Title

Blood and Tissue Biomarkers in Advanced Non-Small Cell Lung Cancer (NSCLC) Patients Treated with Erlotinib (Tarceva?) Alone or Erlotinib and Fulvestrant (Faslodex?): A Phase II Clinical Trial Analysis

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A thesis submitted in partial satisfaction of the requirements for the

Master of Science in Clinical Research

by

Patricia Young

2015

ABSTRACT OF THE THESIS

Blood and Tissue Biomarkers in Advanced Non-Small Cell Lung Cancer (NSCLC) Patients Treated with Erlotinib (Tarceva®) Alone or Erlotinib and Fulvestrant (Faslodex®): A Phase II Clinical Trial Analysis

by

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Master of Science in Clinical Research

University of California, Los Angeles, 2015

Professor Elliot M. Landaw, Chair

Purpose:

Preclinical models suggest bidirectional signaling between estrogen receptor (ER) and epidermal growth factor receptor (EGFR) pathways in lung cancer. This phase II randomized clinical trial of erlotinib, an EGFR inhibitor, and fulvestrant, an ER antagonist, versus erlotinib alone, evaluated efficacy in advanced stage non-small cell lung cancer (NSCLC) patients. The purpose of this investigation is to evaluate the influence of various clinical, biochemical, and histopathological features as predictive or prognostic markers.

Methods:

Patients who met eligibility criteria for the study were enrolled in a 2:1 ratio to combination erlotinib and fulvestrant or erlotinib alone. Primary endpoint was objective tumor response rate, while secondary analysis included overall survival (OS) and progression free survival (PFS). Analysis included blood estrogen (estradiol and estrone) run in duplicate, and a panel of relevant biomarkers obtained at the beginning of the first three 28-day cycles. Pre-treatment tumor tissue was obtained and evaluated

using immunohistochemistry (IHC) for estrogen receptor (ER)-α, ER-β, and progesterone receptor.

Results:

106 patients were randomized to a treatment arm between March 2006 and June 2011 with 100 patients receiving at least one dose of therapy. Clinical response data were available for 82 patients. Median PFS was 4.3 and 1.9 months for the erlotinib plus fulvestrant arm versus the erlotinib alone arm (HR 0.64, 95% CI 0.34-1.08). Median OS was 11.6 and 8.6 months for the erlotinib plus fulvestrant versus the erlotinib alone arm (HR 0.9876 95% CI 0.60-1.6).

Blood analysis showed that after 1 cycle of treatment, placental growth factor (PlGF) and transforming growth factor-α (TGF-α) were higher in patients that had progressive disease (PD) compared to partial response (PR) plus stable disease (SD), p=0.02 and p=0.02, respectively. In the tissue, ER-α or progesterone receptor positivity did not demonstrate a significant difference in response for patients treated with the combination erlotinib and fulvestrant, Chi-squared test, p=0.96. Moreover, cytoplasmic ER-β H score was higher in patients with PD versus PR + SD (median 90 versus 20 respectively, p=0.01). The dataset did not demonstrate statistical significance by treatment arm (median 60 versus 65 in the erlotinib versus erlotinib plus fulvestrant, p=0.59).

Conclusion:

Combination erlotinib and fulvestrant in this study did not show superiority over erlotinib alone with respect to OS and PFS. Blood estrogen and estrone levels did not correlate with response or treatment arm. Biomarkers that may be prognostic include cytoplasmic ER-β H score, while higher Cycle 2 Day 1 PlGF and TGF-α levels may suggest a poor response.

The thesis of Patricia Young is approved.

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Introduction:

Although novel immunotherapeutic agents are in development for non-small cell lung cancer (NSCLC), this disease is currently the second most common malignancy in the United States and the leading cause of cancer death. In 2015, it is estimated there will be more than 220,000 new cases of lung cancer in the United States, with more than 158,000 deaths [1]. Gender differences in the risk of developing lung cancer remain controversial as epidemiologic studies have conflicting results, suggesting more susceptibility to tobacco carcinogens yet improved survival outcomes for women [2-4]. Multiple mechanisms have been suggested to explain the potential gender susceptibility in lung cancer, including polymorphisms that lead to the carcinogen metabolism pathway in women [5] and hormonal mechanisms with estrogen signaling leading to lung cancer progression [6].

The role of tumor estrogen receptors (ER) and serum estrogen has been implicated in the pathogenesis of lung cancer as evidenced by estrogens stimulating growth in both normal and lung tumor cells [7-9]. The two isoforms of ER, ER-α and ER-β, differ in their tissue distribution, and have been detected in lung cancer and normal tissue [7, 8, 10, 11]. More importantly, the serum estrogen and ER positivity in lung cancer tumors have had implications in NSCLC survival [12]. Fulvestrant, an ER antagonist, competitively binds to ER on tumor and other tissue targets, leading to down-regulation of ER and tumor growth inhibition. Currently, fulvestrant is approved to treat hormone receptor positive metastatic breast cancer, but the role of anti-estrogens in lung cancer may also prove beneficial.

Furthermore, epidermal growth factor receptor (EGFR) has been implicated in lung cancer pathogenesis [13] with approximately 15% harboring an EGFR mutation. 90% of EGFR mutations are accounted for by deletions within exon 19 and a mutation in exon 21 [14]. In Asian regions, the incidence of EGFR mutations ranges from 22-62%,

more common in nonsmokers and females [15]. In previously treated NSCLC patients, erlotinib therapy demonstrated an OS benefit compared to best supportive care (6.7 versus 4.7 months, HR=0.7; p<0.001) [16]. EGFR is clearly an important therapeutic target, and preclinical models have suggested interactive cross talk signaling between ER and the EGFR pathways [17].

The cell membrane steroid receptors and the intracellular signaling pathway may play an important role in the mechanism of action of the hormone [18]. In breast cancer cell lines and under estrogen-supplemented conditions, the effect of the EGFR inhibitor, gefitinib, in combination with fulvestrant, had an additive antitumor effect [19]. Furthermore, *in vitro* and *in vivo* data demonstrates fulvestrant and EGFR inhibitors can inhibit cell proliferation, induce apoptosis, and have downstream signaling effects [20, 21]. A pilot study of gefitinib, and fulvestrant in 22 post-menopausal women with advanced NSCLC demonstrated both safety and disease activity [22]. These combined data provide the rationale for this randomized phase II clinical trial of erlotinib and fulvestrant compared to erlotinib alone in advanced or metastatic NSCLC. Primary analysis of this trial include objective tumor response rate (ORR). The secondary analysis includes overall survival (OS), progression free survival (PFS), and assessment of biological correlates in either blood or tissue that may predict outcome.

Methods:

Efficacy Analysis

Patients were accrued from March 2006 to June 2011 and stratified by gender and performance status (ECOG 0,1 vs 2). Inclusion criteria for the primary analysis included patients with advanced (Stage IIIb or IV) NSCLC, age $\geq$ 18 years, Eastern Cooperative Oncology Group performance status (ECOG) 0-2, measurable disease and adequate organ function. Exclusion criteria included therapy (either chemotherapy or non-cytotoxic investigational agents) within 4 weeks of initiating treatment, prior EGFR

inhibitor or anti-estrogen therapy, active CNS metastasis, or significant conditions felt by the investigator to impede participation. The rationale for including all patients rather than limiting to postmenopausal women was that although the levels of estrogen would be low, the estrogenic effects in men may still play a role.

Patients were randomly assigned (2:1) to receive erlotinib (150 mg PO daily) plus fulvestrant (500 mg IM loading dose on day 1 and 15 of cycle 1 and then day 1 of subsequent cycles) or erlotinib alone (150 mg PO daily). 2:1 randomization was performed for the biomarker analysis and because this was the only arm that would be evaluated for the primary endpoint of response rate. The study was not powered to make formal comparisons to the erlotinib alone arm and instead would be compared to historical controls. Additionally, it was felt that more patients would be inclined to participate with an intervention arm (fulvestrant) that would not otherwise have been available outside of a clinical trial. A one-sided exact test, 68 patients with an alpha = 0.10 would yield a power of 82% to detect 100% improvement in response rate with the combination arm (expected RR = 20%) compared to the historical data for erlotinib alone.

Each cycle consisted of 28 days. Response was defined using Response Evaluation Criteria in Solid Tumors (RECIST) 1.0 criteria [23]. The clinical benefit rate was defined as patients achieving a partial response (PR) or stable disease (SD). These groups were analyzed together compared to patients with progressive disease (PD). Patients continued on treatment until there was radiographic evidence of PD, unacceptable toxicity, consent withdrawal, or death.

Blood Biomarker Analysis:

Several exploratory serum and plasma biomarkers were obtained during treatment. Samples were obtained at the start of therapy (C1D1) and during treatment on Cycle 2 Day 1 (C2D1) and Cycle 3 Day 1 (C3D1) if available. Estrogen (estradiol and

estrone) biomarkers were run in duplicate. Exploratory biomarkers obtained during C1D1 and C2D1 included PlGF (placental growth factor), sFLT-1 (soluble FMS-like tyrosine kinase-1), VEGF (vascular endothelial growth factor), AREG (amphiregulin), IL-6 (interleukin-6), TGF-α (transforming growth factor-α), HGF (hepatocyte growth factor), and testosterone. bFGF (basic fibroblast growth factor) was obtained during C2D1 only.

Tissue Biomarker Analysis:

Pre-treatment tumor samples were obtained from patients and were formalin fixed paraffin-embedded. The tissue blocks were evaluated for ER-α, and progesterone receptor. Only slides that were recently cut from blocks were considered reliable. ER-α and progesterone receptor were given a percentage score. If the value was scored as "<1%," the value used for the analysis was 0. The pathologist also categorized ER-α and progesterone receptor as positive, negative or "borderline." If the value was considered "borderline," this was classified as positive for the analysis.

In a separate analysis, ER-β was evaluated with immunohistochemistry. ER-β staining was given a percentage score of cells that stained weakly, moderately, and strongly (1+, 2+, and 3+). An H-score or "histo score" was then obtained to weight the extent of nuclear or cytosplasmic immunoreactivity. The H score is obtained as:

H-score = 3 X (% cells 3+) + 2 X (cells 2+) + 1 X (% cells 1+)

where the range is 0 to 300

EGFR Status

Available tissue samples were sequenced for mutations in the EGFR tyrosine kinase domain (exon 18-21). EGFR results in exon 19 and 21 were considered complete. EGFR mutational status was evaluated at the University of Pittsburgh with the Stabile Group using pretreatment tissue samples. If a patient had a previous EGFR analysis, repeating the test was not required.

Statistical Analysis:

Wilcoxon rank-sum test was applied as specified in figure legends to determine the level of significance using JMP Pro 11 software. Kaplan Meier survival and progression free curves were generated, and the log-rank test was used to compare survival and progression free distributions. Chi-squared test was used to test proportions between two groups. Data were considered statistically significant at $p<0.05$. Refer to the statistical appendix (Chapter 2) for further details on the statistical tests and potential alternatives.

Results:

Patient Characteristics:

106 patients were randomly assigned to receive erlotinib or erlotinib plus fulvestrant at the University of California, Los Angeles and through the Translational Oncology Research Institute (TORI) network. Six patients withdrew consent prior to initiation of therapy. Available followup data and tissue/blood biomarker data were available for 82 patients. 27 patients had received erlotinib alone and 55 patients had received erlotinib plus fulvestrant. Baseline characteristics are presented in **Table 1**.

Efficacy:

Overall PFS and OS did not demonstrate a significant difference between the two treatment arms. Median PFS was 4.3 versus 1.9 months (HR 0.64, 95% CI 0.34-1.08), while median OS was 11.6 versus 8.6 months (HR 0.9876 95% CI 0.60-1.6), for the erlotinib plus fulvestrant versus erlotinib arms, respectively.

EGFR status was available on sixty of the 82 patients. Sixteen of 60 (27%) patients were EGFR mutant while 44 of 60 (73%) patients were EGFR wildtype, similar to population estimates. Despite the small sample size from the available data, the median survival for EGFR mutant compared to EGFR wildtype was superior and

achieved statistical significance at 16.6 and 1.8 months (HR 0.2525, 95% CO 0.13-0.40), respectively.

PFS and OS were also evaluated according to gender. The median PFS for females versus males was 3.5 versus 1.9 months (HR 0.66 95% CI 0.38-1.0), respectively. The median OS for females and males was 24.1 and 10.7 months (0.67, 95% CI 0.37-1.17), respectively.

Kaplan Meier curves for PFS and OS by treatment arm, EGFR mutation status, and gender are shown in **Figure 1**. A summary table for the median PFS and OS is shown in **Table 2.**

Estrogen Blood Biomarker Analyses:

Estradiol and estrone from the blood were obtained at C1D1, C2D1, and C3D1 when available. Estradiol and estrone values less than 1 were below the level of detection by the assay method and assigned a value of 0. Each sample was run in duplicate and the correlation coefficient for estradiol and estrone at C1D1, C2D1, and C3D1 showed a close association between the two values. Therefore, the average was obtained between the duplicate measures **(Table 3)**.

There was no significant association between the average estradiol or estrone serum levels and response at any of the time points obtained (C1D1, C2D1, C3D1) **(Figure 2)**. A second analysis evaluating estradiol or estrone using the individual analyses similarly showed no association with response. The change in estradiol and estrone levels between C2D1 and C1D1 also did not show an association with response (not shown).

The average estradiol and estrone blood levels were compared by treatment arm and there was no statistical difference in the level of either blood biomarker between the treatment arms (**Figure 3)**. Although fulvestrant acts as an anti-estrogen and down-regulates ER, there may still be high levels of estradiol and estrone in the blood.

Furthermore, there may be false positives of estradiol for patients on fulvestrant as reported in the literature [24] although likely rare.

Other Exploratory Blood Biomarker Analyses:

A total of 9 blood biomarkers were obtained at the start of Cycle 1 and/or 2. These included bFGF, PlGF, sFLT-1, VEGF, AREG, IL-6, TGF-α, HGF, and testosterone. The collective results for the blood biomarkers are shown in **Table 4.** A higher C2D1 PlGF level was associated with PD patients compared to PR + SD patients by the Wilcoxon rank-sum test (p=0.02). Similarly, a higher C2D1 TGFα was associated with PD compared to patients with PR + SD by the Wilcoxon rank-sum test (p=0.02). **Figure 4** illustrates the two significant biomarkers by response.

When evaluating by treatment arm at C2D1, the erlotinib alone treated group had predictive implications for response for PlGF (median 0.118 vs 0.072 pg/mL for PD versus PR + SD respectively, p=0.01), VEGF (median 7.16 vs 2.54 pg/mL for PD versus PR + SD respectively, p=0.03), and TGF-α (median 6.125 vs 0 pg/mL for PD versus PR + SD respectively, p=0.002). In the erlotinib plus fulvestrant groups there was no statistical difference by response groups.

The calculated difference in blood biomarker between Cycle 1 and 2 was also analyzed and this did not demonstrate a statistical difference by response (**Figure 5)**.

ER-α and Progesterone Receptor Tissue Biomarker Analyses:

ER-α and progesterone receptor score was interpreted as either positive or negative. Cut point values for ER-α and progesterone receptor positivity was >1%. Cases interpreted as “borderline” were made positive for the analysis. Three of the 45 cases (7%) were considered “borderline” for progesterone receptor, and three of 45 cases (7%) were considered “borderline” for ER.

There were a total of 45 cases tested for ER-α and progesterone receptor by immunochemistry. 18 of the 45 cases (40%) were classified as positive for either ER-α or progesterone receptor. Among the ER-α or progesterone receptor positive cases, 8 of 18 (44%) had PD while 10 of 18 (56%) had PR or SD. Similarly, there were 27 of 45 cases (60%) that tested negative for either ER-α or progesterone receptor. Among the negative cases, 12 of 27 (44%) were PD while 15 of 27 (56%) had PR or SD. Using the Chi squared test, there was no significant difference between cases tested as positive or negative for either ER-α or progesterone receptor and response (p=1) **(Figure 6A)**.

When analyzing cases in treatment arm B (erlotinib + fulvestrant), there were a total of 30 cases. 13 of the 30 cases (43%) were classified as positive for either ER-α or progesterone receptor. Among the ER-α or progesterone receptor positive cases, 6 of 13 cases (46%) had PD while 7 of 13 cases (54%) had PR or SD. Similarly, there were 17 of 30 cases (57%) that tested negative for either ER-α or progesterone receptor. Among the negative cases, 8 of 17 (47%) were PD while 9 of 17 (53%) had PR or SD. Using the Chi squared test, there was no significant difference between cases that tested positive or negative for either ER-α or progesterone receptor and response (p=0.96) in treatment arm B (**Figure 6B).**

ER-β Tissue Biomarker Analyses:

ER-β was analyzed using IHC and assigned an H score as described above for both the nuclear and cytoplasmic ER-β staining. When all cases were analyzed there was no statistical difference in response (PD versus PR + SD) and H score for the nuclear ER-β (p=0.34) staining. However, there was a statistical difference in H score for the cytosplasmic ER-β H score and response, (median H score 90 versus 20 for PD and PR + SD respectively, p=0.01). When analyzing the EGFR wildtype group only, there also was no significant difference in response and H score for the nuclear ER-β.

There was a trend toward significance in the cytoplasmic ER-β H score and response (median H score 90 versus 20 for PD and PR + SD respectively, p=0.07) (**Figure 7**).

Discussion:

The association between EGFR mutation and female hormones remains controversial. Preclinical *in vivo* data suggests estrogen promotes lung adenocarcinoma cell metastasis through PI3K/AKT signaling pathway, leading to worse outcomes [25]. Stabile *et al* reported an interaction between ER and the EGFR pathways in NSCLC [17, 20]. These studies reported downregulation of EGFR in response to estrogen and upregulation in response to fulvestrant *in vitro.* These data provide significant rationale for the dual combination of ER antagonist therapy and EGFR inhibitors. A smaller pilot study of the EGFR inhibitor gefitinib and fulvestrant was reported showing the combination was well tolerated with disease activity; PFS, OS, and 1 year OS was reported as 12 weeks, 38.5 weeks, and 41% respectively [22].

This randomized phase II clinical trial of erlotinib alone or erlotinib plus fulvestrant in advanced NSCLC sought to evaluate efficacy and potential blood and tissue biomarkers for response. Although there was no statistical difference in either PFS or OS between the two treatment arms, this could be related to the relatively small sample size. Furthermore, the dataset was not powered to compare treatment groups directly; rather the erlotinib arm alone was included for the correlative analysis between the treatment groups.

This dataset did demonstrate a PFS and OS difference between EGFR mutant and wildtype groups, (HR=0.25 (0.13-0.4), $p<0.01$; HR=0.32 (0.16-0.60), $p<0.01$, respectively). The PFS and OS difference is expected based on data in the literature [16], although in a meta-analysis of 13 phase III trials there was no OS advantage seen [26].

Blood estradiol and estrone levels did not significantly differ between the two treatment arms. This may not necessarily correlate with fulvestrant activity as these levels may still be elevated despite the ER antagonistic effects of the drug. There was similarly no statistical difference in the estradiol or estrone levels at any of the time points measured. This may be explained by fulvestrant acting on ER rather than blood estrogen levels directly.

ER-α and ER-β clearly have different tissue distributions, with ER-β shown to be expressed and functional in most human NSCLC cell lines [7, 17, 27, 28]. Conflicting studies of ER-β expression, either cytoplasmic or nuclear, report these markers as having a poorer [29, 30], better [31, 32], or no prediction of survival [33]. Our analysis showed that nuclear ER-β H score overall did not correlate with response, but patients with PD did have higher cytoplasmic ER-β H scores (median 90 vs 20 for PD versus PR + SD respectively, p=0.01). This may be related to a small sample size and the method of using the H score in this analysis.

This dataset has been quite informative in both the blood and tissue data available. The interactions between EGFR and ER signaling in the preclinical setting have not been fully elucidated, but have provided encouraging results for a potential therapeutic combination of EGFR inhibitors and anti-estrogen therapy. Here, the number of patient samples obtained is relatively small, and further prospective studies will need to be conducted in order to establish the efficacy of combination therapy as well as the prognostic and predictive value of both the blood and tissue biomarkers.

CHAPTER 2: STATISTICAL ANALYSIS PLAN

Efficacy Analysis:

Randomization for the two groups was 2:1 and carried out in a random permuted web-based block design. Randomizing twice as many patients to the combination arm was to evaluate the primary endpoint of response rate as the study was not powered to compare directly to the erlotinib alone group. The combination arm would be compared to historical controls, and 2:1 randomization would also serve as an incentive for patients to be randomized to an arm with an intervention that would be available outside of a clinical trial. The erlotinib alone arm was included to make comparisons to the combination arm in the correlative analysis. Patients were stratified by gender and ECOG performance status.

For the efficacy analysis, OS and PFS were assessed. Median OS and PFS were found using Kaplan-Meier curves with stratified log-rank test to evaluate differences in PFS and OS between groups. Patients with an unknown cause for death were censored, and survival time was based on the last date the patient was known to be alive. Patients who did not have a date for progressive disease were also censored, and the PFS time was based on their last response assessment although they may have been in PR or SD. This was the available data, and was a more conservative approach, but would ensure that the data would not overestimate PFS or OS.

In a separate analysis, a more liberal approach was used in censoring the data and finding the date of PFS and OS. In this analysis, patients with an unknown date of PD had their PFS time used as the date they had died and they were not censored if they had died. This assumes that the overall survival is the date they had progressed. Furthermore, if the date of death was not due to PD, this was assumed to be PD, and this was not made a censored event. This was a more liberal approach but the analysis was run in order to find any differences in the outcomes data. This sensitivity analysis

proved that this more liberal approach did not make a difference in the overall results (data not shown).

The best response was also analyzed with SD and PR groups analyzed together and compared to patients with PD. Analysis was also carried out separately for all 3 groups and did not show a clinically significant difference in results. The reason for analyzing with clinical best response compared to PD patients was that the analysis was more straightforward comparing two groups and was also more clinically relevant as those with both SD and PR would have some benefit to treatment.

Estrogen Blood Biomarker Analyses:

The blood estrogen biomarkers were run in duplicate, and although there were some discrepant values, this was evaluated statistically to ensure using the average was appropriate. Some of the results from the analyses were below the lower limit of detection of the assay and resulted as <1 pg/mL. In these instances, the value was made 0 pg/mL. Another approach would have been to use a higher threshold of 0.9 to ensure there was no difference in the analysis, but having an estrogen value <1 pg/mL is assumed to be clinically irrelevant in terms of estrogen effects. Similarly, the analysis could have been run with values marked as <1 pg/mL as 0 pg/mL and then as 1 pg/mL in order assess whether this would change the result.

The correlation coefficient was found for the estradiol and estrone values at each of the cycles (C1D1, C2D1, C3D1) and R^2 ranged from 0.75-0.99. Therefore, the duplicate values were reasonably close and the average of the duplicates was used in the analysis. Another approach would be to use the intercorrelation coefficient or the Bland-Altman test.

Furthermore, to ensure that the average was a safe approach, the analyses had been run using both the first run and the second run independently, and again there was not a meaningful difference in the analysis. Another consideration for evaluating the

blood estrogen data was to divide the values into tertiles or quartiles; however, the majority of values are clustered together and this approach would not have been possible. Dividing the data into tertiles or quartiles may have been useful if the association with outcome was similar for patients with low or high values and different for patients with intermediate values. However with blood estrogen data, it seems more reasonable that the association of estrogen would have a direct or inverse relationship with outcome.

Other Exploratory Blood Biomarker Analyses:

The other tested blood biomarkers including PlGF, sFLT-1, VEGF, AREG, IL-6, TGFα, HGF and testosterone demonstrated statistical significance in some cases. Specifically, C2D1 PlGF and TGFα in all patients were higher in PD patients compared to SD + PR. The majority of values for TGFα were 0 for both response groups. Of the 8 non-zero TGFα values, 7 were in 32 PD patients while only 1 was in 36 PR + SD patients, resulting in a significant difference (p=0.022 by Fisher's exact test) between the response groups consistent with the Wilcoxon rank-sum test **(Figure 4B)**. Because the data is far from Gaussian, the Wilcoxon rank-sum test rather than a t-test for parametric data was used.

Furthermore, C2D1 PlGF, VEGF, and TGFα in the erlotinib alone group was higher in PD patients compared to SD + PR patients. The significance of this finding is unclear, as when evaluated in the combination of erlotinib and fulvestrant, there was no longer a statistically significant finding. These findings may be related to the effect of multiple comparisons, and when a more conservative correction such as Bonferroni correction is applied the significance may likely be lost.

The difference in C2D1 and C1D1 of the various biomarkers was also assessed

to evaluate whether it was a change in these values that may be more significant, but the significance of the above biomarkers was lost when evaluating in this fashion.

ER-α, ER-β and Progesterone Receptor Tissue Biomarker Analyses:

ER-α and PR score were classified dichotomously. Either ER-α or PR positivity was felt to be informative because although ER levels may be very low in some tumors, if it is active it can induce progesterone receptor expression. This can serve as a marker of ER activity although IHC may not be sensitive enough to detect ER positivity. Therefore, either ER-α and/or progesterone receptor positivity was considered. Despite this, the analysis did not demonstrate a difference in response with ER-α or progesterone receptor positivity. This was consistent when all cases were considered, and also when the combination erlotinib and fulvestrant arm was analyzed. Again this may be related to the small number of cases with only 45 cases with adequate tissue available for testing.

ER-β staining was evaluated looking at staining in the cytoplasm and the nucleus. The H score for each was obtained as described above, and correlated with response. The H score was used in order to weight the intensity of staining. Future analysis can find a discriminatory threshold for which a correlation with outcome can be found. Furthermore, this was evaluated stratified by EGFR wildtype patients using the Wilcoxan rank-sum test for the reasons listed above.

Limitations of the Analysis:

Although 100 patients were enrolled, clinical response data was available for only 82 patients thereby limiting the power of our analyses. Furthermore, out of the 100 patients, estrogen blood levels (both for estradiol and estrone) were available for 92 patients at C1D1, 71 patients at C2D1, and 30 patients at C3D1. For the other biomarkers, among the 100 patients approximately 70% of patients had available blood

data and yet not all of these patients had available response data. For the tissue samples, although a biopsy was required not all patients had available tissue. Only 45 patients had ER-α and progesterone receptor staining performed while ER-β staining was done on 58 of the cases.

During the processing of ER-α staining at the University of Southern California it was found that the sample was only reliable if tissue was cut fresh. This resulted in having to repeat the staining using only fresh cut tissue on the ER-α samples. This may also be true for ER-β as well, but repeat staining was not performed for ER-β. Furthermore, there is some debate in the literature about the validity of the ER-β antibody, resulting in potential erroneous results in the data for ER-β. ER-α and progesterone receptor were both tested using validated antibodies.

Interestingly, at the time the study was initiated the plan was to use gefitinib and fulvestrant. Four patients had initially been enrolled on the trial, but the study was changed to erlotinib and fulvestrant, and those four patients were not included in the analysis. At the time of the study, gefitinib was being studied and though some patients had benefit, patients could not be identified who would benefit from treatment. Furthermore, during patient accrual from 2006-2011, the guidelines for EGFR inhibitors changed from therapy after two lines of therapy to include after one or more lines of therapy in EGFR wildtype patients and as frontline therapy for EGFR mutant patients [34]. Therefore, with these recommendations, more EGFR mutant patients were enrolled at UCLA and which may have led to a higher response rate. Still, among the patients analyzed only 16 of 60 patients were EGFR mutant.

The key features of this analysis were to identify predictive and prognostic biomarkers that may provide further insight into EGFR inhibitors and ER antagonist therapy. Statistical issues in biomarker research include confounding and multiple

testing and are of course relevant in the current study. EGFR mutational status and the multiple biomarker panel specifically illustrate these problems. Despite this, exploring both the tissue and blood biomarkers has the potential to reveal useful information in the biological understanding of EGFR and ER interactions in NSCLC.

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Tables:

Table 1: Patient Characteristics

Characteristic	Erlotinib (n=27)	Erlotinib plus Fulvestrant (n=55)
Age		
Median	66	67
Standard Deviation	12	11
Range	(42, 86)	(37, 86)
Gender		
Male (%)	13 (48)	21 (38)
Female (%)	14 (52)	34 (62)
Ethnicity		
Asian (%)	6 (22)	14 (26)
Black (%)	1 (4)	3 (5)
Hispanic (%)	4 (15)	5 (9)
White (%)	16 (59)	33 (60)
ECOG		
0 (%)	11 (41)	25 (45)
1 (%)	12 (44)	24 (44)
2 (%)	4 (15)	6 (11)
Smoker		
Yes (%)	20 (74)	40 (73)
No (%)	7 (26)	15 (27)
EGFR		
Mutant (%)	6 (22)	10 (18)
Wildtype (%)	12 (45)	32 (58)
Missing (%)	9 (33)	13 (24)
Histology		
Adenocarcinoma (%)	20 (74)	39 (71)
Squamous (%)	6 (22)	13 (23)
Other* (%)	1 (4)	2 (4)
Missing (%)	0 (0)	1 (2)
Stage at Enrollment		
IIIB (%)	1 (4)	9 (16)
IV (%)	26 (96)	46 (84)
Stage IV: Number of Prior Treatments		
0 (%)	10 (37)	25 (45)
1 (%)	10 (37)	16 (29)
2 (%)	4 (37)	10 (18)
3 (%)	2 (7)	1 (2)
4 (%)	0 (0)	2 (4)
Missing (%)	1 (1)	1 (2)
Cycles Completed		
Mean	4.9	6
Standard Deviation	7.7	7.7
Range	(0, 36)	(0, 35)

*includes large cell and poorly differentiated

Table 2: Median PFS and OS for treatment arm, EGFR status, and gender.

Overall	**Erlotinib**	**Erlotinib + Fulvestrant**	**p-value**
n (%)	27 (33)	55 (67)	
PFS (median, months)	1.95	4.3	0.1
HR (95% CI)	0.64 (0.34-1.08)		
OS (median, months)	8.6	11.6	0.96
HR (95% CI)	0.99 (0.60-1.62)		
By EGFR	**Mutant**	**Wildtype**	**p-value**
n (%)	16 (27)	44 (73)	
PFS (median, months)	16.6	1.8	**<0.01**
HR (95% CI)	0.25 (0.13-0.40)		
OS (median, months)	30.9	10.7	**<0.01**
HR (95% CI)	0.32 (0.16-0.61)		
By Gender	**Female**	**Male**	**p-value**
n (%)	48 (59)	34 (41)	
PFS (median, months)	3.5	1.9	0.07
HR (95% CI)	0.66 (0.38-1.0)		
OS (median, months)	24.1	10.7	0.16
HR (95% CI)	0.67 (0.37-1.17)		

Table 3: Median estradiol and estrone levels by treatment arm with correlation coefficient for duplicate runs. Wilcoxon rank-sum test was used to compare estrogen levels between treatment arms.

	Erlotinib	**Erlotinib + Fulvestrant**	**p-value**	***R^2**
C1D1_Estradiol	7.45 (n=25)	5.1 (n=51)	0.89	0.97
C1D1_Estrone	19 (n=25)	20.5 (n=51)	0.53	0.86
C2D1 Estradiol	6.35 (n=21)	0.975 (n=46)	0.81	0.99
C2D1 Estrone	7.5 (n=21)	6.05 (n=46)	0.74	0.96
C3D1 Estradiol	0.65 (n=3)	0 (n=27)	0.74	0.98
C3D1 Estrone	1.95 (n=3)	10.15 (n=27)	0.16	0.75

*for duplicate runs of estradiol and estrone

Table 4: Biomarker analysis of lung cancer response. Median, interquartile range, and number of cases analyzed for each marker noted for Cycle 1, 2 and the difference between Cycle 2 and 1. Wilcoxon rank-sum test was used for the statistical analysis. Significant markers are bolded.

		PD		PR + SD		
Biomarker	**Cycle**	**Median (IQR)**	**n**	**Median (IQR)**	**n**	**p- value**
PlGF	1	0.08 (0.06, 0.12)	38	0.08 (0.05, 0.10)	38	0.350
	2	0.09 (0.07, 0.12)	29	0.07 (0.05, 0.08)	34	**0.015**
	Delta	0.00 (-0.01, 0.01)	29	-0.01 (-0.27, 0.01)	32	0.348
sFLT-1	1	1 (0.67, 1.89)	38	0.67 (9.74, 0.91)	38	0.418
	2	0.99 (0.65, 1.69)	29	1.03 (0.76, 1.39)	33	0.778
	Delta	-0.01 (-0.65, 0.44)	29	0.07 (-0.17, 0.35)	31	0.311
VEGF	1	2.81 (1.12, 5.62)	38	2.19 (1.46, 3.87)	38	0.712
	2	2.36 (1.44, 6.73)	29	2.36 (1.47, 4.42)	33	0.617
	Delta	-0.09 (-1.04, 2.87)	29	0.04 (-0.97, 0.90)	31	0.947
AREG	1	0 (0, 15.56)	32	0 (0, 20.28)	37	0.619
	2	0 (0, 3.57)	27	0 (0,43.47)	35	0.213
	Delta	0 (0,0)	23	0 (-3.17, 0)	31	0.152
IL-6	1	59 (7.55, 135.60)	33	19.80 (0, 104.82)	37	0.127
	2	84.18 (17.73, 387.48)	26	35 (0, 120.95)	33	0.118
	Delta	12 (-6.18, 137.91)	22	0 (-18.15, 73.86)	29	0.412
TGF-alpha	1	0 (0,0)	37	0 (0,0)	40	0.248
	2	0 (0,0)	32	0 (0,0)	36	**0.016**
	Delta	0 (0,0)	31	0 (0,0)	35	0.130
HGF	1	168.14 (106.13, 218.31)	35	133.81 (94.37, 184.64)	41	0.117
	2	158.57 (122.23, 286.93)	26	139.62 (90.72, 184.37)	39	0.120
	Delta	12.96 (-28.81, 67.04)	23	3.14 (-25.19, 57.50)	38	0.994
Testosterone	1	2.02 (0.74, 3.84)	37	1.39 (0.61, 3.44)	40	0.632
	2	1.08 (0.77, 4.66)	29	1.4 (0.46, 3.58)	39	0.535
	Delta	0.16 (-0.28, 0.54)	28	0.03 (0.26, 0.51)	37	0.587
bFGF	2	0.04 (0.02, 0.10)	29	0.03 (0.01, 0.05)	35	0.232

Figures:

Figure 1: Kaplan Meier curves for PFS and OS by treatment arms (A,B), EGFR status (C,D), and gender (E,F). Log-rank test was used for the statistical analysis.

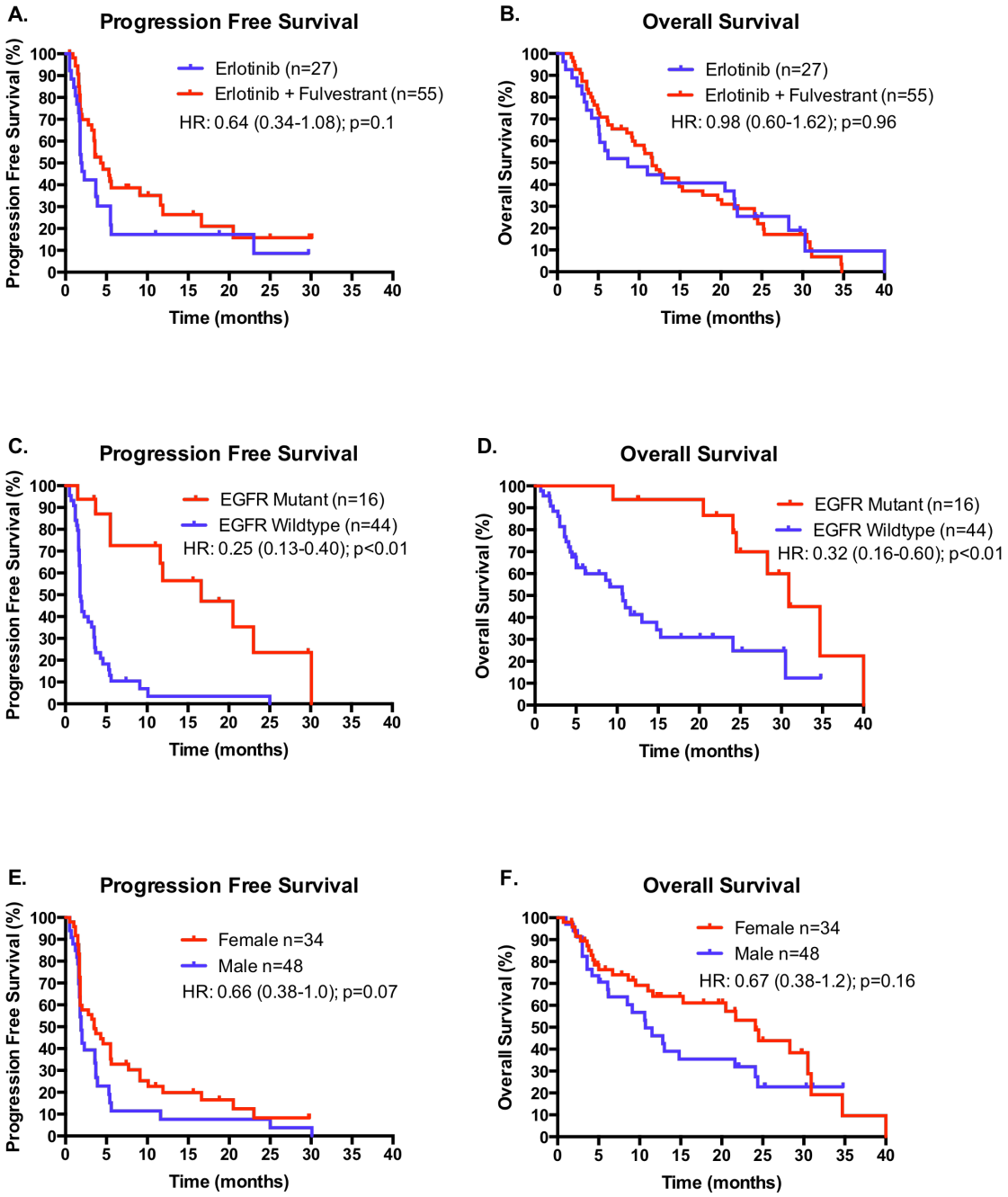

Figure 2: Estradiol and estrone levels for C1D1 (A,B), C2D1 (C,D), C3D1 (E,F) by response. Median and interquartile range are shown. Wilcoxon rank-sum test was used for the statistical analysis.

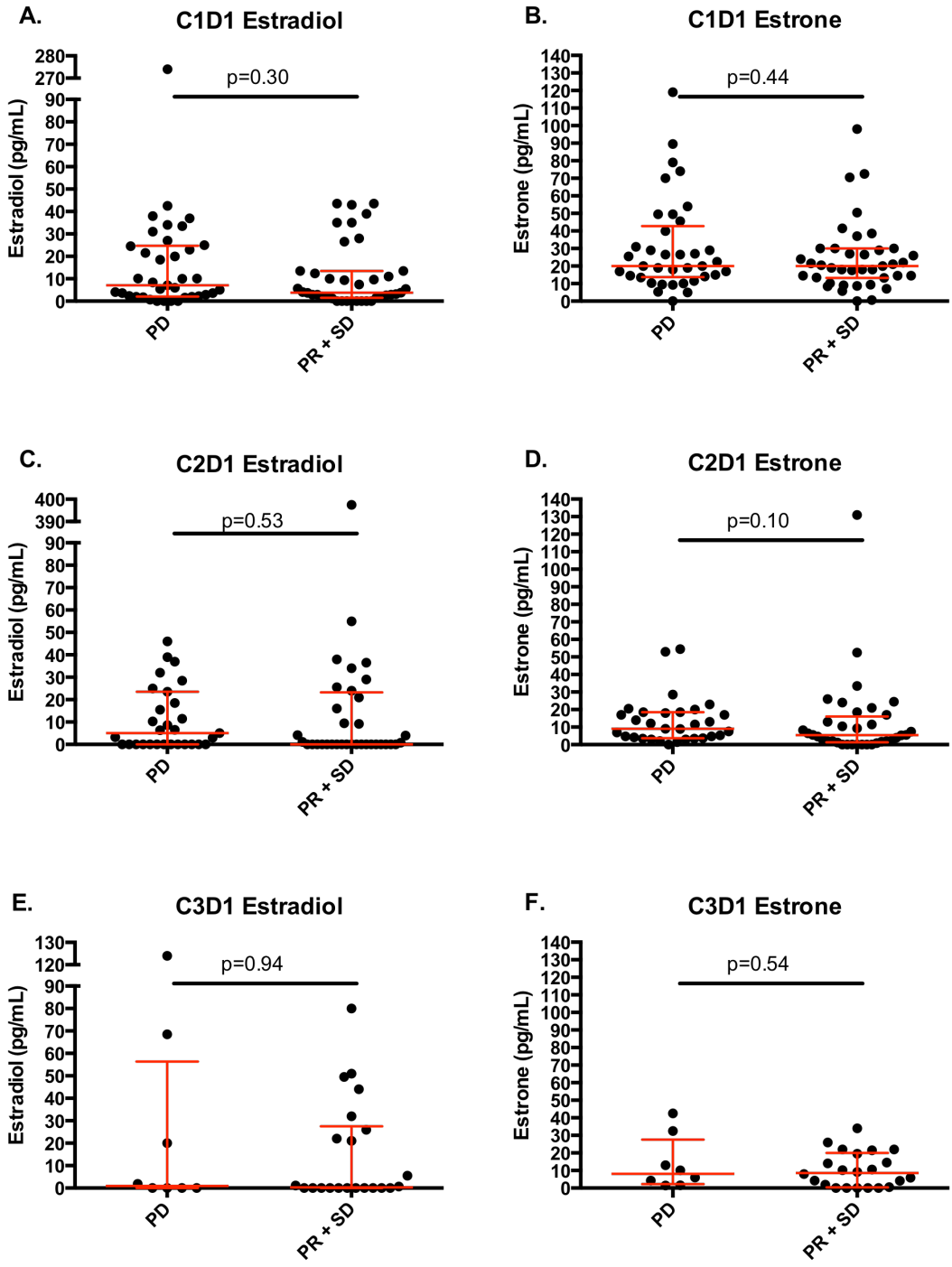

Figure 3: Estradiol and estrone levels for C1D1 (A,B), C2D1 (C,D), C3D1 (E,F) by treatment arm. Median and interquartile range are shown. Wilcoxon rank-sum test was used for the statistical analysis.

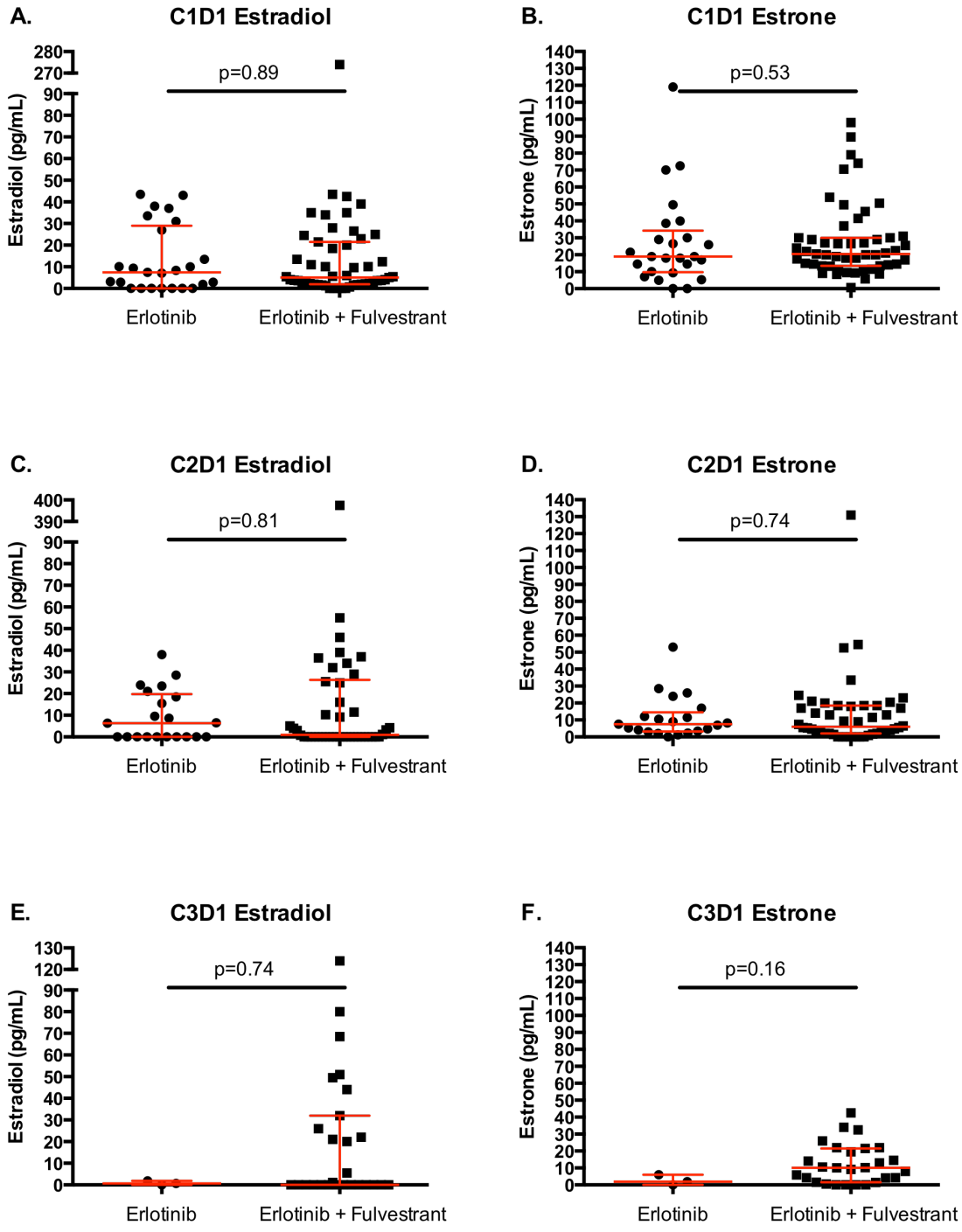

Figure 4: C2D1 biomarker levels for A.) PlGF and B.) TGFα by response. Median and interquartile range are shown. Wilcoxon rank-sum test was used for the statistical analysis.

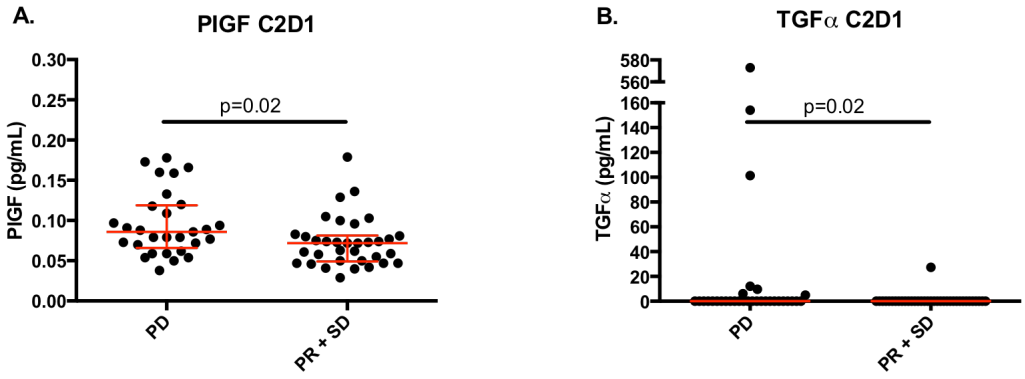

Figure 5: Calculated differences in individual biomarkers between C1D1 and C2D1 by response. Median and interquartile range are shown. Wilcoxon rank-sum test was used for the statistical analysis.

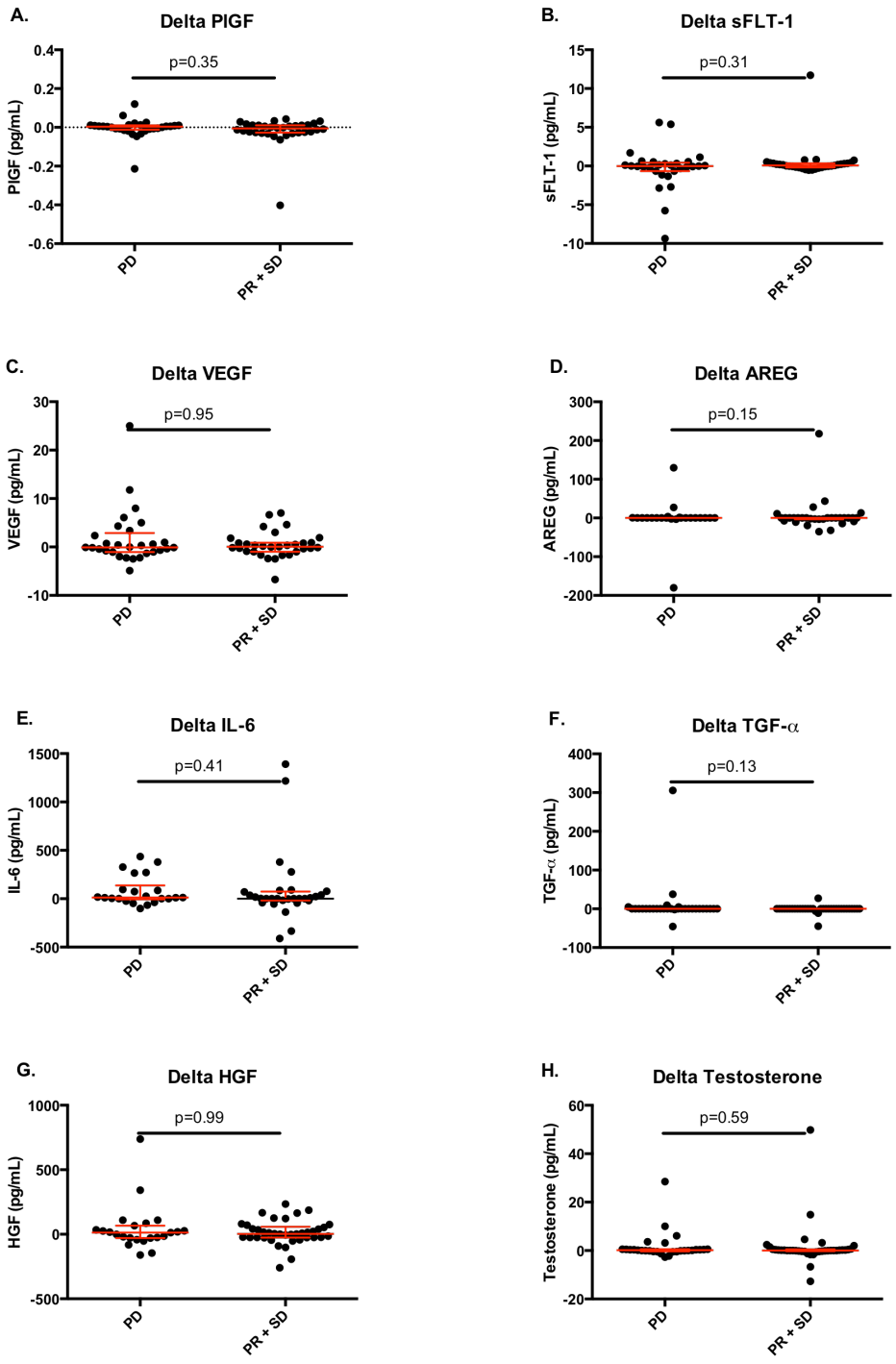

Figure 6: Tissue ER-α and/or progesterone receptor positive expression in relation to response for A.) all cases tested and for B.) erlotinib + fulvestrant arm. Chi-squared test was used for the statistical analysis.

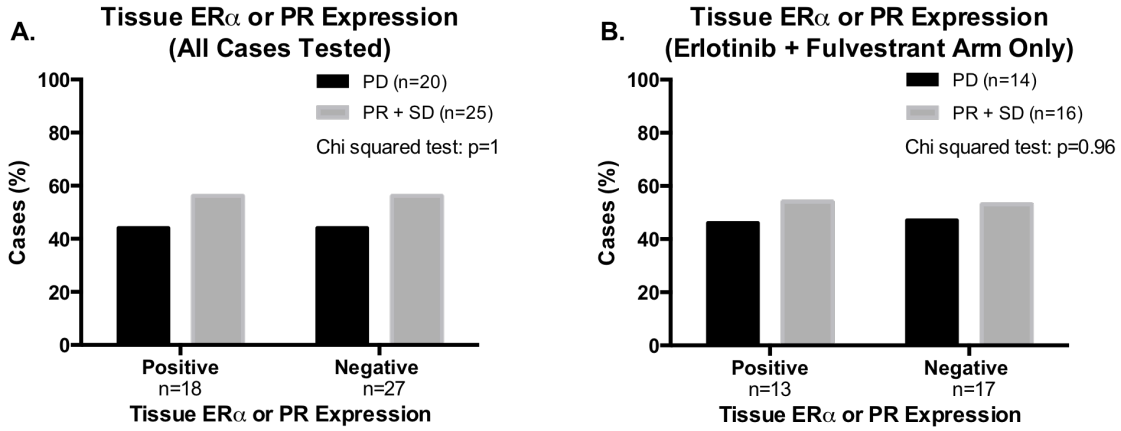

Figure 7: Nuclear and cytoplasmic ER-β H score for A-B) all cases and for C-D) EGFR wildtype group. Median and interquartile range are shown. Wilcoxon rank-sum test was used for the statistical analysis.