# Contribution of the integrative pathology to the discovery of biomarkers for non-small cell lung cancer : integrative pathology and lung cancer

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# T H E S E

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## APPORT DE LA PATHOLOGIE INTEGRATIVE DANS L'IDENTIFICATION DE BIOMARQUEURS DANS LES CARCINOMES PULMONAIRES NON A PETITES CELLULES

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## CONTRIBUTION OF THE INTEGRATIVE PATHOLOGY TO THE DISCOVERY OF BIOMARKERS FOR NON-SMALL CELL LUNG CANCER

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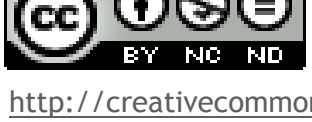

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“To see far is one thing, going there is another.”

Constantin Brancusi


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# RESUME

Le concept de la **pathologie dite « intégrative »** dans le domaine de l'oncologie est centré sur la recherche pré clinique et/ou sur la recherche translationnelle, assurant le lien entre la recherche biologique cognitive et la recherche clinique, pour une mise en œuvre des connaissances les plus récentes de la recherche à la pratique médicale, dans le cadre de l'offre de soins aux patients. S'appuyant sur les connaissances physiopathologiques elle fait ainsi le lien entre l'identification de cibles pertinentes et la sélection de patients atteints de cancer, afin de générer un modèle cohérent pour une compréhension précise du processus néoplasique et une prédiction reproductible de son évolution et sa réponse aux différentes options thérapeutiques. Ces données sont fournies par l'identification et l'étude de «biomarqueurs» tissulaires, cellulaires, et/ou présents dans les liquides biologiques, en particulier le sang.

Notre modèle d'étude est représenté par le cancer pulmonaire non à petites cellules (CNPC). En effet, il s'agit de la première cause de décès par cancer dans le monde. Ce cancer est souvent découvert tardivement, il est agressif, récidivant rapidement, donnant naissance à des métastases et il est chimio-résistant. La découverte de biomarqueurs fiables et validés pourraient représenter une percée majeure pour la prise en charge de ces patients, en facilitant le diagnostic, le pronostic et orienter vers le choix du traitement le plus approprié.

C'est dans ce contexte que nous avons exploré plusieurs aspects liés à la carcinogenèse et à la progression tumorale dans le but d'identifier de nouveaux biomarqueurs dans les CNPC. Premièrement, nous avons démontré un impact antagoniste sur l'évolution des CNPC des anhydrases carboniques IX (CAIX) et XII (CAXII), induites par l'hypoxie caractérisant le microenvironnement tumoral. Nous avons démontré qu'une forte expression tissulaire et plasmatique de CAIX était un biomarqueur de mauvais pronostic et de récidive précoce chez les patients présentant un CNPC, tandis que l'expression tissulaire de CAXII était prédictive d'une évolution plus favorable. De plus, nous avons montré sur un modèle cellulaire *in vitro*, que la réoxygénation dynamique des tumeurs serait à l'origine de cet antagonisme. Deuxièmement, la composante cellulaire du microenvironnement tumoral a été étudiée et plus particulièrement nous avons démontré qu'un recrutement par les cellules tumorales des neutrophiles exprimant un marqueur spécifique CD66b pourrait avoir un signal positif au cours de la progression tumorale des CNPC. Troisièmement, le compartiment sanguin a été évalué en relation avec l'évolution des tumeurs et plus particulièrement nous avons démontré la valeur pronostique des cellules tumorales circulantes (CTCs) chez les patients atteints d'un CNPC. Les CTC semblent représenter également le support idéal pour la mise en évidence de biomarqueurs théranostiques dans les CNPC.

En conclusion, les travaux présentés dans ce travail de thèse explorent les possibilités et les limites de l'identification de biomarqueurs, en soulignant aussi l'importance de la qualité des échantillons et des données cliniques associées afin d'obtenir des résultats pouvant être reproductibles. Nos travaux explorent les possibilités et les limites de l'identification de biomarqueurs dans les CNPC, en soulignant l'importance de l'intégration de la biopathologie dans le domaine de la recherche translationnelle, grâce à la combinaison des connaissances intégrées et des innovations technologiques.

# ABSTRACT

The concept of the so called “Integrative pathology” represents a bridge between basic and clinical research, for the implementation of knowledge from research to medical practice. Relying on the pathophysiological knowledge it is the link between the identification of relevant targets and selection of cancer patients, in order to generate a consistent model for a reliable understanding of cancer and a reproducible prediction of the evolution and response to various treatment options. These data are provided by the identification and study of "biomarkers".

Our study model was the non-small cell lung cancer (NSCLC), which is the leading cause of cancer-related deaths in the world. Reliable and validated biomarkers could represent a possible breakthrough in the management of this tumour type, by facilitating diagnosis, refining prognosis and providing guidance towards the choice of the most appropriate therapy.

In this thesis we approach the field of NSCLC biomarkers by exploring several aspects related to carcinogenesis and tumour progression. First, we have demonstrated a dual impact of two proteins activated by hypoxia, carbonic anhydrases IX and XII, on the outcome of NSCLC patients. We have demonstrated that high tissue and plasma CAIX expression may be a biomarker of poor prognosis and early relapse in NSCLC patients, whereas the CAXII tissue expression would be predictive of a favourable evolution. In addition, we have shown that dynamic reoxygenation of tumours might be at the origin of this antagonism. Furthermore, the cellular component of the tumour microenvironment has been studied and more particularly we have demonstrated that recruitment by the tumour cells of a subpopulation of neutrophils expressing a specific marker CD66b could have a positive signal during tumour progression of NSCLC. Thirdly, the blood compartment has been evaluated in relationship with the evolution of tumours and in particular we have demonstrated the major diagnostic and prognostic value of circulating tumour cells (CTCs) in patients with NSCLC. Finally, the CTC seem to represent the ideal tool for the detection of biomarkers theranostic in NSCLC.

In conclusion, the works presented in this thesis explore possibilities and limitations of biomarker research in NSCLC, highlighting the importance of implementing biopathology in the translational research field, through the combination of integrated knowledge and technological innovations.

## ABBREVIATIONS

| | |
|---|---|
| AKT/PKB | = Protein kinase B |
| ADC | = Adenocarcinoma |
| ALK | = Anaplastic lymphoma kinase |
| CAIX | = Carbonic anhydrase IX |
| CAXII | = Carbonic anhydrase XII |
| CNHC | = Circulating non haematological cell |
| CT | = Computed tomography |
| CTC | = Circulating tumour cells |
| DNA | = Deoxyribonucleic acid |
| EGFR | = Epidermal growth factor receptor |
| ELCAP | = Early Lung Cancer Action Program |
| EMEA | = European Medicines Agency |
| FDA | = Food and Drug Administration |
| FISH | = Fluorescent *in situ* hybridization |
| FFPE | = Formalin-fixed paraffin-embedded |
| HRE | = Hypoxia responsive element |
| IASLC | = International Association for the Study of Lung Cancer |
| IHC | = Immunohistochemistry |
| LCC | = Large cell carcinoma |
| MDSCs | = Myeloid derived suppressor cells |
| miRNA | = microRNA |
| NLST | = National Lung Screening Trial |
| NPV | = Negative predictive value |
| NSCLC | = Non-small cell lung cancer |
| PET | = Positron emission tomography |
| PPV | = Positive predictive value |
| ROC | = Receiver operating characteristic |
| RT-PCR | = Real time-polymerase chain reaction |
| SCC | = Squamous cell carcinoma |
| SCLC | = Small cell lung cancer |
| SBRT | = Stereotactic body radiotherapy |
| SEER | = Surveillance, Epidemiology, and End Results |
| UICC | = International Union against Cancer |
| TAN | = Tumour-associated neutrophils |
| TKI | = Tyrosine kinase inhibitor |
| TMA | = Tissue microarray |
| TNM | = Tumour Node Metastasis |

# BREVE INTRODUCTION

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## BREVE INTRODUCTION

Le cancer du poumon est la première cause de décès par cancer en France et dans le monde. Chez la femme, il est en progression constante, notamment en France: son incidence a triplé ces 20 dernières années, le tabac étant de loin le premier facteur de risque, alors qu'on note une décroissance de l'incidence chez l'homme (Source "Institut National du Cancer"; http://lesdonnees.e-cancer.fr/). Avec environ 37,000 nouveaux cas estimés en 2010 dont 73% survenant chez l'homme, le cancer du poumon se situe au quatrième rang des cancers, tous sexes confondus. Pour les hommes, le cancer bronchique occupe le 2ème rang en termes d'incidence annuelle et le 3ème pour les femmes (Globocan 2008 http://globocan.iarc.fr/) (Jemal et al., 2011). Le taux de survie relative à 5 ans du cancer du poumon reste inférieur à 15%, ce qui le place au premier rang des cancers les plus agressifs (Jemal et al., 2011). La survie à 5 ans est étroitement liée au stade auquel le cancer broncho-pulmonaire est diagnostiqué. En effet, 50% des patients possédant une tumeur pulmonaire localisée, et donc opérable, sont en vie 5 ans après le diagnostic. Inversement, pour une tumeur métastatique (Stade IV), moins de 3% des patients seront en vie 5 années après le diagnostic (Blanchon et al., 2006).

Différentes classifications existent pour caractériser les cancers pulmonaires. On distingue la classification histologique de la classification pathologique (pTNM). Dans tous les cas, de telles classifications servent à préciser le pronostic, à adapter la thérapeutique à la clinique, ainsi qu'à comparer les résultats thérapeutiques entre groupes de malades le plus homogènes possibles.

La classification anatomopathologique sert à caractériser les différents cancers pulmonaires selon des critères histologiques relativement simples, de manière à être reproductible et donc utilisable par tous les pathologistes. C'est le seul examen indispensable au diagnostic de cancer, il conditionne le traitement du patient, et sert également de base aux études biologiques et épidémiologiques. Selon la classification internationale proposée par l'OMS (www.who.int), environ 85% des cancers du poumon peuvent être rassemblés sous le terme de «carcinomes pulmonaires non à petites cellules» (CNPC) par opposition aux «carcinomes à petites cellules » (CPC) qui représentent 15 à 20 % des cancers du poumon (Travis WD, 2004). Cette distinction est nécessaire, ces cancers ayant des caractéristiques différentes (temps de doublement cellulaire, histologie, phénotype, fréquence des métastases, sensibilité à la chimiothérapie et à la radiothérapie). Ainsi, les CPNPC ont un temps de doublement tumoral plus lent de l'ordre de quelques mois, sont peu sensibles aux cytotoxiques, le traitement curatif reposant uniquement sur la chirurgie (Travis WD, 2004). La famille des CNPC contient plusieurs sous-types histologiques différents: les carcinomes épidermoïdes, les adénocarcinomes et les carcinomes à grandes cellules, ou plus généralement, toute autre entité ne présentant pas de caractéristiques "à petites cellules". Les adénocarcinomes bronchiques représentent le type histologique le plus fréquemment diagnostiqué, soit 40% des cancers bronchiques. Son incidence croissante en Europe correspond à l'augmentation du tabagisme. Il s'agit de la forme histologique la plus fréquente de l'homme jeune (moins de 50 ans), de la femme de tout âge et du non-fumeur. Un changement important dans la classification anatomopathologique du cancer du poumon a eu lieu avec la publication en 2011 d'une nouvelle classification des

adénocarcinomes pulmonaires sous le parrainage du Centre International de Recherche sur le Cancer (CIRC), et de la Société Européenne des Maladies Respiratoires (Travis et al., 2011a; Travis et al., 2011b). La classification pathologique TNM (pTNM) représente la classification d'extension tumorale, qui intervient après le diagnostic histologique et sert de référence pour le clinicien pour adapter la thérapeutique, constituant la base pour toute étude épidémiologique (Mountain, 1997). Elle permet d'apprécier le stade histopathologique d'extension d'une tumeur après les examens macroscopiques et microscopiques de la pièce de résection par le pathologiste. Récemment, le CIRC a publié une proposition visant à mettre à jour cette classification (Goldstraw, 2009). La proposition a été acceptée par l'Union internationale contre le Cancer et incluse dans la 7ème édition de la classification TNM des tumeurs (Travis et al., 2011b) (Figure 1). Une nouvelle classification histologique modifiée est en cours d'élaboration par un panel de pathologistes internationaux et devra voir le jour en 2014.

**Caractéristiques moléculaires des CNPC**

La transformation d'une cellule épithéliale bronchique normale en cellule cancéreuse résulte de la rupture de l'équilibre entre l'expression de gènes "prolifératifs", favorisant le déroulement de la division ou de la différenciation cellulaire (proto-oncogènes), et de gènes "antiprolifératifs" (gènes suppresseurs de tumeurs), freinant le cycle cellulaire ou induisant la mort cellulaire programmée (apoptose). Une accumulation séquentielle, sur une période relativement longue, d'anomalies génétiques et épigénétiques dérégule ainsi l'homéostasie de la cellule épithéliale de l'arbre respiratoire. Ces anomalies se traduisent par des mutations ponctuelles à l'échelle nucléotidique, ou par des anomalies qualitatives (réarrangements, délétions, amplification génique) ou quantitatives (aneuploïdie) à l'échelle chromosomique. Le concept de carcinogenèse par étapes successives (carcinogenèse bronchique multi-étapes) s'illustre ainsi parfaitement dans le cancer du poumon. En effet, les études histologiques ont révélé un véritable continuum lésionnel au niveau de l'épithélium bronchique, allant de l'hyperplasie basale au carcinome invasif, en passant par des stades de métaplasie malpighienne, de dysplasies légères à sévères, puis de carcinome *in situ* (Weinstein, 2002).

Le modèle de carcinogenèse bronchique correspond au modèle de carcinogenèse générale décrit par *Hanahan et Weinberg* selon lequel les anomalies génétiques accumulées sont responsables de l'acquisition par la cellule bronchique normale, des six propriétés élémentaires qui vont en faire une cellule tumorale (Hanahan and Weinberg, 2011):

- L'indépendance vis-à-vis des facteurs de croissance (ou «autosuffisance») qui permet aux cellules de rester dans un état de prolifération active et soutenue,
- L'insensibilité vis-à-vis des signaux inhibiteurs de croissance
- La capacité d'échapper à la mort cellulaire programmée ou apoptose
- L'acquisition d'un potentiel réplicatif illimité par division illimitée des chromosomes (échappement à la sénescence réplicative)
- La capacité à induire un réseau de néovascularisation (pour les apports de nutriments et d'oxygène)
- L'acquisition d'un phénotype "mobile" et invasif, donnant à la cellule tumorale la capacité de s'implanter à distance de son tissu d'origine (pouvoir métastatique).

Au cours de la dernière décennie, d'importants progrès thérapeutiques ont été réalisés avec le développement et l'application de nouvelles thérapies ciblant des voies de signalisation impliquées dans la croissance et la survie des cellules cancéreuses (thérapies ciblées) et adaptées en fonction des caractéristiques morphologiques et moléculaires de la tumeur (thérapies personnalisées). Un nombre croissant d'agents ciblant ces voies de signalisation est actuellement disponible ou en cours de développement. Il s'agit généralement de petites molécules (par exemple : inhibiteurs de tyrosine kinase (TKI) : géfitinib, erlotinib et afatinib pour l'*EGFR* ; crizotinib pour *ALK-EML4*) agissant à «l'intérieur» de la cellule, ou d'anticorps monoclonaux (par exemple : cétuximab pour l'*EGFR* et bévacizumab pour le VEGF), agissant à «l'extérieur» de la cellule. L'efficacité de ces thérapies ciblées dépend directement de l'état d'activation/inactivation de différentes voies de signalisation (et de leurs diverses molécules) dans les cellules tumorales ainsi que de l'affinité du médicament avec sa cible, influencés par la présence de certaines altérations génomiques (Ilie and Hofman, 2012; Janku et al., 2011).

Le gène *EGFR*, situé sur le bras court du chromosome 7 (7p11.2), comporte 28 exons et code pour une protéine transmembranaire comportant un site extra-membranaire (récepteur) et un domaine cytoplasmique avec une activité tyrosine kinase. L'EGFR fait partie de la famille de récepteurs tyrosine kinase HER/erbB comprenant quatre protéines (EGFR/HER-1, HER-2/neu, HER-3, HER-4) avec une structure moléculaire similaire. La liaison du ligand avec l'EGFR entraîne une activation du système tyrosine kinase et une transduction du signal en aval contrôlant la prolifération, l'apoptose, l'angiogenèse, l'invasion tumorale et le phénomène métastatique. Les mutations de l'*EGFR* sont présentes dans environ 10% des CNPC. Elles sont plus fréquentes dans les adénocarcinomes (15-20%), en particulier chez les patients de sexe féminin, non-fumeurs et d'origine asiatique (environ 40-50%). Elles sont associées à un meilleur pronostic (survie globale de 37 mois). Il s'agit majoritairement de mutations des exons 18-21 avec dans 90% des cas des délétions de l'exon 19 (associées à 70-100% de réponses aux TKI) ou des mutations ponctuelles dans l'exon 21 (associées à 20-70% de réponses aux TKI). Les cellules cancéreuses avec ces mutations de l'EGFR ont une sensibilité augmentée aux TKI, d'une part, parce que leur survie dépend de cette voie de signalisation et, d'autre part, parce que les TKI ont vraisemblablement une plus grande affinité avec l'*EGFR* muté. L'évaluation de l'efficacité potentielle des traitements anti-**EGFR** chez des patients avec un CNPC est malheureusement compliquée du fait qu'il existe de nombreuses mutations différentes de l'EGFR. Certaines mutations de l'*EGFR* comme T790M (primaire ou secondaire), en diminuant l'affinité du médicament avec l'EGFR, prédisent à l'inverse une résistance à la thérapie. Par ailleurs, une résistance acquise s'observe chez les patients après traitement par des anti-*EGFR* de type TKI, due au développement de nouvelles mutations (par exemple : T790M) (Kosaka et al., 2011). Des anti-*EGFR* de deuxième génération sont en cours d'évaluation clinique pour ces patients (Ilie and Hofman, 2012; Janku et al., 2011).

KRAS est une GTPase qui se trouve en aval de la voie de signalisation d'EGFR (Figure 2). Bien qu'il s'agisse de la mutation la plus fréquente (25-30%), il n'existe pour l'instant pas de thérapie ciblée ciblant ce gène. Typiquement chez les patients fumeurs, elle est associée à une résistance aux anti-

*EGFR* et à un mauvais pronostic (survie globale de quinze mois) (Custodio et al., 2012). Les mutations du gène *BRAF* codant pour une kinase située immédiatement en aval de KRAS dans la voie de signalisation, se retrouvent dans environ 3% des adénocarcinomes avec *EGFR* et *KRAS* non mutés. Elles sont aussi associées à une résistance aux anti-*EGFR* (Paik et al., 2011). Des inhibiteurs sélectifs de *BRAF* sont en cours d'évaluation pour leur efficacité thérapeutique. Le gène de fusion *ALK-EML4* (Anaplastic Lymphoma receptor tyrosine Kinase-Echinoderm microtubule-associated protein-like 4) sur le chromosome 2p est présent dans environ 3-7% des adénocarcinomes (Soda et al., 2007). Ces derniers ne répondant pas non plus aux TKI, ont démontré une réponse spectaculaire suite au traitement par un inhibiteur sélectif des récepteurs tyrosine kinase ALK et MET/HGF et leurs variantes oncogéniques (crizotinib) (Kwak et al., 2010).

A l'ère des thérapies ciblées, un diagnostic générique de CNPC est devenu très insuffisant pour la prise en charge thérapeutique et le pathologiste doit pouvoir, si possible, s'orienter entre l'adénocarcinome et le carcinome épidermoïde. En effet, ce dernier n'est pas éligible pour une thérapie ciblée anti-*EGFR* et anti-VEGF (bévacizumab) comme l'adénocarcinome ; il existe sous traitement par bévacizumab, un risque d'hémorragie pulmonaire fatale chez les patients avec un carcinome épidermoïde (souvent central et massif). L'immunocytochimie à l'aide de plusieurs anticorps (par exemple : TTF-1, p63, cytokératines 7 et 5/6), utilisée de manière complémentaire sur des frottis cytologiques déjà colorés et/ou sur des cytoblocs, permet dans la majorité des cas de faire pencher la balance d'un côté ou de l'autre lorsque la morphologie du CNPC est ambiguë (Faratian et al., 2009; Govindan et al., 2012; Imielinski et al., 2012). Les adénocarcinomes pulmonaires sont typiquement positifs pour TTF-1 et les cytokératines 7 et sont négatifs pour p63 et les cytokératines 5/6. Les carcinomes épidermoïdes ont typiquement le profil inverse. Néanmoins, cette analyse réalisée à partir d'un nombre limité de cellules tumorales soulève le problème de la représentativité et de l'hétérogénéité tumorale sur le plan morphologique mais également moléculaire (par exemple : mutations). Les carcinomes adénosquameux (au moins 10% de l'une des deux composantes en histologie) sont plus rares. Une fois le diagnostic d'adénocarcinome (ou de CNPC compatible avec un adénocarcinome) posé, la réalisation de tests moléculaires (*EGFR, KRAS*, autres) peut être alors entreprise (Faratian et al., 2009; Govindan et al., 2012; Imielinski et al., 2012).

**Pathologie intégrative / Biopathologie**

La «Pathologie intégrative» (ou «Biopathologie»), nouveau champ biomédical, a été créé par l'intégration de données moléculaires à la morphologie en pathologie clinique. Ces données sont apportées par l'identification et l'étude de «biomarqueurs tissulaires et/ou liquides», c'est-à-dire de paramètres biologiques objectifs issus de fragments tissulaires ou de biofluides (sains ou tumoraux) et présentant un intérêt diagnostique, pronostique ou thérapeutique. Ces biomarqueurs sont de nature très diverse. Ils peuvent être identifiés à partir de l'étude des chromosomes (ploïdie, anomalies chromosomiques, anomalies de copies d'ADN en CGH), du génome (mutation, variation, amplification génique, perte d'hétérozygotie), des ARNs (transcrit de fusion, profil d'expression, profil d'épissage, microARNs) ou des protéines tissulaires (protéine anormale, profil protéique,

immunohistochimie). Les biomarqueurs tissulaires ou plasmatiques peuvent avoir une vocation diagnostique (transcrits de fusion) ou pronostique (amplification du NMYC). Le plus grand intérêt de la pathologie intégrative est la désignation de cibles moléculaires ou le guidage des thérapeutiques ciblées (marqueurs «théranostique») (Bhatt et al., 2010; Biomarkers Definitions Working Group, 2001). Une contrainte majeure reste toutefois l'extraction des acides nucléiques et des protéines des prélèvements reçus et stockés dans les laboratoires de biopathologie. Cependant, de nouvelles technologies permettent maintenant d'étudier ces biomolécules à partir d'un matériel très peu abondant (cellules isolées, cellules tumorales circulantes, frottis cytologiques, ponction à l'aiguille fine) ou à partir de fragments tissulaires fixés en formol et inclus en paraffine, et ceci malgré leur dégradation partielle. Il reste néanmoins d'importants obstacles à la découverte de nouveaux biomarqueurs, comme l'hétérogénéité tumorale ou la plasticité du génome tumoral et de la régulation de son expression (Ilie and Hofman, 2012).

L'enjeu actuel pour la prise en charge d'un CPNPC, en particulier de stade précoce, est donc de mettre en évidence des facteurs clinico-biologiques de mauvais pronostic, qui pourrait permettre de sélectionner rapidement les patients devant bénéficier d'un traitement complémentaire post chirurgical (ajduvant) ou préopératoire (néoadjuvant).

La classification pTNM, l'âge et le «performance status» des patients sont actuellement les facteurs pronostiques reconnus qui conditionnent la prise en charge thérapeutique des CPNPC. Cependant, ces facteurs pronostiques «classiques» ne sont pas suffisants pour évaluer le potentiel agressif et évolutif d'une tumeur donnée. Les résultats obtenus à ce jour dans le traitement du cancer du poumon par les nouvelles thérapies ciblées, telles que les inhibiteurs de l'*EGFR*, restent décevants à moyen et à long terme et il est nécessaire d'établir de nouveaux facteurs prédictifs à une réponse thérapeutique. Il n'existe pas en fait de biomarqueur pronostic tissulaire ou plasmatique ayant fait la preuve d'être utilisable en biopathologie et en oncologie thoracique.

Ainsi, le dépistage précoce des formes débutantes et de petite taille, d'une façon simple, rapide, fiable et peu coûteuse est une approche cruciale dans la prise en charge des CPNPC.

**Il est de ce fait important de trouver des «biomarqueurs» plasmatiques et/ou tissulaires nouveaux permettant de cibler une population atteinte de cancer pulmonaire susceptible de bénéficier d'investigations plus agressives, d'une surveillance plus importante et surtout d'un traitement curatif précoce.**

**Ainsi, l'objectif de ce travail de thèse a été d'étudier le potentiel diagnostique, pronostique et théranostique de certains aspects relatifs à la carcinogenèse broncho-pulmonaire et à la progression tumorale, en particulier, les anhydrases carboniques CAIX et CAXII, les cellules tumorales circulantes et les neutrophiles intratumoraux.**

## Biomarqueurs de l'hypoxie tumorale

L'expression élevée de HIF-1α (facteur de transcription de l'hypoxie) est impliquée dans la résistance à la radiothérapie, à la chimiothérapie et dans la sélection de variantes métastatiques (Wykoff et al., 2000). L'anhydrase carbonique (CA)IX est un gène induit exclusivement par HIF-1α dans les cultures cellulaires ainsi que dans les modèles tumoraux. Il existe 25 isoformes de CA chez l'homme, mais CA IX se localise au niveau membranaire, présentant son domaine catalytique à l'extérieur de la cellule (Pouysségur et al., 2006). L'acidification du milieu extracellulaire est alors associée à la recapture de $HCO_3^-$, et le pH intracellulaire devient plus alcalin avec la CAIX. Cet effet induit le maintien d'un niveau suffisant d'ATP et la CAIX permet ainsi la survie cellulaire dans un microenvironnement hostile par la sélection de différents clones tumoraux via l'hypoxie (Chiche et al., 2010). Une autre isoforme de la famille des anhydrase carboniques située elle aussi à la membrane plasmique est CAXII. Cette isoforme serait également activée par l'hypoxie (Pouysségur J. et al. 2006). Cependant, l'impact de CAIX et de CAXII sur la survie des patients atteints de CNPC a été peu ou pas évalué.

Les études immunohistochimiques réalisées à l'aide d'anticorps polyclonaux dirigés contre CAIX sont nombreuses et montrent que cette protéine est surexprimée dans de nombreux cancers chez l'homme. Une corrélation significative entre la quantité de protéine CAIX et la mortalité a été ainsi signalée dans plusieurs cancers : cancer du rein, du sein, et colorectal (Trastour C. et al., 2007; Korkeila E. et al., 2009). Peu d'études ont analysé à ce jour l'impact pronostique de l'expression de CAIX et de CAXII dans les CNPC broncho-pulmonaires. Ces travaux présentent des limites, en particulier sur les données cliniques précises des cohortes analysées: pas de précision sur le bilan préopératoire, les modalités de la chirurgie, le nombre et la nature des récidives, les causes de décès. Il existe également une forme soluble de CAIX de 50/54 kDa, qui peut être «relarguée» dans le milieu de culture cellulaire ou encore dans le plasma des patients (Zavada et al., 2003). Ce phénomène de «relarguage» dans la circulation du domaine extracellulaire de CAIX, encore appelé «shedding», est dépendant du clivage protéolytique par l'enzyme de conversion du TNF alpha (ADAM17/TACE) (Zatovicova et al., 2005). L'activité et l'expression de la CAIX sont aussi fortement induites en condition hypoxique via le motif HRE (hypoxia responsive element) du promoteur du gène *TACE*. Ainsi, le clivage protéolytique de ses substrats devient plus marqué en conditions hypoxiques et pourrait induire une agressivité tumorale accrue (Hyrsl et al., 2009; Zavada et al., 2003).

Notre objectif principal au cours de notre travail a été d'analyser l'expression protéique de CAIX et de CAXII dans une large cohorte de patients opérés pour un carcinome broncho-pulmonaire non à petites cellules à différents stade pTNM et de corréler le niveau d'expression de ces deux protéines à la survie globale et à la survie spécifique. Le niveau d'expression de la CAIX recherché dans le plasma des patients par une technique ELISA a été également corrélé au stade pTNM et au suivi évolutif de ces patients (survie globale et survie spécifique). Une deuxième approche *in vitro* a été

réalisée sur des lignées carcinomateuses d'adénocarcinome pulmonaire afin d'analyser l'effet de la réoxygénation dynamique sur l'expression de CAIX et CAXII en relation avec le cycle cellulaire.

## Cellules tumorales circulantes

La détection et la caractérisation des cellules tumorales circulantes (CTC) pourraient participer idéalement au diagnostic précoce des carcinomes pulmonaires, principalement des CNPC.

Cette mise en évidence pourrait également permettre d'évaluer le pronostic de ces cancers, mais aussi, en analysant les altérations génétiques de ces cellules, de pouvoir prédire la réponse tumorale aux thérapeutiques ciblées et personnalisées (Hofman et al., 2011a; Liu et al., 2011; Maheswaran et al., 2008; Krebs et al., 2012; Krebs et al., 2011). Cependant, comme pour les autres pathologies cancéreuses, se pose encore la question d'utiliser la technique idéale pour mettre en évidence les CTC, affirmer leur potentiel métastatique et définir des biomarqueurs présents dans ces cellules afin de mieux les identifier. Ainsi, depuis plusieurs années, nombreuses sont les méthodes développées pour essayer de répondre à ces questions (Parkinson et al., 2012). En oncologie thoracique, ces méthodes ont été ou sont utilisées, soit en phase préopératoire pour des patients dont la tumeur sera a priori totalement enlevée, soit en phase postopératoire dans le but de corréler la présence des CTC à des facteurs pronostiques (épisode de récidive, d'évolution métastatique, survie globale et survie spécifique). Les CTC peuvent aussi être recherchées chez des patients non opérables en phase métastatique d'un CNPC dans le but de suivre l'effet des différentes stratégies thérapeutiques. La détection de CTC pourrait aussi être «prometteuse» dans deux autres situations cliniques : en phase préopératoire de CNPC de stades I-II et la présence de CTC conduirait alors à instaurer un traitement néoadjuvant; en phase postopératoire (bien qu'il faille alors déterminer à quelle période cette détection est utile) et la présence de CTC pourrait alors conduire à administrer un traitement adjuvant, même en cas de résection complète. Enfin, bien que n'ayant pas ou peu actuellement été réalisée dans ce sens, la mise en évidence de CTC dans des situations pré-néoplasiques (bronchopneumopathie chronique obstructive) et/ou chez des sujets à haut risque de développer un cancer du poumon (forte exposition tabagique) aurait potentiellement un intérêt majeur dans le cadre de campagnes de dépistage très précoce de cancer du poumon.

Les méthodes utilisées pour la détection des CTC chez des patients ayant un carcinome pulmonaire font appel, soit à des techniques indirectes, soit plus rarement à des techniques directes (Parkinson et al., 2012). Il est certain que chacune de ces techniques présente des avantages et des inconvénients et que la meilleure technique serait celle qui apporterait le moins de faux positif et le moins de faux négatif. Cette technique idéale n'existe probablement pas à l'heure actuelle. Ainsi, parmi les méthodes indirectes les plus utilisées, la méthode CellSearch présente l'inconvénient de ne pas détecter les CTC qui présentent une transition épithélio-mésenchymateuse complète (Parkinson et al., 2012). Il est aussi démontré, à l'inverse, que certains macrophages peuvent exprimer des cytokératines, et qu'un certain nombre de cellules épithéliales non

néoplasiques peuvent circuler, comptabilisées alors à tort comme des «CTC». Parmi les méthodes directes, la méthode d'isolement des cellules épithéliales tumorales par des critères de taille et par filtration sanguine sur un filtre muni de pores, ou méthode ISET (Isolation by Size of Epithelial Tumor cell), est une méthode intéressante (Parkinson et al., 2012). Elle peut permettre une confrontation cytomorphologique et phénotypique. Elle présente aussi des inconvénients, comme la nécessité de devoir filtrer rapidement le sang après la ponction, une sensibilité potentiellement plus faible, et une difficulté pour quantifier de façon automatisée les cellules tumorales présentes sur le filtre de polycarbonate. Enfin, l'analyse cytomorphologique des CTC identifiés par cette technique présente les mêmes limites que l'interprétation cytologique des produits de cytoponction ou des étalements cytologiques dans le cadre du diagnostic ou du dépistage des cancers. On peut aussi envisager que la combinaison de deux ou trois méthodes différentes, permettant la détection des CTC, augmenterait de façon significative la possibilité d'identifier plus spécifiquement les CTC chez des patients développant un CNPC.

Il est certain que la mise en évidence d'altérations génétiques dans les CTC de patients atteints de CBPNPC est actuellement un challenge majeur en oncologie thoracique (Maheswaran et al., 2008; Punnoose et al., 2012). Il peut s'agir de la mise en évidence de mutations ponctuelles (sur les exons 18 à 21 du récepteur de l'EGF; sur les codons 12 ou 13 de *KRAS*, par exemple) ou de la mise en évidence de réarrangements chromosomiques (sur le gène *ALK*, par exemple) (Ilie et al., 2012b). Ces mises en évidence peuvent se faire après isolement et caractérisation des CTC, extraction d'ADN puis séquençage, et aussi dans certains cas par hybridation *in situ*. Compte tenu de «l'explosion» actuelle des essais thérapeutiques ciblant les altérations moléculaires et de l'autorisation de la mise sur le marché de molécules administrées en fonction de la présence ou de l'absence d'altérations génétiques moléculaires, cette orientation biopathologique sur les CTC est actuellement en plein développement.

Tout comme pour les autres cancers, la mise en évidence des CTC dans les cancers du poumon est très probablement une voie importante dans le cadre de la prise en charge des patients (traitement et monitoring). On doit toutefois se poser plusieurs questions non résolues à ce jour : est-ce que les cellules détectées par les approches citées ci-dessus sont réellement les cellules les plus agressives ou est-ce que des cellules non détectées ou non détectables ne sont-elles pas plutôt celles dont le pouvoir invasif est le plus marquée (Wicha et Hayes, 2011)? Enfin, au-delà des progrès considérables réalisés dans le domaine de la détection des cellules tumorales circulantes, en particulier chez les patients atteints d'un cancer du poumon, comment évaluer le véritable impact actuel de cette approche sur le bénéfice apporté aux patients (Kaiser, 2010; Lianidou, 2012; Parkinson et al., 2012)?

## Neutrophiles intratumoraux

Les interactions entre les cellules du stroma tumoral et les cellules carcinomateuses pulmonaires participent à la progression tumorale et à la dissémination métastatique (Fridlender et al., 2009; Mantovani et al., 2011; Peranzoni et al., 2010; Piccard et al., 2012). Au sein de ce stroma, l'action de plusieurs cellules impliquées dans la carcinogenèse a été largement analysée précédemment (notamment les populations lymphocytaires T, les fibroblastes, les macrophages, et les cellules endothéliales). Le rôle des cellules myéloïdes (cellules très peu étudiées en cancérologie), est activement étudié par de rares équipes (Fridlender et Albelda, 2012). De façon similaire à la population macrophagique intratumorale, il existerait ainsi au sein des carcinomes, des «tumor-associated neutrophils» de type 1 (TAN1) pro tumoraux, et des TAN2 anti tumoraux, dont la modulation pourrait selon le cas accélérer ou diminuer la progression tumorale et métastatique (Fridlender et al., 2009).

On connaît relativement peu le rôle joué par neutrophiles dans les cancers humains. Un nombre important de patients atteints de cancer à un stade avancé démontrent des niveaux élevés de neutrophiles dans le sang périphérique (Schmidt et al., 2007). Les mécanismes par lesquels la neutrophilie est induite par les tumeurs sont inconnus, bien que la production de GM-CSF soit impliquée dans ce mécanisme (Fridlender et Albelda, 2012). La neutrophilie a été associée à un pronostic plus mauvais dans de nombreux cancers, notamment le carcinome broncho-alvéolaire et le mélanome métastatique (Bellocq et al., 1998; Schmidt et al., 2007). De plus, il y a peu de données sur l'impact des neutrophiles intratumoraux dans la progression tumorale. Les neutrophiles intratumoraux se sont avérées être un facteur pronostique pour la survie sans récidive, ainsi que la survie spécifique et globale dans les carcinomes à cellules claires du rein, les carcinomes épidermoïdes de la tête et du cou, et enfin dans les CNPC opérables (Donskov and von der Maase, 2006; Ilie et al., 2012a; Jensen et al., 2009; Jensen et al., 2012). L'infiltration tissulaire de neutrophiles serait corrélée avec le grade histologique des gliomes et avec des sous-types plus agressifs des tumeurs pancréatiques (Fossati et al., 1999 ; Reid et al., 2011). A l'inverse dans certaines tumeurs (le cancer de l'estomac), un nombre élevé de neutrophiles a été associé à une évolution favorable. Une autre équipe a récemment rapporté qu'une densité élevée de neutrophiles intratumoraux CD66b+ et de macrophages CD163+ était corrélée avec des facteurs pronostiques défavorables et des marqueurs de l'inflammation systémique, mais pas directement corrélée à la survie des patients atteints de CNPC (Carus et al., 2013 ; Caruso et al., 2002). Plusieurs effets des cellules tumorales humaines sur les neutrophiles ont été démontrés *in vitro*. De manière intéressante, l'interleukine 8 (IL-8), sécrétée par les cellules tumorales peut jouer un rôle important en attirant des neutrophiles dans le microenvironnement tumoral (Sparmann et Bar-Sagi, 2004). Ces études suggèrent un rôle important pour l'IL-8 dans la progression tumorale et le processus de métastase en favorisant la prolifération cellulaire et l'angiogenèse dans les CNPC (Sparmann et Bar-Sagi, 2004). En outre, des études récentes ont montré que la surexpression d'IL-8 induite par l'oncogène *KRAS* pourrait favoriser la migration et la croissance cellulaire et contribuer à un phénotype plus agressif dans les CNPC (Sparmann et Bar-Sagi, 2004; Sunaga et al., 2012).

# CHAPTER 1 INTRODUCTION

## 1.1 Non-Small Cell Lung Cancer

### 1.1.1 Brief summary of epidemiology, histopathology and treatment

Lung cancer is the most commonly diagnosed cancer worldwide and its incidence continues to grow. In 2008, an estimated 1.5 million new cases of lung cancer were diagnosed globally, accounting for approximately 12% of the global cancer burden. In addition, it is the leading cause of cancer-related deaths in both men and women, causing approximately 1.2 million deaths per year (Globocan 2008 http://globocan.iarc.fr/) (Jemal et al., 2011). Among all cancers, lung cancer now has the highest mortality rate in most countries, with industrialized regions such as North America and Europe having the highest rates. The possibility that a patient with lung cancer will be alive after five years from diagnosis is approximately 25% across all stages of disease (Blanchon et al., 2006).

#### Pathophysiology

Both exposure (environmental or occupational) to particular agents and an individual's susceptibility to these agents are thought to contribute to the risk of developing lung cancer. The primary risk factor for the development of lung cancer is cigarette smoking, which is estimated to account for approximately 90% of all cases, while the occupational exposures to carcinogens account for approximately 9-15% of lung cancer cases (Cokkinides et al., 2006).

Smoking consumption is undoubtedly the first cause of lung cancer and, given the poor treatment possibilities, the only real breakthrough in the fight against this disease is the control of smoking by government-mediated acts. The incidence of lung cancer is directly associated with smoking exposure, which is measured with an index known as "*pack-year*". One pack-year corresponds to the consumption of 1 pack of cigarettes (20 cigarettes) each day for one year or alternatively half a pack for 2 years and so on. The risk of developing lung cancer for a current smoker of one pack per day for 40 years is approximately 20 times that of someone who has never smoked (Sun et al., 2007a). Generally, global lung cancer trends have followed the trends in smoking, with a lag time of several decades. In France, death rates from lung cancer have recently begun to decline in men, reflecting a decrease in smoking. However, the death rate in women continues to rise (Source "Institut National du Cancer"; http://lesdonnees.e-cancer.fr/). Notably, despite a very low rate of smoking, Chinese females have a higher incidence of lung cancer than European females.

Tobacco smoke contains more than 300 harmful substances with at least 40 known potent carcinogens (Pfeifer et al., 2002). Poly-aromatic hydrocarbons and the nitrosamine-NNK are known to cause DNA damage by forming DNA adducts in animal models. Benzo-A-pyrine also appears to induce molecular signalling such as AKT, as well as inducing mutations in p53 and other tumour suppressor genes (Kometani et al., 2009).

The most common occupational risk factor for lung cancer is exposure to asbestos (O'Reilly et al., 2007). Studies have shown radon exposure to be associated with 10% of lung cancer cases, while outdoor air pollution accounts for perhaps 1-2%. In addition, pre-existing non-malignant lung

diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and tuberculosis have all been shown to be associated with increased lung cancer rates (Molina et al., 2008).

### HISTOPATHOLOGICAL CLASSIFICATION

Approximately 95% of primary lung cancers are epithelial in origin and these are categorised on the basis of histological appearance into two main groups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for the majority (~85%) of cases and is further subdivided into several major histological types: adenocarcinoma (ADC), squamous cell carcinoma (SCC), large cell carcinoma (LCC), large cell neuroendocrine and sarcomatoid carcinomas as well as mixed species (i.e. adenosquamous carcinoma) and some rare tumour types. Furthermore carcinoid tumours as well as the group of salivary gland tumours have to be considered regarding epithelial neoplasms of the lung (Travis WD, 2004).

ADC is the most frequent (~60%) NSCLC sub-type representing 35-40% of all lung cancers. Pulmonary ADC alone thereby has to be considered the most frequent tumour in men in the Western world leading to death and ranks second regarding the frequency of lethal tumours in women. It is more fatal than colon cancer (in both sexes) as well as prostate cancer. It usually occurs in a peripheral location within the lung. ADC is the most common histologic subtype, and may manifest as a "scar carcinoma." This tumour type is the most common in non-smokers and women and it is more frequently associated with pleural effusions and distant metastases. A significant change in the pathologic classification of lung cancer occurred with the publication in 2011 of a new lung adenocarcinoma classification under the sponsorship of the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS) (Travis et al., 2011a; Travis et al., 2011b). The classification outlines many paradigm shifts that affect clinical practice and opens new avenues for research. Pathologists now play an important role in personalized medicine for patients with lung cancer as a result of the newly recognized importance of histologic classification and molecular testing in stratifying patients for specific therapies. This is a central theme of the new classification. Because the lung cancer field is rapidly evolving with new advances occurring on a frequent basis, particularly in the molecular arena, this classification provides a much needed standard for pathologic diagnosis not only for patient care but also for clinical trials (Travis et al., 2013).

SCC accounts for 25-30% of all lung cancers, and is found most often in the central parts of the lung. SCC shows a remarkable dose-dependence with cigarette smoking (Spiro and Porter, 2002). SCC cells are large, flattened and stratified with a high cytoplasm to nucleus ratio. Key diagnostic features include the presence of intracytoplasmic keratin which may be linked to the presence of intercellular bridges and squamous pearl formation. Most SCC arise centrally within the main, lobar, and segmental or sub segmental bronchi but some occur more peripherally. The tumour mass generally extends into the lumen of the airway with invasion into the underlying wall.

LCC is an undifferentiated NSCLC that lacks the cytological and architectural features of small cell carcinoma and glandular or squamous differentiation. LCC accounts for approximately 9% of all lung cancers in most studies. Large cell neuroendocrine carcinoma accounts for about 3% of lung cancer. All types predominate in smokers, except lymphoepithelioma-like carcinoma. LCC except basaloid carcinoma, occur preferentially in the lung periphery, so that tumours may be accessible by transthoracic fine needle aspiration biopsy as well at bronchoscopy. Specific diagnosis of LCC and variants can only be reliably achieved on surgical material (Travis WD, 2004).

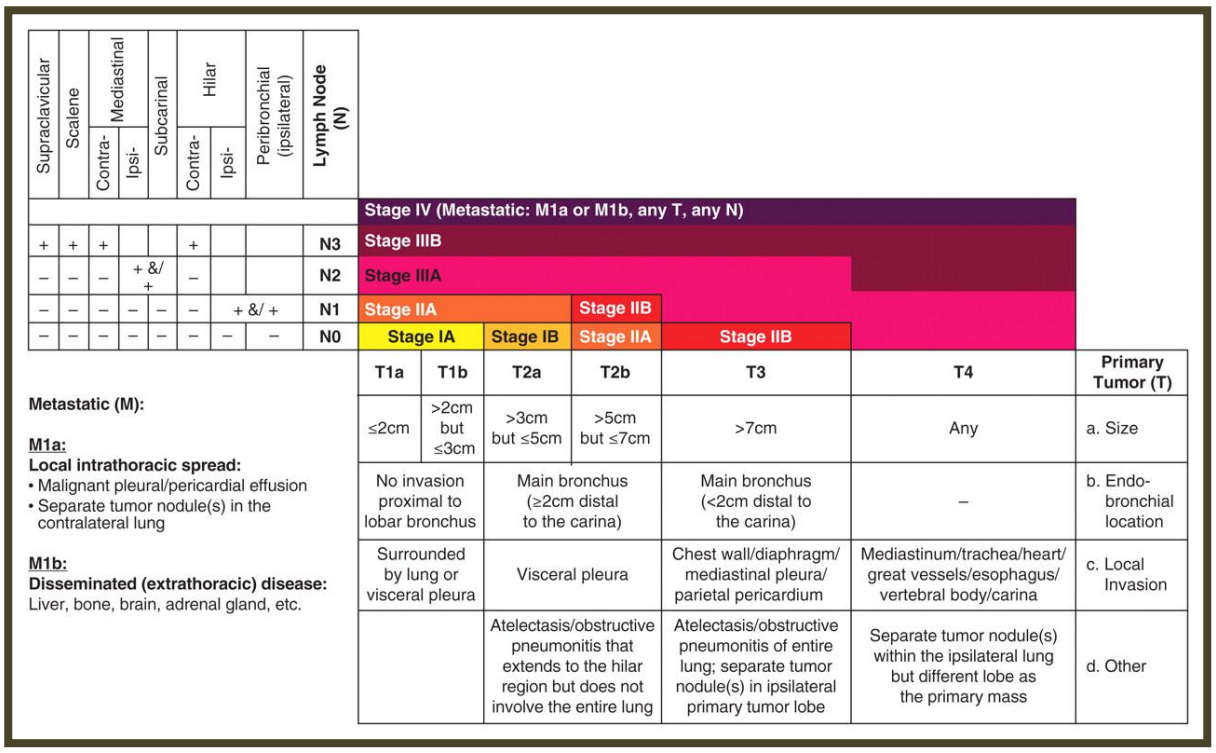
### DIAGNOSIS, STAGING AND PROGNOSIS

Lung cancer diagnosis and staging is mainly based on the following procedures; imaging techniques (chest X-ray, CT scan and PET scan, nowadays usually in combination with CT to merge metabolic and anatomical information), minimally invasive approaches (bronchoscopy, endobronchial ultrasound, fine-needle and middle-needle aspiration biopsies) and invasive techniques (mediastinoscopy, video assisted thoracoscopy or open thoracotomy). All these procedures aim at correctly defining the extension of the disease (stage), and to obtain a tumour sample for proper histological diagnosis. There are currently no generally implemented screening programs for lung cancer. Recently, the first results from the randomized NCI-sponsored National Lung Screening Trial (NLST) were published. Subjects between 55-74 years of age with a history of heavy smoking were randomly assigned to a yearly low-dose CT or regular chest radiography. Although the results are premature and there is a concern of overdiagnosis, there was significantly reduced lung cancer mortality in the CT screened population (Aberle et al., 2011).

Since early lung cancers often are asymptomatic, the debuts of symptoms often indicate advanced disease. Today, a lung tumour is either an incidental finding on a chest X-ray/CT or a finding after dedicated investigation. The goal of the diagnostic procedures is to establish a sufficient evidence of the disease (histology) and clinical stage. A chest CT including the upper abdomen is usually required for a proper identification of a lung tumour and ruling out regional metastases in the chest as well as distant metastases in the liver and suprarenal glands. To establish a diagnosis, sampling of tumour tissue through tissue biopsy and/or brush cytology is usually obtained by bronchoscopy. However, peripheral tumours often require CT guided biopsy. After a confirmed diagnosis of NSCLC, further staging procedures are necessary to establish the extent of disease burden. Brain MRI and a bone scan are often done to rule out apparent brain and bone metastasis. PET scans are helpful and are increasingly becoming available to evaluate if there is mediastinal or distant metastasis and to define the tumour volume for treatment planning in radiotherapy (Goldstraw, 2009). Patients also undergo lung function tests to determine operability. Prediction of prognosis as well as prediction of the response to different therapeutic regimens (surgery, radiation, chemotherapy) is of high importance for therapy planning in almost every tumour entity. Tumour staging is the most important factor in this regard.

NSCLC is staged according to the international TNM (*Tumour Node Metastasis*) classification (Mountain, 1997). The combination of the T, N and M descriptors define each stage and categorize NSCLC into three main prognostic and treatment groups; early stage (I and II), locally advanced (stage III) and metastatic (stage IV). Recently, the IASLC has released a proposal to update the staging system (Goldstraw, 2009). The proposal has been accepted by the International Union against Cancer (UICC) and it was included in the 7th edition of the TNM classification of tumours (Travis et al., 2011b) (Figure 1).

**Figure 1.** Chart illustrates the descriptors from the 7th edition of the TNM staging system for lung cancer.




This classification, developed on more than 130.000 cases and externally validated on >32.000 cases from the SEER database, has contributed to improve the prognostic definition of stage. Prognosis is mainly determined by disease stage and patient performance status, with increasing stage associated with a progressive decline in survival. Unfortunately, the majority of patients present with advanced stage disease, for which no cure is currently available, explaining the poor overall survival (Table 1) (De Petris, 2010).

**Table 1.** Summary of clinical data by stage in NSCLC patients.

| Stage | % of patients at initial diagnosis | Treatment Strategy | 5-year survival |
|---|---|---|---|
| I | 17% | Surgery or SBRT with curative intent in non-operable cases | 65% |
| II | 13% | Surgery + adjuvant chemotherapy | 35% |
| III | 25% | Chemotherapy + radiotherapy | 15% |
| IV | 45% | Palliative chemotherapy or biological agents | <5% |

### TREATMENT MODALITIES

The treatment of choice for early stage NSCLC (Stages I and II) is radical surgical resection of the primary tumour, obtained with the removal of the lung lobe or sometimes of the entire lung, together with a systematical dissection of mediastinal lymph nodes (Table 1) (Patchell et al., 1990). When the tumour size is <5 cm and there are no nodal or distant metastases, but the patient cannot undergo surgery due to co-morbidities or reduced respiratory function, high dose hypofractionate stereotactic body radiotherapy (SBRT) can be a suitable and effective treatment option, leading to disease control and survival outcomes similar to surgery (Billing et al., 2001). In case of stage II disease, the administration of cisplatin-based postoperative (adjuvant) chemotherapy has shown to improve patient survival by 5% at five years. On the other hand the use of adjuvant chemotherapy is detrimental in stage IA tumours (size <3 cm) and is still a questionable approach in stage IB tumours (Bonnette et al., 2001). Locally advanced NSCLC is treated with a combination of chemotherapy and radiotherapy, which can be administered sequentially or concurrently. With this treatment, objective tumour response (reduction of at least 25% of tumour volume) is obtained in approximately 65% of cases and median survival time is approximately 15 months (Chidel et al., 1999). In case or resectable IIIA disease (resectable primary tumour with the presence of ipsilateral N2 mediastinal lymph node metastases) treatment with neoadjuvant (preoperative) chemotherapy ± radiotherapy followed by surgery may improve local tumour control. However, randomized trials have not shown any advantage for this strategy as compared with chemoradiation administered with curative doses (Girard et al., 2006; Hu et al., 2006).

The majority of clinical trials on lung cancer have been conducted in advanced/metastatic Stage IV NSCLC (Louie et al., 2009). In brief, the current clinical practice guidelines suggest a first-line treatment consisting of a combination of a platinum compound (either cisplatin or carboplatin) with a third generation drug (taxanes, gemcitabine, vinorelbine). All combinations have different toxicity profiles, but are equivalent in terms of efficacy. For at least 10 years, the response rate to first-line

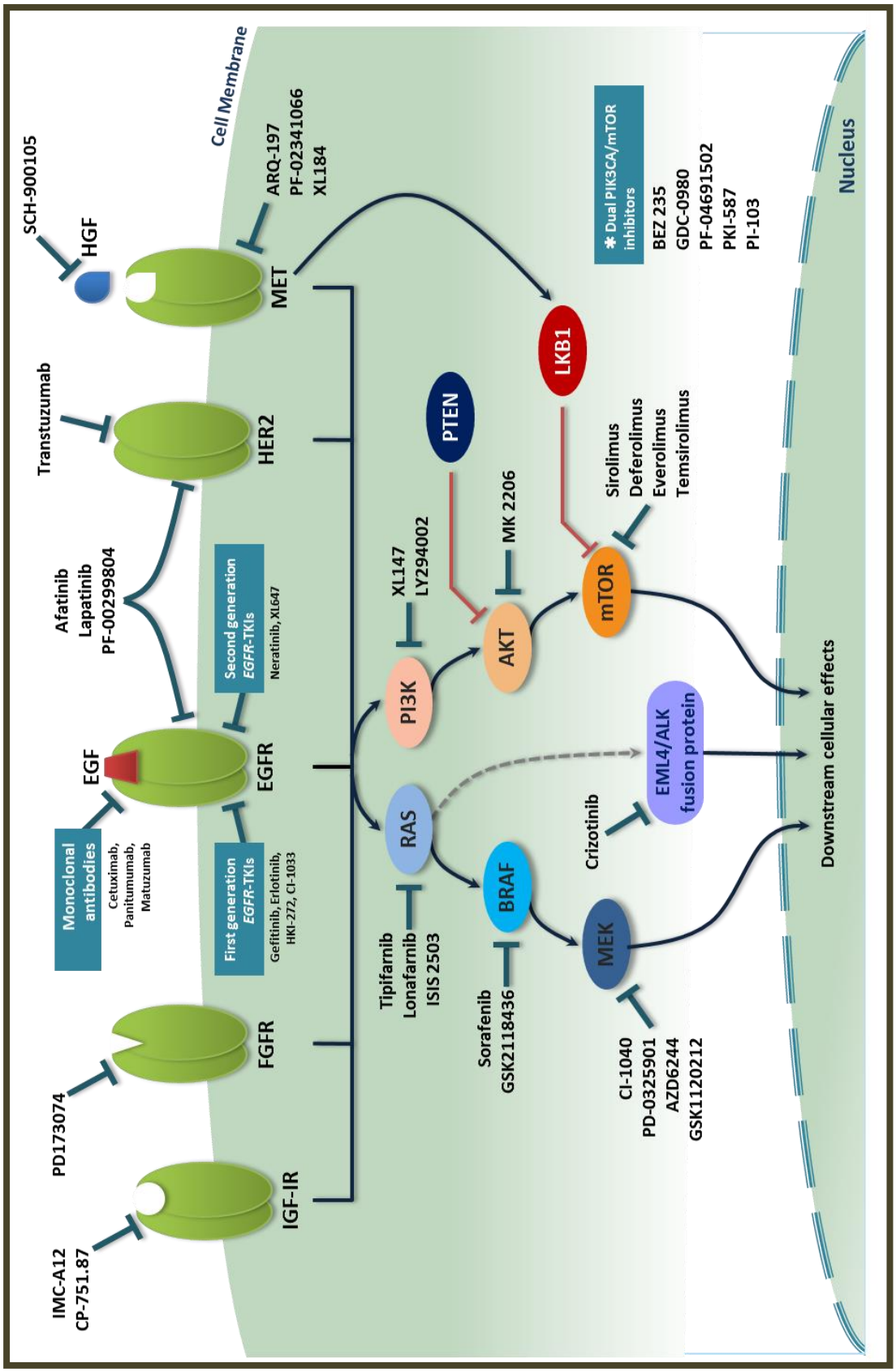
chemotherapy and the median survival time have not gone beyond the disappointing levels of 35% and 7 months, respectively (Ceresoli et al., 2004; Le Chevalier et al., 2005). Recently, new drugs have been developed and the attitude towards treatment choice has also changed (See Chapter 1.1.2). One of the novel agents is pemetrexed, a multitargeted antifolate. Registration trials showed that the combinations cisplatin/pemetrexed and cisplatin/gemcitabine are equally effective for the first-line treatment of NSCLC (Fekrazad et al., 2007). Moreover, single agent pemetrexed is equivalent to docetaxel for second-line NSCLC, namely as salvage therapy once the tumour has progressed after a first-line intervention (Gridelli et al., 2007). However, subgroup analyses of those trials showed that pemetrexed is superior to the comparative regimens in NSCLC with non-squamous histology (ADC and LCC), and detrimental in squamous tumours (Hotta et al., 2004). This may be ascribed to the expression levels of the enzyme thymidylate synthase, a target of pemetrexed, which are higher in squamous compared to non-squamous tumours (Namba et al., 2004). Following this analysis, the American and European regulatory agencies (FDA and EMEA, respectively) approved the use of the drug only for the treatment of non-squamous NSCLC. Another agent is bevacizumab, a monoclonal antibody against the vascular endothelial growth factor (VEGF). In combination with chemotherapy, bevacizumab improves survival of lung cancer patients compared to chemotherapy alone (Wu et al., 2007). This evidence was acquired on non-squamous tumours only, because of safety issues. By inhibiting VEGF, in fact, bevacizumab exposes patients to higher risk of bleeding, and is therefore not indicated in subjects with squamous tumours.

Finally, a novel class of compounds, the inhibitors of the tyrosine kinase domain of the Epidermal Growth Factor receptor (EGFR-TKI), and the ALK/MET tyrosine kinase inhibitor has also emerged as potential breakthrough in NSCLC therapy (See Chapter 1.1.2).

### 1.1.2 The Lung Cancer Molecular Disease Model

In most tumours, a plethora of genomic alterations synergistically cause the malignant phenotype. In most cases inhibition of one gain-of-function aberration alone is therefore not sufficient to inhibit tumour progression. However, in a fraction of tumours, one genomic aberration is driving the whole malignant phenotype and rendering the whole tumour solely dependent on this one aberration (Weinstein, 2002). These alterations are mostly gain-of-function mutations of tyrosine kinases (e.g., *EGFR* mutations), as well as fusion of two protein parts (e.g., *EML4-ALK*). Thus each tumour harbours a unique set of genomic alterations leading to a set of abilities a tumour requires to become fully transformed. Although many abnormalities are found in tumours only a handful of genes have currently high strength of evidence and capable of serving as the dominant oncogene and putative point of intervention for therapy. Recent advances in the understanding of cancer biology and the discovery of multiple molecular mechanisms underlying the development, progression, and prognosis of lung cancer created new opportunities for targeted therapy and improved outcome (Figure 2).

**Figure 2.** Currently available therapies targeting relevant oncogenic pathways in NSCLC.



In addition, the current classification of NSCLC integrated to the morphological analysis, supported in some instances by immunohistochemistry, new "molecular subtypes" of lung cancer based on specific actionable genetic aberrations (Travis et al., 2013; West et al., 2012). Therapeutic decisions for advanced NSCLC patients are currently based on the molecular analysis of tissue specimens. The development of personalized medicine has challenged the routine integration into pathology laboratories of somatic genetic testing, with molecular assessment being regarded as a powerful supplement to the histopathological diagnosis (Ilie and Hofman, 2012; Janku et al., 2011).

Epidermal growth factor receptor (*EGFR*) gene mutations are commonly found among molecular abnormalities analysed in non-small-cell lung cancers (NSCLCs), particularly in lung adenocarcinomas (Lynch et al., 2004; Paez et al., 2004). The epidermal growth factor receptor (EGFR, HER-1/ErbB1) is a receptor tyrosine kinase (TK) of the ErbB family, which consists of four closely related receptors: HER-1/ErbB1, HER-2/neu/ErbB2, HER-3/ErbB3 and HER-4/ErbB4. Upon ligand binding, receptor homo- or hetero-dimerization and phosphorylation activate the EGFR signal downstream of the PI3K/AKT pathway, which is involved in cell survival, or activate the RAS/RAF/MAPK pathway leading to cell proliferation (Herbst et al., 2008; Ladanyi and Pao, 2008). Patients harbouring activating EGFR mutations demonstrate response rates higher than 70%, 14-month progression-free survival and 27-month median overall survival when treated with *EGFR*-tyrosine kinase inhibitors (*EGFR*-TKIs; gefitinib, erlotinib) (Figure 2) (Mok, 2011; Mok et al., 2009; Rosell et al., 2009).

Since 2009, gefitinib (Iressa™, AstraZeneca, Macclesfield, Cheshire, UK) was the only licensed oral preparation for use in adult patients with locally advanced or metastatic NSCLC with activating *EGFR* mutations in all lines of therapy (Mok et al., 2009). Recently, erlotinib (Tarceva™, Roche Group) has been granted European and US approval for the use as a first-line monotherapy in patients with locally-advanced or metastatic NSCLC with *EGFR* activating mutations. This molecule was already FDA and EMEA approved for use in maintenance and second-line treatment of NSCLC (Brugger et al., 2011).

The short in-frame deletions in exon 19 and the exon 21 L858R point mutation account for approximately 90% of all *EGFR* mutations and are the most predictive of *EGFR*-TKIs efficacy in advanced lung adenocarcinomas (Sharma et al., 2007; Yang et al., 2008; Zhu et al., 2008). However, several *EGFR* mutations contribute to primary or acquired resistance to *EGFR*-TKIs treatment (Kosaka et al., 2011). The most conserved (~49% NSCLC cases) mechanism of resistance to TKIs is associated with the emergence of a single recurrent missense mutation *T790M* within the *EGFR* kinase domain (Godin-Heymann et al., 2008). Other secondary *EGFR* gene mutations such as *D761Y*, *L747S*, and *T854A* mutations have been associated to TKIs resistance, but with extremely low frequencies (Kosaka et al., 2011).

Intense research has led to a more detailed understanding of mechanisms of resistance in these tumours (Pal et al., 2010). There is emerging evidence that mutations in other genes of the *EGFR* family (*HER2*) or related tyrosine-kinase receptors (*cMET*) as well as their downstream genes (in

particular, *KRAS*, *BRAF, PIK3CA, AKT1, MEK1*) are present in NSCLC (Ding et al., 2008). The presence of these mutations can be associated with a lack of response to the first-generation *EGFR*-TKIs in the treatment of NSCLC (Gandhi et al., 2009; Marks et al., 2008; Sequist et al., 2011c). The amplification or mutation of *cMET* (20%) and *PIK3CA* gene mutations (5%) are among the most frequent "bypass mechanisms" which may determine resistance to TKIs, with continue activation of critical intracellular signalling pathways, despite continued EGFR inhibition. (Sequist et al., 2011c). In addition, with the greater understanding of tumour biology, agents that specifically target these oncogenes are currently under development and are being evaluated into clinical trials (Figure 2).

Recent studies have suggested that resistance to *EGFR*-TKIs may be mediated through *cMET* amplification or point mutations (Bonanno et al., 2011). About 20% of patients with an *EGFR* mutation who initially respond to an oral *EGFR* inhibitor and finally progress are found to have a *cMET* amplification or somatic mutation (Custodio et al., 2012). Several agents targeting *cMET* are currently under clinical investigation as single agents as well as in combination regimens (Figure 2) (Sattler et al., 2011). The selective, non-ATP competitive orally administered *MET* inhibitor, tivantinib (ARQ-197), has recently completed a phase II clinical trial and demonstrated an important progression-free survival improvement when combined with erlotinib, particularly among patients with non-squamous histology, *EGFR* wild-type status and *KRAS* mutations (Sequist et al., 2011b). Cabozantinib (XL184), a multikinase inhibitor that targets *cMET, VEGFR2, AXL, KIT, TIE2, FLT3*, and *RET*, dramatically decreased tumour cell proliferation coupled with increased apoptosis and dose-dependent inhibition of tumour growth in breast, lung, and glioma tumour *in vitro* models (Yakes et al., 2011). Finally, the dual *cMET* and *ALK* inhibitor, crizotinib (PF-02341066), demonstrated tumour and metastasis inhibitory effects in both *cMET* and *ALK*-positive patients (Kijima et al., 2011; Ou et al., 2011). This compound is under investigation in a phase I study that combines this multikinase *cMET* inhibitor with the irreversible pan-HER inhibitor PF-00299804 (Sierra and Tsao, 2011).

*HER2* (*ErbB2*) gene mutations are found in approximately 2% of NSCLC (Pao and Girard, 2011). The mutations are predominantly small, in-frame insertions in exon 20 and lead to constitutive activation of the mutant HER2 kinase. In NSCLC, activating mutations of *EGFR* and *HER2* occur in a mutually exclusive manner (Perera et al., 2009). Therefore, tumours harboring *HER2* mutations do not respond to treatment with anti-*EGFR* inhibitors or to anti-*HER2* antibody therapy alone (eg., Transtuzumab; (Figure 2) (Pao and Girard, 2011). Instead, *HER2* insertions potentially predict sensitivity to treatment with pan-HER molecules that target *EGFR* and *HER2* (e.g., Lapatinib) (Janne et al., 2011). Moreover, anti-tumour effects were observed when afatinib (BIBW 2992) that inhibits *EGFR* and *HER2* was used in combination with mTOR inhibitors (eg., Sirolimus; (Figure 2) (Pao and Girard, 2011). The pan-*HER* inhibitor PF-00299804, with affinity for *EGFR*, *HER2*, and *HER4*, has demonstrated activity in a phase II study, and seems to have activity in preclinical models of gefitinib resistance. However, it may not overcome resistance generated by *cMET* amplification (Pal et al., 2010). In addition, several small-molecule TKIs that inhibit receptors such as *VEGFR-2, EGFR, MET, PDGFR*, and *KIT* simultaneously have demonstrated clinical value over agents with single targets (Custodio et al., 2012).

Additionally, intense efforts to target mutant *KRAS* are under way, including dual inhibition of the critical downstream RAS effector pathways PI3K/AKT/mTOR and RAS/RAF/MEK (Figure 2) (Roberts et al., 2010).

The frequency of *KRAS* gene mutations varies according to tumour histology (15%–35% of adenocarcinomas), patient ethnicity (more frequent in Caucasians than Asian patients) and smoking history (more frequent in smokers than never smokers). In addition, *KRAS* mutations are non-overlapping with other oncogenic mutations found in NSCLC (Gaughan and Costa, 2011). It seems that *KRAS* mutations work better as a negative predictor of response to *EGFR*-TKIs than EGFR mutations do as a positive predictor (Riely et al., 2009; Roberts et al., 2010). A number of agents targeting *KRAS* have been developed and are currently under clinical investigation. Farnesyl transferase inhibitors (FTIs; tipifarnib and lonafarnib), antisense molecules (e.g. ISIS 2503), and peptide vaccines are being tested in combination with cytotoxic therapy in clinical trials in NSCLC (Custodio et al., 2012).

Somatic *BRAF* mutations are associated with increased kinase activity and as part of the MAP kinase pathway, are involved in cell proliferation, differentiation, and transcriptional regulation (Figure 2). *BRAF* mutations have been initially identified in melanomas (Davies et al., 2002). The *BRAF* mutations prevalence in NSCLC is approximately 1–3%, most of which are adenocarcinomas (Pao and Girard, 2011). In contrast to melanoma where the punctual V600E mutation in *BRAF* kinase domain is the most prevalent (~90%) somatic alteration, NSCLCs can harbour mutations at other positions V600E (50%), G469A (39%), and D594G (11%) (Paik et al., 2011). *BRAF* mutations are non-overlapping with other oncogenic mutations found in NSCLC (e.g. *EGFR* or *KRAS* mutations, *ALK* rearrangements) (Pao and Girard, 2011). In lung cancer *in vitro* models *BRAF* mutations predicted decreased sensitivity to the *EGFR*-TKIs (Gandhi et al., 2009). Most of the current clinical data concerning the *BRAF* inhibitors comes from promising studies conducted with these molecules in melanoma. Improved rates of overall survival and progression-free survival have been reported in a phase III trial comparing vemurafenib to dacarbazine in previously untreated, metastatic melanoma harbouring *BRAF* V600E mutation (Chapman et al., 2011). The activity of sorafenib, the multikinase inhibitor of *BRAF*, *VEGFR-1, -2, -3*, and *PDGFR* was evaluated in NSCLC, but showed no significant difference in survival of patients (Pao and Girard, 2011). However, the potential impact of *BRAF* mutations as predicting biomarkers of response to selective *BRAF* or *MEK* inhibitors is currently under investigation in NSCLC.

MEK1 (also known as MAP2K1) is a serine-threonine protein kinase downstream of BRAF and is a central mediator in the MAP kinase signalling pathway involved in cellular growth and proliferation (Figure 2). The frequency of somatic *MEK1* mutations is low (~1%) in NSCLC and these are more common in adenocarcinoma than squamous cell carcinoma. *MEK1* mutations are mutually exclusive to *EGFR*, *KRAS*, *HER2*, and *BRAF* mutations (Pao and Girard, 2011). The presence of *MEK1* mutations has been associated with *in vitro* resistance to *EGFR*-TKIs (Marks et al., 2008). Inhibitors of *MEK*, which target further downstream along the RAS/RAF pathway, have recently been developed (CI-

1040, PD-0325901, and AZD6244) (Figure 2). Preclinical and clinical studies with these agents have shown promising antitumor activity in the treatment of NSCLC (Marks et al., 2008).

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases with a key role in the mediation between growth factor receptors and intracellular downstream signalling pathways (Figure 2). Preclinical data support the major role of the PI3K pathway in cell proliferation, growth, apoptosis, cytoskeletal rearrangement, disease progression and resistance to chemo- and radiotherapy in NSCLC cell lines (Marinov et al., 2007; Vivanco and Sawyers, 2002). The PI3K/AKT/mTOR signalling pathway may be activated in cancer through multiple mechanisms including mutations in *PIK3CA*, which encodes the catalytic subunit of PI3K, loss or mutation of phosphatase and tensin homolog (*PTEN*), *AKT* mutations and deregulation of mammalian target of rapamycin (mTOR) complexes (Samuels et al., 2004; Wallin et al., 2011). Somatic mutations in *PIK3CA* have been identified in 1-4% of all NSCLC (Kawano et al., 2006). These mutations occur more frequently within two "hotspot" areas within exon 9 and exon 20 (Sequist et al., 2011a). *PIK3CA* mutations appear to be increased in squamous cell carcinomas than in adenocarcinomas and occur in both never smokers and ever smokers (Sequist et al., 2011a). *PIK3CA* mutations may occur concurrently with *EGFR, KRAS*, *BRAF* and *ALK* abnormalities (Chaft et al., 2011). Pre-clinical data demonstrated that *PIK3CA* activating mutations are sensitive to the dual PIK3CA/mTOR inhibition (Figure 1) (Wallin et al., 2011). In exchange, recent studies suggested that coexisting *KRAS* and *PIK3CA* mutations may be associated with resistance to PIK3CA/mTOR inhibitors (Janku et al., 2010). Multiple PI3K inhibitors are in early clinical development, but thus far the response rates to single agents are low (Pao and Girard, 2011). In addition, *PIK3CA* mutations have been detected in *EGFR* mutant lung cancers with acquired resistance to EGFR TKIs therapy (Sequist et al., 2011c; Zou et al., 2012).

AKT is a downstream effector of PI3Ks and is constitutively activated in NSCLCs (Figure 2) (Sun et al., 2007b). The prevalence of *AKT1* mutations in NSCLC is about 1%, and they have only been identified in squamous-cell carcinoma (Malanga et al., 2008). Recently, pre-clinical data suggested that the combination treatment with selective MEK (AZD6244) and AKT inhibitors (MK2206) had a significant synergistic effect on tumour growth *in vitro* and *in vivo* leading to increased survival rates in mice bearing advanced human lung tumours (Meng et al., 2010).

*PTEN* is as tumour suppressor gene by negatively regulating the PI3K/AKT signalling (Figure 2). *PTEN* may be down regulated through several mechanisms, including mutations, loss of heterozygosity, methylation, and protein instability, which contributes to lung carcinogenesis (Custodio et al., 2012). *PTEN* somatic mutations were identified in 4.5% of all NSCLCs. *PTEN* mutations were found in ever-smokers and were significantly more frequent in squamous cell carcinoma than in adenocarcinoma In pre-clinical studies, *PTEN* loss in *EGFR* mutant lung tumours is associated with decreased sensitivity to *EGFR*-TKIs (Jin et al., 2010).

mTOR plays a critical role in transducing proliferative signalling mediated through the PI3K and AKT signalling pathways, that is essential for cancer cell growth and proliferation (Figure 2) (Pal et al., 2010). It's inappropriate activation is involved in the pathogenesis of numerous tumour types,

including NSCLC (Custodio et al., 2012). Numerous molecules interfere with the PI3K/AKT/mTOR pathway at multiple levels. Some of them, such as the mTOR inhibitors temsirolimus and everolimus, are already approved by the FDA and EMEA for other indications, such as renal-cell carcinoma based on previously published phase III randomized trials. mTOR inhibition demonstrated promising results in *KRAS*-mutated cell lines (Janku et al., 2010).

Frequently mutated tumour suppressor genes in NSCLC include *TP53* (~50%), *CDKN2A* (p16) (~17%), and *LKB1* (*STK11*) (8%). *TP53* mutations are more common in squamous cell carcinoma (62%) than in adenocarcinoma (39%), with the most frequent mutations occurring in exons 5–8 (Lee et al., 2010). *TP53* mutations are more commonly found in the presence of *EGFR* mutations in never-smokers patients (Sanders and Albitar, 2010). *LKB1* is more frequently mutated in adenocarcinoma than in squamous cell carcinoma (15% vs. 5%), in Caucasian vs. Asian patients (17% vs. 5%), and rather limited to male smokers. *LKB1* mutations may co-exist with *KRAS* or *BRAF* mutations (Gill et al., 2011; Koivunen et al., 2008). Notably, cell line studies have shown that NSCLC tumours with concurrent mutations in *LKB1* and *KRAS* demonstrate sensitivity to mTOR and MAPK inhibition that is not apparent with either mutation alone (Mahoney et al., 2009). Moreover, studies using gene therapy by replacement of tumour suppressors in preclinical, and in some early-phase clinical trials for NSCLC, have been performed. In this regard, the most evaluated strategy was that of restoring wild-type *TP53* expression in lung tumour cells (Vachani et al., 2010). In general, these trials have demonstrated safety, with low efficacy. Although, some phase I studies of *TP53* replacement with adenoviral vectors suggested clinical responses with a few partial responses, phase II studies failed to demonstrate difference in response rates for Ad.*TP53*/chemotherapy-treated lesions chemotherapy alone (Schuler et al., 2001). There are no current trials on-going in the United States or Europe using this approach in lung cancer. In the opinion of the researchers, the lack of strong bystander effects, along with the low transfection efficiency of adenoviral vectors, limited the potential application in lung cancer, unless more efficient vectors are developed (Vachani et al., 2010). In recent years, the field of gene therapy in NSCLC has shifted toward "immune-gene therapy". This strategy requires enough gene transduction to stimulate an endogenous immune response and to create a strong bystander effect. Although these strategies seem to be successful in initiating anti-tumour immunes responses, it is generally recognized that there some limits remain (e.g. large tumour volumes; significant immune-inhibitory networks created by the tumours involving cytokines such as TGF-ß, interleukin-10, prostaglandin E2, and vascular-endothelial cell growth factor; and inhibitor cells such as T-regulatory cells and myeloid-derived suppressor cells) (Kim et al., 2006).

Recent data showed that fibroblast growth factor receptor 1 (*FGFR1*) may be a new promising molecular target for the treatment of smoking-associated lung cancer (Flemming, 2011). *FGFR1* controls a wide range of biological functions in embryogenesis, development, wound healing, angiogenesis and metabolism, by regulating cellular proliferation, survival, migration and differentiation (Turner and Grose, 2010). High-resolution genomic profiling demonstrated that the chromosomal region at 8p12 spanning the *FGFR1* gene locus is amplified in up to ~20% of squamous

cell lung carcinoma, and is a rare event (1-2%) in lung adenocarcinoma (Dutt et al., 2011; Weiss et al., 2010). *FGFR1* copy number aberration can be detected by several techniques, including fluorescent *in situ* hybridization (FISH) analysis (Weiss et al., 2010). The treatment of mice with *FGFR1*-amplified squamous cell lung cancer xenografts with a small anti-*FGFR1* molecule (PD173074) resulted in significant tumour shrinkage *in vivo* (Weiss et al., 2010). In addition, this pathway may function as a mechanism of resistance to anti-EGFR and anti-VEGF treatment (Marek et al., 2009; Semrad and Mack, 2011).

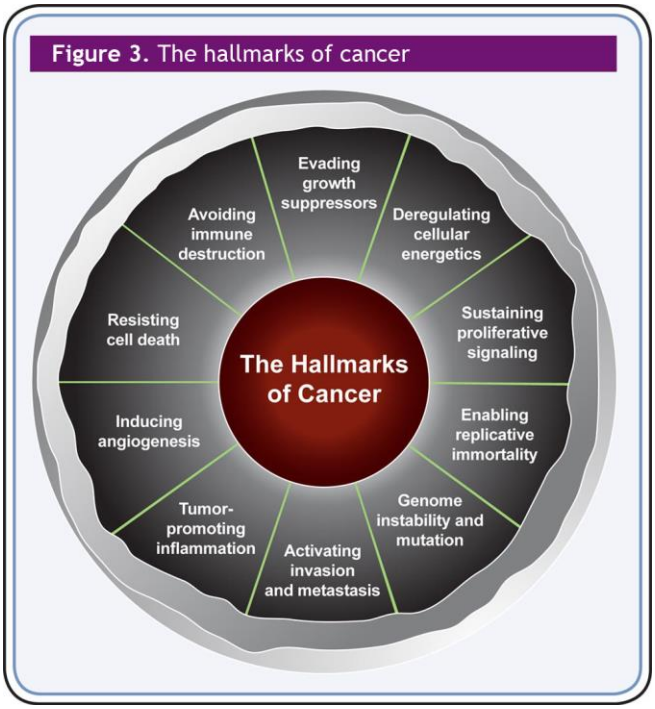
In 2007, the echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) fusion gene was identified in NSCLC (Soda et al., 2007). *EML4-ALK* fusion results in protein oligomerization and constitutive switch on the RAS/RAF signalling pathway (Janku et al., 2010). Transgenic mice expressing *EML4-ALK* under the control of a lung epithelial cell promoter develop multiple lung adenocarcinomas, demonstrating the oncogenic nature of this fusion gene (Pao and Girard, 2011). *EML4-ALK* fusion is a rare abnormality detected in approximately 2-7% of unselected patients with NSCLC, a frequency that increases (13%) in a population of patients with at least two of the following characteristics: female sex, young adults, Asian ethnicity, never (<100 cigarettes in a life time) or light (≤15 pack-year) smoking history, and adenocarcinoma histology (Shaw et al., 2009). *EML4-ALK* rearrangements are generally found in tumours with wild-type *EGFR*, *KRAS* and *BRAF* (Wong et al., 2009). As for *EGFR*-TKIs, *ALK* inhibitors have been found to be highly effective in lung cancers that have this translocation (Toyooka et al., 2011). The small molecule TKI crizotinib (PF02341066; Pfizer, New York, NY, USA) is an orally *ALK* inhibitor of phosphorylation and signal transduction. This inhibition is associated with G1-S phase cell cycle arrest and induction of apoptosis in positive cells *in vitro* and *in vivo* (Custodio et al., 2012). In a phase 1–2 trial, disease control was achieved in 47 (57%) of 82 patients and 27 (33%) patients with *ALK*-fusion-positive tumours had stable disease (Kwak et al., 2010). These dramatic findings led to two subsequent clinical trials of PF-02341066. The first is a randomized phase III trial of PF-02341066 compared with standard second line chemotherapy by pemetrexed or docetaxel in *EML4-ALK* rearranged NSCLC. The second is a phase II clinical trial of single agent PF-02341066 in *EML4-ALK* positive NSCLC designed for patients not eligible for the phase III trial or patients randomized to chemotherapy who subsequently developed progressive disease (Custodio et al., 2012). Therefore, in August 2011, crizotinib (Xalkori®, Pfizer, Inc., New York, USA) was approved by the FDA for the treatment of patients with locally advanced or metastatic NSCLC that express the ALK rearrangement. The FDA also approved the Vysis ALK Break-Apart FISH Probe Kit (Abbott Molecular, Inc., Des Moines, IL, USA) concurrently with the crizotinib approval (Shaw et al., 2011). Moreover, patients who harbor this fusion gene do not benefit from *EGFR*-TKIs and should be directed to trials of *ALK*-targeted agents (Shaw et al., 2009).

In addition, the interest in exploring chromosomal rearrangements other than *ALK* as potential drivers in lung cancer has led to the discovery of *ROS1*, a gene shown to be involved in chromosomal translocations in lung cancer (Rikova et al., 2007). ROS1 is a receptor tyrosine kinase of the insulin receptor family. Chromosomal rearrangements involving the *ROS1* gene were originally described in

glioblastomas, where *ROS1* (chromosome 6q22) is fused to the *FIG* gene (chromosome 6q22 immediately adjacent to ROS1), and have been shown to be transforming in transgenic mice (Charest et al., 2006). More recently, *ROS1* fusions were identified as potential driver mutations in an NSCLC cell line (HCC78; SLC34A2-ROS1) and an NSCLC patient sample (CD74-ROS1) (Rikova et al., 2007; Rimkunas et al., 2012). These fusions lead to constitutive kinase activity and are associated with sensitivity *in vitro* to TKIs (Bergethon et al., 2012).

Finally, an area of growing interest is the development of rationale combinations of conventional cytotoxic drugs with molecularly targeted therapies, or for combining molecular targeted alone to increase the therapeutic potential by blocking cancer cell survival mechanisms. However, nowadays no general guidelines to deal with such combinations exist. Here, we reported several positive results with some combinations, although most of them were reported in pre-clinical studies. The drug interaction patterns observed in vitro may not be similar to those observed clinically. As previously noted, the synergy between cytotoxic and targeted therapies cannot always be reliably predicted from preclinical models and inevitably requires clinical validation (Pal et al., 2010). Recently, in the INTACT-1, INTACT-2, TALENT, and TRIBUTE clinical trials, the addition of gefitinib or erlotinib to first-line chemotherapy failed to improve survival of NSCLC patients (Cheng et al., 2011). Some hypotheses were proposed to explain these disappointing results. First, further research efforts are necessary to select biomarkers that may predict response to targeted therapies. Second, all trials applied chemotherapy and targeted drugs simultaneously (Zwitter, 2010). Recently, a pharmacodynamic separation model was proposed to bypass this issue: *EGFR*-TKIs primarily cause cell cycle arrest and accumulation of cells in G1; and thus, when administered concurrently with chemotherapy, may push tumour cells to the dormant phases of the mitotic cycle and render them resistant to classic cytotoxic agents (Davies et al., 2006). Therefore, as no guidelines are available for the moment, the definition of the optimal schedule of administration of chemotherapy with molecularly targeted therapeutic agents largely remains a controversial clinical issue.

## 1.2 Integrative pathology / Biopathology




Cancer is a very complex, dynamic disease characterized by the ability to become autonomous in growth, evade death, sustain angiogenesis, invade surrounding tissues, and to metastasize (Figure 3) (Hanahan and Weinberg, 2011).

Currently, pathologists generate data from a single pathological snapshot, which acts as a surrogate of the intrinsic biology of the tumour, and forms part of the clinical record from which treatment decisions are made. This approach is remarkably powerful and has stood the test of time for prognostication; however, it does not meet the challenge of predicting individual outcome or responses to treatment. The most common classification of tumour stage is the TNM classification. The tumour stage gives an estimation of the amount of time, or degree of tumour progression, between tumour initiation and resection. The longer the time period between tumour initiation and resection, the greater the chance that the tumour will have undergone clonal evolution and acquired a set of unfavourable characteristics, such as greater size, ability to invade lymphatics or blood vessels, or ability to metastasize to distant sites (Faratian et al., 2009). Therefore, from static measurements alone pathologists are good at estimating dynamic processes, such as prognosis, through the integration of tumour stage. Data generation from tissue has been further refined by advances in molecular classification of cancers by whole-genome expression and analyses of gene amplification or gene loss, and pathologists can now make even more detailed assessment of intrinsic pathway biology on the basis of morphological features alone (Faratian et al., 2009; Govindan et al., 2012; Imielinski et al., 2012). Therefore, traditional morphological pathology can already comprehensively define the molecular pathology of the tumour in these cases, and this information can be used to plan the management of patients, and be exploited for targeted and combinatorial therapy (Li et al., 2013).

With the advent of targeted therapies, accurate characterization of the underlying oncogenic signalling pathways is needed. We need to know not only the structure of these pathways, but also how manipulation of part of a signalling pathway by a drug might affect the dynamics at the cellular and tumour level. To date, this approach has relied on static pathological measurements. Morphology alone is powerful, but reflects pathway biology only in a very limited sense, owing to the remarkable heterogeneity of solid tumours, lack of distinct clinicopathological entities, and lack of comprehensive molecular classification of the majority of diseases. Although traditional

histopathology has enabled rigorous validation of histological prognostic features that correlate with clinical end points such as relapse and survival, the management of patients with cancer based on prediction of how the patient will respond to any given treatment is now almost as heterogeneous as the tumour it is designed to treat (Faratian et al., 2009). Cancer is dynamic and heterogeneous, and therefore requires integration of molecular and cellular biology and pathology data in new predictive frameworks to embrace the dynamic nature of this disease.

One way to understand the complexity of biological systems is based on the concept of levels of organization, starting with the cell as the basic unit of life and disease, followed by tissues as a functional group of cells, organ systems, and ultimately the whole organism (Rodriguez-Canales et al., 2011). The historical evolution of anatomic pathology and cancer research echoes the levels of organization of biology (Figure 4).

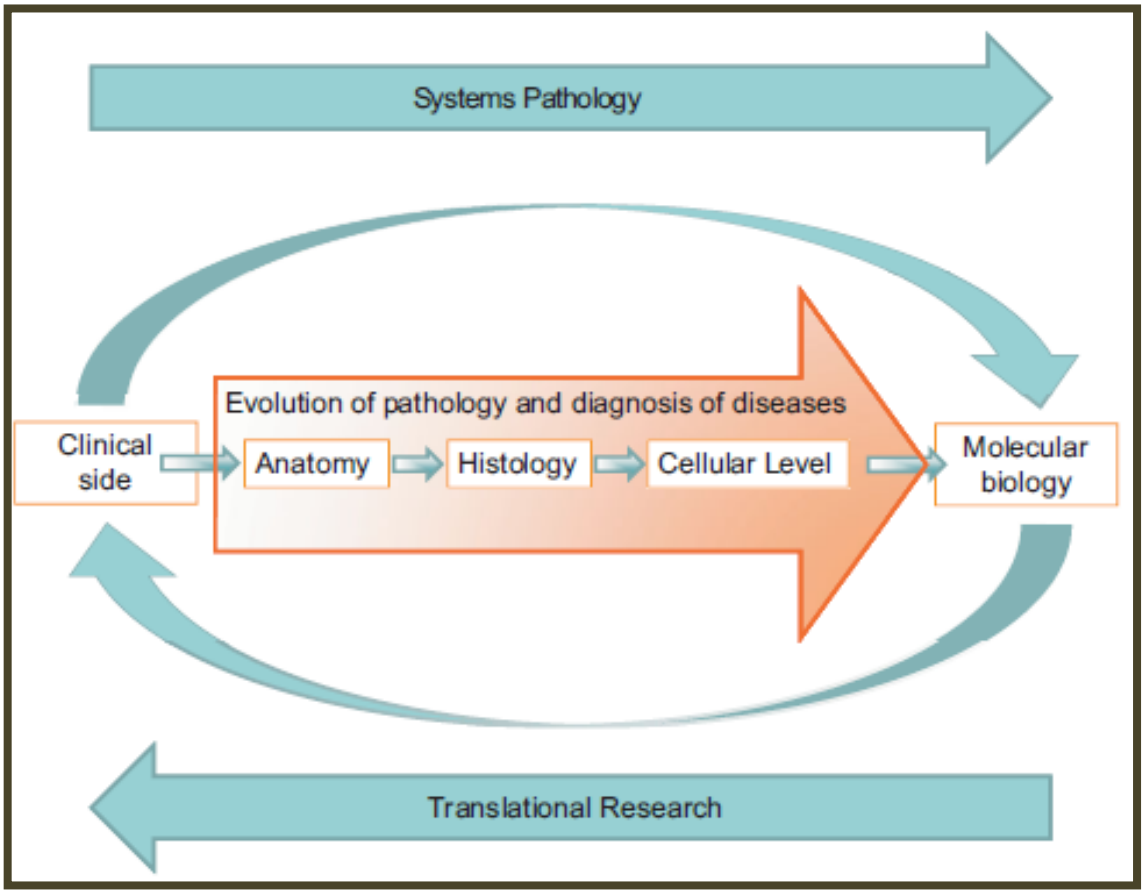


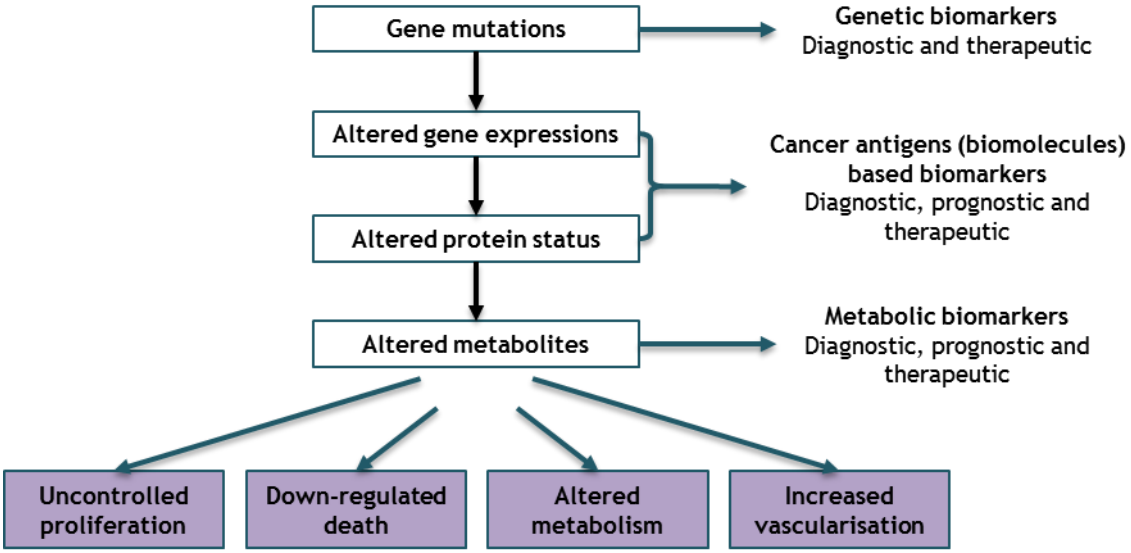

**Figure 4.** Schematic diagram showing the evolution of pathology from the anatomical level to the molecular level, in which clinical information is integrated with morphological changes at the anatomical and histological levels and with the alterations at the molecular level. Translational research allows novel biomarkers, discovered or validated in pathological tissues using molecular studies, to be used in the clinic as potential markers for diagnosis, prognosis, or therapy. Finally, a new systems pathology approach is needed to enhance integration of molecular biology and pathology. (From Rodriguez-Canales et al., 2011).

For instance, diseases were first characterized as clinical entities at the organism level, and then correlated with alterations of anatomy and organs, and finally the study of disease at the tissue and cellular level was introduced. Today the revolution of cellular biology has opened another new

window: a molecular-level mechanistic understanding of pathological processes (Rodriguez-Canales et al., 2011).

During the process of carcinogenesis, cellular and molecular alterations influence the machinery of cancer cells as well as non-cancerous cells in the tumour microenvironment (Figure 5) (Egeblad et al., 2010; Kerkar and Restifo, 2012; Orimo and Weinberg, 2006). Because of the complex structure of cancer and its functional interaction with multiple non-cancer cell types, tumours can be considered as organs (Rodriguez-Canales et al., 2011). Recognizing and understanding this biological complexity promises to provide important insights into the etiology of cancer that can be useful for early detection, diagnosis, and treatment.

**Figure 5.** The process of carcinogenesis showing opportunities of identifying biomarkers.




**Integrative pathology** is an approach to integrate qualitative, quantitative, dynamic and static data to generate a coherent model for a reliable understanding of a disease and a reproducible prediction of its expected course and its response to different therapeutic options. These data are provided by the identification and study of "**biomarkers**". Translational research can be defined as the effort to ''transform scientific discoveries arising from laboratory, clinical, or population studies into clinical applications to reduce cancer incidence, morbidity, and mortality'' (www.cancer.gov/researchandfunding/trwg/TRWGdefinition-and-TR-continuum, National Cancer Institute). In a general way, it is a biomedical effort to apply new knowledge generated from research studies into clinical practice, often referred to as ''from bench to bedside''. As a multidisciplinary approach, translational research can efficiently close the gap between basic science and medical practice as it aims to explain both the complex mechanisms of cancer as a biological entity, and the clinical applications that benefit the patient from the therapeutic standpoint (Figure 4). In addition, **biopathology** represents the study of molecular alterations in tumours.

According to the definition developed by the US National Institutes of Health, a biomarker is "*a characteristic that is objectively measured and evaluated as an indicator of normal biologic*

*processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*". Thus, biomarkers are measured in order to acquire relevant knowledge concerning diverse aspects of a disease, to be converted into clinically useful information that may serve to improve the disease management and, ultimately, the patient outcome (Bhatt et al., 2010; Biomarkers Definitions Working Group, 2001).

### BIOMARKER CLASSIFICATION AND APPLICATION IN CANCER

Biomarkers can be classified based on different parameters. They can be classified based on their **characteristics** such as imaging biomarkers (i.e. metabolic activity of a tumour, measured by the standard uptake value (SUV) of images obtained during PET examination) or molecular biomarkers. Molecular biomarkers can be used to refer to non-imaging biomarkers that have biophysical properties, which allow their measurements in biological samples (e.g., plasma, serum, cerebrospinal fluid, bronchoalveolar lavage, biopsy) and include nucleic acids-based biomarkers such as gene mutations or polymorphisms and quantitative gene expression analysis, peptides, proteins present in the tumour or released by the tumour in the bloodstream, lipids metabolites, epigenetic modifications, and other small molecules (i.e., free circulating nucleic acids or exosomal microRNAs) but even tumour and immune cells such as the circulating tumour cells, neutrophils, T-regulatory cells, or the cancer stem cells may as well serve as biomarkers.

Biomarkers can also be classified based on their **clinical application** such as **diagnostic biomarkers** (directly correlated with the presence of the disease), **prognostic biomarkers** (that predict disease outcome without further treatment), **predictive biomarkers** (that foretell response to a specific therapy), and **pharmacodynamic biomarkers** (that help decide on the optimal dose of a drug for an individual patient).

In this thesis I have evaluated the potential as diagnostic, prognostic or predictive biomarkers in NSCLC patients of several candidates such as cancer proteins (Articles 1-3), circulating tumour cells (Articles 4-8), and CD66b-positive neutrophil-to-CD8-positive T-cell ratio (Article 9), gene alterations (Articles 8 and A9); and circulating microRNAs (Article A2-Annex).

### CLINICAL FUNCTIONS OF BIOMARKERS IN NSCLC

**Diagnostic biomarkers**

More than 60% of NSCLC patients are diagnosed at advanced stages when a cure is unlikely (Siegel et al., 2012). Five-year survival rate for patients with advanced disease is less than 10%, whereas 5-year survival rate in patients with stage I disease is greater than 70% (Jemal et al., 2011). The annual mortality rate for lung cancer exceeds the annual rate for breast, prostate, and colon cancer combined, all of which have successful clinical screening tools for the detection of early-stage disease (Brenner and Normolle, 2007). For this reason, the search for diagnostic strategies for early lung cancer detection has intensified in the last years. Clinicians and scientists continued to

hypothesize that the earlier lung cancer is diagnosed, the opportunity for improved survival increases. For instance, the Early Lung Cancer Action Program (ELCAP) was a large lung cancer screening trial started in the 1990s using chest CT imaging (Henschke et al., 2006). It showed an improved detection rate and survival of early-stage lung cancers, which prompted the design of a large randomized National Lung Screening Trial (NLST). Exciting results of the recently completed study showed a 20% reduction in lung cancer–specific mortality using low-dose CT screening for patients at high risk for lung cancer after a median follow up of 6.5 years, compared with chest x-ray (Aberle et al., 2011; Henschke et al., 2006). This is the first large randomized screening study of lung cancer by low-dose chest CT to show an improvement in overall survival, thus giving new hope in the survival for this cancer. Extrapolating from the NLST results, a screening method that reduces lung cancer–specific mortality by 20% could save an estimated 11,074 lives annually in the United States., which is far greater than 2,303, the number currently estimated to be saved with adjuvant chemotherapy, therefore providing a strong rationale to pursue efforts in early detection (Hassanein et al., 2012).

To be successful at improving lung cancer detection, biomarkers must address a specific clinical question. Two pressing clinical needs are identified, biomarkers that will address the risk of developing lung cancer and others that are diagnostic in nature and will distinguish malignant from benign nodules or between the diverse lung cancer histology types.

Early detection involves a high-risk population, a screening test, and a testing schedule. Within this context, one must distinguish populations of individuals at-risk before or after the disease becomes measurable (Figure 6).

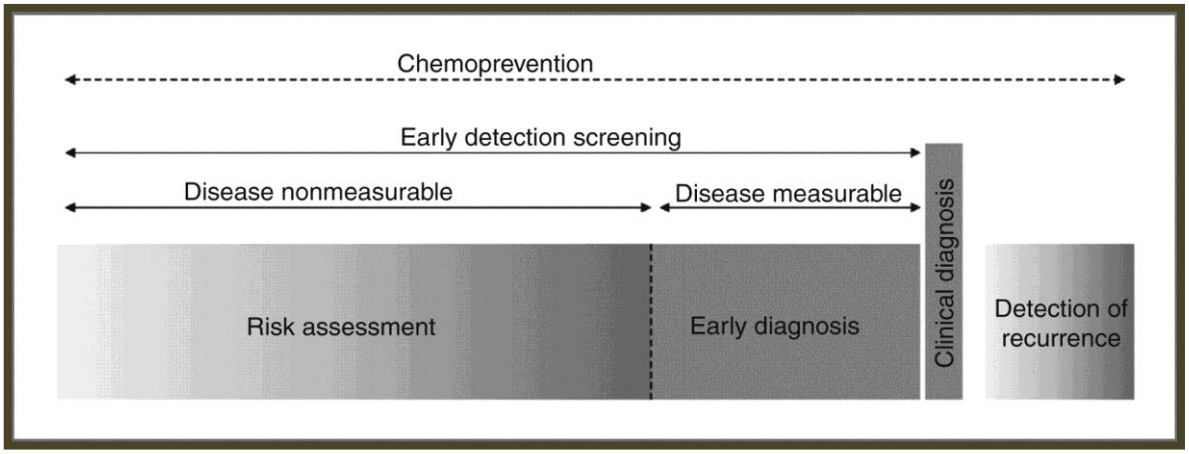



**Figure 6.** Clinical contexts for biomarker development in early detection of lung cancer. This diagram illustrates 4 clinical contexts within 4 windows of time. The period during which lung cancer is non-measurable and precedes the diagnosis characterizes the context of *risk assessment*. It represents a long window of time during which the disease develops and corresponds to an opportunity for chemoprevention. When the disease becomes measurable but remains asymptomatic, we enter the context of *early diagnosis*. Two other clinical contexts relate to *clinical diagnosis*, that is, when the disease is measurable and patients symptomatic, and to *detection of recurrence*. These windows of time correspond to the different contexts for which different biomarker targets can be developed (Hassanein et al., 2012).

A distinction is made between risk biomarkers to assess the risk of developing lung cancer (individuals at risk but with no measurable disease) and diagnostic biomarkers to determine whether cancer is present (individuals at risk with measurable asymptomatic disease such as lung nodules).

Biomarkers of risk for lung cancer have the potential to improve early detection beyond the use of CT scans that suffer from lack of sensitivity (particularly among never-smokers), specificity (high false-positive rate), and from high cost. Identifying a risk biomarker for developing lung cancer would further define the at-risk population, decrease the overall number of screening CTs conducted, and ultimately limit the downstream consequences of discovering these "false positive" nodules.

### Benchmarks for clinical utility

To be useful in the clinical setting, biomarkers go through careful phases of development as discussed below and should respond to specific criteria. The biomarkers should (i) be quantifiable and reproducible, (ii) have good testing performance [with good positive predictive value (PPV) and negative predictive value (NPV)], (iii) be measurable in accessible material, in small amounts and with little preparation, (iv) indicate a disease state, (v) have proven clinical use, (vi) be adopted by the community-at-large to take advantages of the benefits testing affords, (vii) be cost-effective; and (viii) be reimbursed by health insurers. The ideal cancer diagnostic biomarker should be found uniquely in the malignant tissue and should generate a positive signal that can be measured without confounding noise from normal tissues or other non-malignant pathologies (Brooks, 2012).

Such biomarkers may be assessed on tumour samples. However, the large majority of diagnostic biomarker research is performed on serum or plasma. Blood is an ideal source material for biomarker analysis. It is easy to sample, relatively cheap, and multiple samplings can be performed on the same subject. In addition, in preclinical research it is not uncommon to encounter interesting molecules which can be actively released in the bloodstream by tumour cells, or leak out as a consequence of the high cell turnover rate typical of tumours. The strategy most frequently used to discover NSCLC diagnostic biomarkers has been to compare the profile between tumour specimens and specimens of normal lung. The normal lung samples usually consist of a corresponding normal tissue from the same patient. In addition, another strategy has been to directly study serum or plasma from cancer cases and controls. One interesting strategy to find cancer specific biomarkers has been to follow the carcinogenic process from pre-malignant lesions to invasive cancer (Rahman et al., 2005). However, this approach is hampered by the extreme difficulty to acquire relevant specimens from patients (De Petris, 2010).

To identify diagnostic biomarker, a proper control group must be chosen. This should be represented by patients affected by benign diseases or by other tumour forms. Healthy donors should be avoided, partly because they do not constitute a population in which the biomarker will be tested once eventually available in the clinic, partly because of the risk to falsely consider as potential biomarkers factors which are instead related to the chronic inflammatory status that is

usually associated to the tumoral disease or co-morbidities. Healthy donors may be used to determine which cut-off limit should be used when comparing the cancer and control groups. One possibility is to accept as limit the 95$^{th}$ percentile in blood donors, supposing that in healthy people the biomarker should be normal (or negative) in at least 95% of cases.

In addition to the control group, the cancer cohort must be carefully chosen. Usually it is composed of patients with advanced disease who have maybe already received some kind of treatment. However, results obtained in such a cohort may not be equally useful in patients with a disease at earlier stages and much smaller tumour burden, who actually also represent the target of most of diagnostic procedures involving biomarkers. The best cohort to perform diagnostic biomarker validation in cancer is represented by patients' candidate to receive surgery. Usually this is a population of relatively young people, without severe co-morbidities and with a relatively small tumour burden. Besides, in such patient population, the kinetic of the biomarker may be also tested after the removal of the tumour lesion and monitored until disease recurrence. The disadvantage is that the risk to obtain negative results is extremely high, due to several issues, as for example excessive dilution of the marker, sample complexity and matrix effects due to high abundant background molecules. Once the cut-off has been defined it is possible to proceed to determine the diagnostic performance of the biomarker. Cancer and control patients correctly diagnosed by the biomarker are true positives and true negatives, respectively, whereas those who have been incorrectly diagnosed are false negatives and false positives, respectively. The percentage of cancer cases and of control cases that are correctly diagnosed represent the sensitivity and specificity of the test, respectively. The percentage of all cases correctly diagnosed is the diagnostic accuracy. In contrast, the percentage of cases with a positive test result who have cancer, and the percentage of cases with a negative test result who pertain to the control group are the positive and negative predictive values (PPV and NPV), respectively. Of course, a test retaining 100% specificity and 100% sensitivity has also 100% NPV and PPV. However, NPV and PPV depend on the relative quantity of cancer and control cases in the study, and ultimately on the prevalence of the disease in the population, provided that the study is properly designed (Altman and Bland, 1994).

In the case that a cohort of blood donors is not available for testing, the biomarker may still be analysed in the two groups (cancer vs. controls), and results plotted into a receiver operating characteristic (ROC) curve, which is a function of logistic regression. A ROC curve can be used to determine the best cut-off, estimated by the index of Younden, which calculates the cut off level that retains the highest sum of sensitivity + specificity – 1. In addition, with the ROC curve one may compare the diagnostic performance of diverse biomarkers, by comparing the area under the curve (AUC) for each biomarker. Finally, it should be considered that the choice of the cut-off may be adjusted depending on the clinical question. In theory the aim of a diagnostic test would be to detect as many tumours as possible, and this may be obtained by decreasing the cut-off and increasing the sensitivity of the test. However, this approach is not feasible because it must be taken into consideration the number of cases that will have a false positive result and for which the use of expensive and sometime invasive procedures needed to make the diagnosis will be totally

useless. The issue of the cut-off definition is actual and concrete in the field of diagnostic biomarkers (De Petris, 2010).

## CURRENT STATUS OF DIAGNOSTIC BIOMARKERS FOR NSCLC

### *Tissue-based candidate biomarkers*

Tissue-based biomarkers that reflect the molecular changes associated with specific histologic subtypes of NSCLC may provide the means to differentiate tumours originating in the lung from metastases from other organ sites. Furthermore, using immunohistochemical profiling of lung cancer tissue markers in conjunction with well-established histologic examination can provide more accurate sub classification of lung malignancies and thus may directly impact the clinical decision making of antitumor therapy. The molecular changes associated with progression from normal to malignant tissue may lead to the discovery of novel markers that can be detected in circulation or other biofluids. A number of immunohistochemical markers are available to distinguish between the diverse lung cancer histology types in case the morphological examination alone may not be sufficient to achieve diagnosis, as it may be the case in cytological analysis. Adenocarcinoma and undifferentiated large-cell carcinoma frequently express TTF-1 and CK7 and are usually negative for p63/ΔNp63 and high molecular weight CKs (CK5/6). Conversely, p63/ΔNp63 and CK5/6 are highly expressed in squamous-cell carcinoma, which show weak or negative staining for CK7 and TTF-1 (Bishop et al., 2012; Rossi et al., 2009). Finally, lung tumours are usually negative for CK20, and this feature may be used to distinguish between metastases from intestinal tumours, which express CK20, and primary lung adenocarcinoma (Kummar et al., 2002). All these biomarkers are currently used in clinical practice.

In contrast, there are currently no validated tissue-based biomarkers for early lung cancer detection. Extensive efforts have been however made for the development of useful and novel biomarkers in recent studies, briefly described here. For more detailed review about this topic, see reference Hassanein et al., 2012, Table 1 and Table 2 (Annex).

Numerous studies have adapted large-scale analytic approaches to profile the full spectrum of molecular aberrations associated with lung cancer malignancy in tumours (Table 1 – Annex). These studies have yielded valuable information that has unravelled several key molecular events of lung cancer tumorigenesis, including mapping the genomic loci associated with high risk of developing lung cancer, hypermethylation of a number of tumour suppressor genes (Anglim et al., 2008; Belinsky, 2004; Richards et al., 2011), regions of chromosomal amplification (Lee et al., 2011), mRNA expression variation (Wilkerson et al., 2010), the differential expression of several microRNAs (Yanaihara et al., 2006), and the proteomic signature of invasive and preinvasive lesions in lung tissues (Rahman et al., 2011; Yanagisawa et al., 2007). Although much of the early biomarker discovery efforts have used fresh-frozen samples as a primary source, acquiring these specimens is costly and laborious. Because surgical pathology specimens stored as formalin-fixed paraffin-embedded (FFPE) blocks are widely available, many researchers are attempting to profile genomic

and proteomic aberrations in such specimens. Some of these aberrations include hypermethylation of genes and microRNA (miRNA) expression (Lebanony et al., 2009), which may be extracted as candidate biomarkers.

***Biofluids-based markers***

The underlying premise of biofluids-based biomarker research is that molecular alterations of tumour cells lead to the synthesis of distinct molecular species that can be detected in biofluids. Biofluids-based detection strategies are an attractive approach for screening, namely due to their ease of acquisition. Biofluids including peripheral blood and its components (circulating cells, plasma, and serum) offer non-invasive access to large quantities of samples available for analysis. These alterations can lead to the generation of disease-specific molecular species such as altered or methylated DNA, overexpressed mRNA, miRNA, or proteins that can potentially be released into the extracellular microenvironment. Therefore, molecular analyses of early-stage lung cancer-related biofluids represent an attractive choice for the discovery and validation of diagnostic biomarkers (Hanash et al., 2008).

Blood is a complex and dynamic medium whose components can reflect various physiologic or pathologic states such as the presence of some cancers. Detectable moieties of the blood are currently the subject of many investigations and include cellular elements such as circulating tumour cells (CTC), cell-free DNA and RNA, proteins, peptides, and metabolites. Changes of the cell-free genomic components of the blood, including DNA methylation (48, 49), DNA amplification, and gene expression (50), have been reported in the circulation of patients with lung cancer (Table 2 – Annex).

Despite the overlap with healthy controls and patients with benign diseases, highly elevated concentrations of cytokeratin 19 fragment (CYFRA 21-1), tissue polypeptide antigen (TPA) and squamous cell carcinoma antigen (SCCA) in non-small cell lung cancer (NSCLC) particularly for squamous cell carcinoma, carcinoembryonic antigen (CEA) and cancer antigen 125 (CA-125) in adenocarcinoma or large cell lung cancer, as well as progastrin-releasing peptide (ProGRP) and neuron-specific enolase (NSE) in small cell lung cancer are suggestive biomarkers for lung cancer (Hassanein et al., 2012).

More recently, miRNAs have also been identified in the blood of patients with lung cancer (Chen et al., 2008). In an effort to test the validity of miRNA as biomarkers able to predict lung tumor development, diagnosis, and prognosis, an extensive miRNA profiling was conducted in paired lung tumor and normal lung tissue and in plasma collected at the time of diagnosis by spiral CT. A signature of 15 miRNAs present in the blood was able to identify subjects at high risk of developing lung cancer in 2 independent cohorts of patient with 80% sensitivity and 90% specificity (Boeri et al., 2011). These results suggest that miRNA expression ratios may be molecular predictors of lung cancer development and aggressiveness and may have clinical implication for lung cancer management in the future. In a separate study, a test included 34 serum miRNAs that could identify

patients with early-stage NSCLCs in a population of asymptomatic high-risk individuals with 80% accuracy (Bianchi et al., 2011).

Finally, the ability to capture and study CTCs is an emerging and interesting development in the field that carries the potential to become a non-invasive tool for early detection and diagnosis of cancer, measuring response to therapy, as well as for understanding the basic biology of cancer progression and metastasis (See Chapter 1.4) (Hofman et al., 2012b; O'Flaherty et al., 2012).

In respect to the discovery of novel biomarkers, my thesis works have identified plasma carbonic anhydrase IX (CAIX), circulating tumour cells and plasma circulating miRNAs as potentially useful biofluids-based markers to diagnose lung cancer (Articles 1, 6, 7, and A2).

**Prognostic biomarkers**

Prognostic biomarkers are correlated with the natural history of the disease, in terms of metastatic potential, likelihood of tumour progression and probability of patient survival, regardless of treatment. When the expression of a protein, gene or other molecules is directly correlated with an aggressive phenotype this may give important information about the biology of the disease and what pathways are activated when the phenotype is more aggressive. In addition, from the clinical point of view, the availability of validated strong and independent prognostic tumour markers may be used to stratify patients in randomized clinical trials aiming at evaluating the effect of diverse drugs. A proper patient stratification would indeed allow the composition of balanced groups, avoiding that cases with a worse prognosis may end up in only one patient group and introduce bias in the final results.

**Benchmarks for clinical utility**

When research is performed on *in vitro* models it is not uncommon to claim that a molecule which is up-regulated when the cells proliferate and express an aggressive phenotype may be a potential prognostic biomarker. However, prognosis is a function of how a disease affects patient survival. In addition, it should be taken into account that patient survival depends upon a number of known and unknown variables, and that statistical methods used to calculate this endpoint require that patients are dead (events), because living cases (censors) are not informative. It is worth noting that in the attempt to standardize the conduction of prognostic marker studies, in 2005 a NCI-EORTC combined working group released a consensus document suggesting guidelines to perform studies on tumor prognostic biomarkers, known as the REporting recommendations for tumor MARKer prognostic studies (REMARK) (McShane et al., 2005a, b). These recommendations are presented in Table 3 (Annex).

The most common strategy currently implemented to evaluate prognostic biomarkers in lung cancer has been to assess tumour expression of the biomarkers and correlate it to patient survival. An alternative approach has been to compare groups of patients with tumours at different clinical

stages of disease, for example tumours without lymph node metastasis (stage I), with tumours that have already metastasized to the lymph nodes (stages II and III). This strategy is based on the assumption that a tumour at a later clinical stage is more aggressive than a tumour that has not yet macroscopically spread to other organs, and that the late stage tumours may express biomolecules that drive the metastatic process (Lehtio and De Petris, 2010).

The survival of different patient groups, expressing high or low levels of a biomarker, may be plotted together in Kaplan-Meier curves, and the difference between the curves can be calculated using several methods, the most common being the log-rank test. This initial univariate analysis provides a first indication about the potential prognostic relevance of a biomarker. However, this is not sufficient to establish that a biomarker has prognostic value, because, beside the biomarker expression, the two populations may have differential survival because of other confounding factors. At this stage, a common multivariate method, named Cox proportional hazard, is used (Hall et al., 1996). This method is in principle a multivariate regression analysis and calculates the relative risk of death (hazard ratio, HR) for one or more groups compared to another group chosen as reference. For example, in a patient cohort, cases with low biomarker expression are used as reference, and the HR for this group (against itself) will be 1. Cases with a high biomarker expression will have a HR <1 if the biomarker is a positive prognostic factor (risk of death lower than the group with low expression) or conversely >1 if the biomarker is a negative prognostic factor (risk of death higher than the reference group). If the 95% confidence interval of the HR does not include the unit the difference between the two groups is statistically significant. After loading all covariates of interest, which may include one or more biomarkers together with other clinical variables, the model will attest what factors are still independently prognostic and, conversely, what factors are not. These methods have been largely implemented in papers 1 to 8 and A2 of the present thesis. To avoid over fitting of the model, i.e., to avoid that the model describes noise or random errors instead of intrinsic relationships, a good rule for multivariate survival analysis is that the number of variables to be included in the model should not exceed 10% of the number of dead cases (events) present in the sample size. This may be a serious problem in multiple biomarker evaluation and in the case where the majority of patients are still alive due to inadequate follow-up (De Petris, 2010).

### CURRENT STATUS OF PROGNOSTIC BIOMARKERS FOR NSCLC

Huge effort has been spent in the past years for the identification of prognostic biomarkers in NSCLC and many potential biomarkers have been discovered, none of which unfortunately achieved sufficient clinical validation. More specifically, several molecules have shown to be prognostic in a variety of clinical lung cancer settings, but none could be established as a stratification factor in prospective clinical trials, and none is used to determine the phenotypic aggressiveness of a tumour.

In fact, the number of retrospective studies assessing the prognostic role of some histological features or circulating biomarkers is so large that for some biomolecules it has been possible to review all results within meta-analyses. This has been the case for p53 protein expression or TP53 gene mutation (Mitsudomi et al., 2000; Steels et al., 2001), TTF-1 (Berghmans et al., 2006), EGFR overexpression (Meert et al., 2002; Nakamura et al., 2006), Ki-67 (Martin et al., 2004), Bcl-2 (Martin et al., 2003), COX-2 (Mascaux et al., 2006), HER-2 (Meert et al., 2003), KRAS mutation or p21 overexpression (Mascaux et al., 2005) and CYFRA 21-1 (Pujol et al., 2004). P53, KRAS/p21, Ki-67 and CYFRA 21-1 have been associated with a worse prognosis; Bcl-2 and TTF-1 tumour expression are related to a better outcome; for COX-2, EGFR, and HER-2 no definite conclusion could be obtained.

The main reasons why results have been inconclusive and not implemented into clinics so far are the non-reproducibility of laboratory data, often obtained with different methods, and the inconsistency of clinical endpoints, including short follow-up time and incomplete clinical data. In addition, even in the case when it was possible to describe a clear prognostic role for a molecule by reviewing all available data, this was usually based on a general lung cancer population, from stage I to stage IV, whereas data were not clear enough to extrapolate results for a specific clinical setting, such as resectable disease only, locally advanced tumours or first-line chemotherapy.

The era of novel OMICS technologies has led to the discovery of an even higher number of potential prognostic markers. The leading role in this field has been played by genomics, and especially by mRNA/miRNA microarray platforms. In a recent review, 16 studies exploring the prognostic role of gene signatures in early-stage NSCLC were summarized (Subramanian and Simon, 2010). The authors conclude that there still is little evidence supporting the clinical use of genomic signatures to prognosticate patient survival in this setting. The most common flaws of those studies were the inadequate adherence to guidelines for tumour sample handling; patient selection based on the availability of tumour samples and not on the clinical question; unclear comparison between the genomic signature and known clinical prognostic factors, such as completeness of surgical resection and tumour size; and lack of external validation. As for this latter aspect, only one out of four gene signatures submitted to external validation proved to have a statistically significant prognostic role in NSCLC, but it was lost after adjustment for clinical covariates (Sun et al., 2008; Tumor Analysis Best Practices Working, 2004).

Although current discovered prognostic biomarkers are not quite ready for clinical use, their discovery and clinical implementation learning curves are paving the way and represent a major step toward biology-based prediction of NSCLC patients' outcome.

In respect to the discovery of novel biomarkers, I have evaluated during my thesis several biomolecules and have identified carbonic anhydrase IX (CAIX) and XII (CAXII), circulating tumour cells, intratumoral CD66b-positive neutrophil-to-CD8-positive T-cell ratio and plasma circulating miRNAs as potentially useful biomarkers to predict the outcome of lung cancer patients (Articles 1-5, 9 and A2).

**Predictive biomarkers**

A biomarker is defined as predictive when the efficacy of a specific therapy on the patients can be predicted using the biomarker. The efficacy can be defined in terms of response to treatment (i.e. tumour shrinkage) or survival benefit from treatment. Such predictive factors can be used to identify subpopulations of patients who are most likely to benefit from a given therapy. Ideally, after testing for a panel of predictive biomarkers for different treatment regimens, one could administer the most effective drug only to those patients who, on the basis of the results from the biomarker analysis, will likely benefit most from that specific treatment. Biomarkers can also be used to predict toxicity and be particularly valuable for choosing between drugs with the same activity, but different toxicity profiles, as it is the case for regimens implemented in lung cancer treatment (Lehtio and De Petris, 2010).

**Benchmarks for clinical utility**

When working on *in vitro* or *in vivo* animal models it is not uncommon to claim to have found predictive biomarkers. For example, if two cell lines harbouring a mutated or a wild type gene, or expressing high levels or low levels of a protein, are treated with a drug or radiation and only one of the two cell lines respond to treatment, it is reasonable to think that such molecular feature could be used as a predictive biomarker of treatment effect.

However, a strict clinical validation is needed for a biomarker to be defined as predictive of benefit from a targeted treatment. Some requirements must be fulfilled in order to determine if the biomarker is really predictive or rather prognostic. Predictive biomarker validation therefore is more complex and requires the same standards of evidence as is needed to adopt a new therapeutic intervention (Mandrekar and Sargent, 2009). This implies that a predictive marker validation is prospective in nature, and the obvious strategy is to conduct a prospectively designed randomized controlled trial to test for a biomarker by treatment interaction. In some instances, where such prospective trial is not possible due to ethical and logistical (large trial and long time to complete) considerations, a well-conducted retrospective validation can also aid in bringing forward effective treatments to marker defined patient subgroups in a timely manner (Mandrekar and Sargent, 2009).

In a single-arm trial, only predictive markers of tumour response can be identified. To identify predictive factors for survival benefit from treatment, an untreated control group should be included in a study, because a good survival identified in a single-arm trial might simply result from prognostic factors within the cohort, and might not be a result of treatment. The strategy most commonly used to discover such biomarkers has been to compare clinical samples from patients responding or not responding to a certain treatment, and then validate the findings on selected cohorts. When exploring predictive biomarkers it is very relevant to have proper control groups, such as patients in a similar clinical condition who receive another treatment or no treatment at all, to define whether the biomarkers are really predictive of treatment effect and not simply prognostic of disease progression (De Petris, 2010).

Predictive biomarker research to individualize the therapy is the most interesting clinical application of biopathology in lung oncology. However, this area is limited by the need for extremely well characterized samples from patients receiving a uniform treatment in a cohort where the effects of therapy in terms of response are accurately monitored. Moreover, when it comes to chemotherapy and biological agents, these are currently most often used in the advanced disease setting, where an adequate amount of tumour material is often not available because the surgical removal of the primary tumour is not performed. Great interest has therefore again been focused on plasma/serum as a source material for such studies.

Other critical components required for the validation of biomarkers include the choice of an appropriate clinical trial design, the choice of an adequate marker assessment method (IHC, FISH, RT-PCR, high dimensional microarray and proteomics-based classifiers, etc.), the reliability and reproducibility of the assay, and the costs involved with assessing marker status (Mandrekar and Sargent, 2010). The ultimate clinical utility of a biomarker hinges on the added value of the marker assessment in every patient in relation to the prevalence of the marker, specifically the incremental benefit of treatment selection based on the marker compared with the added costs and complexity induced by the measurement of such markers. Moreover, the question of the effectiveness of the new treatment in all patients regardless of the marker status (the magnitude of benefit may differ within the marker-defined subgroups) versus just in the marker defined subgroup(s) needs careful consideration (Mandrekar and Sargent, 2010).

### CURRENT STATUS OF PREDICTIVE BIOMARKERS FOR NSCLC

The current interest in predictive biomarker determination in NSCLC was boosted by the discovery of genes that have proven to be of clinical relevance such as the *EGFR* gene mutations and *ALK* rearrangements. Euphoria is now somewhat tempered because the discovery of other "emerging druggable" biomarkers translates rather slowly into clinical applicability. One reason for this is the fact that the course of malignancy is the consequence of a number of essential alterations in tumour cells rather than a single mutation (Hanahan and Weinberg, 2011). In addition, the limited size of most studies and variable techniques used for biomarker determination plays a role.

The most representative cases of predictive biomarkers in NSCLC has already been described (See chapter **1.1.2 The Lung Cancer Molecular Disease Model**). In respect to the benchmarking of predictive biomarkers, I have developed during my thesis several theranostic tests through FISH analysis of *ALK* rearrangement on CTCs (Article 8), IHC anti-EGFR, anti-ALK or anti-*BRAFV600E* mutations on NSCLC tissue (Articles A6-A9).

In addition, targeting DNA repair can be a therapeutic strategy in itself, notably using mechanism-based approaches, such as synthetic lethality, chemo-sensitivity or radio-sensitivity. Several DNA-repair biomarkers, such as ERCC1, BRCA1, RAP80, RRM1, PARP1, MSH2 or DNA-PK, could be used to customize NSCLC therapy and substantially improve patient outcomes. For comprehensive reports,

two excellent reviews have been recently published by S Postel-Vinay, et al. (Postel-Vinay et al., 2012) and by B Besse (Besse et al., 2013).

**Pitfalls and limitations for biomarker identification and clinical validation**

Since the emergence of the so-called OMICS technology, thousands of potential biomarkers have been identified and reported, which have dramatically increased the opportunities for developing more effective therapeutics. These opportunities can have profound benefits for patients and for the economics of healthcare. However, the identification and transfer of biomarkers from discovery to clinical practice is still a process filled with lots of pitfalls and limitations, mostly limited by conceptual problems and practical challenges.

**Conceptual issues**

Central to conceptual issues is indeed heterogeneity which may occur at two levels: one as multiple clones/phenotypes within an individual (such as within a tumour or between several tumours from the same patient—**specimen heterogeneity**), and the other between individuals (**disease heterogeneity**).

In general, cancer pathogenesis is a downhill disruption of cellular differentiation. The disruption involves the alteration of tissue-specific gene expression (Winter et al., 2004). The alterations are reversible in temporary illnesses, and irreversible due to mutations and permanently dysregulated expressions in cancer and degenerative diseases. Difficulty with the discovery of valuable biomarkers is due to the heterogeneous nature of disease initiation and progression where multiple intertwined processes may produce the same disease phenotype and the inclusion within the definition of disease the blurry boundaries between normal and abnormal health conditions. The collection of disease symptoms may be produced by hundreds or thousands of heterogeneous cellular and molecular variations (Maher, 2008). The asynchronous, homoplastic, and heterotachous expression patterns of genes and proteins among diseased specimens further complicate the state of disease heterogeneity (Abu-Asab et al., 2011).

Cancer incipience and progression are driven by random mutations (Heng et al., 2010). This randomness, compounded with selective pressure within tumours, produces intra- and inter-specimen heterogeneity, which in cancer is the basis for selection to maximize the tumour's success (Gerlinger et al., 2012). Heterogeneity is based on a mixture of ''clonal'' (driver) and ''non-expanded'' (passenger) mutations and may be also on non-genetic individuality (Brock et al., 2009; Swanton, 2012). Only clonal mutations are the potential biomarkers, because they systematically characterize a larger number of specimens, whereas non-expanded ones are restricted to fewer specimens and would have limited utility (Abu-Asab et al., 2011).

More specifically, recent comprehensive analyses of NSCLC genomes have revealed a great matter of heterogeneity in the spectrum of mutations and structural alterations within the different NSCLC histology subtypes (Imielinski et al., 2012; Perez-Moreno et al., 2012). Therefore assays of any

single structural alteration or mutation will identify only a fraction of putative biomarkers, necessitating development of multiplex assays to interrogate entire pathways or many chromosome loci. Furthermore, these detection strategies must allow sensitive detection of mutated sequences against a background of wild-type sequences that are found in any clinical sample (Brooks, 2012).

At the cellular level, the network of interactions between the various signalling and regulatory pathways governs cell behaviour. In general, the interactions between the components of the cell network are nonlinear (Faratian et al., 2009). This means that small uncertainties in relationships between pathways and the defining parameters of their interactions leads to disproportionate errors in predicted dynamics (Kholodenko and Kolch, 2008). Given that we will potentially never know the exact details of the network, and that these will vary between patients and at different times in the progression of the disease, it seems improbable for the moment that precise definition of metabolic and signalling pathways in cancer is possible.

While gene expression profiling has revealed hundreds or even thousands of genes expressed at higher levels in malignant compared with benign tissues, virtually no transcripts or proteins have been identified that are uniquely elevated in cancer. Many candidate biomarkers belong to pathways intrinsic to normal cells and tissues, such as those mediating proliferation, apoptosis, differentiation, angiogenesis, cell death, and inflammation (Hanahan and Weinberg, 2011). Moreover the complex mixture of normal and pathological cell types requires a careful histopathological analysis as part of the research project to ensure that cells are accurately identified (Bova et al., 2005). Specifically, histopathological evaluation helps to identify the presence and amount of tumour cells, including type, patterns of invasion, and grade, as well as viability (vital vs. necrotic), and the presence and amount of other cell populations such as inflammatory cells and fibroblasts (Rodriguez-Canales et al., 2011). Some biomarkers have failed because their cognate protein levels, which are the preferred analyte for most clinical assays, do not correlate with transcript levels. Other candidate transcripts or proteins show only a relative increase in expression in cancer compared with normal tissue and therefore fail as biomarkers because low-level expression from the parent normal tissue, from other organ sites, or from non-malignant pathologies effectively drowns out the signal from the malignancy. Candidate biomarkers expressed in the nucleus or cytoplasm are not accessible to clinical assays since most biomarkers currently in use are cell surface or secreted proteins (Brooks, 2012).

The phenotype of a tumour is not governed only by the epithelial component but also by the tumour environment, that is, other cells in contact with the tumour, the mesenchyme, and the inflammatory infiltrate (Egeblad et al., 2010; Fridlender et al., 2009). Indeed, an important body of knowledge has been obtained via the study of cells retrieved from patients with cancer and grown in the laboratory, i.e. cell lines (Borrell, 2010). As a consequence, the study of tumours in their natural environment is of vital importance in cancer research and is a valuable complement to studying cancer cell lines and other *in vitro* model systems (Figure 7).

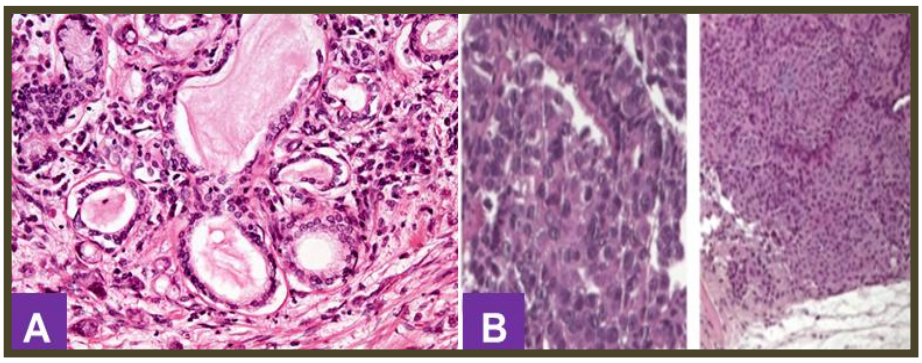



**Figure 7.** Comparison of phenotypic features of cancer cells in their natural environment versus a xenograph implanted into a mouse. **A)** Acinar lung adenocarcinoma, a common histological subtype of NSCLC. The tumour epithelial cells are in intimate relation with the stroma that is composed of multiple non-tumour cell types including blood vessels, fibroblasts, and inflammatory cells. **B)** Morphological phenotype of human lung adenocarcinoma isolated and cultured as a cell line, and subsequently implanted as a xenograft into a mouse. The morphology of this model system is distinctly different from that observed in patients; here the adenocarcinoma cells grow as solid sheets, showing higher atypia, increased mitotic activity, and a different shape and size than in their natural tissue environment. The functional structure of the tumour microenvironment is lost in this model.

These components determine the net inputs to the cell, which include ligands, cell–cell adhesion molecules, metabolites, oxygen, and drugs. It is important to know how to characterize and quantify the spatial heterogeneity at a given time to inform our understanding of the dynamics. This includes the collection and interpretation of measurements of both the intracellular and extracellular environment. On a finer scale, the intracellular regulatory network is imbedded in a complex spatial context within the cell. Many key processes occur on the cell surfaces or across membranes and this spatial compartmentalization within cells has a profound effect on the dynamics of signalling within cells and, therefore, their response to the environment. Relatively little information is available on the spatial organization of key proteins within cells, although new imaging techniques offer the potential of high-resolution measurements of the spatial-temporal dynamics of large numbers of proteins (Cohen et al., 2008; Faratian et al., 2009).

**Practical issues**

Tissue specimens are an important resource for both primary research efforts and for validation of biological findings that are made in the laboratory (Compton, 2007). However, the reliability of biomolecule analysis on tissues presents major challenges to investigators.

The first is **sample collection**, including the ethical and legal rules that protect patient privacy and confidentiality. Tissue samples obtained through biopsies and surgical resections from patients, or even at autopsy, have a diagnostic priority. Clinical specimens can be used for research only when: (i) the patient consents after being carefully informed about the research study, (ii) the planned studies have been officially approved by an Institutional Review Board (IRB), and (iii) the clinical analyses of the specimen have been fully completed. As a practical matter, the acquisition of tissue samples is best facilitated in centres where biological researchers and pathologists interact as a multidisciplinary team and can closely coordinate their efforts (Ilie and Hofman, 2012).

A second challenge is the **sampling** of the tissue for histology and research. After the removal of a tissue specimen from a patient, a gross inspection first needs to be performed. The pathologist ensures that appropriate processing and sampling of the specimen is completed for histological examination and potential molecular testing (Bova et al., 2005). Since tissues and organs that contain tumours are highly heterogeneous with distinct areas of cancer, necrosis, inflammation and normal tissue, gross inspection, and precise sampling are essential for accurate diagnosis. One sampling variable of particular interest is the time of ischemia, which can profoundly affect the nucleic acids integrity. This includes the time of warm ischemia (from the time of blood vessel ligation to surgical excision) and the time of cold ischemia (from excision to freezing or fixation). Ideally, the length of both times should be kept to a minimum and both should be recorded (Riegman et al., 2008). Thus, good coordination between the surgeon and the pathologist can help to reduce the time of cold ischemia, i.e. after the tissue sample is removed and before fixation, resulting in better molecular preservation (Riegman et al., 2008).

A third challenge is the selection of the proper **preservation** technique for the tissue specimen (Ilie and Hofman, 2012). Importantly, preservation is an ‘‘irreversible step’’ that will determine the usefulness of the specimen for histopathological diagnosis and subsequent molecular testing and research (Aisner and Marshall, 2012). The two primary preservation techniques are chemical fixation, usually with a formalin solution followed by paraffin wax embedding, and snap freezing. The microscopic examination of formalin-fixed, paraffin-embedded tissue samples thin-sectioned onto glass slides and then stained with haematoxylin and eosin (H&E) is the gold standard diagnostic technique for surgical pathology (Rosai, 2007). Unfortunately, formalin induces significant chemical alterations that lead to loss of integrity of the biomolecules in the specimens (i.e. integrity of the RNA and (phosphoproteins) and compromises many downstream molecular tests (West et al., 2009). Alternatively, snap-freezing tissue samples better preserves the integrity of tissue biomolecules; however, frozen tissues require special conditions for storage and handling, and also result in lower histological quality, thus the pathological evaluation of frozen sections can be difficult for many diseases. Cancer biomarker development will therefore not come of age until the value of fresh tissue collection is engraved in the minds of a new generation of molecular pathologists. In the meantime, existing technologies can be optimized to extract as much relevant biomarker information as possible out of FFPE-fixed tissues (Bernards, 2010).

The potential use of biomarkers is also highly dependent on the **accessibility** of the biospecimens. The development of tests for biomarkers requires access to biological resources where samples are carefully processed, stored and documented. Information on the patients and access to anonymous medical records of the donors are needed. If biobanks are flowed with extensive data, appropriate softwares are required to support the capture of these datasets. Beyond the issue of information, the problem is that most biobanks provide samples for research projects that were not initially defined. Therefore good practices requirements should be met and standardisation is deemed necessary (Cambon-Thomsen et al., 2011; Hofman et al., 2012a).

Biomarker discovery relies heavily on **data acquisition and analysis** and the amount of medically relevant data that are available electronically increases dramatically. However, these data are generated in complex forms and are acquired and recorded in various ways. Incompatibility among research databases, electronic medical records and laboratory information management systems is then an issue. The challenge is to organise electronic data and to make them usable for research. Open and standardised databases are required to make use of all available data in order to identify stratification biomarkers and to stratify clinical trial recruitment (Rodriguez-Canales et al., 2011).

Another challenge is to deal with the **management** of large amount of **concomitant and complex data** generated by OMICS, phenotyping and imaging technologies (multiple readouts). Traditional statistical methods are based on single signal assays. There is a need to implement new statistical methods to cope with multi-signal assays

Although biomarkers can be identified in clinical phases, the discovery of new biomarkers is often ground in knowledge generated by academic basic research. A better understanding of disease mechanisms is crucial in this respect. It is obvious that neither the pharmaceutical industry nor the diagnostic manufacturers can support alone the continuum that leads to stratified medicine. Therefore, a stronger **cooperation between public and private** sectors is needed.

Furthermore, on the basis of grading and staging, the rate of proliferation and the invasiveness of tumours are major determinants of prognosis. The behaviour of cancer is most often characterized pathologically in terms of **data collected** at a **single time point** (for example, from a treatment-naïve diagnostic biopsy sample or resection specimen) or at relatively few time points during *in vitro* cell-line studies. These data are considered with other data such as imaging and clinical staging. In essence, clinicians infer underlying dynamics from single, or at best relatively few, snapshots of the system.

In addition, most early stage (phase II) clinical trials, in which biomarkers are typically discovered, enrol patients with **metastatic disease**. The number of genomic changes in these advanced cancers is extraordinary high, making it difficult to identify critical early changes that could be used as diagnostic biomarkers. To be meaningful in a screened population, diagnostic biomarkers must be discovered in early-stage, non-metastatic cancers since biomarker expression can change over the course of a disease (Brooks, 2012).

The use of tissue samples can also affect the performance of prognostic biomarkers, leading to subsequent failure to validate the biomarker in another patient population (Ioannidis and Panagiotou, 2011). Quite commonly, cancer prognostic biomarkers are tested in patient samples from cases with early treatment failure or death, and these are compared with cases without recurrence many years after their treatment. This design, however, pits the worst cases against the very best. Clinical practice encompasses patients with a spectrum of risk, and biomarkers developed on samples from the tails of the bell-shaped curve are destined to fail. Therefore, development and validation of biomarkers need to be performed in the context of a discretely defined clinical question, with appropriately selected patients and adequate statistical power.

Working on patients with metastatic cancer often limits access to tumour tissue through biopsies due to the anatomical locations of the metastases. A potential solution is the use of **non-invasive technologies** to assess tumour biomarkers. For example, collecting **CTCs** can be an alternative to tissue sampling, acting as a "liquid biopsy", and can also provide real-time information about the disease but the numbers of cells present in the circulation is often very small (Hofman et al., 2011c; Nieva and Kuhn, 2012). Alternatively, biomarkers can be assessed through the analysis of biomolecules in **fluids** (i.e., plasma, serum). The advantage of using serum over plasma is its reduced complexity and lower protein concentration, however the clotting process is not uniform, unlike the preparation of plasma, and interesting proteomic and metabolomic features may be lost with the insoluble blood clot. Additionally, an array of proteases are activated when clotting occurs, generating degradation products, meaning that the protein profile of serum is quite different from that of plasma. As a consequence of this the Human Proteome Consortium has recommended that plasma is the fluid of choice, as opposed to serum, for proteomic studies (Omenn et al., 2005). Plasma potentially contains elements of all proteins in the body meaning plasma has a high potential for biomarker discovery. Interestingly, recent studies have demonstrated that genomic alterations in solid cancers can be characterized by massively parallel sequencing of circulating cell-free tumour DNA released from cancer cells into plasma, representing a non-invasive liquid biopsy (Forshew et al., 2012).

Difficulty in locating **single biomarkers** is giving way to **profiling** as an alternative to single biomarkers for prognostic and predictive purposes. Small tumours may release low abundance proteins in the blood that are outside of the dynamic range of detection of most conventional assays, but they also alter the expression pattern of normal proteins; profiling of the latter change may therefore be more useful as early disease biomarker than measurement of a single circulating factor that cannot reliably distinguish between individuals with and without cancer (Oved et al., 2009). Similarly, profiling of gene expression on the basis of two to several thousand genes provides diagnostic, prognostic, or predictive information about tumours (Chen et al., 2011; Thomas et al., 2013).

The selection of useful biomarkers must be carefully assessed and depends on different important parameters, such as on **sensitivity** (it should correctly identify a high proportion of true positive rate), **specificity** (it should correctly identify a high proportion of true negative rate), **predictive value** etc. Unfortunately, biomarkers with ideal specificity and sensitivity are difficult to find (Drucker and Krapfenbauer, 2013). To improve sensitivity and specificity, there are different strategies as follows: (i) improve the assay (e.g. antibody with a higher specificity and/or in combination with detection conjugate with a higher sensitivity), (ii) combine several markers, (iii) check for subpopulations and stratify population (e.g. matched by gender, age, pathology) (Drucker and Krapfenbauer, 2013). Moreover, the **method** used to measure the biomarker must be **standardized** and **reproducible**.

Quantification issues, as well as the determination of a cut-off value which discriminates biomarker-positive vs. biomarker-negative cases may be troublesome even in commonly used methods, such as IHC.

In addition, a characteristic of high throughput technologies is the discovery of biomarkers based on the combination of different analytes instead of single molecules. In such a case, an algorithm based on all interesting variables useful to correctly define biomarker-positive vs. negative samples must be developed before proceeding to clinical validation. To obtain a reliable algorithm, early during the discovery phase over fitting of data should be controlled by proper multivariate data analysis, such as Principal Components Analysis (PCA).

Furthermore, for an analyte to be defined as a biomarker and be introduced in the clinical practice, it is essential that it successfully undergoes clinical validation. A high number of potential biomarkers are indeed discovered daily, which do not reach clinical practice because of insufficient validation.

For instance, patients for biomarker studies should be carefully selected by a specialist (e.g. oncologist for cancer studies or a pathologist for tissue samples) to insure the **presence or absence of diseases**. Unfortunately, predictive curve values of biomarkers with no or less overlapping of diseased vs. non-diseased cohorts are difficult to find. There exist always more or less overlapping areas between healthy and diseased cohort. The overlapping area allows the analyst to calculate the proportion of patients whose diagnosis was correctly predicted by the model (true positives for sick patients and true negatives for healthy patients) or false negative or false positive values (Waerner et al., 2010). Generally, the number of patients and control subjects in published studies is very small to give an acceptable statistical value. Also, many of the potential proposed markers have not been confirmed or validated in a high-quality manner. Body fluids and tissues are collected from a group of patients of different disease stages, and results are compared with a group of healthy persons. The effect of a disease stage on sensitivity of a single biomarker should be taken into consideration because sensitivity improves with increase in disease stage (Drucker and Krapfenbauer, 2013).

The **number of samples** in the diseased and healthy control groups in order to be compared with a variety of analytical approaches is often insufficient. A minimum of 15 samples in the discovery phase is necessary to get a reasonable representative selection basis for marker candidates. In addition, an unbiased genome-wide search for association between gene expression and disease outcome requires at least 40 patients whose cancer responds to treatment and an equal number of non-responders, as smaller sample sizes will lead to spurious associations between gene activity and disease outcome. If the number is limited for practical reasons (resources, cohort and time lines), then the observed differences between the two sets of specimens are in danger of being over-interpreted when extrapolated to generalised cohorts (Bernards, 2010; Drucker and Krapfenbauer, 2013).

Finally, the identification, qualification, and application of diagnostic, prognostic and predictive biomarkers remain the “holy grail” of the biopathology field. Despite the setbacks, the quest for biomarkers goes on and the expectations are still holding. Researchers keep a watchful eye for any gene, protein, or metabolite expressions that could serve as biomarkers indicative of early disease phenotypes and sub phenotypes, or predictive of disease progression and outcome. More highly desirable are biomarkers that can be tagged to drug targets and therapy.

## 1.3 Hypoxia targets as biomarkers

### THE HYPOXIC TUMOUR PHENOTYPE

Oxygen is a vital part of human metabolism as the presence of oxygen enables the cells to retrieve around 16 times more energy out of glucose than what is otherwise possible under anaerobic conditions (Brahimi-Horn et al., 2007). Ambient air contains 21% O2 (150 mm Hg). Most tissues are at 2-9% O2, with tissue hypoxia usually defined at ¤2%. In lung tumours, median tumour oxygen tension has been measured at 2.2% (range 0.1-6%), indicating hypoxia to be a prevalent feature (Le et al., 2006).

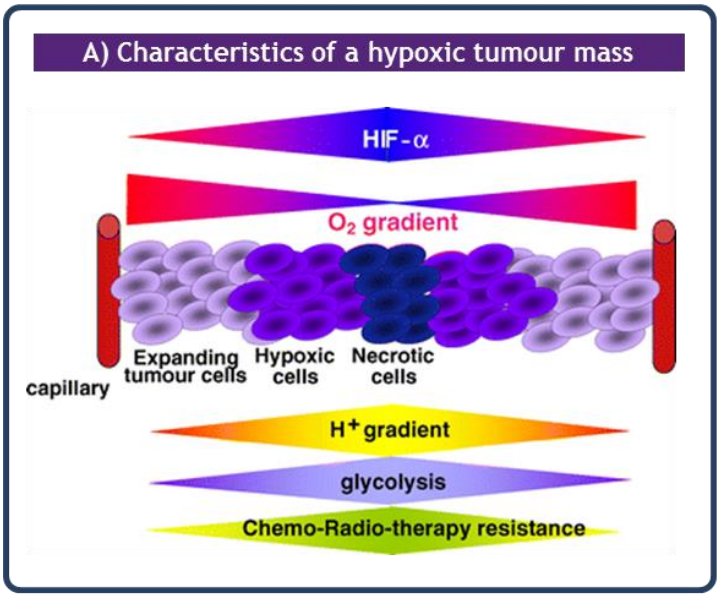
Robust tumour growth requires the presence of a local vascular network that supplies both oxygen and nutrients to tumour cells. Initially, tumour hypoxia arises because of oxygen diffusion limitations in avascular primary tumours or their metastases, but the tumour microvasculature (induced in part as a response to this hypoxia) is highly abnormal and often fails to rectify the oxygen deficit (Jain, 2005; Pries et al., 2009). This persistent hypoxia reflects the spatial disorganization of tumour vascular networks, leading to inter-capillary distances that are often beyond the diffusion range of oxygen (which is up to ~200 μm, depending on the local oxygen concentration in blood plasma) (Figure 8) (Wilson and Hay, 2011). On histological examination, tumours often show a central core of necrotic cells, which has been suggested to result from a drop in the oxygen availability to conditions of severe hypoxia and glucose deprivation resulting in cell death (Figure 8).

In addition to this diffusion-limited hypoxia, temporally unstable blood flow in tumour micro-vascular networks also leads to fluctuating perfusion-limited hypoxia (Dewhirst et al., 2008). The expanding tumour mass distances certain tumour areas from local blood vessels, thereby limiting the oxygen supply. Additionally, at later stages of tumour progression, when a tumour establishes an extensive vasculature to increase oxygen supply, the defects in blood vessel structure contribute to re-oxygenation of certain tumour areas (Carmeliet, 2005).

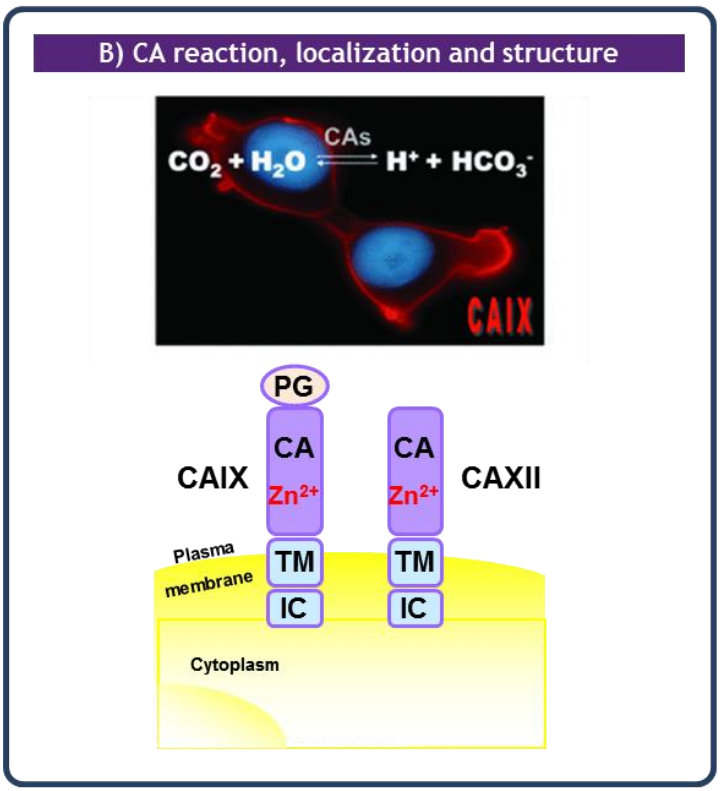
**Hypoxia-inducible factor is the molecular key to hypoxia**

Hypoxia activates an alpha/beta heterodimeric transcription factor termed appropriately the hypoxia-inducible factor 1 (HIF-1). Activation resides in the inhibition of posttranslational hydroxylation of the alpha subunit that permits stabilization, heterodimerisation and binding to hypoxia-response elements (HRE) in target genes. Posttranslational hydroxylation by oxygen-dependent oxygenases, prolyl hydroxylase domain proteins and factor inhibiting HIF (FIH) destabilize and inactivate, respectively, HIF-1α; the former, by favouring von Hippel-Lindau (VHL) E3 ubiquitin ligase-mediated proteasomal degradation, and the latter, by inhibiting interaction with co-activators such as p300/CBP (Brahimi-Horn et al., 2007).

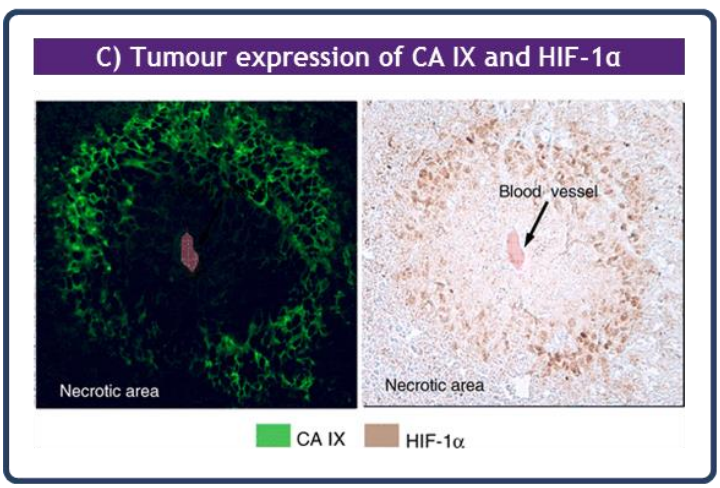
## Figure 8




**A)** Blood capillaries carry oxygen to tissues, but since oxygen has a diffusion limit, its concentration decreases as the distance from capillaries increases. Macroscopic examination of solid tumours reveals the presence of expanding tumour cells in proximity to capillaries and a central region of necrotic cells. This gradient of cell viability parallels that of a decreasing gradient of oxygen, which is accompanied by an increase in HIF-1α levels, a decrease in the extracellular pH and an increase in the resistance to radio- and chemo-therapy.




**B)** CAs catalyse the reversible hydration of $CO_2$ to $H^+$ and $HCO_3^-$. CAIX and CAXII are cell membrane located, as demonstrated by immunofluorescence of CAIX-expressing cells. Domain organization of the membrane-bound hypoxia inducible CAIX and CAXII: PG proteoglycan-like domain, CA catalytic domain, TM transmembrane domain, IC intracellular C-terminal tail.




**C)** Immunohistochemical detection of CAIX and HIF-1α colocalized in hypoxic regions of a section of a human colon adenocarcinoma (LS174) grown in nude mice. Note the hypoxic gradient that develops away from the blood vessel and the necrotic area around the most hypoxic ring.

Adpated from Chiche J, *et al*. 2010.

Hypoxia, through the action of HIF-1α and its targets, influences many aspects of the biology of tumours. The effects of hypoxia on tumour biology include: the anabolic switch in central metabolism, pH regulation, adhesion extracellular matrix remodelling, selection of genotypes favouring cell survival under hypoxia–re-oxygenation injury; pro-survival changes in gene expression that suppress apoptosis and support autophagy. Hypoxia also enhances receptor tyrosine kinase-mediated signalling, tumour angiogenesis, the epithelial-to-mesenchymal transition, induction of CTC resistance to anoikis, invasiveness and metastasis, as well as suppressing immune reactivity. In addition, hypoxia contributes to loss of genomic stability through the increased generation of reactive oxygen species (ROS) and the down-regulation of DNA repair pathways (Azab et al., 2012; Cairns et al., 2011; Pouyssegur et al., 2006; Semenza, 2000; Wang and Ohh, 2010; Yotnda et al., 2010).

### THE HYPOXIA-INDUCED MEMBRANE-ASSOCIATED CARBONIC ANHYDRASES ARE KEY ENZYMES INVOLVED IN PH HOMEOSTASIS, CELL SURVIVAL AND MIGRATION IN A HYPOXIC/ACIDIC MICROENVIRONMENT

As already stated, increased metabolic acids (carbonic and lactic acids) are produced and excreted resulting in acidification of the microenvironment (pHe) of a tumour (Brahimi-Horn and Pouyssegur, 2007; Cardone et al., 2005; Chiche et al., 2009; Gatenby and Gillies, 2004). This poses a stress on the cells in that the extracellular acidification can ultimately lead to a decrease in pHi. However, pHi must be maintained within a narrow range to prevent disruption of basic cell functions including membrane permeability, enzyme activity, cellular metabolism, ATP maintenance, cell proliferation, and apoptotic mechanisms among others (Brooks et al., 2005). A variety of pH measurement techniques have demonstrated that cancer cells maintain a more alkaline intracellular pHi compared to other non-cancerous cells despite their acidic surroundings (Gillies et al., 2002). This property is even more apparent in more aggressive tumours and the reversal of the pH gradient in tumour cells (high pHi vs. low pHe) compared to normal cells is one of the main defining characteristics of tumour cells (Cardone et al., 2005). Consequently the current consensus in the literature is that cancer cells possess efficient membrane transport machinery that extrudes $H^+$ and imports $HCO_3^-$ to maintain an elevated pHi. The overall pHi regulating mechanism may include the Na+/H+ exchanger 1 (NHE1), $Cl^-/HCO_3^-$ exchangers (CBEs), $Na^+/HCO_3^-$ co-transporters (NBCs), $H^+$/lactate co-transporters (monocarboxylate transporters, MCTs), and CAIX and CAXII working in a co-ordinated fashion (Brahimi-Horn et al., 2011; Cardone et al., 2005; Parks et al., 2011; Pouyssegur et al., 2006). It has been proposed that tumour cells develop an enhanced acid resistance to survive in the microenvironment where normal cells will die (Fang et al., 2008).

The CAs are a family of zinc metalloenzymes that reversibly catalyse the hydration of cell-generated $CO_2$ to $H^+$ and $HCO_3^-$ ions (Figure 8) (Supuran, 2008). Thirteen active and three inactive isoforms of CAs are expressed in mammalian cells. The active isoforms contain a conserved active site and variable levels of expression and activity. They differ in their tissue distribution and cellular localization. CA isoforms such as CAIX and CAXII are plasma-membrane localized and have been identified to possess an extracellular catalytic site (Figure 8). The mature CAIX protein is a

unique member among the CAs since it contains a proteoglycan-like (PG) domain, a CA domain (CA), a small transmembrane domain (TM) and a short intracellular C-terminal tail (IC) (Pastorek et al., 1994). In contrast CAXII and the other CAs lack the PG-like domain (Figure 8). In multiple epithelial tumour types, only the expression of the membrane-associated CAIX and CAXII is controlled by oxygen levels through a HIF-1-mediated mechanism and only the two hypoxia-induced CAs, CAIX and CAXII, have been proposed to contribute to tumour pH homeostasis based on the catalytic reaction (Chiche et al., 2010; Wykoff et al., 2000).

### CAIX REGULATION AND EXPRESSION

The predominant full-length carbonic anhydrase 9 (*CA9*) was cloned in HeLa cells, in the 1990s, before the discovery that it was one of the most highly hypoxia-induced genes (Opavsky et al., 1996; Pastorek et al., 1994). In addition, recent RT-PCR detection has revealed an alternative spliced variant, lacking exons 8/9 among 11 exons, which is constitutively expressed, that does not depend on hypoxia (Barathova et al., 2008). Chromatin immunoprecipitation assays have shown that both HIF-1α and HIF-2α can bind to the hypoxia responsive element (HRE) of the full-length *CA9* [148]. However, *CA9* has been identified as a specific HIF-1-induced gene, while it fails to be expressed in cells in which hypoxia is not mediated by HIF-1 or when the *CA9* promoter is methylated (Dayan et al., 2006; Jakubickova et al., 2005). The mechanism of such a level of selectivity remains unclear, but it was suggested that the *CA9* promoter is the most sensitive sensor of HIF-1 activity (Kaluz et al., 2009).

Oncogenic pathways also contribute to *CA9* regulation. Inhibitors of the ERK pathway down-regulate CAIX expression; however, in HeLa cells, CAIX expression and ERK activity were inversely correlated (Kaluz et al., 2006; Kopacek et al., 2005). The MAPK activity may influence *CA9* transcription by an increase in HIF-1 activity indirectly through p300/CBP or SP1/SP3 (Kaluz et al., 2006; Sang et al., 2003). The regulation of *CA9* by others environmental factors such as acidosis, glucose deprivation or bicarbonate depletion with the concurrent activation of HIF-1 has also been reported (Rafajova et al., 2004).

Under physiological conditions, the expression of CAIX is essentially restricted to a few tissues including the gastric mucosa, biliary tree, crypt cells of the duodenum, and the epithelium of the small intestine (Ivanov et al., 2001). CAIX is expressed during embryonic development as shown in the mouse, but is more highly expressed in many cancers such as oligodendroglial brain tumours, colorectal cancer, ovarian tumours, gastric cancer, pancreatic cancer, breast cancer associated with c-erbB2 overexpression, and NSCLC (Chiche et al., 2010; Ilie et al., 2010; Kallio et al., 2006).

In most cases, CAIX expression correlates with the pattern of HIF-1α expression in cancers in hypoxic/perinecrotic regions of the tumour, distant from the blood vessel (Figure 8) (Chiche et al., 2009). However, exceptions have been observed where expression of CAIX is detected in the absence of HIF-1α staining (Mayer et al., 2005). These observations could be interpreted as the existence of dynamic cycles of acute hypoxia/ rapid reoxygenation within the tumour, and the short

half-life of HIF-1α compared to CAIX where the latter is highly stable once induced (Rafajova et al., 2004).

### CAXII REGULATION AND EXPRESSION

The cDNA of *CA12* was cloned in 1998 from renal tumours (Tureci et al., 1998). Its chromosomal localization mapped differently to that of *CA9*. The *CA12* gene is also induced in hypoxia, via a HIF-1-dependent mechanism (Chiche et al., 2009; Ivanov et al., 2001). However, the HRE of *CA12* has not been identified. The fold induction of *CA12* is lower than that for *CA9* because the basal level of *CA12* mRNA and protein is higher than for *CA9* in tumour cells in normoxia. *CA12* mRNA is up-regulated in VHL-defective renal tumours due to constitutive stabilization and activation of HIF-1 (Ivanov et al., 1998). In breast cancer, oestrogen receptor (ER-α) activation leads to transcriptional up-regulation of *CA12* via a hormone-responsive enhancer (Barnett et al., 2008). An alternatively spliced variant of *CA12*, lacking an exon, was detected in infiltrating astrocytomas (Haapasalo et al., 2008). The shorter form that was found to predominate in brain tumours was lacking residues that are located in the transmembrane domain. Thus, this spliced isoform may affect the quaternary structure of dimeric CAXII and signalling pathways involving protein kinase C and A, due to disruption in a potential phosphorylation site of these kinases in the C-terminal tail of CAXII. CAXII is a 39 kDa protein with a conserved Zn-binding catalytic site, a transmembrane domain and a short intracellular C-terminal extension containing potential phosphorylation sites for casein II kinase, protein kinase C, and c-AMP-dependent kinase (Figure 8) (Tureci et al., 1998). Unlike CAIX expression the endogenous expression of CAXII is less tissue specific and is not only restricted to normal epithelia. CAXII is expressed in a variety of normal tissues but its expression becomes stronger in tumours. CAXII expression was found in normal human pancreas, kidney, gut and the gastrointestinal tract (Hynninen et al., 2006; Kivela et al., 2000; Kivela et al., 2005). CAXII was originally identified as a protein overexpressed in renal cancer cells, but it is also known to be overexpressed in different human cancers, such as diffuse astrocytomas and colorectal, gastrointestinal, breast, pancreatic, ovarian, and renal carcinomas (Haapasalo et al., 2008; Hynninen et al., 2006; Kivela et al., 2000; Kivela et al., 2005; Parkkila et al., 2000; Wykoff et al., 2001).

### THE ACTIVITY AND FUNCTIONS OF CAIX AND CAXII

The two hypoxia-inducible CAIX and CAXII are not highly homologous in their protein sequence (30% sequence homology only), but they share the same catalytic site with the three conserved zinc-binding histidine residues. Recent biochemical characterization of the hypoxia-induced CAIX showed it was the most active carbonic anhydrase isoenzyme with a surprisingly high CA activity, which was associated with the presence of the PG-like domain of CAIX (Hilvo et al., 2008). However, the mechanism by which the PG-like domain contributes to an increase in the $CO_2$ hydration activity of the CA is still unknown. It was suggested that metal ions, such as $Zn_2^+$ might bind to the catalytic site and probably relieve the electrostatic repulsion of the PG-like domain. CAIX was also shown to have a higher activity for $CO_2$ hydration in an acidic environment (around pHe 6.5) compared to in a neutral environment (pHe 7.4) (Chiche et al., 2010; De Simone and Supuran, 2010). The activity of

CAXII was reported to be moderate compared to that of CAIX (Pastorekova et al., 2008b). One of the major challenges in the study of HIF-induced CAs is to understand the advantage tumour cells derive from overexpression of enzymes that catalyse an already spontaneous reaction that hydrates $CO_2$ to generate $H^+$ and $HCO_3^-$. CAIX expression was clearly shown to contribute to extracellular acidification (decreased pHe) of tumour cells in hypoxia, in a way that does not depend on lactic acid production (Svastova et al., 2004). Moreover, the expressed CAIX seems functionally active only when cells were exposed to a hypoxic environment (Pastorekova et al., 2008a).

Several studies have shown that soluble CAIX is being shed from the tumour cells into the culture medium and plasma or urine of renal cancer patients. This corresponds to the extracellular part of the CAIX molecule, which is composed of the proteoglycan (PG)-like and CA domain that are cleaved off the plasma membrane (Hyrsl et al., 2009; Zavada et al., 2003). Our group was the first to evaluate the value as prognostic factor of CAIX plasma level in patients with NSCLC (Ilie et al., 2010).

While the role of CAIX in tumour pH homeostasis has been largely investigated, the contribution of CAXII was not examined until recently. A conjugated role of CAIX and XII in tumour pHi regulation and tumour growth has been recently suggested. Knockdown of *CA9* did not change the rate of catalysis of acidification and pHi regulation in an acidic environment due to compensation by CAXII expression (at the mRNA and protein level) and activity in hypoxia (Chiche et al., 2009). Even in absence of changes in pH (pHe and pHi), silencing of *CA9* reduced spheroid and tumour growth in nude mice as shown in *in vitro* cell proliferation assays in bicarbonate-containing media (Chiche et al., 2009; Robertson et al., 2004). Combined silencing of both membrane-associated hypoxia-inducible CAIX and CAXII led to a reduction in extracellular acidification and pHi regulation, which correlated with a dramatic decrease in the rate of xenograft tumours growth (Chiche et al., 2009). Both CAIX and XII play a key role in pHi regulation in hypoxia by contributing to removal of metabolically generated $CO_2$. Thus, such regulation is a key event controlling cell viability, and *in vivo* tumour growth in a hostile acidic and hypoxic microenvironment. The possible implication of these CAs as signalling molecules, independent of their function as pH-regulating enzymes, is another important point that has not been extensively investigated and may explain the partial decrease in xenograft growth of *CA9* silenced cells despite maintenance of the CA catalytic activity due to up-regulation of CAXII. Signalling through the EGF pathway by phosphorylation of a cytoplasmic tyrosine residue of CAIX may either activate CAIX or enhance its expression by increasing translation of HIF-1α (Dorai et al., 2005). In addition, phosphorylation activates PI3-K resulting in phosphorylation of Akt and cell survival. CAIX expression was shown to interact with ß-catenin and thus was suggested to play an important role in the modulation of E-cadherin mediated cell adhesion [209]. When renal carcinoma cells were treated with acetazolamide, an inhibitor of carbonic anhydrases, their capacity to invade was diminished (Svastova et al., 2003). In addition, CAIX expression was strongly associated with the development of metastasis in patients with cervical tumours, while CAXII expression was not associated (Robertson et al., 2004).

Accordingly, CAIX has been proposed as a biomarker of an aggressive malignant phenotype of solid tumours, since a strong link between its expression and poor patient survival has been established by many groups (Bui et al., 2003; Choi et al., 2008; Hedley et al., 2003; Klatte et al., 2009; Korkeila et al., 2009; Trastour et al., 2007). Conversely, the impact of CAXII on survival is less obvious and tissue-dependent, with CAXII tumour expression being associated with either poor survival in infiltrating astrocytic gliomas or with better outcome in invasive breast carcinomas or NSCLC (Haapasalo et al., 2008; Ilie et al., 2011; Watson et al., 2003). It is interesting to note that the CAXII in astrocytomas is an alternatively spliced shorter variant with eleven fewer amino acids than CAXII in other tissues raising the possibility that this CAXII functions in a different manner (Haapasalo et al., 2008). These data seem to contradict the experimental data discussed above. In fact this contradiction is only on the surface. If CAIX and CAXII share almost identical pH-regulatory functions, their expression in tumours is strikingly different. Indeed our study of a large cohort of patients has revealed that CAXII is a marker for good prognosis in resectable NSCLC whereas expression of CAIX in this cohort was associated with a very poor prognosis (Ilie et al., 2010; Ilie et al., 2011). The interesting point is that the 19% subset of NSCLC that overexpress CAXII is associated with low grade and well-differentiated features, a characteristic that contrasts with the subset of tumours expressing strongly CAIX. We believe that the expression of CAIX, but not of the more promiscuous CA XII, gives a selective advantage for tumour survival that occurs only when the tumour has reached an advanced grade of dedifferentiation. Before this stage hypoxia cannot ‘‘turn-on’’ the normally repressed *CA9* gene. These latest aspects on the dual prognostic value of CAIX and CAXII were analysed in Articles 1, 2 and 3 of this thesis.

## 1.4 Cells as biomarkers – Circulating tumour cells

Cancer-related death is in general the consequence of tumour cells spreading from the primary tumour and forming metastases in resident organs (Joosse and Pantel, 2013). In routine cancer diagnostics by histopathology and high-resolution imaging technology, it remains unclear whether early tumour spread has taken place until the manifestation of overt metastasis. Therefore, administration for systemic adjuvant therapy for cancer metastasis intervention is presently based on personal statistical risk, resulting in overtreatment of many patients. A novel method to estimate the risk for metastatic relapse or progression is the detection of circulating tumour cells (CTC) in cancer patients' blood (Joosse and Pantel, 2013).

### TERMINOLOGY AND DEFINITIONS

The term « circulating metastatic cell » is not adapted to the field of CTC as, at present; it is not possible to assess the "metastatic potential" of CTCs, in particular using immunological, morphological, phenotypical and/or genotype analyses. The term "circulating tumour cells" is largely employed in the literature, but the word "tumour" may not refer to a "diagnostic" analysis in this context. In fact, several methodological approaches use "epithelial-specific" and not "tumour-specific" markers (Hayes and Smerage, 2010; Paterlini-Brechot, 2011; Paterlini-Brechot and Benali, 2007). As an example, the largely used CellSearch method isolates and identifies cells from blood using epithelial-specific antibodies and markers. The test is thus not "diagnostic" for tumour cells in blood and the term "circulating tumour cells" is inappropriate. In this setting the term "Circulating Epithelial Cells" (CEpC) would be more adapted. This situation has historical reasons. At the beginning of the "circulating tumour cell" era, in the years 1985-2000, the view was that "epithelial cells do not circulate in blood unless they become "tumorous". It was thus "plausible" that detection of circulating epithelial cells could be, in some way, referred to as CTCs. However, our knowledge in the field of circulating rare cells has evolved since then. It is now established that non tumour epithelial cells having benign epithelial morphological characteristics may circulate in the blood of patients (Paterlini-Brechot, 2011). In addition, we now know that the most malignant CTCs lose their epithelial antigens, which additionally questions the use of the term "circulating tumour cells" applied to "circulating epithelial cell" detection and of course the related potential clinical utility of the approach (Barriere et al., 2012; Bonnomet et al., 2010). Cytopathology is used in clinical oncology to study and identify morphological features associated with the characteristics of a "tumour". It is a difficult domain, especially when used for diagnostic purposes (for examples for cervix cancer, bladder cancer etc.), as it cannot be backed up by aspects of the "tissue" structure that are related to cell transformation. However, we have shown that cytopathological criteria can be applied to cells isolated from blood, in combination with immunolabeling for cell characterization, provided that the isolation pre-analytic step carefully maintains the cell morphology (Hofman et al., 2011b; Hofman et al., 2012b). We have also shown that the classical limitations of cytopathology (e.g. cells from thyroid and parathyroid adenomas)

are still valid in the context of cells isolated from blood (Hofman et al., 2011c). To clarify this point, we have used the term "circulating non haematological cell" (CNHC) referring to circulating cells without blood- or bone marrow-specific characteristics (Hofman et al., 2012b). In this group we then identified cells with clearly defined "tumour" morphological and/or immune-morphological characteristics, which can be defined as true "circulating tumour cells". It is clear that the future advent of validated high throughput alternative approaches to identify a cell as a "tumour cell", like molecular genetic assays, could bring benefit to the domain of CTCs. However, as yet we do not have alternative diagnostic markers or approaches for the identification of tumour cells in blood (Katz et al., 2010). The cytomorphological study of CTCs reveals that they can form aggregates or sheets of several cells. In this case, the term "circulating tumour microemboli" (CTMs) should be employed instead of "blood micrometastasis" as the metastatic potential of these sheets of CTCs, while probable, is not demonstrated. Finally, we have to underline that the difficulty of culturing CTCs isolated from the blood of patients has hampered, up to now, investigations aimed at studying their proliferative, invasive, transforming and tumorigenic potential. In this review, we will use the term "circulating epithelial cells" (CEpC) and Circulating Tumour Cells (CTCs) according to the method used for their detection.

## NEW INSIGHTS INTO LUNG CANCER DISSEMINATION

It has been well-established for a couple of years that the metastatic dissemination of lung cancer takes place through spread into the blood of tumour cells that invade the vessels after migration from the primary tumour. The transendothelial migration of tumour cells and their subsequent blood vessel intravasation is an early event in the natural history of carcinogenesis and may occur in lung tumours of a small size. For instance, we have demonstrated that CTCs can be detected in stage I NSCLC before surgical resection (See Articles 4 and 5) (Hofman et al., 2011a). During surgery for lung cancer, a large number of tumour cells can be shed into the blood stream but the behaviour of these CTCs is unknown, in particular it has not yet been demonstrated that these cells may be involved in development of future metastases (Okumura et al., 2009; Sawabata et al., 2007). A large number of tumour cells circulate into the bloodstream but a variable proportion of them are apoptotic or die because of shearing forces and/or are eliminated by the immune system. In agreement with this view, animal models have shown that within 24 hours of intravenous administration of tumour cells, around 0.1% of the cells are still viable and that 0.01% of these surviving CTCs may give rise to metastases (Langley and Fidler, 2007).

The passage of CTCs into the blood stream benefits substantially from a change in the tumour cell phenotype. This phenotypical change is characterized by a loss, more or less complete, of the epithelial markers (in particular cytokeratin filaments and E-cadherin) and the increase or the gain of some mesenchymal markers. This progressive transformation, called the epithelio-mesenchymal transition (EMT) phenomenon, gives substantial plasticity to the tumour cells and increases their migration and invasion (Figure 9).

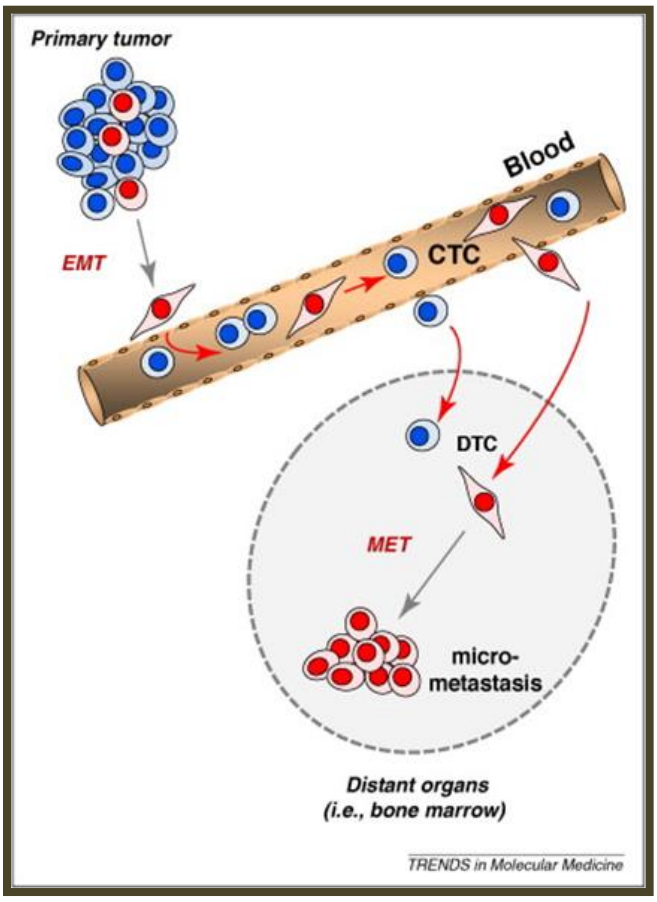



**Figure 9.** Putative roles of EMT and mesenchymal-to-epithelial transition (MET) in tumour cell dissemination. In the primary tumour, a subpopulation of tumour cells (red) acquires a mesenchymal-like migratory phenotype during tumour progression. They lose their epithelial properties (i.e. downregulate EpCAM and CK) through the EMT process and enter the bloodstream. These specific CTCs are thought to have stem cell properties. After extravasation into distant organs, these tumour cells (DTCs) have to re-express their epithelial properties through MET to form tumour cell clusters (micrometastases). Another subpopulation of CTCs, which is not able to undergo EMT (blue), can also disseminate through the bloodstream into distant organs but lacks cancer stem cell properties and, therefore, does not form (micro)metastases. (From Pantel K, & Alix-Panabières C. Trends Mol Med. 2010).

It probably also gives CTCs a better resistance to high blood pressure and to anoïkis. As recently described for breast carcinoma, the population of mesenchymal CTCs in lung cancer patients could also be associated with disease progression (Yu et al., 2013).

Moreover, a recent insight derived from CTC analyses has involved finding clusters of CTCs, called circulating tumour microemboli (CTMs), in the circulation of patients with advanced cancers (Hou et al., 2011; Hou et al., 2012). It is not clear whether these aggregates are artefacts of sample processing, or if the detection of clusters has previously been limited by the technologies used for CTC isolation, most of which are purification approaches likely to disrupt clusters. Early studies suggest that CTC clusters may be relatively protected from cell death and the harsh environment and shear stresses of the vascular circulation; they may also be clinically significant; particularly the number, size, or composition of the clusters (Hou et al., 2011; Hou et al., 2012; Parkinson et al., 2012). The presence of clusters may be a better biomarker for increased metastatic potential than single CTCs (Hou et al., 2011; Hou et al., 2012). The study of the phenotypical profile of tumour cells belonging to CTMs shows that some of them do not express any epithelial biomarkers,

but have gained a mesenchymal phenotype, which could help collective migration. In fact, when organized as CTMs, the CTCs may benefit from a survival advantage over single CTCs via cell-cell contact-related survival signalling. They can also bring their own "soil" helping them to metastasize to distant organs (Duda et al., 2010; Friedl and Gilmour, 2009).

It is unknown if CTCs represent more aggressive cells than those in the tumour of origin. However, phenotypic assessments to date suggest that at least some subset of CTCs may represent viable metastatic precursor cells capable of initiating a clonal metastatic lesion (Heitzer et al., 2013; Joosse et al., 2012). In this context, the importance of tumour-stroma interactions in generation and/or maintenance of the cancer "stem"/ tumorigenic cell status (tumour plasticity) should not be underestimated. The resulting molecular and phenotypical alterations are complex and may vary by cancer sub-type, over time, and by stage of the disease. These complexities introduce additional challenges for interpreting CTC analysis results.

The interaction between the CTCs and blood leukocytes in the blood stream has been poorly investigated to date. Different studies have demonstrated that neutrophils may facilitate the transendothelial migration of tumour cells (Wu et al., 2001). Neutrophils could also help the formation of CTMs. A relationship seems to exist between the number of lymphocytes, the number of CTCs and the overall survival of patients (De Giorgi et al., 2012; Zhang et al., 2013). Moreover, some cytokines released by lymphocytes may indirectly have an impact on the behaviour of CTCs. An elevated neutrophil to lymphocyte blood ratio has been associated with worse prognosis in cancer patients, in particular in lung cancer patients who present with lower disease free survival, an early recurrence and a worse overall survival (Ilie et al., 2012a). However, direct impact of leukocytes on CTC behaviour has not been established.

A few studies have demonstrated that platelets may play a role in survival of CTCs and in their metastatic behaviour (Labelle et al., 2011; Varon et al., 2012). In fact, platelet-tumour cell interactions may prime tumour cells for subsequent metastatic formation *in vivo* by activating the TGFβ/Smad and NF-κB pathways in cancer cells and transition to an invasive and metastatic mesenchymal-like phenotype (Labelle et al., 2011). Once located in distant organs, the CTCs may return to the epithelial phenotype, thereby allowing them to cross the endothelial barrier and to proliferate in the parenchyma. Thus a subpopulation of CTCs having a high "plasticity" should exist and may be able to switch from EMT to MET and vice-versa depending on a combination of conditions including re-expression of E-cadherin and/or EMT-inhibitory miR-200 and local microenvironment-dependent factors (Korpal et al., 2011). Additionally, the expression of chemokine receptor on CTCs could favor specific metastatic implementation in a distant organ (Fusi et al., 2012). Finally, neutrophils may also facilitate CTC implantation in metastatic sites (Huh et al., 2010).

It has been highlighted recently that CTCs can re-colonize the primary tumour site from which they were initially detached (Comen et al., 2011; Kim et al., 2009). This phenomenon, called "tumour self-seeding", allows the primary tumour to keep growing. CTCs interact preferentially with the

tumoral stroma via certain integrins (Kim et al., 2009). Tumour reactivation following an apparently successful treatment of the primary mass with initial therapies (such as surgery or systemic therapy) is a well-known phenomenon. This metastatic rebirth is preceded by an interlude, termed "dormancy", when the cancer "sleeps" and remains undetected for periods that can last years or even decades until reactivation of tumour cell proliferation occurs, thereby revealing long-term tumour recurrence (Almog, 2010; Uhr and Pantel, 2011). In this setting, it has been postulated that the CTCs could be involved in this phenomenon of dormancy (Almog, 2010; Sleeman et al., 2011).

## General principles of the detection and characterization of CTCs

The methods for CTC detection are numerous and are based on many different technical approaches (Parkinson et al., 2012). Most detection techniques are preceded by enrichment steps (removal of red blood cells, separation of CTCs from leukocytes using immunomagnetic beads or immunofluorescence-based separation of mononuclear cells) (Figure 10).

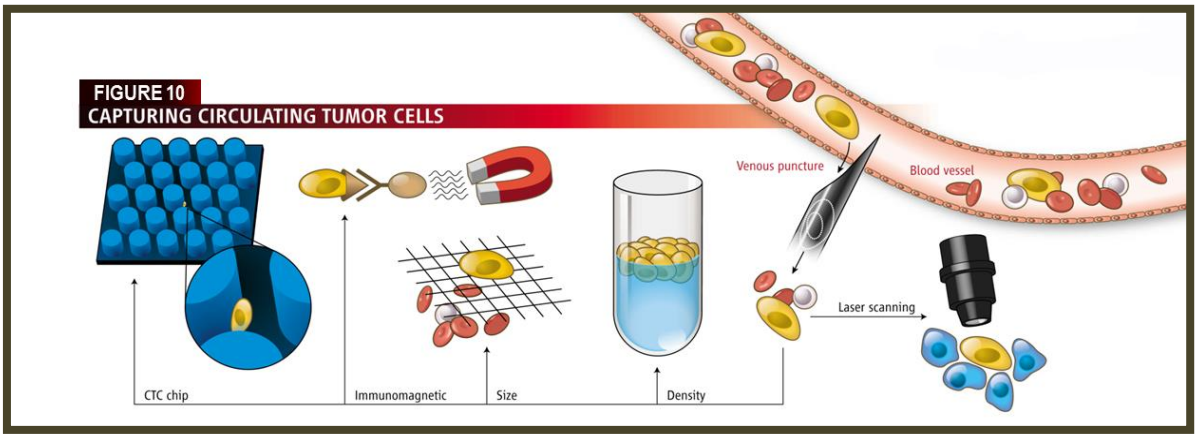



**Figure 10.** Devices for separating the rare tumour cells (yellow) in a blood sample include a silicon chip studded with microscopic posts, magnetic beads coated with antibodies, filters, density-based centrifuges, and laser detection. Antibodies or genetic analyses are then used to identify and characterize the cells (From Kaiser J. Science. 2010).

In fact, the most current methods can be distinguished according to three general principles for CTC isolation: methods based on antigen expression by the target cells, also called "immunological methods" for CTC isolation (they use antigens presumably not expressed on blood cells, but not specific to tumour cells (e.g. epithelial antigens); methods based on cell density, which is thought to be higher in CTCs than in blood cells; and, methods based on cell size, which is defined by cytopathological criteria and is distinctly larger in CTCs compared to blood cells (Parkinson et al., 2012). Additionally, two main approaches to CTC detection can be distinguished: 1) direct methods based on CTC cytomorphology, complemented when needed by immunomediated characterization, which allow diagnostic identification of CTCs and, 2) indirect methods based on immunological and/or biochemical cell characterization without cytopathological analysis (Alix-Panabieres et al., 2012; Parkinson et al., 2012).

Following their isolation by different approaches, CEpCs and CTCs can be further characterized using different complementary tools. Indeed, different genomic alterations or molecular characteristics can be performed on CEpCs and CTCs after nucleic acid (DNA, RNA, microRNA) extraction and characterization using several molecular biology analyses (PCR, RT-PCR, microarrays) (Alix-Panabieres et al., 2012; Parkinson et al., 2012). Immunocytochemical and *in situ* hybridization methods performed on CEpCs and CTCs can also detect expression of a number of macromolecules of interest (Alix-Panabieres et al., 2012; Parkinson et al., 2012). Finally, some methods are able to isolate non fixed viable CEpCs and CTCs, to grow them in a culture medium and to further check their phenotype, including drug resistance or sensitivity.

The different methods for CTC isolation and detection hold advantages and disadvantages (Alix-Panabieres et al., 2012; Parkinson et al., 2012). Some of these methods are relatively easy to perform and "cost effective", but others are more complex and expensive (both for equipment and consumables). Some methods have been widely used and are commercially available, while others are still «confidential» and under investigation and/or not commercialized, and thus difficult to set up for routine use in a laboratory (Alix-Panabieres et al., 2012; Parkinson et al., 2012).

The cellular and molecular characterization of the detected CTCs is certainly the most critical step at this stage. However, this step is mandatory for optimization of this field of interest in thoracic oncology, especially with the increasing development of targeted therapies based on the identification of different genomic alterations. Indeed, this step of CTC characterization is crucial to help lung cancer diagnosis, to better define prognosis and to optimize personalized treatment during the time of disease progression.

### IMPACT OF CTC DETECTION FOR DIAGNOSIS AND PROGNOSIS OF LUNG CANCER PATIENTS

Most of the studies related to CTC detection in lung cancer patients have targeted patients developing NSCLC (Table 4 - Annex). A few studies have demonstrated that CTC detection can be diagnostic for these lung cancer patients (Hofman et al., 2011a; Hofman et al., 2012b; Tanaka et al., 2009) (Table 4 - Annex). In NSCLC patients, recent studies have demonstrated that the efficiency of the chemotherapy directly correlates with a decrease in the number of detected CEpCs following treatment (Hirose et al., 2012; Krebs et al., 2011). The presence of CEpC/CTCs has been shown by several studies, using different technologies, to be an independent poor prognostic factor in NSCLC patients (Table 4 - Annex) (Krebs et al., 2012; Krebs et al., 2011; Sher et al., 2005; Wu et al., 2009). In this regard, CEpC/CTCs detected before, during or after resected tumours as well as CEpC/CTCs detected in non-resected lung cancer correlated with worse prognosis (Table 4 - Annex).

The field of CTCs is potentially relevant in patients with NSCLC. However, there is a strong need for standardization of sensitive CTC isolation methods without losing CTCs because of the EMT phenomenon and for diagnostic CTC identification and characterization. In fact, there are clear limitations in using CEpC as a prognostic biomarker in certain lung cancer patients as some patients,

although negative for CEpCs, still develop metastases. Moreover, a significant fraction of patients with overt metastasis have surprisingly low CEpCs counts. Thus, CEpCs that are capable of forming metastasis may remain undetected by some detection methods.

### MOLECULAR CHARACTERIZATION OF CTCS IN NSCLC PATIENTS

The current interest in molecular characterization of CTCs isolated from lung cancer patients is strongly linked to the advent of targeted therapies and to the concept of personalized medicine in thoracic oncology. It is now well-established that genomic alterations (mutations and gene rearrangements) occurring in lung tumour cells can be "druggable", thus driving targeted treatments, as described above. The list of the potential genomic alterations of interest is long, reflecting the great molecular heterogeneity of NSCLC. Some reports have shown that *EGFR* mutations can also be detected either in CEpCs or in plasma free DNA (Liu et al., 2011; Maheswaran et al., 2008). However, some *EGFR* mutations detected in plasma free DNA have been reported to be associated with false negative results, thus highlighting the interest of detecting mutations specifically in CTCs (Liu et al., 2011). The analysis of *EGFR* mutations in CEpCs isolated from lung cancer patients has been performed using different methods (Maheswaran et al., 2008; Punnoose et al., 2012). These studies are of major potential interest in NSCLC patients care as the CTC-based approach is non-invasive, rapid and can be proposed in vulnerable patients. Moreover, it is possible to repeat the test during follow-up to adapt the therapeutic choice by screening the onset of secondary resistant mutations such as T790M. Among other druggable genomic alterations currently being actively looked for in adenocarcinoma lung cancer patients, the rearrangement of the *ALK* gene can be detected in CTCs by FISH and ICC analysis, thus guiding treatment with crizotinib (Ilie et al., 2012b). Our group found a good correlation for the *ALK* status in NSCLC tissues and in CTCs isolated by ISET (Ilie et al., 2012b).

### CTC IDENTIFICATION IN THE CLINICAL DAILY PRACTICE FOR BETTER MANAGEMENT OF LUNG CANCER PATIENTS: FACT OR FANCY?

The increasing number of publications, comments and general reviews in the CTC field shows the growing interest in this subject by the scientific and medical community. However, the clinical impact of CTCs is still under question as contrasting results have been obtained due to technical differences in specificity and sensitivity and due to limitations including the high cost and labour needed, which limit the use of some approaches for large cohorts of patients and in the daily clinical practice (Kaiser, 2010; Lianidou, 2012; Parkinson et al., 2012). Moreover, the results of these different studies of NSCLC patients are difficult to compare due to the different technologies. Moreover, the numbers of patients studied are often too small and the clinicopathological characteristics are not homogenous from one study to another, which makes it difficult to give robust conclusions. As mentioned above, the most widely used approach for CEpC detection, the CellSearch technology has been shown to have prognostic FDA approved value only in metastatic breast, colon and prostate cancer patients. However, as for other methods, the Cellsearch technical

approach is based on epithelial markers (epithelial cell adhesion molecule and keratin), which prevents the detection of most CTCs since epithelial markers tend to be down regulated during tumour cell dissemination, especially in lung cancer patients.

The major challenging goals in oncology, especially in lung oncology, are early detection of the primary cancer; with or without metastasis, determining the prognosis of a patient and predicting the response of an individual to treatment. In addition, the possibility of detecting early on secondary resistance to treatment so as to quickly adapt the therapy or to appreciate the treatment efficacy or disease recurrence through blood sampling is critical since access to tissue or cytological can be difficult in fragile patients. Moreover, the presence of CTCs in early stage operated patients may help to define the prognosis of these patients and for the positive population to propose an adjuvant treatment even if this strategy deserves to be validated with large prospective trials. Furthermore, in the case of carcinoma of unknown primitive origin, the molecular analysis of CTCs, in particular by using a new genomic sequencing method or a transcriptomic approach could provide informative results regarding the primitive tumour site (Gervasoni et al., 2008; Punnoose et al., 2010).

Despite the objectives and the promises claimed by the scientific community concerning the value of CEpC/CTC detection and characterization in thoracic oncology, there is uncertainty concerning as to when and how CEpC/CTC translational results will be implemented into the daily health care (Kaiser, 2010; Lianidou, 2012). In this context, there is a striking contrast between the high number of international publications related to the CTC field and the real usefulness for oncologists in their daily decision-making and thus the benefit to lung cancer patients. Nonetheless, studies on CTCs have considerably increased knowledge concerning lung cancer pathophysiology and the mechanisms involved in metastasis.

In fact, doubts persist among some oncologists who question whether the results obtained with CTC detection in lung cancer patients are meaningful. Clearly, issues concerning specificity, sensitivity and reproducibility of the different technical approaches, their ability to isolate and identify the "most malignant" CTCs, the optimal blood volume for study, the possibility to find different genomic alteration in the tumour tissues versus the CTCs and the cost policy allowing implementation of CTC testing in public and private hospitals, are still to be addressed (Gorges and Pantel, 2013; Wicha and Hayes, 2011). An additional concern, related to the low number of CTCs in the blood, is the need to develop methods and tools compliant with ISO norms for their suitable transfer to the hospitals' biology laboratories. This will help avoiding pitfalls that occur during the pre-analytical and analytical phases, which lead to technical errors with impact on the quality and robustness of the results, especially for interpretation of genomic alterations and detection in lung cancer patients (Ilie and Hofman, 2012; Long et al., 2013).

However, despite all these open questions and unmet needs, studies on CTCs have increased considerably the knowledge of lung cancer pathophysiology and of mechanisms involved in metastasis. In this setting, methods allowing the isolation of non-fixed CTCs which can be engaged

in cell multiplication processes, although difficult to apply in current clinical practice, are being continuously developed. These viable cells could be further injected into mice to form xenografts, thereby allowing the study of their invasive potential *in vivo*.

## 1.5 Cells as biomarkers - Tumour-associated neutrophils

Inflammatory cells and mediators are a key component of the tumour microenvironment and cancer-related inflammation has been proposed to represent the seventh hallmark of cancer (Figure 3) (Hanahan and Weinberg, 2011; Mantovani, 2009). Of all cell types residing within the tumour microenvironment, neutrophils have received the least amount of attention. Although commonly encountered, only recently have these short-lived professional phagocytes been considered more than an inconsequential bystander. This is somewhat surprising given that neutrophils possess substances known to alter tumour growth when elaborated from other sources. However, cynics would point to their short half-life and lack of "intellect," rendering them incapable of meaningfully impacting tumour cell behaviour (Houghton, 2010).

Neutrophils are the predominant circulating leukocyte population in humans, accounting for 50–70% of circulating leukocytes. They have been seen *in vivo* in close association with tumour cells and within tumour vasculature (Fridlender and Albelda, 2012). However, the exact role of neutrophils in the tumour cell microenvironment is the subject of controversy. The interest in the role of neutrophils in cancer increased as recent data suggested important and significant roles for neutrophils in tumour biology (Mantovani et al., 2011; Piccard et al., 2012).

Neutrophils play a well-established role in host defence, where they extravasate from the circulation and enter tissues. There, they phagocytose and kill invading microorganisms (such as bacteria and fungi) by releasing activating cytokines [e.g. tumour necrosis factor (TNF)-a, interleukin (IL)-1, interferons (IFNs), etc.] and defensins, along with toxic substances and reactive oxygen species. Although neutrophils are traditionally considered in the context of their antibacterial functions, it is becoming clear that tumour-associated neutrophils (TAN) and their myeloid precursors [peripheral neutrophils and granulocytic myeloid-derived suppressor cells (G-MDSC)] in the spleen, bone marrow and blood play an important role in cancer biology (Fridlender et al., 2009; Mantovani et al., 2011; Peranzoni et al., 2010; Piccard et al., 2012).

In contrast to the well-described ability of inflammatory neutrophils to engulf bacteria, activate the immune system and induce tissue damage in infections, it has now become apparent that myeloid cells can also function as immunosuppressive cells in the context of tumours (Nagaraj et al., 2010). This property has been very well described in recent years for the so-called "myeloid-derived suppressor cells" found in large quantities in the spleens of tumour-bearing animals and for tumour-associated macrophages that develop an "M2" or tumour-supportive phenotype (Gabrilovich and Nagaraj, 2009; Hao et al., 2012; Movahedi et al., 2008).

### NEUTROPHIL POLARIZATION: N1 AND N2 TAN

Recently, Fridlender et al. provided evidence for the existence of N1 (antitumoral) and N2 (protumoral) tumour-associated neutrophils, in analogy to M1 and M2 macrophage polarization (Allavena et al., 2008; Fridlender et al., 2009). This neutrophil plasticity is regulated by molecules in the tumour microenvironment (Figure 11).

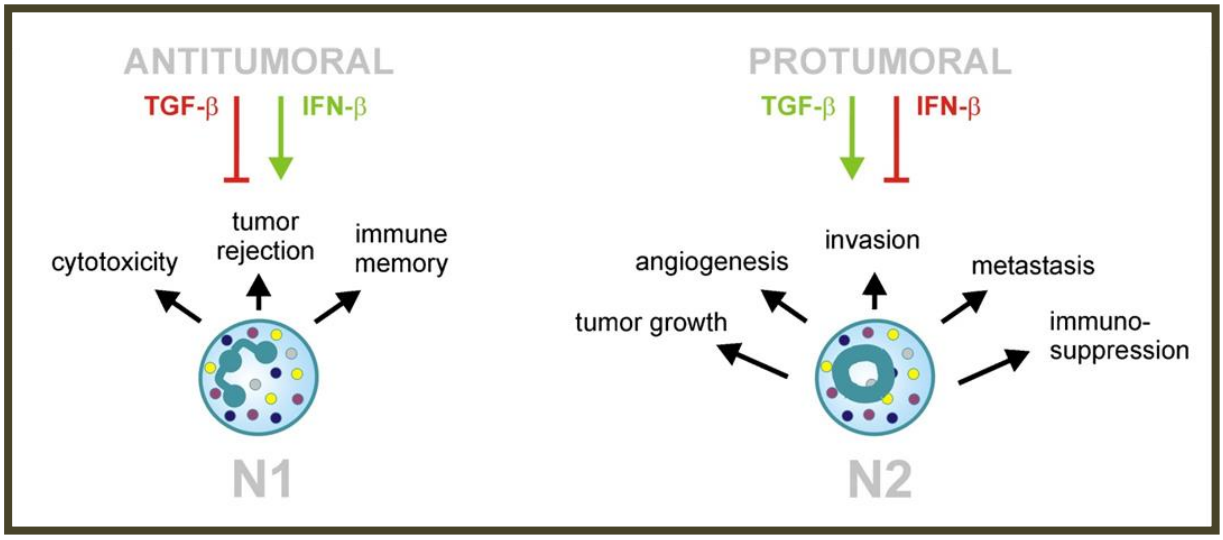



**Figure 11.** Simplified scheme of polarization of tumour-associated neutrophils (TAN). Neutrophils exert both antitumoral and protumoral functions in cancer development and progression. This phenomenon is denominated "neutrophil polarization" and is influenced by the cytokines TGF-¤ and IFN-¤. Neutrophils are antitumoral effector cells by inducing cytotoxicity and by mediating tumour rejection and antitumoral immune memory (N1 phenotype). In contrast, neutrophils support tumour progression by promoting angiogenesis, invasion, metastasis and immunosuppression (N2 phenotype). Adapted from (Piccard et al., 2012).

Neutrophils are induced by the immunosuppressive cytokine transforming growth factor-ß (TGF-ß) to acquire an N2 protumoral phenotype and the presence of TGF-ß prevents the generation of antitumorigenic N1 neutrophils. The antitumoral activities of N1 neutrophils (upon TGF-ß blockade) include the enhanced expression of immunoactivating cytokines and chemokines, lower levels of arginase, a higher capacity to kill tumour cells and the activation of cytotoxic T lymphocytes (CTLs) (Fridlender et al., 2009). Moreover, neutrophils exert tumour angiogenic activity that is inhibited by endogenous type I IFN (Jablonska et al., 2010). These recent data introduced the concept of host neutrophil polarization by a particular cytokine milieu, shine a new light on tumour-associated neutrophils and might help to explain the dual roles of neutrophils in cancer at the molecular level. These alterations in neutrophil phenotypes might be based on different degrees of neutrophil activation, in which neutrophils release more of a given substance, rather than different molecules (Houghton, 2010). It is a key question whether N1 neutrophils are simply more activated than N2, or whether these cells are alternatively activated by agonists, in line with phenomena observed for M1/M2 macrophages (Houghton, 2010).

In addition, neutrophils carry functional receptors for other cytokines than IFN-ß and TGF-ß. For instance, IL-1ß, TNF-α and IL-10 are known to act on neutrophils and it is well established that IL-1ß

and TNF-α are pro-inflammatory and immunostimulating cytokines, whereas IL-10 has the capacity to dampen inflammation and to be immunosuppressive (Moore et al., 2001).

It is worth considering the relationship between TAN and the granulocytic fraction of MDSC. G-MDSC, a heterogeneous population of immune suppressive cells that are produced at high levels in cancer, are defined in mice on the basis of expression of the surface markers CD11b and Gr-1 and by their ability to inhibit T-lymphocyte activation. The CD11b+/GR1+ MDSC population is comprised of at least two subsets—granulocytic (Ly6G+) and monocytic cells (Ly6C+), possibly with different immunosuppressive properties (Peranzoni et al., 2010). There is substantial agreement on the immunosuppressive activity of the monocytic MDSC subset. However, there is still contrasting evidence on the role of the granulocytic fraction. Whereas some have shown that granulocytic MDSC have immunosuppression properties similar to the monocytic fraction, others have recently demonstrated that they are less immunosuppressive (Dolcetti et al., 2010; Gabrilovich and Nagaraj, 2009; Peranzoni et al., 2010). It has been shown previously that adoptively transferred MDSC can enter tumours and differentiate to mature tumour associated macrophages or neutrophils (TAN) (59). However, little is known in animals about whether MDSC leave the spleen and circulate. It is thus not clear whether the majority of TAN are actually G-MDSC that were attracted to the tumour or whether they are bone marrow/blood-derived neutrophils that were then converted to N2 TAN by the tumour microenvironment. A recent transcriptomic study comparing the phenotype of TAN to naive neutrophils from the bone marrow (NN) and to the G-MDSC, clearly showed that TAN are not "tissue-based G-MDSC" but are a distinct population of neutrophils, differing markedly in their genetic profile from both NN and G-MDSC, with the NN and G-MDSC being more closely related to each other than to TAN (Fridlender et al., 2012). In addition, the CD66b+ antigen was expressed specifically on neutrophils and may be assigned to a limited, activated subtype of neutrophils (Jensen et al., 2009; Murdoch et al., 2008). The CD66b antigen belongs to the family of carcinoembryonic antigen-related cell adhesion molecules that exhibit rapid up-regulation after neutrophil activation (Elghetany, 2002).

## DUAL ROLES OF TAN WITHIN THE MULTIPLE STEP PARADIGM OF CANCER

After neoplastic transformation due to genetic alterations, tumour-host interactions promote various molecular and cellular processes underlying multiple interrelated steps that define cancer progression (Vogelstein and Kinzler, 2004). Neutrophils are associated with and make functional contributions to many of these different steps. This approach links the antitumoral (N1) and protumoral (N2) phenotypes of neutrophils with the promotion of particular steps in tumour biology and immunological processes (Figure 11). Note that cytotoxicity, tumour cell apoptosis, immunologically mediated tumour rejection and antitumoral immune memory may counteract tumour progression at every stage (Piccard et al., 2012).

### THE TUMOUR-SUPPORTIVE ROLES OF TAN

TAN appear to be involved in **tumorigenesis** and **tumour growth** through multiple mechanisms: initiation, carcinogenesis and tumour growth (Fridlender and Albelda, 2012). Neutrophils have a dual role in the initiation process of tumours, mainly by affecting the extracellular matrix and the neoplastic cells microenvironment (Piccard et al., 2012). In addition to its effects on matrix, it has been shown that MMP-9 secreted from neutrophils (among other bone marrow cells) prevents apoptosis of tumour cells in the lung (Acuff et al., 2006). Another potential direct effect of neutrophils on tumour growth is through secretion of neutrophil elastase that enters tumour cells, where it binds IRS-1 allowing increased activation of AKT (Houghton et al., 2010).

Mounting evidence supports the role of neutrophils in the important pro-tumorigenic process of **angiogenesis** and **neovascularization**, at least partially mediated by the secretion of chemokines and MMPs (Jablonska et al., 2010; Tazzyman et al., 2009).

Studies have shown indirectly that neutrophils are associated with more lung metastases in skin squamous cell carcinoma and in melanoma (Schaider et al., 2003). More than two decades ago, Welch et al. demonstrated that neutrophils elicited from tumours (nowadays called TAN) secrete high levels of the basement membrane degrading enzymes collagenase-IV and heparanase, assisting **tumour cell extravasation** during the metastatic process (Welch et al., 1989).

In addition, bronchoalveolar adenocarcinoma is characterized by intrapulmonary spread and a strong inflammatory reaction, composed of macrophages and neutrophils. Neutrophils induce cell detachment (**"tumour shedding"**) and several molecules are involved in this process e.g. intercellular adhesion molecule (ICAM)-1/lymphocyte function-associated antigen (LFA)-1, TNF-α/TNF-α receptor inhibitor, IL-1α/IL-1α receptor and neutrophil elastase. Shedding was detected in tumour tissue samples and was associated with a high neutrophil count in bronchoalveolar lavage and reduced patient survival (Wislez et al., 2007).

Another mechanism by which neutrophils promote metastasis has been demonstrated recently *in vivo*. CTCs were shown to directly anchor to the vascular endothelium, facilitating transendothelial migration of tumour cells and extravasation. Thus, these tumour cells may enter tissues and establish metastases or continue their journey. Neutrophils are shown to facilitate the extravasation of CTCs (Huh et al., 2010).

The ability of neutrophils to influence CD8+ T cells has been suggested in infections and in cancer (Di Carlo et al., 2001; Tvinnereim et al., 2004). In cancer, whereas N1 TAN were shown to promote recruitment and activation of immunocytes, recent data demonstrated that N2 neutrophils can inhibit T-cell effector functions; neutrophil depletion of untreated tumour-bearing animals (i.e. removal of N2 TAN) increased the activation status of CD8+ T cells, supporting the idea that N2 TAN can function in an immunosuppressive fashion in the same way that has been proposed for M2 tumour associated macrophages (Fridlender et al., 2009; Movahedi et al., 2008; Rodriguez et al., 2004). A possible suggested mechanism for this suppression of T-cell proliferation and

responsiveness to stimulation is by the secretion of stored arginase 1 that degrades extracellular arginine, a factor needed for the proper activity of T cells (Rotondo et al., 2009).

### THE ANTITUMOR EFFECTS OF N1 TAN

Despite the broad literature on the pro-tumour effects of TAN, there are several studies reporting antitumor roles for these cells, mostly with engineered tumour cell lines or following specific therapies (Gregory and Houghton, 2011).

Neutrophils were shown to be capable of **protecting against tumour development**, by secreting MMP-8. Loss of MMP-8, mainly arising from neutrophils, increased skin susceptibility to chemical carcinogens in mice. Ironically, this effect seems to be mediated by preventing an influx of neutrophils to the carcinogen injection site (Balbin et al., 2003).

The **direct killing of tumour cells** by neutrophils was demonstrated *in vitro* and *in vivo* (Gerrard et al., 1981; Katano and Torisu, 1982). Ishihara et al. reported that neutrophils from tumour-bearing animals have an enhanced cytotoxicity profile as measured by superoxide anion generation and phagocytosis, inducing a marked decrease in the size and number of metastatic foci in the lung (Ishihara et al., 1998a; Ishihara et al., 1998b). Others have demonstrated that oxidative damage caused by reactive oxygen species secreted from neutrophils is capable of inducing tumour cell lysis (Fridlender et al., 2009; Zivkovic et al., 2007). Interestingly, there seems to be a difference between the cytotoxicity of neutrophils to primary versus metastatic cells, the latter being less affected (Schaider et al., 2003). Furthermore, it is possible that tumour-entrained neutrophils can actually **inhibit metastatic seeding** in the lungs, inducing a neutrophil-mediated inhibitory process at the metastatic site (Granot et al., 2011).

A second mechanism by which neutrophils were shown to be capable of directly inhibiting tumour cells is by mediating Fas-ligand-associated apoptosis (Chen et al., 2003; Fridlender et al., 2009). A third mechanism of killing mediated by neutrophils, mostly shown following treatment, is antibody-dependent cellular cytotoxicity for example, as part of the mechanism of the EGFR antibodies—panitumumab and zalutumumab (Hubert et al., 2011; Piccard et al., 2012; Schneider-Merck et al., 2010).

The ability of the **adaptive immune system**, and specifically the CD8+ cytotoxic T lymphocytes, to reject tumours is a key process for the success of any immunotherapy. As previously suggested, N2 neutrophils can be major inhibitors of T-cell effector functions in a similar way previously proposed for M2 tumour associated macrophages (Di Carlo et al., 2001; Fridlender et al., 2009). However, N1 neutrophils can actually be immunostimulatory, supporting tumour rejection. These pro-inflammatory N1 neutrophils can promote CD8+ recruitment and activation by producing T-cell attracting chemokines (e.g. CCL-3, CXCL9 and CXCL10) and pro-inflammatory cytokines (e.g. IL-12, TNF-α and GM-CSF) (Fridlender et al., 2009).

## TAN IN HUMAN CANCERS

Relatively little is known about neutrophils in human cancers. Many patients with advanced cancer show high levels of blood neutrophilia (Schmidt et al., 2007). The mechanisms by which neutrophilia is induced by tumours is uncertain, although GM-CSF production has been implicated in some tumour systems, such as lung, melanoma, pancreas and breast (Fridlender and Albelda, 2012). Several additional cytokines secreted from tumours and stroma cells have been suggested to contribute to neutrophilia and to the induction of suppressive properties of these neutrophils. These include, among others, G-CSF, vascular endothelial growth factor (VEGF), IL1-ß and IL-6 (Lechner et al., 2010). Neutrophilia has been associated with poorer prognosis in many cancers, including bronchoalveolar carcinoma and metastatic melanoma (Bellocq et al., 1998; Schmidt et al., 2007).

There is surprisingly little data about the presence of neutrophils within human tumours. Intratumoral neutrophils were shown to be a strong, independent prognostic factor for recurrence free, as well as cancer-specific and overall survival in metastatic and in localized clear cell renal cell carcinoma, head and neck squamous cell carcinoma, stage I/II melanoma and finally in resectable NSCLC, as demonstrated by our team (Donskov and von der Maase, 2006; Ilie et al., 2012a; Jensen et al., 2009; Jensen et al., 2012). Infiltration of neutrophils was found to correlate with tumour grade in human gliomas and to be related to more aggressive types of pancreatic tumours (Fossati et al., 1999; Reid et al., 2011). However, this observation is not universal. In some tumours (e.g. gastric cancer), a high neutrophil count has been associated with a favourable prognosis, whereas another group recently reported that the densities of tumour-associated CD66b+ neutrophils and CD163+ macrophages were correlated with adverse prognostic factors and systemic blood inflammation markers, but not directly correlated with survival of NSCLC patients (Carus et al., 2013; Caruso et al., 2002).

Several effects of human tumour cells on neutrophils were demonstrated *in vitro*. Interestingly, IL-8 secreted by the tumour cells may play an important role in attracting neutrophils to the tumour microenvironment (Sparmann and Bar-Sagi, 2004). Increasing evidence suggests an important role for IL-8 in tumour progression and metastasis by promoting cell proliferation and angiogenesis in NSCLC. Furthermore, recent studies have reported that oncogenic KRAS-induced interleukin-8 overexpression may promote cell growth and migration and contributes to aggressive phenotypes of NSCLC (Sparmann and Bar-Sagi, 2004; Sunaga et al., 2012).

# CHAPTER 2 RESULTS

## 2.1 Hypoxia targets as biomarkers

### *Article 1*

High levels of carbonic anhydrase IX in tumour tissue and plasma are biomarkers of poor prognostic in patients with non-small cell lung cancer.

**Ilie M**, Mazure NM, Hofman V, Ammadi RE, Ortholan C, Bonnetaud C, Havet K, Venissac N, Mograbi B, Mouroux J, Pouysségur J, Hofman P.

*Br J Cancer. 2010;102:1627-1635.*

### *Article 2*

Overexpression of carbonic anhydrase XII in tissues from resectable non-small cell lung cancers is a biomarker of good prognosis

**Ilie MI**, Hofman V, Ortholan C, Ammadi RE, Bonnetaud C, Havet K, Venissac N, Mouroux J, Mazure NM, Pouysségur J, Hofman P.

*Int J Cancer. 2011;128:1614-1623.*

### *Article 3*

Response of CAIX and CAXII to re-oxygenation: contribution to clinical outcome in NSCLC.

**Ilie MI**, Hofman V, Zangari J, Mouroux J, Mazure NM, Pouysségur J, Brest P, Hofman P.

*Under revision. Lung Cancer.*

## RESULTS

Considering the amount of evidence for the most hypoxia-induced membrane-associated carbonic anhydrases as key enzymes involved in pH homeostasis, cell survival and migration, we conducted in parallel two “a priori” studies to determine whether CAIX and CAXII could represent new diagnostic or prognostic biomarkers in NSCLC.

The first study evaluated the prognostic value of the tumour expression of CAIX from patients with NSCLC. Tissue microarrays (TMA) containing 555 NSCLC tissue samples were generated for quantification of CAIX expression. Secondly, we evaluated the diagnostic and prognostic value of the plasma level of CAIX as determined by ELISA in 209 NSCLC patients and in 58 healthy individuals. Finally, the CAIX tissue immunostaining and plasma levels were correlated with clinicopathological factors and patient outcome.

We showed that 24.3% NSCLC tumours overexpress CAIX. There was a significantly higher expression of CAIX in the non-adenocarcinoma histological subtypes ($P < 0.001$). In addition, we showed that CAIX tissue overexpression correlated with shorter overall survival (OS) ($P=0.05$) and disease-specific survival (DSS) of patients ($P=0.002$). Multivariate Cox analyses revealed that high CAIX tissue expression ($P=0.002$) was an independent biomarker of poor prognosis in patients with resectable NSCLC. Moreover, we showed evidence for the first time that the plasma CAIX level in NSCLC patients was significantly higher than in healthy individuals ($P < 0.001$). Our data showed that the CAIX ELISA test had a good sensitivity (84%) and specificity (95%). Moreover, even for tumours inferior to 1 cm in size, the mean value of CAIX was significantly higher when compared with the mean value of CAIX plasma in the control group. A high level of CAIX in the plasma of patients was associated with shorter OS ($P<0.001$) and DSS ($P<0.001$), mostly in early stage I+II NSCLC. Multivariate Cox analyses revealed that high CAIX tissue expression ($P=0.002$) was a factor of poor prognosis in patients with resectable NSCLC. In addition, a high CAIX plasma level was an independent variable predicting poor OS ($P < 0.001$) in patients with NSCLC.

This study demonstrated that CAIX tumour tissue expression as detected by immunohistochemistry on TMA, can serve as predictor for survival in patients with NSCLC. Moreover, we showed that the plasma CAIX level is an independent prognostic factor in early-stage NSCLC. Our results support the high specificity and the potentiality of plasma CAIX as a helpful clinical biomarker for detection of NSCLC at an early stage.

Furthermore, we assessed the prognostic significance of CAXII tumour tissues expression in patients with NSCLC. An immunohistochemical assay on TMA was developed and the results were correlated with clinicopathological parameters and outcome of patients. CAXII overexpression was present in 19% cases and was associated with tumours of lower grade ($P=0.015$) and squamous cell histological subtype ($P<0.001$). High CAXII expression correlated with better overall and disease-specific survival of patients with resectable NSCLC in univariate ($P<0.001$) and multivariate survival analyses

($P$<0.001). This was the first study demonstrating that high CAXII tumour tissue expression is related to a better outcome of patients with resectable NSCLC.

These findings were quite intriguing and seemed to contradict the experimental data previously published. To explore this contradiction, we further investigated two mechanisms that would potentially clarify these associations: the temporal relationship between the expression of CAIX and CAXII and hypoxia or re-oxygenation, as well as the expression in two human lung adenocarcinoma cell lines of their splicing variants. In addition, we have evaluated the frequency of expression of both CAIX and CAXII in NSCLC patients and questioned whether the balance in expression of these two CA isoenzymes could modify the prediction of clinical outcome of these patients.

Firstly, it is known that both CA9 and CA12 have alternatively spliced (AS) isoforms with different distributions in response to cellular hypoxia along with a dissimilar relationship to clinical outcome. In our study, the results of quantitative RT-PCR showed a constant full-length (FL) to alternatively-spliced ratio in two human lung adenocarcinoma cells cultured under hypoxic or re-oxygenated conditions when compared to normoxia. In contrast, CA12 expression seemed to be dependent on the phenotype, as both FL and AS CA12 forms were induced by hypoxia exclusively in A549 cells.

Most importantly, the initial response of lung adenocarcinoma cells to hypoxia was the stabilization of HIF-1α along with marked induction of CAIX. We were not able to detect CAXII expression in H1975 cells neither in normoxia nor in hypoxia. These findings supported our immunohistochemical data that showed only a slight overlap (3%) between high CAIX and high CAXII expression in NSCLC patients. Twenty-seven % of tumours had either high CAIX with low CAXII expression or conversely low CAIX together with high CAXII expression, suggesting mutually exclusive detection of these two isoforms in NSCLC. Moreover, no significant correlation was noted between CAIX and CAXII expression in tumour tissue samples. We hypothesized that the difference in distribution may reflect different temporal responses to cellular hypoxia and re-oxygenation. While CAIX showed marked induction in hypoxia of 32 h CAXII required 48 h of hypoxia in A549 cells. Whereas the transcript and protein levels of CAIX remained high during re-oxygenation for at least 24 h those of CAXII declined rapidly. Moreover, in cells exposed to hypoxia we observed decreased cell cycle arrest. Because fluctuations in oxygen pressure occur in tumours, we investigated the effect of re-oxygenation on CAIX and CAXII expression in arrested hypoxic cells. Following 8 h of re-oxygenation, the S/G2 phase was restored and the restart-associated CAIX expression was higher relative to normoxia. Our findings suggest that once CAIX is transcriptionally induced by hypoxia, lung cancer cells can maintain CAIX stability in re-oxygenated conditions, consistent with the half-life of CAIX. We demonstrate for the first time a connection between the post-translational stability of CAIX and the replicative restart of hypoxia arrested cells. Furthermore, CAXII expression seemed to have a minimum effect on cell proliferation upon re-oxygenation as the mRNA and protein levels were rapidly down-regulated by the shift to normoxia. Thus, these observations strengthen our hypothesis that the dynamic CAIX and CAXII response to hypoxia-re-oxygenation may promote aggressive tumour growth. Cancer cells may therefore require hypoxia-inducible CAXII expression at early steps

in oncogenesis, yet CAIX expression may be more relevant in increasing the metastatic potential of NSCLC. Our observations were supported by our findings in NSCLC patients. The strong tumour CAIX expression associated with weak CAXII expression had an unfavourable impact on relapse and survival. Surprisingly, either the co-expression of high CAIX and high CAXII or the simultaneous low CAIX and low CAXII tumour expression had an intermediate impact on relapse and patient outcome. Conversely, low tumour CAIX expression associated with CAXII overexpression, significantly correlated with a lower level of relapse and better prognosis. This study allowed us to stratify NSCLC patients into groups with different survival rates according to the tumour CAIX and CAXII expression subgroups.

## 2.2 Cells as biomarkers – Circulating tumour cells

### *Article 4*

Preoperative circulating tumour cell detection using the isolation by size of epithelial tumour cell method for patients with lung cancer is a new prognostic biomarker.

Hofman V(Au), Bonnetaud C(Au), **Ilie MI(Au),** Vielh P, Vignaud JM, Fléjou JF, Lantuejoul S, Piaton E, Mourad N, Butori C, Selva E, Poudenx M, Sibon S, Kelhef S, Vénissac N, Jais JP, Mouroux J, Molina TJ, Hofman P.

*Clin Cancer Res. 2011;17:827-835.*

### *Article 5*

Detection of circulating tumour cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch Assay™ and the isolation by size of epithelial tumour cell method.

Hofman V(Au), **Ilie MI(Au),** Long E, Selva E, Bonnetaud C, Molina T, Vénissac N, Mouroux J, Vielh P, Hofman P.

*Int J Cancer. 2011;129:1651-1660.*

### *Article 6*

Cytopathologic detection of circulating tumor cells using the isolation by size of epithelial tumor cell method: promises and pitfalls.

Hofman VJ, **Ilie MI**, Bonnetaud C, Selva E, Long E, Molina T, Vignaud JM, Fléjou JF, Lantuejoul S, Piaton E, Butori C, Mourad N, Poudenx M, Bahadoran P, Sibon S, Guevara N, Santini J, Vénissac N, Mouroux J, Vielh P, Hofman PM.

*Am J Clin Pathol. 2011;135:146-156.*

### *Article 7*

Morphological analysis of circulating tumour cells in patients undergoing surgery for non-small cell lung carcinoma using the isolation by size of epithelial tumour cell (ISET) method.

Hofman V, Long E, **Ilie M,** Bonnetaud C, Vignaud JM, Fléjou JF, Lantuejoul S, Piaton E, Mourad N, Butori C, Selva E, Marquette CH, Poudenx M, Sibon S, Kelhef S, Vénissac N, Jais JP, Mouroux J, Molina TJ, Vielh P, Hofman P.

*Cytopathology. 2012;23:30-38.*

### *Article 8*

ALK-gene rearrangement: a comparative analysis on circulating tumour cells and tumour tissue from patients with lung adenocarcinoma.

**Ilie M,** Long E, Butori C, Hofman V, Coelle C, Mauro V, Zahaf K, Marquette CH, Mouroux J, Paterlini-Bréchot P, Hofman P.

*Ann Oncol. 2012;23:2907-2913.*

## RESULTS

Our first aim was to determine the diagnostic potential of the ISET method for preoperative detection of CTC in resectable NSCLC patients and to correlate the presence and number of CTCs with different clinicopathological variables and outcome of patients. For this purpose, we prospectively screened 208 patients with resectable NSCLC, with a large majority having early-stage tumours. Also, blood samples from 39 healthy volunteers (all smokers, without knowledge of neoplastic disease) were used as negative controls. For these patients we filtered the blood by ISET. Stained spots were examined by light microscopy. As this field was quit new even for pathologists, we designed the study as a blind multicentre study for the cytopathological analysis, in which 10 cytopathologists from 8 French centres accepted to participate. The end-point of our study was the patient outcome, including overall and disease-free survival. Cytomorphological criteria have been established by the panel of cytopathologists for classification of detected circulating nonhematologic cells into 3 groups: (i) circulating non haematological cells with malignant features (CNHC-MF), (ii) CNHC with uncertain malignant features (CNHC-UMF), and (iii) CNHC with benign features (CNHC-BF). The following criteria were taken into account to characterize the detected cells such as irregularity and size of the nucleus, anisonucleosis, nuclear hyperchromatism, nucleocytoplasmic ratio, size and number of nucleoli, and presence of tridimensional sheets. CNHC-MFs were characterized by the presence of at least 3 of these criteria; the uncertain malignant features were retained when less than 3 of these criteria were present. Cells with benign features were characterized in the absence of these criteria. In all cases, at least 5 of the 10 cytopathologists agreed with the final diagnosis for each patient. Overall, by using ISET, CNHCs were present preoperatively in 49% of patients undergoing surgery for NSCLC. However, the cytopathological features of CNHC were undistinguishable between the histology NSCLC subtypes. No correlation was observed between the detection of CNHC and the clinicopathological variables of patients such as the disease stage, age, gender, tobacco exposure, tumour size, histologic subtype, histologic grade, percentage of epithelial tumour cells in the primary tumour, pleural invasion, presence of intratumoral emboli, and TTF1 staining. On the other hand, a high number of CTCs was significantly associated with shorter OS and DFS of patients. In addition, the level of 50 or more CNHCs was significantly associated with worse DFS in later-stages NSCLCs, but more interestingly the presence of CTCs was a poor prognosis factor in the early-stages as well.

We further evaluated the specificity of ISET in another study. We screened more than 800 patients with malignant, benign, inflammatory diseases or healthy subjects. Our results showed that different morphologic subtypes of CNHCs circulate in the blood of patients, but only one, defined as CNHC-MF, represents “true” CTCs because they were never found in patients with non tumoral diseases or in healthy subjects. However, there may be some false-positive results as cells with MFs were found in 10 patients with benign thyroid and parathyroid pathologies, which are known to be diagnostically challenging in fine needle aspiration cytopathology.

With regard to these results, we further wanted to take advantage of the combination of two methods for CTC isolation, one indirect and one direct to compare the efficacy of these technologies to detect CTCs. In addition, each method was evaluated to correlate the presence of CTCs and the prognostic value. For this purpose, we included a population of 210 patients with resectable NSCLC. These patients were screened for CTCs in parallel by the CellSearch system and by ISET. CTCs were detected in 50% patients using the ISET method, independently of the CS method. On the other hand, only 39% patients had CTC according to the CS method, independently of the ISET method. Moreover, CTCs were detected in 30% patients when using the ISET method and not detected by CS and in 19% patients when using the CS method and not detected by ISET. Only 20% of patients showed CTCs detected both by the CS and ISET methods. The number of CTCs detected by CS varied from 1 to 23 cells (mean: 12 cells), whereas the number of CTCs detected by ISET varied from 1 to 150 cells (mean: 34 cells). We further performed immunocytochemistry on CTC isolated by ISET. The immunostained cells expressed cytokeratin alone in 26% of cases or in association with vimentin in 53%. However, in 21% of patients, CTCs were only positive for vimentin but these latter cells clearly showed cytological malignant features. Furthermore, the presence of CTCs detected by these two methods was independent of the histological subtypes of carcinomas as well as of the disease stage. Finally, patients without CTCs had a significantly longer DFS compared to patients with CTCs detected by CS or ISET alone. Interestingly, this significance was somewhat even higher in patients with CTCs detected by both methods. Subsequently, the presence of CTCs as detected by CS or ISET or by both methods together was an independent prognostic factor for shorter DFS, as demonstrated by the multivariate survival analysis using the Cox's regression model. Overall, we demonstrated the interest of CTC detection for the prognosis of patients with NSCLC by using two methods of isolation. Moreover, the detection of CTCs seems to have good sensitivity and specificity for discriminating cancer patients from individuals without neoplastic disease. CTC could therefore represent an ideal biomarker with high clinical impact. However, we and others think that the most promising applications lie beyond the prognostic enumeration of CTCs. The current major interest of molecular characterization of CTCs isolated in lung cancer patients is certainly strongly linked to the advent of targeted therapies and to the concept of personalized medicine in the lung oncology field. In this context, the aim of our next study was to assess the ALK status in CTCs detected in lung adenocarcinoma patients and to correlate these results with the ALK status in the corresponding tumour tissue. In parallel, we have performed immunohistochemistry or immunocytochemistry with the 5A4 antibody, and FISH analysis to determine ALK status, either on FFPE samples or on the corresponding CTCs. Positive ALK immunostaining was found in five tumours mostly corresponding to adenocarcinomas with a solid pattern. These five cases showed strong positive cytoplasmic staining for all tumour cells, with membrane reinforcement in a couple of cells. FISH analysis performed on the same paraffin block on serial sections, demonstrated ALK fusion. Positive ALK immunostaining was also found in CTCs isolated in five patients, corresponding to the same patients having ALK-fusion in their lung tumours. The immunocytochemistry using the 5A4 clone showed strong cytoplasmic staining of all the CTCs on filters. Further, we have successfully performed FISH analysis on CTCs which confirmed the presence of the ALK translocation

in these cases. Finally, for these five patients, filters stained with MGG showed cells with clear malignant features. The clinicopathological analysis demonstrated that the ALK positive patients were Caucasian never-smoker patients, having lung adenocarcinoma mostly with solid architecture. ALK rearrangement was detected in three advanced tumours, as well as in two patients with IIA stage NSCLC. Thus, we demonstrated the feasibility of the non-invasive detection of ALK fusion on CTCs isolated by ISET.

## 2.3 Cells as biomarkers - Tumour-associated neutrophils

### *Article 9*

Predictive clinical outcome of the intratumoral CD66b-positive neutrophil-to-CD8-positive T-cell ratio in patients with resectable nonsmall cell lung cancer.
**Ilie M**, Hofman V, Ortholan C, Bonnetaud C, Coëlle C, Mouroux J, Hofman P.
*Cancer. 2012;118:1726-1737.*

### RESULTS

In this study, we wanted to explore the conflicting ways by which tumour-associated neutrophils may operate on the outcome of NSCLC patients. We were the first to evaluate the impact of intratumoral neutrophils and the intratumoral neutrophil to- lymphocyte ratio (iNTR) (i.e., the ratio of CD66b-positive neutrophils to CD8-positive T lymphocytes) as a prognostic biomarker in NSCLC patients.

Expression levels of MPO, CD66b and CD8 were evaluated by immunohistochemistry on TMA consisting of 632 NSCLC specimens from patients who underwent curative surgery. The relation between clinicopathological variables and patient outcome was assessed.

CD66b-positive neutrophils either were localized within the blood vessels of tumors or were scattered diffusely throughout the tumor. The distinction between intratumoral CD66b-positive neutrophils and vessel-only CD66b-positive neutrophils was made by manually excluding positive cells located in vessels from tumors. Patients who had high intratumoral CD66b-positive neutrophil density had a significantly increased cumulative incidence of relapse (CIR) and had a trend toward decreased OS. We observed that MPO-positive cells were greater in number than CD66b-positive cells. However, we did not observe any correlation between MPO-positive neutrophils and outcome. Only the CD66b-positive phenotype was associated significantly with poor survival, clearly identifying a subgroup of high-risk patients with NSCLC. The CD66b-positive antigen is expressed specifically on neutrophils and may be assigned to a limited, activated subtype of neutrophils. In contrast, the amount of MPO-positive cells may not be an accurate marker of activated intratumoral neutrophils. Moreover, we identified no significant correlation between MPO-positive neutrophils and the clinical outcome of patients with NSCLC.

Furthermore, given the major role played by tumor-promoting neutrophils on the inhibition of the cytotoxic response of the CD8-positive T cells, we sought to determine whether this balance in cells of the immune system is modified in a tumor tissue microenvironment. In the current study, high

levels of intratumoral CD66b-positive neutrophils were associated with low levels of CD8-positive T cells. We demonstrated that the iNTR was strongly correlated with a high CIR and poor OS. Moreover, in the current study, it is noteworthy that the iNTR predicted significantly poor OS and a high CIR in early-stage NSCLC. Our study raised the possibility that the recruitment of neutrophils may be doubly beneficial for the developing tumor by directly promoting tumor progression while simultaneously affording a means to evade immune destruction. Given the exploratory nature of our study, further investigations are needed to clarify the mechanism of action of intratumoral neutrophils.

# CHAPTER 3 CONCLUSIONS AND PERSPECTIVES

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# CONCLUSIONS AND PERSPECTIVES

The overall aim of this thesis was to determine the potential and interest of the integrating (bio) pathology concept into the discovery and validation of new biomarkers in NSCLC. For this purpose, we explored several aspects related to NSCLC carcinogenesis and tumour progression, mostly by using human bio-resources collected both in a pathology laboratory and in a hospital-related oncology biobank. In particular, the works presented here have been broken down into three main fields, namely:

- Assessment of the diagnostic and prognostic value of two hypoxia targets
- Assessment of the diagnostic, prognostic and predictive value of circulating tumour cells
- Assessment of the prognostic value of tumour-associated neutrophils

Between these three large "chapters" runs a joint theme, with all the works presented aiming to integrate biopathology into the translational research field related to biomarker discovery.

The general conclusions of this thesis are summarized as follows:

- High tissue and plasma CAIX expression is a biomarker of poor prognosis and early relapse in NSCLC patients
- High CAIX plasma level significantly discriminates between cancer patients and healthy individuals
- High CAXII tissue is predictive of a more favourable evolution in NSCLC patients
- The antagonistic impact of CAIX and CAXII on the outcome of NSCLC patients might be an effect of the dynamic reoxygenation of tumours
- High intratumoral CD66b-positive neutrophil density and high intratumoral CD66b-positive neutrophil to-CD8-positive lymphocyte ratio were correlated with increased cumulative incidence of relapse and poor survival of NSCLC patients
- Levels of CTC detected by using two methods of isolation were associated with poor outcome of patients with NSCLC
- The detection of CTCs has good sensitivity and specificity for discriminating cancer patients from individuals without neoplastic disease
- We demonstrated the feasibility of the non-invasive detection of *ALK* fusion gene on CTCs

The field of lung cancer biomarker research is very broad and in continuous development and as such it is premature to make conclusive statements. Nevertheless, there are some aspects which need some further general considerations.

One of the main objectives of integrative (bio)pathology in lung cancer is to identify biomarkers that discriminate between low- versus high-risk individuals and between non-neoplastic and

neoplastic lung diseases. Ultimately, these biomarkers can potentially be translated to non-invasive, simple, low cost, and reliable diagnostic tests for early detection of lung cancer. The underlying assumption behind these strong efforts is that tumour-specific or overexpressed proteins can be detected simply and accurately in complex clinical samples such as surrogate tissues and biofluids. The intensive research in genomics and proteomics aimed at identifying these biomarkers has yielded a large number of potential diagnostic biomarkers, although very few have progressed to the level of approval for clinics.

This disappointingly slow pace of lung cancer biomarkers discovery and validation is attributed to a host of technologic and methodological factors. The gap between promise and product can partially be explained by the fact that the current discovery methods are neither really reliable nor efficient. In this regard, biomarker research needs to be standardized in its structural aspects. The clinical question, outcomes and criteria for patient selections must be clearly defined before starting any research projects. Sufficient information on experimental methodology should be provided, as well as an accurate description of statistical methods, which should include proper adjustments and corrections for multiple testing. Another quandary is the quite limited capacity to usually verify and validate analytically existing candidate markers in a high-throughput manner. The lack of available quality reagents such as antibodies or methodologies to translate the discovery of candidates in tissue specimens and measure their concentration in the circulation remains a great challenge. Therefore, it is possible that biomarkers have already been "discovered" but not yet validated. Furthermore, once a long list of candidate biomarkers is compiled, no current standardized method exists for selecting those that are most promising for systematic validation. In addition, the reproducibility of biomarker data has been flawed because of the poor design, model over-fitting, and the lack of cross-validation and independent validation in different cohorts of patients. Changing technology, and low concentration of signals combined with few prospective studies make the area of lung cancer biomarker research still highly challenging to date.

Lung cancer is recognized as a complex and heterogeneous disease, not only at the biochemical level (genes, proteins, metabolites) but also at the tissue, biofluid, organism, and population level. In such complex context, there is a need to apply new knowledge generated from research studies into clinical practice. For instance, a biofluid-based molecular test may improve the selection of high-risk individuals for CT screening, distinguish those with malignant nodules from benign "lesions", and identify patients with particularly aggressive cancer. Clinical benefit could include further reductions in mortality and thus provide significant cost-savings to the health care system.

As suggested throughout my thesis, the isolation, development and characterization of CTCs as a non-invasive predictive biomarker have a huge clinical potential and can increase our knowledge of lung cancer pathophysiology. In early stage NSCLC patients the occult metastatic disease correlates with disease recurrence. Thus, sensitive and specific detection of CTCs in the blood has the potential to become a relevant prognostic biomarker for patients with resectable NSCLC.

Indeed, the main goal of preoperative detection of CTCs is to identify NSCLC patients with a high risk of recurrence after surgery in order to adopt the best therapeutic strategy and follow-up.

However, the clinical use of CTC detection as a new biomarker has to be very carefully validated. Indeed, despite the report of a large number of studies in lung cancer patients on CTC detection, methodological aspects concerning sensitivity, specificity and reproducibility have prevented a clear appraisal of the clinical impact. Numerous technologies have now been set up to improve CTC detection in lung cancer patients, some of them appear to be very sophisticated. Despite the large enthusiasm in method development, in the different translational research studies and some clinical trials, and despite the impressive number of recent publications concerning the CTC field, it is quite paradoxical to note that this approach is rarely taken into consideration by the clinical oncologists regarding their therapeutic choice for lung cancer patients. Indeed, the technology transfer from research to the hospital's biology laboratories is still difficult. CTC detection in lung cancer patients, especially for theranostic application, is currently a very competitive domain with different approaches also targeting non cellular blood biomarkers, such as free circulating tumour DNA. Thus, the identification of genomic alterations from free circulating DNA isolated from lung cancer patients could be an attractive and probably less costly option for some specific indications. Finally, the use of non-invasive approaches based on CTCs and/or other blood biomarkers to develop so called «liquid biopsy» applications could allow, in the future, monitoring of lung cancer patients on therapy in order to diagnose early recurrence or disease progression, but also to decipher the molecular mechanisms of resistance for a rapid treatment adjustment.

The concept of a liquid biopsy needs to be considered in tight association with the different parameters obtained from the pathology laboratory (data from the surgical specimen, bronchial and trans-parietal biopsies and/or from bronchial cytology). These latter parameters include the morphology, the immunohistochemical and the molecular pathology data. *Only a combined approach that integrates the pathologist, the cytopathologists and the biologists will allow optimisation of the interpretation of the results of the liquid biopsy while avoiding the different pitfalls.* Thus developing new training options of expertise as a "biopathologist" could be a good way to obtain a global view of the lung cancer disease both for diagnosis and for a better understanding of the pathophysiology of the disease.

The field of molecular characterization of CTCs is vast as virtually all genomic alterations detected in lung cancer tissues can be detected in CTCs. In the future, next generation sequencing methods applied to CTCs from lung cancer patients could probably explore several hundred mutations and may be more on different genes. Furthermore, the development of antibodies and probes specific to mutated nucleic acids and proteins could be used in FISH and immunolabeling assays to speed up detection in CTCs of druggable genomic alterations. In addition to the theranostic impact, molecular characterization of CTCs and in particular transcriptomic analyses may be of great help in studying the origin of occult lung cancer in patients without detectable imaging of the primary tumour.

Finally, it is becoming increasingly clear that tumour-associated neutrophils play a major role in cancer biology. TAN are a distinct population of neutrophils, and recent evidence demonstrated the dual roles, protumoral and antitumoral, of TAN within the multiple step paradigm of cancer. In addition, it is noteworthy that cancer cells in bronchoalveolar carcinoma, a subtype of lung adenocarcinoma, are able to recruit neutrophils to the tumour microenvironment by producing interleukin-8, a chemo-attractant of neutrophils. In addition, *KRAS* mutant lung tumours in mice recruit neutrophils through CXC chemokine release. With regard to the impact of *KRAS* mutations on the survival of patients with NSCLC, it may be of interest to perform further studies comparing the different *KRAS* mutations with intratumoral CD66b-positive neutrophils in a large series of NSCLC. Neutrophils are thus an important underappreciated cell population in cancer biology, and their functions need to be better characterized. A more complete understanding of the way these cells support or fight cancer will be important to develop strategies to direct the immune system against tumours.

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# ANNEX

## Tables (Annex)

**Articles (Annex)**

**Article A1.** Expression of a truncated active form of VDAC1 in lung cancer associates with hypoxic cell survival and correlates with progression to chemotherapy resistance. Cancer Res. 2012

**Article A2.** Two Panels of Plasma MicroRNAs as Non-Invasive Biomarkers for Prediction of Recurrence in Resectable NSCLC. PLoS One. 2013

**Article A3.** Significance of circulating tumor cell detection using the CellSearch system in patients with locally advanced head and neck squamous cell carcinoma. Eur Arch Otorhinolaryngol. 2013

**Article A4.** Usefulness of Immunocytochemistry for the Detection of the BRAF(V600E) Mutation in Circulating Tumor Cells from Metastatic Melanoma Patients. J Invest Dermatol. 2013

**Article A5.** Pitfalls in lung cancer molecular pathology: how to limit them in routine practice? Curr Med Chem. 2012

**Article A6.** Usefulness of tissue microarrays for assessment of protein expression, gene copy number and mutational status of EGFR in lung adenocarcinoma. Virchows Arch. 2010

**Article A7.** Immunohistochemistry to identify EGFR mutations or ALK rearrangements in patients with lung adenocarcinoma. Ann Oncol. 2012.

**Article A8.** Diagnostic value of immunohistochemistry for the detection of the BRAFV600E mutation in primary lung adenocarcinoma Caucasian patients. Ann Oncol. 2013

**Article A9.** The Potential Value of Immunohistochemistry as a Screening Tool for Oncogenic Targets of Personalized Lung Cancer Therapy. J Oncopathol. 2013

**Article A10.** Autophagy plays a critical role in the degradation of active RHOA, the control of cell cytokinesis and genomic stability. Cancer Res. 2013 May 23. [Epub ahead of print].

This thesis has also resulted in the following oral and poster presentations:

## ORAL COMMUNICATIONS - INTERNATIONAL CONGRESSES

### (*FIRST AUTHOR*)

1. M. Ilie, J. Pouysségur, V. Hofman, N. Mazure, C. Butori, S. Lassalle, C. Bonnetaud, J. Mouroux, N. Vénissac, P. Hofman. High level of carbonic anhydrase IX (CAIX) in tumour tissue and plasma is associated with non-small cell lung carcinoma (NSCLC) progression and poor survival of patients. 22nd European Congress of Pathology, Florence 2009 (abstract OP22.8. Virchows Archiv 2009: S70).
2. Ilie M, Hofman V, Butori C, Marquette Ch, Vénissac N, Mouroux J, Hofman P The presence of intra-tumoral neutrophils is an independent prognostic factor in early stage non-small cell lung carcinomas. Intercongress Meeting of The European Society of Pathology, Krakow, Poland, 2010.
3. Ilie M, Long E, Butori C, Hofman V, Coelle C, Mauro V, Zahaf K, Marquette C.H., Mouroux J, Paterlini-Bréchot P, Hofman P. ALK-gene rearrangement, a comparative analysis on circulating tumour cells and tumour tissue from lung adenocarcinoma patients. 24th European Congress of Pathology, Prague 2012. (OFP-18-002. Virchows Archiv 2012; 461:S1-S332).
4. M. Ilie, V. Hofman, C. Butori, C. Bonnetaud, J. Mouroux, N. Mazure, J. Pouysségur, P. Brest, P. Hofman. Dynamic responses of carbonic anhydrase isoforms IX and XII during tumour reoxygenation: contribution to an aggressive phenotype and discrimination of clinical outcome in non-small cell lung cancer (NSCLC). 24th European Congress of Pathology, Prague 2012. (OFP-18-001. Virchows Archiv 2012; 461:S1-S332).
5. Ilie M, Blot L, Hofman V, Long E, Nunes M, Butori C, Selva E, Merino-Trigo A, Vénissac V, Mouroux J, Vrignaud P, Hofman P. Usefulness of linking biobanking field and animal model: High successful rate of human primary non-small cell lung carcinoma (NSCLC) xenografts in a model system separated by distance and time. 24th European Congress of Pathology, Prague 2012. (OFP-10-010. Virchows Archiv 2012; 461:S1-S332).
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## ORAL COMMUNICATIONS - NATIONAL CONGRESSES

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1. M. Ilie, V. Hofman, N. Mazure, S. Lassalle, C. Butori, E. Selva et al. Expression tissulaire et plasmatique de l'anhydrase carbonique IX (CA IX) dans les carcinomes pulmonaires non à petites cellules. Carrefour Pathologie 2008, Société Française de Pathologie, Maison de la Chimie, Paris (Ann Pathol 2008; 28 : S144).
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7. Ilie M, Long E, Butori C, Hofman V, Coelle C, Mauro V, Zahaf K, Marquette C.H., Mouroux J, Paterlini-Bréchot P, Hofman P. Le réarrangement du gène ALK-EML4 : Analyse comparative sur les cellules tumorales circulantes et le tissu tumoral à partir de 84 cas d'adénocarcinomes broncho-pulmonaires. Carrefour Pathologie 2012, Société Française de Pathologie, Maison de la Chimie, Paris (Ann Pathol 2012 ; 32, S1-S189).
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1. Hofman V, Ilie M, Butori C, Selva E, Vignaud JM, Thivolet F, Vénissac N, Mouroux J, Brambilla E, Barbry P, Mari B, Hofman P. Gene Expression Profiling of the Tumor Microenvironment during Non-Small Cell Lung Cracinoma (NSCLC) Progression. United States and Canadian Academy of Pathology. Washington DC. Mod Pathol, 2010, 1802; 403A.
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## ORAL COMMUNICATIONS - NATIONAL CONGRESSES

### (*COAUTHOR*)

1. V. Hofman, E. Selva, C. Bonnetaud, E. Long, C. Butori, P. Vielh, T. Molina, J-M Vignaud, J-F Fléjou, S. Lantuejoul, E. Piaton, N. Mourad, M. Poudenx, M Ilie et al. Détection préopératoire des cellules circulantes non hématologiques dans les carcinomes pulmonaires non à petites cellules par la Méthode ISET. Carrefour Pathologie 2008, Société Française de Pathologie, Maison de la Chimie, Paris (Ann Pathol 2008; 28 : S144).
2. V. Hofman, E. Long, M. Ilie, E. Selva, C. Bonnetaud, V. Lespinet, C. Coelle, T. Molina, P. Vielh, J. Mouroux, N. Vénissac, P. Paterlini-Bréchot, P. Hofman. Détection préopératoire des cellules tumorales circulantes (CTC) chez des patients atteints d'un carcinome pulmonaire non à petites cellule : résultats préliminaires d'une étude comparative des méthodes CellSearch et ISET à partir d'une série consécutive de 116 patients. Carrefour Pathologie 2009, Société Française de Pathologie, Maison de la Chimie, Paris (Ann Pathol 2009; 29: S152).
3. C. Ortholan, V. Hofman, O. Bordone, M. Ilie, V. Virolle, T. Virolle, N. Vénissac, J. Mouroux, P. Barbry, B. Mari, P. Hofman. Identification d'une signature microARN prédictive d'une récidive tumorale précoce des carcinomes broncho-pulmonaires non à petites cellules de stade I. Etude préliminaire à partir de tissus congelés ou de tissus fixés en formaldéhyde. Carrefour Pathologie 2009, Société Française de Pathologie, Maison de la Chimie, Paris (Ann Pathol 2009; 29 : S120).
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5. V. Hofman, M. Ilie, E. Long, V. Lespinet, C. Bonnetaud, CH. Marquette, J. Mouroux, P. Hofman. Analyse du statut mutationnel du gène *KRAS* à partir de 411 adénocarcinomes broncho-pulmonaires d'une population française caucasienne : Corrélation avec le statut tabagique, le sous types histologiques, le stade pTNM et

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## POSTER COMMUNICATIONS - INTERNATIONAL CONGRESSES

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7. P. Fouret, M. Ilie, S. Roux, M. Alifano, V. Hofman, F. Leroy-Ladurie, I. Rouquette, P. Validire, F. Vaylet, P. Hofman. Specificity and sensitivity of immunohistochemistry for detecting *EGFR* mutations in lung cancer cells. 23rd European Congress of Pathology, Helsinki 2011.
8. O. Bordone, M. Ilie, E. Selva, V. Hofman, V. Gavric-Tanga, E. Pedrazzi, C. Lagardére, P. Mayer, J. Mouroux, C. Chabannon, P. Hofman. Improvement of the "pre-analytical" phase control in a molecular pathology laboratory using Radio Frequency IDentification (RFID)-based system. Experience of a single institution (CHU Nice, France). 23rd European Congress of Pathology, Helsinki 2011.
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## POSTER COMMUNICATIONS - NATIONAL CONGRESSES

1. E Selva, M Mari, V Hofman, S Lassalle, C Butori, C Bonnetaud, M Ilie, O Bordone, K Havet, C Coelle, V Gavric-Tanga, Y Bille, P Grier, D Sadoulet, O Carrugi, V Lespinet, A Dridi, MC Gaziello, L Lacour, B Brion, N Lerda, P Hofman. La plateforme de biopathologie du Laboratoire de Pathologie Clinique et Expérimentale (LPCE) du CHUN. « Journée Plateformes de recherche en sciences du vivant. De la stratégie nationale aux enjeux régionaux », Cancéropôle PACA, Marseille 2009.
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7. M. Ilie, V. Hofman, C. Butori, C. Bonnetaud, J. Mouroux, N. Mazure, J. Pouysségur, P. Brest, P. Hofman. Dynamic responses of carbonic anhydrase isoforms IX and XII during tumour reoxygenation: contribution to an aggressive phenotype and discrimination of clinical outcome in non-small cell lung cancer (NSCLC). The 4th EMBO Meeting. Nice 2012.
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