Gene expression profiling of sequential metastatic biopsies for biomarker discovery in breast cancer

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ABSTRACT

The feasibility of longitudinal metastatic biopsies for gene expression profiling in breast cancer is unexplored. Dynamic changes in gene expression can potentially predict efficacy of targeted cancer drugs.

Patients enrolled in a phase III trial of metastatic breast cancer with docetaxel monotherapy versus combination of docetaxel + sunitinib were offered to participate in a translational substudy comprising longitudinal fine needle aspiration biopsies and Positron Emission Tomography imaging before (T1) and two weeks after start of treatment (T2). Aspirated tumor material was used for microarray analysis, and treatment-induced changes (T2 versus T1) in gene expression and standardized uptake values (SUV) were investigated and correlated to clinical outcome measures.

Gene expression profiling yielded high-quality data at both time points in 14/18 patients. Unsupervised clustering revealed specific patterns of changes caused by monotherapy vs. combination therapy ($p = 0.021$, Fisher's exact test). A therapy-induced reduction of known proliferation and hypoxia metagene scores was prominent in the combination arm. Changes in a previously reported hypoxia metagene score were strongly correlated to the objective responses seen by conventional radiology assessments after 6 weeks in the combination arm, Spearman's $\rho = 1$ ($p = 0.017$) but not in monotherapy, $\rho = -0.029$ ($p = 1$). Similarly, the Predictor Analysis of Microarrays 50 (PAM50) proliferation metagene correlated to tumor changes merely in the combination arm at 6 and 12 weeks ($\rho = 0.900$, $p = 0.083$ and $\rho = 1$, $p = 0.017$ respectively). Reductions in mean SUV were a reliable early predictor of objective response in monotherapy, $\rho = 0.833$ ($p = 0.008$), but not in the combination arm $\rho = -0.029$ ($p = 1$).

Gene expression profiling of longitudinal metastatic aspiration biopsies was feasible, demonstrated biological validity and provided predictive information.

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1. Introduction

In early breast cancer, gene expression has emerged as a method for classifying into biologically and clinically relevant subtypes, defining prognosis and selecting patients for treatment (Prat et al., 2012). In metastatic breast cancer (MBC) the data on gene expression and its impact on treatment effect or survival are scarce. Classical prognostic and predictive biomarkers that are determined on primary tumor are used. Accumulating evidence suggests that this strategy is suboptimal as the metastatic disease is often different from the primary, owing to clonal evolution, tumor heterogeneity and effect of adjuvant treatments (Foukakis et al., 2012).

This limitation has been recognized, and both retrospective (Lindstrom et al., 2012) and prospective (Amir et al., 2012; Thompson et al., 2010) studies have demonstrated that determining diagnosis and estrogen receptor and human epidermal growth factor 2 (HER2) on metastatic biopsies have an impact on treatment selection and in some cases on survival (Botteri et al., 2012). Several efforts have focused on identifying surrogate biomarkers in peripheral blood, not limited to proteins by classical biochemical assays, but also circulating tumor cells (Bidard et al., 2013) and more recently circulating tumor DNA (Dawson et al., 2013). All these are however in early stage of development and their clinical validity and utility remain to be proven. Similarly, evolving imaging methods using ^{18}F-2-fluoro-2-deoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) appear promising for therapy monitoring and prediction but are to date not utilized clinically (Cachin et al., 2006; De Giorgi et al., 2009; Dose Schwarz et al., 2005).

Sunitinib is a multi-targeted tyrosine kinase inhibitor with antiangiogenic and tumor inhibitory effects both in vivo and in vitro (Abrams et al., 2003; O'Farrell et al., 2003). Targets of sunitinib include vascular endothelial growth factor receptors, platelet-derived growth factor receptors and stem cell factor receptor. In MBC, single agent sunitinib demonstrated antitumoral effect with overall response rate of 11% in a phase II trial (Burstein et al., 2008). In a subsequent phase III trial the addition of sunitinib to standard first line treatment with docetaxel was assessed (Bergh et al., 2012). The objective response (OR) rate was higher with the combination than with monotherapy (55% vs. 42%). However, this did not translate into improvements in median progression-free survival (PFS, 8.6 vs. 8.3 months) or overall survival (24.8 vs. 25.5 months). The patients included in the trial at Karolinska Hospital were also offered to participate in a translational substudy utilizing sequential metastatic fine needle aspiration biopsies (FNAB) and FDG-PET/CT. Here, we report the results of the substudy, as a proof of concept for this strategy aiming at understanding drug action and identifying potential biomarkers.

2. Materials and methods

2.1. The docetaxel ± sunitinib phase III clinical trial

The randomized phase III trial of docetaxel ± sunitinib in MBC has been reported in detail elsewhere (see Supplementary Methods for the full study protocol) (Bergh et al., 2012). Briefly, it was a prospective, multicenter, randomized, open-label, phase III study, comparing docetaxel (75 mg/m^2 intravenously every 3 weeks (q3w)) combined with sunitinib (37.5 mg orally once daily from days 2—15 q3w) versus docetaxel alone (100 mg/m^2 q3w), as first line therapy in patients with HER2 negative MBC. Patients were stratified based on the number of metastatic sites, estrogen receptor status and disease-free interval after prior adjuvant treatment. Measurable disease by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0 or bone-only disease was allowed. The primary endpoint was PFS, defined as the time from the date of randomization to the first evidence of progression or death from any cause. Conventional radiological tumor assessments were performed at baseline and at 6 weeks intervals (based on RECIST) and were evaluated by the investigators and retrospectively by an independent central review laboratory blinded to treatment assignment (RadPharm, Princeton, NJ). OR were confirmed at least 6 weeks after having been initially documented.

2.2. The exploratory substudy at Karolinska

All patients included in the phase III trial at Karolinska University Hospital were offered to participate in this exploratory substudy (see Supplementary Methods for the main study protocol including the substudy amendment). The main objective of the study was to determine whether gene expression profiling is feasible using sequential, intra-patient FNAB. Secondary objectives were to identify potential biomarkers of early response by gene expression and/or FDG-PET/CT and to explore drug action in vivo by changes in gene expression.

Patients were subjected to baseline FDG-PET/CT assessment, followed by Ultrasound/CT-guided FNAB of one tumor lesion prior to start of treatment (time point T1). The aspirated material allowed the morphological confirmation of the lesion and RNA isolation. FDG-PET/CT and FNAB of the same lesion were repeated at Day 14 ± 1 (time point T2). Patients were treated and evaluated according to the main study protocol. The randomization algorithm of the phase III trial was not stratified by site and was not affected by the participation in the substudy.

The study was approved by the ethics committee of the Karolinska Institutet. A separate written informed consent for the substudy was obtained from all patients.

2.3. Functional imaging with 18F-FDG-PET/CT

The methodology, technical details and equipment used for the 18F-FDG-PET/CT are detailed in the Supplementary Methods. Tumor assessments were performed at T1 and T2, using the same radiopharmaceutical and activity at both times. The radioactive uptake in the selected regions of interest and of the same tumor areas was calculated and evaluated by the investigators who were blinded to study arm. Three-dimensional isocontour-ROI's were used to assess standardized uptake values (SUV) of the lesion targeted for FNAB. PET response was stratified by the metabolic response criteria

using a threshold of >25% reduction in maximum SUV (SUV-max) and mean SUV (SUVmean) at T2 compared to baseline.

2.4. Metastatic lesion biopsies and gene expression profiling

Ultrasound/CT-guided FNAB from a selected metastasis was obtained at T1 and T2 allowing for morphological confirmation of the lesion and gene expression profiling at both times. To perform gene expression analysis, RNA extraction was performed using the RNeasy mini kit (Qiagen, Hilden, Germany) according to a standard protocol. In brief, biopsies preserved in RNAlater were centrifuged and the pellets were transferred into RLT buffer (RNeasy Lysis Buffer, Qiagen, Hilden, Germany). Proteinase K was added and the samples were treated for 10 min at 55 °C (Egyházi et al., 2004). Total RNA was then isolated using Qiagen's microspin technology. DNase was also added to the samples. The quality of the RNA was assessed by measuring the 28S:18S ribosomal RNA ratio using an Agilent 2100 bioanalyzer (Agilent Technologies, Rockville, MD, USA). All samples with RNA of high quality were stored at −80 °C until used in microarray experiments. Due to the low amounts of RNA retrieved from FNAB samples, a two-cycle cDNA synthesis protocol was used to generate labeled cRNA for hybridization. Samples were hybridized in the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, CA, USA) containing 54,675 probesets. A summary of the handling of the FNAB according to the Biospecimen Reporting for Improved Study Quality (BRISQ) (Moore et al., 2011) recommendations is provided in the Supplementary Table S1.

2.5. Gene expression data analysis

All gene expression data analysis was done in R/Bioconductor. Probe intensities were extracted from Affymetrix CEL files and background corrected, normalized and summarized to probeset expression using the rma function from the affy package with the default settings (Gautier et al., 2004). The quality of gene expression data was assessed with the arrayQualityMetrics package (Kauffmann et al., 2009). Control of RNA integrity and correction of RNA quality effects was performed using the AffyRNADegradation package (Fasold and Binder, 2013). Probesets were mapped to Entrez Gene IDs using the hgu133-plus2.db package and expression data was collapsed to gene level using a non-specific filter keeping only the probesets with highest interquartile range in the case of multiple mappings to the same Entrez Gene ID. An exception of this was the hypoxia metagene, which is based on the same platform. Intra-patient change from T1 to T2 for each gene was also derived. Expression levels are log2 transformed intensity values, hence a difference in expression by one unit corresponds to a two-fold mean change in probe intensities.

For unsupervised clustering, a further non-specific filtering was applied to keep only the top 1000 genes with highest interquartile range of expression across samples − or intrapatient change from T1 to T2 when these quantities were explored. Cluster heatmaps were drawn where rows and columns of the data matrix were arranged based on unsupervised clustering using the Euclidean distance metric and average-linkage hierarchical clustering algorithm. In each cluster heatmap, the leaves of the dendrograms were reordered according to the column and row means of the matrix, respectively, maintaining constrains of the dendrograms from the hierarchical clustering. Clustering patterns were further visualized using multidimensional scaling plots in which a Euclidian-based distance metric was used. Finally, an unsupervised 2-means clustering of the change from baseline values was performed.

Two previously published metagenes were studied: The 11 proliferation genes in the Predictor Analysis of Microarrays 50 (PAM50) gene set - the PAM50-proliferation index (Nielsen et al., 2010) (proliferation metagene) and the 51 HG-U133 Plus 2 probesets in the common hypoxia metagene by Buffa et al. (Buffa et al., 2010). For each sample and metagene, a metagene score was calculated as the average expression of the genes/probesets in the metagene.

Group-level treatment induced changes in gene expression from T1 to T2 in each arm were quantified with moderated paired t-test statistics using the limma package. Gene set enrichment analyses (GSEA) of the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene set collections in the Molecular Signatures Database (Broad Institute, version 4.0) was performed using the GSEA Software (Broad Institute, version 2.0.13) (Subramanian et al., 2005) with genes pre-ranked according to the moderated paired t-test statistics.

Array data were deposited at the Gene Expression Omnibus Database under accession number GSE54323.

2.6. Statistical analysis

All statistical analysis was done in R version 3.0.2. Spearman's rank correlation coefficient and Fisher's exact test were used to assess association between two continuous variables and two categorical variables, respectively. The median PFS was calculated using the R package survival with the default settings. All statistical tests were two-sided.

3. Results

3.1. Patient characteristics and clinical outcome

Twenty-one patients were included in the docetaxel ± sunitinib trial at Karolinska and 18 of them agreed to participate in the substudy. Of the 18 women enrolled, 8 were randomly assigned to sunitinib plus docetaxel and 10 to docetaxel alone (Figure 1). Demographic and baseline disease characteristics were well balanced between the treatment arms (Table 1).

Efficacy results based on independent central review were available for 17 patients (one patient in the monotherapy arm was lost to follow up). An OR was seen in 11 patients (all confirmed partial responses), 5 with partial response and 3 with stable disease in combination arm (OR rate = 63%) and 6 with partial response, 2 with stable disease and 1 with progressive disease in the monotherapy arm (OR rate = 67%). Median PFS was 7 months (95% Confidence interval = 5.36 − not reached) in the combination arm and 8.2 months (95% Confidence interval 3.94 − not reached) in the docetaxel arm.
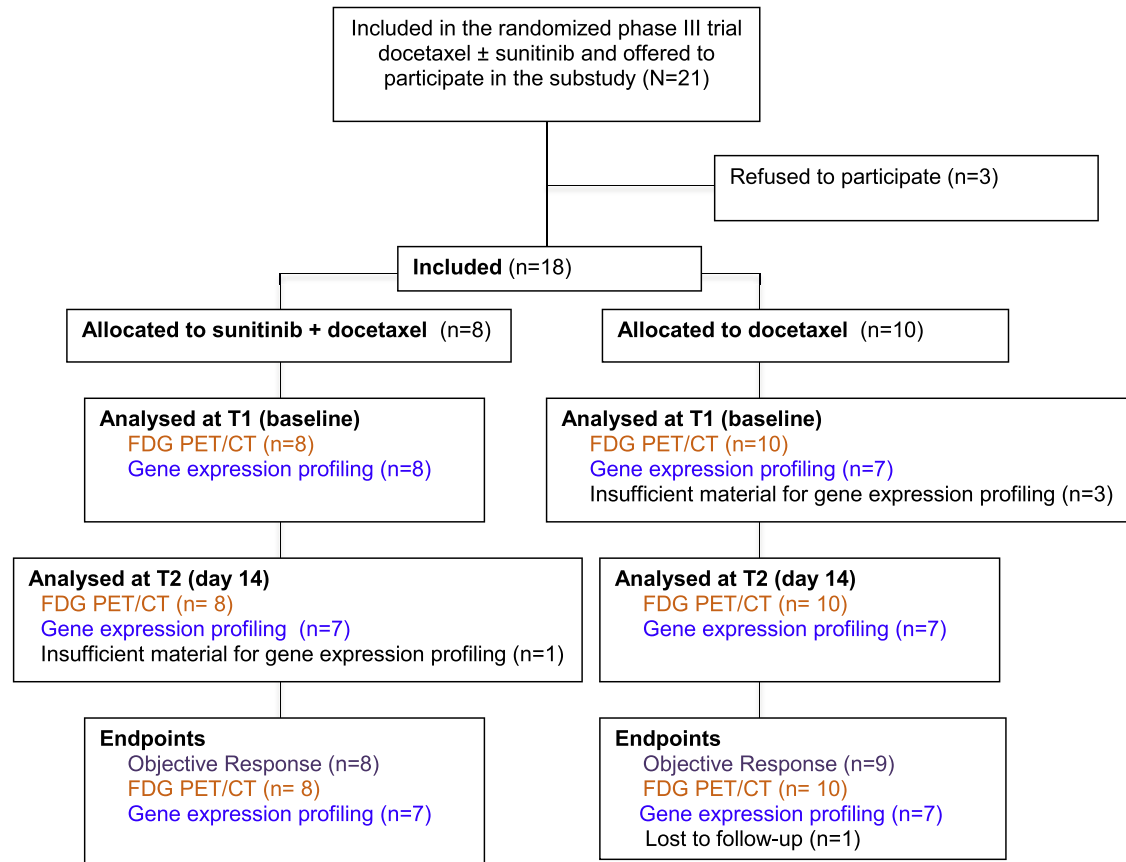
Figure 1 – Consort diagram of the Karolinska substudy in the docetaxel ± sunitinib phase III trial of metastatic breast cancer. FDG PET/CT = ^{18}F-2-fluoro-2-deoxyglucose positron emission tomography/computed tomography.

Table 1 – Baseline characteristics of the 18 patients included in the study.

Variable	Sunitinib + docetaxel	Docetaxel
No. of patients	8	10
Age		
Median	59	52
Range	33–66	28–62
Extend of disease		
Metastatic	7	10
Locally recurrent[a]	1	0
Location of metastasis		
Visceral	5	9
Bone only	2	1
Histology		
Ductal	8	10
Other	0	0
Estrogen receptor status		
Positive	6	8
Negative	2	2
Recurrence-free interval		
≤12 months	0	2
>12 months	8	8

a Non-operable.

3.2. Treatment-induced changes in 18F-FDG PET/CT

All 18 patients had a positive FDG-PET/CT uptake in the regions of interest at baseline before starting treatment, measured by SUV. Changes in SUV_{max} and SUV_{mean} between T1 and T2 were investigated in the two treatment arms (Supplementary Table S2). The result of serial-assessment in regions of interest shows that the reduction of median SUV_{max} was greater than 25% in both groups, and median SUV_{mean} was reduced by 24% in the combination arm and 30% in the monotherapy arm.

3.3. Feasibility of FNAB and gene expression profiling

Metastatic FNAB were carried out in 17 of the 18 patients at both time points with no complications reported. The biopsy sites are seen in Figure 2. Representative tumor material, sufficient for RNA extraction both in T1 and T2 was obtained in 14 cases, 7 in each arm (Figure 1). The quality of RNA was approved for microarray experiments in all samples. A quality control of the expression data indicated low levels of RNA degradation with no confounding pattern among the samples (Supplementary Figure S1). Correction of RNA quality effects using the AffyRNADegradation package did not affect further analyses (data not shown) and all results presented are generated using the original, uncorrected data. Furthermore, gene

expression profiles were consistent when a different RNA amplification kit or RNA from another lesion was used (study subject 10, Supplementary Figure S2).

3.4. Unsupervised clustering of gene expression profiles

Unsupervised clustering of the 29 gene expression profiles (15 at T1 and 14 at T2) demonstrated a substantial inter-patient variation and a strong tendency of paired samples from the same patient to cluster together − 5 of 7 in monotherapy and 2 of 7 in the combination arm (Figure 2A and 2B). Furthermore, a large distinct cluster of down-regulated genes (upper right part of Figure 2A) was formed, overrepresented by genes related to immune response (gene to biological process ontology conditional hyper-geometric test for overrepresentation, data not shown). Interestingly, also samples from the same metastatic site (liver, bone or lymph node) formed distinct clusters (Figure 2A). However, when intra-patient change of gene expression from T1 to T2 was analyzed, patterns specific to therapy emerged, with more pronounced changes seen in the combination arm (Figure 2C and 2D).

There was a significant agreement between treatment arm and the unsupervised 2-means clustering based on change from T1 to T2 (p = 0.021, by Fisher's exact test). An explorative GSEA indicated that treatment led to up-regulation of immune response related genes and down-regulation of cell cycle related genes in both arms (Supplementary Table S3).

3.5. Treatment-induced changes in hypoxia and proliferation

The effect of treatment in gene expression was further explored using the previously published hypoxia and PAM50-proliferation metagenes. A decrease in both metagene scores from T1 to T2 was seen in the majority of cases (Figure 3). There was a high correlation between the hypoxia and proliferation metagene scores (Spearman's $\rho = 0.929$, $p = 0.007$ in the combination arm and $\rho = 0.821$, $p = 0.034$ in the monotherapy arm), despite that there is no overlap in the genes that comprise these signatures. The decrease in both hypoxia and proliferation scores was more pronounced in the combination arm (Figure 3).
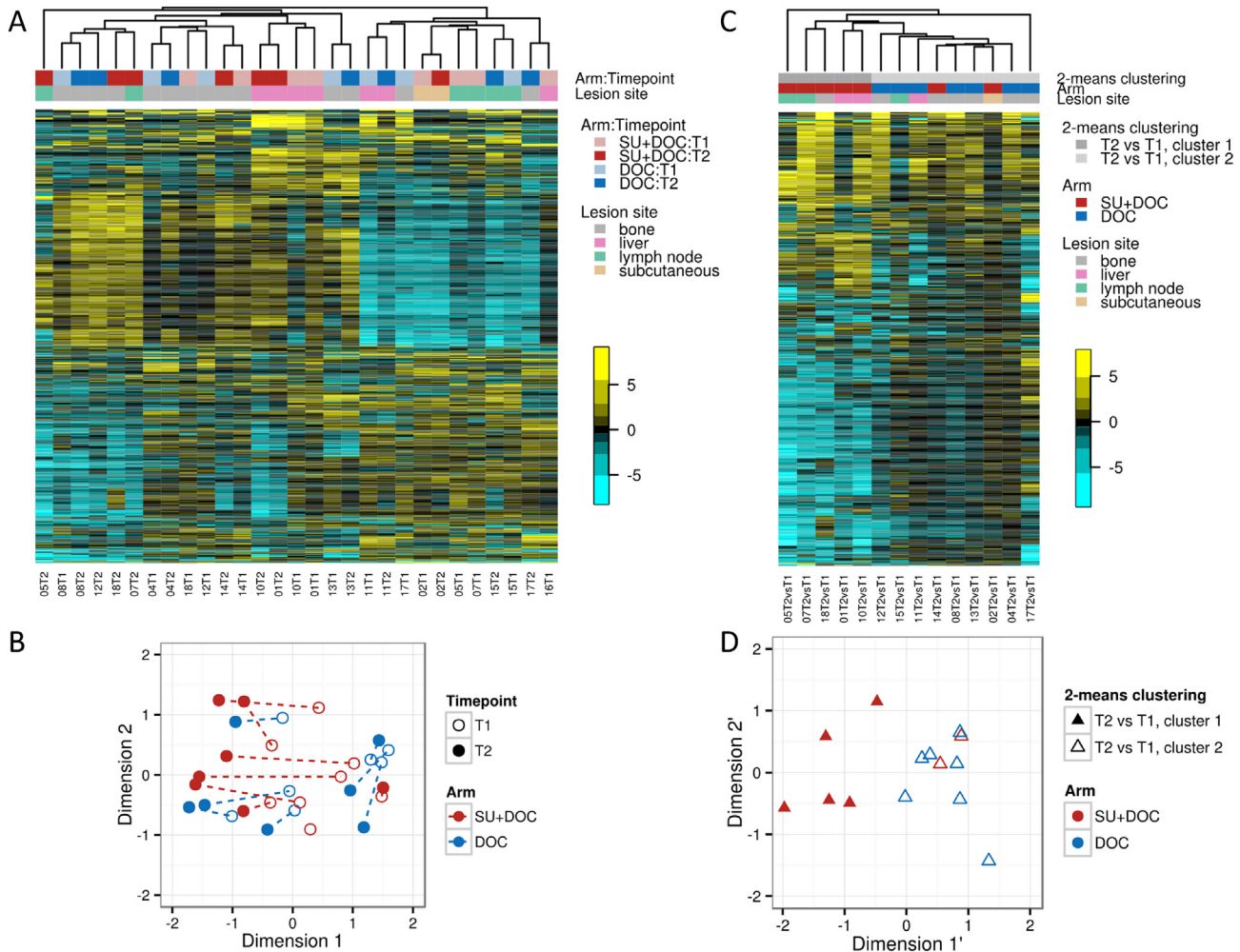

Figure 2 − Unsupervised clustering of gene expression profiles. Cluster heatmap of gene expression (median centered by rows) for top 1000 most varying genes in all 29 samples (A), and corresponding multidimensional scaling plot where distances on the plot can be interpreted in terms of leading log2 fold change (B). The paired samples from the same patient are indicated by a dotted line. Cluster heatmap of change from baseline gene expression to day 14 for top 1000 most varying genes in all 14 paired samples (C), and corresponding multidimensional scaling plot (D). T1 = baseline; T2 = day 14; DOC = docetaxel, SU = sunitinib.

3.6. FDG-PET/CT and gene expression changes as early response markers

Variations in SUV and in hypoxia and proliferation metagenes from T1 to T2 were plotted against the changes of target lesions in CT scan at 6 weeks versus baseline, as assessed by RECIST (Figure 4). A significant correlation was observed between SUV_{mean} and tumor size changes in the monotherapy arm, Spearman's $\rho = 0.833$, $p = 0.008$, but not in the combination arm $\rho = -0.029$, $p = 1$ (Figure 4A). Reductions in the hypoxia metagene score were highly correlated to OR in the combination arm, $\rho = 1$, $p = 0.017$, but not in the monotherapy arm, $\rho = -0.029$, $p = 1$ (Figure 4B). These findings remained essentially unchanged when OR was evaluated at 12 weeks (Supplementary Figure S3). Similar to hypoxia, the PAM50 proliferation metagene was well correlated to tumor changes merely in the combination arm at 6 and 12 weeks ($\rho = 0.900$, $p = 0.083$ and $\rho = 1$, $p = 0.017$ respectively, Supplementary Figure S3). Due to the small number of patients and the censoring of patients that stopped therapy without progressive disease, correlation between gene expression or SUV changes and PFS or overall survival could not be assessed.

4. Discussion

Despite the introduction of new drugs, MBC has a dismal prognosis (Foukakis et al., 2011). It is widely accepted that new therapeutic strategies and methods of drug development are needed in order to achieve a clinically meaningful improvement of survival in MBC, without compromising quality of life and with a sustainable cost of care (Jonsson and Bergh, 2012). Especially with novel targeted drugs, it is of utmost importance to personalize treatment and select patients in whom the target in question is active (Bergh, 2009).

To this direction, designing clinical trials with mandatory tissue biopsies is a promising approach. Previous data show high acceptance from patients, feasibility and safety of the procedure (Gomez-Roca et al., 2012; Seah et al., 2013). Little is known however about the scientific and clinical usefulness of such methods, especially for high-throughput molecular analysis such as gene expression profiling. To our knowledge, this is the first report of a clinical trial in MBC utilizing sequential, metastatic FNAB for gene expression analysis in combination with functional imaging. We demonstrate that this trial design is feasible and despite the small number of patients in this pilot study, can give high yields of information related to drug action and potential predictive biomarkers.

The use of sequential biopsies did not compromise the acceptance rate (85%), or safety as no complications were reported in the trial. In 82% of the cases representative and adequate material for gene expression profiling was obtained at both time points. The generated data were approved in comprehensive quality controls and dynamic changes in the relevant processes of cell proliferation and response to hypoxia highlight the biological validity of the gene expression data.

The intra-individual longitudinal design facilitated the discovery of potential biomarkers and very robust correlations of both SUV and gene expression signatures with treatment effect were observed. Changes in hypoxia and proliferation metagene scores after 2 weeks of treatment were a strong predictor of the response documented at 6 and 12 weeks, exclusively in patients treated with sunitinib. This finding clearly demonstrates that in vivo observed changes in relevant biological pathways could be used as early response markers. Preliminary data show that in addition to efficacy, also safety outcomes may be predicted using this approach (Foukakis et al., 2014).

Decrease of SUV_{mean} seen in FDG-PET/CT after the first treatment cycle was highly indicative of response in patients treated with docetaxel, similar to findings in neoadjuvant treatment of breast cancer (Gebhart et al., 2013). Although
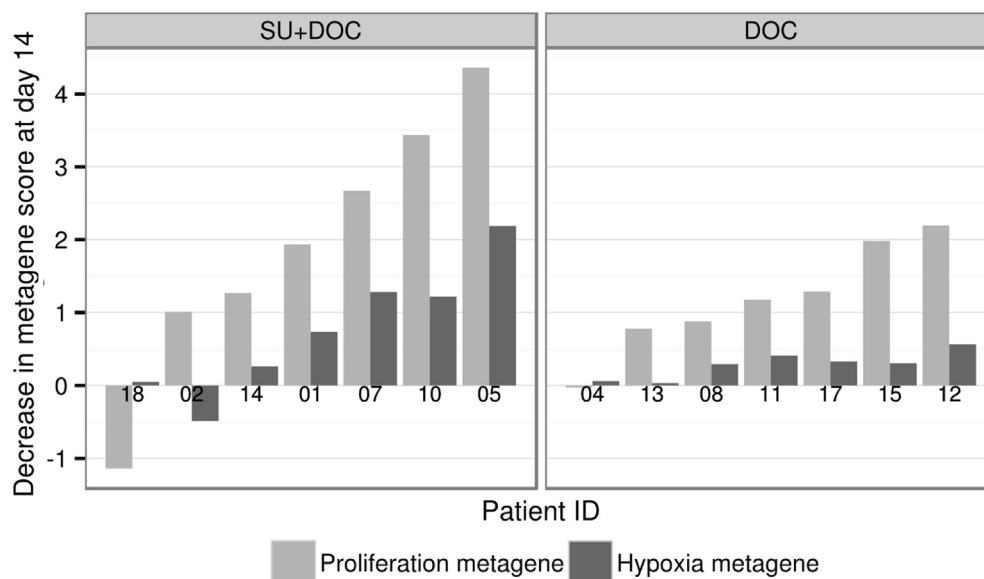

Figure 3 − Treatment-induced changes in the PAM50-proliferation metagene and hypoxia metagene scores in the combination (SU + DOC) and monotherapy (DOC) arms. A decrease in metagene scores by one unit corresponds to a 2-fold mean decrease in probe intensities from baseline to day 14. Patients are ordered on the x-axis according to their decrease in PAM50-proliferation metagene score.
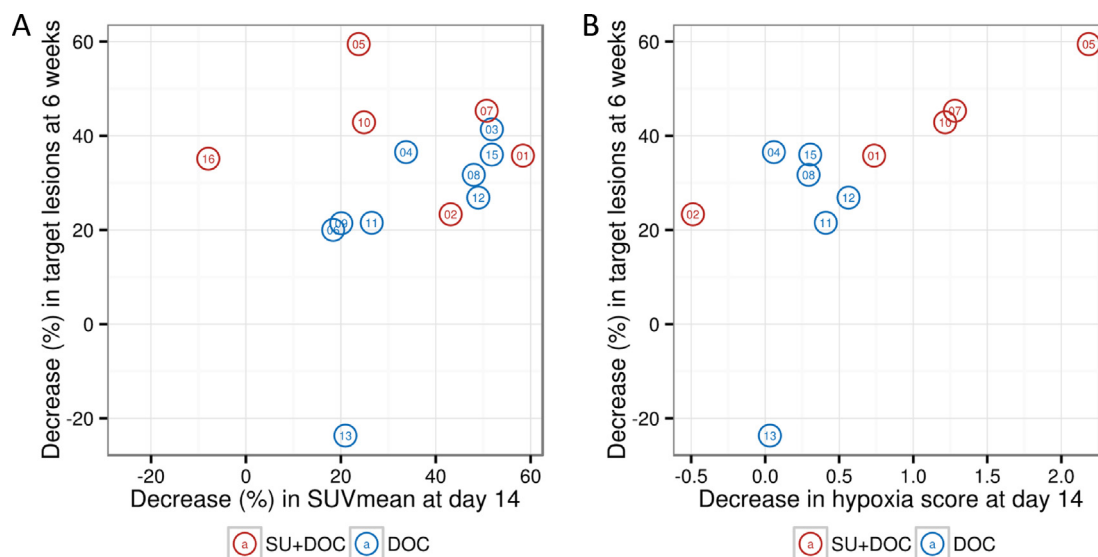
Figure 4 – Treatment-induced changes in SUV_{mean} (A) and hypoxia metagene score (B) in relation to the objective response (OR) seen after 6 weeks of treatment by Computed Tomography scan (independent review by RECIST v 1.0) in the combination (SU-DOC, red circles) and monotherapy (DOC, blue circles) arms. Patient ID is seen in the center of each circle; only patients with measurable disease are included in this analysis. A decrease in hypoxia metagene scores by one unit corresponds to a 2-fold mean decrease in probe intensities from baseline to day 14. A high correlation is seen between SUV_{mean} and OR in the DOC arm (Spearman's $\rho = 0.833$, $P = 0.008$) and between hypoxia metagene score and OR in the SU + DOC arm (Spearman's $\rho = 1$, $P = 0.017$).

PET did not add to the predictive information in the combination arm, its role in the proposed trial design should be further investigated, probably using tracers more specific to the drug in question. Whole-body functional imaging could provide predictive information per se or complement biopsies by addressing the potential caveats of tumor heterogeneity and differential responses of metastatic lesions to therapy.

In summary, this pilot study is a proof of concept for a novel design of clinical trials, incorporating longitudinal metastatic biopsies and functional imaging. In contrast to classical trials investigating mean therapy effects in large groups of patients, the intra-patient comparisons applied here give insights into drug action mechanisms and predictive biomarkers in individual patients. Application of this strategy in oncology drug development may lead to enrichment of trials with patients gaining more from treatment and drug efficacy being rapidly evaluated.

Disclosure of potential conflicts of interest

Jonas Bergh has received a research grant by Pfizer paid to Karolinska for conducting the study. The other authors disclosed no potential conflicts of interest.

Appendix A.
Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.03.011.

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