



ANGIOPREDICT PROJECT FINAL REPORT CORE OF REPORT

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1. Final publishable summary report

1.1 *Executive summary*

Mechanistically, it has been hypothesized that resistance to angiogenesis inhibition may be Intrinsic or Adaptive in nature. ANGIOPREDICT (www.angiopredict.com) has specifically studied mechanisms of resistance to combination bevacizumab therapy in metastatic colorectal cancer (mCRC) patients building on an SME led modular platform for the integrated discovery and validation of predictive pharmacogenomics biomarkers for combination therapy. Uniquely, the consortium engaged an integrated state-of-the-art genomic discovery approach to identify and validate both germline and tumor-derived predictive genomic markers of combination [chemotherapy + bevacizumab] therapy in the first line setting. The goal to optimize in vitro diagnostic tests through exploitation of proven SME strategies ultimately ensured a cross-sectoral benefit to the cancer patient, prescribing clinician and more broadly across European and global public health networks.

The IRISH CLINICAL ONCOLOGY RESEARCH GROUP (ICORG) oversaw development of a tissue biorepository which provided both retrospective and prospective tissue for downstream omic analyses. ICORG further worked with clinical collaborators at the UNIVERSITY OF HEIDELBERG (UHEI) to complete recruitment on the AC-ANGIOPREDICT trial. A large tissue cohort from the consortium retrospective and prospective biorepositories was processed for DNA & RNA extraction by partners at UNIVERSITY COLLEGE DUBLIN (NUID UCD) and ONCOMARK LTD (ONCOMARK). Through collaboration of partners at VIB Leuven (VIB), NUID UCD and VREI UNIVERSITY MEDICAL CENTRE AMSTERDAM (VUMC) an exome-sequencing approach was implemented to generate pan'omic datasets and identify novel predictive biomarker signatures from combination bevacizumab treated patient samples. EPIGENOMICS (EPI) optimized DNA Methylation Companion IVD assays. Several marker candidates were deemed to have significant utility as valuable biomarkers for diagnostic and/or prognostic purposes in colon and other cancers. Thus, priority filing of a patent application (January 2016) was completed. The search for novel predictive signatures was performed in both whole tumor tissues (NUID UCD) and isolated tumor cell compartments from metastatic colorectal cancer (mCRC) samples (SOM & VUMC). The predictive potential of recently published multi-gene signatures (molecular subtypes) was also assessed, and novel response prediction molecular clusters described. Through previously described methods, pioneered by researchers at SOM, tumor compartments from CRC patients were isolated, including tumor endothelial cells (TEC), tumor associated macrophages (TAM) and isolated tumor cells (TC). Cells from different compartments were profiled for gene expression differences (VUMC). Candidate response prediction genes, at both tumor tissue and TEC-specific levels, were verified on retrospectively collected materials using in situ hybridization (ISH) (ONCO). Identified candidates were further evaluated using qRT-PCR assays by ONCO. The ROYAL COLLEGE OF SURGEONS IN IRELAND (RCSI) provided the consortium with a fully optimised relational MySQL database for both retrospective and prospective omics and clinical data sets. RCSI further employed statistical analysis and systems biology approaches to analyse and identify individual biomarkers and combinations of biomarkers that predict combination bevacizumab therapy success. An unbiased approach (network based stratification) defined a new method to stratify Angiopredict patients into two subtypes with significantly different survival outcomes (PFS). Moreover, application of an integrative statistical model over two data sets (CNA and somatic SNPs) also defined two patient clusters having a significantly better treatment outcome. A cellular automata multi-scale agent based model developed by RCSI predicted that chemotherapy given before Bev shows greater efficacy than Bev given before chemotherapy. These findings were validated using in vivo models and molecular imaging. This CA model further incorporated genomic signalling information to analyse effects of gene mutations on tumour behaviour and treatment response. Moreover, molecularly subtyped (expression) colorectal cancer cell line xenograft models were implemented to further demonstrate differential response to combination avastin therapy in vivo.

In conclusion, ANGIOPREDICT has united world-class molecular diagnostic SMEs [EPI, SOM, ONCO] with leading clinical [UHEI, VUMC, ICORG] and academic [VIB, UCD, RCSI] partners to identify a new generation of individualized methods for stratification of mCRC patients receiving combination bevacizumab therapy.

1.2 A summary description of project context and objectives

Mechanistically, it has been hypothesized that resistance to angiogenesis inhibition may be Intrinsic or Adaptive in nature with several hypothesized mechanisms having been investigated. ANGIOPREDICT (www.angiopredict.com) has specifically studied mechanisms of resistance to combination bevacizumab therapy in metastatic colorectal cancer (mCRC) patients building on an SME led modular platform for the integrated discovery and validation of predictive pharmacogenomics biomarkers for combination therapy. Uniquely, the consortium engaged an integrated state-of-the-art genomic discovery approach to identify and validate both germline and tumor-derived predictive genomic markers of combination [chemotherapy + bevacizumab] therapy in the first line setting. The project interrogated the tumor as a discrete tissue, but also through isolation of individual Tumor Cell (TC) tumor endothelial cell (TEC) and Tumor associated Macrophage (TAM) compartments. Computational modelling approaches facilitated data integration across 'omic datasets. The goal to optimize in vitro diagnostic tests through exploitation of proven SME strategies ultimately ensured a cross-sectoral benefit to the cancer patient, prescribing clinician and more broadly across European and global public health networks. ANGIOPREDICT united world-class molecular diagnostic SMEs [EPI, SOM, ONCO] with leading clinical [UHEI, VUMC, ICORG] and academic [VIB, UCD, RCSI] entities to identify a new generation of individualized methods for stratification of mCRC patients who currently receive combination bevacizumab therapy.

Main Objectives:

1. Provision of ANGIOPREDICT Retrospective Sample Biorepository: Access was provided to retrospectively collected primary tumor tissue samples [Fresh Frozen (FF) and Formalin Fixed Paraffin Embedded (FFPE)] and associated clinical follow-up data from patients treated with combination bevacizumab therapy, or chemotherapy alone.
2. Analysis of retrospective germline DNA or tumor-derived DNA/RNA linked with clinical follow up response data, from patients treated with combination bvz therapy to identify putative predictive/prognostic genomic DNA and expression biomarkers of response.
3. Prospectively collected samples from the prospective AC-ANGIOPREDICT (NCT01822444) and CAIRO 2 clinical trials used for subsequent target validation.
4. To exploit a multi-variant 'omics platform towards the identification & validation of DNA associated pharmacogenomic biomarker signatures. Approaches to include exome sequencing of both normal and tumor tissue & genome wide DNA methylation profiling /target validation.
5. To employ a whole tumor and isolated tumor cell (TC), tumor endothelial cell (TEC) and tumor associated macrophage (TAM) screening strategy to identify pharmacogenomic expression biomarker gene signatures in responders vs. non-responders. Predictive gene signatures to be validated in whole tissues.
6. To create a fully integrated molecular analysis bioinformatics database portal. To implement this portal to exchange, analyse and integrate data sets emerging from pharmacogenomic DNA and expression biomarker discovery platforms. To complement conventional statistical analysis approaches with state of the art computational modelling strategies.
7. To optimization robust, reliable and standardized tissue-based DNA methylation and Expression Signature assays for translation into validated companion in vitro diagnostic (IVD) tests, leveraging proven SME-driven strategies.
8. To implement an SME led commercial development strategy to allow timely protection of emerging IPR of primary relevance to participating companies.

1.3 A description of the main S&T results/foregrounds

1.3.1 Introduction

In ANGIOPREDICT, academic cancer biologists and industry-based biotechnology researchers have worked together with clinicians to explore biomarkers and their potential for use to predict whether individual metastatic colorectal cancer patients respond positively to Avastin® combination therapies. Our ultimate goal has been to help meet the challenges of (i) clinical decision-making in mCRC, so that the right treatment is delivered to the right patient at the right time and (ii) delivering more focused and cost-effective care.

In the project ANGIOPREDICT partners have used a number of different technologies to analyse tissue samples from patients receiving Avastin® combination therapy or chemotherapy, to explore potential predictive biomarkers. The results of this exploratory work was then validated using other tissue samples (e.g. from the AC-ANGIOPREDICT clinical trial), where patients received Avastin® combination therapy.

The main research undertaken and results achieved are described below under the following headings:

- WP2 Biorepository
- WP3 Genomic Biomarker focused Phase 2 Clinical Study: AC-ANGIOPREDICT Trial
- WP4 Germline SNP profiling & target validation
- WP5 Somatic Mutation Analysis & Validation
- WP6 DNA Methylation based Predictive Methods
- WP7 Predictive Pharmacogenomic Expression methods
- WP8 Bioinformatics Database & Exchange Portal
- WP9 Integrated analysis of data using statistical inference and computational systems approaches.

1.3.2 WP2 Biorepository

1.3.2.1 Introduction

The ANGIOPREDICT consortium identified the discovery and validation of predictive pharmacogenomic methods for combination therapy [FOLFOX/FOLFIRI+bvz] in first line treatment of metastatic colorectal cancer (mCRC) patients as a primary goal. In the discovery phase of the project, a retrospective sample collection of fresh frozen (FF) and formalin fixed paraffin embedded (FFPE) tissues from patients with CRC was established. These patients underwent combination bvz therapy or chemotherapy alone. In the validation phase of the project prospectively collected tumour tissue, plus serially collected peripheral blood samples were provided from the prospective clinical trial (detailed in WP3) to the ANGIOPREDICT central biorepository, housed at UHEI. Tissue samples at the ANGIOPREDICT central biorepository were assessed by consortium pathologist Prof Timo Gaiser (or by partner pathologist Prof Elaine Kay locally at RCSI and Dr. Nicole van Grieken, VUMC) where cancer diagnosis and tumour content was confirmed and normal tissue delineated. Samples were then distributed to NUID UCD for DNA/RNA extraction and downstream “-omic” analyses as described in WP 4-7. Coding of samples and collation of clinical data was overseen by ICORG for integrative analyses as described in WP8/9.

1.3.2.2 Work Undertaken and Results Achieved

Task 1 Selection of tumour samples for molecular analyses [UHEI]

A protocol for the molecular analysis of both a retrospective and prospective sample collection was established.

For the **retrospective** biorepository a total of 335 samples were provided from the tissue bio-banks of RCSI Beaumont Hospital (32), UHEI (107), VUMC (88) and the Dutch Colorectal Cancer Group (DCCG) (108). The collection included FFPE and FF tissue from mCRC patients having received first line combination bvz therapy or chemotherapeutic alone.

For quality control a central pathology review of tissues was performed by consortium pathologist Prof Gaiser [UHEI] (or by partner pathologist Prof Elaine Kay locally at RCSI and Dr Nicole van Grieken, VUMC). In this review cancer diagnosis was confirmed and adjacent normal tissue was delineated. For the molecular analysis at least 30% tumour cell content, as judged by H&E staining, had to be confirmed. Such threshold for tumour cell content is commonly used e.g. for the FDA-approved MammaPrint molecular diagnostics assay. For analysis of gene mutations, as described in WP5, only tumour blocks with at least 50% tumour cells, as judged by H&E staining, can be used to allow multiplex sequencing runs. In addition, sample volume has been determined in order to allow successful isolation of DNA and RNA in sufficient quantities for the molecular analyses described in the other WPs. Reviewed slides were subsequently sent to NUID UCD for RNA and DNA extraction. In a subset of patients fresh frozen samples were available and these provided the basis for the direct comparison of DNA and RNA quality in fresh frozen and FFPE tissue. Standard operating procedures were established for RNA and DNA extraction.

Of the 107 patients from UHEI, all were FFPE tissue mostly originating from primary tumour, and a subset of 29 patients also had FF tissue. In this cohort 85 patients that received bvz in combination with chemotherapy in first line or in following lines and 22 patients with chemotherapeutic alone were identified.

All 32 patients from the RCSI, Beaumont Hospital cohort had received bvz either alone or in combination with chemotherapy in first line or in following lines and all samples consisted of FFPE blocks.

From the VUMC cohort 43 patients had received bvz in combination with chemotherapy in first line and 45 patients received chemotherapy alone. All samples consisted of FFPE blocks.

All 108 samples provided by DCCG were collected through the CAIRO-2 trial (patients received Xelox and avastin) and consisted of FFPE blocks. Retrospective clinical data collection started in February 2012. The observation period differed between the participating centres. UHEI data time frame span from July 2004 to December 2014; for RCSI data from August 2004 to June 2015, for VUMC data from September 2004 to July 2013 and for DCCG data from August 2005 to October 2010. The final data collection for retrospective samples was continued and completed by January 2015. **A publication with title “Combination Bevacizumab therapy in elderly vs. young patients with colorectal cancer: A pooled retrospective analysis of three European cohorts from the ANGIOPREDICT initiative” was submitted to Journal of Cancer Research and Clinical Oncology in March 2016.**

For the **prospective** biorepository tissue samples were collected through the AC-ANGIOREPDICT trial. From 76 patients enrolled in the trial 72 FFPE blocks (2 patients had withdrawn their consent, 2 blocks were not available) were collected and sent to the Angiopredict biorepository at UHEI. 66 blocks have undergone the quality control step performed by consortium pathologist Prof Timo Geiser: 57 blocks contained tumour material (27 biopsies and 22 resections, 8 not identified), 7 blocks did not contain enough tumour material, one block contained rectal poly tissue and one block contained bone metastasis (which cannot be cut). All blocks containing tumour material were cut and sections were sent to NUID UCD for RNA and DNA extraction. Serial peripheral **blood** samples were collected from each participating patient (where possible) at 5 different time points (before treatment, 6 weeks, 3, 6 and 12 months after start of treatment), processed to serum, plasma and cellular components and sent to consortium partner Prof Diether Lambrechts at VIB.

Task 2 Sample Coding & Clinical Data [ICORG]

Samples for the retrospective sample collection and patients enrolled in the prospective trial were registered with ICORG using a standardized Registration Form and were assigned a unique study number. The decoding key is kept at each hospital site. Clinical data for the retrospective samples were requested on the registration form, while clinical data of participating trial patients were collected on study-specific Case Report Forms, which needed to be filled in before the treatment phase, 6 weeks, 3, 6, 9, and 12 months after start of treatment and every 3 months in the 1 year follow up phase. The ANGIOPREDICT clinical database is held at ICORG.

Task 3 Distribution of samples [UHEI]

Tissue samples from the retrospective and prospective biorepository at UHEI were sent to NUID UCD for DNA/RNA extraction. Further distribution to relevant ANGIOREDICT partners for genomic analyses was monitored through a tissue tracking tool developed by NUID UCD.

Significant Results

This WP succeeded in providing a database of clinical data and a valuable retrospective and prospective biorepository containing FFPE and FF tissue, serum, plasma and cellular components, available for the omics studies described in WPs 4, 5, 6 and 7. Remaining material can be used for future studies after having received renewed ethics approval. In more detail the following activities have been performed:

- a. Collection of retrospective (tissue) and prospective (tissue and blood) samples from well characterized patients with metastatic colorectal cancer that underwent combination bvz therapy or chemotherapy alone.
- b. Coordinated retrieval, pathology review and distribution of retrospective and prospective tissue samples.
- c. Coordinated collection of blood samples from participating trial patients and transfer to consortium partner VIB.
- d. Collection of retrospective and prospective clinical data and storage in the ICORG database.
- e. Analysis of retrospective clinical data (RCSI, VUMC, UHEI) with respect to overall survival, progression-free survival as well as age-related differences in chemotherapy response. **A publication with title “Combination Bevacizumab therapy in elderly vs. young patients with colorectal cancer: A pooled retrospective analysis of three European cohorts from the ANGIOPREDICT initiative” was submitted to Journal of Cancer Research and Clinical Oncology in March 2016.**

Significant results of the analysis of the retrospective cohort

Patient characteristics

A total of 172 patients were included in this analysis. Median age at the start of treatment with bevacizumab was 65 years (mean 63.7, range 27-84). 80 patients (47%) were older than 65 years, 90 patients (53%) were 65 years or younger. Fifty-eight patients (34%) received an irinotecan doublet chemotherapy backbone, 83 (48%) an oxaliplatin doublet. Twenty-four patients (14%) received bevacizumab together with a fluoropyrimidine monotherapy, 7 patients (4%) were treated with bevacizumab and irinotecan or with bevacizumab as monotherapy. The three included cohorts differed significantly with respect to gender, age, T-classification, grading, location of the primary tumor, treatment line and chemotherapy backbone. Median follow-up time was 48.1 months (95% CI. 40.5 – 56.2). Median follow-up times of the UHEI, RCSI and VUMC cohorts were 30.9, 60.0 and 60.0 months, respectively. 217 patients (80%) experienced disease progression. At the end of follow-up, 104 (60%) deaths had occurred, while 68 patients (40%) were censored with respect to OS.

Survival analysis

Patients with CRC that underwent combination therapy with bevacizumab had a median PFS of 9.67 months (95% CI: 9.18-10.56) and a median OS of 27.4 months (95% KI: 22.9-32.7). Patients from UHEI, RCSI and VUMC cohorts had median PFS of 9.9, 9.2 and 9.7 months, respectively. OS of patients from UHEI, RCSI and VUMC were 34.0, 20.5 and 25.1 months, respectively. Patients that were older than 65 years at the start of treatment with bevacizumab had significantly shorter PFS (9.5 months vs. 9.8 months, $P=0.01$), but no significant difference in OS was noted (27.4 months vs. 27.5 months, $P=0.43$) under treatment with bevacizumab.

Conclusion

This work package was designed to establish a central biorepository for the retrospective and prospective sample collection for the ANGIOPREDICT consortium. Retrospective and prospective tissue samples were collected, diagnosis was reconfirmed, cancer containing areas were defined and the tissue samples were distributed for further molecular analysis. Serial blood samples were collected from each participating patient (where possible) and transferred to consortium partners for analysis. Clinical information for all samples registered in the biorepository was collected and a database established. Retrospective clinical data were analysed and results are summarized in a manuscript, which has been submitted for publication. Prospectively collected samples and available clinical data have been used for Mass Sequenom orthogonal validation studies as described in WP4 and WP5 periodic reports.

1.3.3 WP3 Genomic Biomarker focused Phase 2 Clinical Study: AC-ANGIOPREDICT Trial

1.3.3.1 Introduction

The objective of WP3 was to conduct a phase 2 clinical trial comprising biomarker analysis of oxaliplatin plus infusional fluorouracil/leucovorin (FOLFOX) in combination with bvz in first line treatment of mCRC expressing mutant k-ras [Principal Investigators: Prof. M. Ebert, UHEI and Prof. Seamus O'Reilly, Cork University Hospital]. This was a multi-centre translational trial co-ordinated by ICORG; clinical centres were in Germany, including UHEI, and across the island of Ireland. The primary objective of the trial was to identify and validate genomic markers for combination bvz therapy and assess their value as response predictors in K-ras mutant mCRC. Secondary objectives were to test the efficacy of bvz in combination with FOLFOX in patients with newly diagnosed mCRC K-ras mutant colorectal cancer and to determine the progression free and overall survival of patients under first line FOLFOX+/- bvz in mCRC. Tissue and blood samples were obtained prior to therapy and treatment response assessed by standard radiologic imaging (CT).

1.3.3.2 Work Undertaken and Results Achieved

Task 1: Development of the clinical Protocol for AC-ANGIOPREDICT Phase 2 Trial [UHEI, ICORG]

The AC-ANGIOPREDICT protocol was collaboratively developed by a team from UHEI and ICORG and version 1 was finalised and signed off on 20th July 2012 by ICORG according to its protocol development and quality control (QC) procedures. The protocol was assigned the ICORG protocol number 12-16.

The protocol was submitted to each local/regional ethics committee and subsequently initiated over a period of 5 months with the activation of the first participating hospital in November 2012.

During the initiation process and the initial recruitment phase a number of critical issues arose, which made two amendments of the study protocol necessary. The inclusion and exclusion criteria were changed to be less restrictive (advanced and metastatic patients as well as disease progression were now allowed in the study, Kras mutation was removed from the requirements, bvz combination treatment with leucovorin, fluorouracil and oxaliplatin (FOLFOX) or capecitabine and oxaliplatin (XELOX) or leucovorin, fluorouracil and irinotecan (FOLFIRI) or capecitabine and irinotecan (XELIRI) were included, fresh frozen tissue was not mandatory anymore and the screening/registration phase was extended to 6 weeks). The amended protocol version 2 was finalised and signed off on 10th December 2012. Approval from all ethics committees was received by July 2013 and version 2 was implemented at all participating hospitals. Version 3 was finalised and signed off on 23 September 2013 and ethics approval for all sites was in place by 1st April 2014.

Task 2: Standardized SOPs for Tissue Collection & Processing of [ICORG]:

ICORG developed 5 Angiopredict-specific SOPs:

- SOP1 Tissue Processing – Fresh Frozen.
- SOP2 Blood Sampling – Serum.
- SOP3 Blood Sampling – Plasma.
- SOP4 DNA Isolation from FFPE and Frozen Tissue.
- SOP5 RNA Isolation from FFPE and Frozen Tissue.

The objectives of the SOPs were to standardize the sample processing and ensure the highest possible quality. SOPs 1-3 are part of the clinical trials protocol and were followed by clinical staff at the various hospitals, where the trial was open. SOPs 4 and 5 were followed by the relevant partners that are processing retrospective and prospective material.

Task 3: Initiation of AC-ANGIOPREDICT trial [UHEI, ICORG]

There were 10 participating centres in Ireland (Figure 1): Cork University Hospital (CUH); Bon Secours Hospital Cork (BonS Cork); University Hospital Galway (UHG); University Hospital Waterford (UHW); St Vincent's University Hospital public and private (SVUH and SVPH); St James's Hospital (SJH); Mater Misericordiae University Hospital public and private (MMUH and MPH); Beaumont Hospital (BH), Sligo General Hospital (SGH), and Adelaide & Meath Hospital incorporating the National Children's Hospital (AMNCH); and 8 participating centres in Germany (Figure 2): University Hospital Mannheim; Medizinische Klinik and Poliklinik, Mainz; Klinikum Ludwigsburg; Private Practice Oncology, Speyer; Klinikum Deggendorf, Deggendorf; University Hospital Aachen; University Hospital Saarland, Homburg; and Oncology Centre, Lebach.

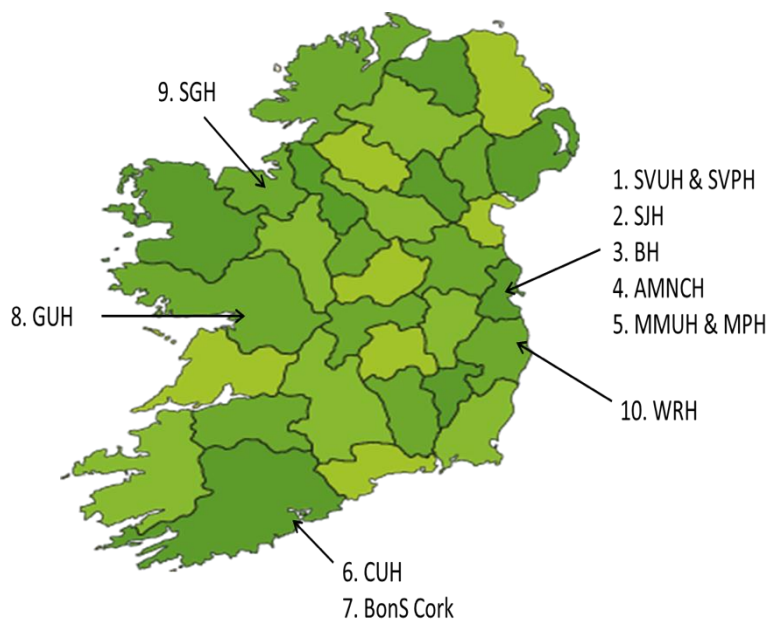


Figure 1 Irish Hospitals

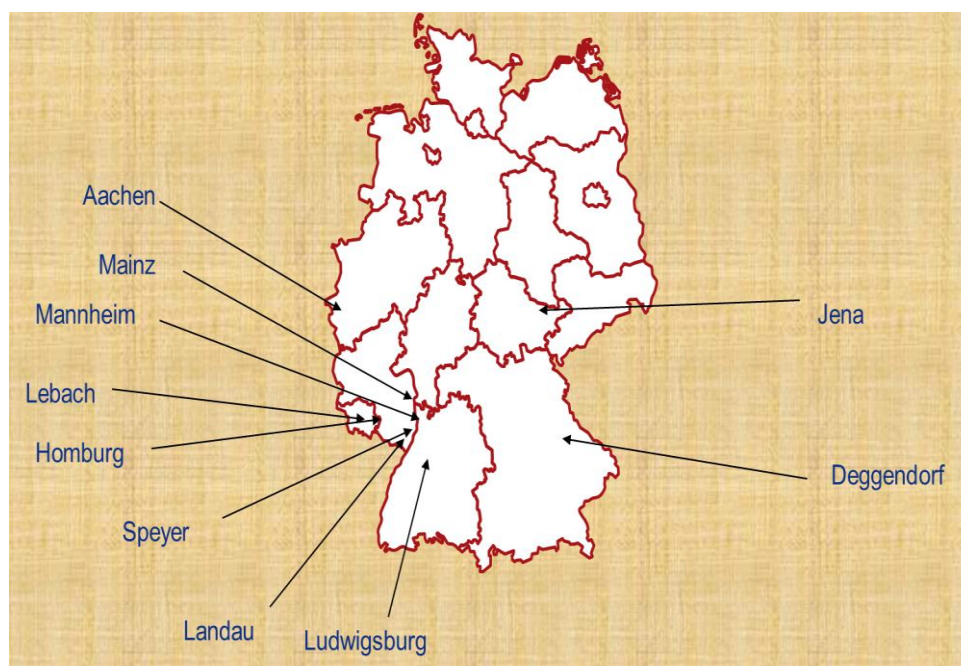


Figure 2 German Hospitals

The following list details the initiation in the 18 participating hospital sites:

1. UHEI 23rd October 2012
2. SGH 06th November 2012
3. SVUH and SVPH 27th November 2012
4. AMNCH 27th November 2012
5. SJH 28th November 2012
6. University Hospital Saarland, Homburg 14th March 2013
7. Klinikum Deggendorf, Deggendorf 15th March 2013
8. BonS Cork 20th March 2013
9. CUH 21st March 2013
10. GUH 9th May 2013
11. Klinikum Ludwigsburg 12th June 2013
12. Oncology Centre, Lebach 13th June 2013
13. University Hospital Aachen 14th June 2013
14. BH 26th June 2013
15. Private Practice Oncology Speyer, 11th September 2013
16. Medizinische Klinik and Poliklinik Mainz 12th September 2013.
17. WRH 21st October 2013
18. MMUH/MPH 24th July 2014

At initiation, study training was given for the protocol procedures, SOP for sample processing and any other study materials like the clinical datasheets. All study materials were provided in an investigator site file.

Closure of Accrual Period and Study Timeline: The accrual to the AC-ANGIOPREDICT trial was closed by EoB on 28th February 2015. The total number of patients accrued to the trial was 76, 48 patients at Irish hospitals and 28 at German Hospitals (Figure 3)

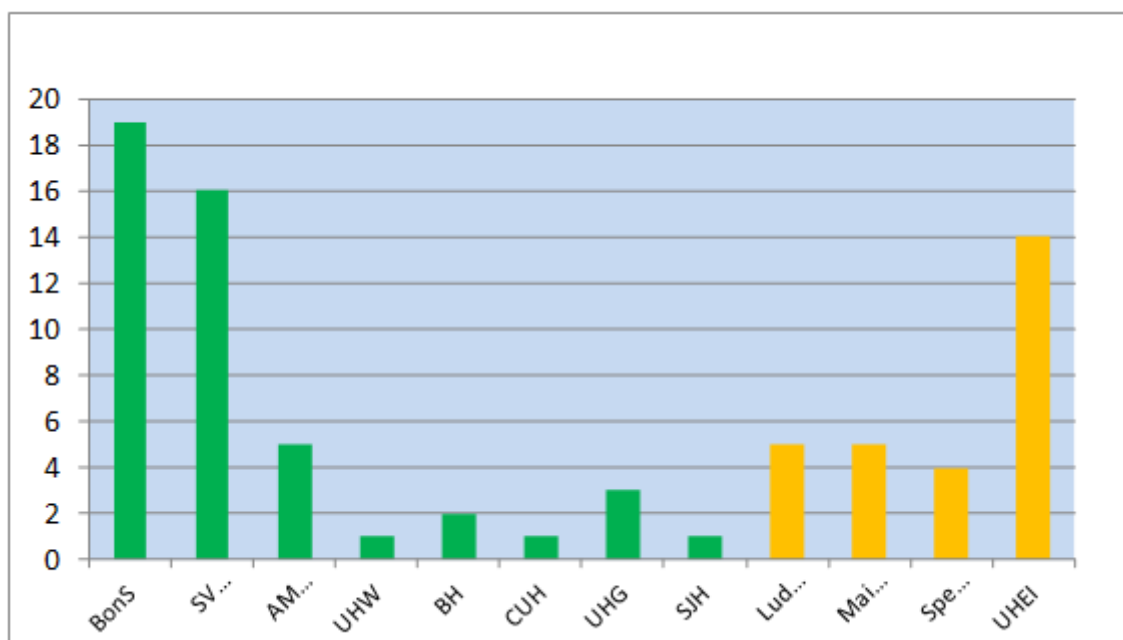


Figure 3 Accrual at each participating hospital: Green represents Irish hospitals, yellow are German Hospitals. Hospital sites not mentioned in the graph have not enrolled any patient.

The study time line of the translational trial (Figure 4) spans from Nov 2012 (activation of the first site) until 30th April 2017 when all clinical data collected through Case report Forms (CRFs) will have been analysed and the final study report will be available for publication. Nevertheless, and as mentioned above, all prospective clinical samples have to date (March 2016) been provided to ANGIOPREDICT partners along with clinical follow-up data available as of March 2016.

These prospective samples and available clinical data have been used for Mass Sequenom orthogonal validation studies as described in WP4 and WP5 reports.

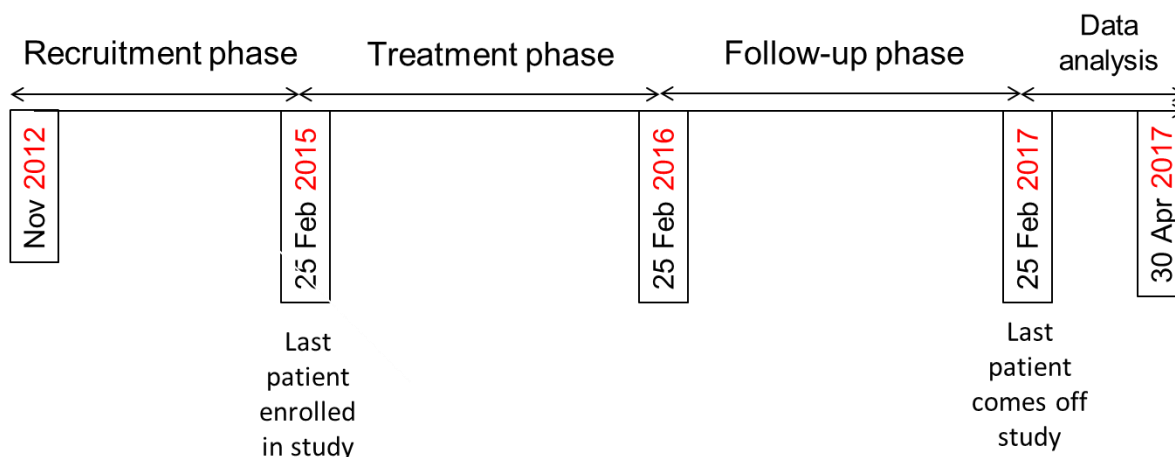


Figure 4 Study Timeline

During the trial a number of patients came off study (death, withdrawal, treating doctor’s decision, lost to follow-up), which is detailed below and summarized in Table 1.

Out of 76 patients

- 51 patients reached the 10 month time point of the treatment phase (time frame for response); 25 patients had come off study due to death (15), withdrawal of consent (5), lost to follow up (2) and treating doctor’s decision (3).
- By Jan 2016, 26 patients had come off study due to death (16), withdrawal of consent (5), lost to follow up (2) and treating doctor’s decision (3).
- In Jan 2016, 11 patients had completed or were completing the trial.

Table 1 Status and Estimation of Patient Status

	Close of Enrolment	10M response time point			
	Feb 2015	Oct 2015	Jan 2016	Aug 2016	Feb 2017
Number of patients still enrolled in the study	63	51	39		
Number of patients off-study (prematurely)	16	25	26		
Number of patients completed/completing the trial			11	33*	50*

**estimates (no further off-study events added).*

Collection of Clinical Data

The collection of clinical data was performed through study specific Case Report Forms (CRFs), which were filled in at set time points:

- Baseline (before treatment).
- On Treatment Assessments: week 6, month 3, month 6, month 12 (after starting treatment).
- Follow Up Assessments: month 15, 18, 21 and 24 (after starting treatment).
- Off Study Form at the end of trial participation (either at completion or when coming off the trial because of withdrawal of consent, death, lost to follow up or treating doctors decision).

CRFs were requested from hospital sites as soon as they become available and data checked for correctness and completeness by ICORG data management and subsequently inputted in the prospective ANGIOPREDICT database held in ICORG.

Collection of biological samples

FFPE tissue (from diagnostic biopsy or resection) was collected from 72 patients (two patients withdrew consent, 2 blocks were not available). 66 blocks were sent to UHEI and reviewed by consortium pathologist Prof Timo Geiser. The remaining 6 blocks were sent in March 2016. The result of the pathological assessment of the first 66 blocks was detailed in Deliverable 3.2.

In summary: From a total of 66 blocks

- 57 blocks contained tumour material (27 biopsies and 22 resections, 8 not identified)
- 7 blocks did not contain enough tumour material
- 1 block contained rectal polyp tissue
- 1 block contained bone metastasis (which cannot be cut)

All blocks containing tumour material were cut into 5micron sections and sent to NUID UCD, Dublin, for further extraction and analysis.

Blood samples: All patients reached the end of the treatment phase in February 2016. From each participating patient (where possible) 2 blood samples were collected at 5 different time points: one non-EDTA blood sample, which was processed to serum and one EDTA blood sample, which was processed to plasma and cellular components. The 5 time points are before start of treatment, 6 weeks, 3, 6 and 12 month after start of treatment. These samples were temporarily stored in -80°C freezers at the hospital sites until batch transport to the national collection location (UHEI for German samples and BH, Dublin, for Irish samples).

A first batch transport of processed samples was sent in Q3 2015 from UHEI and from BH to VIB for further extraction and analysis.

The remaining blood samples were collected from the hospital sites in March 2016 and sent to VIB.

1.3.3.3 Conclusion

This work package was designed to develop a translational trial, to collect biological samples and clinical information from patients diagnosed with colorectal cancer and who were foreseen to receive bevacizumab combination therapy with either FOLFOX, XELOX, FOLFIRI or XELIRI. There were 76 patients enrolled in the study. With the biological samples (tissue, serum, plasma and cellular components) a prospective biorepository was established and samples were made available to the various work packages of the ANGIOPREDICT consortium for omic analyses.

1.3.4 WP4 Germline SNP profiling & target validation

1.3.4.1 Introduction

Single nucleotide polymorphisms (SNPs) in germ-line DNA can have an effect on a patients' outcome following chemotherapy. Within WP4 of ANGIOPREDICT, we sought to identify SNPs that are related to outcome, following combination bvz therapy. Germ-line SNPs were determined using a customized whole-exome sequencing approach and each of these SNPs was subsequently correlated individually with progression free survival. The top SNPs correlating with progression free survival were thereafter orthogonally validated using prospectively collected patients of the AngioPredict AC cohort and an independent technology (i.e. Sequenom MassARRAY).

1.3.4.2 Work Undertaken and Results Achieved

We decided to use massive-parallel sequencing, rather than SNP arrays or targeted re-sequencing, to generate all data required for WP4 and WP5 (partners VIB, UCD, and VUMC). Briefly, we designed a custom exome capture kit, which in addition to the regular exome also captures promoter regions and 5' or 3'UTRs from a number of key angiogenic factors. This kit was used to capture both tumor and matched germ-line DNA of patients from the retrospective AngioPredict discovery cohort. Captured DNA was subsequently subjected to massive parallel sequencing on an Illumina HiSeq2000 to detect single nucleotide variants in the germ-line samples (WP4) and somatic variants in the tumor samples (WP5). The original DNA libraries were furthermore used to perform low copy-number sequencing, allowing the unbiased identification of somatic copy number alterations (WP5). The entire sample workflow is shown in Figure 5. *This change in methodology, which is an important and valuable evolution of our original plan, was previously agreed with the ANGIOPREDICT project officer.*

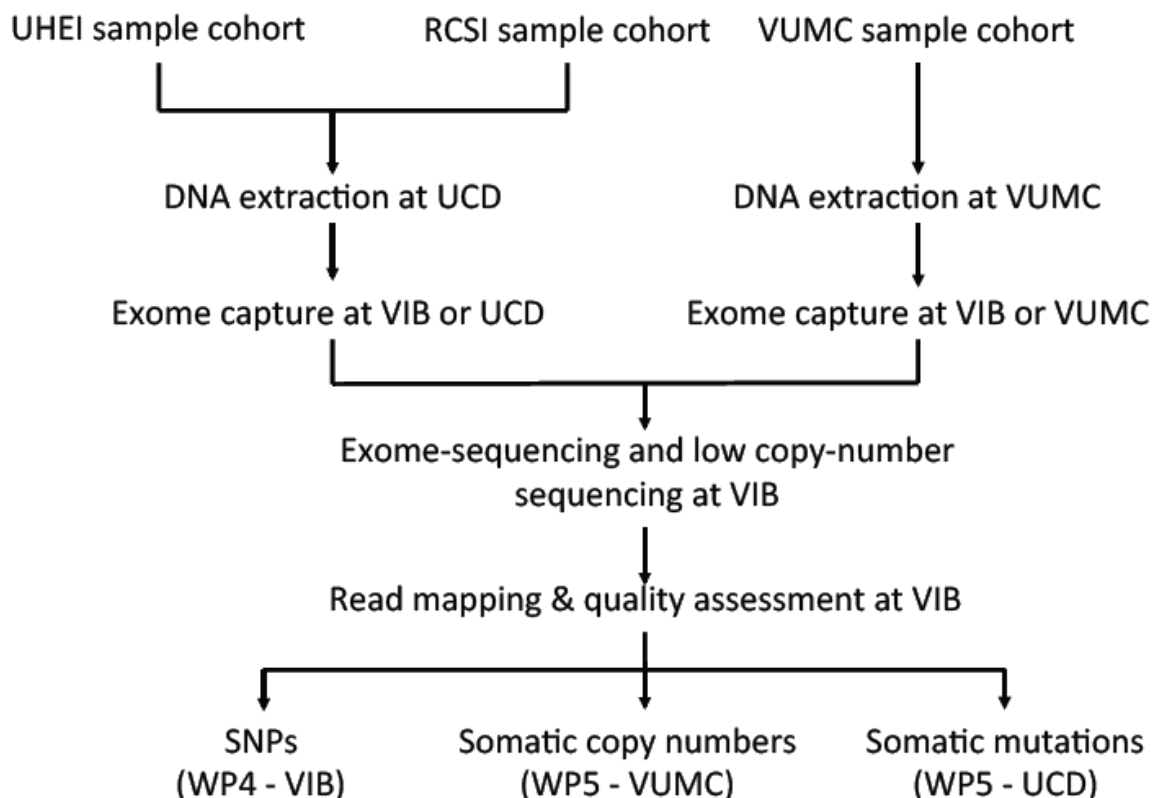


Figure 5 Workflow of the samples. Samples were collected by UHEI, RCSI and VUMC and processed for whole-exome and low-coverage whole-genome sequencing at VIB. After quality assessment at VIB the data was distributed among the relevant partners.

Design of a specific exome capture kit to cover angiogenic effector genes

To make sure that the most relevant germ-line SNPs in angiogenic pathway effectors were indeed assessed, we decided to perform whole-exome sequencing using the Nimblegen SeqCapV3 exome capture kits in combination with the HiSeq2000 sequencing technology (VIB). One of the reasons for choosing this technology, is that the standard Nimblegen SeqCapV3 kit can be expanded with customized probes. Therefore, in an effort not only to detect variants located in the exonic sequences of angiogenic effector genes (Table 2), we expanded the capture of the standard Nimblegen SeqCapV3 kit by also including the 5' and 3' untranslated and promoter regions of these genes. Overall, this approach will not only allow us to detect common variants ($f > 0.05$) in angiogenic effector genes, as proposed in the initial project proposal, but additionally, it will allow us to assess the impact of a number of rare exonic variants (i.e., out-of-frame insertions or deletions in angiogenic effector genes).

Table 2 List of genes for which 5' and 3' UTR and promoter regions have been added to the standard Nimblegen SeqCapV3 exome capture design kit.

ANG	EGLN2	HGF	IL8	PPP2R2A	TIMP2
ANGPT1	EGLN3	HIF1A	KDR	PRL	TL1
ANGPT2	EPAS1	HIF1AN	LTA	PTGS2	TNF
ANGPT4	EPO	HIF3A	NOS3	PTN	TNNI1
COL18A1	F5	IFNA1	NOTCH1	SELE	TNNI2
CSF2	FAM105B	IFNG	NOTCH2	SERPINC1	TNNI3
CSF3	FCGR2A	IGF1	NOTCH4	SERPINE1	TP53
CXCL10	FGF1	IL10	NRP1	SERPINF1	TYMP
CXCL12	FGF2	IL10RA	NRP2	TEK	TYMS
CXCR1	FGFR1	IL12A	PDGFA	TGFA	UGT1A1
CXCR2	FGFR2	IL12B	PDGFB	TGFB1	UGT1A10
CXCR4	FIGF	IL1B	PDGFC	TGFB2	VCAM1
DLL4	FLT1	IL2	PDGFD	TGFB3	VEGFA
DVL2	FLT4	IL3	PF4	THBS1	VEGFB
EGF	GSTM1	IL4	PFKFB3	THBS2	VEGFC
EGFR	GSTP1	IL5	PGF	TIE1	VHL
EGLN1	GSTT1	IL6	PLG	TIMP1	

Overall, 270.000 exonic common SNPs can be derived from standard exome-sequencing experiments. By expanding the captured regions with 5' and 3'UTR and promoter regions in the selected genes, an additional 14.000 SNPs could be genotyped.

Optimization of exome-sequencing for FFPE-extracted DNA

Our retrospective collection of tumor and normal DNA samples were mainly derived from samples stored in FFPE and since exome-sequencing on FFPE samples was not yet fully standardized or commercially available, optimization of the exome-sequencing protocol was required. First of all, we developed a macrodissection protocol whereby both tumor and matched normal DNA were extracted from a number of consecutive glass slides. Importantly, we then determined the quantity and quality of the extracted DNA from FFPE slides using different methods, such as Nanodrop and Picogreen, qPCR and standard PCR. These analyses revealed a large variation in quality between the samples. Overall, we established that the amount of double-stranded DNA present in the DNA samples had to be taken as the reference amount of input DNA (optimally 1000 ng and preferably more) to ensure that a gDNA library of sufficient quality was constructed. Furthermore we optimized the protocols by including several steps to ensure sufficient library quantity and quality prior to whole-exome sequencing.

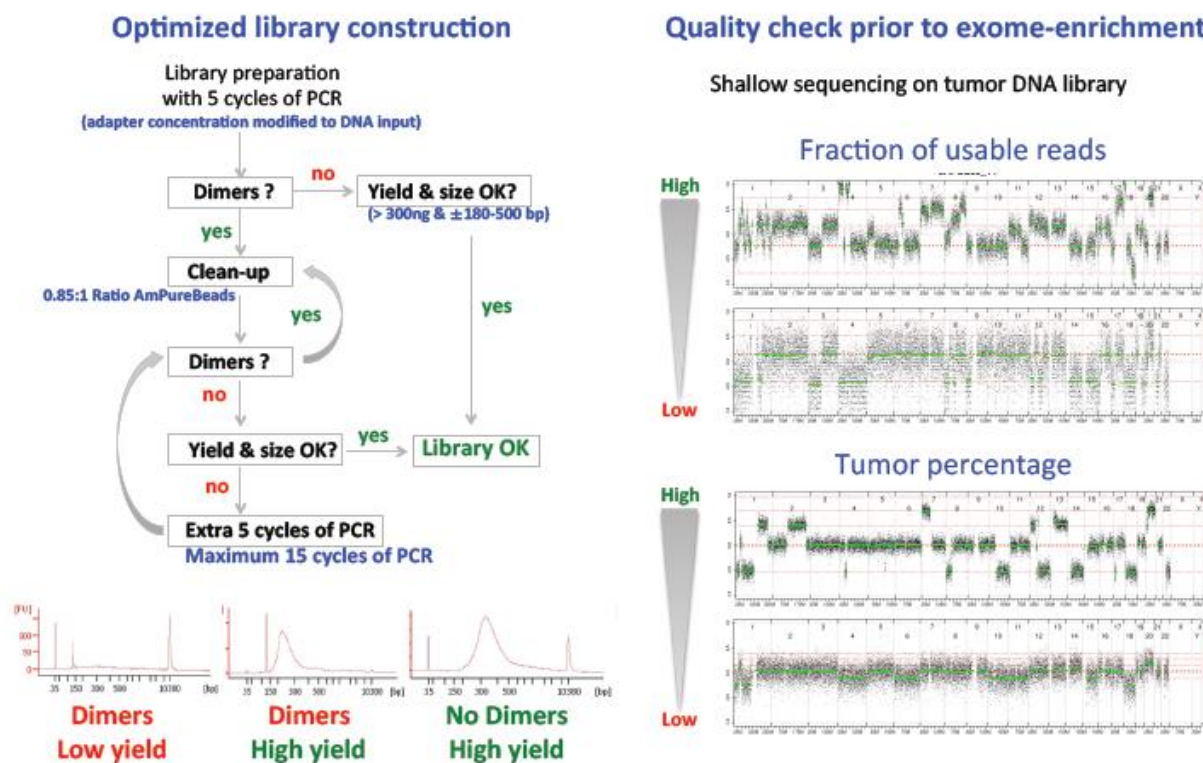


Figure 6 Optimization of library preparation protocols for sequencing on FFPE samples and quality assessment of tumor samples using low-coverage whole-genome sequencing on tumor samples prior to whole-exome enrichment and sequencing.

Library preparations for and sequencing data quality assessment for whole-exome and low-coverage whole-genome sequencing

Shot-gun whole genome libraries were prepared using KAPA library preparation kit (KAPA Biosystems). Since the DNA was extracted from FFPE tissue blocks, whole genome DNA libraries from matched normal and tumor tissue samples were created according to the manufacturer's instructions with some modifications to the protocol as described in the previous section. After confirmation of successful library construction, whole exome enrichment was performed using the customized SeqCapV3 exome enrichment kit (Roche) following the manufacturer's instructions. The resulting whole-exome libraries were then sequenced on a HiSeq2000 using a V3 flowcell generating 2 x 100 bp paired end reads.

To assess data quality, raw sequencing reads were mapped to the reference genome using the Burrow-Wheeler Aligner algorithm (BWA) and after removing duplicate sequencing reads using the Picard packages the resulting coverages and duplicate rates were assessed for each of the samples (Figure 7).

Overall, after patient selection and after performing extensive quality assessment of the extracted DNA, 556 samples were selected for library preparation. For each of these 556 samples, a library construction was attempted, but since this failed for some DNA samples, additional constructions were performed for these samples. In total, 694 library constructions were attempted resulting in 448 libraries that were eventually of sufficient quality to undergo exome-sequencing. All these samples were processed for sequencing and after data processing and quality assessment, data was available for 388 paired tumor-normal samples (194 patients), 11 tumor samples and 11 normal samples. Additionally, we performed low-coverage whole-genome sequencing using the original DNA libraries on a total of 257 tumor samples.

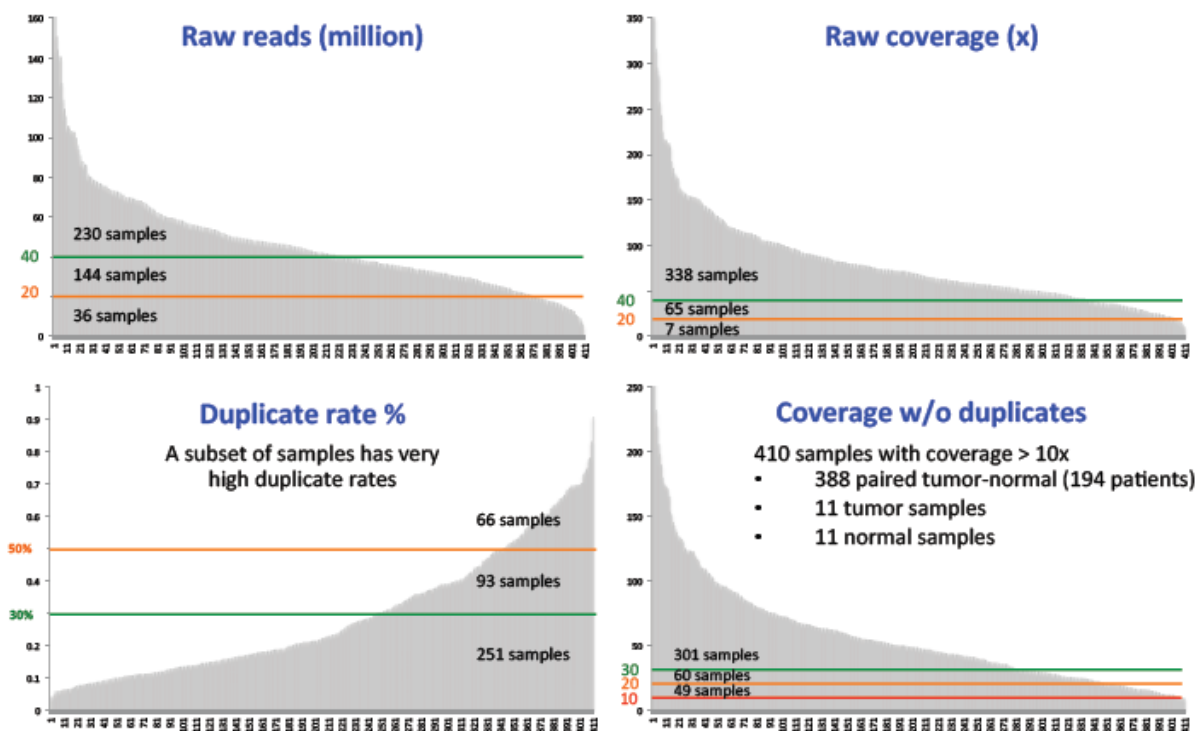


Figure 7 Sequencing metrics of the whole-exome sequencing experiments. The final cohort with whole-exome data available consists of 388 paired tumor-normal samples (194 patients), 11 tumor samples and 11 normal samples.

Germ-line SNP profiling and target identification

In a next step, we identified all variants and indels that differed relative to the hg19 reference genome (Figure 7). Raw sequencing reads were mapped to the reference genome using the Burrow-Wheeler Aligner algorithm (BWA). Data quality was then improved by removing duplicate sequencing reads, recalibrating the base quality scores and realigning the reads around indels using Genome Analysis Toolkit (GATK) and Picard packages. Variant and indel calling on high quality bases is then performed using Bed Tools, GATK and Dindel, the detected variants and indels are then annotated using Annovar. To obtain individual genotypes of the 300.000 selected SNPs, we also generated a new script that automatically extracts genotypes from vcf-files, which contain all bases called by The Genome Analysis Toolkit (GATK), the observed allelic frequencies, quality score and zygosity. Based on these characteristics, our script can very easily and accurately call the individual genotype for each of these selected SNPs. In case sequencing data are of poor quality in a region that contains a given SNP of interest, the algorithm will indicate this and will not identify a genotype call.

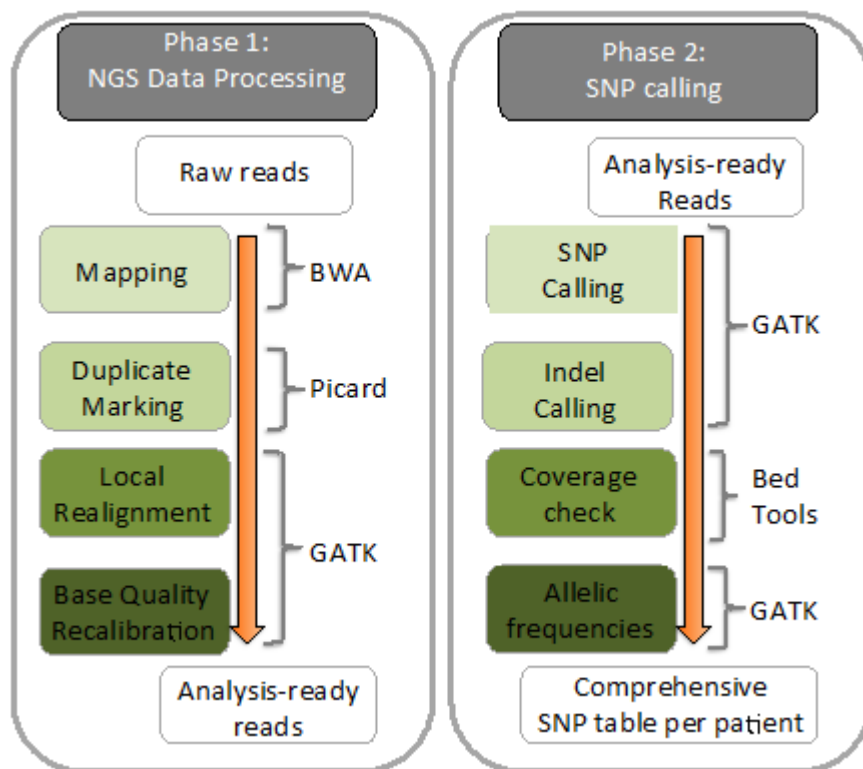


Figure 8 Sequencing data assembly and germ-line variant calling

Using the 205 germ-line DNA exomes and script described above we converted sequencing data into SNP genotypes. We identified a total of 441,440 SNPs. Subsequently we selected for common SNPs only using the following criteria: 1) The SNP has to be present in at least 10% and less than 100% of all the patients ($n = 97,105$ SNPs). 2) The SNP has to be present in at least 5% of the European individuals of the 1000 genomes project ($n = 83,080$ SNPs). 3) The position of the SNP has to be covered in at least 90% of the patients ($n = 78,697$ SNPs). 4) The SNPs require a p -value $> 1.10^{-4}$ when departing from the Hardy-Weinberg equilibrium resulting in a total of 73,535 SNPs.

Each of these individual 73,535 SNPs was correlated separately with the progression-free survival (PFS) obtained for those patients treated with combination avastin using a Cox-regression analysis. This resulted in the discovery of 44 SNPs in angiogenic effector genes and 2,743 SNPs in other genes that were significantly associated with PFS. This set of 2787 SNPs formed the basis for orthogonal validation in the prospective cohort.

Selection and assay design of germ-line SNPs for orthogonal validation

We selected a total of 60 SNPs for validation in the additional cohort. At first instance, we focused on the 44 SNPs that were identified in angiogenic effector genes as these genes are most likely to have an effect upon treatment of a patient with combination avastin. Additionally 15 lead SNPs were selected from the non-angiogenic effector genes as well as a SNP in MUC2 as this gene has been linked to Regorafenib (an anti-angiogenic TKI) resistance in the CRC setting. All of these SNPs were prioritized according to significance, a complete list of the selected SNPs can be found in Table 3 (angiogenic effector genes) and Table 4 (SNPs in other genes). Selected SNPs were designed in multiple assays and optimized for genotyping on the Sequenom iPLEX platform (Sequenom Inc, San Diego, CA, USA).

In brief, using the Assay Design Suite v2.0 software, the sequences surrounding the SNP of interest were retrieved and formatted. Subsequently, proximal SNPs are determined and annotated in the extracted sequence after which the optimal primer areas are identified. In a last step, amplification and extension primers were designed using the MassARRAY Assay Design v3.1 software, which allows primers for different SNPs to be combined in a single PCR reaction with a maximum of 40 SNPs per assay.

We were able to successfully combine primers for 53 out of 60 SNPs in 2 different assays (Table 3 and Table 4). No appropriate primer triplets were found for 3 SNPs in angiogenic effector genes and 4 SNPs from other genes due to high false extension primer potentials for the located amplicons (n=5) or the presence of multiple extension primer hits for the scanned primer triplets (n=2).

Table 3 The top 44 SNPs in angiogenic effector genes that were selected for validation in the prospective cohort

Gene	SNP ID	Assay	Ref	Alt	HR (CI 95)	Pr(> z)	GxT	MAF	Mean PFS homozygous	Mean PFS heterozygous	Mean PFS wild type
FLT1	rs7987649	1	A	G	1.67 (1.32-2.13)	2.63E-05	1.09E-01	0.66	345.9	343	342.7
NRP1	rs10827226	1	C	T	1.63 (1.25-2.13)	3.47E-04	9.46E-01	0.2	210.4	314.7	367.6
NRP1	rs2284938	2	A	G	1.55 (1.17-2.05)	2.12E-03	8.44E-01	0.81	365	312.4	231.8
THBS2	rs62435222	1	G	A	1.55 (1.17-2.05)	2.19E-03	9.90E-01	0.81	350.4	333.7	261.4
THBS2	rs9718074	1	G	C	1.44 (1.14-1.83)	2.67E-03	9.33E-01	0.74	368.4	317	309.8
NRP2	rs1983343	2	T	C	0.69 (0.54-0.89)	4.19E-03	8.72E-02	0.66	364.9	330.4	321.7
NOS3	rs2566508	2	T	G	1.5 (1.12-1.99)	5.73E-03	6.59E-01	0.81	305.3	404.2	549.8
PF4	rs394408	1	C	T	1.57 (1.12-2.18)	8.35E-03	1.04E-01	0.12	140.5	331.1	351.9
PF4	rs447206	1	G	A	1.55 (1.11-2.16)	9.82E-03	6.21E-02	0.12	140.5	329.2	352.6
SELE	rs1534904	1	G	T	0.75 (0.6-0.95)	1.73E-02	2.94E-01	0.35	335.6	336.5	353.3
FLT1	rs12877323	1	T	G	0.63 (0.42-0.92)	1.87E-02	1.18E-03	0.9	352.5	312.2	NA
CXCL12	rs728143	2	T	A	1.42 (1.06-1.91)	2.00E-02	5.03E-01	0.82	337.4	373.1	164.8
ANG	rs61976879	1	T	G	0.7 (0.52-0.95)	2.02E-02	1.91E-01	0.81	336.8	355.8	448.5
ANG	rs17516133	1	G	C	0.7 (0.52-0.95)	2.02E-02	1.91E-01	0.81	336.8	355.8	448.5
PDGFD	rs10895597	2	G	A	1.38 (1.05-1.81)	2.09E-02	1.91E-02	0.79	344.5	327.2	547
IFNG	rs7968411	2	A	G	1.32 (1.04-1.68)	2.15E-02	3.70E-01	0.37	412.2	337.2	333.8
SELE	rs10753792	1	A	G	0.76 (0.6-0.96)	2.26E-02	1.49E-01	0.34	327.7	333.9	358.8
VHL	rs112248386	NA	C	T	0.71 (0.52-0.95)	2.28E-02	1.64E-01	0.81	349.8	332.6	354.7
CXCR4	rs10191360	1	C	T	0.79 (0.64-0.97)	2.39E-02	4.82E-01	0.49	349.1	368.1	303.4

ANGPT4	rs62187504	1	T	C	0.71 (0.52-0.96)	2.45E-02	4.29E-01	0.83	326	374.4	424
TEK	rs2273719	2	G	A	1.42 (1.05-1.94)	2.48E-02	7.53E-01	0.86	360.7	297.5	379.7
PTGS2	rs689466	2	T	C	0.71 (0.53-0.96)	2.65E-02	2.83E-01	0.81	341.6	352.9	333.7
HIF3A	rs116478928	2	C	T	0.73 (0.56-0.97)	2.74E-02	3.97E-01	0.82	345.9	326	381.1
COL18A1	rs2236468	1	A	G	1.32 (1.03-1.69)	2.96E-02	5.95E-01	0.61	359.9	314.5	396.5
KDR	rs9998950	2	C	T	0.73 (0.54-0.97)	3.14E-02	5.39E-01	0.78	349.4	339.6	313
PDGFD	rs924562	1	C	T	1.48 (1.03-2.11)	3.18E-02	9.50E-01	0.88	340.1	361.5	319
VEGFA	rs3025033	1	A	G	1.42 (1.03-1.95)	3.20E-02	6.43E-01	0.86	341.6	356.9	316.3
TEK	rs9987817	2	G	A	1.43 (1.03-1.99)	3.26E-02	8.73E-01	0.85	364.4	296.3	364.5
COL18A1	rs7499	1	G	A	1.31 (1.02-1.67)	3.26E-02	6.85E-01	0.64	359.3	317.4	391.8
CXCR4	rs12691874	1	A	G	0.8 (0.65-0.99)	3.60E-02	4.60E-01	0.48	354.7	365.5	297.7
TGFA	rs3771523	1	C	T	1.48 (1.03-2.14)	3.63E-02	2.18E-01	0.88	341.8	353.4	NA
PFKFB3	rs1064891	2	T	C	0.77 (0.61-0.99)	3.82E-02	2.39E-01	0.58	325	364.9	314.6
IGF1	rs35765	1	G	T	0.67 (0.46-0.98)	4.07E-02	9.88E-02	0.1	134	342.5	346.9
VEGFA	rs3025030	1	G	C	1.43 (1.01-2.01)	4.10E-02	6.33E-01	0.89	344.6	348.5	316.3
TNNI1	rs1107256	2	G	A	0.78 (0.61-0.99)	4.13E-02	1.52E-01	0.72	344.9	350.8	315.1
TGFA	rs3771527	1	A	T	1.47 (1.01-2.12)	4.16E-02	2.23E-01	0.88	341.2	353.4	NA
CXCL12	rs1029153	1	A	G	1.29 (1.01-1.64)	4.23E-02	2.96E-01	0.72	344.9	341.8	358.6
ANGPT4	rs1997923	1	C	T	0.8 (0.64-0.99)	4.30E-02	4.44E-01	0.6	355.2	327.7	358.8
KDR	rs6837735	2	C	T	0.75 (0.56-0.99)	4.42E-02	5.09E-01	0.83	339.1	372.8	207.4
F5	rs6012	1	C	T	1.34 (1-1.78)	4.69E-02	1.44E-01	0.82	328.2	397.4	255.9
VHL	rs112782301	NA	C	T	0.74 (0.55-1)	4.74E-02	1.93E-01	0.82	349.3	346	255.5
EGF	rs4698756	1	G	A	0.79 (0.62-1)	4.79E-02	8.63E-01	0.68	352.8	362.3	237.3
IFNG	rs2069705	1	A	G	1.29 (1-1.67)	4.87E-02	3.83E-01	0.31	354.1	345.9	341.8
PFKFB3	rs590278	NA	C	T	0.81 (0.66-1)	4.93E-02	4.28E-01	0.57	374.4	328.6	313.5

Table 4 The 16 top SNPs in other genes selected for validation in the prospective cohort

Gene	SNP ID	Assay	Ref	Alt	HR (CI 95)	Pr(> z)	GxT	MAF	Mean PFS homozygous	Mean PFS heterozygous	Mean PFS wild type
SPTB	rs229592	1	A	G	1.82 (1.4-2.38)	1.00E-05	5.72E-01	0.74	341.5	354.3	309.5
MMADHC	rs12232959	1	G	C	1.82 (1.39-2.37)	1.20E-05	1.55E-01	0.25	204.9	356.2	350.3
COL11A2	rs2855438	NA	A	T	2.1 (1.5-2.95)	1.60E-05	1.71E-01	0.85	339.2	359.2	317.0
CACNA1C	rs10848683	1	T	C	1.93 (1.43-2.61)	2.10E-05	9.26E-01	0.18	593.0	351.8	331.2
FAM86EP	rs34505493	1	T	C	1.93 (1.41-2.63)	3.70E-05	4.98E-01	0.21	350.2	362.2	335.2
CACNA1C	rs10774053	2	G	A	1.9 (1.4-2.57)	3.80E-05	9.29E-01	0.17	593.0	356.7	329.8
HLA-DQA1	rs9272736	NA	G	A	1.74 (1.33-2.27)	4.60E-05	6.01E-02	0.81	344.6	284.4	506.4
ALDH4A1	rs4592275	1	G	A	1.64 (1.29-2.09)	5.50E-05	3.80E-01	0.74	362.4	322.7	304.0
FAM86EP	rs34583792	1	T	C	1.63 (1.28-2.06)	5.80E-05	9.90E-01	0.27	427.3	342.2	335.0
HLA-DQA1	rs9272737	NA	G	A	1.72 (1.32-2.25)	6.10E-05	1.35E-01	0.81	346.1	284.4	506.4
DUSP27	rs267745	1	G	C	0.59 (0.46-0.77)	6.20E-05	3.13E-03	0.52	345.0	364.1	297.6
RABGGTA	rs941502	2	A	T	0.59 (0.45-0.76)	6.80E-05	2.61E-02	0.66	347.7	330.5	386.9
HLA-DRB5	rs1136633	NA	G	A	1.77 (1.33-2.35)	7.50E-05	2.58E-01	0.85	358.9	310.3	286.8
C5AR1	rs11670330	2	G	A	1.74 (1.32-2.29)	7.60E-05	5.64E-01	0.73	379.8	292.6	380.0
NLRP1	rs12150220	1	A	T	0.63 (0.5-0.79)	8.00E-05	8.44E-02	0.54	340.8	346.2	345.7
MUC2	rs34367348	1	C	T	1.63 (1.26-2.13)	2.60E-04	6.30E-01	0.73	365.4	353.6	223.0

SNP-genotyping using the Sequenom MassARRAY

In a first step, the sequence surrounding the SNPs of interest was amplified using the previously described primers in a multiplexed PCR. After removing dNTPs using shrimp alkaline phosphatase, a primer adjacent to the site of interest is extended in an allele specific manner. The resulting extension products differ in mass and are analysed with MALDI-TOF MS. Automated genotyping calls were generated using the MassARRAY RTM software v4.0 and were validated by manual review of the raw mass spectra. A schematic presentation of the process is shown in Figure 9.

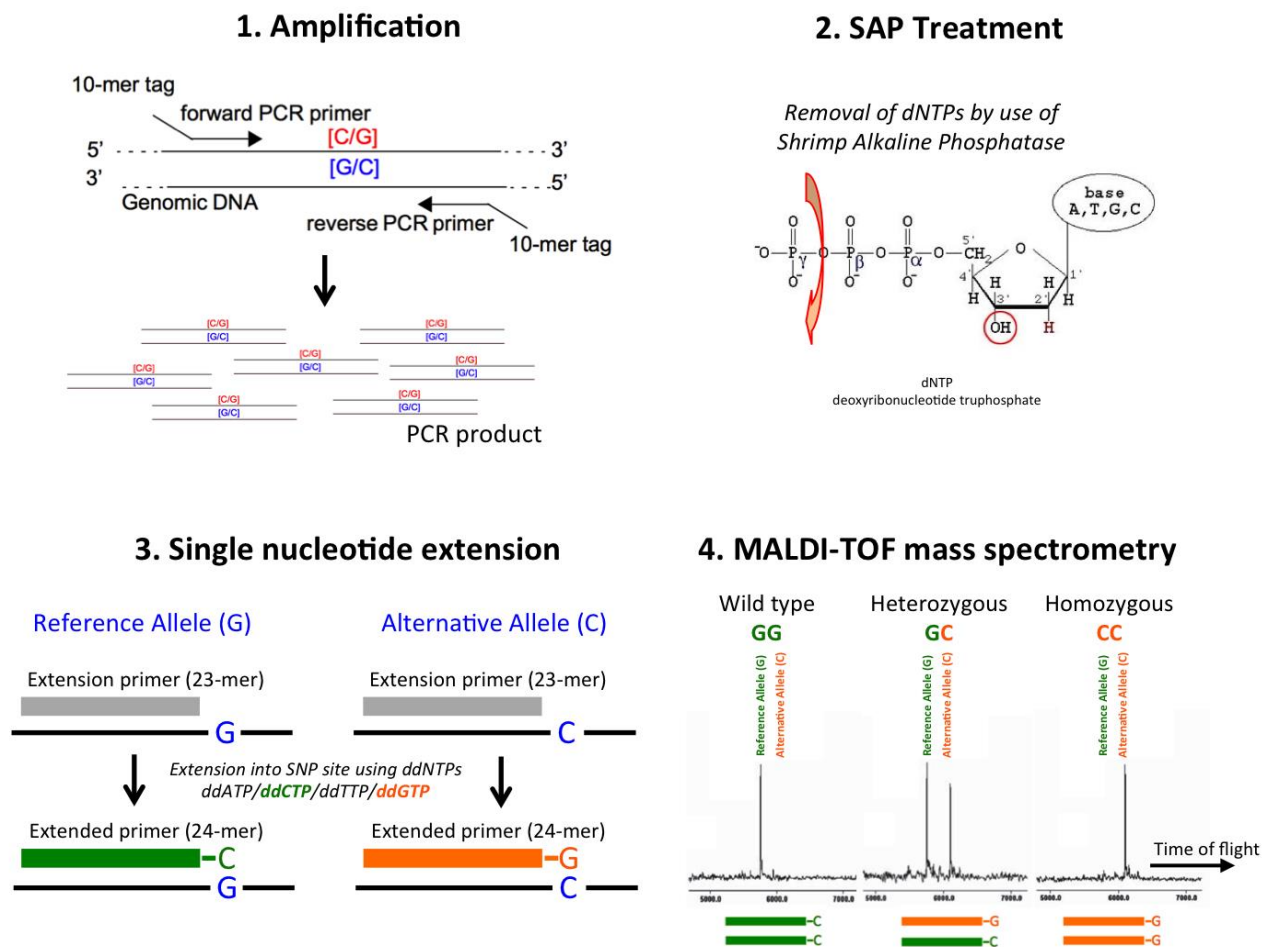


Figure 9 Schematic presentation of the primer extension used in combination with MALDI-TOF. (1) The region of interest is amplified. (2) dNTPs are removed using Shrimp Alkaline Phosphatase. (3) Single nucleotide extension of the SNP of interest using extension primers. (4) Mass spectrum for a wild type, heterozygous or homozygous SNP.

Resulting germ-line SNPs and correlation with progression-free survival

A total of 135 patients were selected for orthogonal validation. Currently 76 patients are enrolled in the AC-Angiopredict prospective study. Since not all patients from the retrospective cohort were included in the target identification analysis (i.e. not included in the final discovery cohort for germ-line SNP detection since whole-exome sequencing was not possible) we also included these retrospective patients for orthogonal validation using the Sequenom MassARRAY.

After manual review of the raw mass spectra resulting from the Sequenom experiments, the results were assembled in a matrix containing all the genotype calls per SNP for each patient. 3 patients were removed from the results since genotyping failed for more than 90% of the selected SNPs, indicating that the integrity of the DNA was not sufficient for analysis. 3 SNPs had a success rate less than 50% and were excluded from the further analysis (rs12232959, rs4592275 and rs6837735). The average genotyping success rate of the SNPs was 98.7±1.1% (range 94.7-100%).

For each SNP we first determined the Hardy-Weinberg Equilibrium (HWE) to assess whether the distribution of SNPs was normal in this validation cohort. 6 SNPs showed a strong deviation ($P < 10^{-4}$), and were removed from further analysis (rs34367348, rs34505493, rs34583792, rs62187504, rs7499 and rs941502). In a next step we calculated p-values and HR-ratio's for each of the SNPs to determine the correlation with progression-free survival in a similar manner as performed for the discovery analysis. Using a Cox-regression analysis, each individual SNP was correlated separately with the progression-free survival (PFS) obtained for those patients treated with a combination Avastin

regimen. Overall, 110 patients received combination Avastin treatment and were included in the survival analysis. 10 SNPs in Angiogenic effector genes (NOS3, CXCR4, PF4, IGF1, EGF, PDGFD, NRP1, CXCL12, PF4 and TNNT1) and 1 SNP in the gene DUSP27 correlated significantly with PFS (marked in grey in Table 5). Kaplan-Meier plots for these 11 SNPs are shown in Figure 10.

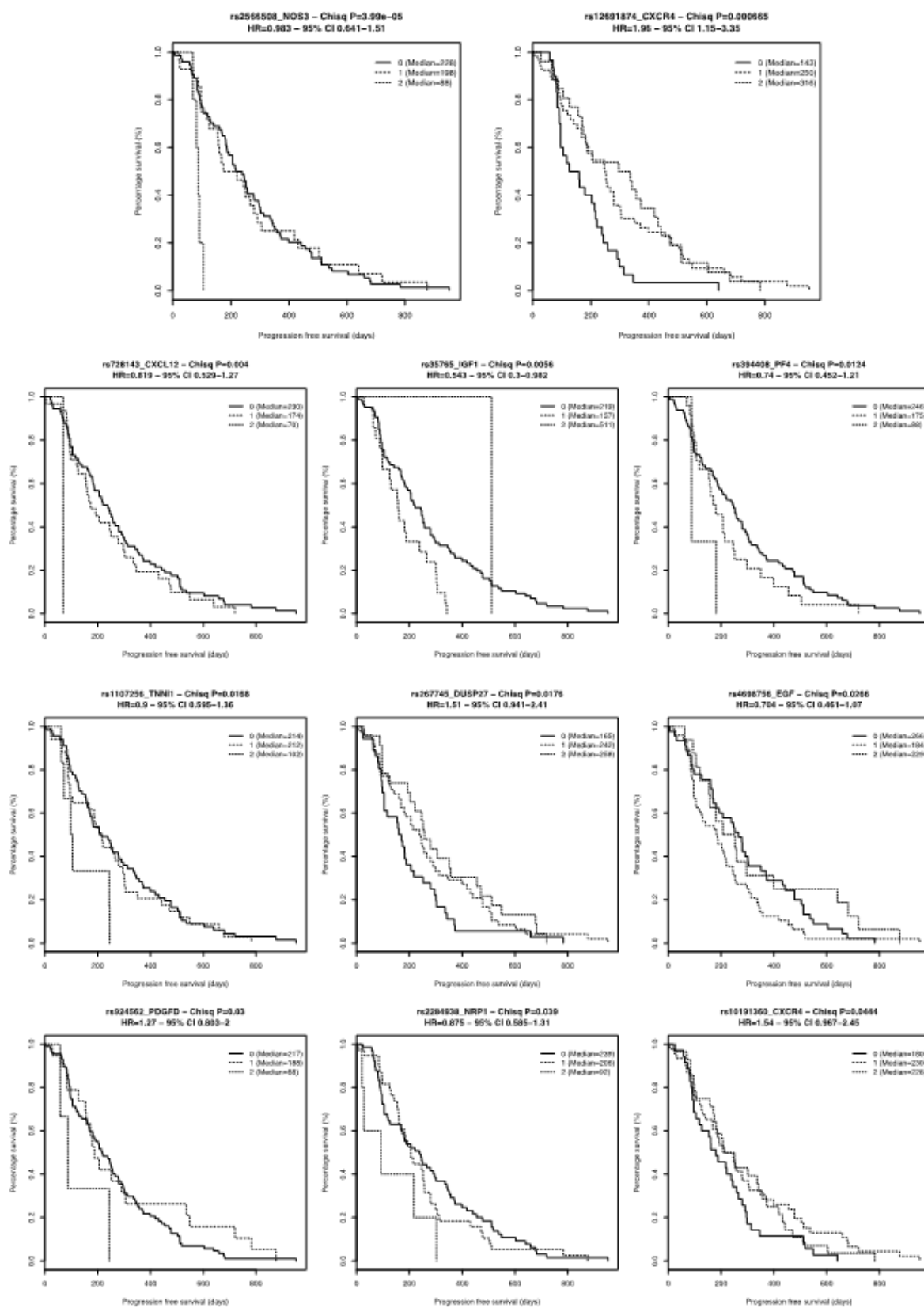


Figure 10 Kaplan-Meier plots of the 11 SNPs that correlate with progression-free survival in the prospective validation cohort

Table 5 Correlations of validated SNPs with PFS

SNP ID	Gene	Number of wild-type patients	Number of heterozygous patients	Number of homozygous patients	KM P-value	HR (CI 95)	Mean PFS WT (days)	Mean PFS Heterozygous (days)	Mean PFS Homozygous (days)
rs2566508	NOS3	87	37	5	3.99163E-05	0.98 (0.64-1.51)	228	198	88
rs12691874	CXCR4	37	61	29	0.000664661	1.96 (1.15-3.35)	143	250	316
rs728143	CXCL12	88	38	2	0.004000049	0.82 (0.53-1.27)	230	174	70
rs35765	IGF1	102	25	1	0.005599427	0.54 (0.3-0.98)	219	157	511
rs394408	PF4	98	29	4	0.01241053	0.74 (0.45-1.21)	246	175	88
rs1107256	TNNI1	78	40	10	0.01677849	0.9 (0.6-1.36)	214	212	102
rs267745	DUSP27	42	58	29	0.0176165	1.51 (0.94-2.41)	165	242	258
rs4698756	EGF	51	60	16	0.02656866	0.7 (0.46-1.07)	266	184	229
rs924562	PDGFD	101	25	4	0.02996755	1.27 (0.8-2)	217	188	88
rs2284938	NRP1	82	41	6	0.03899854	0.87 (0.58-1.31)	239	206	92
rs10191360	CXCR4	43	56	32	0.0444192	1.54 (0.97-2.45)	180	230	228
rs447206	PF4	98	30	2	0.05084174	0.75 (0.46-1.2)	246	160	135
rs10827226	NRP1	93	28	9	0.07877945	1.16 (0.75-1.79)	204	255	210
rs689466	PTGS2	82	39	5	0.09323874	0.96 (0.64-1.44)	206	247	140
rs116478928	HIF3A	77	38	14	0.1324838	0.75 (0.47-1.18)	248	170	186
rs10774053	CACNA1C	81	45	4	0.1435074	0.96 (0.64-1.44)	221	214	126
rs10895597	PDGFD	82	37	12	0.152637	1.02 (0.67-1.56)	230	186	148
rs9718074	THBS2	76	45	9	0.1777137	0.77 (0.51-1.17)	214	194	206
rs1983343	NRP2	47	63	18	0.1785889	1.31 (0.86-1.99)	184	216	250
rs2069705	IFNG	57	59	15	0.2089598	0.9 (0.6-1.36)	207	203	324
rs3025033	VEGFA	97	31	1	0.2209054	1.24 (0.82-1.86)	190	280	168
rs9998950	KDR	72	49	9	0.2361601	1.16 (0.78-1.74)	188	239	294
rs7987649	FLT1	72	44	13	0.2888014	0.81 (0.54-1.22)	214	204	280

rs1064891	PFKFB3	52	52	23	0.2899777	1.22 (0.8-1.86)	239	221	171
rs11670330	C5AR1	74	48	7	0.3303332	1.21 (0.81-1.79)	182	239	280
rs10753792	SELE	61	47	22	0.3871891	0.97 (0.64-1.46)	217	187	250
rs12877323	FLT1	105	25	0	0.4030607	0.81 (0.47-1.39)	216	147	NA
rs10848683	CACNA1C	75	51	4	0.4057602	0.93 (0.63-1.38)	221	206	180
rs17516133	ANG	76	42	11	0.4197741	0.95 (0.63-1.43)	242	186	175
rs3771527	TGFA	111	16	3	0.4222823	1.17 (0.7-1.97)	206	239	378
rs6012	F5	91	33	5	0.4676468	1.05 (0.68-1.64)	206	252	187
rs3025030	VEGFA	105	20	5	0.495831	1.09 (0.66-1.79)	206	286	242
rs12150220	NLRP1	44	64	23	0.5000804	0.97 (0.64-1.46)	170	252	239
rs1029153	CXCL12	72	49	8	0.504166	0.89 (0.59-1.33)	230	182	206
rs3771523	TGFA	100	31	0	0.5321701	1.15 (0.75-1.74)	204	252	NA
rs7968411	IFNG	53	55	22	0.5328465	0.88 (0.59-1.33)	221	193	258
rs229592	SPTB	76	46	9	0.5918998	0.97 (0.65-1.45)	216	221	156
rs62435222	THBS2	95	31	4	0.5995628	0.92 (0.59-1.43)	214	206	213
rs1534904	SELE	74	40	17	0.6213721	1 (0.66-1.52)	208	206	257
rs2236468	COL18A1	51	51	25	0.6260136	1.06 (0.69-1.61)	184	258	214
rs61976879	ANG	79	35	16	0.6577263	0.93 (0.6-1.43)	217	194	188
rs1997923	ANGPT4	52	57	21	0.6837568	0.93 (0.62-1.41)	204	200	280
rs2273719	TEK	91	30	3	0.6974594	1.1 (0.7-1.72)	200	250	280
rs9987817	TEK	95	32	2	0.7158577	0.92 (0.58-1.47)	206	218	280

1.3.4.3 Conclusion

To identify single nucleotide polymorphisms (SNPs) in germ-line DNA with an effect on a patients' outcome following combination bvx therapy we determined germ-line SNPs using a customized whole-exome sequencing approach on a cohort of retrospectively collected patients. After optimizing the experiments for use with FFPE-derived DNA we successfully genotyped 196 patients and identified a total of 441,440 SNPs. To facilitate integrative analysis of the data from these 196 germ-line DNA exomes with data generated by other platforms (i.e. somatic mutations and copy

number alterations from WP5), we have generated genetic variant data files (in GVCF-format), which are currently being transferred to the AngioPredict database (WP8).

73,535 of these 441,440 SNPs met the inclusion criteria for subsequent survival analysis and each of these SNPs was subsequently correlated individually with progression free survival, resulting in the discovery of 44 SNPs in angiogenic effector genes and 2,743 SNPs in other genes that were significantly associated with PFS. This set of 2787 SNPs formed the basis for orthogonal validation in a cohort of 132 patients, which consisted of prospective patients expanded with retrospective patients for whom no whole-exome data was available yet.

The 44 SNPs that were identified in angiogenic effector genes were selected for validation with Sequenom MassARRAY, as these genes are most likely to have an effect upon treatment of a patient with combination avastin. Additionally 15 lead SNPs were selected from the non-angiogenic effector genes as well as a SNP in MUC2 as this gene has been linked to Regorafenib (an anti-angiogenic TKI) resistance in the CRC setting. Of the 60 resulting SNPs, we were able to successfully genotype 44 SNPs in an additional cohort of 132 samples. We identified 11 SNPs (in NOS3, CXCR4, PF4, IGF1, EGF, PDGFD, NRP1, CXCL12, PF4, TNN1 and DUSP27) for which we could confirm a significant correlation with PFS in patients receiving Avastin.

As expected, rigid validation substantially decreased the number of candidate SNP biomarkers identified in the first cohort. **More importantly, however, we could identify 11 SNPs that showed a predictive effect in the replication cohort of 132 CRC patients. The latter SNPs are very interesting, and could be used to stratify patients and predict response to Avastin treatment. As not all patients recruited in the prospective trial had a disease progression event, we will perform an updated analysis once these data are available. Should effects remain significant, we will consider these SNPs for further development, possibly as a companion diagnostic test.**

1.3.5 WP5 Somatic Mutation Analysis & Validation

1.3.5.1 Introduction

One of the primary goals of the ANGIOPREDICT consortium was the discovery and validation of predictors using pharmacogenomic methods for combination bevacizumab (Avastin) therapy in first line treatment of Kras mutant metastatic colorectal cancer (mCRC). Therefore, retrospective frozen and FFPE tumor samples from mCRC patients having received first line combination bvz therapy or chemotherapeutics alone were provided to the ANGIOPREDICT central biorepository housed at UHEI from the bio-banks at RCSI, UHEI and VUMC. This tissue bank was then used to provide patient tumour and 'normal' adjacent colorectal material for DNA sequencing. Due to the high cost of 'whole genome sequencing' (WGS; ca. 3 billion nucleotides) an 'exome' sequencing strategy (investigating only approximately 2% of the whole genome) was used to identify somatic mutations.

Somatic mutations are common alterations observed across various cancer types including mCRC. These alterations are of particular interest from a biomarker discovery perspective, ultimately allowing identification of variants that can be effectively used to predict patient response to therapy. Somatic single nucleotide variant (SNV) mutations can be orthogonally validated (i.e. using a different technology). These validated mutations can then be exploited based on occurrence correlated with treatment response.

Along with somatic mutations, 'copy number aberrations' (CNA) can be determined from DNA sequence. Copy number varies in tumours due to an increasingly studied and important phenomenon termed 'genomic instability' whereby chromosomes are more often affected by rearrangements during cell divisions than in non-cancerous tissue. Chromosomal copy number aberration is one of the main mechanisms by which cancerous cells acquire their hallmark characteristics and as such are necessary to investigate how the disease forms and/or progresses.

1.3.5.2 Work Undertaken and Results Achieved

Task 5.1: Exome Re-sequencing: Mutation Identification

DNA extraction and exome sequencing from mCRC patient samples (tumour and normal tissues) treated with chemotherapy +/- bvz was carried out and data analysis conducted for 421 patient samples (see Figure 11 for

schematic overview). A new and dynamic bioinformatics pipeline was set up for this purpose using current best practices and conducted on a dedicated server hosting both raw data and results files. The analysis allowed clear quality assessment of the sequencing data generated, from which we were able to call consensus somatic mutations using two complementary methods. The output from this deliverable was identification of a set of somatic SNV, in total 135,759, to which we were able to apply a variety of filters to remove SNV of poor quality and that were also found in the germline mutation analysis (WP4) resulting in a final total set of 32,688 of which 291 were novel and nonsynonymous and found in more than two patients. **This 'single 'omics dataset' analysis did not find any correlation with overall survival and bvz treatment. However somatic mutation data was further analysed within the context of WP9.1 by integrating with gene interaction networks to provide a novel combination avastin predictive strategy.**

Mutations from ctDNA were detected in the tumour biopsy with only a minority ($4.8 \pm 2.2\%$) occurring uniquely in the ctDNA. Overall, $71.8 \pm 20.4\%$ of the detected mutations were present in both the tumour biopsy and the plasma sample indicating that nearly all mutations in these 3 CRC patients were detected by sequencing the tumour biopsy. **These results have shown that nearly all mutations characteristic for the bulk tumour can reliably be detected by sequencing the tumour biopsy.**

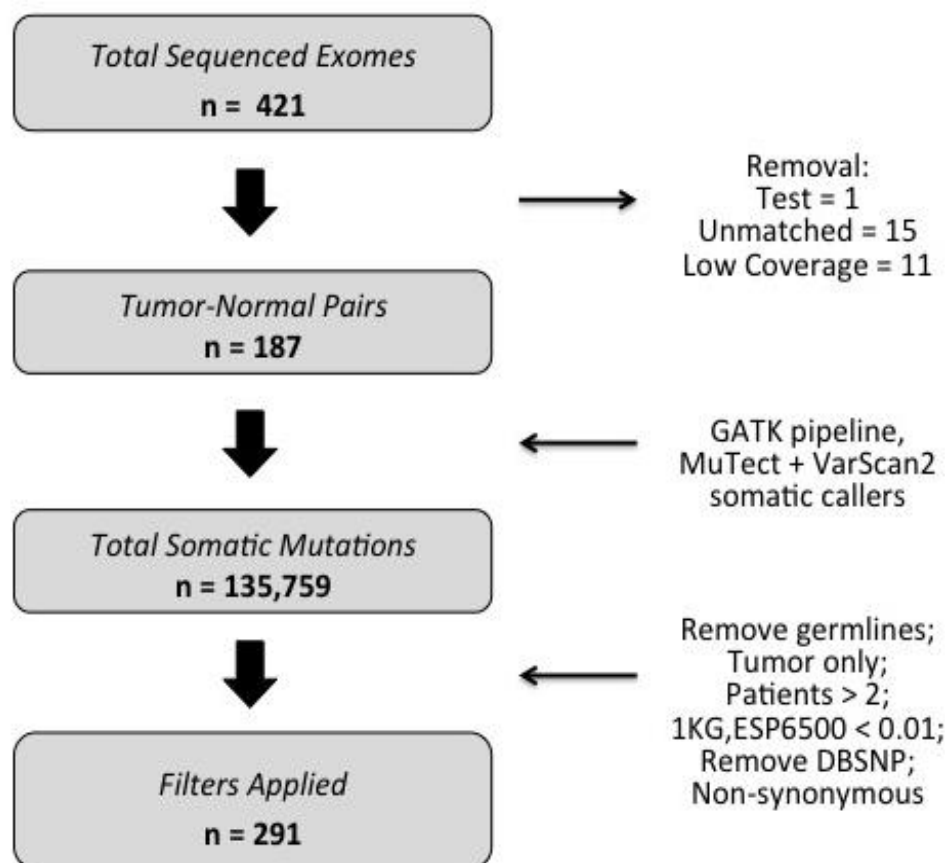


Figure 11 Schematic Overview of Exome DNA Sequencing. The final exome sequencing set included 421 patients, resulting in 374 'exomes', or 187 tumour/normal matched pairs representing that number of patients.

Task 5.2: Exome Re-sequencing: Mutation Validation

The validation by Sequenom mass spectrometry was unsuccessful for identifying the same mutations as discovered by next generation sequencing. Given that our circulating tumour DNA analysis can identify up to 94% of mutations

found in the bulk tumour sample through next-generation sequencing, we conclude that the failure of the Sequenom analysis is a function of the fragmented nature of FFPE-derived DNA.

Task 5.3: Development of Commercialization Plan for Findings

To date, no individual somatic alterations that significantly correlate with response to bevacizumab have been identified that warrant further commercialization. **However, ongoing work, integrating the breadth of omics data generated during the Angiopredict programme, has identified novel mutator phenotypes that hold promise for future development.** Additional studies and validation in an independent cohort are ongoing in order to ascertain whether a discrete, exploitable diagnostic tool can be developed from these signatures.

Additional research work carried out at VUMC on **copy number alteration profiling (relating to Task 5.4) has further delivered promising results.** Additional validation studies are ongoing and will be completed prior to protection of any new intellectual property.

Task 5.4: Copy Number Alteration Profiling: Identification

In total, 152 tumor patient samples were processed for our novel shallow-sequencing methodology (see Figure 12 for schematic overview). After passing all our inclusion criteria the final dataset comprised sequence data of 89 tumor samples. Of these tumor samples 73 samples were from patients receiving bvz combination therapy and 16 of the patients were treated with chemotherapy alone.

Copy numbers were correlated with outcome for combination bvz-treated mCRC tumors (overall survival). Only copy number aberrations that occur frequently (e.g. in >10% of mCRC tumors) were considered relevant. Only aberrations that correlated significantly with overall survival to bvz ($p < 0.05$) were selected for a more detailed statistical analysis such as correlation with overall survival in the bvz mCRCs while correcting for prognostic variables, and correlation for effect on treatment response (RECIST) and progression-free survival.

To compensate the diminished number of samples that could be included for this project, VUmc provided 216 samples (tumor and normal DNA) from the CAIRO2 clinical trial (Haan et al 2015, doi: 10.1038/ncomms6457).

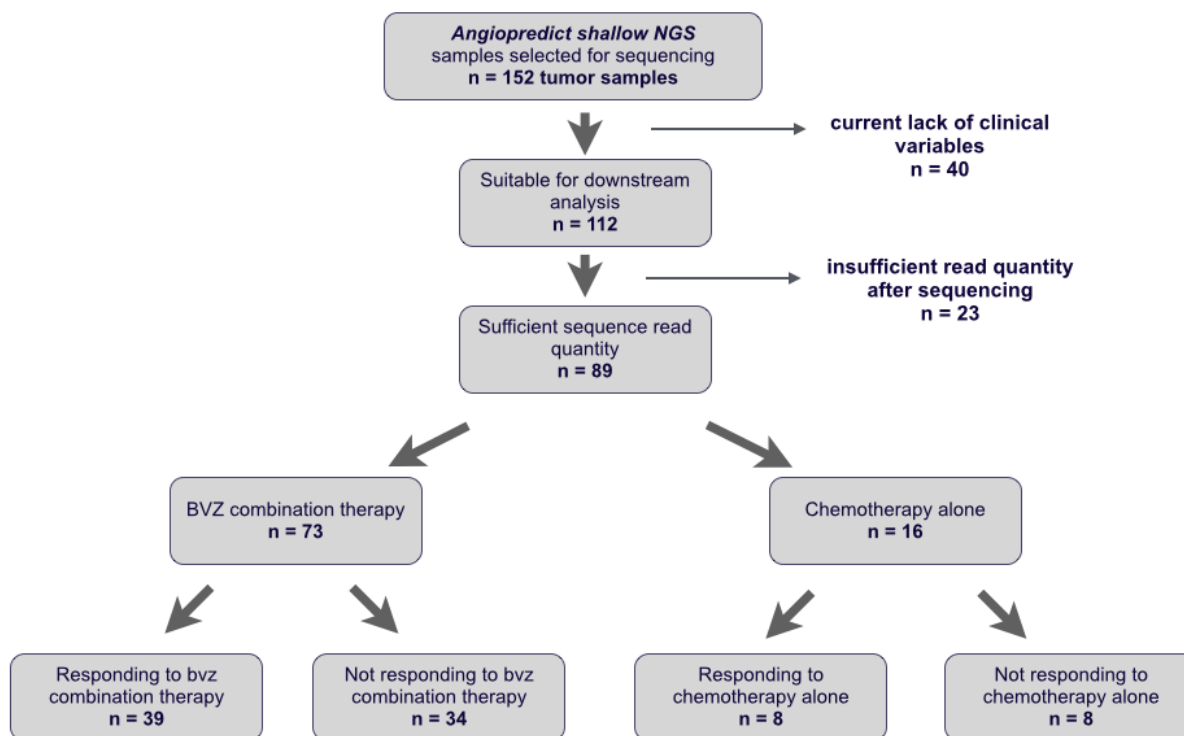


Figure 12 Schematic Overview. Selection criteria used for formation of the final CNA profiles including clinical variables and sequence read quantity.

Task 5.5: Copy Number Alteration Profiling: Validation

For this study retrospective FFPE tumor samples from 260 mCRC patients having received first line combination bvz therapy or chemotherapy alone were selected and provided to the ANGIOPREDICT project. For 286 unique samples, 517 DNA library with sufficient DNA for both shallow and exome next generation sequencing were available. After sequencing of 478 tumor libraries, 192 samples were pooled to create a final dataset of 286 patients with copy number data. From this dataset 182 patients were derived from the Angiopredict cohort. VUmc provided 104 additional samples from the CAIROII clinical trial armA as a validation cohort. Final results from this validated copy number profile found 449 regions of copy number gain or loss.

Frequency of copy number gain and loss in bvz+chemotherapy (and chemotherapy alone) treated samples was analysed using logrank statistical testing (10,000 permutations). 10 regions significantly associated with progression free survival (PFS) in the combination bvz treated Angiopredict cohort (Figure 13, blue line) were noted. A further 7 regions were found to be significantly associated with PFS in the CAIRO2 bvz treated cohort. These 7 regions were evaluated for association as prognostic or predictive candidates in the Angiopredict cohort without bvz treatment (chemotherapy alone; Figure 13, red line).

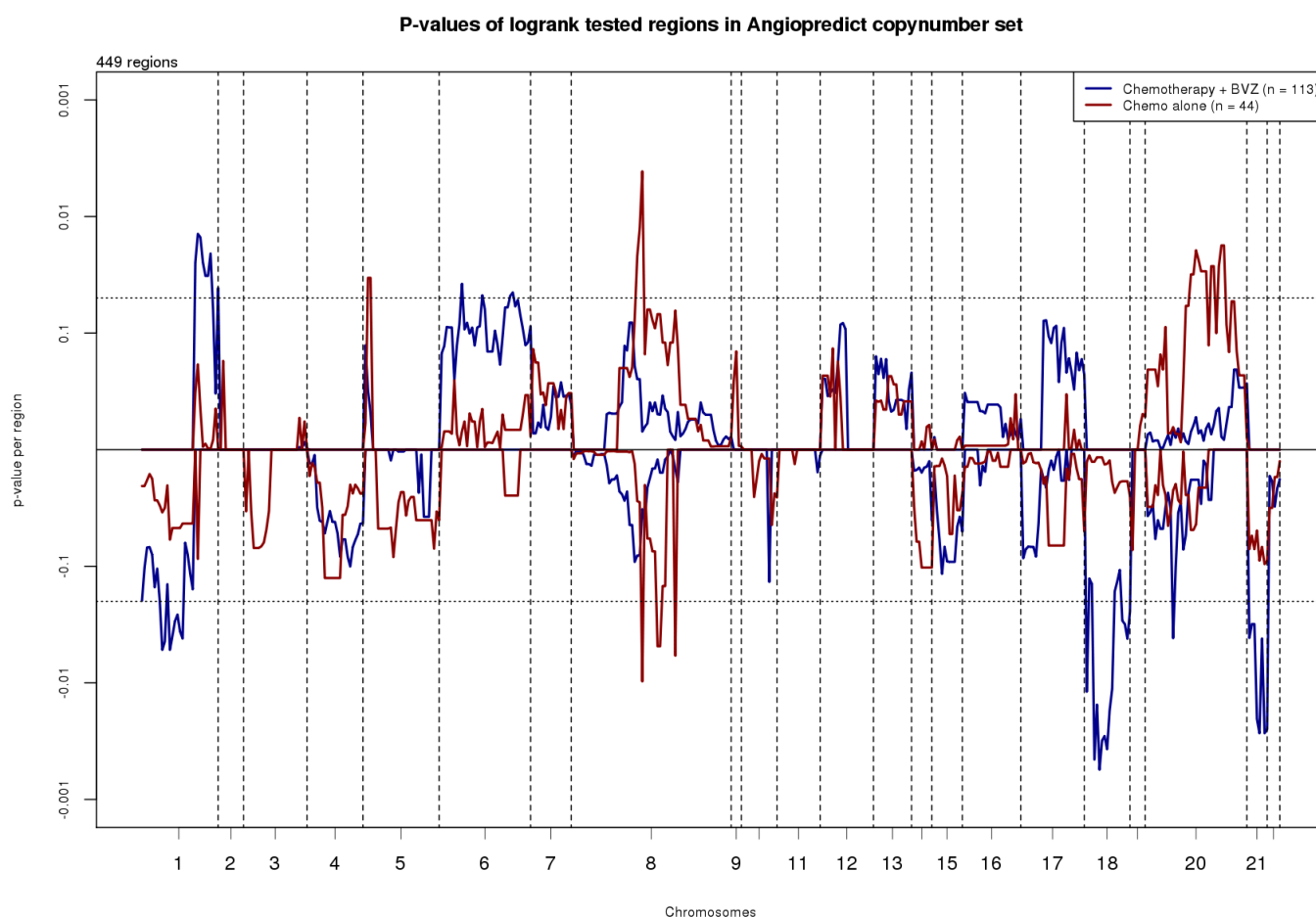


Figure 13 Gains and Losses Relating to Treatment. The x-axis displays regions sorted on chromosomal position. The horizontal dotted lines display the p-values of gains (above zero) or losses (below zero). Boundaries of chromosomes are indicated by dotted lines. The patient group receiving chemotherapy + bvz (n=113) depicted in blue and chemotherapy alone (n=44) in red.

Lastly we also explored a second alternative method to analyze the copy number data.

Apart from the copy number profiles for the samples described above, we downloaded additional publicly available copy number data for a cohort of 205 patients from the CAIRO I trial that were treated with Irinotecan-Capecitabine (CAPIRI) or capecitabine (CAP) only (Agilent oligonucleotide hybridization arrays; GSE36864). After using GISTIC to identify the most frequent and overrepresented copy number aberrations (CNAs) in the resulting 362 tumors we performed unsupervised hierarchical Ward clustering using the status of each of these recurrent CNAs for each patient and identified 5 clusters to which patients could be assigned.

Survival analysis shows that these subgroups can be correlated to progression-free and overall survival (Figure 14). More importantly, 3 out of 5 groups have an additional benefit when treated with combination-bvz therapy compared to patients that are treated with the chemotherapy backbone only (Figure 15). This suggests that molecular subtype classification of tumors can be used to predict bevacizumab response.

We are currently further characterizing the different subgroups at the copy number level (but also additional levels such as somatic mutations and clinical characteristics) to determine whether a simple, low-cost and non-invasive test (i.e. on circulating tumor DNA) can be used to classify a tumor to its corresponding subtype and in this way provide an effective and cheap method to not only predict additional benefit in the response on bevacizumab but also monitor cancer progression in mCRC patients. We are also validating these findings in 150 metastatic colorectal samples for which DNA was provided to us by the MOMA clinical trial (NCT02271464).

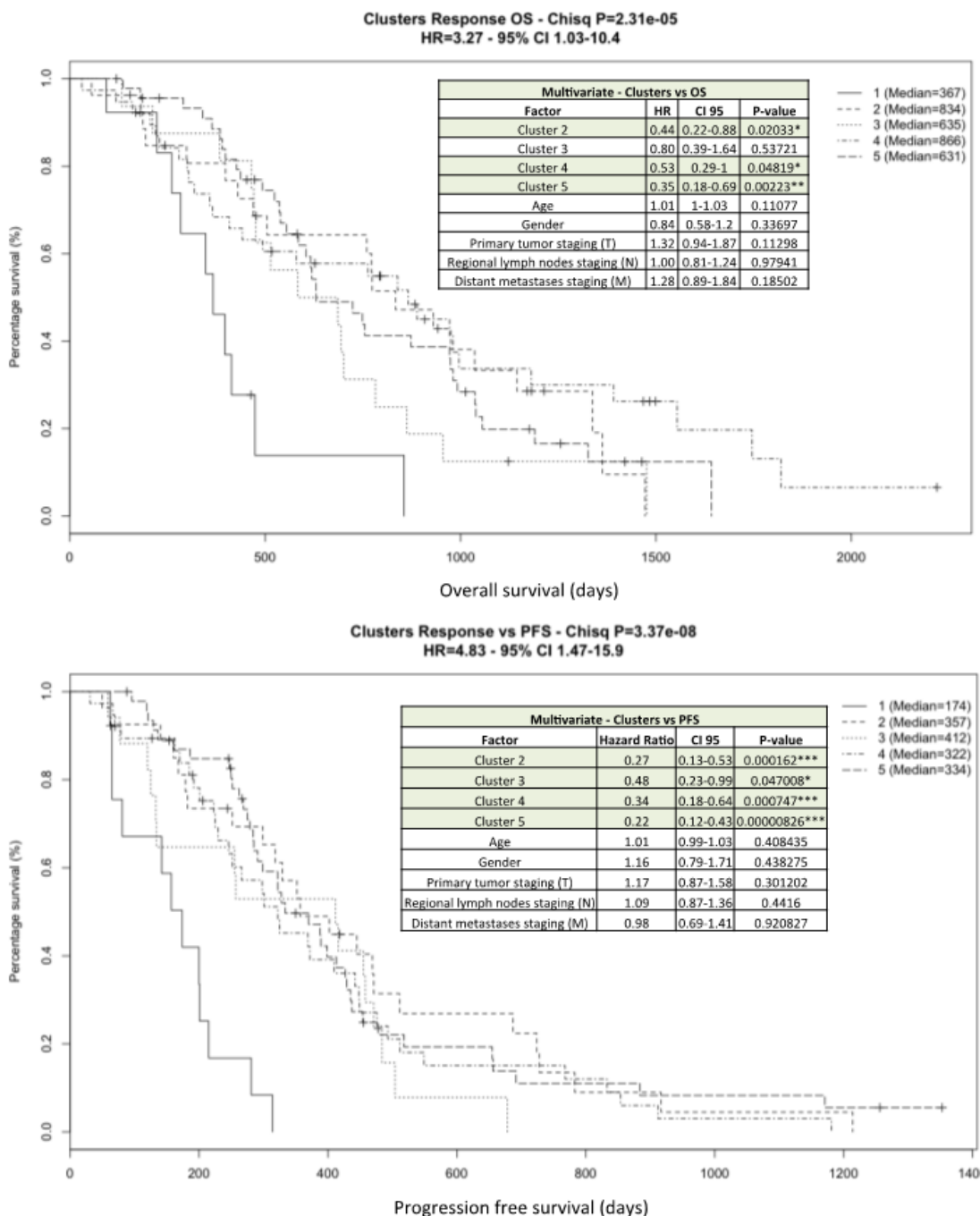
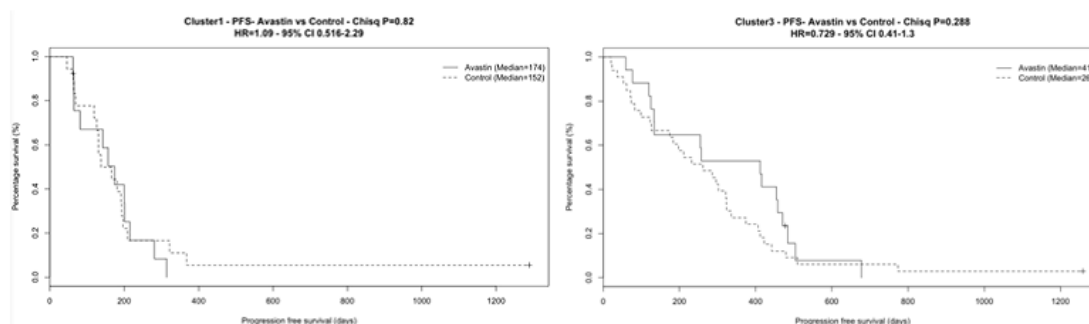


Figure 14 Univariate and multivariate correlation of the different clusters with OS (upper panel) and PFS (lower panel). Clusters 1 and 3 correlate with worse survival, clusters 2, 4 and 5 with better survival. This effect is independent of clinical factors such as age, gender and TNM-staging

Clusters 1 and 3



Clusters 2, 4 and 5

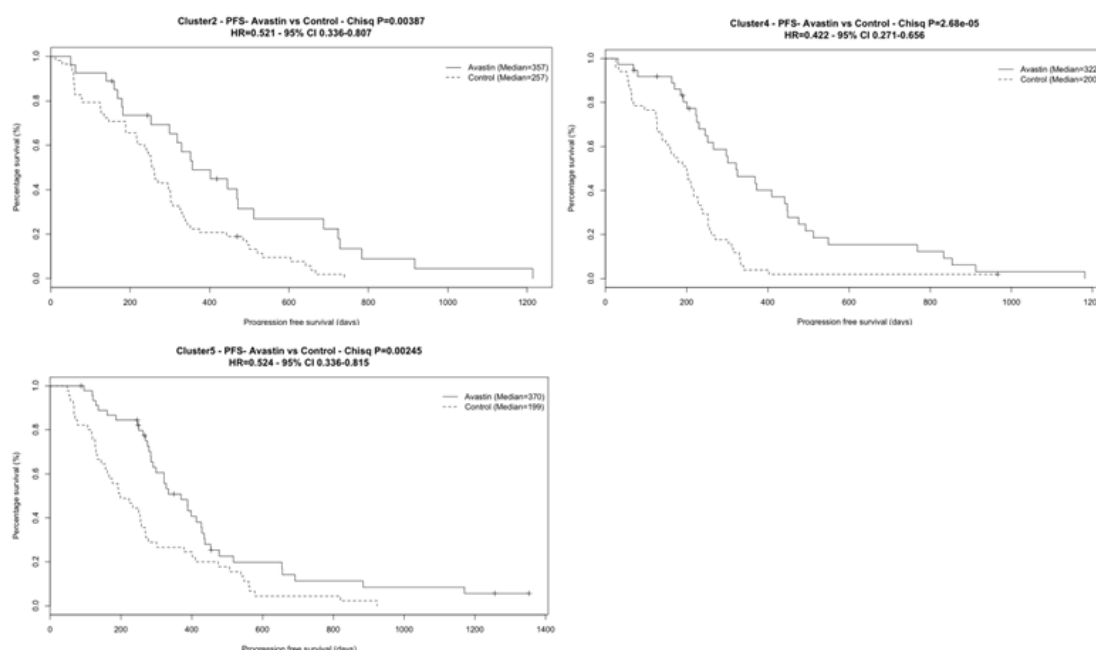


Figure 15 Comparison of patients treated with combination Avastin to those not treated with Avastin for each of the clusters and the effect on PFS. Patients from clusters 2, 4 and 5 show additional benefit when treated with Avastin compared to patients not treated with Avastin.

1.3.5.3 Conclusion

We have conducted exome (Task 5.1) and shallow whole genome sequencing (Task 5.3) for somatic mutation and copy number aberration analysis respectively. This has resulted in two large genomic datasets. We have orthogonally validated exome somatic mutations using the Sequenom platform (Task 5.2) which were determined to be involved in relevant functional pathways including angiogenesis, found as part of WP9 (D9.1). We have also provided this exome data which has proved invaluable for integrative omic analysis in other work packages of ANGIOPREDICT.

Copy number aberration data analysis (Tasks 5.4, 5.5) has identified 10 regions of copy number gains and losses associated with combination bvz treatment response as measured by PFS. This is a significant result for WP5, and highlights the value of both the novel shallow-sequencing methodology and the utility of this data. **Further, and of undeniable importance, is the validation of our Angiopredict results in the CAIRO2 bvz treated cohort accessed by**

VUMC. This indicates the robustness of the sequencing and analytical methodologies that have come directly from ANGIOPREDICT and will be useful not only to this project, but to the wider scientific community.

Additionally, a new area for research has emerged from an alternative analysis performed on the copy number profiles. **Copy number aberrations can be used to classify tumors into 5 different subtypes of which 3 show an additional benefit when comparing patients treated with combination-bvz therapy to those that receive the chemotherapy backbone only. Upon closer investigation and characterization, these 5 different subtypes could be linked to the recently described Consensus Molecular Subtypes of colorectal cancer (Guinney et al., Nature, 2015). This suggests that classifying tumors into appropriate subtypes may have an important role in the field of precision cancer medicine and drug treatment stratification.**

1.3.6 WP6 DNA Methylation based Predictive Methods

1.3.6.1 Introduction

WP 6, *DNA Methylation based Predictive Methods*, aimed to identify DNA methylation signatures that may serve as a predictive biomarker of Avastin combination treatment response and to further develop putative markers into companion diagnostic assays. Furthermore WP6 sought to establish tissue based assays first based on material from tumour biopsies, and then to further optimize it towards a blood based predictive genomic biomarker assay if applicable. Tasks associated with WP6 included: Task 6.1 Genome wide DNA methylation profiling from selected populations using Differential Methylation Hybridization [DMH] array technology on retrospectively collected frozen samples to be correlated with clinical outcome to generate a list of marker candidates; Task 6.3 Optimization of pre-analytics including capturing of low-concentration, fragmented DNA from tissue biopsies and adaptation of work steps for automation of work flows for DNA extraction and bisulfite conversion from blood-plasma. Task 6.4 Design, development and establishment of new bisulfite specific real-time PCR assays with high specificity for detection and quantification of methylated/unmethylated marker DNA. Establishment of real-time multiplex PCR assay prototypes for biomarker candidates identified in combination with assessment of tumour and total DNA load in the background. Task 6.5 Optimization of the assays to a degree that would enable the transfer prototypes to product development. Technical characterization of the marker candidate assays and Task 6.2 validation on independent sample sets to assess their potential as predictive markers.

1.3.6.2 Work Undertaken and Results Achieved

The main results and achievements of WP 6 (see also Table 6) are linked to the marker candidates that were found and assays that were established in the project with many significant achievements on the technological side, for pre-analytics and for analytics. The different results and achievements from the tasks in WP6 are not independent of each other (see Figure 16) and many interim results, are not only usable achievements on their own, but also were pre-conditions for other items or next steps.

DNA Methylation Marker Candidates and Markers

At the beginning of the project, the marker candidate discovery in task 6.1 used the retrospective samples already collected before the project. This resulted in a quality controlled data set consisting of DNA methylation patterns for 383k sites on 86 biological samples (comprising 28 colorectal cancer, 28 paired normal adjacent tissue samples thereof, 20 patients with usable progression free survival (PFS) data, and 30 PBL samples from patients without cancer). From this data set, a subset of sites was generated that were likely to be unmethylated in lymphocytes and could serve as basis for Identifying marker candidates for a blood based test assessing methylation patterns of free circulating tumour DNA (details are found in the Deliverable D6.1 Epigenetic Marker Discovery Report).

A list of 197 DNA-methylation marker candidates which had the potential to become predictive assays detecting DNA-methylation patterns in tumour tissue or on free circulating tumour DNA in blood were generated. To do so, we used a set of meaningful criteria and survival analysis on data from 20 patients from the full data set and the subset (details are found in the report documenting the achievement of Milestone MS7 Validated list of 50-100 ranked DNA methylation biomarker candidates predictive for response to comb).

In a stepwise process, the 197 candidates were further filtered based on biological and technical parameters and developed into assays in Task 6.4. These assays were further optimized in Task 6.5. The approach led to a set of sensitive and precise assays for 6 marker candidates, 2 of them applicable to blood plasma (see also Deliverable 6.3, Epigenetic Assays Report). The overall strategy for finding, filtering and streamlining the markers and assays - as documented in D 6.1 and D6.3 – is currently being written up as a Methods style article and will be submitted for publication in the coming months.

The 6 marker candidates were assessed on plasma and tissue samples from CRC patients in Task 6.2 in order to verify their potential as biomarkers predictive for combination avastin treatment response (see also Deliverable D6.2 Diagnostic Accuracy Report). None of the marker candidates could be confirmed to provide predictive information for combination avastin treatment in sample sets independent from the discovery samples.

Despite the ostensible lack of utility for predicting combination avastin treatment response, the two resulting markers CORO6 and FOXF2 and their assays, represented interesting candidates for diagnostic and other applications in the field of cancer (they are negative in blood plasma from most healthy patients, and positive in free circulating tumor from diseased patients and in tumour tissue DNA from various cancers). Due to their potential, IP was filed on these markers in January 2016.

Technologies - pre-analytics and analytics

At the beginning of the project, work was commenced on pre-analytical methods to provide the basis for the DNA methylation marker candidate assessment based on blood plasma and biopsy samples collected and available later in the project. A clear objective was the automated processing of blood plasma on a robot in order to provide a reproducible pre-analytical workflow that is independent from biases introduced by different users or sites. The automated workflow was successfully established on the Invigenius™ robot from Stratec in a step by step approach and became the default method in WP6 to provide bisulfite DNA from technical samples (e.g. DNA from cell lines in blood-plasma like matrix), technical samples based on patient material (e.g. bulk plasma spiked with methylated DNA) and on clinical blood plasma samples. LOD experiments and a confirmation study on clinical samples using mSept9 marker demonstrated its valid usability as an alternative to the manual workflow.

Many existing sample collections that are interesting for DNA methylation analysis (within Angiopredict and beyond) consist of limited amounts of archived sample material. Therefore - in parallel with the automation of blood plasma workflow - a manual workflow was established that combined DNA extraction, purification and bisulfite conversion from single needle biopsies (formalin fixed paraffin embedded tissue (FFPET) on glass slides) with measurable amounts of template for realtime bisulfite PCR to be recovered. Commercially available methods used in a comparison were not able to yield comparable amounts from the same type of source.

The successful establishment of a HQM (quantitative heavyMethyl) duplex assay in the first half of the project was a precondition for measuring the marker candidates (as triplex) later in the study. It enables to set quantitative methylation marker measurements in relation to the amount of tumour and/or total DNA. The HQM assay is an adaption/extension of the HeavyMethyl (HM) assay technology that uses methylation specific blockers to sensitively measure methylated DNA and combines two HM assays, one for methylated DNA (e.g. tumour marker as in WP6, based on mSept9 as a surrogate marker for colorectal tumour DNA load) and for unmethylated background DNA measured at the same locus – both in parallel on independent bisulfite strands. Such an assay can safely be applied to DNA from sources in which measurements at different loci might distort proportion due to copy number effects. The duplex assay was optimized for later use until sensitivity for methylated DNA around 6 pg was achieved (equivalent of DNA from one cell). The HQM was successfully combined with the marker candidates found and assessed in the later parts of WP6, leading to triplex assays capable of sensitively determining background DNA, total DNA, tumour DNA and the DNA methylation marker candidate in the same assay.

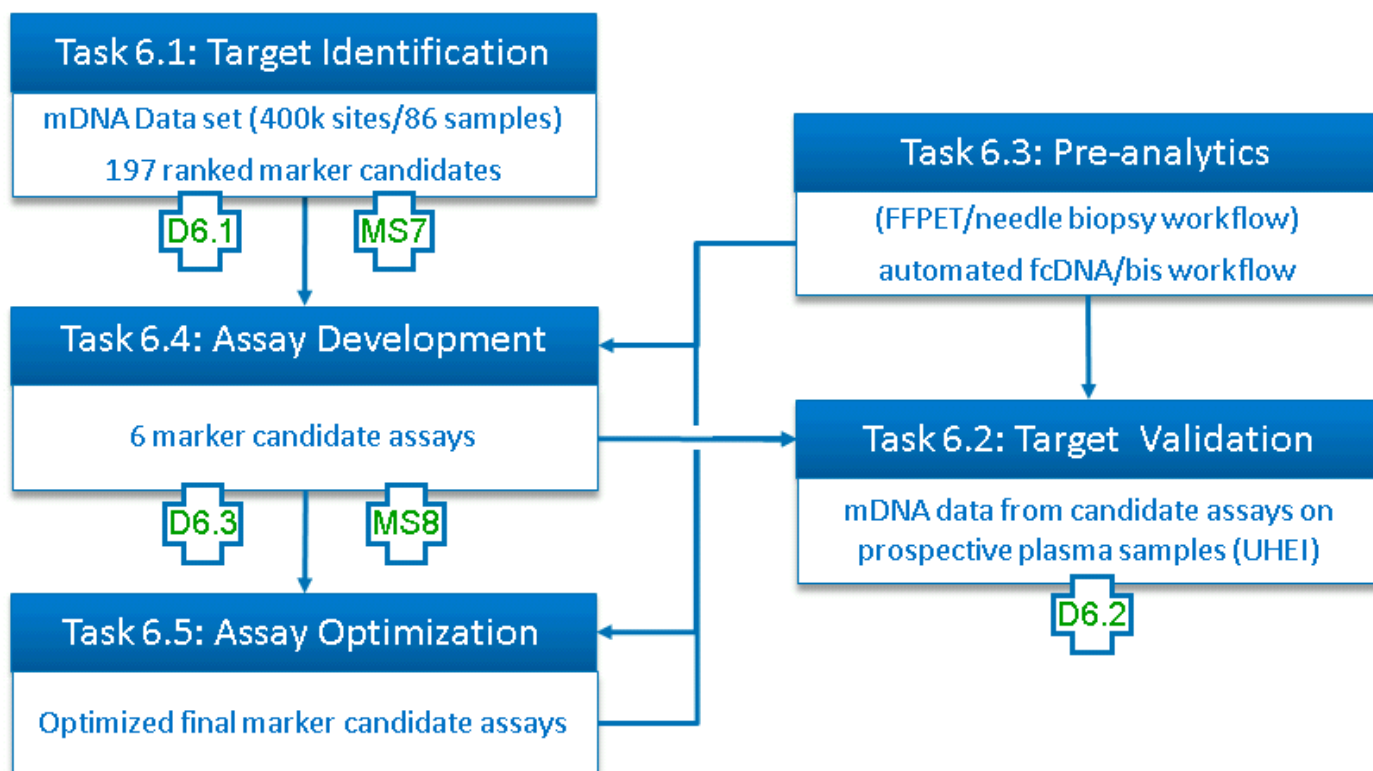


Figure 16 WP6 task Overview by Flowchart including the most relevant results of each task, dependencies, milestones and deliverables.

Table 6 WP6 - summary and listing of main results, achievements, deliverables and milestones by task.

<p>Task 6.1 Genome-Wide DNA Methylation Profiling: Target Identification [EPI]</p> <ul style="list-style-type: none"> • DMH-III based DNA methylation data set of about 400k genomic sites on 86 tissue samples (after QC) • Deliverable D6.1: Epigenetic Marker Discovery Report. Report on epigenetic marker discovery including list of marker candidates. • List of 197 ranked marker candidates for follow-up task 6.4 (MS7: Validated list of 50-100 ranked DNA methylation biomarker candidates predictive for response)
<p>Task 6.3 DNA Methylation Companion IVD: Pre-analytics Optimization [EPI]</p> <ul style="list-style-type: none"> • Protocol for a pre-analytical procedure for obtaining amounts of measurable bisulfite DNA from single needle biopsies removed from PET-glass slides • Automated workflow on the Invigenius™ for pre-analytical processing of blood plasma to gain bisulfite fc-DNA - used on technical and biological samples in follow-up experiments in task 6.2, 6.4, 6.5
<p>Task 6.4 DNA Methylation Companion IVD: Assay Development [EPI]</p> <ul style="list-style-type: none"> • Establishment of a HQM duplex measuring methylated fc tumour-DNA and unmethylated background DNA from blood plasma that became the base for all marker candidate triplex assays. • Triplex assay for 6 marker candidate assays surviving the step-by-step assay establishment. • 2 marker candidate assays usable in blood plasma (negative background), 4 in tissue. Used for optimization in task 6.5 and for measurement of patient samples in 6.2: Provision of 5-10 prototype methylation assays available for validation on clinical samples) Deliverable D6.3: Report on epigenetic real-time assays for 5-10 marker candidates.
<p>Task 6.5 DNA Methylation Companion IVD: Assay Optimization [EPI]</p> <ul style="list-style-type: none"> • Optimized oligomer compositions for assays used in task 6.2 • Optimized dye/quencher combinations for triplex assays used in task 6.2 • Assay characterization (LOD, LOQ) and assessment with digital PCR

- Applicability of other pre-analytic RUO workflows that are not IVD products.
- Marker characterization for cancer specificity/cross-reactivity with other cancers.

Task 6.2 Genome-Wide DNA Methylation Profiling: Target Validation [EPI/UHEI]

- DNA methylation data of 6 marker candidate assays on a) prospectively collected samples from blood plasma of CRC patients from UHEI, b) FFPE tissue samples of CRC patients from UCD, c) fresh frozen tissue samples of CRC patients from UHEI, and d) cell compartments from SOM did not confirm any correlation of DNA methylation with treatment response classification of patients.
- Deliverable D6.2 - Diagnostic Accuracy Report: Report on diagnostic accuracy of methylation marker panel in retrospective and prospective tissue collections

1.3.6.3 Conclusion

Despite the fact that potentially predictive DNA methylation marker candidates identified in the discovery phase could not be confirmed for such application in independent samples, the work package was a success leading to several results that are valuable for R&D, for medical applications and for commercial purposes, just to mention the three most important ones:

- **Two cancer biomarkers were found that due to the unmethylated background DNA in healthy individuals are measurable on fcDNA in blood plasma. Both are worth follow-up, from the clinical as well from the commercial view, and therefore IP was filed.**
- Automation of a previously manual IVD workflow based on exiting IVD pre-analytic kits that is now available for current and future applications for measuring DNA methylation markers in fcDNA in blood plasma.
- A duplex realtime PCR assay (HQM) that measures total DNA and estimates colorectal tumour DNA load down to one cell equivalent per ml, which can serve as basis for different triplex assays in which other third markers are to be quantified in relation to total and/or tumor DNA.

1.3.7 WP7 Predictive Pharmacogenomic Expression methods

1.3.7.1 Introduction

A primary aim of this WP was to identify predictive pharmacogenomic gene expression signatures in combination Bevacizumab (Bvz) treated responders vs. non-responders, by generating gene expression signatures that reflect the activation state of resistance pathways. The search for novel predictive signatures was performed in both whole tumour samples (UCD) and isolated tumour compartments from mCRC samples (SOM/VUMC). Tumour compartments were isolated [tumour endothelial cells (TEC), tumour associated macrophages (TAM) and isolated tumour cells (TC)] by SOM. The cells from different compartments were examined via gene expression profiling (VUMC). Candidate predictive gene signatures, at both tumour tissue and TEC-specific levels, were orthogonally verified on retrospectively collected materials using ISH (ONCO). The utility of putative multi-gene signatures were then assessed using retrospectively collected Angiopredict samples.

1.3.7.2 Work Undertaken and Results Achieved

7.1 Predictive gene signatures in whole tumors

RNA sequencing was conducted on 135 tumors, 104 from formalin-fixed paraffin-embedded (FFPE) tissues and 31 fresh-frozen (FF) tissues. Sequence data was quality controlled and aligned to the human reference genome (build hg19). Mean total reads was 69,020,000 (SD = 32,062,140), mean mapped was 62,200,000 (SD = 29,831,478), mapped following duplicate removal was 38,080,000 (SD = 17,643,251), and counts was 17,070,000 (SD = 8,400,217). To test whether FFPE and FF derived sequence were significantly different in numbers of reads we applied a Chi squared test to reads in each group (total, mapped, duplicate-removed and counted), which found

significant deviation from the expected distribution. **Response status including location in the linear model identified 28 differentially expressed genes in FFPE following false discovery rate (FDR) adjustment of significance to a 5% level.** Subtyping was conducted using the signature gene set from the CMS consortium (Guinney et al 2015), the most recent and definitive subtype set in CRC comprised of 4 classes. We have found that 37 samples have a 'predicted' CMS subtype. The CMS2 and 4 classes make up the majority with 16 and 18 respectively. In the total set the same trend is apparent, with 46 and 49 respectively. The CMS consortium has shown CMS2 and CMS4 to be the most abundant classes (24-31% and 26-56%).

RNA sequence data analysis has yielded count and FPKM data that have been submitted to the database for archiving. **Differential gene expression based on response status using FFPE data shows 28 genes have significantly altered expression based on response.** We used FPKM for molecular subtyping/classification using the CMS consortium defined gene panel (Guinney et al 2015). The RNAseq expression profiles for 37 samples with 'predicted' CMS class separated in a PCA into 2 clusters based on a large component of 32%; CMS2 is defined by the CMS consortium as being canonical, epithelial, marked WNT and MYC signalling activation associated; CMS4 is defined as mesenchymal, prominent transforming growth factor- β activation, stromal invasion and angiogenesis associated. FF data resulted in similar separation. **While direct stratification of Angiopredict samples using recently published CMS classifiers (Guinney et al 2015), did not define distinct Responder Vs Non-Responder clusters, expression data were further analysed and clustered as described in D 5.1. This analysis identified new CNV/CMS-related subtype classifiers with ability to stratify patients and predict response to combination avastin therapy in an mCRC setting.**

7.2/7.3 Isolation of Tumor cell compartments and Predictive gene signatures in tumor compartments

As TEC's are highly dependent on VEGF, in contrast to normal EC's, it was hypothesized that tumour vs normal cell compartment comparison analysis may reveal VEGF-induced/regulated genes predictive for response to combination Avastin therapy. Also tumour-associated macrophages (TAM's) have been described as pro-angiogenic by secreting growth factors. It was decided to isolate and profile these cells specifically to explore Avastin biomarkers. Therefore, in **task 7.2** tumours were separated in three different cell compartments: (i) EC's, (ii) TC's and (iii) TAM's. This approach identified genes that could not be detected using whole tumour comparisons as they accounted for only a small fraction of the whole tumour population.

Since tumour compartment isolation from frozen samples was not feasible, SOM initiated a strategy to collect fresh (i.e. unfrozen) CRC tissue from locally (VUMC) operated patients. For FACs cell sorting, single cell suspensions were stained with corresponding antibodies to sort EC's (CD31⁺CD45⁻) and epithelial/TC's (CD31⁻, CD45⁻, EPCAM⁺). Bulk samples were taken upfront for cell sorting. Due to technical difficulties in sorting, we adopted an alternative method for TAM isolation. In this method, bulk cells were transferred to a cell culture flask thereby enabling TAM's to adhere to the plastic, in contrast to all other cells. Non-attached cells were removed and attached TAM's were lysed with Trizol for RNA isolation. RNA from these samples, bulk samples and sorted EC/TC fractions with an RNA integrity number (RIN) above 6 were included for Agilent array hybridization by VUMC.

Comparing 13 tumour versus 10 normal endothelial cell samples revealed a list of 2610 probesets ($p < 0.05$). We focused on the top 500 genes with the greatest fold change and further selected genes based on known biological function, localization and expression distribution. We ended up with a candidate list of 10 genes. These were further validated (WP7.4, 7.5). For epithelial cells, we could compare 16 tumour versus 8 normal samples (4138 genes $p < 0.05$). For TAM's we compared 3 tumour versus 3 normal samples. This list includes the literature described TAM markers CD163 and CD68 and CD204 (MSR1).

7.4 Verification/validation of predictive TEC-derived gene signature via In Situ hybridisation

ONCOMARK established the in situ hybridization (ISH) platform to verify and validate target genes that were identified in other work packages. For this work, extensive research was performed initially evaluating which RNA ISH method would be appropriate for FFPE tissue. Two targets (CD34 a vascular endothelial cell marker and CDX-2 a marker for colorectal cancer) were used for initial optimization of the protocol to detect ISH signals on FFPE CRC tissue samples using ViewRNA technology (Periodic report October 2012). C7orf42 and TEM-8 are two markers that showed promising results as described in the periodic report (1 August 2013 to 31 January 2015). Later more careful evaluation revealed that these markers were not purely vascular specific markers. SOM further identified several

specific TEC markers, which have been validated by ONCO on normal/tumor pairs using qRT-PCR (Task 7.5). From this study, two markers showed increased expression in patients that responded to bvz treatment in comparison to non-responders by qRT-PCR. Therefore, these two markers were selected for further ISH studies. For this, we used RNAscope 2.0 Red Assay (Advanced Cell Diagnostics, Hayward, CA) as this assay has been significantly improved in terms of detection sensitivities by the vendor over the last few years (described in deliverable submitted 29/01/2016). Although the new assay resulted in excellent staining outcomes in CRC full-face test sections, unfortunately, several ANGIOPREDICT samples generated varying staining patterns with poor reproducibility. As we could not perform ISH on the existing ANGIOPREDICT samples due to technical limitations (relating to archive FFPE tissue), we decided to explore marker protein expression by immunohistochemistry to confirm differential expression between responders and non-responders. Fifteen responders and 15 non-responder tissue sections, along with corresponding healthy control tissue sections, were immunostained using commercial antibodies against the targets of interest.

Marker 1 expression was observed mainly in colonic epithelial cells and vascular endothelial cells. There were significant over expression of both markers in tumour colon tissue compared to normal colon tissue. Image analysis calculated the number of red dots in defined areas from both tumour and normal regions of stained sections. On average, 1050 mRNA positive red dots were detected for tumour colon tissue compared to only 50 dots from the normal tissue area for four out of five samples tested. In order to use image analysis software for the ISH signal detection, we developed and used a new algorithm called ISH-MARK. **Our IHC data indicates that protein of marker 1 is significantly overexpressed in tumour tissues from non-responders, as observed in our previous qRT-PCR findings (D7.3 last deliverable report). Although marker 2 mRNA was previously found to be overexpressed in tumour tissue from non-responders, this observation could not be confirmed at the protein level. However, we found that marker 2 protein is over-expressed in normal tissue from non-responders.**

7.5 Optimization of multiplex quantitative RT-PCR companion diagnostic assay

In this final report we summarise the optimisation of a candidate tumour tissue-derived predictive multi-gene signature through a quantitative RT-PCR-based (qRT-PCR) companion diagnostic assay. Taqman low density arrays (TLDA) were utilised to facilitate and contribute to the reproducibility of molecular diagnostics, which allow up to 24 targets to be measured simultaneously across 8 specimens. Validation of putative qRT-PCR companion diagnostic assays were performed on collected fresh frozen (FF) and formalin fixed paraffin embedded (FFPE) samples. Final gene expression signatures from equivalent FF and FFPE materials were cross-referenced; to further determine the robustness of the signature under different sample collection conditions. The relative quantification of target gene expression was achieved using the comparative C_T (delta-delta- C_T) method in which a two-fold discrimination at 99.7% confidence level is detectable. Final data analysis was performed using qBase+, which is the real-time PCR data-analysis software, based on the proven geNorm and qBase technology. No significant difference in expression between Bvz responders and Bvz non-responders could be observed for any of the genes assessed. However, several targets did show significant difference in gene expression in colorectal cancer tumour samples compared to normal tissues, which are described in the periodic report above. These expression signatures originating from FF or FFPE materials were cross-referenced and show robustness of these signatures under different sample collection conditions.

An initial gene expression signature of potential tumor-specific biomarkers and bvz response biomarkers were assessed on macro-dissected regions of pathologist determined tissue sections as part of the AngioPredict study. This pilot study was performed on a cohort of matching tumour versus normal samples originating from 30 patients. Two genes showed significantly increased expression in FFPE CRC tumour samples from bvz responders compared to bvz non responders. These 2 potential biomarkers were further validated in an expanded patient cohort via qRT-PCR using specific primer probes.

To further validate the initial findings, a larger patient cohort was tested by qRT-PCR for two sets of genes. The specific targets were identified by 2 partners. One set of targets originated from NUID UCD and identified targets through coupling RNAseq data to clinical response data from patients treated with bvz. The other set of targets provided by SomantiX distinguishes tumour versus normal tissues. These final experiments and results were generated to validate targets to either determine bvz response or differentiate between tumours versus normal

tissue samples. All data has been analysed according to study design, as well as origin of samples. The identified significant targets can potentially be useful as markers in further ISH experimentation.

7.6 Development of Commercialization Plan for Findings

ONCO is focused on developing biomarkers in the oncology space, therefore are particularly interested in biomarkers that could predict response to Avastin. Here ONCO worked closed with NUID UCD and SOM to evaluate potential biomarkers from their respective analysis using ISH and qRT-PCR. Two biomarkers showed some early promise, however further validation on large cohorts would be required before we would consider commercialisation. Another potentially commercial outcome for this project was the development of ISH-MARK software. This is based on ONCOs IHC-MARK software for quantifying IHC staining. This software could be used by researchers to aid in developing ISH based biomarkers, and will be included as part of the companies suite of software tools.

1.3.7.3 Conclusion

In WP7 NUID UCD have conducted RNA sequencing on 135 tumour samples and conducted a thorough alignment and analysis from this data. We have found a number of genes to be differentially expressed based on response category defined by 10 months PFS which were available for validation by partners. We have also generated a set of expression data useful to partners (WP5 & WP9) and critical to the identification of novel combination avastin classification subtypes described.

SOM were able to isolate a sufficient number of cells of bulk, EC, TC and TAM fractions from a descent number of patients. Top 500 genes were identified out of total 2610 genes detected in the endothelial cell fractions. Several candidates were picked from this list based on their biological function, localization and expression pattern; they were tested by qRT-PCR for Avastin responder vs non responder expression differences. For TAM, several well characterized markers such as CD 204, CD163 and CD68 were also identified. From 18 of these samples, DNA was send to Epigenomics for methylation profiling.

ONCO confirms that markers identified by transcriptomic profiling can be verified by ISH. Unfortunately, ISH is challenging on FFPE tissue samples, especially those preserved for a long time. Our established methods for marker ISH can be used on newer sets of patient samples from bvz treated patients, avoiding samples that have been stored for long. We have also developed a new software approach, ISH-MARK, specifically designed to facilitate quantification of ISH signals. ONCO has also optimized the qRT-PCR assay by post qPCR quality control data, geNorm analyses for each origin of sample set (FFPE or FF) for each experiment (bvz response or tumour versus normal studies). Two separate study designs were set up for each set of targets provided by partners for either the bvz response study as well as the tumour versus normal tissue study. Several interesting targets have been identified for the tumour versus normal tissue study and, pending further validation of these targets, they could be potentially useful as diagnostic markers/novel therapeutic targets.

Both ONCO and SOM have developed a commercialization plan based on this work package. ONCO will focus on perusing the validation of these two markers on a large cohort of samples. SOM is currently developing therapeutic monoclonal antibodies against targets described in this work package.

This work package has generated data for four manuscripts; most of them are in preparation currently. ONCO and SOM are working together on a joint manuscript titled 'predictive biomarkers 1 and 2 for Avastin responsiveness in CRC patients'. The first draft of the manuscript is already prepared and expected to be submitted in May/June 2016. Below is a table for expected publications from this work package.

Table 7 Expected Publications from WP7

<i>Title</i>	<i>Organization/WP</i>	<i>Expected submission date</i>
Colon tumour vascular gene signatures reveal novel CRC subtypes	Somantix/ WP 7.2; 7.3	Dec 2016
New biomarkers for avastin-responsiveness in colon cancer patients	Somantix/ WP 7.2; 7.3	Dec 2016
Expression Biomarkers for Colorectal cancer using ISH & IHC	Oncomark/ WP 7.4	June 2016
Expression Biomarkers for Avastin response in Colorectal cancer using qPCR	Oncomark/ WP 7.5	June 2016

1.3.8 WP8 Bioinformatics Database & Exchange Portal

1.3.8.1 Introduction

Objectives of this work package were to establish an efficient means of data storage and transfer between the partners, and also facilitate data querying, pooling, comparing and joint analyses. The aim was thus to enter clinical data, as well as data from germline mutations, somatic mutations, whole tumor expression signatures, DNA methylation signatures, expression signatures for TEM, TAM, TC.

Connections / dependencies to other WPs:

- WP2: Patient and samples, (ICORG).
- WPs 4 and 5: Germline /Somatic mutations and Copy Number Variations, all assessed by exome sequencing, (VIB, UCD and VUMC respectively).
- WP6: Methylation data in both whole tumour samples and in isolated tumour compartments, assessed by custom methylation-specific microarrays, EPI.
- WP7: Gene expression data in whole tumour samples, assessed by RNA sequencing, (UCD) and gene expression data in isolated tumour compartments (tumor endothelial cells, tumor associated macrophages and isolated tumor cells), assessed by Agilent microarrays, (SOM).

1.3.8.2 Work Undertaken and Results Achieved

A consistent and unified clinical retrospective data set was produced to align together 4 retrospective cohorts. This clinical data set is central to the project and has been used to perform analysis of all individual omics' data sets, as well as integrative analysis with multiple 'omics data sets. We have also put together a prospective data set in the same format as the retrospective data set. The prospective data set is used for validation of the omics' assays results.

A relational MySQL database was made available for the retrospective data set (both patients and samples), integrated with the results from the following omics' data: CNV, somatic SNPs, germline SNPs. The MYSQL database contains partial information related with the methylation and RNAseq data sets. Figure 17 shows the overlaps of the 'omics datasets (apart from the Methylation assay data set which is smaller). 173 patients had matched somatic SNPs, germline SNPs and CNV data while 40 patients had somatic SNPs, germline SNPs, CNV and RNAseq for integrative analysis.

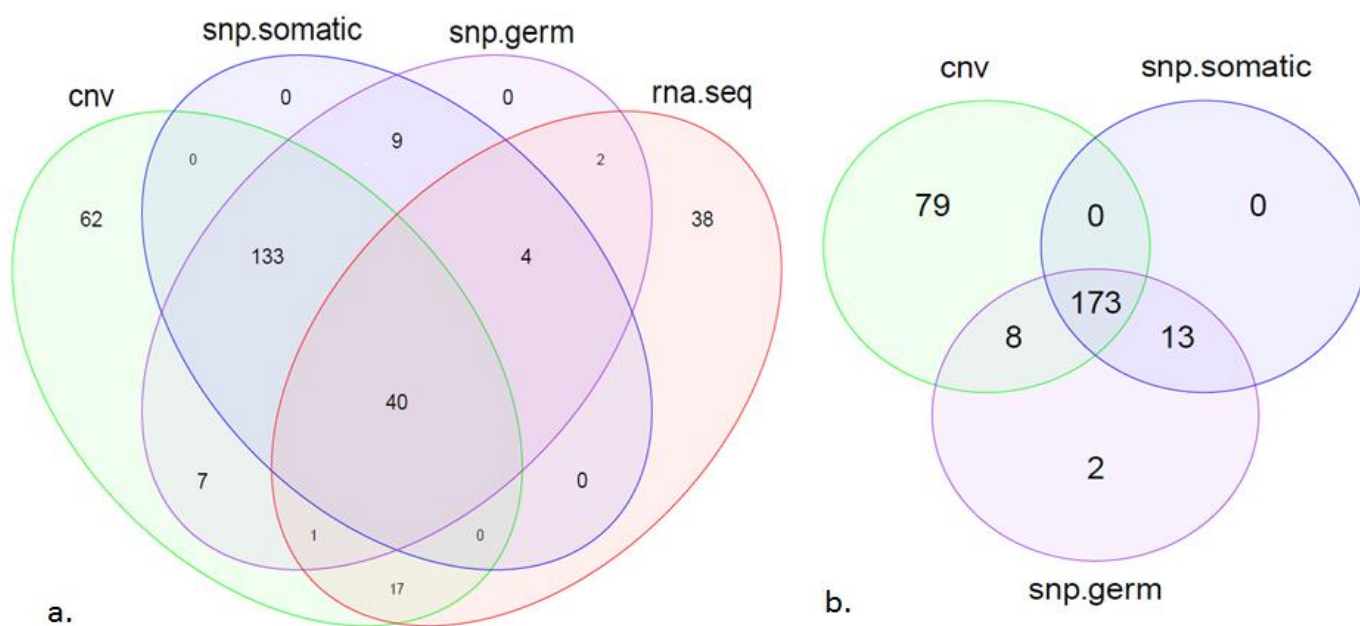


Figure 17 Overlap between various ‘omics data sets, produced during the Angiopredict project. **a.** Overlaps among CNV, somatic SNP, germline SNP and RNAseq (expression data). **b.** Overlap among the 3 largest data sets (CNV, somatic SNP, germline SNP) that have been introduced in the MySQL data base.

Figure 18 shows how individual omics’ data sets were represented in terms of cohort, samples with known response as well as number of examined features and data storage details.

	CAIRO	RCSI	UHEI	VUMC	All	Nb features	MySql	File System
SNPs all	100	20	43	24	187	32456	+	+
SNPs known resp.	99	17	27	23	166			
Germ all	105	21	45	26	197	78697	+	+
Germ known resp.	104	18	29	25	175			
CNVs all	103	18	60	79	260	449	+	+
CNVs known resp.	102	16	39	77	235			
RNAseq all	0	18	84	0	102	48683	-	+
RNAseq known resp.	0	14	60	0	74			
Meth	0	0	28	0	28	404594	-	+
Meth known resp.	0	0	16	0	16			

Figure 18 Information of how individual Omics’ data sets were represented in terms of cohort, samples with known response as well as number examined features and data storage details.

All data sets, including Agilent expression profiles for tumour compartments made available by SOM are maintained in an organised file system available at RCSI. Both MySQL backups and the folder with all data sets' files are available from RCSI to all partners. A data querying interface prototype is available for the MySQL database and is focused on visualising germline and somatic SNPs and linking SNPs annotations to other online data bases. The interface also permits data extracts downloads. This interface was coded in PHP and was designed to be used locally. The code of this interface is also available at RCSI.

1.3.8.3 Conclusion

Data has been collected and processed from Angiopredict work packages. Data has been imported in a relational MySQL database (using PHP scripts) and all data is available in a file system repository in classical formats ready for analysis. Efforts in this work package were further deployed to create a unified clinical dataset integrating four cohorts. This data set was used for analysis of all individual omics' data sets (WP4, 5, 6, 7) and also for integrative analysis of the omics' data sets (WP9).

1.3.9 WP9 Integrated analysis of data using statistical inference and computational systems approaches.

1.3.9.1 Introduction

The aim of the WP9 was to use statistical and systems biology approaches to analyse and identify individual biomarkers and combinations of biomarkers that predict therapy success.

1.3.9.2 Work Undertaken and Results Achieved

Task 9.1 Predictive Gene Cluster Report

The aim for Task 9.1 was to superpose omics' data issued from AP patients with gene / protein interaction networks in order to derive combined knowledge, particularly in the form of clinically relevant patient stratification.

The work undertaken in Task 9.1 consisted of using an unbiased method (Network Based Stratification, NBS) relying on combining pan-genomic somatic SNPs identified in 187 Angiopredict patients with publically available gene / protein interaction networks. This method is based on the hypothesis that that a somatic SNP related to a gene and impacting the quality/quantity of the encoded protein, will also have an impact on the proteins that are in the same gene-protein interaction network. The main problem with whole exome (or whole genome) mutation profiles, like the one described here, is that they are very sparse (are arranged in a matrix containing too many 0s compared to the 1s) because somatic mutations are relatively rare. This kind of data set is difficult to employ for collective analysis such as clustering. Integrating SNP data with gene interaction networks data not only enriches the data set with new knowledge, but also leads to a more tightly packed, continuous data set, (see Figure 19). This new matrix contained what is called 'smoothed' mutation profiles.

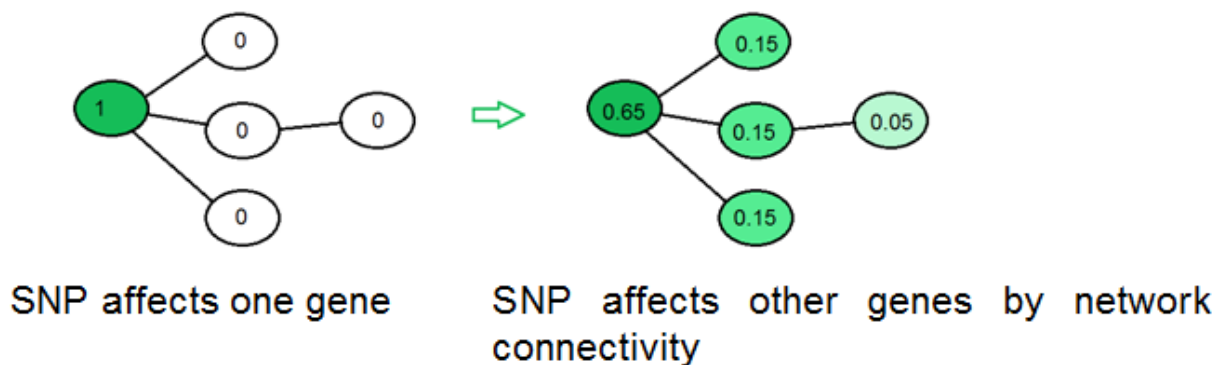


Figure 19 Principle of propagation a mutation effect on a gene interaction network neighbourhood

Applying this method, two clinically informative patient subtypes were identified, designated NBS1 and NBS2, of which NBS2 is characterised by significantly improved PFS compared to the NBS1 ($p=0.006$). Furthermore, we identified molecular network modules commonly mutated in each of the subtypes. Strikingly, NBS2 was associated with a gene interaction network module containing many of the main angiogenesis effectors and genes associated to angiogenesis-related cellular processes. These results suggest that several of the detected mutations may have physiological impact, affecting the main angiogenesis pathways in ways relating to improved response to combination therapy. A panel of 14 mutations which are specific of NBS2 and occur more frequently than other mutations were selected for a potential collective panel biomarker on the basis of mutation annotations and is subject to further validation.

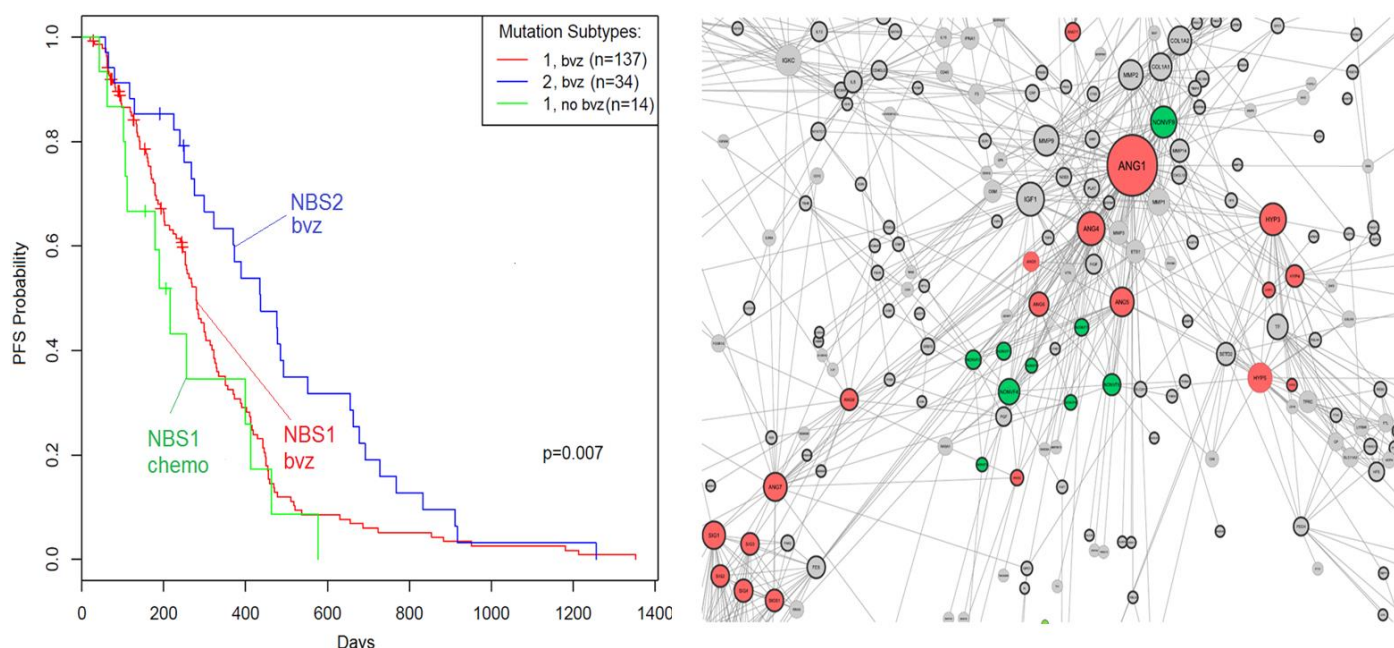


Figure 20 Left: Association of NMF consensus-clustering based subtypes (with the number of predefined subtypes $k=2,3$ and 4) with patient progression-free survival time: Kaplan–Meier survival plots for NBS subtypes for 171 AP patients treated with combination bvz therapy and 15 AP patients treated with chemotherapy only. P-value - computed using the logrank test. Right: A gene-interaction network module, involving the main angiogenesis pathways, is found associated with the better PFS patient subtype (NBS2). Thicker contour lines around nodes – genes that actually carry mutations. Red: genes in the VEGF-axis angiogenesis pathway. Green: genes belonging to the non-VEGF modulators of angiogenesis.

Results from Task 9.1 were used as input into WP9.3, in order to introduce individual patient information to the Cellular Automata (CA) model for spatiotemporal tumour proliferation, by integrating a molecular pathway-based layer to the overall model. This is used to study effect of multiple mutation interplay in overcoming tumour's compensatory mechanisms to develop resistance to combined bvz therapy.

Task 9.2 Hybrid Models Report

The main aim of this task was to identify biomarkers via integrative statistical and systems biology approaches for successful application of combination Bevacizumab therapy in CRC patients. The input for this task was from AP WPs 3, 4, 5, 6 and 7 which have been pre-processed by collaborators and accessed through AP data base (WP8). **The main output from this task included a list of biomarkers responsible for Bevacizumab therapy success.** Boolean logic modelling was initially proposed for this task, however, Boolean models have been increasingly criticised in recent years as these are qualitative rather than quantitative in nature. We have therefore implemented hybrid statistical and network based integrative methods for ANGIOPREDICT high-throughput 'omics data sets.

(i) Integrative statistical model

Principle component analysis (PCA) is one of the commonly used statistical methods while dealing with high-throughput 'omics data sets although it has some disadvantages, including the inability to associate a probabilistic model or provide any further insight into data structure (Nyamundanda, Brennan et al. 2010). For this reason we introduced a sparse Bayesian factor analysis model (sBFAC) to integrate AP 'omics data which has the advantages of both PCA and probabilistic models. The sBFAC model was initially performed over all combination sets of AP data sets (although, the methylation data set was later excluded due to small overlap). **Amongst all the different combination sets, the sBFAC model performed with copy number alteration (CNA), somatic mutation data sets and phenotypic information plus the clinical data gave rise to 4 patient clusters which included two patients clusters with better response compared to the other two ($p = 0.00586$). Further analysis of these clusters resulted in identification of 387 CNA regions and 23 somatic SNPs that are significantly correlated with therapy outcome.**

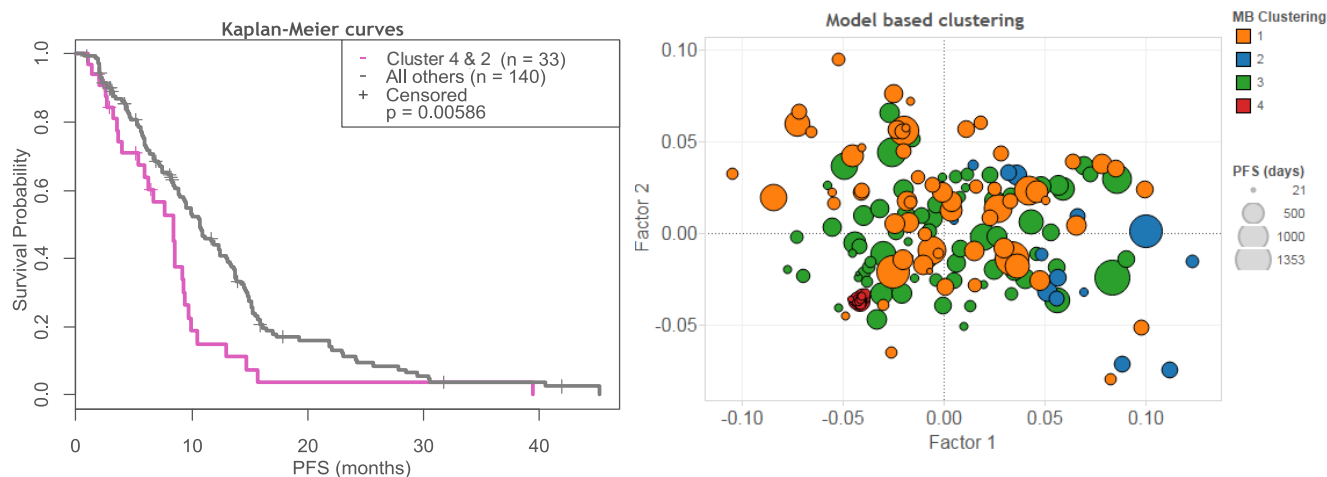


Figure 21 sBFAC result from integrating CNA and somatic mutations data sets and PFS Kaplan-Meier curves of cluster 2 and 4 against two other clusters. Patients were groups in 4 clusters by sBFAC model using CNA and somatic mutation data sets. If patients from cluster 2 and 4 are pooled together, the new group have a significantly worse progression free survival ($p = 0.00585$).

(ii) Integrative network based model

Network modelling techniques are important tools for analysing genomics data but with increasing numbers of 'omics data sets, they are facing a time limiting problem. Methods that are based on Sparse Inverse Covariance Selection (SICS) overcome the big data problem, as they are much faster than other methods (Kling, Johansson et al. 2015). The result from applying this technique to AP data sets was an interconnected network that firstly gave information about important hubs in Angiopredict cohort landscape, and secondly could be used as a tool for investigating differences between patients outcome. The result from integrating mRNA, CNA and somatic mutations data sets using this method was a network consisted of one main interconnected network with 297 nodes and three distinctive sub-networks. One of these sub-networks consists of 129 mRNA and 2 somatic mutation nodes. Gene ontology enrichment of this sub-network using DAVID database (Huang da, Sherman et al. 2009) showed enrichment for ontology terms such as regulation of blood vessel size, vascular process in circulatory system, blood circulation and regulation of blood pressure, making it highly related to angiogenesis.

Task 9.3

The CA model of avascular tumour growth was firstly developed and validated with experimental data by good adjustment of model parameters. The model was further developed to include angiogenesis and due to high computational burden of solutions for the CA model a slice of tumour model was simulated instead of the whole tumour. Once the CA model for the slice of tumour was established, the effects of treatments with FOLFOX and

Bevacizumab were added into the model at certain time points. In particular combination of the two treatment regimes in different schedules was introduced in the CA model. The following figure shows the simulated tumour growth response to control, Bevacizumab, FOLFOX, Bevacizumab, therapy followed by FOLFOX, or FOLFOX followed by Bevacizumab.

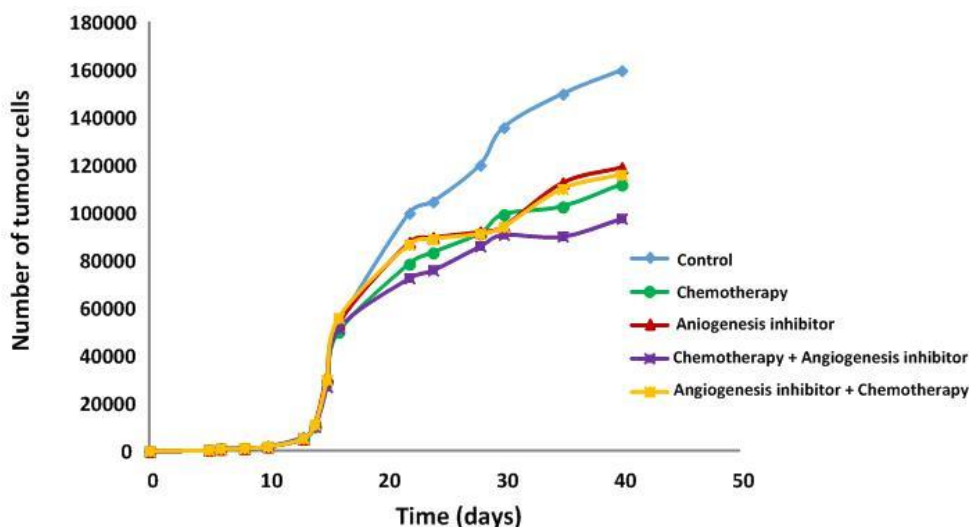


Figure 22 Simulated tumour growth curve and response to treatment with the FOLFOX and Bevacizumab. The model was simulated once due to requirement of high computational resources.

The tumours being treated on all drug regimens were smaller than the control tumour. The combination of FOLFOX and Bevacizumab inhibited tumour growth, while the others showed similar effects on tumour development. The results suggested that FOLFOX may enhance the effect of Bevacizumab when given before Bevacizumab and that Bevacizumab therapy may reduce influx of FOLFOX if given before FOLFOX. The model predictions for tumour growth were validated by HCT116 xenograft model studies (Task 9.4) in both drug schedules. The CA model then incorporated genomic signalling information to predict tumour growth based on gene mutations. One gene (NRP1-) was selected and incorporated into the model with the resulting following tumour behaviour:

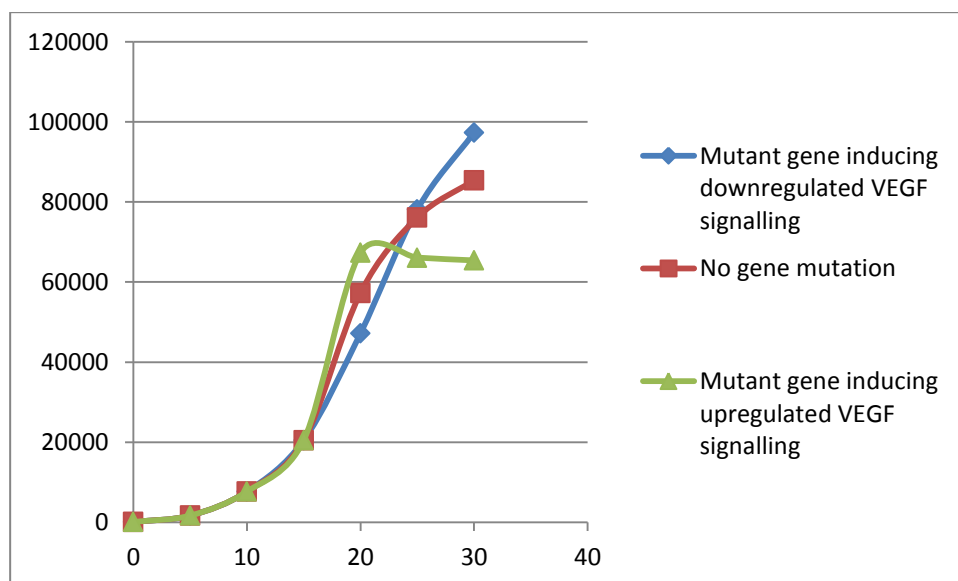


Figure 23 Simulated tumour growth curve responding to mutant gene when treatment with combination of FOLFOX and Bevacizumab was introduced.

It was shown that (NRP-1) mutation inducing gain of VEGF signalling resulted in reduced tumour volume when combination of FOLFOX and Bevacizumab was applied, indicating importance of this gene mutation.

Task 9.4

The ability of CA models (as described above) to predict response to combination Bev therapy was assessed and validated using *in vivo* tumour growth, survival and immunohistochemical data from clinically relevant mCRC xenograft studies. Model simulation data was also compared with histological findings both from small animal xenograft studies and is currently undergoing comparison with retrospective/ prospectively collected clinical samples through analysis of microvessel density (MVD), tumor cell proliferation (Ki 67 staining) or cell death.

FOLFOX/ Avastin efficacy/ validation study: Following on from a successful pilot study confirming the dosage of FOLFOX and Bev, 48 Balb C nu/nu mice were implanted with 2×10^6 HCT116 in the right flank as previously described and tumours were allowed to develop until tumours reached 200mm^3 .

Treatment Schedule 1 Avastin followed by Folfox:

Subsequently, animals were divided into groups ($n=12$) and treated with either vehicle (5% glucose and PBS) or the previously determined clinically relevant doses of Bev [10mg/kg, IP once a week] and FOLFOX [Folinic acid 13.4 mg/kg, 5-FU: 40 mg/kg, Oxaliplatin: 2.4 mg/kg., IP once a week 24hrs after Bev] either alone or in combination for 4 weeks (see Figure 24 for outline of time line of treatment). Tumour response was assessed by calliper measurement. At 4 weeks all animals were euthanized and tissue was collected for further immunohistochemical analysis.

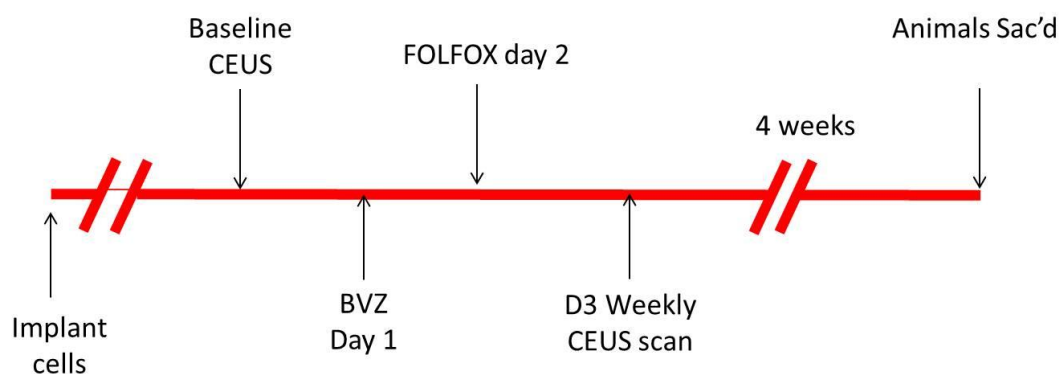


Figure 24 Experimental time line. Tumours were allowed to develop to 200mm^3 and the treatment regimens commenced. Avastin was given at day one and 24hrs later the FOLFOX cocktail was administered. This cycle of dosing 4 weeks (4 cycles of therapy) and then animals were euthanized and the tumours removed for future analysis.

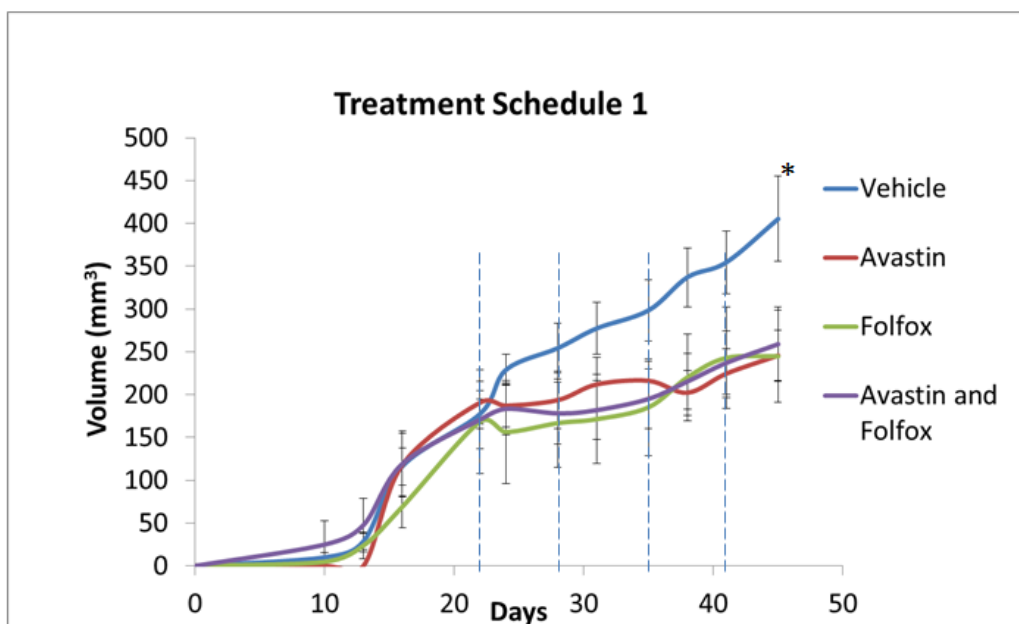


Figure 25 Treatment Schedule 1: HCT116 tumour growth curve and response to treatment with the chemotherapeutic agents Bev, FOLFOX and a combination of both drugs (n=12 per group) Avastin: 10mg/kg Folinic acid 13.2 /kg, 5-FU: 40mg/kg, Oxaliplatin: 2.4 mg/kg. Treatment commenced on day 23 was continued once weekly until end of experiment. *p<0.05 n=12 per group.

Treatment Schedule 1: Figure 25 indicates the response of the HCT116 mouse model to weekly dosing of Vehicle, Bev, FOLFOX, or a combination of Bev and Folfox beginning at day 23 post implantation of tumour cells. Tumours in the control animals were larger than those being treated on all drug regimens. The combination of Bev and FOLFOX significantly (p=0.0483) inhibited tumour growth. However, this response was not significantly different to treatment with Bev alone.

As data from this initial study did not replicate efficacy as seen in the clinic, we re-considered our experimental design and changed the drug sequencing to better reflect the clinical treatment paradigm.

Treatment Schedule 2 (TS2):

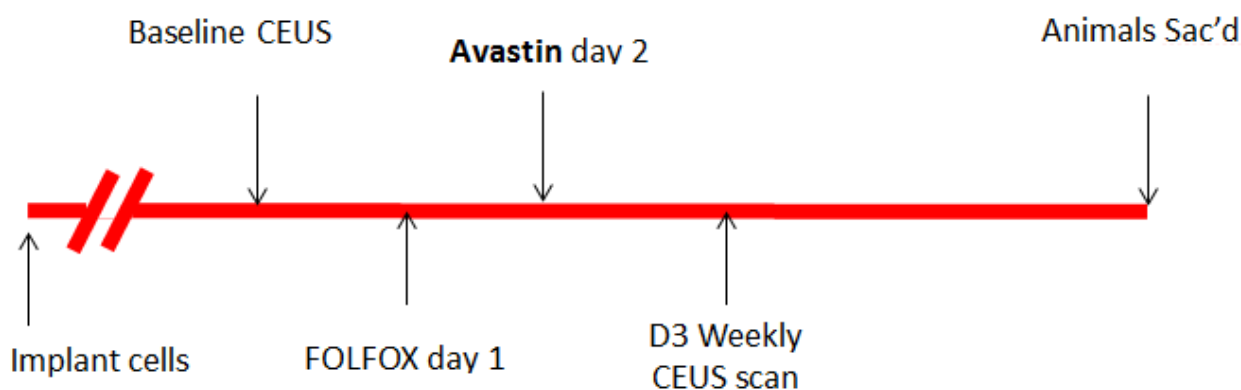


Figure 26 Treatment Schedule 2: Experimental time line for TS2 efficacy study. Tumours were allowed to develop to 200 mm³ and the treatment regimens commenced. FOLFOX was given at day one and 24hrs later Bev was administered. 24hrs after dosing weekly CEUS was performed. This cycle of dosing continued for 4 weeks (4 cycles of therapy) and then animals were euthanized and the tumours removed for future analysis.

This new dosing regimen follows the approach of oncologists in the clinic, enhancing the relevance of our model.

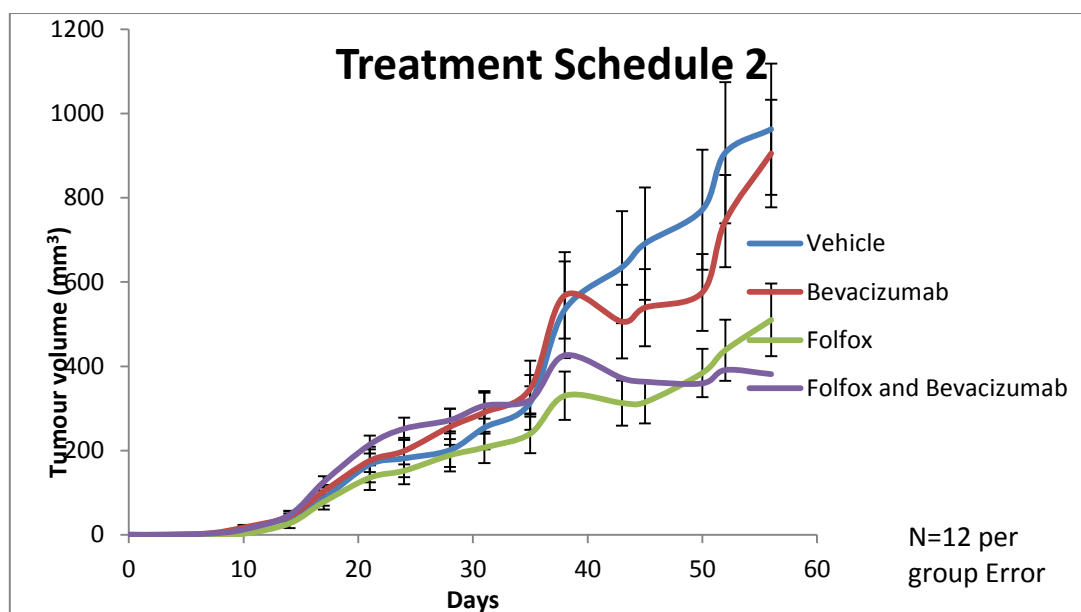


Figure 27 HCT116 tumour growth curve and response to treatment with the chemotherapeutic agents Bev, FOLFOX and a combination of both drugs (n=12 per group) Avastin: 10mg/kg Folinic acid 13.2 /kg, 5-FU: 40mg/kg, Oxaliplatin: 2.4 mg/kg. However this plot shows the effect of giving FOLFOX 24hrs before Avastin*= $p < 0.05$ n=10 per group.

Figure 27 indicates the response of the HCT116 mouse model to weekly dosing of Vehicle, Bev, FOLFOX, or a combination of both (TS2). This cell line model accurately replicates the response seen in the clinic in patients. Tumours in the control animals were larger than those being treated on all drug regimens. The combination of FOLFOX and Bev significantly ($p < 0.05$) inhibited tumour growth. However, in TS2 the combination of FOLFOX before Bev was significantly different from the mono therapies. Both FOLFOX alone and Bev alone show signs of stasis after one dose but then continue to grow by the second dose of drug. The combination displays decrease of tumour volume and then a static growth curve for the rest of the therapy. The growth curves are suggestive, however, of FOLFOX having a great effect when given before Bev compared to TS1. With these data validated CA model predictions (Task 9.3), in the previous experiment (Treatment Schedule 1), it is possible that vascular shut down occurs following Bev treatment with a resultant reduced influx of FOLFOX into the tumour.

1.3.9.3 Conclusion

Task 9.1

Integration of whole-exome based somatic SNPs from 187 AP patients with publicly available gene/protein interaction networks revealed two clinically relevant patient subtypes (NBS1 and NBS2), one (NBS2) relating to better PFS. Each subtype has been related to specific modules of gene interaction networks and of particular interest is that NBS2 was related to an angiogenesis-effector enriched gene interaction network module. Mutations more frequent in NBS2 have been annotated with multiple information and a mutation panel has been put forward for further study as a combined biomarker set for improved PFS for bevacizumab combination therapy.

Task 9.2

Both modelling approaches from task 9.2 have shown the importance of multiple 'omic integrative methods. **The statistical model gave rise to a set of biomarker somatic SNPs and CNA regions for Bevacizumab therapy and the network based model results can be used as a frame work for constructing a predictive mathematical model of**

angiogenesis signalling pathway. Such predictive models can potentially be used in the future by medical professionals as decision making tools for implementing combination Bevacizumab therapy in late stage colorectal cancer patients.

Task 9.3

The cellular automata model accurately predicts the growth of an in vivo CRC xenograft model in two drug schedules. The CA model predicts that chemotherapy given before Bev shows greater efficacy than Bev given before chemotherapy. The CA model displays similar efficacies of combination therapies as with monotherapies in treatment schedule 1. Finally, the CA model suggests that mutant NRP-1 inducing upregulated VEGF signalling may be important for response to Bevacizumab.

Task 9.4

We have established a model (Treatment schedule 2) which accurately replicates clinical response to combination Bev treatment. The cellular automata model accurately predicts the growth of the HCT116 xenograft model in both given drug schedules. The CA model predicts that chemotherapy given before Bev shows greater efficacy than Bev given before chemotherapy.

1.3.10 S&T Overall Conclusion

The IRISH CLINICAL ONCOLOGY RESEARCH GROUP (ICORG) has developed a tissue biorepository which provided both retrospective and prospective tissue for downstream omic analyses. ICORG further worked with clinical collaborators at the UNIVERSITY OF HEIDELBERG (UHEI) to complete recruitment on the AC-ANGIOPREDICT trial. A large tissue cohort from the consortium retrospective and prospective biorepositories was processed for DNA & RNA extraction by partners at UNIVERSITY COLLEGE DUBLIN (NUID UCD) and ONCOMARK LTD (ONCOMARK). Through collaboration of partners at VIB Leuven (VIB), NUID UCD and VREI UNIVERSITY MEDICAL CENTRE AMSTERDAM (VUMC) an exome-sequencing approach was implemented to generate pan'omic datasets and identify novel predictive biomarker signatures from combination bevacizumab treated patient samples. EPIGENOMICS (EPI) optimized DNA Methylation Companion IVD assays. Several marker candidates were deemed to have significant utility as valuable biomarkers for diagnostic and/or prognostic purposes in colon and other cancers. Thus, priority filing of a patent application (January 2016) was completed. The search for novel predictive signatures was performed in both whole tumor tissues (NUID UCD) and isolated tumor cell compartments from metastatic colorectal cancer (mCRC) samples (SOM & VUMC). The predictive potential of recently published multi-gene signatures (molecular subtypes) was also assessed, and novel response prediction molecular clusters described. Through previously described methods, pioneered by researchers at SOM, tumor compartments from CRC patients were isolated, including tumor endothelial cells (TEC), tumor associated macrophages (TAM) and isolated tumor cells (TC). Cells from different compartments were profiled for gene expression differences (VUMC). Candidate response prediction genes, at both tumor tissue and TEC-specific levels, were verified on retrospectively collected materials using in situ hybridization (ISH) (ONCO). Identified candidates were further evaluated using qRT-PCR assays by ONCO. The ROYAL COLLEGE OF SURGEONS IN IRELAND (RCSI) provided the consortium with a fully optimised relational MySQL database for both retrospective and prospective omics and clinical data sets. RCSI further employed statistical analysis and systems biology approaches to analyse and identify individual biomarkers and combinations of biomarkers that predict combination bevacizumab therapy success. An unbiased approach(network based stratification) defined a new method to stratify Angiopredict patients into two subtypes with significantly different survival outcomes (PFS).Moreover, application of an integrative statistical model over two data sets (CNA and somatic SNPs) also defined two patient clusters having a significantly better treatment outcome. A cellular automata multi-scale agent based model developed by RCSI predicted that chemotherapy given before Bev shows greater efficacy than Bev given before chemotherapy. These findings were validated using in vivo models and molecular imaging. This CA model further incorporated genomic signalling information to analyse effects of gene mutations on tumour behaviour and treatment response. Moreover, molecularly subtyped (expression) colorectal cancer cell line xenograft models were implemented to further demonstrate differential response to combination avastin therapy in vivo. ANGIOPREDICT has thus united world-class molecular diagnostic SMEs [EPI, SOM, ONCO] with leading clinical

[UHEI, VUMC, ICORG] and academic [VIB, UCD, RCSI] partners to identify a new generation of individualized methods for stratification of mCRC patients receiving combination bevacizumab therapy.

1.4 *Impact, dissemination and exploitation.*

1.4.1 Impact and exploitation

1.4.1.1 *Overview*

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females in the developed world. Currently, an important treatment for these patients is the drug Avastin® (bevacizumab), which inhibits tumour blood vessel formation and thus prevents tumour growth. Up to now, the underlying genomic mechanisms that determine whether or not a patient will respond to Avastin® combination therapy have been unknown.

ANGIOPREDICT has focused on research that works towards the realisation of a precision medicine paradigm for patients with advanced colorectal cancer. In the project, academic cancer biologists and industry based biotechnology researchers have worked together with clinicians to identify biomarker strategies that predict whether individual colorectal cancer patients will respond positively to Avastin® combination therapy. The ultimate goal is to help develop diagnostic tests using these biomarkers, in order to provide clinicians with the means to predict patient treatment responses in the future. There are obvious benefits to this approach, including the prevention of unnecessary treatment, the avoidance of possible side effects, the saving of critical time in patient care (so that treatments more appropriate to the individual may be pursued), the improvement of quality of life and cost efficiencies for the health system.

There exists an urgent need for predictive combination *bvz* biomarkers in mCRC patients to better progress towards a more personalised treatment paradigm. Through the application of an integrative approach ANGIOPREDICT is uniquely poised to play a major role in this space. The project also offers participating SMEs several key commercial opportunities for consolidation and extension of current activities. The perceived impact of ANGIOPREDICT at the level of the patient, physician, regulatory body and public health agency is thus significant and will likely in the future impact on several cancer indications for which treatment with *bvz* and other anti-angiogenic agents has become standard of care. This collaborative paradigm supersedes any individual clinical, basic science, or industry based attempts to date at developing predictive genomic methods in these complex scenarios.

ANGIOPREDICT has the identification of a predictive genomic biomarker for combination *bvz* therapy as a primary objective. However, it should be noted that the secondary objective of the trial is to prospectively examine the efficacy of FOLFOX+ *bvz* therapy as a first line therapy for k-ras mutant mCRC patients. Efficacy data to date for this combination regimen has been extracted from large scale Phase 3 trials having alternative primary or secondary objectives. Currently there are no other data available with regard to the role of *bvz* in this combination first line treatment. Thus, an important ultimate outcome of the AC-ANGIOPREDICT trial will be to inform mCRC patient treatment protocols.

1.4.1.2 *Key achievements*

Angiopredict has achieved significant advances towards the ultimate goal of delivering biomarkers and diagnostic tests to predict response to Avastin® combination therapy. Our research has involved the use of several different and complex technologies, about which we have also developed new knowledge during the course of the project. Some of our research results and exploitation plans must naturally remain confidential at the present time. Full details were provided in D1.4 (Exploitation Plan - a confidential report provided to the Commission). However, we can confirm that significant successes achieved include:

- Identification of novel predictive Copy Number Alternation biomarker signatures.
- Priority filing of a patent application (January 2016) to protect valuable methylation biomarkers for diagnostic and/or prognostic purposes in colon and other cancers.
- Identification of novel predictive genes and confirmation of the predictive potential of recently published multi-gene signatures (molecular subtypes) was also assessed, and novel response prediction molecular clusters described.

- New NBS method to stratify Angiopredict patients into subtypes with significantly different survival outcomes (PFS).
- Application of an integrative statistical model over two data sets (CNA and somatic SNPs) also defined two patient clusters having a significantly better treatment outcome.

In addition to the above advances that the project has delivered, Angiopredict has opened up a number of new avenues for possible further research. These include:

Table 8 Avenues for Further Research

New Opportunity	Status at end of AngioPredict	WP	Partners
Improved definition and identification of SNPs as bvz therapy response predictors	Strong baseline data; project plan not yet fully developed. H2020 prospect	4	VIB
Use of bio-informatics with AngioPredict and other data sets to define and validate MTRs for CRC sub-types	Project plan under development; key resources already available.	5	UCD
Verification of promising markers for pan-cancer liquid biopsy platform.	Priority patenting underway. The potential of the markers to become part of a commercial product is further assessed at EPI	6	EPI
New targets in tumour vasculature, for CRC	ITN/EID grant secured; research already begun	7	SOM
TEC targets for improved drug delivery module	Ongoing EU proposal	7	SOM
TEC signature as marker for metastatic CRC.	Exciting data and results. Awaiting suitable funding opportunity	7	SOM
CNA for patient stratification and specific therapy response prediction	Excellent research results; strong opportunity for new research project (H2020)	7	VIB, UHEI, ICORG, VUMC, UCD, RCSI

1.4.1.3 Conclusion

We believe that Angiopredict has achieved significant advances towards the ultimate goal of delivering biomarkers and diagnostic tests to predict response to Avastin® combination therapy. Ultimately the project will contribute to more focused treatment, better care and quality of life for patients and reduced healthcare costs.

It will be clear from the foregoing that the project can report a strong list of exploitable outputs, including assets already on the patent pathway, as well as research results that have excellent potential for development into patentable IP. In a related success, the project has developed data and outputs that form excellent foundations for further high-impact research.

1.4.2 Dissemination

The team undertook a comprehensive dissemination programme over the course of the project. Our core target audience included academics, researchers, clinicians, health professionals and related industry, but we also engaged with the general public, in an educational role. Our aim has been to raise awareness about the project, our research and results. Below we describe the dissemination activities undertaken including online, peer reviewed publications, conference presentations and project materials.

1.4.2.1 Website and on-line

The Angiopredict project website (<https://www.angiopredict.com>) was established in August 2012 and played a central role as a public dissemination tool and as a means of communication and data sharing within the consortium. Different sections of the website were aimed at different audiences, including scientists, industry, researchers, students, media and the general public. The website includes the following sections:

- Homepage.
- About Us – featuring a project overview, summaries of each work package and the project newsletters and flyer for download.
- Partners – introducing the consortium, team leaders, links to partner pages and contact emails.
- Key Technologies – providing an overview of the key technologies used in the project.
- News & Events – featuring updates about the project and events of interest.
- Clinical Trials – details about the Angiopredict clinical trial.
- Related Projects – links to related projects.
- Education Section – providing information on colorectal cancer, cancer treatment and the impact of ANGIOPREDICT, aimed at the general public.
- Publications – where publications generated by the project team are posted.
- Contact Us – providing the contact details of the project coordinator and a project contact form.
- Secure Area – accessible to partners only.

The Education Section is designed to be accessible to the general public and includes an overview of colorectal cancer; a summary of treatment options; answers to the question what will Angiopredict do; answers to the question how will the project achieve its aims and information about the background to the project (including a videoed presentation by Coordinator, Dr Annette Byrne).

In addition to the website, the project has also maintained twitter and facebook accounts as part of our on-line and social media strategy. There are links to our facebook and twitter accounts from our website.

[Login](#) | [Contact Us](#)

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Home

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Key Technologies

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Clinical Trials

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Welcome to AngioPredict

ANGIOPREDICT - an FP7-funded project enabling personalised medicine for patients with metastatic colorectal cancer.

About AngioPredict

In ANGIOPREDICT, academic cancer biologists and industry-based biotechnology researchers will work together with clinicians to identify biomarkers to *predict* whether individual metastatic colorectal cancer patients will respond positively to Avastin® combination therapy. Diagnostic tests using these biomarkers will also be developed to provide clinicians with the means to predict patient treatment responses in the future.

Recently Published

Project Flyer

Project Newsletter Vol I

Project Newsletter Vol II

Project Newsletter Vol III

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 278981

CORDIS

web design by ionic

Figure 28 Screenshot from website homepage <https://www.angiopredict.com/>

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Education

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- [About Us](#)
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- Education**
 - [Overview of Colorectal Cancer](#)
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 - [What Will AngioPredict Do?](#)
 - [How Will AngioPredict Achieve Goal?](#)
 - [Background and Aims](#)
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Education

 print version
  send to a friend

Welcome to the Education Section of the AngioPredict Site.

Please view our most recent public lecture for an informative overview of the Project.



Overview of Colorectal Cancer
Treatment Options
What Will AngioPredict Do?
How Will AngioPredict Achieve this goal?
Angiopredict – Background and aims



web design by ionic

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 278981

CORDIS

Figure 29 Screenshot from Angiopredict Education Page <https://www.angiopredict.com/go/education>

Traffic to the website has grown steadily over the course of the project. Relevant details are set out below (Table 9).

Table 9 Summary of Visits to website

Year	No of Sessions	No of Users (new and returning)	Page Views
1 Feb 2012 – 31 July 2013	272	178	1,396
1 Aug 2013 – 31 Jan 2015	1,965	1,497	6,548
1 Feb 2015 – 31 Jan 2016	4,840	4,332	7,869

1.4.2.2 Project Materials

Project materials produced over the course of the project included:

- Project Flyer - a short introduction to Angiopredict, with details of the project background, goals, key technologies and partners.
- Newsletter first edition – the first edition of the project newsletter was produced in May 2013 and included an overview of the project, a summary of results from the first year of our research, project news and details of work over the coming months.
- Newsletter second edition – the second edition of the project newsletter was produced in early 2015 and focused on project publications. The newsletter also included a reminder of the project's key aims and objectives, details of recent developments and events and a summary of what to expect in the months ahead.
- Newsletter third edition – the third and final edition of the newsletter was produced in January 2016 and included a short recap about the project, an update on some recent publications, details of conferences, presentations and events and the researchers behind the project.

The project materials have all been uploaded to the project website and are readily available for download at <https://www.angiopredict.com/>.



ANGIOPREDICT

ANGIOPREDICT WILL PREDICT WHICH CANCER PATIENTS WILL RESPOND POSITIVELY TO COMBINATION AVASTIN TREATMENT

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females in the developed world. Currently, an important treatment for these patients is the drug Avastin® (bevacizumab), which inhibits tumour blood vessel formation and thus prevents tumour growth. The underlying genomic mechanisms that determine whether or not a patient will respond to Avastin® combination therapy are currently unknown.

ANGIOPREDICT is a research project working towards personalised medicine for patients with advanced colorectal cancer.

In ANGIOPREDICT, academic cancer biologists and industry-based biotechnology researchers will work together with clinicians through an ICORG led trial to identify biomarkers to predict whether individual colorectal cancer patients will respond positively to Avastin® combination therapy. Diagnostic tests using these biomarkers will also be developed to provide clinicians with the means to predict patient treatment responses in the future.

AngioPredict will identify biomarkers to predict whether individual advanced colorectal cancer patients will respond positively to Avastin® combination therapy. This will prevent unnecessary treatment, save critical time in patient care and improve quality of life.

KEY TECHNOLOGIES

GERMLINE SNP PROFILING	SOMATIC MUTATION ANALYSIS
DNA METHYLATION BASED PREDICTION MODELS	PREDICTIVE PHARMACOGENOMIC EXPRESSION METHODS
INTEGRATED SYSTEMS BASED DATA ANALYSIS	IMAGING VALIDATED SYSTEMS MODELLING

PROJECT GOALS

- 1 AngioPredict will identify potential predictive biomarkers using genomic analysis on previously collected samples from patients receiving Avastin® combination therapy.
- 2 AngioPredict will validate the predictive ability of potential biomarkers using tissue samples from the AC-ANGIOPREDICT clinical trial, where patients will receive combination Avastin® therapy.
- 3 AngioPredict will use validated, predictive biomarkers to develop diagnostic tests that can be used by clinicians to identify which patients will benefit from Avastin combination therapy.

FIND OUT MORE AT WWW.ANGIOPREDICT.COM

The Angiopredict project is funded by the EU's Framework Programme Seven (FP7) under contract #306021. Please visit www.angiopredict.com for more information.

 **RCSI**
 ROYAL COLLEGE OF SURGEONS IN IRELAND
 COLAISTE RÍOGA NA MÁINLEA IN ÉIRINN

 **VIB**
 FLANDERS INTERUNIVERSITY INSTITUTE FOR BIOTECHNOLOGY

 finding cancer early
epigenomics

 **SomantiX**

 **oncomark**

 **VUmc**
 VU University Medical Center Amsterdam
 Striving together for excellence

 **UMM**
 UNIVERSITÄTSMEDIZIN MAINZ

 **UCD**
 CONWAY INSTITUTE OF BIOMOLECULAR & BIOMEDICAL RESEARCH

 **ICORG**
 All Ireland cooperative oncology research group

 **Pintail Ltd**

 **SEVENTH FRAMEWORK PROGRAMME**

Figure 30 The Project Flyer

Predictive Genomic Biomarkers Methods for Combination Bevacizumab (Avastin) Therapy in Metastatic Colorectal Cancer



ANGIOPREDICT NEWSLETTER WINTER 2016

ANGIOPREDICT

WELCOME TO THE WINTER 2016 NEWSLETTER FOR THE EU PROJECT ANGIOPREDICT

IN THIS ISSUE

Angiopredict Quick Guide – reminder of our key aims and objectives	2
Essential Reading – an update on some of our recent publications	3
At the lecturn – recent conferences, presentations and events	4
Meet the Team – the researchers behind the project	6



The Angiopredict team at the Berlin plenary meeting on 25 June 2015.

FOR FURTHER INFORMATION ABOUT ANGIOPREDICT PLEASE SEE THE PROJECT WEBSITE [HTTPS://WWW.ANGIOPREDICT.COM](https://www.angiopredict.com)

AngioPredict is funded by the European Community's Framework Programme Seven (FP7) under contract No. 278981 'AngioPredict'.
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Figure 31 Third Edition of the Project Newsletter

1.4.2.3 Publications

Publications in peer-reviewed journals have been an important part of the ANGIOPREDICT dissemination strategy. We have achieved a total of 16 publications in key scientific journals to date. A selection of these papers are set out below:

- *“Colorectal Tumour Simulation using Agent Based Modelling and High Performance Computing”*. (Guiyeom Kang et al., *Future Generation Computer Systems (In Press)*). This article details some of the successes achieved in WP9.
- *“Integrating Colon Cancer Microarray Data: Associating Locus-Specific Methylation Groups to Gene Expression-Based Classifications”* (A. Barat et al., *Microarrays 2015 4(4)*, pages 630-646, 23 November 2015). Changes in gene expression and abnormal DNA-methylation are both known to play an important role in cancer onset and development. Here publicly available microarray based gene expression and methylation data sets are used to characterize expression subtypes with respect to locus-specific methylation.
- *“Evaluation of efficacy and safety markers in a phase II study of metastatic colorectal cancer treated with aflibercept in the first-line setting”* (Diether Lambrechts et al., *British Journal of Cancer 113*, pages 1027-1034, 29 September 2015). This article constitutes a novel first biomarker study in mCRC treated with aflibercept and concludes that IL8 may act as a potentially predictive biomarker of aflibercept treatment outcome.
- *“Biomarker-driven Studies in Metastatic Colorectal Cancer (mCRC): Challenges and Opportunities”* (Van Cutsem, Eric et al., *The Journal of OncoPathology, Vol 2:4*, pages 37-45(9). November 2014). This paper explores the search for molecular biomarkers that predict the likely response to targeted treatments for metastatic colorectal cancer (mCRC).
- *“Genetic variability of VEGF pathway genes in six randomized phase III trials assessing the addition of bevacizumab to standard therapy”* (S. de Haas et al. *Angiogenesis, Vol 17:4*, pages 909-920. October 2014). This paper describes a meta-analysis of individual patient data from six randomized phase III trials, exploring relationships between 195 common genetic variants in the vascular endothelial growth factor (VEGF) pathway and bevacizumab treatment outcome. Potential value for predicting treatment outcome across tumor types was identified.
- *“DNA copy number analysis of fresh and formalin-fixed specimens by shallow whole-genome sequencing with identification and exclusion of problematic regions in the genome assembly”* (Sheinin et al., *Genome Research, 24:12* pages 2022-2032, 18 September 2014). Detection of DNA copy number aberrations by shallow whole-genome sequencing (WGS) faces many challenges. This article presents a robust, cost-effective WGS method for DNA copy number analysis that addresses many of the most significant challenges faced by currently available procedures.
- *“Patient-Derived Xenograft Models: An Emerging Platform for Translational Cancer Research”* (M. Hidalgo et al., *Cancer Discovery, 4:9* Pages 998-1013. September 2014). Recently, there has been an increasing interest in the development and characterization of patient-derived tumor xenograft (PDX) models for cancer research. These models are useful for drug screening, biomarker development, and the preclinical evaluation of personalized medicine strategies. This article summarizes the current state of the art in this field, including methodologic issues, available collections, practical applications, challenges, shortcomings and future directions, and introduces a European consortium of PDX models.
- *“Bevacizumab in the treatment of metastatic colorectal cancer - a retrospective analysis with a focus on the elderly”* (J Betge et al., *Z Gastroenterol 52 - KG171*, August 2014). This paper analyses the survival times of 87 patients with mCRC who began chemotherapy in

combination with bevacizumab from March 2005 to August 2008 in the University Hospital Mannheim.

- *“Genetic markers of bevacizumab-induced hypertension”*, (Lambrechts et al., *Angiogenesis* 17:3:685). Here the authors address whether genetic variation in vascular endothelial growth factor-A (VEGF-A) pathway or hypertension-related genes are associated with bevacizumab-induced hypertension.
- *“Markers of response for the antiangiogenic agent bevacizumab.”* (Lambrechts D. et al., *Angiogenesis*, Vol 17:3, pages 685-694. July 2014). This article discusses progress towards finding robust biomarkers that can guide selection of mCRC patients for whom bevacizumab therapy is most beneficial. The current challenge identified is to expand the set of candidate markers emanating from recent studies and to validate and implement them into clinical practice.

In addition to the above list we also have concrete plans to pursue several other publications. We anticipate a further c. 144 papers will be published in peer review journals in the coming months. Work on many of these publications is already well advanced. Moreover, [a consortium Publication Planning Workshop will take place at VIB Leuven on May 13 2016.](#)

1.4.2.4 Conferences and Events

The Angiopredict team has also reached its core scientific audience by presenting at numerous key conferences and events. There are a total of 61 dissemination activities entered on the EU portal for the project, most of which relate to conference presentations. A selection of these are set out below:

- Presentation by Dr Annette Byrne (RCSI) to international angiogenesis research community on 2 October 2012 in San Diego, USA at 10th International M. Judah Folkman Conference.
- Prof John Prehn (RCSI) presented on *“Application of systems biology models of apoptosis signalling in a clinical setting; towards novel patient stratification tools”* at the Europe-Brazil Meeting on Systems and Synthetic Biology, in Natal, Brazil, on 10 March 2014.
- Project Coordinator Dr Annette Byrne (RCSI) and Scientific Coordinator Prof Diether Lambrechts (VIB) presented during the Genentech/Roche sponsored Angiogenesis Biomarkers session at the 5th International Meeting on Angiogenesis at Vrije University Medical Centre, Amsterdam on March 14th, 2014.
- On 13 May 2014 Prof Dr Bauke Ylstra (VU-VUMC) presented an invited lecture at the International Society of Cellular Oncology, which focussed on copy number and quality assurance with next generation sequencing from FFPE material. Prof Ylstra also presented on this topic at the American Society of Cancer Research ASCR (San Diego, 8 April 2014) and at the 4th International Symposium on Translational Oncology (19, September 2014, Barretos, Brazil).
- Project Coordinator Dr Annette Byrne and Prof Heinrich Huber (formally of RCSI Centre for Systems Medicine) presented at the European Research Workshop in Digestive Oncology in Barcelona on 24 June 2014. The workshop focused on biomarker-driven studies in metastatic colorectal cancer (mCRC) and examined the challenges and opportunities offered by biomarker-driven studies.
- Prof Diether Lambrechts (Scientific Co-ordinator, VIB) presented on 6 March 2015 in Orlando, Florida at the ACCR Conference - Tumor Angiogenesis and Vascular Normalization: Bench to Bedside to Biomarkers. He also presented at the 18th International AEK Cancer Congress 2015 (Heidelberg) on 19 March 2015, (presentation on *“Angiogenesis biomarkers in solid cancers”*); on 19 April 2015 at the AACR Annual Conference (*“Biomarkers of Antiangiogenic*

Therapy: Are We Lost in Translation?"), Philadelphia, USA and on 8 May 2015 at the 9th International Experts Panel Meeting "Antiangiogenic Therapies In NSCLC: Reality And Hopes" in Sperlonga, Italy, presentation entitled "*Biomarkers for antiangiogenetics*".

- Angiopredict Coordinators Dr Annette Byrne (RCSI) and Prof Diether Lambrechts (VIB) presented at the EU-LIFE Scientific Workshop on Epigenetics and Disease in Copenhagen, on 12- 13th May 2015. The event was organised by BRIC (the Biotech Research and innovation Centre) and brought together scientists and clinicians/translational researchers from EU-LIFE institutes. Prof Lambrechts presented "*Tumor hypoxia causes DNA epimutations by reducing TET activity*" and Dr Byrne presented "*Angiopredict: Predicting response to combination bevacizumab therapy in metastatic colorectal cancer*".
- On 28 June 2015 researchers from partner VU presented on Q-DNAseq at the EACR Conference Series 2015, (2nd Special Conference: Cancer Genomics). Dr Bauke Ylstra (VU) also made a poster presentation at the AACR Precision Medicine Series: Integrating Clinical Genomics and Cancer Therapy in Utah, USA on 13 June 2015 (title: *Q-DNAseq: a bioinformatics pipeline that yields DNA copy numbers from shallow whole genome sequencing with noise levels near the statistical limit imposed by read counting*).
- Dr Sudipto Das (partner RCSI) recently made a number of significant presentations focused on understanding different strategies to optimise both experimental and bioinformatic workflow, including at the European Society of Human Genetics annual meeting, Glasgow, UK in May, 2015 and at the Genome Sciences Meeting 2015, in Birmingham, U.K., in August, 2015.
- On 30 October 2015 Prof Diether Lambrechts (VIB) presented on "*Genetic traits predicting the efficacy of antiangiogenic therapy*" at the International Symposium on Metastatic Colorectal Cancer held in Heidelberg. Prof. Lambrechts also presented "*Molecular profiling: Personalizing your cancer treatment*" at the Global Nintedanib (BIBF 1120) Consultancy Meeting on CRC, 20 November 2015, Boehringer-Ingelheim, Germany.
- Angiopredict Co-ordinator Dr Annette Byrne (RCSI) was invited to discuss the use of pre-clinical ultrasound to interrogate angiogenesis inhibitors during the 47th Annual British Medical Ultrasound Meeting held in Cardiff, UK on December 8-11th 2015. Dr Byrne also presented at the inaugural ESMO-Asia Congress held in Singapore, on December 18th-21st 2015, speaking on "*Angiogenesis across Tumor Types*".
- Dr. Ian Miller (RCSI), recently presented the Angiopredict initiative at the Coordinating Action Systems Medicine (CASYM) workshop entitled "European Systems Medicine: Are we there yet?" held in Brussels 18th February 2016. The aim of this strategic workshop was to bring together key investigators and scientists from the European Systems Medicine community. It provided a forum to showcase state of the art and novel approaches to systems medicine, discuss challenges and reflect on "success" stories of current projects which implement a systems medicine approach. The Angiopredict presentation was well received and was noted for its successes in developing integrative systems medicine approaches to identify predictive biomarkers for Bevacizumab. The Angiopredict approach will be included in the upcoming white paper publication "The CASYM roadmap for Systems Medicine" to be submitted to the European Commission in the coming weeks.

Work completed during the course of the project will also lead to other conference presentations in the coming months e.g. Dr Nicole van Grieken (VUMC) will present at the ESMO World Congress on Gastrointestinal Cancer 2016 in Barcelona, Spain (29 Jun - 02 Jul 2016).

During the project the Angiopredict team have also engaged with the general public e.g. (i) on 8 April 2014 Angiopredict researchers Dr Alice O'Farrell and Dr Ian Miller hosted visits to RCSI in

Dublin by local secondary school students (the presentations made covered general information about research science, aspects of the Angiopredict project and an introduction to non-invasive imaging) and (ii) on 26th February 2014 a lecture on the subject of cancer therapies was delivered free of charge to the public at the Royal College of Surgeons in Ireland as part of the RCSI Minimed Open Lecture Series. The lecture was entitled '*Tackling Cancer by Targeting Tumour Blood Vessels*'.

1.4.2.5 Other

Building networks and collaborations has played an important part in our dissemination strategy and in the success of the project overall. The Angiopredict team has built connections with numerous stakeholders and initiatives working in sectors aligned with our research.

We have engaged productively with the project's Advisory Board, which is comprised of key international experts in disciplines of direct relevance to the project. Our continued engagement with the Advisory Board has helped to keep us in touch with the cutting edge of science in tumour angiogenesis, the treatment of colorectal cancer and related technologies.

The team has also collaborated with the Dutch Colorectal Cancer Group (DCCG) (on behalf of the sponsored study known as CAIRO 2); the VAMPIRE (EU ITN/EID) project; the international Colorectal Cancer Subtyping Consortium (CRCSC); Dr Anguraj Sadanandam (Team Leader, Laboratory of Systems and Precision Cancer Medicine, Division of Molecular Pathology, The Institute of Cancer Research, UK) and the EuroPDX consortium. The project is also closely aligned with a number of other projects that are focused on similar research and which have some partners in common e.g.:

- Apo-Decide - using systems medicine to deliver personalised medicine for colorectal cancer, EU FP7 project;
- AngioTox - Histopathologic & Mechanistic Assessment of Angiogenesis Inhibitor Related Toxicities: An EU FP7 Industry-Academia Partnerships & Pathways (IAPP) Funded Project;
- MErCuRIC - A Phase Ib/II study of MEK1/2 inhibitor PD-0325901 with cMET inhibitor PF-02341066 in KRASMT and KRASWT (with aberrant c-MET) Colorectal Cancer Patients, funded under the EU FP7 programme and
- ColoForetell - A Xenopatient Discovery Platform for the integrated Systems based Identification of Predictive Biomarkers for Targeted Therapies in Metastatic Colorectal Cancer), funded by Science Foundation Ireland (SFI).

1.4.2.6 Conclusion

Throughout the Angiopredict project we have followed a targeted dissemination strategy. We have grown the project's presence online through the project website in particular. We have issued project materials (available for download from <https://www.angiopredict.com/>) and have achieved several significant publications (with many more in preparation). Members of the team have presented on our research (and related topics) at numerous key conferences. Finally, we have built a network of contacts working in tumour angiogenesis, the treatment of colorectal cancer and related disciplines, which has added further breadth and depth to our reach.

We believe that our work on dissemination has delivered a tangible awareness of the project's research and results within our target scientific audience, which will yield future opportunities and collaborations. We have also reached out to the public (e.g. through the accessible information made available on our website and through public facing events).

We believe that the work undertaken in Angiopredict will continue to bear fruit beyond the life of the project and that our results and findings have real potential to impact on clinical decision-making in

mCRC in the future, so that the right treatment can be delivered to the right patient at the right time, in a more focused and cost-effective manner.

1.5 Website and contact details

The ANGIOPREDICT website is at <https://www.angiopredict.com/>

ANGIOPREDICT

Home

Home

About Us

Partners

Key Technologies

News & Events

Clinical Trials

Related Projects

Education

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Contact Us

Welcome to AngioPredict

ANGIOPREDICT - an FP7-funded project enabling personalised medicine for patients with metastatic colorectal cancer.

About AngioPredict

In ANGIOPREDICT, academic cancer biologists and industry-based biotechnology researchers will work together with clinicians to identify biomarkers to predict whether individual metastatic colorectal cancer patients will respond positively to Avastin® combination therapy. Diagnostic tests using these biomarkers will also be developed to provide clinicians with the means to predict patient treatment responses in the future.

Recently Published

Project Flyer

Project Newsletter Vol I

Project Newsletter Vol II

Project Newsletter Vol III

web design by ionic

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 278981

CORDIS

Figure 32 Screenshot of the Angiopredict website – homepage

Partner Details are set out below.

 <p>RCSI Royal College of Surgeons in Ireland (RCSI) http://www.rcsi.ie/ <i>Coordinator of Angiopredict</i></p>	 <p>VIB Flanders Interuniversity Institute for Biotechnology (VIB) http://www.vib.be/ <i>(Scientific Coordination)</i></p>	
<p>— finding cancer early epigenomics</p> <p>Epigenomics AG (EPI) http://www.epigenomics.com/</p>	 <p>SomantiX SomantiX B.V. (SOM) http://www.somantix.com/</p>	 <p>oncomark</p> <p>OncoMark Limited (ONCO) http://www.oncomark.com/</p>
 <p>VUmc VU University Medical Center Amsterdam</p> <p>Stichting VU-VUMC http://www.vumc.com</p>	 <p>UMM UNIVERSITÄTSMEDIZIN MANNHEIM</p> <p>Ruprecht-Karls-Universität Heidelberg, University of Heidelberg (Mannheim University Hospital) (UHEI) http://www.umm.de</p>	 <p>UCD Conway Institute of Biomolecular and Biomedical Research</p> <p>University College Dublin (National University of Ireland, Dublin) (UCD). Conway Institute of Biomolecular and Biomedical Research http://www.ucd.ie/conway</p>
 <p>ICORG All Ireland cooperative oncology research group</p> <p>Irish Clinical Oncology Research Group Limited (ICORG) http://www.icorg.ie/</p>	 <p>Pintail</p> <p>Pintail Limited http://www.pintail.eu/</p>	

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