712	0.469-1.082	0.111	0.800	0.540-1.187	0.268
PS						
0 vs. 1	2.554	1.361-4.792	0.003*	1.783	0.982-3.234	0.057
Metastasis						
Local vs. Distant	1.031	0.547-1.945	0.924	1.001	0.544-1.843	0.997

Results obtained using a univariate Cox regression method. HR, hazard ratio; LN, lymph node; OS, overall survival; PFS, progression free survival; PS, performance status; EGFR, epidermal growth factor receptor; *$p < 0.05$.

Univariate analysis of clinicopathological variables showed that older patients, as well as those with a PS of 1, have worse OS ($p = 0.034$ and $p = 0.003$ respectively). However, no other significant association was found for the rest of the variables. Univariate analysis showed that none of the genes analysed in this part of the study had any prognostic value for advanced NSCLC when analysed individually (Table 41). This lack of association with survival could be explained by the low number of advanced NSCLC patients analysed. Therefore, these markers should be analysed in a larger cohort of patients where any possible differences between patients with low or high levels of these markers are more likely to be detected. The next step was to perform combined analysis with markers related to the same immunoregulatory cells or processes. From the different combinations tested, we observed that advanced NSCLC

patients with high CD25 and low CD127 expression levels in blood samples had worse OS (4.7 vs. 9.8 $p = 0.039$) and shorter PFS (2.4 vs. 5.4 months, $p < 0.001$; Figure 62).

Table 41. Survival analysis results obtained according to immunoregulatory marker expression.

	OS			PFS		
Variable	**HR**	**[95% CI]**	***p*-value**	**HR**	**[95% CI]**	***p*-value**
CD4 *High vs. Low*	0.961	0.521-1.772	0.898	1.236	0.690-2.213	0.476
CD8 *High vs. Low*	0.913	0.494-1.685	0.771	0.947	0.530-1.692	0.855
CD25 *High vs. Low*	1.110	0.601-2.052	0.739	1.195	0.669-2.136	0.547
CD127 *High vs. Low*	0.749	0.405-1.382	0.355	0.649	0.362-1.164	0.147
FOXP3 *High vs. Low*	1.154	0.609-2.189	0.660	1.391	0.775-2.499	0.269
TGFB1 *High vs. Low*	1.744	0.926-3.285	0.085	1.167	0.651-2.090	0.604
CD97 *High vs. Low*	1.146	0.620-2.119	0.663	1.000	0.560-1.785	0.999
CSF1R *High vs. Low*	1.046	0.568-1.927	0.884	1.419	0.792-2.541	0.240
IL4R *High vs. Low*	1.319	0.717-2.426	0.373	0.949	0.529-1.701	0.860
ITGAM *High vs. Low*	1.137	0.616-2.098	0.681	1.001	0.560-1.791	0.996
ITGB2 *High vs. Low*	0.796	0.432-1.467	0.465	0.717	0.401-1.284	0.264

Gene expression levels dichotomised according to their medians. Results from univariate Cox regression method. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; *$p < 0.05$.

As previously explained in this thesis, FOXP3 is a specific Treg marker, but the combination of high levels of CD25 and low levels of CD127 are also considered to be characteristic of Tregs. Therefore, this result may indicate that advanced NSCLC patients with a Treg phenotype, defined by high mRNA levels of CD25 and low levels of CD127 in blood, had worse survival than the rest of the patients. However, since we are detecting mRNA markers from all the nucleated cells in blood (including leukocytes and other non-haematological cells) we cannot elucidate the cellular origin of these markers. This combination of markers has previously been assessed in blood from patients with other types of cancer, but using other methodologies such as flow cytometry. In one of these studies the higher presence of $CD4^{+}$ $CD25^{high}$ $CD127^{low}$ cells in patients with gastric cancer indicated patients with advanced stage of the disease (Shen et al., 2009).

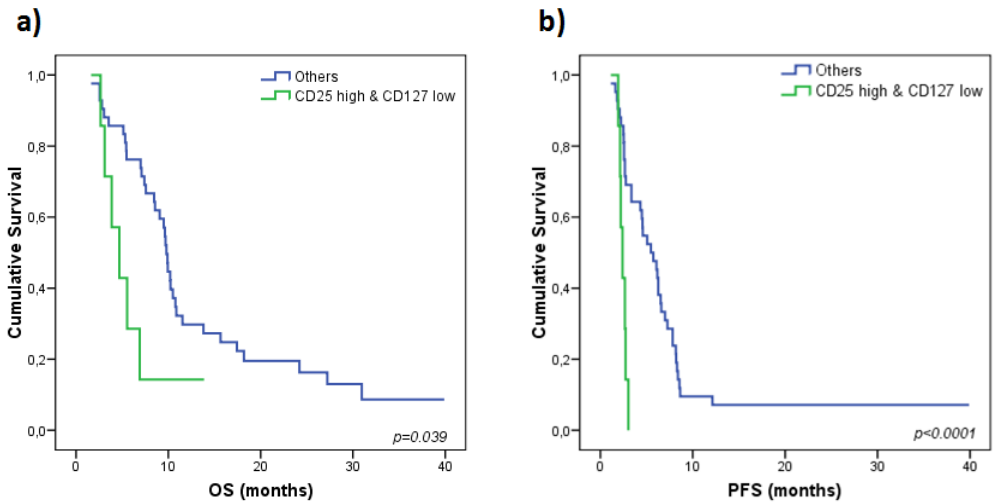


Figure 62. Kaplan-Meier plots according to the combination of CD25 and CD127 expression in blood samples from advanced NSCLC patients. a) OS, and b) PFS. The green line represents patiens with high levels of CD25 and low levels of CD127 in the stroma while the blue line represents other combinations of these two markers. *P*-values were calculated using the Kaplan-Meier test.

Finally, multivariate analysis was performed for OS, using the CD25 and CD127 marker combination and the age and PS variables which were significant from the univariate analysis. The results indicated that PS [HR, 2.67; 95% CI, 1.41-5.06; p = 0.003] and $CD25^{high}$ $CD127^{low}$ expression levels [HR, 2.01; 95% CI, 1.08-6.75; p = 0.045] were independent prognostic factors in advanced NSCLC. As for PFS, the only variable that was associated with this survival parameter was the CD25 and CD127 combination, and so it was not necessary to assess its independency from any other variable. The combination of high CD25 and low CD127 expression levels was also associated with survival when analysed in the stroma compartment of resected NSCLCs. In this case the expression was assessed in FFPE tissue, in the stroma adjacent to tumour nest. We therefore concluded that the combination of CD25 and CD127 might be a surrogate marker for the possible presence of Tregs in the tumour microenvironment.

C) INTEGRATION OF RESULTS FROM STUDY II

In this study, the expression of immunoregulatory biomarkers was assessed in peripheral blood from advanced NSCLC patients. A liquid biopsy, or blood sample, can be obtained from patients in a non-invasive way, at any time during disease. It provides a clearer picture of tumour evolution over time and it can be used to address the tumour heterogeneity problem. The analysis of biomarkers in advanced NSCLC could provide useful information regarding adequate treatments and could also predict patient outcome.

We analysed the expression of 11 genes in a cohort of advanced NSCLC patients, before and after treatment with three cycles of chemotherapy with docetaxel and cisplatin, and in a cohort of matched healthy patients. Gene expression analysis revealed differences in the expression of some of the biomarkers analysed. A decrease in genes related to immune response and an increase in markers related to MDSCs were observed in advanced NSCLC patients. In fact, the expression of one of these markers, ITGAM, was able to distinguish between patients and healthy controls with adequate sensitivity and specificity. Although some differences in the expression of MDSC gene markers were observed after treatment, none of the biomarkers correlated with treatment response and therefore no predictive biomarker was found.

As for survival analysis, the combination of high CD25 and low CD127 expression levels identified a group of patients with worse OS and PFS. This combined marker was an independent prognostic factor. This combination, which might indicate a possible presence of the Treg phenotype in patient blood, could be used as a surrogate marker of tumour status in advanced NSCLC.

V. CONCLUSIONS

1. Unsupervised hierarchical analysis of fresh-frozen specimens from resected NSCLCs according to their gene expression patterns revealed two clusters with different prognostic values.

2. We demonstrated that in resected samples an immune checkpoint score, defined by CTLA4 and PD1 expression, was an independent prognostic factor; patients with high immune checkpoint scores had significantly longer survival times.

3. FFPE samples from resected NSCLC tumour and stroma areas were separately analysed. We observed that combined biomarkers defined by high FOXP3 expression levels in the stroma and low expression of CD4 or CD8 in the tumour compartment were associated with worse survival and these were validated in situ by IHC.

4. High infiltration of $CD8^+$ cells in the tumour compartment was associated with longer survival, whereas high infiltration of $FOXP3^+$ cells, also defined as Tregs, in the stroma was associated with a worse outcome, and both variables were independent prognostic factors.

5. In blood samples from advanced NSCLC patients, ITGAM was found to have a diagnostic value with adequate sensitivity and specificity, and the combination of high CD25 and low CD127 expression level, which are characteristic of a Treg phenotype, identified a subgroup of patients with worse outcomes.

6. The integration of our results indicated that high infiltration rates of $CD8^+$ cells into the tumour, which was associated with better survival, was correlated with gene expression patterns related to immune response and immunoregulation.

Altogether, the results presented in this thesis suggest the existence of two possible immune-scenarios in resected NSCLCs. The first scenario is that of an inflamed microenvironment in which the tumour might be recognised by the immune system, thus activating the immunoregulatory pathways. This scenario correlates with a better prognosis, with the exception of Treg markers, which identify a group of patients with worse survival rates in both resected and advanced NSCLC. The second scenario presents no inflammation, which might indicate that the immune system does not recognise the tumour, and is associated with worse patient outcomes.

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VII. APPENDICES

1. SUPPLEMENTARY TABLES

Supplementary table 1. Efficiency results for the assays used in this study. The efficiency of each TaqMan® assay was evaluated by carrying out serial dilutions of a reference cDNA.

Gen	Slope	Efficiency	Percentage Efficiency
ACTB	-3.123	2.090	100
CCL2	-3.095	2.104	100
CCL22	-3.375	1.978	99
CCL5	-3.469	1.942	97
CD1C	-2.9951	2.157	100
CD209	-3.273	2.021	100
CD25	-3.534	1.919	95
CD33	-3.537	1.918	96
CD34	-3.557	1.911	96
CD4	-3.465	1.944	97
CD40	-3.307	2.006	100
CD40LG	-2.510	2.090	100
CD44	-3.643	1.881	94
CD8	-3.0247	2.141	100
CD80	-3.242	2.035	100
CD86	-3.492	1.934	97
CD97	-3.1091	2.097	100
CDKN1B	-3.705	1.862	93
CSF1R	-4.745	1.625	82
CSF3R	-3.132	2.086	100
CTLA4	-3.373	1.979	99
CXCL12	-3.541	1.916	96
CXCR4	-3.539	1.917	96
FOXP3	-3.387	1.974	99
GAPDH	-2.818	2.264	100
GITR	-3.371	1.980	99
GUS	-2.829	2.257	100
HPRT	-3.569	1.906	95
IDO1	-3.559	1.910	95
IFNG	-2.780	2.289	100
IL10	-3.348	1.989	99
IL12B	-2.528	2.487	100
IL13	-3.397	1.969	98
IL23	-3.302	2.009	100
IL4R	-3.358	1.985	99

IL8	-3.481	1.938	97
ITGAM	-3.327	1.998	100
ITGB2	-3.433	1.955	97
LAG3	-3.579	1.903	95
LGALS1	-3.396	1.970	99
LGALS2	-3.464	1.944	97
MMP2	-3.898	1.805	90
NRP1	-3.708	1.861	93
PD1	-3.249	2.032	100
PDL1	-3.681	1.869	94
TGFB1	-3.617	1.890	95
THBD	-3.539	1.917	96
TNF	-3.343	1.991	100

Efficiency values were measured using the CT slope method. This method involves generating a dilution series of the target template and determining the CT value for each dilution. A plot of CT versus log cDNA concentration is constructed.

Supplementary Table 2. Survival analysis results obtained according to immunoregulatory markers expression in ADC and SCC separately using FFPE samples. Results from univariate survival analysis (using a Cox regression method) based on gene expression levels.

	OS			PFS		
			ADENOCARCINOMA			
Variable	**HR**	**[95% CI]**	***p*-value**	**HR**	**[95% CI]**	***p*-value**
Tumoural CD4 *High vs. Low*	0.265	0.101-0.694	0.007*	0.318	0.143-0.706	0.005*
Tumoural CD8 *High vs. Low*	0.292	0.118-0.723	0.008*	0.290	0.130-0.650	0.003*
Tumoural FOXP3 *High vs. Low*	0.339	0.120-0.953	0.040*	0.496	0.216-1.141	0.099
			SQUAMOUS CELL CARCINOMA			
Stromal CD8 *High vs. Low*	0.287	0.106-0.776	0.014*	0.325	0.136-0.776	0.011*
Tumoural CD209 *High vs. Low*	0.214	0.061-0.749	0.016*	0.239	0.080-0.719	0.011*

Gene expression levels dichotomised according to their medians. Results from univariate Cox regression method. CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression free survival; *$p < 0.05$.

2. APPROVAL FROM THE INSTITUTIONAL ETHICAL AND SCIENTIFIC REVIEW BOARD

CONSORCI HOSPITAL GENERAL UNIVERSITARI VALENCIA

FUNDACIÓ INVESTIGACIÓ HOSPITAL GENERAL UNIVERSITARI VALÈNCIA

Consorcio Hospital General Universitario de Valencia

Comisión de Investigación

APROBACIÓN PROYECTO DE INVESTIGACIÓN

Esta Comisión tras evaluar en su reunión de 29 de abril de 2014 el Proyecto de Investigación:

Título:	Análisis de biomarcadores de inmunoregulación en cancer de pulmón no microcítico		
I.P.:	Marta Uso Marco	Servicio/Unidad	Laboratorio Oncología Molecular

Acuerda respecto a esta documentación:

- Que cumple con los requisitos exigidos por esta Comisión para su realización, por tanto se decide su APROBACIÓN.

Los miembros que evaluaron esta documentación:

		Presente	Ausente	Disculpa
Presidente	Dr. Ricardo Guijarro Jorge	X		
Vocales	Dr. Julio Cortijo Gimeno		x	
	Dra. Goizane Marcaida Benito			x
	Dr. Carlos Sánchez Juan	X		
	D. Federico Palomar Llatas	X		
	Dr. Emilio López Alcina			x
	Dr. Alfonso Berrocal Jaime		x	
	Dr. Julio Álvarez Pitti	x		
	Dr. Miguel Armengot Carceller			X
	Dña. Ángela Garrido Bartolomé			x
	Dr. Miguel Sanfeliu Giner		x	
	Dr. Manuel Navarro Villena			x
	Dra. Amparo Esteban Reboll	x		
Secretario	D. Carlos Gil Santiago		x	

Lo que comunico a efectos oportunos a miércoles, 30 de abril de 2014:	Fdo. Dr. Ricardo Guijarro Jorge Presidente de la Comisión de Investigación:

Consorcio Hospital General Universitario de Valencia

Comité Ético de Investigación Clínica

APROBACIÓN PROYECTOS DE INVESTIGACIÓN

- ANEXO 11 -

Este CEIC tras evaluar en su reunión de 29 de mayo de 2014 el Proyecto de Investigación:

Título:	Análisis de biomarcadores de inmunoregulación en cancer de pulmón no microcítico		
I.P.: Tutor:	Marta Uso Marco Eloisa Jantus Lewintre	Servicio/Unidad	Laboratorio Oncología Molecular FIHGUV

Acuerda respecto a esta documentación:

- Que el Proyecto de Investigación y Hoja de Información al Paciente y Consentimiento Informado presentado reúnen las condiciones exigidas por este CEIC, por tanto se decide su APROBACIÓN.

Los miembros que evaluaron esta documentación:

		Presente	Ausente	Disculpa
Presidente	Dr. Severiano Marín Bertolín	X		
	D. Ernesto Bataller Alonso		X	
	D. Alejandro Moner González		X	
	Dña. Mª Teresa Jareño Roglan			X
Miembros Lego	Dña. Encarna Domingo Cebrián			X
	D. Jaime Alapont Pérez	X		
	Dña. Carmen Sarmiento Cabañes	X		
	D. Antonio Baltasar Olivas Nevado	X		x
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4. NATIONAL AND INTERNATIONAL CONGRESS COMMUNICATIONS

- Marta Usó, Eloisa Jantus-Lewintre, Rafael Sirera, Silvia Calabuig, Enrique Pastor, Jerónimo Forteza, Carlos Camps. "Immune checkpoint expression score is an independent prognostic biomarker in resectable non-small cell lung cancer". **American Association for Cancer Research Annual Meeting 2015.** Philadelphia, Pennsylvania, USA, 18-22 April 2015. Abstract number: 4330
- M. Usó, E. Jantus-Lewintre, R. Sirera, S. Calabuig-Fariñas, A. Blasco, R. Guijarro, J. Forteza, C. Camps. "Immune checkpoints score and $CD8^+$ t cells infiltration are independent prognostic biomarkers in resected NSCLC". **European Lung Cancer Conference 2015.** Geneva, Switzerland 15-18 April 2015. Annals of Oncology Official Journal of the European Society for Medical Oncology and the Japanese Society of Medical Oncology Volume 26, 2015 Supplement 1 39O.
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5. PUBLICATIONS

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