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1 # Mass spectrometry-based quantitation of Her2 in
2 # gastroesophageal tumor tissue: Comparison to IHC and FISH

3

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18 gastroesophageal adenocarcinoma; stomach cancer; SRM-MS, selected reaction monitoring mass
19 spectrometry; companion diagnostic; clinical biomarker assay; multiplex protein expression
20 analysis in FFPE tissue

21 **Nomenclature:** HER2 – reference to the pathway in general;

22 *HER2* – reference to the gene;

23 Her2 - reference to the protein.

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Abstract

Background: Trastuzumab showed survival benefit for Her2-positive gastroesophageal cancers (GEC). Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) currently determine eligibility for trastuzumab-based therapy. However, both assays are low throughput with various limitations.

Methods: We developed a selected reaction monitoring mass spectrometric (SRM-MS) assay and quantified levels (amol/ug) of Her2-SRM in cell lines (n=27) and GEC tissues (n=139). We compared Her2-SRM expression with IHC/FISH, seeking to determine optimal SRM expression cut-offs to identify *HER2* amplification.

Results: After demonstrating assay development, precision, and stability, Her2-SRM measurement was observed to be highly concordant with *HER2/CEP17* ratio, particularly in a multivariate regression model adjusted for SRM-expression of Met, Egfr, Her3, and HER2-heterogeneity covariates, and their interactions (cell lines r^2=0.9842; FFPE r^2=0.7643). In GEC tissues, Her2-SRM was detected in 71.2% of cases, and 12.3% were identified as 'HER2+'. ROC curves demonstrated HER2-SRM levels to have high specificity (100%) at an upper-level cut-off of >750 amol/µg and sensitivity (75%) at lower-level cut-off of <450 amol/ug. We observed an 'equivocal-zone' between 450-750 amol/ug, analogous to 'IHC2+', but less frequent (9-16% of cases versus 36-41%).

Significance: Compared to IHC, SRM-MS provided more objective and quantitative Her2 expression with excellent *HER2/CEP17* FISH correlation and fewer 'equivocal' cases. Along with the multiplex capability for other relevant oncoproteins, these results demonstrated a refined HER2 expression assay for clinical application.

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Introduction

The human epidermal growth factor receptor-2 (HER2, *ERBB2*) is a receptor tyrosine kinase promoting cell development, differentiation and survival.[1, 2] Aberrant HER2 activity due to gene amplification and consequent protein overexpression results in a HER2-driven oncogenic phenotype.[1, 2] HER2 is amplified/overexpressed in various cancers including breast (~20%), gastroesophageal (GEC) (~10-15%) and endometrial cancers (~12%).[3] HER2 positivity is higher in esophageal/esophagogastric adenocarcinomas (~15%), compared to distal gastric adenocarcinomas (~10%).[4, 5] The '**T**rastuzumab in the treatment **O**f **GA**stric cancer' ('ToGA') trial reported survival benefit among 'HER2-positive' GEC patients treated with trastuzumab-based therapy in comparison to standard chemotherapy, with ensuing widespread incorporation of immunohistochemistry (IHC) and/or fluorescence in situ hybridization (FISH) testing into routine GEC care (**Supplementary Figure 1**).[4]

'ToGA' trial eligibility defined 'HER2 positivity' as either 'FISH+/any IHC' or 'anyFISH/IHC3+'. However, patients within 'FISH+/IHC 0-1+' subgroups (n=131 or 22% of enrolled patients) derived no benefit from trastuzumab (**Supplementary Figure 2**). In 'ToGA', FISH positivity was defined as '*HER2/CEP17* ratio of ≥2'. IHC validation on GEC samples led to modification of the breast scoring system to account for basolateral membranous immunoreactivity and/or higher rates of intra-tumoral heterogeneity[6]. Now, 'HER2+' is clinically defined as IHC3+ or IHC2+/FISH+ (**Supplementary Figure 1,2**).[6-9] Subsequent phase III "HER2-selective" GEC trials in the first line ('LOGiC')[10] and second line ('TyTAN')[11] metastatic settings evaluated the HER2/Egfr specific oral tyrosine kinase inhibitor, lapatinib, versus placebo along with chemotherapy; both trials were negative for the primary endpoint (overall survival) in the intention-to-treat populations. Interestingly, 'TyTAN' enrolled 261 patients of which 31% were FISH+/IHC 0-1+.[11] Moreover, the IHC3+/FISH+ subset demonstrated a statistically significant survival advantage (14 vs 7.6 months, HR 0.59, p=0.0175).[11] Recently, a report suggested that the degree of *HER2* amplification/expression may better predict

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1 therapeutic benefit from anti-HER2 therapy.[12] These observations suggest the need for revised HER2
2 criteria/diagnostics, and also implications regarding optimal therapeutic strategies *within* classic 'HER2+'
3 groups.[13, 14]

4 Despite noted utility of HER2 IHC/FISH, various reports detail numerous limitations.[15-20] IHC is
5 semi-quantitative attempting to incorporate staining intensity and extensity into a '0-3+' scoring system.
6 IHC is notoriously subjective, and sensitive to antigen instability in formalin fixed paraffin embedded
7 (FFPE) unstained-sections, as recently demonstrated.[21-24] 'HER2-equivocal' (IHC2+) scores require
8 reflex FISH analysis – accounting for almost 30% (159/584) of FISH+ enrolled cases in 'ToGA'
9 (**Supplementary Figure 2A**), not including undocumented IHC2+/FISH- screen failures. Reflex FISH
10 testing is also laborious, time-consuming (especially serially after IHC), expensive, and remains operator
11 dependent/subjective, particularly in molecularly heterogeneous cases.[7, 25-28] Both assays are low-
12 throughput.[13] A consequence of these limitations is false positive/ negative and delayed results and
13 uneconomical use of limited tissue samples.[15, 19, 26] Refinement of HER2 diagnostic methods is welcomed.

14 The mass spectrometry-based selected-reaction-monitoring (SRM-MS) assay has gained broad
15 acceptance as a specific and sensitive technology for measuring absolute levels of specific protein
16 targets,[29-31] however the ability to apply this technology to FFPE tissues has been technically challenging.
17 A multiplexed and quantitative Liquid Tissue-SRM method to quantify proteins in FFPE tissues based on
18 unique peptide sequences does not have the same technical limitations as IHC/FISH, as recently
19 described.[21-24]

20 We sought to evaluate a clinical role of Her2-SRM expression for GEC. Herein we describe
21 application of the Her2-SRM assay to 27 cell lines and uniquely to 139 FFPE GEC tumors. We first
22 tested precision and temporal reproducibility of the assay. We next assessed correlation of Her2-SRM
23 with Her2 IHC/FISH scores, along with multivariate modeling accounting for HER2-heterogeneity and
24 SRM-expression of other relevant GEC oncoproteins Met, Egfr, and Her3. We determined optimal Her2-

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SRM expression cut-off values for clinical application using ROC curves correlating with *HER2/CEP17* FISH ratio. Finally, we present clinical cases to substantiate advantages of the 'GEC-plex' assay with the capability of quantifying multiple oncoproteins simultaneously, addressing issues surrounding the current molecular profiling hurdles of inter- and intra-patient molecular heterogeneity.

Materials and Methods

GEC Clinical Samples and Cell Lines

Patient samples and cell lines were obtained and processed from the University of Chicago (Chicago, IL) under preapproved protocols as previously described.[23, 32]

Cell line mixing studies with *HER2* amplified OE-19 and *HER2* non-amplified MKN-1 to demonstrate dilutional effects of molecular subclones were performed in six lysate conditions with varying ratios (0/100, 20/80, 40/60, 60/40, 80/20, 100/0).

Sample Preparation and Her2-SRM Assay Development

Laser microdissection isolated cells were obtained from FFPE tumor sections as previously described.[21-24] Total protein content for lysates was measured using Micro-BCA assay (Thermo Fisher Scientific Inc, Rockford, IL). Her2-SRM assay development followed previously described methods.[21-24] Briefly, recombinant Her2 protein (UniProtKB accession number P04626) was digested with trypsin and the resultant peptides analyzed using a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nanoAcquityLC system (Waters, Milford, MA). Peptides containing methionine or cysteine-residues were excluded due to their propensity to undergo unpredictable oxidation. Tryptic peptides analysis based on reproducible peak heights, retention times, chromatographic ion intensities, and distinctive/reproducible transition ion ratios identified the optimal SRM peptide ELVSEFSR, comprising residues 971-978 within the protein's intracellular domain, to be unique to Her2. Light (ELVSEFSR) and heavy (ELVSEFSR [$^{13}C_6$,$^{15}N_4$]) versions of this peptide were synthesized to

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1 develop and perform the assay (Thermo Scientific, San Jose, CA). SRM transitions, chromatography and
2 mass spectrometer conditions used for the quantification were specified in our previous report[21]
3
4 ***Her2-SRM Assay Precision & Temporal Reproducibility***
5 Assay precision and temporal stability of the Her2-SRM assay was performed as previously described.[23,
6 24] To demonstrate assay precision, eight breast cancer and eleven GEC FFPE tissues were analyzed in
7 triplicate independently by two different operators on two platforms ("System R" and "System S") on
8 different days, blinded to the others' results. "System R" was comprised of a nanoAcquity LC coupled to
9 a TSQ Vantage mass spectrometer and "System S" was comprised of a Thermo Easy nLC II coupled to a
10 separate TSQ Vantage mass spectrometer.
11 Stability of the MS-based SRM-HER2 assay in FFPE tumor tissue was assessed. Serial tissue sections
12 were cut from eighteen GEC tumor blocks and 11 NSCLC tumor blocks. LM and Liquid Tissue
13 preparation were immediately performed on freshly cut FFPE sections then SRM-HER2 performed.
14 Approximately 13 months later processing of the serial tissue sections was performed and SRM-HER2
15 levels compared to the previous measurements.
16
17 ***Quantitative Analysis and Validation of Her2 in Clinical GEC Tissues and Cell lines***
18 Her2-SRM for 139 GEC FFPE samples and 27 cell lines was calculated from the ratio of area under the
19 curve (AUC) for the endogenous and isotopically-labeled standard peptide multiplied by the known
20 amount of isotopically-labeled standard peptide spiked into the sample before analysis, as previously
21 described.[21-24]
22
23 ***HER2 Fluorescence in situ hybridization (FISH)***
24 FISH results were obtained through routine clinical testing for *HER2/CEP17* ratio. The majority of
25 samples which had clinical FISH testing were those with an initial Her2 IHC 2+ score, per routine

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1 standards. FISH was retrospectively performed on available IHC0/1/3+ samples missing FISH results, as
2 previously described.[32, 33]

3 *HER2* Heterogeneity: FISH *HER2* heterogeneity (hetero+) was defined as 10-50% of enumerated nuclei
4 having *HER2/CEP17* ratio $\geq$2.[26] HER2 negative (HER2-) was defined as <10% of scored nuclei having
5 ratio $\geq$2, and HER2+ (non-heterogeneous) was defined as >50% of scored nuclei having ratio $\geq$2.[26]
6

7 ***Her2 Immunohistochemistry (IHC)***
8 IHC Her2 scores were obtained per routine clinical care using the Hercept Test kit from DAKO.[6]
9 (**Supplementary Figure 1,2**). For samples without clinical Her2 IHC (eg. archived curative-intent
10 resections), when tissue was available, DAB-labeled dextrose-based polymer complex bound to
11 secondary antibody (Leica Microsystems Inc., Buffalo Grove, IL) was performed as previously
12 described.[34]
13

14 ***Statistical Methods***
15 To examine association between Her2-SRM and *HER2* gene copy number (GCN) or *HER2/CEP17* ratio
16 in cell lines and tissues, we used univariate and multivariate linear regression models with Her2-SRM as
17 the independent and *HER2* GCN or *HER2/CEP17* ratio as the dependent variable. In multivariate models,
18 we included SRM expression for Met, Egfr, and Her3, and their interaction terms, given their putative
19 association with HER2 signaling. Analyzing the tissue sample data, *HER2* FISH heterogeneity was
20 additionally included in the models. To compare IHC to either FISH or Her2-SRM in GEC tissues, we
21 included indicators for IHC2+ and IHC3+ (IHC0/1+ reference category) in the regression model. To
22 assess the most effective cutoff value for Her2-SRM to identify *HER2/CEP17* ratio, we computed a
23 receiver-operating-characteristic (ROC) curve. All analyses were performed using R software (www.r-
24 project.ort), version 3.0.1.
25

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1 **Results**

2 ***SRM Assay Development***

3 For development of the Her2 Liquid Tissue-SRM assay, multiple peptides obtained from a tryptic digest
4 of recombinant Her2 were measured using MS. The resulting three candidate peptides, SLTEILK,
5 VLQGLPR, and ELVSEFSR, were then extensively screened in multiple formalin-fixed cell lines and
6 FFPE clinical samples. The peptide ELVSEFSR provided the most reproducible peak heights, retention
7 times, chromatographic ion intensities, clean elution profile, and distinctive/reproducible transition ion
8 ratios; therefore, this peptide was selected for clinical assay development. SRM transitions used for the
9 quantification of Her2 was selected based on the representative MS fragmentation spectrum for peptide
10 ELVSEFSR [$^{13}C_6$,$^{15}N_4$] (**Figure 1A**). A calibration curve was built in formalin-fixed PC3 cell lysates to
11 assess the linearity and the limits of detection and quantitation (LOD, LOQ) of the assay (**Figure 1B**).
12 Coefficients of variation (CV's) ranged from 2.20-14.04% for samples (5 replicates). The LOD/LOQ was
13 150/200 amol, respectively, with a linear regression value of r^2 = 0.9998. The linearity and tight %CV
14 over the concentration range demonstrated the accuracy, reproducibility, and quantitative resolution of the
15 assay. The total ion chromatograms for the light/heavy isotopically-labeled peptides (**Figure 1C**), and the
16 transition ions (**Figure 1D**) are shown. SRM-Fgfr2 and SRM-Her3 are detailed in **Supplementary**
17 **Figure 3**.

18

19 ***Precision of HER2-SRM Assay***

20 To test assay precision, Her2 was measured in eight human breast cancer and eleven human GEC tissues.
21 Using two different LC-MS systems and operators, all breast cancer samples expressed Her2, ranging
22 395.1-18896.7 amol/ug, CVs ranging 3.7-10.4%. Nine of eleven GEC samples expressed Her2 ($\geq$LOD)
23 ranging 306-767.7 amol/ug, CVs ranging 7.5-14.6%. The two operating systems showed very good
24 concordance, r^2 = 0.9978, as demonstrated previously (**Figure 1E, Supplemental Table 1**).[21, 23]

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1 ***Temporal Reproducibility of Her2-SRM Assay***

2 To test the assay's temporal reproducibility, two sections from 18 GEC and 9 NSCLC samples were
3 processed 13 months apart. Very good correlation (r^2=0.8332) supported the reproducibility of Her2-SRM
4 results in archival FFPE sections **(Figure 1F, Supplemental Table 2).**

5

6 ***Her2-SRM Correlation with FISH in Cell Lines & HER2-heterogeneity Mixing Studies***

7 The correlation of Her2-SRM expression and *HER2* GCN or ratio was assessed in 27 GEC and breast
8 cancer lines. (**Figure 2, Supplemental Table 3).** Her2-SRM ranged from <150-21896.7 amol/ug (**Figure**
9 **2A**). Her2-SRM results correlated well with *HER2* GCN and ratio in univariate analyses (r^2 = 0.6096 and
10 0.7493, respectively) (**Figure 2B,C**). Adjusting for SRM-expressions for Met, Egfr, and Her3, the
11 multivariate regression model resulted in an improved and very good correlation between Her2-SRM and
12 *HER2* GCN or ratio (r^2 = 0.8829 and 0.9842, respectively) (Supplementary **Figure 4**, **Supplementary**
13 **Table 5**). Using a preliminary cut-off of 1175 amol/ug, derived from these data, Her2-SRM discerned cell
14 lines with *HER2* amplification versus non-amplification with 100% sensitivity (5/5) and specificity
15 (22/22).

16 One cell line, CAT-14a, was observed to be 'HER2+/hetero+', having 30% of nuclei with ratio $\geq$2; Her2-
17 SRM level was 969.33amol/ug, below the established cut-off of 1175 amol/ug. To demonstrate effects of
18 both i) stromal elements and ii) clonal sub-populations within tumor masses,[14, 23, 25-28, 34] a mixing study of
19 *HER2*-amplified and non-amplified cell lines (OE-19/MKN-1, respectively) exemplified a dilutional
20 effect of HER2 expression upon lowering OE-19 concentrations, likely recapitulating consequences of
21 intra-tumoral HER2-heterogeneity, such as within CAT-14a (**Figure 2D**).

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1 ***SRM, IHC and FISH on FFPE Samples***

2 Her2-SRM results were obtained for 139 GEC samples. Among this cohort, Her2 IHC was available for
3 122 samples. Among these 122 IHC cases, 51 had FISH *HER2/CEP17* ratio results, and 42 with absolute
4 GCN scores.

5

6 ***Her2-SRM Expression in FFPE Samples & Comparison to HER2 FISH***

7 Her2-SRM levels were quantitated in 139 GEC tumors (**Supplemental Table 4**), and expression was
8 above the LOD in 99/139 (71.2 %) samples, ranging 150-24,671amol/µg (**Figure 3A**).

9 Among cases having both Her2-SRM and either FISH GCN (n=42) or FISH ratio (n=54) results,
10 univariate analyses demonstrated fair/moderate correlation (r^2 = 0.3615 and r^2 = 0.5354, respectively)
11 (**Figure 3B**). After incorporating *HER2* FISH heterogeneity along with SRM co-expression of Met, Egfr,
12 and Her3 (Supplementary **Figure 4, Supplementary Table 5**) there was improvement in the fit of the
13 regression model, and good correlation between HER2-SRM and FISH GCN (r^2 = 0.7345) or FISH ratio
14 (r^2 = 0.7643) was observed.

15 Optimal Her2-SRM cutoffs corresponding with *HER2/CEP17* ratio $\geq$2 were determined using a ROC
16 curve (**Figure 3C**). Exploring various cut-offs, 450amol/ug was 92.86% specific [95%CI 83.33-100] and
17 75% sensitive [95%CI 55-95] to identify *HER2* amplification by FISH; alternatively, a cut-off level of
18 750 amol/ug was 100% specific [95%CI 100-100] and 55% sensitive [95% CI 30-75].

19 While most tumors demonstrated Her2-SRM levels < 450 amol/ug (112/139 (80.1%)), a few samples had
20 values >750 amol/µg (14/139 (10.1%)). Of 11/14 of samples >750amol/ug that were available for FISH
21 testing, all (100%) were FISH+ (mean ratio 9.28) - a positive predictive value (PPV) of 100% . A
22 ‘double cut-off’ level, or ‘equivocal’ zone, was applied to better identify marginal HER2 positive cases
23 (not unlike IHC2+ ‘equivocal’). Within the identified SRM ‘equivocal zone’ of 450-750 amol/ug, there
24 were 13 (9.4%) samples (**Figure 3C, 5B**). In terms of identifying FISH ratio$\geq$2, the performance of the

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1 upper/lower boundaries of this equivocal zone was evaluated on the 54 cases having both Her2-SRM and
2 FISH ratio results (**Figure 3D, 5B**). Of 7 samples (7/54, 12.9%) between 450-750 amol/ug, there were 3/7
3 (42.9%) that were deemed FISH- (mean FISH ratio 1.28). Therefore, the PPV was 4/7 (57.1%). A lower
4 mean ratio (3.04) was noted for these FISH+ samples falling within the Her2-SRM equivocal zone than
5 samples with Her2-SRM >750amol/ug (**Figure 5B**). Of 36 samples <450amol/ug (mean ratio 1.387), 5
6 (13.8%) were FISH+ (mean ratio 2.758), one (2.78%) was FISH 'equivocal', and the remaining 30
7 samples were FISH-, demonstrating a negative predictive value (NPV) of 83.3%. Depicting IHC, SRM
8 and FISH results sorted by IHC explicitly demonstrates the wide range of SRM expression within the
9 IHC 2+ and 3+ categories, and the large proportion of samples with very low SRM expression within
10 these two groups, potentially leading to better predictive capacity with respect to benefit from anti-HER2
11 therapy (**Figure 5C**). In summary, using the two SRM expression boundaries, the sensitivity of the lower
12 boundary was 75% and the specificity of the upper boundary was 100% to identify *HER2* FISH ratio$\geq$2,
13 and values within the Her2-SRM equivocal zone showed a FISH+ PPV of 57.1%. (**Figure 3D table**)
14
15 ***Comparison of Her2 IHC2+ Status to FISH Ratio and Her2-SRM in Tissues***
16 Among tumors having concomitant HER2 IHC/FISH/SRM testing, there were 20/54 (37%) that were
17 IHC 2+, of which 15 (75%) were FISH- and 5 (25%) were FISH+, demonstrating a PPV of 25% (**Figure**
18 **3E**). By Her2-SRM, 18 (90%) of these IHC2+ samples were <450 amol/ug. Among all IHC samples,
19 44/122 (36%) were IHC2+, with PPV for FISH+ of 37.5% (**Figure 5A**). The PPV for Her2-SRM was
20 comparatively higher (57%) within the 450-750amol/ug 'equivocal' zone (**Figure 3D, 5B**).
21
22 ***Comparison of IHC to FISH or HER2-SRM in Tissues***
23 Among samples having both Her2 IHC and *HER2* FISH GCN (n=42) or *HER2/CEP17* FISH ratio (n=52),
24 poor correlation was observed (r^2=0.1959 and r^2=0.1242, respectively) (**Figure 4A,B**). However, while

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1 IHC2+ (as referenced to IHC0/1+) was not associated with either FISH GCN or ratio, IHC3+ was
2 significantly associated (p=0.00578 and p=0.016, respectively).
3
4 The subset of GEC samples (n=122) having both Her2 IHC (categorical variable) and Her2-SRM (linear
5 variable) demonstrated very poor correlation, r^2=0.0355 (**Figure 4C)**. However, when comparing IHC to
6 SRM both as categorical variables, there was noted dependence, Chi Square p=0.02219 (**Figure 4C**
7 **table**). To demonstrate the differences in sensitivity, specificity, and resolution between Her2 IHC and
8 Her2-SRM, we compared the LODs for IHC (≥1+) versus Her2-SRM (≥150amol/ug) (**Figure 4D**). Of
9 122 cases, 108 (88.5%) were ≥IHC 1+, while 89 (73%) samples were ≥150amol/ug. IHC was 89.9%
10 sensitive in identifying cases ≥150amol/ug, but only 15.2% specific in discerning SRM-negative cases. A
11 score of 'IHC2+' was observed in 36% of all cases (44/122) (**Figure 5A**), or 41% (21/51) of cases
12 available for a three-way comparison of IHC, SRM and FISH ratio. These results revealed that Her2-
13 SRM better correlated with FISH ratio compared to IHC, with better sensitivity and specificity in
14 identifying FISH ratio≥2 (**Figure 4E**).
15
16 ***Multivariate regression model with HER2 heterogeneity and multi-plex SRM analysis of oncoproteins***
17 To test whether the correlation between Her2-SRM expression and *HER2* FISH would improve after
18 adjusting for the covariates 'HER2-hetero+', Met-SRM, Egfr-SRM, and Her3-SRM for both the cell line
19 and tissue analyses, we evaluated a multivariate regression model (Supplementary **Figure 4A,B,**
20 **Supplementary Table 5**). Interactions were observed with Her3-SRM and Egfr-SRM, (positive-
21 interactions) as well as Met-SRM and HER2-hetero+ (negative-interactions) on the association of Her2-
22 SRM with FISH GCN as well as with FISH ratio. These interactions were observed when either FISH
23 status or Her2-SRM was the outcome variable.
24
25
26

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Clinical Correlation with Patient Vignettes

Patient 1, GEC181 (**Figure 6A**), was diagnosed clinically as stage IV HER2+ gastric cancer, yet Her2-SRM demonstrated low baseline expression (not detected, **Supplementary Table 4** sample #2) and extremely high Fgfr2-SRM. Upon rapid progression on anti-Her2 therapy, the patient responded to second line Fgfr-specific tyrosine kinase inhibition.

Patient 2, GEC159 (**Figure 6B**), was diagnosed stage IV HER2+ esophago-gastric cancer, with extremely high baseline Her2-SRM levels observed (24671 amol/ug, **Supplementary Table 4** sample #139). Upon interval treatments, serial primary tumor biopsies revealed interesting SRM-expression evolutionary patterns. Upon initial trastuzumab exposure, a dramatic decrease (-78.1%) in Her2-SRM was noted at first tumor progression. After treatment with trastuzumab and lapatinib, elevation of Her3-SRM (+30.8%) was observed (+58.9% from baseline). Subsequently, addition of pertuzumab led to the most recent clinical and biochemical responses with improved dysphagia/tumor markers. The patient is maintained on this therapy 35 months from diagnosis.

Discussion

Current HER2 diagnostics have recognized limitations, and there is urgent need for more objective, expedient, and 'tissue economic' assays in order to optimize clinical outcomes for patients.[8, 13, 16, 20, 23, 35] We developed a Her2-SRM assay within a multiplex proteomic quantification assay, and demonstrated its precision, stability, and reproducibility in cell lines and clinical FFPE samples, along with correlation with IHC/FISH and other relevant oncoproteins (Her3/Egfr/Met).

We observed a wide range of Her2-SRM expression not only within the entire GEC cohort (N=139), but also *within* the subgroups of 'IHC3+' or 'FISH+' samples (ranging <150-21896.7 amol/ug). Her2-SRM correlated well with *HER2* FISH amplification status, reliably identifying highly amplified samples. The degree of *HER2* amplification (*HER2/CEP17* ratio) linearly correlated with Her2-SRM, as

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1 was previously reported,[36] just as the absolute *HER2/CEP17* ratio was recently shown to correlate with
2 the degree of anti-Her2 therapeutic benefit.[12] One criticism of SRM technolgy in FFPE tissue is the low
3 sensitivity to identify very low expression levels (ie <LOD). However, when considering gene amplified
4 proteins, it was evident that the expression levels were dramatically higher than non-amplified expression
5 levels, and therefore this limitation does not appear to be relevant when applying the technology to
6 identify gene amplified tumors. We have noted this across various genes/proteins of interest.[22, 23]

7 Most IHC2+ cases had little Her2-SRM expression. The ‘equivocal’ zone that we defined for
8 Her2-SRM expression (450-750 amol/ug), which lacked good correlation with *HER2/CEP17* ratio,
9 represented approximately 10-15% of cases, substantially lower than semi-quantitative IHC2+ scoring
10 (~35-40%). Within respective ‘equivocal zones’, the PPV for Her2-SRM was 57% compared to 25-37.5%
11 with IHC2+; others have demonstrated a PPV within IHC2+ as low as 13%.[36] Our IHC2+ rate is similar
12 to that observed in the ‘TOGA’ trial, particularly if including their IHC2+/FISH- undocumented cases.
13 These IHC2+ rates represent the current experience in routine clinical care. Evaluating he performance of
14 IHC versus Her2-SRM, as contrasted in **Figure 5,** demonstrated the superiority of SRM over IHC in
15 identifying truly FISH+ positive HER2 samples, importantly relying less on reflex FISH testing.

16 In previous years, HER2 cut-offs for IHC and FISH rendering eligibility for anti-Her2 therapy
17 erred towards lower thresholds, likely intending to avoid missing potential benefit of anti-Her2 therapies
18 in patients who would otherwise be given standard cytotoxics alone. However, lack of benefit for these
19 low-expressing subgroups is now recognized.[4, 11] Patient 1 was clinically ‘HER2+’ (IHC2+/FISH+
20 equivocal), yet Her2-SRM was low (<450) - this ultimately predicted lack of benefit from anti-Her2
21 therapy. Although further prospective validation is required in independent datasets (which is underway),
22 the ability to further stratify HER2 status with SRM *within* currently clinically accepted HER2+ patients
23 will have significant treatment implications by judiciously assigning anti-HER2 therapy to those patients
24 most likely to benefit, while sparing those likely to not benefit from both the clinical and financial toxicity
25 of such therapy. Moreover, multiplex SRM-testing to globally survey biomarkers may allow the optimal

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1 treatments towards most-likely tumor 'drivers' to be administered as early as possible. In this example,
2 very high Fgfr2 expression (consistent with *FGFR2* amplification) might have trumped borderline/low
3 Her2 expression. Trials testing *prioritized* personalized treatment algorithms based on higher-throughput
4 molecular profiling, including SRM-MS are ongoing.[14, 37]

5 Spatial intratumoral heterogeneity of FISH/IHC resulted in an observed dilutional Her2-SRM
6 measurement, similar to the exemplary cell line mixing study. It is likely that the identified Her2-SRM
7 'cut-off' for cell lines was higher than that of tissues (>1175 versus >750 amol/ug) due to less subclonal
8 and stromal influences. Supporting this, the CAT-14a cell line, which demonstrated HER2-heterogeneity,
9 demonstrated Her2-SRM levels (969.33 amol/ug) lower than the 1175 amol/ug cell line cut-off. As such,
10 the Her2-SRM assay on FFPE samples inherently captured intra-tumoral HER2 clonal heterogeneity by
11 effectively providing an objective aggregate Her2 expression level representing all the tumor sampled via
12 microdissection using standard H&E staining. As hypothesized, an improved correlation between Her2-
13 SRM level and *HER2/CEP17* ratio, was observed when including the HER2-heterogeneity status into the
14 multivariate linear regression model. The improved correlation between SRM and FISH after adjusting
15 for FISH-heterogeneity was likely due FISH scores reflecting certain select areas of tumor (eg. invasive
16 front, areas with higher FISH ratio than others), while SRM selects all H&E tumor indiscriminately. A
17 significant negative interaction between Her2-SRM level and presence of *HER2*FISH heterogeneity was
18 therefore demonstrated (lower Her2-SRM level with presence of HER2-heterogeneity).

19 Additionally, Her2 functional interactions with Met, Her3, and Egfr have been described. [26, 36, 38-
20 43] After adjusting for these three covariates (Met/Her3/Egfr-SRM co-expression), a stronger linear
21 correlation between Her2-SRM and FISH *HER2/CEP17* ratio was observed. Although the mechanisms
22 for each of these interactions are not clearly defined, ultimately, gene amplification is a surrogate marker
23 for protein overexpression. Specifically, the relationship between gene amplification and protein
24 expression is likely multifactorial, and may be influenced by the expression of other key oncoproteins
25 within the cell. *HER2* amplified tumors tended to have relatively lower Her2-SRM levels if they were

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1 also highly expressing SRM-Met, compared to *HER2* amplified tumors that were not highly expressing
2 SRM-Met. Supporting our findings, Met overexpression has been linked with resistance to anti-Her2
3 therapy for *HER2* amplified tumors, and vice-versa.[38, 40] On the other hand, Her3- and Egfr-SRM levels
4 were observed to be positively associated with Her2-SRM levels, and both receptors have been implicated
5 in signal transduction of *HER2* amplified tumors. Regardless, further work to understand these
6 associations more clearly is required. Notwithstanding, it is possible that incorporating these co-
7 expression covariates, such as is feasible with SRM-MS multiplex technology, while assessing clinical
8 outcome with anti-Her2 therapies will better identify most-likely responders, and may also direct better
9 future multi-drug targeted regimens.[38, 40, 42, 44] This is currently being assessed prospectively in a clinically
10 linked independent dataset.

11 The ability to evaluate molecular heterogeneity longitudinally through time and treatment was
12 demonstrated using the SRM-multiplex assay in Patient 2. Extremely high Her2-SRM levels appeared to
13 portend for prolonged benefit from anti-Her2 trastuzumab therapy (~12 months, twice the median
14 progression free survival in the ToGA study) before first progression (**Figure 6B** Segment 1-2). At
15 trastuzumab-progression, Her2-SRM levels were ~5-fold lower, offering a potential mechanism of
16 resistance by down-regulating receptor expression - yet it remained well above IHC, FISH, and SRM cut-
17 offs for HER2 positivity. Her2-SRM expression increased slightly after withdrawing trastuzumab for
18 brief anti-PD1 therapy (Segment 2-3), providing more evidence of continued 'HER2-addiction'. This
19 expression trend, along with previous evidence that maintaining therapeutic inhibition beyond progression
20 upon a persistent oncogenic-driver, provided rationale for resuming trastuzumab-based therapy (Segment
21 3-4).[14, 45-48] After an initial response to reintroduction of trastuzumab-based therapy, but upon further
22 progression, 'vertical inhibition' with lapatinib/trastuzumab led to continued response (Segment 4-5).[48, 49]
23 Finally, evolution towards higher Her3-SRM expression suggested another mechanism of resistance;
24 pertuzumab-based therapy was then introduced with clinical benefit (Segments 5-6-present).[36, 43, 50] Serial

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testing in order to 're-target' therapies based on 'real-time' molecular profiles merits further testing in ongoing novel prospective clinical trial designs.[14]

Compared to IHC, SRM-MS provided more quantitative Her2 expression with better *HER2* FISH correlation, and a narrower 'equivocal zone'. Ultimately, FISH testing for *HER2* amplification is a surrogate for Her2 protein overexpression, and we showed that this expression level is influenced by several factors, including not only *HER2/CEP17* ratio, but also HER2-heterogeneity within the sample, along with co-expression levels of various other critical oncoproteins. Therefore, a single Her2-SRM expression cut-off in the context of the SRM-MS 'GEC-plex' may in the future better predict anti-HER2 therapeutic benefit without any reliance on FISH or IHC. This is the subject of ongoing evaluation and validation in a large cohort of clinically-linked samples. Along with the multiplex capability of quantifying other protein biomarkers, these results demonstrate a refined Her2 expression assay for clinical application.

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1 **Figure Legends**

2 **Figure 1. Development of HER2 SRM assay. (A)** The fragmentation spectrum for heavy ELVSEFSR
3 peptide and **(B)** the standard curve generated in human PC3 cell lysate; inset: the standard curve
4 generated without the highest two spiking points (5000 and 25000 amol). Each point injected contained
5 5000 amol heavy (ELVSEFSR [$^{13}C_6$,$^{15}N_4$]) on column. **(C)** The total ion chromatograms for the light and
6 heavy isotopically labeled peptides, with **(D)** the transition ions used to identify and quantitate each
7 peptide. **(E)** Precision assessment for measuring Her2 level in 8 breast cancer (red) and 11 GEC (blue)
8 FFPE tissues. **(F)** Temporal reproducibility of FFPE sections processed and analyzed using LT-SRM at
9 two time points over one year apart (blue, GEC (n=18); red, NSCLC (n=9)).

10 **Figure 2: Her2 expression levels using LT-SRM and correlation with *HER2* gene amplification (by**
11 **FISH) in 27 cell lines. (A)** Quantification of Her2-SRM (amol/ug) for 27 cell lines including GM15677
12 lymphoblast control. *HER2* amplified cell lines (*HER2/CEP7* ratio $\geq$2) indicated in dark red, and
13 heterogenous *HER2* amplification in pink (see methods for hetero+ scoring). **(B)** Her2-SRM and FISH
14 gene copy number (GCN) univariate correlations: The left y-axis (blue plus sign) represents the mean
15 *HER2* GCN per nucleus and the right y-axis (red triangle) indicates *HER2:CEP17* ratio (see text for
16 multivariate analysis including Egfr-, Her3-, and Met-SRM coexpression, GCN R^2=0.8829 and Ratio R^2=
17 0.9824) and **(C)** scatter plot of *HER2* GCN/ratio (by FISH) (blue diamond, red square, respectively) and
18 Her2-SRM expression in samples where Her2 expressions are < 1500 amol/ug represented by the black
19 box in (3B). A preliminary Her2-SRM cut-off, from these cell line data, correlating with *HER2*
20 amplification was determined to be $\geq$1150 amol/ug. **(D)** Her2-SRM (red) and Her3-SRM (blue) levels in
21 a cell line mixing study (OE-19: *HER2* amplified / MKN-1: *HER2* non-amplified), modeling intra-tumor
22 clonal heterogeneity.

23 **Figure 3: Absolute levels of Her2 in GEC tissues and correlation of Her2-SRM levels with *HER2***
24 **gene amplification**. **(A)** Her2-SRM analysis of clinical FFPE GEC tissues (n=139) ranging <150-24617

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1 amol/ug. Red highlighted samples are verified by FISH to be *HER2* amplified (*HER2/CEP17* ratio$\geq$2);
2 non-amplified samples are labeled as green, and samples in black were not FISH tested. **(B)** Univariate
3 correlation of Her2-SRM and *HER2* FISH GCN (n=42, blue, r^2 = 0.3615) and ratio (n=54, red,
4 r^2=0.5354). Multivariate analysis revealed stronger correlations when incorporating Met-SRM, Egfr-
5 SRM, Her3-SRM and *HER2* FISH heterogeneity in the model: Her2-SRM:*HER2* GCN r^2 = 0.7345 and
6 Her2-SRM:*HER2/CEP17*ratio r^2=0.7643 (54 Her2-SRM cases had absolute FISH ratio available). **(C)**
7 Optimal Her2-SRM cutoff values determined by receiver operating characteristics (ROC) curve with
8 respect to HER2/CEP17 ratio $\geq$2 . Using one cut-off level, a value of 450 amol/ug was 75% sensitive and
9 93% specific to identify 'amplification'; alternatively, a cut-off level of 750 amol/ug was 55% sensitive
10 and 100% specific. **(D)** Using two cut-points (analogous to IHC 0/1+ = Her2 negative, and IHC 3+ =
11 Her2 positive), with values in between (analogous to IHC 2+) = 'equivocal', an upper SRM level bound
12 of 750 amol/ug and lower bound of 450 amol/ug created an equivocal range 450-750 amol/ug. The two
13 red lines (1 and 2) represent the Her2-SRM expression falling into this equivocal range (n=9/54, 16%).
14 (54 cases were available with binary FISH ratio data ($\geq$2 or <2). Currently, it is recommended that these
15 SRM-equivocal cases undergo confirmatory FISH testing. **(E)** Among tumors exhibiting Her2 IHC 2+
16 with FISH results (n=20), 15 tumors (75%) were FISH- and 5 (25%) were FISH+. Her2-SRM expression
17 levels are superimposed, demonstrating that the majority (18, 90%) of these IHC2+ samples were below
18 the 450 amol/ug SRM cut-off.

19 **Figure 4**. **Her2 IHC correlations with FISH assay or SRM.** Correlation of Her2 IHC to **(A)** FISH GCN
20 in 42 GEC tumors (r^2=0.1959), **(B)** *HER2/CEP17* ratio in n=52 GEC tumors (r^2=0.1242) (52 cases with
21 IHC results had absolute FISH ratio results); and **(C)** Her2-SRM (amol/µg) in 122 GEC tumors
22 (r^2=0.0355). **(D)** IHC compared to Her2-SRM with inset 2X2 table; and (E) three-way comparison of
23 Her2-IHC, Her2-SRM and *HER2/CEP17* ratio by FISH in 51 GEC tumors where all three values were
24 available. Inset tables demonstrate the comparison of the lower boundary for each assay (left, IHC2+
25 versus SRM 450amol/ug) and IHC2+ versus FISH (right). Red bar-SRM, green bar-IHC, and black dot-

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1 *HER2/CEP17* ratio by FISH. Inset tables assess sensitivity/specificity of IHC assuming SRM (D,E) and
2 FISH (B,E) as the comparative standards.

3 **Figure 5. Her2 status assessment of GEC cases by IHC (A) and Her2-SRM (B) to identify HER2**
4 **'positive' and 'negative' cases, as determined by underlying FISH *HER2/CEP17* ratio.** Both assays
5 resulted in an equivocal zone in identifying underlying *HER2* amplification (by ratio>2). However, the
6 incidence of the Her2-SRM equivocal zone (450-750amol/ug) was much lower (9.4%) compared to
7 IHC2+ (36%). Within these Her2-SRM equivocal cases, the PPV was relatively high (57%) compared to
8 IHC2+ (PPV 37.5%). Her2-SRM cases >750amol/ug demonstrated a high mean *HER2/CEP17* ratio
9 (9.28) and all were FISH amplified, compared to IHC3+ (4.16); mean ratio for Her2-SRM equivocal
10 cases that were FISH positive was 3.04, compared to 5.15 in IHC2+/FISH+. Fewer equivocal cases
11 requiring less reflex FISH testing along with better stratification of *HER2/CEP17* ratio demonstrated
12 superiority of Her2-SRM over IHC. **(C)** Depicting these same IHC, SRM and FISH results now sorted by
13 IHC category demonstrated the wide range of SRM expression within each IHC category, particularly the
14 large proportion of samples with very low SRM expression within IHC2+ and IHC3+ groups, potentially
15 leading to better predictive capacity of Her2-SRM with respect to benefit from anti-HER2 therapy. Four
16 cases were scored clinically as IHC2-3+ cases (represented with '*) (**Supplementary Table 4**), and all
17 four had FISH ratio >2, with three cases having Her2-SRM >750 amol/ug and one case between 450-750
18 amol/ug; these cases were included in the IHC 2+ category given the pathologist's uncertainty of scoring
19 (ie equivocal) and requirement for reflex FISH.

20 **Figure 6 (A). Molecular profiling and a call for treatment prioritization based on *degree* of**
21 **genomic/proteomic aberration.** A GEC patient diagnosed clinically as 'HER2+' based on IHC (2+) and
22 FISH, yet a more appropriate drug pairing was suggested by SRM multiplex testing (Fgfr2). Her2-SRM
23 was observed to be <150 amol/ug (the range observed in all preclinical/clinical samples to date is <150-
24 26170 amol/ug) while Fgfr2-SRM was observed to be 384 amol/ug (above the 95 percentile of
25 documented Fgfr2 expression). NGS, next generation sequencing. **(B) Serial Her2-SRM, Her3-SRM,**

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1 **and Egfr-SRM levels over time/treatment of a Her2 overexpressing and *HER2* gene amplified**
2 **esophageal tumor of a 39 year old man**. **Segment 1 to 2:** First line cisplatin/irinotecan-trastuzumab 6
3 cycles (D1,D8 q 21 days) not well tolerated; RT to lumbar metastases; Second line FOLFIRI –
4 trastuzumab 12 doses, SD but increasing tumor markers/bleeding; **Segment 2 to 3:** Embolization to
5 bleeding primary tumor; Third Line –anti-PD1 antibody (PDL1+ IHC), 3 biweekly doses then PD (new
6 multiple liver metastases); **Segment 3 to 4:** Fourth Line docetaxel-trastuzumab 10 cycles, then recurrent
7 primary tumor bleeding but SD; **Segment 4 to 5:** RT 20Gy 1/2014-2/2014 to bleeding primary tumor.
8 Fifth Line lapatinib/paclitaxel plus trastuzumab (15 biweekly cycles then PD on CT as well as PD
9 clinically and by tumor markers); **Segment 5 to 6:** Sixth line FOLFOX-trastuzumab/pertuzumab – stable
10 disease on CT with slight progression of tumor markers. **Segment 6 to present:** FOLFIRINOX-
11 trastuzumab/pertuzumab for three cycles, response in tumors markers and PR on CT**.** Biopsy time points
12 (all via EGD of primary tumor, except #6 – liver core biopsy): **1)** 5/22/2012 **2)** 6/10/2013 **3)** 10/30/2013
13 **4)** 1/14/2014 **5)** 10/7/2014 **6)** 12/17/15. Diagnosed with symptoms 1/2012, ultimate tissue diagnosis
14 5/2012 with stage IV disease (bone, M1 lymph nodes) - now almost 36 months from onset of symptoms,
15 33 months from biopsy (as of 2/2015).

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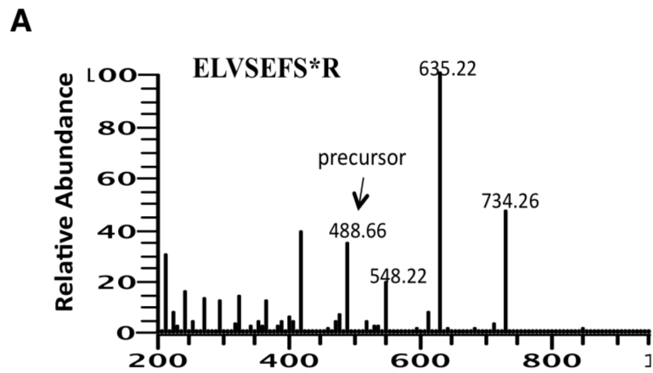

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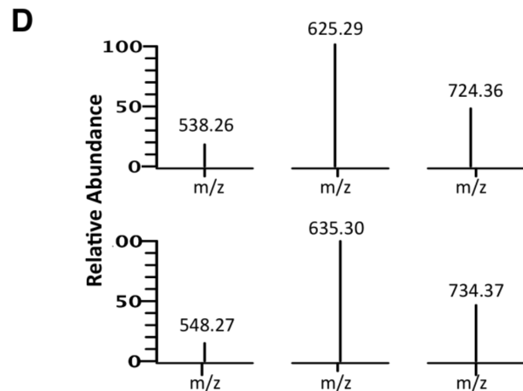

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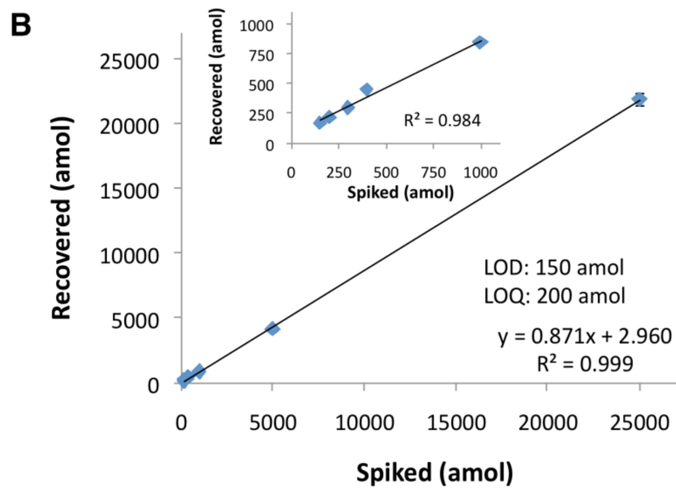

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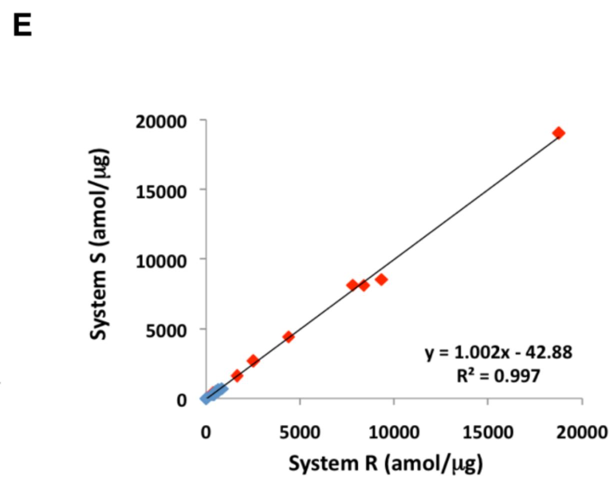

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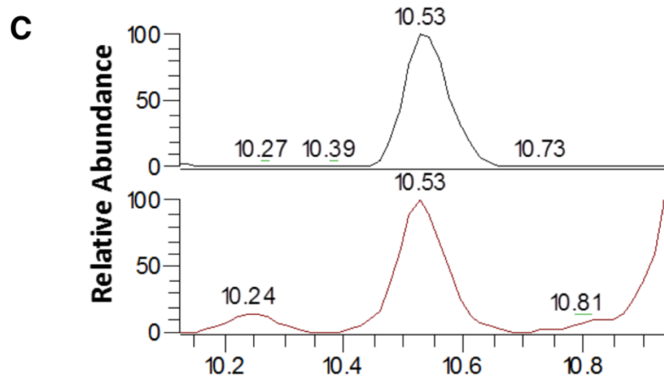

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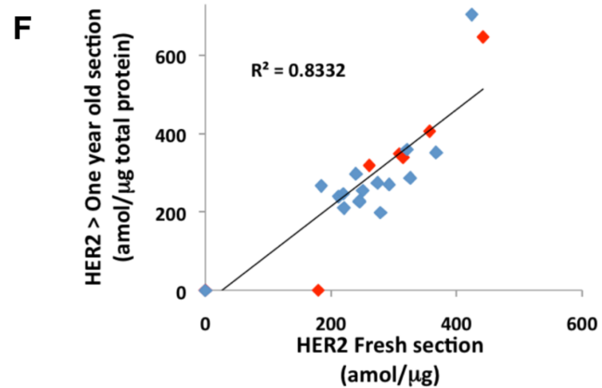

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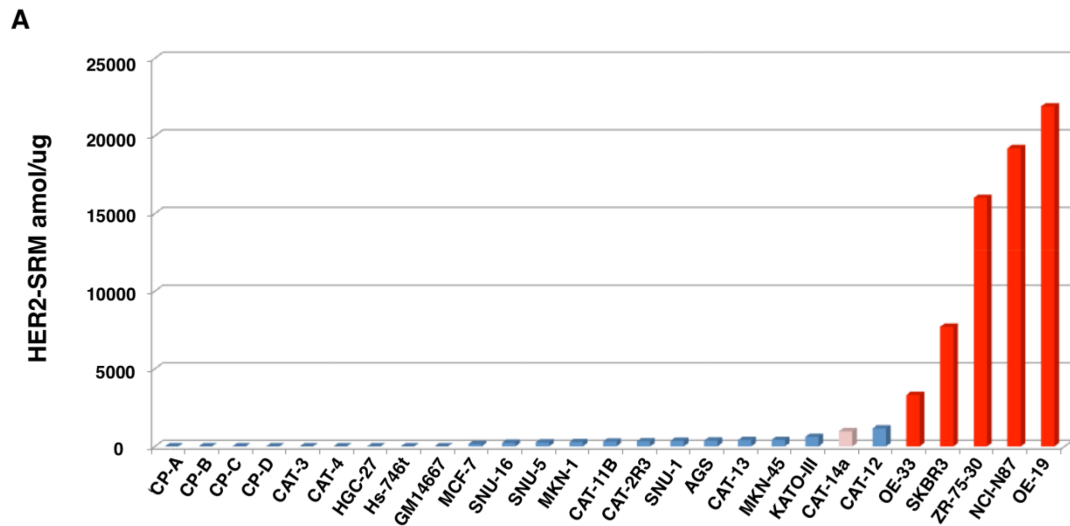

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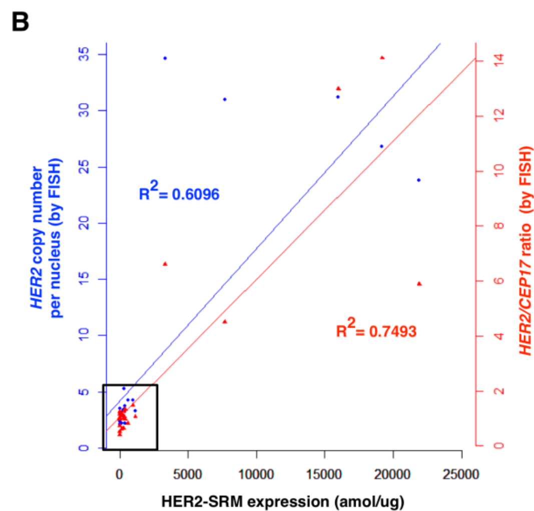

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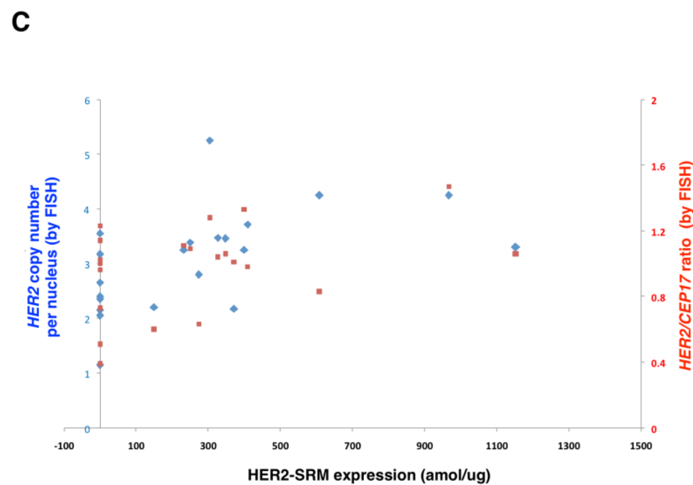

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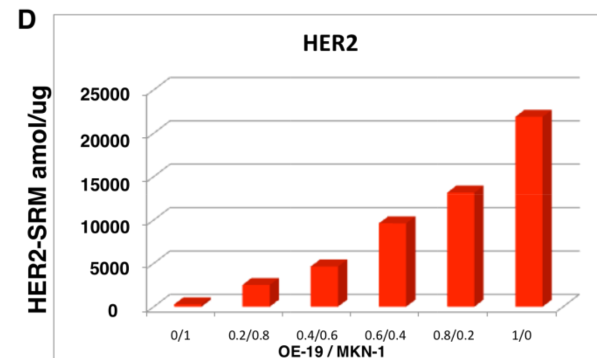
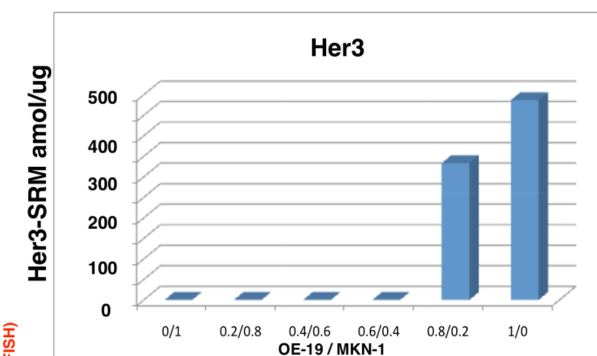
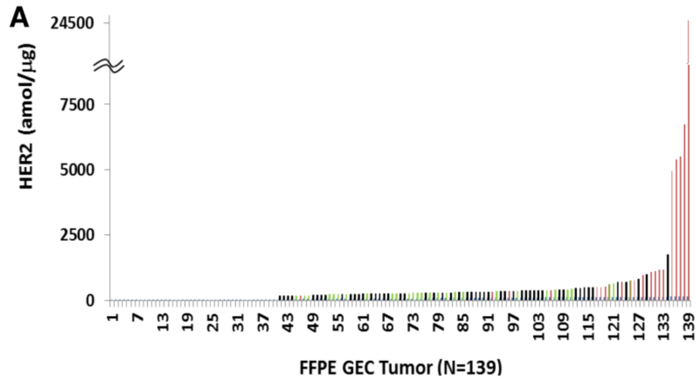
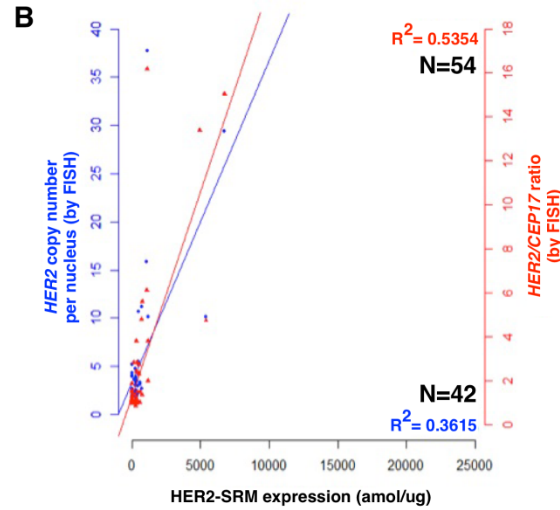
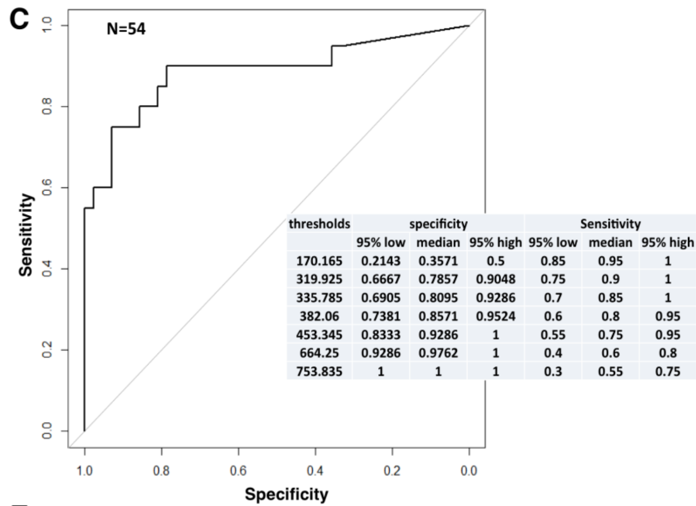




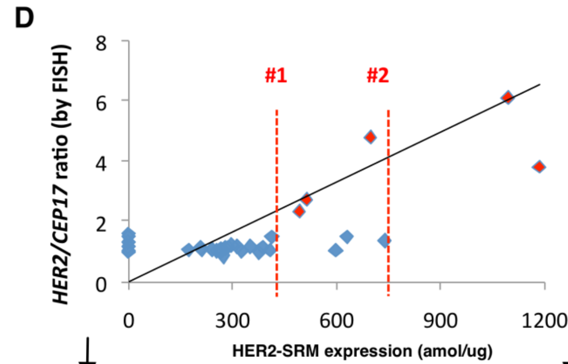
OE-19/MKN-1	HER2	HER3
0/1	274.38	0.00
0.2/0.8	2511.33	0.00
0.4/0.6	4633.17	0.00
0.6/0.4	9608.33	0.00
0.8/0.2	13150.00	333.05
1/0	21896.7	486.97





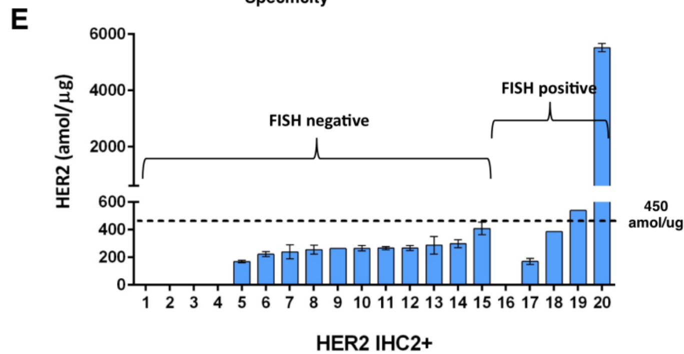


thresholds	specificity			Sensitivity		
	95% low	median	95% high	95% low	median	95% high
170.165	0.2143	0.3571	0.5	0.85	0.95	1
319.925	0.6667	0.7857	0.9048	0.75	0.9	1
335.785	0.6905	0.8095	0.9286	0.7	0.85	1
382.06	0.7381	0.8571	0.9524	0.6	0.8	0.95
453.345	0.8333	0.9286	1	0.55	0.75	0.95
664.25	0.9286	0.9762	1	0.4	0.6	0.8
753.835	1	1	1	0.3	0.55	0.75



HER2/CEP17 ratio (by FISH)

N=54	HER2 SRM (amol/ug)		
	<450	450-750	>750
HER2/CEP17 FISH-	30	3	0
HER2/CEP17 FISH+	5	4	11
Equivocal	1	0	0
Total	36	7	11
	Sensitivity: 75%		Specificity: 100%



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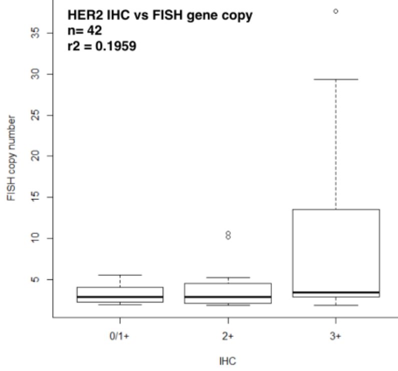
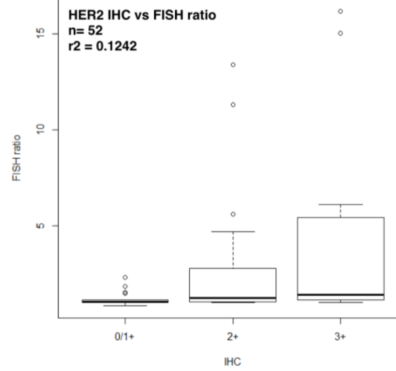


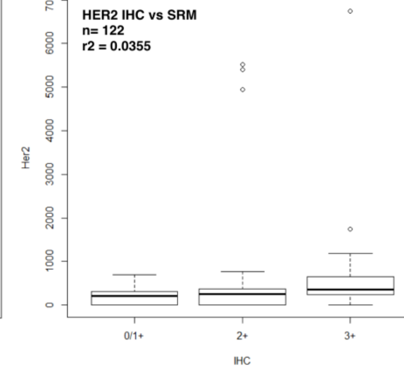
IHC to FISH		estimate	p-value	R^2
copy number	0/1+ (ref)			0.1959
	2+	0.8334	0.7319	
	3+	7.2197	0.00578	
ratio	0/1+ (ref)			0.1242
	2+	1.595	0.1461	
	3+	3.32	0.0116	

B



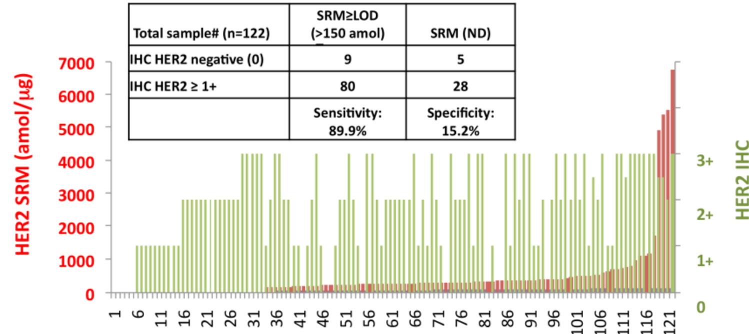
	FISH			
IHC	positive	negative	sensitivity	specificity
positive (3+)	6	8	85.7%	70.4%
negative(0/1+)	1	19		

C



	HER2-SRM <450	HER2-SRM 450-750	HER2-SRM >750	
IHC 3+	23	5	7	35
IHC 2+	36	4	4	44
IHC 0/1+	40	3	0	43
missing	13	1	3	17
	112	13	14	139

D



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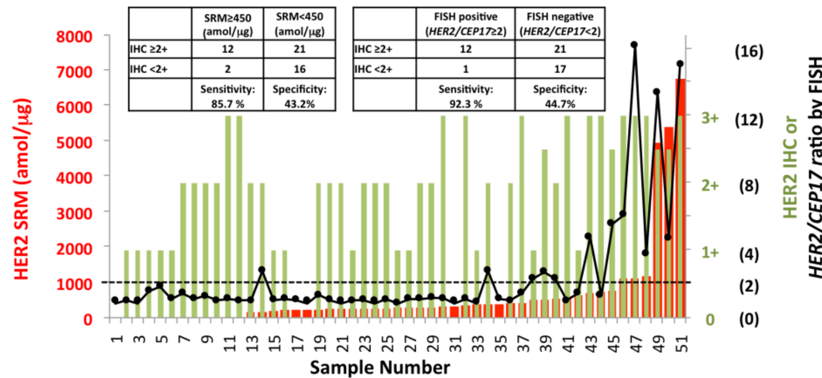
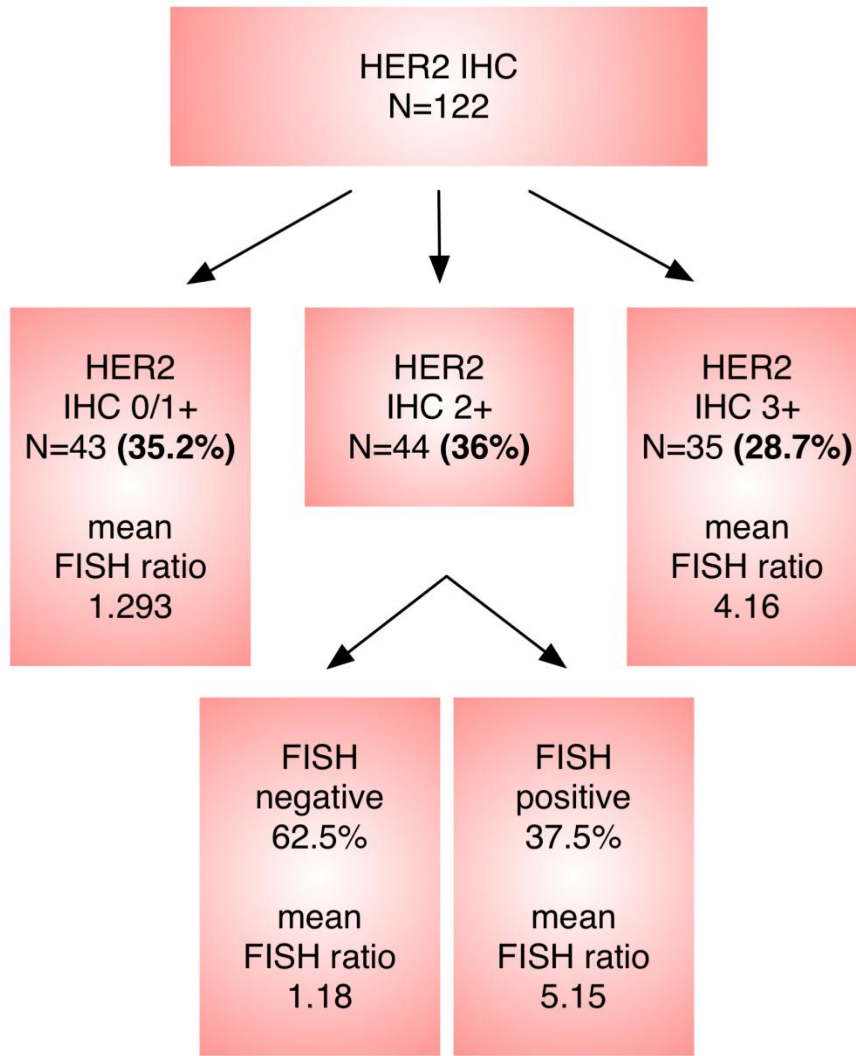
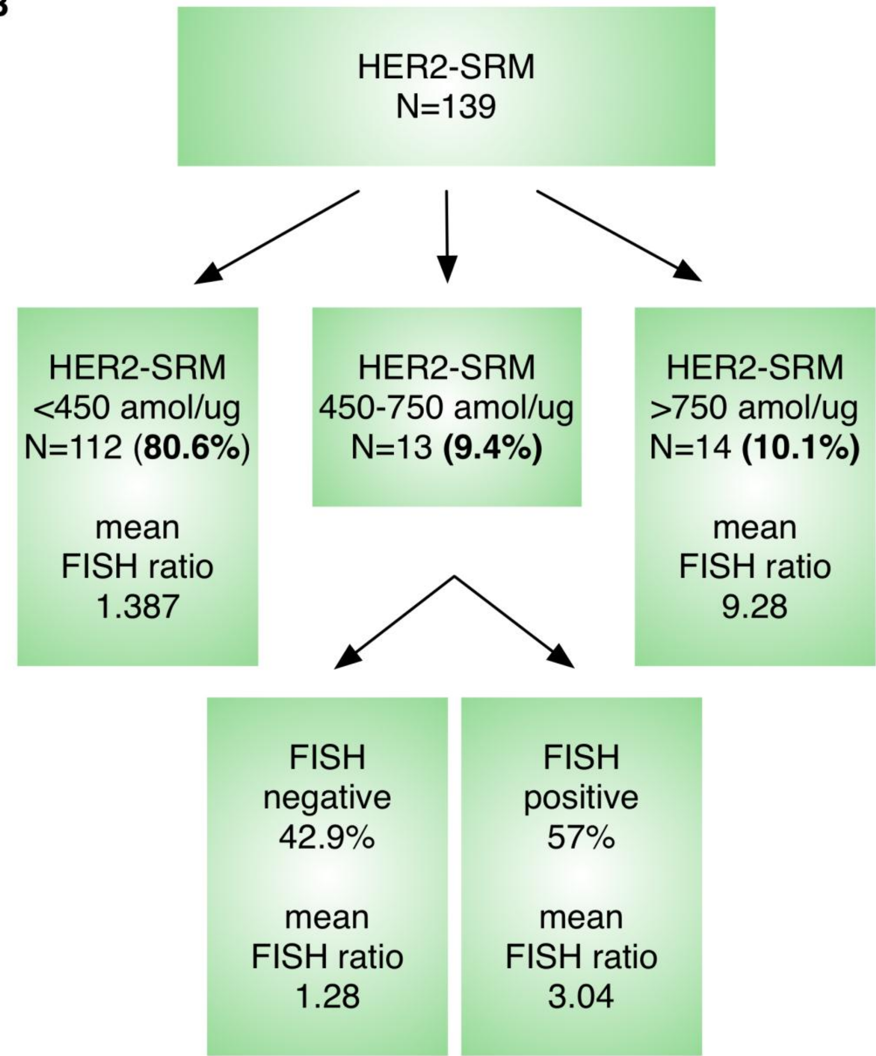
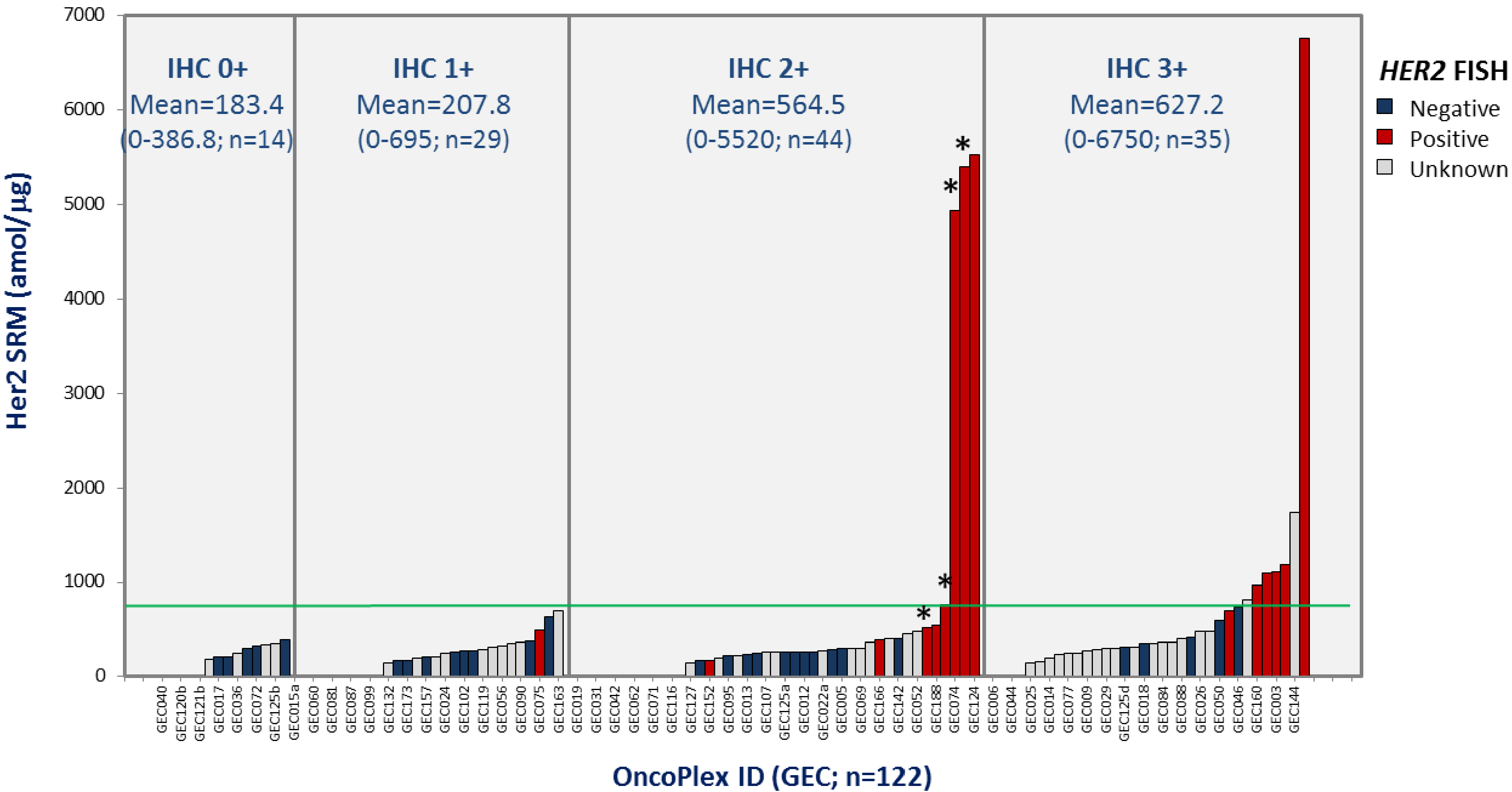


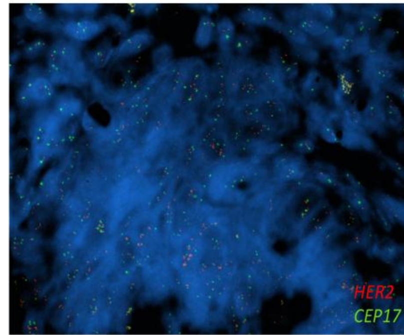

A
HER2 IHC
N=122
HER2
IHC 0/1+
N=43 (35.2%)
mean
FISH ratio
1.293
HER2
IHC 2+
N=44 (36%)
HER2
IHC 3+
N=35 (28.7%)
mean
FISH ratio
4.16
FISH
negative
62.5%
mean
FISH ratio
1.18
FISH
positive
37.5%
mean
FISH ratio
5.15

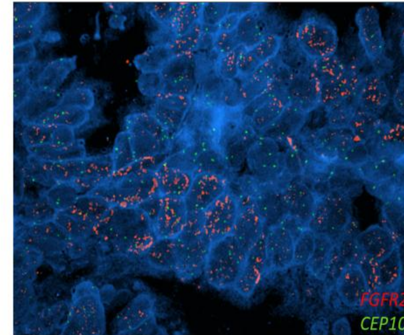

B
HER2-SRM
N=139
HER2-SRM
<450 amol/ug
N=112 (80.6%)
mean
FISH ratio
1.387
HER2-SRM
450-750 amol/ug
N=13 (9.4%)
HER2-SRM
>750 amol/ug
N=14 (10.1%)
mean
FISH ratio
9.28
FISH
negative
42.9%
mean
FISH ratio
1.28
FISH
positive
57%
mean
FISH ratio
3.04

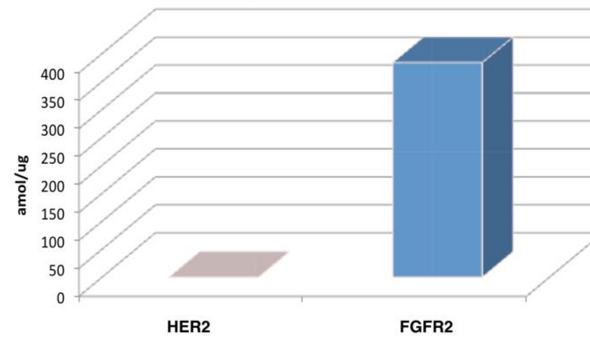


IHC 0+
Mean=183.4
(0-386.8; n=14)
IHC 1+
Mean=207.8
(0-695; n=29)
IHC 2+
Mean=564.5
(0-5520; n=44)
IHC 3+
Mean=627.2
(0-6750; n=35)
HER2 FISH
Negative
Positive
Unknown
Her2 SRM (amol/µg)
OncoPlex ID (GEC; n=122)
0
1000
2000
3000
4000
5000
6000
7000
GEC040
GEC120b
GEC121b
GEC017
GEC036
GEC072
GEC125b
GEC015a
GEC060
GEC081
GEC087
GEC099
GEC132
GEC173
GEC157
GEC024
GEC102
GEC119
GEC056
GEC090
GEC075
GEC163
GEC019
GEC031
GEC042
GEC062
GEC071
GEC116
GEC127
GEC152
GEC095
GEC013
GEC107
GEC125a
GEC012
GEC022a
GEC005
GEC069
GEC166
GEC142
GEC052
GEC188
GEC074
GEC124
GEC006
GEC044
GEC025
GEC014
GEC077
GEC009
GEC029
GEC125d
GEC018
GEC084
GEC088
GEC026
GEC050
GEC046
GEC160
GEC003
GEC144

A







HER2									FGFR2					
FISH	*HER2* Copy Number	*CEP17* Copy Number	Mean Ratio *HER2:CEP17*	NGS Gene Copy	% of Cells with Ratio ≥ 2 (5-50%)	*HER2* Heterogeneity	IHC	HER2-SRM	FISH	FGFR2 Copy Number	CEP10 Copy Number	Mean Ratio FGFR2:CEP10	NGS Gene Copy	FGFR2-SRM
Her2+ (equivocal)	4.5	3.00	1.5	6	20%	Hetero+	2+	<150	FGFR2+	>20	3.00	>20	237	384