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The role of the Renin-Angiotensin-Aldosterone System and Hypoxia in Lung Cancer Progression

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THE ROLE OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM AND HYPOXIA IN LUNG CANCER PROGRESSION

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List of Abbreviations

4E-BP1 - Eukaryotic translation initiation factor 4E-binding protein 1

ACE - Angiotensin-converting enzyme

ACE2 - Angiotensin-converting enzyme type 2

ADAM - Disintegrin and metalloproteinase

AGT - Angiotensinogen

AGTR1 - Angiotensin II Receptor Type 1 gene

AGTR2 - Angiotensin II Receptor Type 2 gene

AGXT - Alanine--Glyoxylate and Serine--Pyruvate Aminotransferase

AJCC – American Joint Committee on Cancer

ALK - Anaplastic lymphoma kinase

AMNA - Aminopeptidase A

AMNN - Aminopeptidase N

Ang – Angiotensin

Ang 1-9 - Angiotensin-(1–9)

Ang 2–8 - Angiotensin III

Ang 3–8 - Angiotensin IV

Ang I - Decapeptide angiotensin I

Ang II - Angiotensin II

ANGPT2 - Angiopoietin 2

AT1 - Angiotensin II type 1

AT2 - Angiotensin II type 2

AT4 - Angiotensin II type 4

BAD - BCL2 associated agonist of cell death

BCL2 - B-cell leukaemia/lymphoma 2;

BIRC5 - Baculoviral IAP Repeat Containing 5

BRCA2 –Breast cancer 2

CAFs - Cancer-associated fibroblast

CAGE - Chymostatin-sensitive angiotensin II-generating enzyme

Cas – Caspase

CAV1 - Caveolin-1

CHECK2 - Checkpoint kinase 2

CHRNA3 - Cholinergic Receptor Nicotinic Alpha 1 Subunit

CHRNA5- Cholinergic Receptor Nicotinic Alpha 5 Subunit

COPD - Chronic obstructive pulmonary disease

COVID-19 - Coronavirus disease

COX-2 - Cyclooxygenase 2

CPNPC – Carcinoma do Pulmão de Não Pequenas Células

c-SRC - Cellular Proto-oncogene tyrosine-protein kinase

CXCR4 - C-X-C chemokine receptor type 4

DAG – Diacylglycerol

DNA – Deoxyribonucleic acid

EBUS - Endobronchial ultrasound

ECOG PS - Eastern Cooperative Oncology Group performance status

EGFR- Epidermal growth factor receptor

EMT - Epithelial to mesenchymal transition

ERK1/2 - Extracellular signal-regulated protein kinase

EUS - Endoscopic ultrasound

FAK - Focal adhesion kinase

FGF - Fibroblast growth factor

FGFR - Fibroblast growth factor receptor

FPKM – Kilobase of transcript per million

GADD45 - Growth Arrest and DNA Damage 45

GSTM1 - Glutathione S-transferase M1

GSTP1 - Glutathione S-transferases P1

GTP - Guanosine triphosphate

HIF1a - Hypoxia-inducible factor 1-alpha

ICAM1 - Intercellular Adhesion Molecule 1

IHC – Immunohistochemistry

IKKB - Inhibitor of nuclear factor kappa-B kinase subunit beta

IL-1 β - Interleukin 1 β

IL-6 - Interleukin 6

JAK - Janus kinase
KRAS - Kirsten rat sarcoma viral
LUAD – Lung Adenocarcinoma
M2 - Macrophage polarized towards M2
MAPK - Mitogen activated protein kinase
MAPK - Mitogen activated protein kinase
MASR - Mas receptor
MEK1/2 - Dual-specificity kinases that activate ERK1 and ERK2
MLC - Myosin light chain
MLC - Myosin light-chain kinase
MMP - Matrix metalloproteinases
mRNA - Messenger Ribonucleic Acid
mTOR - Mammalian target of rapamycin
NADPH - Hydro-nicotinamide adenine dinucleotide phosphate
NEP – Neprilysin
NFAT - Nuclear factor of activated T-cells
NF-kB - Nuclear Factor-kB
NGS - Next generation sequencing
NO – Nitric oxide
NSCLC - Non-small cell lung cancer
OS - Overall survival
PD-L1 – Programmed Death-Ligand 1
PFS – Progression Free Survival
PI3K/Akt - Phosphoinositide 3-kinase/protein kinase B
PKC - Protein kinase C
PLCb - Phospholipase C beta
PLCg/PKC - Phospholipase C gamma/protein kinase C
PlGF - Placental growth factor
PP2A - Protein phosphatase 2A
PTP - Phosphotyrosine phosphatase
Rab - Ras analog in brain

Rac1 - Rac Family Small GTPase 1gene

RAS – Renin-Angiotensin System

RECIST - Response Evaluation Criteria in Solid Tumours

RhoA - Ras homolog family member A

ROCK1/2 - Rho associated kinase ½

RTK - Receptor tyrosine kinase

S6K -Ribosomal S6 kinase

SARS-CoV - Severe acute respiratory syndrome coronavirus 1

SARS-CoV 2 - Severe acute respiratory syndrome coronavirus 2

SDF1 - Stromal cell-derived factor 1

SDF-1 α - Stromal cell derived factor-1 α

SHP2 - Src homology region 2 domain-containing phosphatase-2

Smad - Mothers against decapentaplegic homolog

Snail1 - Snail Family Transcriptional Repressor 1

SNP – Single Nucleotide Polymorphism

SRA – Sistema Renina Angiotensina

SREBP - Sterol regulatory element binding proteins

STAT - Signal transduction and activator of transcription

TAMs - Tumour-associated macrophages

TCGA – The Cancer Genome Atlas Program

TERT - Telomerase Reverse Transcriptase

TGF- β 1 - Transforming growth factor beta 1

TNFA - Tumour necrosis factor

t-PA - tissue-plasminogen activator

VCAM1 - Vascular cell adhesion molecule 1

VEGF - Vascular endothelial growth factor.

Abstract

Introduction: The RAS is involved in cell proliferation, immunoinflammatory response, hypoxia and angiogenesis, which are critical biological processes in lung cancer. The variants of genes involved in RAS and its impact in NSCLC prognosis, remains to be established. *AGXT* gene codes for the enzyme alanine glyoxylate aminotransferase, which is involved in hepatic peroxisomal metabolism of platinum-based chemotherapeutic agents. The association of genetic variant *AGXT* rs34116584 on the clinical outcome and response to chemotherapy of patients with NSCLC is unknown. Our aim was to study the association of putatively functional genetic polymorphisms in genes coding for proteins involved in RAS, hypoxia, angiogenesis and *AGXT* with NSCLC prognosis.

Methods: Genotyping of 52 germline variants from genes of the RAS and hypoxic/angiogenic factors/receptors was performed using MassARRAY iPLEX Gold in a retrospective cohort ($n = 167$) of advanced NSCLC patients. Validation of the resulting genetic markers was conducted in an independent group ($n = 190$), matched by clinicopathological characteristics. TCGA analysis was performed for the genes coding for RAS peptides.

Results: Multivariate analysis on the discovery set revealed that *MME* rs701109 C carriers were protected from disease progression in comparison with homozygous T (hazard ratio (HR) = 0.5, 95% confidence interval (CI) = 0.2-0.8, $p = 0.010$). Homozygous A and T genotypes for *KDR* rs1870377 were at increased risk for disease progression and death compared to heterozygous (HR = 1.7, 95% CI = 1.2-2.5, $p = 0.005$ and HR = 2.1, 95% CI = 1.2-3.4, $p = 0.006$, respectively). Carriers of homozygous genotypes for *ACE2* rs908004 presented increased risk for disease progression, only in the subgroup of patients without tumour actionable driver mutations (HR = 2.9, 95% CI = 1.3-6.3, $p = 0.010$). Importantly, the association of homozygous genotypes in *MME* rs701109 with risk for disease progression was confirmed after multivariate analysis in the validation set. Furthermore, multivariate analysis showed shorter PFS for *AGXT* rs34116584 T carriers [HR=2.0, 95% CI, 1.4-3.0, $p<0.0001$] and shorter OS [HR=1.8, 95% CI, 1.1-3.0, $p=0.017$] globally, as well as in a subgroup of patients ($n=144$) treated with first line platinum-based chemotherapy [HR=2.0, 95% CI, 1.3-3.1, $p=0.001$] and [HR=1.8, 95% CI,

1.1-3.1, $p=0.026$], respectively. TCGA analysis showed that there was significantly less quantity mRNA for *REN*, *ACE*, *AGTR1*, *AGTR2*, *MME*, *MAS1*, *CMA1* in lung adenocarcinoma tissue when compared with normal adjacent tissue from 500 patients. The opposite was found for *AGT* expression. Higher expression of *REN*, *ACE*, *AGTR1*, *CMA1* was associated with better OS.

Conclusion: This study provides evidence that *MME* rs701109, *KDR* rs1870377, *ACE2* rs908004 and *AGXT* rs34116584 polymorphisms may modulate PFS in advanced NSCLC. Present genetic variations findings will foster basic, translational, and clinical research on their role in NSCLC.

Resumo

Introdução: O SRA está envolvido na proliferação celular, resposta imuno-inflamatória, hipóxia e angiogénese, que são processos biológicos envolvidos no cancro do pulmão. As variantes de genes que codificam proteínas do SRA e o seu impacto no prognóstico do CPNPC ainda precisam ser estabelecidas. O gene *AGXT* codifica a enzima alanina glioxilato aminotransferase, que está envolvida no metabolismo peroxissómico hepático de agentes quimioterápicos à base de platina. A associação da variante genética *AGXT* rs34116584 no prognóstico e resposta à quimioterapia em doentes com CPNPC é desconhecida. O nosso objetivo foi estudar a associação de polimorfismos genéticos potencialmente funcionais em genes que codificam proteínas envolvidas no SRA, hipóxia, angiogénese e *AGXT* com o prognóstico do CPNPC.

Métodos: A genotipagem de 52 variantes da linha germinativas do SRA e fatores/recetores hipóxicos/angiogénicos foi realizada através da técnica MassARRAY iPLEX Gold numa coorte retrospectiva (n = 167) de doentes com CPNPC avançado. A validação dos marcadores genéticos resultantes foi realizada num grupo independente (n = 190) com as mesmas características clínico-patológicas. A análise de TCGA (*The Cancer Genome Atlas*) foi realizada para os genes que codificam os peptídeos do SRA.

Resultados: A análise multivariada revelou que portadores do *MME* rs701109 C tiveram maior tempo até progressão da doença em comparação com homozigotos T (*Hazard Ratio* (HR) = 0,5, intervalo de confiança de 95% (IC) = 0,2-0,8, p = 0,010). Genótipos homozigotos A e T para o *KDR* rs1870377 apresentaram risco aumentado para progressão da doença e morte em comparação aos heterozigotos (HR = 1,7, 95% CI = 1,2-2,5, p = 0,005 e HR = 2,1, 95% CI = 1,2-3,4, p = 0,006, respetivamente). Portadores de genótipos homozigotos para *ACE2* rs908004 apresentaram risco aumentado para progressão da doença, apenas no subgrupo de doentes sem mutações clinicamente acionáveis (HR = 2,9, IC 95% = 1,3-6,3, p = 0,010). É importante ressaltar que a associação de genótipos homozigotos *MME* rs701109 com risco de progressão da doença foi confirmada após análise multivariada na coorte de validação. Além disso, a análise multivariada mostrou sobrevivência livre de progressão inferior para portadores *AGXT* rs34116584 T [HR=2,0, 95% CI, 1,4-3,0, p<0,0001] e sobrevivência global inferior [HR=1,8,

95% CI, 1,1-3,0, $p=0,017$], bem como no subgrupo de doentes ($n=144$) tratados com quimioterapia em primeira linha à base de platina [HR=2,0, 95% CI, 1,3-3,1, $p=0,001$] e [HR=1,8, 95% CI, 1,1 -3,1, $p=0,026$], respetivamente. A análise de TCGA (*The Cancer Genome Atlas*) mostrou uma quantidade significativamente menor de mRNA para *REN*, *ACE*, *AGTR1*, *AGTR2*, *MME*, *MAS1*, *CMA1* no tecido de adenocarcinoma do pulmão quando comparado com o tecido adjacente normal de 500 doentes. O oposto foi observado para a expressão de *AGT*. A maior expressão de *REN*, *ACE*, *AGTR1*, *CMA1* foi associada a uma melhor sobrevivência global.

Conclusão: Este estudo fornece evidências de que os polimorfismos *MME* rs701109, *KDR* rs1870377, *ACE2* rs908004 e *AGXT* rs34116584 podem ter impacto na sobrevivência livre de progressão no CPNPC avançado. As descobertas atuais de mutações germinativas promoverão mais estudos translacionais e clínicos sobre o seu papel no CPNPC.

I. Introduction

Lung cancer is one of the most common malignancies and the most frequent cause of cancer deaths in the past few decades, with over one million people diagnosed worldwide each year (1, 2). Notably, the five-year survival rate is the lowest compared with other frequent malignancies (3). Its poor prognosis fosters the need for research, namely understanding its aggressiveness mechanisms, in order to improve prevention and treatment.

The renin-angiotensin-aldosterone system is an established primary regulator of blood pressure, homeostasis, and natriuresis (4). Nevertheless, recent evidence indicates that angiotensin peptides also might have a role in tumour cell proliferation, inflammation and hypoxia, which have been recognized as hallmarks of cancer, actively participating in lung tumour microenvironment regulation (5). Here, we review current knowledge on the renin-angiotensin system (RAS), considering its potential modulatory role on cancer cell phenotype and on immune surveillance, while exploring the mechanistic association with lung cancer. Here, we also set the state-of-the-art that grounds experimental design and scientific biological rationale for developing subsequent translational studies that embody this thesis.

1. The Renin-Angiotensin pathway: an intricate regulatory mechanism in lung disease physiopathology

The RAS was first discovered and studied in the physiological regulation of blood pressure, fluid homeostasis and pathogenesis of hypertension (6). Angiotensin II (Ang II), the final effector of the system, causes vasoconstriction, both directly and indirectly, by stimulating Ang II type 1 receptor (AT1 receptor) present on the vasculature, and by increasing sympathetic tone and arginine vasopressin release. Chronically, Ang II regulates blood pressure by modulating renal sodium and water reabsorption directly, by stimulating AT1 receptors in the kidney, or indirectly, by stimulating the production and release of aldosterone from the adrenal glands, or stimulating the sensation of thirst in the central nervous system (7). The enzymatic cascade by which Ang II is produced consists of renin, which cleaves angiotensinogen (AGT) to form the decapeptide angiotensin I (Ang I). Ang I is then further cleaved by angiotensin-converting enzyme

(ACE) to produce the octapeptide Ang II, the physiologically active component of the system. Further degradation by aminopeptidase A and N produces angiotensin III (Ang 2–8), and angiotensin IV (Ang 3–8), respectively (7) (Figure 1). The actions of Ang II result from its binding to specific receptors (AT1 and AT2). Ang II, via its AT1 receptor, is also involved in cell proliferation, left ventricular hypertrophy, nephrosclerosis, vascular media hypertrophy, endothelial dysfunction, neointima formation, and processes leading to atherothrombosis (8). Ang II, via its AT2 receptor, is involved in cell differentiation, tissue repair, apoptosis and vasodilation (8). Other receptors have been described in relation to the RAS. For instance, an AT4 receptor binding site has been identified, and in contrast to the other AT receptors, it does not seem to be a G protein-coupled receptor (9). This receptor binds Ang IV (3–8) preferentially, has been localized in various mammalian tissues, and suggested to cause vasodilatation (9).

Recently, a role for local tissue-based RAS has been described, both as modulator of inflammation and of the injury/repair response (10). In addition, supportive evidence claims that tissue RAS is capable of working synergistically or independently of the systemic RAS, and locally generates angiotensin peptides with regulatory homeostatic functions, thus contributing to dysfunction and/or disease (11) (Figure 1). Within the RAS, the generation of angiotensin peptides is controlled by complex interactions: renin, which is released from the juxtaglomerular apparatus of the kidney, converts the angiotensinogen released by the liver to form angiotensin I. In fact, the liver is the primary source of circulating AGT, a minor but significant fraction of which is filtered through the glomerulus and reabsorbed by proximal tubule cells. It was shown that liver-specific angiotensinogen knockout mice had nearly abolished plasma and renal angiotensinogen protein and renal tissue Ang II (12). On its turn, the ACE plays a central role in generating Ang II from Ang I (13). The capillary blood vessels in the lung are one of the major sites of ACE expression and Ang II production in the human body (14). Alterations in RAS expression were shown to be involved in multiple lung diseases: idiopathic pulmonary fibrosis (15), sarcoidosis (16), pulmonary hypertension (17), acute respiratory distress syndrome (17), and lung cancer (18). Understanding the involvement of intra-pulmonary RAS on inflammation or fibrosis may open new therapeutic possibilities for the treatment of respiratory diseases. In fact, the increased

ACE expression observed in several interstitial lung diseases supports the existence of a pulmonary RAS and establishes a putative role for Ang II in the response to lung injury and fibrosis (10). Concurringly, findings from a substantial number of *in vivo* studies demonstrated that ACE inhibitors were capable of attenuating experimental pulmonary fibrosis (19). Captopril, enalapril, lisinopril and perindopril (20-22), could block the effects of bleomycin-, γ irradiation-, amiodarone- and paraquat- induced pulmonary fibrosis in rats. A recent post hoc analysis of data from the placebo arms of phase 3 trials including patients with idiopathic pulmonary fibrosis, showed a slower disease progression in patients under therapy with ACE inhibitors (23). Therefore, local upregulation of the expression of either Ang II or TGF- β 1, which might influence each other's activity or act in synergy, should be blocked to delay the progression of lung fibrotic disease.

Ang I can also be converted to Ang II through the action of chymase, a chymotrypsin-like enzyme that is expressed in the secretory granules of mast cells (24). In fact, chymase is the most efficient Ang II-forming enzyme in the human body and has been implicated in a wide variety of human diseases that also implicate its many other protease actions: 1. cleavage of matricellular proteins and peptides, including laminin and fibronectin important for cell survival; 2. activation of peptide and enzyme precursors, including matrix metalloproteinases (MMPs), such as MMP-9, TGF- β , stem cell factor, kallikrein, IL-6 and IL-1 β , preproendothelin I and IL-18 (25). Chymase activity was noticeably increased in the lungs of animals following bleomycin instillation (26). A recent report showed the prominent accumulation of chymase-expressing mast cells in idiopathic pulmonary arterial hypertension and idiopathic pulmonary fibrosis lungs, and their presence was near the regions with marked TGF- β 1 expression (26). Furthermore, tumour sections from 32 cases of adenocarcinoma and 13 cases of squamous cell carcinoma revealed significantly higher counts of chymase-positive mast cells in the central region than those in the normal regions (27). Chymase has been associated with TGF- β activation and endothelin-1 formation from preproendothelin-1 in pulmonary fibrosis and chronic obstructive pulmonary disease (25), and in proliferation, adhesion, regulation of E-cadherin expression, and modulation of immunosuppressive microenvironment in lung carcinoma (28, 29). This effect is likely

due to TGF- β inhibitory effect on T-cell proliferation and effector activity. Moreover, TGF- β subverts T cell immunity by favouring regulatory T-cell differentiation, further reinforcing immunosuppression at the tumour microenvironment (30).

Ang II is a ligand for two classes of seven trans-membrane G protein-coupled receptors, angiotensin II type 1 (AT1) and angiotensin II type 2 (AT2) receptors (24). The cellular distribution of AT1 and AT2 receptors in the normal and pathological human lung has been previously described (31). Whereas *AGTR1* transcript and encoded protein, the AT1 receptor, localized at vascular smooth muscle cells, alveolar macrophages and in the stroma underneath the airway epithelium, *AGTR2* expression, at both mRNA and protein levels, of AT2 receptors were found in bronchial epithelia and endothelial cells (32). The components of RAS are frequently differentially expressed in various cancers including brain, lung, pancreatic, breast, prostate, colon, skin and cervical carcinomas in comparison with their corresponding non-malignant tissue (33). A large-scale study of estrogen receptor-positive breast cancer tumours revealed an increase on *AGTR1* mRNA expression. Contradictory results were observed in lung adenocarcinoma tissues since *AGTR1*, *AGTR2* as well as ACE mRNA expression, were under-expressed. Notably, AGT was overexpressed in lung adenocarcinoma tissue (34). Notably, several physiological and pathophysiological effects of Ang II are mediated mainly through the AT1 receptor (35). Upon its activation, a pleiotropic regulatory role is induced at the lung, resulting in vasoconstriction, cell proliferation, angiogenesis, and augmentation of pro-inflammatory cytokine production, inflammatory cell chemotaxis, epithelial cell apoptosis, increased oxidative stress and fibrosis (36). Several in vitro studies demonstrated that the epithelial to mesenchymal transition (EMT) induced by TGF- β 1 resulted in an increase in angiotensinogen and AT1 receptor expression, in human lung fibroblasts (37-39). Moreover, active TGF- β 1 expression by human lung myofibroblasts was downregulated by AT1 receptor blockade and accompanied the inhibition of collagen synthesis (19). In contrast, the activation of the AT2 receptor induces opposite effects, although some proinflammatory responses are mediated through the activation of the Nuclear Factor-kB (NF-kB) pathway (36). Ang II can also be converted into Angiotensin-(1-7) (Ang (1-7)) by the enzymatic activity of angiotensin converting enzyme type 2 (ACE2) or from Ang I, via

Angiotensin (1–9) (Ang-(1–9)), a pathway that involves both ACE2 and ACE (33, 40) (Figure 1). In the human lung, ACE2 is expressed in endothelial and smooth muscle cells of large and small blood vessels, as well as in alveolar epithelial cells type I and II, and bronchial epithelial cells (41). ACE2 has a multiplicity of physiological roles that revolve around its trivalent function: a negative regulator of the RAS, facilitator of amino acid transport, and is the entry receptor for SARS-CoV and SARS-CoV-2 (42).

Alternatively, Ang-(1–7) might be produced through hydroxylation of Ang I by the enzyme neprilysin (43). Neprilysin is a cell membrane-bound zinc metalloprotease that catalyses the degradation of a number of endogenous vasodilator peptides, such as atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide (44). Physiological actions of neprilysin depend on the balance of its action on the breakdown of vasodilators and vasoconstrictors (45). Neprilysin has been involved in malignancies, such as prostate, renal and lung cancer (46). Ang-(1–7) binds to the G protein-coupled receptor Mas, counteracting cardiovascular and baroreflex effects of Ang II through AT1 receptor, and has been suggested to have vasodilator properties and to act as an inhibitor of cell proliferation (47). It was reported that ACE2 upregulation can reduce lung injury, whereas ACE2 or Ang-(1–7) have a role in the prevention of acute respiratory distress (48-50). ACE2 has recently been identified as the SARS-CoV-2 receptor, the infective agent responsible for COVID-19, providing a critical link between immunity and inflammation (42). Furthermore, some studies reported that the ACE2/Ang-(1–7)/Mas axis counteracted the ACE/Ang II/AT1 axis in different models of cancer, including lung cancer (51-53).

regulates Ang II, ACE, and AT1 receptor, while downregulating ACE2 and AT2 receptor. Local angiotensin II predominantly exists in the hypoxic regions of tumours, while these tumour cells produce Ang II by a chymase-dependent mechanism. Subsequently, the hypoxic tumour microenvironment induces the upregulation of the ACE/Ang II/AT1R axis, which is associated with the hallmarks of cancer, while it downregulates the ACE2/Ang-(1–7)/Mas receptor axis. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AMNA, aminopeptidase A; AMNN, aminopeptidase N; Ang, angiotensin; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; AT4R, angiotensin receptor 4; CAGE, chymostatin-sensitive angiotensin II-generating enzyme; COX-2, cyclooxygenase 2; EMT, epithelial-to-mesenchymal transition; HIF1 α , hypoxia-inducible factor 1-alpha; MASR, Mas receptor; NADPH, hydro-nicotinamide adenine dinucleotide phosphate; TGF β 1, transforming growth factor beta 1; t-PA, tissue-plasminogen activator; VEGF, vascular endothelial growth factor.

2. Renin-Angiotensin pathway and the hallmarks of cancer: application to Lung Tumours

The RAS is an important signalling system within the tumour microenvironment, promoting both malignant and stromal cell proliferation (5). Notably, it is also involved in the regulation of metabolic homeostasis and of tumour-associated phenotypes, such as angiogenesis, migration and invasion (5, 11, 54).

2.1 Cell Proliferation, Invasion and Migration

2.1.1. ACE/Ang II/AT1 Receptor Axis

Ang II is an important growth promoter in a variety of cell types, where it activates the phosphatidylinositol signalling via AT1 receptor and increases cytosolic Ca²⁺, a process linked to mitogenesis (55). Another intracellular mechanism by which Ang II controls cell growth is through the modulation of protein tyrosine kinases (PTK) (56). These proteins are coupled to several growth factor receptors, particularly epidermal growth factor receptor (EGFR), and transduce growth factor-induced signals through protein kinase C (PKC), or through MMPs-mediated mechanisms, which include a disintegrin and metalloproteinase (ADAM) family members. This crosstalk was shown to contribute towards cancer (5) (Figure 2). Ang II was shown to stimulate the migration of fibroblasts through activation of AT1 receptor in an EGFR activation-dependent process and be mediated by shedding of heparin-binding-EGF (57). Noteworthy, previous experiments have shown that EGFR inhibition partially blocked Ang II-

induced proliferation of breast intraductal carcinoma cells (58). Although the exact molecular mechanism underlying the transactivation of EGFR through Ang II-AT1 receptor binding in lung tumour cells is not completely understood, previous clinical studies evidenced an effect of ACE inhibitors or angiotensin type 1 receptor (ATR1) blockers on lung cancer patients, in combination with chemotherapy or EGFR inhibitors (59, 60).

c-Fos and c-jun genes are members of the activator protein-1 (AP-1) transcription factor family and are well known targets of the ERK1/2 mitogen activated protein kinase (MAPK) pathway (61). Ang II increased the phosphorylation of the extracellular signal-regulated protein kinase 1/2, c-jun-N-terminal kinase 1/2, or p38 mitogen-activated protein kinases, signalling pathways involved on cell proliferation and particularly relevant for cancer development (62, 63) (Figure 2). Notably, in fibroblasts, Ang II binding to AT1R results in Smad2 and Smad3 phosphorylation via the ERK/p38/MAPK pathway, increasing the activation of the TGF β 1/Smad2/Smad3/Smad4 signalling (64). Telmisartan, an AT1 receptor antagonist, significantly inhibited the growth of the non-small cell lung cancer (NSCLC) A549 cell line, in a time- and dose-dependent manner and led to an increase of the pro-apoptotic proteins caspase-3 and Bcl-xL (65). Attoub S and colleagues demonstrated that captopril significantly inhibited tumour growth using a non-small cell lung carcinoma LNM35 pre-clinical model (66).

The establishment of distant metastatic disease is associated with poor prognosis in patients with lung cancer. Tumour metastasis involves several biological steps, such as cell-to-cell and cell-to-extracellular matrix (ECM) interactions, degradation of ECM components, and new vessels formation (67), and some studies suggest that cancer stem cells (CSC) contribute, directly and indirectly, to the generation of metastases (68-70). Ang II/ATR1 pathway activation has been shown to promote EMT in cancer cells, which is a hallmark of cancer aggressiveness (71), while chymase, a protease that converts Ang I to Ang II, increased the expression of MMP-9 in a dose dependent manner in two lung cancer cell lines (A549 and H520) (29). Moreover, it was shown that Ang II induced CSC marker expression and facilitated tumour cell survival in NSCLC cells line (72). Recent data from a breast cancer model revealed an additional mechanism whereby ATR1 promotes tumour cell contraction, migration and invasion by upregulating the C-X-C

chemokine receptor type 4 (CXCR4)/Stromal cell derived factor-1 α (SDF-1 α) signalling through the Focal adhesion kinase (FAK)/Ras homolog family member A (RhoA)/Rho associated kinase 1/2 (ROCK1/2)/Myosin light-chain kinase (MLC) pathway, ultimately inducing metastatic disease (73). Taken together, these findings support a role for mediators of the Ang II/ATR1 axis as lung cancer modulators, particularly influencing malignant cell growth, dedifferentiation, and migration (Figure 2).

2.1.2. AT2 Receptor

In contrast to the pro-tumour effects of AT1 receptor signalling, activation of the AT2 receptor has been shown to be protective, often acting as opposite to AT1 receptor. The AT2 receptor inhibited cell proliferation while stimulating apoptosis in a variety of cell types, including vascular smooth muscle cells, cardiomyocytes, endothelial cells, and malignant prostate and lung cells (74-77). Furthermore, AT2 receptor overexpression induced apoptosis independent of Ang II, through p38 MAPK, caspase 8 and caspase 3 activation, and was mediated through an extrinsic cell death-signalling pathway partially dependent on p53 (78, 79). Notably, nanoparticle-based AT2 receptor gene transfection, which markedly increased AT2 receptor expression, resulted in increased cell death of the human lung cancer cell line A549 (76). The activation of AT2 receptor signalling has been described to produce antiproliferative, survival-promoting, as well as migratory, pro-invasive effects due to phosphatase-mediated (phosphotyrosine phosphatase PTP, protein tyrosine phosphatase 1B, PTP1B, and protein phosphatase 2A, PP2A) dephosphorylating events, through an inhibitory effect on Ras and MEK1/2 proteins of the Smad and MAPK pathways (PTP and PP2A) and by PTP1B-mediated novel Caveolin-1 (CAV1) dephosphorylation and inhibition of the CAV1/Rab5/Rac-1 pathway (64, 80) (Figure 2).

2.1.3. ACE2/Ang (1-7)/Mas Receptor Axis

The branch of the ACE2/Ang-(1-7)/MasR axis connected to the RAS has been associated with anti-proliferative and anti-metastatic properties. It was shown that overexpression of ACE2 inhibited the proliferation of lung cancer cells in vitro and reduced tumour growth in vivo, in a mouse lung xenograft model (81). Furthermore,

with human lung tumour xenografts evidenced that treatment with Ang-(1-7) reduces tumour volume in mice and inhibits cell proliferation through reduction on Cyclooxygenase-2 (COX-2) activity (82). Moreover, it was demonstrated that Ang-(1-7) reduced lung cancer cell migratory and invasive abilities through the decrease of expression and activity of matrix metalloproteinases MMP-2 and MMP-9, and inactivation of the PI3K/Akt, P38 and JNK signalling pathways (83). Noteworthy, it was recently reported that Ang-(1-7) inhibited human lung cancer cell EMT through the reduction of Cdc6 expression, dependent on the agonism of Mas receptor. An anti-metastatic mechanism, induced upon activation of ACE2/Ang-(1-7)/Mas axis has been recently described in a breast cancer model that includes repression of store-operated calcium entry (SOCE)-induced PAK1/NF- κ B/Snail1 signal pathway, ultimately resulting in decreased expression of Snail1 and increased production of E-cadherin (54). Further data from cardiomyocytes supports a nitric oxide/guanosine 3',5'-cyclic monophosphate-dependent pathway, which modulated the activity of the transcription factor NFAT (nuclear factor of activated T-cells), preventing its translocation to the nucleus (84), and impairing the transcriptional regulation of genes involved in malignant cell proliferation, migration and metastasis (85). However, the crosstalk between ACE2/Ang-(1-7)/Mas axis and NFAT upregulation in lung cancer has never been explored and the relationship to tumours is speculative based on studies in other tissues.

Taken together, this data provides a link to support the ACE2/Ang-(1-7)/Mas as a beneficial and targetable system for the treatment of lung cancer, reducing cell proliferation and preventing metastasis (Figure 2).

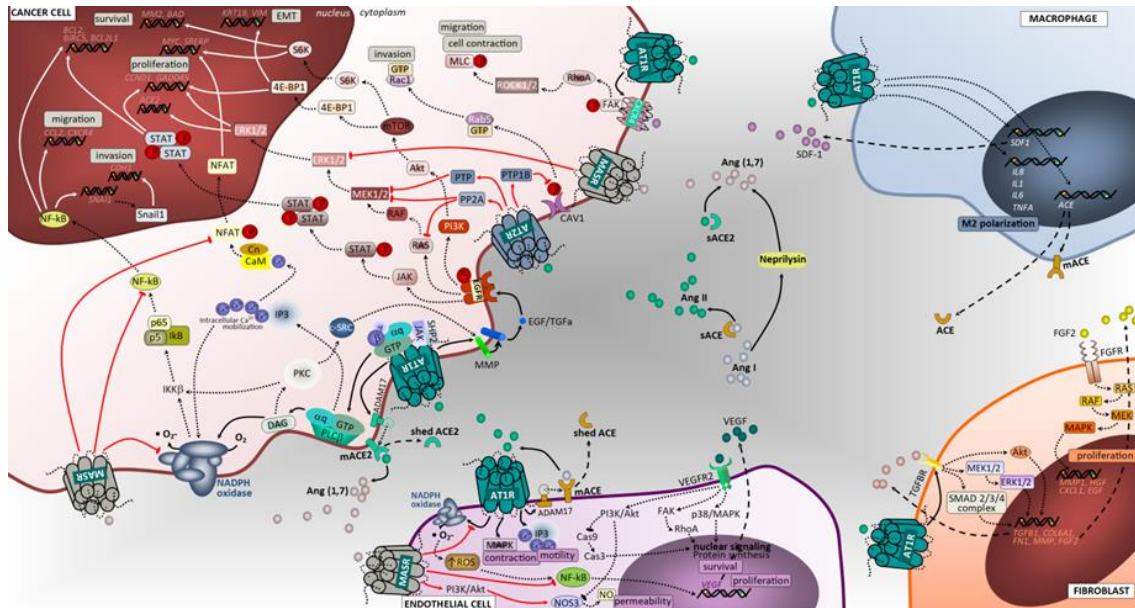


Figure 2. Angiotensin-associated pathways associated with cell proliferation, invasion, and migration in lung tumour microenvironment. Membrane-bound and soluble ACE and ACE2 catalyse the production of angiotensin II or angiotensin (1,7), ligands that exert regulatory functions in tumour microenvironment cells. The effects of activating Ang II/AT1R, Ang II/AT2R and Ang (1,7)/MasR axes' signalling has been mostly studied in tumour cells, including lung cancer cells. Overall, in contrast to the Ang II/AT1R that mediates several pathological events associated with activated RAS, the Ang (1,7)/MasR and Ang II/AT2R pathways are thought to antagonize many of the cellular actions of the Ang II-AT1R axis. In cancer cells, upon binding of Ang II to AT1R, a pleiotropic downstream signalling cascade is triggered, ultimately causing, either directly or indirectly, upregulation of cell proliferation, survival, motility, migration, invasion and EMT. Activated AT1R subunits stimulate PLC β , that hydrolyses membrane lipids, activates PKC and mobilization of intracellular Ca²⁺, while free G β and G γ subunits bind and gate ion channels. Activated G α q/11 units also activate the JAK-SHP2/STAT pathway and receptor tyrosine kinase (RTK) transactivation. Activation of RTK, depicted in the figure as EGFR, occurs through second messenger's stimulation of ADAM family and MMPs to cleave its ligands (in the figure EGF and TGF α) that bind and activate RTK. Alternatively, the AT1R-mediated activation of MMPs can follow a PLC β /DAG/PKC/c-SRC signalling mechanism to elicit increased ligands for RTK. Subsequent signalling upon activation of EGFR is represented in the figure using dashed arrows. Other intracellular cascades mediated by Ang II/AT1R include the activation of CXCR4/SDF-1 signalling through FAK/RhoA/ROCK1-2/MLC increasing cell contraction, migratory potential and tumour invasion. The Ang (1,7)/MasR activation inhibits NFAT transcriptional regulation that reduces proliferation. Notably, this pathway blocks the NF- κ B molecule formation thereby impacting EMT (including Snail1-mediated), invasion and survival. In addition, the inhibitory effect over ERK1/2 and NAPH oxidase signalling pathways significantly impact cell proliferation. The Ang II/AT2R pathway signals are mediated through protein phosphatases PTP1B, PTP and PP2A. The inhibition of CAV-1 phosphorylation stops the

Rab5/Rac1/GTP migratory potential of malignant cells, thus suppressing invasion. Furthermore, AT2R-associated increase in PTP and PP2A exerts blocking effects in RTK-mediated signals of the RAS/RAF/MEK1-2/ERK1-2 pathway at the level of MERK1/2 and RAS molecules, reducing cell proliferation. Macrophages, endothelial cells and fibroblasts are important components of the tumour microenvironment and capable of generating and expressing RAS components. These cells, beyond their functional ligands and receptors that are altered in tumour microenvironment, and reflect the crosstalk between all cell constituents, also use the RAS signalling pathway (mostly AT1R, but also MasR in endothelial cells) to yield functional characteristics that ultimately may favour the cell itself and enhance tumour growth. BIRC5, surviving gene; BCL2, B-cell leukemia/lymphoma 2; BAD, BCL2 associated agonist of cell death; GADD45, Growth Arrest and DNA Damage 45; SREBP, Sterol regulatory element binding proteins; ROCK1/2, Rho-associated protein kinase 1/2; Rab5, Ras-related protein Rab-5; S6K, ribosomal S6 kinase; PTP, protein tyrosine phosphatase; PP2A, Protein phosphatase 2; 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; IKKB, Inhibitor of nuclear factor kappa-B kinase subunit beta; c-SRC, cellular Proto-oncogene tyrosine-protein kinase; SHP2, Src homology region 2 domain-containing phosphatase-2; PTP1B, Protein tyrosine phosphatase 1B; MLC, myosin light chain; CAV1, caveolin 1; IL1, interleukin 1; TNFA, tumour necrosis factor; Snail1, Zinc finger protein SNAI1; *SNAI1*, Snail Family Transcriptional Repressor 1; JAK, janus kinase; STAT, signal transduction and activator of transcription; GTP, guanosine triphosphate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; CXCL1, The chemokine (C-X-C motif) ligand 1; IL8, interleukin 8; NF-kB, Nuclear Factor-kappa B; SDF1, stromal cell-derived factor 1; IL6, interleukin 6; HGF, hepatocyte growth factor; RAS, oncogene protein p21; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; Cas9, caspase 9; Cas3, caspase 3; ERK1/2, extracellular signal-regulated kinase ; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; MEK1/2, mitogen activated protein kinase kinase; HIF1 α , hypoxia inducible factor 1 alpha; NO, nitric oxide; RhoA, Ras homolog family member A; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; IP3, inositol trisphosphate; DAG, diacylglycerol; M2, macrophage polarized towards M2; ADAM17, Desintegrin and metalloproteinase domain-containing protein 17; p38/MAPK, protein 38/mitogen activated protein kinase; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; MasR, G-protein coupled *Mas receptor*; VEGF, vascular endothelial growth factor; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; mACE, membrane angiotensin converting enzyme; sACE, soluble angiotensin converting enzyme; mACE2, membrane angiotensin converting enzyme 2; sACE2, soluble angiotensin converting enzyme 2; TGFBR, transforming growth factor beta receptor; VEGFR2, vascular endothelial growth factor receptor 2; TGF α , transforming growth factor alpha; Ang (1,7), angiotensin 1,7; PLC β , phospholipase C beta; FAK, Focal adhesion kinase; NFAT, nuclear factor of activated T cells.

2.2. Hypoxia and Promotion of Tumour Angiogenesis

Hypoxia is typically present on solid tumours, such as lung cancer, and is known to enhance tumour aggressiveness, disease progression and therapy resistance. The effects of hypoxia are largely mediated by hypoxia-inducible factors (HIFs), particularly HIF-1 α and HIF-2 α (86). Importantly, RAS activation at the tumour microenvironment through Ang II has been described to induce hypoxia through production of reactive oxygen species (ROS), subsequently leading to pro-inflammatory and pro-angiogenic signals (Figure 3) (11, 87). Conversely, hypoxia regulates RAS-related proteins expression in somatic tissues and cells, up-regulating Ang II, ACE, and AT1 receptor, while down-regulating ACE2 and AT2 receptor in lung carcinoma cells (88-90).

2.2.1. ACE/Ang II/AT1 Receptor Axis and AT2 Receptor

Several studies indicated that HIF-1 α may be activated by Ang II upon binding to AT1 receptor (87, 91, 92). Consequently, HIF-1 α not only activates vascular endothelial growth factor (VEGF) expression, but also upregulates ACE expression, which then increases endogenous Ang II formation and enhances AT1 receptor activation (93). A significant reduction on VEGF-A mRNA and protein, in association with attenuated tumour growth, was also observed in Lewis lung tumours following candesartan administration, an ACE inhibitor. Furthermore, it was shown that treatment of Lewis lung carcinoma cells with Ang II caused a significant increase in VEGF-A mRNA and protein, which was prevented with administration of an AT1 receptor antagonist (94). Further evidence from molecular in vitro studies demonstrated that Ang II elicited the expression of placental growth factor (PlGF) at both mRNA and protein expression in human vascular endothelial and smooth muscle cells, mediated through the activation of protein kinase C, extracellular signal-regulated kinase 1/2 (ERK1/2) and PI3-kinase (95) intracellular signalling pathways. PlGF has an important role in tumour angiogenesis (96, 97), although a stimulatory effect was also reported for MMP-9-mediated NSCLC cancer cell invasion (98). In contrast to AT1 receptor, the role of AT2 receptor in cancer angiogenesis is less clear. Ex-vivo experiments in Lewis lung tumour xenografts revealed a significant decrease in angiogenesis after inhibition of AT2

receptor or in AT2 receptor-knockout mice with impaired production of proangiogenic factors included VEGF (99).

2.2.2. ACE2/Ang (1–7)/Mas Receptor Axis

ACE2 and Ang-(1–7) are known counter-regulators of ACE and Ang II activity. In vitro analysis in murine endothelioma revealed the inhibition of tumour growth after Ang-(1–7) treatment, which was related with the reduction of HIF-1A and PLGF gene expression (100). Previous studies demonstrated that Ang-(1–7) inhibits in vitro growth of human lung cancer cells and tumour angiogenesis in vivo, through activation of the Mas Receptor (81, 101). ACE2 overexpression or ACE2-Ang-(1–7)-MasR activation may reduce angiogenesis, either through the inhibition of VEGF α production in NSCLC, or downregulation of the expression of VEGFR in nasopharyngeal carcinoma, respectively (102, 103).

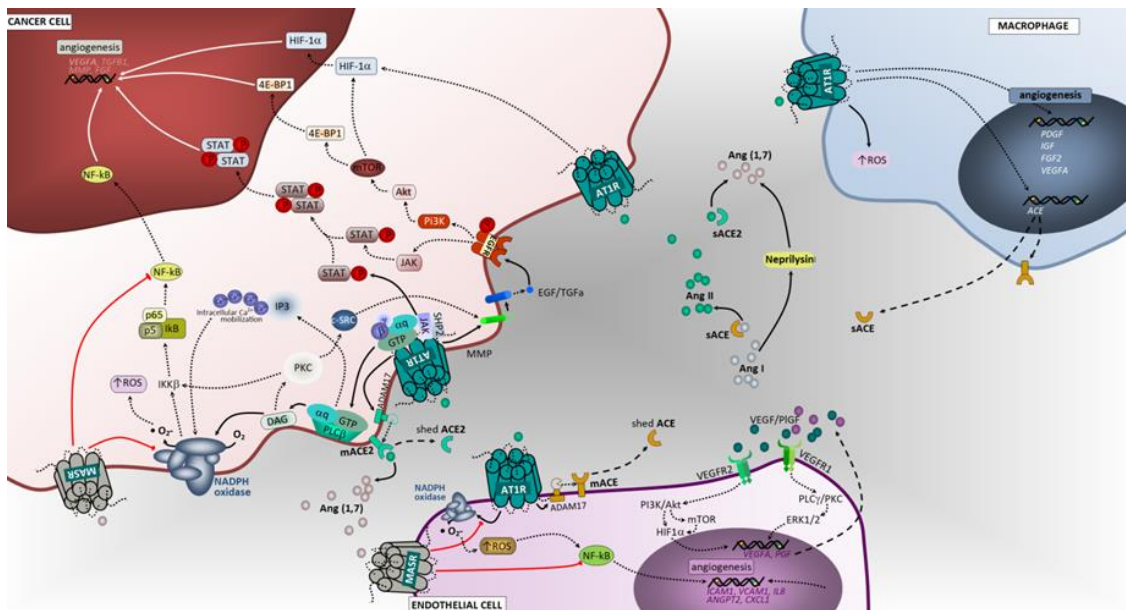


Figure 3. Angiotensin-associated pathways involved on hypoxia and angiogenesis in lung tumour microenvironment. In cancer cells, upon binding of Ang II to AT1R, a pleiotropic downstream signalling cascade is triggered, ultimately causing, either directly or indirectly, upregulation of angiogenesis and metastasis. Activated AT1R subunits trigger potent oxidant signalling through NADPH complex, which is involved in angiogenesis. Activated Gq/11 units also activate the JAK-SHP2/STAT pathway and receptor tyrosine kinase (RTK) transactivation. Subsequent signalling upon activation of EGFR is herein represented. Macrophages, endothelial cells and fibroblasts are important components of the tumour microenvironment and capable of

generating and expressing RAS components. These cells, beyond their functional ligands and receptors that are altered in tumour microenvironment, and reflect the crosstalk between all cell constituents, also use the RAS signalling pathway (mostly AT1, but also MasR in endothelial cells) to yield functional characteristics that ultimately may favour the cell itself and enhancing tumour angiogenesis and metastasis. 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; IKKB, Inhibitor of nuclear factor kappa-B kinase subunit beta; c-SRC, cellular Proto-oncogene tyrosine-protein kinase; SHP2, Src homology region 2 domain-containing phosphatase-2; IL1, interleukin 1; JAK, janus kinase; STAT, signal transduction and activator of transcription; Ang I, angiotensin I; Ang II, angiotensin II; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ANGPT2, angiopoietin 2; ICAM1, Inter cellular Adhesion Molecule 1; VCAM1, *vascular cell adhesion molecule 1*; NF- κ B, Nuclear Factor-kappa B; ROS, reactive oxygen species; ERK1/2, extracellular signal-regulated kinase ; PLC γ /PKC, phospholipase C gamma/protein kinase C; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; MEK1/2, mitogen activated protein kinase kinase; HIF1 α , hypoxia inducible factor 1 alpha; NO, nitric oxide; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; IP3, inositol trisphosphate; DAG, diacylglycerol; M2, macrophage polarized towards M2; ADAM17, Disintegrin and metalloproteinase domain-containing protein 17; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; MasR, G-protein coupled *Mas receptor*; VEGF, vascular endothelial growth factor; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; mACE, membrane angiotensin converting enzyme; sACE, soluble angiotensin converting enzyme; mACE2, membrane angiotensin converting enzyme 2; sACE2, soluble angiotensin converting enzyme 2; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2 Ang (1,7), angiotensin 1,7; PLC β , phospholipase C beta.

2.3. *Inflammation*

In lung pathologies, chronic obstructive pulmonary disease (COPD), lung fibrosis and lung inflammation are considered relevant events for cancer development (104). Chronic inflammation is known to contribute towards the tumour microenvironment—rich in inflammatory cells, ROS, recurrent DNA damage, growth factors, and other growth-supporting stimuli. Inflammation has been postulated to play a key role in lung carcinogenesis. Polymorphisms in genes coding for inflammatory signalling molecules have also been involved in lung cancer development (105). Engels and colleagues assessed lung cancer risk in relation to a large number of candidate polymorphisms in inflammation-related genes, namely *IL1A* and *IL1B* genes (106). Along with a predisposing germline genetic background, environmental exposure of agents associated with elevated lung cancer risk, such as tobacco, silica or asbestos, damage the

lung through the modulation of chronic inflammation. Individuals with tuberculosis, HIV infection associated with chronic lung infections, COPD and interstitial lung disease (106-108) seem to have an increased risk of lung cancer, reflecting the effects of underlying inflammatory disorders.

2.3.1. ACE/Ang II/AT1 Receptor and AT2 Receptor

Several studies have shown the influence of RAS in inflammation (109-112). Activation of AT1 receptor through Ang II results in potent oxidant signalling through NADPH complexes, which are involved in inflammation (11). Within the tumour microenvironment, the major components of RAS are expressed by cancer cells as well as by stromal cells, such as macrophages and cancer-associated fibroblasts (113). Through the upregulation of key inflammatory molecules, tumour cells invoke a chronic inflammatory state that also induces tumour-supporting myeloid cells, such as tumour-associated macrophages (TAMs) (114, 115). Notably, macrophages can exert pro-inflammatory (M1) or anti-inflammatory (M2) effects according to their polarization status. M1 macrophages are tumoricidal and highly phagocytic, promoting a Th1 response. Moreover, the tumour microenvironment can polarize M1 to M2 macrophages that have anti-inflammatory activities and promote angiogenesis, proteolysis, and tissue repair (116). In patients with NSCLC, the M1 macrophage subset has been associated to extended survival time (117). A report showed that Ang II influences macrophage behaviour, fostering cancer growth in an in vivo non-small cell lung cancer model, and restraining macrophage amplification after Ang II blockade (118). Shen and colleagues demonstrated that ACE overexpression reduced the number of blood and splenic myeloid-derived suppressor cells, in a spontaneous tumour model and in a model of chronic inflammation (119). Furthermore, macrophages from mice with myeloid ACE overexpression were more pro-inflammatory, exhibiting enhanced tumor-killing activity compared to their wild-type mice counterparts (119).

Although there is some controversy in the field, Ang II/AT1 receptor signalling stimulates the expression of cytokines and growth factors, which enhance lung cancer-associated inflammation. Activation of AT1 receptor signalling cascade leads to ROS, prostaglandin E2 and nitric oxide production (120). In addition, Ang II induced *Cox-2*

gene expression by activating the calcineurin/NFAT signalling pathway in endometrial cells and by malignant cells (Figure 4) (121, 122). Little is known about the contribution of AT2 receptor. Its functions remain controversial, as it has been reported to either inhibit or promote inflammation in different experimental settings (123-126), albeit most studies support that AT2 receptor mainly exerts an anti-inflammatory action (127).

2.3.2. ACE2/Ang (1–7)/Mas Receptor Axis

In opposite from AT1 receptor activation by Ang II, the Ang-(1–7) binding to Mas receptor triggers a down regulation of pro-oxidant pathways, preventing or attenuating the oxidative stress-induced cellular damage (109). In addition, Ang-(1–7) can also inhibit Ang II-activated inflammation through a deregulatory effect in lectin-like oxidized low-density lipoprotein receptor-1 (128). Menon and colleagues showed that Ang-(1–7) reduces tumour volume and inhibits cell proliferation via the reduction of COX-2 activity in a human lung tumour xenograft model (82). Moreover, compelling evidence suggests that the Mas receptor activation may counterbalance Ang-II-mediated proinflammatory effects, likely through macrophage function. A recent study reported that Mas signalling affected macrophage polarization, migration, and mediated T-cell activation, in two different chronic inflammatory animal models (129), providing the rationale that Mas may have an important role in regulating inflammatory processes, likely influencing macrophage behaviour at the cancer microenvironment, namely in lung cancer. Additional experimental work in diabetes showed that administration of Ang-(1–7) limited the activation of NADPH oxidase through downregulation of

molecular components of the NADPH oxidase complex (130), thus controlling ROS production and subsequent inflammation.

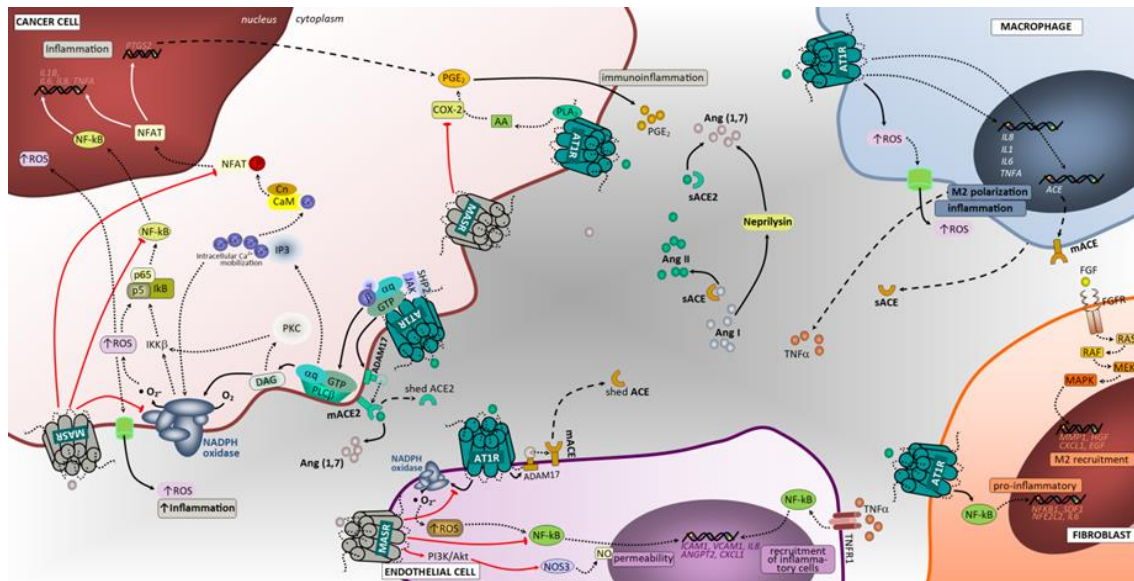


Figure 4. Angiotensin-associated immunoinflammatory pathways in lung tumour microenvironment. Extracellular proteins of the RAS, ligands (Ang I, Ang II and Ang 1,7) and enzymes (ACE, ACE2 and neprilysin), are represented with distinct colours in the tumour microenvironment. The expression and cleavage of ACE and ACE2 are represented in tumour and endothelial cells, showing the AT1R signalling-associated activation of ADAM17 that cleaves the ectodomain of mACE and mACE2 shedding the enzymes into sACE and sACE2. The Ang II/AT1R-mediated direct intracellular signalling is represented with full black arrows as a first step after AT1R triggering in the cascade. The subsequent steps of signalling cascades are depicted, up to moving towards intranuclear space, using dashed black arrows. Intranuclear signalling from these pathways mainly results in transcriptional regulatory effects and is represented by full white arrows. The Ang II/AT2R and Ang (1,7)/MasR-mediated counter-regulatory mechanisms are depicted using black arrows and when adequate the suppressive step is represented by blunt-ended red arrows. In cancer cells, upon binding of Ang II to AT1R, a pleiotropic downstream signalling cascade is triggered, ultimately causing, either directly or indirectly inflammation immune cells recruitment. Activated AT1R triggers potent oxidant signalling through NADPH complex, which is involved in inflammation and angiogenesis. Other intracellular cascades mediated by Ang II/AT1R include the activation of PLA2/AA/COX-2/PGE2 signalling. The Ang (1,7)/MasR activation elicits downstream signalling through PI3K/Akt/NOS3 or NOS1 to produce NO that inhibits NFAT transcriptional regulation that reduces inflammation. Notably, this pathway blocks the NF-κB molecule formation thereby impacting inflammation. In addition, the inhibitory effect over COX-2 significantly impact inflammation. Macrophages, endothelial cells and fibroblasts are important components of the tumour microenvironment and

capable of generating and expressing RAS components. These cells, beyond their functional ligands and receptors that are altered in tumour microenvironment, and reflect the crosstalk between all cell constituents, also use the RAS signalling pathway (mostly AT1, but also MasR in endothelial cells) to yield functional characteristics that ultimately may favour the cell itself and enhance tumour growth and aggressiveness. PLA2, phospholipase A2; PTP, protein tyrosine phosphatase; PP2A, Protein phosphatase 2; IKKB, Inhibitor of nuclear factor kappa-B kinase subunit beta; CARMA3, CARD recruited membrane associated protein 3; MALT1, Mucosa-associated lymphoid tissue lymphoma translocation protein 1; BCL-10, B-cell lymphoma/leukemia 10; c-SRC, cellular Proto-oncogene tyrosine-protein kinase; SHP2, Src homology region 2 domain-containing phosphatase-2; AA, arachidonic acid; COX-2, cyclooxygenase 2; PGE2, prostaglandin E2; IL1, interleukin 1; TNFA, tumour necrosis factor; Ang I, angiotensin I; Ang II, angiotensin II; GTP, guanosine triphosphate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ANGPT2, angiopoietin 2; ICAM1, Intercellular Adhesion Molecule 1; VCAM1, *vascular cell adhesion molecule 1*; CXCL1, The chemokine (C-X-C motif) ligand 1; IL8, interleukin 8; NF-kB, Nuclear Factor-kappa B; NFKB1, Nuclear Factor-kappa B Subunit 1; IL6, interleukin 6; NFE2L2, Nuclear Factor, Erythroid 2 Like 2; HGF, hepatocyte growth factor; RAS, oncogene protein p21; ROS, reactive oxygen species; NOS3, nitric oxide synthase 3 endothelial; NOS1, nitric oxide synthase 1 neuronal; Cas9, caspase 9; Cas3, caspase 3; ERK1/2, extracellular signal-regulated kinase; PLC γ /PKC, phospholipase C gamma/protein kinase C; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; TNFR1, tumour necrosis factor receptor 1; c-Raf, kinase Raf-1; MEK1/2, mitogen activated protein kinase kinase; HIF1 α , hypoxia inducible factor 1 alpha; NO, nitric oxide; RhoA, Ras homolog family member A; IP3, inositol trisphosphate; DAG, diacylglycerol; M2, macrophage polarized towards M2; ADAM17, Disintegrin and metalloproteinase domain-containing protein 17; p38/MAPK, protein 38/mitogen activated protein kinase; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; MASR, G-protein coupled *Mas receptor*; VEGFA, vascular endothelial growth factor A; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; mACE, membrane angiotensin converting enzyme; sACE, soluble angiotensin converting enzyme; mACE2, membrane angiotensin converting enzyme 2; sACE2, soluble angiotensin converting enzyme 2; PDGFR β , platelet derived growth factor receptor beta; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; TGF α , transforming growth factor alpha; Ang (1,7), angiotensin 1,7; PLC β , phospholipase C beta; FAK, Focal adhesion kinase; NFAT, nuclear factor of activated T cells; PDGF, platelet derived growth factor.

2.4. Tumour Immunological Response

Recent cancer immunotherapies, including immune-checkpoint blockade, have produced durable clinical effects in some patients with various advanced cancers,

namely in NSCLC (131). tumour anti-programmed death-ligand 1 (PD-L1) expression is associated with increased tumour-infiltrating lymphocytes in lung cancer. Unresponsiveness to the immune-checkpoint blockade therapies may be mediated by numerous immunosuppressive mechanisms that inhibit T-cell responses and T-cell infiltration into tumours (132, 133). In the lung cancer microenvironment, the cooperation between cancer cells namely airway epithelial cells, macrophages, and other peripherally-recruited innate immune cells can determine the fate of lung tumours at different stages, of both metastatic and primary disease, including pre-neoplastic, early, and late lesions (134). Among the hematopoietic suppressor cells of interest are M2 macrophages, splenic myeloid-derived suppressor cells, and regulatory T cells (Treg), which have been associated with a poor prognosis in many cancers, and with unfavourable clinical response to anti-PD-1/PD-L1 (132). Furthermore, cancer-associated fibroblasts (CAFs) can manipulate the immune system, directly by inhibiting T and NK (natural killer) cell functions, promoting accumulation of suppressive cell types, and maintaining a pro-tumorigenic milieu. CAFs also induce tumour fibrosis, which represents a physical barrier to T cell infiltration (135).

Neutrophils make up a significant portion of the inflammatory cells infiltrate in the tumour, whereby they show high functional plasticity displaying both anti-tumour and pro-tumour activities (136). Studies in early NSCLC have shown that neutrophils are pivotal to tumour cell clearance by stimulating adaptive immunity. In contrast, the role of neutrophils during later stages of primary lung cancer progression frequently appears to be pro-tumorigenic, as neutrophils represent the most abundant recruited cells in more advanced NSCLC (134). Tumour-derived signals induce a pro-tumour phenotype in neutrophils, which supports cancer cell invasion and metastasis (N2 neutrophils). N2 polarized neutrophils promote the proliferation, migration, and invasion of tumour cells, stimulate angiogenesis, as well as mediate immunosuppression (136). Dendritic cells are crucial for the activation of antigen-specific CD4 T lymphocytes, a pivotal step in the initiation of innate and adaptive immune responses, which are essential for lung tumour cell clearance (137). Previous studies demonstrated that lung tumours dynamically exclude functional DCs from the tumour region to support malignant progression. Furthermore, clinical trials have proven that dendritic cell function is

reduced in lung cancer patients (137). At the tumour microenvironment, the major components of RAS are also expressed both by resident and infiltrating cells, such as endothelial cells, fibroblasts, monocytes, macrophages, neutrophils, dendritic and T cells (11). RAS signalling in these cells can facilitate or hinder growth and dissemination and has been shown to affect cell proliferation, migration, invasion, metastasis, apoptosis, angiogenesis, cancer-associated inflammation, immunomodulation, and tumour fibrosis/desmoplasia (137).

2.4.1. Targeting RAS to Improve Anti-Tumour Immunity

A recent study using 4T1 and CT26 syngeneic mouse tumour models, showed that Ang II generates infiltration of fibroblasts and macrophages, contributing to the reduction of CD8⁺ T cell infiltration, resulting in an immunosuppressive environment (138). Importantly, Nakamura and colleagues, demonstrated, in a tumour bearing mouse model of colorectal carcinoma, that the antagonism of AT1 receptor decreased the expression of immunosuppressive factors, such as chemokine ligand 12 and nitric oxide synthase 2 in CAFs, and that the combination of antagonists of AT1-receptor and PD-L1 antibodies resulted in significant augmentation of anti-tumour effects in a CD8⁺ T cell-dependent way (113). Furthermore, RAS-activated CAFs secrete collagen and other ECM components which produce fibrosis and consequently tumour desmoplasia (139). Notably, tumour desmoplasia can be either a physical barrier to immune cell infiltration or provide the substratum to interstitial cell migration (139). It was demonstrated that the antagonism of AT1 receptor-inhibited collagen I production by carcinoma-associated fibroblasts isolated from breast cancer biopsies (140). Additionally, this led to a dose-dependent reduction of stromal collagen in desmoplastic models of human breast, pancreatic, and skin tumours in mice (140). Contrary to the Ang II effect in tumour immunity response, interesting findings also showed that Ang-(1–7) targets the tumour microenvironment to inhibit CAF growth and tumour fibrosis in orthotopic breast tumours (141). TGF- β suppresses the differentiation and function of T helper, CD8⁺ cells, Natural Killer cells, and tumour-associated neutrophils, tumour associated macrophages and myeloid-derived suppressor cells (142). Tumour supporting cytokines are released from tumour and stromal cells upon AT1 receptor activation via Ang II

including, TGF- β and Interleukins (IL-1 α , IL-1 β , IL-6, IL-8) (135). Immunomodulatory cytokines may up-regulate immunosuppressive pathways, i.e., COX-2 via prostaglandin E2 synthesis, and impair dendritic cell function by reducing their migration (143). Ang II/AT1R signalling induces reactive oxygen species generation and related proteins such as inducible nitric oxide synthase in tumour and stroma cells. Exposure to ROS in the tumour microenvironment can impair T cell function while enhancing T regs and tumour associated macrophages (135). Treatment with the candesartan (AT1 receptor blocker) diminishes ROS generation (139). Ang II/AT1R signalling is also important for myeloid differentiation and functional maturation. Cultured bone marrow from ACE 10/10 mice, a mouse line overexpressing ACE in monocytic cells, demonstrates increased myeloid maturation and reduced splenic myeloid-derived suppressor cells production. In addition, macrophages from these mice have an enhanced proinflammatory phenotype and antitumor activity compared to those from wild-type mice (119). Similarly, tumour-bearing ACE 10/10 mice showed enhanced immune responses, which ultimately resulted in a reduced tumour growth. ACE inhibitors reversed the beneficial effects on tumour growth, but AT1 receptor blockade did not, suggesting that the effects of ACE overexpression were not dependent of Ang II/AT1 receptor signalling (144). Hence, these reports suggest that targeting RAS could alleviate immunosuppression and enhance the outcome of immunotherapy. Nevertheless, it is necessary, through experimental and clinical research, to highlight the role of RAS in lung cancer and its influence on immunotherapy response.

2.5 Clinical Studies

Pre-clinical studies demonstrated an association between RAS activation and increased invasion, angiogenesis, inflammation, and modulation of immune response at the lung tumour microenvironment. The putative impact on cancer hallmarks, through AT1 receptor blockade or activation of the ACE2/Ang-(1–7)/Mas receptor axis, has been linked to reduced tumour growth and vascularization in lung cancer, thus fostering the need for clinical studies.

2.5.1 Clinical Trials

Despite basic and pre-clinical evidence concerning the impact of RAS in cancer hallmarks, the information available regarding clinical trials remains scarce, particularly in lung cancer. The primary objective of a Phase II Randomized Trial (NCT00077064, Table 1) was to test the ability of captopril to alter the incidence of pulmonary damage at 12 months after the completion of radiation in patients with non-small cell lung cancer or limited-stage small cell lung cancer (145). Due to the unmet accrual/randomization goals, it was not possible to adequately test the hypothesis that captopril ameliorates radiotherapy-induced pulmonary toxicity. Another double-blind placebo-controlled randomized trial (NCT01880528, Table 1) enrolled 23 patients with lung cancer to study the putative protective effect of lisinopril in pneumonitis induced by radiotherapy (146). The results of this clinical trial suggest that there was a clinical signal for safety and possibly beneficial in concurrently administering lisinopril during thoracic radiotherapy to mitigate or prevent radiation-induced pulmonary distress (146). However, larger-scale randomized phase 3 trials are needed in the future to confirm these results. Notably, a multicentre clinical trial showed that losartan stabilized lung function in patients with idiopathic pulmonary fibrosis over 12 months (NCT00879879, Table 1), reinforcing the importance of AT1 receptor blockers in the impairment of fibrosis progression (147). Moreover, treatment with AT1 receptor blockers might potentiate the response to immunotherapy in lung cancer patients due to a hypothetical impairment of the fibrotic immunosuppressive microenvironment. Nevertheless, there is no study still to prove this hypothesis.

A small Phase I study (NCT00471562, Table 1) of Ang-(1–7) as a first-in-class anti-angiogenic drug targeting Mas receptor, was undertaken in 18 patients with advanced solid tumours refractory to standard therapy with only one patient with lung cancer included (148). Dose-limiting toxicities encountered at the 700 µg/kg dose included stroke (grade 4) and reversible cranial neuropathy (grade 3). Other toxicities were generally mild. Clinical benefit, defined as disease stabilization for more than three months, was observed in two of the three patients with metastatic sarcoma and it was associated with reduction of PlGF plasma levels (148). A Phase II study failed to confirm PlGF as biomarker of response to Ang-(1–7) administration (149). However, two patients

with vascular sarcomas demonstrated prolonged disease stabilization of 10 months (hemangiopericytoma) and 19 months (epithelioid hemangioendothelioma) under Ang-(1–7) treatment, thereby requiring further investigation (149).

Identifier	Condition	Design	Intervention	Primary endpoint	Status
NCT00077064	Captopril in treating patients with non-small cell lung cancer or limited-stage small cell lung cancer that has been previously treated with radiation therapy with or without chemotherapy	Phase II Randomized Trial	Captopril or placebo	Incidence of pulmonary damage after radiotherapy	Terminated
NCT01880528	Lisinopril in reducing shortness of breath caused by radiation therapy in patients with lung cancer	Double-Blind Randomized	Lisinopril or placebo	Incidence of grade 3 or higher hypotension, acute kidney injury, allergic reaction, or anaphylaxis	Completed
NCT01754909	Mitigation of radiation pneumonitis and fibrosis	Phase II Randomized Trial	Enalapril or placebo	Occurrence and grade of radiation pneumonitis	Completed
NCT00879879	Losartan in treating patients with idiopathic pulmonary fibrosis	Multicentre Clinical Trial	Losartan	Stable or improved forced vital capacity (FVC) response at 1 year in idiopathic pulmonary fibrosis	Completed
NCT00471562	Angiotensin-(1-7) in treating patients with metastatic or unresectable solid tumors	Phase I Non-Randomized Clinical Trial	Angiotensin-(1-7) sequentially increasing doses 50-1000 mcg/kg	Maximum tolerated dose and toxicity	Completed
NCT00886353	Safety and tolerability study of APN01 (recombinant human angiotensin converting enzyme 2)	Phase II Randomized Trial	APN01 i.v. or placebo	Safety and tolerability profile of APN01	Completed
NCT00880386	Losartan in treating pulmonary fibrosis in patients with Stage I, II, or III Non-Small Cell Lung Cancer	Multicentre Clinical Trial	Losartan and radiotherapy	Change in carbon monoxide diffusing capacity (DLCO) at 6 months	Withdrawn

Table 1. Clinical Trials of renin angiotensin blockers and its role in lung cancer and pulmonary fibrosis

(adapted from *clinicaltrials.gov*).

2.5.2. Observational Studies

A retrospective cohort study that included 228 patients with advanced NSCLC and 73 with early-stage disease revealed that those under therapeutics with ACE inhibitors (iACE) or AT1 receptor blockers, in combination with standard chemotherapy or tyrosine kinase inhibitors, had a positive effect on progression-free survival and overall survival, regardless of lung cancer stage (60). A retrospective study with 117 metastatic NSCLC patients demonstrated that the intake of AT1 receptor blockers, during erlotinib treatment, may prolong overall survival (150). Another study including 287 advanced NSCLC patients, demonstrated a survival advantage for patients under ACE inhibitors or AT1 receptor blockers (151). Notably, the reduced risk for cancer progression in patients submitted to ACE inhibitors or AT1 receptor blockers has not been seen with other antihypertensive medications, suggesting the existence of an adjuvant effect for angiotensin blockers (151). A recent systematic review and meta-analysis showed that AT1 receptor blockers are significantly associated with reduced lung cancer risk (152). Conversely, a study on a large population-based cohort that used ACE inhibitors suggested an increased risk for developing lung cancer, particularly among patients undergoing treatment for more than five years. Nevertheless, the risk for lung cancer was not confirmed in patients under AT1 receptor blockers (153). These findings might rely on ACE inhibitors-associated accumulation of bradykinin in the lung, rather than a decrease in Ang II signalling, especially when higher dosages of ACE inhibitors are taken into consideration (154). When analysed together, these results from clinical and pre-clinical studies suggest that AT1 receptor blockers, in contrast to ACE inhibitors, might be putative therapeutic tools to impair lung cancer progression. Further larger prospective studies with well-designed clinical trials are required to confirm whether targeting RAS improves lung cancer treatment.

3. Association of Genetic Polymorphisms related to Renin-Angiotensin and Angiogenesis Pathways in Cancer

The presence of polymorphic gene variants in the human genome provides extensive genetic variation affecting both normal physiological mechanisms and cancer

pathogenesis (155). Single-nucleotide polymorphism (SNP) are the most common, accounting for 90% of genetic variation (156). Nucleotide substitution, according to its location in germline DNA, may elicit functional modifications, ultimately with repercussion at protein level, contributing to inter-individual variability, and correlating with relevant phenotypic modification. The associations between genetic polymorphisms and cancer susceptibility, disease progression, aggressiveness and death have been thoroughly described in the literature (155, 156).

3.1 Single Genetic Polymorphisms of the Renin-Angiotensin Pathway

As a gatekeeper system, RAS is responsible for more than one function, including cell cycle regulation, inflammation, angiogenesis and fibrosis (157). Loss of RAS homeostasis, either genetically or environmentally-driven, might contribute to the deregulation of intersected axes, thereby modulating lung cancer aggressiveness (158).

ACE plasma levels depend on a 287 bp insertion/deletion (I/D) polymorphism in the *ACE* gene located on chromosome 17q23. Homozygotes for the D allele have the highest ACE plasma levels, ID heterozygotes intermediate and I homozygotes have the lowest. Several studies found an association between *ACE* I/D polymorphism and lung cancer, despite others revealed conflicting results (159-161).

The human gene encoding angiotensinogen (*AGT*) is located on chromosome 1q42.3. The SNPG-6A nucleotide substitution at position 6, upstream from the initial transcription start codon, has been studied particularly. The A allele has been associated *in vitro* with an increased expression of the *AGT* gene and with higher AGT synthesis (162). A previous report showed that the G-6A polymorphism of the *AGT* gene is associated with increased angiotensin production and idiopathic pulmonary fibrosis progression (162). Another polymorphism, the *AGT* M235T conferred risk for developing breast cancer in post-menopausal women (159).

In the later years, it was demonstrated that neprilysin (NEP), an enzyme encoded by the gene *MME* gene, located on chromosome 3q25.1–q25.2, is implicated in oncogenesis as modulator of tumour microenvironment (163). Several reports suggested a protective role for neprilysin in solid cancers (163, 164), although other studies showed that neprilysin is a possible marker for tumour progression and metastasis in a number

of solid cancers including colorectal, head and neck squamous cell carcinoma, breast and lung cancer (165-168). There are no studies that demonstrate the influence of genetic polymorphisms of neprilysin in tumoral progression of cancer patients. Previous studies demonstrated that the SNP *MME* rs701109 has been associated with Alzheimer's disease in Spanish and Finnish patients (169, 170).

Genetic variation of the *ACE2* gene which is located on chromosome Xp22 is also a strong candidate for differences in *ACE2* activity (171). A functional variant of *ACE2* gene (rs2106809 T allele), leads to reduced activity of the enzyme and is known to increase the risk of hypertension, reduced response to anti-hypertensive drugs and downregulating the production of *ACE2* (172). There are also studies showing different polymorphisms in *ACE2* gene (rs1514283, rs4646176, rs879922, rs1514282, rs4646174) in association with blood pressure and its response to cold stress or to dietary sodium intervention (173, 174). The genetic variations in *ACE2*, *AGTR1*, *AGTR2*, *MAS1* and its association with lung cancer remains largely unknown.

3.2 Single Genetic Polymorphisms of Hypoxia and Angiogenesis pathways

One of the most important features of tumours is hypoxia. Reduced levels of oxygen in tumour tissues induce a molecular response, which drives the activation of transcription factors, among these, hypoxia-inducible factors (175). Studies that ascertained the association between *HIF1A* polymorphisms and cancer risk, revealed conflicting results, hence requiring further investigation (176-178). Two single-nucleotide polymorphisms (SNPs) in *HIF1A* which result in proline to serine (rs11549465) and alanine to threonine (rs11549467) amino acid substitutions, respectively, were found within the coding region of N- terminal activation domain of *HIF1A* (179). It was demonstrated that variants in those two polymorphisms significantly enhanced transcription activities and were associated with higher disease stage in head and neck squamous cell carcinoma (180). Liu and colleagues (2014) studied another two functional SNPs (rs2057482 and rs2301113) in *HIF1A* gene and observed increased risk on mortality and recurrence in NSCLC patients with early T-stage tumors for rs2301113, as well as in patients without lymph node involvement (N0 stage) for rs2057482 (181).

Angiogenesis is a crucial step for lung cancer progression (182), and is closely related to both hypoxia and RAS axis. Furthermore, studies have shown that NSCLC patients presented with higher serum VEGF than healthy controls (183, 184). The *VEGF* gene is located on chromosome 6p21.3 and consists of eight exons that undergo alternate splicing to form a family of proteins (185). This gene is highly polymorphic, with multiple common SNPs in the promoter, 5'-untranslated and 3'- untranslated regions (186) which are regions of primary interest in cancer susceptibility and outcome because they may modulate VEGF expression (187). *VEGF* polymorphisms have been studied in relation to risk and histopathologic characteristics of various cancers (188, 189). Five functional polymorphisms at positions -2,578C>A, -1498T>C, -1154G>A, -634G>C, and 936C>T have been associated with serum VEGFA and cancer risk, including breast cancer risk and its aggressiveness (186).

FLT1 encodes the VEGF receptor 1 (VEGFR-1), a mediator of tumour endothelial function (190). Its level of phosphorylation in response to VEGFA is low (191), and its soluble form is expressed by endothelial tumour cells, sequestering VEGFA through the formation of a complex (192). Soluble VEGFR-1 also inhibits VEGFA in a dominant manner by heterodimerizing with the ligand binding region of VEGFR-2 (193), the most potent receptor of VEGFA which mediates most of the pro-angiogenic effects of VEGFA. Enhanced *in vivo* expression of soluble VEGFR-1 by tumour cells inhibits solid tumour growth, impedes metastatic nodule development, and extends host survival (190). A previous study demonstrated that the SNP rs9582036 of *FLT1* gene was associated with overall survival in patients with metastatic pancreatic cancer under bevacizumab (194). Another report showed that the variant allele (C) of the *FLT1* SNP rs9582036 was associated with shorter relapse-free survival in stage I-III NSCLC patients (195).

KDR encodes VEGFR-2, the key receptor mediating endothelial cell proliferation and sprouting, leading to angiogenesis and tumour promotion upon binding and signalling through the VEGFA pathway (196). *KDR* gene is located on chromosome 4q12 and contains 30 exons (197). Interestingly, previous study suggested that the 889C>T polymorphism in the coding region of the *KDR* gene influenced the prognosis of patients with liver cancer treated with sorafenib (198). Simultaneously, polymorphism in *KDR* gene was significantly related to the objective response rate of bevacizumab combined

with oxaliplatin in the treatment of European and American patients with advanced colorectal carcinoma patients with advanced colorectal carcinoma (199). A recent study demonstrated that patients with advanced NSCLC receiving first-line bevacizumab plus chemotherapy regimens, who simultaneously carry CT/TT genotype of *KDR* V297L polymorphism, had worse progression free survival (PFS) and overall survival (OS) compared with CC genotype (197). The CT/TT genotype was associated with higher *KDR* mRNA expression (197).

Placenta growth factor (PlGF), the second member of VEGF family, is encoded by *PGF* gene mapped to chromosome 14q24 (200). Placental growth factor induces various biological effects by affecting a wide range of different cell types. PlGF can stimulate vessel growth and maturation directly by affecting endothelial and mural cells, and indirectly by recruiting pro-angiogenic cell types (96, 201, 202). Accumulating reports have suggested that PlGF might be a useful prognostic marker of cancer progression. Plasma PlGF levels are upregulated and have been correlated with tumor grade and survival in renal-cell carcinoma (203). Moreover, PlGF mRNA and protein from tumour tissues have been found to be correlated with tumour stage in lung cancer (204). Only one recent study assessed the association between polymorphisms in *PGF* and cancer, which demonstrated that *PGF* rs2268615 was a risk factor for cervical cancer (205).

4. Non-Small Cell Lung Cancer: the current state-of-art

Lung cancer is one of the most common malignancies worldwide and the most common cause of cancer deaths in the past few decades, with over one million subjects yearly diagnosed (1, 2). Notably, the five-year survival rate is the lowest compared with other frequent malignancies (3). The two major types of lung cancer are non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), with approximately 85% and 15% of all lung cancers, respectively (206). NSCLC is further categorized into adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma and large cell carcinoma (206).

4.1 Risk Factors

The primary risk factor for the development of lung cancer is cigarette smoking, which is estimated to account for approximately 90% of all lung cancers (207). The risk

of lung cancer increases with both the number of cigarettes smoked per day as well as the lifetime duration of smoking (208). The intensity of exposure to environmental tobacco smoke (ie, passive or "secondhand" exposure) is far less than that which occurs with active smoking. On the other hand, exposure to environmental tobacco smoke can begin earlier in life than it does with active smoking, and the exposure to carcinogens occurs over a longer period of time. There is a dose-response relationship between intensity of exposure and relative risk of lung cancer (209). The risk for the development of lung cancer in response to "secondhand" exposure may be influenced by genetics. One study found a significant increase in polymorphisms in the gene *glutathione S-transferase M1* (*GSTM1*) among 51 non-smoking females with tobacco exposure who developed lung cancer compared with 55 non-smoking females with lung cancer who did not have exposure (210). In another population-based study among never-smokers, in those with high tobacco exposure, *GSTM1* and *GSTP1* polymorphisms were associated with over a fourfold increased risk of developing lung cancer (211). Familial risk of lung cancer has been reported in several registry-based studies after careful adjustment for smoking (212). Genome-wide association studies (GWAS) have identified lung cancer susceptibility loci including *CHRNA3*, *CHRNA5*, *TERT*, *BRCA2*, *CHECK2* and the human leukocyte antigen (HLA) region (213-215).

Numerous occupational and environmental carcinogens increase the risk of lung cancer. The best-known factors are asbestos and radon (216). Other exposures that have been associated with lung cancer include arsenic, bis(chloromethyl) ether, chromium, formaldehyde, ionizing radiation, nickel, polycyclic aromatic hydrocarbons, hard metal dust, and vinyl chloride (217-219).

Various "benign" lung diseases have been associated with an increased risk of lung cancer, which appears to be mediated through chronic inflammation. Notably, individuals with diffuse pulmonary fibrosis have an 8- to 14-fold increased risk for lung cancer, even when age, gender, and smoking history are taken into consideration (220).

4.2 Diagnosis

The majority of patients with lung cancer have advanced disease at clinical presentation. This may reflect the aggressive biology of the disease and the frequent

absence of symptoms until locally advanced or metastatic disease is present. High-risk patients may be diagnosed while asymptomatic through screening with low-dose computed tomography. The preferred approach uses imaging as a road map and invasive biopsy as a tool to confirm both the histopathological diagnosis and the stage of disease. When feasible, diagnosis and staging should be established concurrently by targeting for invasive biopsy the abnormality that would yield the most advanced stage. A complete medical history with comorbidities, weight loss, Eastern Cooperative Oncology Group performance status (ECOG PS) (221) and physical examination must be recorded. A contrast-enhanced CT scan of the chest and upper abdomen including complete assessment of liver, kidneys and adrenal glands should be carried out. Imaging of the central nervous system is relevant for disease staging. Magnetic resonance imaging (MRI, preferably with gadolinium enhancement) is more sensitive than CT scan. FDG PET/CT combines anatomic data with functional and metabolic information. The anatomic detail provided by CT, such as tumour size and local aggressiveness, is complemented by the metabolic information of PET. Although conventional CT is still widely used for staging NSCLC, assessment of nodal metastases is limited with this modality (222). MRI may complement or improve the diagnostic staging accuracy of FDG-PET-CT imaging, particularly in assessing local chest wall, vascular or vertebra invasion and is also effective for identification of nodal and distant metastatic disease (223).

Bronchoscopy is a technique ideally suited to large, central lesions and offers the advantage of minimal morbidity. Bronchoscopy can be used for bronchial washing, brushing, bronchial and transbronchial biopsy, with a diagnostic yield of 65%–88% (224). By combining direct bronchoscopic airway visualization with ultrasound-guided biopsy of the lesion, endobronchial ultrasound (EBUS) provides a diagnostic yield of 75%–85% in large, centrally located lesions (225). Fibre optic bronchoscopy allows for the evaluation of regional lymph nodes by EBUS and/or endoscopic ultrasound (EUS), essential for mediastinal staging (226).

Histological diagnosis of NSCLC is crucial to many treatment decisions and should be as exact and detailed as the samples and available technology allow. Diagnosis should be based upon the WHO classification criteria (227). After morphological diagnosis, the

next consideration is therapy-predictive biomarker testing (223). Several molecular drivers for oncogene addiction represent strong predictive biomarkers and excellent therapeutic targets. Multiple-gene sequencing (next generation sequencing, NGS) has the potential to concurrently detect single nucleotide variants, small insertions, deletions, copy number alterations, structural variants, gene fusions, or chromosomal rearrangements (228), thus reducing the costs and sparing the time for a comprehensive molecular assessment. NGS allows to identify co-occurring genetic alterations in a specific subgroup of molecularly classified patients and better define the genomic complexity and the prognosis of patients with cancer, as in the case of *KRAS* mutant or *EGFR* mutant NSCLC tumours (229).

The approval of the anti-programmed cell death protein 1 (PD-1) agent pembrolizumab as a standard-of-care first-line treatment in selected patients has made programmed death-ligand (PD-L1) immunohistochemistry (IHC) a mandatory test in all patients with advanced NSCLC (223). Although the PD-L1 IHC 22C3 assay was the only test validated in clinical trials of pembrolizumab, extensive technical comparison studies suggest that trial-validated commercial kit assays based on the 28-8 and SP263 PD-L1 IHC clones may be alternative tests (230).

4.3 Treatment of metastatic NSCLC

Patients with advanced NSCLC receive palliative therapies with systemic treatments namely chemotherapy (ChT), targeted therapies and immune checkpoint inhibitors. The type of systemic therapy depends on tumour histology, presence or absence of driver mutations in tumours, performance status of patients and other factors (231).

Standard-of-care first-line chemotherapy for advanced NSCLC without actionable driver mutations or low expression of programmed death-ligand 1 (PD-L1) has historically been platinum-doublet, cisplatin or carboplatin, with or without maintenance therapy (232). Despite its wide acceptance and use, platinum-based chemotherapy presents poor clinical outcomes and efficacy varies across patients. Currently, the combination of immune checkpoint inhibitors with chemotherapy in advanced driver mutation-negative NSCLC and tumour PD-L1 expression under 50%,

has replaced the regimen of only platinum-based chemotherapy in first line treatment (231). Beyond clinical and pathologic features, genetic variation is also considered a factor associated with treatment efficacy and prognosis (233). Germline mutations of genes involved in DNA repair (*EXCC1*, *XPA*, *XPB*, *XPD*, *XPG*, *XRCC1* and *XRCC3*), drug influx and efflux (*MDR1*), metabolism and detoxification (*GSTP1*) and DNA synthesis (*MTHFR*), might be considered as potential prognostic biomarkers for assessing objective response and progression risk in NSCLC patients receiving platinum-based regimens (233). Notably, the polymorphism *AGXT* rs34116584, that codes for the enzyme alanine glyoxylate aminotransferase, was shown to be associated with toxicity induced by oxaliplatin and progression-free survival (PFS) in patients with metastatic colorectal cancer (234).

The recent development of immune checkpoint inhibitors provided the proof of principle that immunotherapy can play an important role in the treatment of patients with lung cancer. The phase III KEYNOTE-024 study has established the role for pembrolizumab as first-line treatment in patients with untreated, advanced NSCLC and tumour characterized by PD-L1 expression $\geq 50\%$, in absence of *EGFR* mutation or *ALK* translocations (235). In KEYNOTE-189, patients with metastatic non-squamous NSCLC, PS 0–1, without sensitizing *EGFR* or *ALK* mutations and regardless of PD-L1 status, were randomized to receive pemetrexed and cisplatin or carboplatin plus either 200 mg of pembrolizumab or placebo every 3 weeks for 4 cycles, followed by pembrolizumab or placebo for up to a total of 35 cycles plus pemetrexed maintenance therapy (236). The median overall survival (OS) in the pembrolizumab/ChT arm was 22.0 months (95% CI 19.5–25.2) versus 10.7 months (95% CI 8.7–13.6) in the ChT arm (HR 0.56, 95% CI 0.45–0.70, $P < 0.00001$) (237). The addition of frontline pembrolizumab to platinum-based doublet chemotherapy has also improved outcomes in squamous cell cancers with PD-L1 $< 50\%$, without adding substantial toxicity (238).

An improved understanding of the molecular pathways that drive malignancy in NSCLC has led to the development of agents that target specific molecular pathways in malignant cells. These agents have been a significant step forward in the treatment of patients whose tumours contain specific mutations in these pathways. Epidermal growth factor receptor (*EGFR*) mutation is the best-established oncogenic target for

management of advanced stage NSCLC (239). EGFR tyrosine kinase inhibitors (TKIs) significantly improve outcomes in patients with advanced NSCLC that contains an activating mutation in EGFR compared with platinum-based chemotherapy doublets (240, 241). Although first-generation (erlotinib, gefitinib) and second-generation (afatinib) TKIs have been standard of care for initial management of EGFR, newer data suggest improved outcomes with front-line treatment with the third-generation agent, osimertinib (242).

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase that can be aberrantly expressed in several tumour types. In NSCLC, chromosomal rearrangements involving the *ALK* gene loci on chromosome 2 are found in approximately 3 to 5 percent of NSCLC tumours (243). The antitumor activity of crizotinib was initially demonstrated in two multicentre single-arm studies, with significant overall response rate and PFS advantages, as well as a survival advantage, compared with other treatment options (223). Currently, ceritinib and alectinib are second-generation ALK inhibitors that have shown robust antitumor efficacy, along with intracranial activity, in patients with *ALK*-rearranged NSCLC (223). Brigatinib is another next-generation ALK inhibitor that is approved for clinical use in patients with *ALK*-positive metastatic NSCLC who have progressed on or are intolerant to crizotinib (244).

A number of other less common driver mutations have been identified, which define specific subsets of patients with NSCLC. Examples include c-ROS oncogene 1 (*ROS1*), *BRAF*, *RET*, *NTRK*, *MET*, and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (223). For most of these, specific inhibitors are available, and these should be integrated into the overall sequence of treatments for a given patient.

Response evaluation is recommended after 2–3 cycles of chemotherapy or immunotherapy, using the same initial radiographic investigation that demonstrated tumour lesions (223). The same procedure and timing (every 6–9 weeks) should be applied for the response evaluation in patients treated with targeted therapies and/or immunotherapy. Measurement of lesions should follow Response Evaluation Criteria in Solid Tumours (RECIST) v1.1 (245).

4.4 Prognosis

The tumour, Node, Metastasis (TNM) stage at presentation in patients with NSCLC is the factor that has the greatest impact on prognosis. Survival decreases progressively with more advanced disease, according to both the previous and the eighth edition staging systems (figure 5) (246). Other clinical and pathological factors present at the time of diagnosis can predict survival independent of the disease stage. These factors were identified in studies that primarily included patients with advanced NSCLC and early stages with surgical approach: age (247), performance status (248), smoking cessation (247), histology (249), degree of differentiation (250), lymphatic invasion (251), occult lymph node metastases (252), intense tumour lymphocytic infiltration (253), driver mutations (254) and expression of tumour PD-L1 (255).

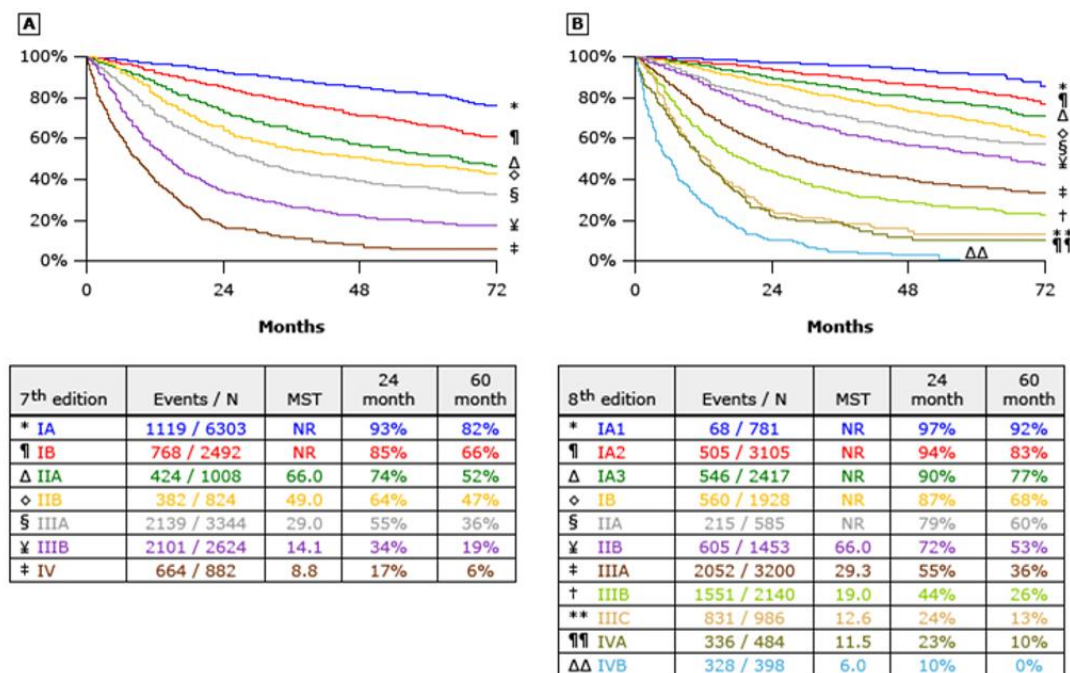


Figure 5. Overall survival by clinical stage according to the seventh edition (A) and the eighth edition (B) groupings using the entire database available for the eighth edition. Survival is weighted by type of database submission: registry versus other. N: number of patients; MST: median survival time; NR: not reached. Reproduced from: Goldstraw P, Chansky K, Crowley J, et al. The IASLC Lung Cancer Staging Project: Proposals for Revision of the TNM Stage Groupings in the Forthcoming (Eighth) Edition of the TNM Classification for Lung Cancer. *J Thorac Oncol* 2016; 11:39.

II. Aim

The aim of the investigation presented in this thesis was to study the association of putatively functional genetic polymorphisms in renin-angiotensin pathway, hypoxia and angiogenesis with lung cancer aggressiveness, response to systemic therapy, and its predictive and prognostic value in patients with locally advanced and advanced NSCLC.

The specific objectives are:

1. Evaluate the association of a selected panel of putatively functional genetic polymorphisms in genes coding for renin-angiotensin pathway proteins (*ACE*, *ACE2*, *AGT*, *AGTR1*, *AGTR2*, *CMA1*, *MME*, *MAS1*), hypoxia (*HIF1A*) and angiogenesis genes (*VEGFA*, *PGF*, *KDR* and *FLT1*), with clinicopathological variables, disease progression and overall survival in stage IIIA-IVB NSCLC at diagnosis.
2. Analyse whether a RA/hypoxia/angiogenesis-based multilocus genetic risk analysis combined with relevant clinicopathological covariates outperforms classical clinicopathological variables in predictive and prognostic values (endpoint 1: disease progression; endpoint 2: overall survival). This study included a discovery set and an independent validation set.
3. Evaluate whether the functional genetic polymorphism *AGXT* Pro11Leu was associated with clinical outcomes in NSCLC patients, under platinum-based chemotherapy regimen.

III. Material and Methods

1.1 Study Population

1.1.1 Discovery and Validation Set

This study comprised a retrospective cohort of histologically confirmed NSCLC patients ($n = 167$) with clinical advanced stage at diagnosis (IIIA-IVB), between 2012 and 2018. A nested group was prospectively followed since the first anti-neoplastic treatment ($n=28$). Participants were consecutively recruited during routine follow-up visit, since August 2017 to October 2018 from Coimbra University Hospital (discovery set). The validation set comprised an independent cohort of NSCLC patients ($n = 190$) selected from the Lung Biobank Heidelberg, member of the Biomaterialbank Heidelberg (BMBH) and the biobank platform of the German Centre for Lung Research (DZL). All patients were recruited at Thoraxklinik (University Hospital of Heidelberg) between 2015–2018, using the same matched clinical and pathological criteria from the discovery set.

Histopathological diagnoses in both sets were made by experienced pathologists following the 2009 and 2015 WHO classification criteria for lung cancer, accordingly to the year of patient's diagnosis. The inclusion criteria for both cohorts were: 1) diagnosis of NSCLC (stage IIIA-IVB) confirmed histologically at the institutions; 2) expected survival of at least 1 month to the date of inclusion; 3) patients aged 18 years or older; 4) possibility of collecting peripheral blood (in a vein peripheral or central venous catheter); 5) signed informed consent. The absence of the inclusion criteria and the existence of concomitant primary tumours in another organ were considered as exclusion criteria for both cohorts.

Clinical data was retrieved from clinical charts on pathological background, previous medication, Eastern Cooperative Oncology Group performance status (ECOG PS) at diagnosis, TNM staging according to 8th classification, tumour mutational status, type of cancer treatment, disease progression/death by clinical authors. Information on chemotherapy related febrile neutropenia (grade 3-4) in patients admitted to hospital wards was retrieved from clinical charts. Informed consent was obtained from each participant and all procedures are in agreement with Helsinki Declaration.

In the discovery set, somatic mutations in *EGFR*, *ALK* and *ROS1* were determined using fluorescence in situ hybridization (FISH), in patients with adenocarcinoma stage IVA-IVB, whereas for the validation set, somatic mutations were determined through panel-based next generation sequencing in advanced NSCLC patients (256). In both cohorts, targeted therapies were administered to carriers of actionable driver mutations, whereas checkpoint inhibitors were used as second-, or third-line therapy for patients with PD-L1 expression on tumour cells below 50%. The primary endpoint was PFS and the time-to-disease progression was calculated in months, from the date of first line chemotherapy/target therapy, until the date of progression according to RECIST criteria. OS was included as secondary endpoint, and the time-to-death was computed in months from the date of first line chemotherapy/target therapy until the date of death/date of last visit.

1.2 Selection of Genetic Polymorphisms

Fifty-two SNP were included here from genes *AGXT*, *ACE*, *ACE2*, *AGT*, *AGTR1*, *AGTR2*, *MME*, *CMA1*, *MAS1*, *HIF1A*, *VEGFA*, *KDR*, *PGF* and *FLT1* (Table 2). They were selected from search in genomic public databases, in silico analysis and review of scientific literature, to identify putatively functional polymorphisms (169, 173, 181, 257-265). Variants with minor allele frequency below 1% were excluded.

Genes	Single Nucleotide Polymorphisms
<i>AGXT</i>	rs34116584
<i>ACE</i>	rs121912703, rs4316, rs4317
<i>ACE2</i>	rs199951323, rs4646116, rs7890520, rs908004
<i>AGT</i>	rs699, rs121912923, rs4762
<i>AGTR1</i>	rs1800766, rs380400, rs5186, rs5185, rs5189, rs5187, rs12721226
<i>AGTR2</i>	rs11091046, rs5191, rs35474657, rs5194
<i>MME</i>	rs652438, rs701109, rs989692, rs777476150
<i>CMA1</i>	rs5250, rs7142020, rs140295781, rs201312283
<i>MAS1</i>	rs147267209, rs11968301, rs138263796, rs139579440
<i>HIF1A</i>	rs52801082, rs2057482, rs11549465, rs11549467
<i>VEGFA</i>	rs2010963, rs3025039, rs25648, rs10434, rs41282644
<i>KDR</i>	rs7667298, rs1870377, rs2305948
<i>PGF</i>	rs8185, rs12411, rs140032352
<i>FLT1</i>	rs2296283, rs7326277, rs9554314

Table 2: Representative single nucleotide polymorphisms of *AGXT* gene, genes involved in RAS (*ACE*, *ACE2*, *AGT*, *AGTR1*, *AGTR2*, *MME*, *CMA1*, *MAS1*) and SNPs of genes that express hypoxia-inducible factor 1-alpha, vascular endothelial growth factor and its receptor, placental growth factor and its receptor.

1.3 Genotyping

1.3.1 Discovery Set

Each patient donated a sample of blood (~4 mL) for research, collected to EDTA-Vacutainer tubes, at the same time of blood collection for routine analytic follow-up. The collected blood was centrifuged to obtain the buffy coat, which was used to isolate and purify DNA by the EZ1 DNA Blood kit in the EZ1 BioRobot (QIAGEN, Hilden, Germany). The selected SNPs were genotyped using the Sequenom Mass ARRAY matrix-assisted laser desorption/ionization time-of-flight mass spectrometry platform (Sequenom, San Diego, CA, USA). Primers were designed using semi-automated Assay Design 3.1

Software (Sequenom). The global genotyping success rate was 99.85% with 100% replication agreement among one third of the samples.

1.3.2 Validation Set

Buffy coat samples were provided by Lung Biobank Heidelberg from patients diagnosed with advanced NSCLC (clinical stage IIIA-IVB). DNA extraction and purification were conducted using the FlexiGene DNA Kit (QIAgen). The resulting genetic polymorphisms, associated with disease progression or overall survival from multivariate analysis in the discovery set, were genotyped in the validation set using Real-Time PCR. Allelic discrimination was obtained using TaqMan SNP genotyping assays: *ACE2* rs908004 (Assay ID: C__8816953_20), *MME* rs701109 (Assay ID: C__964850_10) and *KDR* rs1870377 (Assay ID: C__11895315_20) (all from Thermo Fisher Scientific, Waltham, MA, USA). Quality control included non-template controls in all runs and blind replicate assessment in 5% of the samples. Genotype was assigned to 96.3% of all samples tested.

1.3.3 Statistical Analysis

Descriptives were depicted as average $\pm$ standard deviation or median (interquartile range) according to departure from normality (Shapiro-Wilk test). Central tendency data was compared using the *t*-test, Mann-Whitney or Kruskal-Wallis tests according to normality and stratification of independent variables. Categorical variables were expressed as frequency (percentage) and difference in proportions tested using the chi-square test. The difference in median months of PFS and OS from both cohorts was calculated through Kaplan-Meier curve with log rank test.

Four genetic models were considered in this study: each SNP was stratified according to the wild type allele into additive ("AA" genotype versus "Aa" versus "aa"), dominant ("AA" genotype versus "Aa" and "aa" genotypes), recessive ("Aa" and "AA" versus "aa" genotype) and heterosis ("Aa" versus "AA" and "aa" genotypes). The wild-type allele for each SNP was designated in agreement with Ensembl (<https://www.ensembl.org/index.html>). Empirical analyses were conducted on clinicopathological and genetic variables to test the association with time-to-outcome, using Cox regression analyses. After univariate analyses, $p < 0.10$ and an effect $> 30\%$ in

hazard ratio (HR) were used as criteria for inclusion in a multivariate Cox with stepwise regression model. Genetic models with under-represented variant genotype ($n < 4$) were excluded from the multivariate analysis, and only the genetic model with the highest likelihood ratio for each SNP was included in the multivariate model to avoid collinearity. The resulting multivariate model was further analysed with bootstrapping (MonteCarlo simulations, 1000 iterations), to elect the resulting relevant markers to be tested in the validation set. To investigate the possible predictive value of each SNP in treatment response for first line therapy, and time-to-progression or time-to-death, exploratory analyses were conducted examining the effect of genotype in a subgroup of patients treated with first line platinum doublet chemotherapy and in another subgroup of wild-type *EGFR* (epidermal growth factor receptor) patients, using the same survival analysis methods. Sample size, power, effect size and number of events for survival analyses (assuming $\alpha = 0.05$ and power > 0.8) were estimated in both cohorts. Statistical analyses were performed on STATA software version 16.0 (StataCorp, College Station, TX, USA).

1.3.4 Ethics Approval

This project has been reviewed and approved by Coimbra University Hospital's Ethical Committee (ref. no. 0111/CES) and by the Portuguese National Committee for data protection (ref. no. 2588/2017). The biobank protocol of Lungbiobank Heidelberg was approved by the ethics committee of the University of Heidelberg (S-270/2001).

1.4 Bioinformatic Analysis of the Renin-Angiotensin Pathway Transcriptome

The Cancer Genome Atlas (TCGA; <https://cancergenome.nih.gov/>) dataset of lung adenocarcinoma (LUAD) was analysed and survival plots generated in SPSS (version 26) using Kaplan Meier curves. "High" and "low" gene expression levels of RAS were calculated taking into account the fragments per kilobase of transcript per million (FPKM) values that yields maximal difference with regard to survival between the two groups, at the lowest log-rank P value. Transcript levels and clinical information were both provided by the TCGA consortium for a total of 500 patients with lung

adenocarcinoma. RAS related transcripts were compared in matched samples of normal adjacent tumoral tissue and the tumour tissue of TCGA LUAD dataset.

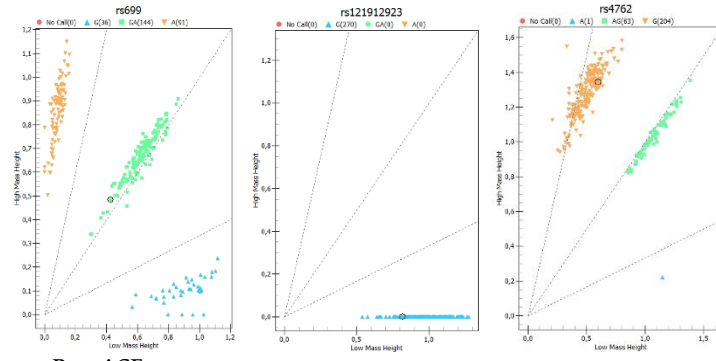
IV. Results

1.1 Impact of genetic polymorphisms of Renin-Angiotensin pathway and angiogenic factors in NSCLC prognosis

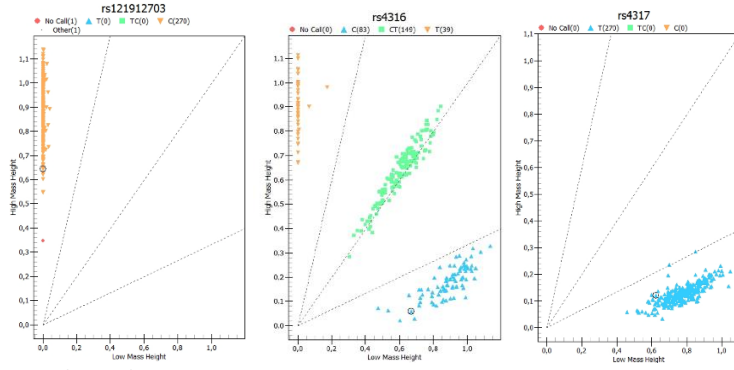
The clinicopathological characteristics of participating subjects from discovery and validation sets are described in Table 3. Regarding mutational status from discovery set (n = 167), we observed that 10.8% of patients (n = 18) had *EGFR* mutation (exon 19 deletions or exon 21 mutation), whereas 3.0% (n = 5) had rearrangements in the gene encoding anaplastic lymphocyte kinase (*ALK*). Mutations of *ROS1* were not detected. From the validation set (n = 190), 15.3% (n = 29) of NSCLC patients had *EGFR* mutation (exon 19 deletions or exon 21 mutation), 13.7% (n = 26) were *KRAS* mutated, 4.2% (n = 8) had *EML4-ALK* fusion, 2.6% (n = 5) had *BRAF* V600E mutation. No other targetable mutations were identified. Concerning respiratory co-morbidities from discovery set, twenty patients (12.0%) had moderate-severe COPD with lung emphysema and one patient (0.6%) had sarcoidosis, whereas in the validation set there were fifty patients (26.3%) with moderate-severe COPD and lung emphysema, and five patients (2.6%) had idiopathic pulmonary fibrosis.

The description of clinical-pathological variables is depicted in Table 4 with report of univariate analyses for PFS and OS. Genotypic distribution of functional SNPs in genes of the renin-angiotensin pathway, hypoxic and angiogenic factors using additive, dominant, recessive and heterosis models from discovery set are described in Table 5, together with hazard and survival univariate analyses. SNPs without variation in this population included *ACE* rs4317, *ACE* rs121912703, *ACE2* rs4646116, *ACE2* rs1999951323, *AGT* rs121912923, *AGTR1* rs12721226, *AGTR1* rs5187, *AGTR2* rs5191, *AGTR2* rs35474657, all SNPs studied for *MAS1*, *HIF1A* rs11549467, *PGF* rs140032352, *CMA1* rs140295781, *CMA1* rs201312283, *CMA1* rs7142020, *MME* rs777476150. We also verified the following Tag SNPs: *AGTR2* (rs5194, rs11091046), *FLT1* (rs9554314, rs11549465), *HIF1A* rs52801082, *KDR* (rs1531289, rs2239702, rs7667298, rs2305948, rs1870377). The analysis of genetic polymorphisms through mass spectrometry, included in this study, are represented in plots (figures 6, 7, 8).

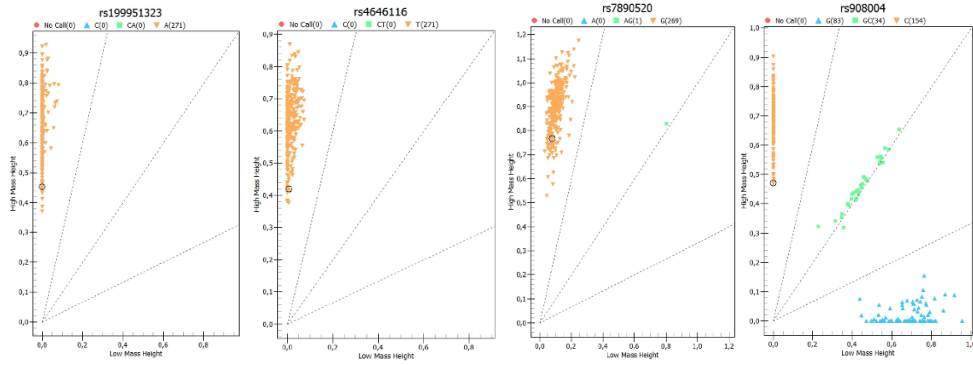
A. AGT



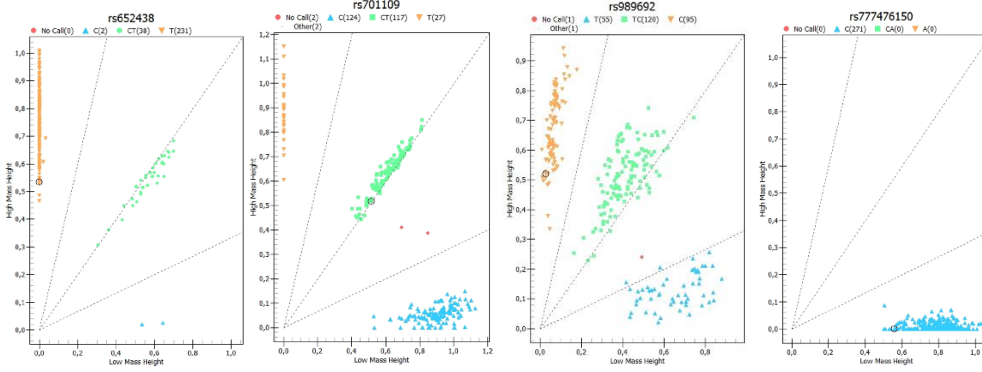
B. ACE



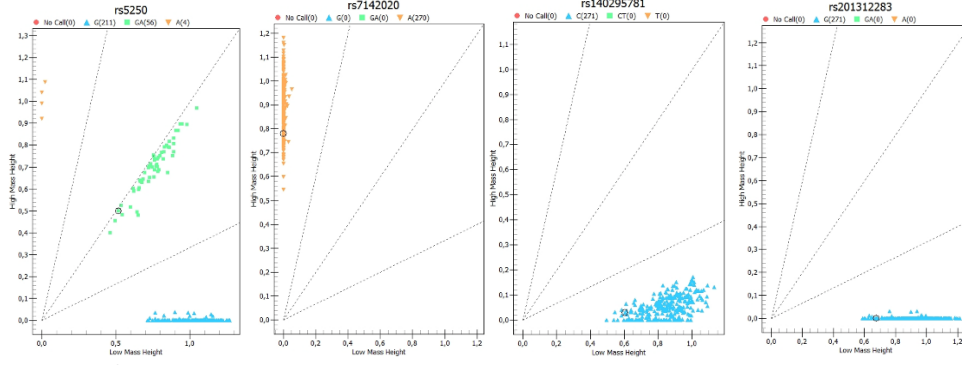
C. ACE2



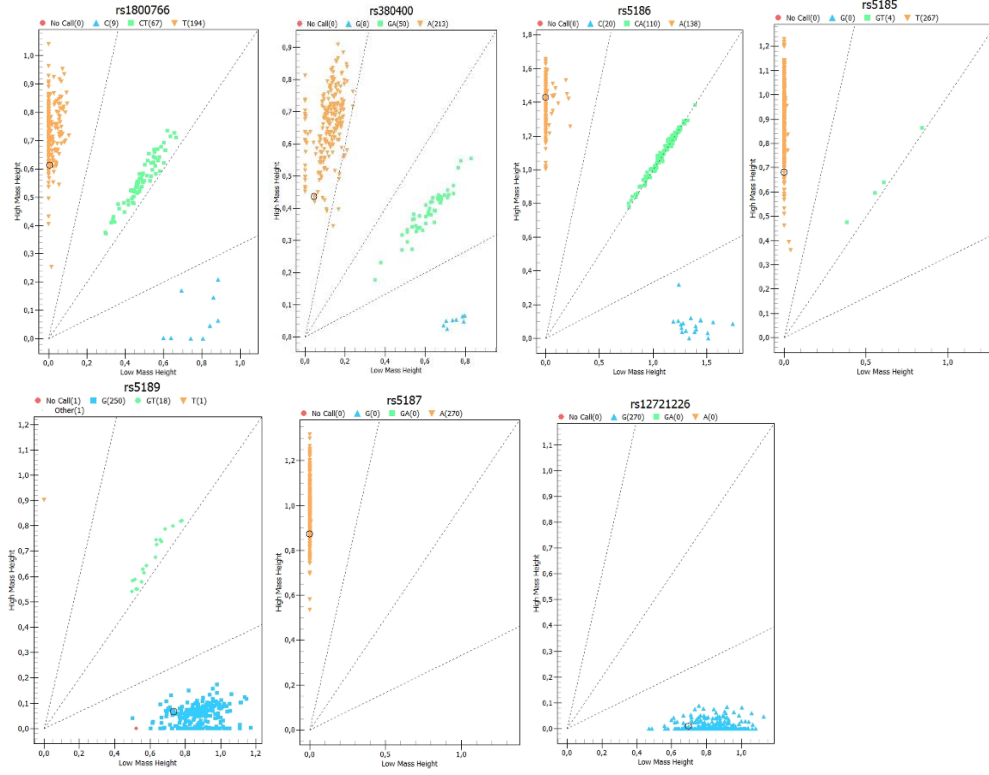
D. MME



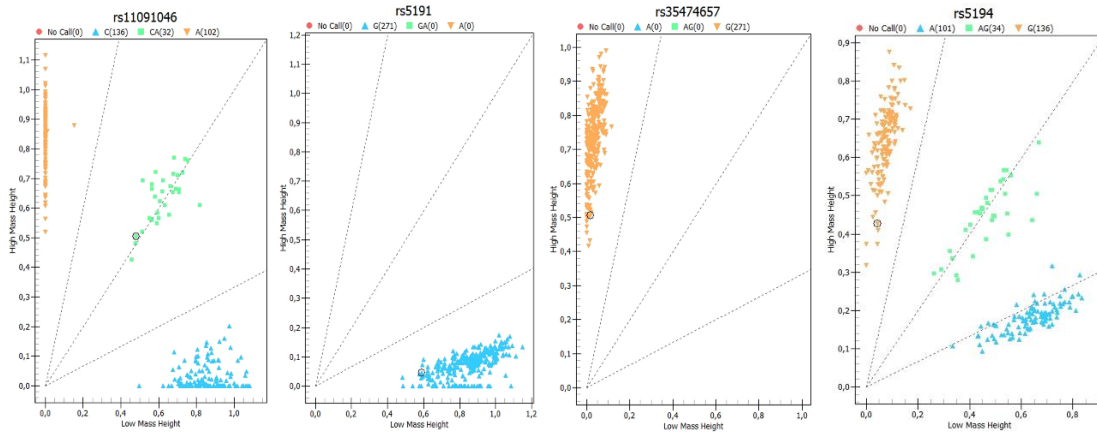
E. CMA1



F. AGTR1



G. AGTR2



H. MAS1

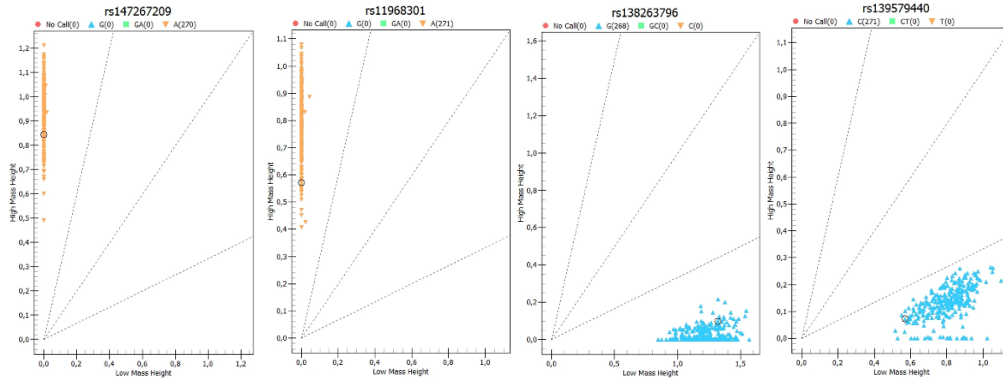
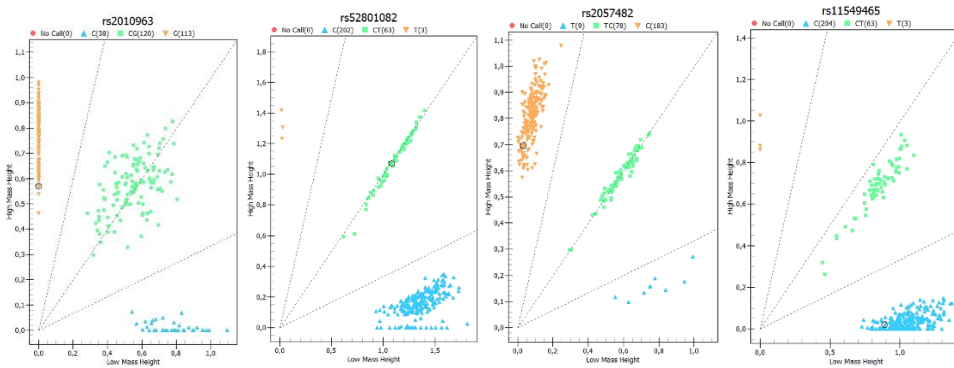
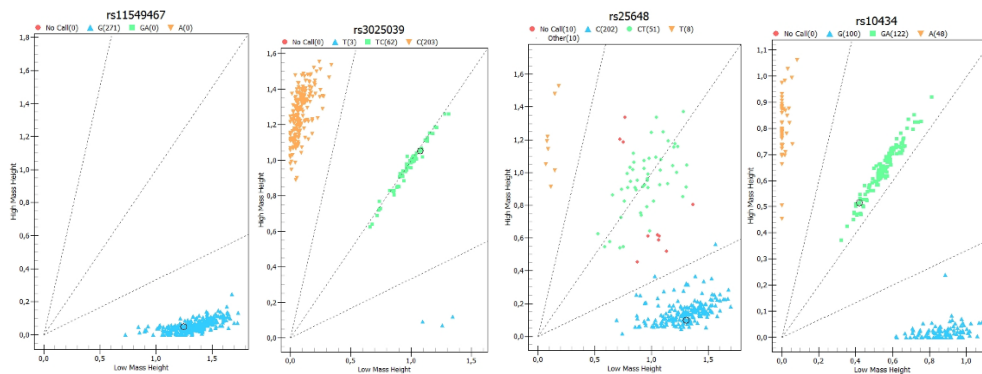


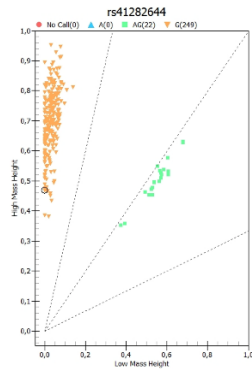
Figure 6. Analysis of genetic polymorphisms through mass spectrometry. A) *AGT* rs699, rs121912923, rs4762; B) *ACE* rs121912703, rs4316, rs4317; C) *ACE 2* rs199951323, rs4646116, rs7890520, rs908004; D) *MME* rs652438, rs701109, rs989692, rs777476150; E) *CMA1* rs5250, rs7142020, rs140295781, rs201312283 F) *AGTR1* rs1800766, rs380400, rs5186, rs5185, rs5189, rs5187, rs12721226; G) *AGTR2* rs11091046, rs5191, rs35474657, rs5194; H) *MAS1* rs147267209, rs11968301, rs138263796, rs139579440.

A. HIF1A

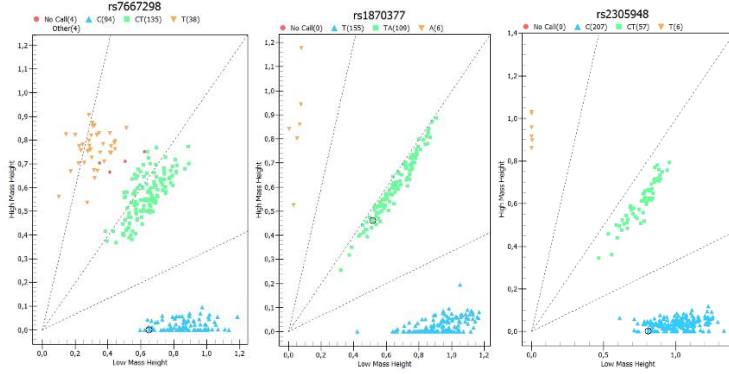


B. VEGFA

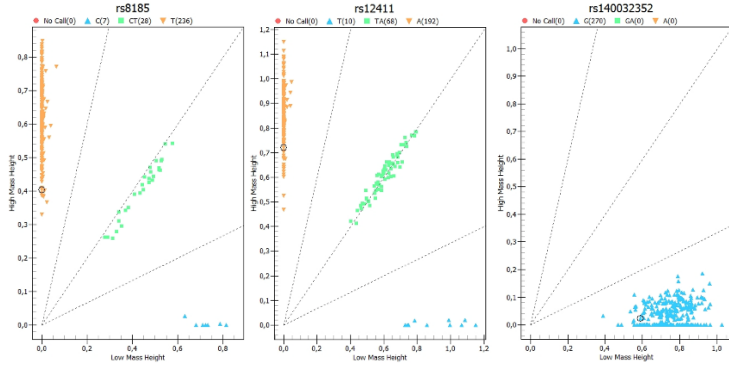




C. *KDR*



D. *PGF*



E. *FLT1*

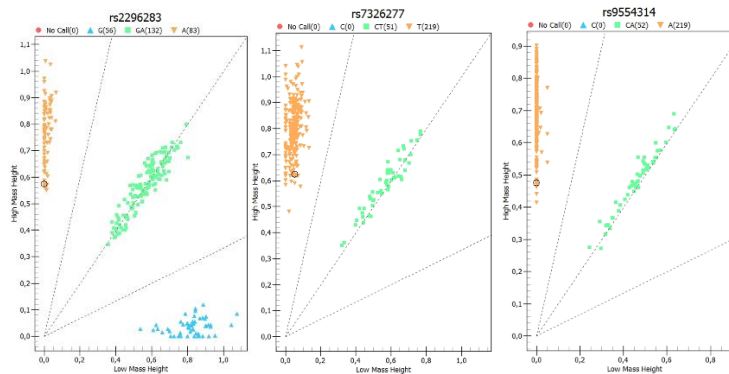


Figure 7. Analysis of genetic polymorphisms through mass spectrometry. A) *HIF1A* rs52801082, rs2057482, rs11549465, rs11549467; B) *VEGFA* rs2010963, rs3025039, rs25648, rs10434, rs41282644; C) *KDR* rs7667298, rs1870377, rs2305948; D) *PGF* rs8185, rs12411, rs140032352; E) *FLT1* rs2296283, rs7326277, rs9554314.

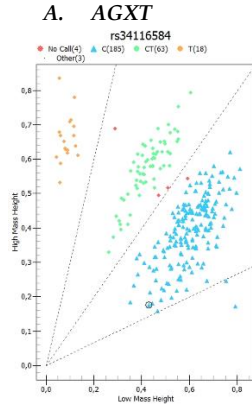


Figure 8. Analysis of genetic polymorphisms through mass spectrometry. A) AGXT 34116584.

The univariate analysis showed that carriers of allele T ($n = 112$, 67.1%) for the SNP rs4316 in *ACE* gene had a significant protection to disease progression (Hazard ratio (HR) = 0.6, 95% confidence interval (CI) (0.4–0.9), $p = 0.011$). Homozygous GG/CC for *ACE2* rs908004 ($n = 151$, 90.4%) had increased risk for disease progression (HR = 2.1, 95% CI (1.1–3.9), $p = 0.021$). Another SNP from *MME* gene that encodes for neprilysin, showed a tendency between the dominant genetic model of *MME* rs701109 and PFS, having the heterozygous CT and homozygous CC protection to disease progression (HR = 0.6, 95% CI (0.3–1.0), $p = 0.064$). Univariate analysis also showed a significant association between the functional genetic variant of *VEGFA* rs25648 with time to tumour progression, being the homozygous C and T ($n = 127$, 78.9%) associated with worse prognosis (HR = 1.7, 95% CI (1.1–2.6), $p = 0.030$). The data showed a tendency between the heterosis genetic model of *KDR* rs1870377 and time to tumour progression, having the homozygous A and T increased risk to disease progression (HR = 1.4, 95% CI (1.0–2.0), $p = 0.054$) and to death (HR = 1.7, 95% CI (1.0–2.7), $p = 0.034$).

Clinical variables	Discovery set (N=167)	Validation set (N=190)	P
Gender, n			
Males	123 (73.7%)	133 (70.0%)	0.445*
Females	44 (26.3%)	57 (30.0%)	
Age (years)			
Median (IQR)	64.0 (58.0-72.0)	65.0 (58.0-73.0)	0.773**
Smoking Status, n (%)			
Never smokers	31 (27.7%)	20 (10.5%)	<0.0001*
Previously smokers	68 (60.7%)	97 (51.1%)	
Smokers	13 (11.6%)	73 (38.4%)	
Hypertension, n (%)			
Yes	67 (48.6%)	91 (47.9%)	0.911*
No	71 (51.4%)	99 (52.1%)	
Anti-hypertension drugs, n (%)			
No	86 (65.2%)	126 (67.0%)	0.728*
iACE/ARB	46 (34.8%)	62 (33.0%)	
ECOG PS, n (%)			
0-2	163 (97.6%)	188 (98.9%)	0.153*
>2	4 (2.4%)	2 (1.1%)	
Histology, n (%)			
Adenocarcinoma	116 (69.5%)	126 (66.3%)	0.066*
Squamous cell carcinoma	42 (25.1%)	62 (32.6%)	
Adeno+Squamous cell	9 (5.4%)	2 (1.1%)	
cTNM Stage, n (%)			
IIIA	20 (12.0%)	27 (14.2%)	0.597*
IIIB	32 (19.2%)	36 (18.9%)	
IIIC	21 (12.6%)	18 (9.5%)	
IVA	65 (38.9%)	66 (34.7%)	
IVB	29 (17.4%)	43 (22.6%)	
Treatment modality, n (%)			
Surgery+ChT	12 (7.2%)	0 (0.0%)	<0.0001*
ChT+RT	31 (18.6%)	81 (42.6%)	
ChT	124 (74.3%)	109 (57.4%)	
First-line treatment, n (%)			
Platinum based ChT	145 (86.8%)	155 (81.6%)	0.194*
TKI	22 (12.8%)	35 (18.4%)	
PFS (months)			
Median, 95% CI	7.5 (6.1-9.0)	8.1 (6.8-9.4)	0.415***
OS (months)			
median, 95% CI	30.0 (16.9-43.2)	20.6 (16.3-24.9)	0.036***

Table 3. Patient's clinical characteristics. IQR – interquartile range; iACE – inhibitors of angiotensin-converting enzyme, ARB – AngiotensinII receptor blocker, ECOG PS – Eastern cooperative Oncology Group Performance Status; ChT – chemotherapy; RT – radiotherapy; TKI – tyrosine kinase inhibitors; CI95% – confidence interval. PFS – progression-free survival, OS – overall survival. *Pearson Chi Square test. **Mann-Whitney U test. *** Kaplan Meier curve with log rank test.

Table 4	PFS				OS		
	N	HR (95CI)	P *	Ptrend	HR (95CI)	P	Ptrend
<i>Clinical variables</i>							
Age							
≤64.0	85 (0.51)	Referent			Referent		
>64.0	82 (0.49)	0.9 (0.7-1.3)	0.678	-	1.6 (1.0-2.5)	0.047	-
Gender							
Male	123 (0.74)	Referent			Referent		
Female	44 (0.26)	0.9 (0.6-1.3)	0.539	-	0.6 (0.4-1.1)	0.105	-
Histology							
Adenocarcinoma	116 (0.70)	Referent			Referent		
Squamous cell	42 (0.25)	1.4 (0.9-2.1)	0.116		1.8 (1.1-3.0)	0.027	
Others *	9 (0.05)	0.8 (0.4-1.7)	0.571	0.680	0.9 (0.4-2.4)	0.902	0.291
T							
1	26 (0.16)	Referent			Referent		
2	46 (0.27)	1.3 (0.7-2.3)	0.339		0.5 (0.2-1.2)	0.138	
3-4	95 (0.57)	2.1 (1.3-3.6)	0.004	0.001	1.4 (0.8-2.7)	0.276	0.047
N							
N0-N2	63 (0.38)	Referent			Referent		
N3	103 (0.62)	1.4 (0.9-1.9)	0.099	-	1.1 (0.7-1.7)	0.815	-
Distant Metastasis							
No	73 (0.44)	Referent			Referent		
Yes	94 (0.56)	1.7 (1.2-2.4)	0.003	-	2.5 (1.5-4.2)	<0.001	
Type_Therapy							
Surgery+ChT	12 (0.07)	Referent			Referent		
ChT	124 (0.74)	2.9 (1.3-6.6)	0.012		2.7 (0.8-8.6)	0.099	
ChT+RT	31 (0.19)	3.1 (1.3-7.6)	0.011	0.026	3.0 (0.9-10.2)	0.080	0.127
ECOG PS							
Good (0-2)	163 (0.98)	Referent			Referent		
Poor (3-4)	4 (0.02)	1.4 (0.5-3.8)	0.501	-	2.1 (0.6-6.6)	0.223	-
Anti-hypertensive drugs							
No	86 (0.65)	Referent			Referent		
iACE/ARB	46 (0.35)	0.8 (0.6-1.3)	0.442	-	1.2 (0.7-2.1)	0.538	-
Type_SysTher							
Platinum based	145 (0.87)	Referent			Referent		
TKI	22 (0.13)	0.5 (0.3-0.9)	0.014	-	0.6 (0.3-1.4)	0.218	-

Table 4. Clinical-pathological variables and progression-free survival (PFS) and overall survival (OS) in discovery set (n=167) – Univariate analysis. HR – Hazard Ratio. ChT – chemotherapy, RT – radiotherapy, TKI - tyrosine kinase inhibitor, iACE – inhibitors of angiotensin-converting enzyme, ARB – AngiotensinII receptor blocker, Type_SysTher – type of systemic therapy.

Table 5									
Genetic variants	Genetic model	Genotype	Progression-free Survival				Overall Survival		
			N (freq.)	HR (95CI)	P *	Ptrend	HR (95CI)	P *	Ptrend
ACE rs4316	Additive	CC	55 (0.33)	Referent			Referent		
		CT	82 (0.49)	0.6 (0.4-0.9)	0.013		0.6 (0.4-1.1)	0.089	
		TT	30 (0.18)	0.7 (0.4-1.1)	0.113	0.049	0.8 (0.4-1.2)	0.541	0.308
	Dominant	CC	55 (0.33)	Referent			Referent		
		CT/TT	112 (0.67)	0.6 (0.4-0.9)	0.011	-	0.7 (0.4-1.1)	0.114	
	Recessive	CC/CT	137 (0.82)	Referent			Referent		
		TT	30 (0.18)	0.9 (0.6-1.4)	0.676	-	1.0 (0.6-1.9)	0.896	
	Heterosis	CT	82 (0.49)	Referent			Referent		
		CC/TT	85 (0.51)	1.4 (1.0-2.0)	0.058	-	1.5 (0.9-2.3)	0.116	
ACE rs4317 *		TT	167 (1.0)	-	-	-	-	-	-
ACE rs121912703 *		CC	167 (1.0)	-	-	-	-	-	-
ACE2 rs908004	Additive	GG	55 (0.33)	Referent			Referent		
		GC	16 (0.09)	0.4 (0.2-0.9)	0.018		0.5 (0.2-1.1)	0.089	
		CC	96 (0.58)	0.9 (0.6-1.3)	0.566	0.794	0.9 (0.5-1.4)	0.587	0.681
	Dominant	GG	55 (0.33)	Referent			Referent		
		GC/CC	112 (0.67)	0.8 (0.6-1.2)	0.236	-	0.8 (0.5-1.3)	0.320	
	Recessive	GG/GC	71 (0.42)	Referent			Referent		
		CC	96 (0.58)	1.1 (0.8-1.6)	0.539	-	1.0 (0.7-1.7)	0.849	
	Heterosis	GC	16 (0.10)	Referent			Referent		
		GG/CC	151 (0.90)	2.1 (1.1-3.9)	0.021	-	2.0 (0.9-4.9)	0.109	
ACE2 rs4646116 *		TT	167 (1.0)	-	-	-	-	-	-
ACE2 rs7890520		GG	166 (0.99)	Referent					
		AG	1 (0.01)	-	-	-	-	-	-
ACE2 rs1999951323*		A	167 (1.0)	-	-	-	-	-	-
AGT rs121912923 *		GG	167 (1.0)	-	-	-	-	-	-
AGT rs4762	Additive	GG	135 (0.81)	Referent			Referent		
		GA	31 (0.18)	1.0 (0.6-1.5)	0.964		0.8 (0.5-1.5)	0.495	0.495
		AA	1 (0.01)	-	-	-	-	-	-
	Dominant	GG	135 (0.81)	Referent			Referent		
		GA/AA	32 (0.19)	1.0 (0.6-1.5)	0.917	-	0.8 (0.4-1.4)	0.389	
	Recessive	GG/GA	166 (0.99)	Referent			Referent		
		AA	1 (0.01)	-	-	-	2.0 (0.3-14.5)	0.492	
	Heterosis	GA	31 (0.19)	Referent			Referent		
		GG/GA	136 (0.81)	1.1 (0.7-1.7)	0.758	-	1.4 (0.7-2.7)	0.308	
AGT rs699	Additive	GG	23 (0.14)	Referent			Referent		
		GA	87 (0.52)	0.8 (0.5-1.3)	0.370		1.6 (0.7-3.7)	0.303	
		AA	57 (0.34)	0.9 (0.5-1.6)	0.710	0.997	1.6 (0.7-4.0)	0.272	0.361
	Dominant	GG	23 (0.14)	Referent			Referent		
		GA/AA	144 (0.86)	0.8 (0.5-1.4)	0.464	-	1.6 (0.7-3.7)	0.271	
	Recessive	GG/GA	110 (0.66)	Referent			Referent		
		AA	57 (0.34)	1.1 (0.8-1.6)	0.622	-	1.1 (0.7-1.8)	0.634	
	Heterosis	AG	87 (0.52)	Referent			Referent		
		GG/AA	80 (0.48)	1.2 (0.8-1.7)	0.350	-	0.9 (0.6-1.5)	0.780	
AGTR1 rs12721226 *		GG	167 (1.0)	-	-	-	-	-	-
AGTR1 rs1800766	Additive	TT	115 (0.69)	Referent			Referent		
		TC	46 (0.27)	1.1 (0.7-1.6)	0.641		0.7 (0.4-1.3)	0.245	
		CC	6 (0.04)	1.0 (0.4-2.7)	0.999	0.726	0.4 (0.1-2.7)	0.372	0.135
	Dominant	TT	115 (0.69)	Referent			Referent		
		TC/CC	52 (0.31)	1.1 (0.7-1.6)	0.666	-	0.7 (0.4-1.2)	0.162	
	Recessive	TT/TC	161 (0.96)	Referent			Referent		
		CC	6 (0.04)	1.0 (0.4-2.6)	0.959	-	0.4 (0.1-2.9)	0.366	
	Heterosis	TC	46 (0.27)	Referent			Referent		
		TT/CC	121 (0.73)	0.9 (0.6-1.3)	0.639	-	1.4 (0.8-2.5)	0.284	
AGTR1 rs5187 *		AA	167 (1.0)	-	-	-	-	-	-
AGTR1 rs380400	Additive	GG	5 (0.03)	Referent			Referent		
		GA	31 (0.19)	0.4 (0.1-1.1)	0.067		0.3 (0.1-0.9)	0.037	
		AA	131 (0.78)	0.5 (0.2-1.3)	0.148	0.778	0.4 (0.1-1.2)	0.093	0.932
	Dominant	GG	5 (0.03)	Referent			Referent		
		GA/AA	162 (0.97)	0.5 (0.2-1.2)	0.124	-	0.3 (0.1-1.1)	0.073	
	Recessive	GG/GA	36 (0.22)	Referent			Referent		
		AA	131 (0.78)	1.2 (0.8-1.8)	0.455	-	1.2 (0.7-2.2)	0.524	
	Heterosis	AG	31 (0.19)	Referent			Referent		
		GG/AA	136 (0.81)	1.3 (0.8-2.1)	0.224	-	1.5 (0.8-2.8)	0.228	
AGTR1 rs5186	Additive	AA	87 (0.52)	Referent			Referent		
		AC	66 (0.40)	1.0 (0.7-1.5)	0.901		0.9 (0.6-1.5)	0.755	
		CC	14 (0.08)	1.3 (0.7-2.6)	0.370	0.513	1.9 (0.8-4.2)	0.131	0.461
	Dominant	AA	87 (0.52)	Referent			Referent		
		AC/CC	80 (0.48)	1.1 (0.8-1.5)	0.715	-	1.0 (0.6-1.6)	0.892	
	Recessive	AA/AC	153 (0.92)	Referent			Referent		
		CC	14 (0.08)	1.3 (0.7-2.5)	0.371	-	1.9 (0.9-4.3)	0.102	
	Heterosis	AC	66 (0.39)	Referent			Referent		
		AA/CC	101 (0.61)	1.0 (0.7-1.4)	0.939		1.2 (0.7-1.9)	0.535	
AGTR1 rs5185		TT	164 (0.98)	Referent					
AGTR1 rs5189	Additive	TG	3 (0.02)	0.2 (0.0-1.4)	0.102	-	-	-	-
		TT	1 (0.01)	Referent			Referent		
		TG	12 (0.07)	-	-	-	-	-	-
	Dominant	GG	154 (0.92)	-	-	-	1.2 (0.4-3.4)	0.665	0.665
		TT	1 (0.01)	Referent			Referent		
	Recessive	TG/GG	166 (0.99)	-	-	-	-	-	-
		TT/TG	13 (0.08)	Referent			Referent		
	Heterosis	GG	154 (0.92)	1.0 (0.5-1.8)	0.928	-	1.7 (0.5-5.4)	0.375	
		GT	12 (0.07)	Referent			Referent		
AGTR2 rs5191 *		TT/GG	155 (0.93)	1.1 (0.5-2.1)	0.817	-	2.6 (0.6-10.5)	0.190	
AGTR2 rs5194		GG	167 (1.0)	-	-	-	-	-	-
AGTR2 rs5194	Additive	GG	85 (0.51)	Referent			Referent		
		GA	20 (0.12)	1.1 (0.6-1.9)	0.795		1.1 (0.5-2.3)	0.801	
		AA	62 (0.37)	1.0 (0.7-1.5)	0.856	0.848	0.9 (0.5-1.5)	0.692	0.706
	Dominant	GG	85 (0.51)	Referent			Referent		
		AG/AA	82 (0.49)	1.0 (0.7-1.5)	0.804	-	0.9 (0.6-1.5)	0.815	

	Recessive	GG/AG	105 (0.63)	Referent			Referent		
		AA	62 (0.37)	1.0 (0.7-1.5)	0.910	-	0.9 (0.5-1.4)	0.628	
	Heterosis	AG	20 (0.12)	Referent			Referent		
AGTR2 rs11091046		GG/AA	147 (0.88)	0.9 (0.6-1.6)	0.828	-	0.9 (0.4-1.7)	0.702	
	Additive	AA	62 (0.37)	Referent			Referent		
		AC	20 (0.12)	1.0 (0.6-1.8)	0.893		1.2 (0.6-2.6)	0.615	
		CC	85 (0.51)	1.0 (0.7-1.4)	0.856	0.848	1.1 (0.7-1.8)	0.692	0.706
	Dominant	AA	62 (0.37)	Referent			Referent		
		AC/CC	105 (0.63)	1.0 (0.7-1.4)	0.910	-	1.1 (0.7-1.8)	0.628	
	Recessive	AA/AC	82 (0.49)	Referent			Referent		
		CC	85 (0.51)	1.0 (0.7-1.3)	0.804	-	1.1 (0.7-1.7)	0.815	
	Heterosis	AA	20 (0.12)	Referent			Referent		
		AA/CC	147 (0.88)	0.9 (0.6-1.6)	0.828	-	0.9 (0.4-1.8)	0.702	
AGTR2 rs35474657 *		AA	167 (1.0)	-	-	-	-	-	-
MAS1 rs11968301 *		AA	167 (1.0)	-	-	-	-	-	-
MAS1 rs138263796 *		GG	167 (1.0)	-	-	-	-	-	-
MAS1 rs139579440 *		CC	167 (1.0)	-	-	-	-	-	-
MAS1 rs147267209 *		AA	167 (1.0)	-	-	-	-	-	-
VEGFA rs10434	Additive	GG	55 (0.33)	Referent			Referent		
		GA	81 (0.48)	1.1 (0.8-1.7)	0.486		1.0 (0.6-1.6)	0.851	
		AA	31 (0.19)	1.3 (0.8-2.2)	0.238	0.236	1.1 (0.6-2.1)	0.718	0.785
	Dominant	GG	55 (0.33)	Referent			Referent		
		GA/AA	112 (0.67)	1.2 (0.8-1.7)	0.332	-	1.0 (0.6-1.6)	0.985	
	Recessive	GG/GA	136 (0.81)	Referent			Referent		
		AA	31 (0.19)	1.2 (0.8-1.9)	0.330	-	1.2 (0.7-2.0)	0.626	
	Heterosis	GA	81 (0.48)	Referent			Referent		
		GG/AA	86 (0.52)	1.0 (0.7-1.4)	0.830	-	1.1 (0.7-1.8)	0.712	
VEGFA rs2010963	Additive	GG	67 (0.40)	Referent			Referent		
		GC	73 (0.44)	1.2 (0.8-1.7)	0.436		1.2 (0.7-2.0)	0.459	
		CC	27 (0.16)	1.5 (1.0-2.5)	0.088	0.099	1.0 (0.5-1.9)	0.886	0.895
	Dominant	GG	67 (0.40)	Referent			Referent		
		GC/CC	100 (0.60)	1.2 (0.9-1.8)	0.213	-	1.1 (0.7-1.8)	0.608	
	Recessive	GG/GC	140 (0.84)	Referent			Referent		
		CC	27 (0.16)	1.4 (0.9-2.2)	0.125	-	0.9 (0.5-1.7)	0.667	
	Heterosis	GC	73 (0.44)	Referent			Referent		
		GG/CC	94 (0.56)	1.0 (0.7-1.4)	0.868	-	0.8 (0.5-1.3)	0.394	
VEGFA rs25648	Additive	CC	122 (0.76)	Referent			Referent		
		CT	34 (0.21)	0.6 (0.4-0.9)	0.029		1.0 (0.5-1.7)	0.913	
		TT	5 (0.03)	0.9 (0.3-2.5)	0.837	0.072	1.9 (0.6-6.2)	0.277	0.603
	Dominant	CC	122 (0.76)	Referent			Referent		
		CT/TT	39 (0.24)	0.6 (0.4-1.0)	0.036	-	1.1 (0.6-1.8)	0.838	
	Recessive	CC/CT	156 (0.97)	Referent			Referent		
		TT	5 (0.03)	1.0 (0.4-2.7)	0.997	-	1.9 (0.6-6.2)	0.269	
	Heterosis	CT	34 (0.21)	Referent			Referent		
		CC/TT	127 (0.79)	1.7 (1.1-2.6)	0.030	-	1.1 (0.6-1.9)	0.847	
VEGFA rs3025039	Additive	CC	123 (0.74)	Referent			Referent		
		CT	42 (0.25)	1.0 (0.7-1.5)	0.768		0.8 (0.4-1.4)	0.545	-
		TT	2 (0.01)	-	-	-	-	-	-
	Dominant	CC	123 (0.74)	Referent			Referent		
		CT/TT	44 (0.26)	1.0 (0.7-1.5)	0.940	-	0.8 (0.5-1.4)	0.433	
	Recessive	CC/CT	165 (0.99)	Referent			Referent		
		TT	2 (0.01)	2.4 (0.6-9.8)	0.225	-	2.5 (0.3-17.9)	0.373	
	Heterosis	CT	42 (0.25)	Referent			Referent		
		CC/TT	125 (0.75)	1.0 (0.7-1.5)	0.875	-	1.3 (0.7-2.3)	0.343	
VEGFA rs41282644		GG	153 (0.92)	Referent			Referent		
		GA	14 (0.08)	1.3 (0.7-2.4)	0.454	-	1.0 (0.5-2.4)	0.910	-
	Additive	CC	123 (0.74)	Referent			Referent		
KDR rs2305948		CT	40 (0.24)	1.0 (0.7-1.5)	0.903		1.4 (0.8-2.4)	0.186	
		TT	4 (0.02)	0.7 (0.3-2.0)	0.508	0.735	0.3 (0.4-2.2)	0.239	0.974
	Dominant	CC	123 (0.74)	Referent			Referent		
		CT/TT	44 (0.26)	1.0 (0.7-1.4)	0.913	-	1.2 (0.7-2.0)	0.488	
	Recessive	CC/CT	163 (0.98)	Referent			Referent		
		TT	4 (0.02)	0.7 (0.3-1.9)	0.498	-	0.3 (0.0-2.0)	0.211	
	Heterosis	CT	40 (0.24)	Referent			Referent		
		CC/TT	127 (0.76)	1.0 (0.6-1.4)	0.841	-	0.7 (0.4-1.1)	0.145	
KDR rs1870377	Additive	TT	93 (0.56)	Referent			Referent		
		TA	68 (0.40)	0.7 (0.5-1.0)	0.082		0.6 (0.4-1.0)	0.064	
		AA	6 (0.04)	1.9 (0.8-4.8)	0.163	0.347	4.1 (1.4-11.8)	0.010	0.517
	Dominant	TT	93 (0.56)	Referent			Referent		
		TA/AA	74 (0.44)	0.8 (0.5-1.1)	0.143	-	0.7 (0.4-1.1)	0.160	
	Recessive	TT/TA	161 (0.96)	Referent			Referent		
		AA	6 (0.04)	2.2 (0.9-5.5)	0.088	-	4.9 (1.7-14.0)	0.003	
	Heterosis	TA	68 (0.41)	Referent			Referent		
		TT/AA	99 (0.59)	1.4 (1.0-2.0)	0.054	-	1.7 (1.0-2.7)	0.034	
KDR rs7667298	Additive	TT	24 (0.14)	Referent			Referent		
		TC	84 (0.51)	1.1 (0.6-1.8)	0.806		1.5 (0.7-3.0)	0.274	
		CC	57 (0.35)	1.0 (0.6-1.7)	0.927	0.990	0.9 (0.4-2.0)	0.859	0.482
	Dominant	TT	24 (0.14)	Referent			Referent		
		TC/CC	141 (0.86)	1.0 (0.6-1.7)	0.849	-	1.2 (0.6-2.3)	0.600	
	Recessive	TT/TC	108 (0.65)	Referent			Referent		
		CC	57 (0.35)	1.0 (0.7-1.4)	0.899	-	0.7 (0.4-1.1)	0.155	
	Heterosis	TC	84 (0.51)	Referent			Referent		
		TT/CC	81 (0.49)	1.0 (0.7-1.3)	0.793	-	0.6 (0.4-1.0)	0.067	
FLT1 rs2296283	Additive	GG	32 (0.19)	Referent			Referent		
		GA	80 (0.48)	1.4 (0.9-2.3)	0.149		1.4 (0.7-2.7)	0.378	
		AA	55 (0.33)	1.2 (0.7-1.9)	0.583	0.785	1.2 (0.6-2.5)	0.638	0.810
	Dominant	GG	32 (0.19)	Referent			Referent		
		GA/AA	135 (0.81)	1.3 (0.8-2.1)	0.245	-	1.3 (0.7-2.5)	0.445	-
	Recessive	GG/GA	112 (0.67)	Referent			Referent		
		AA	55 (0.33)	0.9 (0.6-1.3)	0.564	-	0.9 (0.6-1.5)	0.798	-
	Heterosis	GA	80 (0.48)	Referent			Referent		
		GG/AA	87 (0.52)	0.8 (0.5-1.1)	0.136	-	0.8 (0.5-1.3)	0.415	-
FLT1 rs9554314		AC	34 (0.20)	Referent			Referent		
		AA	133 (0.80)	0.7 (0.5-1.1)	0.119	-	0.7 (0.4-1.2)	0.167	-

FLT1 rs7326277		TT	133 (0.80)	Referent			Referent		
		TC	34 (0.20)	1.4 (0.9-2.1)	0.119	-	1.4 (0.9-2.4)	0.167	-
HIF1A rs11549465		CC	124 (0.74)	Referent			Referent		
<i>Additive</i>		CT	42 (0.25)	1.0 (0.7-1.5)	0.875		1.4 (0.9-2.4)	0.164	
		TT	1 (0.01)	-	-	-	-	-	-
		CC	124 (0.74)	Referent			Referent		
		CT/TT	43 (0.26)	0.9 (0.6-1.4)	0.699	-	1.3 (0.8-2.2)	0.251	
<i>Recessive</i>		CC/CT	166 (0.99)	Referent			Referent		
		TT	1 (0.01)	-	-	-	-	-	-
		CT	41 (0.25)	Referent			Referent		
		CC/TT	126 (0.75)	1.0 (0.7-1.5)	0.966	-	0.7 (0.4-1.1)	0.144	
HIF1A rs11549467 *		GG	167 (1.0)	-	-	-	-	-	-
HIF1A rs2057482		TT	6 (0.04)	Referent			Referent		
<i>Additive</i>		TC	53 (0.31)	1.6 (0.6-4.4)	0.384		1.3 (0.3-5.5)	0.729	
		CC	108 (0.65)	1.6 (0.6-4.4)	0.355	0.524	1.0 (0.2-4.0)	0.966	0.346
		TT	6 (0.04)	Referent			Referent		
		TC/CC	161 (0.96)	1.6 (0.6-4.3)	0.357	-	1.1 (0.3-4.4)	0.925	
<i>Recessive</i>		TT/TC	59 (0.35)	Referent			Referent		
		CC	108 (0.65)	1.1 (0.7-1.5)	0.718	-	0.8 (0.5-1.2)	0.268	
		TC	53 (0.31)	Referent			Referent		
		TT/CC	114 (0.69)	1.0 (0.7-1.4)	0.964	-	0.8 (0.5-1.2)	0.243	
HIF1A 52801082		CC	124 (0.74)	Referent			Referent		
<i>Additive</i>		CT	41 (0.25)	1.0 (0.7-1.5)	0.875		1.4 (0.9-2.4)	0.164	
		TT	2 (0.01)	-	-	-	-	-	-
		CC	124 (0.74)	Referent			Referent		
		CT/TT	43 (0.26)	0.9 (0.6-1.4)	0.699	-	1.3 (0.8-2.2)	0.251	
<i>Recessive</i>		CC/CT	165 (0.99)	Referent			Referent		
		TT	2 (0.01)	0.3 (0.0-2.2)	0.241	-	-	-	-
		CT	41 (0.25)	Referent			Referent		
		CC/TT	126 (0.75)	1.0 (0.7-1.5)	0.966	-	0.7 (0.4-1.1)	0.144	
PGF rs12411		TT	6 (0.04)	Referent			Referent		
<i>Additive</i>		TA	44 (0.26)	1.8 (0.7-4.7)	0.206		1.4 (0.5-4.0)	0.587	
		AA	117 (0.70)	1.5 (0.6-3.8)	0.347	0.913	1.2 (0.4-3.5)	0.691	0.937
		TT	6 (0.04)	Referent			Referent		
		TA/AA	161 (0.96)	1.6 (0.7-4.0)	0.296	-	1.3 (0.5-3.5)	0.654	
<i>Recessive</i>		TT/TA	50 (0.30)	Referent			Referent		
		AA	117 (0.70)	0.9 (0.6-1.3)	0.687	-	1.0 (0.6-1.6)	0.891	
<i>Heterosis</i>		TA	44 (0.26)	Referent			Referent		
		TT/AA	123 (0.74)	0.8 (0.6-1.2)	0.309	-	0.9 (0.5-1.5)	0.683	
PGF rs140032352 *		GG	167 (1.00)	-	-	-	-	-	-
PGF rs8185		TT	147 (0.88)	Referent			Referent		
<i>Additive</i>		TC	16 (0.10)	1.1 (0.6-1.9)	0.845		1.3 (0.6-2.7)	0.472	
		CC	4 (0.02)	0.6 (0.2-1.9)	0.363	0.544	0.6 (0.2-2.6)	0.517	0.889
		TT	147 (0.88)	Referent			Referent		
		TC/CC	20 (0.12)	0.9 (0.5-1.6)	0.756	-	1.1 (0.6-2.1)	0.828	
<i>Recessive</i>		TT/TC	163 (0.98)	Referent			Referent		
		CC	4 (0.02)	0.6 (0.2-1.8)	0.358	-	0.6 (0.1-2.5)	0.495	
<i>Heterosis</i>		TC	16 (0.10)	Referent			Referent		
		TT/CC	151 (0.90)	0.9 (0.5-1.6)	0.800	-	0.8 (0.4-1.6)	0.446	
CMA1 rs140295781 *		CC	167	-	-	-	-	-	-
CMA1 rs201312283 *		GG	167	-	-	-	-	-	-
CMA1 rs5250		GG	129 (0.77)	Referent			Referent		
<i>Additive</i>		GA	36 (0.22)	0.9 (0.6-1.3)	0.565		1.1 (0.6-1.8)	0.792	
		AA	2 (0.01)	-	-	-	-	-	-
		GG	129 (0.77)	Referent			Referent		
		GA/AA	38 (0.23)	0.8 (0.6-1.3)	0.412	-	1.0 (0.6-1.7)	0.921	
<i>Recessive</i>		GG/GA	165 (0.99)	Referent			Referent		
		AA	2 (0.01)	0.8 (0.2-3.4)	0.805	-	0.6 (0.1-4.0)	0.563	
<i>Heterosis</i>		GA	36 (0.22)	Referent			Referent		
		GG/AA	131 (0.78)	1.2 (0.8-1.8)	0.447	-	0.9 (0.5-1.6)	0.752	
CMA1 rs7142020 *		AA	167 (1.0)	-	-	-	-	-	-
MME rs777476150 *		CC	167 (1.0)	-	-	-	-	-	-
MME rs652438		TT	141 (0.85)	Referent			Referent		
<i>Additive</i>		TC	24 (0.14)	1.4 (0.9-2.2)	0.178		1.4 (0.8-2.7)	0.266	
		CC	2 (0.01)	-	-	-	-	-	-
		TT	141 (0.85)	Referent			Referent		
		CT/CC	26 (0.15)	1.2 (0.8-1.9)	0.404	-	1.4 (0.7-2.5)	0.304	
<i>Recessive</i>		TT/TC	165 (0.99)	Referent			Referent		
		CC	2 (0.01)	0.3 (0.0-2.3)	0.259	-	0.9 (0.1-6.5)	0.917	
<i>Heterosis</i>		TC	24 (0.14)	Referent			Referent		
		TT/CC	143 (0.86)	0.7 (0.4-1.1)	0.142	-	0.7 (0.4-1.3)	0.264	
MME rs701109		TT	17 (0.10)	Referent			Referent		
<i>Additive</i>		CT	72 (0.44)	0.6 (0.3-1.2)	0.134		0.7 (0.3-1.4)	0.277	
		CC	77 (0.46)	0.5 (0.3-0.9)	0.045	0.074	0.6 (0.3-1.3)	0.189	0.271
		TT	17 (0.10)	Referent			Referent		
		TC/CC	149 (0.90)	0.6 (0.3-1.0)	0.064	-	0.6 (0.3-1.3)	0.203	
<i>Recessive</i>		TT/TC	89 (0.54)	Referent			Referent		
		CC	77 (0.46)	0.8 (0.6-1.1)	0.200	-	0.8 (0.5-1.4)	0.489	
<i>Heterosis</i>		TC	72 (0.43)	Referent			Referent		
		CC/TT	94 (0.57)	0.9 (0.7-1.3)	0.688	-	1.0 (0.6-1.6)	0.987	
MME rs989692		TT	39 (0.23)	Referent			Referent		
<i>Additive</i>		TC	69 (0.42)	0.9 (0.6-1.4)	0.669		1.4 (0.7-2.5)	0.327	
		CC	58 (0.35)	0.8 (0.5-1.3)	0.364	0.358	1.3 (0.7-2.5)	0.385	0.421
		TT	39 (0.23)	Referent			Referent		
		TC/CC	127 (0.77)	0.9 (0.6-1.3)	0.473	-	1.3 (0.8-2.4)	0.306	
<i>Recessive</i>		TT/TC	108 (0.65)	Referent			Referent		
		CC	58 (0.35)	0.9 (0.6-1.2)	0.419	-	1.1 (0.7-1.8)	0.729	
<i>Heterosis</i>		TC	69 (0.42)	Referent			Referent		
		TT/CC	97 (0.58)	1.0 (0.7-1.4)	0.863	-	0.9 (0.5-1.4)	0.557	

Table 5. Discovery Set (n=167): progression-free survival (PFS) and overall survival (OS) – Univariate analysis. * SNPs without variation in this population. HR, hazard ratio; 95%CI, 95% confidence interval.

Multivariate analysis included the relevant variables (both clinical-pathological and genetic) from univariate empirical analysis (Table 6, Table 7). The C carriers (n = 149, 89.7%) for *MME rs701109* had significantly decreased risk for disease progression than homozygotes T (n = 17, 10.3%), regardless of relevant clinical-pathological variables, namely tumour size, distant metastasis, type of systemic therapy, treatment modality or from other relevant SNPs (Table 6). Homozygous A and T for *KDR rs1870377* (n = 99, 59.3%) had significantly lesser PFS and OS compared to heterozygous (n = 68, 40.7%), regardless of relevant clinical and pathological variables (Table 6 and Table 7). Notably, these results maintained its significance after bootstrapping, as a statistical method for internal validation.

	PFS (Multivariate stepwise Cox proportional hazards model)		Bootstrap	
	HR (95%CI)	P	HR (95%CI)	P
T				
T1	Referent		Referent	
T2				
T3-4	1.7 (1.3-2.2)	<0.0001*	1.7 (1.3 - 2.3)	<0.0001*
Distant metastasis				
No	Referent		Referent	
Yes	1.7 (1.2-2.4)	0.005	1.7 (1.1 - 2.5)	0.009
Type of Therapy				
Surgery+ChT	Referent		Referent	
ChT				
ChT+RT	1.5 (1.1-2.1)	0.012*	1.5 (1.0 - 2.3)	0.053*
Type of Systemic Therapy, 1st line				
Platinum based ChT	Referent		Referent	
TKi	0.4 (0.2-0.8)	0.003	0.4 (0.2 - 0.7)	0.002
<i>KDR rs1870377</i> , Heterosis Model				
TA	Referent		Referent	
TT/AA	1.7 (1.2-2.5)	0.005	1.8 (1.2 - 2.7)	0.003
<i>MME rs701109</i> , Dominant Model				
TT	Referent		Referent	
TC/CC	0.5 (0.3-0.9)	0.018	0.5 (0.2 - 0.9)	0.030

Table 6. Multivariate analysis for progression-free survival (PFS) in the discovery cohort (n=167). Analyses were conducted using stepwise Cox regression, followed by Bootstrap analysis (Montecarlo simulations with 1000 iterations). HR, hazard ratio, 95%CI, confident interval, ChT, chemotherapy, RT, radiotherapy, TKi, tyrosine kinase inhibitors. *P trend value.

	OS (Multivariate stepwise Cox proportional hazards model)		Bootstrap	
	HR (95%CI)	P	HR (95%CI)	P
T				
T1	Referent		Referent	
T2				
T3-4	1.5 (1.1 – 2.2)	0.018*	1.5 (1.0 - 2.2)	0.066*
Distant metastasis				
No	Referent		Referent	
Yes	2.5 (1.5 - 4.3)	0.001	2.6 (1.5 - 4.5)	0.001
Age median				
≤ 64.0	Referent		Referent	
> 64.0	1.7 (1.0 - 2.7)	0.031	1.6 (1.0 - 2.8)	0.069
<i>KDR rs1870377</i> , Heterosis Model				
TA	Referent		Referent	
TT/AA	2.1 (1.2 – 3.4)	0.006	2.0 (1.2 – 3.3)	0.012

Table 7. Multivariate analysis of relevant variables for overall survival (OS) in the discovery set (n=167) using stepwise Cox regression, followed by Bootstrap analysis (Montecarlo simulations with 1000 iterations). HR, hazard ratio, 95% CI, confident interval. *P trend value.

Exploratory subgroup analyses were conducted among NSCLC patients from discovery set that included only patients under first line platinum-based doublet chemotherapy without actionable driver mutations ($n = 145$) or *EGFR* wild-type ($n = 149$). Within the *MME* (rs701109) and *KDR* (rs1870377) polymorphisms, we found evidence for an independent effect at PFS and OS respectively, across all subgroups. Notably, CT and CC patients for *MME* (rs701109) under first line platinum-based doublet chemotherapy or *EGFR* wild-type had protection for disease progression (HR = 0.4, 95% CI (0.2–0.7), $p = 0.005$ and HR = 0.4, 95% CI (0.2–0.8), $p = 0.006$, respectively). Homozygous A and T for *KDR* rs1870377 had increased risk to death also in patients under first line platinum doublet chemotherapy or *EGFR* wild-type (HR = 1.9, 95% CI (1.1–3.2), $p = 0.019$ and HR = 2.0, 95% CI (1.2–3.4), $p = 0.011$, respectively). Moreover, from the multivariate analysis conducted in the subgroup of patients treated with first line platinum-based doublet chemotherapy without actionable driver mutations ($n = 145$), we also found that homozygous for *ACE2* rs908004 had increased risk for disease progression, regardless of relevant clinical-pathological covariates (HR = 2.9, 95% CI (1.3–6.3), $p = 0.010$).

The validation set included an independent matched cohort of NSCLC patients (Table 3) to validate the resulting genetic SNPs from the discovery set. Results from univariate analysis of clinicopathological variables in association with PFS and OS are described in Table 8.

Table 8		PFS			OS		
	N	Univariate (Cox) HR (95CI)	P *	Ptrend	Univariate (Kaplan-Meier) HR (95CI)	P *	Ptrend
Clinical variables							
Age							
<65.0	89 (0.47)	Referent			Referent		
>65.0	101 (0.53)	1.3 (0.9-1.8)	0.133	-	1.6 (1.0-2.5)	0.027	-
Gender							
Male	133 (0.70)	Referent			Referent		
Female	57 (0.30)	0.6 (0.4-0.9)	0.015	-	0.9 (0.6-1.3)	0.586	-
Histology							
Adenocarcinoma	126 (0.66)	Referent			Referent		
Squamous cell	62 (0.33)	1.5 (1.1-2.1)	0.14		1.7 (1.1-2.2)	0.021	
Others *	2 (0.01)	3.2 (0.8-13.3)	0.103	0.006	-	-	0.066
T							
T1	16 (0.08)	Referent			Referent		
T2	33 (0.18)	1.3 (0.6-2.7)	0.485		1.6 (0.5-4.9)	0.424	
T3/T4	141 (0.74)	1.3 (0.7-2.4)	0.473	0.595	1.7 (0.6-4.7)	0.288	0.308
N							
N0-N2	114 (0.60)	Referent			Referent		
N3	76 (0.40)	1.2 (0.9-1.7)	0.268	-	1.2 (0.8-1.8)	0.376	-
Distant Metastasis							
No	81 (0.43)	Referent			Referent		
Yes	109 (0.57)	1.3 (1.0-1.9)	0.085	-	1.1 (0.7-1.7)	0.669	-
ECOG PS							
Good (0-2)	188 (0.99)	Referent			Referent		
Poor (3-4)	2 (0.01)	18.9 (4.1-86.3)	<0.0001	-	22.3 (5.0-100.9)	<0.0001	-
Type_Therapy							
ChT	109 (0.57)	Referent			Referent		
ChT+RT	81 (0.43)	0.7 (0.5-1.0)	0.052	-	0.7 (0.5-1.1)	0.163	-
Type_SysTher							
Platinum based ChT	155 (0.82)	Referent			Referent		
TKI	35 (0.18)	0.5 (0.3-0.8)	0.003	-	0.5 (0.3-0.9)	0.020	-
Anti-hypertensive drugs							
No	126 (0.67)	Referent			Referent		
iACE/ARB	62 (0.33)	1.1 (0.8-1.6)	0.441	-	1.0 (0.6-1.5)	0.892	-

Table 8. Clinical-pathological variables, progression-free survival (PFS) and overall survival (OS) in validation set (n=190) – Univariate analysis. HR – Hazard Ratio. ChT – chemotherapy, RT – radiotherapy, TKI - tyrosine kinase inhibitor, iACE – inhibitors of angiotensin-converting enzyme, ARB – AngiotensinII receptor blocker.

The genotype distribution of *MME* rs701109, *KDR* rs1870377 and *ACE2* rs908004 are presented in plots (figure 9). The univariate analysis according to additive, dominant, recessive or heterosis models of *MME* rs701109, *KDR* rs1870377 and *ACE2* rs908004 for hazard and survival analysis are depicted in Table 9. The genetic distribution of genotypes in *MME* rs701109, *KDR* rs1870377 and *ACE2* rs908004 are similar among discovery and validation sets ($p = 0.204$, $p = 0.846$ and $p = 0.251$, respectively) (Pearson Chi-square test).

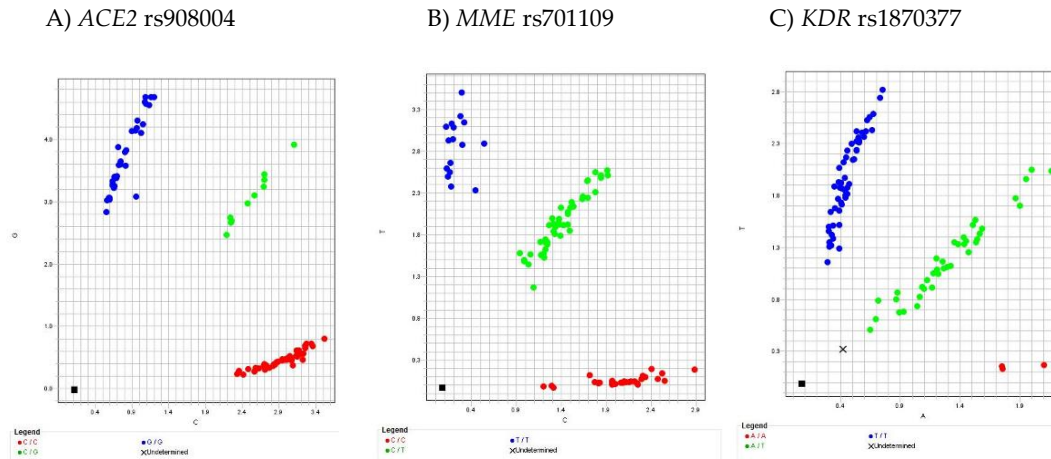


Figure 9. Allelic discrimination plots. A) *ACE2* rs908004 G/C; B) *MME* rs701109 C/T; C) *KDR* rs1870377 A/T.

Table 9			Progression-free survival				Overall survival			
			Univariate (Cox)			Univariate (Cox)				
Genetic variants	Genetic model		N	HR (95CI)	P *	Ptrend	HR (95CI)	P	Ptrend	
ACE2 rs908004	Additive	G	61 (0.32)	Referent			Referent			
		GC	29 (0.15)	1.1 (0.6-1.7)	0.819		0.8 (0.4-1.5)	0.443		
		C	99 (0.53)	0.9 (0.6-1.2)	0.459	0.425	0.8 (0.5-1.2)	0.281	0.299	
	Dominant	G	61 (0.32)	Referent			Referent			
		GC/C	128 (0.68)	0.9 (0.6-1.3)	0.587	-	0.8 (0.5-1.2)	0.256	-	
	Recessive	G/GC	90 (0.48)	Referent			Referent			
	C	99 (0.52)	0.8 (0.6-1.2)	0.350	-	0.8 (0.6-1.3)	0.421	-		
	Heterosis	GC	29 (0.15)	Referent			Referent			
		G/C	160 (0.85)	0.9 (0.6-1.4)	0.531	-	1.1 (0.6-2.0)	0.735	-	
	Additive	TT	108 (0.58)	Referent			Referent			
		TA	74 (0.39)	0.9 (0.7-1.4)	0.932		1.1 (0.7-1.7)	0.674		
		AA	5 (0.03)	1.0 (0.3-3.3)	0.959	0.958	1.2 (0.3-5.1)	0.632	0.632	
	Dominant	TT	108 (0.58)	Referent			Referent			
TA/AA		79 (0.42)	1.0 (0.7-1.4)	0.941	-	1.1 (0.7-1.7)	0.652	-		
	Recessive	TT/TA	182 (0.97)	Referent			Referent			
		AA	5 (0.03)	1.0 (0.3-3.3)	0.951	-	1.2 (0.3-4.8)	0.815	-	
	Heterosis	TA	74 (0.40)	Referent			Referent			
		TT/AA	113 (0.60)	1.0 (0.7-1.4)	0.927	-	0.9 (0.6-1.4)	0.699	-	
	MME rs701109	Additive	TT	29 (0.15)	Referent			Referent		
			TC	87 (0.46)	0.7 (0.4-1.1)	0.115		0.8 (0.4-1.4)	0.381	
CC			73 (0.39)	1.0 (0.6-1.5)	0.882	0.665	1.0 (0.6-1.8)	0.954	0.698	
Dominant		TT	29 (0.15)	Referent			Referent			
		TC/CC	160 (0.85)	0.8 (0.5-1.2)	0.308	-	0.9 (0.5-1.5)	0.628	-	
Recessive		TT/TC	116 (0.61)	Referent			Referent			
	CC	73 (0.39)	1.3 (0.9-1.8)	0.165		1.2 (0.8-1.9)	0.347	-		
	Heterosis	TC	87 (0.46)	Referent			Referent			
		TT/CC	102 (0.54)	1.4 (1.0-1.9)	0.041	-	1.3 (0.8-2.0)	0.207	-	

Table 9. Validation Set (n=190) – Univariate analysis. HR, hazard ratio; 95%CI, 95% confidence interval.

After univariate Cox regression analyses, the values for retention were $P < 0.10$ and more or less than 30% effect in HR, in order to be included into the multivariate Cox proportional hazards model. Only one genetic model was included in the model to avoid collinearity.

The multivariate model for PFS in the validation cohort is presented in Table 10. Homozygous ($n = 102$, 53.9%) for *MME* rs701109 had significantly increased risk to disease progression compared to CT genotype ($n = 87$, 46.0%), regardless of histology, type of systemic therapy or treatment modality (chemotherapy or chemo and

radiotherapy). There was no significant effect on overall survival for *MME* rs701109, *KDR* rs1870377 and *ACE2* rs908004.

Subgroup analyses were also conducted in this set, for first line platinum-based doublet chemotherapy ($n = 155$) and *EGFR* wild-type patients ($n = 161$). The *MME* rs701109 polymorphism was associated with PFS only in the subgroup of patients with wild-type *EGFR*, being the homozygous at increased risk to disease progression (HR = 1.7, 95% CI (1.1–2.5), $p = 0.009$).

	PFS (Multivariate stepwise Cox proportional hazards model)		Bootstrap	
	HR (95%CI)	P	HR (95%CI)	P
Histology				
Adenocarcinoma	Referent		Referent	
Squamous cell	1.7 (1.2-2.4)	0.003**	1.7 (1.2 - 2.5)	0.003**
Other *	-		-	
Type of Therapy				
ChT	Referent		Referent	
ChT+RT	0.4 (0.3-0.6)	<0.0001	0.4 (0.3 – 0.6)	<0.0001
Type of Systemic Therapy, 1st line				
Platinum based ChT	Referent		Referent	
TKi	0.4 (0.3-0.7)	0.001	0.4 (0.3 – 0.7)	0.002
<i>MME</i> rs701109, Heterosis Model				
TC	Referent		Referent	
TT/CC	1.6 (1.2-2.3)	0.005	1.6 (1.1 – 2.4)	0.007

Table 10. Multivariate analysis of relevant variables for disease progression using stepwise Cox regression, followed by Bootstrap analysis with 1000 iterations from validation set ($n=190$). PFS – progression-free survival, HR – hazard ratio, CI – confident interval, ChT – chemotherapy, RT – radiotherapy, TKi – inhibitors of tyrosine kinase. *Adenocarcinoma+squamous cell. **P trend value.

1.2 Pharmacogenetics of advanced lung cancer: Predictive value of functional genetic polymorphism AGXT: Pro11Leu in clinical outcome?

The participants from this study are those included in the discovery set. Their clinicopathological characteristics are described in Table 3. The anatomical localization of distant metastases at diagnosis ($n=94$) was distributed as pleura and lung (62.8%), extra-thoracic (29.8%) and multiple (7.4%). Regarding mutational status, we observed that 8.3% of patients ($n=14$) had *EGFR* mutation (exon 19 deletions or exon 21 mutation), whereas 3.0% ($n=5$) had rearrangements in the gene encoding anaplastic lymphocyte kinase. Platinum-based doublet chemotherapy was administered to 85.7% of NSCLC patients, most frequently the cisplatin combination. Adjuvant chemotherapy was

administered in twelve patients. In a subgroup of patients with chronic renal disease (n=24) the doublet chemotherapy with carboplatin was the first choice. Fifty-one patients underwent checkpoint inhibitors as second-, third- and fourth-line therapy. The median time-to-disease progression and the median time-to-death was 7.5 (CI 95%, 6.1-9.0) and 30.0 months (CI 95%, 16.9-43.2), respectively.

The *AGXT* rs34116584 genetic polymorphism distribution in this cohort of NSCLC patients was 71.7% C homozygous, 23.5% heterozygous and 4.8% T homozygous. Genotyping was successfully performed in 166 patients, with two missing genotyping. Sample size and number of events were calculated assuming $\alpha=0.05$ and $\text{power}>0.8$. For both endpoints, the effect size was calculated from the resulting Hazard Ratio of *AGXT* variable in multivariate analysis. The minimal sample size for PFS was n=62 with an estimated number of events of n=50, whereas for OS, the calculated sample size was n=173 and the estimated number of events n=77.

The median time-to-endpoint, hazard and survival univariate analyses of the empirical statistical procedure are depicted in Table 11. In the dominant genetic model, there was a significantly shorter PFS for T-allele carriers [5.4 months (CI 95% 4.3-6.4) versus 9.4 (CI 95%, 7.2-11.7), $p<0.0001$] and a shorter OS [22.2 months (CI 95% 13.6-30.8) versus 43.6 months (20.3-66.9), $p=0.015$] (Figure 10). Notably, despite the *AGXT* rs34116584 T-carriers had shorter PFS than CC homozygous both in the subset of mutated (n=14, $p=0.028$) and wild-type (n=154, $p<0.0001$) *EGFR*, those *AGXT* carriers only presented shorter OS in wild-type ($p=0.022$) but not for mutated *EGFR* ($p=0.692$). Additionally, in a subset of patients with information on PD-L1 expression (n=98, 33.7% without and 66.3% with PD-L1 expression $\geq 1\%$), Kaplan-Meier plots with Log-Rank tests showed that T-carriers had shorter time-to-progression independently of PD-L1 positivity ($p=0.010$ and $p=0.040$, respectively).

	N	Progression-free survival		Overall survival	
		Median (95%CI)	P *	Median (95%CI)	P *
Age					
<65.4	86	7.2 (5.8-8.7)	0.541	43.6 (8.5-78.7)	0.078
>65.4	82	8.6 (5.1-12.1)		23.6 (15.3-31.8)	
Gender					
Male	124	7.2 (5.3-9.1)	0.547	28.1 (21.7-34.4)	0.102
Female	44	8.9 (5.5-12.3)		82.5 (15.1-150.0)	
Histology					
Adenocarcinoma	117	7.8 (5.7-9.9)	0.201	44.0 (18.6-69.4)	0.069
Squamous cell	42	5.7 (4.4-7.0)		24.6 (17.0-32.2)	
Others *	9	9.5 (3.4-15.7)		31.3 (19.3-43.4)	
T					
1	26	10.2 (4.1-16.3)	0.008	30.0 (14.1-46.0)	0.011
2	46	9.4 (4.0-14.9)		67.4 (46.7-88.2)	
3	18	4.7 (1.4-8.1)		26.9 (19.5-34.3)	
4	78	5.5 (4.0-7.1)		25.4 (16.7-34.2)	
N					
N0	13	9.0 (4.2-13.8)	0.151	-	0.790
N1	18	7.1 (2.3-12.0)		82.5 (22.9-142.1)	
N2	34	9.5 (2.8-16.2)		26.9 (18.5-35.3)	
N3	103	6.6 (4.6-8.6)		31.3 (12.9-50.0)	
M					
no	74	9.6 (5.2-14.0)	0.003	78.7 (53.0-104.4)	<0.0001
yes	94	5.4 (4.5-6.2)		22.2 (17.3-27.1)	
Type Therapy					
Surgery+ChT	12	20.5 (0.0-49.2)	0.024	-	0.188
ChT	125	6.6 (4.9-8.2)		26.9 (21.0-33.0)	
ChT+RT	31	8.9 (4.9-12.8)		34.9 (7.2-62.5)	
ECOG PS					
Good (0-1)	125	8.0 (6.1-9.9)	0.171	44.0 (21.2-66.8)	<0.0001
Poor (2-4)	43	5.4 (2.7-8.1)		12.9 (9.8-16.0)	
Systemic Therapy					
Platinum based	144	6.2 (4.7-7.8)	0.005	28.1 (20.0-36.2)	0.183
Target therapy	24	13.3 (0.2-26.3)		-	
AGXT rs34116584					
Additive model					
CC	119	9.4 (7.2-11.7)	<0.0001	43.6 (20.3-66.9)	0.009
CT	39	5.7 (5.0-6.4)		17.8 (10.0-25.7)	
TT	8	4.0 (3.4-4.6)		24.6 (21.9-27.2)	
Dominant model					
CC	119	9.4 (7.2-11.7)	<0.0001	43.6 (20.3-66.9)	0.015
CT/TT	47	5.4 (4.3-6.4)		22.2 (13.6-30.8)	
Recessive model					
CC/CT	158	7.8 (6.3-9.2)	0.025	31.3 (16.7-45.9)	0.615
TT	8	4.0 (3.4-4.6)		24.6 (21.9-27.2)	

Table 11. Univariate analyses of AGXT rs34116584 and clinical variables with time-to-progression and time-to-death. ChT, chemotherapy; ECOG PS, ECOG performance status; OS, overall survival; PFS, progression-free survival; RT, radiotherapy. * Log-Rank test. ** others: pleomorphic, combined squamous and adenocarcinoma. 95%CI, 95% confidence interval.

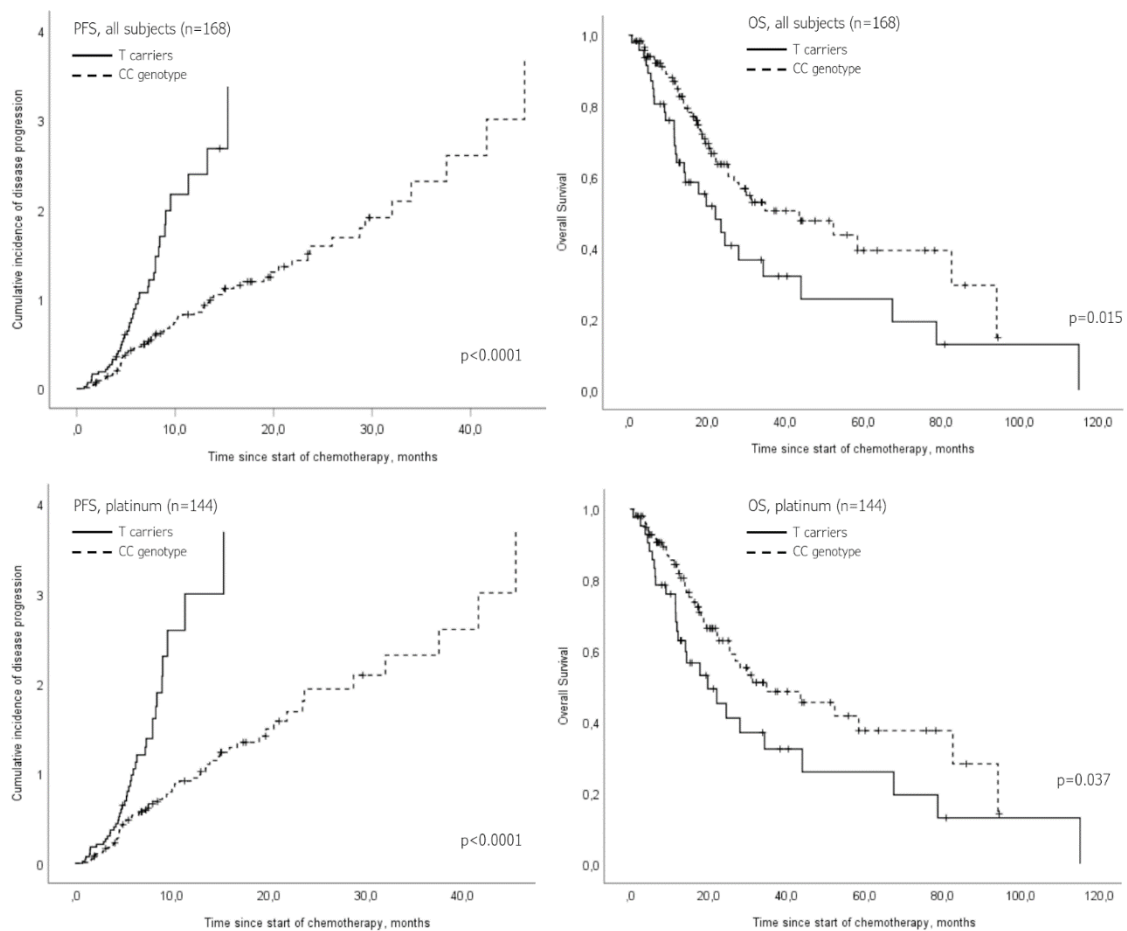


Figure 10. Kaplan-Meier plots with Log-Rank tests for AGXT dominant genetic models in association with progression-free survival (PFS) and with overall survival (OS) for all NSCLC patients (n=168) and those treated with platinum-based chemotherapy (n=144).

The statistically significant covariates from univariate analysis were included in a Cox proportional-hazards multivariate model. This data showed for AGXT T-carriers an increased risk for progression (HR=2.0; 95% CI, 1.4-3.0; $p<0.0001$) and for cancer-specific death (HR=1.8; 95% CI, 1.1-3.0; $p=0.017$), regardless of tumour size, distant metastasis at diagnosis, type of systemic therapy and type of treatment modality (Table 12). To test the hypothesis that AGXT rs34116584 was associated with the response to platinum-based chemotherapy, the analysis was conducted in the group of patients treated with first line platinum-based doublet chemotherapy (n=144). In this subgroup, there were no identifiable actionable driver mutations at the diagnosis. Univariate analysis showed longer PFS in C homozygous (median 8.6, CI 95%, 6.1-11.1 months) in comparison with T-carriers (median 5.1, CI 95%, 4.2-6.0 months) ($p<0.0001$) (Figure 10). Concordantly, the

time-to-death was also longer in CC (median 34.9, CI 95%, 12.1-57.6 months) compared to T-carriers (median 19.8, CI 95%, 8.9-30.7 months) ($p=0.037$) (Figure 10). On multivariate analysis T-carriers had higher risk for disease progression (HR=2.0, 95% CI, 1.3-3.1, $p=0.001$) independently of relevant clinicopathological covariates. In platinum-treated patients, those with febrile neutropenia ($n=20$) exhibited more frequently the T-allele compared to non-febrile neutropenia (35% versus 29%, respectively), despite the lack of association of the SNP with myelotoxicity (OR=1.34, 95% CI, 0.49-3.64, $p=0.566$).

	Progression-free survival		Overall survival	
	HR (95%CI)	P	HR (95%CI)	P
cT (TNM)				
T1	Referent		Referent	
T2	1.6 (0.9-2.8)	0.131	0.6 (0.3-1.4)	0.278
T3	2.2 (1.1-4.6)	0.026	0.9 (0.4-2.2)	0.856
T4	2.1 (1.2-3.7)	0.007	1.6 (0.8-3.1)	0.159
Distant metastasis				
No	Referent		Referent	
Yes	1.6 (1.5-2.3)	0.010	2.1 (1.3-3.7)	0.005
Systemic Therapy				
Platinum	referent		-	
Target therapy	0.4 (0.2-0.8)	0.003	-	-
ECOG PS				
Good (0-1)	-		Referent	
Poor (2-4)	-	-	2.3 (1.4-3.7)	0.001
Type of therapy				
Surgery+ChT	Referent		-	
ChT	2.7 (1.1-6.7)	0.026	-	
ChT+RT	2.8 (1.1-7.0)	0.027	-	-
AGXT rs34116584				
Dominant model				
CC	Referent		Referent	
CT/TT	2.0 (1.4-3.0)	<0.0001	1.8 (1.1-3.0)	0.017

Table 12. Multivariate Cox regression including only the significant covariates after empirical analysis, for PFS and OS. ChT, chemotherapy; ECOG PS, ECOG performance status; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; RT, radiotherapy; 95%CI, 95% confidence interval.

1.3 Survival analysis according to RAS genes expression based on TCGA LUAD

To understand the relevance of gene expression of renin-angiotensin pathway's genes and its impact in lung adenocarcinoma prognosis, we used TCGA public database. The mRNA expression level for *REN*, *AGT*, *ACE*, *ACE2*, *MME*, *CMA1*, *AGTR1*, *AGTR2* and *MAS1* genes was analysed in lung adenocarcinoma tissue and tumour-adjacent normal tissue from patients submitted to lobectomy or pneumectomy. We hypothesized

that altered gene expression in RAS genes impact overall survival. Therefore, survival analysis was conducted according to high or low mRNA levels.

Five-hundred patients were included (270 female, 54,0% and 230, 46,0% male), with a mean age of $65,3 \pm 10,1$ years-old. The TNM staging according to the AJCC 7th edition, identified 134 patients in stage IA (27,2%), 134 in stage IB (27,2%), 199 in stage IIA (40,4%) and 25 in stage IIIA (5,1%). The median time-to-death in the cohort was 49,9 months (CI 95% 42,6-57,2).

When compared with adjacent normal tissue, the lung adenocarcinoma presented with significantly lower quantity of transcripts for *REN*, *ACE*, *AGTR1*, *AGTR2*, *MME*, *MAS1*, *CMA1* (figure 11). Conversely, *AGT* expression was upregulated in lung cancer (figure 11). No statistically significant difference was observed for *ACE2* expression (figure 11).

In agreement, higher expression of *REN*, *ACE*, *AGTR1*, *CMA1* was significantly associated with better overall survival when compared with patients with lower expression (table 13 and figure 12). The *AGT*, *ACE2*, *AGTR2*, *MAS1*, *MME* expression profiles were not related to overall survival (table 13 and figure 12).

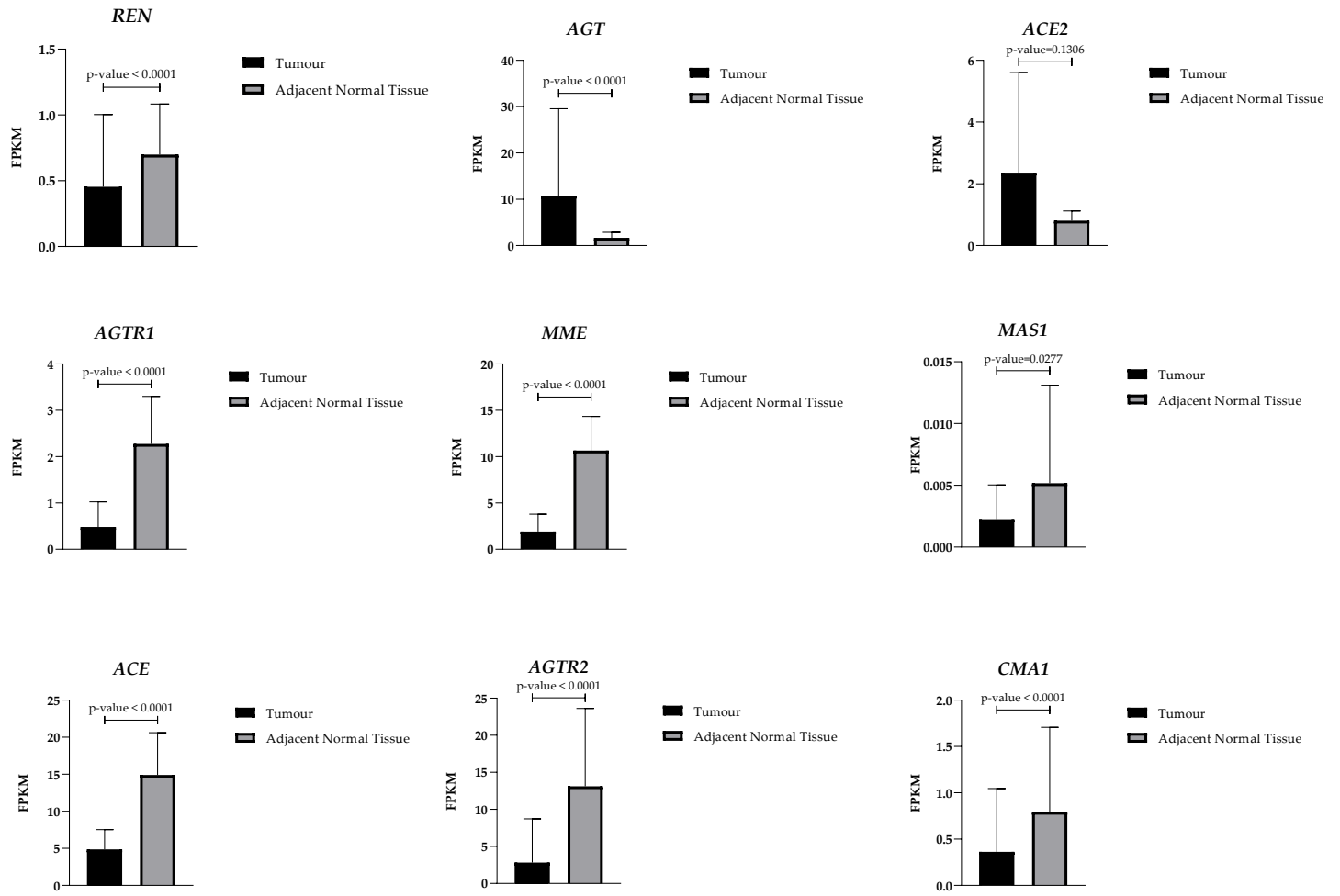


Figure 11. mRNA expression of *REN*, *AGT*, *ACE*, *ACE2*, *MME*, *CMA1*, *AGTR1*, *AGTR2* and *MAS1* from lung adenocarcinoma tissue and adjacent normal tissue. FPKM - kilobase of transcript per million. *Mann-Whitney U test.

	Overall survival	
	Median (95%CI)	P *
<i>REN</i>		
Low expression	41.0 (42.0-63.4)	0.018
High expression	50.0 (77.9-113.7)	
<i>AGT</i>		
Low expression	50.5 (71.2-110.8)	0.327
High expression	42.3 (51.7-82.2)	
<i>ACE</i>		
Low expression	40.5 (55.9-107.2)	0.021
High expression	55.1 (72.2-110.0)	
<i>ACE2</i>		
Low expression	43.1 (60.0-118.5)	0.178
High expression	50.5 (72.4-108.4)	
<i>AGTR1</i>		
Low expression	45.2 (60.2-93.4)	0.010
High expression	105.6 (76.3-111.2)	
<i>AGTR2</i>		
Low expression	42.9 (54.8-110.8)	0.123
High expression	53.3 (72.4-109.7)	
<i>MAS1</i>		
Low expression	50.9 (68.4-107.3)	0.533
High expression	42.9 (63.3-115.1)	
<i>MME</i>		
Low expression	50.0 (67.5-103.6)	0.393
High expression	49.7 (74.4-132.4)	
<i>CMA1</i>		
Low expression	37.2 (52.1-96.9)	0.002
High expression	55.1 (75.3-115.4)	

Table 13. Univariate analyses of *REN*, *AGT*, *ACE*, *ACE2*, *MME*, *CMA1*, *AGTR1*, *AGTR2* and *MAS1* and high and low expression with time-to-death or time-to-last visit. * Kaplan Meier curves. 95%CI, 95% confidence interval

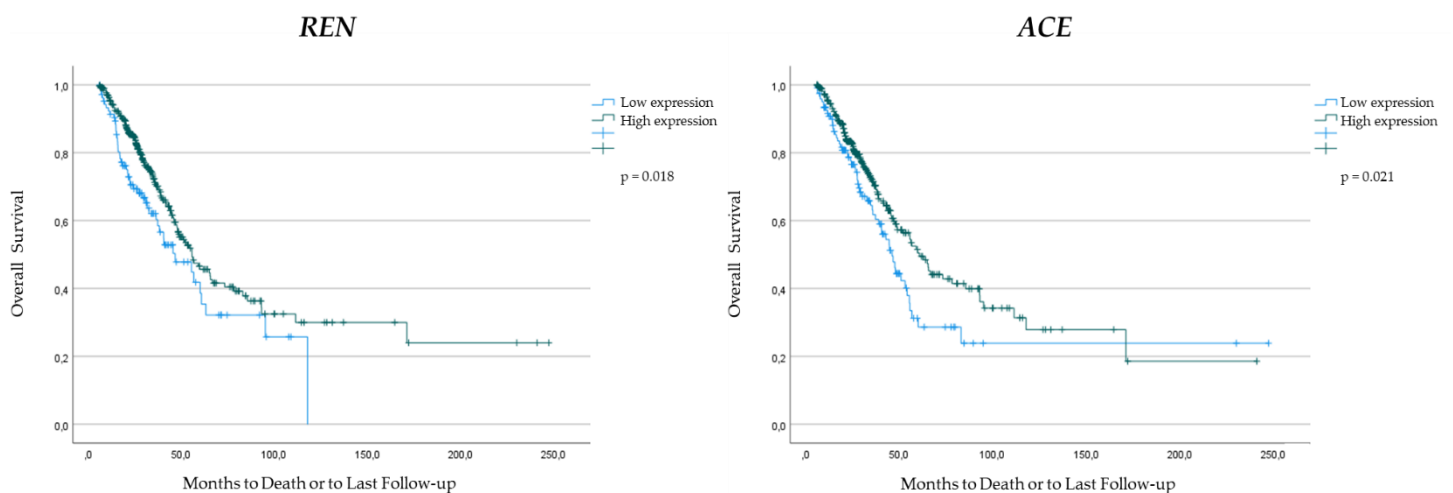


Figure 12. Kaplan-Meier plots with Log-Rank test for high and low expression of *REN*, *ACE*, *AGTR1*, *CMA1*, *AGT*, *ACE2*, *AGTR2*, *MAS1*, *MME* in association with overall survival for all LUAD patients (n=500).

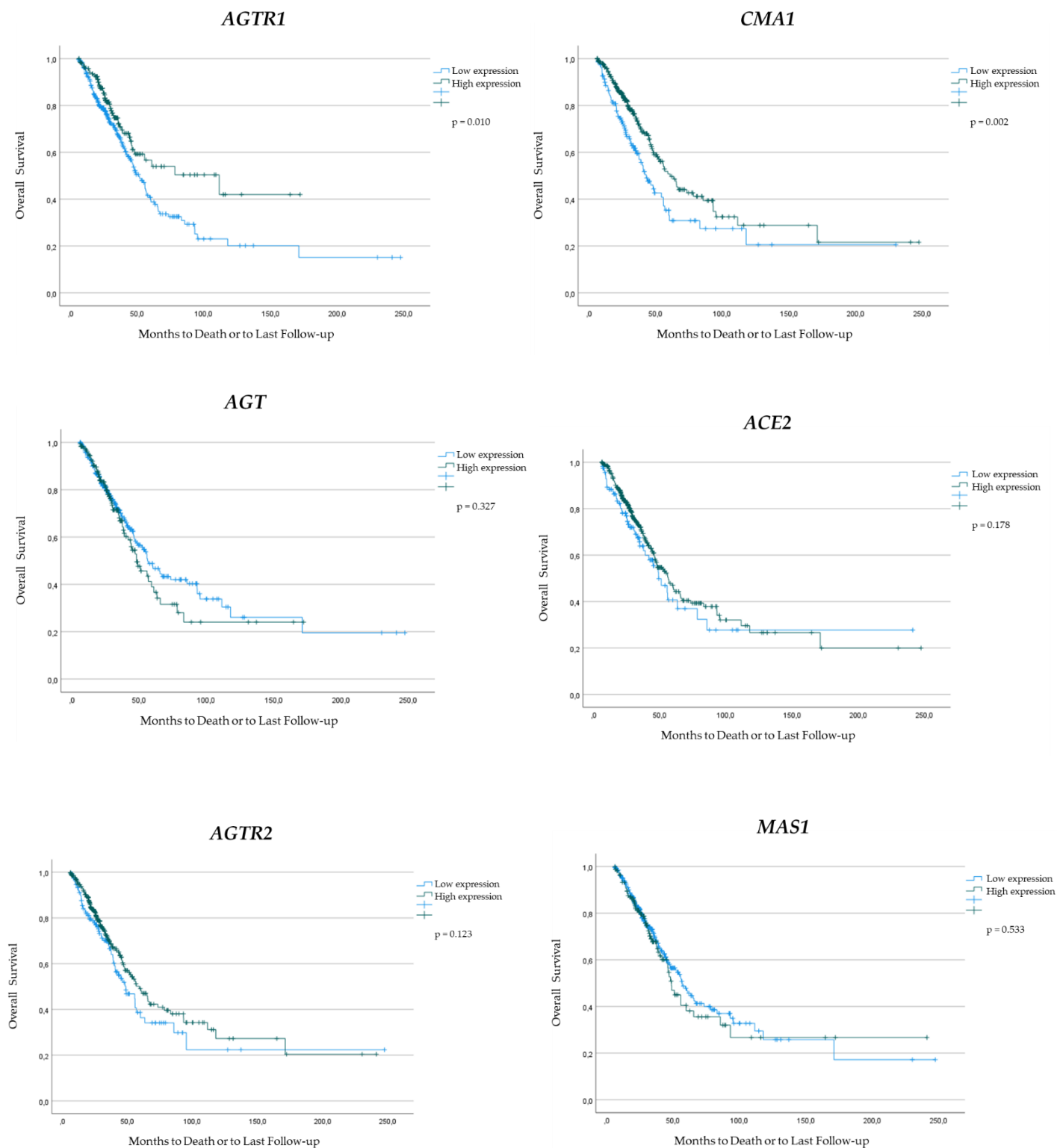


Figure 12 (continued). Kaplan-Meier plots with Log-Rank test for high and low expression of *REN*, *ACE*, *AGTR1*, *CMA1*, *AGT*, *ACE2*, *AGTR2*, *MAS1*, *MME* in association with overall survival for all LUAD patients (n=500).

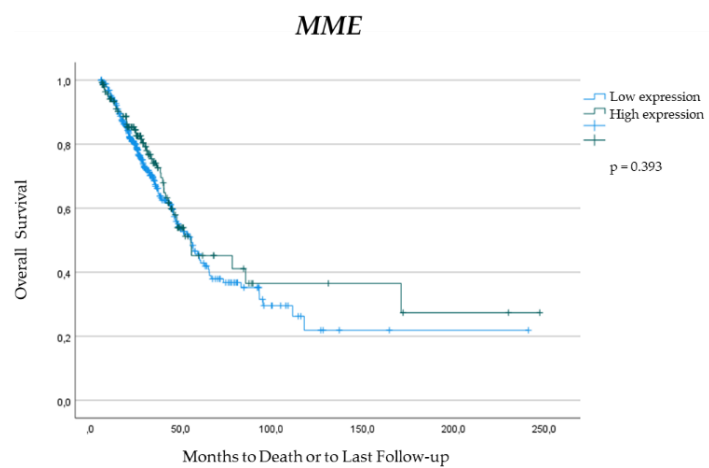


Figure 12 (continued). Kaplan-Meier plots with Log-Rank test for high and low expression of *REN*, *ACE*, *AGTR1*, *CMA1*, *AGT*, *ACE2*, *AGTR2*, *MAS1*, *MME* in association with overall survival for all LUAD patients (n=500).

V. General Discussion

Lung cancer management has changed in the last decade (266). Advances in molecular tools and targeted therapies have provided new insight for the field of oncology (267). Despite these advancements, surgery is still the only effective curative approach, whether used alone or alongside a combination of other treatment modalities, such as radiotherapy and chemotherapy. In light of the current knowledge, clinicians have begun selecting optimized populations that could receive improved benefits from these innovative therapeutic modalities. Tumour carcinogenesis pathways are now of primary interest in the field of oncology. The hallmarks of cancer comprise several biological capabilities acquired during the multistep development of human tumours. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (268).

The renin-angiotensin-aldosterone system is an established primary regulator of blood pressure, homeostasis, and natriuresis. Nevertheless, recent evidence indicates that angiotensin peptides also might have a role in tumour cell proliferation, inflammation and hypoxia, which have been recognized as hallmarks of cancer, actively participating in lung tumour microenvironment regulation (157).

Underlying the hallmarks of cancer are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions (268). Furthermore, the association between genetic polymorphisms and cancer susceptibility, progression, aggressiveness and death has been thoroughly described in the literature (156). SNPs are the most common type of polymorphisms, accounting for 90% of genetic variations (156). Nucleotide substitution, according to its location in germline DNA, may elicit functional modifications, ultimately at protein level, contributing to inter-individual variability, and correlating with relevant phenotypic alterations.

Lung cancer is a multifactorial disease, as such, an improved understanding of the interplay of environmental and genetic polymorphisms at multiple loci may help identify individuals who are at increased risk for lung cancer and for lung cancer

progression. The work developed here sought to test the hypothesis that variants in genes coding for the renin–angiotensin pathway and for interacting hypoxia/angiogenesis key molecules might have predictive and prognostic value, respectively for NSCLC progression and survival (Annex II). Moreover, we demonstrated an association of the genetic variant *AGXT* rs34116584 on the clinical outcome and response to chemotherapy of patients with locally advanced and metastatic NSCLC (Annex III). To the best of our knowledge, this is the first time that it was established an association between some genetic variants of genes involved in renin-angiotensin pathway, angiogenesis and *AGXT* polymorphisms in NSCLC prognosis.

1.1 Impact of genetic polymorphisms of Renin-Angiotensin pathway in NSCLC

RAS was first discovered and related with the physiological regulation of systemic arterial pressure and involved in hypertension pathogenesis (6). As a gatekeeper system, RAS is responsible for more than one function, including cell cycle regulation, inflammation, angiogenesis and fibrosis (5). The capillary blood vessels in the lung are one of the major sites of ACE expression and Ang II production in the human body (14). In fact, the increased ACE expression observed in several interstitial lung diseases supports pulmonary RAS and establishes a putative role for Ang II in the response to lung injury and fibrosis (157). A previous report showed that the G-6A polymorphism of the *AGT* gene is associated with increased angiotensin production and idiopathic pulmonary fibrosis progression (162), which is associated with increased risk of lung cancer (269). Furthermore, the insertion/deletion (I/D) polymorphism of *ACE* gene has been linked to the pathogenesis and progression of human cancers (161, 270) and the lowest serum ACE levels were correlated with poorer prognosis and higher relapse rates in lung cancer (51). We found that the association between *ACE* rs4316 genetic variant and PFS, despite being significant in univariate, the finding was not confirmed on multivariate analysis. It is an I/D polymorphism and is localized at intron 16 of *ACE* gene. This SNP has been associated with plasma ACE levels, with DD genotype carriers independently associated to higher expression (271), albeit no effect was noted on AngII (271). Thus, it seems unlikely this SNP might impact the ACE/AngII/ATR1 axis, which has been associated with tumour cell growth and angiogenesis, partially justifying the

absence of clinical impact in NSCLC patients with advanced stage disease. There are other possible reasons for the failure to replicate the prior findings. Many of the initial reports were present only in retrospectively defined subsets of the subjects studied and the main hypotheses investigated were related to cancer risk. Another possibility is that varied ethnic background of study populations may result in a different genetic background which confers a different biologic significance to a given functional polymorphism.

ACE2 is responsible for converting AngII to Ang-(1-7), an agonist of Mas receptor (MasR). In a mouse lung xenograft model, it was previously shown that ACE2 overexpression inhibited both lung cancer cell proliferation *In vitro* and reduced tumour growth *in vivo* (81). The ACE2 rs908004 is an intronic variant at the X-linked ACE2 gene (272). Findings from the discovery cohort revealed that ACE2 rs908004 heterozygous carriers presented shorter time-to-progression, only in the subgroup of patients treated with first line platinum-based chemotherapy without actionable driver mutations. We hypothesize that heterozygous produces reduced levels of ACE2, contributing to the up-regulation of ACE/Ang II/AT1 receptor axis, or to linkage with another genomic region that might be relevant for this clinical phenotype. These hypotheses need to be confirmed in further studies. Furthermore, as with all gene association studies, subgroup analyses can lead to spurious associations and might justify our result. Nevertheless, there is lack of studies on genotype–phenotype relationship with this SNP in lung cancer, opening new opportunities for further investigations.

Ang-(1-7) has opposite effects to Ang II upon binding to MasR and might be produced through hydroxylation of Ang I by neprilysin (NEP), an enzyme encoded by the gene *MME* (43). NEP, also called CD10, has been described as a tumour-specific antigen for lymphocytic leukemia (163). In the later years, it was demonstrated that NEP functions are implicated in oncogenesis and tumour microenvironment (163). Moreover, alterations in NEP protein expression have also been reported in solid tumours, including lung cancer (273). Both in discovery and validation cohorts, NSCLC subjects carrying the heterozygous TC genotype were at decreased risk for disease progression, albeit in the discovery set the protection was conferred to carriers of the TC/CC combined genotypes (dominant model), whereas in the validation cohort it was

conferred only to TC (heterosis model). Heterosis for a single gene refers to a heterozygote that has a different phenotypic trait than for either allele homozygotes (274), and heterozygosity has been associated with human healthy aging (275). Despite insufficient knowledge on the molecular basis of heterosis, an accepted mechanism is related to environmentally-driven epigenetic modifications, namely methylation and microRNAs (miRNAs) (276). MicroRNAs regulate gene expression by binding to target sequences mostly at the 3'-UTR of mRNAs, and evidence exist for their functional effect in candidate SNPs of various types of cancer (277). Noteworthy, recently reported data demonstrated such a mechanism with functional repercussion, the SNP rs6665, located downstream of rs701109 in the 3'-UTR region sequence of *MME* (278).

We conducted an *in-silico* proof-of-principle analysis using the MicroSNiPer database (<http://vm24141.virt.gwdg.de/services/microsniper/>) (279) to predict *MME* rs701109 SNP effects on putative microRNA targets, followed by study of the candidate miRNAs using the RNAhybrid program (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid>) (280) to calculate the minimum free energy (MFE) of hybridization between miRNAs and target sequence (3'-UTR sequence of *MME* extracted from Ensembl. The strongest binding affinity was found for hsa-miR-362-3p, which overlaps the locus of rs701109 at 3'-UTR region. A stronger predicted pairing affinity between the hsa-miR-362-3p miRNA and the target mRNA of *MME* was observed for C-allele (-26.1 kcal/mol) in comparison to T-allele (-23.2 kcal/mol), stronger interaction with the miR-362-3p, resulting in lower expression of *MME*, and consecutively decreased risk for disease progression. Notably, previous data confirmed that hsa-miR-362-3p is expressed in lung tissue, aberrantly expressed in lung adenocarcinoma, and a promoter of metastasis (281). The differences among cohorts, particularly due to distinct therapeutics, genotype distribution and environmental stimuli, might account for different epigenetic regulation. Experimental studies in lung cancer tissues and *in vitro* with lung cancer organoids are warranted to further validate current findings. The *MME* rs701109 variant consists in a nucleotide substitution of T-by-C, that has been shown to associate with lower NEP protein expression in the brain of patients with Alzheimer's disease (282). Notably, previous studies revealed that higher NEP expression associated with poorer prognosis in NSCLC and that tumour

NEP was an independent predictor of recurrence in lung adenocarcinomas (165, 273). Taking into consideration that increased NEP expression in lung tumours associates with worst prognosis, and that TC and CC genotype carriers secrete less protein, our findings showing an independent protection for disease progression in TC carriers likely reflect the influence of this SNP in decreasing NEP availability. This result strengthens the involvement of NEP in NSCLC and supports the putative interest of including this genetic biomarker in a multilocus model for predicting relapse. Nevertheless, despite the known ability of NEP to degrade mitogenic peptides, the lack of Ang II degradation, as well as reduced production of Ang-(1-7) by NEP, might account for the growth of lung cancer cells (51). Taken together, accumulated data and current findings support the need to foster research in neprilysin actions on cancer, particularly in lung cancer. These results might open new opportunities to improve biomarkers predictive values and therapeutics in lung cancer patients. There is also a need for further genotype-phenotype studies to better clarify the impact of these genetic variants in protein expression. We did not find association with PFS and OS for the remaining germline genetic mutations included in this study. These findings might be related to the lack of biologic relevance of the SNPs included and probably due to the size of the study population. Furthermore, we verified that there were some SNPs without genotype variance for the genes *CMA1*, *MAS1*, *AGTR1*, *AGTR2*, being impossible to analyse the impact of genetic variants in NSCLC prognosis.

As such, this study provides evidence that SNPs coding for molecules involved in the RAS (*ACE2* rs908004, *MME* rs701109) might influence PFS and OS in advanced NSCLC patients, although only *MME* rs701109 was validated in an independent cohort. Candidate SNP studies searching associations with clinically relevant endpoints, have been reported to yield false-positive findings. Here, the multivariate model was first developed in a cohort of consecutively recruited advanced NSCLC patients. Furthermore, we included a robust data analyses setting that included: (i) sample size and number of events in both cohorts were within adequate estimations, (ii) initial empirical analyses to include only the most relevant variables in the multivariate models, (iii) bootstrapping using Montecarlo simulations with 1000 iterations to strengthen internal validation in each cohort, and (iv) validation of important SNPs from

the discovery set in an independent cohort of lung cancer patients matched for demographic and pathological characteristics. Nevertheless, despite matching for clinicopathological characteristics, differences between discovery and validation cohorts existed, and this could partially explain the validation of a single polymorphism. Particularly, the validation set was selected from a larger lung cancer database and somatic mutational analyses were determined using distinct methods, revealing different mutational status for both cohorts, which likely influenced the course of disease and the subsequent therapeutic decisions. In addition, differences in respiratory co-morbidities, which may have conducted to mismatched treatment modalities (surgery with adjuvant chemotherapy versus chemo with radiotherapy for stages IIIA–IIIB), combined with the genetic background may also add to explain failure to validate the other genetic polymorphisms.

Bioinformatics methods are used to construct genetic prognosis assessment model for cancer patients and to screen biomarkers that affect the occurrence and prognosis of cancer. When exploring lung tumour databases, we demonstrated that there was significantly less quantity of mRNA for *REN*, *ACE*, *AGTR1*, *AGTR2*, *MME*, *MAS1*, *CMA1* in the lung adenocarcinoma tissue when compared with the adjacent normal tissue. Furthermore, patients with the lowest values of transcripts for *REN*, *ACE*, *AGTR1*, *CMA1* had worse overall survival. These findings are in agreement with the low expression of *REN*, *ACE*, *AGTR1*, *CMA1* in lung adenocarcinomas, conferring the worse prognosis.

As described in the literature, the lowest serum ACE levels were correlated with poorer prognosis and higher relapse rates in lung cancer (51). Prochazka *et al.* observed a reduction in ACE activity in primary human lung tumours as compared to normal lung tissue (283); a decrease in serum ACE in patients with lung cancer is likely reflective of increased tumour burden, leading to reduced pulmonary epithelial cells which are the primary source for circulating ACE, as well as diminished ACE production by the lung cancer cells. Treatment of patients with chemotherapy or radiotherapy reduces the tumour load resulting in increased ACE production. Taken together, these results suggest that plasma ACE activity may serve as a biomarker for patients with poor prognosis as well as an indicator of responders to therapeutic intervention. A previous study showed that *AGTR1*, *ACE* and *MME* were significantly under-expressed in lung

adenocarcinoma (34). The ability of the AT1 receptor to promote cancer cell growth has been described in the literature (5). However, the reduced expression of the AT1 receptor gene in lung tumour cells, suggests that AT1 receptor stimulation of the tumour cells may not be a driving force for their oncogenic activity. Conversely, the slightly greater reduction in AT2 receptor gene expression could contribute to the oncogenic activity of lung tumour cells by reducing the inhibitory actions on cell growth that Ang II causes by activating the AT2 receptor. Indeed, there is evidence that when AT2 receptors are overexpressed in lung adenocarcinoma cells, their growth is inhibited (76). In our bioinformatic study, we found that there was a significantly low expression of *AGTR2* in lung adenocarcinoma, however we did not find an association with OS.

Furthermore, we also found that decreased transcripts levels of *CMA1* in lung adenocarcinoma was correlated with decreased OS. These results are in conflict with a previous study from Ibaraki T. *et al.*, where it was described higher counts of chymase-positive mast cells in the central region of adenocarcinoma and squamous cell lung cancer tissues than those in the normal regions (27). Another recent report showed that higher NEP expression was associated with poorer prognosis in NSCLC and that tumour NEP was an independent predictor of recurrence in lung adenocarcinomas (51). Our findings are at odds with this bioinformatic study, since we found decreased levels of *MME* in lung adenocarcinoma tissue and there was no association with OS. Further studies, in NSCLC patients at early and advanced stages, are needed to clarify the role of chymase and neprilysin in lung cancer oncogenesis and evaluate their relevancy as therapeutical targets.

Concerning the precursors of RAS, increased expression of angiotensinogen in lung tumour tissue suggests that there may be an increased activation of the RAS in lung tumour cells (34). However, the transcript levels did not have an association with overall survival. There is also lack of evidence of the role of *REN* expression in lung tumours, opening new opportunities for further studies.

In summary, these studies emphasize the involvement of components of the RAS (both direct and extended) in the development and progression of lung cancer. Moreover, it will foster conducted genome-wide expression studies to identify novel

therapeutic targets for NSCLC. This will decrease the cost burden of investigating new drug entities and possibly allow for the repurposing of already approved drugs.

1.2 *Impact of genetic polymorphisms of genes involved in hypoxia and angiogenesis in NSCLC prognosis*

Hypoxia is a hallmark of solid tumours, and consequently related to enhanced aggressiveness, risk of disease progression and therapy resistance, effects known to be largely mediated by hypoxia-inducible factors (284). The downstream upregulated vascular endothelial growth factor (VEGF) further contributes to cancer growth and metastasis, directly targeting tumour cells. Furthermore, angiogenesis is a crucial step for lung cancer progression (182). The creation of new vessels from pre-existing vessels influences tumour growth, invasion, and metastasis (285) and increased VEGF expression and/or circulating VEGF levels have been consistently reported in lung cancer (286).

Noteworthy, the activation of RAS in the tumour microenvironment usually involves Ang II, which has been described to induce hypoxia through reactive oxygen species production, and subsequent activation of pro-inflammatory and pro-angiogenic signals (11).

Albeit no clinical impact was demonstrated for polymorphisms in *HIF1A*, *PGF* and *FLT1* genes, associations were observed in the discovery set for *VEGFA* rs25648 with PFS (univariate analysis) and *KDR* rs1870377 with PFS and OS (multivariate analysis). Koukourakis et al. (287) described that inherited *VEGF* sequence variations were strong determinants of the molecular VEGF and VEGF-downstream phenotype of NSCLC, being the substitution C > A at locus -2578 associated with higher levels of VEGF. Moreover, genetic variants in *VEGFR-1* and *VEGF-A* and plasma levels of *VEGF-A* were associated with clinical benefit, progression-free survival, or overall survival in a cohort of advanced non-squamous non-small-cell lung cancer patients receiving chemotherapy plus antiangiogenic therapy (288). Another study showed that *VEGFA* rs25648 (-73C > T) was a prognostic biomarker for PFS and OS in patients with hepatocellular carcinoma, with C-allele conferring improved prognosis, despite not confirmed in multivariate analysis (289). In the discovery cohort, lung cancer patients had a different outcome since

the homozygous C for *VEGF* rs25648 had lower time until disease progression in univariate analysis. The apparently conflicting results might be due to particularities related with different cancer models. Notably, studies investigating the potential association between *VEGF* polymorphisms and circulating VEGF levels also led to controversial results (290-292), opening new opportunities for further investigations to clarify the role of germline VEGF variations in NSCLC progression and its repercussion in VEGF levels.

The *KDR* rs1870377 was significantly associated with PFS and OS in the discovery set. Homozygous T and C had increased risk for tumour progression PFS and death, independent of clinicopathological variables and after bootstrap analysis. Findings were not replicated in the validation cohort. *KDR* encodes VEGFR-2, the key receptor mediating endothelial cell proliferation and sprouting, leading to angiogenesis and tumour promotion upon binding and signalling through the VEGFA pathway (196). The *KDR* rs1870377 (c.1719A > T) variant induces a missense alteration in exon 11 that results in the substitution A > T and in the amino acid modification Gln472His. This substitution was previously described to alter the function of VEGF-KDR signalling pathway, enhancing ligand/receptor binding efficacy and upregulated signal transduction, ultimately leading to increased microvessel density (293). This phenotypic correlation with the *KDR* rs1870377 might support T homozygous findings, hypothetically by promoting tumour vascular density for homozygous carriers. Previous findings reported that microvessel count, reflecting tumour neoangiogenesis, appeared to be a poor prognostic factor for survival in NSCLC patients (294), despite some conflicting results (295).

In 2011, experimental and translational research concerning angiogenesis and NSCLC was published to assess the role of VEGFR2 in tumour behaviour (196). In vitro, VEGFR2 copy number gains were significantly associated with resistance to platinum chemotherapy as well as increased levels of nuclear HIF-1 α in both NSCLC tumour specimens and cell lines (296). VEGFR2 genetic variation (Q472H, -2854 C, and -2455 A) in three populations had an impact on tumour VEGFR2 expression and vascularization (196). In light of these data, it is understandable that the role of angiogenesis and NSCLC

is not completely elucidated. Clinicians should expect further improved research about enhanced approaches involving anti-angiogenic therapies and NSCLC.

Inhibition of VEGF by bevacizumab confers immune modulatory effects that, when combined with chemotherapy, may further enhance the T-cell-mediated killing of cancer cells by atezolizumab through reversal of VEGF-mediated immunosuppression and chemotherapy-induced cell death (297). The phase III Impower150 trial assessed the efficacy and safety of bevacizumab + atezolizumab + chemotherapy as first-line therapy in patients with stage IV or recurrent metastatic nonsquamous NSCLC, including patients with EGFR/ALK mutations. The results showed that first-line treatment with atezolizumab + bevacizumab + carboplatin and paclitaxel significantly prolonged PFS and OS compared with bevacizumab + carboplatin and paclitaxel in patients with non-squamous NSCLC, including those with EGFR mutations (297). There is a need for further research concerning genetic biomarkers in patients treated with bevacizumab. Therefore, variants of angiogenic VEGF genes might be useful to predict the best responders to bevacizumab treatment.

Ramucirumab is a human IgG1 monoclonal antibody that targets the extracellular domain of VEGFR-2 (298). Patients with squamous or non-squamous NSCLC who had progressed during or after a first-line platinum-based chemotherapy regimen were included in the multicentre, double-blind, randomised phase 3 trial (REVEL) (298). Patients were randomly allocated (1:1) to receive docetaxel 75 mg/m² and either ramucirumab (10 mg/kg) or placebo on day 1 of a 21 days cycle until disease progression, unacceptable toxicity, withdrawal, or death. The results showed that ramucirumab plus docetaxel improves survival as second-line treatment of patients with stage IV NSCLC (298).

Our study showed that KDR rs1870377 was significantly associated with PFS and OS. This finding might contribute to foster further research in the field of pharmacogenomics in patients with advanced NSCLC treated with ramucirumab.

In summary, the results of this study support previous findings regarding the influence of SNPs on angiogenic VEGF genes clinical outcomes of patients with NSCLC. In most cases, consistent results across studies are lacking, warranting further research

on the subject before molecular biomarkers can be useful in selecting patients with NSCLC and being included in treatment algorithms for antiangiogenic agents.

1.3 *Impact of genetic polymorphism of AGXT in NSCLC prognosis*

During the last years, advances in genetic knowledge about lung cancer mutational landscape, together with development of targeted therapies, led to a paradigm shift in the treatment of NSCLC. Nevertheless, platinum-containing regimens remain the appropriate treatment for most patients (299). Clinical management of resistance or toxicity to chemotherapy in NSCLC patients would benefit from the identification of predictive and prognostic molecular biomarkers, including functional genetic polymorphisms.

The *AGXT* gene, located in chromosome 2q37.3 region, encodes the alanine-glyoxylate aminotransferase, whose activity is largely confined to peroxisomes in the liver (300). This enzyme catalyses the transamination between L-alanine and glyoxylate to produce pyruvate and glycine using pyridoxal 5'-phosphate as cofactor (301). A missense genetic variant (*AGXT* rs34116584), with a proline-to-leucine substitution located at codon 11 of exon 1, occurs with a frequency of 15-20% in European and North American population (302). This polymorphism was primarily studied in primary hyperoxaluria type I (303-305). A recent report explored its role in cancer, showing an association with disease progression and death in metastatic colon cancer patients treated with oxaliplatin (234). Reports are sparse concerning the association of this SNP with cancer and have never been explored in lung cancer patients.

Herein, the *AGXT*-rs34116584 genetic polymorphism was analysed in locally advanced/metastatic NSCLC patients, using as outcomes the PFS and OS. Multivariate analyses revealed an independent increased risk for disease progression and for death in *AGXT* rs34116584 T-carriers, after adjustment for tumour size, distant metastasis, ECOG PS, treatment modality or systemic therapy. Previous molecular in vitro studies showed that the C-to-T substitution results in an amino acid modification at position 11 and creates a conformational alteration that ultimately leads to a significant decrease in alanine-glyoxylate aminotransferase's activity and subsequent accumulation of oxalate (306, 307). Both oxalate and glyoxylate generate reactive oxygen species (ROS) (308, 309),

which have been associated with increased mutational burden, tumour progression and dissemination (310). Since T-allele carriers have higher levels of oxalate (311) and consequently are prone to increased ROS production, the worst prognosis described for TT/TC might be an oxidative stress-mediated deregulation induced by *AGXT* rs34116584 SNP. This effect might be exponentiated upon exposure to hypoxia and oxidative stress causing DNA damage, or during concomitant administration to cytotoxic therapies (312).

Furthermore, a significantly shorter time-to-disease progression was found for T-allele carriers independent of *EGFR* mutational status, although no relation was observed with OS for subjects with *EGFR* tumour mutation. These findings could be aligned with a minor clinical relevance for *AGXT* rs34116584 SNP in comparison to *EGFR* mutation status that impacts a longer-term endpoint. Notably, tyrosine kinase inhibitors (TKIs) improve survival in NSCLC patients with *EGFR* mutation (313), modifying the natural history of disease, and possibly impacting the association of the genetic polymorphism.

In patients under first line platinum-based doublets, we verified that T-allele carriers had shorter PFS and OS; regardless of tumour size, distant metastasis, ECOG PS and treatment modality. These well-established prognostic covariates, were shown to influence NSCLC clinical outcomes (314). Here, the *AGXT* rs34116584 association with response to platinum-based chemotherapy remained significant, despite adjustment for these factors, suggesting that this SNP might add significant information to traditional clinical predictive and prognostic factors. The *AGXT* rs34116584 C>T substitution, induces a decrease of alanine-glyoxylate aminotransferase activity and is responsible for the mistargeting of the enzyme from the peroxisomes to the mitochondria, where the enzyme cannot work properly (315). These changes were predicted to have significant effects in oxalate synthesis and excretion, and the deposition of insoluble calcium oxalate in the kidney and urinary tract (316), which could be associated with increased toxicity and lesser efficacy of platinum based chemotherapy.

Moreover, cisplatin causes a number of significant side effects including nausea and vomiting, neutropenia, ototoxicity, neurotoxicity, and renal function impairment (317). Despite efforts to identify genetic predictors of the effectiveness and toxicity of cytotoxic

therapies, to this point there are no robust data that can be used in clinical practice to guide the best subgroup of patients to receive cisplatin (317). Although carboplatin induces nephrotoxicity to a lesser extent, it induces more myelotoxicity (318). No association was found in our study for the *AGXT* rs34116584 SNP with febrile neutropenia, although the low number of subjects included in this analysis limits conclusions. Further studies, in larger independent populations, are required to confirm these results. Despite inherent size limitations, in this study patients were recruited from a homogeneous cohort, the analysed SNP was selected based on functional biological relevance, and the study design and statistics accounted for important risk factors in NSCLC.

VI. Conclusions and future perspectives

Germline genetic variants are common genomic events, putatively inducing phenotypic modifications affecting both normal physiology and cancer physiopathology. Data presented here supports the rationale for RAS mechanistic involvement and angiogenic genes variants in NSCLC, whereas the external validated polymorphism *MME* rs701109 might prove to be a useful biomarker for NSCLC progression. These findings will foster further and larger studies to confirm the role of RAS, and particularly NEP in NSCLC, as well as to determine the usefulness of *MME* rs701109 as a molecular marker for disease progression. Taken together, these results can lead to a better understanding of NEP influence in the NSCLC biological mechanisms, contributing towards the definition of a genetic profile for this disease and eventually serve as guidance for therapeutic decisions. Finally, in order to confirm whether targeting the RAS has the potential to reprogram cancer cell immunogenicity and the immunosuppressive lung tumour microenvironment, preclinical research that combine immune checkpoint inhibitors with RAS agonists/antagonists is needed.

Moreover, the genetic variants of angiogenic genes might open new opportunities for further research in the field of pharmacogenomics, especially in patients with NSCLC treated with anti-angiogenic factors, contributing for their inclusion in the treatment algorithms.

Our study also showed that the *AGXT* rs34116584 might have an influence in platinum metabolism, with impact on toxicity and tumour aggressiveness, being associated with worse prognosis. This polymorphism seems to have also an impact on NSCLC progression, opening new perspectives for its inclusion as a biomarker or as a pharmacogenetic predictor of response to platinum-based chemotherapy. Future studies are needed to further strengthen the genotype-phenotype association of the SNPs that had clinical impact in NSCLC patients.

VII. References

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VIII. Annex

1.1 Annex I



Review

Renin-Angiotensin System in Lung Tumor and Microenvironment Interactions

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Abstract: The mechanistic involvement of the renin-angiotensin system (RAS) reaches beyond cardiovascular physiopathology. Recent knowledge pinpoints a pleiotropic role for this system, particularly in the lung, and mainly through locally regulated alternative molecules and secondary pathways. Angiotensin peptides play a role in cell proliferation, immunoinflammatory response, hypoxia and angiogenesis, which are critical biological processes in lung cancer. This manuscript reviews the literature supporting a role for the renin-angiotensin system in the lung tumor microenvironment and discusses whether blockade of this pathway in clinical settings may serve as an adjuvant therapy in lung cancer.

Keywords: lung cancer; renin-angiotensin; hypoxia; tumor microenvironment

1. Introduction

Lung cancer is one of the most common malignancies and the most frequent cause of cancer deaths in the past few decades, with over one million people diagnosed worldwide each year [1,2]. Notably, the five-year survival rate is the lowest compared with other frequent malignancies [3]. Its poor prognosis fosters the need for research, namely understanding its aggressiveness mechanisms, in order to improve prevention and treatment.

The renin-angiotensin-aldosterone system is an established primary regulator of blood pressure, homeostasis, and natriuresis [4]. Nevertheless, recent evidence indicates that angiotensin peptides also might have a role in tumor cell proliferation, inflammation and hypoxia, which have been recognized as hallmarks of cancer, actively participating in lung tumor microenvironment regulation [5]. Here, we review current knowledge on the renin-angiotensin system (RAS), considering its potential modulatory role on cancer cell phenotype and on immune surveillance, while exploring the mechanistic association with lung cancer.

2. The Renin-Angiotensin System: An Intricate Regulatory Mechanism in Lung Disease Physiopathology?

The RAS was first discovered and studied in the physiological regulation of blood pressure, fluid homeostasis and pathogenesis of hypertension [6]. Angiotensin II (Ang II), the final effector of the system, causes vasoconstriction, both directly and indirectly, by stimulating Ang II type 1 receptor (AT1 receptor) present on the vasculature, and by increasing sympathetic tone and arginine vasopressin release. Chronically, Ang II regulates blood pressure by modulating renal sodium and water reabsorption directly, by stimulating AT1 receptors in the kidney, or indirectly, by stimulating the production and release of aldosterone from the adrenal glands, or stimulating the sensation of thirst in the central nervous system [7]. The enzymatic cascade by which Ang II is produced consists of renin, which cleaves angiotensinogen (AGT) to form the decapeptide angiotensin I (Ang I). Ang I is then further cleaved by angiotensin-converting enzyme (ACE) to produce the octapeptide Ang II, the physiologically active component of the system. Further degradation by aminopeptidase A and N produces angiotensin III (Ang 2–8), and angiotensin IV (Ang 3–8), respectively [7] (Figure 1). The actions of Ang II result from its binding to specific receptors (AT1 and AT2). Ang II, via its AT1 receptor, is also involved in cell proliferation, left ventricular hypertrophy, nephrosclerosis, vascular media hypertrophy, endothelial dysfunction, neointima formation, and processes leading to atherothrombosis [8]. Ang II, via its AT2 receptor, is involved in cell differentiation, tissue repair, apoptosis and vasodilation [8]. Other receptors have been described in relation to the RAS. For instance, an AT4 receptor binding site has been identified, and in contrast to the other AT receptors, it does not seem to be a G protein-coupled receptor [9]. This receptor binds Ang IV (3–8) preferentially, has been localized in various mammalian tissues, and suggested to cause vasodilatation [9].

Recently, a role for local tissue-based RAS has been described, both as modulator of inflammation and of the injury/repair response [10]. In addition, supportive evidence claims that tissue RAS is capable of working synergistically or independently of the systemic RAS, and locally generates angiotensin peptides with regulatory homeostatic functions, thus contributing to dysfunction and/or disease [11] (Figure 1). Within the RAS, the generation of angiotensin peptides is controlled by complex interactions: renin, which is released from the juxtaglomerular apparatus of the kidney, converts the angiotensinogen released by the liver to form angiotensin I. In fact, the liver is the primary source of circulating AGT, a minor but significant fraction of which is filtered through the glomerulus and reabsorbed by proximal tubule cells. It was shown that liver-specific angiotensinogen knockout mice had nearly abolished plasma and renal angiotensinogen protein and renal tissue Ang II [12]. On its turn, the ACE plays a central role in generating Ang II from Ang I [13]. The capillary blood vessels in the lung are one of the major sites of ACE expression and Ang II production in the human body [14]. Alterations in RAS expression were shown to be involved in multiple lung diseases: idiopathic pulmonary fibrosis [15], sarcoidosis [16], pulmonary hypertension [17], acute respiratory distress syndrome [18], and lung cancer [19]. Understanding the involvement of intra-pulmonary RAS on inflammation or fibrosis may open new therapeutic possibilities for the treatment of respiratory diseases. In fact, the increased ACE expression observed in several interstitial lung diseases supports the existence of a pulmonary RAS and establishes a putative role for Ang II in the response to lung injury and fibrosis [10]. Concurring, findings from a substantial number of *in vivo* studies demonstrated that ACE inhibitors were capable of attenuating experimental pulmonary fibrosis [20]. Captopril, enalapril, lisinopril and perindopril [21–23], could block the effects of bleomycin-, γ irradiation-, amiodarone- and paraquat-induced pulmonary fibrosis in rats. A recent post hoc analysis of data from the placebo arms of phase 3 trials including patients with idiopathic pulmonary fibrosis, showed a slower disease progression in patients under therapy with ACE inhibitors [24]. Therefore, local upregulation of the expression of either Ang II or TGF- β 1, which might influence each other's activity or act in synergy, should be blocked to delay the progression of lung fibrotic disease.

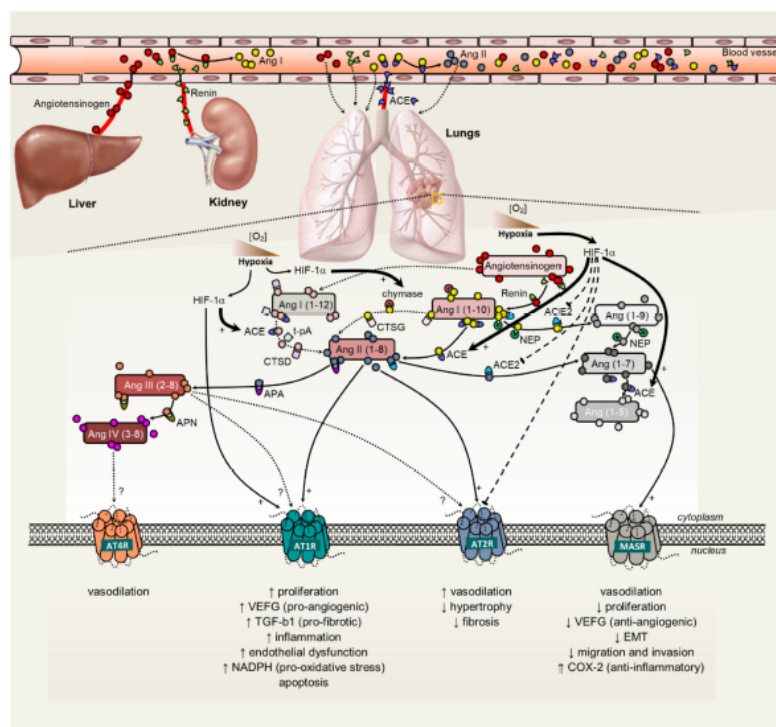


Figure 1. Schematic representation of the renin-angiotensin system, its interaction with tumor hypoxia and angiogenesis and subsequent functional impact on tumor phenotype. Full line arrows represent positive effects, whereas dashed lines with blunt ends represent inhibitory effects. Angiotensinogen, a precursor of angiotensin peptides, is the only known naturally occurring renin substrate. Ang I is processed by ACE or ACE2 to produce Ang II and Ang-(1-7), respectively. Alternatively, Ang I can be converted to Ang II through chymase. Ang-(1-7) can be generated from Ang I, via Ang-(1-9), a pathway that utilizes both ACE2 and ACE, or by neprilysin that hydrolyzes Ang I directly to Ang-(1-7). Ang II acts in cells through two classes of seven trans-membrane G protein-coupled receptors: the angiotensin type I (AT1) and angiotensin type II (AT2) receptors. Upon binding of Ang II to AT1R, a downstream intracellular cascade is induced that results in cell proliferation, fibrosis and hypoxia by generating reactive oxygen species and subsequently pro-inflammatory and pro-angiogenic signals. The AT1 receptor is overexpressed in neoplastic tissues, suggesting its involvement in carcinogenesis. The role of AT2 receptors in cancer remains controversial, although including pro-apoptotic and anti-proliferative effects. Ang-(1-7) binds to MasR and counteracts the resulting effects of Ang II/AT1 receptor activation, preventing tissue remodeling, improving vascular and cardiac function, and promoting the downregulation of cell proliferation, migration and metastasis. Hypoxia up-regulates Ang II, ACE, and AT1 receptor, while downregulating ACE2 and AT2 receptor. Local angiotensin II predominantly exists in the hypoxic regions of tumors, while these tumor cells produce Ang II by a chymase-dependent mechanism. Subsequently, the hypoxic tumor microenvironment induces the upregulation of the ACE/Ang II/AT1R axis, which is associated with the hallmarks of cancer, while it downregulates the ACE2/Ang-(1-7)/Mas receptor axis. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AMNA, aminopeptidase A; AMNN, aminopeptidase N; Ang, angiotensin; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; AT4R, angiotensin receptor 4; CAGE, chymostatin-sensitive angiotensin II-generating enzyme; COX-2, cyclooxygenase 2; EMT, epithelial-to-mesenchymal transition; HIF1 α , hypoxia-inducible factor 1-alpha; MASR, Mas receptor; NADPH, hydro-nicotinamide adenine dinucleotide phosphate; TGF β 1, transforming growth factor beta 1; t-PA, tissue-plasminogen activator; VEGF, vascular endothelial growth factor.

Ang I can also be converted to Ang II through the action of chymase, a chymotrypsin-like enzyme that is expressed in the secretory granules of mast cells [25]. In fact, chymase is the most

efficient Ang II-forming enzyme in the human body and has been implicated in a wide variety of human diseases that also implicate its many other protease actions: (1) cleavage of extracellular proteins and peptides, including laminin and fibronectin important for cell survival; (2) activation of peptide and enzyme precursors, including matrix metalloproteinases (MMPs), such as MMP-9, TGF- β , stem cell factor, kallikrein, IL-6 and IL-1 β , preproendothelin I and IL-18 [26]. Chymase activity was noticeably increased in the lungs of animals following bleomycin instillation [27]. A recent report showed the prominent accumulation of chymase-expressing mast cells in idiopathic pulmonary arterial hypertension and idiopathic pulmonary fibrosis lungs, and their presence was near the regions with marked TGF- β 1 expression [27]. Furthermore, tumor sections from 32 cases of adenocarcinoma and 13 cases of squamous cell carcinoma revealed significantly higher counts of chymase-positive mast cells in the central region than those in the normal regions [28]. Chymase has been associated with TGF- β activation and endothelin-1 formation from preproendothelin-1 in pulmonary fibrosis and chronic obstructive pulmonary disease [26], and in proliferation, adhesion, regulation of E-cadherin expression, and modulation of immunosuppressive microenvironment in lung carcinoma [29,30]. This effect is likely due to TGF- β inhibitory effect on T-cell proliferation and effector activity. Moreover, TGF- β subverts T cell immunity by favoring regulatory T-cell differentiation, further reinforcing immunosuppression at the tumor microenvironment [31].

Ang II is a ligand for two classes of seven trans-membrane G protein-coupled receptors, angiotensin II type 1 (AT1) and angiotensin II type 2 (AT2) receptors [25]. The cellular distribution of AT1 and AT2 receptors in the normal and pathological human lung has been previously described [32]. Whereas *AGTR1* transcript and encoded protein, the AT1 receptor, localized at vascular smooth muscle cells, alveolar macrophages and in the stroma underneath the airway epithelium, *AGTR2* expression, at both mRNA and protein levels, of AT2 receptors were found in bronchial epithelia and endothelial cells [33]. The components of RAS are frequently differentially expressed in various cancers including brain, lung, pancreatic, breast, prostate, colon, skin and cervical carcinomas in comparison with their corresponding non-malignant tissue [34]. A large-scale study of estrogen receptor-positive breast cancer tumors revealed an increase on *AGTR1* mRNA expression. Contradictory results were observed in lung adenocarcinoma tissues since *AGTR1*, *AGTR2* as well as ACE mRNA expression, were underexpressed. Notably, AGT was overexpressed in lung adenocarcinoma tissue [35]. Notably, several physiological and pathophysiological effects of Ang II are mediated mainly through the AT1 receptor [36]. Upon its activation, a pleiotropic regulatory role is induced at the lung, resulting in vasoconstriction, cell proliferation, angiogenesis, and augmentation of pro-inflammatory cytokine production, inflammatory cell chemotaxis, epithelial cell apoptosis, increased oxidative stress and fibrosis [37]. Several in vitro studies demonstrated that the epithelial to mesenchymal transition (EMT) induced by TGF- β 1 resulted in an increase in angiotensinogen and AT1 receptor expression, in human lung fibroblasts [38–40]. Moreover, active TGF- β 1 expression by human lung myofibroblasts was downregulated by AT1 receptor blockade and accompanied the inhibition of collagen synthesis [20]. In contrast, the activation of the AT2 receptor induces opposite effects, although some proinflammatory responses are mediated through the activation of the Nuclear Factor- κ B (NF- κ B) pathway [37].

Ang II can also be converted into Angiotensin-(1–7) (Ang (1–7)) by the enzymatic activity of angiotensin converting enzyme type 2 (ACE2) or from Ang I, via Angiotensin (1–9) (Ang-(1–9)), a pathway that involves both ACE2 and ACE [34,41] (Figure 1). In the human lung, ACE2 is expressed in endothelial and smooth muscle cells of large and small blood vessels, as well as in alveolar epithelial cells type I and II, and bronchial epithelial cells [42]. ACE2 has a multiplicity of physiological roles that revolve around its trivalent function: a negative regulator of the RAS, facilitator of amino acid transport, and is the entry receptor for SARS-CoV and SARS-CoV-2 [43].

Alternatively, Ang-(1–7) might be produced through hydroxylation of Ang I by the enzyme neprilysin [44]. Neprilysin is a cell membrane-bound zinc metalloprotease that catalyzes the degradation of a number of endogenous vasodilator peptides, such as atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide [45]. Physiological actions of neprilysin depend on the balance

of its action on the breakdown of vasodilators and vasoconstrictors [46]. Neprilysin has been involved in malignancies, such as prostate, renal and lung cancer [47]. Ang-(1–7) binds to the G protein-coupled receptor Mas, counteracting cardiovascular and baroreflex effects of Ang II through AT1 receptor, and has been suggested to have vasodilator properties and to act as an inhibitor of cell proliferation [48]. It was reported that ACE2 upregulation can reduce lung injury, whereas ACE2 or Ang-(1–7) have a role in the prevention of acute respiratory distress [49–51]. ACE2 has recently been identified as the SARS-CoV-2 receptor, the infective agent responsible for COVID-19, providing a critical link between immunity and inflammation [43]. Furthermore, some studies reported that the ACE2/Ang-(1–7)/Mas axis counteracted the ACE/Ang II/AT1 axis in different models of cancer, including lung cancer [52–54].

The circulating RAS might also be important in providing the substrates for local modulation of angiotensin peptides with influence in lung diseases. Genetic and epidemiological evidences provide support that germline mutations of RAS components contribute to the risk of developing idiopathic pulmonary fibrosis, acute lung injury and certain malignancies. For example, the human gene encoding angiotensinogen (*AGT*) is located on chromosome 1q42.3, where a large number of single-nucleotide polymorphisms (SNPs) has been described. Among them, the SNPG-6A nucleotide substitution at position 6, upstream from the initial transcription start codon, has been studied particularly. The A allele has been associated in vitro with an increased expression of the *AGT* gene and with higher *AGT* synthesis [55]. A previous report showed that the G-6A polymorphism of the *AGT* gene is associated with increased angiotensin production and idiopathic pulmonary fibrosis progression [55]. The *AGT* M235T variant conferred a risk for developing breast cancer in post-menopausal women [56]. Moreover, ACE plasma levels depend on a 287 bp insertion/deletion (I/D) polymorphism of the ACE gene located on chromosome 17q23. Homozygotes for the D allele have the highest ACE plasma levels, while homozygotes for the I allele have the lowest, and ID heterozygotes have intermediate levels. Several studies found an association between ACE I/D polymorphism and lung cancer, albeit some conflicting results [56–58].

Thus, the altered regulation of RAS homeostasis, either genetically or environmentally-driven, might contribute to the deregulation of intersected axes, thereby modulating lung cancer aggressiveness.

3. Renin-Angiotensin System and the Hallmarks of Cancer: Application to Lung Tumors

The RAS is an important signaling system within the tumor microenvironment, promoting both malignant and stromal cell proliferation [5]. Notably, it is also involved in the regulation of metabolic homeostasis and of tumor-associated phenotypes, such as angiogenesis, migration and invasion [5,11,59] (Figure 2).

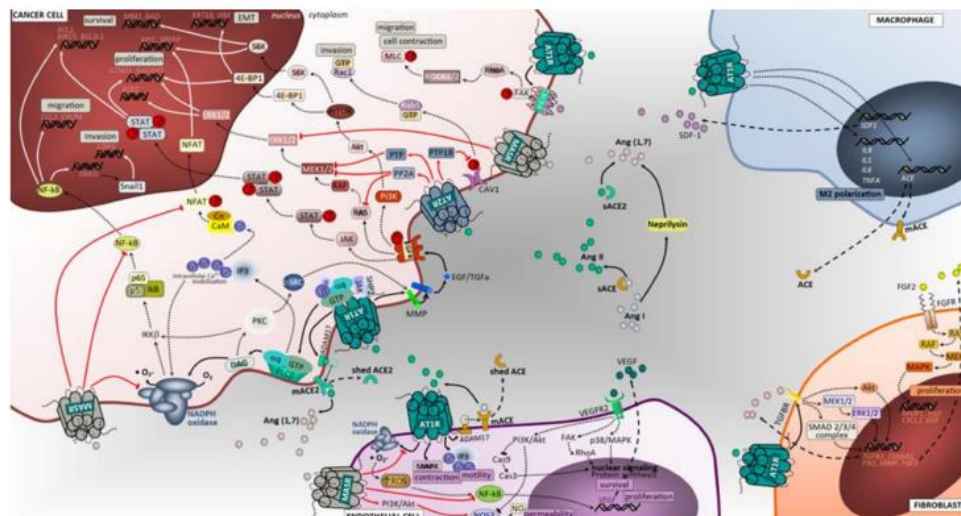


Figure 2. Angiotensin-associated pathways associated with cell proliferation, invasion, and migration in lung tumor microenvironment. Membrane-bound and soluble ACE and ACE2 catalyze the production of angiotensin II or angiotensin (1,7), ligands that exert regulatory functions in tumor microenvironment cells. The effects of activating Ang II/AT1R, Ang II/AT2R and Ang (1,7)/MasR axes' signaling has been mostly studied in tumor cells, including lung cancer cells. Overall, in contrast to the Ang II/AT1R that mediates several pathological events associated with activated RAS, the Ang (1,7)/MasR and Ang II/AT2R pathways are thought to antagonize many of the cellular actions of the Ang II-AT1R axis. In cancer cells, upon binding of Ang II to AT1R, a pleiotropic downstream signaling cascade is triggered, ultimately causing, either directly or indirectly, upregulation of cell proliferation, survival, motility, migration, invasion and EMT. Activated AT1R subunits stimulate PLC β , that hydrolyses membrane lipids, activates PKC and mobilization of intracellular Ca²⁺, while free G β and G γ subunits bind and gate ion channels. Activated G α /11 units also activate the JAK-SHP2/STAT pathway and receptor tyrosine kinase (RTK) transactivation. Activation of RTK, depicted in the figure as EGFR, occurs through second messenger's stimulation of ADAM family and MMPs to cleave its ligands (in the figure EGF and TGF α) that bind and activate RTK. Alternatively, the AT1R-mediated activation of MMPs can follow a PLC β /DAG/PKC/c-SRC signaling mechanism to elicit increased ligands for RTK. Subsequent signaling upon activation of EGFR is represented in the figure using dashed arrows. Other intracellular cascades mediated by Ang II/AT1R include the activation of CXCR4/SDF-1 signaling through FAK/RhoA/ROCK1-2/MLC increasing cell contraction, migratory potential and tumor invasion. The Ang (1,7)/MasR activation inhibits NFAT transcriptional regulation that reduces proliferation. Notably, this pathway blocks the NF- κ B molecule formation thereby impacting EMT (including Snail1-mediated), invasion and survival. In addition, the inhibitory effect over ERK1/2 and NADPH oxidase signaling pathways significantly impact cell proliferation. The Ang II/AT2R pathway signals are mediated through protein phosphatases PTP1B, PTP and PP2A. The inhibition of CAV-1 phosphorylation stops the Rab5/Rac1/GTP migratory potential of malignant cells, thus suppressing invasion. Furthermore, AT2R-associated increase in PTP and PP2A exerts blocking effects in RTK-mediated signals of the RAS/RAF/MEK1-2/ERK1-2 pathway at the level of MEK1/2 and RAS molecules, reducing cell proliferation. Macrophages, endothelial cells and fibroblasts are important components of the tumor microenvironment and capable of generating and expressing RAS components. These cells, beyond their functional ligands and receptors that are altered in tumor microenvironment, and reflect the crosstalk between all cell constituents, also use the RAS signaling pathway (mostly AT1R, but also MasR in endothelial cells) to yield functional characteristics that ultimately may favor the cell itself and enhance tumor growth. BIRC5, surviving gene; BCL2, B-cell leukemia/lymphoma 2; BAD, BCL2 associated agonist of cell death; GADD45, Growth Arrest and DNA Damage 45; SREBP, Sterol regulatory element binding proteins; ROCK1/2, Rho-associated protein

kinase 1/2; Rab5, Ras-related protein Rab-5; S6K, ribosomal S6 kinase; PTP, protein tyrosine phosphatase; PP2A, Protein phosphatase 2; 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; IKKB, Inhibitor of nuclear factor kappa-B kinase subunit beta; c-SRC, cellular Proto-oncogene tyrosine-protein kinase; SHP2, Src homology region 2 domain-containing phosphatase-2; PTP1B, Protein tyrosine phosphatase 1B; MLC, myosin light chain; CAV1, caveolin 1; IL1, interleukin 1; TNFA, tumor necrosis factor; Snail1, Zinc finger protein SNAI1; *SNAI1*, Snail Family Transcriptional Repressor 1; JAK, janus kinase; STAT, signal transduction and activator of transcription; GTP, guanosine triphosphate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; CXCL1, The chemokine (C-X-C motif) ligand 1; IL8, interleukin 8; NF-kB, Nuclear Factor-kappa B; SDF1, stromal cell-derived factor 1; IL6, interleukin 6; HGF, hepatocyte growth factor; RAS, oncogene protein p21; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; Cas9, caspase 9; Cas3, caspase 3; ERK1/2, extracellular signal-regulated kinase; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; MEK1/2, mitogen activated protein kinase kinase; HIF1 α , hypoxia inducible factor 1 alpha; NO, nitric oxide; RhoA, Ras homolog family member A; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; IP3, inositol trisphosphate; DAG, diacylglycerol; M2, macrophage polarized towards M2; ADAM17, Disintegrin and metalloproteinase domain-containing protein 17; p38/MAPK, protein 38/mitogen activated protein kinase; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; MasR, G-protein coupled Mas receptor; VEGF, vascular endothelial growth factor; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; mACE, membrane angiotensin converting enzyme; sACE, soluble angiotensin converting enzyme; mACE2, membrane angiotensin converting enzyme 2; sACE2, soluble angiotensin converting enzyme 2; TGFBR, transforming growth factor beta receptor; VEGFR2, vascular endothelial growth factor receptor 2; TGF α , transforming growth factor alpha; Ang (1,7), angiotensin 1,7; PLC β , phospholipase C beta; FAK, Focal adhesion kinase; NFAT, nuclear factor of activated T cells.

3.1. Cell Proliferation, Invasion and Migration

3.1.1. ACE/Ang II/AT1 Receptor Axis

Ang II is an important growth promoter in a variety of cell types, where it activates the phosphatidylinositol signaling via AT1 receptor and increases cytosolic Ca²⁺, a process linked to mitogenesis [60]. Another intracellular mechanism by which Ang II controls cell growth is through the modulation of protein tyrosine kinases (PTK) [61]. These proteins are coupled to several growth factor receptors, particularly epidermal growth factor receptor (EGFR), and transduce growth factor-induced signals through protein kinase C (PKC), or through MMPs-mediated mechanisms, which include a disintegrin and metalloproteinase (ADAM) family members. This crosstalk was shown to contribute towards cancer [5] (Figure 2). Ang II was shown to stimulate the migration of fibroblasts through activation of AT1 receptor in an EGFR activation-dependent process and be mediated by shedding of heparin-binding-EGF [62]. Noteworthy, previous experiments have shown that EGFR inhibition partially blocked Ang II-induced proliferation of breast intraductal carcinoma cells [63]. Although the exact molecular mechanism underlying the transactivation of EGFR through Ang II-AT1 receptor binding in lung tumor cells is not completely understood, previous clinical studies evidenced an effect of ACE inhibitors or angiotensin type 1 receptor (ATR1) blockers on lung cancer patients, in combination with chemotherapeutics and EGFR inhibitors [64,65].

c-Fos and c-jun genes are members of the activator protein-1 (AP-1) transcription factor family and are well known targets of the ERK1/2 mitogen activated protein kinase (MAPK) pathway [66]. Ang II increased the phosphorylation of the extracellular signal-regulated protein kinase 1/2, c-jun-N-terminal kinase 1/2, or p38 mitogen-activated protein kinases, signaling pathways involved on cell proliferation and particularly relevant for cancer development [67,68] (Figure 2). Notably, in fibroblasts, Ang II binding to AT1R results in Smad2 and Smad3 phosphorylation via the ERK/p38/MAPK pathway, increasing the activation of the TGF β 1/Smad2/Smad3/Smad4 signaling [69]. Telmisartan, an AT1 receptor antagonist, significantly inhibited the growth of the non-small cell lung cancer (NSCLC) A549 cell line, in a time- and dose-dependent manner and led to an increase of the pro-apoptotic proteins

caspase-3 and Bcl-xL [70]. Attoub S and colleagues demonstrated that captopril significantly inhibited tumor growth using a non-small cell lung carcinoma LNM35 pre-clinical model [71].

The establishment of distant metastatic disease is associated with poor prognosis in patients with lung cancer. Tumor metastasis involves several biological steps, such as cell-to-cell and cell-to-extracellular matrix (ECM) interactions, degradation of ECM components, and new vessels formation [72], and some studies suggest that cancer stem cells (CSC) contribute, directly and indirectly, to the generation of metastases [73–75]. Ang II/ATR1 pathway activation has been shown to promote EMT in cancer cells, which is a hallmark of cancer aggressiveness [76], while chymase, a protease that converts Ang I to Ang II, increased the expression of MMP-9 in a dose dependent manner in two lung cancer cell lines (A549 and H520) [30]. Moreover, it was shown that Ang II induced CSC marker expression and facilitated tumor cell survival in NSCLC cells line [77]. Recent data from a breast cancer model revealed an additional mechanism whereby ATR1 promotes tumor cell contraction, migration and invasion by upregulating the C-X-C chemokine receptor type 4 (CXCR4)/Stromal cell derived factor-1 α (SDF-1 α) signaling through the Focal adhesion kinase (FAK)/Ras homolog family member A (RhoA)/Rho associated kinase 1/2 (ROCK1/2)/Myosin light-chain kinase (MLC) pathway, ultimately inducing metastatic disease [78]. Taken together, these findings support a role for mediators of the Ang II/ATR1 axis as lung cancer modulators, particularly influencing malignant cell growth, dedifferentiation, and migration (Figure 2).

3.1.2. AT2 Receptor

In contrast to the pro-tumor effects of AT1 receptor signaling, activation of the AT2 receptor has been shown to be protective, often acting as opposite to AT1 receptor. The AT2 receptor inhibited cell proliferation while stimulating apoptosis in a variety of cell types, including vascular smooth muscle cells, cardiomyocytes, endothelial cells, and malignant prostate and lung cells [79–82]. Furthermore, AT2 receptor overexpression induced apoptosis independent of Ang II, through p38 MAPK, caspase 8 and caspase 3 activation, and was mediated through an extrinsic cell death-signaling pathway partially dependent on p53 [83,84]. Notably, nanoparticle-based AT2 receptor gene transfection, which markedly increased AT2 receptor expression, resulted in increased cell death of the human lung cancer cell line A549 [81]. The activation of AT2 receptor signaling has been described to produce antiproliferative, survival-promoting, as well as migratory, pro-invasive effects due to phosphatase-mediated (phosphotyrosine phosphatase PTP, protein tyrosine phosphatase 1B, PTP1B, and protein phosphatase 2A, PP2A) dephosphorylating events, through an inhibitory effect on Ras and MEK1/2 proteins of the Smad and MAPK pathways (PTP and PP2A) and by PTP1B-mediated novel Caveolin-1 (CAV1) dephosphorylation and inhibition of the CAV1/Rab5/Rac-1 pathway [69,85] (Figure 2).

3.1.3. ACE2/Ang (1–7)/Mas Receptor Axis

The branch of the ACE2/Ang-(1–7)/MasR axis connected to the RAS has been associated with anti-proliferative and anti-metastatic properties. It was shown that overexpression of ACE2 inhibited the proliferation of lung cancer cells *in vitro* and reduced tumor growth *in vivo*, in a mouse lung xenograft model [86]. Furthermore, with human lung tumor xenografts evidenced that treatment with Ang-(1–7) reduces tumor volume in mice and inhibits cell proliferation through reduction on Cyclooxygenase-2 (COX-2) activity [87]. Moreover, it was demonstrated that Ang-(1–7) reduced lung cancer cell migratory and invasive abilities through the decrease of expression and activity of matrix metalloproteinases MMP-2 and MMP-9, and inactivation of the PI3K/Akt, P38 and JNK signaling pathways [88]. Noteworthy, it was recently reported that Ang-(1–7) inhibited human lung cancer cell EMT through the reduction of Cdc6 expression, dependent on the agonism of Mas receptor. An anti-metastatic mechanism, induced upon activation of ACE2/Ang-(1–7)/Mas axis has been recently described in a breast cancer model that includes repression of store-operated calcium entry (SOCE)-induced PAK1/NF- κ B/Snail1 signal pathway, ultimately resulting in decreased expression of Snail1 and increased production of E-cadherin [59]. Further data from cardiomyocytes supports a

nitric oxide/guanosine 3',5'-cyclic monophosphate-dependent pathway, which modulated the activity of the transcription factor NFAT (nuclear factor of activated T-cells), preventing its translocation to the nucleus [89], and impairing the transcriptional regulation of genes involved in malignant cell proliferation, migration and metastasis [90]. However, the crosstalk between ACE2/Ang-(1–7)/Mas axis and NFAT upregulation in lung cancer has never been explored and the relationship to tumors is speculative based on studies in other tissues.

Taken together, this data provide a link to support the ACE2/Ang-(1–7)/Mas as a beneficial and targetable system for the treatment of lung cancer, reducing cell proliferation and preventing metastasis (Figure 2).

3.2. Hypoxia and Promotion of Tumor Angiogenesis

Hypoxia is typically present on solid tumors, such as lung cancer, and is known to enhance tumor aggressiveness, disease progression and therapy resistance. The effects of hypoxia are largely mediated by hypoxia-inducible factors (HIFs), particularly HIF-1 α and HIF-2 α [91]. Importantly, RAS activation at the tumor microenvironment through Ang II has been described to induce hypoxia through production of reactive oxygen species (ROS), subsequently leading to pro-inflammatory and pro-angiogenic signals (Figure 3) [11,92]. Conversely, hypoxia regulates RAS-related proteins expression in somatic tissues and cells, up-regulating Ang II, ACE, and AT1 receptor, while down-regulating ACE2 and AT2 receptor in lung carcinoma cells [93–95].

3.2.1. ACE/Ang II/AT1 Receptor Axis and AT2 Receptor

Several studies indicated that HIF-1 α may be activated by Ang II upon binding to AT1 receptor [92,96,97]. Consequently, HIF-1 α not only activates vascular endothelial growth factor (VEGF) expression, but also upregulates ACE expression, which then increases endogenous Ang II formation and enhances AT1 receptor activation [98]. A significant reduction on VEGF-A mRNA and protein, in association with attenuated tumor growth, was also observed in Lewis lung tumors following candesartan administration, an ACE inhibitor. Furthermore, it was shown that treatment of Lewis lung carcinoma cells with Ang II caused a significant increase in VEGF-A mRNA and protein, which was prevented with administration of an AT1 receptor antagonist [99]. Further evidence from molecular in vitro studies demonstrated that Ang II elicited the expression of placental growth factor (PLGF) at both mRNA and protein expression in human vascular endothelial and smooth muscle cells, mediated through the activation of protein kinase C, extracellular signal-regulated kinase 1/2 (ERK1/2) and PI3-kinase [100] intracellular signaling pathways. PLGF has an important role in tumor angiogenesis [101,102], although a stimulatory effect was also reported for MMP-9-mediated NSCLC cancer cell invasion [103]. In contrast to AT1 receptor, the role of AT2 receptor in cancer angiogenesis is less clear. Ex-vivo experiments in Lewis lung tumor xenografts revealed a significant decrease in angiogenesis after inhibition of AT2 receptor or in AT2 receptor-knockout mice with impaired production of proangiogenic factors included VEGF [104].

3.2.2. ACE2/Ang (1–7)/Mas Receptor Axis

ACE2 and Ang-(1–7) are known counter-regulators of ACE and Ang II activity. In vitro analysis in murine endothelioma revealed the inhibition of tumor growth after Ang-(1–7) treatment, which was related with the reduction of HIF-1A and PLGF gene expression [105]. Previous studies demonstrated that Ang-(1–7) inhibits in vitro growth of human lung cancer cells and tumor angiogenesis in vivo, through activation of the Mas Receptor [86,106]. ACE2 overexpression or ACE2-Ang-(1–7)-MasR activation may reduce angiogenesis, either through the inhibition of VEGF α production in NSCLC, or downregulation of the expression of VEGFR in nasopharyngeal carcinoma, respectively [107,108].

3.3. Inflammation

In lung pathologies, chronic obstructive pulmonary disease (COPD), lung fibrosis and lung inflammation are considered relevant events for cancer development [109]. Chronic inflammation is

known to contribute towards the tumor microenvironment—rich in inflammatory cells, ROS, recurrent DNA damage, growth factors, and other growth-supporting stimuli. Inflammation has been postulated to play a key role in lung carcinogenesis. Polymorphisms in genes coding for inflammatory signaling molecules have also been involved in lung cancer development [110]. Engels and colleagues assessed lung cancer risk in relation to a large number of candidate polymorphisms in inflammation-related genes, namely *IL1A* and *IL1B* genes [111]. Along with a predisposing germline genetic background, environmental exposure of agents associated with elevated lung cancer risk, such as tobacco, silica or asbestos, damage the lung through the modulation of chronic inflammation. Individuals with tuberculosis, HIV infection associated with chronic lung infections, COPD and interstitial lung disease [111–113] seem to have an increased risk of lung cancer, reflecting the effects of underlying inflammatory disorders.

3.3.1. ACE/Ang II/AT1 Receptor and AT2 Receptor

Several studies have shown the influence of RAS in inflammation [114–117]. Activation of AT1 receptor through Ang II results in potent oxidant signaling through NADPH complexes, which are involved in inflammation [11]. Within the tumor microenvironment, the major components of RAS are expressed by cancer cells as well as by stromal cells, such as macrophages and cancer-associated fibroblasts [118]. Through the upregulation of key inflammatory molecules, tumor cells invoke a chronic inflammatory state that also induces tumor-supporting myeloid cells, such as tumor-associated macrophages (TAMs) [119,120]. Notably, macrophages can exert pro-inflammatory (M1) or anti-inflammatory (M2) effects according to their polarization status. M1 macrophages are tumoricidal and highly phagocytic, promoting a Th1 response. Moreover, the tumor microenvironment can polarize M1 to M2 macrophages that have anti-inflammatory activities and promote angiogenesis, proteolysis, and tissue repair [121]. In patients with NSCLC, the M1 macrophage subset has been associated to extended survival time [122]. A recent report showed that Ang II influences macrophage behavior, fostering cancer growth in an in vivo non-small cell lung cancer model, and restraining macrophage amplification after Ang II blockade [123]. Shen and colleagues demonstrated that ACE overexpression reduced the number of blood and splenic myeloid-derived suppressor cells, in a spontaneous tumor model and in a model of chronic inflammation [124]. Furthermore, macrophages from mice with myeloid ACE overexpression were more pro-inflammatory, exhibiting enhanced tumor-killing activity compared to their wild-type mice counterparts [124].

Although there is some controversy in the field, Ang II/AT1 receptor signaling stimulates the expression of cytokines and growth factors, which enhance lung cancer-associated inflammation. Activation of AT1 receptor signaling cascade leads to ROS, prostaglandin E2 and nitric oxide production [125]. In addition, Ang II induced *Cox-2* gene expression by activating the calcineurin/NFAT signaling pathway in endometrial cells and by malignant cells (Figure 4) [126,127]. Little is known about the contribution of AT2 receptor. Its functions remain controversial, as it has been reported to either inhibit or promote inflammation in different experimental settings [128–131], albeit most studies support that AT2 receptor mainly exerts an anti-inflammatory action [132].

3.3.2. ACE2/Ang (1–7)/Mas Receptor Axis

In opposite from AT1 receptor activation by Ang II, the Ang-(1–7) binding to Mas receptor triggers a down regulation of pro-oxidant pathways, preventing or attenuating the oxidative stress-induced cellular damage [114]. In addition, Ang-(1–7) can also inhibit Ang II-activated inflammation through a deregulatory effect in lectin-like oxidized low-density lipoprotein receptor-1 [133]. Menon and colleagues showed that Ang-(1–7) reduces tumor volume and inhibits cell proliferation via the reduction of COX-2 activity in a human lung tumor xenograft model [87]. Moreover, compelling evidence suggests that the Mas receptor activation may counterbalance Ang-II-mediated proinflammatory effects, likely through macrophage function. A recent study reported that Mas signaling affected macrophage polarization, migration, and mediated T-cell activation, in two different chronic inflammatory animal

models [134], providing the rationale that Mas may have an important role in regulating inflammatory processes, likely influencing macrophage behavior at the cancer microenvironment, namely in lung cancer. Additional experimental work in diabetes showed that administration of Ang-(1–7) limited the activation of NADPH oxidase through downregulation of molecular components of the NADPH oxidase complex [135], thus controlling ROS production and subsequent inflammation.

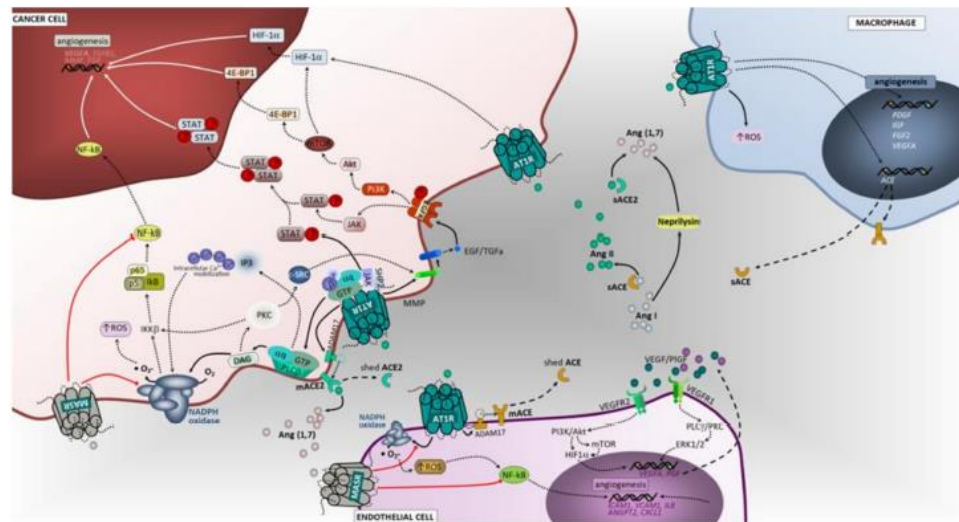


Figure 3. Angiotensin-associated pathways involved on hypoxia and angiogenesis in lung tumor microenvironment. In cancer cells, upon binding of Ang II to AT1R, a pleiotropic downstream signaling cascade is triggered, ultimately causing, either directly or indirectly, upregulation of angiogenesis and metastasis. Activated AT1R subunits trigger potent oxidant signaling through NADPH complex, which is involved in angiogenesis. Activated Gq/11 units also activate the JAK-SHP2/STAT pathway and receptor tyrosine kinase (RTK) transactivation. Subsequent signaling upon activation of EGFR is herein represented. Macrophages, endothelial cells and fibroblasts are important components of the tumor microenvironment and capable of generating and expressing RAS components. These cells, beyond their functional ligands and receptors that are altered in tumor microenvironment, and reflect the crosstalk between all cell constituents, also use the RAS signaling pathway (mostly AT1, but also MasR in endothelial cells) to yield functional characteristics that ultimately may favor the cell itself and enhancing tumor angiogenesis and metastasis. 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; IKKB, Inhibitor of nuclear factor kappa-B kinase subunit beta; c-SRC, cellular Proto-oncogene tyrosine-protein kinase; SHP2, Src homology region 2 domain-containing phosphatase-2; IL1, interleukin 1; JAK, janus kinase; STAT, signal transduction and activator of transcription; Ang I, angiotensin I; Ang II, angiotensin II; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ANGPT2, angiopoietin 2; ICAM1, Intercellular Adhesion Molecule 1; VCAM1, vascular cell adhesion molecule 1; NF-κB, Nuclear Factor-kappa B; ROS, reactive oxygen species; ERK1/2, extracellular signal-regulated kinase; PLCγ/PKC, phospholipase C gamma/protein kinase C; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; MEK1/2, mitogen activated protein kinase kinase; HIF1α, hypoxia inducible factor 1 alpha; NO, nitric oxide; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; IP3, inositol trisphosphate; DAG, diacylglycerol; M2, macrophage polarized towards M2; ADAM17, Disintegrin and metalloproteinase domain-containing protein 17; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; MasR, G-protein coupled Mas receptor; VEGF, vascular endothelial growth factor; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; mACE, membrane angiotensin converting enzyme; sACE, soluble angiotensin converting enzyme; mACE2, membrane angiotensin converting enzyme 2; sACE2, soluble angiotensin converting enzyme 2; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2 Ang (1,7), angiotensin 1,7; PLCβ, phospholipase C beta.

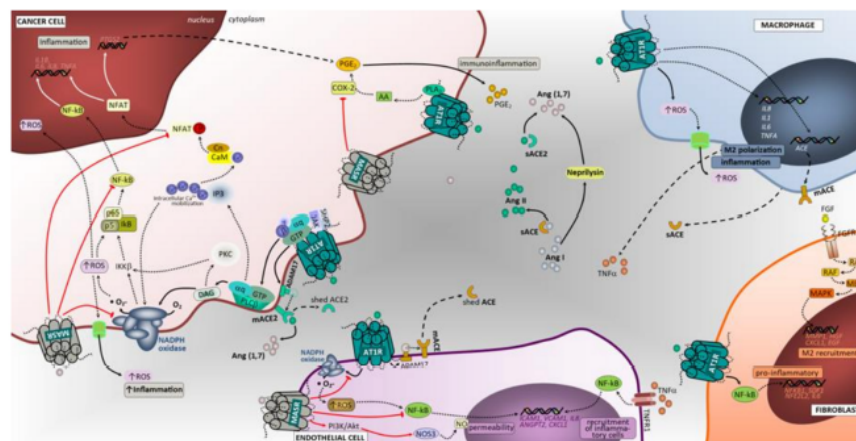


Figure 4. Angiotensin-associated immunoinflammatory pathways in lung tumor microenvironment. Extracellular proteins of the RAS, ligands (Ang I, Ang II and Ang 1,7) and enzymes (ACE, ACE2 and neprilysin), are represented with distinct colors in the tumor microenvironment. The expression and cleavage of ACE and ACE2 are represented in tumor and endothelial cells, showing the AT1R signaling-associated activation of ADAM17 that cleaves the ectodomain of mACE and mACE2 shedding the enzymes into sACE and sACE2. The Ang II/AT1R-mediated direct intracellular signaling is represented with full black arrows as a first step after AT1R triggering in the cascade. The subsequent steps of signaling cascades are depicted, up to moving towards intranuclear space, using dashed black arrows. Intranuclear signaling from these pathways mainly results in transcriptional regulatory effects and is represented by full white arrows. The Ang II/AT2R and Ang (1,7)/MasR-mediated counter-regulatory mechanisms are depicted using black arrows and when adequate the suppressive step is represented by blunt-ended red arrows. In cancer cells, upon binding of Ang II to AT1R, a pleiotropic downstream signaling cascade is triggered, ultimately causing, either directly or indirectly inflammation immune cells recruitment. Activated AT1R triggers potent oxidant signaling through NADPH complex, which is involved in inflammation and angiogenesis. Other intracellular cascades mediated by Ang II/AT1R include the activation of PLA2/AA/COX-2/PGE2 signaling. The Ang (1,7)/MasR activation elicits downstream signaling through PI3K/Akt/NOS3 or NOS1 to produce NO that inhibits NFAT transcriptional regulation that reduces inflammation. Notably, this pathway blocks the NF-κB molecule formation thereby impacting inflammation. In addition, the inhibitory effect over COX-2 significantly impact inflammation. Macrophages, endothelial cells and fibroblasts are important components of the tumor microenvironment and capable of generating and expressing RAS components. These cells, beyond their functional ligands and receptors that are altered in tumor microenvironment, and reflect the crosstalk between all cell constituents, also use the RAS signaling pathway (mostly AT1, but also MasR in endothelial cells) to yield functional characteristics that ultimately may favor the cell itself and enhance tumor growth and aggressiveness. PLA2, phospholipase A2; PTP, protein tyrosine phosphatase; PP2A, Protein phosphatase 2; IKKB, Inhibitor of nuclear factor kappa-B kinase subunit beta; CARMA3, CARD recruited membrane associated protein 3; MALT1, Mucosa-associated lymphoid tissue lymphoma translocation protein 1; BCL-10, B-cell lymphoma/leukemia 10; c-SRC, cellular Proto-oncogene tyrosine-protein kinase; SHP2, Src homology region 2 domain-containing phosphatase-2; AA, arachidonic acid; COX-2, cyclooxygenase 2; PGE2, prostaglandin E2; IL1, interleukin 1; TNFA, tumor necrosis factor; Ang I, angiotensin I; Ang II, angiotensin II; GTP, guanosine triphosphate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ANGPT2, angiotensin 2; ICAM1, Intercellular Adhesion Molecule 1; VCAM1, *vascular cell adhesion molecule* 1; CXCL1, The chemokine (C-X-C motif) ligand 1; IL8, interleukin 8; NF-κB, Nuclear Factor-kappa B; NFKB1, Nuclear Factor-kappa B Subunit 1; IL6, interleukin 6; NFE2L2, Nuclear Factor, Erythroid 2 Like 2; HGF, hepatocyte growth factor; RAS, oncogene protein p21; ROS, reactive oxygen species; NOS3, nitric oxide synthase 3 endothelial; NOS1, nitric oxide synthase 1 neuronal;

Cas9, caspase 9; Cas3, caspase 3; ERK1/2, extracellular signal-regulated kinase; PLC γ /PKC, phospholipase C gamma/protein kinase C; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; TNFR1, tumor necrosis factor receptor 1; c-Raf, kinase Raf-1; MEK1/2, mitogen activated protein kinase kinase; HIF1 α , hypoxia inducible factor 1 alpha; NO, nitric oxide; RhoA, Ras homolog family member A; IP3, inositol trisphosphate; DAG, diacylglycerol; M2, macrophage polarized towards M2; ADAM17, Disintegrin and metalloproteinase domain-containing protein 17; p38/MAPK, protein 38/mitogen activated protein kinase; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; MASR, G-protein coupled Mas receptor; VEGFA, vascular endothelial growth factor A; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; mACE, membrane angiotensin converting enzyme; sACE, soluble angiotensin converting enzyme; mACE2, membrane angiotensin converting enzyme 2; sACE2, soluble angiotensin converting enzyme 2; PDGFR β , platelet derived growth factor receptor beta; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; TGF α , transforming growth factor alpha; Ang (1,7), angiotensin 1,7; PLC β , phospholipase C beta; FAK, Focal adhesion kinase; NFAT, nuclear factor of activated T cells; PDGF, platelet derived growth factor.

3.4. Tumor Immunological Response

Recent cancer immunotherapies, including immune-checkpoint blockade, have produced durable clinical effects in some patients with various advanced cancers, namely in NSCLC [136]. Tumor anti-programmed death-ligand 1 (PD-L1) expression is associated with increased tumor-infiltrating lymphocytes in lung cancer. Unresponsiveness to the immune-checkpoint blockade therapies may be mediated by numerous immunosuppressive mechanisms that inhibit T-cell responses and T-cell infiltration into tumors [137,138]. In the lung cancer microenvironment, the cooperation between cancer cells namely airway epithelial cells, macrophages, and other peripherally-recruited innate immune cells can determine the fate of lung tumors at different stages, of both metastatic and primary disease, including pre-neoplastic, early, and late lesions [139]. Among the hematopoietic suppressor cells of interest are M2 macrophages, splenic myeloid-derived suppressor cells, and regulatory T cells (Treg), which have been associated with a poor prognosis in many cancers, and with unfavorable clinical response to anti-PD-1/PD-L1 [137]. Furthermore, cancer-associated fibroblasts (CAFs) can manipulate the immune system, directly by inhibiting T and NK (natural killer) cell functions, promoting accumulation of suppressive cell types, and maintaining a pro-tumorigenic milieu. CAFs also induce tumor fibrosis, which represents a physical barrier to T cell infiltration [140]. Neutrophils make up a significant portion of the inflammatory cells infiltrate in the tumor, whereby they show high functional plasticity displaying both anti-tumor and pro-tumor activities [141]. Studies in early NSCLC have shown that neutrophils are pivotal to tumor cell clearance by stimulating adaptive immunity. In contrast, the role of neutrophils during later stages of primary lung cancer progression frequently appears to be pro-tumorigenic, as neutrophils represent the most abundant recruited cells in more advanced NSCLC [139]. Tumor-derived signals induce a pro-tumor phenotype in neutrophils, which supports cancer cell invasion and metastasis (N2 neutrophils). N2 polarized neutrophils promote the proliferation, migration, and invasion of tumor cells, stimulate angiogenesis, as well as mediate immunosuppression [141]. Dendritic cells are crucial for the activation of antigen-specific CD4 T lymphocytes, a pivotal step in the initiation of innate and adaptive immune responses, which are essential for lung tumor cell clearance [142]. Previous studies demonstrated that lung tumors dynamically exclude functional DCs from the tumor region to support malignant progression. Furthermore, clinical trials have proven that dendritic cell function is reduced in lung cancer patients [142]. At the tumor microenvironment, the major components of RAS are also expressed both by resident and infiltrating cells, such as endothelial cells, fibroblasts, monocytes, macrophages, neutrophils, dendritic and T cells [11]. RAS signaling in these cells can facilitate or hinder growth and dissemination and has been shown to affect cell proliferation, migration, invasion, metastasis, apoptosis, angiogenesis, cancer-associated inflammation, immunomodulation, and tumor fibrosis/desmoplasia [142].

Targeting RAS to Improve Anti-Tumor Immunity

A recent study using 4T1 and CT26 syngeneic mouse tumor models, showed that Ang II generates infiltration of fibroblasts and macrophages, contributing to the reduction of CD8⁺ T cell infiltration, resulting in an immunosuppressive environment [143]. Importantly, Nakamura and colleagues, demonstrated, in a tumor bearing mouse model of colorectal carcinoma, that the antagonism of AT1 receptor decreased the expression of immunosuppressive factors, such as chemokine ligand 12 and nitric oxide synthase 2 in CAFs, and that the combination of antagonists of AT1-receptor and PD-L1 antibodies resulted in significant augmentation of anti-tumor effects in a CD8⁺ T cell-dependent way [118]. Furthermore, RAS-activated CAFs secrete collagen and other ECM components which produce fibrosis and consequently tumor desmoplasia [144]. Notably, tumor desmoplasia can be either a physical barrier to immune cell infiltration or provide the substratum to interstitial cell migration [144]. It was demonstrated that the antagonism of AT1 receptor-inhibited collagen I production by carcinoma-associated fibroblasts isolated from breast cancer biopsies [145]. Additionally, this led to a dose-dependent reduction of stromal collagen in desmoplastic models of human breast, pancreatic, and skin tumors in mice [145]. Contrary to the Ang II effect in tumor immunity response, interesting findings also showed that Ang-(1–7) targets the tumor microenvironment to inhibit CAF growth and tumor fibrosis in orthotopic breast tumors [146]. TGF- β suppresses the differentiation and function of T helper, CD8⁺ cells, Natural Killer cells, and tumor-associated neutrophils, tumor associated macrophages and myeloid-derived suppressor cells [147]. Tumor supporting cytokines are released from tumor and stromal cells upon AT1 receptor activation via Ang II including, TGF- β and Interleukins (IL-1 α , IL-1 β , IL-6, IL-8) [140]. Immunomodulatory cytokines may up-regulate immunosuppressive pathways, i.e., COX-2 via prostaglandin E2 synthesis, and impair dendritic cell function by reducing their migration [148]. Ang II/AT1R signaling induces reactive oxygen species generation and related proteins such as inducible nitric oxide synthase in tumor and stroma cells. Exposure to ROS in the tumor microenvironment can impair T cell function while enhancing T regs and tumor associated macrophages [140]. Treatment with the candesartan (AT1 receptor blocker) diminishes ROS generation [144]. Ang II/AT1R signaling is also important for myeloid differentiation and functional maturation. Cultured bone marrow from ACE 10/10 mice, a mouse line overexpressing ACE in monocytic cells, demonstrates increased myeloid maturation and reduced splenic myeloid-derived suppressor cells production. In addition, macrophages from these mice have an enhanced proinflammatory phenotype and antitumor activity compared to those from wild-type mice [124]. Similarly, tumor-bearing ACE 10/10 mice showed enhanced immune responses, which ultimately resulted in a reduced tumor growth. ACE inhibitors reversed the beneficial effects on tumor growth, but AT1 receptor blockade did not, suggesting that the effects of ACE overexpression were not dependent of Ang II/AT1 receptor signaling [149]. Hence, these reports suggest that targeting RAS could alleviate immunosuppression and enhance the outcome of immunotherapy. Nevertheless, it is necessary, through experimental and clinical research, to highlight the role of RAS in lung cancer and its influence on immunotherapy response.

4. Clinical Studies

Pre-clinical studies demonstrated an association between RAS activation and increased invasion, angiogenesis, inflammation, and modulation of immune response at the lung tumor microenvironment. The putative impact on cancer hallmarks, through AT1 receptor blockade or activation of the ACE2/Ang-(1–7)/Mas receptor axis, has been linked to reduced tumor growth and vascularization in lung cancer, thus fostering the need for clinical studies.

4.1. Clinical Trials

Despite basic and pre-clinical evidence concerning the impact of RAS in cancer hallmarks, the information available regarding clinical trials remains scarce, particularly in lung cancer.

The primary objective of a Phase II Randomized Trial (NCT00077064, Table S1) was to test the ability of captopril to alter the incidence of pulmonary damage at 12 months after the completion of radiation in patients with non-small cell lung cancer or limited-stage small cell lung cancer [150]. Due to the unmet accrual/randomization goals, it was not possible to adequately test the hypothesis that captopril ameliorates radiotherapy-induced pulmonary toxicity. Another double-blind placebo-controlled randomized trial (NCT01880528, Table S1) enrolled 23 patients with lung cancer to study the putative protective effect of lisinopril in pneumonitis induced by radiotherapy [151]. The results of this clinical trial suggest that there was a clinical signal for safety and possibly beneficial in concurrently administering lisinopril during thoracic radiotherapy to mitigate or prevent radiation-induced pulmonary distress [151]. However, larger-scale randomized phase 3 trials are needed in the future to confirm these results. Notably, a multicenter clinical trial showed that losartan stabilized lung function in patients with idiopathic pulmonary fibrosis over 12 months (NCT00879879, Table S1), reinforcing the importance of AT1 receptor blockers in the impairment of fibrosis progression [152]. Moreover, treatment with AT1 receptor blockers might potentiate the response to immunotherapy in lung cancer patients due to a hypothetical impairment of the fibrotic immunosuppressive microenvironment. Nevertheless, there is no study still to prove this hypothesis.

A small Phase I study (NCT00471562, Table S1) of Ang-(1–7) as a first-in-class anti-angiogenic drug targeting Mas receptor, was undertaken in 18 patients with advanced solid tumors refractory to standard therapy with only one patient with lung cancer included [153]. Dose-limiting toxicities encountered at the 700 µg/kg dose included stroke (grade 4) and reversible cranial neuropathy (grade 3). Other toxicities were generally mild. Clinical benefit, defined as disease stabilization for more than three months, was observed in two of the three patients with metastatic sarcoma and it was associated with reduction of PlGF plasma levels [153]. A Phase II study failed to confirm PlGF as biomarker of response to Ang-(1–7) administration [154]. However, two patients with vascular sarcomas demonstrated prolonged disease stabilization of 10 months (hemangiopericytoma) and 19 months (epithelioid hemangioendothelioma) under Ang-(1–7) treatment, thereby requiring further investigation [154].

4.2. Observational Studies

A retrospective cohort study that included 228 patients with advanced NSCLC and 73 with early stage disease revealed that those under therapeutics with ACE inhibitors (iACE) or AT1 receptor blockers, in combination with standard chemotherapy or tyrosine kinase inhibitors, had a positive effect on progression-free survival and overall survival, regardless of lung cancer stage [65]. A retrospective study with 117 metastatic NSCLC patients demonstrated that the intake of AT1 receptor blockers, during erlotinib treatment, may prolong overall survival [155]. Another study including 287 advanced NSCLC patients, demonstrated a survival advantage for patients under ACE inhibitors or AT1 receptor blockers [156]. Notably, the reduced risk for cancer progression in patients submitted to ACE inhibitors or AT1 receptor blockers has not been seen with other antihypertensive medications, suggesting the existence of an adjuvant effect for angiotensin blockers [156]. A recent systematic review and meta-analysis showed that AT1 receptor blockers are significantly associated with reduced lung cancer risk [157]. Conversely, a study on a large population-based cohort that used ACE inhibitors suggested an increased risk for developing lung cancer, particularly among patients undergoing treatment for more than five years. Nevertheless, the risk for lung cancer was not confirmed in patients under AT1 receptor blockers [158]. These findings might rely on ACE inhibitors-associated accumulation of bradykinin in the lung, rather than a decrease in Ang II signaling, especially when higher dosages of ACE inhibitors are taken into consideration [159]. When analyzed together, these results from clinical and pre-clinical studies suggest that AT1 receptor blockers, in contrast to ACE inhibitors, might be putative therapeutic tools to impair lung cancer progression. Further larger prospective studies with well-designed clinical trials are required to confirm whether targeting RAS improves lung cancer treatment.

5. Future Perspectives

A significant body of knowledge supports a role for the RAS as modulator of the tumor microenvironment through direct and indirect influences on tumors and surrounding cells, including immunological mediators. In vitro and in vivo studies demonstrate that the activation of the ACE/Ang II/AT1 receptor axis promotes cancer cell proliferation, invasion, angiogenesis and reduced immunosurveillance, albeit opposing effects were found when the ACE2/Ang-(1–7)/Mas receptor was activated. Nevertheless, those pathways remain largely unexplored at translational and clinical levels, prompting the need for further research. The major challenge in clinical context is the complex nature of RAS signaling, which seems to be variable when considering several factors, making the response to RAS therapeutics, either individually or in combination with other drugs, difficult to predict [11]. Furthermore, dysregulation of RAS can occur due to germline [160] or somatic mutations [161], or due to hypoxia within the tumor microenvironment [95]. Putative functional genetic variants coding for proteins of RAS might have predictive and/or prognostic value in lung cancer, opening novel opportunities to guide clinical reasoning and therapeutics in lung cancer patients.

Moreover, to confirm whether targeting the RAS has the potential to reprogram cancer cell immunogenicity and the immunosuppressive lung tumor microenvironment, preclinical research that combines immune checkpoint inhibitors with RAS agonists/antagonists is needed. Finally, the intra-tumor expression of the components of the RAS may also add potential to predict the response to RAS therapeutics.

6. Conclusions

Malignant transformation and lung tumor progression is associated with at least six acquired, functional capabilities: sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, and limitless replication potential [162]. The RAS regulates all these capabilities, although most prominent are its effects on angiogenesis, invasion, pro-survival signaling and proliferation. Inevitably, many of these processes are interdependent and cooperative [162]. Preclinical studies have provided compelling evidence that the Ang II/AT1R axis regulates almost all hallmarks of cancer. It is also clear that the Ang II/AT1R signaling contributes to the immuno-suppressive tumor microenvironment in multiple ways. The immuno-suppressive milieu is a major barrier for immunotherapy and may explain why immune checkpoint inhibitors failed in NSCLC patients [140]. The high expression of the gene encoding the AT1 receptor in lung tumors suggests that Ang II acting upon the AT1 receptor plays an important role in cell growth, invasion, fibrosis and angiogenesis. The low level of expression of the genes encoding the components of the ACE2/Ang 1–7/MasR axis in lung tumors promotes the tumorigenic effects of Ang II, while reducing the stimulation of the Mas receptor by Ang 1–7, that has contradictory Ang II/ATR1 mediated effects [35]. Multiple clinical studies have also revealed that RAS inhibitors may have beneficial effects in a broad range of malignancies. The gain in survival is tumor type-and-stage-dependent and ranged from 3 months (advanced NSCLC) to more than 25 months (metastatic colorectal cancer) in retrospective studies [140]. Finally, taking the large amount of preclinical and clinical data suggesting a beneficial effect of RAS inhibitors in different cancer types, including lung cancer, into account, we indicate that RAS inhibitors have a great potential to become an adjunct within the oncological armamentarium.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/6/1457/s1>, Table S1: Clinical Trials of renin angiotensin blockers and their role in lung cancer and pulmonary fibrosis. (adapted from clinicaltrials.gov).

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Article

Germline Genetic Variants of the Renin-Angiotensin System, Hypoxia and Angiogenesis in Non-Small Cell Lung Cancer Progression: Discovery and Validation Studies

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Simple Summary: The presence of polymorphic gene variants in the human genome provides extensive genetic (and eventually phenotypic) variation affecting both normal physiological mechanisms and cancer pathogenesis. Functional genetic polymorphisms might have predictive and/or prognostic value in lung cancer, opening novel opportunities to improve prediction and guide clinical reasoning and therapeutics in lung cancer patients. Recent knowledge pinpoints a pleiotropic role for renin-angiotensin system, particularly in the lung and mainly through locally regulated alternative molecules and secondary pathways. Dysregulation of this system play a role in cell proliferation, hypoxia and angiogenesis, which processes are involved in lung cancer progression. Here we suggest that polymorphic variants in genes coding for renin-angiotensin system might play a role in Non-Small Cell Lung Cancer progression.

Abstract: Introduction: The renin-angiotensin system (RAS) is involved in cell proliferation, immunoinflammatory response, hypoxia and angiogenesis, which are critical biological processes in

lung cancer. Our aim was to study the association of putatively functional genetic polymorphisms in genes coding for proteins involved in RAS, hypoxia and angiogenesis with non-small cell lung cancer (NSCLC) prognosis. Methods: Genotyping of 52 germline variants from genes of the RAS and hypoxic/angiogenic factors/receptors was performed using MassARRAY iPLEX Gold in a retrospective cohort ($n = 167$) of advanced NSCLC patients. Validation of the resulting genetic markers was conducted in an independent group ($n = 190$), matched by clinicopathological characteristics. Results: Multivariate analysis on the discovery set revealed that *MME* rs701109 C carriers were protected from disease progression in comparison with homozygous T (hazard ratio (HR) = 0.5, 95% confidence interval (CI) = 0.2–0.8, $p = 0.010$). Homozygous A and T genotypes for *KDR* rs1870377 were at increased risk for disease progression and death compared to heterozygous (HR = 1.7, 95% CI = 1.2–2.5, $p = 0.005$ and HR = 2.1, 95% CI = 1.2–3.4, $p = 0.006$, respectively). Carriers of homozygous genotypes for *ACE2* rs908004 presented increased risk for disease progression, only in the subgroup of patients without tumour actionable driver mutations (HR = 2.9, 95% CI = 1.3–6.3, $p = 0.010$). Importantly, the association of homozygous genotypes in *MME* rs701109 with risk for disease progression was confirmed after multivariate analysis in the validation set. Conclusion: This study provides evidence that *MME* polymorphism, which encodes neprilysin, may modulate progression-free survival in advanced NSCLC. Present genetic variation findings will foster basic, translational, and clinical research on their role in NSCLC.

Keywords: genetic polymorphisms; lung cancer; renin–angiotensin; hypoxia; angiogenesis

1. Introduction

Lung cancer is one of the most common malignancies worldwide and the most common cause of cancer death, with over one million people diagnosed worldwide each year [1].

The renin–angiotensin system (RAS) is an established primary regulator of blood pressure, homeostasis, and natriuresis [2]. Recent evidences indicate that angiotensin peptides might have a role in tumour cell proliferation, inflammation, immune surveillance, hypoxia and angiogenesis, which have been recognized as hallmarks of cancer, and thereby actively participating in lung tumour microenvironment regulation [3,4]. The enzymatic cascade leading to Angiotensin II (Ang II) production includes renin, which cleaves angiotensinogen (AGT) to form angiotensin I (Ang I). Angiotensin-converting enzyme (ACE) cleaves Ang I to produce Ang II [2]. The chymase, which might catalyse the conversion from Ang I to Ang II, has been associated with activation of proliferation, adhesion, regulation of E-cadherin expression, and modulation of the immunosuppressive microenvironment in lung carcinoma [5,6]. Angiotensin-(1-7) (Ang-(1-7)) has opposite effects to Ang II through binding to Mas receptor (MasR), and is obtained by catalysis from the enzymes ACE type 2 (ACE2) or neprilysin (NEP) [7]. On its turn, NEP has been associated with some malignancies, such as prostate, renal and lung cancer [8].

In vitro and in vivo studies showed that activation of the ACE/Ang II/ angiotensin II type 1 receptor (ATR1) axis promotes cancer cell proliferation, invasion, angiogenesis and reduced immunosurveillance [9–11]. Conversely, anti-tumour effects were demonstrated for the ACE2/Ang-(1-7)/MasR axis [12–14]. Upregulated expression of *ATR1* transcripts in lung tumours suggests that Ang II, acting upon the ATR1, plays an important role in cell growth and invasion [15], whereas decreased expression of genes encoding for ACE2/Ang-(1-7)/MasR axis proteins in lung tumours halt MasR signalling, which is contrary to Ang II/ATR1 [16]. Notably, clinical studies revealed that RAS inhibitors combined with chemotherapy and Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors prolonged progression-free survival in non-small cell lung cancer (NSCLC) patients [17–19].

Activity and modulation of the RAS are dependable of germline genetic variations [20], somatic mutations [21], environmental stimulus and from hypoxic lung tumour microenvironment [22]. Additionally, the RAS is causally and consequently associated with hypoxia and angiogenesis, considered in continuum as branches of RAS [4]. Notwithstanding, the RAS remains largely unexplored at translational and clinical levels in cancer, particularly NSCLC. In this study, we sought to evaluate whether functional genetic variants coding for proteins of RAS and hypoxic/angiogenic factors/receptors have predictive and/or prognostic value in NSCLC using progression-free survival (PFS) and overall survival (OS) as clinical endpoints.

2. Material and Methods

2.1. Study Population: Discovery and Validation Sets

This study comprised a retrospective cohort of histologically confirmed NSCLC patients ($n = 167$) with clinical advanced stage at diagnosis (IIIA-IVB), between 2012 and 2018. Participants were consecutively recruited during routine follow-up visit, since August 2017 to October 2018 from Coimbra University Hospital (discovery set). The validation set comprised an independent cohort of NSCLC patients ($n = 190$) selected from the Lung Biobank Heidelberg, member of the Biomaterialbank Heidelberg (BMBH) and the biobank platform of the German Centre for Lung Research (DZL). All patients were recruited at Thoraxklinik (University Hospital of Heidelberg) between 2015–2018, using the same matched clinical and pathological criteria from the discovery set.

Histopathological diagnoses in both sets were made by experienced pathologists following the 2009 and 2015 WHO classification criteria for lung cancer, accordingly to the year of patient's diagnosis. The existence of concomitant primary tumours in another organ was considered as exclusion criteria for both cohorts. Clinical data was retrieved from clinical charts on pathological background, previous medication, Eastern Cooperative Oncology Group performance status (ECOG PS) at diagnosis, TNM staging according to 8th classification, tumour mutational status, type of cancer treatment, disease progression/death by clinical authors. Informed consent was obtained from each participant and all procedures are in agreement with Helsinki Declaration.

In the discovery set, somatic mutations in *EGFR*, *ALK* and *ROS1* were determined using fluorescence in situ hybridization (FISH), in patients with adenocarcinoma stage IVA-IVB, whereas for the validation set, somatic mutations were determined through panel-based next generation sequencing in advanced NSCLC patients [23]. In both cohorts, targeted therapies were administered to carriers of actionable driver mutations, whereas checkpoint inhibitors were used as second-, or third-line therapy for patients with PD-L1 expression on tumour cells below 50%.

The primary endpoint was PFS and the time-to-disease progression was calculated in months, from the date of first line chemotherapy/target therapy, until the date of progression according to RECIST criteria. OS was included as secondary endpoint, and the time-to-death was computed in months from the date of first line chemotherapy/target therapy until the date of death/date of last visit.

2.2. Selection of Genetic Polymorphisms

Fifty-two single nucleotide polymorphisms (SNP) included in the present study from the genes *ACE*, *ACE2*, *AGT*, *AGTR1*, *AGTR2*, *MME*, *CMA1*, *MAS1*, *HIF1A*, *VEGFA*, *KDR*, *PGF* and *FLT1* (Table S1) were selected from search in genomic public databases, in silico analysis and review of scientific literature, to identify putatively functional polymorphisms [24–37]. Polymorphisms with minor allele frequency below 1% were excluded [37].

2.3. Genotyping

2.3.1. Discovery Set

Each patient donated a sample of blood (~4 mL) for research, collected to EDTA-Vacutainer tubes, at the same time of blood collection for routine analytic follow-up. The collected blood was centrifuged to obtain the buffy coat, which was used to isolate and purify DNA by the EZ1 DNA Blood kit in the EZ1 BioRobot (QIAGEN, Hilden, Germany). The selected SNPs were genotyped using the Sequenom Mass ARRAY matrix-assisted laser desorption/ionization time-of-flight mass spectrometry platform (Sequenom, San Diego, CA, USA). Primers were designed using semi-automated Assay Design 3.1 Software (Sequenom). The global genotyping success rate was 99.85% with 100% replication agreement among one third of the samples.

2.3.2. Validation Set

Buffy coat samples were provided by Lung Biobank Heidelberg from patients diagnosed with advanced NSCLC (clinical stage IIIA-IVB). DNA extraction and purification were conducted using the FlexiGene DNA Kit (QIAGEN). The resulting genetic polymorphisms, associated with disease progression or overall survival from multivariate analysis in the discovery set, were genotyped in the validation set using Real-Time PCR. Allelic discrimination was obtained using TaqMan SNP genotyping assays: *ACE2* rs908004 (Assay ID: C__8816953_20), *MME* rs701109 (Assay ID: C__964850_10) and *KDR* rs1870377 (Assay ID: C__11895315_20) (all from Thermo Fisher Scientific, Waltham, MA, USA). Quality control included non-template controls in all runs and blind replicate assessment in 5% of the samples. Genotype was assigned for 96.3% of all samples tested.

2.4. Statistical Analysis

Descriptives were depicted as average $\pm$ standard deviation or median (interquartile range) according to departure from normality (Shapiro-Wilk test). Central tendency data was compared using the *t*-test, Mann-Whitney or Kruskal-Wallis tests according to normality and stratification of independent variables. Categorical variables were expressed as frequency (percentage) and proportions tested using the chi-square test. The difference in median months of PFS and OS from both cohorts was calculated through Kaplan-Meier curve with log rank test.

Four genetic models were considered in this study: each SNP was stratified according to the wild type allele into additive ("AA" genotype versus "Aa" versus "aa"), dominant ("AA" genotype versus "Aa" and "aa" genotypes), recessive ("Aa" and "AA" versus "aa" genotype) and heterosis ("Aa" versus "AA" and "aa" genotypes). The wild-type allele for each SNP was designated in agreement with Ensembl [37]. Empirical analyses were conducted on clinicopathological and genetic variables to test the association with time-to-outcome, using Cox regression analyses. After univariate analyses, $p < 0.10$ and an effect $> 30\%$ in hazard ratio (HR) were used as criteria for inclusion in a multivariate Cox with stepwise regression model. Genetic models with under-represented variant genotype ($n < 4$) were excluded from the multivariate analysis, and only the genetic model with the highest likelihood ratio for each SNP was included in the multivariate model to avoid collinearity. The resulting multivariate model was further tested with bootstrapping (MonteCarlo simulations, 1000 iterations), to elect the resulting relevant markers to be tested in the validation set. To investigate the possible predictive value of each SNP in treatment response for first line therapy, and time-to-progression or time-to-death, exploratory analyses were conducted examining the effect of genotype in a subgroup of patients treated with first line platinum doublet chemotherapy and in another subgroup of wild-type *EGFR* (epidermal growth factor receptor) patients, using the same survival analysis methods. Sample size, power, effect size and number of events for survival analyses (assuming $\alpha = 0.05$ and power > 0.8) were estimated in both cohorts. Statistical analyses were performed on STATA software version 16.0 (StataCorp, College Station, TX, USA).

2.5. Ethics Approval

This project has been reviewed and approved by Coimbra University Hospital's Ethical Committee (ref. no. 0111/CES) and by the Portuguese National Committee for data protection (ref. no. 2588/2017). The biobank protocol of Lungbiobank Heidelberg was approved by the ethics committee of the University of Heidelberg (S-270/2001).

3. Results

The clinicopathological characteristics of participating subjects from discovery and validation sets are described in Table 1. Regarding mutational status from discovery set ($n = 167$), we observed that 10.8% of patients ($n = 18$) had *EGFR* mutation (exon 19 deletions or exon 21 mutation), whereas 3.0% ($n = 5$) had rearrangements in the gene encoding anaplastic lymphocyte kinase (*ALK*). Mutations of *ROS1* were not detected. From the validation set ($n = 190$), 15.3% ($n = 29$) of NSCLC patients had *EGFR* mutation (exon 19 deletions or exon 21 mutation), 13.7% ($n = 26$) were *KRAS* mutated, 4.2% ($n = 8$) had *EML4-ALK* fusion, 2.6% ($n = 5$) had *BRAF* V600E mutation. No other targetable mutations were identified. Concerning respiratory co-morbidities from discovery set, twenty patients (12.0%) had moderate-severe COPD with lung emphysema and one patient (0.6%) had sarcoidosis, whereas in the validation set there were fifty patients (26.3%) with moderate-severe COPD and lung emphysema, and five patients (2.6%) had idiopathic pulmonary fibrosis.

Table 1. Patient's clinical characteristics.

Clinical Variables.	Discovery Set (N = 167)	Validation Set (N = 190)	p
Gender, n			
Males	123 (73.7%)	133 (70.0%)	0.445 *
Females	44 (26.3%)	57 (30.0%)	
Age (years)			
Median (IQR)	64.0 (58.0–72.0)	65.0 (58.0–73.0)	0.773 **
Smoking Status, n (%)			
Never smokers	31 (27.7%)	20 (10.5%)	<0.0001 *
Previously smokers	68 (60.7%)	97 (51.1%)	
Smokers	13 (11.6%)	73 (38.4%)	
Hypertension, n (%)			
Yes	67 (48.6%)	91 (47.9%)	0.911 *
No	71 (51.4%)	99 (52.1%)	
Anti-hypertension drugs, n (%)			
No	86 (65.2%)	126 (67.0%)	0.728 *
iACE/ARB	46 (34.8%)	62 (33.0%)	
ECOG PS, n (%)			
0–2	163 (97.6%)	188 (98.9%)	0.153 *
>2	4 (2.4%)	2 (1.1%)	
Histology, n (%)			
Adenocarcinoma	116 (69.5%)	126 (66.3%)	0.066 *
Squamous cell carcinoma	42 (25.1%)	62 (32.6%)	
Adeno + Squamous cell	9 (5.4%)	2 (1.1%)	
cTNM Stage, n (%)			
IIIA	20 (12.0%)	27 (14.2%)	0.597 *
IIIB	32 (19.2%)	36 (18.9%)	
IIIC	21 (12.6%)	18 (9.5%)	
IVA	65 (38.9%)	66 (34.7%)	
IVB	29 (17.4%)	43 (22.6%)	
Treatment modality, n (%)			
Surgery + ChT	12 (7.2%)	0 (0.0%)	
ChT + RT	31 (18.6%)	81 (42.6%)	

Table 1. Cont.

ChT	124 (74.3%)	109 (57.4%)	<0.0001 *
First-line treatment, n (%)			
Platinum based ChT	145 (86.8%)	155 (81.6%)	
TKI	22 (12.8%)	35 (18.4%)	0.194 *
PFS (months)			
Median, 95% CI	7.5 (6.1–9.0)	8.1 (6.8–9.4)	0.415 ***
OS (months)			
median, 95% CI	30.0 (16.9–43.2)	20.6 (16.3–24.9)	0.036 ***

IQR—interquartile range; iACE—inhibitors of angiotensin-converting enzyme, ARB—AngiotensinII receptor blocker, ECOG PS—Eastern cooperative Oncology Group Performance Status; ChT—chemotherapy; RT—radiotherapy; TKI—tyrosine kinase inhibitors; 95% CI—confidence interval. PFS—progression-free survival, OS—overall survival.

* Pearson Chi Square test. ** Mann-Whitney U test. *** Kaplan Meier curve with log rank test.

The description of clinical-pathological variables is depicted in Table S2 with report of univariate analyses for PFS and OS. Genotypic distribution of functional SNPs in genes of the renin-angiotensin pathway, hypoxic and angiogenic factors using additive, dominant, recessive and heterosis models from discovery set are described in Table S3, together with hazard and survival univariate analyses. The univariate analysis showed that carriers of allele T ($n = 112$, 67.1%) for the SNP rs4316 in *ACE* gene had a significant protection to disease progression (Hazard ratio (HR) = 0.6, 95% confidence interval (CI) (0.4–0.9), $p = 0.011$). Homozygous GG/CC for *ACE2* rs908004 ($n = 151$, 90.4%) had increased risk for disease progression (HR = 2.1, 95% CI (1.1–3.9), $p = 0.021$). Another SNP from *MME* gene that encodes for neprilysin, showed a tendency between the dominant genetic model of *MME* rs701109 and PFS, having the heterozygous CT and homozygous CC protection to disease progression (HR = 0.6, 95% CI (0.3–1.0), $p = 0.064$). Univariate analysis also showed a significant association between the functional genetic variant of *VEGFA* rs25648 with time to tumour progression, being the homozygous C and T ($n = 127$, 78.9%) associated with worse prognosis (HR = 1.7, 95% CI (1.1–2.6), $p = 0.030$). The data showed a tendency between the heterosis genetic model of *KDR* rs1870377 and time to tumour progression, having the homozygous A and T increased risk to disease progression (HR = 1.4, 95% CI (1.0–2.0), $p = 0.054$) and to death (HR = 1.7, 95% CI (1.0–2.7), $p = 0.034$).

Multivariate analysis included the relevant variables (both clinical-pathological and genetic) from univariate empirical analysis (Table 2, Table 3). The C carriers ($n = 149$, 89.7%) for *MME* rs701109 had significantly decreased risk for disease progression than homozygotes T ($n = 17$, 10.3%), regardless of relevant clinical-pathological variables, namely tumour size, distant metastasis, type of systemic therapy, treatment modality or from other relevant SNPs (Table 2). Homozygous A and T for *KDR* rs1870377 ($n = 99$, 59.3%) had significantly lesser PFS and OS compared to heterozygous ($n = 68$, 40.7%), regardless of relevant clinical and pathological variables (Tables 2 and 3). Notably, these results maintained its significance after bootstrapping as a statistical method for internal validation.

Table 2. Multivariate analysis for progression-free survival (PFS) in the discovery cohort ($n = 167$).

Variables	PFS (Multivariate Stepwise Cox Proportional Hazards Model)		Bootstrap	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
T				
T1	Referent		Referent	
T2				
T3-4	1.7 (1.3–2.2)	<0.0001 *	1.7 (1.3–2.3)	<0.0001 *
Distant metastasis				
No	Referent		Referent	
Yes	1.7 (1.2–2.4)	0.005	1.7 (1.1–2.5)	0.009
Type of Therapy				
Surgery + ChT	Referent		Referent	
ChT				
ChT + RT	1.5 (1.1–2.1)	0.012 *	1.5 (1.0–2.3)	0.053 *
Type of Systemic Therapy, 1st line				
Platinum based				
ChT	Referent		Referent	
TKi	0.4 (0.2–0.8)	0.003	0.4 (0.2–0.7)	0.002
KDR rs1870377, Heterosis Model				
TA	Referent		Referent	
TT/AA	1.7 (1.2–2.5)	0.005	1.8 (1.2–7)	0.003
MME rs701109, Dominant Model				
TT	Referent		Referent	
TC/CC	0.5 (0.3–0.9)	0.018	0.5 (0.2–0.9)	0.030

Analyses were conducted using stepwise Cox regression, followed by Bootstrap analysis (Montecarlo simulations with 1000 iterations). HR, hazard ratio, 95% CI, confident interval, ChT, chemotherapy, RT, radiotherapy, TKi, tyrosine kinase inhibitors. * *p* trend value.

Table 3. Multivariate analysis of relevant variables for overall survival (OS) in the discovery set ($n = 167$) using stepwise Cox regression, followed by Bootstrap analysis (Montecarlo simulations with 1000 iterations).

Variables	OS (Multivariate Stepwise Cox Proportional Hazards Model)		Bootstrap	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
T				
T1	Referent		Referent	
T2				
T3-4	1.5 (1.1–2.2)	0.018 *	1.5 (1.0–2.2)	0.066 *
Distant metastasis				
No	Referent		Referent	
Yes	2.5 (1.5–4.3)	0.001	2.6 (1.5–4.5)	0.001
Age median				
≤64.0	Referent		Referent	
>64.0	1.7 (1.0–2.7)	0.031	1.6 (1.0–2.8)	0.069
KDR rs1870377, Heterosis Model				
TA	Referent		Referent	
TT/AA	2.1 (1.2–3.4)	0.006	2.0 (1.2–3.3)	0.012

HR, hazard ratio, 95% CI, confident interval. * *p* trend value.

Exploratory subgroup analyses were conducted among NSCLC patients from discovery set that included only patients under first line platinum-based doublet chemotherapy without actionable driver mutations ($n = 145$) or *EGFR* wild-type ($n = 149$). Within the *MME* (rs701109) and *KDR* (rs1870377) polymorphisms, we found evidence for an independent effect at PFS and OS respectively, across all subgroups. Notably, CT and CC patients for *MME* (rs701109) under first line platinum-based doublet chemotherapy or *EGFR* wild-type had protection for disease progression (HR = 0.4, 95% CI (0.2–0.7), $p = 0.005$ and HR = 0.4, 95% CI (0.2–0.8), $p = 0.006$, respectively). Homozygous A and T for *KDR* rs1870377 had increased risk to death also in patients under first line platinum doublet chemotherapy or *EGFR* wild-type (HR = 1.9, 95% CI (1.1–3.2), $p = 0.019$ and HR = 2.0, 95% CI (1.2–3.4), $p = 0.011$, respectively). Moreover, from the multivariate analysis conducted in the subgroup of patients treated with first line platinum-based doublet chemotherapy without actionable driver mutations ($n = 145$), we also found that homozygous for *ACE2* rs908004 had increased risk for disease progression, regardless of relevant clinical-pathological covariates (HR = 2.9, 95% CI (1.3–6.3), $p = 0.010$).

The validation set included an independent matched cohort of NSCLC patients (Table 1) to validate the resulting genetic SNPs from the discovery set. Results from univariate analysis of clinicopathological variables in association with PFS and OS are described in Table S4. The genotype distribution and univariate analysis according to additive, dominant, recessive or heterosis models of *MME* rs701109, *KDR* rs1870377 and *ACE2* rs908004 for hazard and survival analysis are depicted in Table S5. The genetic distribution of genotypes in *MME* rs701109, *KDR* rs1870377 and *ACE2* rs908004 are similar among discovery and validation sets ($p = 0.204$, $p = 0.846$ and $p = 0.251$, respectively) (Pearson Chi-square test).

The multivariate model for PFS in the validation cohort is presented in Table 4. Homozygous ($n = 102$, 53.9%) for *MME* rs701109 had significantly increased risk to disease progression compared to CT genotype ($n = 87$, 46.0%), regardless of histology, type of systemic therapy or treatment modality (chemotherapy or chemo and radiotherapy). There was no significant effect on overall survival for *MME* rs701109, *KDR* rs1870377 and *ACE2* rs908004.

Table 4. Multivariate analysis of relevant variables for disease progression using stepwise Cox regression, followed by Bootstrap analysis with 1000 iterations from validation set ($n = 190$).

Variables	PFS (Multivariate Stepwise Cox Proportional Hazards Model)		Bootstrap	
	HR (95% CI)	p	HR (95% CI)	p
Histology				
Adenocarcinoma	Referent		Referent	
Squamous cell	1.7 (1.2–2.4)	0.003 **	1.7 (1.2–2.5)	0.003 **
Other *	-		-	
Type of Therapy				
ChT	Referent		Referent	
ChT + RT	0.4 (0.3–0.6)	<0.0001	0.4 (0.3–0.6)	<0.0001
Type of Systemic Therapy, 1st line				
Platinum based	Referent		Referent	
ChT	Referent		Referent	
TKi	0.4 (0.3–0.7)	0.001	0.4 (0.3–0.7)	0.002
<i>MME</i> rs701109, Heterosis Model				
TC	Referent		Referent	
TT/CC	1.6 (1.2–2.3)	0.005	1.6 (1.1–2.4)	0.007

PFS—progression-free survival, HR—hazard ratio, 95% CI—confident interval, ChT—chemotherapy, RT—radiotherapy, TKi—inhibitors of tyrosine kinase. * Adenocarcinoma+squamous cell. ** p trend value.

Subgroup analyses were also conducted in this set, for first line platinum-based doublet chemotherapy ($n = 155$) and *EGFR* wild-type patients ($n = 161$). The *MME* rs701109 polymorphism was associated with PFS only in the subgroup of patients with wild-type *EGFR*, being the homozygous at increased risk to disease progression (HR = 1.7, 95% CI (1.1–2.5), $p = 0.009$).

4. Discussion

The work developed here sought to test the hypothesis that variants in genes coding for the renin–angiotensin system and for interacting hypoxia/angiogenesis key molecules might have predictive and prognostic value, respectively for NSCLC progression and survival. SNPs are the most common type of polymorphisms, accounting for 90% of genetic variations [38]. Nucleotide substitution, according to its location in germline DNA, may elicit functional modifications, ultimately at protein level, contributing to inter-individual variability, and correlating with relevant phenotypic alterations. The association between genetic polymorphisms and cancer susceptibility, progression, aggressiveness and death has been thoroughly described in the literature [38].

RAS was first discovered and related with the physiological regulation of systemic arterial pressure and involved in hypertension pathogenesis [39]. As a gatekeeper system, RAS is responsible for more than one function, including cell cycle regulation, inflammation, angiogenesis and fibrosis [3]. The capillary blood vessels in the lung are one of the major sites of ACE expression and Ang II production in the human body [40]. In fact, the increased ACE expression observed in several interstitial lung diseases supports pulmonary RAS and establishes a putative role for Ang II in the response to lung injury and fibrosis [4]. A previous report showed that the G-6A polymorphism of the *AGT* gene is associated with increased angiotensin production and idiopathic pulmonary fibrosis progression [41], which is associated with increased risk of lung cancer [42]. Furthermore, the insertion/deletion (I/D) polymorphism of *ACE* gene has been linked to the pathogenesis and progression of human cancers [43,44] and the lowest serum ACE levels were correlated with poorer prognosis and higher relapse rates in lung cancer [15]. We found that the association between *ACE* rs4316 genetic variant and PFS, despite being significant in univariate, the finding was not confirmed on multivariate analysis. It is an I/D polymorphism and is localized at intron 16 of *ACE* gene. This SNP has been associated with plasma ACE levels, with DD genotype carriers independently associated to higher expression [45], albeit no effect was noted on AngII [45]. Thus, it seems unlikely this SNP might impact the ACE/AngII/AT1 axis, which has been associated with tumour cell growth and angiogenesis, partially justifying the absence of clinical impact in NSCLC patients with advanced stage disease. There are other possible reasons for the failure to replicate the prior findings. Many of the initial reports were present only in retrospectively defined subsets of the subjects studied and the main hypotheses investigated were related to cancer risk. Another possibility is that varied ethnic background of study populations may result in a different genetic background which confers a different biologic significance to a given functional polymorphism.

ACE2 is responsible for converting AngII to Ang-(1-7), an agonist of Mas receptor (MasR). In a mouse lung xenograft model, it was previously shown that ACE2 overexpression inhibited both lung cancer cell proliferation *In vitro* and reduced tumour growth *in vivo* [46]. The *ACE2* rs908004 is an intronic variant at the X-linked *ACE2* gene [47]. Findings from the discovery cohort revealed that *ACE2* rs908004 heterozygous carriers presented shorter time-to-progression, only in the subgroup of patients treated with first line platinum-based chemotherapy without actionable driver mutations. We hypothesize that heterozygous produces reduced levels of ACE2, contributing to the up-regulation of ACE/Ang II/AT1 receptor axis, or to linkage with another genomic region that might be relevant for this clinical phenotype. These hypotheses need to be confirmed in further studies. Furthermore, as with all gene association studies, subgroup analyses can lead to spurious associations and might justify our result. Nevertheless, there is lack of studies on genotype–phenotype relationship with this SNP in lung cancer, opening new opportunities for further investigations.

Ang-(1-7) has opposite effects to Ang II upon binding to MasR and might be produced through hydroxylation of Ang I by neprilysin (NEP), an enzyme encoded by the gene *MME* [48]. NEP, also called CD10, has been described as a tumour-specific antigen for lymphocytic leukemia [49]. In the later years, it was demonstrated that NEP functions are implicated in oncogenesis and tumour microenvironment [49]. Moreover, alterations in NEP protein expression have also been reported in solid tumours, including lung cancer [50]. Both in discovery and validation cohorts, NSCLC subjects carrying the heterozygous TC genotype were at decreased risk for disease progression, albeit in the discovery set the protection was conferred to carriers of the TC/CC combined genotypes (dominant model), whereas in the validation cohort it was conferred only to TC (heterosis model). Heterosis for a single gene refers to a heterozygote that has a different phenotypic trait than for either allele homozygotes [51], and heterozygosity has been associated with human healthy aging [52]. Despite insufficient knowledge on the molecular basis of heterosis, an accepted mechanism is related to environmentally-driven epigenetic modifications, namely methylation and microRNAs (miRNAs) [53]. MicroRNAs regulate gene expression by binding to target sequences mostly at the 3'-UTR of mRNAs, and evidence exist for their functional effect in candidate SNPs of various types of cancer [54]. Noteworthy, recently reported data demonstrated such a mechanism with functional repercussion, the SNP rs6665, located downstream of rs701109 in the 3'-UTR region sequence of *MME* [55].

We conducted an *in-silico* proof-of-principle analysis using the MicroSNiPer database [56,57] to predict *MME* rs701109 SNP effects on putative microRNA targets, followed by study of the candidate miRNAs using the RNAhybrid program [58,59] to calculate the minimum free energy (MFE) of hybridization between miRNAs and target sequence (3'-UTR sequence of *MME* extracted from Ensembl [37]). The strongest binding affinity was found for hsa-miR-362-3p, which overlaps the locus of rs701109 at 3'-UTR region. A stronger predicted pairing affinity between the hsa-miR-362-3p miRNA and the target mRNA of *MME* was observed for C-allele (−26.1 kcal/mol) in comparison to T-allele (−23.2 kcal/mol), stronger interaction with the miR-362-3p, resulting in lower expression of *MME*, and consecutively decreased risk for disease progression. Notably, previous data confirmed that hsa-miR-362-3p is expressed in lung tissue, aberrantly expressed in lung adenocarcinoma, and a promoter of metastasis [60]. The differences among cohorts, particularly due to distinct therapeutics, genotype distribution and environmental stimuli, might account for different epigenetic regulation. Experimental studies in lung cancer tissues and *In vitro* with lung cancer organoids are warranted to further validate current findings.

The *MME* rs701109 variant consists in a nucleotide substitution of T-by-C, that has been shown to associate with lower NEP protein expression in the brain of patients with Alzheimer's disease [61]. Notably, previous studies revealed that higher NEP expression associated with poorer prognosis in NSCLC and that tumour NEP was an independent predictor of recurrence in lung adenocarcinomas [50,62]. Taking into consideration that increased NEP expression in lung tumours associates with worst prognosis, and that TC and CC genotype carriers secrete less protein, our findings showing an independent protection for disease progression in TC carriers likely reflect the influence of this SNP in decreasing NEP availability. This result strengthens the involvement of NEP in NSCLC and supports the putative interest of including this genetic biomarker in a multilocus model for predicting relapse. Nevertheless, despite the known ability of NEP to degrade mitogenic peptides, the lack of Ang II degradation, as well as reduced production of Ang-(1-7) by NEP, might account for the growth of lung cancer cells [15]. Taken together, accumulated data and current findings support the need to foster research in neprilysin actions on cancer, particularly in lung cancer.

Hypoxia is a hallmark of solid tumours, and consequently related to enhanced aggressiveness, risk of disease progression and therapy resistance, effects known to be largely mediated by hypoxia-inducible factors [63]. The downstream upregulated vascular endothelial growth factor (VEGF) further contributes to cancer growth and metastasis, directly targeting tumour cells. Increased VEGF expression and/or circulating VEGF levels have been consistently reported in lung cancer [64]. Noteworthy, the activation of RAS in the tumour microenvironment usually involves Ang II, which has

been described to induce hypoxia through reactive oxygen species production, and subsequent activation of pro-inflammatory and pro-angiogenic signals [65]. Albeit no clinical impact was demonstrated for polymorphisms in *HIF1A*, *PGF* and *FLT1* genes, associations were observed in the discovery set for *VEGFA* rs25648 with PFS (univariate analysis) and *KDR* rs1870377 with PFS and OS (multivariate analysis). Koukourakis et al. [66], described that inherited *VEGF* sequence variations were strong determinants of the molecular VEGF and VEGF-downstream phenotype of NSCLC, being the substitution C > A at locus −2578 associated with higher levels of VEGF. Another study showed that *VEGFA* rs25648 (−73C > T) was a prognostic biomarker for PFS and OS in patients with hepatocellular carcinoma, with C-allele conferring improved prognosis, despite not confirmed in multivariate analysis [67]. In the discovery cohort, lung cancer patients had a different outcome since the homozygous C for *VEGF* rs25648 had lower time until disease progression in univariate analysis. The apparently conflicting results might be due to particularities related with different cancer models. Notably, studies investigating the potential association between *VEGF* polymorphisms and circulating VEGF levels also led to controversial results [68–70], opening new opportunities for further investigations to clarify the role of germline *VEGF* variations in NSCLC progression and its repercussion in VEGF levels.

The *KDR* rs1870377 was significantly associated with PFS and OS in the discovery set. Homozygous T and C had increased risk for tumour progression PFS and death, independent of clinicopathological variables and after bootstrap analysis. Findings were not replicated in the validation cohort. *KDR* encodes VEGFR-2, the key receptor mediating endothelial cell proliferation and sprouting, leading to angiogenesis and tumour promotion upon binding and signalling through the *VEGFA* pathway [71]. The *KDR* rs1870377 (c.1719A > T) variant induces a missense alteration in exon 11 that results in the substitution A > T and in the amino acid modification Gln472His. This substitution was previously described to alter the function of VEGF-*KDR* signalling pathway, enhancing ligand/receptor binding efficacy and upregulated signal transduction, ultimately leading to increased microvessel density [72]. This phenotypic correlation with the *KDR* rs1870377 might support T homozygous findings, hypothetically by promoting tumour vascular density for homozygous carriers. Previous findings reported that microvessel count, reflecting tumour neoangiogenesis, appeared to be a poor prognostic factor for survival in NSCLC patients [73], despite some conflicting results [74].

This study provides evidence that SNPs coding for molecules involved in the RAS (*ACE2* rs908004, *MME* rs701109, *KDR* rs1870377) might influence PFS and OS in advanced NSCLC patients, although only *MME* rs701109 was validated in an independent cohort. Candidate SNP studies searching associations with clinically relevant endpoints, have been reported to yield false-positive findings. Here, the multivariate model was first developed in a cohort of consecutively recruited advanced NSCLC patients. Furthermore, we included a robust data analyses setting that included: (i) sample size and number of events in both cohorts were within adequated estimations, (ii) initial empirical analyses to include only the most relevant variables in the multivariate models, (iii) bootstrapping using Montecarlo simulations with 1000 iterations to strengthen internal validation in each cohort, and (iv) validation of important SNPs from the discovery set in an independent cohort of lung cancer patients matched for demographic and pathological characteristics. Nevertheless, despite matching for clinicopathological characteristics, differences between discovery and validation cohorts existed, and this could partially explain the validation of a single polymorphism. Particularly, the validation set was selected from a larger lung cancer database and somatic mutational analyses were determined using distinct methods, revealing different mutational status for both cohorts, which likely influenced the course of disease and the subsequent therapeutic decisions. In addition, differences in respiratory co-morbidities, which may have conducted to mismatched treatment modalities (surgery with adjuvant chemotherapy versus chemo with radiotherapy for stages IIIA–IIIB), combined with the genetic background may also add to explain failure to validate the other genetic polymorphisms.

5. Conclusions

Germline genetic variants are common genomic events, putatively inducing phenotypic modifications affecting both normal physiology and cancer physiopathology. Data presented here supports the rationale for NEP and RAS mechanistic involvement in NSCLC, whereas the polymorphism *MME* rs701109 might prove to be a useful biomarker for NSCLC progression. These findings will foster further and larger studies to confirm the role of RAS, and particularly NEP in NSCLC, as well as to determine the usefulness of *MME* rs701109 as a molecular marker for disease progression. Taken together, these results can lead to a better understanding of NEP influence in the NSCLC biological mechanisms, contributing towards the definition of a genetic profile for this disease and eventually serve as guidance for therapeutic decisions.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/12/3834/s1>, Table S1: Representative single nucleotide polymorphisms (SNPs) of genes involved in RAS (*ACE*, *ACE2*, *AGT*, *AGTR1*, *AGTR2*, *MME*, *CMA1*, *MAS1*) and SNPs of genes that express hypoxia-inducible factor 1-alpha, hypoxia-inducible factor 1-beta, hypoxia-inducible factor 2-alpha, hypoxia-inducible factor 2-beta, vascular endothelial growth factor and its receptor, placental growth factor and its receptor, Table S2. Clinical-pathological variables and progression-free survival (PFS) and overall survival (OS) in discovery set ($n = 167$)—Univariate analysis, Table S3: Discovery Set ($n = 167$): progression-free survival (PFS) and overall survival (OS)—Univariate analysis, Table S4. Clinical-pathological variables and progression-free survival (PFS) and overall survival (OS) in validation set ($n = 190$)—Univariate analysis, Table S5. Validation Set ($n = 190$)—Univariate analysis.

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Table S1. Representative single nucleotide polymorphisms (SNPs) of genes involved in RAS (*ACE*, *ACE2*, *AGT*, *AGTR1*, *AGTR2*, *MME*, *CMA1*, *MAS1*) and SNPs of genes that express hypoxia-inducible factor 1-alpha, hypoxia-inducible factor 1-beta, hypoxia-inducible factor 2-alpha, hypoxia-inducible factor 2-beta, vascular endothelial growth factor and its receptor, placental growth factor and its receptor.

Genes	Single Nucleotide Polymorphisms
<i>ACE</i>	rs121912703, rs1799752, rs4340, rs4316, rs4317
<i>ACE2</i>	rs199951323, rs4646116, rs7890520, rs908004
<i>AGT</i>	rs699, rs34116584, rs121912923, rs4762
<i>AGTR1</i>	rs1800766, rs380400, rs5186, rs5185, rs5189, rs5187, rs12721226, rs12721225
<i>AGTR2</i>	rs11091046, rs5191, rs35474657, rs5194
<i>MME</i>	rs652438, rs701109, rs989692, rs777476150
<i>CMA1</i>	rs5250, rs7142020, rs140295781, rs201312283
<i>MAS1</i>	rs147267209, rs11968301, rs138263796, rs139579440
<i>HIF1A</i>	rs52801082, rs17834641, rs2057482, rs11549465, rs11549467
<i>VEGFA</i>	rs2010963, rs3025039, rs25648, rs10434, rs3025053, rs41282644
<i>KDR</i>	rs7667298, rs1870377, rs2305948
<i>PGF</i>	rs8185, rs12411, rs140032352, rs11542848
<i>FLT1</i>	rs2296283, rs7326277, rs9554314

Table S2. Clinical-pathological variables and progression-free survival (PFS) and overall survival (OS) in discovery set (n=167).

	PFS				OS		
	N	HR (95CI)	P *	Ptrend	HR (95CI)	P	Ptrend
<i>Clinical variables</i>							
Age							
≤64.0	85 (0.51)	Referent			Referent		
>64.0	82 (0.49)	0.9 (0.7-1.3)	0.678	-	1.6 (1.0-2.5)	0.047	-
Gender							
Male	123 (0.74)	Referent			Referent		
Female	44 (0.26)	0.9 (0.6-1.3)	0.539	-	0.6 (0.4-1.1)	0.105	-
Histology							
Adenocarcinoma	116 (0.70)	Referent			Referent		
Squamous cell	42 (0.25)	1.4 (0.9-2.1)	0.116		1.8 (1.1-3.0)	0.027	
Others *	9 (0.05)	0.8 (0.4-1.7)	0.571	0.680	0.9 (0.4-2.4)	0.902	0.291
T							
1	26 (0.16)	Referent			Referent		
2	46 (0.27)	1.3 (0.7-2.3)	0.339		0.5 (0.2-1.2)	0.138	
3-4	95 (0.57)	2.1 (1.3-3.6)	0.004	0.001	1.4 (0.8-2.7)	0.276	0.047
N							
N0-N2	63 (0.38)	Referent			Referent		
N3	103 (0.62)	1.4 (0.9-1.9)	0.099	-	1.1 (0.7-1.7)	0.815	-
Distant Metastasis							
No	73 (0.44)	Referent			Referent		
Yes	94 (0.56)	1.7 (1.2-2.4)	0.003	-	2.5 (1.5-4.2)	<0.001	
Type_Therapy							
Surgery+ChT	12 (0.07)	Referent			Referent		
ChT	124 (0.74)	2.9 (1.3-6.6)	0.012		2.7 (0.8-8.6)	0.099	
ChT+RT	31 (0.19)	3.1 (1.3-7.6)	0.011	0.026	3.0 (0.9-10.2)	0.080	0.127
ECOG PS							
Good (0-2)	163 (0.98)	Referent			Referent		
Poor (3-4)	4 (0.02)	1.4 (0.5-3.8)	0.501	-	2.1 (0.6-6.6)	0.223	-
Anti-hypertensive drugs							
No	86 (0.65)	Referent			Referent		
iACE/ARB	46 (0.35)	0.8 (0.6-1.3)	0.442	-	1.2 (0.7-2.1)	0.538	-

Type_SysTher							
Platinum based	145 (0.87)	Referent			Referent		
TKI	22 (0.13)	0.5 (0.3-0.9)	0.014	-	0.6 (0.3-1.4)	0.218	-

Univariate analysis. HR – Hazard Ratio. ChT – chemotherapy, RT – radiotherapy, TKI - tyrosine kinase inhibitor, iACE – inhibitors of angiotensin-converting enzyme, ARB – AngiotensinII receptor blocker.

Table S3. Discovery Set (n=167): progression-free survival (PFS) and overall survival (OS) – Univariate analysis.

Genetic variants	Genetic model	Genotype	Progression-free Survival				Overall Survival		
			N (freq.)	HR (95CI)	P *	Ptrend	HR (95CI)	P *	Ptrend
ACE rs4316	Additive	CC	55 (0.33)	Referent			Referent		
		CT	82 (0.49)	0.6 (0.4-0.9)	0.013		0.6 (0.4-1.1)	0.089	
		TT	30 (0.18)	0.7 (0.4-1.1)	0.113	0.049	0.8 (0.4-1.2)	0.541	0.308
	Dominant	CC	55 (0.33)	Referent			Referent		
		CT/TT	112 (0.67)	0.6 (0.4-0.9)	0.011	-	0.7 (0.4-1.1)	0.114	
	Recessive	CC/CT	137 (0.82)	Referent			Referent		
ACE2 rs908004	Additive	TT	30 (0.18)	0.9 (0.6-1.4)	0.676	-	1.0 (0.6-1.9)	0.896	
		CT	82 (0.49)	Referent			Referent		
		CC/TT	85 (0.51)	1.4 (1.0-2.0)	0.058	-	1.5 (0.9-2.3)	0.116	
	Dominant	TT	167 (1.0)	-	-	-	-	-	-
		CC	167 (1.0)	-	-	-	-	-	-
	Recessive	GG	55 (0.33)	Referent			Referent		
ACE2 rs4646116 *	Additive	GC	16 (0.09)	0.4 (0.2-0.9)	0.018		0.5 (0.2-1.1)	0.089	
		CC	96 (0.58)	0.9 (0.6-1.3)	0.566	0.794	0.9 (0.5-1.4)	0.587	0.681
		GG	55 (0.33)	Referent			Referent		
	Dominant	GC/CC	112 (0.67)	0.8 (0.6-1.2)	0.236	-	0.8 (0.5-1.3)	0.320	
		GG/GC	71 (0.42)	Referent			Referent		
	Recessive	CC	96 (0.58)	1.1 (0.8-1.6)	0.539	-	1.0 (0.7-1.7)	0.849	
ACE2 rs7890520	Additive	GC	16 (0.10)	Referent			Referent		
		GG/CC	151 (0.90)	2.1 (1.1-3.9)	0.021	-	2.0 (0.9-4.9)	0.109	
		TT	167 (1.0)	-	-	-	-	-	-
	Dominant	GG	166 (0.99)	Referent			Referent		
		AG	1 (0.01)	-	-	-	-	-	-
	Recessive	A	167 (1.0)	-	-	-	-	-	-
ACE2 rs1999951323*	Additive	GG	167 (1.0)	-	-	-	-	-	-
		GG	135 (0.81)	Referent			Referent		
		GA	31 (0.18)	1.0 (0.6-1.5)	0.964		0.8 (0.5-1.5)	0.495	0.495
	Dominant	GG	167 (1.0)	-	-	-	-	-	-
		GA	31 (0.18)	1.0 (0.6-1.5)	0.964		0.8 (0.5-1.5)	0.495	0.495
	Recessive	GA	31 (0.18)	1.0 (0.6-1.5)	0.964		0.8 (0.5-1.5)	0.495	0.495

		AA	1 (0.01)	-	-	-	-		
	Dominant	GG	135 (0.81)	Referent			Referent		
		GA/AA	32 (0.19)	1.0 (0.6-1.5)	0.917	-	0.8 (0.4-1.4)	0.389	
	Recessive	GG/GA	166 (0.99)	Referent			Referent		
		AA	1 (0.01)	-	-	-	2.0 (0.3-14.5)	0.492	
	Heterosis	GA	31 (0.19)	Referent			Referent		
		GG/GA	136 (0.81)	1.1 (0.7-1.7)	0.758	-	1.4 (0.7-2.7)	0.308	
	Additive	GG	23 (0.14)	Referent			Referent		
		GA	87 (0.52)	0.8 (0.5-1.3)	0.370		1.6 (0.7-3.7)	0.303	
		AA	57 (0.34)	0.9 (0.5-1.6)	0.710	0.997	1.6 (0.7-4.0)	0.272	0.361
	Dominant	GG	23 (0.14)	Referent			Referent		
		GA/AA	144 (0.86)	0.8 (0.5-1.4)	0.464	-	1.6 (0.7-3.7)	0.271	
	Recessive	GG/GA	110 (0.66)	Referent			Referent		
		AA	57 (0.34)	1.1 (0.8-1.6)	0.622	-	1.1 (0.7-1.8)	0.634	
	Heterosis	AG	87 (0.52)	Referent			Referent		
		CG/AA	80 (0.48)	1.2 (0.8-1.7)	0.350	-	0.9 (0.6-1.5)	0.780	
AGTR1 rs12721226 *		GG	167 (1.0)	-	-	-	-	-	-
	Additive	TT	115 (0.69)	Referent			Referent		
		TC	46 (0.27)	1.1 (0.7-1.6)	0.641		0.7 (0.4-1.3)	0.245	
		CC	6 (0.04)	1.0 (0.4-2.7)	0.999	0.726	0.4 (0.1-2.7)	0.372	0.135
	Dominant	TT	115 (0.69)	Referent			Referent		
		TC/CC	52 (0.31)	1.1 (0.7-1.6)	0.666	-	0.7 (0.4-1.2)	0.162	
	Recessive	TT/TC	161 (0.96)	Referent			Referent		
		CC	6 (0.04)	1.0 (0.4-2.6)	0.959	-	0.4 (0.1-2.9)	0.366	
	Heterosis	TC	46 (0.27)	Referent			Referent		
		TT/CC	121 (0.73)	0.9 (0.6-1.3)	0.639	-	1.4 (0.8-2.5)	0.284	
AGTR1 rs5187 *		AA	167 (1.0)	-	-	-	-	-	-
	Additive	GG	5 (0.03)	Referent			Referent		
		GA	31 (0.19)	0.4 (0.1-1.1)	0.067		0.3 (0.1-0.9)	0.037	
		AA	131 (0.78)	0.5 (0.2-1.3)	0.148	0.778	0.4 (0.1-1.2)	0.093	0.932
	Dominant	GG	5 (0.03)	Referent			Referent		
		GA/AA	162 (0.97)	0.5 (0.2-1.2)	0.124	-	0.3 (0.1-1.1)	0.073	
	Recessive	GG/GA	36 (0.22)	Referent			Referent		
		AA	131 (0.78)	1.2 (0.8-1.8)	0.455	-	1.2 (0.7-2.2)	0.524	
	Heterosis	AG	31 (0.19)	Referent			Referent		
		CG/AA	136 (0.81)	1.3 (0.8-2.1)	0.224	-	1.5 (0.8-2.8)	0.228	
	Additive	AA	87 (0.52)	Referent			Referent		
		AC	66 (0.40)	1.0 (0.7-1.5)	0.901		0.9 (0.6-1.5)	0.755	
		CC	14 (0.08)	1.3 (0.7-2.6)	0.370	0.513	1.9 (0.8-4.2)	0.131	0.461
	Dominant	AA	87 (0.52)	Referent			Referent		
		AC/CC	80 (0.48)	1.1 (0.8-1.5)	0.715	-	1.0 (0.6-1.6)	0.892	
	Recessive	AA/AC	153 (0.92)	Referent			Referent		
		CC	14 (0.08)	1.3 (0.7-2.5)	0.371	-	1.9 (0.9-4.3)	0.102	
	Heterosis	AC	66 (0.39)	Referent			Referent		
		AA/CC	101 (0.61)	1.0 (0.7-1.4)	0.939		1.2 (0.7-1.9)	0.535	
AGTR1 rs5185		TT	164 (0.98)	Referent			-	-	-
		TG	3 (0.02)	0.2 (0.0-1.4)	0.102	-	-	-	-
	Additive	TT	1 (0.01)	Referent			Referent		
		TG	12 (0.07)	-	-	-	-	-	-
		GG	154 (0.92)	-	-	-	1.2 (0.4-3.4)	0.665	0.665
	Dominant	TT	1 (0.01)	Referent			Referent		
		TG/GG	166 (0.99)	-	-	-	-	-	-
	Recessive	TT/TG	13 (0.08)	Referent			Referent		
		GG	154 (0.92)	1.0 (0.5-1.8)	0.928	-	1.7 (0.5-5.4)	0.375	
	Heterosis	GT	12 (0.07)	Referent			Referent		
		TT/GG	155 (0.93)	1.1 (0.5-2.1)	0.817	-	2.6 (0.6-10.5)	0.190	
AGTR2 rs5191 *		GG	167 (1.0)	-	-	-	-	-	-
	Additive	GG	85 (0.51)	Referent			Referent		
		GA	20 (0.12)	1.1 (0.6-1.9)	0.795		1.1 (0.5-2.3)	0.801	
		AA	62 (0.37)	1.0 (0.7-1.5)	0.856	0.848	0.9 (0.5-1.5)	0.692	0.706
	Dominant	GG	85 (0.51)	Referent			Referent		
		AG/AA	82 (0.49)	1.0 (0.7-1.5)	0.804	-	0.9 (0.6-1.5)	0.815	
	Recessive	GG/AG	105 (0.63)	Referent			Referent		
		AA	62 (0.37)	1.0 (0.7-1.5)	0.910	-	0.9 (0.5-1.4)	0.628	
	Heterosis	AG	20 (0.12)	Referent			Referent		
		GG/AA	147 (0.88)	0.9 (0.6-1.6)	0.828	-	0.9 (0.4-1.7)	0.702	
	Additive	AA	62 (0.37)	Referent			Referent		
		AC	20 (0.12)	1.0 (0.6-1.8)	0.893		1.2 (0.6-2.6)	0.615	
		CC	85 (0.51)	1.0 (0.7-1.4)	0.856	0.848	1.1 (0.7-1.8)	0.692	0.706
	Dominant	AA	62 (0.37)	Referent			Referent		
		AC/CC	105 (0.63)	1.0 (0.7-1.4)	0.910	-	1.1 (0.7-1.8)	0.628	
	Recessive	AA/AC	82 (0.49)	Referent			Referent		
		CC	85 (0.51)	1.0 (0.7-1.3)	0.804	-	1.1 (0.7-1.7)	0.815	
	Heterosis	AC	20 (0.12)	Referent			Referent		
		AA/CC	147 (0.88)	0.9 (0.6-1.6)	0.828	-	0.9 (0.4-1.8)	0.702	
AGTR2 rs35474657 *		AA	167 (1.0)	-	-	-	-	-	-

MAS1 rs11968301 *	AA	167 (1.0)	-	-	-	-	-	-
MAS1 rs138263796 *	GG	167 (1.0)	-	-	-	-	-	-
MAS1 rs139579440 *	CC	167 (1.0)	-	-	-	-	-	-
MAS1 rs147267209 *	AA	167 (1.0)	-	-	-	-	-	-
VEGFA rs10434	Additive	GC	55 (0.33)	Referent			Referent	
		GA	81 (0.48)	1.1 (0.8-1.7)	0.486		1.0 (0.6-1.6)	0.851
		AA	31 (0.19)	1.3 (0.8-2.2)	0.238	0.236	1.1 (0.6-2.1)	0.718 0.785
	Dominant	GG	55 (0.33)	Referent			Referent	
		GA/AA	112 (0.67)	1.2 (0.8-1.7)	0.332	-	1.0 (0.6-1.6)	0.985
		GG/GA	136 (0.81)	Referent			Referent	
	Recessive	AA	31 (0.19)	1.2 (0.8-1.9)	0.330	-	1.2 (0.7-2.0)	0.626
		GA	81 (0.48)	Referent			Referent	
		GG/AA	86 (0.52)	1.0 (0.7-1.4)	0.830	-	1.1 (0.7-1.8)	0.712
	Additive	GG	67 (0.40)	Referent			Referent	
		GC	73 (0.44)	1.2 (0.8-1.7)	0.436		1.2 (0.7-2.0)	0.459
		CC	27 (0.16)	1.5 (1.0-2.5)	0.088	0.099	1.0 (0.5-1.9)	0.886 0.895
VEGFA rs2010963	Dominant	GG	67 (0.40)	Referent			Referent	
		GC/CC	100 (0.60)	1.2 (0.9-1.8)	0.213	-	1.1 (0.7-1.8)	0.608
		GG/GC	140 (0.84)	Referent			Referent	
	Recessive	CC	27 (0.16)	1.4 (0.9-2.2)	0.125	-	0.9 (0.5-1.7)	0.667
		GC	73 (0.44)	Referent			Referent	
		GG/CC	94 (0.56)	1.0 (0.7-1.4)	0.868	-	0.8 (0.5-1.3)	0.394
	Additive	CC	122 (0.76)	Referent			Referent	
		CT	34 (0.21)	0.6 (0.4-0.9)	0.029		1.0 (0.5-1.7)	0.913
		TT	5 (0.03)	0.9 (0.3-2.5)	0.837	0.072	1.9 (0.6-6.2)	0.277 0.603
	Dominant	CC	122 (0.76)	Referent			Referent	
		CT/TT	39 (0.24)	0.6 (0.4-1.0)	0.036	-	1.1 (0.6-1.8)	0.838
		CC/CT	156 (0.97)	Referent			Referent	
VEGFA rs25648	Recessive	TT	5 (0.03)	1.0 (0.4-2.7)	0.997	-	1.9 (0.6-6.2)	0.269
		CT	34 (0.21)	Referent			Referent	
		CC/TT	127 (0.79)	1.7 (1.1-2.6)	0.030	-	1.1 (0.6-1.9)	0.847
	Heterosis	CC	123 (0.74)	Referent			Referent	
		CT	42 (0.25)	1.0 (0.7-1.5)	0.768		0.8 (0.4-1.4)	0.545 -
		TT	2 (0.01)	-	-	-	-	-
	Dominant	CC	123 (0.74)	Referent			Referent	
		CT/TT	44 (0.26)	1.0 (0.7-1.5)	0.940	-	0.8 (0.5-1.4)	0.433
		CC/CT	165 (0.99)	Referent			Referent	
	Recessive	TT	2 (0.01)	2.4 (0.6-9.8)	0.225	-	2.5 (0.3-17.9)	0.373
		CT	42 (0.25)	Referent			Referent	
		CC/TT	125 (0.75)	1.0 (0.7-1.5)	0.875	-	1.3 (0.7-2.3)	0.343
VEGFA rs41282644	Additive	GG	153 (0.92)	Referent			Referent	
		GA	14 (0.08)	1.3 (0.7-2.4)	0.454	-	1.0 (0.5-2.4)	0.910 -
		CC	123 (0.74)	Referent			Referent	
	KDR rs2305948	CT	40 (0.24)	1.0 (0.7-1.5)	0.903		1.4 (0.8-2.4)	0.186
		TT	4 (0.02)	0.7 (0.3-2.0)	0.508	0.735	0.3 (0.4-2.2)	0.239 0.974
		CC	123 (0.74)	Referent			Referent	
	Dominant	CT/TT	44 (0.26)	1.0 (0.7-1.4)	0.913	-	1.2 (0.7-2.0)	0.488
		CC/CT	163 (0.98)	Referent			Referent	
		TT	4 (0.02)	0.7 (0.3-1.9)	0.498	-	0.3 (0.0-2.0)	0.211
	Heterosis	CT	40 (0.24)	Referent			Referent	
		CC/TT	127 (0.76)	1.0 (0.6-1.4)	0.841	-	0.7 (0.4-1.1)	0.145
		TT	93 (0.56)	Referent			Referent	
KDR rs1870377	Additive	TA	68 (0.40)	0.7 (0.5-1.0)	0.082		0.6 (0.4-1.0)	0.064
		AA	6 (0.04)	1.9 (0.8-4.8)	0.163	0.347	4.1 (1.4-11.8)	0.010 0.517
		TT	93 (0.56)	Referent			Referent	
	Dominant	TA/AA	74 (0.44)	0.8 (0.5-1.1)	0.143	-	0.7 (0.4-1.1)	0.160
		TT/TA	161 (0.96)	Referent			Referent	
		AA	6 (0.04)	2.2 (0.9-5.5)	0.088	-	4.9 (1.7-14.0)	0.003
	Recessive	TA	68 (0.41)	Referent			Referent	
		TT/AA	99 (0.59)	1.4 (1.0-2.0)	0.054	-	1.7 (1.0-2.7)	0.034
		TT	24 (0.14)	Referent			Referent	
	Additive	TC	84 (0.51)	1.1 (0.6-1.8)	0.806		1.5 (0.7-3.0)	0.274
		CC	57 (0.35)	1.0 (0.6-1.7)	0.927	0.990	0.9 (0.4-2.0)	0.859 0.482
		TT	24 (0.14)	Referent			Referent	
KDR rs7667298	Dominant	TC/CC	141 (0.86)	1.0 (0.6-1.7)	0.849	-	1.2 (0.6-2.3)	0.600
		TT/TC	108 (0.65)	Referent			Referent	
		CC	57 (0.35)	1.0 (0.7-1.4)	0.899	-	0.7 (0.4-1.1)	0.155
	Recessive	TC	84 (0.51)	Referent			Referent	
		TT/CC	81 (0.49)	1.0 (0.7-1.3)	0.793	-	0.6 (0.4-1.0)	0.067
		GG	32 (0.19)	Referent			Referent	
	Additive	GA	80 (0.48)	1.4 (0.9-2.3)	0.149		1.4 (0.7-2.7)	0.378
		AA	55 (0.33)	1.2 (0.7-1.9)	0.583	0.785	1.2 (0.6-2.5)	0.638 0.810
		GG	32 (0.19)	Referent			Referent	
	Dominant	GA/AA	135 (0.81)	1.3 (0.8-2.1)	0.245	-	1.3 (0.7-2.5)	0.445 -
		GG/GA	112 (0.67)	Referent			Referent	
		AA	55 (0.33)	0.9 (0.6-1.3)	0.564	-	0.9 (0.6-1.5)	0.798 -

	<i>Heterosis</i>	GA	80 (0.48)	Referent			Referent		
		GG/AA	87 (0.52)	0.8 (0.5-1.1)	0.136	-	0.8 (0.5-1.3)	0.415	-
		AC	34 (0.20)	Referent			Referent		
<i>FLT1 rs9554314</i>		AA	133 (0.80)	0.7 (0.5-1.1)	0.119	-	0.7 (0.4-1.2)	0.167	-
		TT	133 (0.80)	Referent			Referent		
<i>FLT1 rs7326277</i>		TC	34 (0.20)	1.4 (0.9-2.1)	0.119	-	1.4 (0.9-2.4)	0.167	-
		CC	124 (0.74)	Referent			Referent		
<i>HIF1A rs11549465</i>	<i>Additive</i>	CT	42 (0.25)	1.0 (0.7-1.5)	0.875		1.4 (0.9-2.4)	0.164	
		TT	1 (0.01)	-	-	-	-	-	-
		CC	124 (0.74)	Referent			Referent		
	<i>Dominant</i>	CT/TT	43 (0.26)	0.9 (0.6-1.4)	0.699	-	1.3 (0.8-2.2)	0.251	
		CC/CT	166 (0.99)	Referent			Referent		
	<i>Recessive</i>	TT	1 (0.01)	-	-	-	-	-	-
		CT	41 (0.25)	Referent			Referent		
	<i>Heterosis</i>	CC/TT	126 (0.75)	1.0 (0.7-1.5)	0.966	-	0.7 (0.4-1.1)	0.144	
		GG	167 (1.0)	-	-	-	-	-	-
<i>HIF1A rs2057482</i>	<i>Additive</i>	TT	6 (0.04)	Referent			Referent		
		TC	53 (0.31)	1.6 (0.6-4.4)	0.384		1.3 (0.3-5.5)	0.729	
		CC	108 (0.65)	1.6 (0.6-4.4)	0.355	0.524	1.0 (0.2-4.0)	0.966	0.346
	<i>Dominant</i>	TT	6 (0.04)	Referent			Referent		
		TC/CC	161 (0.96)	1.6 (0.6-4.3)	0.357	-	1.1 (0.3-4.4)	0.925	
	<i>Recessive</i>	TT/TC	59 (0.35)	Referent			Referent		
		CC	108 (0.65)	1.1 (0.7-1.5)	0.718	-	0.8 (0.5-1.2)	0.268	
	<i>Heterosis</i>	TC	53 (0.31)	Referent			Referent		
		TT/CC	114 (0.69)	1.0 (0.7-1.4)	0.964	-	0.8 (0.5-1.2)	0.243	
		CC	124 (0.74)	Referent			Referent		
<i>HIF1A 52801082</i>	<i>Additive</i>	CT	41 (0.25)	1.0 (0.7-1.5)	0.875		1.4 (0.9-2.4)	0.164	
		TT	2 (0.01)	-	-	-	-	-	-
		CC	124 (0.74)	Referent			Referent		
	<i>Dominant</i>	CT/TT	43 (0.26)	0.9 (0.6-1.4)	0.699	-	1.3 (0.8-2.2)	0.251	
		CC/CT	165 (0.99)	Referent			Referent		
	<i>Recessive</i>	TT	2 (0.01)	0.3 (0.0-2.2)	0.241	-	-	-	-
		CT	41 (0.25)	Referent			Referent		
	<i>Heterosis</i>	CC/TT	126 (0.75)	1.0 (0.7-1.5)	0.966	-	0.7 (0.4-1.1)	0.144	
		TT	6 (0.04)	Referent			Referent		
<i>PGF rs12411</i>	<i>Additive</i>	TA	44 (0.26)	1.8 (0.7-4.7)	0.206		1.4 (0.5-4.0)	0.587	
		AA	117 (0.70)	1.5 (0.6-3.8)	0.347	0.913	1.2 (0.4-3.5)	0.691	0.937
		TT	6 (0.04)	Referent			Referent		
	<i>Dominant</i>	TA/AA	161 (0.96)	1.6 (0.7-4.0)	0.296	-	1.3 (0.5-3.5)	0.654	
		TT/TA	50 (0.30)	Referent			Referent		
	<i>Recessive</i>	AA	117 (0.70)	0.9 (0.6-1.3)	0.687	-	1.0 (0.6-1.6)	0.891	
		TA	44 (0.26)	Referent			Referent		
	<i>Heterosis</i>	TT/AA	123 (0.74)	0.8 (0.6-1.2)	0.309	-	0.9 (0.5-1.5)	0.683	
		GG	167 (1.00)	-	-	-	-	-	-
<i>PGF rs140032352 *</i>	<i>Additive</i>	TT	147 (0.88)	Referent			Referent		
		TC	16 (0.10)	1.1 (0.6-1.9)	0.845		1.3 (0.6-2.7)	0.472	
		CC	4 (0.02)	0.6 (0.2-1.9)	0.363	0.544	0.6 (0.2-2.6)	0.517	0.889
	<i>Dominant</i>	TT	147 (0.88)	Referent			Referent		
		TC/CC	20 (0.12)	0.9 (0.5-1.6)	0.756	-	1.1 (0.6-2.1)	0.828	
	<i>Recessive</i>	TT/TC	163 (0.98)	Referent			Referent		
		CC	4 (0.02)	0.6 (0.2-1.8)	0.358	-	0.6 (0.1-2.5)	0.495	
	<i>Heterosis</i>	TC	16 (0.10)	Referent			Referent		
		TT/CC	151 (0.90)	0.9 (0.5-1.6)	0.800	-	0.8 (0.4-1.6)	0.446	
<i>CMA1 rs140295781 *</i>		CC	167	-	-	-	-	-	-
<i>CMA1 rs201312283 *</i>		GG	167	-	-	-	-	-	-
<i>CMA1 rs5250</i>	<i>Additive</i>	GG	129 (0.77)	Referent			Referent		
		GA	36 (0.22)	0.9 (0.6-1.3)	0.565		1.1 (0.6-1.8)	0.792	
		AA	2 (0.01)	-	-	-	-	-	-
	<i>Dominant</i>	GG	129 (0.77)	Referent			Referent		
		GA/AA	38 (0.23)	0.8 (0.6-1.3)	0.412	-	1.0 (0.6-1.7)	0.921	
	<i>Recessive</i>	GG/GA	165 (0.99)	Referent			Referent		
		AA	2 (0.01)	0.8 (0.2-3.4)	0.805	-	0.6 (0.1-4.0)	0.563	
	<i>Heterosis</i>	GA	36 (0.22)	Referent			Referent		
		GG/AA	131 (0.78)	1.2 (0.8-1.8)	0.447	-	0.9 (0.5-1.6)	0.752	
<i>CMA1 rs7142020 *</i>		AA	167 (1.0)	-	-	-	-	-	-
<i>MME rs777476150 *</i>		CC	167 (1.0)	-	-	-	-	-	-
<i>MME rs652438</i>	<i>Additive</i>	TT	141 (0.85)	Referent			Referent		
		TC	24 (0.14)	1.4 (0.9-2.2)	0.178		1.4 (0.8-2.7)	0.266	
		CC	2 (0.01)	-	-	-	-	-	-
	<i>Dominant</i>	TT	141 (0.85)	Referent			Referent		
		CT/CC	26 (0.15)	1.2 (0.8-1.9)	0.404	-	1.4 (0.7-2.5)	0.304	
	<i>Recessive</i>	TT/TC	165 (0.99)	Referent			Referent		
		CC	2 (0.01)	0.3 (0.0-2.3)	0.259	-	0.9 (0.1-6.5)	0.917	
	<i>Heterosis</i>	TC	24 (0.14)	Referent			Referent		
		TT/CC	143 (0.86)	0.7 (0.4-1.1)	0.142	-	0.7 (0.4-1.3)	0.264	

MME rs701109	Additive	TT	17 (0.10)	Referent			Referent		
		CT	72 (0.44)	0.6 (0.3-1.2)	0.134		0.7 (0.3-1.4)	0.277	
		CC	77 (0.46)	0.5 (0.3-0.9)	0.045	0.074	0.6 (0.3-1.3)	0.189	0.271
	Dominant	TT	17 (0.10)	Referent			Referent		
		TC/CC	149 (0.90)	0.6 (0.3-1.0)	0.064	-	0.6 (0.3-1.3)	0.203	
		TT/TC	89 (0.54)	Referent			Referent		
	Recessive	CC	77 (0.46)	0.8 (0.6-1.1)	0.200	-	0.8 (0.5-1.4)	0.489	
		TC	72 (0.43)	Referent			Referent		
		CC/TT	94 (0.57)	0.9 (0.7-1.3)	0.688	-	1.0 (0.6-1.6)	0.987	
MME rs989692	Additive	TT	39 (0.23)	Referent			Referent		
		TC	69 (0.42)	0.9 (0.6-1.4)	0.669		1.4 (0.7-2.5)	0.327	
		CC	58 (0.35)	0.8 (0.5-1.3)	0.364	0.358	1.3 (0.7-2.5)	0.385	0.421
	Dominant	TT	39 (0.23)	Referent			Referent		
		TC/CC	127 (0.77)	0.9 (0.6-1.3)	0.473	-	1.3 (0.8-2.4)	0.306	
		TT/TC	108 (0.65)	Referent			Referent		
	Recessive	CC	58 (0.35)	0.9 (0.6-1.2)	0.419	-	1.1 (0.7-1.8)	0.729	
		TC	69 (0.42)	Referent			Referent		
		TT/CC	97 (0.58)	1.0 (0.7-1.4)	0.863	-	0.9 (0.5-1.4)	0.557	

* SNPs without variation in this population. HR, hazard ratio; 95%CI, 95% confidence interval. After univariate Cox regression analyses, the values for retention were P<0.10 and more or less than 30% effect in HR, in order to be included into the multivariate Cox proportional hazards model. Only one genetic model was included in the model to avoid collinearity. Tag SNPs: *AGTR2* (rs5194, rs11091046), *FLT1* (rs9554314, rs11549465), *HIF1A* rs52801082, *KDR* (rs1531289, rs2239702, rs7667298, rs2305948, rs1870377).

Table S4. Clinical-pathological variables and progression-free survival (PFS) and overall survival (OS) in validation set (n=190).

	PFS				OS		
	Univariate (Cox)				Univariate (Kaplan-Meier)		
	N	HR (95CI)	P *	Ptrend	HR (95CI)	P *	Ptrend
Clinical variables							
Age	89 (0.47)						
<65.0	101	Referent		-	Referent	0.027	-
>65.0	(0.53)	1.3 (0.9-1.8)	0.133		1.6 (1.0-2.5)		
Gender	133	Referent	0.015	-	Referent	0.586	-
Male	(0.70)	0.6 (0.4-0.9)			0.9 (0.6-1.3)		
Female	57 (0.30)						
Histology	126	Referent	0.14	0.006	Referent	0.021	0.066
Adenocarcinoma	(0.66)	1.5 (1.1-2.1)	0.103		1.7 (1.1-2.2)		
Squamous cell	62 (0.33)	3.2 (0.8-13.3)			-	-	
Others *	2 (0.01)						
T	16 (0.08)	Referent	0.485	0.595	Referent	0.424	0.308
T1	33 (0.18)	1.3 (0.6-2.7)	0.473		1.6 (0.5-4.9)	0.288	
T2	141	1.3 (0.7-2.4)			1.7 (0.6-4.7)		
T3/T4	(0.74)						
N	114	Referent	0.268	-	Referent	0.376	-
N0-N2	(0.60)	1.2 (0.9-1.7)			1.2 (0.8-1.8)		
N3							
	76 (0.40)						
Distant Metastasis	81 (0.43)	Referent	0.085	-	Referent	0.669	-
No	109	1.3 (1.0-1.9)			1.1 (0.7-1.7)		
Yes	(0.57)						
ECOG PS	188	Referent	<0.0001	-	Referent	<0.0001	-
Good (0-2)	(0.99)	18.9 (4.1-86.3)			22.3 (5.0-100.9)		
Poor (3-4)	2 (0.01)						
Type_Therapy	109	Referent	0.052	-	Referent	0.163	-
ChT	(0.57)	0.7 (0.5-1.0)			0.7 (0.5-1.1)		
ChT+RT	81 (0.43)						
Type_SysTher	155	Referent	0.003	-	Referent	0.020	-
Platinum based ChT	(0.82)	0.5 (0.3-0.8)			0.5 (0.3-0.9)		
TKI	35 (0.18)						
Anti-hypertensive drugs	126	Referent	0.441	-	Referent	0.892	-
No	(0.67)	1.1 (0.8-1.6)			1.0 (0.6-1.5)		
iACE/ARB	62 (0.33)						

Univariate analysis. HR – Hazard Ratio. ChT – chemotherapy, RT – radiotherapy, TKI – tyrosine kinase inhibitor, iACE – inhibitors of angiotensin-converting enzyme, ARB – AngiotensinII receptor blocker

Table S5. Validation Set (n=190).

			Progression-free survival				Overall survival		
			Univariate (Cox)			Univariate (Cox)			
Genetic variants	Genetic model		N	HR (95CI)	P *	Ptrend	HR (95CI)	P	Ptrend
ACE2 rs908004	Additive	G	61 (0.32)	Referent	0.819	0.425	Referent	0.443	0.299
		GC	29 (0.15)	1.1 (0.6-1.7)	0.459		0.8 (0.4-1.5)		
		C	99 (0.53)	0.9 (0.6-1.2)	0.8 (0.5-1.2)				
	Dominant	G	61 (0.32)	Referent	0.587	-	Referent	0.256	
		GC/C	128 (0.68)	0.9 (0.6-1.3)	0.8 (0.5-1.2)	-			
	Recessive	G/GC	90 (0.48)	Referent	0.350	-	Referent	0.421	
C		99 (0.52)	0.8 (0.6-1.2)	0.8 (0.6-1.3)	-				
	Heterosis	GC	29 (0.15)	Referent	0.531	-	Referent	0.735	-
		G/C	160 (0.85)	0.9 (0.6-1.4)	1.1 (0.6-2.0)				
	Additive	TT	108 (0.58)	Referent	0.932	0.958	Referent	0.674	0.632
		TA	74 (0.39)	0.9 (0.7-1.4)	0.959		1.1 (0.7-1.7)	0.632	
		AA	5 (0.03)	1.0 (0.3-3.3)	1.2 (0.3-5.1)				
	Dominant	TT	108 (0.58)	Referent	0.941	-	Referent	0.652	
TA/AA		79 (0.42)	1.0 (0.7-1.4)	1.1 (0.7-1.7)					
Recessive	TT/TA	182 (0.97)	Referent	0.951	-	Referent	0.815	-	
	AA	5 (0.03)	1.0 (0.3-3.3)	1.2 (0.3-4.8)					
	Heterosis	TA	74 (0.40)	Referent	0.927	-	Referent	0.699	-
		TT/AA	113 (0.60)	1.0 (0.7-1.4)	0.9 (0.6-1.4)				
	Additive	TT	29 (0.15)	Referent	0.115	0.665	Referent	0.381	0.698
		TC	87 (0.46)	0.7 (0.4-1.1)			0.8 (0.4-1.4)		
		CC	73 (0.39)	1.0 (0.6-1.5)			1.0 (0.6-1.8)		
	Dominant	TT	29 (0.15)	Referent	0.308	-	Referent	0.628	
TC/CC		160 (0.85)	0.8 (0.5-1.2)	0.9 (0.5-1.5)					
Recessive	TT/TC	116 (0.61)	Referent	0.165		Referent	0.347	-	
	CC	73 (0.39)	1.3 (0.9-1.8)			1.2 (0.8-1.9)			
Heterosis	TC	87 (0.46)	Referent	0.041	-	Referent	0.207	-	
	TT/CC	102 (0.54)	1.4 (1.0-1.9)			1.3 (0.8-2.0)			

Univariate analysis. HR, hazard ratio; 95%CI, 95% confidence interval. After univariate Cox regression analyses, the values for retention were $P < 0.10$ and more or less than 30% effect in HR, in order to be included into the multivariate Cox proportional hazards model. Only one genetic model was included in the model to avoid collinearity.



ORIGINAL ARTICLE

Pharmacogenetics of advanced lung cancer: Predictive value of functional genetic polymorphism AGXT Pro11Leu in clinical outcome?



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Abstract

Introduction: AGXT gene codes for the enzyme alanine glyoxylate aminotransferase, which is involved in hepatic peroxisomal metabolism of platinum-based chemotherapeutic agents. The association of genetic variant AGXT rs34116584 on the clinical outcome and response to chemotherapy of patients with non-small cell lung cancer (NSCLC) remains to be established. Our aim was to evaluate the association of functional AGXT gene polymorphism in NSCLC progression, considering as primary and secondary endpoint, progression free survival (PFS) and overall survival (OS), respectively.

Methods: Genotyping of the AGXT rs34116584 genetic polymorphism was performed by mass spectrometry on 168 DNA samples from patients with NSCLC (stages IIIA–IVB). Univariate survival analysis included the study of Kaplan–Meier curves with the Log-Rank test, while Cox regression was used as a multivariate analysis.

Results: Multivariate analysis showed shorter PFS for T carriers [HR = 2.0, 95% CI, 1.4–3.0, $p < 0.0001$] and shorter OS [HR = 1.8, 95% CI, 1.1–3.0, $p = 0.017$] globally, as well as in a subgroup of patients ($n = 144$) treated with first line platinum-based chemotherapy [HR = 2.0, 95% CI, 1.3–3.1, $p = 0.001$] and [HR = 1.8, 95% CI, 1.1–3.1, $p = 0.026$], respectively.

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Conclusion: This polymorphism seems to have an impact on NSCLC progression, opening new perspectives for its inclusion as a pharmacogenetic predictor of response to platinum-based chemotherapy.

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Introduction

Lung cancer is one of the most common malignancies worldwide and the most common cause of cancer deaths in the past few decades, with over one million subjects yearly diagnosed¹. The 5-year survival rate is the lowest compared with other frequent malignancies². Among all primary lung cancers, non-small cell lung cancer (NSCLC) represents approximately 85% of cases. The 5-year relative survival rate has been increasing over the last years, particularly due to progress in treatment over the years³.

Although targeted therapies have redefined treatment options for patients with molecularly defined NSCLC (eg, epidermal growth factor receptor [EGFR]-mutant, anaplastic lymphoma kinase [ALK]-rearranged NSCLC), these therapies are ineffective in those whose tumours lack such genetic alterations, which comprise the majority of NSCLC patients⁴.

Standard-of-care first-line chemotherapy for advanced NSCLC without actionable driver mutations or low expression of programmed death-ligand 1 (PD-L1) has historically been platinum-doublet, cisplatin or carboplatin, with or without maintenance therapy⁵. Despite its wide acceptance and use, platinum-based chemotherapy presents poor clinical outcomes and efficacy varies across patients. Currently, the combination of immune checkpoint inhibitors with chemotherapy in advanced driver mutation-negative NSCLC and tumour PD-L1 expression under 50%, has replaced the regimen of only platinum-based chemotherapy in first line treatment⁶.

Beyond clinical and pathologic features, genetic variation is also considered a factor associated with treatment efficacy and prognosis⁷. Single-nucleotide polymorphisms (SNP), account for 90% of genetic polymorphisms, with some responsible for distinct molecular roles, contributing to inter-individual functional variability, correlating with relevant phenotypic variations in medicine⁸. The AGXT gene codes for the enzyme alanine glyoxylate aminotransferase, localized in hepatic peroxisomes, which is known to participate in glyoxylate detoxification⁹. Mutations in this gene have been reported to alter subcellular targeting and have been associated with type I primary hyperoxaluria¹⁰. A polymorphism in AGXT gene (rs34116584) is responsible for a C>T substitution at locus +32 that results in Pro-Leu substitution located at codon 11 of exon 1¹¹. The amino acid substitution at position 11 creates a conformational change that is related to decreased activity¹¹. The polymorphism AGXT rs34116584 was shown to be associated with progression-free survival (PFS) in patients with metastatic colorectal cancer in response to oxaliplatin¹². Here, we

sought to evaluate whether this genetic variant was associated with clinical outcomes in NSCLC patients, under the platinum-based chemotherapy regimen.

Material and methods

Population

This study comprises a retrospective cohort of histologically confirmed NSCLC patients (n = 168), which were recruited between August 2017 and October 2018 from Coimbra University Hospital. Subjects with concomitant primary tumour in another organ were excluded. Clinical information was retrieved from clinical charts on pathological background, medications, stage, Eastern Cooperative Oncology Group performance status (ECOG PS), tumour mutational status, type of cancer treatment and disease progression/death. Targeted therapies were administered to carriers of genetic alterations in EGFR and ALK, whereas checkpoint inhibitors were used as salvage therapy. Information on chemotherapy-related febrile neutropenia (grade 3–4) in patients admitted to hospital stay was retrieved from clinical charts. The primary endpoint was progression-free survival (PFS) and the time-to-disease progression was calculated in months from the date of first line chemotherapy until the date of progression according to RECIST criteria. Overall survival (OS) was included as secondary endpoint, and the time-to-death was computed in months from the date of first line chemotherapy until the date of death/date of last visit. The research was reviewed and approved by the Coimbra University Hospital's Ethical Committee (ref. 0111/CES) and by the Portuguese National Committee for data protection (number 2588/2017). Informed consent was obtained from each participant in agreement with the Helsinki Declaration.

AGXT genetic polymorphism and genotyping

The single nucleotide polymorphism included in the present study (AGXT rs34116584) was selected after reviewing public databases, *in silico* analysis and review of scientific literature to identify this functional polymorphism with minor allele frequency above 1%^{8,10,11}. Each patient donated a sample of blood (~8 mL) for research, collected to EDTA-Vacutainer tubes, at the same time of blood collection for routine analytic follow-up. The collected blood was separated into plasma and buffy coat and stored at –80 °C until further analysis. DNA was isolated and purified from diluted buffy coats, using EZ1 BioRobot and EZ1 DNA Blood kit (QIAGEN). AGXT rs34116584 was

genotyped using the Sequenom Mass ARRAY matrix-assisted laser desorption/ionization time-of-flight mass spectrometry platform (Sequenom, San Diego, CA, USA). Primers were designed using semi-automated Assay Design 3.1 Software (Sequenom).

Statistical analysis

Statistical analyses were performed on SPSS statistics software V.25.0 and *P* values below 0.05 were considered statistically significant. Continuous variables were depicted as average $\pm$ standard deviation or median (interquartile range) according to departure from normality using Shapiro-Wilk test. Additive (CC vs. CT vs. TT), recessive (CC/CT vs. TT) and dominant (CC vs. CT/TT) genetic models were stratified according to wild type allele C. The time-to-outcome for AGXT genotypes was tested using Kaplan-Meier curves and Log-rank test in univariate and Cox proportional hazard model for multivariate analyses. The univariate empirical analyses included AGXT genetic models as well as other clinicopathological covariates. A *p*-value <0.05 was used as criteria for inclusion of a clinical variable in the multivariate Cox regression analysis, whereas the genetic model to include was determined using the likelihood ratio. The estimates of sample size, power, and effect size (regression coefficient) for survival analyses that use Cox proportional hazards models were conducted using STATA 16.0. It also reports the number of events (failures) required to be observed in the study. Sample size and number of events were calculated assuming $\alpha=0.05$ and $\text{power}>0.8$. For both endpoints, the effect size was calculated from the resulting Hazard Ratio of AGXT variable in multivariate analysis. The minimal sample size for PFS was $n=62$ with an estimated number of events of $n=50$, whereas for OS, the calculated sample size was $n=173$ and the estimated number of events $n=77$.

Results

The clinicopathological characteristics of participating subjects are described in Table 1. The anatomical localization of distant metastases at diagnosis ($n=94$) was distributed as pleura and lung (62.8%), extra-thoracic (29.8%) and multiple (7.4%). Regarding mutational status, we observed that 8.3% of patients ($n=14$) had *EGFR* mutation (exon 19 deletions or exon 21 mutation), whereas 3.0% ($n=5$) had rearrangements in the gene encoding anaplastic lymphocyte kinase. Platinum-based doublet chemotherapy was administered to 85.7% of NSCLC patients, most frequently the cisplatin combination. Adjuvant chemotherapy was administered in twelve patients. In a subgroup of patients with chronic renal disease ($n=24$) the doublet chemotherapy with carboplatin was the first choice. Fifty-one patients underwent checkpoint inhibitors as second-, third- and fourth-line therapy. The median time-to-disease progression and the median time-to-death was 7.5 (CI 95%, 6.1–9.0) and 30.0 months (CI 95%, 16.9–43.2), respectively.

The AGXT rs34116584 genetic polymorphism distribution in this cohort of NSCLC patients was 71.7% C homozygous, 23.5% heterozygous and 4.8% T homozygous. Genotyping was successfully performed in 166 patients, with two miss-

Table 1 Clinical and oncological characteristics of the patients (N=168).

Clinical Variables	
Age, Mean $\pm$ SD	64.8 $\pm$ 10.7
Gender, N (%)	
Male	124 (73.8%)
Female	44 (26.2%)
Smoking history, N (%)	
No	31 (18.5%)
Smoker	13 (7.7%)
Previous smoker	68 (40.5%)
pTNM 8 th edition, N (%)	
IIIA	20 (11.9%)
IIIB	33 (19.6%)
IIIC	21 (12.5%)
IVA	65 (38.7%)
IVB	29 (17.3%)
ECOG performance status at diagnosis, N (%)	
0	39 (23.2%)
1	86 (51.2%)
2	39 (23.2%)
3	4 (2.4%)
4	0 (0%)
Histology, N (%)	
Adenocarcinoma	117 (69.9%)
Squamous cell carcinoma	42 (25.9%)
Adenosquamous	6 (3.6%)
Others	3 (1.8%)
First line systemic therapy, N (%)	
Platinum-based doublet chemotherapy	144 (85.7%)
Cisplatin	121 (84.0%)
Carboplatin	23 (16.0%)
Targeted therapy	24 (14.3%)

ing genotyping. The median time-to-endpoint, hazard and survival univariate analyses of the empirical statistical procedure are depicted in Table 2. In the dominant genetic model, there was a significantly shorter PFS for T-allele carriers [5.4 months (CI 95% 4.3–6.4) versus 9.4 (CI 95%, 7.2–11.7), $p<0.0001$] and a shorter OS [22.2 months (CI 95% 13.6–30.8) versus 43.6 months (20.3–66.9), $p=0.015$] (Fig. 1). Notably, despite the AGXT rs34116584 T-carriers had shorter PFS than CC homozygous both in the subset of mutated ($n=14$, $p=0.028$) and wild-type ($n=154$, $p<0.0001$) *EGFR*, those AGXT carriers only presented shorter OS in wild-type ($p=0.022$) but not for mutated *EGFR* ($p=0.692$). Additionally, in a subset of patients with information on PD-L1 expression ($n=98$, 33.7% without and 66.3% with PD-L1 expression $\geq 1\%$), Kaplan-Meier plots with Log-Rank tests showed that T-carriers had shorter time-to-progression independently of PD-L1 positivity ($p=0.010$ and $p=0.040$, respectively).

The statistically significant covariates from univariate analysis were included in a Cox proportional-hazards multivariate model. This data showed for AGXT T-carriers an increased risk for progression (HR=2.0; 95% CI, 1.4–3.0; $p<0.0001$) and for cancer-specific death (HR=1.8; 95% CI, 1.1–3.0; $p=0.017$), regardless of tumour size, distant metastasis at diagnosis, type of systemic therapy and type

Table 2 Univariate analyses of AGXT rs34116584 and clinical variables with time-to-progression and time-to-death.

	Progression-free survival			Overall survival	
	n	Median (95%CI)	P *	Median (95%CI)	P *
Age					
<65.4	86	7.2 (5.8-8.7)	0.541	43.6 (8.5-78.7)	0.078
>65.4	82	8.6 (5.1-12.1)		23.6 (15.3-31.8)	
Gender					
Male	124	7.2 (5.3-9.1)	0.547	28.1 (21.7-34.4)	0.102
Female	44	8.9 (5.5-12.3)		82.5 (15.1-150.0)	
Histology					
Adenocarcinoma	117	7.8 (5.7-9.9)	0.201	44.0 (18.6-69.4)	0.069
Squamous cell		5.7 (4.4-7.0)		24.6 (17.0-32.2)	
Others *	429	9.5 (3.4-15.7)		31.3 (19.3-43.4)	
T					
1	26	10.2 (4.1-16.3)	0.008	30.0 (14.1-46.0)	0.011
2	46	9.4 (4.0-14.9)		67.4 (46.7-88.2)	
3	18	4.7 (1.4-8.1)		26.9 (19.5-34.3)	
4	78	5.5 (4.0-7.1)		25.4 (16.7-34.2)	
N					
N0	13	9.0 (4.2-13.8)	0.151	-	0.790
N1	18	7.1 (2.3-12.0)		82.5 (22.9-142.1)	
N2	34	9.5 (2.8-16.2)		26.9 (18.5-35.3)	
N3	103	6.6 (4.6-8.6)		31.3 (12.9-50.0)	
M					
no	74	9.6 (5.2-14.0)	0.003	78.7 (53.0-104.4)	<0.0001
yes	94	5.4 (4.5-6.2)		22.2 (17.3-27.1)	
Type Therapy					
Surgery+CT	12	20.5 (0.0-49.2)	0.024	-	0.188
CT	125	6.6 (4.9-8.2)		26.9 (21.0-33.0)	
CT+RT	31	8.9 (4.9-12.8)		34.9 (7.2-62.5)	
ECOG PS					
Good (0-1)	125	8.0 (6.1-9.9)	0.171	44.0 (21.2-66.8)	<0.0001
Poor (2-4)	43	5.4 (2.7-8.1)		12.9 (9.8-16.0)	
Systemic Therapy					
Platinum based	144	6.2 (4.7-7.8)	0.005	28.1 (20.0-36.2)	0.183
Target therapy	24	13.3 (0.2-26.3)		-	
AGXT rs34116584					
Additive model					
CC	119	9.4 (7.2-11.7)	<0.0001	43.6 (20.3-66.9)	0.009
CT	39	5.7 (5.0-6.4)		17.8 (10.0-25.7)	
TT	8	4.0 (3.4-4.6)		24.6 (21.9-27.2)	
Dominant model					
CC	119	9.4 (7.2-11.7)	<0.0001	43.6 (20.3-66.9)	0.015
CT/TT	47	5.4 (4.3-6.4)		22.2 (13.6-30.8)	
Recessive model					
CC/CT	158	7.8 (6.3-9.2)	0.025	31.3 (16.7-45.9)	0.615
TT	8	4.0 (3.4-4.6)		24.6 (21.9-27.2)	

CT, chemotherapy; ECOG PS, ECOG performance status; OS, overall survival; PFS, progression-free survival; RT, radiotherapy. * Log-Rank test. ** others: pleomorphic, combined squamous and adenocarcinoma. 95%CI, 95% confidence interval.

of treatment modality (Table 3). To test the hypothesis that AGXT rs34116584 was associated with the response to platinum-based chemotherapy, the analysis was conducted in the group of patients treated with first line platinum-based doublet chemotherapy (n=144). In this subgroup, there were no identifiable actionable driver mutations at the diagnosis. Univariate analysis showed longer PFS in C homozygous (median 8.6, CI 95%, 6.1–11.1 months) in com-

parison with T-carriers (median 5.1, CI 95%, 4.2–6.0 months) ($p < 0.0001$) (Fig. 1). Concordantly, the time-to-death was also longer in CC (median 34.9, CI 95%, 12.1–57.6 months) compared to T-carriers (median 19.8, CI 95%, 8.9–30.7 months) ($p = 0.037$) (Fig. 1). On multivariate analysis T-carriers had higher risk for disease progression (HR = 2.0, 95% CI, 1.3–3.1, $p = 0.001$) independently of relevant clinico-pathological covariates. In platinum-treated patients, those

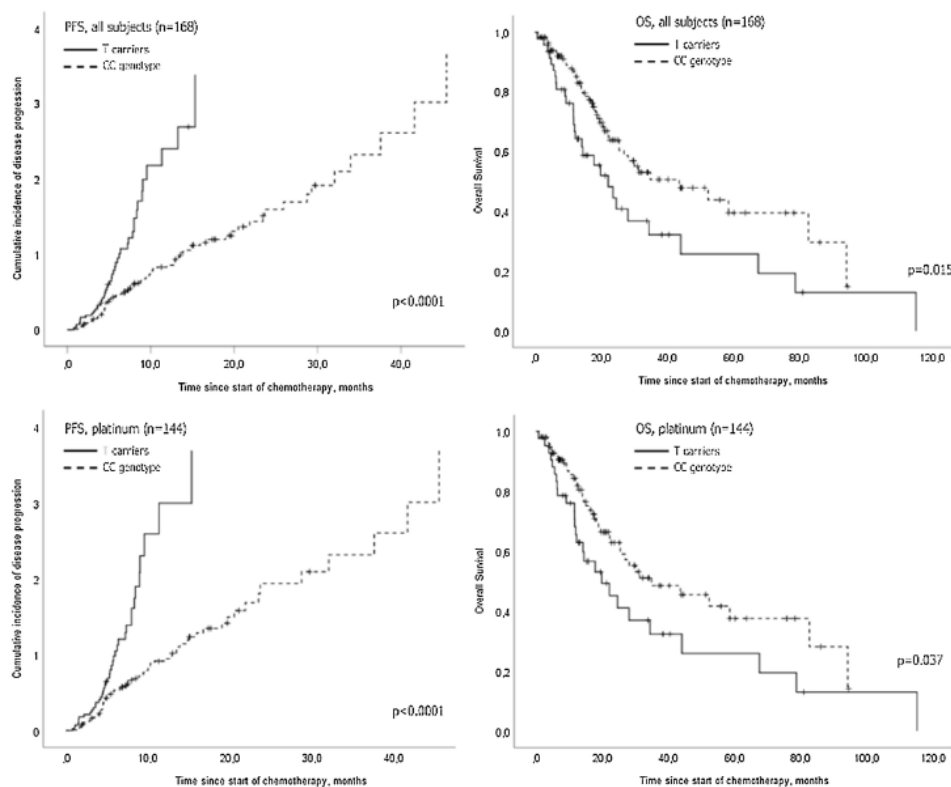


Fig. 1 Kaplan-Meier plots with Log-Rank tests for AGXT dominant genetic models in association with progression-free survival (PFS) and with overall survival (OS) for all NSCLC patients ($n = 168$) and those treated with platinum-based chemotherapy ($n = 144$).

with febrile neutropenia ($n = 20$) exhibited more frequently the T-allele compared to non-febrile neutropenia (35% versus 29%, respectively), despite the lack of association of the SNP with myelotoxicity (OR = 1.34, 95% CI, 0.49–3.64, $p = 0.566$).

Discussion

In the past, advances in genetic knowledge about lung cancer mutational landscape, together with development of targeted therapies, led to a paradigm shift in the treatment of NSCLC. Nevertheless, platinum-containing regimens remain the appropriate treatment for most patients¹³. Clinical management of resistance or toxicity to chemotherapy in NSCLC patients would benefit from the identification of predictive and prognostic molecular biomarkers, including functional genetic polymorphisms.

The AGXT gene, located in chromosome 2q37.3 region, encodes the alanine-glyoxylate aminotransferase, whose activity is largely confined to peroxisomes in the liver¹⁴. This enzyme catalyses the transamination between L-alanine and glyoxylate to produce pyruvate and glycine using pyridoxal 5'-phosphate as cofactor¹⁵. A missense genetic variant (AGXT rs34116584), with a proline-to-leucine substitution located at codon 11 of exon 1, occurs with a frequency

of 15–20% in European and North American population¹¹. This polymorphism was primarily studied in primary hyperoxaluria type I^{16–18}. A recent report explored its role in cancer, showing an association with disease progression and death in metastatic colon cancer patients treated with oxaliplatin¹². Reports are sparse concerning the association of this SNP with cancer and have never been explored in lung cancer patients.

Herein, the AGXT-rs34116584 genetic polymorphism was analysed in locally advanced/metastatic NSCLC patients, using as outcomes the PFS and OS. Multivariate analyses revealed an independent increased risk for disease progression and for death in AGXT rs34116584 T-carriers, after adjustment for tumour size, distant metastasis, ECOG PS, treatment modality or systemic therapy. Previous molecular *in vitro* studies showed that the C-to-T substitution results in an amino acid modification at position 11 and creates a conformational alteration that ultimately leads to a significant decrease in alanine-glyoxylate aminotransferase activity and subsequent accumulation of oxalate^{19,20}. Both oxalate and glyoxylate generate reactive oxygen species (ROS)^{21,22}, which have been associated with increased mutational burden, tumour progression and dissemination²³. Since T-allele carriers have higher levels of oxalate²⁴ and consequently are prone to increased ROS production, the

Table 3 Multivariate Cox regression including only the significant covariates after empirical analysis, for PFS and OS.

	Progression-free survival		Overall survival	
	HR (95%CI)	P	HR (95%CI)	P
cT (TNM)				
T1	Referent		Referent	
T2	1.6 (0.9-2.8)	0.131	0.6 (0.3-1.4)	0.278
T3	2.2 (1.1-4.6)	0.026	0.9 (0.4-2.2)	0.856
T4	2.1 (1.2-3.7)	0.007	1.6 (0.8-3.1)	0.159
Distant metastasis				
No	Referent		Referent	
Yes	1.6 (1.5-2.3)	0.010	2.1 (1.3-3.7)	0.005
Systemic Therapy				
Platinum	referent		-	
Target therapy	0.4 (0.2-0.8)	0.003	-	-
ECOG PS				
Good (0-1)	-		Referent	
Poor (2-4)	-	-	2.3 (1.4-3.7)	0.001
Type of therapy				
Surgery+CT	Referent		-	
CT	2.7 (1.1-6.7)	0.026	-	
CT+RT	2.8 (1.1-7.0)	0.027	-	-
AGXT rs34116584				
Dominant model				
CC	Referent		Referent	
CT/TT	2.0 (1.4-3.0)	<0.0001	1.8 (1.1-3.0)	0.017

CT, chemotherapy; ECOG PS, ECOG performance status; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; RT, radiotherapy; 95%CI, 95% confidence interval

worst prognosis described for TT/TC might be an oxidative stress-mediated deregulation induced by AGXT rs34116584 SNP. This effect might be exponentiated upon exposure to hypoxia and oxidative stress causing DNA damage, or during concomitant administration to cytotoxic therapies²⁵.

Furthermore, a significantly shorter time-to-disease progression was found for T-allele carriers independent of *EGFR* mutational status, although no relation was observed with OS for subjects with *EGFR* tumour mutation. These findings could be aligned with a minor clinical relevance for AGXT rs34116584 SNP in comparison to *EGFR* mutation status that impacts a longer-term endpoint. Notably, tyrosine kinase inhibitors (TKIs) improve survival in NSCLC patients with *EGFR* mutation²⁶, modifying the natural history of disease, and possibly impacting the association of the genetic polymorphism.

In patients under first line platinum-based doublets, we verified that T-allele carriers had shorter PFS and OS; regardless of tumour size, distant metastasis, ECOG PS and treatment modality. These well-established prognostic covariates, were shown to influence NSCLC clinical outcomes²⁷. Here, the AGXT rs34116584 association with response to platinum-based chemotherapy remained significant, despite adjustment for these factors, suggesting that this SNP might add significant information to traditional clinical predictive and prognostic factors. The AGXT rs34116584 C > T substitution, induces a decrease of alanine-glyoxylate aminotransferase activity and is responsible for the mistargeting of the enzyme from the peroxisomes to the mitochondria, where the enzyme cannot work properly¹⁰. These changes were predicted to have significant effects

in oxalate synthesis and excretion, and the deposition of insoluble calcium oxalate in the kidney and urinary tract²⁸, which could be associated with increased toxicity and lesser efficacy of platinum based chemotherapy.

Moreover, cisplatin causes a number of significant side effects including nausea and vomiting, neutropenia, ototoxicity, neurotoxicity, and renal function impairment²⁹. Despite efforts to identify genetic predictors of the effectiveness and toxicity of cytotoxic therapies, up to now there are no robust data that can be used in clinical practice to guide the best subgroup of patients to receive cisplatin²⁹. Although carboplatin induces nephrotoxicity to a lesser extent, it induces more myelotoxicity³⁰. No association was found in our study for the AGXT rs34116584 SNP with febrile neutropenia, although the low number of subjects included in this analysis limits its conclusions.

To the best of our knowledge, this is the first report describing the prognostic impact of functional AGXT polymorphism in lung cancer patients. As such, further studies in larger independent populations are required to confirm these results. Despite inherent size limitations, in this study patients were recruited from a homogeneous cohort, the analysed SNP was selected based on functional biological relevance, and the study design and statistics accounted for important risk factors in NSCLC.

Conclusion

The functional impact of the AGXT rs34116584 SNP in decreasing the peroxisomal activity of the enzyme alanine

glyoxylate aminotransferase influence oxalate accumulation. This effect might have an influence in platinum metabolism, with impact on toxicity and tumour aggressiveness, being associated with worse prognosis. This polymorphism seems to have an impact on NSCLC progression, opening new perspectives for its inclusion as a biomarker or as a pharmacogenetic predictor of response to platinum-based chemotherapy.

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Ethics approval

This project has been reviewed and approved by Coimbra University Hospital's Ethical Committee (reference number 0111/CES; date of approval: 27th July 2017) and was also approved by the National Committee for data protection (number 2588/2017; date of approval: 6th March 2017).

Conflicts of interest

All authors declare that they have conflict of interest.

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