CHEMORES
TUMOUR CHEMOTHERAPY RESISTANCE

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CHEMORES

Molecular mechanisms underlying chemotherapy resistance, therapeutic escape, efficacy and toxicity

Integrated Project
Priority 1

Publishable Final Activity Report

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CHEMORES is an integrated project involving clinicians and scientists in eight European countries. The purpose of the project is to improve cancer treatment by obtaining increased knowledge on mechanisms of chemotherapy resistance. It is estimated that the two diseases that are studied, lung cancer and melanoma, caused over 350,000 deaths in Europe in 2002. An important contributing factor in cancer mortality is the fact that the most common types of cancer do not respond well to systemic chemotherapy in the advanced stages. Increased understanding of the underlying processes will contribute to the development of predictors of both therapy response and toxicity, and in the end more efficient and personalised therapy.

The project has spanned more than five years and the results generated have been presented in > 230 scientific publications. The main achievements in the different workpackages (WPs) are summarised below:

WP1 and WP3 – Clincal trials on melanoma patients

Work in WP3 (Constitution of a Biological Resources Centre) overlaps with that of WP1 and WP2. The collection of the CHEMORES biobank is one of the most valuable outcomes of the project and a summary of the clinical materials collected can be found in Table 1. In addition to the data generated during the course of the project, this unique material will continue to be used for studies on treatment response and other key issues in melanoma and lung cancer biology.

The main objectives of WP1 was to use clinical data and biobank material from large randomized trials in melanoma patients to study the molecular mechanisms for sensitivity and resistance to interferon (IFN) and the DNA alkylating agents dacarbazine (DTIC) and temozolomide (TMZ).

Autoimmune antibodies in sera of melanoma patients on adjuvant interferon (IFN) treatment have previously been reported to be important for prognostics and possibly also in prediction of outcome (Gogas et al. N Engl J Med 2006; Satzger et al. Int J Cancer 2007). In CHEMORES the collection and test of sera for the presence of autoimmune antibodies has been performed in three large IFN adjuvant trials (Eggermont et al. Lancet 2005; Eggermont et al. Lancet 2008; Hansson et al. Lancet Oncol 2011). These analyses clearly show that the presence and/or emergence of autoimmune antibodies is not a (strong) prognostic factor and not a predictive factor for IFN-sensitivity and outcome in relation to IFN treatment (Bouwhuis et al. J Natl Cancer Inst. 2009; Bouwhuis et al. J. Clin. Oncol. 2010).

Additional studies addressed other candidate serum markers and showed that high preoperative serum YKL-40 levels are associated with poor survival in untreated patients, but not in IFN treated patients. Increases in serum YKL-40 during treatment or follow-up is associated with poor prognosis (Krogh et al. presented at ASCO 2010). IL-1β and IL-6 serum levels were determined by using and comparing two different techniques. We could not confirm the predictive value of initial high IL-6 levels nor the importance of IL-1β levels and improved outcome in IFN-treated patients. This study illustrates the complexity of cytokine determination in sera and stresses the importance of validating assays.

IFN induces increased ferritin and decreased C-reactive protein (CRP) levels; however, a possible association with the treatment effect had previously not been studied. This question was addressed in one of the cohorts, where IFN induced an increase in ferritin levels but not in CRP levels. Ferritin and CRP ratios showed no prognostic value regarding distant metastasis-free survival (Bouwhuis et al. Melanoma Res. 2011).

Both ulceration of primary melanoma and tumour stage have been identified as predictive factors for the efficacy of adjuvant interferon (IFN)/PEG-IFN therapy in two of the three randomized trials. Within WP3, formalin-fixed paraffin embedded (FFPE) material from the three IFN study cohorts were collected and used in WP4 in the search for novel predictive markers for adjuvant IFN treatment in melanoma, also considering ulceration status, and tissue microarrays (TMAs) were constructed for the purpose of validation.

With regard to response to DNA alkylating agents, less biopsies than expected were performed in a randomized trial of Stage IV melanoma patients comparing daily prolonged scheduling of TMZ *vs*. classic scheduling of DTIC. This initially delayed the project. However, with the combined efforts of the CHEMORES melanoma centres, biopsy samples were also collected from other studies and analyzed in WP4 for discovery and validation of predictive markers. This material consists of fresh frozen tumour samples acquired pre- and/or post-treatment with DTIC or TMZ, as well as several TMAs constructed from FFPE material used for validation studies (WP3). Several candidate predictive markers were investigated by immunohistochemistry in 50-120 tumour samples and the data support a role for the melanogenesis related proteins MITF and GPR143 as resistance factors.

Work performed in WP3 also includes the production of antibodies for validation studies. Three antibodies were made for investigation of chemoresponse relevant proteins in melanoma (WPs 1 and 10), four for mouse studies in WP7 and one for the clinical lung cancer studies (WPs 2 and 4).

To summarize, the work in CHEMORES WP1 has clearly ruled out that the presence and/or emergence of autoimmune antibodies would be a significant prognostic factor or a predictive factor for IFN-sensitivity and outcome in relation to IFN treatment. The studies of the clinical melanoma materials have resulted in many interesting leads with regard to prediction of response to chemotherapy and IFN. However, additional clinical validation is required before any of these markers can be used in the clinical practice.

The data generated in WP1 have resulted in several high impact publications and have been presented at scientific meetings and CHEMORES workshops, directed towards clinicians, researchers, students and patient organisations. Several of the participants in the CHEMORES melanoma studies are part of the major international melanoma networks that influence the clinical management of the disease, for example the European consensus-based interdisciplinary guideline for diagnosis and treatment of melanoma (Garbe et al. European Journal of Cancer (2012) 48, 2375– 2390). Thus, the knowledge generated in the project and follow-up studies will be incorporated in future clinical guidelines.

Table 1. Summary of the CHEMORES clinical materials used for discovery and validation studies

STUDY	TYPE OF MATERIAL	APPROXIMATE NUMBER OF PATIENTS
DTIC/TMZ melanoma cohort	Pre- and/or post-treatment melanoma tumour biopsies, FFPE material and FNAs. Normal tissue for some patients.	260
Sorafenib/TMZ melanoma cohort	Pre- and/or post-treatment melanoma tumour biopsies.	36

EORTC Adjuvant IFN melanoma cohort (EORTC 18952 and 18991)	FFPE material and serum samples.	537
Nordic Adjuvant melanoma cohort	FFPE material and serum samples.	>320
Retrospective lung cancer cohort	Pre-treatment NSCLC tumour biopsies and normal tissue.	123 + additional
Prospective lung cancer cohort	Pre- and/or post-treatment NSCLC and SCLC tumour biopsies and blood samples.	815
International Adjuvant Lung Cancer Trial (IALT) cohort	FFPE material.	48

WP2 and WP3– Clinical trials on lung cancer patients

The overall aim of WP2 was to generate biological material (blood and tumour specimens) and clinical data for patients with non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) who are treated with chemotherapy. The major achievements include:

1. A change in culture for lung cancer translational research.

2. A biospecimen bank for validation of molecular biomarkers arising from preclinical and clinical studies.

3. Discovery and clinical evaluation of circulating tumour cells as a valid biomarker for lung cancer treatment and prognosis

1. A change in culture for lung cancer translational research.

Progress in understanding metastatic lung cancer biology and treatment resistance mechanisms has been significantly hampered by insufficient tissue biopsy specimens for research. There has been a culture that due to the very poor prognosis of patients with metastatic lung cancer it would be unethical to request participation in research of no direct therapeutic benefit. This workpackage aimed to promote a cultural change by requesting patients to prospectively donate serial blood and tissue samples for research. The ethical approvals in Manchester and in Paris did underwent lengthy review but eventually were agreed. At the start of the CHEMORES project the concept of serial sampling was largely unexplored. Now serial sampling has become a high priority in patients with lung cancer (Sequist 2011) and is becoming a standard of care. The CHEMORES project has provided a step change in the way translational research can be conducted in lung cancer and has identified quality measures and standard operating procedures that can be applied in future projects and clinical treatment algorithms requiring serial tissue biopsies and samples.

2. A biospecimen bank for validation of molecular biomarkers arising from preclinical and clinical studies (WP3)

Retrospective samples:

Cases of retrospective non-small cell lung cancer (NSCLC) tumours (stages I-IIIA, non-metastatic) with matched normal / non-malignant lung tissue and complete clinical follow-up data were identified. Over 1000 case files were screened to identify suitable cases in the archive. After exclusion of samples with degraded RNA, 123 cases remained. The histology of these samples was reviewed independently prior to delivery of samples to the genomics, proteomics and toxicity workpackages (WPs 4, 5 and 12).

The use of pairs of normal and tumour tissues from the same patients is a unique feature of the CHEMORES project, which allows us to achieve increased statistical power. We decided to compare lung tumour with normal lung tissue for each patient and then make the comparison between the treatment groups based on this. The major advantage of our strategy is that we reduce artifacts related to genetic variability between individuals. This reduction in background noise significantly decreases the number of samples needed to identify chemoresistance-associated cancer biomarkers.

Additional cases of retrospective lung tumours with complete follow up data were also collected. Moreover, tissue microarrays with tumour material from in total >950 lung cancer patients were constructed.

Prospective samples:

In total 815 patients with lung cancer have been recruited to provide clinical samples. Clinical data on demographics, smoking history, treatment received, toxicity and outcomes (response and survival) have been collated. Samples have been collected from patients with non-small cell and small cell histology. To our knowledge this is one of the largest prospective collections for metastatic lung cancer patients on standard of care therapies. Samples from this collection have been used for discovery and validation of toxicity and chemoresistance markers and this resource will continue to be used in future studies addressing response and resistance to treatment.

3. Discovery and clinical evaluation of circulating tumour cells as a valid biomarker for lung cancer treatment and prognosis

The prospective sample collection included blood samples used to assess for the presence of circulating tumour cells. Ultimately serial blood samples are easier to obtain than serial tumour samples. This work has generated novel publications on the presence, clinical significance and molecular characteristics of circulating tumour cells in patients with small cell and non-small cell lung cancer. At the start of the CHEMORES project circulating tumour cell technology did not allow detailed molecular characterization. In the last 6 months of the project it has been possible to optimize circulating tumour cell sample processing to allow molecular comparison of samples obtained before and after treatment. In this way selected molecular markers (particularly RNA transcripts) from preclinical studies (WP7, WP8) and clinical studies on chemoresistance candidates (WP4) can be evaluated more easily than on biopsy tissue.

There is currently intense interest in personalized medicine for lung cancer. This aims to avoid exposing patients to potentially harmful and ineffective treatments, and to select the best treatment for the individual according to molecular characteristics of the cancer and the patient. Blood based tests will ultimately be more practical in the clinic for serial analysis of cancer markers and treatment selection. In CHEMORES we have conducted the first clinical studies of circulating cancer cells in lung cancer. The results highlight the potential for blood borne cancer cells to be used to guide and select treatment decision making, and to determine

the molecular mechanisms that result in the dissemination of cancer and resistance to treatment.

As a consequence of the knowledge on circulating tumour cells (CTCs) gained, several prospective, randomized clinical trials in Europe and in the US are now incorporating CTCs as a biomarker. Through the course of CHEMORES multiple so-called oncogenic 'driver mutations' have been identified. The feasibility of molecular testing for these markers in small biopsy samples in order to guide therapy has been demonstrated in addition to new knowledge gained on the correlation between molecular biomarkers and response to standard chemotherapy treatments.

WP4 – Genome analysis

The following was accomplished during the project in the genome analysis workpackage:

(1) A complete molecular profiling of a series of 123 pairs of lung NSCLC tumour biopsies and matched normal tissues from a retrospective collection. Fresh frozen samples were collected after curative surgery. Profiling included comparative genomic hybridization, (CGH), gene expression and microRNA profiling, and sequencing of a panel of key genes (including TP53 and KRAS). For promoter DNA-methylation studies, a pilot investigation of four selected biomarkers, using bisulfite transformation of DNA and quantitative PCR, was performed on the 123 paired patient tissue samples. After this quality assurance, the global promoter methylation status was assessed for a subset of 20 patients. In addition, 16 patients were analyzed with whole exome sequencing. At DNA, mRNA and miRNA levels we could identify (in cooperation with WP6), molecular markers that discriminated significantly between the various clinicopathological entities of NSCLC. We reported proofs of distinct molecular profiles that contribute to distinguishing NSCLC tumour subtypes even in small biopsies. The manuscript for this study has been submitted. Integrative analysis found signatures that defined a molecular subtype with a strong prognostic significance. A publication describing this work is under review.

(2) Technology development for investigation of gene expression and miRNA in formalin fixed paraffin embedded (FFPE) tissue.

(3) Molecular profiling of a series of 40 NSCLC FFPE samples from a retrospective cohort of the International Adjuvant Lung Cancer Trial (IALT) clinical trial.

(4) Gene expression profiling of paired sequential biopsies from metastatic melanomas, pre- and post-treatment with sorafenib/TMZ, or DTIC/TMZ, as well as pre-treatment biopsies from patients treated with DTIC/TMZ. Molecular signatures that distinguish between responders and non-responders were identified and validation studies were performed for several candidate predictive markers for therapy response. To date, 33 candidates have been validated by an alternative methodology, for 21 candidates the gene expression differences have been confirmed at protein level and 7 candidates have been investigated in extended sample sets. Further validation studies, including functional studies using melanoma cell lines, are ongoing.

(5) Gene expression and microRNA profiling of an FFPE collection of melanomas from the EORTC 18952 and 18991 adjuvant interferon clinical trials.

(6) Microarray analyses of cancer cell lines with or without expression of the aspartyl protease cathepsin D.

(7) Mutation analyses in melanoma and lung cancer

In addition to increased knowledge regarding potential biomarkers with high clinical relevance, this WP has contributed with unique datasets that are or will soon be publicly available, and that are potentially very useful for the lung cancer research community (see below).

WP5 – Proteome analysis

The work in this workpackage has pushed the boundaries on analysis of proteome changes in human cancers by generating novel methods and applying them on biological and clinical cancer research. These proteomics methods and results will help us to understand how cancer cells and tumours respond to therapy and how molecular resistance to cancer treatment is obtained by tumours.

Novel methods for improved human proteome analysis

A key issue to obtain reproducible results on tissue material is the sample preparation. We have successfully developed a method to prepare tissue samples for quantitative mass spectrometry (MS) based proteomics to avoid plasma contamination. We optimized the method using a tissue which is difficult to study due to extensive vascularization, namely lung tissue and lung cancer tissue. The results are published in De Petris, Proteome Sci. 2010. Another area of improvement in clinical proteomics is the ability to analyse larger sample series. We evaluated a method utilizing the improved ultra pressure liquid chromatography coupled to MS to obtain robust biomarker discovery and validation workflow. The results are published in Lengqvist J., J Chrom., 2009. As we have gained knowledge and improved the peptide IEF method, we have realized the novel possibilities which predictable fraction using peptide pI allows. We used the data to investigate protein post translational modification (PTM) and here for the first time we describe the usefulness of the IEF fraction for data analysis to detect PTMs: Lengqvist J., Amino Acids. 2010.

MS-based proteomics generates huge amounts of data, which today is underexploited, largely due to lack of analysis methods. We set out to improve the quantification of proteins in proteomics experiments. This was achieved by a novel algorithm (protein quality control by peptide quantitation, PQPQ) that uses correlation analysis of quantitative signals from different peptides detected and derived from the same proteins. We can improve the quantitative accuracy of proteomics experiment and use the algorithm to detect protein variants, for example splice isoforms, on protein level. This method was published in the leading proteomics journal, MCP and is already in use in many laboratories round the world (Forshed, Molecular and cellular proteomics, 2011).

In addition, we published another method (pathway analysis method) and its application on detection of cancer therapy related alterations, which was published in the same journal (Ståhl, MCP 2009). We have also developed and demonstrated the use of multivariate supervised data analysis to connect several proteomics data sets to detect prognostic cancer markers by meta-analysis (Hultin-Rosenberg, BMC Bioinformatics. 2010).

Finally, to combine different omics data, we have contributed with a novel statistical method to combine genomics and proteomics data in WP6 (Tan, BMC Bioinformatics. 2009).

All together, these methods have provided new tools for improved quantitative analysis of complex proteome analysis.

Application of methods to cancer biology research

The proteomics and bioinformatics methods that we have developed were successfully applied in several applications. To study cellular response to therapy in lung cancer we performed proteomics study and discovered involvement of S100-proteins (S100A6 and S100A4) in response to DNA damaging treatment (Orre et al, MCP, 2007).

Further, we used the IEF-LC-MS/MS method to study membrane proteome components effecting cellular response to chemotherapy and identified SERCA2 and several other proteins with potential effect on therapy sensitivity (Eriksson et al, Proteomics. 2008).

Another application is a focused study on post translational protein modifications, namely proteome phosphorylation changes in lung cancer cells, describing interplay and down streams effects between the cell surface receptor EphA2 and its ligand ephrin B3 (Ståhl, J Proteome Res. 2011). In collaborations with other groups, we have also applied the developed proteomics method on a number of other applications.

Clinical applications

One of the main goals of our research is to apply the methods on clinical proteomic studies to improve cancer treatment by personalizing the selection of the most effective drugs for each patient. It is also equally crucial in cancer to detect the disease when it is still in a curable state. We used our IEF-LC-MS/MS method funded by this grant to screen marker candidates between inflammatory lung disease and lung cancer using plasma and pleural effusion samples. This study generated putative marker candidates, such as A2M, SERPINA1, EFEMP1, CLEC3B, for further validation and also described for the first time plasma and pleural effusion proteome from same individuals (Pernemalm, Proteomics, 2009).

As described above, in a previous in vitro study we showed that S100A4 and S100A6 were post-translationally modified, upregulated and relocalized from the nucleus to the cytoplasma in response to irradiation in lung cancer cells with functional TP53 (Orre et al MCP 2007). We continued with clinical validation and observed that the tumour expression of S100A6 (n=103) was a prognostic factor in p53-negative non-small cell lung cancer (NSCLC) samples (De Petris et al., Lung Cancer, 2009). During the last year of the project we have expanded the study to several hundred patients and included eight S100-protein family members in the analysis (S100A2, S100A4, S100A6, S1007, S100A8, S100A9, S100A10 and S100A11). This analysis confirms the prognostic role of S100A6 (De Petris et al., Manuscript).

Finally, we carried out protein marker analysis in a larger clinical cohort to asses the clinical information that a number of markers could provide. Two cytokeratins, CK18 and a fragment of 19, were analysed in plasma from 300 lung cancer patients. Seventy-eight patients had locally advanced or metastatic disease treated with chemoradiotherapy or first-line chemotherapy. The results suggest a diagnostic role of the CK19 fragment and a prognostic role of CK18 levels, independent of the therapeutical intervention (De Petris, Eur J Cancer. 2010).

An ambitious tumour proteomics study on 16 lung adenocarcinomas (eight with relapse and eight non-relapse cases) was performed, with identification of more than 3600 proteins (Figure 1) (manuscript in preparation). Nine candidate protein level changes were identified and further validated on 100 cases on tissue microarray. In parallel, genomic analyses were performed on the same cohort of lung adenocarcinomas in WP4 with combined data analysis (manuscript in preparation).

To identify a melanoma patient cohort that is in risk of relapse during ongoing therapy, we studied tumour proteome using quantitative proteomics. Several chemoresponse candidates were identified, including some that were previously identified by gene expression profiling (manuscript in preparation). In addition, proteomics on melanoma cell lines with induced resistance to BRAF inhibitors was performed, as described in WP10.

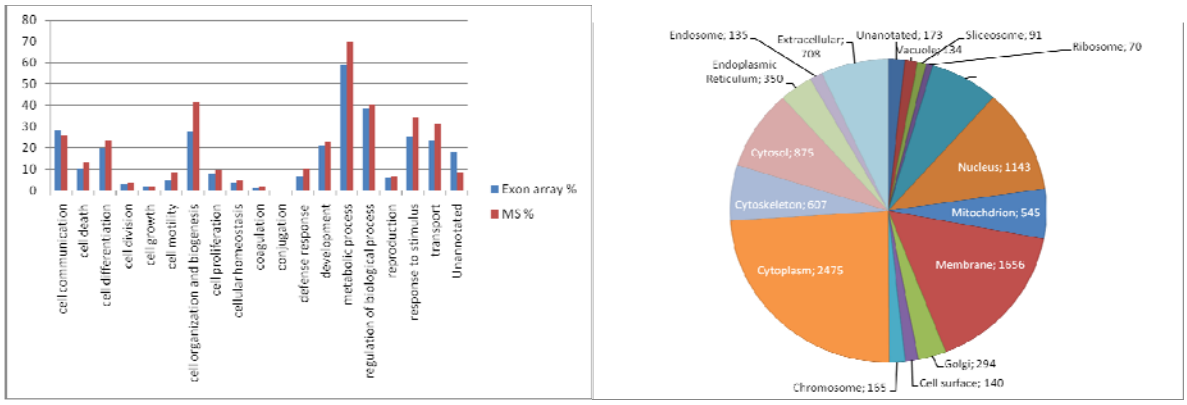

Figure 1. *Distribution of gene ontology (GO) categories comparing mass spectrometry data (red) and exon array transcriptomics data (blue) from the same lung cancer tumour samples, percent of total number of expressed exons/ proteins. Right panel: circle diagram showing molecular localization GOSlim annotations for the proteins identified and quantified in the study.*

To summarize, during the CHMEORES project we have published methods and applied these to provide valuable biological and clinical information on proteome level. A major breakthrough was made during the fall of 2010, when we further developed our proteomics method by performing ultra resolution IEF. Using this improved method we have achieved two landmarks 1) we have profiled quantitatively the human proteome and detected a similar amount of peptides as the analysis of the transcriptome in the same lung cancer samples (estimated by RNAseq read out) and 2) used the method to generate novel proteome information, including the discovery of proteins from genes previously not known to be expressed. Hence, we are eager to explore the possibilities that the improved method provide in cancer biology and clinical proteomics. The findings during this project on WP5 on the significant protein expression level changes (S100A6, SERCA2, EphA2 etc.) indicating drug resistance and/or poor prognosis will be validated further and form the basis for coming research on molecular response on cancer treatment in our group.

WP6 – Bioinformatics - Biostatistics

The main objectives for WP6 were to integrate the collection of clinical data and the statistical analytical approaches. The following was accomplished during the project:

With regard to the integration of the collection of clinical data we designed, implemented and tested a web-based data-entry and data-base of the clinical data for the CHEMORES prospective and retrospective lung cancer study. A copy of the Data Entry Manual can be found on the CHEMORES website: www.chemores.org. The rest of the achievements below refer to integration of the statistical analytical approaches:

We developed a general algorithm to search for 'interesting' molecular subtypes, where 'interesting' is defined by the clinical objective such as prognosis or chemoresistance. The motivation comes from the fact that cancer is a heterogeneous disease, so different subgroups may use different molecular mechanisms for progression. We applied the algorithm to the retrospective lung cancer patient cohort (described in WPs 2 and 4) and found signatures that defined a molecular subtype with a strong prognostic significance. A publication describing this work is under review. A similar approach was applied for analyzing the gene expression profiling data from melanoma pre- and post-treatment tumour samples (described in WPs 1 and 4).

We also developed a network enrichment analysis (NEA) to identify pathways that were activated in an experimentally derived gene set. This is a powerful general extension of the gene-set enrichment analysis. The method allows us to exploit rich biological knowledge available on gene or protein networks in order to characterize any set of genes found to be significant/interesting in a study. In CHEMORES NEA was applied at patient level; the results conveyed the diversity of cancer progression across patients in terms of which pathways were used by the cancer cells in their growth. The activation score for each pathway was correlated with clinical phenotypes, such as histology, and with patient prognosis. A publication describing the methodology is published in BMC Bioinformatics.

Finally, we also developed a general method to integrate several omics data, in particular to apply this to available retrospective CHEMORES samples, containing CGH, mRNA and microRNA data. The method is summarized in Figure 2. One output from this analysis is an integrated molecular portrait of NSCLC. NSCLC represents a heterogeneous group of neoplasms, mostly comprising squamous cell carcinoma (SCC), adenocarcinoma (AC) and large-cell carcinoma (LCC). We provided a systems biology insight into this current clinical classification. Comparative genomic hybridization followed by mutational analysis, gene expression and miRNA microarray profiling were performed on 123 paired tumour and non-tumour tissue samples from patients with NSCLC. Using integrated systems biology approaches, we sought to find out if combining data types from different levels of biology would improve clinical assessment of NSCLC. At DNA, mRNA and miRNA levels we could identify molecular markers that discriminated significantly between the various clinicopathological entities of NSCLC. We reported proofs of distinct molecular profiles that contribute to distinguishing NSCLC tumour subtypes even in small biopsies. The manuscript for this study has been submitted.

Candidate markers for early diagnosis and therapy monitoring were identified based on genes that were differentially expressed between tumour and normal tissues. There were a total of 625 probes with tumour>normal expression in more than 75% of samples. Fold change (FC max ~3) of 2054 probes were >1.5 and the tumour was up-regulated >75% samples. Various validation works are still ongoing. The full list of candidate markers is too extensive to show in this report, but they are all available in this website:

http://www.meb.ki.se/~yudpaw/misc/TumourNormal-comparisons.html

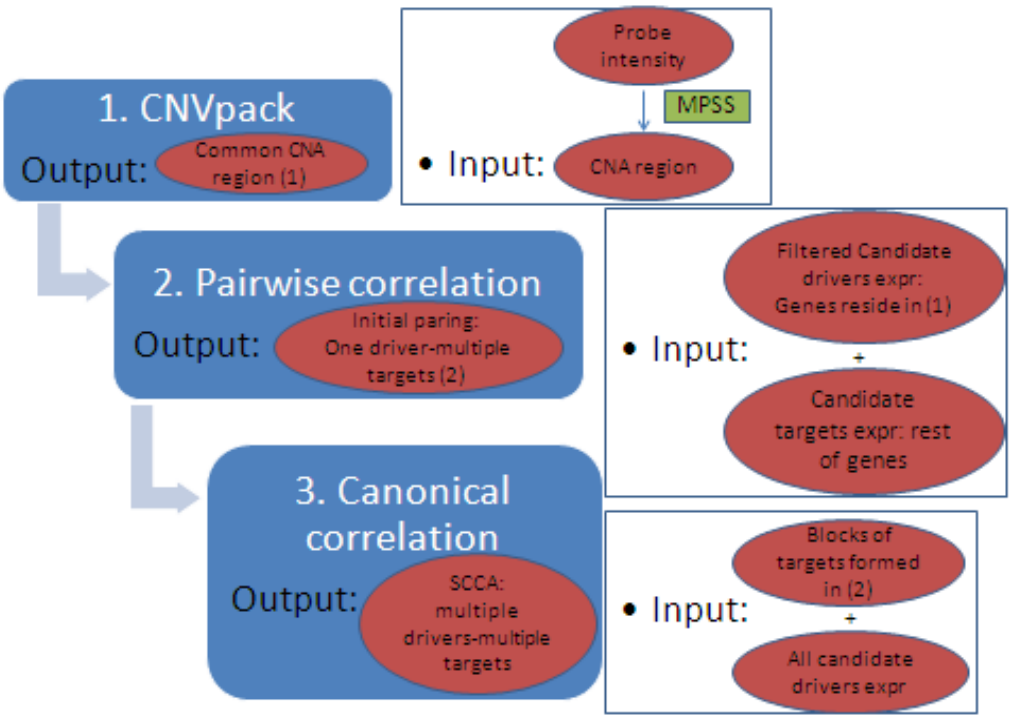

Figure 2. *Flowchart of our algorithm. Driver and target genes are identified in a three step process as shown. In the first step we use the multi-platform smoothed segmentation (MPSS) algorithm (Teo et al, 2011) to the array CGH data in order to identify copy-number altered (CNA) regions. The CNVpack algorithm (Teo et al, 2010) is used to identify recurrent CNAs across multiple individuals. Candidate driver genes are then defined as those genes belonging to these recurrent CNAs. In Step 2, the genes in the non-altered regions, designated as potential target genes, are grouped into modules based on the closest correlation so the candidate driver genes. In Step 3, we expand the number of drivers in each module by performing a sparse canonical correlation analysis (SCCA, Lee et al, 2011).*

Almost all recent genetic signatures are based on a single omic platform, such as gene expression or SNP panels. It seems obvious that combining omics data would help in prediction or prognostic modelling, but this is still not commonly done because of lack of data and lack of established methodology. The development of analytical methodology for high-throughput data always lags the rapid development in measurement technology. The work in WP6 has contributed significantly to the analyses of multiple high-throughput molecular data in CHEMORES, and the set up provides a model for future studies. All of the general algorithms for high-throughput data analyses developed and implemented throughout this project are made freely available for academic researchers at:

http://www.meb.ki.se/~yudpaw/

WP7 – Animal tumor models

The aim of WP7 was to use animal tumour models that reproduce the natural history of malignant melanoma, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) to reveal the molecular mechanisms responsible for chemotherapy resistance. Pre-

clinical validation of potential druggable targets for the treatment of NSCLC was performed. To this end we have generated several genetically engineered mouse models that faithfully recapitulate human disease:

We have found in our model of NSCLC the existence of a synthetic lethal interaction between the K-Ras oncogene and Cdk4 (Puyol et al., 2010). Activation of K-Ras^{G12V} in Cdk4$^{-/-}$ mice results in a major reduction in tumour formation whereas it proceeds unaffected in the absence of the closely related Cdk2 or Cdk6. Importantly, in this study, we also showed that conditional ablation of Cdk4 in lung adenocarcinomas detectable by imaging techniques (computed tomography) induced rapid senescence leading to tumour regression of most of the tumours. This was also validated pharmacologically using the PD-0332991 Cdk4 inhibitor from Pfizer.

Furthermore, we have initiated a long-term project to systematically examine, by genetic means, the contribution of the druggable Raf/Mek/Erk cascade of kinases in the K-*Ras* driven NSCLC model. Our studies have revealed that the Erk kinases, Erk1 and Erk2, display compensatory activities that allow tumour development in the presence of either Erk1 or Erk2 (Blasco et al., 2011). However, elimination of both kinases completely prevents K-Ras^{G12V}-driven NSCLC tumour development. Similar results have been obtained with the Mek kinases, Mek1 and Mek2. Surprisingly, the results obtained with the Raf kinases, c-Raf and B-Raf, were quite unexpected. Ablation of B-Raf had no effect on tumour development, a result that could be explained by the compensatory effects of c-Raf and A-Raf. However, ablation of c-Raf completely prevented tumour development even in the presence of normal levels of expression of A-Raf and B-Raf (Blasco et al., 2011). Similarly to Cdk4, we are now conducting a therapeutic approach to evaluate the impact of c-Raf elimination in lung adenocarcinomas. Preliminary results suggest that this approach also results in tumour reduction. We are now characterizing the molecular mechanisms that underlie this response and also further validating the approach in more aggressive / metastatic lesions resulting from the loss of the tumour suppressor TP53.

In addition, we have generated a conditional knock out mouse for A-Raf to determine whether this kinase is also essential for the development of K-Ras^{G12V}-driven NSCLC. The effect of ablating A-Raf could not be tested since germ line deletion of A-Raf alleles results in early postnatal lethality. These experiments are currently ongoing.

During cancer therapy, inhibitors block the kinase activity without eliminating the target. To address the issue of tumour development in the presence of a dead kinase, to mimic this type of pharmacological intervention, we have generated a strain of mice that expresses a c-Raf kinase dead isoform in an inducible fashion. To this end, we engineered a knock-in consisting of a c-Raf minigene (made up of cDNA sequences) that expresses the wild type kinase flanked by loxP sites so it can be removed in a timely and spatially controlled manner upon expression of the Cre recombinase. In addition, these targeted mice will carry a knocked in mutation (D468A) in the corresponding exon that completely inactivates the c-Raf kinase (exon 13). These mice will express the normal c-Raf kinase during embryonic and postnatal development from the knocked-in minigene until these sequences are removed by the Cre recombinase. At that time, the kinase dead isoform will be expressed from the targeted endogenous locus. A similar approach is ongoing for Cdk4 by introducing a D140A mutation in the Cdk4 locus that causes complete inactivation of the Cdk4 kinase activity. Needless to say, depending on the results obtained with these strains, we will combine both mutant isoforms to determine whether they display additive or synergistic activities. Finally, we are conducting parallel studies with c-Raf and Cdk4 inhibitors to establish a correlation between genetic and pharmacological approaches, and to define the therapeutic conditions to eventually translate this information to patients with NSCLC positive for K-Ras oncogenes.

For the melanoma studies, we generated a mouse model for metastatic melanoma carrying a conditional knock-in for the $Braf^{V600E}$ mutation and a conditional deletion for the PTEN tumour suppressor. The specific activation of these two mutations in melanocytes is achieved with Tyrosinase-CreERT2 strain upon skin topic treatment with 4OHT tamoxifen. We have induced the development of melanoma in this compound strain [$PTEN^{lox/lox}$; $B\text{-}Raf^{+/LSLV600E}$; $Tyr\text{-}CreERT2^{+/T}$] and treated melanoma-bearing mice with the $Braf^{V600}$ specific inhibitor PLX4032 (vemurafenib) for two weeks. Treated mice showed a substantial reduction in their tumour burden as well as lower metabolic activity as measured by PET scan. Unfortunately $PTEN^{lox/lox}$; $B\text{-}Raf^{+/LSLV600E}$; $Tyr\text{-}CreERT2^{+/T}$ developed skin and oral papillomas that caused an early death. This made it impossible to do long term follow up of treated-mice in order to monitor disease recurrence and potential drug resistance.

In this project we have also investigated the chemotherapy response of various additional genetically engineered mouse models that mimic cancer in humans. This comprises mouse models for lung cancer, breast cancer, and mesothelioma. In particular, we have treated animals carrying tumours with defects in homology-directed DNA repair with the maximum tolerable dose (MTD) of topoisomerase I and II inhibitors, taxanes, or DNA crosslinking agents; anti-cancer drugs that are regularly used in the clinic. We also tested the novel therapeutic agents poly (ADP-ribose) polymerase inhibitors.

We found that defects in homology-directed DNA repair (*e.g.* due to lack of BRCA1, BRCA2) have an enormous impact on therapy response. Despite the high drug sensitivity that we observed in the BRCA1/2-deficient models, tumours are usually not eradicated, even not by dose-dense treatment. Our data strongly suggests that this drug tolerance is caused by tumour-initiating cells that are lying low in the G0/G1 phase of the cell cycle.

Acquired resistance to docetaxel, doxorubicin, topotecan or olaparib of relapsing tumours was frequently due to increased expression of the drug efflux transporters ABCB1 or ABCG2 (Rottenberg et al., 2007; Rottenberg et al., 2008; Pajic et al., 2009; Zander et al., 2010). In addition, new resistance mechanisms that are independent of drug efflux were identified: for example loss of 53BP1 results in partial restoration of homologous recombination in the absence of BRCA1, and this mechanism causes olaparib and topotecan resistance. In addition, our studies suggest that the BRCA1 RING function is essential for tumour suppression but dispensable for therapy resistance (Drost et al., 2011).

In the course of the project we have also made an attempt to identify predictive markers for chemotherapy response in our models by genome-wide expression profiling. Even in such genetically homogeneous tumour system, we did not find a specific gene expression signature predicting docetaxel response (Rottenberg et al., 2012). Our results show why it is difficult to develop predictive markers, based on genome-wide expression arrays: only if the response to a drug is primarily determined by the expression level of a gene in most tumours, one can expect that gene to show up in the array-based gene expression analyses. For example, low expression of the *Xist* gene correlated with cisplatin hypersensitivity in most tumours, and it also predicted long recurrence-free survival of HER2-negative, stage III breast cancer patients treated with intensive platinum-based chemotherapy (Rottenberg et al., 2012).

In this project we have shown that the new generation mouse models are useful to address major clinical problems, such as validation of potential targets for therapeutic intervention, validation of mechanisms of drug resistance, predicting chemotherapy response or characterizing the nature of residual tumour cells that escape eradication.

WP8 – Lung cancer and melanoma stem cells

The cancer stem cell (CSC) model proposes that tumour progression, metastasis and relapse after therapy may be driven by a subset of tumour cells that possess stem cell capacity to self-renew. The identification of lung and melanoma CSC and associated markers may be useful for optimization of therapeutic approaches and to provide predictive and prognostic information in lung cancer and melanoma patients.

Lung cancer stem cells

Concerning lung cancer, we have reported (Bertolini G. et al., PNAS 2009) the presence of a highly tumourigenic CD133+ subpopulation displaying stem-like features and chemoresistance to conventional drugs in primary non-small cell lung tumours (NSCLC). CD133, a surface glycoprotein linked to organ-specific stem cells, was described as a marker of cancer-initiating cells in different tumour types. For the isolation and characterization of lung CSC, we took advantage of pre-clinical *in vivo* models derived from the direct implant of patients' lung tumours in immunocompromised mice (tumourgrafts) that closely resemble the features of primary tumours. We found that a CD133+EpCAM+ population was increased in primary NSCLC compared to normal lung tissue and displayed higher tumourigenic potential in SCID mice and increased expression of genes involved in stemness, adhesion, motility and drug efflux than the CD133- counterpart. Cisplatin treatment of lung cancer cells *in vitro* resulted in enrichment of the CD133+ fraction both after acute cytotoxic exposure and in cells with a stable cisplatin-resistant phenotype. Subpopulations of CD133+ABCG2+ and CD133+CXCR4+ cells were spared by in vivo cisplatin treatment of lung tumourgrafts, a finding that may explain restoration of the tumour after chemotherapy cessation. A tendency towards shorter progression-free survival was observed in CD133+ NSCLC patients treated with platinum-containing regimens. These results indicate that chemoresistant populations with highly tumourigenic and stem-like features are present in lung tumours.

In an attempt to identify novel drugs capable of overcoming CSC-induced resistance to conventional cytotoxic compounds, we focused our study on combination therapy with cisplatin and differentiating agent (i.e. all-trans-retinoic acid, ATRA). To test wheter ATRA was able to deplete the tumour stem-like pool, we treated two different tumourgraft models with ATRA in combination with cisplatin. ATRA treatment could partially deplete the component of CD133+/CXCR4+ cells as shown by FACS analysis, an effect that may account for the increased latency in tumour re-growth after combination therapy compared to cisplatin treatment alone. Moreover, *in vivo* serial transplantation assays of cells treated with combination therapy indicated a progressive reduction in CSC pool, thereby suggesting a decreased ability to initiate tumours.

We then investigated the recent hypothesis of a direct link between epithelial-mesenchymal transition (EMT) and generation of CSC. We proved that EMT activation in a primary lung cancer cell line provides cells with mesenchymal traits as well as stem-like properties, as supported by up-regulation of stemness genes, expansion of the CD133+ cell fraction, enhanced tumourigenic ability and increased resistance to cisplatin treatment in vitro. Within the EMT-generated CD133+ cells, we identified a subset of CD133+/CXCR4+/EpCAM- cells endowed with a mesenchymal and migratory phenotype, that we hypothesised were involved in the dissemination process and metastasis initiation.

Analysis of disseminated cells to lungs of tumourgrafts-bearing mice indicated an enrichment for the subset of CD133+/CXCR4+/EpCAM- cells compared to parental tumours, strengthening the hypothesis of a direct role for this cells fraction in the dissemination process. In vitro invasion assay of tumourgraft cells, confirmed that the invading tumour cell fraction was enriched for CD133+/CXCR4+/EpCAM- cells. Finally, we proved in the clinical

setting that CD133+/CXCR4+/EpCAM- cells were enriched in patient's lymph node metastasis compared to parental lung tumours.

Inhibition of CXCR4 signalling using a peptide antagonist of CXCR4 could reduce the invasion capability of tumourgrafts cells, preferentially blocking the invasion of CD133+/CXCR4+ cells, as evaluated in *in vitro* invasion assays. Moreover, exploiting an *in vivo* model of spontaneous lung metastases generated from subcutaneous injection of the H460 cell line, we verified that CXCR4 signalling inhibition in combination with cisplatin was able to prevent the spreading of chemoresistant CD133+/CXR4+ cells induced by cisplatin treatment, resulting in a decrease of metastasis formation. Thus, a distinct subset of CD133+ migrating CSCs, possibly generated through the EMT process, could play a major role in metastasis formation and may represent an amenable target for a rationale therapeutic strategy in the clinical management of lung cancer patients.

Melanoma cancer stem cells

Concerning melanoma CSC we provided evidence that further sustains the notion that human melanomas contain cells endowed with features of CSC (Perego et al. J Invest Dermatol. 2010;130(7):1877-86.). Melanoma stem cells were selected *in vitro* as melanospheres, i.e. melanoma cells growing as non-adherent colonies. Melanospheres were highly tumourigenic and intradermal injections in SCID mice of as few as 100 cells generated tumours that maintained tumourigenic potential into subsequent recipients. Primary and serially transplanted tumourgrafts recapitulated the phenotypic features of the original melanoma of the patient. Melanospheres displayed a heterogeneous phenotype for the expression of stem cell markers. However, they displayed an enhanced expression of the embryonic markers Nanog and SOX3/4 as compared to adherent melanoma cells lines. Moreover melanospheres contained a discrete fraction of cells expressing the NGFR or CD133 markers. In addition, our data clearly indicated that culture conditions that include Fetal Calf Serum (FCS) are still able to maintain the melanoma stem cells originally contained in melanoma specimens, but with a lower efficiency as compared to propagation in stem cell medium. All together our data confirmed that melanospheres are an appropriate model to investigate melanoma complexity. These issues have been extensively discussed in a note recently published (Perego et al., J Invest Dermatol. 2011;131(2):546-7). Using immunohistochemistry we showed that in human tumours, *in vivo* primary as well as metastatic melanomas, do contain a discrete subpopulation of tumour cells stained positive for the NGFR or CD133 stem-associated markers identified in melanospheres (Perego et al. J Invest Dermatol. 2010;130(7):1877-86).

However, there is evidence suggesting that CSC is not a static compartment but rather that stemness features can be acquired by tumour cells in response to environmental signals. Thus our research was also focused on dissecting the reciprocal interaction between melanoma initiating cells and the immune system, with the aim of defining any role of immune-related factors in shaping the fraction of tumour cells responsible for tumour maintenance. Analysis of the secretion profile of melanospheres revealed that melanospheres displayed a secretory capacity quantitatively and qualitatively different from that of melanoma adherent cells or melanocytes. We focused our attention on IL-10, produced by one of our melanosphere cultures, and on IL-6 that was selectively produced by melanoma cells when cultured as adherent cells and by melanocytes. Since melanospheres express the receptors for these cytokines, we tested their effect in modulating melanosphere proliferation and self-renewal. Specifically, IL-6 exerted its effects both by reducing the number of live cells when added to the melanosphere culture medium and by diminishing their self-renewal and clonogenic ability. Conversely, IL-10 strongly promoted melanosphere self-renewal. Interestingly, only

melanospheres were responsive to these soluble factors, while adherent cells were not influenced by the exposure to these same cytokines. These data underlined a strong relation between the immune system and melanoma stem cells, and provided a possible model in which cytokines working at different levels may be responsible for influencing the modality of asymmetric cell divisions of cancer stem cells. Data collected in this project also provide new perspectives for melanoma therapy, by showing that an approach directly targeting the immune-related microenvironment, such as IL-6 or IL-10 cytokines, may potentially be helpful in limiting the *in vivo* expansion of the tumour mass. Of note antibodies directed against human IL-6 or its receptor are already available for clinical use.

The molecular features of these cells may provide the rationale for more specific therapeutic targeting and for defining predictive factors in the clinical management of this lethal disease.

To summarise, in WP8 the CSC model has been investigated in NSCLC and melanoma. The major achievements include:

Lung cancer:

CD133 has emerged as a marker for lung tumour cells endowed with tumourigenic potential and resistance to cisplatin treatment. The identification of a subpopulation of tumour cells co-expressing CD133 and ABCG2 or CD133 and CXCR4, involved in restoration of the tumour after chemotherapy cessation, suggest new therapeutic strategies aimed at preventing drug resistance and tumour dissemination.

Establishment of pre-clinical in vivo models (tumourgrafts) that recapitulate primary tumour features, useful for testing the efficacy of new drugs able to overcome the chemoresistance of CSC.

Identification of a stem cells marker (CD133) predictive of poor outcome for lung cancer patients after chemotherapy.

Indication of the possible use of differentiation therapy in combination with standard therapy to prevent the enrichment of the chemoresistant CD133+ CSC fraction, which in turn could drive tumour recurrence.

Verification of a direct link between the EMT process, which could be triggered by the tumour microenvironment, and generation of a disseminating CSC population, involved in the metastatic process. Blocking the EMT process through tumour microenvironment targeting could represent an innovative strategy to prevent the generation of the metastasis-initiating cells.

Evidence for an enhanced metastatic spreading of CD133+/CXCR4+ CSC induced by chemotherapy, that could be prevented by blocking the CXCR4 pathway. Thus, the CXCR4/SDF1 axis could be a new promising target to counteract metastasis formation in combination with standard therapy in lung cancer.

Melanoma:

Establishment of suitable *in vitro* culture conditions to maintain and expand melanoma stem cells thus available for further biological characterization.

Indication of a new direction for possible clinical intervention in melanoma therapy, targeting the network between cancer stem cells and the immunological environment. Identification of IL-6 and IL-10 as possible targets.

WP9 – Novel therapeutic strategies

The aim of WP9 was to develop novel therapeutic strategies to treat cancers with intrinsic or acquired resistance to anticancer drugs, using the following approaches:

1.Exploring lysosomal cathepsins as therapeutic targets of cells that are resistant to conventional chemotherapy

2. Inhibiting the function of proton pumps

1. Exploring lysosomal cathepsins as therapeutic targets of cells that are resistant to conventional chemotherapy

Lysosomes are involved in certain forms of cell death (e.g. induced by TNF-α and TRAIL). Permeabilization of lysosomes leads to release of cathepsins to the cytosol where they cleave the BH3-only protein Bid, leading to apoptosis. We have previously reported that lysosomal cell death is a common mechanism for agents that induce p53-independent apoptosis (Erdal et al., PNAS 102, 192, 2005). The lysosomal aspartic protease cathepsin D is overexpressed and hypersecreted by cancer cells. We have shown that, in apoptosis-induced conditions, cathepsin D overexpressed by cancer cells is released in the cytoplasm and enhances apoptosis-dependent chemo-sensitivity (Beaujouin et al., Oncogene 25, 1967, 2006). Within CHEMORES, two groups collaborated to screen a chemical library for agents that induce cathepsin-dependent apoptosis. For that purpose, we developed an assay by transiently silencing cathepsin D or cathepsin B in cancer cells. Four agents were identified that were found to be dependent on cathepsin D, but not of cathepsin B, for induction of apoptosis (Berndtsson et al., Int J Cancer 124, 1463, 2009). Cathepsin-dependent agents were found to be effective on a panel of drug resistant cell lines and to show increased efficiency on a cell line known to contain a large acidic vesicle compartment and high levels of cathepsin-B. In order to repeat the screening for cathepsin-independent compounds using another library and other cells with or without permanent extinction of cathepsin D, we have developed cell lines with stable extinction of cathepsin D using an shRNA approach.

Inhibitors of vacuolar ATPases (proton pump inhibitors, PPIs) have been found to induce tumour cell death (apoptosis) and to increase the cellular content of acidic vesicles. To investigate whether PPI-induced apoptosis is cathepsin-D-dependent, we have established new model cell lines that stably overexpress cathepsin-D. In parallel, cells were cultivated under unbuffered conditions, and the results indicate that these cells can acidify the extracellular pH from 7.4 to 6.7 within 7 days, thus providing the acidic conditions for a better activation of the PPI. We studied the effect of PPI on cell viability in this new model, with or without expression of cathepsin D, but did not detect any difference.

Identification of a novel drug target in oncology – the deubiquitinating activity of the 19S proteasome.

The work described above, lead to the identification of 4 different small molecular weight compounds that induced apoptosis by a mechanism dependent on cathepsin D (Berndtsson et al., Int J Cancer 2009, op cit). Two of these compounds are chemically related and we proceeded to characterize the mechanism of action of these.

We concentrated on the small molecule b-AP15. We found that b-AP15 inhibits the ubiqutin-

proteasome system (UPS) by a novel mechanism. In contrast to the clinically used drug bortezomib (Velcade®), b-AP15 does not inhibit the activity of the proteasome but instead inhibits the activity of the deubiquitinating enzymes USP14 and UCHL5 of the 19S proteasome. The work was recently published (D'Arcy et al., Nature Medicine 17, 1636, 2011) and has attracted significant interest in the field (Nat Rev Drug Discov. 2012 Jan 3;11:23).

Bortezomib (Velcade®) is an inhibitor of the 20S proteasome used for treatment of multiple myeloma and mantle cell lymphoma. Cancer cells develop resistance to bortezomib. A major mechanism of resistance is mutations in the proteasomal subunit PSMB5; another mechanism is overexpression of Bcl-2. Since b-AP15, in distinction to bortezomib, does not inhibit 20S proteasome enzymatic activity but inhibits the deubiquitinating enzymes of the 19S RP, mutations in PSMB5 will not confer resistance to this compound. Furthermore, and interestingly, b-AP15 is able to induce apoptosis of cells overexpressing Bcl-2 (D'Arcy et al., Nature Medicine 2011). The drug is therefore expected be effective in the treatment of malignancies that are bortezomib-resistant due to Bcl-2 overexpression.

b-AP15 induces an extraordinary proteotoxic response – significantly stronger than bortezomib. This response is associated with a lack of aggresome formation, a physiological response that protects cells from malfolded proteins not degraded by the proteasome. The ability of b-AP15 to induce stronger cytotoxic response compared to bortezomib, is due to a defect in the transport of ubiquitin conjugates to aggresomes.

2. Inhibiting the function of proton pumps

This part has included both pre-clinical and clinical studies. The preclinical achievements were based on two important aspects of the anti-neoplastic activity of proton pump inhibitors:

Proton pump inhibitors (PPI) have a clear anti-neoplastic effect, particularly against human melanoma. Their effects are mediated by the induction of a non-conventional cell death, whose early events is represented by ROS intracellular accumulation and cytosolic acidification. A later event is caspase activation, which, particularly in melanoma cells was proven instrumental to the cell death. The *in vivo* experiments, in human melanoma-SCID mice xenografts, have shown a clear inhibition of the tumour growth, consistent with pH gradients similar to the surrounding normal tissues and with a markedly prolonged survival of the treated animals (De Milito A et al., Int J Cancer 2010). We have also shown that the effect of PPI at the cellular level was mediated by a tentative autophagic reaction of melanoma cells with atypical intracellular pathways (Marino M et al., Cell Death Dis 2011).

More recently, a series of *in vitro* and *in vivo* experiments have shown that PPI, although belonging to a class of generic drugs, show significant differences in their anti-neoplastic activity. Moreover, we have performed experiments aimed at understanding the pH-mediated mechanism underlying melanoma resistance to chemotherapeutics, using cisplatin as a prototype drug. The results has shown that at low pH, only negligible amounts of cisplatin actually enters into human melanoma cells, and that PPI treatment can significantly increase this drug entry. Moreover, an additional and very efficient mechanism of cisplatin resistance is mediated by exosomes that extracellularly eliminate the small amounts of the drug that has entered the melanoma cells. PPI reduces the exosome release and can increase the permanence of the drug intracellularly.

We have also demonstrated that autophagy is induced as a protective response to tumour acidity (Marino et al., J Biol Chem 2012, Epub July 4).

Overall, the work on proton pump inhibitors have demonstrated that these drugs are promising anticancer agents. A clinical trial has been initiated and shown promising results in terms of increased progression-free survival of patients. The work has demonstrated that PPI-induced decreases in tumour acidity leads to increased entry of cisplatin into tumour cells. This observation will form the basis for future trials where PPI is combined with cisplatin.

In conclusion, we believe that the workprogramme has been successful and that significant results have been achieved on a limited budget. The work has led to increased understanding of the role of the protease cathepsin-D in apoptosis. We have been able to identify a novel drug target in oncology (the 19S proteasome), and an effective novel drug. The work on proton pump inhibitors has proceeded into clinical trials and these have generated promising results. In animal experiments, the identified drug is more effective compared to a cancer drug currently used to treat a form of leukemia. We hope that our drug will be useful for this group of patients.

WP10 – Mechanisms involved in resistance to DNA damaging drugs

Several potential DNA damage response target have been identified during the course of the project, both as an outcome from gene expression profiling and other global approaches, and as a result of hypothesis driven investigations. Some of the main findings are described below. The melanoma gene and protein expression profiling and validation studies performed are described in WPs 4 and 5.

We have defined the basis for XPF as a biomarker for oxaliplatin sensitivity in melanoma, using a variety of pre-clinical models. The ERCC1-XPF endonuclease is required for nucleotide excision repair (NER) of helix-distorting DNA lesions, such as adducts produced by cisplatin and oxaliplatin. The dynamic range of the marker in tissue arrays suggests that it is usable as the basis for selecting patients for treatment.

Early in the project, histone deacetylase and insulin-like growth factor receptor inhibitors were identified as therapeutic targets for modulating DNA damage response. IGF1R inhibition was shown to enhance the cytotoxicity of temozolomide in human melanoma cells in vitro, and this combination therapy is currently tested in an animal model.

Acetylation and deacetylation of histone tails is tightly regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation status modulates gene expression and DNA accessibility. We undertook through a genome-wide loss-of-function screen to identify genes that impact on the sensitivity of tumour cells to HDAC inhibitors, identifying HR23B as a potential biomarker. HR23B functions in at least two pathways; nucleotide excision repair and protein targeting to the proteasome. Further studies indicated that it is the ability of HR23B to engage in proteasomal shuttling that underpins its role as an HDAC inhibitor sensitivity determinant, and it is consistent with this idea that proteasomal activity is deregulated in tumour cells treated with HDAC inhibitors. An analysis of HR23B in melanoma, including biopsies taken from patients treated with HDAC inhibitors, suggests that the low levels of HR23B frequently coincide with autophagy. Tumours that harbour autophagocytic cells are associated with poor prognosis disease. Our results define HR23B as an important sensitivity and regulatory determinant that influences the biological outcome and therapeutic effect of HDAC inhibitor treatment.

Previous studies in cell lines have suggested a role for melanosomes and related protein trafficking pathways in melanoma drug response. We have investigated the protein and/or mRNA expression of factors related to melanosomes and other organelles, in pre-treatment tumours from melanoma patients (n>52) with different response to dacarbazine or temozolomide. MITF and GPR143 showed significantly higher protein expression in non-

responders compared to responders, and a similar trend was detected for RAB27A, a chemoresistance candidate previously identified by gene expression profiling. We also performed *in vitro* studies to test the chemosensitivity of melanoma cell lines with different melanosomal content and expression of related proteins. The effects of siRNA inhibition of RAB27A or gp100/PMEL on chemosensitivity were evaluated in the MNT-1 melanoma cell line. While no effect was detected with regard to response to temozolomide, the knock-down of gp100/PMEL could sensitize the cells to paclitaxel and cisplatin. Overall our results suggest that MITF, and several MITF regulated factors, play a role in melanoma chemoresistance.

The DNA repair protein, O6-methylguanine-DNA methyltransferase (MGMT) removes cytotoxic alkyl adducts from O6-guanine, and can thereby inhibit apoptosis of tumour cells and confer resistance to alkylating chemotherapeutic agents, such as dacarbazine and temozolomide. The efficacy of temozolomide chemotherapy in glioblastoma, as studied in many large and well-conducted clinical trials, is increased by inactivation of MGMT, by promoter hypermethylation of the gene. Within CHEMORES, we have evaluated and developed methods to assess MGMT promoter methylation in melanoma tumours, including FFPE material, in a reliable manner. The mRNA expression of MGMT was also investigated, as well as the impact of MGMT promoter methylation as a predictive marker of chemotherapy response in melanoma. Our results suggest that in the dacarbazine or temozolomide monotherapy context, the MGMT hypermethylation has potential predictive value in malignant melanoma.

The relative importance of different DNA repair pathways has been studied, and also the possibility of using DNA repair inhibitors as chemosensitisers. While MGMT has been demonstrated in preclinical studies to be a major resistance factor in melanoma and other tumour types, in phase II clinical trials, the inactivation of MGMT using the pseudosubstrate, lomeguatrib (O6-(4-bromothenyl)guanine did not improve the outcome for melanoma patients treated with temozolomide. This raises the possibility that other repair pathways may be responsible for resistance and this has been investigated in preclinical models. One strategy has involved attempts to block the repair of O6-methylguanine by means of expressing in human melanoma cells, the gene from S.pombe that encodes an O6-methylguanine-binding protein which is a potent inhibitor of MGMT *in vitro*. Another has been to assess the rate or removal of O6-methylguanine from DNA in temozolomide-treated melanoma cells. We have examined cells that express MGMT and lomeguatrib to inactivate this, and also cells that do not express MGMT.

In addition to these approaches, we have recently shown that relatively high levels of expression of the DNA repair enzyme, alkypurine-DNA glycosylase (APNG), is related to poor survival in glioblastome multiforme patients treated with temozolomide. As part of CHEMORES, we have developed pilot drugs that would inhibit APNG and at the same time, MGMT activity, and shown that these are effective, although with very low potency. We have also established robust radioactivity-based assays for APNG and MGMT and also apurinic/apyrimidinic site endonuclease (APE) activity and screened a number of melanoma cell lines for these. Radioactivity-free assays have also been developed, however, these have not yet achieved the sensitivity required for routine screening of tumour biopsies that could be applied in future clinical studies. Extensive training activities have also been performed in WP10, including full research laboratory training of seven undergraduates in Manchester.

Since the CHEMORES project started in 2007, major advances have been made in melanoma therapy with the introduction of CTL4 antibodies (ipilimumab) and the BRAF V600 specific inhibitor vemurafenib. Clinical trials are ongoing for additional inhibitors of the MAPK/PI3K pathways and also other novel forms of immunotherapy. Although material from melanoma

patients receiving these types of therapies is being collected by several of the CHEMORES participants, clinical sample sets of sufficient sizes were not obtained in time for inclusion into the project workprogramme. However, *in vitro* studies of these novel drugs have been performed in several WPs, including WP10. The main outcome includes the identification of mechanisms for induced resistance to vemurafenib (though proteomic, gene expression and gene targeting analyses) and studies of combination therapies of MAPK/PI3K inhibitors and standard chemotherapeutic agents.

Drug resistance is a major clinical challenge in melanoma therapy. Primary resistance to standard chemotherapeutic agents is widely experienced, and, despite many impressive initial responses to inhibitors targeting the MAPK signalling pathway, acquired drug resistance eventually develops to these drugs. Combinations of therapies are likely necessary to overcome this, and targeting of dual pathways, or multiple steps in the MAPK pathway, is currently studied intensely. However, traditional chemotherapeutic drugs could also play an important role in this context, by preventing growth of tumour cells resistant to targeted drugs. These types of combination therapies have not been studied as intensively, and the work performed in CHEMORES WP10 adds to the knowledge of these types of treatments.

To summarise, we have identified and developed two means for selecting melanoma patients for existing treatments: XPF for oxaliplatin and HR23B for histone deacetylase inhibitors. Our understanding of the mechanism behind these observations indicates new treatment combinations that have the potential to improve upon existing therapy.

Previous studies in cell lines have suggested a role for melanosomes and related protein trafficking pathways in melanoma drug response. Our studies are the first, to our knowledge, to investigate these factors in a clinical material and overall our results suggest that MITF, and several MITF regulated factors, play a role in melanoma chemoresistance.

In addition, the outcome from the work in WP10 also includes the development of improved methodology to study DNA repair activity and DNA methylation, which is of great benefit also for other researchers in the field. We have also defined the repair activities of a large number of human melanoma cell lines that will provide a basis for their use in future studies relating to alkylating agent resistance.

WP11 – Apoptosis regulation

Workpackage 11 has dealt with the identification of biomarkers that allow prediction of chemotherapy resistance and susceptibility, as well as with the exploration of novel therapeutic targets for subverting chemotherapy resistance in cancer. The overall strategy to achieve these long-term goals was a mixture of systems biology methods and hypothesis-driven approaches. For this, *in vitro* experiments were performed on cultured human cell lines exposed to chemotherapeutic agents. The consortium has been extremely successful in exploring chemotherapy-induced cell death (which is the therapeutic goal of anti-cancer therapy) and all milestones and deliverables that have been outlined in the project have been achieved, without any deviation from the plan. Several candidate biomarkers and targets have been identified, and the results have been published in high-ranking scientific journals.

These biomarkers consist of microRNAs, mRNAs, proteins and processes that regulate the susceptibility of cancer cells to the induction of chemotherapy-induced cell death. The functional (genetic, biochemical and epistatic) analysis of these microRNAs, mRNAs, proteins and processes has been carried out and has been validated in preclinical studies. These results have allowed the design of strategies for sensitizing cancer cells to chemotherapy-induced cell death, by reducing the expression of resistance factors that protect

cells against cell death inducers. *In vivo* and clinical validation studies of some strategies have also been performed.

We have studied cell death in lung cancer and melanoma models. The modulation of acetylation processes contributes to improve the effect of chemotherapeutic agents employed in the clinical setting for NSCLC, in particular cisplatin. This is evident from data obtained in models in which the *in vivo* modulation of the p53 pathway and of apoptosis was found. These results may have implications on the optimization of platinum-based treatment of NSCLC. Moreover, cell response to treatment can result in modulation of genes exploitable as targets to improve the outcome of treatment. In such a view TRAIL could be effectively employed in drug combinations including arsenic trioxide.

Some of the main findings with regard to melanoma indicate a) a role for matricellular proteins such as CCN3 in regulating apoptosis by the activation of survival signals conferring tumour cells resistance to drug induced apoptosis; b) a role for MET and SRC activated signalling in the primary resistance to BRAF inhibitors, and as additional targets for combined treatment strategies; c) that a molecule of the inhibitors of apoptosis protein (IAP) family (Apollon/BIRC6) and a signalling pathway (calcium/calcineurin/NFATc2 pathway) are new potential therapeutic targets in melanoma. Inhibition of Apollon and of the calcineurin/NFATc2 pathway contribute to rescue melanoma susceptibility to cytotoxic drugs, to target-specific inhibitors (including MEK and BRAFV600E-spcific inhibitors) and to anti-tumour agents as TRAIL.

We have also analyzed mRNA and miRNA expression in relation to lung cancer cell propensity to undergo cell death after DNA damaging treatments. By mRNA expression analyses of NSCLC cells prior and post DNA damaging treatment, we identified Ephrin B3 as a putative driver of treatment resistance. Thus we demonstrated that inhibition of Ephrin B3 expression sensitized NSCLC cells to DNA-damaging treatments, increased pro-apoptotic and senescence cell death signalling and greatly influenced growth factor pathways such as MAPK and PI3K/Akt. Moreover, by applying phosphoproteomic profiling of NSCLC cells with or without Ephrin B3 expression, we showed that Ephrin B3 in fact drives a growth promoting signalling circuit involving the Ephrin A2 receptor (EphA2), Akt, FAK as well as a heat shock protein 90 isoform. These results are of particular interest given recent publications which have linked deregulated EphA2 signalling to metastatic propensity of NSCLC. Hence manipulations of Ephrin B3 signalling may be a future strategy for sensitizing NSCLC to DNA damaging treatments. We also analysed if miRNA expression could be linked to sensitivity to DNA damaging treatments i.e. conventional radiotherapy. The global miRNA profiling identified miR-214 as a putative driver of DNA damage treatment resistance in NSCLC in part as a result of impeded DNA-damage-induced apoptotic signalling. Interestingly, in melanoma as well as in cervical cancer recent publications have demonstrated a role of this miRNA in regulating metastatic propensity. The finding of miR-214 as a driver of DNA-damage resistance in NSCLC may therefore also point to a more general role of this miRNA as an inhibitor of DNA-damage signalling in other tumour types, including malignant melanoma.

To summarise, a detailed analysis of tumour cell response to treatment is relevant to define strategies to improve the treatment of tumours. The pattern of apoptosis-modulated genes found in NSCLC supports the interest of using drug combinations including TRAIL in NSCLC exhibiting resistance to conventional chemotherapeutic agents.

The complexity of signalling in melanoma supports the relevance of genetic and proteomic profiling for classification of melanoma in molecular subtypes to build rational combination treatments for more effective therapies with targeted agents.

Apollon/BIRC6 is a key regulator of apoptosis in advanced melanoma. This defines a new therapeutic target to improve efficacy of anti-tumour agents that activate the intrinsic or the extrinsic apoptosis pathways. In addition, the calcineurin/NFATc2 pathway regulates melanoma survival and its targeting significantly enhances the anti-tumour effects of target-specific drugs, such as MEK- or $BRAF^{V600E}$ inhibitors, and of biological agents with anti-tumour activity, such as TRAIL.

Our results also suggest that manipulations of Ephrin B3 signalling may be a future strategy for sensitizing NSCLC to DNA damaging treatments. We also identified miR-214 as a putative driver of DNA damage treatment resistance in NSCLC. Together with other recent publications, this highlights miR-214's possible role as a predictive/prognostic marker also in other cancer types, including melanoma.

WP12 - Studies of markers of individual chemotherapy-induced toxicity

Workpackage 12 has focused on finding markers to predict adverse drug reactions during chemotherapy. We have pioneered the studies of predictive toxicity, an issue that just during the past years has become recognized as one of the cornerstones for personalized medicine.

We have focused on two treatment regimes used mostly in lung cancer, namely carboplatin in combination with paclitaxel, and gemcitabine in combination with carboplatin. For these drugs we have tried to identify genetic variants affecting both the pharmacokinetics (of especially paclitaxel) and the adverse drug reactions of these drugs.

For paclitaxel we have identified CYP2C8*3 as a predictor for the patients' individual paclitaxel clearance. This was first done on total clearance of paclitaxel (Gréen et al, Basic and Clinical Pharmacology and Toxicology, 2009, 104, 130–137) and later on unbound clearance of paclitaxel (Bergmann et al. The Pharmacogenomics Journal, 2011; 11, 113–120).

Genetic variants in the metabolizing enzymes and transporters of paclitaxel have also been shown to influence the risk of adverse drug reactions for paclitaxel induced toxicity. The genetic variant CYP2C8*3 was found to be associated with neuropathy (Gréen et al, Basic and Clinical Pharmacology and Toxicology, 2009, 104, 130–137), this was later confirmed by Leskela et al. (Pharmacogenomics J 2011;11:121-129).We have also shown that the genetic variants CYP2C8 Hap C, CYP3A5*3 and ABCB1 C3435T are associated with myelosuppression after paclitaxel/carboplatin chemotherapy.

To identify new candidates for paclitaxel/carboplatin induced toxicity we used state of the art bioinformatics tools: To find new candidate genes for toxicity several partners worked together to perform a meta-analysis of public microarray data. The meta-analysis involved downloading all relevant high quality public domain gene expression data and the generation of a list of ranked genes, which are induced or decreased by the drugs and also expressed in tissues important for the toxicity (i.e. the organ were the toxicity occurs and tissues responsible for the elimination of the drugs). We know that paclitaxel is eliminated via the liver and that the dose limiting toxicities are neurotoxicity and myelosuppression, while carboplatin is eliminated via the kidneys and causes myelosuppression. From more than 100 microarray samples we generated a candidate list of genes that are both affected by the drug exposure and present in liver, kidney, bone marrow or relevant neurological tissues. From this list we have selected 10 genes and identified 42 non-synonymous single nucleotide polymorphisms (SNPs) in the coding regions and analyzed most of them in the 33 patients that had been treated with paclitaxel/carboplatin. For 11 of the 42 SNPs we detected genetic variation in the material. Five of these eleven SNPs correlated to toxicity (myelosuppression or neuropathy) in the screening material. These SNPs were then validated in an additional

material. In summary, genetic variants in the ABCA1 gene and the ATM gene were shown to be associated with paclitaxel/carboplatin induced myelosuppression, see Table 2 (Hasmats et al Manuscript accepted in GENE).

Table 2. Correlations between SNPs and paclitaxel/carboplatin induced toxicities.

GENE	SNP	TOXICITY	P-VALUE
ABCA1	rs4149313	Wild type patients had significantly lower nadir platelet counts as compared to the rest of the patients	0.031
ABCA1	rs2230806	28 % of the wild type patients suffered grade 2 neuropathy or higher as compared to 46% of the rest of the patients.	0.083
ATM	rs1800058	Wild type patients had significantly higher nadir platelet counts as compared to the rest of the patients	0.044

In addition, we have conducted a Genome Wide Association Study (GWAS) to identify genetic markers associated with paclitaxel-induced neurotoxicity. In this collaborative effort, 144 patients have been genotyped using the Illumina 660K SNP chip and the genotype correlated to the cumulative dose of paclitaxel until the patient experienced grade 2 of neuropathy. Our top 18 candidate with a p-value below 10^{-5} were then validated in an American material. One of the genetic variants could be validated in both studies with a p-value below 10^{-5} in both studies. These findings indicate that this genetic variant plays a role in chemotherapy induced neuropathy (unpublished results).

We have also identified genetic variants associated with gemcitabine/carboplatin induced myelosuppression. We collected clinical data and DNA from 243 NSCLC patients uniformly treated with carboplatin in combination with gemcitabine. The most relevant clinical toxicities for gemcitabine and carboplatin are neutropenia and thrombocytopenia, both leading to actions needed to be taken by the treating doctor and increased hospitalization costs. About ¼ of the patients do not suffer any of these toxicities (Common Toxicity Criteria, CTC-grade 0-2), ¼ suffer only neutropenia (grade 3-4), ¼ suffer from thrombocytopenia (grade 3-4) and ¼ suffer from both toxicities after the first chemotherapy cycle, see Figure 3. We have conducted a screening study where we have sequenced all human exons (>20 000 genes) using an Illumina HiSeq2000 on an individual basis in 32 patients selected from the 243 individuals. Half of these patients were selected based their blood status being “unaffected” by the treatment (grade 0 in myelosuppression – during the whole treatment) and the other half was selected based on extreme neutropenia (grade 4) and thrombocytopenia (grade 4). For each patient we have identified 5000 to 6800 non-synonymous SNPs and around 100 insertions and deletions affecting the amino-acid sequence. We are now comparing the genotype between these groups to identify potential genetic variants associated with carboplatin and gemcitabine induced myelosuppression.

The comparison of genetic differences is performed using four bioinformatic strategies:

1) We have performed a meta-analysis of gene expression data. This involved downloading all relevant high quality public domain gene expression data (from more than 300 microarrays) concerning platinum analogous and/or gemcitabine and generating a list of ranked genes which are induced or decreased by the drugs and also expressed in tissues important for the toxicity (i.e. the organ where the toxicity occur, bone marrow, and tissues responsible for the elimination of the drugs, kidney). We have compared the distribution of the wild type and variant alleles in the two toxicity groups of all SNPs in the top 100 genes generated by the meta-analysis. One of our top hits is in the HPS4 gene, Hermansky-Pudlak syndrome 4, which has been associated with platelet defects.

2) In addition we have identified 60 candidate genes, i.e. resistance mechanisms, metabolism and transporters, target genes, repair mechanisms and genes involved in specific toxicities. Comparing distribution of the genetic variants in the toxicity groups, the ATP7A gene on chromosome X, an indicated carboplatin transporter, showed the largest difference between the two sets with a p-value of 0.0006.

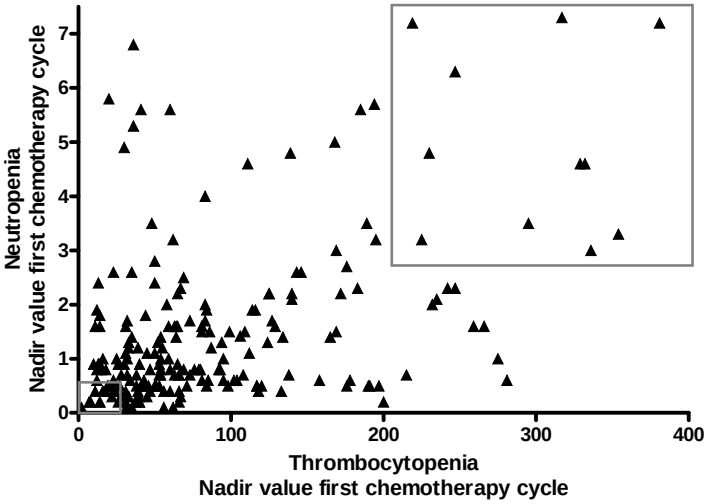

Figure 3. *The distribution of nadir values for neutrophiles and thrombocytes during the first chemotherapy cycle in 234 lung cancer patients that received gemcitabine and carboplatin. Upper Right Square represents patients unaffected by chemotherapy (please note that some will be excluded due to toxicity in later cycles). Lower Left Square represents patients with grade 4 neutropenia and thrombocytopenia*

3) We also do a whole genome comparison based on the number of non-synonymous genetic variants per gene/transcript as well as per position. Locking at SNP with the largest difference among the groups 173 SNPs show a significant difference and a genetic variant in PI3 (elastase specific inhibitor in neutrophils) is our top hit (p=0.0005). We use the fact that these patients are extremes in the sense that they have extremely low or high toxicity. If there is a common genetic variant correlating to the toxicity, and we do have a gene-dose-dependent impact, the heterozygous patients will be missing from the material. We therefore search for genetic variants out of Hardy-Weinberg equilibrium and with a genetic difference between the groups. Using this approach the PRSSL1 gene shows the highest difference between the groups with a p-value of 0.0001, also in this analysis the carboplatin transporter ATP7A comes out in the top ten genes.

4) We also look at pathways with the largest genetic difference between these two groups using three different soft wares: Ingenuity, FunCoup and Nextbio. This will generate a number of pathways with enriched or depleted genetic variants in either group of patient with low or high toxicity.

We have compiled the results and generated a list of 66 potential genetic variants associated with gemcitabine and carboplatin induced toxicity. To generate this list we took the top 20 hits from each bioinformatics strategy and made the union of these genetic variants.

These 66 genetic variants are currently analysed in 318 lung cancer patients treated with gemicitabine and carboplatin by genotyping at an individual level. The individual patients' genotype will then be correlated to the acquired bone-marrow toxicity registered in the clinical data (blood-status collected at days 0, 8, 15 or 21 (day 0 in next cycle). The validation for this set of genetic variants for gemcitabine/carboplatin induced myelosuppression will be completed in 2012.

In summary, this workpackage has generated and validated genetic variants associated with both gemicitabin/carboplatin and paclitaxel/carboplatin induced toxicity using state of the art technologies such as advanced bioinformatic tools, genome wide association studies and exome sequencing. Once these variants have been independently validated they can be introduced into the clinic to identify patients with high risk for adverse drug reactions, i.e.

probably in need of other regimes, premedication or reduced dose, and patients with low risk of toxicity who might benefit from a higher dose and thereby a better response.

Deviations from the project workprogramme:

Less biopsies than expected was performed in the EORTC 18032 trial, because of a large proportion of patients with visceral metastases and a small proportion of patients with biopsiable soft tissue metastases. Although this caused a delay, the additional collection of material from alternative sources within the consortium still allowed for the planned discovery and validation of melanoma chemoresponse candidate biomarkers.

Early in the project it became obvious that revisions of sample sizes for the prospective lung cancer collections were needed, due to challenges of obtaining serial biopsies, and that mainly retrospective samples would be used for discovery. However, during the course of the project, alternative use of the prospective CHEMORES collections has generated significant knowledge on lung cancer chemotherapy biology, thanks to the inclusion of groundbreaking circulating tumour cell analysis and oncogenic driver mutation analysis and correlation with outcome.

Some studies in WP4 were performed on different sample sets than planned in the beginning of the project. This was due to the challenges in acquiring sufficient numbers of paired sequential tumour biopsies pre- and post-treatment that passed histological controls, and where RNA of sufficient quality could be obtained. The major goals of the WP were still met, in particular for melanoma where several drug response candidates were identified and validated in clinical materials. For lung cancer several highly clinically relevant markers for diagnosis, prognosis and histological subtype were identified based on an extensive molecular profiling of 123 patients in the retrospective cohort. In addition, for lung cancer patients treated with cisplatin, expression profiles were identified that can potentially discriminate between ERCC1 negative tumours from patients with long survival versus ERCC1 positive tumours from patients with short survival.

WP5 has fulfilled its objectives with the exception that the planned melanoma plasma study was performed on tumour samples instead of plasma, due to more information rich proteomic read out on tumour tissue. This change allows also correlation of proteomics data with transcriptomics data set on same samples which were generated in WP4.

Some deviations occurred in the studies of mouse models: We found that the responses of the *Adeno-Cre, $Rb^{F/F}$, $p53^{F/F}$* mouse model for small cell lung cancer (SCLC) to the maximum tolerable dose of cisplatin differ from what is observed in the clinic. Instead focus was shifted to the K14cre, Brca1F/F, p53F/F and K14cre, Brca2F/F, p53F/F models to identify clinically relevant molecular mechanisms of chemotherapy resistance. In parallel, continuous efforts were made to further develop lung cancer models that are suitable for drug studies. In addition, we could not complete the melanoma study due to unforeseen problems with the mouse model. Early death of the animals prevented the long term follow up of treated-mice in order to monitor disease recurrence and potential drug resistance.

In the preclinical workpackages there were very few deviations from the programme, except the addition of studies of novel targeted therapies. This was encouraged by the external reviewer of the project.

Dissemination and use

Studies of clinical materials, animal models and *in vitro* models in CHEMORES have resulted in many interesting leads with regard to prediction of response to chemotherapy as well as

novel therapy options. However, additional clinical validation is required before any of these markers can be used in the clinical practice. The collection of the CHEMORES biobank is one of the most valuable outcomes of the project. In addition to the data generated during the course of the project, this unique material will continue to be used for studies on treatment response and other key issues in melanoma and lung cancer biology.

In CHEMORES we have also conducted the first clinical studies of circulating cancer cells in lung cancer. The results highlight the potential for blood borne cancer cells to be used to guide and select treatment decision making, and to determine the molecular mechanisms that result in the dissemination of cancer and resistance to treatment. Blood based tests will ultimately be more practical in the clinic for serial analysis of cancer markers and treatment selection.

The data generated in CHEMORES have resulted in several high impact publications and have been presented at scientific meetings and CHEMORES workshops, directed towards clinicians, researchers, students and patient organisations. Several of the participants in CHEMORES are part of international networks that influence the clinical management of these diseases, for example the European consensus-based interdisciplinary guideline for diagnosis and treatment of melanoma (Garbe et al. European Journal of Cancer (2012) 48, 2375– 2390). Thus, the knowledge generated in the project and follow-up studies will be incorporated in future clinical guidelines.

There have also been significant advances in the development of new methodology within the project, including RNA and DNA methylation analyses of formalin fixed paraffin embedded tumours, assays for DNA repair mechanisms, new tools for improved quantitative analysis of the proteome and novel bioinformatic approaches integrating multiple high-throughput molecular data. This is of great value for others performing similar studies, and all of the general algorithms for high-throughput data analyses developed and implemented throughout this project are made freely available for academic researchers (see: http://www.meb.ki.se/~yudpaw/). Unique data sets from CHEMORES are or will soon be publicly available (see: http://www.meb.ki.se/~yudpaw/misc/TumourNormal-comparisons.html and http://www.scilifelab.se).