João Luís Ribeiro Pereira

Relevance of circulating markers of inflammation and metastization in the NSCLC landscape

The use of Lymphoblastoid Cell Lines from patients

Dissertação de Candidatura ao grau de Mestre em Oncologia - Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Professor Doutor António Manuel Ferreira Araújo

Categoria – Professor Auxiliar Convidado

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientador – Professora Doutora Berta Martins da Silva

Categoria – Professora Associada

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.” Thomas A. Edison

Preface

The project's aims and methodologies were presented in the 2016 Post-Graduation Scientific Seminars in 27th of May, 2016 at ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Porto.

Agradecimentos

Um trabalho de dissertação de Mestrado é considerado e avaliado como um trabalho individual. No entanto, para o seu desenvolvimento, existem várias pessoas e Instituições ligadas ao mesmo que considero ser da mais elementar justiça identificar e realçar. Desejo, pois, de forma muito sincera e verdadeira, expressar os meus agradecimentos:

À Universidade do Porto e em especial ao Instituto de Ciências Biomédicas Abel Salazar – ICBAS, que foi a Instituição que me proporcionou a possibilidade de integrar o Mestrado em Oncologia e que me acolheu durante estes dois anos;

À Sra. Prof.ª Doutora Berta Martins da Silva, e a toda a Comissão Cientifica do Mestrado em Oncologia, pela oportunidade que me foi dada em ingressar neste Mestrado e em perseguir o meu sonho de estudar e trabalhar nesta área que tanto me intriga e motiva;

A todos os Docentes e Convidados que nos acompanharam e lecionaram as várias unidades curriculares durante o primeiro ano deste Mestrado. Foi através de todos eles que nós, alunos, ficámos dotados de um maior conhecimento e espirito crítico e de uma maior motivação para continuar a estudar na área de Oncologia;

Não me posso esquecer de agradecer ao Instituto Português de Oncologia do Porto Francisco Gentil (IPO-Porto) e também à Escola Portuguesa de Oncologia do Porto (EPOP-IPO Porto). Foi, sem dúvida alguma, uma experiência muito enriquecedora a todos os níveis o facto de puder ter contactado com os vários Profissionais e Especialistas que integram as várias equipas e valências do IPO-Porto e da EPOP e também com todo o "dia-a-dia" e trabalho que é realizado. Durante um ano, essas foram também "a minha casa" e não poderia esquecer de agradecer tudo o que lá aprendi não só como estudante mas também como Homem;

Agradeço profundamente ao Sr. Prof. Doutor António Araújo, Diretor do Serviço de Oncologia do Centro Hospitalar do Porto por ter aceitado ser o Orientador da minha dissertação e por toda a sua disponibilidade e ajuda durante todo o processo. Graças a esta oportunidade cresci não só como estudante mas também como pessoa, e devo parte desse desenvolvimento à ação sempre pronta e proactiva do Sr. Professor. É meu dever e, sobretudo, obrigação agradecer-lhe a oportunidade que me foi proporcionada de integrar a sua equipa da Unidade de Investigação em Oncobiologia (UniO – UMIB) e, com toda a competência que lhe é reconhecida, de sempre ter manifestado enorme

disponibilidade, sentido de responsabilidade e exigência no acompanhamento do meu trabalho, motivando em mim o forte desejo de fazer sempre mais e melhor;

Agradeço também a toda a equipa do Serviço de Oncologia do Centro Hospitalar do Porto pois, sem o seu contributo, a realização deste trabalho não seria possível. Agradeço a todos os participantes por terem aceitado entrar para este estudo;

Agradeço novamente à Sra. Prof.ª Doutora Berta Martins da Silva que na qualidade de Diretora do Curso de Mestrado em Oncologia esteve sempre disponível para receber e ajudar todos os alunos. Na qualidade de Docente da unidade curricular em Imunologia e Cancro transmitiu-me uma vontade ainda maior de estudar esta área. Agradeço-lhe a sua ajuda em encontrar um projeto no qual pudesse trabalhar e, com esse propósito, ter promovido o meu contacto com o Sr. Professor Doutor António Araújo. Agradeço também ter aceitado ser Co-orientadora e me ter autorizado a estar em contacto com o ambiente de trabalho no Laboratório de Imunogenética do ICBAS durante cerca de dois meses e meio. Esta foi, sem dúvida, uma experiência enriquecedora e que me motivou num momento particularmente menos bom do meu percurso;

Aproveito para agradecer também a todos os profissionais e estudantes que trabalham no Laboratório de Imunogenética do ICBAS que me acolheram da melhor forma, em especial, às minhas colegas e amigas Sara, Marta e Mafalda e ao Dr. Pedro Madureira.

Agradeço muito sinceramente à Mestre Mónica Gomes por toda a paciência que teve comigo e por toda a ajuda que sempre me deu durante estes meses. Por ter "perdido" horas a ajudar-me e ensinar-me a conseguir desenvolver e completar as várias tarefas do trabalho laboratorial e não só. Pelas palavras motivadoras, de compreensão e principalmente pela disponibilidade que teve para comigo a minha muito sincera gratidão;

Ao Instituto de Genética Médica Doutor Jacinto Magalhães – Centro Hospitalar do Porto, e a todos os seus profissionais, em especial ao grupo de Enzimologia e de Genética, pelo acolhimento e disponibilização do espaço e instrumentos que fizeram com que a realização deste projeto fosse possível;

A toda a equipa organizadora dos Seminários de Pós-graduação UMIB e em especial ao Dr. Marco Alves pelo convite em participar neste evento. Foi uma experiência nova e enriquecedora que não irei esquecer;

Ao Sr. Professor Francisco Coelho que me ajudou na revisão do Inglês e que me motivou a continuar o meu trabalho na persecução dos meus sonhos;

Agradeço a todos os meus colegas e amigos do Mestrado pois sem eles a adaptação a esta nova etapa seria sem dúvidas muito mais difícil para mim;

Aos meus amigos pelo apoio durante este ano e motivação que me deram quando passei por momentos mais difíceis a nível pessoal, de saúde e de trabalho. A vossa força deu-me a garra que faltou em alguns momentos para conseguir ultrapassar as várias dificuldades que passei durante este ano;

E finalmente, mas mais importante que tudo, aos meus Pais! Eles que tudo fizeram e fazem para que possa perseguir os meus sonhos e trabalhar na área que amo. É meu dever dar o meu máximo em tudo a que me proponho fazer mesmo que por vezes possa não ser suficiente. É minha obrigação, não só por querer evoluir e melhorar a todos os níveis, mas também por respeito ao esforço que os meus pais fazem todos os dias. Considero esta experiência e esta Dissertação como um momento importante no meu desenvolvimento como estudante e profissional mas, acima de tudo, como uma grande aprendizagem. Para mim, nada é mais importante que a família e se não fossem as reprimendas mas também as palavras de ânimo e de amparo dos meus pais com certeza que não conseguiria terminar este trabalho.

Abstract

Lung Cancer is the number one cause of death by cancer worldwide in both genders and Non-Small Cell Lung Cancer represents the majority of Lung Cancer cases. The treatment for this cancer is planned with base on the TNM stage, the presence of some alterations that may allow targeted therapies to be used and recently the use of immunotherapies. However, the precise selection of the treatment is still a problem in this cancer as it is for many others. Not all patients benefit from all therapies and there are also differences in patients' responses to the same therapy. In order to have a more efficient and precise therapy selection, the discovery of biomarkers and their influence and action is a vital approach. MMPs and TIMPs are thereby potential candidates to be used in the selection of anti-cancer therapies as they are very important in many processes of carcinogenesis such as inflammation and metastization.

One of the methods used to study drug sensitivity and response is the use of LCLs from the patients. In these studies, the peripheral blood lymphocytes are immortalized using EBV virus in order to have an unlimited DNA and other biomolecules reservoir. It becomes interesting to hypothesize the importance of potential biomarkers such as MMPs and TIMPs in drug sensitivity and response in order to evaluate their influence and eventually consider them as candidates for therapy selection taking into consideration the patients' characteristics. However, the transformation that occurs can raise a barrier to this possibility as the immortalization technique induces alterations to the cells that can change their behavior and biology.

In this project, the mRNA levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 were studied before and after the immortalization technique in order to understand if this method would induce significant changes to the levels of these MMPs and TIMPs. The results suggested that there were significant changes in the levels of MMP-2 and TIMP-2 which means that the bioavailability of these biomolecules is greatly altered by this technique and therefore the study of their influence in drug sensitivity and response using LCLs as a model may not be possible. Both MMP-2 and TIMP-2 mRNA levels were found to be increased after the immortalization technique. This can be explained due to the use of EBV to immortalize the patients' peripheral blood lymphocytes. In further studies it would be interesting to understand if these changes are related not only to cell growth but also to potential invasive and metastatic features in lymphoblastoid cells which would mean that eventually, EBV could induce processes similar to those that happen in carcinogenesis. The study of MMP-2 and TIMP-2 activities and protein levels in LCLs could give more insight on their influence in this context. These changes can not only be

potential limitations in LCLs based studies but also partially responsible for the difficulties on translating findings in LCLs to clinical practice. The levels of MMP-7 and TIMP-4 did not registered any significant change which leaves the possibility for them to be studied in this context further on.

Resumo

O cancro do pulmão é a principal causa de morte por cancro no mundo em ambos os sexos e o cancro do pulmão de não-pequenas células representa a maioria dos casos. A escolha para o tratamento deste cancro baseia-se essencialmente no estadiamento TNM, na presença de algumas alterações que podem permitir o uso de terapias direcionadas e, mais recentemente, a utilização de imunoterapias. No entanto, a seleção do tratamento neste cancro para determinado doente, ainda não é tão efetiva e eficiente como desejaríamos, problema que é comum para muitos outros tipos de cancro. Nem todos os doentes beneficiam de determinadas terapias visto que muitas vezes existem diferenças na resposta dos mesmos face ao mesmo tipo de tratamento. Afim de melhorar e tornar a seleção das terapias mais eficaz, a descoberta de biomarcadores e da sua influência e ação é um tipo de abordagem fundamental. As metaloproteinases de matriz e os seus inibidores naturais são potenciais candidatos para serem utilizados na seleção de terapias contra o cancro uma vez que participam em muitos processos da carcinogénese, tais como a inflamação e a metastização.

Um dos métodos utilizados para estudar a sensibilidade e resposta a fármacos é a utilização de linhas celulares linfoblastóides dos doentes. Nestes estudos, os linfócitos do sangue periférico são imortalizados utilizando o vírus Epstein-Barr com o objetivo de ter uma reserva e fonte ilimitada de DNA e outras biomoléculas. Como tal, torna-se interessante colocar a hipótese de estudar a importância e influência de biomoléculas como as MMPs e TIMPs no estudo da sensibilidade e resposta a fármacos e, eventualmente, considerá-las como potenciais biomarcadores para a seleção de terapias tendo em conta as caraterísticas dos doentes. No entanto, a transformação que ocorre pode ser um entrave visto que a técnica de imortalização induz alterações nas células que podem alterar a sua biologia e o seu comportamento.

Neste projeto, os níveis de mRNA das MMP-2, MMP-7, TIMP-2 e TIMP-4, foram estudados antes e após a técnica de imortalização de modo a esclarecer se esta técnica realmente induz mudanças significativas nos níveis destas MMPs e TIMPs. Os resultados sugeriram que houve mudanças significativas nos níveis de MMP-2 e TIMP-2, o que significa que a disponibilidade destas biomoléculas é, de facto, alterada por este método e, por conseguinte, o estudo da sua influência na sensibilidade e resposta aos fármacos, utilizando as linhas linfoblastóides dos doentes como modelo, pode não ser possível. Ambos os níveis de mRNA das MMP-2 e TIMP-2 aumentaram significativamente após a técnica de imortalização. Isto pode indicar que o uso do vírus EBV para imortalizar

linfócitos do sangue periférico dos doentes provoca alterações significativas nos níveis de mRNA destas biomoléculas. Em estudos futuros, seria interessante perceber se estas mudanças estão relacionadas, não só com o crescimento celular, mas também com potenciais caraterísticas invasivas e metastáticas das células linfoblastóides. Tal significaria que o uso do vírus EBV poderia induzir processos semelhantes aos que acontecem na carcinogénese. O estudo das atividades e níveis proteicos das MMP-2 e TIMP-2 nas linhas linfoblastóides poderia fornecer mais informação e clarificar a influência das MMP-2 e TIMP-2 neste contexto. Estas alterações também podem ser potenciais limitações nos estudos que usam estas linhas celulares e podem justificar, em parte, as dificuldades em traduzir descobertas nestas linhas celulares para a prática clínica. Os níveis de MMP-7 e TIMP-4 não registaram qualquer alteração significativa o que deixa a possibilidade de poderem ser estudados neste contexto no futuro.

List of Abbreviations

ADC – Adenocarcinoma

ALK – Anaplastic lymphoma receptor tyrosine kinase

BRAF – B-Raf proto-oncogene, Serine/Threonine Kinase

cDNA – Complementary Deoxyribonucleic Acid

Ct – Cycle Threshold

CTLA-4 – Cytotoxic T-lymphocyte-associated protein 4

DDR2 – Discoidin Domain Receptor Tyrosine Kinase 2

EBV – Epstein - Barr virus

ECM – Extracellular matrix

EGFR – Epidermal Growth Factor Receptor

EML4 – Echinoderm Microtubule Associated Protein like 4

EMT – Epithelial - Mesenchymal Transition

FGFR1/2 – Fibroblast Growth Factor Receptor 1/2

HER2 – Receptor tyrosine-protein kinase erbB-2

KRAS – Kirsten Rat Sarcoma Viral Oncogene Homolog

LC - Lung Cancer

LCC – Large-Cell Carcinoma

LCL – Lymphoblastoid Cell Line

MMP – Metalloproteinase

mRNA – Messenger Ribonucleic Acid

NF-κB – Factor Nuclear Kappa B

NSCLC – Non-Small Cell Lung Cancer

P53 – Tumor Protein 53

PCR – Polymerase Chain Reaction

PD-1 – Programmed Cell Death 1

PD-L1 – Programmed Cell Death Ligand 1

PI3K – Phosphoinositide 3-kinase

RNA – Ribonucleic Acid

SCC – Squamous Cell Carcinoma

SCLC – Small Cell Lung Cancer

SNP – Single Nucleotide Polymorphism

TIMP – Tissue Inhibitor of Metalloproteinases

TKI – Tyrosine kinase inhibitor

List of Figures

List of Tables

Table of Contents

Chapter 1

Introduction

1 Introduction

1.1 Cancer and Carcinogenesis

Cancer is the 21st century disease, it is a vast, complex and interesting area of research. Its characteristics and statistics often alarm the general population worldwide but they also motivate the scientific community to increase their efforts in order to combat this complex disease. There are much data and information yet to discover but that must be perceived as many opportunities to study and improve our knowledge on cancer and ultimately improve the conditions and prognosis of cancer patients.

The formation of cancer is often called carcinogenesis [1]. At first it occurs initiation which involves alteration, change or mutation of genes spontaneously or induced by exposure to a carcinogenic agent [2]. This leads to dysregulation of signaling pathways associated with cellular proliferation, survival and differentiation [2]. After initiation there is promotion, where the proliferation of preneoplastic cells is continuous and there is an accumulation of preneoplastic cells [2]. Progression is considered to be the stage between premalignant lesions and invasive cancer [2]. It is the final stage of neoplastic transformation where cell proliferation, genetic and phenotypic changes can occur [2]. At this stage there is a fast increase of tumor size where cells undergo into further alterations with invasive and metastatic potential [2]. Finally there is metastasis, which involves the spread of cancer cells from the primary tumor to other parts of the organism through bloodstream or lymphatic system [2].

During the years the number of cancer survivors increased due to the aging and growth of populations and improvements in early diagnosis and treatment [3]. Cancer patients that are diagnosed at early stages tend to have a better chance of survival than those diagnosed at later stages [3].

According to the World Health Organization, cancers are one of the leading causes of morbidity and mortality worldwide with approximately 14 million new cases and 8,2 million cancer related deaths in 2012 [4]. The number of new cases is expected to rise by 70% in the next two decades [4]. A third of cancer deaths are due to behavioral and dietary risks such as high body mass index, tobacco and alcohol use [4]. In fact tobacco smoke is the most important risk factor for cancer, causing around 20% of all cancer deaths and about 70% of lung cancer (LC) deaths [4].

1.2 Lung Cancer

There are two major forms of LC: Non-Small Cell Lung Cancer (NSCLC) about 85% of all LC cases and Small-Cell Lung Cancer (SCLC) counting approximately 15% [5]. The first can be divided in three major histological subtypes: Squamous cell carcinoma (SCC), Adenocarcinomas (ADC) and Large-cell lung cancer (LCC) [5]. The SCC and SCLC are usually developed in central airways and contrastingly, ADCs are developed in peripheral airways [5–7].

LC risk is increased by genetic alterations that can be inherited such as rare mutations in p53 or retinoblastoma or mutations in epidermal growth factor receptor (EGFR) [5, 8–10]. Reduced DNA repair capacity accompanied by exposure to tobacco smoke is also very harmful and can result in driver mutations towards the formation of LC [5, 11].

In general, environmental factors such as tobacco smoke and genetic susceptibility interact to influence carcinogenesis [5, 12]. Nevertheless, there are unrelated factors to smoking which are also important to consider [5, 12].

LC is the number one cause of death by cancer worldwide and the most incident cancer in both genders with 1824701 incidence cases and 1589925 deaths (Figure 1). LC is the most incident and mortal cancer for men with 1241601 incidence cases and 1098702 deaths. As far as women are concerned, it is the third most common cancer with 1671149 incidence cases and the second deadliest with 491223 deaths [13].

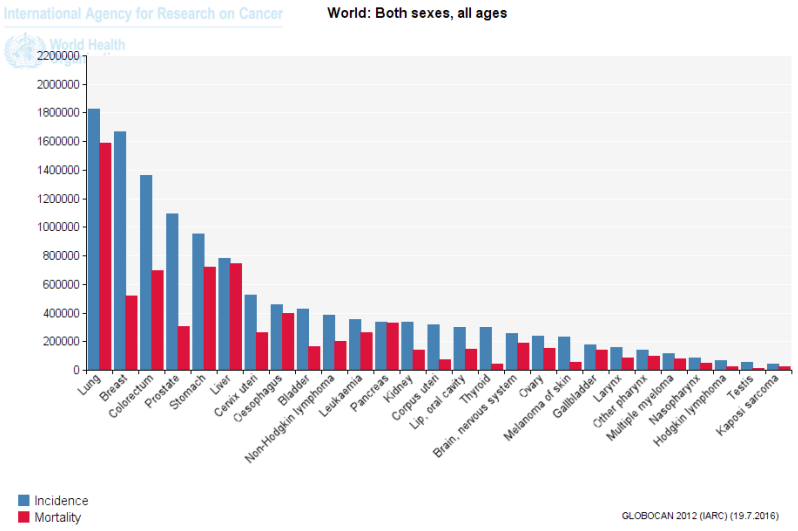

Figure 1 – Lung Cancer incidence and mortality Worldwide, both genders, all ages. GLOBOCAN 2012 (IARC).

In Europe, LC is the deadliest cancer and the third most incident in both genders with 410220 incidence cases and 353848 deaths (Figure 2). It is the leading cause of

death by cancer for men with 254706 deaths and the second most common with 290904 incidence cases. As for women, LC is the third deadliest cancer with 99142 deaths and the third most common cancer 119316 incidence cases [13].

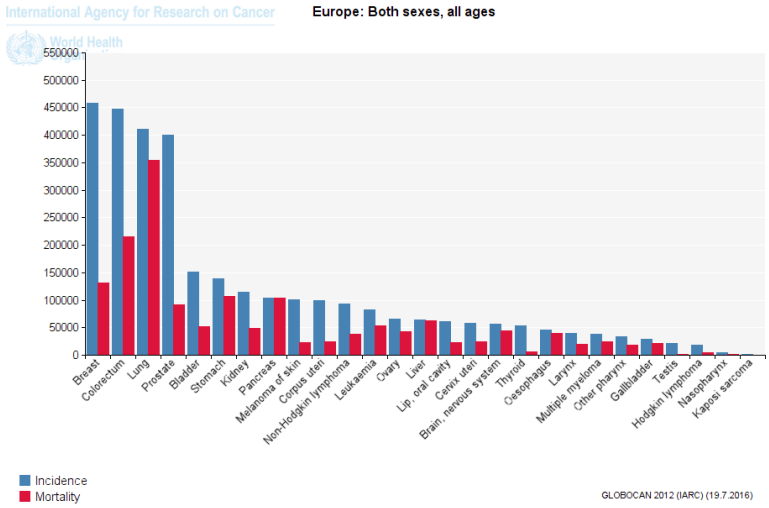

Figure 2 - Lung Cancer incidence and mortality in Europe, both genders, all ages. GLOBOCAN 2012 (IARC).

In Portugal LC is the fourth most incident cancer with 4192 incidence cases but it is the second deadliest with 3441 deaths, once again in both genders (Figure 3). For men, LC is the third most common cancer with 3215 incidence cases and the deadliest cancer with 2638 deaths. For women, LC is the fifth most incident cancer with 977 incidence cases and the fourth deadliest with 803 deaths [13].

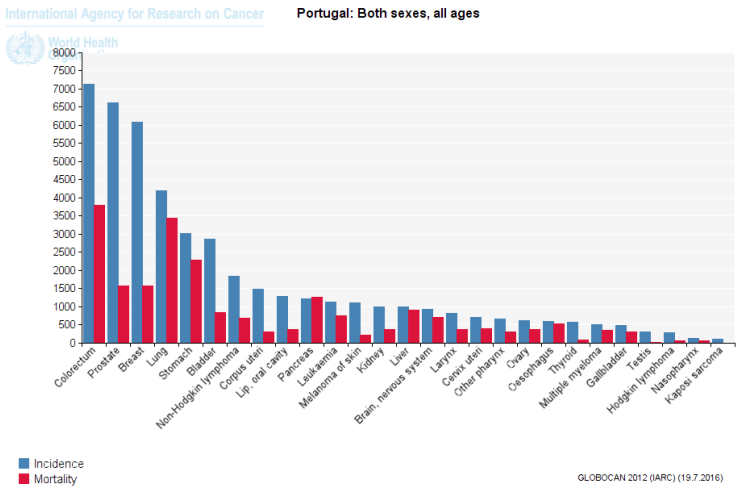

Figure 3 - Lung Cancer incidence and mortality in Portugal, both genders, all ages. GLOBOCAN 2012 (IARC).

The greatest problem with this cancer is that 70% of patients are diagnosed at an advanced stage [14]. More than 50% of patients are diagnosed with a stage IV and the prognosis for all stages combined is still poor with a 5-year survival rate of 15% [15]. As

for patients with an early-stage or localized disease, 5-year-survival rates reach approximately 52% and patients with advanced disease or stage IV have a 5-year survival rate of less than 5% [15].

It is well known that LC is a great cause of severe morbidity and mortality and 80-90% of all LC cases are attributed to cigarrete smoking and could be prevented [16–18]. Smoking has been associated with LC for several years and it is without doubt a major risk factor [16–18]. Involuntary smoking or environmental tobacco smoke exposure is also associated with the risk of developing LC although the risk is not as great as for the active smokers [17].

Environmental or occupational exposures to radiation, chemical agents, air pollution, asbestos, radon and others carcinogens are also associated with LC being important to 10-15% of never smokers who develop LC [17].

Family history of LC, genetic susceptibility, infectious agents and pulmonary diseases such as chronic obstructive pulmonary disease can also be risk factors to develop LC [17].

The treatment options for LC include surgery, radiation therapy, chemotherapy, target therapy and immunotherapy [19, 20]. Their application depends on many factors such as LC histological type and TNM stage in which the size of primary tumor (T), the presence of metastases in regional surrounding lymph nodes (N) and the presence of distant metastases (M) are the main drivers of choice of treatment [21]. Despite all the recent advances and improvements in diagnostic methods, technologies and therapies, in the case of LC they are still unsatisfactory because most patients are diagnosed at an advanced stage and treatment is not as effective as we would want [22].

Most of LC patients are treated with platinum based chemotherapy, many times combined with gemcitabine, vinorelbine, pemetrexed or taxanes but this treatment is often associated with resistance due to acquired mutations [19, 20]. It is also known that factors such as stage of disease, histological type and smoking status can influence the response to treatment [19, 20].

1.3 Non-Small Cell Lung Cancer

There are three main histological subtypes in NSCLC: ADCs which corresponds to approximately 50% of NSCLC cases followed by SCC with approximately 40% of the cases and finally LCC with approximately the remaining 10% [23, 24].

Generally, ADCs arise in distal airways while SCCs arise in more proximal airways and are strongly associated with smoking and chronic inflammation [23, 25, 26]. ADCs often have a glandular differentiation and express biomarkers consistent with the origin in distal areas of the lung [25, 26]. SCCs are characterized by a squamous differentiation and are distinguished from ADCs in immunostaining and/or transcription factors [25–27]. LCC is normally diagnosed by exclusion, if the tumor cells do not appear to be either glandular or squamous in shape and there is no expression of biomarkers connected to them [23].

There are many gene mutations associated with NSCLC. EGFR overexpression is present in 43% to 83% of NSCLC cases and mutations in this gene, are present in 10% to 15% of NSCLC patients. There is also a rearrangement of the ALK gene, most of the times resulting in an EML4-ALK fusion gene, that is present in approximately 5% of NSCLC cases with other reports of percentages between 2% to 11% of NSCLC cases [15, 28–33].

In ADCs, many studies had identified mutations in genes such as KRAS, BRAF, HER2 and EGFR [23, 34–38]. ALK rearrangements are more common in ADCs patients who are either never-smokers or light smokers [15, 39–41]. In SCC many mutations have also been discovered in genes such as DDR2, FGFR1/2 and genes that are part of the PI3K pathway [23, 42].

The predictive 5-year-survival rate for NSCLC is approximately 15%, a number that has only improved in the past few decades [23, 43]. As it would be expected, early-stage tumors normally have a better clinical outcome and early stage NSCLC cases treated with surgical resection have a 5-year survival rate of less than 70%. Even stage I NSCLC patients have a risk of recurrence of approximately 30-40% [24, 32]. About a third of patients with stage I NSCLC can eventually die of recurrent disease despite a successful curative resection which demonstrates that several patients with high risk of relapse can also be found in early stages of NSCLC [24, 44]. The average 5-year survival rate after the diagnosis of advanced NSCLC is very low, only 5% for stage IIIB patients and 1% for stage IV patients [14, 26].

Nowadays, the basic histological distinction between SCLC and NSCLC is no longer considered sufficient to make a correct diagnosis as more specific classification of the tumor is possible [15, 45]. For the correct diagnosis, different approaches are needed: collection and analysis of non-resected small biopsy samples and cytology as well as the use of immunohistochemical techniques and molecular tests [14]. This allows not only to

distinguish ADCs from SCC, but also to detect molecular aberrations implicated in NSCLC pathogenesis that can help in planning of the therapeutic strategies [14].

The treatment of NSCLC is based on the context of the patient. Surgery is a possible treatment when the primary tumor is found at early stages of development [14, 24]. However, in LC patients the standard treatment is the use of chemotherapy, although it is sometimes associated with resistance and toxicity [20]. When metastases are identified, the conventional chemotherapies remain the standard treatment in NSCLC [14, 24].

Targeted therapies are also a possibility for NSCLC patients, for example: the use of EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib [24]. These therapies have shown efficacy in advanced-stage NSCLC and several studies reported that the patients fit for this therapies could benefit more from the use of these TKIs as a first-line treatment than the use of traditional platinum-based chemotherapies [15, 26]. ALK alterations such as EML4-ALK may also be subjected to targeted therapy with drugs such as crizotinib [15, 24]. Finally, immunotherapies are also arising in NSCLC with Anti-CTLA-4 and Anti-PD-1 and PD-L1 therapies having promising results [46].

1.4 Personalized Medicine and Pharmacogenomics

In cancer landscape the term personalized medicine is very important due to the lethal characteristics of cancer, side effects of therapies and also resistance to the therapies that are available [21].

With a precise medicine, there is a customization of healthcare with medical decisions and approaches being directed towards the patient's characteristics [47]. The goal is to tailor the diagnosis and treatment to the patient's biological and molecular profile [48].

It becomes crucial to approach the right patient, at the right time with the right drug, dose and schedule [21]. But the treatment of cancer, in general, continues to be a "one size fits all" strategy rather than an individualize treatment [21]. One of the reasons that this happens is due to the actual treatment selection [21]. The tools to assess which patients benefit from which therapies are still lacking as the selection is normally based in the TNM staging system [21]. Drug resistance is another problem, even patients that seem to respond well to a treatment may develop resistant variants of cancer cells that

are no longer affected by it [49]. These variants become dominant after drug exposure and in some cases can be fought with second or third line options [49].

It becomes important to identify molecular and biological features that allow to predict the patient's response to a therapy. In order to do that, more studies are required to correctly identify these features and translate the data into clinical practice [21].

Most of the advances in personalized medicine in LC is happening in ADCs with Bevacizumab and Pemetrexed having good effective results and Erlotinib/Gefitinib and Crizotinib being also approved [50]. The successful development of these therapies proved the importance of finding markers that can be identified and targeted in order to achieve a new treatment. However, predictive markers of response are also very important and their finding can enhance the identification of patients that will actually benefit from many other therapies as most of first-line therapies do not have response predictive biomarkers identified [51]. In order to achieve that, patient-oriented tests are required, it is necessary to evaluate the drug sensitivity and response of the patient to complement the tumor's characteristics and the presence or absence of biomarkers that can be targetable by therapies [51].

Ultimately, the interaction of genetics, epigenetics, tumor molecular biology and clinical and pathological criteria can result in an improved and more effective combination of therapies taking the patient's characteristics into consideration as well [52].

1.4.1 Pharmacogenomics

Understanding the molecular characteristics of both the tumor and the patient is essential to establish their relationship with drug response. This can be achieved by pharmacogenomic studies that intend to identify biomarkers that are able to predict a clinical outcome such as drug response [19].

Pharmacogenomics is seen as a highly important area, it focus on the identification of genetic variants that influence drug effects in the patients [19, 53, 54]. The study of single nucleotide polymorphisms (SNP) is an example of one of the most important type of studies in pharmacogenomics as many of them have identified toxicity, prognosis and predictive drug response markers [19, 55].

Nowadays, dose adjustments of cytotoxic drugs are often made taking patient's body surface area and response into consideration [19]. As for targeted therapies normally a common standard dose is given to all patients [19]. The study of biomarkers that can predict clinical outcomes can change this landscape [19].

Pharmacogenomic studies have already found some potential biomarkers related to cisplatin sensitivity and targeted therapy in NSCLC [56, 57]. There are some limitations when translating pharmacogenomics findings into clinical practice due to, for example, the number of heterogeneous patients screened [58]. The development of prospective clinical trials in which treatments would be selected based on conventional criteria and compared with specific treatments for patients taking into consideration their own characteristics might overcome the existing reluctance in transferring novel knowledge into clinical practice [59].

Treatment efficacy will always be primary goal when making cancer therapy decisions and choices and in the future, the selection of an optimal and efficient treatment strategy for each patient will eventually be a reality [60].

1.4.2 The Contribute of Lymphoblastoid Cell Lines

Lymphoblastoid Cell Lines (LCLs) are achieved by infecting peripheral blood lymphocytes with Epstein-Barr Virus (EBV), promoting their transformation and immortalization into LCLs [61]. Infection by EBV induces the transformation of lymphocytes into lymphoblastoid cells which can be an unlimited source of patient's DNA and other biomolecules [62]. The use of EBV virus to transform lymphocytes is the method that offers the least genetic changes as the virus stays in a episomal form inside the host cell [61]. The somatic mutation rate of LCLs is very low with only 0,3% and their maintenance is easy which makes this method a very good choice to storage patients' genetic material and variability [62, 63]. The use of LCLs are reported to be fit and suitable for molecular and functional studies as well as genetic studies [62, 64].

The use of LCLs from patients in genetic studies proved to be very useful and showed that genetic material of the patient is conserved and adequate for these studies [62]. Thereby, it maintains the genetic variability and is considered to be a good model to study drug toxicity and, at some extent, drug response [61, 65, 66].

However, there are some potential limitations. After two/three months in culture there is a risk that these cells may show alterations that are not relatable with the patients anymore [61, 62]. Many drug effects that are observed, cannot be translated into the organism as there are many interactions that are not present in a cell culture and it is known that LCLs do not express many CYP450 enzymes and several transporters [66, 67].

In cancer landscape, LCLs have been used in genetic studies, for example, to evaluate the influence of SNPs in drug sensitivity and response [66, 67]. The use of LCLs

has proved to be a good model in phamacogenomic studies and, in many cases, have a successful clinical translation of what is seen *in vitro* [66, 67]. However, it is clear that any biomarker identified using this model needs to be further validated *in vivo* using additional models or samples [65].

Ultimately, in an era where personalized medicine is becoming a reality and subject to many investigations, the use of LCLs may provide a valuable method to better understand the relationship between the patient's characteristics and the clinical outcomes. This can lead to an improvement in personalized medicine as the finding of biomarkers related to drug toxicity and response can eventually help in the treatment approach by the multidisciplinary teams.

1.5 Inflammation, Metastization and Cancer

1.5.1 The Hallmarks of Cancer

In 2011 Hanahan & Weinberg wrote a review article describing the traits and alterations in cell physiology that collectively lead to malignant growth and are referred to as Hallmarks of Cancer (Figure 4) [68].

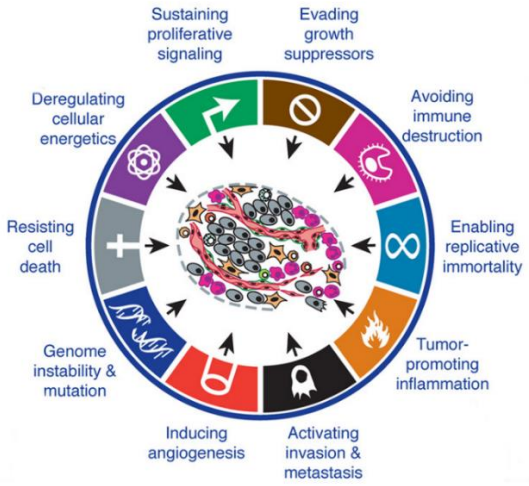

Figure 4 – The Hallmarks of Cancer. Hanahan, D. & Weinberg, Robert A. (2011) [68].

During carcinogenesis the cell must acquire numerous abilities: at first it acquires various genetic and genomic alterations and mutations as well as many epigenetic

changes that lead them to aberrant phenotypes [68–70]. The cell is then able to divide, grow and proliferate in the absence of external growth stimuli or in the presence of exogenous inhibitory signals [68–70]. They can do this by promoting and inducing neoangiogenesis and the secretion and release of growth factors [68–70]. Eventually, they are able to evade apoptosis and immune system response and create an inflammatory microenvironment favorable for their development [68–70]. Finally, they acquire properties of invasion and metastization making them an even bigger threat to the organism [68–70].

The two main hallmarks on which this project has its focus are inflammation and metastization. Both of them are very important in the development and progression of any cancer and in the case of LC, and NSCLC, inflammation and metastization are crucial to understand its formation, progression and mortality.

1.5.2 Inflammation and Lung Cancer

Tumor promoting inflammation is a very important characteristic. Tumors are often associated with infiltrated cells of the immune system and also molecules and mediators such as growth factors, survival factors, ECM modifying enzymes and many others that contribute to tumor progression [68, 71, 72].

Inflammation is normally associated with physiological and pathological processes such as wound healing, tissue injury or infection [73, 74]. It is a multifactorial and complex response of our immune system with the objective of eliminate or neutralize any harmful stimuli in order to allow the healing and repairing of our tissues [73, 74].

The relationship between inflammation and cancer is very old. In 1863 Rudolf Virchow already noted the presence of leukocytes in neoplastic tissues and made a connection between inflammation and cancer [72]. According to his hypothesis, that infiltration reflected the origin of cancer at sites of chronic inflammation [72].

About 50 years after Virchow, Paul Ehrlich suggested that the immune system could act against tumors [75, 76]. It could repress a potentially “overwhelming frequency” of carcinomas but this idea was not pursued for a long time [75, 76]. Later on, Burnet and Thomas supported even more this theory with their work on immunosurveillance hypothesis, they speculated that lymphocytes acted as sentinels in recognizing and eliminating continuously arising nascent transformed cells [76, 77].

It is known that nascent tumor cells can be eliminated by our immune system [78]. The interaction between the immune system and tumor suggests that at first there is an elimination of nascent tumor cells by the immune system followed by a stage of

equilibrium where the immune response can control the tumor expansion and metastasis and finally the escape stage where tumor cells are able to resist and evade the immune system response [76, 79].

In the tumor microenvironment, inflammation and immune system cells exhibit characteristics that can enhance the tumor progression, for example, the presence of tumor associated macrophages such as M2 macrophages capable of suppressing the immune system response and promote tissue remodeling and angiogenesis [80]. Inflammatory mediators such as chemokines and cytokines or Metalloproteinases (MMPs) are also present in the tumor microenvironment and are associated with many processes such as tumor growth, migration, invasion and metastasis [73]. The tumor infiltrating inflammatory cells can also release cytokines, growth factors and proteases that not only modulate inflammation but can also play a role in tumor progression invasion and metastasis [81].

It is clear that inflammation and cancer are very tightly related in many processes of carcinogenesis, as it can be seen in Figure 5: Inflammation combined with oncogenic driver mutations lead to the activation of many inflammatory transcription factors within the tumor cell and tumor microenvironment [82]. Eventually, malignant cells start to produce inflammatory mediators leading to the recruitment of inflammatory cells into the microenvironment [82]. Therefore, all conditions to a cancer related inflammation are achieved and processes such as cell survival and proliferation, immune suppression, angiogenesis, migration, invasion and metastasis are influenced by this inflammatory microenvironment [82].

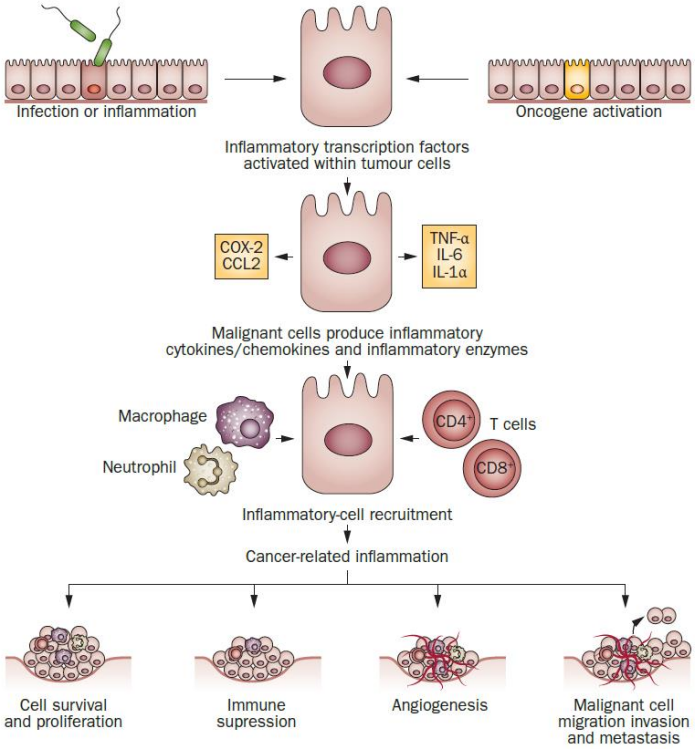

Figure 5 – Summary of the association between inflammation and cancer. [82]

In summary, at very early stages of cancer development there is a proliferation of host immune effector populations in response to nascent transformed cells. But when the tumor surpasses this response and adapts to it, it creates a microenvironment that is favorable for its progression [83].

1.5.3 Metastization and Lung Cancer

Tissue Invasion and Metastasis is one of the most important characteristics of cancer development [68, 84]. Metastasis are accountable for approximately 90% of all cancer related deaths and thereby this process is of great importance to study it [85].

In 1889 Stephen Paget proposed the “seed and soil” hypothesis. From there, many studies were made focusing on metastization. Mechanical forces and factors were discovered, lymphatic and haematogeneous circulation of tumor cells were differentiated, host factors were also found and associated with metastasis, clonal evolution and heterogeneity of tumor cell populations were discovered, the origin of metastases was associated with a few tumor cells with specific characteristics and recently the study of possible therapies against metastases are being made [86, 87].

Metastization is a sequential and interrelated complex multistep process where each step is of great importance [87, 88]. The outcome depends on the cancer cell properties

and host response which means that there is a balance between host-tumor cellular interactions that vary between patients [87]. In order for cancer cells to metastasize they need to have some specific characteristics such as loss of cellular adhesion, increased invasive potential, ability to intravasate and survive in the vascular system, ability to extravasate and survive and proliferate in a new site [89].

During the metastasization process there are several steps (Figure 6), namely 1. Invasion and Migration: Individual metastatic cells detach from the primary tumor and invade an adjacent tissue. During this process many enzymes that degrade the Extracellular Matrix (ECM) and facilitate posterior migration are secreted 2. Intravasation: These cells are capable of entering blood or lymphatic vessels. The neoplastic cells secrete proteolytic enzymes which enable infiltration. 3. Circulation: The cells travel via blood or lymphatic stream and need to resist and endure that circulation and thereby many cells die at this point and only the “fittest” survive 4. Extravasation: Cancer cells leave the blood stream by penetrating the endothelium through proliferation and/or action of proteolytic enzymes. 5. Colonization, Proliferation and Angiogenesis. The neoplastic cell settles at the distant tissue site and establishes a microenvironment in order to proliferate and induce neoangiogenesis to ensure their survival [70, 86, 87]. It is also known that some cells from metastases can also metastasize again [86, 87].

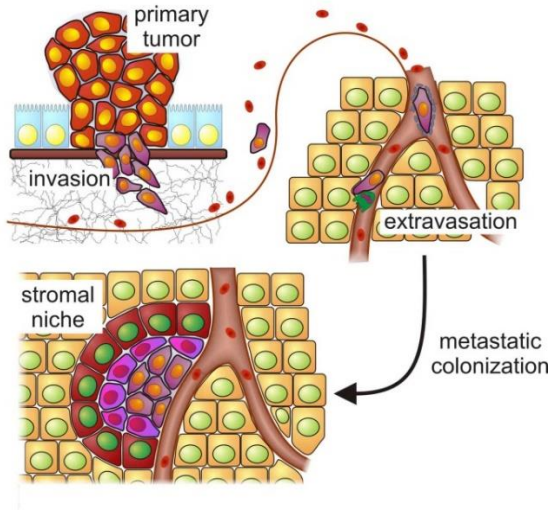

Figure 6 – Schematic view of the main steps of metastization [90].

During metastization, Epithelial to Mesenchymal Transition (EMT) mechanisms have been indicated as responsible for dissemination of single tumor cells from epithelial tumors [91, 92]. During this process, epithelial cells undergo through various alterations regulated by many transcription factors, signaling pathways and mediators that end up promoting a transition where epithelial cells gain mesenchymal properties [70, 91, 93]. EMT can thereby promote invasion, metastasis, resistance to apoptosis and cell survival

[91, 94–99]. The mechanical forces are also important in metastization as they are essential for the metastatic cell to invade, metastasize and survive bloodstream [100].

It is known that LC cells can rapidly acquire properties that grant infiltration and colonization competence which can explain the short time between the primary tumor diagnosis and a metastatic relapse [101].

LCs are able to establish distant macrometastases within months of diagnosis. The most typical sites of metastatic relapse are: bone (34,3%), lung (32,1%) brain (28,4%), adrenals (16,7%) and liver (13,4%) [101–103].

Targeting the metastatic cell is difficult due to the genetic instability and heterogeneity. Ultimately, the treatment should take into consideration the characteristics of metastatic cells and also the characteristics of the host microenvironment [104].

1.6 Metalloproteinases and Tissue Inhibitors of Metalloproteinases

MMPs were identified in 1962 by Gross and Lapiere. Their study described the ability of a soluble factor to lyse purified collagen *ex vivo* [105, 106]. Since then, this family of endopeptidases has been studied and vast information has been discovered about them and their function [105]. During the years, they began to be associated with many pathologies and eventually were associated with tumors and metastasis [105].

MMPs are classified as a calcium dependent, Zn^{2+} containing endopeptidases and can be found in a soluble form or a membrane-bound form [105]. There are 23 human MMPs that can be divided into different groups such as collagenases, gelatinases, stromelysins, matrylisins, elastases and membrane-type MMPs [105, 107–109]. Collectively, they are normally called matrixins and participate in ECM degradation [110–112]. They are also important in tissue homeostasis, host defense, tissue remodeling and repairing, inflammation and many other processes [107, 113]. Their natural inhibitors, the Tissue Inhibitors of Metalloproteinases (TIMPs) are specific inhibitors that regulate and control their activities [110, 114].

Their structure is complex (Figure 7), they contain several domains: a predomain, a propeptide domain, a catalytic domain and a hemopexin domain [115]. They also require zinc in their catalytic domain and are synthetized as inactive zymogens that need to be activated [115, 116].

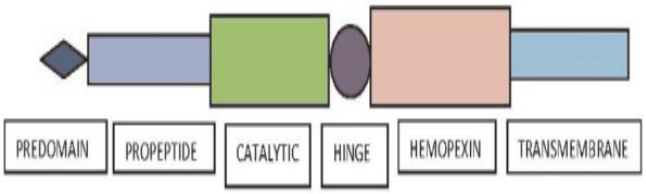

Figure 7 – Overview on MMPs' structure. [115]

The proteolytic activation of MMPs allows the propeptide, called pro-MMP, to achieve its active form [110]. The process of activation ends up with the removal of the propeptide by intermolecular processing [110, 117]. Other processes such as plasmin activation, intracellular activation or cell surface activation can also activate MMPs [110].

The proteolytic activity of MMPs is normally very low in healthy tissues, their transcription levels can be upregulated by action of inflammatory cytokines and growth factors and their expression can also be regulated by hormones, tumor promoters, cell-to-cell and cell-to-ECM interactions [107].

TIMPs can bind to MMPs in order to regulate their activity, there are four TIMPs (TIMP-1-4) identified in vertebrates and their expression is normally upregulated during tissue remodeling [110, 114]. Because of their importance in regulating MMPs' activities, their analysis in pathological conditions associated with MMPs is also important [110]. In fact, TIMPs can form complexes with pro-MMPs in order to regulate their action [118].

1.6.1 MMPs, TIMPs and Lung Cancer

At first MMPs were believed to facilitate metastasis by breaking down physical barriers provided by ECM. But actually, their action in carcinogenesis is important in many other processes such as growth, apoptosis and angiogenesis (Figure 8) [115, 119].

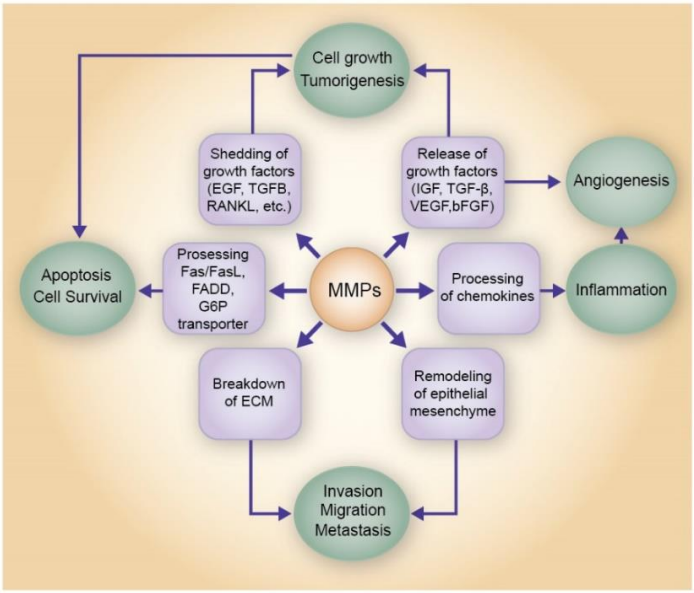

Figure 8 – Summary on MMPs' activities and their role in carcinogenesis. Adapted from Hua, H., et al. (2011) [81].

It is clear that cancer cells are able to stimulate host cells to be a great source of MMPs [120]. MMPs are able to regulate tumor growth by different mechanisms as they can disrupt the balance between growth and anti-growth signals in the tumor microenvironment due to their potential to influence the bioavailability and functionality of multiple factors [115, 121, 122].

In the apoptotic process MMPs can either have apoptotic or anti-apoptotic roles [115, 120]. They can interfere with the induction of apoptosis in malignant cells and can lower the impact of chemotherapy on the tumor [122].

MMPs are also capable of regulate angiogenesis [119] . Once more, MMPs play a dual role in angiogenesis as they can either promote or inhibit angiogenesis through their activity which can promote the release of both pro and anti angiogenic factors [81, 115, 120, 122–126].

The overexpression and high activity levels of MMPs are usually associated with tumor progression [81]. Downregulation of TIMPs may result in an increase of MMPs' activities and thereby a more invasive potential for tumor cells [81]. However, upregulation of TIMPs can actually inhibit tumor invasion and metastasis [81]. TIMPs can participate in many processes of carcinogenesis and commonly act as tumor suppressive agents being dysregulated in many cancers [81].

Tumor cells can produce pro-inflammatory factors and MMPs which can induce an influx of inflammatory cells to the site of the tumor contributing for its progression [119].

Elevated expression and activity of MMPs can be observed in acute or chronic inflammation [81]. They exhibit either pro-inflammatory or anti-inflammatory activities depending on the characteristics and context of the environment [81, 127].

MMPs that degrade ECM may also affect the immune system as some of the proteolytic fragments that result from that process can exert chemotactic properties [122, 128].

Usually, the expression of MMPs in primary tumor sites is increased and can be found in the invasive front or leading edge of the tumor which can be explained by their proteolytic activity which is essential to allow the malignant cells to break physical barriers during expansion, intravasation, extravasation and invasion [115, 119, 120].

MMPs are therefore associated with cell movement due to their proteolytic functions as they can regulate cell-cell and cell-ECM interactions [120]. The production of peptides in the process of degradation of ECM components can also promote cell migration [120, 129–131].

MMPs have also been associated with EMT [120, 121]. This happens due to their proteolytic activity an ability to cleave ECM components [115, 120, 132, 133].

They have many roles in carcinogenesis making them potential therapeutic targets. However, their dual roles in carcinogenesis and importance in the homeostasis of normal tissues difficult this option [120].

1.6.2 MMP-2, MMP-7, TIMP-2 and TIMP-4

In this work, MMP-2, MMP-7, TIMP-2 and TIMP-4 were studied in the context of NSCLC.

MMP-2 (gelatinase A) is associated with many pulmonary diseases [134, 135]. It is capable of degrading a variety of ECM components and is often associated with angiogenesis, cell invasion, migration and induction of EMT. Therefore it is considered to be important in the process of metastization and inflammation and is related to advanced stage and poor prognosis in cancer [81, 120, 122, 136]. It is also capable of suppressing the T-lymphocyte proliferation and reaction against cancer cells [120]. MMP-2 has also been associated with tumor growth and dampen inflammatory properties [81, 107, 113, 127, 137]. It can influence the migration of some immune system cells such as leukocytes and is associated with pro-inflammatory processes [81, 107, 138].

In NSCLC, high expression of MMP-2 in tumor and peritumoural cells and high serum levels in patients with metastases were associated with invasion, metastasis,

increased risk of tumor recurrence and poor prognosis [139–141]. Its overexpression in tumor tissue of patients at an early stage was also observed and related to poor prognosis [142]. MMP-2 expression in NSCLC patients with lymph node metastasis was significantly higher than in patients without lymph node metastasis [140].

MMP-7 (matrylisin) is a proteolytic enzyme responsible for the destruction of ECM and basement membrane components and can be produced by tumor cells. It has been associated with tumor progression, apoptosis, invasion and metastasis [143–145].

In NSCLC, high expression of this MMP in tumor cells is common as well as high levels of MMP-7 mRNA in tumor tissue [143, 145, 146]. This MMP has been associated with chemotherapy response with its overexpression in tumor samples being related with poor response to platinum-derived chemotherapy [144]. It was also considered to be an independent predictor of prognosis in NSCLC patients with overall survival being considerably lower in patients with high MMP-7 expression [144]. A high enzymatic activity by MMP-7 was also found in tumor tissue [145].

In regard of TIMPs, it is known that both MMP-2 and MMP-7 can be inhibited by TIMP-2 and TIMP-4 [81, 118, 135, 147–149]. Both of them can inhibit tumor growth *in vitro* and *in vivo* which would be expected from their ability to inhibit MMPs [150].

TIMP-2 is a multifunctional protein secreted into ECM. Usually, its high levels are associated with a favorable prognosis in NSCLC due to its association with the inhibition of endothelial cell proliferation, migration and angiogenesis [150, 151]. Its overexpression has already been associated with inhibition of tumor growth, invasion and metastasis due to their MMP inhibitory activity [150, 152]. High TIMP-2 expression is not only related to the outcome at early-stage NSCLC, being considered a favorable factor, but is also associated with the outcome at late stages of the disease [151]. The absence of this TIMP accelerated tumor growth and stimulated mediators of angiogenesis and inflammation [153]. Serum levels of TIMP-2 have also been found to be significantly lower in NSCLC patients than in controls [152].

TIMP-4 is the most recent member of the TIMP family. In normal conditions, it is only abundantly expressed in human cardiovascular structures [154]. In the lung, it can be expressed by epithelial and plasma cells and interstitial macrophages [134]. This TIMP was already associated with inflammatory diseases [154]. A study of idiopathic pulmonary fibrosis observed its expression in macrophages and plasma cells [155]. In cancer landscape, studies *in vitro* and *in vivo* have demonstrated TIMP-4 potential to inhibit cell invasiveness, metastatic potential, angiogenesis and tumor growth and can sensitize cervical cancer cells to an apoptotic death [150, 156–159]. In breast cancer cell lines, the

induction of an overexpression of this TIMP was reported to inhibit *in vitro* growth rates, invasive potential and eventually, *in vivo* tumorigenicity and metastasis [158].

Taking into consideration what has been described, it becomes interesting to better understand the relationship between these MMPs and TIMPs and NSCLC. Based on their activity and influence in many processes such as inflammation and metastization, there is much potential in MMPs and TIMPs yet to be investigated particularly in NSCLC.

Chapter 2

Aims

2 Aims

2.1 Main Objectives

1. Study the importance and relevance of circulating markers of inflammation and metastization (MMPs and TIMPs) in the NSCLC landscape.
2. Evaluate if the use of LCLs from patients is a good model to study the influence of MMP-2, MMP-7, TIMP-2 and TIMP-4 in NSCLC.

2.2 Secondary Objectives

1. Study the importance and relevance of MMPs and TIMPs in the NSCLC landscape.
2. Relative quantification of MMP-2, MMP-7, TIMP-2 and TIMP-4 mRNA levels in patients with NSCLC and controls before and after the immortalization technique.
3. Comparison between the mRNA levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 before and after the technique of immortalization.
4. Comparison between the mRNA levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 in NSCLC patients and controls before and after the technique of immortalization.

Chapter 3

Materials and Methods

3 Materials and Methods

3.1 Patient Selection

The patient selection criteria for this study was the following: adult patients diagnosed with NSCLC by either histological or cytological methods, at any TNM stage and without any oncological treatment regime or medical intervention. These patients were diagnosed and recruited to this study from the Service of Medical Oncology at Centro Hospitalar do Porto between May and August of 2016. Their clinical and demographic information was collected from the medical records.

Three controls without any known oncological pathology, residing within the Porto residence area were also recruited for this study from Centro Hospitalar do Porto.

All the participants gave their consent to be included in this study according to the Helsinki declaration.

3.2 Sample Collection and Processing

Blood samples were collected from all participants using the standard intravenous collection method. Approximately 8mL of peripheral blood was collected into EDTA and CPT collection tubes.

EDTA collection tubes were then processed with centrifugations at 10 minutes and 2500 rpm until the peripheral blood cells were separated and then conserved in Tripure® (Roche) at -80ºC. The CPT collection tubes were centrifuged during 30 minutes at 3000 rpm in order to separate the peripheral blood mononuclear cells (PBMCs) from the patients' blood.

3.3 B95-8 Cell Culture and Lymphoblastoid Cell Lines from Patients

In order to study the possible relationship between the molecular targets of interest (MMPs and TIMPs) and NSCLC and possible clinical outcomes, such as treatment toxicity, response or prognosis, it was used the LCLs model.

In this project B95-8 cells (ATCC®, VR-1491) were used in order to have enough supply of EBV virus. B95-8 cell lines were put in culture medium constituted by RPMI 1640 Medium + 20% FBS and 1% Penicillin – Streptomycin.

The PBMCs previously isolated from patients' blood samples were put in contact with the supernatant from the B95-8 cell culture which had the EBV necessary for the

immortalization technique and then were put with “transformation” culture medium containing RPMI 1640 Medium + 20% FBS and 1% Penicillin – Streptomycin + 400ng/mL of cyclosporine. At approximately 30 days of culture, a pellet of LCLs’ cells was made through a 5 minutes at 1500 rpm centrifugation and preserved in Tripure® (Roche) at -80ºC.

3.4 RNA extraction and cDNA synthesis

RNA was extracted from the patients’ peripheral blood cells and LCLs’ pellet, both preserved in Tripure® using the GeneJet RNA Purification Kit (Thermo Scientific ®) and following manufacturer’s instructions. The purity of the isolated RNA was measured at 260 nm and 280 nm using *NanoDrop* ® spectrophotometer.

After that, mRNA samples were used as template for the cDNA synthesis using the High capacity RNA-to-cDNA Kit (Applied Biosystems ®) according to manufactures’’ instructions.

3.5 Relative Quantification of MMP-2, MMP -7, TIMP-2 and TIMP-4

The relative quantification of the transcript levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 was made by Quantitative Real-Time PCR. The reactions were made in a 7500 Fast Real Time PCR system (Apllied Biosystems ®) with the following resources: 1x MasterMix (Applied Biosystems ®), 1x probes (Taqman ® Gene Expression Assay – Hs01548727_m1 MMP-2; Hs01042796_m1 MMP-7; Hs00234278_m1 TIMP-2 and Hs00162784_m1 TIMP-4 - Applied Biosystems ®), cDNA samples from patients’ peripheral blood cells and LCLs and β2M endogenous control (β2M Oligo Mix (REF:4332653) - Applied Biosystems ®) in order to normalize the results observed.

Data analysis was made using 7500 Software v2.0.3 (Applied Biosystems ®) with the same baseline and threshold for each sample in order to have threshold cycle (Ct) values for all samples. The quantification of MMP-2, MMP-7, TIMP-2 and TIMP-4 and β2M was performed in duplicate and negative controls lacking samples were also included.

3.6 Statistical Analyses

All the statistical analyses were made using *IBM® SPSS® statistics* software v 23.0 for Windows ®. The t´Student test and Livak method (2-ΔΔCT) were used to analyze and

evaluate differences between the mRNA normalized expression levels, ρ-values under 0,05 were considered to be statistically significant.

The real-time PCR assays originated Ct (cycle threshold) values for each combination of assay-sample. These values are correspondent to the cycle number of the early exponential phase of the amplification reaction and, therefore, are inversely proportional to the relative expression levels or quantity of the targets in the sample tested. Thus, the mean of the Ct values within each sample type was determined and, in order to normalize the results, the difference between the mean Ct values of the target and the mean Ct values of endogenous control (β2M) was assessed. This number is designated as the ΔCt. The fold change values were calculated by formula $2^{-\Delta\Delta CT}$, following Livak et al. (2001) [160].

Chapter 4

Results

4 Results

4.1 Patients Information

From the patients selection, a total of 16 NSCLC patients, 13 males and 3 females with ages between 39 and 80 years old, a mean of approximately 63 years old (±10,856), all Caucasians from the North Region of Portugal were recruited. All the clinical, pathological and demographic information is described in Table 1.

Table 1 - Clinical, Pathological and Demographic characteristics of the NSCLC cases group.

		Mean	Std. Deviation
Age at Diagnosis		63,44	10,856
		n (n=16)	**%**
Gender			
	Masculin	13	81,25
	Feminin	3	18,75
	Total	16	100,00
Smoking			
	Non-Smokers	3	18,75
	Ex-Smokers	6	37,50
	Smokers	7	43,75
	Total	16	100,00
TNM Stage			
	IIIA	2	12,50
	IIIB	3	18,75
	IV	10	62,50
	No Information	1	6,25
	Total	16	100,00
Histology			
	Adenocarcinoma	12	75,00
	Squamous Cell Carcinoma	4	25,00
	Total	16	100,00

4.2 mRNA Relative Quantification levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 Before and After Immortalization

4.2.1 Relative Quantification of mRNA levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 in NSCLC cases: Before and After Immortalization

At first the NSCLC cases group was evaluated and the relative quantification of mRNA levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 before and after the immortalization technique was done in order to understand if the use of this technique would induce significant changes in the mRNA levels of our molecular targets.

The mRNA levels of MMP-2 were significantly higher after the immortalization technique (ρ=0.006). The mRNA levels of MMP-7 appear to be lower after immortalization. However, this difference cannot be considered to be significant (ρ=0.765). As for the mRNA levels of TIMP-2, after the technique of immortalization the levels were increased but the change was not significant (ρ=0.220). Finally, the mRNA levels of TIMP-4 were lower after the immortalization although that difference was not significant (ρ=0.090).

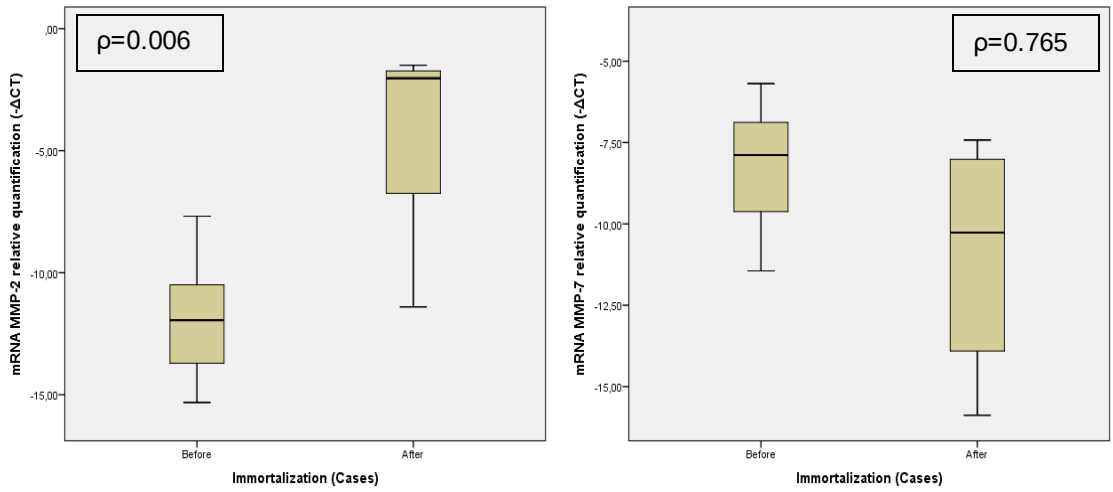

Figure 9 – mRNA Relative Quantification levels of MMP-2 and MMP-7 before and after the immortalization technique in NSCLC cases (-ΔCT).

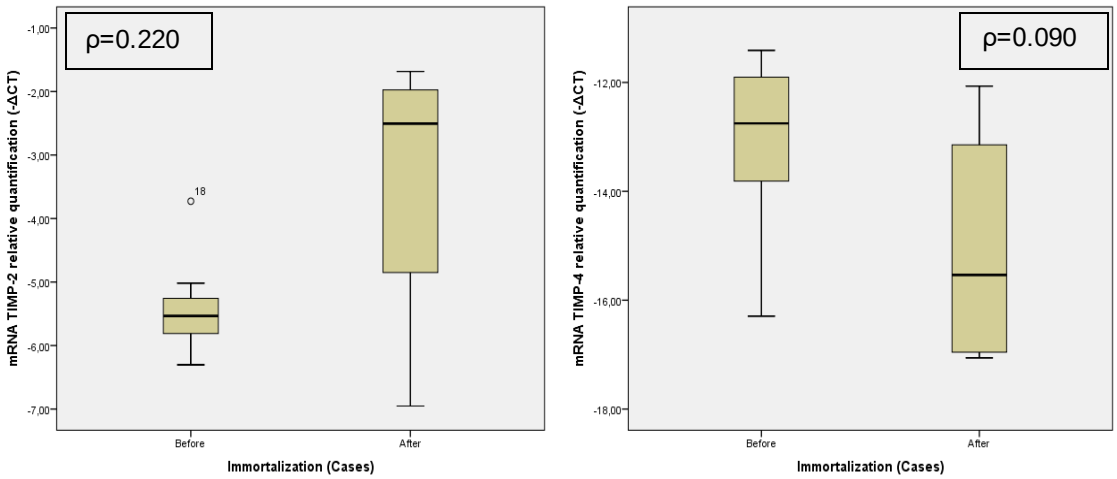

Figure 10 – mRNA Relative Quantification levels of TIMP-2 and TIMP-4 before and after the immortalization technique in NSCLC cases (-ΔCT).

In order to understand the magnitude of these differences the Fold Changes were made (Table 2) and according to them the significant change observed between the MMP-2 levels before and after immortalization corresponds to an increase of the expression levels after the immortalization technique as it was portrayed in Figure 9. The changes observed for the MMP-7 and TIMP-4 indicate higher levels of these targets mRNA before the immortalization technique and TIMP-2 mRNA levels are lower before the technique. Apart for the MMP-2 levels all the other changes were not statistically significant.

Table 2 – Fold Changes of MMP-2, MMP-7, TIMP-2 and TIMP-4 mRNA levels between before and after the immortalization technique in NSCLC cases (2^-ΔΔCT).

Immortalization	**MMP-2**	**MMP-7**	**TIMP-2**	**TIMP-4**
Before vs After	0.012 (ρ=0.006)	1.410 (ρ=0.765)	0.338 (ρ=0.220)	3.662 (ρ=0.090)

4.2.2 Relative Quantification of mRNA levels of MMP -7 and TIMP-2 in controls: Before and After Immortalization

As it happened with the NSCLC cases group, the control group was also evaluated for mRNA levels of our molecular targets due to the importance of clarifying if the presence of NSCLC had any influenced in the changes between the levels before and after immortalization. The relative quantification of mRNA levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 in controls was made before and after the immortalization technique.

However, the mRNA levels of MMP-2 and TIMP-4 could not be compared due to the lack of data.

The mRNA levels of MMP-7 were found to be higher after the immortalization in controls. However, that difference was not significant (ρ=0.365). The difference registered between the levels of TIMP-2 before and after immortalization was found to be statistically significant (ρ=0.001), it was registered an increase of mRNA TIMP-2 levels after the immortalization technique.

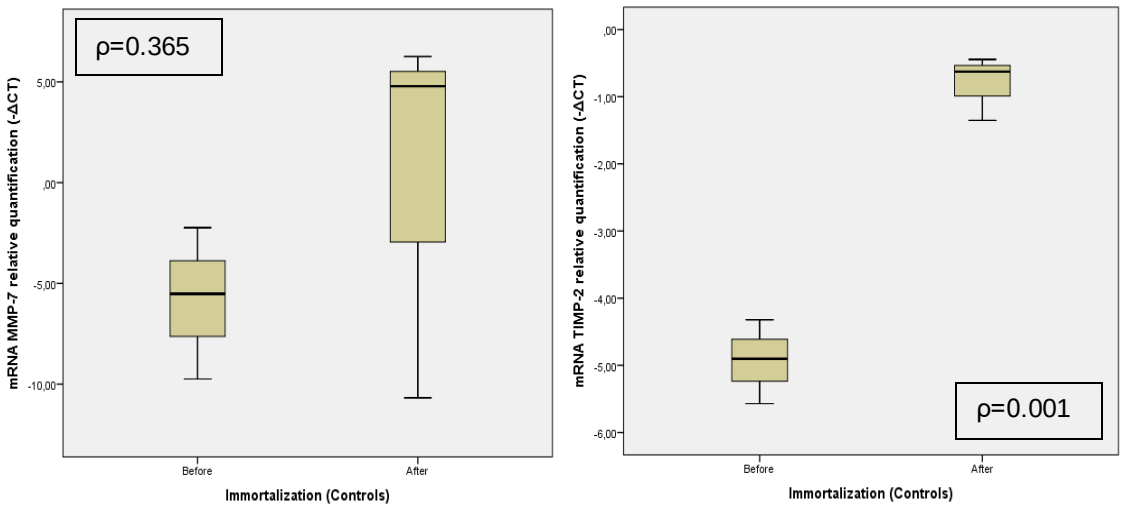

Figure 11 - mRNA Relative Quantification levels of MMP-7 and TIMP-2 before and after the immortalization technique in controls (-ΔCT).

Once again, in order to understand the magnitude of these differences the Fold Changes were also made for the controls group (Table 3) and indicated that the significant change observed in the TIMP-2 mRNA levels before and after immortalization corresponds to an increase of the expression levels after the immortalization technique as it was portrayed in Figure 11. The changes observed for MMP-7 indicated lower levels of mRNA before the immortalization technique but the difference was not significant.

Table 3 –Fold Changes of MMP-7 and TIMP-2 mRNA levels between before and after the immortalization technique in controls (2^-ΔΔCT).

Immortalization	**MMP-2**	**MMP-7**	**TIMP-2**	**TIMP-4**
Before vs After	*	0.016 (ρ=0.365)	0.057 (ρ=0.001)	*
*- Not enough data.				

4.2.3 Relative Quantification of mRNA levels of MMP -7 and TIMP-2 in NSCLC cases and Controls: Differences between NSCLC cases and Controls before and after Immortalization

The comparison between controls and NSCLC cases was made taking into consideration the mRNA levels of MMP-7 and TIMP-2 before and after the immortalization technique in both groups.

According to Figure 12, the mRNA levels of MMP-7 and TIMP-2 before the immortalization were slightly higher in controls compared to NSCLC cases but none of these differences were significant. After the immortalization technique the levels of MMP-7 and TIMP-2 were also found to be higher in controls when compared to NSCLC cases and the difference between the TIMP-2 mRNA levels in controls and NSCLC cases registered after immortalization was considered to be statistically significant (ρ=0.007).

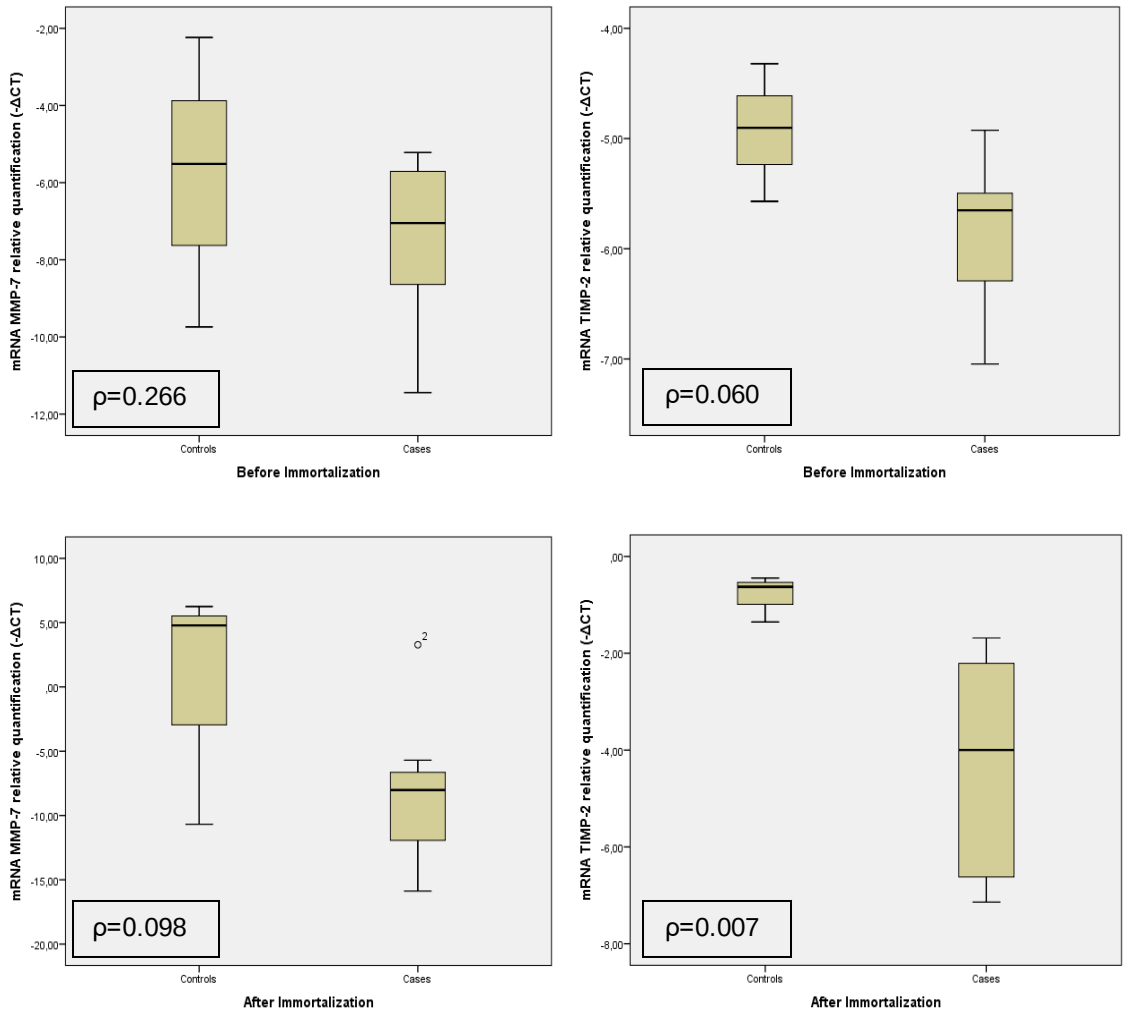

Figure 12 - mRNA Relative Quantification levels of MMP-7 and TIMP-2 in controls and NSCLC cases before and after the immortalization technique (-ΔCT).

The fold changes were also made to better understand the differences between controls and NSCLC cases before and after the immortalization technique (Table 4). Once more, what was portrayed in Figure 12 was confirmed with this test: mRNA levels of MMP-7 and TIMP-2 before immortalization were found to be higher in controls compared to NSCLC cases but were not significant. mRNA levels of MMP-7 and TIMP-2 after immortalization were also found to be increased in controls when compared to NSCLC cases and the TIMP-2 difference was statistically significant.

Table 4 – Fold Changes of MMP-7 and TIMP-2 mRNA levels between controls and NSCLC cases before and after the immortalization technique (2^-ΔΔCT).

Immortalization	**MMP-2**	**MMP-7**	**TIMP-2**	**TIMP-4**
Before				
Controls vs Cases	*	3.062 (ρ=0.266)	1.804 (ρ=0.060)	*
After				
Controls vs Cases	*	336.695 (ρ=0.098)	10.677 (ρ=0.007)	*

*- Not enough data.

Chapter 5

Discussion

5 Discussion

5.1 Changes in the mRNA MMP-2, MMP-7, TIMP-2 and TIMP-4 levels before and after Immortalization in NSCLC cases and in controls

As it was described, both the NSCLC cases and controls were submitted to a relative quantification of the mRNA levels of MMP-2, MMP-7, TIMP-2 and TIMP-4 before and after the immortalization technique in order to understand if the use of LCLs from NSCLC patients would be a good predictive model to study the importance of these molecular targets in the NSCLC landscape namely in drug sensitivity and response.

The mRNA levels of MMP-2 were significantly higher after the immortalization technique (ρ=0.006) in the NSCLC cases group. This could be explained due to the changes in the cell biology and behavior that happen when peripheral blood cells undergo through EBV transformation in order to become a LCL from the patient [61]. It is documented that EBV transformation and culture cell processes can induct cellular and molecular changes such as growth rate and according to this finding the transformation can eventually enhance the cell invasion and metastatic potential due to the difference registered in the MMP-2 mRNA levels considering that this MMP has been associated not only with tumor growth but also with invasion, angiogenesis and metastasis [65, 66, 81]. It is important to notice that in a study of human lymphoproliferative diseases, lymphoblastoid cells were reported to be associated with an increase expression and secretion of MMP-2 and MMP-9 and that was related with invasive, angiogenic and metastasis abilities of these cells [161]. This hypothesis would need further studies to evaluate if the activity of this MMP in LCLs is related to an invasive and metastatic potential of lymphoblastoid cells or its actions do not promote those characteristics in these cells. Another aspect that may be important is that the use of EBV can induce inflammation and the increased levels of MMP-2 after immortalization may suggest that this MMP may be related with that process.

The mRNA levels of MMP-7 appear to be lower after EBV transformation in the NSCLC cases group although it is not a significant change (ρ=0.765). This MMP has also been associated with tumor progression, invasion and metastasis [143, 146]. However, in this case the results suggest that the immortalization technique does not alter the levels of this MMP to a point where the differences between before and after immortalization are significant. This means that in LCLs, MMP-7 could probably be studied in order to evaluate its influence in drug sensitivity tests and response.

TIMP-2 mRNA levels were found to be increased after immortalization in NSCLC cases group but without statistical significance (ρ=0.220). This result suggests that the mRNA levels of this TIMP-2 were not greatly altered in the NSCLC cases group by the immortalization technique.

The TIMP-4 mRNA levels after immortalization appeared to be lower than before immortalization but without any statistical significance (ρ=0.090) in the NSCLC cases group. This result suggests that the levels of TIMP-4 are not greatly altered by the immortalization technique and therefore it may be possible to study this TIMP and its action in LCLs.

In the controls group the levels of MMP-2 and TIMP-4 mRNAs were not evaluated as there was not enough data for a correct analysis.

As for MMP-7, it was observed that in the controls group the levels of MMP-7 increased after the immortalization technique, although that difference was not significant (ρ=0.365). As it happened with the NSCLC cases group, the changes registered of this MMP before and after immortalization were not significant and that can indicate that this MMP can be studied in the context of a LCL based investigation.

The TIMP-2 mRNA levels were found to be significantly higher after the immortalization technique (ρ=0.001) in the controls group. This time, the results were considered to be significant and suggest that EBV transformation can eventually promote changes in the expression of mRNA levels of TIMP-2 therefore altering the cell behavior and offering a barrier to the study of this TIMP in a LCLs context. High levels of TIMP-2 were already associated with the inhibition of angiogenesis, tumor growth, invasion and metastasis [150–152]. If the EBV transformation induces a process similar to carcinogenesis, this increase of TIMP-2 mRNA levels may be an attempt to regain homeostasis.

5.2 Comparison of the mRNA levels of MMP-7 and TIMP-2 between controls and NSCLC cases before and after Immortalization

After the measure of mRNA levels of our molecular targets in both the NSCLC cases group and controls group it became interesting to evaluate the differences between controls and NSCLC cases in the targets available for both of them before and after the Immortalization technique.

mRNA levels of MMP-7 were found to be higher in controls than in NSCLC cases before (ρ=0.266) and after (ρ=0.098) the immortalization technique but none of those differences were considered to be statistically significant. Before immortalization, the difference registered is interesting as MMP-7 is usually related with tumor progression, invasion and metastasis and in this study its levels are not higher in the NSCLC cases group comparing to the controls group. After the transformation, the changes were concordant with the previous data: in NSCLC cases there was a decrease of MMP-7 after immortalization and in controls there was an increase, both without statistical significance. When comparing both groups after immortalization the controls had higher levels than NSCLC cases. It is important to notice that these changes were not significant.

As for the mRNA levels of TIMP-2, there were higher levels in the control group before (ρ=0.060) and after (ρ=0.007) the immortalization technique in comparison with the NSCLC cases group. The difference observed before immortalization was not significant but it demonstrates a statistical tendency that may indicate that lower levels of circulating TIMP-2 are present in NSCLC patients in comparison with controls. Taking into consideration the action of TIMP-2 in inhibiting MMPs and its relation with the inhibition of tumor growth, invasion and metastasis it becomes understandable that this difference may represent an unbalance in NSCLC patients between MMPs/TIMPs. In fact, serum levels of TIMP-2 were already reported to be lower in NSCLC patients than in controls [152]. After the immortalization technique the difference between NSCLC cases and controls was significant. This difference may represent the effects of the immortalization technique in TIMP-2 levels. As it was described earlier, the difference between NSCLC cases and controls before the immortalization was not significant but after that technique it became significant, which shows that the EBV transformation may greatly alter the expression of this TIMP because even though the number of controls is limited we are referring to the control group in all the relative quantifications and therefore if EBV transformation had no part in altering these levels the difference registered after the immortalization would not be significant as it happened before the immortalization. This result suggests that TIMP-2 levels in LCLs are altered and the study of the influence of this TIMP in a LCL based project may not relate to what happens in the organism.

5.3 Limitations of LCLs and their association with MMPs

It is reported that LCLs from patients may present a somatic mutation rate of 0.3% and the whole exome and genome sequencing have shown 99% concordance in DNA

sequence between LCLs and peripheral blood cells from the same individual [63, 65]. LCLs provide an unlimited source of biomolecules such as DNA, RNA or proteins and taking into consideration the similarity described before, it is possible to consider this model to be useful in many pharmacogenomic studies and also immunology and cellular biology studies [61].

However, normal peripheral blood lymphocytes undergo significant transformation to become “immortal” and originate LCLs and that can alter the biology and metabolism of the cell. That must be considered relevant and important in the interpretation of any study that involves the use of LCLs [61]. EBV transformation and cell culture processes might introduce cellular changes such as growth rate that can be responsible for the difficulties encountered when trying to access a biomarker´s importance and influence found in LCLs models in clinical studies and functional assays meaning that phenotypes that are found *in vitro* may not be observed *in vivo* [65, 66] .

Although the role and potential alteration of MMPs and TIMPs in LCLs has yet to be discovered, some associations between EBV and MMPs have already been reported.

The latent membrane protein 1 (LMP1) of EBV is crucial in cell immortalization [61]. It aggregates cellular proteins of the TNF receptor signaling pathway to active transcription factor NF-κB [61]. In sinonasal squamous cell carcinomas, the presence of EBV and LMP1 positive patients was related with lymph node or distant metastasis [162]. Induction of MMP-1 and MMP-3 was also detected in nasopharyngeal cancer patients with LMP1 positive tumors compared with other head and neck cancer patients. The expression of MMP-3 was also associated with initial stages of carcinogenesis and tumor progression [163]. Zta, a lytic transactivator of EBV, has been shown to promote migration and invasion of epithelial cells and once again MMP-3 and MMP-9 were essential for Zta-induced cell invasion [164]. The invasive potential of an EBV positive LCL (NC-37, a Burkitt’s lymphoma cell line used as a target cell for EBV infection studies) was also studied *in vitro* and in this case MMP-2 expression was related with the invasive potential of this cell line [165]. Finally in human lymphoproliferative diseases, lymphoblastoid cells have also been associated with an increase in expression and secretion of MMP-2 and MMP-9 and with invasive, angiogenic and metastasis behaviors [161].

In this study, it was observed that the immortalization technique using EBV in order to have LCLs may greatly alter the mRNA levels of MMP-2 and TIMP-2 and therefore the investigation of these biomolecules in drug sensibility and response using LCLs may not be possible due to the changes that this transformation causes. This can also represent a possible limitation for LCLs studies as the behavior and environment of lymphoblastoid

cells are not similar to the initial patients' cells and it is known that many LCLs studies do not have a successful translation into clinical practice. For MMP-7 and TIMP-4 the changes were not significant which means that it may be possible to study these biomolecules in a LCL based study.

Chapter 6

Conclusions and Future Perspectives

6 Conclusions and Future Perspectives

The results from this study suggest that the mRNA levels of MMP-2 and TIMP-2 are significantly altered by the immortalization technique used which means that the use of EBV in order to transform and immortalize the patients' peripheral blood lymphocytes in order to have LCLs may induce alterations in the bioavailability of these biomarkers. For the first time, to our knowledge, the levels of mRNA of MMP-2, MMP-7, TIMP-2 and TIMP-4 were evaluated in a LCL based study and the comparison between before and after the immortalization technique was made.

Due to the activity of MMP-2 and its association with cell growth, invasion and metastization, it seems that this technique can not only promote the growth of these cells but also an invasive and metastatic behavior on lymphoblastoid cells meaning that this technique may induce processes similar to carcinogenesis. However, this could only be validated in future studies by the evaluation of MMP-2 protein levels using other techniques such as ELISA in order to understand if the mRNA levels are associated with the actual protein levels. As for the processes that may be induced by EBV, levels of inflammatory and angiogenic mediators as well as cell growth rates could indicate if the processes that happen in LCLs may be similar to those that happen in carcinogenesis.

The higher mRNA levels of TIMP-2 in controls compared to NSCLC patients before the immortalization were very close to be significant (ρ=0.060) and demonstrates a statistical tendency which would be expected as TIMPs levels in cancer patients are reported to be usually lower whereas MMPs levels tend to be higher. After the immortalization the difference was significant suggesting that TIMP-2 levels are actually altered by this technique and the increase of mRNA levels of TIMP-2 after the immortalization technique in controls is proof of that significant difference. To validate these findings, the evaluation of TIMP-2 protein levels using, for example, ELISA, could indicate if the differences of mRNA levels are actually accompanied by differences in the actual protein levels.

However, the changes registered in the mRNA levels of MMP-7 and TIMP-4 were not significant leaving the possibility for them to be studied in the context of a LCL based study. This can also mean that some MMPs and TIMPs such as MMP-2 and TIMP-2 tend to be more important in carcinogenesis: if the transformation by EBV virus induces processes similar to those that happen in carcinogenesis the difference registered can be an indicator that MMP-2 and TIMP-2 play more important roles in these processes than MMP-7 and TIMP-4.

In summary, the potential influence of MMPs and TIMPs in drug sensitivity and response is still possible to be studied as they are very important in many processes of carcinogenesis. However, the use of LCLs towards that objective may offer potential limitations as changes between before and after immortalization are registered indicating that this method promotes differences between what is seen *in vivo* and what is observed *in vitro*. This study provided more insight on what happens in LCLs and can indicate a possible reason why the findings in LCLs studies are difficult to be translated into clinical practice.

Chapter 7

References

7 References

1 Pitot HC. (1993) The Molecular Biology of Cancer. *CANCER Supplement*, 72(3), 962–970.

2 Siddiqui IA, Sanna V, Ahmad N, Sechi M, Mukhtar H. (2015) Resveratrol nanoformulation for cancer prevention and therapy. *Annals of the New York Academy of Sciences*, 1348(1), 20–31.

3 DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, et al. (2014) Cancer Treatment and Survivorship Statistics, 2014. *CA: A Cancer Journal for Clinicians*, 64, 252–271.

4 WHO. Cancer. Retrieved August 23, 2016, from http://www.who.int/mediacentre/factsheets/fs297/en/index.html

5 Neal RD, Hamilton W, Rogers TK. (2014) Lung Cancer. *The New England Journal of Medicine*, 349(November), 1367–1380.

6 Mao L, Lee JS, Kurie JM, Fan YH, Lippman SM, Lee JJ, et al. (1997) Clonal Genetic Alterations in the Lungs of Current and Former Smokers. *Journal of the National Cancer Institute*, 87(12), 857–862.

7 Wistuba, Berry J, Behrens C, Maitra A, Shivapurkar N, Milchgrub S, et al. (2000) Molecular changes in the bronchial epithelium of patients with small cell lung cancer. *Clinical Cancer Research*, 6(July), 2604–2610.

8 Hwang S-J, Cheng LS-C, Lozano G, Amos CI, Gu X, Strong LC. (2003) Lung cancer risk in germline p53 mutation carriers: association between an inherited cancer predisposition, cigarette smoking, and cancer risk. *Human Genetics*, 113(3), 238–243.

9 Sanders BM, Jay M, Draper GJ, Roberts EM. (1989) Non-ocular cancer in relatives of retinoblastoma patients. *British Journal of Cancer*, 60(3), 358–365.

10 Bell DW, Gore I, Okimoto R a, Godin-Heymann N, Sordella R, Mulloy R, et al. (2005) Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. *Nature Genetics*, 37(12), 1315–1316.

11 Yu D, Zhang X, Liu J, Yuan P, Tan W, Guo Y, et al. (2008) Characterization of functional excision repair cross-complementation group 1 variants and their association with lung cancer risk and prognosis. *Clinical Cancer Research*, 14(9), 2878–2886.

12 Sun S, Schiller JH, Gazdar AF. (2007) Lung cancer in never smokers — a different disease. *Nature*, 7(October), 778–790.

13 International Agency for Research on Cancer. Globocan 2012. Retrieved July 23, 2016, from http://globocan.iarc.fr/Default.aspx

14 Salgia R. (2015) Diagnostic challenges in non-small-cell lung cancer: an integrated medicine approach. *Future Oncology*, 11(3), 489–500.

15 Aisner DL, Marshall CB. (2012) Molecular pathology of non-small cell lung cancer: A practical guide. *American Journal of Clinical Pathology*, 138(3), 332–346.

16 Torre LA, Siegel RL, Jemal A. (2016) Lung Cancer Statistics. In *Lung Cancer and Personalized Medicine*, pp.1–19.

17 Schwartz AG, Cote ML. (2016) Epidemiology of Lung Cancer. In *Lung Cancer and Personalized Medicine*, pp.21–41.

18 Warner KE, Mendez D. (2010) Tobacco control policy in developed countries: yesterday, today, and tomorrow. *Nicotine & Tobacco Research*, 12(9), 876–887.

19 Rodríguez-Antona C, Taron M. (2015) Pharmacogenomic biomarkers for personalized cancer treatment. *Journal of Internal Medicine*, 277(2), 201–217.

20 Tan X, Moyer AM, Fridley BL, Schaid DJ, Niu N, Batzler AJ, et al. (2011) Genetic varitaiton predicting cisplatin cytotoxicity associated with overall survival in lung cancer patients receiving platinum-based chemotherapy. *Clinical Cancer Research*, 17(17), 5801–5811.

21 Hayes D, Schott A. (2015) Personalized Medicine: Genomics Trials in Oncology. *Transactions of the American Clinical and Climatological Association*, 126, 211–215.

22 Lemjabbar-Alaoui H, Hassan OU, Yang Y-W, Buchanan P. (2015) Lung cancer: Biology and treatment options. *Biochimica et Biophysica Acta*, 1856(2), 189–210. Elsevier B.V.

23 Chen Z, Fillmore CM, Hammerman PS, Kim CF, Wong K-K. (2014) Non-small-cell lung cancers: a heterogeneous set of diseases. *Nature Reviews Cancer*, 14(8), 535–546. Nature Publishing Group.

24 Yu Y, He J. (2013) Molecular classification of non-small-cell lung cancer: diagnosis, individualized treatment, and prognosis. *Frontiers of Medicine*, 7(2), 157–171.

25 Davidson MR, Gazdar AF, Clarke BE. (2013) The pivotal role of pathology in the management of lung cancer. *Journal of Thoracic Disease*, 5(5), S463–S478.

26 Langer CJ, Besse B, Gualberto A, Brambilla E, Soria J-C. (2010) The Evolving Role of Histology in the Management of Advanced Non-Small-Cell Lung Cancer. *Journal of Clinical Oncology*, 28(36), 5311–5320.

27 Lu Y, Futtner C, Rock JR, Xu X, Whitworth W, Hogan BLM, et al. (2010) Evidence that SOX2 overexpression is oncogenic in the lung. *PLoS ONE*, 5(6).

28 Dacic S, Shuai Y, Yousem S, Ohori P, Nikiforova M. (2010) Clinicopathological predictors of EGFR/KRAS mutational status in primary lung adenocarcinomas. *Modern Pathology*, 23(2), 159–168. Nature Publishing Group.

29 Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*, 448(7153), 561–566.

30 Sasaki T, Rodig SJS, Chirieac LLR, Jänne P a. (2010) The biology and treatment of EML4-ALK non-small cell lung cancer. *European Journal of Cancer*, 46(10), 1773–1780.

31 Wong DWS, Leung ELH, So KKT, Tam IYS, Sihoe ADL, Cheng LC, et al. (2009) The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. *Cancer*, 115(8), 1723–1733.

32 Araújo A, Ribeiro R, Azevedo I, Coelho A, Soares M, Sousa B, et al. (2007) Genetic Polymorphisms of the Epidermal Growth Factor and Related Receptor in Non-Small Cell Lung Cancer—A Review of the Literature. *The Oncologist*, 12(August), 201–210.

33 Green John A, Bates V, Greenhalgh J, Boland A, Jain P, Dickson Rumona C, et al. (2013) First-line treatment of advanced epidermal growth factor receptor (EGFR) mutation positive non-squamous non-small cell lung cancer. *Cochrane Database of Systematic Reviews*, 1–87.

34 Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. (2002) Mutations of the BRAF gene in human cancer. *Nature*, 417(6892), 949–954.

35 Santos E, Martin-Zanca D, Reddy EP, Pierotti M a, Della Porta G, Barbacid M. (1984) Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue of the same patient. *Science*, 223(1982), 661–664.

36 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. (2004) Activating Mutations in the Epidermal Growth Factor Receptor Underlying Responsiveness of Non–Small-Cell Lung Cancer to Gefitinib. *The New England Journal of Medicine*, 35(21), 1209–1223.

37 Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*, 316(5827), 1039–1043.

38 Stephens P, Hunter C, Bignell G, Edkins S, Davies H, Teague J, et al. (2004) Lung cancer: intragenic ERBB2 kinase mutations in tumours. *Nature*, 431(7008), 525–526.

39 Takahashi T, Sonobe M, Kobayashi M, Yoshizawa A, Menju T, Nakayama E, et al. (2010) Clinicopathologic features of non-small-cell lung cancer with EML4-ALK fusion gene. *Annals of surgical oncology*, 17(3), 889–897.

40 Pillai RN, Ramalingam SS. (2012) The biology and clinical features of non-small cell lung cancers with EML4-ALK translocation. *Current Oncology Reports*, 14(2), 105–110.

41 Rodig SJ, Mino-kenudson M, Dacic S, Yeap BY, Shaw A, Barletta A, et al. (2009) Unique Clinicopathologic Features Characterize ALK-rearranged Lung Adenocarcinoma in the Western Population. *Clinical Cancer Research*, 15(16), 5216–5223.

42 Network TCGAR. (2014) Comprehensive genomic characterization of squamous cell lung cancers. *Nature*, 489(7417), 519–525.

43 Ettinger DS, Akerley W, Borghaei H, Chang AC, Cheney RT, Chirieac LR, et al. (2013) Non–Small Cell Lung Cancer, Version 2.2013. *Journal of the National Comprehensive Cancer Network* , 11(6), 645–653.

44 Duncavage E, Goodgame B, Sezhiyan A, Govindan R, Pfeifer J. (2010) Use of microRNA expression levels to predict outcomes in resected stage I non-small cell lung cancer. *Journal of Thoracic Oncology*, 5(11), 1755–1763. Elsevier.

45 Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, et al. (2011) International Association for the Study of Lung Cancer / American Thoracic Society / European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma. *Journal of Thoracic Oncology*, 6(2), 244–285.

46 Sundar R, Soong R, Cho B, Brahmer JR, Soo RA. (2014) Immunotherapy in the treatment of non-small cell lung cancer. *Lung Cancer*, 85(2), 101–109. Elsevier Ireland Ltd.

47 Bando H, Takebe N. (2016) Perspectives on research activity in the USA on cancer precision medicine. *Japanese Journal of Clinical Oncology*, 46(2), 106–110.

48 Carneiro BA, Costa R, Taxter T, Chandra S, Chae YK, Cristofanilli M, et al. (2016) Is Personalized Medicine Here ? *Cancer Network*, 1–16.

49 Tursz T, Bernards R. (2015) Hurdles on the road to personalized medicine. *Molecular Oncology*, 9(5), 935–939. Elsevier B.V.

50 Moreira AL, Eng J. (2014) Personalized therapy for lung cancer. *Chest*, 146(6), 1649–1657.

51 Fang B, Mehran RJ, Heymach J V., Swisher SG. (2015) Predictive biomarkers in precision medicine and drug development against lung cancer. *Chinese Journal of Cancer*, 34(7), 1–15. BioMed Central.

52 Raparia K, Villa C, DeCamp MM, Patel JD, Mehta MP. (2013) Molecular profiling in non-small cell lung cancer: a step toward personalized medicine. *Archives of Pathology & Laboratory Medicine*, 137(4), 481–491.

53 Relling M V, Evans WE. (2015) Pharmacogenomics in the clinic. *Nature*, 526(7573), 343–350.

54 Pirmohamed M. (2001) Pharmacogenetics and pharmacogenomics. *British Journal of Clinical Pharmacology*, 52, 345–347.

55 Robert J, Le Morvan V, Giovannetti E, Peters GJ. (2014) On the use of pharmacogenetics in cancer treatment and clinical trials. *European Journal of Cancer*, 50(15), 2532–2543.

56 Rose MC, Kostyanovskaya E, Huang RS. (2014) Pharmacogenomics of cisplatin sensitivity in non-small cell lung cancer. *Genomics, Proteomics and Bioinformatics*, 12(5), 198–209. Beijing Institute of Genomics, Chinese Academy of Sciences and Genetics Society of China.

57 Delaney C, Frank S, Stephanie Huang R. (2015) Pharmacogenomics of EGFR-targeted therapies in non–small cell lung cancer: EGFR and beyond. *Chinese Journal of Cancer*, 34(7), 1–12.

58 Giovannetti E, Toffalorio F, De Pas T, Peters GJ. (2012) Pharmacogenetics of conventional chemotherapy in non-small-cell lung cancer: a changing landscape? *Pharmacogenomics*, 13(9), 1073–1086.

59 Galvani E, Toffalorio F, Peters GJ, De Pas T, Giovannetti E. (2014) Pharmacogenetics of non-small cell lung cancer (NSCLC): time to “work it out”? *Current Pharmaceutical Design*, 20(24), 3863–3874.

60 Hertz DL, Rae J. (2015) Pharmacogenetics of cancer drugs. *Annual Review of*

Medicine, 66(October 2014), 65–81.

61 Hussain T, Mulherkar R. (2012) Lymphoblastoid Cell lines: a Continuous in Vitro Source of Cells to Study Carcinogen Sensitivity and DNA Repair. *International Journal of Molecular and Cellular Medicine*, 1(2), 75–87.

62 Sie L, Loong S, Tan EK. (2009) Utility of lymphoblastoid cell lines. *Journal of Neuroscience Research*, 87(9), 1953–1959.

63 Mohyuddin A, Ayub Q, Siddiqi S, Carvalho-Silva DR, Mazhar K, Rehman S, et al. (2004) Genetic instability in EBV-transformed lymphoblastoid cell lines. *Biochimica et Biophysica Acta*, 1670(1), 81–3.

64 Amoli MM, Carthy D, Platt H, Ollier WER. (2008) EBV immortalization of human B lymphocytes separated from small volumes of cryo-preserved whole blood. *International Journal of Epidemiology*, 37(SUPPL. 1), 41–45.

65 Niu N, Wang L. (2015) In vitro human cell line models to predict clinical response to anticancer drugs. *Pharmacogenomics*, 16(3), 273–285.

66 Moen EL, Godley LA, Zhang W, Dolan ME. (2012) Pharmacogenomics of chemotherapeutic susceptibility and toxicity. *Genome Medicine*, 4(11), 90.

67 Wheeler HE, Dolan ME. (2012) Lymphoblastoid cell lines in pharmacogenomic discovery and clinical translation. *Pharmacogenomics*, 13(1), 55–70.

68 Hanahan D, Weinberg RA. (2011) Hallmarks of cancer: The next generation. *Cell*, 144(5), 646–674. Elsevier Inc.

69 Hahn W, Weinberg R. (2002) Rules for making human tumor cells. *The New England Journal of Medicine*, 347(20), 1593–1604.

70 Leber MF, Efferth T. (2009) Molecular principles of cancer invasion and metastasis. *International Journal of Oncology*, 34, 881–895.

71 Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. (2010) Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene*, 29(8), 1093–1102.

72 Balkwill F, Mantovani A. (2001) Inflammation and cancer: Back to Virchow? *Lancet*, 357(9255), 539–545.

73 Coussens LM, Werb Z. (2002) Inflammation and cancer. *Nature*, 420(6917), 860–867.

74 Shalapour S, Karin M. (2015) Immunity, inflammation, and cancer: An eternal fight

between good and evil. *Journal of Clinical Investigation*, 125(9), 3347–3355.

75 P E. (1909) The current state of cancer research (über den jetzigen stand der karzinomforschung). *Ned Tijdschr Geneeskd*, 5, 273–290.

76 Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nature Immunology*, 3(11), 991–998.

77 Burnet FM. (1970) The Concept of Immunological Surveillance. *Progress in Experimental Tumor Research*, 13, 1–27.

78 Mlecnik B, Bindea G, Pagès F, Galon J. (2011) Tumor immunosurveillance in human cancers. *Cancer and Metastasis Reviews*, 30(1), 5–12.

79 Bremnes RM, Al-Shibli K, Donnem T, Sirera R, Al-Saad S, Andersen S, et al. (2011) The role of tumor-infiltrating immune cells and chronic inflammation at the tumor site on cancer development, progression, and prognosis: emphasis on non-small cell lung cancer. *Journal of Thoracic Oncology*, 6(4), 824–833. International Association for the Study of Lung Cancer.

80 Allavena P, Sica A, Solinas G, Porta C, Mantovani A. (2008) The inflammatory micro-environment in tumor progression: The role of tumor-associated macrophages. *Critical Reviews in Oncology and Hematology*, 66(1), 1–9.

81 Hua H, Li M, Luo T, Yin Y, Jiang Y. (2011) Matrix metalloproteinases in tumorigenesis: An evolving paradigm. *Cellular and Molecular Life Sciences*, 68(23), 3853–3868.

82 Crusz SM, Balkwill FR. (2015) Inflammation and cancer: advances and new agents. *Nature Reviews Clinical Oncology*, 12(10), 1–13.

83 O'Callaghan DS, O'Donnell D, O'Connell F, O'Byrne KJ. (2010) The role of inflammation in the pathogenesis of non-small cell lung cancer. *Journal of Thoracic Oncology*, 5(12), 2024–2036. International Association for the Study of Lung Cancer.

84 Hanahan D, Weinberg RA. (2000) The Hallmarks of Cancer. *Cell*, 100, 57–70.

85 Mehlen P, Puisieux A. (2006) Metastasis: a question of life or death. *Nature reviews. Cancer*, 6(6), 449–458.

86 Fidler IJ. (2003) The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited. *Nature Reviews Cancer*, 3(June), 453–458.

87 Talmadge JE, Fidler IJ. (2010) AACR Centennial Series: The Biology of Cancer Metastasis: Historical Perspective. *Cancer Research*, 70(14), 5649–5669.

88 Poste G, Fidler IJ. (1980) The pathogenesis of cancer metastasis. *Nature*, 283, 139–146.

89 Coghlin C, Murray GI. (2010) Current and emerging concepts in tumour metastasis. *Journal of Pathology*, 222(1), 1–15.

90 Huelsken Lab. Metastasis. Retrieved August 22, 2016, from http://huelsken-lab.epfl.ch/page-126563-en.html

91 Jung H-Y, Fattet L, Yang J. (2015) Molecular Pathways: Linking Tumor Microenvironment to Epithelial–Mesenchymal Transition in Metastasis. *Clinical Cancer Research*, 21(5), 962–968.

92 Craene B, Berx G. (2013) Regulatory networks defining EMT during cancer initiation and progression. *Nature Reviews Cancer*, 13(2), 97–110. Nature Publishing Group.

93 Thiery JP, Acloque H, Huang RYJ, Nieto MA. (2009) Epithelial-Mesenchymal Transitions in Development and Disease. *Cell*, 139(5), 871–890.

94 Ansieau S, Bastid J, Doreau A, Morel A-P, Bouchet BP, Thomas C, et al. (2008) Induction of EMT by Twist Proteins as a Collateral Effect of Tumor-Promoting Inactivation of Premature Senescence. *Cancer Cell*, 14(1), 79–89.

95 Browne G, Sayan AE, Tulchinsky E. (2010) ZEB proteins link cell motility with cell cycle control and cell survival in cancer. *Cell Cycle*, 9(5), 886–891.

96 Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. (2008) The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells. *Cell*, 133(4), 704–715.

97 Morel a P, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux a. (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One*, 3(8), e2888.

98 Derksen PWB, Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, et al. (2006) Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell*, 10(5), 437–449.

99 Valdes F, Alvarez AM, Locascio A, Vega S, Herrera B, Fernandez M, et al. (2002) The Epithelial Mesenchymal Transition Confers Resistance to the Apoptotic Effects of Transforming Growth Factor {beta} in Fetal Rat Hepatocytes. *Molecular Cancer Research*, 1(1), 68–78.

100 Kumar S, Weaver VM. (2009) Mechanics, malignancy, and metastasis: The force journey of a tumor cell. *Cancer and Metastasis Reviews*, 28(1–2), 113–127.

101 Nguyen DX, Bos PD, Massagué J. (2009) Metastasis: from dissemination to organ-specific colonization. *Nature Reviews Cancer*, 9(4), 274–284.

102 Popper HH. (2016) Progression and metastasis of lung cancer. *Cancer Metastasis Reviews*, 35(1), 75–91.

103 Tamura T, Kurishima K, Nakazawa K, Kagohashi K, Ishikawa H, Satoh H, et al. (2015) Specific organ metastases and survival in metastatic non-small-cell lung cancer. *Molecular and Clinical Oncology*, 3(1), 217–221.

104 Fidler IJ, Kripke ML. (2015) The challenge of targeting metastasis. *Cancer and Metastasis Reviews*, 34(4), 635–641.

105 Pulkoski-Gross AE. (2015) Historical Perspective of Matrix Metalloproteases. *Frontiers in Bioscience*, 7(JUNE 2015), 125–149.

106 Gross J, Lapiere CM. (1962) Collagenolytic Activity in Amphibian Tissues: a Tissue Culture Assay. *Proceedings of the National Academy of Sciences of the United States of America*, 48(6), 1014–1022.

107 Nissinen L, Kähäri VM. (2014) Matrix metalloproteinases in inflammation. *Biochimica et Biophysica Acta - General Subjects*, 1840(8), 2571–2580.

108 Nagase H, Visse R, Murphy G. (2006) Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research*, 69(3), 562–573.

109 Hua Y, Nair S. (2015) Proteases in cardiometabolic diseases: Pathophysiology, molecular mechanisms and clinical applications. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1852(2), 195–208.

110 Visse R, Nagase H. (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. *Circulation Research*, 92(8), 827–839.

111 Hideaki Nagase, J. Frederick Woessner J. (1999) Matrix metalloproteinases. *The Journal of Biological Chemistry*, 274(31), 21491–21494.

112 Sternlicht M, Werb Z. (2001) How Matrix metalloproteinases regulate cell behavior. *Annual Review of Cell and Developmental Biology*, 17, 463–516.

113 Khokha R, Murthy A, Weiss A. (2013) Metalloproteinases and their natural inhibitors in inflammation and immunity. *Nature Reviews. Immunology*, 13(9), 649–65. Nature Publishing Group.

114 Brew K, Nagase H. (2010) The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1803(1), 55–71.

115 Yadav L, Puri N, Rastogi V, Satpute P, Ahmad R, Kaur G. (2014) Matrix metalloproteinases and cancer - Roles in threat and therapy. *Asian Pacific Journal of Cancer Prevention*, 15(3), 1085–1091.

116 Rundhaug JE. (2003) Matrix Metalloproteinases , Angiogenesis , and Cancer. *Clinical Cancer Research*, 9(February), 551–554.

117 Nagase H. (1997) Activation mechanisms of matrix metalloproteinases. *Journal of Biological Chemistry*, 378(3–4), 151–160.

118 Brew K, Nagase H. (2010) The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1803(1), 55–71. Elsevier B.V.

119 Deryugina EI, Quigley JP. (2006) Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Reviews*, 25(1), 9–34.

120 Gialeli C, Theocharis AD, Karamanos NK. (2011) Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *The FEBS Journal*, 278(1), 16–27.

121 Egeblad M, Werb Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nature Reviews Cancer*, 2, 161–174.

122 Kessenbrock K, Plaks V, Werb Z. (2010) Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell*, 141(1), 52–67.

123 Stetler-Stevenson WG. (1999) Matrix metalloproteinases in angiogenesis: A moving target for therapeutic intervention. *Journal of Clinical Investigation*, 103(9), 1237–1241.

124 Cornelius L a, Nehring LC, Harding E, Bolanowski M, Welgus HG, Kobayashi DK, et al. (1998) Matrix metalloproteinases generate angiostatin: effects on neovascularization. *Journal of immunology*, 161(12), 6845–6852.

125 Ferreras M, Felbor U, Lenhard T, Olsen BR, Delaissé JM. (2000) Generation and degradation of human endostatin proteins by various proteinases. *FEBS Letters*, 486(3), 247–251.

126 Koolwijk P, Sidenius N, Peters E, Sier CFM, Hanemaaijer R, Blasi F, et al. (2001) Proteolysis of the urokinase-type plasminogen activator receptor by

metalloproteinase-12: Implication for angiogenesis in fibrin matrices. *Blood*, 97(10), 3123–3131.

127 McQuibban CA, Gong J-H, Tam EM, McCulloch CAG, Clark-Lewis I, Overall CM. (2000) Inflammation Dampened by Gelatinase A Cleavage of Monocyte Chemoattractant Protein-3. *Science*, 289(August), 1202–1206.

128 Houghton AM, Quintero PA, Perkins DL, Kobayashi DK, Kelley DG, Marconcini LA, et al. Elastin fragments drive disease progression in a murine model of emphysema. *The Journal of Clinical Investigation*, 1–7.

129 Koshikawa N, Giannelli G, Cirulli V, Miyazaki K, Quaranta V. (2000) Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *Journal of Cell Biology*, 148(3), 615–624.

130 Xu J, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon Yuen S, et al. (2001) Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *Journal of Cell Biology*, 154(5), 1069–1079.

131 Baciu PC, Suleiman EA, Deryugina EI, Strongin AY. (2003) Membrane type-1 matrix metalloproteinase (MT1-MMP) processing of pro-alphav integrin regulates cross-talk between alphavbeta3 and alpha2beta1 integrins in breast carcinoma cells. *Experimental Cell Research*, 291(1), 167–175.

132 Noë V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, et al. (2000) Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *Journal of Cell Science*, 114(114), 111–118.

133 Illman S a, Lehti K, Keski-Oja J, Lohi J. (2006) Epilysin (MMP-28) induces TGF-beta mediated epithelial to mesenchymal transition in lung carcinoma cells. *Journal of Cell Science*, 119(Pt 18), 3856–3865.

134 Kramer F, Dusek A. (2012) Biomarkers of lung tissue remodeling in pulmonary diseases: Implications for clinical practice and research. *Current Respiratory Medicine Reviews*, 8(2), 100–107.

135 Chelladurai P, Seeger W, Pullamsetti SS. (2012) Matrix metalloproteinases and their inhibitors in pulmonary hypertension. *European Respiratory Journal*, 40(3), 766–782.

136 John A, Tuszynski G. (2001) The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. *Pathology Oncology Research*, 7(1), 14–23.

137 Houghton AM, Grisolano JL, Baumann ML, Kobayashi DK, Hautamaki RD, Nehring

LC, et al. (2006) Macrophage elastase (matrix metalloproteinase-12) suppresses growth of lung metastases. *Cancer Research*, 66(12), 6149–6155.

138 Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, et al. (2001) Gelatinase B functions as regulator and effector in leukocyte biology. *Journal of Leukocyte Biology*, 69(June), 851–859.

139 Leinonen T, Pirinen R, Böhm J, Johansson R, Kosma VM. (2008) Increased expression of matrix metalloproteinase-2 (MMP-2) predicts tumour recurrence and unfavourable outcome in non-small cell lung cancer. *Histology and Histopathology*, 23(6), 693–700.

140 Li GH, Cui YS, Wu QY, Zhang XJ, Gao YF. (2013) Clinicopathologic significance of beta-catenin and matrix metalloproteinase-2 expression in non-small cell lung cancer. *Medical Oncology*, 30(1), 437.

141 Kanoh Y, Abe T, Masuda N, Akahoshi T. (2013) Progression of non-small cell lung cancer: Diagnostic and prognostic utility of matrix metalloproteinase-2, C-reactive protein and serum amyloid A. *Oncology Reports*, 29(2), 469–473.

142 Passlick B, Sienel W, Seen-Hibler R, Wockel W, Thetter O, Mutschler W, et al. (2000) Overexpression of matrix metalloproteinase 2 predicts unfavorable outcome in early-stage non-small cell lung cancer. *Clinical Cancer Research*, 6(10), 3944–3948.

143 Leinonen T, Pirinen R, Böhm J, Johansson R, Ropponen K, Kosma VM. (2006) Expression of matrix metalloproteinases 7 and 9 in non-small cell lung cancer: Relation to clinicopathological factors, β-catenin and prognosis. *Lung Cancer*, 51(3), 313–321.

144 Liu H, Zhang T, Li X, Huang J, Wu B, Huang X, et al. (2008) Predictive value of MMP-7 expression for response to chemotherapy and survival in patients with non-small cell lung cancer. *Cancer Science*, 99(11), 2185–2192.

145 Safranek J, Holubec L, Topolcan O, Pesta M, Klecka J, Vodicka J, et al. (2007) Expression of mRNA MMP-7 and mRNA TIMP-1 in non-small cell lung cancer. *Anticancer Research*, 27(4 C), 2953–2956.

146 Safranek J, Pesta M, Holubec L, Kulda V, Dreslerova J, Vrzalova J, et al. (2009) Expression of MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA in lung tissue of patients with non-small cell lung cancer (NSCLC) and benign pulmonary disease. *Anticancer Research*, 29(7), 2513–2517.

147 Wiggins-Dohlvik K, Oakley RP, Han MS, Stagg HW, Alluri H, Shaji C a., et al.

(2016) Tissue inhibitor of metalloproteinase-2 inhibits burn-induced derangements and hyperpermeability in microvascular endothelial cells. *The American Journal of Surgery*, 211(1), 197–205. Elsevier Inc.

148 Kai HST, Butler GS, Morrison CJ, King AE, Pelman GR, Overall CM. (2002) Utilization of a novel recombinant myoglobin fusion protein expression system to characterize the tissue inhibitor of metalloproteinase (TIMP)-4 and TIMP-2 C-terminal domain and tails by mutagenesis. The importance of acidic residues in binding the MMP-2 . *Journal of Biological Chemistry*, 277(50), 48696–48707.

149 Jackson P. (2007) New Developments in Metastasis Suppressor Research.

150 Cox G, Steward WP, O'Byrne KJ. (1999) The plasmin cascade and matrix metalloproteinases in non-small cell lung cancer. *Thorax*, 54, 169–179.

151 Zhu L, Yu H, Liu S, Xiao X, Dong W, Chen Y, et al. (2015) Prognostic Value of Tissue Inhibitor of Metalloproteinase-2 Expression in Patients with Non–Small Cell Lung Cancer: A Systematic Review and Meta-Analysis. *PLOS ONE*, 10(4), e0124230.

152 Suemitsu R, Yoshino I, Tomiyasu M, Fukuyama S, Okamoto T, Maehara Y. (2004) Serum tissue inhibitors of metalloproteinase-1 and -2 in patients with non-small cell lung cancer. *Surgery Today*, 34(11), 896–901.

153 Guedez L, Jensen-Taubman S, Bourboulia D, Kwityn CJ, Wei B, Caterina J, et al. (2012) TIMP-2 targets tumor-associated myeloid suppressor cells with effects in cancer immune dysfunction and angiogenesis. *Journal of Immunotherapy*, 35(6), 502–512.

154 Elias GJ, Ioannis M, Theodora P, Dimitrios PP, Despoina P, Kostantinos V, et al. (2008) Circulating tissue inhibitor of matrix metalloproteinase-4 (TIMP-4) in systemic sclerosis patients with elevated pulmonary arterial pressure. *Mediators of Inflammation*, 2008.

155 Selman M, Ruiz V, Cabrera S, Segura L, Barrios R, Pardo A. (2000) TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 279, 562–574.

156 Pullen NA, Anand M, Cooper PS, Fillmore HL. (2012) Matrix metalloproteinase-1 expression enhances tumorigenicity as well as tumor-related angiogenesis and is inversely associated with TIMP-4 expression in a model of glioblastoma. *Journal of Neuro-Oncology*, 106(3), 461–471.

157 Stratmann B, Farr M, Tschesche H. (2001) MMP-TIMP interaction depends on residue 2 in TIMP-4. *FEBS Letters*, 507(3), 285–287.

158 Wang M, Liu YE, Greene J, Sheng S, Fuchs A, Rosen EM, et al. (1997) Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. *Oncogene*, 14, 2767–2774.

159 Lizarraga F, Ceballos-Cancino G, Espinosa M, Vazquez-Santillan K, Maldonado V, Melendez-Zajgla J. (2015) Tissue Inhibitor of Metalloproteinase-4 Triggers Apoptosis in Cervical Cancer Cells. *PloS one*, 10(8), e0135929.

160 Livak KJ, Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^-ΔΔCT Method. *Methods*, 25, 402–408.

161 Vacca A, Ribatti D, Iurlaro M, Albini A, Minischetti M, Bussolino F, et al. (1998) Human lymphoblastoid cells produce extracellular matrix-degrading enzymes and induce endothelial cell proliferation, migration, morphogenesis, and angiogenesis. *International Journal of Clinical and Laboratory Research*, 28(1), 55–68.

162 Doescher J, Piontek G, Wirth M, Bettstetter M, Schlegel J, Haller B, et al. (2015) Epstein-Barr virus infection is strictly associated with the metastatic spread of sinonasal squamous-cell carcinomas. *Oral Oncology*, 51(10), 929–934. Elsevier Ltd.

163 Lee DCW, Chua DTT, Wei WI, Sham JST, Lau a SY. (2007) Induction of matrix metalloproteinases by Epstein-Barr virus latent membrane protein 1 isolated from nasopharyngeal carcinoma. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 61(9), 520–6.

164 Lan YY, Yeh TH, Lin WH, Wu SY, Lai HC, Chang FH, et al. (2013) Epstein-Barr Virus Zta Upregulates Matrix Metalloproteinases 3 and 9 That Synergistically Promote Cell Invasion In Vitro. *PLoS ONE*, 8(2), 1–10.

165 Hendrix MJ, Seftor EA, Grogan TM, Seftor RE, Hersh EM, Boyse EA, et al. (1992) Expression of type IV collagenase correlates with the invasion of human lymphoblastoid cell lines and pathogenesis in SCID mice. *Molecular and cellular probes*, 6(1), 59–65.