Review and extension of the ACGT clinical studies

Project Number: FP6-2005-IST-026996

Deliverable id: D12.6

Deliverable name: Review and extension of the ACGT clinical studies

Submission Date: 28/11/2008

COVER AND CONTROL PAGE OF DOCUMENT	
Project Acronym:	ACGT
Project Full Name:	Advancing Clinico-Genomic Clinical Trials on Cancer: Open Grid Services for improving Medical Knowledge Discovery
Document id:	D12.6
Document name:	Review and extension of the ACGT clinical studies
Document type (PU, INT, RE)	RE
Version:	1
Submission date:	28/11/2008
Editor: Organisation: Email:	Christine Desmedt IJB christine.desmedt@bordet.be

Document type PU = public, INT = internal, RE = restricted

ABSTRACT:

This document presents the ACGT deliverable **D12.6**: *Review and extension of the ACGT clinical studies*, which follows up on D12.5. This deliverable unfolds into four-(4) main chapters (3, 4, 5 and 6), which present a yearly update of the overall ACGT clinical research activity. The two first chapters will give an update on the status of the ACGT trials on Nephroblastoma and Breast Cancer. The third one will give a brief summary of the in silico oncology study which is based on the above mentioned trials. Finally, the last chapter will focus on the Multi-Centre Multi-Platform scenario, which is an inter-centre Affymetrix-Illumina gene expression microarrays comparative study.

KEYWORD LIST: cancer, breast cancer, nephroblastoma, wilms tumor, clinical trials, in silico oncology, tumor biology

MODIFICATION CONTROL			
Version	Date	Status	Author
1.0	18/11/2008	Draft	C. Desmedt
2.0	25/11/08	Pre-final	C. Desmedt
3.0	28/11/08	Final	C. Desmedt

List of Contributors

- **Norbert Graf**, UdS, Homburg/ Saar, Germany
- **Alexander Hoppe**, UdS, Homburg/ Saar, Germany
- **Georgios Stamatakos**, ICCS/NTUA, Athens, Greece
- **Francesca Buffa**, UOXF, UK
- **Thierry Sengstag**, SIB, Lausanne, Switzerland
- **Christine Desmedt**, IJB, Brussels, Belgium

Contents

CONTENTS....4

1. EXECUTIVE SUMMARY 7

2. INTRODUCTION....9

3. THE ACGT NEPHROBLASTOMA TRIAL....10

3.1. IMPLEMENTATION AND STATUS OF THE ACGT NEPHROBLASTOMMA TRIAL....10

3.1.1. The Antigen Scenario....10

3.1.2. SIOP/GPOH 2001 database and Imaging Study....10

4. THE ACGT BREAST CANCER TRIAL 20

4.1. AIM OF THE TRIAL....20

4.2. STATUS OF THE ACGT TOP TRIAL 20

4.1.1. Amendments 20

4.1.2. End of accrual....21

4.1.3 Patient's characteristics 21

4.3. PRELIMINARY RESULTS 22

4.3.1. Gene expression profiling....22

4.3.2. Evaluation of TOP2A 24

4.4. SHARING OF DATA....25

5. THE ACGT *IN SILICO* ONCOLOGY STUDY....26

5.1. STATUS 26

6. THE MULTI-CENTRE MULTI-PLATFORM (MCMP) STUDY 27

6.1. OVERVIEW....27

6.2. SCOPE OF THE PRESENT STUDY 27

6.3. RELATED WORK....28

6.4. THE MCMP SCENARIO 30

6.4.1. Patient characteristics, data collection and ethical approval 30

6.4.2. Sharing of datal 32

6.4.3. Available samples and microarrays 32

6.4.4. Data processing (the actual scenario)....33

6.4.5. End points of the study 36

6.4.6. Preliminary results....37

7. CONCLUSIONS & PERSPECTIVES....42

Table or Figures

Figure 1: Age Distribution of 133 nephroblastoma patients included in the Antigen scenario 11

Figure 2: Stage distribution of 133 patients enrolled in the Antigen scenario 11

Figure 3: Discrimination between nephroblastoma and neuroblastoma shown for 1 antigen in different sera of patients 12

Figure 4: Discrimination between healthy children, nephroblastoma and neuroblastoma using HOMI20569 13

Figure 5: Overlapping of antigens between healthy children and patients with nephro- and neuroblastoma 14

Figure 6: Frequency of autoantigens found in sera of patients 14

Figure 7: Age distribution of the group with other malignancies than Nephroblastoma (n = 60) 15

Figure 8: Age distribution of the reference group of healthy children without malignant diseases (n=72) 15

Figure 9: Segmentation tool from FhG used for the rendering of a tumour 17

Figure 10: Screenshot showing an example of a segmented nephroblastoma (low resolution) 18

Figure 11: Variability within platforms and across platforms in the MAQC I study 28

Figure 12: The agreed general workflow for the MCMP scenario 30

Figure 13: Distribution of pair-wise non-parametric sample correlation (Spearman) of overall gene expression between Illumina and Affymetrix platforms in 75 samples 37

Figure 14: Reproducibility of breast cancer classification by Affymetrix and Illumina arrays 39

Figure 15: Expression of ESR1 in Illumina and Affymetrix microarrays, and reliability of classification into ER positive and negative samples as predicted by ELISA measurements40

Figure 16: Reproducibility of a prognostic signature of distant metastasis (top panel) in N0 ER+ breast cancer (bottom panel) 41

Table 1: Number of collected sera from patients with nephroblastoma at different time points of the Antigen scenario 10

Table 2: Cellular distribution of the most important clones differentiating between nephroblastoma and neuroblastoma . 13

Table 3: Cancer Types of the group of children with malignant diseases other then nephroblastoma (N=60) 16

Table 4: Overview of available imaging data at the study office of the SIOP 2001/GPOH 18

Table 5: Available imaging studies of patients enrolled in the Antigen scenario 19

Table 6: Summary of patient accrual in the TOP trial across the different centres 21

Table 7: Patient and tumour baseline characteristics 21

Table 8: Patient demographics 31

1. Executive Summary

This document presents ACGT deliverable **D12.6**: *Review and extension of the ACGT clinical studies*, which follows up on D12.5. It unfolds into **four**- (4) main chapters (**3**, **4**, **5 and 6)**, which present the overall ACGT clinical research activity.

- **The ACGT Nephroblastoma trial (3):** Wilms tumor is the most common malignant renal tumor in children. In the SIOP 2001/ GPOH trial clinical data, molecular data and pre- and post chemotherapy DICOM imaging studies are collected, coming from patients out of more than 50 hospitals in Germany. From a limited set of these patients, microarray data are provided. Since June 2006 patients of the SIOP trial were enrolled in the ACGT nephroblastoma trial. The main question of the ACGT nephroblastoma trial is to answer whether molecular biology helps to define new risk groups in Wilms tumor and can be used to stratify treatment of these patients in the future. *ACGT* promotes the integration of the heterogeneous data, facilitates further molecular analysis, the access to tissue banks, provides the necessary analytic tools and allows clinicians to efficiently analyze data that are presently communicated by mail, fax or maintained in flat text files at various remote clinical sites.
- **The ACGT Breast Cancer trial (4):** Carcinoma of the breast remains the most prevalent cancer diagnosed in women in the world. The ACGT trial on breast-cancer investigates pre-operative chemotherapy treatment and responses in order to identify indicative individualised patients' profiles. The whole effort relies, and enhances, the TOP-trial on breast-cancer. Data collected are images of the tumour, clinical information as reported in the case report form, and different types of biological data including microarray gene expression profiles of the tumour.
- **The ACGT *in silico* oncology study (5):** The third action of the ACGT clinical trials concerns the validation, adaptation and optimization of an advanced computational system, the "Oncosimulator" which is being developed within the framework of ACGT. The "Oncosimulator" is able to simulate within defined limits of reliability tumor growth as well as tumor and (to a lesser extent) normal tissue response to therapeutic schedules. The in silico oncology trial is based on the two other clinical trials (nephroblastoma SIOP 2001/GPOH and breast cancer TOP trial) following their considerable enhancement in terms of data collection.
- **The Multi-Centre Multi-Platform Scenario (6)**: The specific aim of this study is to assess the variability in gene expression microarrays, and the reliability of the

prognostic and predictive profiles obtained from this technology, when the arrays are performed using different technological platforms and at different centres. We will report here the status of this study as well as the preliminary results.

2. Introduction

The clinical post-genomic studies, in this work package, target breast cancer and children's nephroblastoma diseases that show intriguing predisposition to genetic background, molecular signatures implicated in the clinical outcome and responses to drugs correlating to both genotypes and molecular classification of the disease. The scope, design and aims of these trials were described in detail in D12.4.

With this document, which is updated on a yearly basis and therefore follows on D12.5, we aim to describe the status of the different trials. Additionally, we will present here the Multi-Centre Multi-Platform scenario, which aims to assess the variability in gene expression microarrays, and the reliability of the prognostic and predictive profiles obtained from this technology, when the arrays are performed using different technological platforms and at different centres. .

3. The ACGT Nephroblastoma Trial

3.1. Implementation and Status of the ACGT Nephroblastoma Trial

The Nephroblastoma study and trial protocol 2001 started accrual in June 2001. In 2006 the first patient did enter the ACGT Nephroblastoma trial. The whole study protocol including CRF`s and treatment is described in D 12.1. which can be found at the BSCW Server (https://bscw.ercim.org/bscw/bscw.cgi/d192904/ACGT_D12.1_FORTH_Final.pdf).

All Case Report Forms (CRFs) that have been received continuously from the local centres were processed by the trial centre in Homburg. Since the last report in D12.5 no new statistical analysis is performed. This will be done on a yearly basis and reported in the next deliverable. This deliverable focuses on the Antigen scenario done within the ACGT Nephroblastoma trial.

3.1.1. The Antigen Scenario

Since the start of the ACGT nephroblastoma trial 281 patients are enrolled in the trial. In 133 patients (47 %) we did receive serum for the Antigen scenario. Altogether 355 sera are collected from 265 patients out of 36 local hospitals. Out of this cohort 72 sera were from healthy children and 60 from patients suffering from other cancers than nephroblastoma. These sera are used as a control groups.

Table 1: Number of collected sera from patients with nephroblastoma at different time points of the Antigen scenario

Distribution of Sera from nephroblastoma patients due to the different time points of the Antigen scenario				
	T1	T2	T3	T4
Total	109	50	51	13

Figure 1: Age Distribution of 133 nephroblastoma patients included in the Antigen scenario

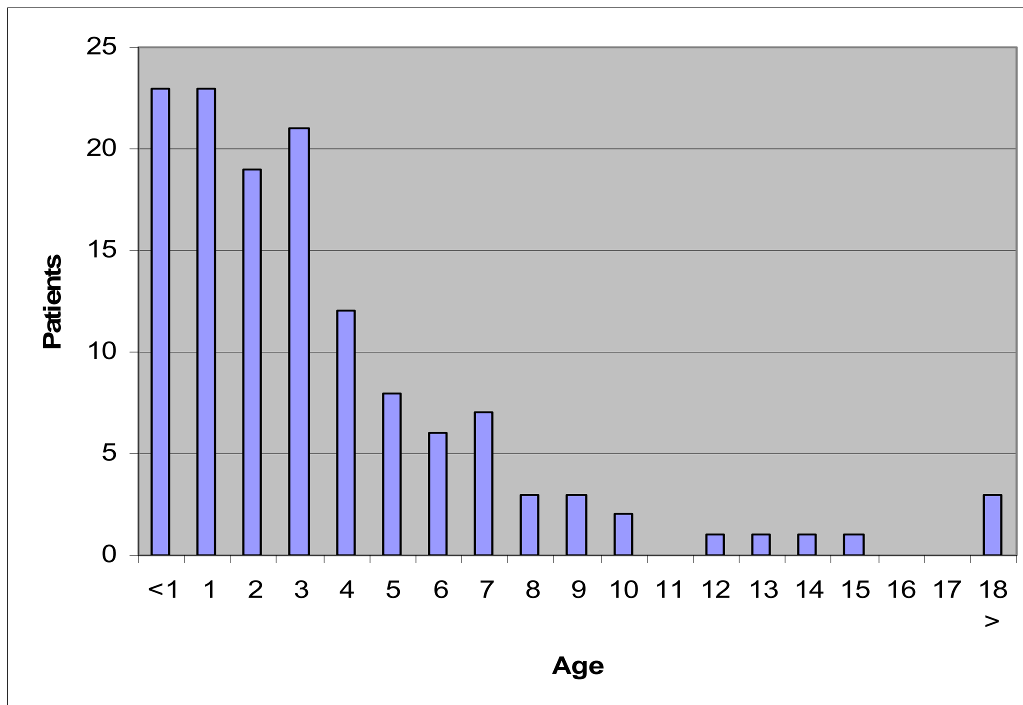
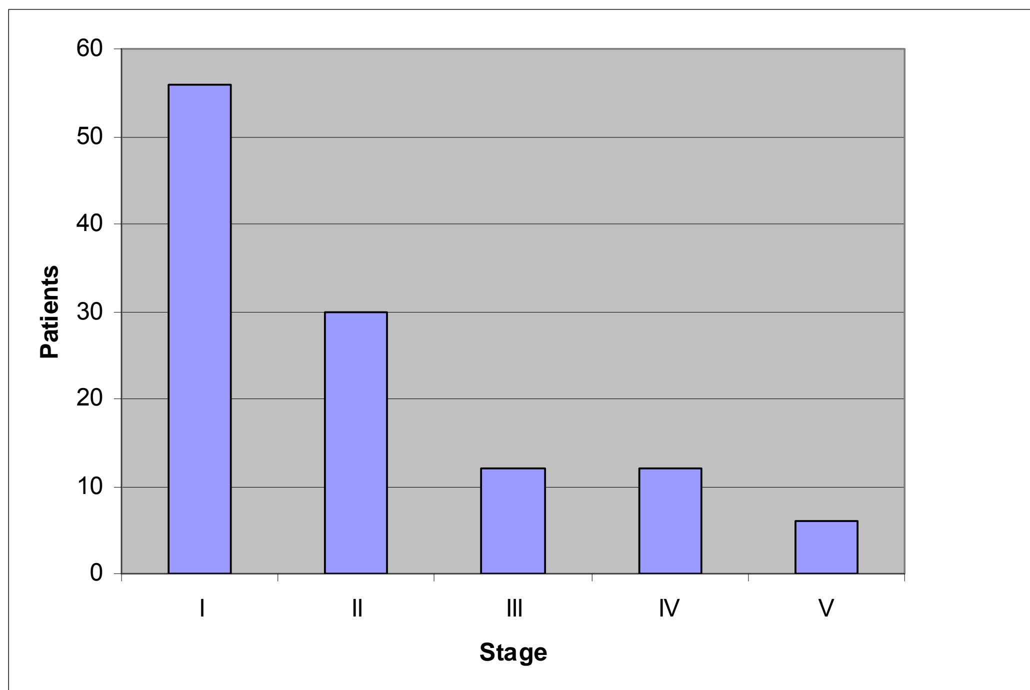


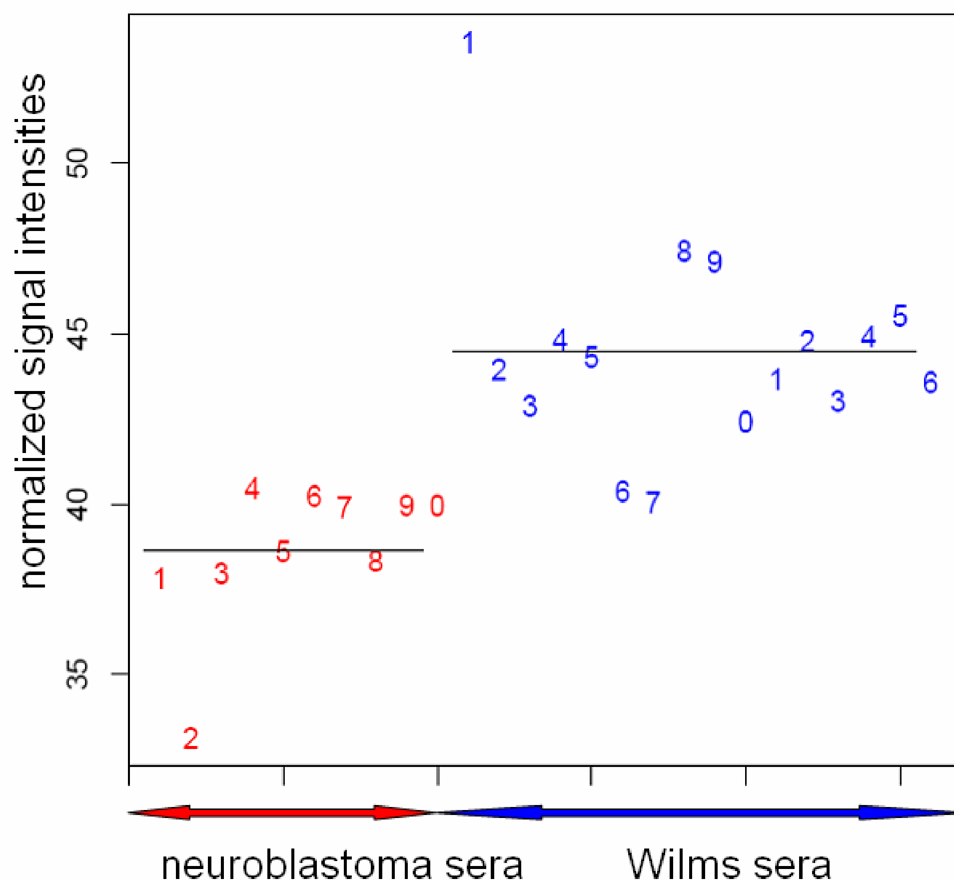
Figure 2: Stage distribution of 133 patients enrolled in the Antigen scenario



Most of the sera are collected at time point 1. Only in few patients we got serum at all 4 different time points. A preliminary analysis of the Antigen Scenario regarding the characterization of found autoantigens against nephroblastoma was reported at the Nephroblastoma meeting in Chamonix, France in March 2008 and at the SIOP conference in Berlin in October 2008[1].

61 clones could be found that discriminates between nephroblastoma and neuroblastoma. 39 of these clones can be selected as best discriminators. Figure 3 shows for a single antigen the discrimination between sera of patients with nephroblastoma and neuroblastoma.

Figure 3: Discrimination between nephroblastoma and neuroblastoma shown for 1 antigen in different sera of patients.



The celluar distribution of the most important clones is given in Table 2.

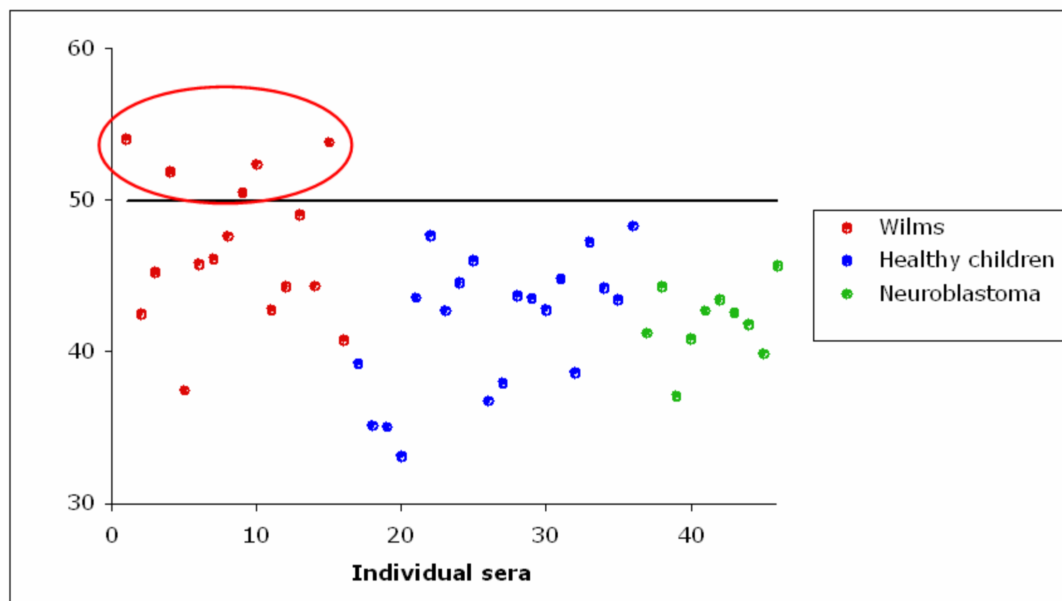
[1] Graf N, Heisel S, Hoppe A Lenhof HP, Meese E. Seroreactivity Patterns in Nephroblastoma. SIOP Abstract Book 2008. 40th Congress of the International Society of Paediatric Oncology, Berlin Germany. October 2-6, 2008. p61-62, O.152

Table 2: Cellular distribution of the most important clones differentiating between nephroblastoma and neuroblastoma.

name	Subcellular localization	AUC
HOML14545	nucleus	0.91
HOMJ05580	cytoplasm, nucleus, cell membrane	0.90
HOMB12601	nucleus	0.90
HOMM19554	cytoplasm	0.89
HOMF23541	nucleus	0.89
HOMC12520	nucleus	0.88
HOMB14528	cell membrane	0.87
HOMF13533	nucleus	0.86
HOMD22538	nucleus	0.86
HOMO19578	mitochondrion	0.85
HOMM03586	cytoplasm, nucleus	0.85
HOMM22541	cell membrane	0.85

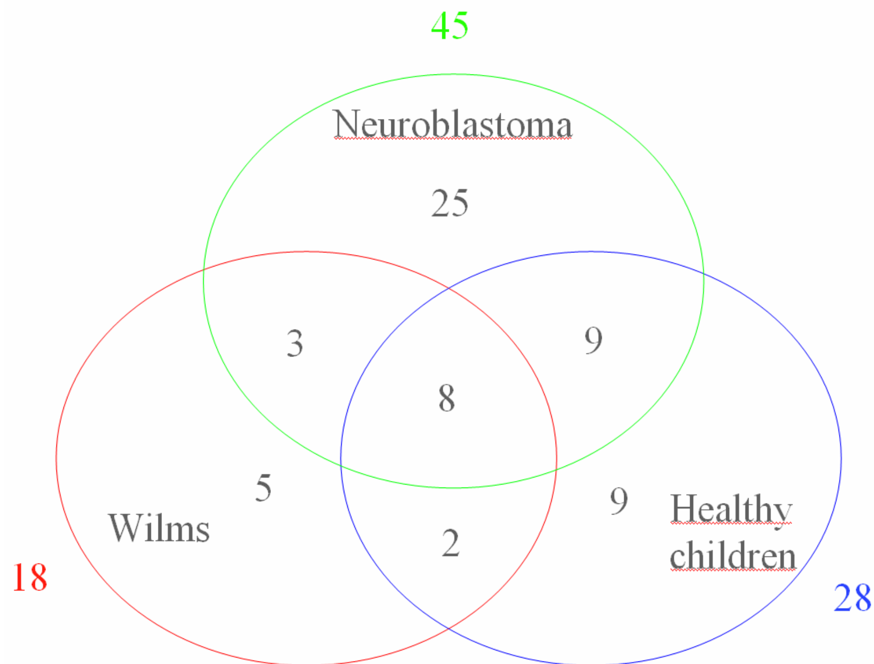
There are different autoantigens that can discriminate between healthy children and those with nephro- or neuroblastoma. As an example the autoantigen HOMI20569 is shown in figure 4.

Figure 4: Discrimination between healthy children, nephroblastoma and neuroblastoma using HOMI20569.



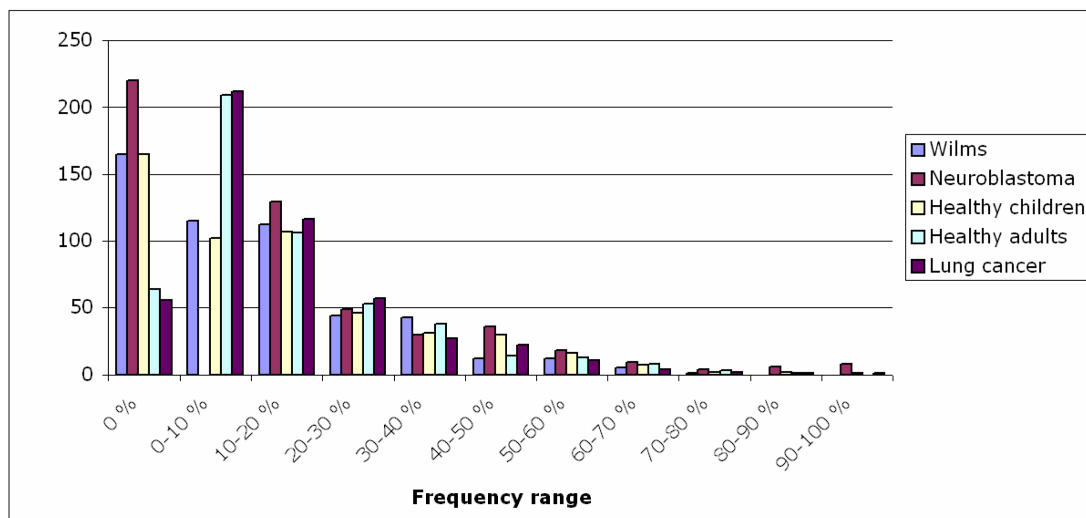
Between healthy children and patients with nephro- or neuroblastoma there is an overlapping of autoantigens. But one can find single autoantigens that are only expressed in single diseases as shown in figure 5.

Figure 5: Overlapping of antigens between healthy children and patients with nephro- and neuroblastoma.



In contrast to adult patients one can find more autoantigens in sera of children. This is shown in figure 6. The same autoantigen can be found in a higher frequency in children and children with cancer than in adult cancer patients.

Figure 6: Frequency of autoantigens found in sera of patients.



For the purpose of validation sera of healthy children and children with other malignant diseases than nephroblastoma are analyzed and compared. Figure 7 and 8 show the age distribution of this cohorts. Table 3 shows the different cancer types of the reference group.

Figure 7: Age distribution of the group with other malignancies than Nephroblastoma (n = 60)

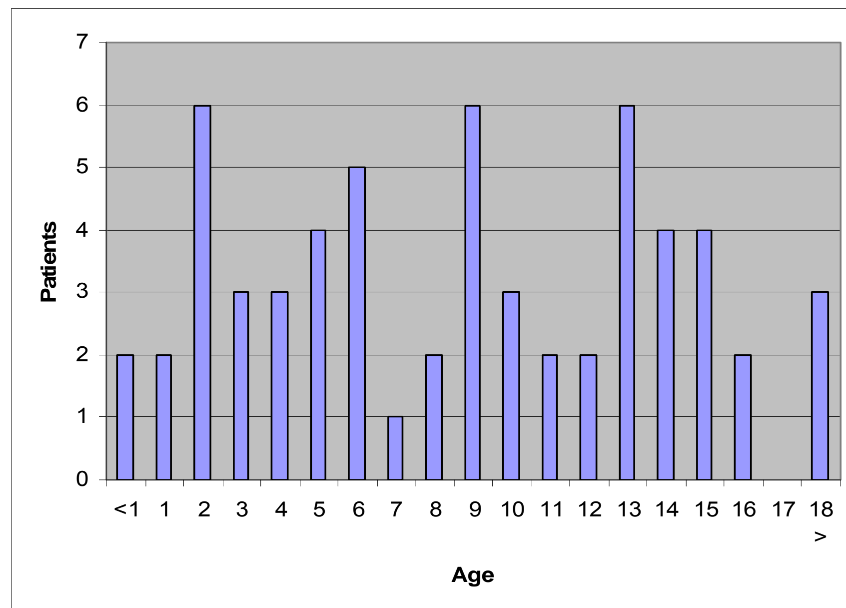


Figure 8: Age distribution of the reference group of healthy children without malignant diseases (n=72)

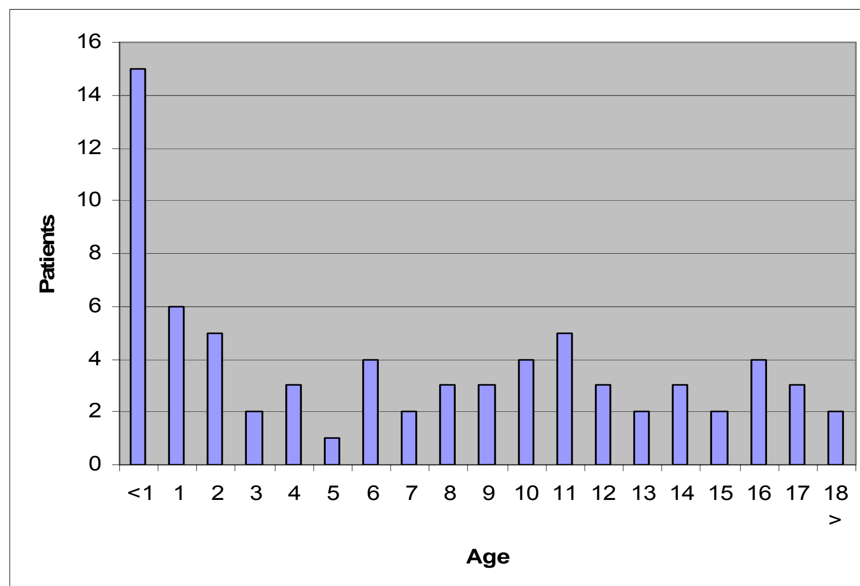


Table 3: Cancer Types of the group of children with malignant diseases other then nephroblastoma (N=60)

Cancer Type	Cases/ Patients
Acute lymphocytic leucaemia	12
Glioma	5
AML (including 1 relapsed patient)	5
Other Hämatological disease	5
Ewing Sarcoma	4
Neuroblastoma	3
Hodgkin disease	2
Rhabdomyosarcoma	2
Neurinoma	2
Chondrosarcoma (extrasceletal)	2
Clear cell sarcoma (kidney)	2
Non Hodgkin lymphoma	2
Clear Cell Sarcoma (lumbal soft tissue)	1
Osteosarcoma	1
Synovial Sarcoma	1
Medulloblastoma	1
Cardiac angiosarcoma	1
Ovarial tumour	1
PNET	1
MDS	1
Spindle cell Tumor	1
Outstanding Histology	5
Total	60

3.1.2. SIOP/GPOH 2001 database and Imaging Study

MRI Imaging studies of 10 patients were anonymized using the CAT tool and will be available for further processing via a FTP server at the University Hospital of Saarland. In two of theses cases the tumour was rendered with the help of a segmentation tool provided by FhG (Fig. 9). This work was carried out in close cooperation with ICCS (Institute of Communications and Computer Systems, Athens) and the University of Amsterdam.

Figure 9: Segmentation tool from FhG used for the rendering of a tumour

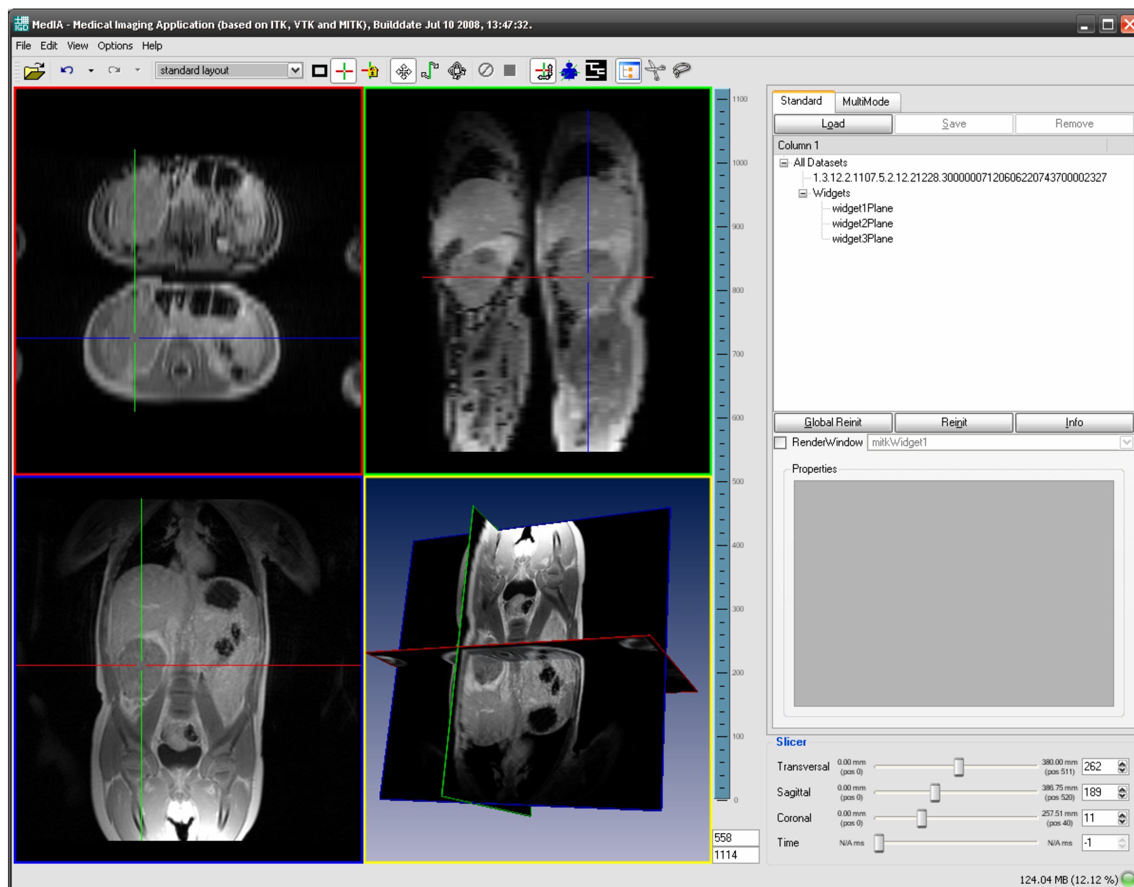
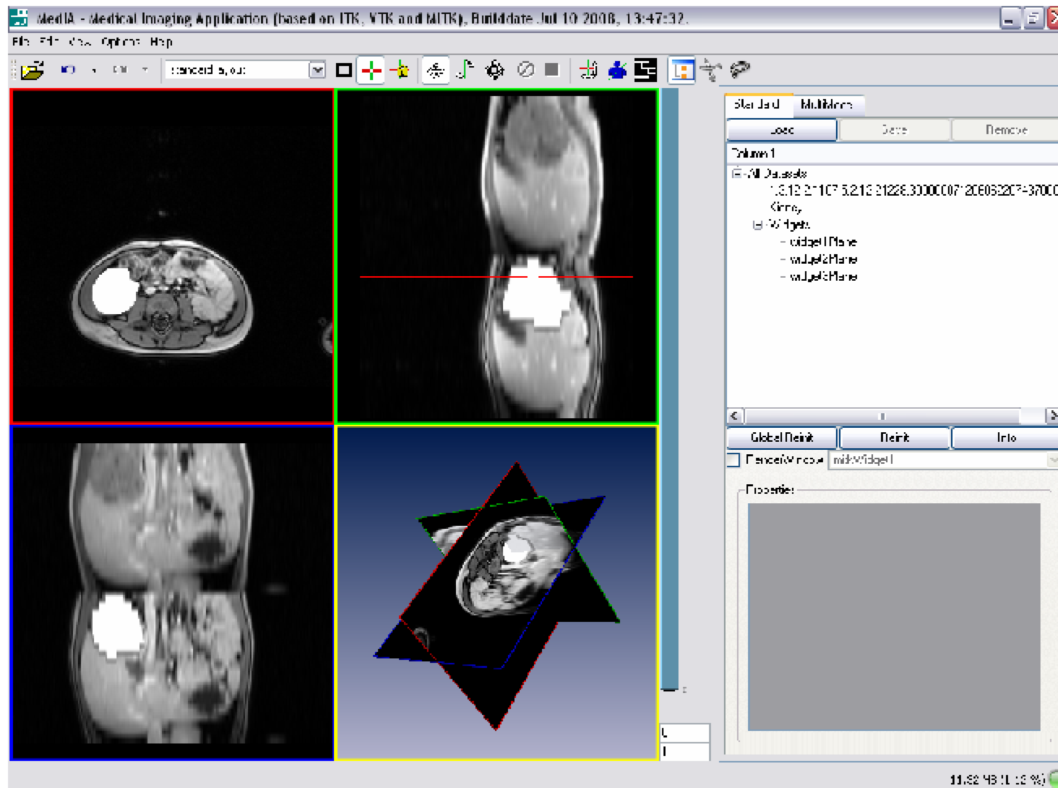


Figure 10: Screenshot showing an example of a segmented nephroblastoma (low resolution)



Imaging data including the rendered tumour volume will be used in the in silico scenario for nephroblastoma together with clinical data, data of the antigen scenario and pharmacokinetic data. The tumour volume after preoperative chemotherapy serves as the real volume for comparison with the virtual volume as a result of the in silico scenario.

Up to now images of 159 patients are available for analysis in different scenarios in ACGT. A detailed overview is given in table 4.

Table 4: Overview of available imaging data at the study office of the SIOP 2001/GPOH

	initial	post chemotherapy	additional
MRI (abdomen)	125	39	16
CT (thorax)	37	13	1
CT (thorax+ abdomen)	23	11	-

Ultrasound (kidney)	39	2	1

A detailed overview of imaging data of patients enrolled in the antigen scenario can be found in table 5.

Table 5: Available imaging studies of patients enrolled in the Antigen scenario**.**

	Initial	Post Chemotherapy	Others/ Additional
MRI	45	15	7
CT Thorax	12	13	5
CT Thorax + Abdomen	3	1	-
Ultrasound	22	2	1

In 35 patients sufficient imaging studies are available at diagnosis and after preoperative chemotherapy. In 8 of these patients serum is available for the antigen scenario. In all of these 35 patients the tumour volume of the nephroblastoma will be measured by using the segmentation tool of FhG. The data together with the data of the antigen scenario in 8 patients will be send to ICCS for use in the in silico scenario.

4. The ACGT Breast cancer trial

4.1 Aim of the trial

The aim of the TOP trial is to identify biological markers associated with pathological complete response to anthracycline therapy (epirubicin), one of the most active drugs used in breast cancer treatment. This is study is unique in the sense that it aims to: 1) determine the predictive factors of response to epirubicin (most studies are assessing response to polychemotherapy); and 2) evaluate the response of patients whose tumours do not express estrogen receptors (ER-negative), which eliminates the confounding effect of indirect ovarian suppression in ER-positive disease. For more details regarding the scope and design of the trial, see D12.4.

4.2 Status of the ACGT TOP trial

4.1.1 Amendments

The first patient of the TOP trial was registered in January 2003. In April 2004, a first amendment was submitted with the purpose to extend the trial also to locally advanced and inflammatory breast cancer patients.

Since it had been decided that the TOP trial would be one of the ACGT pilot studies, a second amendment as well as modifications to the informed consent were needed in order to allow the data to be shared in the context of ACGT. In brief, the main changes reported in this amendment include: 1/ the collection of additional samples for the analysis of circulating tumor cells and predisposal genes; 2/ the modification of the adjuvant treatment; and 3/ the sharing of the data in the context of ACGT. The amendment was accepted by the ethics committee of the Institut Jules Bordet in February 2008. At the Institut Jules Bordet, we are currently re-informing the patients who are already included in the TOP trial protocol with regard to the additional tests done for the predisposal genes and the sharing of the data in the context of the TOP trial. They have then the possibility to agree or not to have these additional tests done and/or to share their data within ACGT. At the time being, 15 patients gave their consent for having their data shared in the context of ACGT.

4.1.2 End of recruitment

During a meeting at the end of July 2008, the team in charge of the TOP trial reviewed thoroughly its status, and after consideration, has decided to stop the accrual of patients in this trial. The decision to prematurely stop including additional patients to this trial was motivated by: 1/ the low recruitment observed during the last months, which unfortunately would not have been improved significantly by the participation of the University of Crete (UoC); 2/ the concern of several investigators that this neo-adjuvant study only includes a monotherapy instead of a poly-chemotherapy, and 3/ the fact that the biological samples which are already collected and will be collected after that the patients have signed the second informed consent should allow to conduct the planned analyses.

4.1.3 Patient's characteristics

Altogether, 149 patients were registered in the TOP trial across 7 different hospitals (Table 6).

Table 6: Summary of patient accrual in the TOP trial across the different centres.

Investigator	Country/Town	Number of pts
Piccart	BE/Bruxelles	103
Maerevoet	BE/Ottignies	6
Kains	BE/Bruxelles	7
Richard	BE/Baudour	1
Vindevoghel	BE/Namur	2
Delaloge	FR/Villejuif	19
Duhem	Luxembourg	11
		149

All Case Report Forms (CRFs) that have been monitored in the different centres were processed by the coordinating centre, the Institut Jules Bordet in Brussels. The following table illustrates the main patient and tumor characteristics of these patients.

Table 7: Patient and tumour baseline characteristics

Data	Patients (n=149)
Patient registered	149
Case Report Forms collected	149
Median age (range)	47 (27-68)
Age $\leq$ 50	91
Age > 50	58

Missing	0
T size (at baseline)	
≤ 2 cm	21
> 2 and ≤ 5 cm	105
> 5 cm	5
T4	17
Missing	1*
N status (at baseline)*	
N0	74
N1	68
N2	3
N3	3
Missing	1*
Histological type*	
ductal	139**
lobular	2
other	8
Missing	1*
Histological grade*	
G1	2
G2	28
G3	110
Gx (unknown)	9
Missing	1*
Type of surgery	
Mastectomy	49
Conservative	88
Other	1

4.3 Preliminary results

4.3.1 Gene expression profiling

Aims:

Using gene expression profiling, we aim to:

1/ identify “de novo” the genes differentially expressed between patients who presented a response vs the others;

2/ investigate whether the gene expression modules based on several biological processes of breast cancer could be associated with response;

3/ investigate whether gene expression modules based on the anthracycline target genes could be associated with response.

Methods:

Frozen sections of the pre-treatment biopsies for the first series of patients were examined centrally by one pathologist and excluded if the tumor cell content is below 70%. Isolation of RNA was performed using the Trizol method (Invitrogen) according to the manufacturer's instructions and purified using RNeasy mini-columns (Qiagen, Valencia, CA). The quality of the RNA obtained from each tumor sample was assessed based on the RNA profile generated by the bioanalyzer (Agilent Inc). We used the Affymetrix Human Genome U133-2.0 plus GeneChips, which contains almost 50,000 probe sets representing more than 47,000 transcripts, derived from approximately 39,500 well-substantiated human genes. Hybridizations were performed at the microarray facility of the Jules Bordet Institute. Gene expression profiles were obtained for 88 patients. Retrieval and gene expression profiling analysis of the remaining samples is ongoing.

Evaluation of the predictive value of gene expression modules related to key biological processes of breast cancer

Recently, IJB performed together with SIB a large comprehensive meta-analysis (>2100 patients) integrating both clinico-pathological and gene expression data summarized in molecular modules representing key biological processes involved in breast cancer. This allowed us to better understand the mechanisms that trigger the different tumors to progress and to characterize the biological foundation of the different published prognostic signatures[2]. Here, we evaluated the potential predictive value of these gene expression modules. We therefore considered the gene expression profiles from the 88 patients for which we already realized the microarray experiments. We found that low values of the tumor invasion module were associated with pathological complete response, defined as the complete disappearance of tumor cells in the breast and the axillary lymph nodes. Interestingly, looking at data collected in the context of a prospective trial formally comparing anthracyclines and taxanes, this module appeared to be correlated with response to anthracyclines only but not with taxanes. This suggests that this module could be regimen-specific.

[2] Desmedt C, Haibe-Kains B, Wirapati P, et al. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Can Res 2008

4.3.2 Evaluation of TOP2A

Aims:

Topoisomerase II alpha (TOP2A) is probably the most representative and promising marker that could predict the activity of anthracycline-based regimens for breast cancer patients. Indeed, TOP2A is a key-enzyme in DNA replication, the molecular target of anthracyclines and its gene is located next to HER2 on the chromosome 17.

Cell lines studies have suggested that cells with high amounts of this enzyme might be more sensitive to anthracyclines. Several groups have investigated this hypothesis during the last decade and controversial results regarding TOP2A amplification/expression and response to anthracyclines in breast cancer patients have been reported.

In order to further refine these findings, we aim here in the context of the TOP trial at:

1/ evaluating the correlation between the gene, mRNA and protein levels of TOP2A since it is not clear at what level this marker should be evaluated;

2/ identifying the genes differentially expressed between TOP2A amplified and not-amplified tumors in order to understand the impact of TOP2A gene aberrations on the breast cancer phenotype;

3/ evaluating the predictive value of TOP2A gene, mRNA and protein levels.

Methods:

Fluorescent in situ hybridization (FISH) assays were done to evaluate the gene status of TOP2A. This was done using the Abott Multi-color TOP2A Spectrum orange, HER2 Spectrum green and CEP17 Spectrum aqua probe. Signals from at least 60 non-overlapping nuclei with intact morphology were evaluated to determine the mean number of signals/cell (ratio between mean number of TOP2A or HER2 signals and the mean number of chromosome 17 centromere signals). Amplification of HER2 and TOP2A genes was defined as a relative copy number ratio ≥2.

TOP2A protein expression was evaluated by immunohistochemistry (IHC) using the KiS1 antibody from Boehringer-Mannheim.).TOP2A mRNA expression was derived from the Affymetrix data. All TOP2A evaluations were carried out in a blinded fashion: TOP2A gene, mRNA and protein evaluations were done independently.

Preliminary results:

FISH and IHC results were available for 83 and 92 patients respectively for these preliminary analyses. We observed a correlation between the mRNA levels and the FISH ratios, but no correlation between the mRNA and IHC levels. We further identified a list of genes differentially expressed between TOP2A amplified and non-amplified tumors. We found that TOP2A amplification, which was exclusively observed in HER2 amplified cases, but not TOP2A mRNA or protein expression, was predictive of response to epirubicin in these ER-negative breast cancer patients. These preliminary results were presented at the annual meeting of the American Society for Clinical Oncology in June 2008[3] and will need to be validated in the globality of the TOP trial patients.

4.4 Sharing of data

In addition to the data which has been shared already initially for 10 patients, the data for the 15 patients which have re-consented is ready for being shared within ACGT. Data from additional patients will become available for ACGT once they have signed the new consent during one of their follow-up visits.

However, before data can be shared, the contracts developed by WP10 need to be signed by the legal department of IJB and other partners wishing to access the data. These contracts are now being refined based on the requirements of both data holders and data users. Once these contracts are signed, IJB will send real data to Custodix for anonymisation. These procedures have been discussed in details with WP10 and WP11 during the consortium meeting in Crete in September 2008 and during a meeting at IJB in November 2008.

5. [3] Desmedt C, Azambuja E, Larsimont D, et al. Investigating the predictive value of topoisomerase II alpha (TOP2A) gene, mRNA and protein levels in anthracycline-treated estrogen receptor (ER) negative breast cancer patients. American Society of Clinical Oncology Meeting, Chicago-Illinois 2008.

5. The ACGT *In Silico* Oncology Trial

5.1. Status

An initial version of the ACGT Oncosimulator i.e. an integrated software system simulating *in vivo* tumour response to therapeutic modalities within the clinical trials environment and aiming at supporting patient individualized optimization of cancer treatment is presented in deliverable D8.3. The document refers to both the basic science and the technology modules of the system whereas a description of the clinical requirements and the types of medical data provided and exploited is included. As deliverable D8.2 contains the foundations of the simulation component only an outline of the major improvements, refinements, extensions and *numerical checks* and *explorations* of the simulation model including a *sensitivity analysis* is included in that document. A relatively detailed description of the technology modules is presented. The latter mainly refer to image processing, the grid execution scenario, cluster execution and parallelization of the simulation code, interactive and virtual reality visualization, subjunctive interfaces and the Oncosimulator component collaboration diagram. A brief outline of a possible future extension of the system which would address the immune system reaction has also been incorporated. The favourable outcome of an initial step towards the *clinical adaptation* and *validation* of the Oncosimulator is presented and discussed[4] [5](1,2).

Inhomogeneous data collection for both nephroblastoma and breast cancer is in progress. Regarding especially the molecular data of breast cancer particular emphasis has been put on the exploitation of the ampification degree of TOP2A since it appears to play a particular role in the response of early breast cancer to epirubicin.

---

[4] E. Ch. Georgiadi, G. S. Stamatakos, N. M. Graf, et al., "Multilevel Cancer Modeling in the Clinical Environment: Simulating the Behavior of Wilms Tumor in the Context of the SIOP 2001/GPOH Clinical Trial and the ACGT Project," Proc. 8th IEEE International Conference on Bioinformatics and Bioengineering (BIBE 2008), Athens, Greece, 2008. In press

[5] E. A. Kolokotroni, G. S. Stamatakos, D. D. Dionysiou, et al. “Translating Multiscale Cancer Models into Clinical Trials: Simulating Breast Cancer Tumor Dynamics within the Framework of the “Trial of Principle” Clinical Trial and the ACGT Project.,” Proc. 8th IEEE International Conference on Bioinformatics and Bioengineering (BIBE 2008), Athens, Greece, 2008. In press

6. The Multi-centre multi-platform (MCMP) study

6.1 Overview

Multi-centre genomic/post-genomic trials are becoming the rule rather than the exception. This is often dictated by population size requirements. In fact, in genomic trials a huge amount of variables is measured and very large numbers are needed in order to be able to resolve the associations amongst these variables and clinical outcome.

In addition, many centres are now able to process the genomic data on-site rather than having to transfer the material to a central facility, and this can potentially cut time, costs and organizational problems related to multi-centre genomic trials. Also, results obtained in trials performed using a specific platform might be used in a clinical context where different, but still state-of-the-art, platforms are available. Furthermore, meta-analyses of trials/clinical studies, which investigate for example rare side-effects, or rare groups of patients, need to merge data collected at different centres and generated using different platforms. Thus, the issue of reproducibility and generality of genomics measurements, it is becoming increasingly important.

6.2 Scope of the present study

In this context, the specific aim of the present study (called here the “MCMP study”, i.e. multi-centric, multi-platform study) is to assess the variability in gene-expression and the reliability of the prognostic and predictive profiles obtained with the microarray technology when gene expression is measured using different technological platforms at different centres. Although this study is not a clinical trial as such, it forms the basis of a scenario to assess the feasibility of mixed-platform trials. The study uses human breast cancer samples, but one of the aims is to set the scenario in a general way, so that it could be transposed to other contexts.

It is assumed that the patients in two (or more) centers have biopsies collected and that each center is using a different microarray platform, namely Affymetrix and Illumina, to measure gene expression in the tumors. In addition, the classical clinical parameters associated to each patient are collected and made available in a relational database. This specifies a hypothetical multi-centric and multi-platform study, with only one microarray per

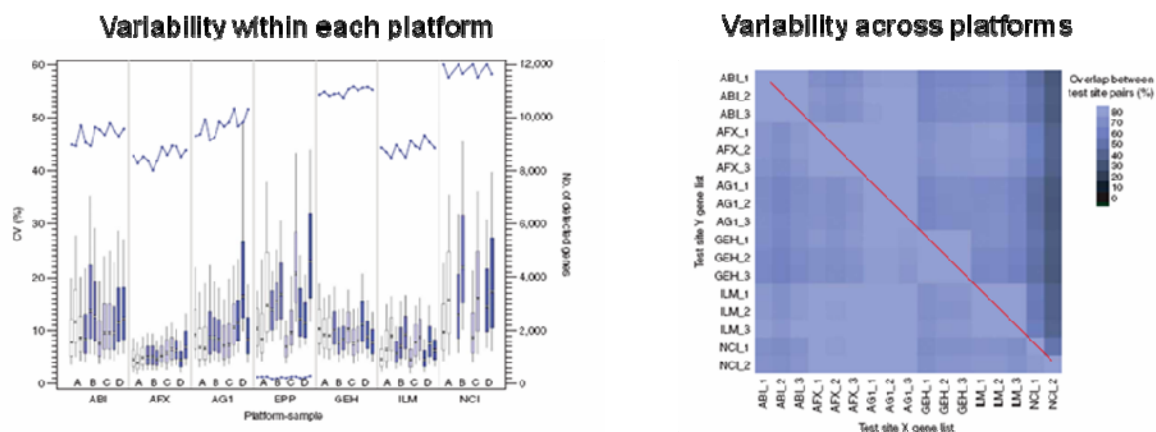
patient. In reality, in the present scenario the RNA extracted from samples from patients have been hybridized on *both* Affymetrix and Illumina platforms, at two different facilities. This unique dataset will allow testing the feasibility of multi-platform studies using the high-performance environment of the ACGT connected grid.

Furthermore, we aim at generating resources and workflows with a high degree of reusability that can be deployed in similar comparative studies.

6.3 Related work

Previous studies have shown an good degree of reproducibility in microarray gene-expression data. In particular a large recent study, the phase I from the MAQC (MicroArray Quality Control) consortium has shown a general good correlation between different array platforms. Specifically, 5 major commercial platforms (Applied Biosystems, Affymetrix, Agilent, GE Healthcare and Illumina) have shown relatively high levels of interplatform concordance in addition to concordance with independent gene expression measurements obtained by RT-PCR assays[6]. In the MAQC study, the Illumina and Affymetrix platforms, which are used in the present scenario, were the two platforms yielding most similar results, from the viewpoint of correlation of expression level, classification of results (Figure 11), and variability. Specifically, the median replicate coefficient of variation (CV) was lower than 12% for all samples across sites. Thus, they are two very credible candidates for a possible inter-platform inter-centre trial.

Figure 11: Variability within platforms and across platforms in the MAQC I study



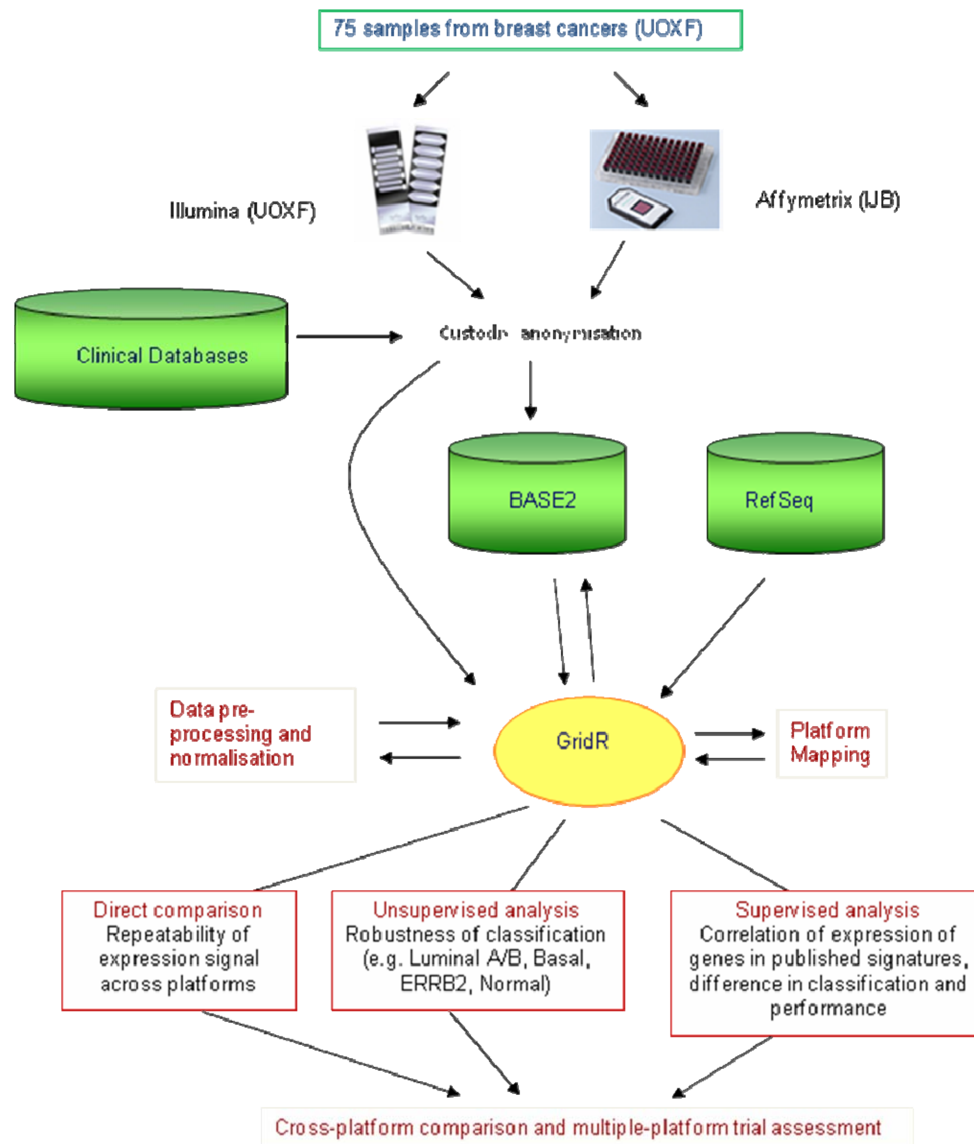
[6] MAQC Consortium. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nature Biotechnology* 24, 1151-1161 (2006)

The MACQ Phase I study was performed on reference biological material and did not address the variability observed in context of multi-centre clinical studies in cancer; nor the stability, generalizability and transportability of gene expression prognostic/predictive classifiers developed in these studies when different platforms are used.

A second phase, the MAQC II, is addressing some of these issues; namely the ones of transportability of classifiers. Informal meetings with MAQC consortium members have taken place to understand differences and similarities. Clearly the problems addressed in the present study are similar to those of MAQC II, and the clinical context and technological platforms used are also similar; however, the number of samples and the design are different. Furthermore here two aspects absent from MAQC will be addressed which are first the assessment of the Grid ACGT environment for these type of comparative studies; and secondly the construction of a re-usable, comprehensive and general, analysis framework that can be applied to other studies of this type. In summary, our aim is to provide a first contribution to establishing workflows for comparative studies scenarios.

The agreed general workflow for this study is illustrated in Figure 12.

Figure 12: The agreed general workflow for the MCMP scenario



6.4 The MCMP scenario

6.4.1 Patient characteristics, data collection and ethical approval

The MCMP study includes samples from a historical series breast cancer patients (n=75) treated in Oxford between 1989 and 1992. Patients received surgery followed by adjuvant hormone therapy or no adjuvant treatment. Tamoxifen was used as endocrine therapy for 5 years for premenopausal and postmenopausal patients if estrogen receptor (ER)–positive. In patients who were <50 years of age, adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil were administered if the tumors were lymph node–positive,

or ER-negative and/or >3 cm in diameter. Patients >50 years of age with ER-negative, lymph node–positive tumors also received cyclophosphamide, methotrexate, and 5-fluorouracil for six cycles, at a thrice weekly intravenous regimen. The data set was complete for age, nodal status, definitive surgery, relapse, and survival. The patient demographics are provided in Table 8. Data were collected from clinical and pathologic records.

Table 8. Patient demographics.

Age	Mean	56.7
	Minimum and Maximum Age	32-79
Estrogen receptor status	Positive	52
	Negative	18
	Missing	5
Nodal involvement	No	61
	Yes	14
Grade	I	6
	II	33
	III	24
	missing	12
Hormonal Adjuvant Treatment	No	40
	Yes	35

Ethical approval for analysis of samples and notes was obtained from the local research ethics committee. In discussion with WP10 and WP1, the patient consent collected at the time of the study was considered to be possibly too broad in scope for EU directives, namely the Directive 2004/23/EC (Human Tissues and Cells Directive) and the Data Protection Directive 95/46/EC. At the same time, re-consenting would not be possible as a significant proportion of the patients have died and this would create a bias in the study. However, it was observed by WP10 that these data can be qualified as non-personal data for any third party, as they have been anonymised in Oxford and can be shared after anonymisation. In this case, the Data Protection Directive and the national implementation are not applicable, and the safety net of the ACGT legal framework is not mined. The data sharing procedure are described below.

6.4.2. Sharing of data

The modality for sharing the data inside the ACGT consortium was discussed with WP10 and 11 to ensure that the legal, ethical and security requirements were respected. To comply with the ACGT policies, the ACGT contracts (deliverable 10.4) will be signed by UOXF and all ACGT partners who will be involved in the MCMP scenario, and will have access to the data.

A first pseudonimization of all the data had already been performed by UOXF. The data were then sent to WP11 who performed a second round of pseudonimization.

The ACGT contracts are under review by UOXF legal department. However, it was decided to make data for the MCMP scenario available to specific partners before the approval and signature of the ACGT contracts, so that the test and development of the scenario and the tools required could proceed. Thus, a “Terms of Use” was developed in association with WP10 and sent to all ACGT partners who would work on the data. This document includes items such as the exclusive use of the data in the context of ACGT, the confidentiality and property of the data. A signed version of this document was sent back from ACGT partners to UOXF; after this, Custodix provided these partners secure access to the pseudonimization data. The partners who have already signed these contracts and have the access to the data are Custodix, Lausanne and Lundt; and Phillips should follow soon.

6.4.3. Available samples and microarrays

RNA extraction

Total RNA was extracted from the tumour samples. RNA was isolated by use of the Trizol method (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions and was purified using RNeasy mini-columns (Qiagen, Valencia, CA). RNA samples from Oxford were shipped on dry ice at the JBI in Brussels, Belgium, for processing. The quality of the RNA obtained from each tumor sample was assessed via the RNA profile generated by the Agilent bioanalyzer. Samples with a total area under the 28S and 18S bands of less than 15% of the total RNA band area, as well as a 28S/18S ratio of less than 1.1, were considered to be degraded and were not analyzed further (approximately 20% of the samples analyzed). Only tumor samples with good quality of RNA were considered for further analysis.

Affymetrix arrays

Affymetrix U133A and B Genechips (Affymetrix, Santa Clara, CA, USA) were used.

RNA amplification, hybridization, and scanning were done according to standard Affymetrix protocols. Image analysis and probe quantification was done with the Affymetrix software that produced raw probe intensity data in the Affymetrix CEL files.

Processing of the CEL files was done using the Bioconductor software, in R, so that any routines or wrokflow developed for the processing ad analysis could be transported to gridR without having to be changed. Expression calculation and normalization was done in two different ways and results compared:-

1) MAS5, using default options, and filtering of the absent probesets as estimated simpleaffy (Bioconductor)

2) gcrma (Bioconductor), using default options, and filtering of the absent probesets as estimated simpleaffy (Bioconductor)

Illumina arrays

Illumina Human RefSeq-8 arrays (illumina inc., San Diego, CA, USA) were used. RNA was amplified using Ambion Illumina Amplification Kit (Catalog #I1755). 850ng of amplified RNA product was hybridised to the Illumina Sentrix Beadchip 8x1 GAP REFSEQ2 using single chamber hybridisation cartridges. Washing and staining were carried out as specified in the Illumina Whole Genome Expression Manual version 1. Beadchips were scanned using the Illumina BeadArray Reader, a confocal-type imaging system with 532 (cy3) nm laser illumination. Expression data was extracted using the Illumina proprietary software BeadStudio, using background substraction, rescaling was used to eliminate negative values, and normalization was done in Bioconductor (R) using quantile normalization with default options.

6.4.4 Data processing (the actual scenario)

Microarray-platform probe/probesets matching

Platform mapping and data pre-processing is done adopting existing standards/methods. Specifically, the probe sequences from each platform are mapped to the NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq/). Probes for which a match was not found were filtered out. A probe is considered as having a match if it perfectly matches a RefSeq sequence and does not perfectly match any other transcript sequence with a different gene ID (http://jura.wi.mit.edu/entrez_gene/). Affymetrix probe sets are

defined as matching if at least 80% of the probes within the probe set are matching. In case of a transcript matching to more than one probe or probesets, the one closest to the 3' end of the transcript is selected. The common set of matching probes between Affymetrix and Illumina is then considered for further analyses and full annotation is retrieved basing on the RefSeq ID. This includes:- GO gene ontology (http://www.geneontology.org/), HUGO symbols (http://www.genenames.org/), Unigene ID (http://www.ncbi.nlm.nih.gov/unigene), Ensembl IDs (http://www.ensembl.org/index.html).

Expression computation and normalisation

Normalisation and pre-processing are carried out both independently and across the two platforms.

In the first case the platforms are processed and normalized using standard recognized methods (see above in the microarray-platform section for details) and then data are merged at a meta-level by calculating common statistics. A review of methods for doing this is for example provided in Ramasamy et al[7].

In the second case the platforms are normalized at the expression level, so that absolute expression levels are directly comparable. Different method have been suggested for this, the most recently published example is Shabalin et al.[8], other methods are described in the same reference and compared. A comparison of these methods, also with respect to the meta-level platform merging described above, will be carried out in the context of our scenario to assess differences and accuracy of results.

Comparative analyses

The platform comparison is based on different criteria:

1) Reliability of gene expression measurement:- comparison of gene expression as measured by Illumina and Affymetrix platforms in the 73 samples
2) Reliability of patient classification:- comparison of published gene expression classifiers and their performance in patient classification when using the two platforms

[7] Ramasamy A, Mondry A, Holmes CC, Altman DG. Key Issues in Conducting a Meta-Analysis of Gene Expression Microarray Datasets. PLoS Med. 2008 Sep 2;5(9):e184.
[8] Shabalin AA, Tjelmeland H, Fan C, Perou CM, Nobel AB. Merging two gene-expression studies via cross-platform normalization. Bioinformatics. 2008 May 1;24(9):1154-60.

3) Reliability of biological/clinical findings:- comparison of biological content of the gene expression classifiers obtained using the two platforms

4) Feasibility of a combined-platform trial:- simulation of a combined platform study and assessment of feasibility of multi-platform studies

The 4th analysis re-uses some of the statistical and bioinformatics methods of analyses 1-3, but a heavy re-sampling of the data is needed and the ACGT grid environment, and GridR performance, is specifically tested using this part of the scenario.

Gene expression profiles and classifiers, both in point 2 and 3, are of two main types:-

A) *Supervised signatures*: a signature that is derived in a supervised analysis, where the signature is trained to be predictive of a given outcome/tumour status and the genes are selected in order to maximize this prediction ability (see deliverable 12.3 for examples of this approach). Under this umbrella we will also include here knowledge-based signatures, where biological or phenotype variables other than clinical variables are used to train the signature, either in clinical datasets or previously collected cell line datasets (this has been referred to as “Hypothesis-driven” approach in deliverable 12.3). In the case that a signature already derived is tested (point 2 above), the signature performance as a predictor of the patients outcome/condition when using Illumina or Affymetrix is compared. This is assessed for example in terms of correlation of the derived score, or using performance evaluator instruments such as ROC curves. When deriving a new supervised signature, as in point 3 above, the content of the signature in term of genes and pathways is also compared between Affymetrix and Illumina analyses. Endpoints considered for this apporach include for example the main clinical covariates, which are estrogen receptor status, grade, nodal status, clinical outcome. Models for all these signatures have been published and these clinical variables are present in our dataset.

B) *Unsupervised signatures*: a signature that is derived in an unsupervised analysis, where the structure of the expression data is considered and samples are grouped basing on this structure and independently from clinical variables or outcome. On classical example of this is the breast cancer classification signature in 5 types Luminal A & B, Basal, ERRB2, Normal classes (see deliverable 12.3 for more

discussion). A more recent one is a breast cancer classification based on ER, HER2, and proliferative status[9]. In these cases, the robustness of classification of the derived signatures when using Illumina or Affymetrix can be compared when carrying out analyses as described in point 2 above; otherwise, the content of the signatures in terms of gene and pathways can be compared when carrying out analyses as described in point 3 above.

6.4.5 End points of the study

In practice the implementation of the MCMP study as part of a demonstration of the ACGT environment aims at reaching the following goals:

- Providing exploratory data analysis (EDA) plots describing the data sets
  - Demographics
  - Affymetrix data
  - Illumina data
- Normalization of microarray data within each platforms
- Normalization of microarray data between platforms
- Identification of probes matching across microarray platforms (see section above for suggested methods)
- Data filtering based on present/absent calls and CV of the expression
- Comparison between Illumina and Affymetrix platforms with respect to the performance of previously published supervised and unsupervised classifiers (see description above in points A & B)
- Construction of supervised and unsupervised classifiers
  - For Affymetrix platform alone
  - For Illumina platform alone
  - For a mixture of Affymetrix and Illumina
- Comparison of constructed classifiers in terms of classification performance and gene/pathways content
- Assessment of the grid functionality when using resampling techniques

A practical implementation of the main points of this study with the ACGT data mining environment is described in Deliverable D13.2: Intermediate Evaluation Report.

---

[9] Wirapati P, Sotiriou C, Kunkel S, et al. Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. *Breast Cancer Res.* 10(4):R65 (2008)

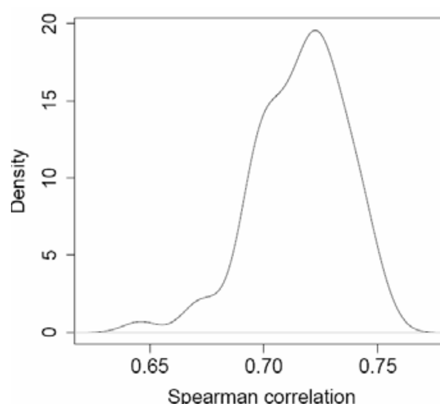
6.4.6 Preliminary results

In what follows some first results and discussions are reported for these comparative analyses, although some of these and further analyses are still in progress.

Reliability of gene expression measurement

The pair-wise non-parametric sample correlation (Spearman) of overall gene expression between Illumina and Affymetrix platforms had a median and mean of 0.72, and a standard deviation of 0.02. This indicates that based on the global expression (i.e. the expression of the transcripts in common, matched as described above) the samples showed a relatively high correlation when one or the other platforms were used, especially accounted for the noise of a clinical dataset, but that this correlation was not perfect.

Figure 13: Distribution of pair-wise non-parametric sample correlation (Spearman) of overall gene expression between Illumina and Affymetrix platforms in 75 samples.



However, this comparison was done using both present and absent genes, and it will improve significantly when only present genes and genes carrying the signal are considered[10]. In fact, below few examples are shown where this correlation is much improved when genes carrying the signal are considered. Specifically, supervised and unsupervised gene signatures were used that have already been shown to be either biologically or clinically relevant from previously published studies. More of this work is under way.

[10] Barnes M, Freudenberg J, Thompson S, Aronow B, Pavlidis P. Experimental comparison and cross-validation of the Affymetrix and Illumina gene expression analysis platforms. Nucleic Acids Research 33, 5914–5923 (2005)

Reliability of patient classification using published signatures

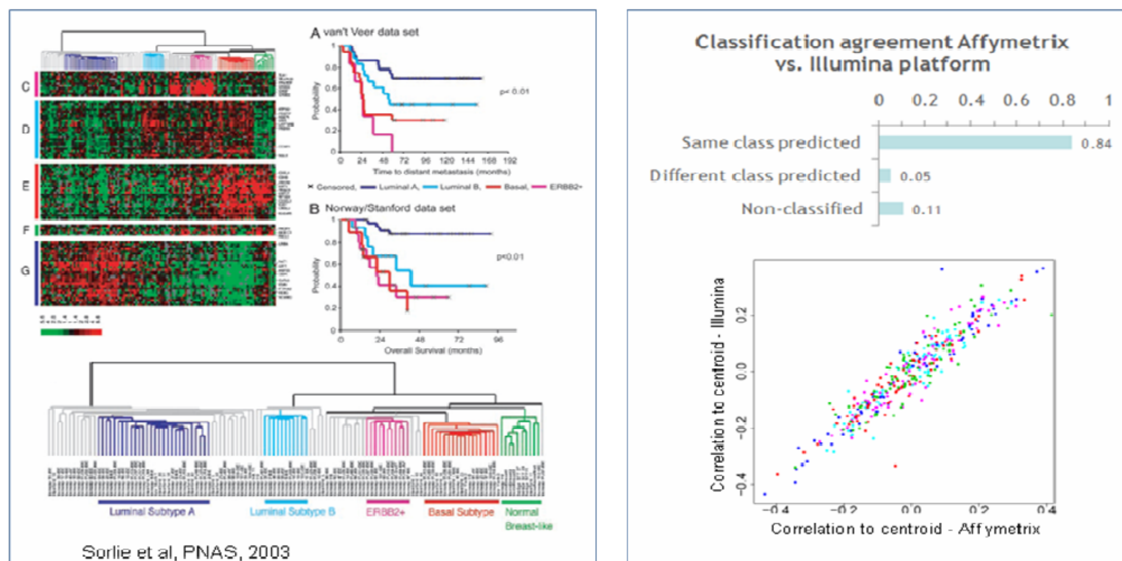
As a first example of unsupervised signature, the 5 group classifier developed (Figure 14, left panel) by Sorlie et al[11] was considered as this was the first one of this kind developed using gene expression microarray data.

The correlation of each sample with the original centroids of the 5 groups (Luminal A & B, ERBB2, Basal and Normal) were computed using non-parametric methods, and the sample assigned to the most similar group (i.e. highest correlation). However, samples with too low correlation with any f the 5 groups were not assigned and left as unclassified. The methods for doing this were the one used in the original publication of Sorlie et al.; the only difference being that non-parametric correlation was used instead of Pearson correlation when classifying the new samples.

In each patient, a very high agreement was observed between the correlation to the 5 centroids calculated using the Illumina and using the Affymetrix gene expression data (Figure 14, right panel). This was reflected in a high agreement in the 5 groups classification. For 95% of the patients the same class was predicted when using Illumina or Affymetrix, and only 5% of the cases the assignment was discordant. Amongst the 95% correct prediction, 11% were tumours assigned to the “unclassified” group both by Illumina and Affymetrix; this percentage of unclassified tumours is in agreement with the original publication by Sorlie et al.

[11] Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A. 2003;100(14):8418-23.

Figure 14: Reproducibility of breast cancer classification by Affymetrix and Illumina arrays.



The expression of estrogen receptor gene (ESR1) and HER2 gene were also compared, as a 3 groups breast cancer classification based on ESR1 and HER2 expression has been suggested[12]. The correlation (non-parametric) of these two genes in Affymetrix and Illumina array was extremely high (significant after any multiple test correction) and it was 0.96 and 0.82 respectively. This indicates that the signatures built on these two genes and the subsequent 3 clusters of samples would be in high agreement. This exercise is in progress and results will be reported in future deliverables.

A previous publication[13] had shown that the amounts of *ESR1* and *ERBB2* mRNA as measured by the Affymetrix GeneChip reliably and reproducibly establish oestrogen-receptor status and ERBB2 status. Here ERBB2 status was not available, but oestrogen-receptor status was measure by ELISA (http://www.medterms.com/script/main/art.asp?articlekey=9100) in these samples. Thus the model developed in the original study for oestrogen-receptor prediction using Affymetrix data was applied to the Illumina data, using the same threshold. The classification of tumours was very accurate, and as accurate as in the original study, and the accuracy seen when using the Affymetrix-derived model on the Illumina data and on the Affymetrix data was very similar

[12] Wirapati P, Sotiriou C, Kunkel S, et al. Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. *Breast Cancer Res.* 10(4):R65 (2008)

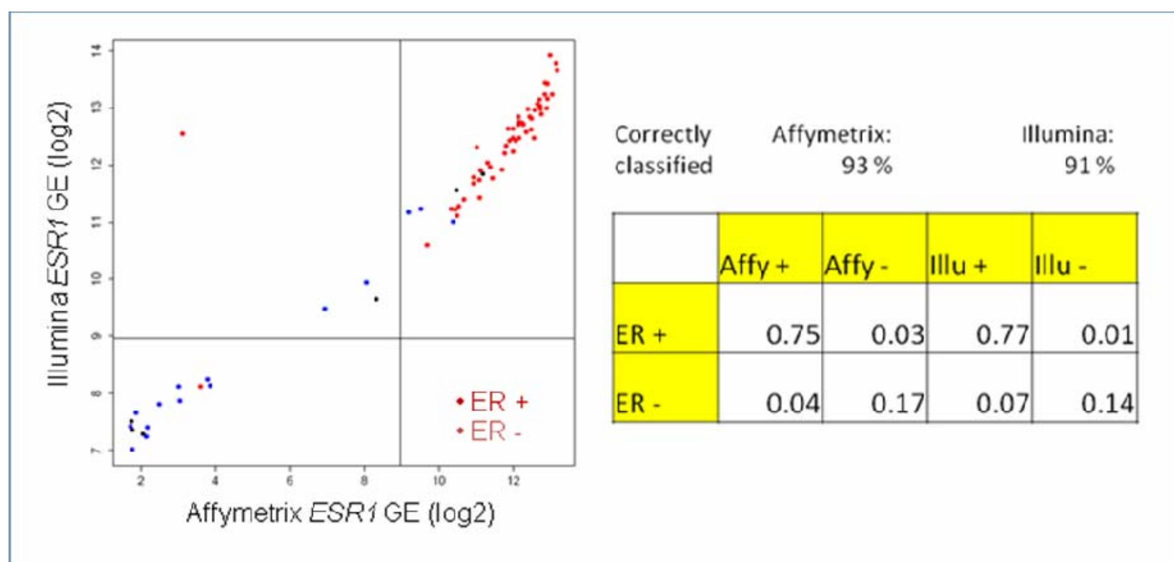
[13] Gong Y, Yan K, Lin F, et al. Determination of oestrogen-receptor status and ERBB2 status of breast carcinoma: a gene-expression profiling study. Lancet Oncol, 8:203-11 (2007)

(Figure 15). However, this result depends partially on the fact the ESR1 has a bimodal distribution, with expression tending to be very high or absent (Figure.15).

As mentioned above, breast cancer molecular subgroups have been recently defined also using the expression of ESR1 and HER2, and their respective gene expression models (i.e. the expression of the genes showing similar expression patterns to each one of them)[14], thus we plan to compare the gene expression modules for these genes and also the reproducibility of this classification when using Illumina and Affymetrix platforms.

As first outcome-related signature, a 15 genes prognostic signature of breast cancer distant metastases in node negative, ER + tumours, was considered [8], and expression of the 15 genes was compared in the two platforms using non-parametric correlation. The agreement between the two platforms was very high for all genes (only 11 could be univocally mapped to both arrays, so only 11 are reported here) but one, with highly significant p values (p <0.0001 in all but one case). When the mean or median expression of these genes was considered the agreement between platforms was even higher, with 0.96 and 0.86 correlation respectively.

Figure 15: Expression of ESR1 in Illumina and Affymetrix microarrays, and reliability of classification into ER positive and negative samples as predicted by ELISA measurements



	Affy +	Affy -	Illu +	Illu -
ER +	0.75	0.03	0.77	0.01
ER -	0.04	0.17	0.07	0.14

[14] Wirapati P, Sotiriou C, Kunkel S, et al. Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. *Breast Cancer Res.* 10(4):R65 (2008)

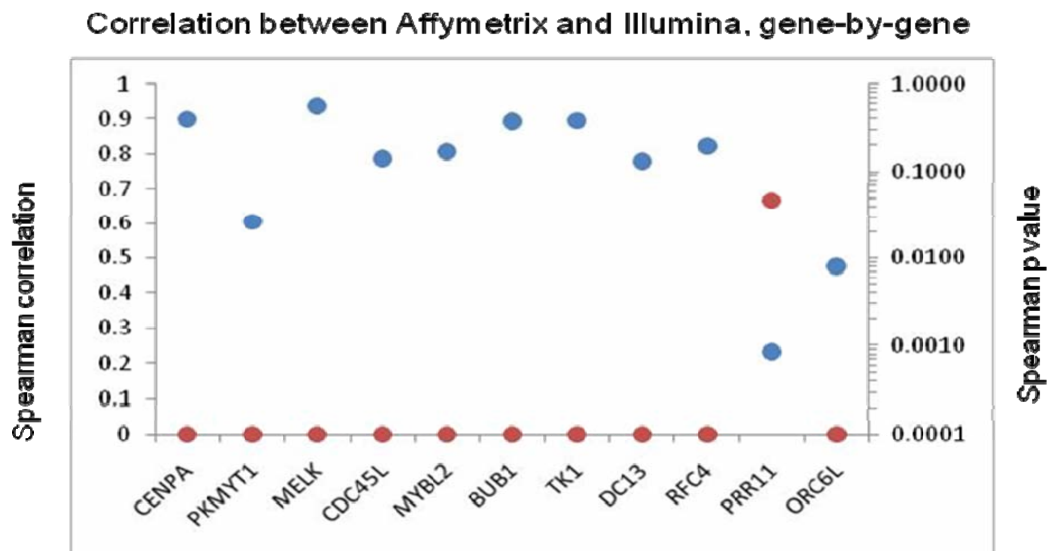
Figure 16: Reproducibility of a prognostic signature of distant metastasis (top panel) in N0 ER+ breast cancer (bottom panel)

Prognostic Signature for Distant Metastasis

Primarily Proliferation Genes

Gene	Paik	van't Veer	Dai	Gene Description
CENPA		x	x	centromere protein A, 17kDa
PKMYT1			x	membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase
MELK		x		maternal embryonic leucine zipper kinase
CDC45L			x	CDC45 cell division cycle 45-like (S. cerevisiae)
MYBL2	x			v-myb myeloblastosis viral oncogene homolog (avian)-like 2
BUB1			x	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)
RACGAP1			x	Rac GTPase activating protein 1
TK1			x	thymidine kinase 1, soluble
UBE2S			x	ubiquitin-conjugating enzyme E2S
DC13		x		DC13 protein
RFC4		x		replication factor C (activator 1) 4, 37kDa
PRR11			x	proline rich 11
DIAPH3		x	x	diaphanous homolog 3 (Drosophila)
ORC6L		x	x	origin recognition complex, subunit 6 homolog-like (yeast)
CCNB1	x			cyclin B1

Lau at al, JCO, 2006 (ASCO meeting)



7. Conclusions & Perspectives

To conclude, in this document we clearly defined and described the implementation status of the two pilot clinical trials considered in the context of ACGT: the SIOP 2001/GPOH trial for nephroblastoma patients and the TOP trial for breast cancer patients. We also give a brief update on the *in silico* oncology study which is based on the ACGT pilot clinical trials. Additionally, we explained into more details the Multi-Centre Multi-Platform study and reported the preliminary results. All these activities are still ongoing. Additionally, potential new trials or scenarios are being evaluated and are described into more details in deliverable 2.4.

.