CONTRACTUAL ACTIVITY REPORT

Period covered: from M1 to M18

Periodic report: 1st ☒ 2nd ☐ 3rd ☐

Status: CONFIDENTIAL

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Project title	**HER Imaging and Molecular Interaction Mapping in Breast Cancer**
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Date of latest version of Annex I against which the assessment will be made	**2012-03-07**
Periodic report	**1st Periodic report**
Period covered	**From M1 (01/05/2011) to M18 (31/10/2012)**
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List of beneficiaries

Beneficiary Number*	Beneficiary name	Beneficiary short name	Country
1	UNIVERSITY COLLEGE LONDON	UCL	United Kingdom
2	UNIVERSITAET ZUERICH	UZH	Switzerland
3	KING'S COLLEGE LONDON	KCL	United Kingdom
4	TOPOSNOMOS LTD	TNL	United Kingdom
5	MAX PLANCK GESELLSCHAFT ZUR FOERDERUNG DER WISSENSCHAFTEN E.V.	MPG	Germany
6	UPPSALA UNIVERSITET	UU	Sweden
7	Innovative Technologies in Biological Systems	INO	Spain
8	FIRALIS S.A.S.	FLS	France
9	MEDIPOLIS GMP OY	M-GMP	Finland
10	NOVAMEN SAS	NOVAMEN	France

Declaration by the scientific representative of the project coordinator

I, as scientific representative of the coordinator of this project and in line with the obligations as stated in Article II.2.3 of the Grant Agreement declare that:

- The attached periodic report represents an accurate description of the work carried out in this project for this reporting period;
- The project (tick as appropriate):

  ☐ has fully achieved its objectives and technical goals for the period;
  ☒ has achieved most of its objectives and technical goals for the period with relatively minor deviations.
  ☐ has failed to achieve critical objectives and/or is not at all on schedule.

- The public website, if applicable
  ☒ is up to date
  ☐ is not up to date
- To my best knowledge, the financial statements which are being submitted as part of this report are in line with the actual work carried out and are consistent with the report on the resources used for the project (section 3.4) and if applicable with the certificate on financial statement.
- All beneficiaries, in particular non-profit public bodies, secondary and higher education establishments, research organisations and SMEs, have declared to have verified their legal status. Any changes have been reported under section 3.2.3 (Project Management) in accordance with Article II.3.f of the Grant Agreement.

Name of scientific representative of the Coordinator: .....**Kerry Chester**.....

Date: ....30...../ .....12..../ .. 2012

For most of the projects, the signature of this declaration could be done directly via the IT reporting tool through an adapted IT mechanism and in that case, no signed paper form needs to be sent

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PUBLISHABLE SUMMARY

SUMMARY OF PROJECT CONTEXT AND OBJECTIVES

IMAGINT is a multidisciplinary project that is developing a range of new tools for imaging and characterizing interactions of the human epidermal growth factor receptor (HER) family of tyrosine kinase cell surface receptors; HER1 (EGFR), HER2, HER3 and HER4. IMAGINT tools are being made from Designed Ankyrin Repeat Proteins (DARPins), which are small, antibody-like proteins based on human protein scaffolds but 10 times smaller than antibodies.

DARPins are ultra stable and bind specific targets with high affinity in monovalent form; they are also readily engineered for site-specific chemical modification to create specific marker-tags and thus allow for the creation of tools that would be difficult or impossible to create from antibodies. IMAGINT is using the DARPins to develop tools for different applications based on current scientific understanding of HER2-related cancer processes and to address areas of unmet need.

The aim is to develop a wide range of tools that will be of use for clinicians to guide treatment regimes and for prediction, diagnosis, monitoring and prognosis of disease. Specific objectives of IMAGINT are: (i) To develop DARPins and HER-reactive antibodies to characterize HER2 homo- and hetero-dimers using 4 novel technologies: Single Molecule Fluorescence, Proximity Ligation, super-resolution microscopy and FRET/FLIM. The collected data will be analyzed with information on clinical outcome to determine which HER2 interactions are associated with resistance to HER2 targeted treatments. (ii) To isolate and characterize protein/RNA complexes. These complexes may be new biomarkers for breast cancer and their characterization is aimed at elucidating mechanisms of transcriptional regulation in response to anti-HER2 treatment. (iii) To identify protein networks associated with HER signaling by imaging clusters of 50-100 different proteins in a single cell or tissue section. This will be achieved with a robot, using large dye-conjugated tag libraries, and automatically bleaching after each imaging cycle. (iv) To develop a new whole body imaging agent, using a radiolabelled anti-HER2 DARPin, to improve specificity and sensitivity of clinical imaging. (v) To create a range of bioinformatic tools, including artificial neural network methods. The bioinformatic tools will be used to analyze the multivariate data obtained by the new IMAGINT technologies in order to determine novel biomarkers that aim to classify disease.

The focus of IMAGINT is on breast cancer, a chronic disease where HER2 is a potent oncoprotein and has become an established target. Because of its central role, IMAGINT emphasis will be placed on tools to dissect HER2-related events. Once developed for breast cancer, the tools will find wide application for other cancers in which HER2 is clinically relevant. For example IMAGINT tools are applicable to gastric cancer, another chronic disease and the world's second leading cause of cancer death.

SUMMARY OF WORK PERFORMED DURING PERIOD 1 (M1-M18)

In the first 1-18 M, the IMAGINT consortium has made good progress towards their ambitious objectives: (i) The first set of high affinity DARPins have been generated to HER1 (EGFR), HER2, HER3 and HER4 (UZH). These DARPins are being applied to study HER2 interactions by proximity ligation, where proof-of-principle has been successfully established with HER2+ve cell lines (UZH). In P2 the DARPins will be evaluated for applicability to other IMAGINT technologies. Super-resolution microscopy (MPG) imaging of HER2 has been demonstrated with HER2+ve cells using standard antibodies and DARPins are being evaluated in the same systems (MPG). Technology for characterising HER2 homo-and hetero-dimers by FRET/FLIM (KCL) has first been developed using standard antibodies to characterize HER2-HER3 hetero-dimers, as these dimers are the most stable and form the most actively signaling pair of receptors within the HER family. The FRET/FLIM assay has been successfully validated on cells and applied to FFPE tissues from invasive breast cancer (KCL). (ii), Immunoprecipitation against endogenous Ago2 protein has been successfully applied to isolate protein/RNA complexes. The method allowed effective isolation of Ago2 complexes from breast cancer cell lines treated and untreated with Trastuzumab. The RNA-protein complexes were analysed by MALDI-TOFF yielding 209 candidate marker proteins and miRNAs which will be further investigated in IMAGINT (INO). (iii) To identify protein networks associated with HER signaling a toponome library of 100 distinct proteins/biomolecules has been calibrated and biologically validated against epidermal tissue. Results indicate that the basal lamina is composed of 6 to 7 distinct, spatially clearly separated and stoichiometrically controlled layers, which have not yet been described in the literature (TNL). The tools will now be applied to analysis of clinical breast cancer tissue. (iv) A lead anti-HER2 DARPin format has been selected for development as clinical imaging agent (UCL, UZH, UU). In pre-clinical *in vivo* studies, the radiolabelled anti-HER2 DARPin gives low background and tumour:blood ratios of over 150:1 at early time points (UCL, UU). A draft clinical protocol has been written and toxicity testing in transgenic mice prepared (UCL). (v) For data capture, the consortium have agreed to create a data store based on a flat-file directory structure to capture raw data (where required) and curated data with associated documentation including data sources, contact details, and file formats. The data store will also hold metadata about the experiments from which the data was collected (KCL and FLS). A Bayesian mathematical Model has been developed to reduce the risk of overfitting during data analysis (KCL and FLS).

SUMMARY OF PROJECT OUTCOMES AND EXPECTED RESULTS

IMAGINT is a 48-month collaborative project addressing the objectives of the HEALTH priority of the FP7 Cooperation programme, and in particular the area “detection, diagnosis and monitoring” applied to cancer. Research has shown that human epidermal growth factor receptor 2 (HER2) is a potent oncoprotein and it has become an established target for breast cancer treatment. There is urgent need for development of safe, efficient non-invasive HER2-related biomarkers to guide treatment regimes and for prediction, diagnosis, monitoring and prognosis of disease. IMAGINT aims to develop such tools by combining bio-imaging and molecular testing biomarkers from RNA, DNA and/or protein and their complexes. The impacts of IMAGINT will be widespread from new biomarkers through molecular discoveries, to new patient treatment methods. The IMAGINT European consortium brings together top-level experts from public and private organizations to cover all the fields required for the development of tools for the identification and the detection of biomarkers in clinical samples and patients.

Metastatic breast cancer is usually diagnosed by a combination of clinical and radiological findings. A successful outcome of IMAGINT could: (i) Permit the identification and detection of biomarkers in clinical samples and patients. (ii) Ensure an earlier detection of metastatic breast cancer and increase early diagnosis. (iii) Avoid some biopsies associated with negative outcomes: pre-procedure anxiety, post-procedural pain. (iv) Optimise the monitoring of therapeutic agents for safer, efficient and quicker results. (v) Allow identification of resistance to anticancer treatments earlier than can be done clinically. (vi) Eliminate morbidity due to unnecessary treatments. (vii) Give patients a realistic expectancy of treatment response. In summary, IMAGINT project will demonstrate the following concept: that targeted oncology is to select the “right patient for the right drug at precisely the right point in their cancer journey” to avoid unnecessary side effects and improve treatment outcomes at efficient costs. Thus, it is expected to have impacts in terms of science, health, health economics, society and European competitiveness. In addition, IMAGINT will contribute to the translational approach from laboratory discovery to clinical tools. The data generated will be shared with the research community through a system of standards and a database which is compatible with FP7 capacities infrastructure.

Breast cancer has a huge incidence in Europe, with more than 430,000 new cases per annum but the IMAGINT tools will have wider application. Once developed for breast cancer the tools can be used for other cancers in which HER2 is clinically relevant and provides a potential target in a definable sub-set of patients. For example, gastric cancer a major cause of death worldwide and pancreatic cancer, a devastating disease, resistant to conventional therapies and a major cause of cancer death, accounting for over 60,000 deaths in a single year in the EU. HER2 has also been reported as a potential target in wide range of other cancers including ovarian cancer, non-small-cell lung cancer and prostate cancer.

ADDRESS OF PUBLIC WEBSITE

http://www.imagint.eu/

PROJECT OBJECTIVES

WP1 – Generation and development of DARPins as tools for quantitative imaging and detection of interacting partners

Task 1.1: Generation of additional DARPins. M1-M24; UZH, INO

Progress toward objectives

- The objective is to develop new, high affinity DARPins specific for all members of the EGFR family.
- These will be used to establish rigorous understanding of the EGF-family receptor pairs and oligomers formed, exploiting single molecule methodology and nanoscopy.
- In later stages of the project, this knowledge will to be converted into the development of tools for diagnostics.
- Good progress has been made as binders for all members of the EGFR family have now been generated.

Significant results

- High affinity binders for all members of the EGFR family have been obtained.
- A particular challenge is HER3, because of its low stability and tendency to dimerize/aggregate. HER3 Binders have been obtained by both phage display and ribosome display. However, their affinity is as yet not as good as those that have been obtained against other members of the family.
- Constructs with mono- and multispecific binders have been cloned and will be evaluated.
- Experiments with ribosome display to affinity-mature the binders have been initiated. Briefly, using random mutagenesis with error prone PCR and base analogs, a library of point mutants is created. Using off-rate selection, binders with slower off-rates will be selected.

Deviations from workplan

- It was necessary for this work to be put on hold because resources had to be moved to Task 5.1, as preparation of this material for a clinical trial is on the critical path.

Impacts (resources, planning, link with other tasks)

- Significant resources were devoted to developing a production process for the G3 DARPin (Task 5.1).
- In addition UZH performed critical biological experiments that were essential for ethical approval (see Task 7.6; Figures 7.6.1 - 7.6.3)

Correctives actions

- After having established large scale production methods for DARPins, with and without a hexahistidine tag (H_6-tag), protein samples were shipped from UZH to other partners.

Task 1.2: Single molecule detection of homo and heterodimeric pairs of the EGF-R superfamily. M1-M48; UZH, MPG

Progress toward objectives

- UZH has worked on labelling DARPins for single molecule detection. In this work important controls were established.
- Since later work will require dual colour detection, controls were established in making doubly labeled DARPins.
- It was very helpful that an orthogonal labelling technology, based on click chemistry was recently developed at UZH, as it allows now to label a DARPin regiospecifically with two dyes.

Significant results

- The detection of labelled DARPins on cells turned out to be more difficult than initially assumed. The main challenge was that the single molecule technology requires low levels of labelling (to have single molecules in the focus). This, in turn, requires that the background is extremely low.
- We found that this problem is almost unsolvable for green dyes and therefore switched completely to red dyes.
- After these problems were be solved, the first encouraging pictures of DARPins bound to cells have been obtained.

Deviations from workplan

- UZH started an orthogonal approach to specifically label the EGFR receptors directly, to have a quantitative real time read-out of receptor homo- and heterodimerization. However, this is still in the cloning phases and will only yield results on the next period.
- The advantage is that this is an independent approach and thus does not require the DARPins as a reporter but rather controls their performance.

Impacts (resources, planning, link with other tasks)

- None.

Correctives actions

- None.

Task 1.3: Nanoscopy of the HER superfamily. M1-M18; MPG, UZH

Progress toward objectives

- Several different cell fixation protocols have been tested for the use in combination with DARPin and antibody labelling. Of these, formaldehyde fixation proved to be the most suited for antibody labelling, whereas live cell labelling with subsequent formaldehyde fixation showed the best results for staining with DARPins.
- For nanoscopy (STED-microscopy), H14-DARPins directed against the extracellular domain of the HER2 protein were provided by UZH. Imaging protocols for STED-microscopy on cultivated SKOV3 cells (HER2 positive ovarian cancer cell line) with the H14-DARPin and antibodies (IgGs) directed against HER2 were developed and refined.

Significant results

- So far, classical IgGs outperform the DARPins in terms of handling, labelling efficiency and specificity as well as stability during the imaging process.
- In preparation for WP2, first attempts to label chemically fixed, paraffin embedded tissue sections of colon cancer with IgGs directed against HER2 have been carried out successfully (Figs 1.3.1, 1.3.2).

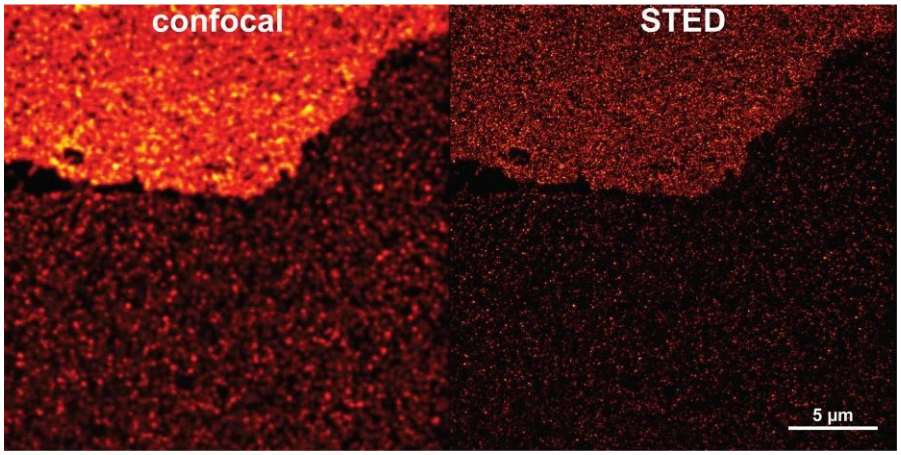


Figure 1.3.1. Image shows section of two adjacent SKOV3 cells labeled with classical IgGs against HER2. Left: Diffraction-limited confocal imaging. Right: STED-microscopy.

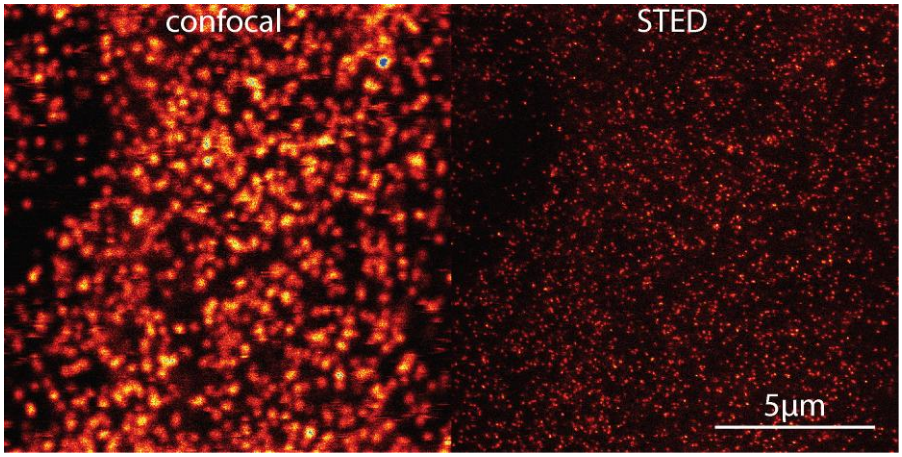


Figure 1.3.2 Image shows section of SKOV3 cell, labeled with H14-DARPin against HER2. Left: Diffraction-limited confocal imaging. Right: STED-microscopy.

Deviations from workplan

- No deviations from the workplan were required.

Impacts (resources, planning, link with other tasks)

- Work so far has been performed with the H14-DARPin supplied from UZH. During the IMAGINT-Meeting in Uppsala we became aware that other members of the consortium are working with a different DARPin (G3). We next plan to use the G3-DARPin which will be tested for expression and fluorophore coupling in our lab.

Correctives actions

- None.

Task 1.4: Proximity ligation and bispecific DARPins for use in tissue samples. M1-M48, UZH, UCL, KCL

Progress toward objectives

- DNA-mediated affinity-based assays, such as immuno-PCR and proximity ligation assays (PLA), have been developed.
- We have successfully established a proof-of-principle using HER2.

Significant results

- DNA-mediated affinity-based assays, such as immuno-PCR and proximity ligation assays (PLA), use oligonucleotides attached to affinity reagents as reporter molecules. Conjugation of oligonucleotides to affinity reagents generally employs chemistries that target primary amines or cysteines. Because of the random nature of these processes neither the number of oligonucleotides conjugated per molecule nor their sites of attachment can be accurately controlled for affinity reagents with several available amines and cysteines. We developed a straightforward and convenient approach to functionalize recombinant affinity reagents for PLA by expressing the reagents as fusion partners with SNAP protein tags.

- This allowed us to conjugate oligonucleotides in a site-specific fashion, yielding precisely one oligonucleotide per affinity reagent. We demonstrated this method using DARPins recognizing the tumour antigen HER2 and we apply the conjugates in different assay formats.

Deviations from workplan

- None.

Impacts (resources, planning, link with other tasks)

- None.

Correctives actions

- None.

WP2 – Tools for a multivariate tumour invasion signature

Task 2.1: Establishing a multiparametric tissue imaging-based metastatic signature. M1-M233; KCL

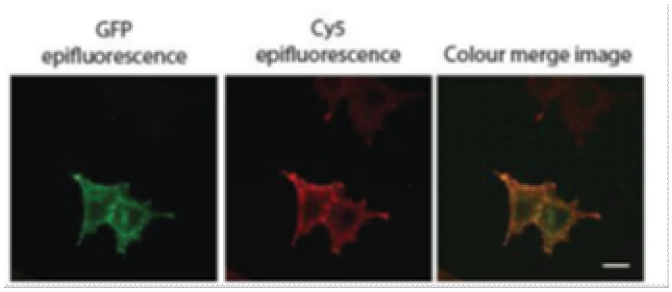
Progress toward objectives

- Dimerization of HER2 with other members of the HER family is important for activation.
- Of these dimers, HER2:HER3 heterodimers are the most stable and form the most actively signaling pair of receptors within the HER family.
- We therefore set to establish new FRET/FLIM based assay for assessment of HER2:HER3 interaction in cells and human breast cancer samples.
- Quantification of this interaction may prove to be a superior prognostic and/or predictive biomarker for the use of HER2-targeted treatment in breast cancer.
- We first validated the antigen specificity of a variety of antibodies in cell lines transfected with the fluorescently tagged protein of interest. Those successfully validated were kindly conjugated with fluorophores by Dr Gilbert Fruhwirth.
- Monoclonal antibodies were selected as they are more specific than polyclonal antibodies which are raised against multiple epitopes on any given antigen. Furthermore, conjugation of monoclonal antibodies with fluorophores is more reliable compared to serum from polyclonal antibodies, which contains a heterogeneous complex mixture of antibodies, which may be conjugated with different dye: protein ratios.
- Once conjugated, the antigen specificity was re-tested as the process of antibody conjugation could lead to a loss of specificity. Furthermore, both antibodies must bind to the protein of interest in a configuration which sterically positions the fluorophores within an appropriate proximity for FRET to occur. Therefore, we first screened a variety of antibodies against HER2 and HER3 in order to provide a panel of antibodies which could then be applied in various combinations to select those which provide the highest level of interaction by FRET/FLIM.

Significant results

- Tested antibodies against HER2 were: clone L26 and N24 (donated by Prof' Yosef Yarden, Weizmann Institute of Science), HER2-Int (engineered by GIbiochem.Ltd) N17 and Ab8 (clone e2-4001, TermoScientific Ltd), #2165 (Cell Signaling) and DARPin (G3, directly labelled with Cy5); against HER3 were: HER3-YY (donated by Prof' Yosef Yarden), MAB 3483 (R&D Systems), RTJ2 (Millipore), 2F12 (TermoScientific Ltd) and B9A11 (Monogram, Ltd). Results are shown below in figures 2.1.1 - 2.1.5.

A



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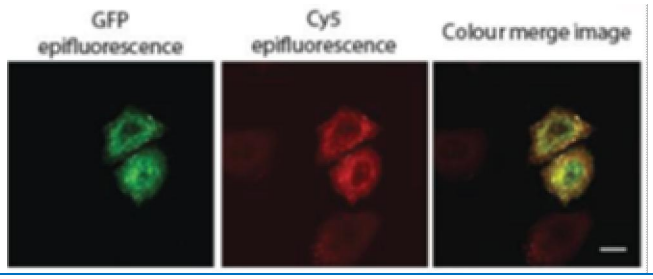


C

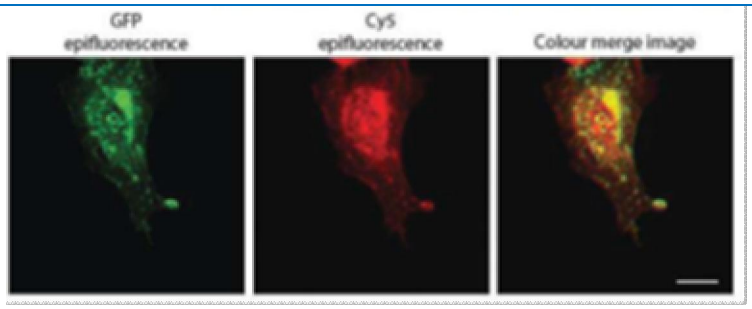
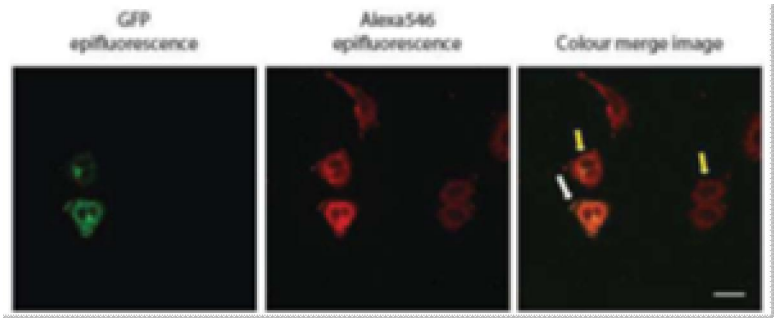


Figure 2.1.1. Shows MCF-7 cells transfected with HER2-GFP and stained with anti-HER2 (N17, clone e2-4001,) and anti-mouse-Cy5 IgG (panel A), stained with N17 directly labelled with Cy5 (panel B) or DARPin (G3-Cy5, panel C). Similar tests were performed for all antibodies listed above.

A



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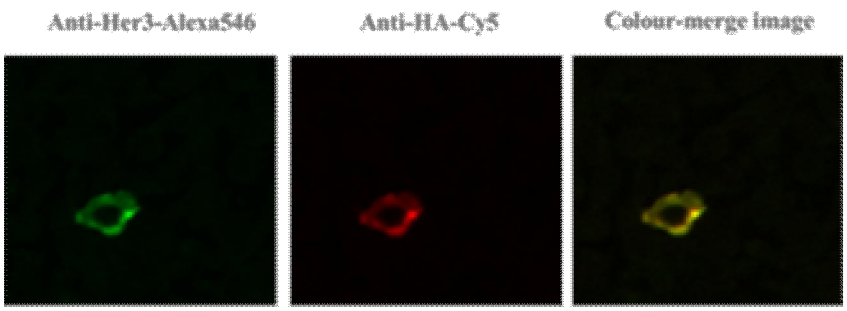


Figure 2.1.2. Shows SKBR3 cells transfected with HER3-GFP and stained with anti-HER3-Alexa546 antibody (panel A, white arrow indicates HER3-GFP and yellow arrows indicate recognition of HER3-GFP by antibody), or cells transfected with HER3-HA and stained with anti-HER3-B9A11-Alexa546 and anti-HA-Cy5 antibodies (panel B).

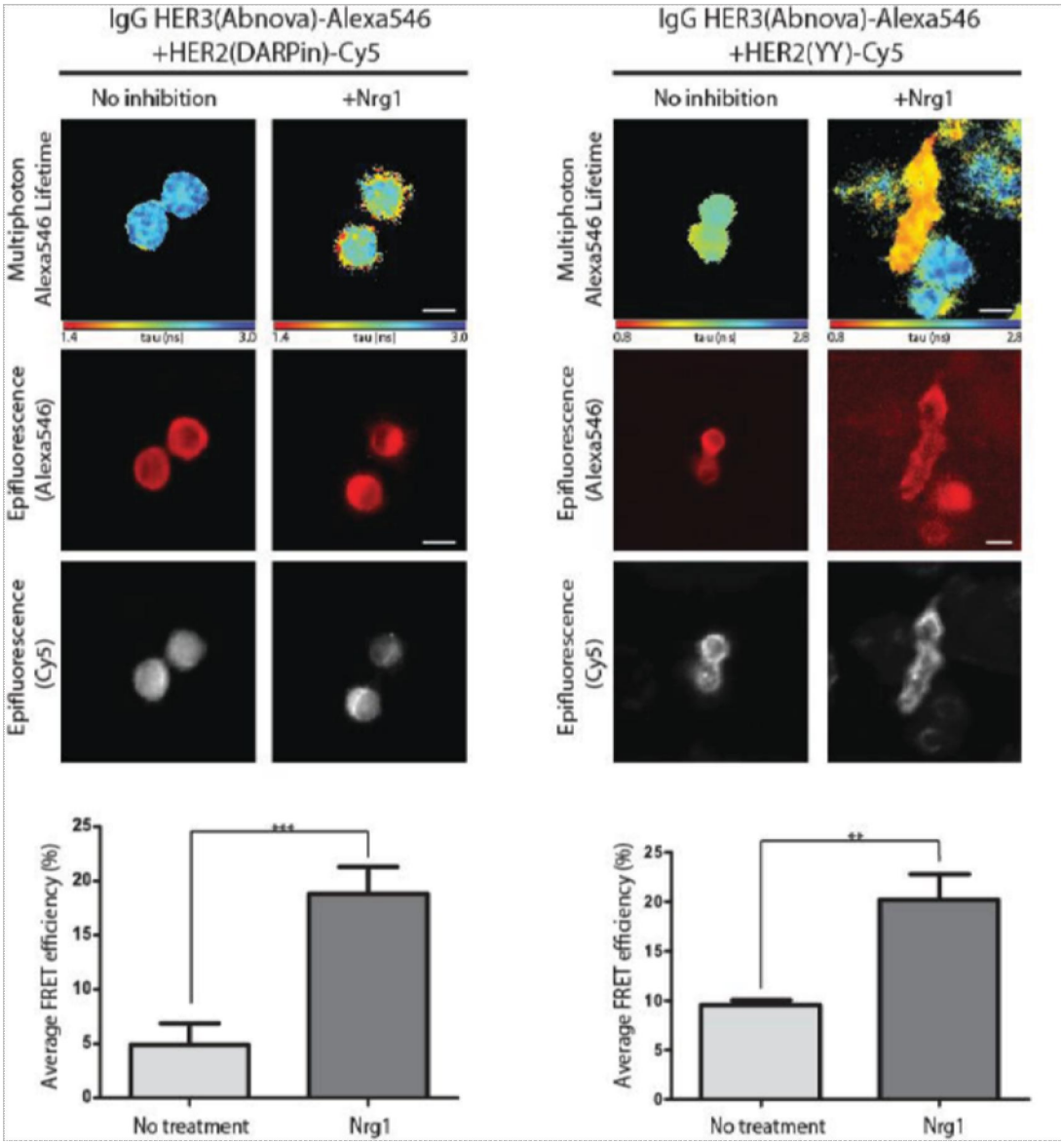


Figure 2.1.3. The FRET pairs (directly labelled antibodies) were tested first in cells transfected with HER2-HA and HER3-HA constructs. Abnova-DARPin and Abnova-N24 pairs were exhibit base line FRET efficiency around 5-10%, which was increased by treatment with natural ligand of HER3 – neuregulin, known to bind to HER3 receptor and promote dimerization with HER2.

- Once our FRET/FLIM assay had been validated in cells, it was then applied to FFPE tumour tissue in order to assess the spectrum of HER2:HER3 dimers detectable. Therefore, we used a section from a composite block of four tumour tissues, each expressing various levels of HER2 protein (3+, 2+, 1+ and 0 or negative, as scored using the HercepTest). Of note, the level of HER3 protein expression in each tumour tissue had not been previously documented, and of course is highly relevant within our assay. The bar graph in Figure 2.1.4 shows the range of lifetimes detected in these tissues, which did not entirely correlate with the IHC scoring of HER2. An antibody raised against an intracellular epitope of HER3, 2F12, was used as a negative control since DARPin binds to an extracellular epitope of HER2, and 2F12, to an intracellular epitope, hence little change in fluorescent lifetime of HER3(2F12)-X546 is expected as the fluorophores are too far apart for FRET to occur. In order to illustrate this point, the mean lifetimes and FRET efficiencies for the 4 tissues for both HER3-X546 (Abnova) and HER3-X546 (2F12) are illustrated.

- A similar result was obtained with FRET pair antibodies targeting intracellular domain (data not shown).

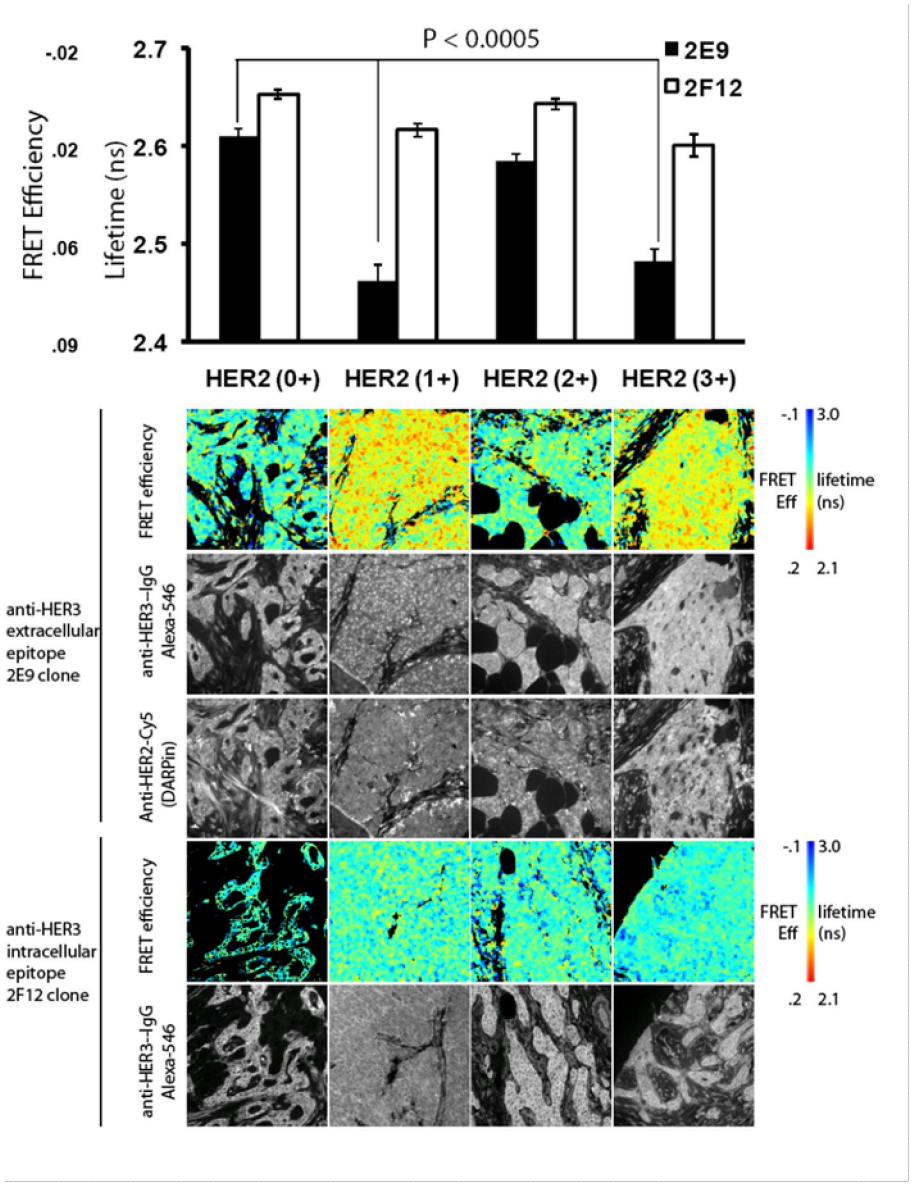
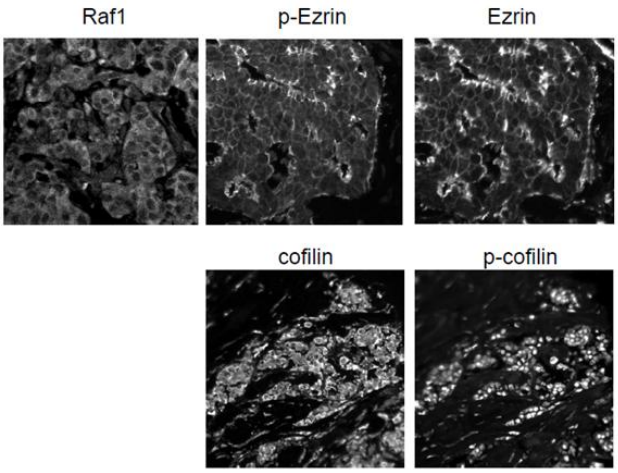


Figure 2.1.4. FRET images from formalin-fixed paraffin-embedded invasive ductal breast tumour samples, stained with HER2(DARPin)-Cy5 and either anti- HER3(2E9)-Alexa546 or anti- HER3(2F12)-Alexa546. The latter was used as negative control since the epitope recognized is on the cytoplasmic portion of HER3 whereas the HER2 DARPin recognizes an extracellular epitope on HER2 (*1*). FRET efficiency=1-lifetime or τ(DA)/ τ (control) where τ (DA) is the lifetime of anti-HER3(2E9)-Alexa546 in the presence of anti-HER2 DARpin-Cy5, whereas τ (control) was that of anti- HER3(2F12)-Alexa546 in the presence of anti-HER2 DARpin-Cy5. Red and blue pixels on the pseudocolor tumour image = low lifetime/high FRET efficiency and high lifetime/low FRET efficiency, respectively. The tumour tissues express various levels of HER2 protein, as scored by immunohistochemistry, ranging from highly positive (3+) to negative (0), according to the HercepTest scoring system. Cumulative analysis of FRET efficiency based on fluorescent lifetime are shown in a bar graph in the upper panel. Error bars represent SEM and significance values are shown.

- In addition to the development of FRET/FLIM assays for HER2:HER3 dimerization (which allow for the first time to assess the interaction between the proteins in human samples) we set up assays for detection and quantification of Raf1, Ezrin and cofilin in human samples, which will provide an additional information for interrogation of EGFR family signaling leading to metastasis (activity of those proteins were implicated in proliferation and motility of cancer cells). Figure 2.1.5. shows representative images of tissue stained with Raf1, p-Ezrin/Ezrin, pCofilin/Cofilin.

Figure 2.1.5.



Deviations from workplan

- None.

Impacts (resources, planning, link with other tasks)

- Task 2.3. Design of new FRET/FLIM assays based on toponomics. The SOPs for imaging protein interactions are now established as per figure 4 and therefore the KCL team is ready to engage with TNL.

Correctives actions

- None.

Acknowledgement: We thanks to Dr.Gargi Patel for running multiple preliminary tests to validate HER2 and HER3 antibodies.

Task 2.2:Toponomics. M1-M48; TNL

Progress toward objectives

Sub task 2.2.1.

- A toponome library of 100 distinct proteins/biomolecules has been calibrated and biologically validated against epidermal tissue [1] The results obtained were extremely interesting and will be mentioned in 6 publications.

Significant results

- A 100-component molecular library was calibrated against epidermal tissue of human skin [1]. The experiments concentrated on the epidermis cells (basal) and the molecular supermolecular structure of the basal lamina, a limiting membrane, which shows rather constant structural features across all tissues (including breast tissue), thereby separating epithelial cells from juxtaposed connective tissue, by forming 3 distinct, electron microscopically distinguishable layers: Lamina lucida, lamina densa, and lamina fibroreticularis (having a diameter of app 120 nm).

- An important advantage of this approach was that we were surprisingly able to detect by simple light microscopy many more layers within this structure, by co-mapping of 100 distinct proteins and carbohydrate structures: we detected that the basal lamina is composed of 6 to 7 distinct, spatially clearly separated and stoichiometrically controlled layers, which have not yet been described in the literature: By direct correlation with transmission electron microscopy we had already shown in this project that TIS robotics combined with a complex real time algorithm, based on a mathematical procedure of so called similarity mapping (SIM) [2], can easily resolve these basal lamina structures at app. 40 nm distance [1], herein referred to as functional super-resolution. In later experiments we were able to detect even more detailed exactly combinatorial stoichiometric molecular codes subdividing the basal lamina into 6 to 7 structures (in real time), thereby showing that the 120 to 150 nm basal lamina is composed of structures at spatial distances of approximately 20 to 30 nm (as derived from comparative TEM).
- Together these approaches allowed us to identify in morphological normal skin how the combinatorial molecular code of this structure is composed quantitatively. We consider this finding an important next step to understand the basic mechanisms operating in early cancer stages on the level of epithelial-basal lamina interactions enabling early cancer cells to crack these basal lamina barriers and penetrate the basal lamina structures.
- A significant result of the above briefly described progress towards our objectives is the functional super-resolution of 100 co-mapped proteins allowing us to distinguish novel toponome structures (Figure 2.2.1).

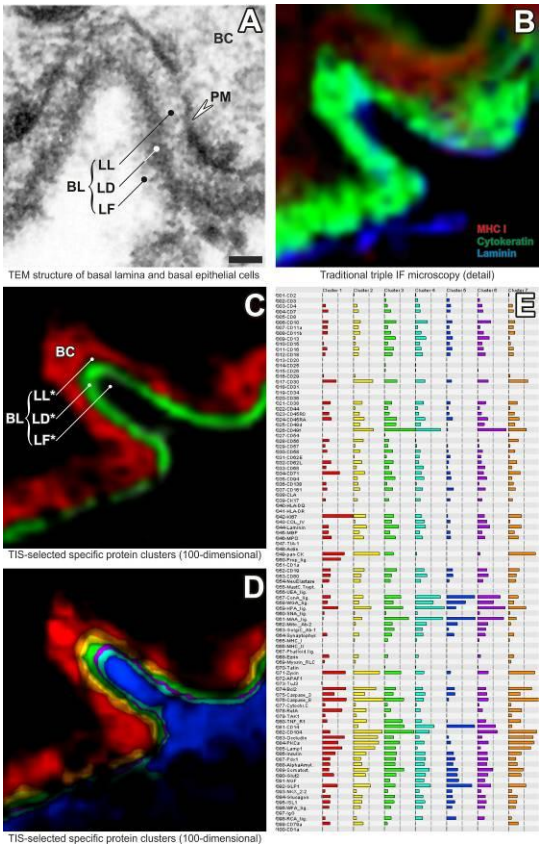


Figure 2.2.1. **A**, Transmission electron micrograph (TEM) of an epithelial-connective tissue junction. BL = Basal lamina; LL = Lamina lucida; LD = Lamina densa; LF = Lamina fibroreticularis. Bar: = 50 nm). **B,** normal triple epipfluorescence (BL: not resolved). **C,** TIS-similarity mapping (SIM) image showing the same 100 co-mapped proteins (note three layers of the BL resolved) [1]. **D,** 6 to 7 distinct layers of the BL resolved by real time TIS-SIM (see corresponding different colors of the corresponding protein profiles of 100-co mapped proteins in **E**.

- The comparison of toponome images in Figure 2.2.1 (A through E) shows the progress of our subtask 2 project: subcellular resolution of approx. 40 nm described in [1] (Figure 2.2.1, C) was improved down to 20 nm (Fig 2.2.1, D), to be published). Note: the total transverse diameter of the basal lamina across all tissues varies between 120 to 150 nm.
- We consider our TIS-SIM based detection of hitherto unknown substructures of the basal lamina on the molecular level as an important result to resolve the very early molecular mechanisms in this limiting structure in breast cancer, and the role of HER associated protein networks.

- Another result of the project was the publication of the ability to identify, interactively and in real time, protein network structures in 3D in any human cell type [3, 4]. A number of results of these recent studies are discussed and documented in detail in 7 separated articles by our group as part of the Encyclopedia of Systems Biology (in press) [5-8].

References:

[1] Schubert W, Gieseler A, Krusche A, Serocka P, Hillert R. Next-generation biomarkers based on 100-parameter functional super-resolution microscopy TIS. N Biotechnol. 2012 Jun 15;29(5):599-610.
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[2] Andreas Dress, Tatjana Lokot, Walter Schubert, and Peter Serocka. Two Theorems about Similarity Maps. Annals of Combinatorics, Volume 12, Number 3, October 2008 , pp. 279-290(12)
http://dx.doi.org/10.1007/s00026-008-0351-4

[3] YouTube-Video: http://www.youtube.com/watch?v=nU9yLY7IXyM

[4] Andrei Barysenka, Andreas W. M. Dress , Walter Schubert. A Comparative Method for Analysing Toponome Image Stacks. East Asian Journal on Applied Mathematics, 1 (2011), pp. 35-48. doi:10.4208/eajam.280909.270410a

[5] Gieseler A. Synaptic Proteins; Synaptic Toponome; Cell Membrane Toponomics. In: (eds. Dubitzky W *et al.*); Encyclopedia of Systems Biology. Springer; in press (2013)

[6] Hillert R. Toponome Analysis; Combinatorial Molecular Phenotype (CMP). In: (eds. Dubitzky W *et al.*); Encyclopedia of Systems Biology. Springer; in press (2013)

[7] Krusche A. TIS robot. In: (eds. Dubitzky W *et al.*); Encyclopedia of Systems Biology. Springer, in press (2013)

[8] Schubert W. Toponomics. In: (eds. Dubitzky W *et al.*); Encyclopedia of Systems Biology. Springer; in press (2013)

Deviations from workplan

- Delays have occurred in obtaining human breast tissue from KCL resulted in lack of suitable material for identification of HER-2 associated toponome clusters (D2.5 due M16). The delay was due to time obtaining ethical permission from KCL and agreeing a material transfer agreement for transport of suitable human breast tissue from KCL to TNL. These issues have now been successfully resolved and tissues will be available at the beginning of 2013.
- TNL addressed the difficulties in obtaining the breast cancer tissues by using a similar epithelial tissue (human skin) as this has the same origin than breast tissue.
- This enabled TNL to answer the principle questions of our subtasks 2.2., namely to calibrate and biologically validate a toponome library of 100 distinct proteins/biomolecules in relevant human tissue.

Impacts (resources, planning, link with other tasks)

- Due to above described delays; TNL was not able to deliver to D2.5 at M16.
- As a consequence the beginning of Task 2.3 might be delayed (interaction KCL, TNL to design new FRET/FLIM assay).

Correctives actions

- Provision of the breast cancer tissues in January 2013 will allow TNL to map these structures and provide signatures of protein complexes embedding the HER-receptor.

Other

- The public-private partnership model of TNL is under discussion with the European Commission. It is intended that University of Magdeburg will enter as third party during P2.

Task 2.3: Design of new FRET/FLIM assays based on toponomics M24-M33; KCL, TNL

Month

Task 2.4: Nanoscopy M19-M48; MPG

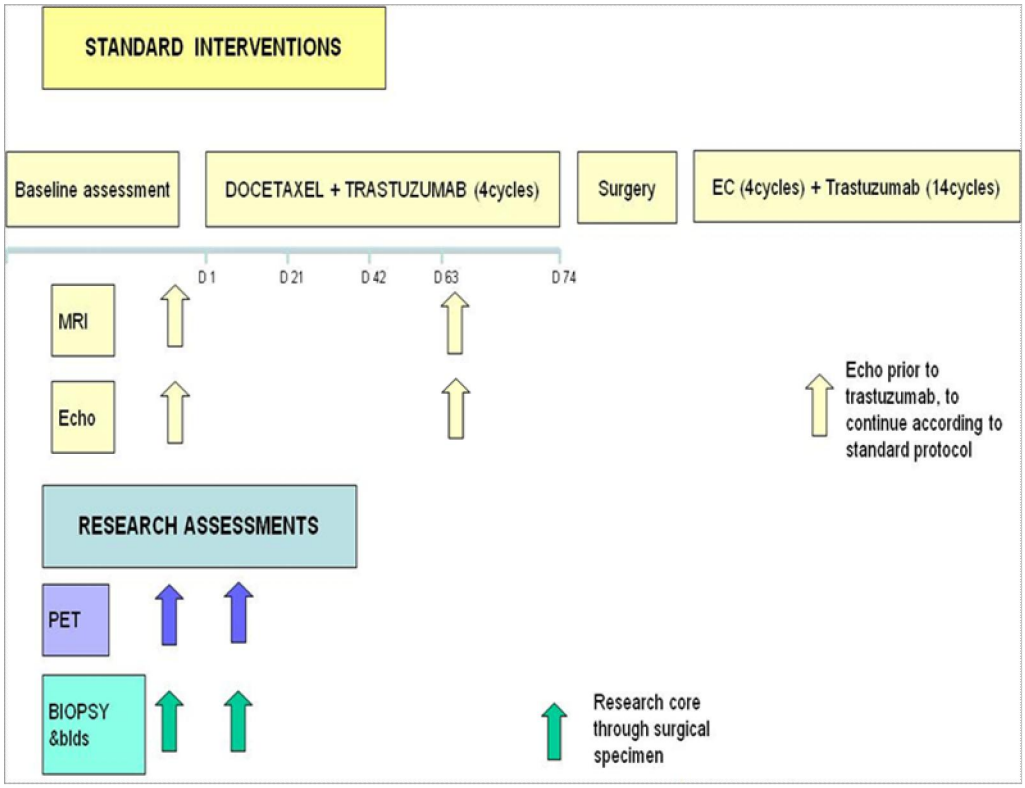
Month

Task 2.5: Combination of medical IMAGINT (PET) and tissue imaging-based diagnostics. M1-M36, KCL, TNL, MPG

Progress toward objectives

- The protocol for a clinical trial entitled " A randomized phase II trial of [18F]fluorothymidine and the standard tracer 18F]Fluorodeoxyglucose in the assessment of HER2-targeted and systemic therapy response in HER2-positive breast cancer and their utility compared to conventional MRI imaging response and biopsy derived biomarkers" is written.
- Primary objective is "To correlate PET imaging response in breast and axillary lymph nodes with residual cancer burden (RCB) at definitive surgery". Schema 2.5.1 shows the rationale and order of tests and treatments along the trial. Initial assessment of the clinical status followed by 4 cycles of treatment with biopsies at the beginning and the end of the treatment. At the end of the first 4 cycles surgery will be performed and will follow by additional 18 cycles of treatment.

2.5. Schema 2.5.1



Material collected in this study will be subjected to newly developed assays for HER2:HER3 dimerization and quantification of phosphorylation status of ezrin and cofilin, as well as expression levels of Raf1.

Significant results

- None.

Deviations from workplan

- Within the same Cancer Imaging Centre (directed by Prof. Tony Ng), there is now a clinical study to investigate a HER2 PET tracer (from GE Healthcare). This imaging trial is favourable for IMAGINT because (unlike FDG/FLT PET) it is HER2-specifc. After discussion with Prof. Chester (Coordinator), it was agreed to pursue the opportunity of using the HER2 PET study (which will be of identical design to that in Schema 2.5.1) to deliver D2.6; Comparison of FRET/FLIM metastatic signature with FDG and/or FLT PET-CT data (M36).

Impacts (resources, planning, link with other tasks)
• None
Correctives actions
• D2.6 will be delivered with a HER2 PET tracer instead of FDG/FLT PET. The new trial will have the advantage of being HER2-specific.

WP3 – Developing methods for isolation characterization of protein/RNA complexes from clinical tissues

Task 3.1: Isolation of RNA/protein complexes using Anti-Ago2 DARPINS. M1-M10; INO, UZH, UCL

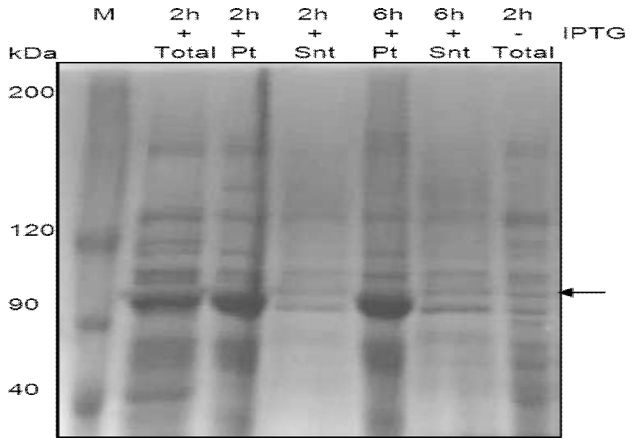
Progress toward objectives

- Human full length Ago2 (NM_012154) has been produced using the baculovirus system in Sf9 cell line as it was not possible to express the protein in either mammal cells or bacteria.

Significant results

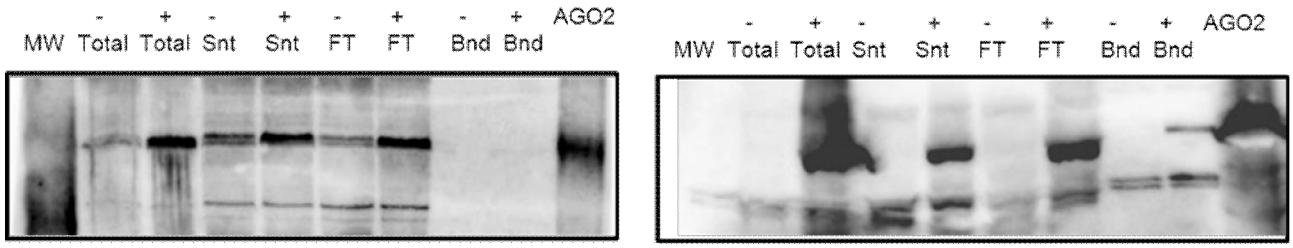
- Human Ago2 cDNA and N-terminal deletion mutant Ago2 cDNA were separately cloned into a bacterial expression plasmid containing an N-terminal Avitag (GLNDIFEAQKIEWHW). The Avitag allows the biotinylation *in vivo* of Ago2 protein, which it is necessary for the process of DARPin generation. Human Ago2 protein and Ago2 mutant protein were correctly expressed in bacteria. However, both proteins are insoluble (Figure 3.1.1) and it was not possible to purify them from the bacterial extracts.

Figure 3.1.1



- To overcome the solubility problem, human Ago2 cDNA was cloned into a bi-cistronic mammalian expression vector containing in the N-terminus an Avitag and the biotin ligase cDNA sequence. The biotin ligase is essential for protein biotinylation process in human cells. Ago2 protein was expressed in HEK293 cells and it was biotinylated *in vivo*. The biotinylated protein was isolated from cellular extracts using Streptavidin–Sepharose. However, the purification yield was not enough to proceed with the DARPINs generation process (Figure 3.1.2).

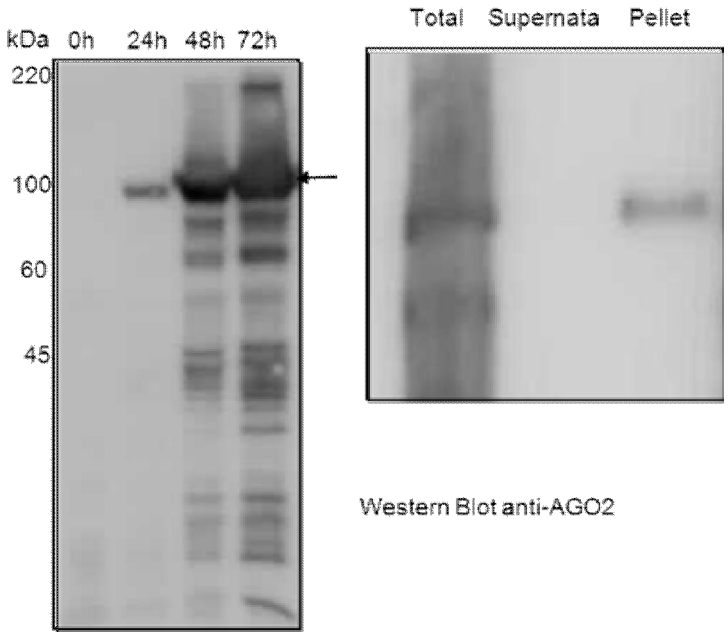
Figure 3.1.2



Western Blot anti-AGO2 Western Blot anti-Biotin

- We have used another strategy in order to obtain the needed amount of Ago2 protein. Ago2 cDNA has been cloned into a baculovirus vector system in order to solve the solubility and the protein purification yield problems. An N-terminal Avitag and a C-terminal hexahistidine-tag (H_6-tag) were added to Ago2 for biotinylation and purification purposes respectively. The Avitag allows the *in vitro* biotinylation of Ago2 protein, which is necessary for DARPINs generation. The H_6-tag allows purifying the protein in its native state.

Figure 3.1.3



- Ago2 protein was successfully expressed in Sf9 cells. However, the resulting protein proved to be mainly insoluble again (Figure 3.1.3), so its purification at a reasonable yield has not been possible.

Deviations from workplan

- Human full length Ago2 (NM_012154) was unable to be expressed in either mammal cells or bacteria. Thus, the protein was produced using the baculovirus system in Sf9 cell line.

Impacts (resources, planning, link with other tasks)

- The inability to produce mgs of purified Ago2 protein will hinder the production of Ago2 DARPins in WP1.

Correctives actions

- The possibility of using another RISC complex protein, isolated from Proteomic results in the task 3.3, will be explored.

Task 3.2: Isolation of RNA complexes using Ago2tagged technology. M1-M12; INO, UCL

Progress toward objectives

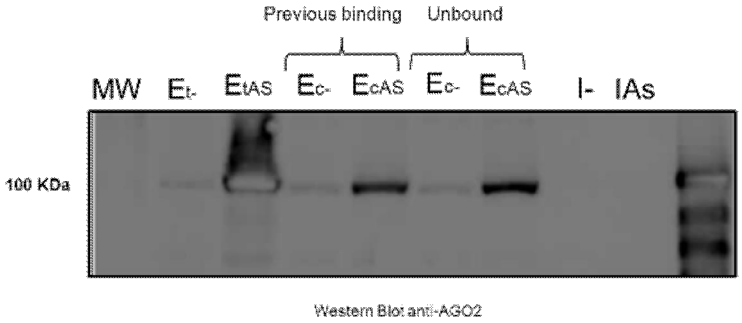
- Good progress has been made. Ago2 protein complexes have been isolated from BT747 cell line by immunoprecipitation. These protein complexes have been characterized by MALDI-TOFF and we are identifying the first candidates to be studied as new disease biomarkers.

Significant results

- Human Ago2 cDNA was cloned in a mammalian expression vector containing an N-terminal Strep-tag. The Strep-tag allows the isolated Ago2/miRNA complexes to maintain its molecular integrity. This construct was transfected into HEK293 cell line. The results showed that the protein expression was correct but it was not possible to isolate it from cellular extracts. Possibly the protein's 3D conformation hides the tag (Figure 3.2.1).

Figure 3.2.1

Et: Total extract. Ec: Cytosolic extract.
I: Immunoprecipitation.
-: Untransfected. AS: Ago2 transfected.

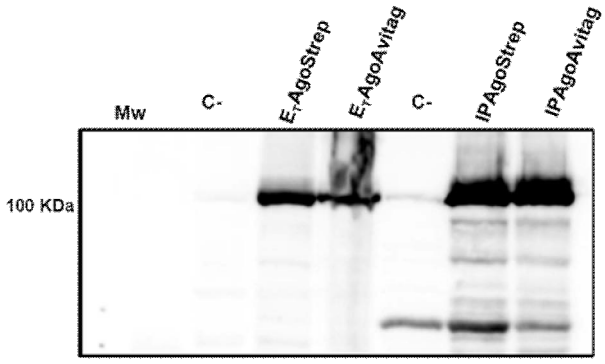


Western Blot anti-AGO2

To overcome this problem, we transfected HEK293 cells with different Ago2 constructs cDNA. The cellular extracts were used to isolate Ago2 protein by immunoprecipitation using an anti-Ago2 antibody (Figure 3.2.2).

Figure 3.2.2

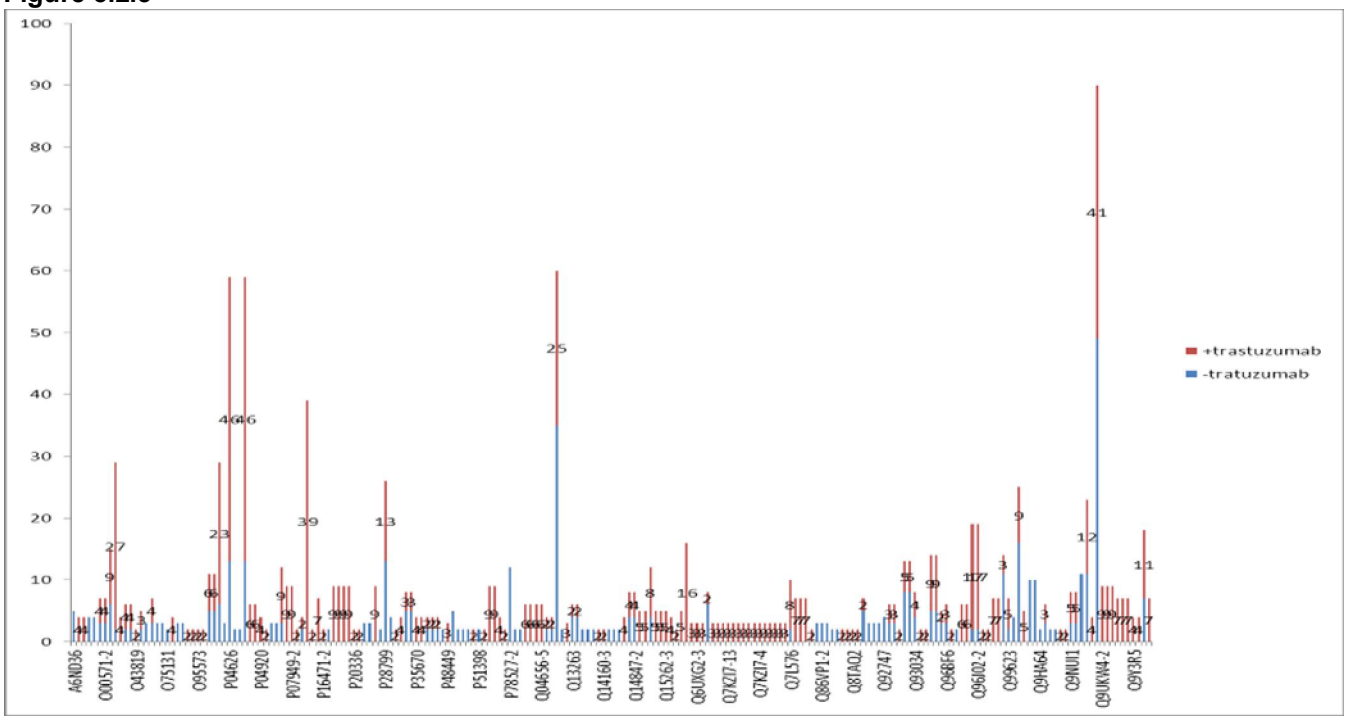
Et: Total extract. IP: Immunoprecipitation.
C-: Untransfected. AgoStrep: Ago + Strep-tag.
AgoAvitag: Ago + Avitag.



Western-Blot anti-AGO2

- The results showed that endogenous Ago2 protein complexes and transfected Ago2 complexes could be isolated using antibodies against Ago2. In addition, the transfected Ago2 protein expression and isolation yields are at least 10 times higher than the endogenous complexes in HEK293.
- These expression and complexes isolation protocols were transferred to human breast cancer cell lines as SKBR3 and BT474. However, both cell lines presented very low transfection efficiency and a high cytotoxicity due to transfection treatment; the immunoprecipitation experiment achieved better results using the endogenous Ago2 protein in BT747 cell line.
- In addition, we have obtained sufficient quantities of protein complexes to use them in the proteomic approaches from BT747 treated or not treated samples with Trastuzumab.
- The two isolated protein complexes have been characterized by MALDI-TOFF (Figure 3.2.3). In total around 1365 high confidence proteins (less than 1% mismatch error) have been identified with at least two peptides matched. This number has been narrowed down to 209 proteins that have been selected to be studied in relative with this project.

Figure 3.2.3



Deviations from workplan

- Due to problems with the expression of tagged version of Ago2, we decided to isolate the protein complexes using antibodies against Ago2 protein.

Impacts (resources, planning, link with other tasks)

- We have used these results in the task 3.3, task 3.4 and task 3.7.

Correctives actions

- None.

Task 3.3:CLIP technology to isolate the miR/RISC complex. M1-M10; INO, UZH, UCL

Progress toward objectives

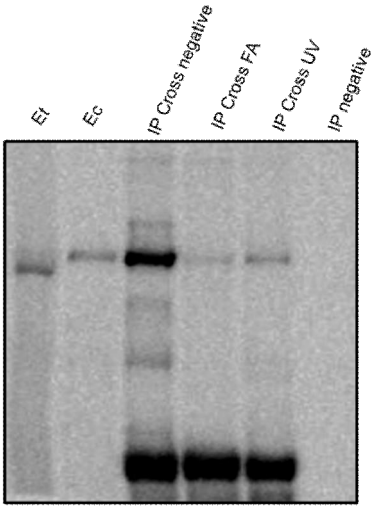
- In order to increase the yield of proteins and miRNAs detected in the immunoprecipitation procedure, we have included a crosslinking step prior to the immunoprecipitation. This broadly used technique (so called CLIP: Crosslinking immunoprecipitation) stabilizes the interactions between the molecules making easier their co-isolation and the downstream analysis. The protein Ago2 complexes were isolated by crosslinking immunoprecipitation (CLIP) using UV irradiation (physical method) or formaldehyde (FA) (chemical method) (Figure 3.3.1). The miRNA have been isolated for further sequencing analysis.

Significant results

- We have performed UV-CLIP and FA-CLIP experiments in Trastuzumab treated or untreated BT747 cells. In addition, the miRNAs and mRNAs associated with Ago2 have been isolated from BT474 cells cross-linked or not with UV radiation or FA .
  These samples will be further analyzed in the tasks 3.8 and 3.9.

Figure 3.3.1

Et: Total extract.
Ec: Cytosolic extract.
IP cross negative: IP with empty resin.
IP cross FA: IP with anti Ago2 resin over FA crosslinked sample.
IP cross UV: IP with anti Ago2 resin over UV crosslinked sample.
IP negative: IP without sample.



Western-Blot anti-AGO2

Deviations from workplan

- We have used anti Ago2 antibodies to isolate the complexes.
- We have also included the FA-crosslinking.

Impacts (resources, planning, link with other tasks)

- The results of this task will be used in tasks 3.8 and 3.9.

Correctives actions

- None.

Task 3.4: Comparison of technologies. M12-M16; INO, FLS, KCL

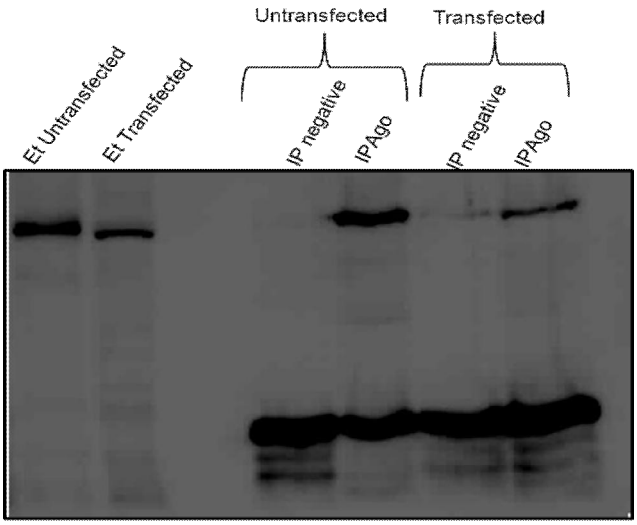
Progress toward objectives

- At this moment, only the immunoprecipitation method has given good results allowing us the RNA-protein complexes isolation.
- It has been impossible for us to obtain enough Ago2 protein to produce anti-Ago2 DARPins although we have tried several strategies to express the protein. Unfortunately in our hands, transfected Ago2 has showed, either low expression level, cytotoxicity or a high tendency to aggregation.

Significant results

- The results indicated that immunoprecipitation using specific antibodies against Ago2 is the most efficient technology. In addition, the transfection of the specific breast cancer cell lines as BT747 cell line and SKBR3 showed that the transfection efficiency is low and affects cell viability. However, immunoprecipitation against the endogenous protein provides enough protein isolation yield to perform the genomic and proteomic experiments (Figure 3.4.1).

Figure 3.4.1



Western Blot with antibody against AGO2

Deviations from workplan

- As stated in task 3.1, it has been impossible to obtain Ago2 or tagged Ago2. We have faced expression yield problems and the expressed protein was prone to aggregation. We have tried to express the protein in different systems and we have switched the tag between N- and C-terminus of the protein in order to avoid this aggregation. None of the modifications that we have performed in the protocol of expression and purification of Ago2, have worked out successfully. There is no way to compare between technologies.

Impacts (resources, planning, link with other tasks)

- None.

Correctives actions

- None.

Task 3.5: P-bodies study during RISC formation complex. M1-M10, INO, UZH

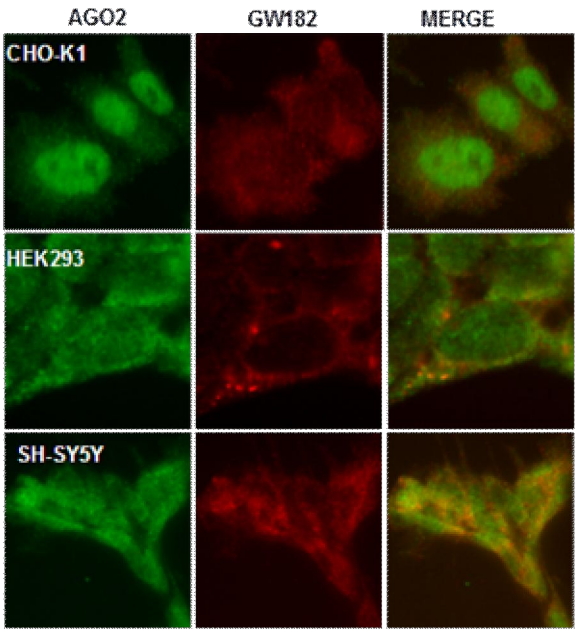
Progress toward objectives

- It is described in the literature that the functionality of the RNA/RISC complex depends on its location in the cell: to be functional, the complex must be part of the P-bodies or Processing-bodies, the place in the cytoplasm where the processing and degradation of the RNA takes place and where the RISC machinery works.
- In order to use the location of the RNA/RISC complexes in the cell as a complementary marker for studying the effect of anti-tumoural compounds, Ago2 and GW182 proteins were detected by immuno-fluorescence. Ago2 is one of the main components of the RNA/RISC complex and GW182 is a protein present in the Processing-bodies and one of the responsible for their stability. The change in the location of the RNA/RISC after a treatment will infer the effect of this treatment in the activity of AGO2 and the complex inside living cells.
- The experimental conditions have been developed to study cellular co-localization of both proteins in human cells.

Significant results

- GW182 and Ago2 protein were stained using specific antibodies, labelled with different fluorochromes and their co-localization has been determined in different cell lines (CHOk1, HEK293 and SH-SY5Y). In these experiments, Ago2 localized in CHOk1 nuclei. On the contrary, it appeared diffused in the cytoplasm and also restricted to P-bodies in HEK293 and SH-SY5Y cells (Figure 3.5.1).

Figure 3.5.1



These experiments were performed to choose the appropriate conditions to analyse the P-bodies with BD Pathway 855 image platform. With this platform the co-localization of both proteins was analyzed.

Deviations from workplan

- There have been no deviations.

Impacts (resources, planning, link with other tasks)

- None.

Correctives actions

- None.

Task 3.6:Transfer the microRNA/mRNA/protein isolation and characterization technology for the antiHER2 treatment cases. M16-M24, INO, UCL

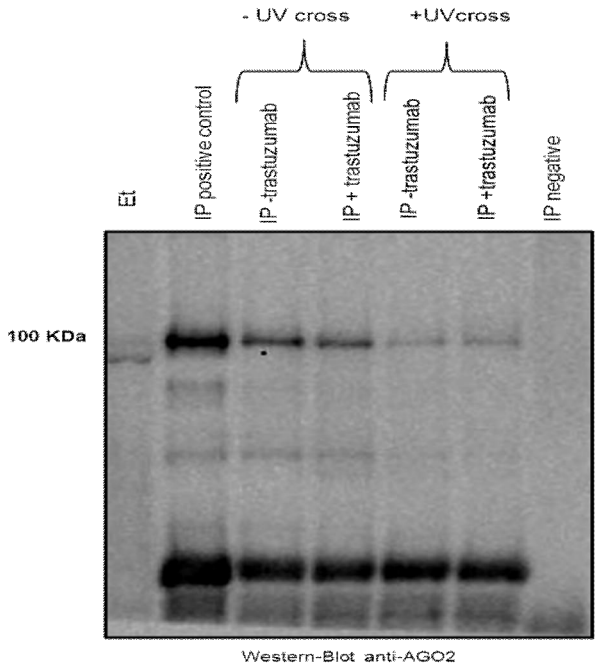
Progress toward objectives

- The Ago2 containing complexes were isolated by cross-linking immunoprecipitation (CLIP) using UV irradiation (physical method) or formaldehyde (FA) (chemical method). The miRNA/mRNA from BT474 cells treated or not treated with Trastuzumab have been isolated to be sequenced.

Significant results

- We have performed UV-CLIP and FA-CLIP experiments in Trastuzumab treated or untreated BT747 cells. The miRNAs and mRNAs associated with Ago2 have been isolated from BT474 cells treated or not treated with Trastuzumab and cross-linked or not with UV radiation (Figure 3.6.1). The miRNAs have been isolated to be sequenced by SOLEXA technology. However, the experiments showed that isolation of the protein complexes was affected by cross-linking process; the complex isolation yield seems to be lower. So, for the proteomic approach we have utilized the protein complexes from not cross-linked samples (Task 3.2).

Figure 3.6.1



Western-Blot anti-AGO2

Deviations from workplan

- The Ago2 protein complexes are isolated with a higher yield from non cross-linked samples. We have analyzed the protein complexes from non cross-linked samples from Task 3.2

Impacts (resources, planning, link with other tasks)

- None.

Correctives actions

- None.

Task 3.7:Transfer the RISC location technology to antiHER2 treated cells. M11-M10, INO

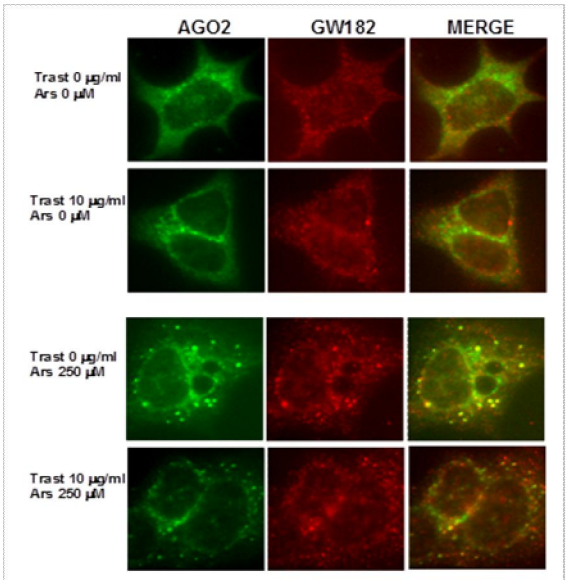
Progress toward objectives

- The previous experimental conditions (task 3.5) were used in two human breast cancer HER2 positive (HER2+ve) cell lines (BT474 and SKBR3) untreated and treated with the anti-tumoural compound Trastuzumab in order to study whether the P-bodies containing Ago2 could be used as biomarker or not. With the BD Pathway 855 image platform the co-localization of both proteins was studied by immuno-fluorescence and number and size of Ago containing P-bodies after the treatment with Trastuzumab were analyzed. This study was done according to different parameters; i.e. the granularity and average area of AGO2 fluorescence and the co-localization with GW182.

Significant results

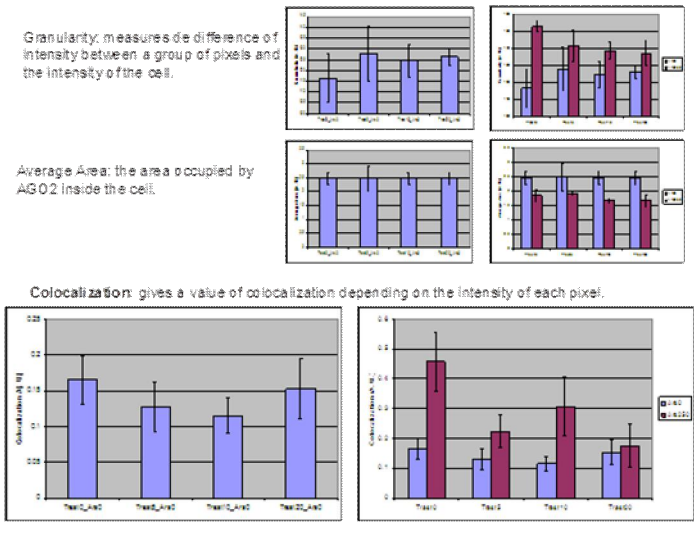
- The immunofluorescence in these cells showed a speckled pattern around the nucleus for Ago2 and GW182 proteins (Figure 3.7.1). The preliminary results are promising.

Figure 3.7.1



Generally, the granularity of AGO2 fluorescence after adding sodium arsenite (a compound that increases the oxidative stress) seems to decrease in the presence of Trastuzumab. The most promising result is the co-localization of AGO2 and GW182 based on the intensity of their fluorescence. After adding arsenite, the co-localization of these proteins increases and when Trastuzumab is added, decreases (Figure 3.7.2). It is possible that Trastuzumab may affect the location, and consequently, the activity of AGO2 and its function as miRNA regulator.

Figure 3.7.2



Deviations from workplan

- The oxidative stress caused by the addition of sodium arsenite increases the number of Ago2 containing bodies and facilitates its analysis.
  We will try to improve co-localization data using Ago3 protein, an Ago family protein that is also part of the P-bodies.

Impacts (resources, planning, link with other tasks)

- None.

Correctives actions

- None.

Task 3.8: The new biomarkers finding process by antiHER2 responsiveness therapies. M24-M30, INO, FLS, UCL, TNL
Month

Task 3.9:Transfer the technology to the human samples. M31-M40, INO, KCL, UCL, FLS
Month

WP4 – Development of radiolabelling and software for quantum imaging

Task 4.1: Further dev of purification protocols for radionuclides M1-M15; UU, UCL, GEHC

Progress toward objectives

- Work flow with microfluidic small scale device has been established

Significant results

- Radiolabelling experiments using a newly developed microfluidic system were carried out, where ^{68}Ga from a ^{68}Ge generator was incorporated into a mixture of monomeric, dimeric and possibly higher aggregate DARPin proteins conjugated to a NOTA chelator.

Deviations from workplan

- None.

Impacts (resources, planning, link with other tasks)

- Small scale handling of radionuclides will be used for radiolabelling.

Correctives actions

- None.

Task 4.2: Development and biological validation of protocols for labelling technology M1-M15; UU, UZH, UCL, GEHC

Progress toward objectives

- Protocol for conjugation of NOTA chelate to DARPins has been established.
- Quality control protocol for conjugation of maleimide to DARPins has been established.

Significant results

Quality control of DARPin protein:

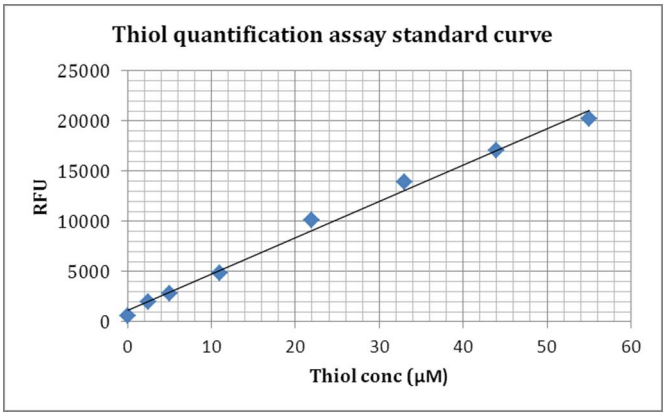
- SDS-PAGE analysis of G3 DARPins obtained from UZH showed a mixture of aggregates readily visualized by silver staining.
- Subsequent handling of this DARPin during the process of conjugation of a NOTA chelator led to considerable precipitation and loss of protein.
- Dilution of the protein to 1/10 of the initial concentration did not noticeably change the ratio of monomer to multimer, suggesting that the aggregation was irreversible.
- The conditions for conjugation were worked out using this material and are described below. A new batch of DARPin was prepared by UZH and delivered for conjugation.
- The procedure of conjugating NOTA followed the same protocol, and considerable precautions were exercised to keep it reduced to avoid dimerization/oxidization.

- A thiol assay was set up to monitor the reduction and conjugation reactions in order to ensure quantitative conjugation. Before conjugation the concentration of free thiols was 11µM, after conjugation there were no free thiols (Figure 4.2.1).

.

Figure 4.2.1

Thiol conc (µM)	RFU
0	603,286
2,5	2133,403
5	2752,91
11	5185,382
22	10457,33
33	13942,82
44	17425,8
55	20422,43



Measured concentrations of G3-Cys DARPin

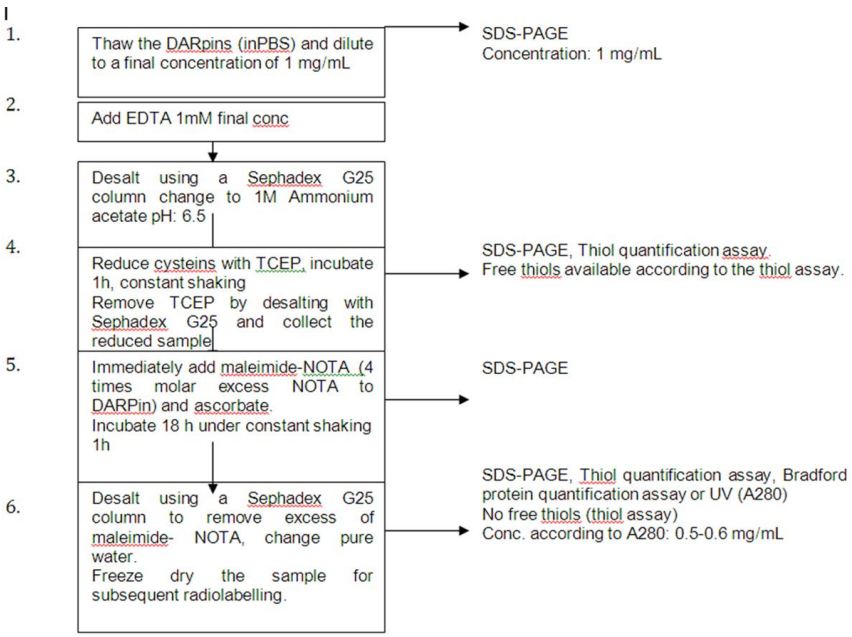
G3-Cys red TCEP, desalted : 4769 ⇒ 11 µM Concentration: 0.375 mg/mL
G3-Cys after conjugation NOTA (1): 597 ⇒ 0 Concentration: 0.5 mg/mL (A280)
G3-Cys after conjugation NOTA (2): 681 ⇒ 0 Concentration: 0.6 mg/mL (A280)

- Procedure for conjugation of DARPins to maleimide-NOTA

All buffers and solutions are degassed and passed through a Chelex column to remove contaminating metal ions prior to use.

- Protocol for NOTA conjugation.

Procedure for conjugation of DARPins to maleimide-NOTA
All buffers and solutions are degassed and passed through a Chelex column to remove contaminating metal ions prior to use.



Radiolabelling:

- We have in several projects and patents showed that it is possible to produce labelled peptides and proteins using conjugated chelates like DOTA and NOTA, in combination with ^{68}Ga obtained from a ^{68}Ge-generator. With this as background we performed a series of labelling approaches using the DARPins obtained, although the chelate coupled proteins were shown to be a mixture of monomers, dimers and possibly higher aggregates.
- We have so far gained a significant knowledge about the parameters, which are crucial for the labelling, aiming to prepare the labelled proteins in high specific radioactivity but using the conjugated chelated dimer as the precursor.
- We are now setting up a technology platform where we are in control of parameters such as protein/peptide concentration, temperature, solvents and buffers, cassette technology and this set up has also been used in our attempts to label the DARPin mixture of monomers, dimers. We have shown good efficiency regarding trapping, release and protein reaction. The platform technology has been focusing on the use of ^{68}Ga but the concept will be useful also for ^{111}In and other radio-metals.
- We are just now entering in a phase where we will have access to other DARPin preparations, which has been coupled to the chelate using modified procedures.

Deviations from workplan

- Progress was delayed due to late delivery of DARPins and because analysis of the initial batch revealed aggregation.

Impacts (resources, planning, link with other tasks)

- We have postponed the biological evaluation until we have been able to perform the labelling under controlled conditions. This has caused delay to D10.

Correctives actions

- UZH, UCL and UU collectively resolved problems of aggregation, including a work visit by Robert Goldstein (UCL) to UU.

Task 4.3: Development of improved QSPECT Imaging for Ga67/In111 M1-M15; UCL, GEHC

Progress toward objectives

- The objective was to determine whether proprietary compensation for collimator geometry ("resolution recovery" (RR)) improves the quantification of ^{111}In uptake by SPECT imaging.
- Improved SPECT quantification of ^{111}In distribution has been achieved by compensation for collimator geometry effects.

Significant results

- Resolution of ^{111}In SPECT was measured using three line sources of (each 10MBq ^{111}In) in air. SPECT data was acquired in 60 projections over 360° using a GE Discovery 670 SPECT-CT system fitted with a MEGP collimator. Data was acquired using two peak windows (171keV±10% and 245keV±10%) and one scatter window (140keV±9%). Recovery Coefficient was measured for a 16.5ml (r=16mm) sphere filled with 20MBq ^{111}In, in a cylindrical water filled phantom (r=111mm) using the same acquisition parameters. Transaxial images were reconstructed using OSEM (10 subsets, 6 iterations, no post filtering) using standard CT attenuation and scatter correction; two sets of images were created, one with RR1 and one without. Resolution was estimated from x and y line profiles for each line on three SPECT non-consecutive slices, the profiles were filled to a Gaussian model and the full width half maximum (FWHM) values calculated. Mean FWHM values were calculated with and without RR. Recovery coefficient (RC) was taken as the number of counts seen inside the sphere as a percentage of total counts in the reconstruction. For the counts in the sphere a spherical VOI was defined using the CT image.

- The line profiles from the RR data were observed to fit the Gaussian model better than the non-RR data, FWHM without RR was 11.7±1.8mm and 8.1±0.9mm with RR, ($p<0.01$). Recovery Coefficient: RC was calculated as 52% without RR and 60% with RR for the sphere of radius 16.5mm. 1RR using the GE Evolution Toolkit.
- Thus the employed resolution recovery technique significantly improves the resolution of ^{111}In -SPECT imaging with a consequent improvement in the Recovery Coefficient. This result is important for the visualization of small lesions.

Deviations from workplan

- None

Impacts (resources, planning, link with other tasks)

- None

Correctives actions

- None

Task 4.4: Optimization of DARPins as imaging agents in spheroids and mice M16-M42; UU, GEHC, UZH, UCL

Progress toward objectives

- Progress has been made in task 5.3.

Significant results

- See 5.3 significant results section and figures 5.3.1/5.3.2.

Deviations from workplan

Delay due to dependence on outcome of task 5.3

Impacts (resources, planning, link with other tasks)

- The decision to evaluate the different DARPin formats (Task 5.3) necessitated Task 4.4 to be put on hold, in order not to waste animals or materials on pre-clinical testing of an irrelevant DARPin.
- UU worked closely with UCL, including UU hosting a visit from Dr Robert Goldstein (UCL) to develop maleimide-mono-amide DOTA conjugation to a unique C-terminal Cysteine on the G3 DARPin.

Correctives actions

- G3 DARPin with HEHEHE (HE_3) will be delivered by UCL
- DOTA will be incorporated
- Radiolabelling of DOTA labelled DARPin with HE_3-tag will commence beginning of 2013
- Spheroid and animal studies to follow as appropriately labelled DARPins have been prepared
- We will use ^{68}G PET instead of SPECT, due to excellent biodistribution data at early time points obtained by UCL (Task 5.3) that indicate that ^{68}Ga PET would be the favourable imaging modality (more sensitive and shorter exposure to radiation).
- We will apply for an amendment to report D4.10 at M26.

WP5 – Phase I trial to assess safety and efficacy of quantitative imaging biomarkers in patients

Task 5.1: Manufacture and analysis of GMP DARPin M1-M18; M-GMP, UZH, UCL, UU/GE, KCL

Progress toward objectives

- Progress has been scientifically rewarding with a necessity to overcome a number of challenges in manufacture of the G3 anti-HER2 DARPin and to test G3 DARPin with different engineered tags; a H_6-tag, an alternation triplicate of histidine and glutamate (HE_3) tag and tag-free (No tag) variant. This required several parallel modes of attack and exploration of different expression platforms.

DARPin construct

- Our original plan was to use H_6-tagged G3 DARPin for IMAGINT and to produce the DARPin in *Escherichia coli* using established laboratory protocols that would be adapted for Good Manufacturing Practice (GMP) production in collaboration with beneficiary 9 (M-GMP).
- The H_6-tag appeared to be a good choice; it is used for purification by immobilised metal affinity chromatography (IMAC) and many H_6-tagged proteins have been successfully used in patients without adverse effects. Furthermore, the His-tag can be used for site specific radio-labelling.
- However, indications from subsequent published work by others that His-tag could enhance liver uptake (Hofstrom *et al*. [J Med Chem 2011; 54;3817–382]) led us to re-think this purification strategy: (i) to minimise the risk of potential unwanted liver uptake and (ii) to avoid possible future restrictions by regulatory bodies on use of H_6-tag. We first addressed the issue by designing G3 with an alternative tag and by introducing a cleavage site into the DARPin construct to allow removal of the His tag following IMAC purification. We also wanted to investigate the possibility of using a HE_3-tag instead of a H_6-tag as it has been indicated that HE_3 could protect from liver uptake with other radiolabelled proteins (Hofstrom *et al*. J Med Chem 2011; 54;3817–382).

Process development:

- It was necessary to develop a GMP process for production of tag-free DARPin and HE_3–tagged DARPin. M-GMP did not become engaged in the project due to internal issues (and have subsequently left IMAGINT). Therefore UZH and UCL joined forces to address the issue.
- Since G3 DARPin production was on the critical path of the project, UZH changed emphasis to bring forward *E.coli* protocols for tag-free DARPin. (UZH source).
- In parallel UCL explored the yeast *P. pastoris* platform.

Summary:

- Loss of M-GMP and necessity for unforeseen process development has delayed the delivery of a process for production of the GMP DARPin by 6-9M. However, good progress has been made and the issues are now largely resolved.

Significant results

E.coli protocols:

- At UZH, a number of protocols were established for large scale *E. coli* expression and purification. This required to carry out the work at scale, and to deliver the protein to the other partners. This repetitive large scale work had not only slowed down progress (as columns at this scale were not yet available at this time), but also added to the expense due to high running costs.
- For this reason a procedure was developed to use 3C protease to cleave off the tag. A procedure using a self-expressed 3C protease with itself a H_6-tag was developed such that this 3C protease can be removed by "inverse IMAC"
- The overall procedures consist of IMAC with removal of endotoxin, ion exchange, gel filtration. It was found important to always keep the protein reduced.

- New vectors were developed (fully sequenced, with kanamycin resistance) and several origins were tested.
- Also, mutations were tested which were suggested to be part of an interaction between G3 monomers. Unfortunately, all of them slightly or significantly decreased affinity, such that this work was not further pursued
- UZH sent a protocol of the worked out procedure to the project partners. (UZH source)

P. pastoris protocols:

- In order to clarify the effect of tags on biodistribution, three variant G3 DARPins have been produced in *P. pastoris* and purified for imaging studies. The variant proteins have a H_6-tag, a HE_3-tag or no tag at the N-terminus. The proteins have a C-terminal Cysteine for site-specific labelling.
- Based on the UZH protocol, a successful removal of the H_6-tag of the cleavable tag variant was achieved with GMP-grade HRV 3C protease. The tag free G3 variant was further purified on ion exchange chromatography and FPLC.
- The general procedure for production of the three variants comprises IMAC as primary capture, followed by a concentration/dialysis step in a tangential flow filtration device and size exclusion chromatography using FPLC. The final polishing step, comprising glucan and endotoxin removal is under development.
- Task 5.3 revealed that the HE_3-tag G3 variant had a favourable biodistribution over the H_6-tag and no tag variants. Therefore, development of GMP protocols of the HE_3-tag G3 variant was taken forward.
- Process development for the HE_3-tag G3 DARPin in a bioreactor reveals that the *P. pastoris* production platform is able to produce sufficient material for the clinical trial.
- The required protocols/documentation (general, QA and QC SOPs) for GMP production of the G3 DARPin are in an advanced state: i.e. finished or being drafted.
- Relevant equipment and disposables for use in the UCL GMP facility have been sourced and/or a supplier has been approved.

Deviations from workplan

- Our original plan was to use H_6-tagged G3 DARPin for IMAGINT and to produce the DARPin in *E. coli* using established laboratory protocols that would be adapted for GMP production in collaboration with beneficiary 9 (M-GMP).
- The H_6-tag appeared to be a good choice as it is used for purification by IMAC and many H_6-tagged proteins have been successfully used in patients without adverse effects. Furthermore, the H_6-tag can be used for site specific radio-labelling.
- However, indications from subsequent published work by others that H_6-tag could enhance liver uptake (Hofstrom *et al*. J Med Chem 2011; 54;3817–382) led us to re-think this purification strategy. We wanted to test whether these observations were applicable to DARPins therefore generated tag-free DARPin and HE_3-tagged DARPin to test their effect on biodistribution.

Impacts (resources, planning, link with other tasks)

- M-GMP did not become engaged in the project due to internal issues (and have subsequently left IMAGINT).
- Loss of M-GMP and necessity for unforeseen process development has delayed the delivery of a process for production of the GMP DARPin by 6-9M.
- UZH changed emphasis to bring forward *E. coli* protocols for tag-free DARPin. (UZH source). Therefore UZH has become much more involved in this workpackage than previously planned, having shifted resources from the other work packages. Even though, in principle, the methods of DARPin production and purification were well worked out, at larger scales new issues appeared that had to be solved. (UZH source)
- D4.10 was delayed because we did not want to use value resources until we had established which construct would be taken forward to the clinic.

Correctives actions

- Since G3 DARPin production was on the critical path of the project UZH and UCL joined forces to meet 5.1 objectives.
- Good progress has been made and the issues are now largely resolved.
- We will apply for an amendment to report D5.12 at M26.
- Preclinical work to test biodistribution of the different DARPin constructs was arranged by UCL with no cost to IMAGINT (Task 5.3).
- We will apply for an amendment to report D4.10 at M30.
- It is planned that UCL will take over GMP production of the imaging DARPin (originally to be delivered by M-GMP who have left the IMAGINT consortium and have not claimed any reimbursement).
- An amendment to incorporate this change will be applied for during P2. UCL has a dedicated facility which can produce clinical grade microbially-expressed recombinant proteins in compliance with GMP. The site license is number 8520, and the site location is number 104812. The most recent inspection by the Medicines and Healthcare products Regulatory Agency (MHRA) was on the 1st of March 2012.

Task 5.2: Collection and Storage and CTCs M1-M42; UCL

Progress toward objectives

- The study was approved by the University College London Hospitals Local Ethics Committee. All patients provided written informed consent. Eligible patients had pathologically confirmed HER2 expressing breast adenocarcinoma determined by a score of 3+ on immunohistochemistry or amplification by fluorescent in situ hybridisation on a primary tumour and or metastasis. At the time of CTC enumeration all patients were receiving Trastuzumab therapy alone or in combination with chemotherapy for advanced breast cancer.

Significant results

- Experiments of healthy volunteer blood spiked with HER2 expressing BT-474 and HER2 negative MDA-MB-468 breast cancer cells, that showed that the CellSearch System can identify cultured HER2 expressing tumour cells in the presence of Trastuzumab
- Analysis of breast cancer patient blood samples for HER2 expressing circulating tumour cells (CTCs) was performed. Six women with HER2 expressing metastatic breast cancer receiving three weekly Trastuzumab alone or in combination with chemotherapy and or bisphosphonates had blood samples taken just prior to Trastuzumab dosing. Five of the six patients had no CTCs at the time of sampling. One patient had 14 CTCs which were all HER2 positive, as assessed by the CellSearch (Veridex LCC) system (Figure 5.2.1). This patient had relapsed 10 months previously with liver and bone metastases, confirmed by a liver biopsy. This biopsy demonstrated HER2 expressing (IHC 3+), oestrogen receptor positive (Quick score 5/8) and progesterone negative (Quick score 0/8) grade 2 breast adenocarcinoma. The patient had undergone breast surgery for right sided breast cancer in 1995 and left sided breast cancer in 2003. At the time of blood sampling the patient had received twelve doses of Trastuzumab in conjunction with zoledronic acid.
- Patient blood sampling, confirmed experiments of healthy volunteer blood spiked with HER2 expressing cells. Therefore it is not necessary to develop the G3 DARPin for identifying HER2 expressing CTCs in the presence of Trastuzumab.

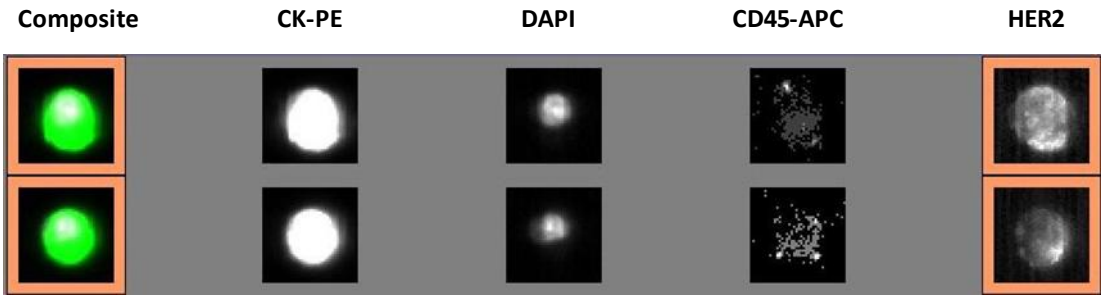


Figure 5.2.1: CellSearch analysis including the fourth channel analysis for HER2 of patient who had fourteen HER2 expressing CTCs. Two of the fourteen CTCs are shown below. Orange boxes in the composite column highlight a circulating tumour cell and the HER2 column identifies a HER2 expressing cell.

Deviations from workplan

- We determined that the CellSearch (Veridex LCC) system is capable of identifying HER2 expressing CTCs in the presence of Trastuzumab. Consequently, it is not necessary to develop the G3 DARPin for this purpose.

Impacts (resources, planning, link with other tasks)

- Personnel have focused on pre-clinical development and biodistribution for the Phase I Clinical trial, (Task 5.3) rather than developing the G3 DARPin for CTC analysis.

Correctives actions

- None required.

Task 5.3: Phase I/II M12-M48; UCL

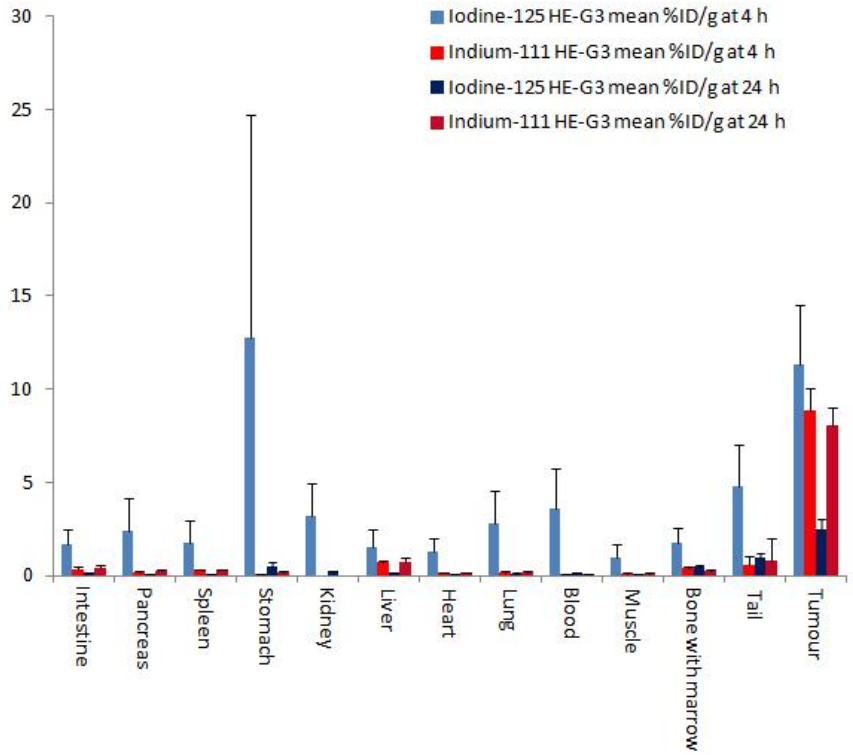
Progress toward objectives

- Indium-111 (chelated via maleimide-mono-amide DOTA site-specifically attached to a c-terminal cysteine) co) and Iodine-125 radiolabelled (Iodogen method) G3 DARPins with 3 different N-terminal domains have been assessed in BALB/c mice including:
  - H_6-tag produced in *E. coli* and *P. pastoris*
  - HE_3-tag produced in *P. pastoris*
  - No tag produced in *E. coli* and *P. pastoris*
- HER2 expressing mice (B6.Cg-Tg(Wap-ERBB2)229Wzw/J) have been obtained for toxicity testing. The mice have been genotyped and are currently being bred at UCL.
- Immunohistochemistry has been performed on human samples to evaluate G3 DARPin interactions with healthy issue.
- A Draft Clinical Trial Protocol has been written at the Joint ECCO - AACR - EORTC - ESMO Workshop on Methods in Clinical Cancer Research, Switzerland.

Significant results

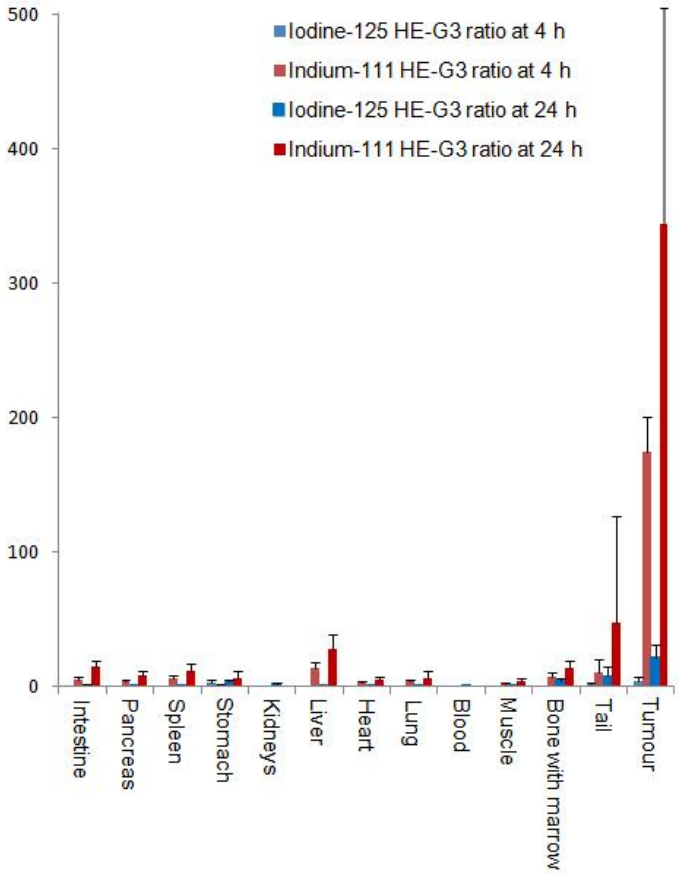
- Site specific attachment of maleimide-mono-amide DOTA is stable and does not reduce the affinity of G3 for HER2
- Biodistribution of the DARPins was evaluated in BALB/c mice at 4 and 24 h. Results showed that ^{111}In-HE_3-G3 had lower or similar uptake to ^{111}In-His_6-G3 and untagged ^{111}In-tag-G3 in 11 different normal tissues tested. Superiority of HE_3-G3 for normal tissue uptake was also observed when the DARPins were labelled with ^{125}I.
- HE_3-G3 was assessed in HER2+ve tumour-bearing mice. The tumour uptake for ^{125}I-HE_3-G3 was approximately 2 fold higher than ^{111}In-HE_3-G3 at 4 hours. However, ^{111}In-HE_3-G3 tumour uptake was better maintained, so that by 24 h ^{111}In-HE_3-G3 tumour uptake was approximate 1.5 fold higher than ^{125}I-HE_3-G3. Normal tissue uptake was generally lower for ^{111}In-HE_3-G3 than ^{125}I-HE_3-G3 at 4 h, except in the kidneys which were considerably higher for ^{111}In-HE_3-G3 throughout. At 24 hours, the differences in normal tissue uptake between ^{111}In-HE_3-G3 and ^{125}I-HE_3-G3 were smaller (Figure 5.3.1).

Figure 5.3.1



In-HE_3-G3 had faster serum clearance than ^{125}I-HE_3-G3, resulting in higher normal tissue:blood ratios for all assessed tissues except stomach. As a consequence, the tumour:blood ratios for ^{111}In-HE_3-G3 were the most impressive, > 150:1 at 4 h, rising to > 300:1 at 24 h . ^{111}In-HE_3-G3 microSPECT/CT imaging demonstrated tumour uptake at 2 and 4 h (Figure 5.3.2).

Figure 5.3.2



- The G3 HER2 DARPin was tested in paraffin-embedded tissue sections and compared to an FDA-approved rabbit monoclonal antibody (clone 4B5; Ventana Medical Systems) in tissue microarrays. The data were correlated with HER-2 amplification status measured by FISH. Immunohistochemistry demonstrated that G3 reacts with HER2 in breast cancer known to be 3+ on the HercepTest but shows no cross-reactivity with the panel of normal tissue studied (see Figure 5.3.3). Thus, the DARPin was able to detect a positive HER2 amplification status with similar sensitivity yet with higher specificity than the FDA approved antibody.

Figure 5.3.3

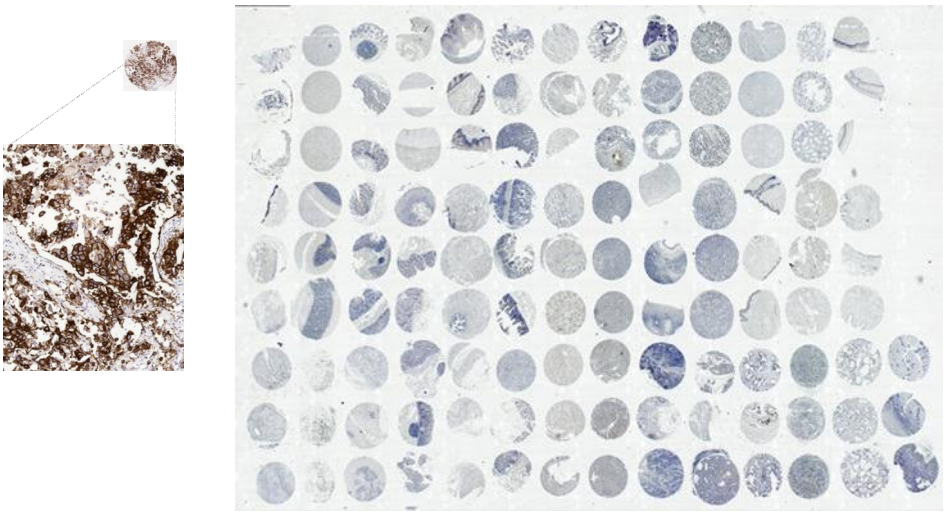


Figure 5.3.3. G3 DARPin staining of a representative tissue microarray of 40 normal tissues (triplicate cores; right hand panel), in comparison with HER2+ve breast cancer (left hand panel).

Conclusions:

- N-terminal tags effect tissue biodistribution of G3.
- DOTA attachment is successful.
- HE_3-G3 radiolabelled with ^{111}In and ^{125}I showed favourably lower uptake in normal tissues compared to untagged or His_6 tagged G3.
- Excellent Tumour:normal tissue ratios were obtained at early timepoints.
- ^{111}In-HE_3-G3 achieved and maintained the highest tumour:blood ratios over 24 h.
- G3 shows appropriate specificity on human tissue staining.

Deviations from workplan

- We had originally planned to use a H_6-tagged G3 DARPin for the first-in-human clinical trial. However, in April 28, 2011, Hofstrom *et al.* [*J Med Chem* 2011; 54;3817–382] reported that the H_6-tag enhances normal liver uptake compared to the HE_3 for a HER2 targeting affibody protein. Thus, prior to embarking upon GMP preparation, we considered it would be important to investigate whether removal of, or changing the H_6-tag on the G3 DARPin would reduce liver uptake and result in a more favourable imaging agent.
- The excellent tumour:blood ratios (> 150:1 at 4 h) at early time points indicate the ^{68}Ga will be the isotope of choice. DOTA is a suitable chelate for this isotope.

Impacts (resources, planning, link with other tasks)

- D4.10 was delayed because we did not did not want to use value resources until we had established which construct would be taken forward to the clinic.

Correctives actions

- Based on its superiority, future development will focus on the radiolabelled C-terminal cysteine-DOTA conjugated HE_3-G3.

WP6 – Data management, integrative Bayesian analysis of data derived from preclinical and clinical studies

Task 6.1: Construct and manage a database for IMAGINT's biomedical M1-M48; KCL

Progress toward objectives

- The WP6 partners (KCL and FLS) agreed to create a data store based on a flat-file directory structure to capture (i) raw data (where required), and (ii) curated data with associated documentation including data sources, contact details, and file formats. The data store will also hold metadata about the experiments from which the data was collected.
- This GIATE standard data store is the repository into which members will contribute their data and also view the updates and progress of work of other members.

Name	HER2
Resource Database	UniProtKb
Accession Number	P04626
Molecule Type	Protein
Ligand Binding Domain	
Work package	Task 1.1
Comments	
Files/URLs	Data stored here → Link to curated data store

Data About Target Molecule

Name	Indium111-labelled Anti HER2 DARPin
Resource Database	None
Accession Number	NA
Molecule Type	DARPin
Post Translational Modifications	None
Components	Indium111, DOTA linker, DARPin, Cys tag
Target	EGFR
GM Produced	No
Reference in work package	Task 1.1

Data About Therapy Agent

Name on WP	Subtask 5.1.2 GMP production of cys-tagged anti-HER2 DARPin
Bond Study (BS)	Data available here
Collaborators	M-GMP, UZH, UCL, UU/GE, KCL
Data Holder	Kerry Chester
Contact Details	k.chester@ucl.ac.uk
Start date	01/01/12
Delivery date	01/07/12
Hypotheses	Process development will be performed by M-GMP using *E. coli*.

Data about experiment

- A simple and flexible database has been initiated using a version control system. The database is initially local to KCL, and can be migrated as requirements evolve.

Significant results

- Created a database entry (Figure 6.1.1) for raw and curated data from an IMAGINT-generated data set (WP2-related) – HER2/HER3 status in breast tumour tissue samples by immunohistochemistry staining on tissue microarrays. The project description, investigator contact details and raw data (.XLS files and descriptive text) are stored, together with curated data, associated curation scripts, and documented data formats. Curated data sets can be taken forward into further analyses, while the data set is traceable to the owners/providers via contact details, descriptive files and/or raw data, as appropriate.

- This provides a use-case for the database which will be adapted and/or migrated to meet databasing and access requirements, as data sets become available to WP6.

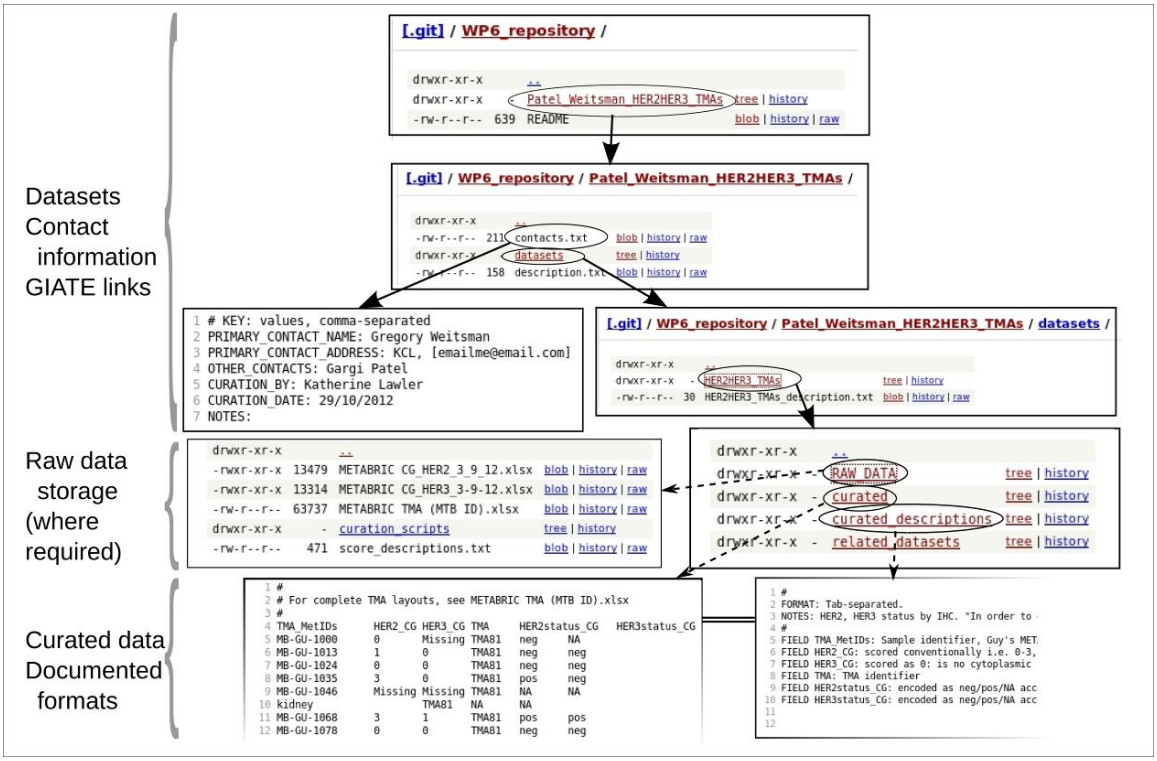


Figure 6.1.1. IMAGINT-generated data set, curated and loaded into a WP6 database (revision 1). Implemented using a version control system on a local machine located within KCL, and to be migrated as per evolving requirements.

Deviations from workplan

- No deviations from plan at this stage.

Impacts (resources, planning, link with other tasks)

- N/A.

Correctives actions

- N/A.

Task 6.2: Construct a generalized version of the BGVLM model with latent variables, tailored to the analysis of data in biomarker discovery M1-M12; KCL

Progress toward objectives

- We have developed a Bayesian mathematical generalization and extension of the Gaussian Process Latent Variable Model (GPLVM) proposed by Lawrence. The GPLVM generates a flexible, non-linear and probabilistic embedding of high dimensional data (such as genetic signals) in terms of (low dimensional) latent variables, while minimising the information loss incurred by the dimension reduction. The primary objective of the dimension reduction, which deliberately makes no use of clinical outcome data, is to reduce the risk of overfitting in subsequent analysis and regression analyses, thus facilitating the reliable extraction of any predictive signals or structure that may exist within a dataset.

Significant results

- We have embedded the GPLVM in a Bayesian model selection framework that enables us to detect automatically the most probable intrinsic dimension of the high-dimensional signals. This optimal number is then chosen as the dimensionality of the reduced representation (i.e. the number of `meta-genes' to represent the genetic information of each patient).
- We have generalized the GPLVM such as to allow for the inclusion and integration of multiple data sources for each patient, with possibly disparate dimensionalities (e.g. gene expression signals versus tumour or whole body imaging parameters or clinical covariates).
- We have developed the formulae required for calculating the latent variable mapping for new patients that were not in the data set on which the GPLVM analysis was performed. This will be important for assessing the transferability of detected patterns across different data sets.
  We have identified various mathematical properties of the latent variable mapping provided by the model

Deviations from workplan

- None.

Impacts (resources, planning, link with other tasks)

- N/A.

Correctives actions

- N/A.

Task 6.3: Numerical implementation of the generalized version of the BGVLM model M6-M18; FLS

Progress toward objectives

- We have carried out an extensive literature study on probabilistic reasoning and Bayesian approaches for biomarker identification, and their numerical implementation in existing software packages for statistical learning.
- We have implemented the Bayesian generalized GPLVM model of task 6.2, in Matlab and in C. This includes CPU-optimised versions that build on accelerated publicly available numerical libraries (such GSL) and that can run in parallelised multi-core mode in HPC servers. At present the C code can be run on any suitable standard GNU/Linux or UNIX system.

Significant results

- Firalis has implemented a multivariate Biomarker prediction suite consisting of a panel of diverse machine learning algorithms including partial least squares discriminant analysis, support vector machine, k-nearest neighbours, naïve bayes, logistic regression, random forest, CN2, classification trees, supervised principal component analysis and other non-linear projection methods, together with a set of different model validation strategies including n-fold cross-validation, leave-one-out validation, and different re-sampling schemes for calibration and test samples.

- Each of these statistical learning approaches has its strengths and weaknesses, and the objective of the Firalis Biomarker prediction suite is to apply all methods in parallel and use a supervisor algorithm to integrate and benchmark the predictions of the individual prediction models. This approach is well known in the machine learning community and is called “Panel of Experts” approach.
- Our Bayesian generalized GPLVM model has been tested and benchmarked extensively on synthetic data with controlled statistical and information-theoretic features. The implementations include different versions that implement different approximations of the Bayesian integrals over micro-parameters, which is the built-in buffer against complexity overfitting in such models. It is found that the implementation of our model is perfectly able to recover correctly the intrinsic dimensionality of data sets, even in the notorious regime where the number of variables per patient is significantly larger than the number of patients in the data set. See figure 6.3.1 for typical examples.

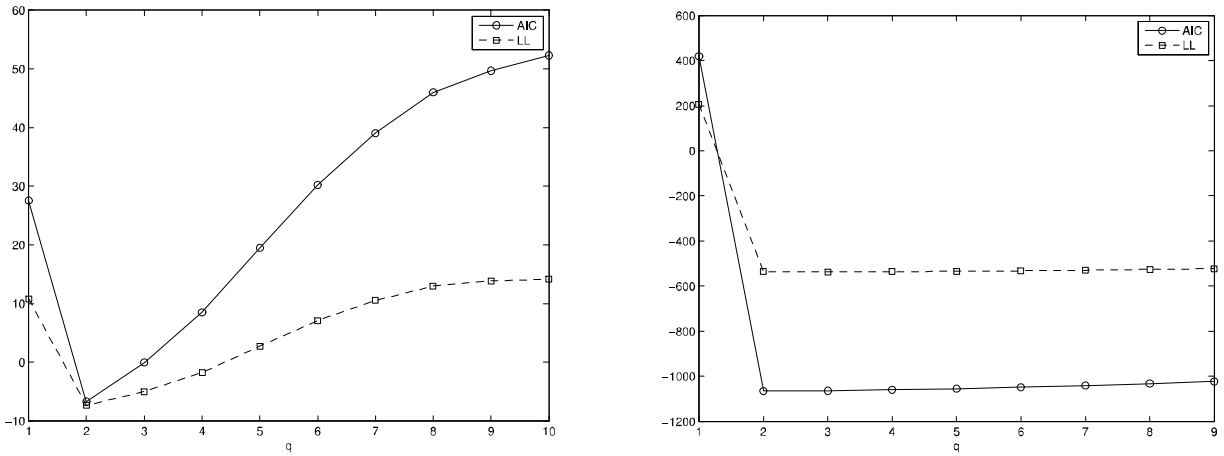


Figure 6.3.1 Identification by the Bayesian generalized GPLVM model of the most probable intrinsic dimension q of data sets with N patients and d variables reported for each. This is done via Bayesian model selection (the different curves refer to different protocols for numerical approximation of micro-variable integrals). Left figure: synthetic data with N=10 patients and d=10 variables each. Right figure: synthetic data with N=10 patients and d=200 variables each (where overfitting is a serious problem). The vertical axis gives minus the rescaled log-likelihood of each possible value of the intrinsic dimension q. The true value q=2 is in both cases correctly recovered, as corresponding to the most probable point. Similar performance is found for large values of N or d.

- The model has been found to also handle data integration tasks adequately. Proper assessment of this feature of the model under controlled conditions is still being carried out, and results will be reported in due course.

Deviations from workplan

- In the initial DoW document (Part B) it was envisaged that a Firalis-hired post-doc would work tightly with Prof. Coolen to contribute to the development of the Bayesian Latent Variable Gaussian Process method and then benchmark it to the existing solutions on the market. We found that the scientific work of this postdoc required a greater presence at KCL, in order for the postdoc interact on a full-time basis with the members of Coolen’s team. After discussion with the IMAGINT partners concerned, the management bureau and the Coordinator, it was unanimously agreed and accepted that Firalis would transfers the budget of one-year post-doc position to KCL to enable this full-time interaction. The postdoc will assist in the integration of the model developed in Coolen’s group into the Firalis prediction suite and to compare the predictive performance of the reduced latent variable representations with that of the standard machine learning approaches. In addition, Firalis will compile a set of test cases of diverse numbers of samples/patients and variables (Nxd) including synthetic data, transcriptomics, proteomics and metabonomics data, clinical data like demographics, medication, morbidity, standard hematologic, biochemistry and urinalysis parameters. Features extracted from imaging procedures will also be included. The objective of this benchmarking approach is to obtain an as unbiased as possible estimate of the predictive performance of the newly developed model.

- While we judge the formal mathematics and the numerical implementation to be correct, we considered it important, prior to application of the method to IMAGINT data, to investigate a number of fundamental aspects of the new methodology that relate to the interpretation of the latent variables and to practicalities of numerical implementations The main aspects are:

  - (i) The reduced latent variable (or `meta-gene') representation of the biomarker signals to which the method is applied is not unique (and cannot be); for instance, any simultaneous rotation of all latent variable vectors would produce an alternative representation with the same information content. We currently investigate which is the most practical definition of optimality in the context of IMAGINT to form the basis of selecting a unique representation.
  - (ii) The search for the Bayes-optimal latent variable representation is CPU intensive, due to the impact of local minima, which limits the size of the data sets to which the method can be applied. We are investigating a number of ideas for more CPU-efficient search code, based on the (temporary) addition of spurious variables, whose effect is to create alternative search paths that reduce the impact of non-optimal local minima.

Impacts (resources, planning, link with other tasks)

- We observed that the scientific position needed a real presence at KCL and interact full time with the Prof. Coolen team members. After the discussion with concerned partners, management bureau and the Coordinator, it has been unanimously accepted that Firalis transfers the budget of one-year post-doc position to KCL.

Correctives actions

- A total budget of 67 K€ of EC grant -meaning 89.333 €- was transferred from Firalis to Prof. Coolen's lab at the KCL. The post-doctoral fellow is now working at the lab of Prof. Coolen, in order to develop the Bayesian methodology and hand over the technology to Firalis for further evaluation and benchmarking.

Task 6.4: Application of existing Bayesian analysis tools to extended FRET/FLIM signatures M12-M15; KCL

Progress toward objectives

- The code that implements existing Bayesian analysis tools has been updated and tested. As soon as the FRET/FLIM signatures are available (these are currently being pre-processed and cleaned) these analyses will be done. Everything is in place.

Significant results

- None yet.

Deviations from workplan

- Waiting for data clean-up and pre-processing to be finalized.

Impacts (resources, planning, link with other tasks)

- N/A.

Correctives actions

- Not necessary.

Task 6.5: Application of generalized BGVLM method to extended FRET/FLIM signatures M15-M18; KCL

Progress toward objectives

- The latent variable model currently operates deliberately in an unsupervised manner, where outcome information is not yet considered (so that overfitting is impossible). The next phase of development, needed to apply the method to extended FRET/FLIM signatures, will be to extend the model to directly link outcome information to the latent variables. This is in principle a straightforward task once the unique reduced latent variable representation is obtained.

Significant results

- None yet.

Deviations from workplan

- It only makes sense to carry out this stage of the project after we have answered the remaining open questions described under 6.3. The deviation of the work plan is for us to answer those questions first.

Impacts (resources, planning, link with other tasks)

- N/A.

Correctives actions

- We focus most of our current work on the remaining open questions described under 6.3. In the meantime we have commenced work initially planned to be carried out later, under Task 6.8 (determination of intrinsic dimensions of biomarker datasets) which can already partially be done with the tools developed so far. The model determines the optimal dimension of the latent variables. Furthermore, multiple data sources may be simultaneously represented in the latent variable space allowing information from several datasets to be quantitatively combined. Any dimensionality mismatch between data sources is adjusted for. It was decided to build these features into the model earlier than anticipated in the original outline of WP6.

Task 6.6: Predicting candidate biomarkers from toponome (protein and microRNA) and protein interaction data M18-M24; FLS, KCL, TNL

Task 6.7: Quantify anti-HER2 treatment effects on biomarker signatures M25-M28; FLS, KCL

Month

Task 6.8: Quantify intrinsic dimensionality and cross-prediction of disjunct data sets M28-M48; FLS, KCL, UCL

Month

Task 6.9: Use of Standards and Construction of a Database for deposition of results and data sharing and dissemination M28-M48; FLS, KCL

Month

WP7 – Dissemination, IPR and ethical issues

Task 7.1: Project communication and dissemination M1-M48; UCL, all partner

Progress toward objectives

- Creation of the IMAGINT logo.
- Creation and launching of the IMAGINT web site: www.imagint.eu

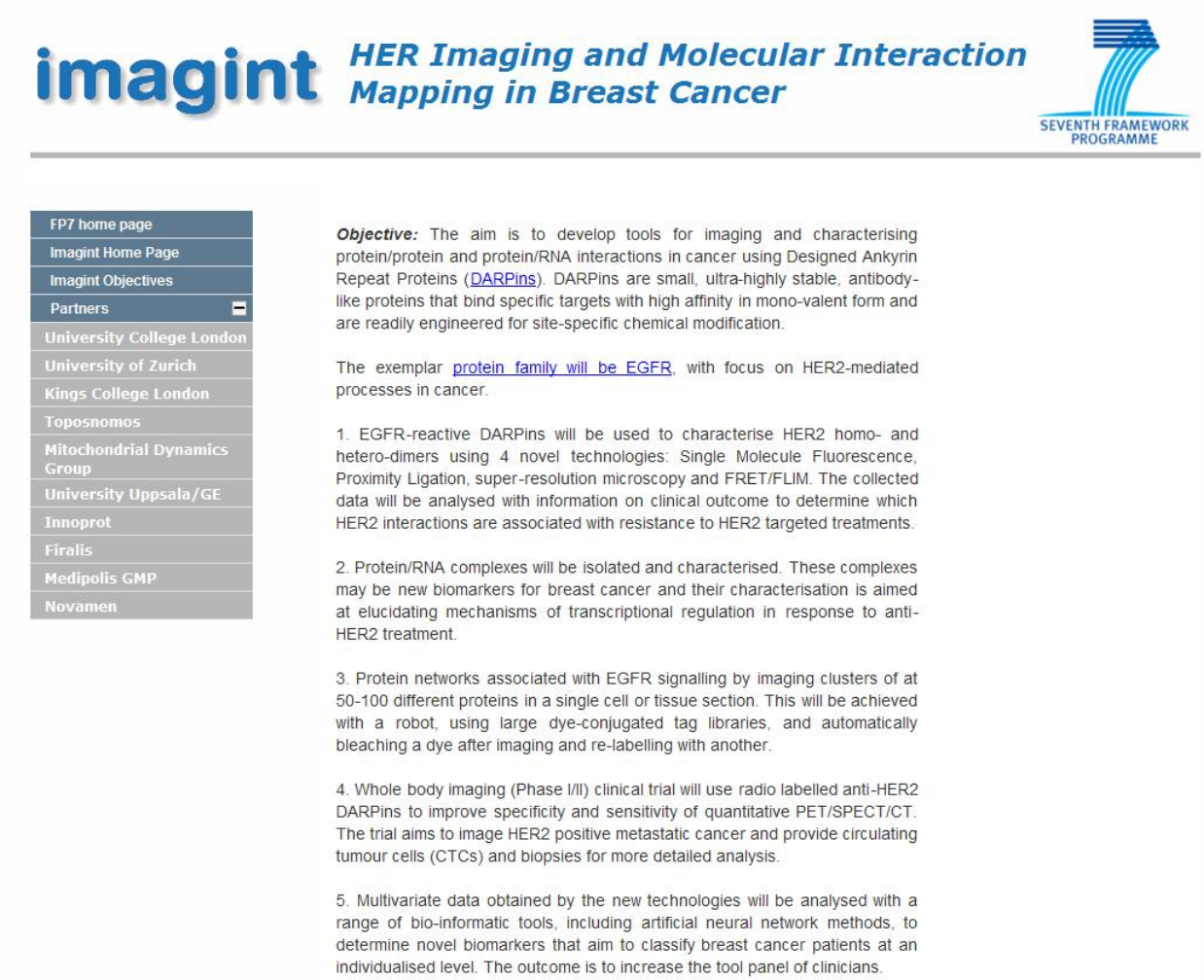
Significant results

- **IMAGINT logo.**

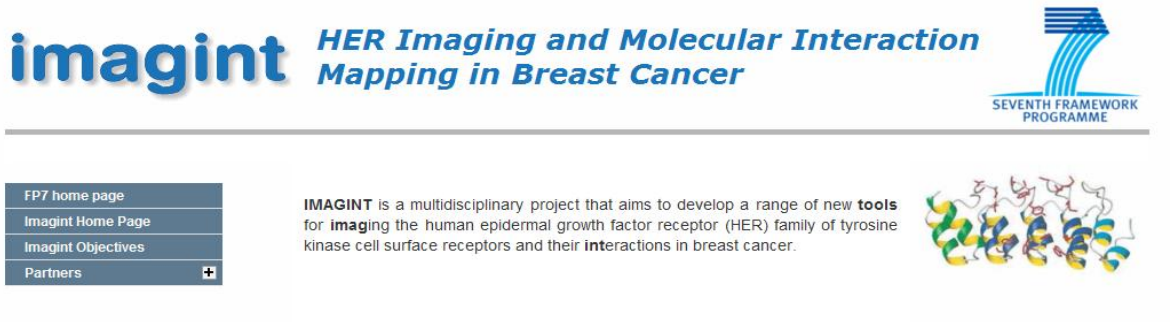
imagint

- **IMAGINT Website:**

The description of the research project of IMAGINT is now accessible to all public.
The objectives of IMAGINT are described and each partner involved is presented.



The website will be regularly updated during period 2 regarding consortium major achievements. The website will also advertise events where IMAGINT participants will be present.



Deviations from workplan

- There is no major deviation from the work plan except
- The annual newsletter that has not been done at M12.
- The internal collaborative portal that has not been created.

Impacts (resources, planning, link with other tasks)

- No impact.

Correctives actions

- Newsletter : A newsletter will be released at M29 (collection of results after the annual meeting M27 + 2 months) when enough relevant results will be collected.
- Internal collaborative portal: so far such a tool is not needed to ensure the good communication between partners (emails, conference call and meetings are sufficient). If requested, such a portal would be put in place.

Task 7.2: IPR/Knowledge management of IMAGINT M1-M48; UCL

Progress toward objectives

- **Intellectual Property Committee (IPC)**
  IMAGINT IPC is under construction. For the moment, three partners have designed a person dedicated to IMAGINT IPR issues. Dr Chris Williams from UCL has been appointed to be responsible of all partner requests concerning the use of results. The final composition of the IPC should be frozen soon.
- **Scientific Advisory Board**
  The composition of the SAB has been finalized during the set-up of IMAGINT. So far, the SAB did not take part of the scientific discussion as no particular scientific problems arose.

Significant results

- **Members of IPC:**

UCL Partner → UCL Business Manager, Dr Chris Williams, Senior Business Manager (Biopharm). UCL Business PLC, The Network Building, 97 Tottenham Court Road, London W1T 4TP Tel: +44 (0)20 7679 9000, Fax: +44 (0)20 7679 9838. Email: c.williams@uclb.com
FIRALIS Partner → Dr Beatrice Molac, 35 rue du Fort, F-68330 Huningue, France, @: **Beatrice.molac@firalis.com**
UZH Partner → Prof. Dr. Andreas Plueckthun, Biochemisches Institut, Universitaet Zurich, Winterthurerstr. 190, CH-8057, Zurich, Switzerland. Tel:(+41)-44-635 5570, FAX:(+41)-44-635 5712, E-mail: plueckthun@bioc.uzh.ch

Deviations from workplan

- The IPC and SAB did not participate to the first Steering Committee meeting.

Impacts (resources, planning, link with other tasks)

- No impact no IP or scientific issues occurred.

Correctives actions

- IPC and SAB members can be contacted at any moment via mail, TC or WebEx if requested.
- IPC and SAB will be present during the second annual meeting (London, 1st week of July 2013).

Task 7.3: Translation of IMAGINT findings into clinical tools M1-M48; UCL, all partners

Progress toward objectives

- Substantial progress has been made in the basic development of the new clinical tools as reported in Tasks 1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.5, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 4.1, 4.2, 4.3, 4.4, 5.1, 5.2, 6.1, 6.2, 6.3, 6.4 and 6.5.
- We aim to translate these findings into clinical tools during the later stages of the IMAGINT project.

Significant results

- Not yet applicable.

Deviations from workplan

- Not yet applicable.

Impacts (resources, planning, link with other tasks)

- Not yet applicable.

Correctives actions

- Not yet applicable.

Task 7.4: European/international IMAGINT workshop to communicate findings of IMAGINT to clinicians and scientists M40-M48; UZH, UCL, all partners

Month

Task 7.5: Set-up the Project Ethics Committee M1-M4; UCL, KCL, UZH, all partners

Progress toward objectives

- **Project Ethic Committee (PEC)**
  IMAGINT PEC has been set-up. Bride Foster from UCL has been appointed to coordinate all ethical questions. PEC members will provide all ethical guidance to the consortium.

Significant results

- **IMAGINT PEC Members (List on next page)**
- **Ethic report**

An ethic report has been placed in annex of this P1 activity report.

Deviations from workplan

- **PEC members** did not participate to the first Steering Committee meeting due to the late setting of PEC.

Impacts (resources, planning, link with other tasks)

- **No impact.**

Despite the late setting of the PEC, the consortium ensure that all ethical standards and guidelines equivalent to, or compatible with, those of FP7 have been be rigorously applied, regardless of the country in which the research has been carried out. All organizations concerned by ethics issues have ensured that an ethical requirement thanks to their internal ethics experts.

Correctives actions

- PEC has been immediately set-up after the first annual meeting. PEC members will meet with the Steering Committee in a specific session during the second annual meeting (London, 1st week of July 2013).
- PEC can be contacted at any moment via mail, TC or webEx if requested.

Affiliation	Title	Lastname	Firstname	Organisation	Expertise	e-mail
UCL	Professor*	Swanton	Charles	UCL	Breast Cancer and Personalised Medicine	c.swanton@ucl.ac.uk
UCL	Ms	Foster	Bride	UCL	Regulatory, GMP QA and Ethics	b.foster@ucl.ac.uk
KCL: Guy's and St Thomas'	Professor	Ellis	Paul	Guy's & St Thomas	Breast Cancer and Lung Cancer	Paul.ellis@gstt.sthames.nhs.uk
KCL. Guy's and St Thomas'	Dr	Gillet	Cheryl	Guy's & St Thomas	Manager of Breast tissue bank	Cheryl.Gillett@gstt.sthames.nhs.uk
External: CRUK	Dr	Williams	Rob	Cancer Research UK Drug Development Office	Chief Drug Development Scientist & Head of Nonclinical Operations, Animal ethics	Robert.Williams@cancer.org.uk
External: Independent Cancer Patient's Voice	Ms	Wilcox	Maggie	Independent Cancer Patients' Voice	Patient Representative (Breast Cancer)	maggie@icpv.org.uk
UCL	Dr	Tolner	Berend	UCL	Scientist and GMP Manager	b.tolner@ucl.ac.uk
KCL	Dr	Irshad	Sheeba	KCL	Clinical Fellow and Oncology SpR	sheeba.irshad@kcl.ac.uk
TNL	Professor	Schubert	Walter	TNL	Chief representative of TNL. Inventor of Toponomics and expert in human tissue staining	toposnomos@gmx.de walter.schubert@med.ovgu.de
MPG	Professor	Jakobs	Stefan	MPG	Lab head and responsible for GMOs in department	sjakobs@gwdg.de
UU	Professor	Balzer	Lars	UU	Radiolabelling and imaging	Lars.Baltzer@biorg.uu.se
INO	Mr	Gamiz	Jorge	Innoprot	Business Development Director	jgamiz@innprot.com
NOVAMEN	Mrs	MUIRAS	Marie-Laure	NOVAMEN	Director for collaborative programs	imagint@novamen.eu

* Has moved to UCL since KOM

Task 7.6: Follow-up of the ethical issues related to the project M4-M48; UCL, KCL, all partners

Progress toward objectives

- Ethical approval for CTC collection = Approval Granted.
- Development of GMP process for DARPin manufacture.
  Delayed due to lack of participation by M-GMP and new information indicating that the presence of H_6-tag (used for purification) can lead to liver uptake of radiolabeled tracers. A cleavable tag system was established. However, superior results were obtained using the HE_3 tag.

Significant results

Preparation for Clinical Trial

- Immunohistochemistry to test for G3 DARPin reactivity with human tissues arrays (total of 85 cores in 2 tissue arrays comprised of 64 normal human tissues and 12 cases each of human breast carcinoma and non-neoplastic breast tissue). Results confirmed that the G3 HER-2 DARPin reacted with HER-2 in breast cancer known to be 3+ on the HercepTest but showed no cross-reactivity with the panel of normal tissue studied.
- Preclinical toxicology studies have been designed in consultation with Cancer Research UK Drug Development Office. The studies will be performed on transgenic mice which express human HER-2 in the mammary gland and brain. The mice are generated with wild-type human HER-2 under the whey acidic protein promoter and are recommended for testing HER-2 vaccines and immunotherapies (Piechocki, *et al.*, J Immunol, 2003. 171:5787-94). B6.Cg-Tg(Wap-ERBB2)229Wzw/J have been ordered from the Jackson Laboratory (http://jaxmice.jax.org/strain/010562.html) and have been successfully bred and genotyped at UCL.
- Safety in terms of biological activity:
  - The G3 DARPin was chosen for imaging because experimental evidence indicates that it has no effect on HER2 signalling. Although it binds effectively and specifically to HER2, there is no evidence that it influences cell functioning. It does not affect cell viability, cell cycling, signalling molecule phosphorylation status or increase receptor shedding.
  - Cell line work performed by UZH confirmed this lack of biological activity by demonstrating that G3 does not affect cell growth (UZH, Figure 7.6.1).
  - Using a biologically inert but effective imaging DARPin will facilitate the safest possible first-in-human testing of radiolabelled anti-HER2 DARPins.

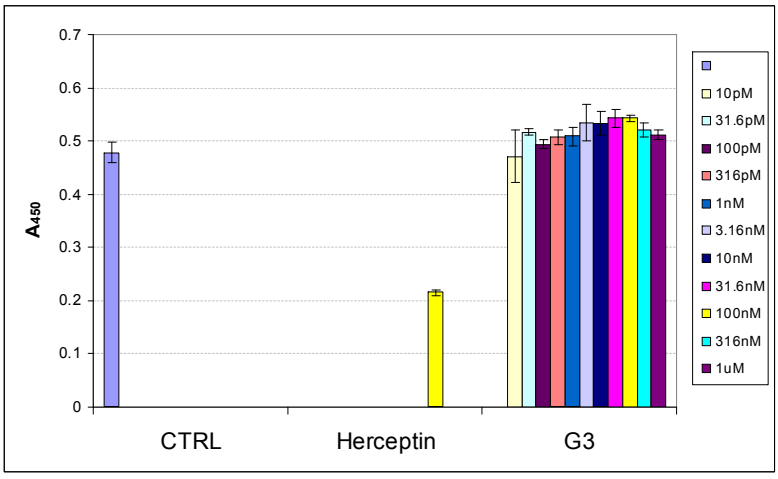


Figure 7.6.1. The G3 DARPin has no effect on cell growth. Cell proliferation assay (XTT) on BT474 cells: Cells were seeded at a density of 10'000 cells per cm^2 in a 96 well-plate, allowed to attach for 24h and then treated for 96 hours with trastuzumab (Herceptin) or DARPin G3 at the indicated concentrations. Comparison to the XTT-signal derived from untreated cells (CTRL) shows, that DARPin G3 – in contrast to Trastuzumab (Herceptin) – does not have any effect on cell growth.

- Non-competition with Trastuzumab (Herceptin):
  - Although the G3 DARPin binds to the same domain as Trastuzumab (domain 4), it has a different and non-overlapping epitope.
  - Thus, UZH demonstrated that the G3 DARPin can bind to HER2 in the presence of Trastuzumab (Figure 7.6.2) which will facilitate its use for imaging even when patients are receiving Trastuzumab treatment.
  - Moreover, UZH demonstrated that the G3 DARPin does not compete with Trastuzumab for binding to HER2 (flow cytometry, BT474 cells) even when presented in 20 fold molar excess and does not inhibit Trastuzumab (Figure 7.6.3).
  - These results are supported by studies using radiolabelled G3 DARPin (Figure 7.6.4). Therefore the DARPin imaging agent should bind in the presence of Trastuzumab and should not interfere with Trastuzumab treatment.

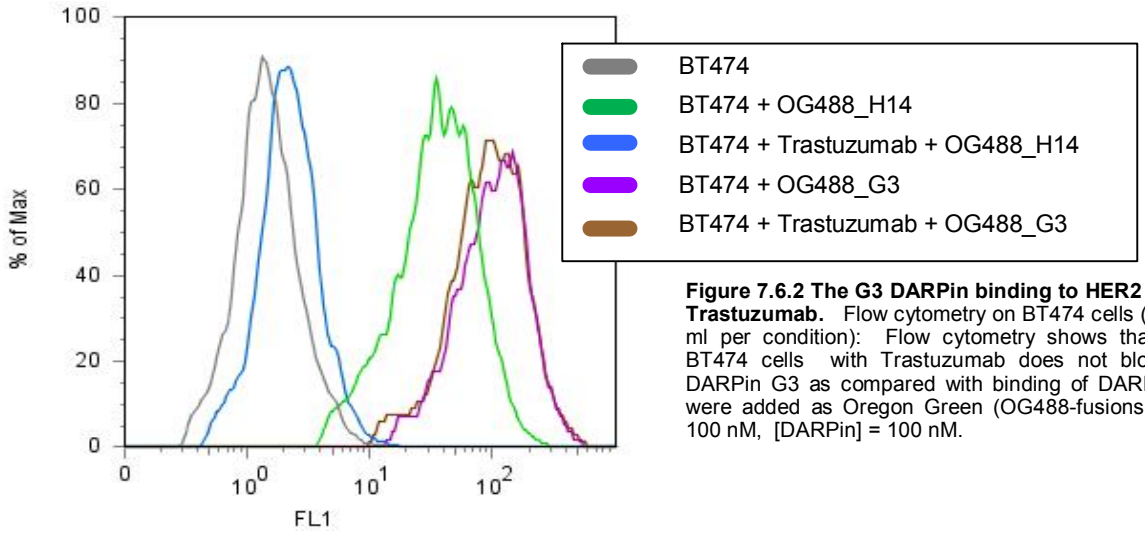


Figure 7.6.2 The G3 DARPin binding to HER2 is not reduced by Trastuzumab. Flow cytometry on BT474 cells (250'000 cells / 500 ml per condition): Flow cytometry shows that pre-treatment of BT474 cells with Trastuzumab does not block binding of the DARPin G3 as compared with binding of DARPin H14 (DARPins were added as Oregon Green (OG488-fusions). [Trastuzumab] = 100 nM, [DARPin] = 100 nM.

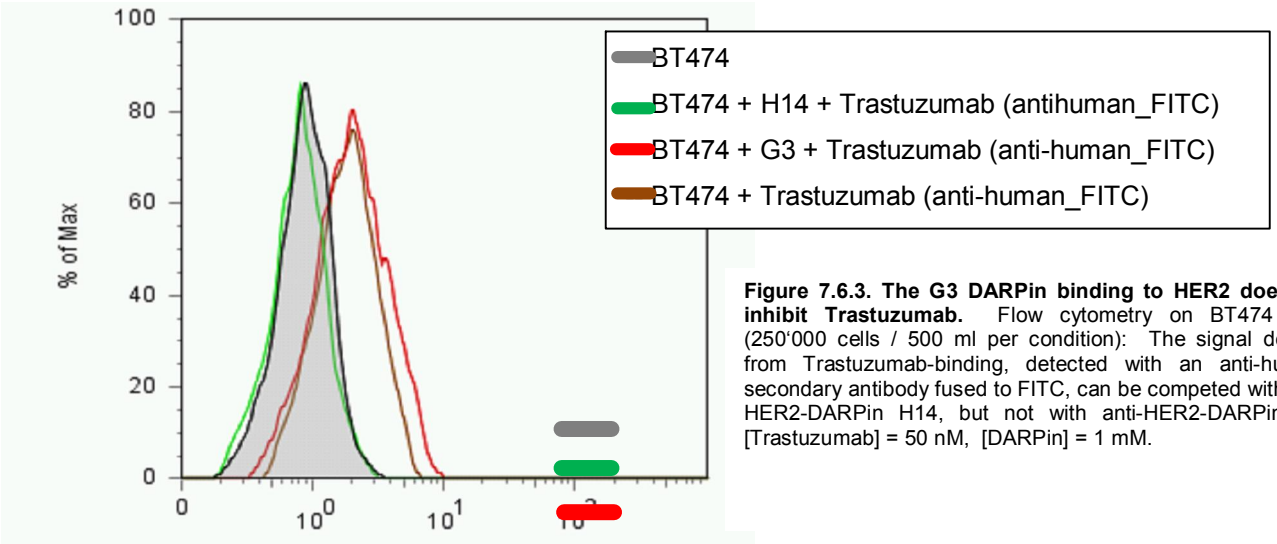


Figure 7.6.3. The G3 DARPin binding to HER2 does not inhibit Trastuzumab. Flow cytometry on BT474 cells (250'000 cells / 500 ml per condition): The signal derived from Trastuzumab-binding, detected with an anti-human-secondary antibody fused to FITC, can be competed with anti-HER2-DARPin H14, but not with anti-HER2-DARPin G3. [Trastuzumab] = 50 nM, [DARPin] = 1 mM.

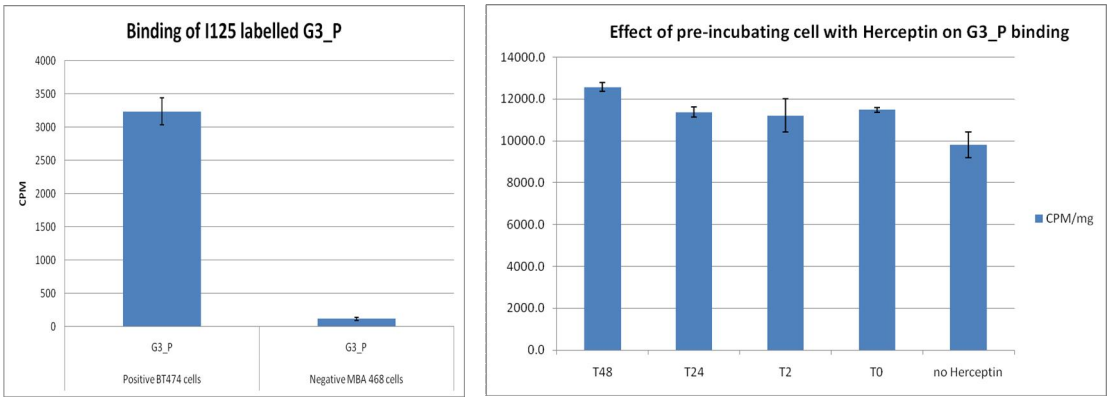


Figure 7.6.4. Binding of ^{125}I-labelled G3 DARPin to HER2-expressing cells. (A) Showing specific binding to HER-2 positive cells. (B) On positive cells only, showing that binding of ^{125}I-labelled G3_P is not reduced by pre-incubation for up to 48 hours (T48) with over 1000 fold molar excess of Trastuzumab (Herceptin). T = time in hours.

Deviations from workplan

- Delay in application for ethical approval for clinical trial due to changes detailed in Tasks 5.1 and 5.3.

Impacts (resources, planning, link with other tasks)

- See Tasks 5.1 and 5.3

Correctives actions

- An amendment to incorporate this change will be applied for during P2

WP8 – Management

Task 8.1: IMAGINT Strategic Chairmanship M1-M48; UCL, IMAGINT Steering Comittee

Progress toward objectives

The Strategic Chairmanship has ensured the consortium adherence to the rules established in the Grant Agreement and the Consortium Agreement from M1-M18.

Significant results

- **Regular monitoring of tasks progresses :**
  - thanks to two internal reports (M6 and M12).
  - thanks to the annual meeting (Uppsala, M17).
  - thanks to regular emails and phone call communication between participants.

- **Corrective actions to facilitate the reach of IMAGINT objectives:**
  - Amendment 1 of the Annex1 (07/03/2012) to transfer funds and tasks from FLS to KCL.
  - Amendment 2 of the Annex1 under construction (termination of M-GMP as participant and entrance of Magdeburg University as third party of TNL).

Deviations from workplan

- There is no deviation from workplan.

Impacts (resources, planning, link with other tasks)

- N/A.

Correctives actions

- N/A.

Task 8.2: IMAGINT Operational Management M1-M48; UCL, ACIES-P2R (NOVAMEN)

Progress toward objectives

- The IMAGINT Management team has ensured the management of all operational aspects of the project from M1-M18.

Significant results

- **Reporting:**

The IMAGINT Management team has provided all templates to collect information for M6 and M12 activity progress reports. The management team has compiled all scientific contributions to produce internal reports for the Strategic Chairmanship to follow the work progress and validate the visible outputs.
The management team has also collected financial data at M12 for the Strategic Chairmanship to follow the overall situation of the financial situation of the IMAGINT project.

- **Meeting:**

The IMAGINT Management team has organized in collaboration with UU the 1st annual meeting of IMAGINT. UU and the management team have arranged the meeting program. The choice of the meeting date as well as the providing of the agenda, were done in accordance with the requirements of the consortium agreement. Attendance sheet templates were provided to the host organization.

A 30 min presentation was done by the operational management team during the meeting to present:
- The procedure of contractual reporting and the preparation of reporting requirements.
- The general and technical objectives of IMAGINT (UCL).
- The communication procedure for any deviations of the project progress.
- Few financial guidelines.

During the meeting, the Management team has also organized a session to vote the potential entry of Magdeburg university as partner in IMAGINT.

- **Communication:**
  - The 2 partners of the operational management team (UCL and NOVAMEN) are in contact at least once a week to follow the management of the project integration.
  - The operational management team communicate regularly via phone call or emails with the different partners of the consortium (see internal meetings p66).

- **Deliverables:**

The operational management team closely follow that deliverables are released on time. Template to fill in work achievement is sent one month before the deadline to the partners. Once the deliverable provided, the management team send it to the coordinator for checking and validation. The management team upload and submit then the deliverable on the dedicated EC website.
So far three deliverables were submitted:
D8.18 Project management manual NOVAMEN (M3)
D2.4 Methods for FRET/FLIM-based HER2/HER3 dimerisation and optical imaging of Raf-Rok pathways KCL (M12)
D6.14 Establishment and analysis of database KCL (M18)

Three other are late:
- D5.12 GMP DARPin procedures, copies of ethical approval and clinical trials protocol (M18) as explained by UCL partner
- D2.5 Identification of HER2-associated toponome protein clusters (M16) as explained by TNL partner
- D4.10 DARPins radiolabelled and tested in *in vitro* and *in vivo* as explained by UU partner

- **Amendment of the DOW.**

The IMAGINT Management team has supported FLS and KCL to proceed to the first Amendment of the DOW. Emails and conference calls were organized between the Operational Management team and the EC to discuss the fund transfer between FLS and KCL regarding the employment of a share post doc. The Operational Management team has collected all information from the two partners (emails and conf call) to describe the tasks transfer and provide a modified version of the part B of the DOW (the post doc will finally be exclusively employed by KCL and not shared between both entities). The Operational Management team has also amended the budget in accordance. The amendment was accepted by the EC.

The IMAGINT Management team has also supported TNL regarding the potential entrance of Magdeburg university as new partner in the IMAGINT project. An email was sent to the project officer to explain the motivation of such a change (the EC agreed in principle if TNL is ensured to not be penalized in term of future IP). A vote session was also organized during the 1st annual meeting for collegial approval. The relevance of the entrance of university of Magdeburg as third party instead of as full partner is under study.
In addition, the Management team is expecting to provide support to the volunteer termination of participation of M-GMP partner.

Deviations from workplan

- No deviation from the workplan can be noticed regarding the role of the operational management team.

Impacts (resources, planning, link with other tasks)

- N/A

Correctives actions

- N/A

Task 8.3: Progress Coordination and Monitoring M1-M48; UCL, ACIES-P2R (NOVAMEN)

Progress toward objectives

- UCL and NOVAMEN have ensured the smooth operation of the IMAGINT project from M1-M18.
- The coordinator has managed the interface between the different modules and has regularly report to the EC.

Significant results

- **Progress Coordination and monitoring:**

The association between UCL and NOVAMEN has ensured a high quality monitoring regarding the project progress. The 2 partners have regular emails and conference call to exchange regarding the advancement of IMAGINT.

For activity reports, UCL and NOVAMEN have defined timelines for templates sending and partner's inputs return deadlines. UCL and NOVAMEN have closely followed the progress of report implementation and have reminded partners if necessary. UCL have monitored the different modules and their scientific and financial coherence.
In parallel, each WP leader monitors have regularly monitored the progress of the different partners involved in her/his WP. Regular report of the WP activity was provided to the coordinator. The correct completion of M6 and M12 internal activity reports has triggered the released of the 2nd and 3rd instalments of pre-financing.

Deviations from workplan

- There is no deviation from workplan as project reports using information provided by partners have been successfully established.

Impacts (resources, planning, link with other tasks)

- N/A.

Correctives actions

- N/A.

Task 8.4: Gender issues M1-M48; UCL, ACIES-P2R (NOVAMEN)

Progress toward objectives

- At equal competencies, female employment was favoured in IMAGINT.

Significant results

- **Women of the IMAGINT project.**

Name	Function	
Kerry Chester	Scientist and Coordinator	UCL
Bride Foster	Ethical expert	UCL
Cheryl Gillet	Ethical expert	Guy's and St Thomas' hospital
Maggie Wilcox	Ethical expert. Patient representative on PEC	Independent Cancer Patient's Voice
Sheeba Irshad	Scientist and PEC member	KCL
Marie-Laure Muiras	Scientist and PEC member	NOVAMEN
Anne-Sophie Belmont	Scientist and management team member	NOVAMEN
Beatrice Molac	Scientist and PEC member	FIRALIS
May Yong	Scientist	UCL
Alison Jones	Consultant Oncologist	UCL
Barbara Pedley	Scientist	UCL
Maria Livanos	Scientist	UCL
AnneMArie Honegger	Scientist	UZH
Sally Barrington	Scientist	KCL
Margaret Cooper	Scientist	KCL
Anne Gieseler	Scientist	TNL
Azita Monazzam	Scientist	UU
Dorina Bratfalean	Scientist	FLS
Pei San Chan	Radio-pharmacist	UCL

IMAGINT PEC is a good reflect of the parity within the consortium. 5 Members on 10 are women.
The above table shows also clearly that the recruitment of employees and collaborators on the IMAGINT project has been done without gender discrimination.

A questionnaire will be circulated during period 2 to established gender indicators and follow the gender equality in the IMAGINT project.

Deviations from workplan

- There is no deviation from action plan as gender balance and equity is ensured in the IMAGINT project.

Impacts (resources, planning, link with other tasks)

- N//A.

Correctives actions

- N/A.

PROJECT MANAGEMENT

Actions conducted for the management of the IMAGINT project are described in WP8.

DELIVERABLES AND MILESTONES

Deliverables

Del N°	Deliverable name	WP N°	Lead beneficiary	Nature	Dissemination level	Delivery date from annex I	Actual/Forecast achievement date	Status	Comments
D1.1	High affinity DARPins to EGF-R, HER2, HER3, HER4	1	2	R	CO	25	25	Not Submitted	75% completed at M18
D1.2	Measurement of HER pairings in relation to tumour outcome	1	2	R	CO	46	46	Not Submitted	35% completed at M18
D2.3	Nanoscopy imaging of HER2 and related proteins	2	5	R	CO	36	36	Not Submitted	50% completed at M18
D2.4	**Methods for FRET/FLIMbased HER2/HER3 dimerisation and optical imaging of Raf-Rok pathways**	**2**	**3**	**R**	**CO**	**12**	**13**	**Submitted**	**28/05/2012**
D2.5	**Identification of HER2- associated toponome protein clusters**	**2**	**4**	**R**	**CO**	**16**	**30**	**Not Submitted**	**40% completed at M18** (Delayed due to lack of breast tissue)
D2.6	Imaging-based metastatic signature established and compared with FDG and/or FLT PET-CT	2	3	R	PU	36	36	Not Submitted	25% completed at M18

D2.7	Full Toponomic analysis of breast cancer	3	4	R	CO	46	46	Not Submitted	20% completed at M18
D3.8	Analysis of Ago proteins in P-bodies and establishment of RISC-miRNA technologies	3	7	R	CO	28	28	Not Submitted	70% completed at M18
D3.9	Application of RISC-miRNA technologies to breast cancer tissues	3	7	R	CO	46	46	Not Submitted	45% completed at M18
D4.10	**DARPins radiolabelled and tested in in vitro and in vivo**	**4**	**6**	**R**	**CO**	**18**	**30**	**Not Submitted**	**50% completed at M18** (Delayed whilst determining lead DARPin)
D4.11	Development and evaluation of SPECT/PET imaging	4	1	R	CO	42	42	Not Submitted	25% completed at M18
D5.12	**GMP DARPin procedures, copies of ethical approval and clinical trials protocol**	**5**	**1**	**R**	**CO**	**18**	**30**	**Not Submitted**	**50% completed at M18** (Delayed whilst determining lead DARPin and by non-engagement of M-GMP)
D5.13	Data from HER2 imaging clinical trial	5	1	R	CO	42	42	Not Submitted	0% completed at M18
D6.14	**Establishment and analysis of database**	**6**	**3**	**R**	**CO**	**18**	**19**	**Submitted**	**13/11/2012**

D6.15	Biomarker analysis and protocol for quantifying relations between data sets	6	8	R	PU	42	42	No Submitted	40% completed at M18
D7.16	Establish communication, IP and ethics strategies	7	1	R	PP	24	24	No Submitted	70% completed at M18
D7.17	Project workshop	7	1	O	PU	48	48	No Submitted	0% completed at M18
D8.18	**Project Management Manual**	**8**	**10**	**R**	**CO**	**3**	**3**	**Submitted**	**30/07/2011**

Milestones

Milestone N°	Milestone name	WP N°	Lead beneficiary	Delivery date from annex I	Achieved	Actual/Forecast achievement date	Comments
MS1	Successful selection of DARPins to all 4 members of the HER family	1	2	24	No	24	75% completed at M18
MS2	Proximity ligation detects HER pairs in tumour cells	1	2	36	No	36	50% completed at M18
MS3	Combined TIS and FRET/FLIM analyses of archived cancer tissues	1, 2,6	3	36	No	36	25% completed at M18
MS4	Combined medical imaging and tissue imaging-based diagnostics	1, 2,6	3	36	No	36	25% completed at M18
MS5	**Production of GMP anti-HER2 DARPins in compliance with EMEA CGMP regulations**	**1,5**	**1**	**18**	**No**	**30**	**50% completed at M18**
MS6	Sucessful completion of Phase I/II clinical	2,4,5,7	1	42	No	42	30% completed at M18

DISSEMINATION ACTIVITIES

PUBLICATION

Two papers on corresponding methodological advances of co-visualizing the cell surface network of proteins (ref 1, below) and on the functional super-resolution of the basal lamina layers at 40 nm have (ref 2, below) been published:

1. Oelze S, Freiler W, Hillert R, Doleisch H, Preim B & Schubert W. Interactive, graph-based visual analysis of high-dimensional, multi-paramter fluorescence microscopy data in toponomics. IEEE Trans Vis Comput Graph. (2011), 17, 1882 – 1891.
2. Schubert W, Gieseler A, Krusche A, Serocka P, Hillert R. Next generation biomarkers based on 100-parameter functional super-resolution microscopy TIS. N Biotechnol (2012), 29, 599-610.

DISSEMINATION INTERNAL MEETINGS

Date & Place	Teleconference/Meetings	Participants
20/12/2012	Telephone: Dr Jean-Luc Sanne was updated on IMAGINT delays and provided general advice on the project, including M-GMP (leaving IMAGINT) and TNL 18M reporting.	Project Officer (Jean-Luc Sanne) and Kerry Chester (UCL)
18/12/2012	Telephone: Mr Volpi provided Prof. Schubert with advice on TNL cost model.	Financial Officer (Emanuele Volpi) and Walter Schubert (TNL)
18/12/2012	Telephone: Mr Volpi provided Prof. Chester with advice on TNL cost model. In particular on the financial guidelines concerning a third party making resources available.	Financial Officer (Emanuele Volpi) and Kerry Chester (UCL)
26/10/2012	Teleconference: Mr Volpi provided Prof. Schubert with advice regarding TNL personnel eligible costs.	Financial Officer (Emanuele Volpi), Walter Schubert (TNL) Anne-Sophie Belmont (Novamen)
02/07/2012- 03/07/2012	Annual IMAGINT Meeting in Upsalla, Sweden	All IMAGINT partners
14/05/2012 - 20/05/2012	Visit to Sweden from Dr Robert Goldstein (UCL) to Prof. Lars Balzer (UU) to receive help with DOTA conjugation to G3 DARPin.	Robert Goldstein (UCL) and Lars Balzer (UU)
21/07 - present	Several visits of Gregory Weitsman (at KCL lab of Prof. Ng, Imagint-supported) to Prof. Chester's lab to discuss and prepare fluorophore-conjugated HER2 DARpin for tissue FRET/FLIM	Gregory Weitsman (KCL) Robert Goldstein (UCL) and Kerry Chester (UCL)
26/04/12	Teleconference to discuss radiolabelling.	Tim Meyer (UCL) Rob Goldstein and Kerry Chester (UCL) Lars Baltzer (UU)
23/02/2012	Teleconference (amendment of the DOW).	Project officer, Novamen
15/12/2011	Teleconference IMAGINT / Project discussion.	Hüseyin FIRAT Peter GRASS
15/12/2011	Teleconference IMAGINT / Project discussion	Hüseyin FIRAT Peter GRASS
22/11/2011	Teleconference IMAGINT / Project discussion.	Hüseyin FIRAT Peter GRASS
10/11/2011	Visit by UCL and UZH to M-GMP (Finland.	Kerry Chester & Berend Tolner (UCL) Andreas Plueckthun (UZH). Ashesh Kumar (M-GMP)
11/2011	Teleconference.	S. Jakobs (MPG) and T. Ng (KCL)
5-6/07/2011 London	Project Kick-off Meeting in London, UK	All partners

DISSEMINATION – CONFERENCES, WORKSHOP, TRAINING

Date & Place	Teleconference/Meetings	Participants
15 November 2012	**Type of dissemination:** Abstract Submission to American Association of Cancer Research (AACR) for 2013 meeting. **Type of audience:** International scientific community. **Title:** Pre-clinical developments of the G3 Designed ankyrin repeat protein (DARPin) for *in vivo* assessment of HER2 expression,	Robert Goldstein, Berend Tolner, Gabriela Nagy, Andreas Plückthun, Tim Meyer, Kerry Chester (UCL and UZH)
17-19 September 2012, London	SMI Conference on New Generation Sequencing. Type of audience: Scientific community Countries addressed: Europe **Type of audience** International scientific community **Countries addressed:** USA, Europe, China, Japan, Canada	FIRALIS
9-12 Oct 2012, Chicago, USA	**Type of activities :** Conference, Keynote lecture Parameter-Unlimited Real Time Stoichiometric Decoding of Five Thousand Protein Cluster Networks in a Single Diagnostic Tissue Section at Nanometer Resolution: The Human Toponome Project. **Type of audience** International scientific community **Countries addressed:** USA, Europe, China, Japan, Canada	Schubert W, TNL

1-5 Oct 2012, Nice, France	**Type of activities :** Conference, Keynote lecture Simultaneous 100-parameter imaging and real time slicing across thousands of protein clusters in a single diagnostic tissue section using TISTM technology at 40nm super-resolution: the human toponome project **Type of audience** International scientific community **Countries addressed:** USA, Europe, China, Japan, Canada	Schubert W, TNL
31 August 2012	**Type of dissemination:** Invited AstraZeneca Personalised Healthcare Biomarkers (PHB) seminar. **Type of audience:** AZ (Global webcast: UK, Europe & US)~150 **Title:** Integrating whole body & protein interaction imaging with genomics to understand cancer heterogeneity : a multidisciplinary translational concept	Tony Ng (KCL & UCL)
26 July 2012	**Type of dissemination:** Invited seminar at Centre for Translational Medicine (CeTM), Cancer Science Institute, Singapore. **Type of audience:** Singapore multidisciplinary scientific community (from across several institutes) ~100 **Title:** Network (gene/protein)-based approach to cancer imaging	Tony Ng (KCL & UCL)
23 - 29 June 2012, Flims, Switzerland	**Workshop** 14th ECCO-AACR-EORTC-ESMO Workshop on Methods in Clinical Cancer Research. **Type of activity** A total of 39 faculty members, all of them top experts from the US and Europe, help selected fellow develop the study concept and complete the writing of the protocol before the end of the workshop.	Robert Goldstein, UCL
GMP Inspection 1st March 2012	**Type of activities :** Presentation on GMP products **Type of audience** Medicines and Healthcare products Regulatory Agency Inspector	Kerry Chester, UCL

15-20 Jul 2011, Dagstuhl, Germany	**Type of activities :** Conference, Keynote lecture Treatment of chronic diseases. A logic of failure. **Type of audience** International scientific community **Countries addressed:** USA, Europe, China, Singapore, Japan, Canada	Schubert W, TNL
05-10 Jun 2012, Ixia, Greece	**Type of activities :** Conference, Keynote lecture Dimension-unlimited super-resolution decoding of subcellular protein networks using TIS technology. **Type of audience** International scientific community **Countries addressed:** USA, Europe, China, Japan, Canada	Schubert W, TNL
15-16 September 2011 Cambridge, UK	**Type of activities:** Cancer Imaging Centre Conference **Presentation title** 'IMAGINT' **Type of audience** UK scientific community	Kerry Chester, UCL
31 May 2011	**Type of activities:** Research Presentation **Presentation title:** Using scFv-based and antibody-like molecules to target cancer treatment. **Type of audience** Scientists at the UCL Cancer Institute, London.	Berend Tolner, UCL

PATENTS

No patent has been established during the first period of the IMAGINT project.

EXPLOITATION FOREGROUNDS LIST

N/A the first period of the IMAGINT project

EXPLANATION OF THE RESOURCES USED

SUMMARY OF ACTUAL TOTAL ELIGIBLE COSTS INCURRED DURING THE FIRST PERIOD FOR EACH ACTIVITY, AND THE CORRESPONDING REQUESTED CONTRIBUTION

	RTD		MANAGEMENT		OTHER		TOTAL	
Partner-Short Name	Total	EC requested contribution	Total	EC requested contribution	Total	EC requested contribution	TOTAL	EC requested contribution
P1 - UCL	267 396,00	200 547,00	45 062,58	45 062,58	14 047,38	14 047,38	**326 505,96**	**259 656,96**
P2 - UZH	479 291,90	359 468,93	0	0	0	0	**479 291,90**	**359 468,93**
P3 - KCL	280 651,20	210 488,40	0	0	0	0	**280 651,20**	**210 488,40**
P4 - TNL	N/A*	N/A*	0	0	0	0	**N/A***	**N/A***
P5 - MPG	189 588,19	142 191,14	0	0	0	0	**189 588,19**	**142 191,14**
P6 - UU	148 580,91	111 435,68	0	0	0	0	**148 580,91**	**111 435,68**
P7 - INO	311 785,60	233 839,20	0	0	0	0	**311 785,60**	**233 839,20**
P8 - FLS	152 685,92	114 514,44	0	0	0	0	**152 685,92**	**114 514,44**
P9 - M-GMP	0	0	0	0	0	0	**0**	**0**
P10 - NOVAMEN	0	0	69 490,17	69 490,17	0	0	**69 490,17**	**69 490,17**
Total	**1 829 979,72**	**1 372 484,79**	**114 552,75**	**114 552,75**	**14 047,38**	**14 047,38**	**1 958 579,85**	**1 501 084,92**

ACTUAL RESOURCES EMPLOYED DURING THE PERIOD FROM M1 TO M18, PER WP AND PER PARTNER

		WP1 RTD	WP2 RTD	WP3 RTD	WP4 RTD	WP5 RTD	WP6 RTD	WP7 OTH	WP8 MGT	TOTAL
P1 - UCL	Planned (M1-M48)	2,00	5,00	4,00	5,00	72,00	5,00	10,00	5,00	108,00
	Spent (M1-M18)					23,70		1,18	2,95	27,83
P2 - UZH	Planned (M1-M48)	96,00	3,00	3,00	12,00	0,00	0,00	3,00	0,00	117,00
	Spent (M1-M18)	44,00	1,00	1,00	6,00			1,00		53,00
P3 - KCL	Planned (M1-M48)	0,00	43,00	0,00	0,00	0,00	57,00	1,00	0,00	101,00
	Spent (M1-M18)		13,82				19,79			33,61
P4 - TNL	Planned (M1-M48)	0,00	40,00	4,00	0,00	0,00	2,00	2,00	0,00	48,00
	Spent (M1-M18)		N/A*							N/A*
P5 - MPG	Planned (M1-M48)	18,00	30,00	0,00	0,00	0,00	6,00	0,50	0,00	54,50
	Spent (M1-M18)	9,00	6,50							15,50
P6 - UU	Planned (M1-M48)	0,00	0,00	0,00	90,00	6,00	0,00	1,00	0,00	97,00
	Spent (M1-M18)				13,41					13,41
P7 - INO	Planned (M1-M48)	5,00	0,00	36,00	0,00	0,00	3,00	3,00	0,00	47,00
	Spent (M1-M18)	5,00		31,00						36,00
P8 - FLS	Planned (M1-M48)	0,00	0,00	60,00	0,00	0,00	36,00	3,00	0,00	99,00
	Spent (M1-M18)						9,49			9,49
P9 - M-GMP	Planned (M1-M48)	0,00	0,00	0,00	5,00	9,00	0,00	1,00	0,00	15,00
	Spent (M1-M18)									0,00
P10 - NOVAMEN	Planned (M1-M48)	0,00	0,00	0,00	0,00	0,00	0,00	1,00	10,00	11,00
	Spent (M1-M18)								7,92	7,92
	Total Planned (M1-M48)	121,00	121,00	107,00	112,00	87,00	109,00	25,50	15,00	697,50
	Total Spent (M1-M18)	58,00	21,32	32,00	19,41	23,70	29,28	2,18	10,87	196,76

JUSTIFICATION OF MAJOR DIRECT COST ITEM

WP	Cost Category	Cost Type	Explanation	Cost	Beneficiary
1	RTD	Personnel	1 Postdoc, 3 PhDs, 1 TA	171,005.44	UZH
1	RTD	Other directs cost	CONSUMABLES: PCR reagents, Oligonucleotides, General chemicals, dyes and crosslinkers, bacteriological media, antibodies	66,560.50	UZH
1	RTD	Other directs cost	TRAVELING: Imagint Kick-off Meeting London 2011 Imagint Meeting Uppsala 2012	1,907.04	UZH
1	RTD	Other directs cost	OTHER: Other personnel costs	1,584.58	UZH
1-2	RTD	Personnel	Peter Illgen, 12,5 PM - WP1 (75%), WP2 (25%)	62,690.90	MPG
1	RTD	Other directs cost	CONSUMABLES: Chemicals	4,688.02	MPG
1-2	RTD	Other directs cost	TRAVELING: Dr. Jakobs, London 07/2011	881.55	MPG
1-2	RTD	Other directs cost	TRAVELING: Dr. Jakobs, Dr Illgen, London 07/2012	1,991.15	MPG
1	RTD	Personnel	Salaries of 4 people with this annual dedication: 15%, 2%, 4%, 11%	24,546.00	INO
1-3	RTD	Other directs cost	CONSUMABLES: 15854.60 / cell culture 7582.36 PCR / 3470.84 cloning and electrophoresis	28,455.00	INO
1-3	RTD	Other directs cost	TRAVELING: 3 flight tickets and the hotel accommodation for 2 people for London and Uppsala meetings.	1,795.00	INO
1-3	RTD	Other directs cost	DURABLE EQUIPMENT: Automated imaging platform depreciation.	12,158.00	INO

2	RTD	Personnel	1 PhD	2,815.39	UZH
2	RTD	Other directs cost	CONSUMABLES: General chemicals	2,080.00	UZH
2-6	RTD	Personnel	PI Professor T Coolen 0.33 PM, PI Professor T Ng 1.94 PM, PI Professor M O'Doherty 0.16 PM, PI Dr C Gillett 0.80 PM, RA G Weitsman 11.35 PM, RA K Lawler 1.03 PM, J Barrett Student 18 PM	125,262.35	KCL
2-6	RTD	Other directs cost	TRAVELING: Prof T Coolen Dinner with Project Partners 18/9/12, Mr J Barrett Conference ECCB12 workshop Basel Switzerland 8-9/9/12, J Barrett Travel Basel Switzerland 27-29/3/12, Prof T Coolen Meeting Uppsala Sweden 13/7/12. Dr G Weitsman Cambidge meeting 17/4/12, Dr G Weitsman Uppsala Conference Sweden 2-3/7/12.	2,250.21	KCL
2-6	RTD	Other directs cost	CONSUMABLES: Laboratory Consumables, Monocional Antibody,E cadiherin Rabbit mAb, Western Blotting subtrate,RNALATER,Antifade reagent, Transfection reagent, cysteamine, Filters, Phospho, Alexa fluor. & antobody's.	29,363.80	KCL
2-6	RTD	Other directs cost	DURABLE EQUIPMENT: Scientific & Computer Equipment	18,530.64	KCL
2	RTD	Personnel	Stefan Stoldt, 3 PM - WP2 (100%)	11,431.12	MPG
3	RTD	Personnel	1 PhD	2,815.39	UZH
3	RTD	Other directs cost	CONSUMABLES: General chemicals	2,160.00	UZH
3	RTD	Personnel	Salaries of 5 people with this annual dedication: 61%, 45%, 32%, 26%, 3%	127,912.00	INO
4	RTD	Personnel	1 TA, 1 Postdoc, 1 Phd	36,901.41	UZH
4	RTD	Other directs cost	CONSUMABLES: PCR reagents, Oligonucleotides, dyes and crosslinkers, antibodies	8,320.00	UZH

4	RTD	Personnel	Salary Lars Baltzer	22,513.78	UU
4	RTD	Personnel	Salary Nawal Badir	5,121.68	UU
4	RTD	Personnel	Salary Thomas Müller	19,036.80	UU
4	RTD	Other directs cost	DURABLE EQUIPMENT: bioanalytical equipment needed for protein radiolabeling in WP4	867.07	UU
4	RTD	Other directs cost	TRAVELING: Lars Baltzer Kick-of meeting in London	1,053.74	UU
4	RTD	Other directs cost	CONSUMABLES: Chemicals 9104,32 Laboratory equipment 31085,74 Chemicals and reagents for the control and verification of purity of Darpins, delivered from Partner 1. Chemicals for radiolabeling of Darpins ultimately to be used for PET Imaging, including linkers, assays for control of completion of conjugation chelating agents for use with Indium and Gallium radioisotopes. Replacement components for analytical equipment used for evaluation of purity of protein conjugates and efficiency of radiolabelling.All costs related to WP4	40,190.07	UU
4	RTD	Other directs cost	OTHER: Conference 3058,37 Repair 462,97 Shipping 558,58 Organization of 1st annual meeting of Imagint in Uppsala, incl lecture hall, refreshments, lunches and conference dinner. Shipment of spare part and repair of PET camera, required for work in WP4.	4,079.93	UU
5	RTD	Personnel	Technician - Mr Gaurav Bhavsar - 4.23 Man Months	18,399.30	UCL
5	RTD	Personnel	Technician - Dr Geoffrey Boxer - 1.72 Man Months	11,443.61	UCL
5	RTD	Personnel	Technician - Dr Maria Livanos - 9 Man Months 21,917.41	21,917.41	UCL

5	RTD	Personnel	PI - Kerry Chester - 0.75 Man Months	6,044.48	UCL
5	RTD	Personnel	Technician - Robert Goldstein - 2 Man Months	9,089.10	UCL
5	RTD	Personnel	Technician - Dr Berend Tolner - 6 Man Months	42,513.04	UCL
5	RTD	Other directs cost	CONSUMABLES: Consumables: to support research work for development of DARPins (antibodies, molecular biology reagents, protein chemistry assays, filters) ready for GMP manufacture, their pre-clinical evaluation and radiolabeling. Validation and maintenance of Research-GMP and GMP facility (probes and pumps and filters). Assays of clinical samples for circulating tmour cell (CTC) assays.	45,167.17	UCL
5	RTD	Other directs cost	DURABLE EQUIPMENT: Equipment for maintenance of GMP facility and to aid GMP research (Pumps and filters and monor works)	10,514.46	UCL
5	RTD	Other directs cost	TRAVELING: Travel and subsistence for research meetings of IMAGINT consortium in UK (London) and Upsaala (Sweden) and to PEGS meeting	2,033.93	UCL
6	RTD	Personnel	Costs for : Hueseyin Firat, institut leader,WP6 leadership Peter Grass,senior scientific expert in data analysis and predictive modelling Pierre Riou , scientific expert in data analysis, modelling Dorina Bratfalean, scientific expert in data analysis, modelling Fuat Firat, Andministrative assistant and financial reporting Regine Will, Andministrative assistant and financial reporting	87,717.39	FLS
6	RTD	Other directs cost	OTHER: Equipment : Prorata amortization costs of equipment of personnel involved in the project (Laptops, licence of Partek analysis software) Travel : Kick-off meeting in London 2011 + WP6 team F2F meeting at Firalis, France and London in 2012 + Consortium F2F meeting in Uppsala in July 2012 + Invited speaker at Biopartnering meeting in Brussels, invitation from CE Director Antoine Mahle, Policy officer.	7,711.31	FLS

7	OTHER	Personnel	Technician - Dr Geoffrey Boxer - 0.54 Man Months	3,598.62	UCL
7	OTHER	Personnel	PI - Kerry Chester - 0.64 Man Months **€**	5,180.99	UCL
7	OTHER	Personnel	1 PhD	1,407.69	UZH
7	OTHER	Other directs cost	CONSUMABLES: Bacteriological media €	2,000.00	UZH
8	MANAG	Personnel	Technician - Dr Geoffrey Boxer - 1.56 Man Months	10,436.00	UCL
8	MANAG	Personnel	PI - Kerry Chester - 1.39 Man Months	11,225.47	UCL
8	MANAG	Other directs cost	CONSUMABLES: Consumables: Conference expenses and booking of meeting rooms and office costs, supporting the IMAGINT Management Team at UCL (Coordinator Professor Kerry Chester and Manager Dr Geoff Boxer). Ensuring WP research targets are met, planning of operational tasks and design of future research	3,354.90	UCL
8	MANAG	Other directs cost	TRAVELING: Travel and Subsistence to attend meetings in UK (London) and Upsaala (Sweden) for Management Team at UCL (Coordinator Professor Kerry Chester and Manager Dr Geoff Boxer). Essential meetings to promote networking of IMAGINT partners, and IMAGINT researchers across Europe.	3,147.74	UCL
8	MANAG	Personnel	Income from project team from M1 to M18	44,717.41	NOVAMEN
8	MANAG	Other directs cost	OTHER: Travel & Accomodation in London for Jean-Luc Roux & Loïc Courtot KOM 5-6.7.11	1,718.46	NOVAMEN
8	MANAG	Other directs cost	OTHER: Travel & accomodation & bills, in Uppsala (annual meeting) for Jean-Luc Roux & Naïma Hamacha, 2-3.7.12	1,663.07	NOVAMEN
8	MANAG	Other directs cost	OTHER: 8 letters couriered by express postmail (Fedex company): Form A (June 2011)	537.69	NOVAMEN

8	MANAG	Other directs cost	OTHER: 7 letters couriered by express postmail (Fedex company): Form A (August 2011)	256.25	NOVAMEN
8	RTD	Other directs cost	OTHER: Conference call (Dec.11)	45.60	NOVAMEN

CERTIFICATES ON FINANCIAL STATEMENT

No certificate on financial statement has been established during the first period of the IMAGINT project.