Toxicity and pharmacokinetic biomarkers for personalized non-small cell lung cancer treatment

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Toxicity and pharmacokinetic biomarkers for personalized non-small cell lung cancer treatment

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During the course of the research underlying this thesis, Anna Svedberg was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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“If we knew what it was we were doing,
it would not be called research,
would it?”

– Albert Einstein

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ABSTRACT

Lung cancer is the leading cause of cancer-related deaths worldwide. Unfortunately, lung cancer is usually discovered at a late stage when the curative treatment options are limited. The treatment can include surgery, radiation, chemotherapy, targeted therapy and now also immunotherapy.

The challenge in cancer treatment is to eradicate cancer by the use of harsh treatments, while still, keeping the patient alive. For this purpose, treatments with severe toxicities are usually accepted but regularly lead to dose reductions or postponed treatment. Large variations in response are generally observed between patients treated with the same drug at the same dose. The dose may be adequate in one patient while ineffective or cause severe adverse drug reactions in other patients. The occurrence of drug-induced toxicities can, however, also be a positive indicator of treatment response. In personalized treatment it is of importance to select the most suitable treatment option and give it at the most favorable dose, to enable the patients to stay on treatment during the time the treatment is able to affect cancer since the tumor commonly develops resistance towards the treatment eventually.

In this thesis, inter-individual variability in pharmacokinetics and toxicity for the targeted therapy erlotinib, associated with the adverse events skin rash and diarrhea was studied. Inter-individual variability in toxicity was also studied for the chemotherapy treatment gemcitabine/carboplatin linked to the hematological toxicities neutropenia and leukopenia.

Erlotinib was studied in papers I-IV. Erlotinib and its metabolite concentrations were determined using a validated LC-MS/MS method. Diarrhea was associated with erlotinib and the metabolite M13, while skin rash was associated with the activity of the erlotinib metabolizing enzyme CYP3A and the ABCG2 single nucleotide polymorphism rs10856870. CYP3A was also shown to be induced during treatment. Additionally, *in vitro* studies showed that genetic variability in ABCG2 contributes to differences in intracellular concentrations. Genes and gene variants were found to be associated with gemcitabine/carboplatin-induced toxicity in paper V. The variants were partially validated, and two models were developed to estimate the risk of leukopenia or neutropenia based on a set of genetic variants.

SVENSK SAMMANFATTNING

Lungcancer är den cancerform som leder till flest antal dödsfall runt om i världen. Tyvärr upptäcks lungcancer oftast i ett sent skede när möjligheten att bota cancer är begränsad. Lungcancer kan behandlas med flera behandlingsmetoder, antingen enskilt eller i kombination, som till exempel kirurgi, strålning, cellgifter, målriktad behandling eller immunoterapi.

Den stora svårigheten vid cancerbehandling är att eliminera cancer samtidigt som patienten klarar av behandlingen. Cancerbehandling innefattar ofta starka läkemedel som vanligtvis kan ge upphov till svåra biverkningar som resulterar i dosreduktion, uppehåll i behandling eller till och med avslutad behandling. Det finns idag flera behandlingsalternativ att välja mellan. Det är därför av stor vikt att välja en behandlingsmetod som cancertumören svarar på, samtidigt som behandlingen ges i rätt dos för nå önskad effekt. Det finns idag stor variation mellan patienter i hur de svarar på cancerbehandling, vissa patienter får bra effekt av behandlingen medan andra patienter inte får någon effekt alls eller får svåra biverkningar. Tumören har en förmåga att efter en tid utveckla resistens. Det är därför viktigt att patienten behandlas effektivt mot cancer under en så lång period som möjligt när det finns en effekt mot tumören.

I den här avhandlingen har två olika behandlingar vid icke-småcellig lungcancer studerats för att bättre förstå vad som orsakar variation mellan patienter. Den ena behandlingen är en målriktad behandling med erlotinib (Tarceva) som vanligtvis ger biverkningar i form av hudutslag eller diarré. Den andra studerade behandlingen är en kombination av cellgifter, gemcitabin tillsammans med karboplatin, som vanligtvis ger biverkningarna leukopeni och neutropeni som leder till försämrat immunförsvar.

I delarbete I-IV studerades erlotinib, antingen via ett modellsystem i form av en cellinje eller från behandlade lungcancerpatienter. Variation av läkemedelskoncentrationer samt genetisk variation i arvsmassan studerades. Läkemedelskoncentrationer i erlotinibpatienters blod analyserades med en utvecklad och validerad kromatografimetod. Diarré visade sig vara kopplat till koncentrationen av erlotinib och metaboliten M13. Hudbiverkningar var associerade med CYP3A aktivitet, som är enzymet som bryter ner erlotinib i kroppen. CYP3A visade sig också att öka sin aktivitet i samband med att man påbörjar erlotinibbehandling. Hudbiverkningar kopplades också till en kvot av metaboliter (OSI-420/didesmethylerlotinib) och en

naturlig genetisk variation i *ABCG2*-genen som är involverad i transport av erlotinib ut ur kroppen.

I delarbete V studerades genetisk variation i gemcitabin/carboplatin behandlade lungcancerpatienter. Då identifierades naturlig genetisk variation i arvsmassan kunna förklara en del av variationen i uppkomsten av biverkningar. Flera genetiska varianter användes för att bygga modeller som kan användas för att förutspå om patienter löper hög risk att drabbas av svåra cellgiftsbiverkningar.

LIST OF PAPERS

- I. A validated liquid chromatography-tandem mass spectrometry method for quantification of erlotinib, OSI-420 and didesmethyl erlotinib and semi-quantification of erlotinib metabolites in human plasma
Anna Svedberg, Henrik Gréen, Anders Vikström, Joakim Lundeberg and Svante Vikingsson.
Journal of Pharmaceutical and Biomedical Analysis (2015)
- II. Erlotinib treatment induces cytochrome P450 3A activity in non-small cell lung cancer patients
Svedberg A, Vikingsson S, Vikström A, Hornstra N, Kentson M, Branden E, Koyi H, Bergman B, Gréen H.
British Journal of Clinical Pharmacology (2019)
- III. Identification of biomarkers in erlotinib treated non-small cell lung cancer patients
Anna Svedberg, Svante Vikingsson, Anders Vikström, Niels Hornstra, Magnus Kentson, Eva Brandén, Hirsh Koyi, Bengt Bergman, and Henrik Gréen.
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- IV. The influence of ABCG2 polymorphism on erlotinib efflux in the K562 cell line
Anna Svedberg, Lianne Jacobs, Svante Vikingsson and Henrik Gréen.
Submitted to pharmacology research and perspectives
- V. A whole-exome sequencing study of gemcitabine/carboplatin induced leukopenia and neutropenia in non-small cell lung cancer patients
Anna Svedberg, Benjamín Sigurgeirsson, Niclas Björn, Sailendra Pradhananga, Eva Brandén, Hirsh Koyi, Rolf Lewensohn, Luigi De Petris, María Apellániz-Ruiz, Cristina Rodríguez-Antona, Joakim Lundeberg and Henrik Gréen.
Manuscript

Other co-authored papers, not included in the thesis:

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ABBREVIATIONS

ABC	ATP-binding cassette
ADR	Adverse drug reaction
AE	Adverse events
AUC	Area under the curve
CDA	Cytidine deaminase
CTCAE	Common toxicity criteria for adverse events
CYP	Cytochrome P450
EGFR	Epidermal growth factor receptor
EYFP	Enhanced yellow fluorescent protein
GWAS	Genome-wide association studies
HLM	Human liver microsome
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LLOQ	Lower limit of quantification
NSCLC	Non-small cell lung cancer
MAF	Minor allele frequency
MS/MS	Tandem mass spectrometry
OS	Overall survival

PBS	Phosphate-buffered saline
PFS	Progression-free survival
TKI	Tyrosine kinase inhibitor
TDM	Therapeutic drug monitoring
SNP	Single nucleotide polymorphism

INTRODUCTION

Non-small cell lung cancer

Lung cancer is the leading cause of cancer-related deaths worldwide, but the mortality rate is decreasing [1]. Yearly, around 4000 Swedes are diagnosed with lung cancer [2], and the incidence of lung cancer is still slightly higher in males but the differences have almost disappeared. Lung cancer is divided into non-small cell lung cancer (NSCLC, 85%) which confers a better prognosis compared to small cell lung cancer [1, 2]. Non-small cell lung cancer is further subgrouped into adenocarcinoma, squamous cell carcinoma and large cell carcinoma.

Several risk factors have been associated with lung cancer [3]. One factor is the individual genetic composition that can increase the lung cancer risk based on natural genetic variation or based on a familial inherited predisposition to lung cancer [4-6]. The main risk factors for developing lung cancer is nevertheless smoking and the duration of smoking [7, 8]. Additionally, exposure to secondhand cigarette smoke increases the risk of developing lung cancer [9, 10]. Other risk factors include, but are not limited to, ionizing radiation, radon, asbestos, silica, polycyclic aromatic hydrocarbons and air pollutions [11-14].

The long term survival of NSCLC strongly depends on the tumor stage at diagnosis. The 5-year relative survival rate for adenocarcinoma is 68.7%,

42.1%, 6.5% in localized, regional and distant stages, respectively [15]. The rates are slightly lower for squamous cell carcinoma and large cell carcinoma.

Treatment options

Depending on the tumor stage and the health of the patient, treatment options alone or in combination can include surgery, radiation, chemotherapy, targeted therapy, or immunotherapy. At the time of lung cancer diagnosis, approximately 70% of the patients have advanced NSCLC (stage III/IV) [16] and the possibility to cure the cancer is limited. The lower stage NSCLCs are usually treated with surgery, chemotherapy, and radiation, either alone or in combination. Treatment options in inoperable advanced NSCLC have increased in the last decades to not only include chemotherapy, but to also include targeted therapy and immunotherapy, Figure 1.

Figure 1: The different treatment options to select from in advanced NSCLC depend on the occurrence of mutations (EGFR and BRAF), translocations (ALK and ROS1) and expression of immune checkpoint proteins (PD-L1) in the tumors.

In common for all the above mentioned anti-cancer drugs are treatment-induced toxicities due to narrow therapeutic windows. The adverse drug reactions vary depending on treatment options and are usually related to the mechanism of action of the drug. For instance, standard chemotherapy acts on rapidly dividing cancer cells, but also on other rapidly dividing cells such as blood cells [17]. Targeted therapies are suggested to inhibit the wild-type target and immunotherapies are associated with immune-related adverse drug reactions [18, 19]. Off-target effects causing adverse drug reactions are, however, also favored because these can be a sign that the treatment is effective [20].

The evaluation of toxicity is usually made according to the common terminology criteria of adverse events (CTCAE) [21]. CTCAE grades adverse events with increasing toxicity from grade 1 being mild to grade 5, if possible, being death caused by an adverse drug reaction, Table 1.

Table 1: Grading of adverse drug reactions according to CTCAE.

	Grade	Intervention
1	Mild	Clinical or diagnostic observations only
2	Moderate	Local or noninvasive intervention indicated
3	Severe	Hospitalization indicated
4	Life-threatening	Urgent interventions indicated
5	Death	Death related to adverse events

Chemotherapy with gemcitabine and carboplatin

There are several different chemotherapy drugs available. Typically, a platinum drug is combined with another agent [22], such as the platinum-based chemotherapy doublet carboplatin in combination with gemcitabine. Gemcitabine and carboplatin are usually given intravenously in cycles of 3 weeks [23]. Gemcitabine is given on days 1 and 8 at a dose of 1250 mg/m³ and the carboplatin is given on day 1 at a dose required to reach area under the curve (AUC) 5 according to the Calvert formula.

Carboplatin is an alkylating agent. Positively charged carboplatin can bind to the negatively charged DNA in the nucleus to the guanine nucleotide forming platinum-DNA complexes. The monoadducts and intra- and interstrand crosslinks created by carboplatin interfere with transcription and/or DNA replication that eventually leads to cell death [24].

Gemcitabine is nucleoside analog to deoxycytidine and is classified as an antimetabolite [25, 26]. Gemcitabine is a probe drug and requires several phosphorylation steps to become a biologically active substance. Gemcitabine diphosphate indirectly contributes to inhibition of DNA synthesis by inhibition of the ribonucleotide reductase enzyme that is responsible for producing the dinucleotides required for DNA synthesis and repair. This leads to a decrease in deoxynucleotides which favor gemcitabine triphosphate in its competition with dCTP for incorporation into DNA. Gemcitabine triphosphate directly inhibits DNA synthesis by masked chain termination. Instead of dCTP, gemcitabine triphosphate incorporates into DNA, followed by one additional incorporated nucleotide. This disables further DNA synthesis and prevents the use of end repair mechanisms.

The dose-limiting toxicity of gemcitabine and carboplatin is myelosuppression, also known as hematological toxicity [27, 28]. This leads to a reduction in the number of blood cells that are essential for immunity (leukocytes and neutrophils), coagulation (thrombocytes), and oxygen transport (red blood cells) and these lead to leukopenia, neutropenia, thrombocytopenia, and anemia, respectively. Signs of myelosuppression prior to initiating a new treatment cycle lead to actions such as dose reduction, treatment delay or treatment discontinuation in severe cases. Severe

adverse events of grade 3-4 according to the CTCAE in patients undergoing gemcitabine/carboplatin treatment have been reported in around 20-70% of the study populations [23, 29-31].

The myelosuppressive toxicities can be managed by blood transfusion to improve the peripheral blood status or by addition of growth factors, such as granulocyte colony-stimulating factor that promotes myeloid cell generation, and in severe cases by bone marrow transplantation [32, 33].

EGFR targeted therapy with erlotinib

Several therapies have been developed to target specific oncogenic driver mutations or translocations in tumors, one of which is to target the epidermal growth factor receptor (EGFR).

Normal EGFR is essential for the development of several epidermal structures in the lung, skin, pancreas, gastrointestinal tract and central nervous system, and developmental defects are seen in EGFR knockout mice and dysregulated and overactive EGFR signaling is commonly seen in several cancer types [34-36]. EGFR signaling is initiated when EGF or other ligands bind to the extra-cellular EGFR. Ligand binding leads to the activation of the intracellular tyrosine kinase domain and ATP phosphorylates the tyrosines on the C-terminal end of the opposite EGFR monomer [37]. The phosphorylated tyrosines initiate further downstream signaling by binding to proteins associated with cellular responses such as cell proliferation, survival, motility, and differentiation. In cancer, EGFR mutations in the tyrosine kinase domain are suggested to destabilize the inactive state of the tyrosine kinase causing it to be in a constantly active state that leads to uncontrolled tumor growth [38, 39].

Dysregulated EGFR signaling in NSCLC can be treated with EGFR tyrosine kinase inhibitors (TKIs), for instance, erlotinib [40]. Unlike chemotherapy that is administered intravenously, erlotinib is taken as a tablet daily at a recommended dose of 150 mg. Erlotinib is approved as a first-line treatment in advanced NSCLC in patients with specific EGFR activating tumor mutations in the tyrosine kinase domain. The most common mutations – L858R and exon 19 deletions – requires a lower erlotinib concentration compared to wild type EGFR in order to inhibit the EGFR signaling. These mutations are present in around 10% of the Caucasian population and in around 35% of the Asian population [41]. Erlotinib acts by preventing the phosphorylation of the tyrosine kinase domain by reversibly binding to the tyrosine kinase domain and blocking the binding site of ATP [42].

Skin rash and diarrhea are the typical adverse reactions to drugs targeting the EGFR signaling and lead to dose reductions and treatment discontinuations. Skin rash of any grade develops in around 75% of patients, while, diarrhea of any grade affects around 50 % of patients [43-45]. EGFR

is essential for proliferation, survival, and differentiation in the skin and gastrointestinal tract [46, 47]. The skin rash appears after around 1 week [48] and is suggested to be due to the off-target effect of erlotinib acting in a dose-dependent manner on wildtype EGFR in the skin, creating an inflammatory response [49]. Diarrhea appears within the first 4 weeks of treatment and is suggested to be due to inhibitions of wildtype EGFR in the gastrointestinal tract causing dysregulated ion transport, inflammation, and mucosal injury [50-52].

Skin rash can be alleviated by the use of sunscreen, emollients and soap substitutes, antibiotics such as tetracyclines that also harbor anti-inflammatory properties, antihistamines or topical corticosteroids [53]. Diarrhea can be managed by changes in diet or antidiarrheal medication upon onset [50]. It is important to start the treatment for the erlotinib induced toxicities as soon as they appear in order to avoid unnecessary patient suffering.

Inter-individual variation in response and toxicity

The response to a drug of the same dose may vary between patients [54]. The dose may be adequate in one patient, but ineffective or cause adverse drug reactions in other patients.

The reason behind interindividual variation in response is usually an inadequate drug concentration that is not within the therapeutic window, which is the drug concentration interval required to obtain the desired therapeutic effect [55]. Drug concentrations below the therapeutic interval might lead to inadequate effects, while concentrations above can cause adverse drug reactions.

The drug concentration that is reached after drug exposure depends on both pharmacokinetic and pharmacodynamic processes [54]. Pharmacokinetic processes include the body's delivery of the drug to the site of action and the subsequent removal of the drug from the site of action over time. These events include absorption, distribution, metabolism, and elimination of the drug. Pharmacodynamic processes describe the action of a drug on its target and how well the target responds to the drug. Subsequently, both pharmacokinetic and pharmacodynamic processes are affected by genetic variations that can cause changes in drug exposure or alterations in the function of the drug target that then affect the drug response [56]. The study of how individual variation in DNA influences the drug response is called pharmacogenetics [57].

In personalized treatment, the aim is to be able to select the most suitable drug and give it at the most appropriate dose. Ideally, the response to a drug will be high while adverse drug reactions will be low. To enable a personalized treatment approach in NSCLC treatment it is important to

study the possible underlying mechanisms that contribute to the interindividual variation in response and adverse drug reactions. This thesis mainly focuses on the variability in erlotinib pharmacokinetics and pharmacogenetics and its association with erlotinib-induced toxicity as well as variability in gemcitabine and carboplatin pharmacogenetics and the association with hematological toxicity.

Inter-individual variability in erlotinib treatment

There is a large inter-individual variation in response and toxicity in erlotinib treatment due to pharmacokinetic and pharmacogenetic variability. Currently, there is evidence that higher erlotinib plasma concentrations correlate with skin rash and occasionally diarrhea in erlotinib treatment [58-60]. Also, the metabolic ratio, erlotinib/OSI-420, has been associated with skin rash [61] and it has been shown that the erlotinib concentration in the skin at the site of rash is higher compared to nearby unaffected skin [62]. Additionally, skin rash has been suggested to be a biomarker for the response because patients with skin rash tend to have longer progression-free survival (PFS) and overall survival (OS) [20, 63-65].

Pharmacokinetic variability in erlotinib treatment

Steady-state plasma trough concentrations of erlotinib are known to vary extensively. The average concentrations at steady-state have been reported to be 800-1600 ng/mL with a standard deviation of at least half the average concentration [40, 59-61]. Several factors influence the erlotinib concentration and some of them are described below.

Erlotinib is taken as a tablet daily at a recommended dose of 150mg. The tablet should be taken under fasting conditions, either 1 hour before or 2 hours after food intake to avoid the impact of food that increases the bioavailability from the expected 60% to as much as 100% [40, 66, 67]. The AUC₀₋₂₄ has been shown to be higher in patients taking erlotinib 2 hours after food intake compared to taking erlotinib 1 hour before food intake, suggesting that the absorption might be influenced by the different gastric emptying states [66].

The actively transporting enzymes ABCB1 and ABCG2 transport erlotinib *in vitro* [68, 69]. ABCB1 and ABCG2 are expressed for example, in the intestine and act by transporting substrates such as erlotinib back to the intestine [70, 71]. In a mouse model, the erlotinib bioavailability was increased in the ABCB1, ABCG2, and ABCC1 knock out mice compared to control [72].

Metabolism of erlotinib is mainly facilitated by enzymes in the CYP family, mostly by CYP3A4 but also by CYP3A5, CYP1A1 and CYP1A2 [73].

Metabolism occurs in the liver as well as outside, for instance, in the intestine by CYP3A and in the lung by CYP1A1 [74]. Erlotinib is metabolized into several metabolites and the main metabolite, OSI-420, is reported to be 12% of the total erlotinib and to consist of two isomers [40]. Patients who smoke during erlotinib treatment show a lower plasma concentration compared to non-smokers due to the induction of CYP1A1 and CYP1A2 by cigarette smoke [75]. The CYP3A4 enzyme is known to exhibit extensive intra- and inter-individual variation influenced by environmental, genetic and physiological factors [76]. The CYP3A activity is, for instance, higher in females compared to males [77, 78]. Co-administration of substances that inhibit or induce CYP3A can also contribute to modulating the erlotinib concentration [79].

Erlotinib is mainly eliminated as metabolites via feces (>90%), and only a small fraction is eliminated via renal excretion (<9%) [74]. Only 2% of erlotinib is eliminated unchanged.

Pharmacogenetic variability in erlotinib treatment

Pharmacogenetic parameters have been widely studied to explain the influence of germline variability on exposure, toxicity, PFS, and OS in treatment with erlotinib; however, no consensus regarding the identified SNPs has been reached. The main focus has been on the variability in the emergence of skin rash. These studies have been conducted mainly using targeted approaches, either focusing on the erlotinib target (EGFR) or on erlotinib metabolizing and transporting enzymes, by studying single nucleotide polymorphisms (SNPs) in genes as well as sequencing of entire genes [58, 60, 65, 80-88].

SNPs in the *EGFR* promoter region and in intron 1 have been shown to influence EGFR expression. In erlotinib treated patients, the promoter SNPs 216G/T (rs712829) and -191C/A (rs712830) have been associated with skin rash [65, 88] and diarrhea [58] and the *EGFR* intron 1 CA repeat has been associated with skin rash [89] and PFS and OS [86].

Genetic variation in *ABCG2* at 34G>A (rs2231137) and 421C>A (rs2231142) are well studied. The 34G>A change does not alter the EGFR expression while 421C>A is known to lower the expression of *ABCG2* [90, 91]. Studies on erlotinib treated patients have identified the *ABCG2* SNPs, -15622C/T, and 1143C/T which are associated with lower *ABCG2* expression to correlate with higher plasma concentrations [58], that 34G>A is associated with OS [84] and that 421C>A is associated with diarrhea [60]. The *ABCB1* haplotype 1236TT-2677TT-3435TT has been associated with skin rash and with erlotinib plasma concentration [87].

Several metabolizing enzymes have been studied, for instance, have SNPs in *CYP27B1* (rs8176345), *CYP4F11* (rs1064796) and *UGT3A1* (rs10045685)

have been associated with skin rash and adverse drug reactions in erlotinib treated patients [81, 83].

Additionally, an unhypothesized genome-wide association study (GWAS) was performed on never-smoking Asian females diagnosed with adenocarcinoma treated with EGFR-TKIs (mainly gefitinib), suggesting the 4q12 region to be important for PFS [92].

Pharmacogenetic variability in gemcitabine/carboplatin treatment

The pharmacogenetics studies on gemcitabine/carboplatin-induced toxicity have until recently focused on the genetic variability in specific targets in genes involved in the mechanism of action of each drug, for instance, transporters required for the drug to enter the cells, metabolizing enzymes, and DNA repair enzymes. However, the results are contradictory.

One prominently studied gemcitabine related-gene is cytidine deaminase (CDA), which catalyzes the inactivation of most of the administered gemcitabine, and it is known that low CDA activity correlates with a higher response rate, longer time to progression and OS [93]. One of the most frequently studied *CDA* SNPs, 79A>C (rs2072671), results in a lower CDA activity and lower gemcitabine clearance [94, 95]. Associations with neutropenia have been identified but the results have been contradictory [96-100]. Moreover, SNPs associated with hematological toxicity in DNA repair pathways have been extensively studied in several platinum-based chemotherapy combinations [100-105]. These genes are mainly located in the nucleotide excision repair pathway responsible for repairing intra-strand crosslinks. SNPs in *ERCC2*, *IL16*, *MMS19L*, *RAD18*, *XPC*, *XPB*, and *XRCC1* have for instance been associated with either hematological toxicity or leukopenia [101, 103, 105-108].

Additionally, a few hypothesis-free GWAS have been performed [109-111]. These studies have identified associations with two SNVs protective against platinum induced myelosuppression (rs13014982 and rs9909179) [109] and an additional, four SNVs associated with gemcitabine-induced leukopenia and neutropenia (rs11141915, rs1901440, rs12046844, and rs11719165) [110]. The largest GWAS (n = 13122) included several chemotherapy combinations and identified several SNPs associated with gemcitabine (rs9961113, rs2547917 and rs12900463) and carboplatin (rs11071200, rs3822735 and rs1623879) induced leukopenia and neutropenia [111]. The variants identified in the hypothesis-free approach haven't confirmed the association to regions identified in the knowledge-based candidate approach but rather identified SNPs in regions not previously studied.

AIM

The overall aim of this thesis was to obtain a better understanding of the inter-individual pharmacokinetic and pharmacogenetic variation in patients undergoing NSCLC treatment as well as to identify potential biomarkers for personalized NSCLC treatment.

The specific aims were to:

- Develop an LC-MS/MS method with the ability to quantify erlotinib and its metabolites in order to study pharmacokinetic variability in erlotinib treated NSCLC patients
- Evaluate the CYP3A activity as a potential biomarker in erlotinib treated NSCLC patients
- Study pharmacokinetic and pharmacogenetic variability in erlotinib-induced skin rash and diarrhea.
- Study the influence of genetic variability in ABCG2 on erlotinib transport *in vitro*
- Study the influence of pharmacogenetic variability in gemcitabine/carboplatin-induced leukopenia and neutropenia.

METHODS

In this section, the study populations and methods used in papers I-V are presented and discussed, and more details about the methods are available in the respective papers.

Patient cohorts and study designs

This thesis includes two studies on NSCLC patients, Table 2. The EM11-study included patients with advanced NSCLC treated with the EGFR-TKI erlotinib. Data obtained from the EM11-study were analyzed in papers I-III. The second study included NSCLC patients of mixed stages treated with gemcitabine and carboplatin in either an adjuvant or advanced setting. Data obtained from this study were analyzed in paper V. Both studies were performed after approval by the regional ethics committee in Linköping or Stockholm. Written informed consent was obtained from all patients prior to study entry.

The EM11-study

EM11 was a prospective observational study of advanced NSCLC patients, performed between 2013 and 2018 in Linköping, Kalmar, Jönköping, Gävle, and Göteborg. Patients diagnosed with advanced NSCLC scheduled for erlotinib treatment were eligible for inclusion, which led to 65 included patients in the study.

The average age of the study population was 68 years, and 68% were females and all were diagnosed with adenocarcinoma. EGFR tumor mutations were confirmed in 78% of the cohort. Among the patients, 5% were current smokers and 49% were former smokers. In total, 70% and 49% of the cohort experienced skin rash and diarrhea of any grade, respectively.

The study was designed to draw blood samples monthly from the patients in the first three months of treatment and to monitor toxicity up until 12 months of treatment, Figure 2. A sample for extraction of DNA was obtained at baseline. CYP3A activity was assessed before treatment start and after 2 months of treatment. Samples for erlotinib and metabolite plasma trough concentrations were drawn after 1, 2, and 3 months. Drug-induced toxicities, skin rash, and diarrhea were registered monthly up until 12 months of treatment according to CTCAE version 3.0.

Figure 2: Study design for the EM11-study that was analyzed in papers I-III.

In paper I, erlotinib plasma trough concentrations were analyzed after 1 month of erlotinib treatment in the first included patients (n=4). In paper II, a subset of 32 patients that had provided CYP3A activity samples were analyzed. CYP3A activity at baseline and after 2 months of treatment was available in 13 patients, and single point CYP3A activity was available in 19 patients, including 7 at baseline and 12 during treatment. In paper III, the entire study population was analyzed (n=50 patients) and 10 patients were excluded due to poor compliance or lack of clinical data and 5 patients were excluded because they were treated with the second-generation EGFR-TKI afatinib.

Table 2: Overview of the patients included in each paper.

Paper	Therapy	Number of patients	Time	Centers	Methods
I	Erlotinib	4	2013–2014	Linköping	LC-MS/MS
II	Erlotinib	32	2013–2016	Linköping Kalmar Jönköping Gävle Göteborg	HPLC
III	Erlotinib	50	2013–2018	Linköping Kalmar Jönköping Gävle Göteborg	LC-MS/MS HPLC Genotyping Gel electrophoresis
V	Gemcitabine/ Carboplatin	212	2006–2008	Stockholm	Whole exome sequencing

The gemcitabine/carboplatin study

In paper V, 215 NSCLC patients scheduled for gemcitabine/carboplatin chemotherapy treatment were included in the study in Stockholm between 2006 and 2008. The patients received at least one cycle of gemcitabine on days 1 and 8 (1,250 mg/m²) and carboplatin on day 1 (AUC = 5).

The median age of the study population was 64 years, 53% were females and adenocarcinoma was the most prevalent diagnosis. Among the patients, 43% were current smokers and 47% were former smokers. In total, 70% and 63% of the cohort experienced leukopenia and neutropenia of any grade, respectively.

The study was designed to draw a blood sample for DNA analysis and to monitor the drug-induced hematological toxicities of neutropenia, and leukopenia at baseline and weekly during the first cycle of 21 days. The nadir values were defined as the lowest leukocyte and neutrophil values measured on days 8, 14, and 21.

Three DNA samples were identified as outliers due to contamination and inadequate sequencing and subsequently removed from the statistical analysis.

Liquid Chromatography

Liquid chromatography (LC) methods with fluorescence and tandem mass spectrometry (MS/MS) detection were used in papers I-IV for the determination of CYP3A activity and for quantification of erlotinib and its metabolites, respectively.

In general, the components in an LC instrument include a mobile phase that is continuously pumped through the system to which a sample can be injected [112, 113]. The sample is transported with the mobile phase to the column where the separation takes place and the separated analytes are then further transported to the detector, Figure 3.

Figure 3: An overview of the included components in an LC system.

The separation of analytes in the column can be based on polarity. In the most common reverse phase chromatography, the column represents the non-polar stationary phase and the mobile phase corresponds to the polar phase [112, 113]. Substances with similar polarities are drawn together. The metabolites are in general more polar endogenous analytes compared to the main substance and are not as highly attracted to the column. This results in the elution of the metabolites prior to the main substance. The mobile phase is usually applied as a gradient, with increasing concentrations of organic solvent that change the polarity of the mobile phase to become more non-polar, in order to more rapidly elute the main compounds.

Different detectors can be used in combination with an LC system, for instance, UV and, fluorescence detectors and mass spectrometers. Fluorescence detection has greater sensitivity than UV detection but is limited because only a few analytes fluoresce [112]. The analytes are irradiated by a light providing excitation energy and the excited analytes emit light of lower energy that is detected at a 90° angle to the excitation wavelength.

The mass spectrometer operates under vacuum and is less dependent on chromatographic separation because the analytes are separated and detected based on mass to charge (m/z) ratios [112, 114]. Single gaseous ions are produced by electrospray ionization and directed towards the quadrupoles. The mass spectrometer consists of two quadrupoles, with a varying electric field only allowing ions of the desired parent and daughter m/z ratios to pass. Fragmentation takes place between the quadrupoles, as the ions collide with neutral gas molecules, for instance, argon. The signal that is passed through the mass spectrometer is registered by the detector.

Assessment of CYP3A activity

Both genetic and environmental factors influence CYP3A activity. To determine the real-time CYP3A activity, the phenotype is usually assessed using a probe drug [115]. The probe drug is a substance exclusively metabolized by CYP3A that is monitored in combination with the CYP3A generated metabolite in urine and/or plasma. The acquired ratio between the main substance and the metabolite is utilized as a measure of the CYP3A activity *in vivo*.

Quinine as a probe for CYP3A activity

Quinine was the probe drug used in the EM11 study to assess CYP3A activity, as reported in papers II and III. Quinine is hydroxylated only by CYP3A into the metabolite 3-OH-quinine, and the metabolic ratio, quinine/3-OH-quinine, is used as a measure of CYP3A activity [116], representing both hepatic and intestinal CYP3A activity [117]. Several other probe drugs are available for determination of CYP3A activity, for instance, oral or intravenous midazolam/1-OH-midazolam [118], or the endogenous CYP3A activity biomarkers cholesterol/4 β -hydroxycholesterol [119] and cholesterol/6 β -hydroxycholesterol [120].

In the EM11 study, a tablet of 250 mg of quinine was taken 16 ± 2 h before sampling. The quinine/3-OH-quinine ratio has been shown to be stable for 96 hours [116]. Consequently, if the tablet was not taken according to instruction, it is not likely to have an impact on the metabolic ratio. Another advantage of quinine as a biomarker for CYP3A activity is that sampling is only required once.

The HPLC method with fluorescence detection

An HPLC-method was setup and modified from a previously published article [121]. The method was partially validated for analysis of CYP3A activity in imatinib treated chronic myeloid leukemia patients [122]. The samples were analyzed using an HPLC system with fluorescence detection with

excitation and emission wavelengths of 350 nm and 450 nm, respectively, and the analytes were separated on an Xbridge C18 column at 60°C. The mobile phase was 0.1 M acetate buffer and acetonitrile, kept at a flow rate of 0.8 mL/min. The total run time was 12.5 min and included two linear gradients – from 10% to 14% acetonitrile from 0 to 5 min, followed by 14% to 26% acetonitrile from 5 min to 9.4 min, and finally returning to 10% acetonitrile from 9.4 min to 12.5 min. A typical chromatogram is illustrated in Figure 4.

Quinine and 3-OH-quinine were quantified in the range of 100–10,000 nM and 10–2,000 nM, respectively. Quality control samples were prepared at three concentration levels for quinine (150, 1000, and 7,500 nM) and 3-OH-quinine (20, 150, and 1,500 nM).

Figure 4: A typical chromatogram showing the peaks of 3-OH-quinine (4.9 min) and quinine (9.1 min).

Quantification of erlotinib and its metabolites

An LC-MS/MS method for quantification of erlotinib, OSI-420, and didesmethyl erlotinib and for semi-quantification of metabolites was developed in paper I. The method was further used in papers I and III to analyze patient samples and in paper IV to analyze cell lysates.

Metabolite production using human liver microsomes

Several of the metabolites in this method lacked reference substances and were instead produced using human liver microsomes, Figure 5, which are vesicles from the endoplasmic reticulum in human livers that are prepared by homogenization following differential centrifugation [123]. Phase I metabolism, for instance, hydroxylation by CYP enzymes, can be studied by the addition of the cofactor NADPH. Phase II metabolism can be studied

by the addition of the cofactor UGDPA to facilitate glucuronidation (addition of sugar molecule) of molecules harboring a hydroxyl group.

Figure 5: Suggested phase I and phase II metabolism of erlotinib by hydroxylation to OSI-420 or M13 and further glucuronidation to M8 or M10.

The LC-MS/MS method

Erlotinib and its metabolites were analyzed on an LC-MS/MS instrument, Figure 6. The analytes were separated on an XBridge C18 column at 55°C. The mobile phase was 5 mM ammonium acetate and acetonitrile kept at a flow rate of 0.7 mL/min. The total run time was 7 min, with a gradient of 10–50% acetonitrile from 0 to 5 min, followed by 90% acetonitrile from 5 min to 6 min that finally returned to 10% acetonitrile from 6 min to 7 min.

The calibration curves were 25–5,000 ng/mL, 0.5–500 ng/mL, and 0.15–10 ng/mL for erlotinib, OSI-420, and didesmethyl erlotinib, respectively. Quality control samples at five concentration levels for erlotinib (25, 75, 400, 1,200 and 3,750 ng/mL), OSI-420 (0.5, 1.5, 20, 100 and 375 ng/mL), and didesmethyl erlotinib (0.15, 0.45, 1.5, 4 and 7.5 ng/mL) were used.

The method was performed in a positive electrospray ionization mode, and all analytes were monitored by multi-reaction-monitoring. To facilitate the quantification of all analytes in one analysis of 7 min, with average metabolite concentrations around 0.2% of the average erlotinib concentration, erlotinib was suboptimally quantified using the ¹³C-isotope ion (M+1) instead of the commonly occurring ¹²C-isotope. This was not enough to achieve a linear calibration curve but erlotinib could be fitted with a

quadratic curve. This modification solved the initial problem, but it was still a challenge to fit a linear calibration for OSI-420, which can be seen in the inter-batch precision with a lower limit of quantification (LLOQ) of 17%. The reason for the high LLOQ was because the calibration curve tended to be more quadratic than linear.

The metabolites were identified using either a targeted or an untargeted approach. The targeted approach utilized existing literature [124], while the untargeted approach scanned the 200–600 m/z range and masses of possible metabolites were further studied in daughter scans. Optimal mass spectrometry settings were identified by stepwise modifications in cone voltage and collision energy. Metabolite structures were suggested based on similarities in fragmentation pattern with already known metabolite structures. The metabolites (M2, M3, M6, M20, M17, M8/M10, M21, M7, M11, M19, M16, and M13) were semi-quantified using the calibration curve from either OSI-420 or didesmethyl erlotinib depending on the metabolite intensities. The semi-quantified metabolites were only validated based on selectivity, precision, and stability. The concentrations obtained from semi-quantified metabolites can further only be used for comparison between samples analyzed with this method.

Figure 6: Chromatograms showing metabolites of low intensities (A) and high intensities (B) after a human liver microsome incubation with erlotinib. Figure modified from paper I, figures 2B and 2C.

In vitro studies of ABCG2 mediated erlotinib transport

In vitro studies were performed in paper IV to study the transport of erlotinib by ABCG2 in the K562 cell line. The K562 suspension cell line was developed in 1970 and originates from a 53-year-old female diagnosed with chronic myeloid leukemia [125].

The K562 cell line was considered a suitable model for studies of erlotinib transport because the expression of EGFR and ABCG2 is very low or is absent. Erlotinib is, therefore, unable to exert its antitumoral effect on the K562 cells and ABCG2 and ABCG2 polymorphisms can be inserted in the cells to specifically study the transport mediated by the transduced ABCG2.

The two most commonly studied polymorphisms of ABCG2 – 34G/A and 421C/A – were chosen to be studied in this project. Previously, recombinant cell lines were created using stable retroviral gene transductions [126]. Briefly, vectors containing ampicillin resistance and human wild-type ABCG2 cDNA were grown in *Escherichia coli* by the addition of ampicillin. The wildtype ABCG2 gene was edited and amplified using PCR-based site-directed mutagenesis with a primer with the point mutation of interest. The edited genes were cut out of the vector and inserted into a retroviral vector (MIY) also containing enhanced yellow fluorescent protein (EYFP) [127]. The packing cell line, 293T, was transfected with MIY-ABCG2 together with helper vectors VSVG and POL-GAG that are essential for the production of infectious particles. The viral supernatant was used for the transduction of the K562 cell line by spinfection. The MIY vector was randomly integrated into the K562 genome and expression of the ABCG2 gene was further mediated by the host.

The established K562 cell lines were carriers of wildtype ABCG2, ABCG2 34G/A, or ABCG2 421C/A, and a control cell line only containing an empty MIY vector.

To study differences in erlotinib transport between the differently transduced ABCG2-expressing cell lines, intra-cellular erlotinib concentrations were measured in each cell line after incubation with 1 μ M of erlotinib for 1 hour. The incubation was stopped by rapidly removing the erlotinib-containing media. Media residues were removed in two washing steps with cold PBS. Different washing conditions were evaluated and the washing method using 5 mL of cold PBS was selected because the variation between replicates within each cell line were lower compared to when washing with 1.5 mL PBS. Washing with 5 mL PBS did however generate lower erlotinib concentrations, but they were still within the measurable range for the LC-MS/MS method. Washing with cold PBS instead of warm PBS additionally

reduced the variation within the cell lines. The cells were further lysed and the proteins were denatured with methanol and ammonium acetate before analysis of the erlotinib concentration using LC-MS/MS.

Assessment of genetic variability by genotyping and sequencing

Genotyping array

In paper III, genotyping was performed using an Infinium Global Screening Array BeadChip from Illumina containing 665,608 SNPs [128]. Genotyping with Illumina arrays generally starts with the amplification of genomic DNA [129], followed by fragmentation. The DNA fragments are precipitated before being hybridized to a complementary probe of 50 nucleotides attached to a silica bead on a BeadChip, Figure 7A [130]. Hybridized fragments are extended with a single-base extension method using all four terminator nucleotides. The incorporated nucleotide is detected in a two-color system of red and green, where ddATP and ddUTP are labeled with dinitrophenyl and ddCTP and ddGTP is labeled with biotin [131]. Dinitrophenyl and biotin are further labeled with red and green fluorescent antibodies, respectively, in a multi-layer immunohistochemical sandwich assay. The bead chip is scanned using an iScan system that creates images of the emitted light from the excited fluorophores, Figure 7B. Bead intensities are extracted from the image to determine the genotype of each SNP, Figure 7C.

Figure 7: Steps involved in the genotyping assay. (A) Hybridization of DNA fragments to a complementary strand attached to a bead. Extension of the complementary strand by a single base and antibody staining before amplification of the signal. (B) Fluorescent image of the chip. (C) Determination of genotypes based on the red and green intensities. Adapted with permission from Springer Nature: Steemers, F.J., et al., Whole-genome genotyping with the single-base extension assay. *Nature Methods*, 2006. 3(1): p. 31-33.

Fragment analysis and capillary electrophoresis.

In paper III, a dinucleotide repeat in intron 1 of the *EGFR* gene was analyzed using capillary electrophoresis. Capillary electrophoresis is a technique that separates single-stranded fluorescently labeled DNA-fragments in a polymer-filled capillary [112]. An applied voltage separates the fragments by size. The detector registers the fluorescence and the fragment size is determined by comparing peaks with a simultaneously analyzed ladder with known fragment size as illustrated in Figure 8. Stutter peaks are common in dinucleotide repeats due to slipped strand extension by Taq DNA polymerase during PCR amplification [132].

Figure 8: Electropherogram of CA repeats in a homozygous individual.

Sequencing

Whole exome sequencing was performed in paper V using the Illumina platform. Illumina short-read sequencing generally starts with enzymatic fragmentation of DNA [133] followed by ligation of adaptors containing different adaptor sequences at each end of the fragment that is being amplified. The fragments are further amplified using solid-phase amplification [134, 135], where the fragments are immobilized on a flow cell. The fragments are denatured to single-strand fragments and annealed to a complementary oligonucleotide attached to the surface of the flow cell and amplified. The attached fragments are further amplified by bridge amplification when the adaptor sequence of the attached fragment is ligated to a complementary oligonucleotide on the flow cell creating a bridge that is amplified. This is repeated numerous times to create a cluster of fragments that can be detected in the sequencing reaction.

Sequencing is described as sequencing by synthesis by cyclic reversible termination [136, 137]. In this process, a DNA polymerase incorporates a single 3'-modified nucleotide labeled with a fluorophore that prevents further incorporation of nucleotides [135]. All four nucleotides, labeled with different dyes, are added simultaneously to the flow cell. Excessive nucleotides are washed away, followed by fluorescence imaging of the flow cell.

The last step of the cycle involves cleavage and removal of the inhibiting group and the fluorescent dye. The process is repeated a predetermined number of cycles. In order to obtain paired-end sequencing, the flow cell bound fragments are bridged and amplified in order to be able to sequence the complementary strand the same way as the first strand.

A base-calling algorithm determines each nucleotide in a cluster based on the fluorescence image along with a quality score for each nucleotide (Phred score), creating a read from each cluster [138]. By using different software, the reads undergo initial quality control before being mapped to the human reference genome. Aligned reads are further processed before the variants can be determined using variant calling.

RESULTS & DISCUSSION

Chromatography method development

Quantification of erlotinib and its metabolites (paper I)

To be able to study erlotinib and its metabolites in plasma in detail, an LC-MS/MS method was developed in paper I. The method was used in papers I, III, and IV to analyze patient samples as well as cell lysates. The method was able to quantify erlotinib and the active metabolites OSI-420 and didesmethyl erlotinib as well as to semi-quantify several metabolites. The LC-MS/MS method was validated according to international guidelines from the EMEA and FDA [139, 140].

Figure 9: The structures of the erlotinib metabolite isomers OSI-420 (3.56 min) and M13 (3.67 min).

The main metabolite has been reported to be around 10% of the erlotinib concentration [40, 141]. The main metabolite is usually referred to as OSI-420; however, it consists of two isomers but they are usually analyzed combined as one analyte, Figure 9. This can be confusing in different publications because this is not always clearly stated. The minor isomer is on average 20% of the major isomer. In this method, the isomers OSI-420 (minor) and M13 (OSI-413, major), are chromatographically separated and quantified independently, Figure 10, as also described in a previously reported method [141]. The isomers share identical analytical settings and differ only in the retention time. The substance that further on is referred to as OSI-420 is the analyte eluting after 3.56 min, as displayed by the OSI-420 reference substance used in paper I, and M13 was subsequently assigned to the analyte eluting after 3.67 min.

Figure 10: A typical chromatogram from a patient sample after 1 month of erlotinib monotherapy. The figure has been modified from paper I, Figure 1C.

Several quantification methods with different advantages are available for quantification of erlotinib alone [142-144], quantification of erlotinib and one or two metabolites [141, 145-149], or quantification of erlotinib in combination with other EGFR-TKIs [150-154]. The advantage of this particular method is that it is relatively broad with the ability to quantify erlotinib, to quantify and separate the main metabolites OSI-420 and M13 as well as quantify didesmethyl erlotinib and semi-quantify several more metabolites. The drawback of the method is that the analysis time of 7 min is longer than many other methods.

Erlotinib *in vitro*

ABCG2 transport (paper IV)

In paper IV, the influence of genetic variability in ABCG2 on the intra-cellular erlotinib concentration was studied in the K562 cell lines. The two common *ABCG2* SNPs – 34G>A and 421C>A – and the wild-type *ABCG2* were compared with a control cell line containing an empty plasmid without ABCG2 expression. The K562 cell line was a convenient model to use for intra-cellular concentration studies because the K562 naturally has very low levels or not at all express ABCG2 or EGFR (www.proteinatlas.org) [155].

The genetic variant 421C>A has generally been associated with low ABCG2 expression [91, 156] and no difference in erlotinib intra-cellular concentrations was previously observed between a cell line harboring 421C>A and the control cell line without ABCG2 [68], which was confirmed in our K562 model, Figure 11.

Figure 11: Intra-cellular erlotinib concentrations. Differences in mean erlotinib intra-cellular concentrations evaluated using one-way ANOVA with the Tukey HSD post-hoc test. Note: *** $p \leq 0.001$. Modified figure from paper V, Figure 3A.

The transport of erlotinib by ABCG2 harboring the genetic variant 34G>A was previously unknown; however, the 34G>A has generally been shown to possess similar transport activity as wild-type ABCG2 [91]. This was also observed in our experiments, and the 34G>A variant showed reduced intracellular concentrations of erlotinib similar to wild-type ABCG2, Figure 11. When taking differences in ABCG2 expression into account, the 34G>A

variant was able to transport more erlotinib with a lower expression of ABCG2 on the cell surface. This finding should, however, be viewed with caution because this might be due to limitations in the modeling system.

Other limitations of this study were that the cell experiments were only carried out in one cell line and only at a concentration of $1\mu\text{M}$, which is at the lower end of the clinically relevant concentration interval.

Erlotinib pharmacokinetics *in vivo*

In the EM11 study presented in paper II and III, erlotinib-induced diarrhea was found to be associated with erlotinib and M13 exposure. Skin rash, on the other hand, was associated with CYP3A activity and erlotinib and metabolite ratios, including the metabolite OSI-420. Males experienced more severe skin rash than females and this might be linked to reduced CYP3A activity in males compared to females.

Gender differences (paper III)

The severity of erlotinib-induced skin rash was shown to differ between the genders in paper III, which has also been reported previously [61], and males were found to experience more severe skin rash compared to females, Figure 12. Diarrhea displayed no gender differences, and was instead, influenced by age.

Figure 12: The severity of maximum skin rash (CTC) split by gender after 3 months of erlotinib treatment (Fisher's exact test).

Erlotinib plasma trough concentrations (paper III)

Previously reported average plasma concentrations of erlotinib in European patients after 1 month of treatment have been reported to be 700–1,300 ng/mL [59, 61], which is in line with the average result after 1 month in this study (1,083 ng/mL). Tiseo et al. (2015) also measured the erlotinib concentration after 1 week of treatment, showing that the average concentration was almost twice as high compared to after 1 month of treatment [59].

In paper III, the exposure of erlotinib and a metabolite was found to be associated with diarrhea. The concentration of erlotinib and the major metabolite M13 both correlated to diarrhea after 1 and 2 months of treatment and suggestively after 3 months of treatment. Trends towards an association with diarrhea were also observed for the concentration of didesmethyl erlotinib and M6. Also, when summarizing all active analytes, a suggestive association was observed after 2 and 3 months.

Previous studies have mostly shown no correlations between erlotinib exposure and diarrhea [58, 157, 158], but one study has [60]. For other EGFR-TKIs such as afatinib and osimertinib, correlations between diarrhea and drug exposure have been observed [159, 160].

The mechanism behind EGFR-TKI-induced diarrhea is yet to be determined, but several different mechanisms or combinations of mechanisms have been proposed, for instance, dysregulated ion transport, inflammation, and mucosal injury [50–52].

CYP3A activity (paper II and III)

CYP3A is the major metabolizer of several xenobiotics as well as of erlotinib [73], and its activity can vary 40-fold [161]. In paper II, CYP3A activity was found to be induced during treatment, generating a lower quinine metabolic ratio compared to the ratio at baseline, Figure 13A. Trends pointing in that direction have previously been reported [59, 162], and our findings thus complicate the use of baseline CYP3A activity as a possible biomarker during treatment. Also in paper II, females were shown to have higher CYP3A activity compared to males, Figure 13A, as previously identified [78, 163].

In paper III, additional clinical data from the EM11 study were added and the association of CYP3A activity with erlotinib pharmacokinetics and toxicity was investigated. No correlations were identified between baseline CYP3A activity and erlotinib pharmacokinetics or toxicity, probably due to the previously identified induction.

Figure 13: Figure modified from papers II and III, illustrating associations identified with CYP3A activity highlighting differences between females (grey) and males (black). (A) Induction of CYP3A activity was observed after 2 months of treatment as well as gender differences in CYP3A activity (Mann-Whitney U-test). (B) Lower CYP3A activity was seen in patients with an increased grade of skin rash (Kruskal-Wallis test). (C) A correlation was identified between CYP3A activity and the erlotinib metabolite ratio OSI-420/didesmethyl erlotinib (non-parametric correlations).

The CYP3A activity after 2 months of erlotinib treatment was suggestively associated with skin rash, Figure 13B. CYP3A activity was also associated with erlotinib and metabolite ratios erlotinib/OSI-420, M13/OSI-420, and OSI-420/didesmethyl erlotinib at one or more time points during erlotinib treatment, Figure 13C. Steffens et al. (2016) previously showed that the ratio of erlotinib/OSI-420 best explained the severity of skin rash [61]. It should be noted that OSI-420 in Steffens et al. (2016) includes both isomers, which correspond to OSI-420 and M13, in our data. Erlotinib/(OSI-420+M13) was not found to be associated with skin rash in our study but was suggestively associated with CYP3A activity.

Several studies have shown that higher erlotinib plasma trough concentrations correlate to skin rash [59, 61]. A possible reason why this was not observed in our data could be due to the small sample size. The time of sampling could also be an additional explanation. Tiseo et al. 2015, showed that average erlotinib plasma concentrations were almost twice as high after 1 week compared to 1 month of erlotinib monotherapy, and the association between skin rash and erlotinib concentration was identified after 1 week of treatment [59]. Erlotinib is expected to reach a steady-state within 8 days and a possible explanation as to why the erlotinib concentration is higher after 1 week compared to 1 month of erlotinib treatment could be that the CYP3A activity has not yet been significantly induced at the earlier time point. Because skin rash previously has been correlated to erlotinib exposure several times, patients with interrupted treatment prior to the first sampling time may have had a higher erlotinib plasma concentration compared to those that did not discontinue their treatment due to toxicity in this study. Additionally, the gender differences in skin rash observed in this study could potentially be a result of the differences in CYP3A activity between females and males.

In summary, the pharmacokinetic data from the EM11 study showed that erlotinib-induced diarrhea was associated with erlotinib and M13 exposure while skin rash was associated with CYP3A activity and erlotinib and metabolite ratios including OSI-420.

Pharmacogenetics in NSCLC

Erlotinib-induced skin rash and diarrhea (paper III)

Paper III studied the genetic variability associated with erlotinib induced skin rash and diarrhea. In total, 171 SNPs located in the *EGFR*, *CYP3A4*, *CYP3A5*, *ABCG2*, *ABCB1*, *CYP27B1*, *PIK3CA* and *AKT1* passed the quality control. The genes were selected because one or more SNPs in these genes were previously found to be associated with skin rash or diarrhea [58, 60, 65, 82, 83, 86-88].

Only SNPs associated with skin rash were identified after the analyses. The rs10856870 in *ABCG2* was associated with skin rash after 1, 2 and 3 months of treatment and the association was after 1 month of treatment still significant after multiple corrections ($p=0.040$), Figure 14. Additionally, three *ABCG2* intronic SNPs (rs2904185, rs75048878 and rs2127863) were identified to be associated with skin rash ($p < 0.05$ at all time points). There is no previous knowledge about the rs10856870 intron SNP; however, other SNPs in *ABCG2* influencing the expression and activity of *ABCG2* have previously been associated with increased plasma concentration [58], response and OS [84] and gefitinib induced skin rash [164].

Figure 14: The SNP rs10856870 in *ABCG2* was associated with skin rash after 1, 2, and 3 months of treatment, showing the TT genotype to be protective against skin rash. The bars represent the mean + SD.

After 2 and 3 months of erlotinib treatment, SNPs in *ABCB1* and *CYP27B1* were associated with skin rash. The correlations to SNPs in *ABCB1* and *CYP27B1* could first be identified after 2 months of treatment and the correlation became clearer after 3 months. After 3 months of treatment, the SNPs were suggestive of association after multiple corrections. These

findings might be influenced by the reduced number of patients in the analysis further on into the treatment or related to the induction of CYP3A activity.

Gemcitabine/carboplatin induced leukopenia and neutropenia (paper V)

Paper V studied the genetic variability associated with gemcitabine and carboplatin induced leukopenia and neutropenia, Figure 15. In total, 71,374 variants (minor allele frequency (MAF) > 0.01), located in or near a gene passed the quality control and were used in the statistical analysis. The decrease in leukopenia and neutropenia – as the nadir values normalized to baseline – was the studied phenotype during the first treatment cycle.

The currently available studies on chemotherapy-induced hematological toxicity have usually determined the phenotypes leukopenia and neutropenia slightly different from what was performed in paper V. In common for all studies is that the leukocyte and neutrophil counts have been analyzed to generate a quantitative value that is not subjected to interpretation or evaluation by different individuals. In paper V, the continuous leukocyte and neutrophil values adjusted for the respective baseline values were used in the statistical analyses instead of categorizing the blood status according to the CTCAE grading system before analysis. The choice to use the decrease phenotype was made based on the observation that the lowest FDR rate was achieved when adjusting for baseline values compared to only using the nadir alone. The FDR signal was most prominent for the decrease in leukopenia. Another difference between paper V and previous studies was when in time of the chemotherapy cycle the blood sampling was performed, which usually was performed at the beginning of a new treatment cycle; however, in paper V, the blood status was monitored weekly and only during the first cycle. The blood status measurement that was assigned the nadir value in paper V was usually the leukocyte or neutrophil count determined after 1 or 2 weeks. Because this study only covers hematological toxicity during the first cycle in order to identify which patients were at risk of leukopenia and neutropenia, the phenotype used in paper V also differs from others studies that have studied the lowest CTC grade experienced from all treatment cycles or the time it takes to reach a predetermined CTC grade [110, 111].

No single variants passed multiple corrections after the association analyses, which could be an indicator that the effect of a single variant could not explain the severity of leukopenia or neutropenia and that more than one variant might contribute to the observed variation in toxicity. A p-value cut-off was determined, based on where the false discovery rate was the lowest in order to obtain variants with a suggestive association. To combine the small effects of several variants, variants residing in genes were merged

into gene sets, and gene-based tests were performed. The gene-based tests showed that two leukopenia-associated genes – SVIL and EFCAB2 – passed multiple corrections.

Figure 15: An overview of the whole-exome sequencing study. Variants and genes associated with leukopenia and neutropenia were identified, and the variants were validated using an external GWAS data set. Validated variants, variants in genes and variants were merged in a weighted genetic risk model.

Due to the lack of validation data, the results from a GWAS were used to study linkage disequilibrium between top hit SNPs. The validation data originated from a study on ovarian and lung cancer patients treated with paclitaxel and carboplatin. Subsequently, the choice of validation data was not ideal because the GWAS analyzes tag-SNPs which are usually intronic SNPs, and the whole exome covers the coding regions. To overcome the fact that the positions of the top hit variants in each data set did not agree, the pair-wise linkage disequilibrium was studied between exome-sequenced top hits and GWAS associated top hits. Because carboplatin is the drug that our NSCLC cohort and the validation cohort had in common, the variants

that could be suggestively validated could only be linked to carboplatin-induced leukopenia or neutropenia. The influence of gemcitabine-induced leukopenia and neutropenia could not be validated. The validation could verify a few SNPs associated with leukopenia and neutropenia, for instance, two SNPs in *EFCAB2* and three SNPs in *SVIL* were suggestively validated.

Figure 16: The weighted genetic risk model applied to the exome sequenced data set and illustrated using the untransformed decrease phenotype (ANOVA with Sidak's multiple comparisons test). Error bars illustrate the mean $\pm$ 95% CI.

To utilize the small effect from several variants, two prediction models based on weighted genetic risk scores were created, one for each phenotype. Variants from the validation, from the gene-based and single-variant association analyses, were merged into one model, Figure 16. The models showed that the average decrease in leukocytes and neutrophils were statistically different between all risk groups except for neutropenia between the risk group 4 and 5.

CONCLUSIONS

In conclusion, the results from papers I–V indicate that pharmacokinetic and pharmacogenetic variability has an influence on the inter-individual variation observed in NSCLC patients.

More specifically from the *in vitro* erlotinib experiments and the EM11-study, we can conclude the following:

- Erlotinib transport was influenced by genetic variation in the *ABCG2* gene *in vitro*
- CYP3A activity was induced in erlotinib treated patients after 2 months of erlotinib treatment.
- Skin rash was associated with CYP3A activity and CYP3A activity was associated with the erlotinib metabolic ratio OSI-420/didesmethyl erlotinib. Skin rash was also associated with the *ABCG2* SNP rs10856870.
- Erlotinib-induced diarrhea correlated with erlotinib and metabolite M13 exposure.
- Males experienced more severe skin rash compared to females.

From the gemcitabine/carboplatin study we can conclude the following:

- Genetic variants and genes were found to be associated with leukopenia and neutropenia.
- Variants were suggestively validated using an external GWAS dataset.
- Risk prediction models for leukopenia and neutropenia based on weighted genetic risk scores created by the use of the cumulative effect of several genetic variants showed a difference in the mean decrease parameter between the risk groups.

The findings in this thesis need to be further validated in order to establish their clinical relevance.

FUTURE ASPECTS

In the EM11 study, CYP3A activity and genetic variability were found to be important for the occurrence of skin rash, and the erlotinib and M13 exposures were important for diarrhea. Validation of these findings in a larger study would be of great interest in order to determine the usefulness of these findings in personalized erlotinib treatment.

To further understand the role of CYP3A activity in erlotinib treatment, it would be of interest to follow the induction of CYP3A activity more closely, at several times points during the first two months of treatment and if possible using an endogenous CYP3A activity probe, while also further exploring the gender differences.

To further improve treatment with erlotinib and other EGFR-TKIs, it would be of interest to keep track of steady-state plasma concentrations of erlotinib and its metabolites in patients in order to ensure that adequate erlotinib concentrations are reached, perhaps by using therapeutic drug monitoring. This is especially the case early in treatment when toxicities usually arise and after dose adjustments. Routine monitoring could be combined with toxicity reporting in order to establish reliable concentration intervals for the patients to ideally reside within. This could generate more efficient erlotinib treatment since treatment with erlotinib only is possible during a limited time due to the development of resistance by the tumor. The separation of the isomeric metabolites has shown that the

metabolites are linked to different toxicities, ratios of OSI-420 showed association to CYP3A activity while M13 correlated to diarrhea, which supports that the metabolites should be determined separately.

In a larger erlotinib study, it would be of interest to identify the patients who are at risk of early treatment discontinuation due to toxicity. Unfortunately, the majority of these patients only provided baseline samples in the EM11-study. Closer monitoring of CYP3A activity and erlotinib and metabolite concentrations early on in the treatment could possibly explain why these patients discontinue their treatment. One way to avoid discontinuation and severe toxicities would be to start all patients at a lower dose, which in combination with therapeutic drug monitoring of erlotinib could be used to adjust the dose in order to reach adequate concentrations. This would also allow the CYP3A activity to adapt to the treatment.

Additionally, the genetic variability should be taken into account because the SNPs identified in the EM11 study were found in genes associated with the transport of erlotinib such as ABCG2 and ABCB1. Genetic variability in ABCG2 was additionally shown to influence the transport of erlotinib *in vitro*. The successful personalization of erlotinib treatment will most likely include combining both pharmacokinetic and pharmacogenetic parameters.

In the gemcitabine/carboplatin study, pharmacogenetic information from a whole-exome sequencing study was used to build two prediction models. The model utilizes the cumulative effect of several genetic variants to foresee the severity of the reduction of leukocytes and neutrophils during gemcitabine/carboplatin treatment. It would be of interest to further develop and refine the models, perhaps by the addition of other clinical parameters. It would also be interesting to validate the models in a larger data set in order to prove their clinical benefit.

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