Biotransformation of Trastuzumab and Pertuzumab in Breast Cancer Patients Assessed by Affinity Enrichment and Ion-Exchange Chromatography[S]

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ABSTRACT

Therapeutic proteins (TPs) are known to be heterogeneous due to modifications that occur during the production process and storage. Modifications may also occur in TPs after their administration to patients due to in vivo biotransformation. Ligand binding assays, which are widely used in the bioanalysis of TPs in body fluids, are typically unable to distinguish such modifications. Liquid chromatography coupled to mass spectrometry is being increasingly used to study modifications in TPs, but its use to study in vivo biotransformation has been limited until now. We present a novel approach that combines affinity enrichment using Affimer reagents with ion-exchange chromatography (IEX) to analyze charge variants of the TPs trastuzumab and pertuzumab in plasma of patients undergoing therapy for HER2-positive breast cancer. Affimer reagents were immobilized via engineered Cys tags to maleimide beads, and the TPs were eluted under acidic conditions followed by rapid neutralization. The enriched TPs were analyzed by cation-exchange chromatography (IEX)

using pH-gradient elution, resulting in the separation of about 20 charge variants for trastuzumab and about five charge variants for pertuzumab. A comparison between in vitro stressed TPs spiked into plasma, and TPs enriched from patient plasma showed that the observed profiles were highly similar. This indicates that in vitro stress testing in plasma can mimic the situation in patient plasma, as far as the generation of charge variants is concerned.

SIGNIFICANCE STATEMENT

This research attempts to elucidate the modifications that occur in therapeutic proteins (TPs) after they have been administered to patients. This is important because there is little knowledge about the fate of TPs in this regard, and certain modifications could affect their efficiency. Our results show that the modifications discovered are most likely due to a chemical process and are not patient specific.

Introduction

Bioanalysis is an important aspect of drug discovery and development, involving the identification and quantification of a drug and its metabolites in biologic fluids such as blood, urine, or tissue extracts (Pandey et al., 2010). Therapeutic protein (TP) drugs are heterogeneous and exist as ensembles of proteoforms due to their expression in cells as

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well as due to modifications occurring during production and storage (Creamer et al., 2014). Proteoforms may also be generated through modifications such as deamidations and oxidations that occur in patients after TPs have been administered (Yang et al., 2018). This process, which is termed biotransformation, may affect therapeutic efficacy and safety due to decreased target binding, a shorter half-life, or increased immunogenicity (Yao et al., 2018). Modifications may also affect the response in bioanalytical assays, leading to variable results (Yao et al., 2018). So far, only a few studies have been done on the biotransformation of TPs. Initial insights have been obtained in a study by Bults et al. (2016), who reported the in vivo deamidation of trastuzumab in plasma samples of breast cancer patients. To gain a more comprehensive view, it is necessary to develop methods that can successfully enrich and separate proteoforms resulting from the biotransformation of TPs to characterize them and assess their biologic activity.

Trastuzumab and pertuzumab are monoclonal antibodies used in the therapy of human epidermal growth factor receptor-2 (HER2)-positive breast cancer (Scheuer et al., 2009; Swain et al., 2015; von Minckwitz et al., 2017), opening new treatment options and increasing overall survival (Nahta and Esteva, 2007; Gianni et al., 2016; Ishii et al., 2019). However, certain patients have been reported to exhibit a poor response

(Shang et al., 2022) after being treated with these antibodies (Nahta and Esteva, 2006; Rimawi et al., 2015; de Melo Gagliato et al., 2016). Mechanisms that involve resistance to therapy include steric hindrance of trastuzumab from binding to the HER2 surface receptor by increased expression of the membrane-associated glycoprotein MUC4, elevated levels of serum HER2 extracellular domain, activation of the PTEN and PI3K signaling pathways, and increased signaling through the insulin-like growth factor-I receptor (IGF-IR) (Nahta and Esteva, 2006). Since biotransformation has been mentioned earlier to result in reduced efficacy of TPs (Yao et al., 2018), it is of interest to understand whether there is a link to a poor therapeutic response in certain patients. However, the methodology to study this is currently lacking.

Ligand binding assays (LBAs) are the conventional method of quantifying TPs (An et al., 2014) in biologic samples by making use of the highly specific interaction between an affinity ligand and its target protein (Hulme and Trevethick, 2010). Although LBAs have been shown to provide the necessary sensitivity and specificity toward their targets (Ezan et al., 2009), they have certain disadvantages. For example, the selectivity of LBAs may be affected by components in the biologic matrix, making them matrix dependent (An et al., 2014; Spengler et al., 2015). This is because LBAs may be affected by competing ligands such as circulating fragments of the target protein, antidrug antibodies, or the nature of the affinity ligand (e.g., monoclonal versus polyclonal antibodies) (Zheng et al., 2014; Bults et al., 2016; Gao et al., 2018). LBAs are furthermore unable to differentiate between the proteoforms of a TP, such as its biotransformation products (An et al., 2014).

Liquid chromatography coupled to mass spectrometry (LC-MS) is an alternative method that is beginning to complement LBAs in the bioanalysis of TPs (van den Broek et al., 2013; van de Merbel, 2019). In comparison with LBAs, LC-MS can distinguish between a parent drug and its biotransformation products as it provides chemical information at the molecular level (Zheng et al., 2014), but for many proteins, it is still unable to offer sensitivities that are comparable to those of LBAs (van den Broek et al., 2013). It is also important to note that affinity enrichment is often necessary prior to LC-MS to improve sensitivity, especially in the bioanalysis of TPs in matrices such as plasma or serum, which contain high levels of endogenous proteins (van de Merbel, 2019).

The mass spectrometry (MS) analysis of TPs may be approached in different ways. Bottom-up MS is the most widely used approach, in which proteases such as trypsin are used to generate peptides from TPs that are subsequently quantified as a measure for the original intact protein (Chait, 2006). It requires the selection of one or more peptides that are unique to the protein of interest for identification and quantification (Higdon and Kolker, 2007; Zhao and Lin, 2010). The increase in sample complexity, because of the digestion process, presents a disadvantage of bottom-up MS (Gregorich et al., 2014). The focus on peptides results in a reduced percentage of the protein sequence being analyzed, with the risk that vital information on modifications (e.g., PTMs or modifications due to biotransformation) may be lost. Another important drawback of bottom-up MS is that modifications are taken out of the context of the respective proteoform. Unless a proteoform has been purified to homogeneity, it is thus impossible to assign a particular modification to a given proteoform (Catherman et al., 2014; Gregorich et al., 2014). In top-down MS, intact proteins are directly analyzed without digestion (Chait, 2006). This technique allows assigning modifications in the context of the entire protein. However, top-down MS is still under development, and the large diversity in physicochemical properties of intact proteins, when compared with peptides, still poses a major challenge (Gregorich et al., 2014). Other challenges include a reduced sensitivity due to broad isotopic distributions and multiple charge states of intact proteins (Catherman et al., 2014). Top-down MS has been able to analyze some heterogeneity in smaller-sized proteins (Wood et al.,

1995; Robinson et al., 2006) but has been of limited value for the analysis of entire antibodies and their proteoforms. Middle-down MS is an approach that represents a compromise between bottom-up and top-down MS. It involves the limited digestion of proteins to generate large peptides or protein fragments before MS analysis (Sidoli et al., 2015). Larger peptides retain more information about modifications in their original sequence context while limiting the challenges of having to analyze proteins of very high molecular weights as in top-down MS (Sidoli et al., 2017). Although some studies have analyzed antibodies by middle-down MS (Wang et al., 2013; Srzentić et al., 2014; Biacchi et al., 2015; Pan et al., 2016; Sokolowska et al., 2017), none of them has yet attempted to follow their biotransformation in patients.

Due to the limitations of the methods described above in terms of studying the heterogeneity of TPs, we describe a novel approach to analyzing proteoforms of trastuzumab and pertuzumab in plasma samples of breast cancer patients. Our method combines affinity enrichment using tailor-made Affimer reagents with ion-exchange chromatography (IEX) for proteoform separation due to charge variants. We have previously detailed the efficiency of an affinity enrichment method for trastuzumab and pertuzumab in a study by Olaleye et al. (2021) as well as an IEX method for separating charge variants of trastuzumab in another study by Spanov et al. (2021). The ability of this combined approach in detecting proteoforms in patients provides insight into the fate of these TPs once they have been administered. A future application of this approach has the goal of establishing a possible link between some of the observed modifications and response to therapy.

Materials and Methods

Trastuzumab(Herceptin, Lot. no. N7185H03) and pertuzumab (Perjeta; lot. no. H0319H03) were purchased from Roche (Almere, The Netherlands). Anti-trastuzumab and antipertuzumab Affimer reagents (antitrastuzumab 386_737_C3 and anti-pertuzumab 00557_709213) were produced and supplied by Avacta Life Sciences (Wetherby, UK). Gibco Dulbecco's PBS (10×; cat. no. 14200-067) was purchased from Thermo Fisher Scientific (Roskilde, Denmark). Human plasma (K$_2$EDTA) was obtained from BioIVT (Burgess Hill, UK). SoluLyse Reagent for Bacteria (cat. no. L100125) was obtained from Genlantis (San Diego, California). Maleimide Irreversible Thiol-Coupling SepFast MAG 4HF beads (cat. no. 390201—5 mL) were purchased from BioToolomics (Durham, UK). DMSO (cat. no. 34943—1 L), Eppendorf Deepwell Plates, Protein LoBind, 96-wells(cat. no. 0030504119), Eppendorf Protein LoBind tubes (0.5 mL—cat. no. 0030108434 and 2 mL—cat. no. 0030108132), sodium tetraborate (Na$_2$B$_4$O$_7$; cat. no. 71997—100 G), sodium acetate (cat no. S2889—250 G), acetic acid (cat no. A6283—1 L), Tween 20 (cat. no. P5927—500 mL), 2-Hydroxy-3-morpholinopropanesulfonic acid (MOPSO; cat. no. M8389—100 G), HEPES (cat no. H4034—100 G), N,N-bis(2-hydroxyethyl)glycine (bicine; cat. no. B3876—25 G), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO; cat no. C2278—100 G), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; cat. no. C6070—100 G), and HCl (≥37%; cat. no. 30721—1 L) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Glycine (analytical grade; cat. no. 23390.04) was obtained from Serva (Heidelberg, Germany).

Patient Sample Analysis. Blood samples were collected from female patients with stage II–III HER2-positive breast cancer, treated at the Netherlands Cancer Institute, who were participating in a nationwide clinical trial (NCT03820063/BOOG 2018–01 TRAIN-3). Breast cancer diagnosis was histologically proven with core biopsies, and HER2 status was locally assessed according to the American Society of Clinical Oncology/College of American Pathologists guideline(Wolff et al., 2013). All patients received 3–9 cycles of trastuzumab and pertuzumab combined with chemotherapy as neoadjuvant treatment. Both hormone receptor–negative (estrogen receptor and progesterone receptor expression < 10%) and -positive (estrogen receptor and/or progesterone receptor expression ≥ 10%) patients were eligible. Trastuzumab (6 mg/kg, with a loading dose of 8 mg/kg at the first treatment cycle only) and pertuzumab (420 mg, with an 840-mg loading dose at the first treatment cycle only) were administered intravenously every 3 weeks for 1 year. Blood withdrawal took place 3 weeks after the previous dose, before the first

TABLE 1

Female patient characteristics

	Participants (n = 8)
Age in years, median (IQR)	54.0 (41–59)
Menopausal status, n (%)	
Pre/perimenopausal	5 (62.5%)
Post	3 (37.5%)
Clinical tumor stage, n (%)	
cT0	0 (0%)
cT1	2 (25%)
cT2	6 (75%)
Clinical lymph node status, n (%)	
Negative	4 (50%)
Positive	4 (50%)
Stage, n (%)	
II	6 (75%)
III	2 (25%)
HER2 status, n (%)	
3+ at IHC	8 (100%)
ER status, n (%)	
Positive (≥10%)	4 (50%)
Negative (<10%)	4 (50%)
PR status, n (%)	
Positive (≥10%)	2 (25%)
Negative (<10%)	6 (75%)
Tumor grade, n (%)	
Grade 2	1 (12.5%)
Grade 3	7 (87.5%)
Histology, n (%)	
NST/ductal	8 (100%)
Pathologic outcome, n (%)	
Pathologic complete response (ypT0/is, N0)	5 (62.5%)
No pathologic complete response	3 (37.5%)

ER, estrogen receptor; IHC, immunohistochemistry; IQR, interquartile range; NST, no special type; PR, progesterone receptor.

day of the next cycle. Plasma was harvested by centrifugation immediately after blood collection in EDTA-containing blood collection tubes and stored at −70°C until analysis. Patients were referred for surgery in case of a radiologic complete remission, nine cycles of chemotherapy, or in case of limiting toxicity. Radiologic response evaluation was performed every three cycles and included dynamic contrast-enhanced magnetic resonance imaging ofthe breast and cytology or histology of the baseline marked pathologic lymph node in the case of clinical lymph node—positive disease. In hormone receptor–positive cases, vacuum-assisted core biopsies were also part of the response evaluation after three and six cycles. Surgical response was locally assessed, and pathologic complete response was defined as the absence of invasive breast cancer cells in the breast and axilla, irrespective of the presence of in situ lesions. Table 1 summarizes the clinical characteristics of the patients in the study. All patients included in this analysis provided written informed consent for participation in the clinical trial and specifically for blood withdrawal for translational research purposes. The study protocol and all amendments were approved by the medical ethics committee of the Netherlands Cancer Institute. Plasma samples from eight patients were prioritized for analysis based on the concentrations of trastuzumab and pertuzumab, respectively, as measured by LC-MS in the selected reaction monitoring modeaccording to Bults et al. (2022). Subjects having a concentration of at least 80 μg/mL at various timepoints were selected for the enrichment process. The median age at registration of the study was 54 years, and most patients were pre- or perimenopausal. Half of the patients were lymph node–positive, and most (75%) were breast cancer stage II. All tumors showed HER2 overexpression (immunohistochemistry 3+), and half were estrogen receptor–negative. Half of the estrogen receptor–positive tumors were also progesterone receptor–positive. All patients had invasive carcinoma of no special type. Five out of the eight patients had a pathologic complete response at surgery after neoadjuvant chemotherapy plus HER2-directed antibodies of varying duration.

In Vitro Stressing of Trastuzumab and Pertuzumab. Both antibodies were spiked separately at 2 mg/mL in PBS (pH 7.4), after which the corresponding samples were aliquoted into 100 μL portions in Eppendorf protein LoBind tubes. The samples were incubated at 37°C, with aliquots removed after 1 and 2 weeks. Aliquots were stored at −80°C until analysis.

Enrichment Process. Maleimide beads were washed three times with coupling buffer (5% DMSO in 50 mM sodium borate, pH 9), and 40 μL was then incubated with 12 μg of Affimer reagent (300 μg/mL) in coupling buffer overnight at room temperature and 700 rpm in the dark. The coupling process was performed separately for the antitrastuzumab and antipertuzumab Affimer reagents in one batch with the coupling mixture (coupling buffer containing
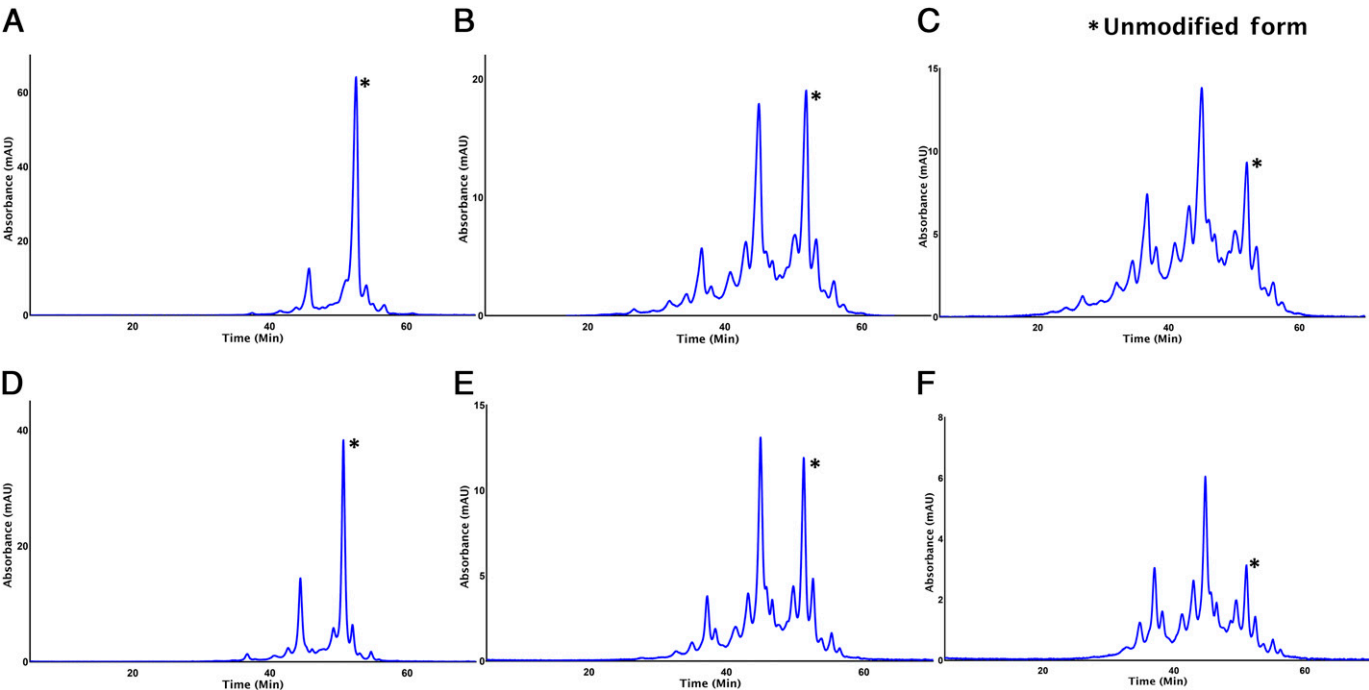

Fig. 1. IEX analysis of trastuzumab charge variants resulting from in vitro stressing in PBS at 37°C. (A), (B), and (C) are from the direct injection of unstressed and 1 week- and 2 week–stressed preparations, and (D), (E), and (F) are from the same preparations after spiking into plasma followed by capture with an antitrastuzumab Affimer reagent.
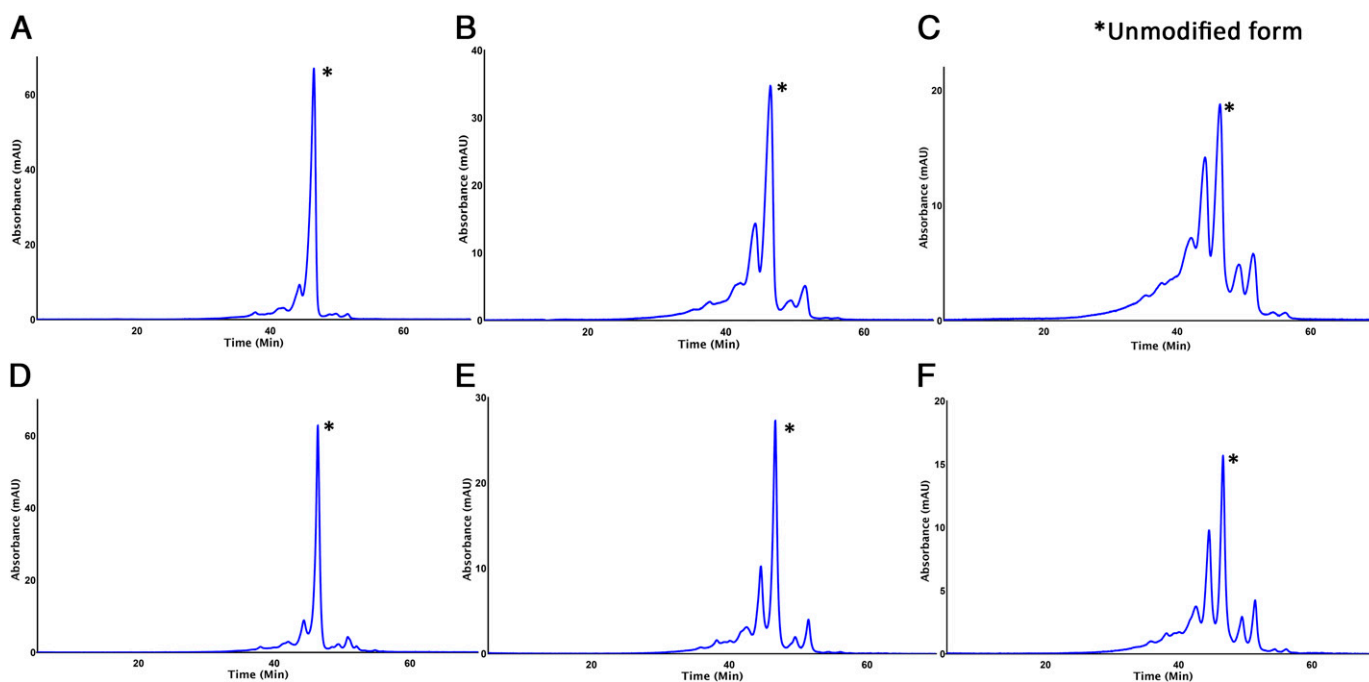
Fig. 2. IEX analysis of pertuzumab charge variants resulting from in vitro stressing in PBS at 37°C. (A), (B), and (C) are from the direct injection of unstressed and 1 week- and 2 week–stressed preparations, and (D), (E), and (F) are from the same preparations after spiking into plasma followed by capture with an antipertuzumab Affimer reagent.

Affimer reagents), constituting 50% of the bead volume. After the coupling process, the maleimide beads were washed three times with coupling buffer and then incubated with 50 mM cysteine in coupling buffer for 1 hour at room temperature and 700 rpm to block unreacted sites. After blocking, the beads were washed with Tris-HCl plus 0.5M NaCl (pH 8) followed by 0.1 M sodium acetate buffer (pH 4) containing 0.5 M NaCl and, finally, with wash buffer (0.05% Tween-20 in PBS). PBS was added to the plasma samples in a 1:1 ratio, and 100 μL of this mixture was added to 40 μL antitrastuzumab Affimer (No. 386_737_C3) functionalized beads in protein Lobind plates and incubated for 1.5 hours at room temperature and 700 rpm to capture trastuzumab. The samples were then transferred to another plate containing 40 μL antipertuzumab Affimer reagent (No. 00557_709213) functionalized beads and incubated for 1.5 hours at room temperature and 700 rpm to capture pertuzumab. After the capture process, beads were washed three times with wash buffer (transferred to fresh protein Lo-Bind plates after the second wash step) and an additional three times with 1x PBS to remove excess Tween-20.

Elution of Captured TPs. The beads were transferred to 2 mL protein Lo-bind tubes and incubated with 20 μL of 50 mM glycine HCl (pH 3) at room temperature and 350 rpm for 15 minutes. The resulting supernatant was transferred to 0.5 mL protein Lobind tubes containing 10 μL of 1.5 M MOPSO (pH 7.5) for neutralization. The elution process was repeated 6 times, producing a final volume of 370 μL for each sample.

IEX Analysis. An Agilent 1200 high-performance liquid chromatography system containing a MabPac SCX-10 column (4 × 250 mm, 5 μm, Thermo Fisher Scientific, cat. no. 078655) was used for the separation of trastuzumab and pertuzumab charge variants with pH gradient buffers. pH gradient buffers were prepared as described by Lingg et al. (2013) and Spanov et al. (2021). Buffer A (HEPES, Bicine, CAPSO, CAPS) had a pH of 8.0, and buffer B had a pH of 10.5 (Bicine, CAPSO, CAPS), respectively. Charge variants were eluted with a linear gradient of B, changing from 0% to 60% over 10 column volumes at 0.5 mL/min (62.8 minutes at a flow rate of 0.5 mL/min) and room temperature. The autosampler temperature was set to 10°C. UV absorbance was measured at 280 nm. A pH/C-900 unit (Amersham Biosciences) with a pH electrode and flow cell was coupled after the UV detector to follow the pH change online over the gradient. The processed data were exported for visualization in Graph-Pad Prism 8 (GraphPad Software, Inc., San Diego, CA).

Bioinformatics and Statistical Analysis. Liquid chromatography (LC) traces were exported in comma-separated values format. An in-house MATLAB script was used for the linear interpolation of all traces to the time vector of one reference trace and to align all possible pairs of interpolated LC traces using correlation-optimized warping (Tomasi et al., 2004). The interpolated, aligned traces between 10 and 60 minutes were used to calculate a similarity score. The similarity scores are based on the overlapping peak area between two interpolated, aligned LC traces divided by the geometric mean of the area under the curve of the two traces. The similarity scores were arranged in a matrix in row and column indexes corresponding to the pairs of LC traces that were used for the similarity score calculation. This matrix was used to visualize similarities in a heatmap after rearranging the order of LC traces in the matrix by hierarchical clustering using average linkage. Processing was done using MATLAB 2021b on a PC equipped with 64 GB RAM and an Intel Core-i 8700K CPU with a 1TB SSD.

Results and Discussion

We have previously reported the application of pH gradient cation-exchange chromatography to study the change in charge variant composition of trastuzumab upon stressing under physiologic conditions (Spanov et al., 2021). After 3 weeks of stressing trastuzumab in PBS (pH 7.4), approximately 84% of charge variants eluted earlier in the pH gradient than the main form (acidic variants), and 16% eluted later (basic variants). The primary source of charge heterogeneity was due to modifications in the Fab region of trastuzumab, namely deamidation of asparagine in the light (Lc-Asn-30) and heavy (Hc-Asn-55) chains, aspartic acid isomerization in the heavy chain (Hc-Asp-102), and N-terminal pyroglutamate formation in the heavy chain. Two acidic and two basic variants were observed when pertuzumab was stressed in PBS (pH 7.4) at 37°C for up to 2 weeks (unpublished data). Acidic variants were due to deamidations in the Fc domain, whereas basic variants were due to N-terminal pyroglutamate formation in the heavy chain of pertuzumab.

The ability of the Affimer reagents to bind trastuzumab and pertuzumab variants produced during in vitro stressing in PBS was assessed to
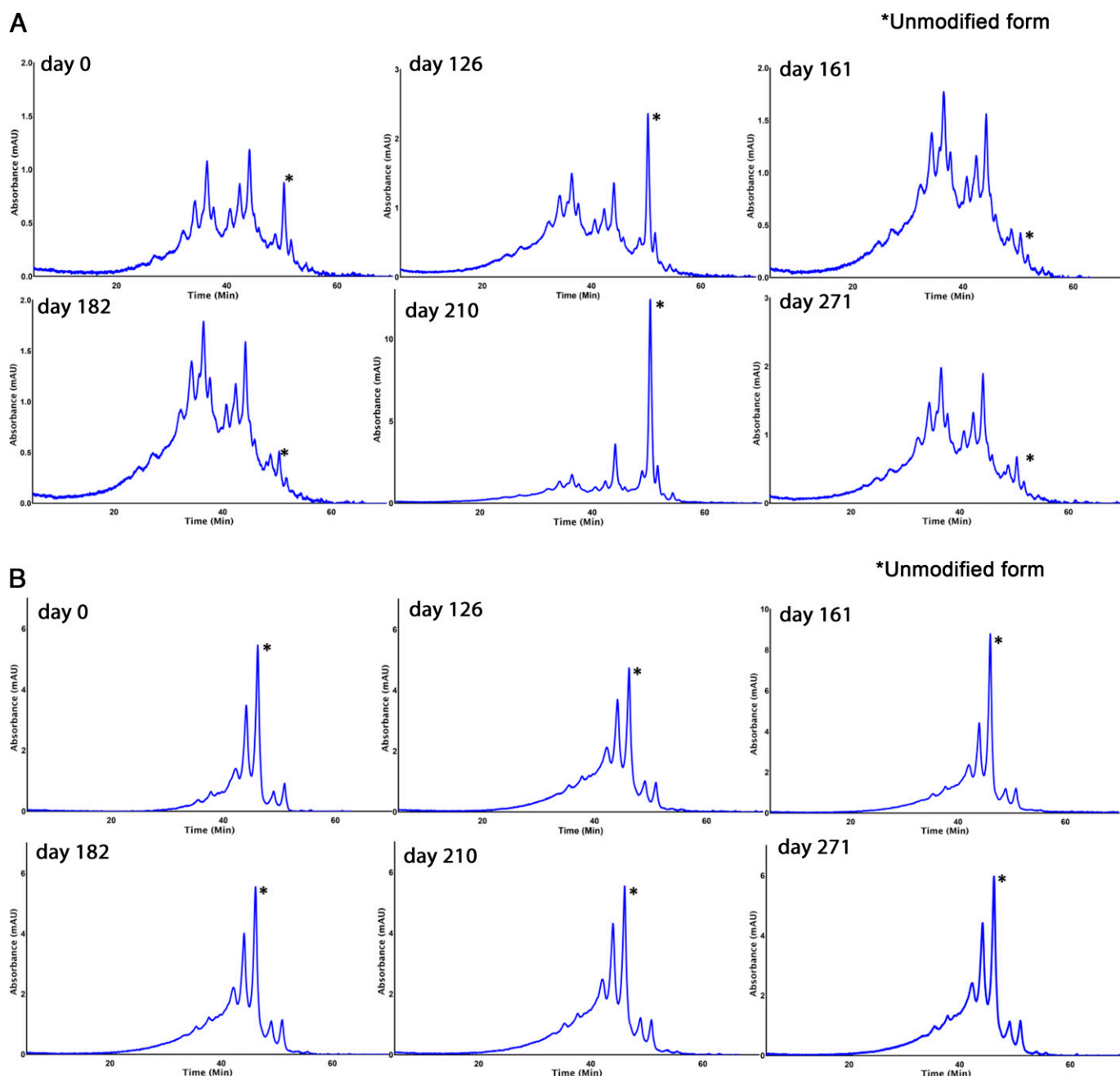
Fig. 3. IEX analysis of plasma samples from one patient at six timepoints after enrichment of trastuzumab (A) and pertuzumab (B) with the Affimer reagents. Day 0 refers to the day when the first plasma sample was collected from this patient (not a predose sample).

provide insight into how well these Affimer reagents will bind to charge variants of both antibodies that may result from biotransformation in breast cancer patients. To study this, unstressed, 1 week- and 2 week–stressed trastuzumab and pertuzumab were spiked in plasma at 150 μg/mL. The spiked antibodies were then captured, eluted, and analyzed by IEX, and the results were compared with the direct analysis of the unstressed, 1 week- and 2 week–stressed trastuzumab and pertuzumab (40 μg) preparations used for spiking. Figure 1 shows the chromatograms of trastuzumab, and Fig. 2 shows the corresponding chromatograms of pertuzumab. For both therapeutic antibodies, very similar charge variant profiles were obtained with and without capturing by the Affimer reagents, indicating that the Affimer reagents captured all charge variants that were generated during in vitro stressing. It is

noteworthy that significantly more charge variants of trastuzumab resulted from the stressing process when compared with pertuzumab.

Analysis of Patient Samples. Plasma samples from patients were analyzed at various timepoints throughout the treatment period. Figure 3 shows the chromatograms obtained from the analysis of one patient as an example (panel A: trastuzumab; panel B: pertuzumab). As observed during in vitro stress testing, in vivo biotransformation results in considerably more charge variants for trastuzumab than for pertuzumab. Although not identical, profiles at the various timepoints are rather similar, except for day 126 and, notably, day 210 (Fig. 3A), which show mainly the unmodified form of trastuzumab. The profiles for pertuzumab at the different timepoints are all very similar, comprising far fewer charge variants than for trastuzumab (Fig. 3B). Although the samples were taken
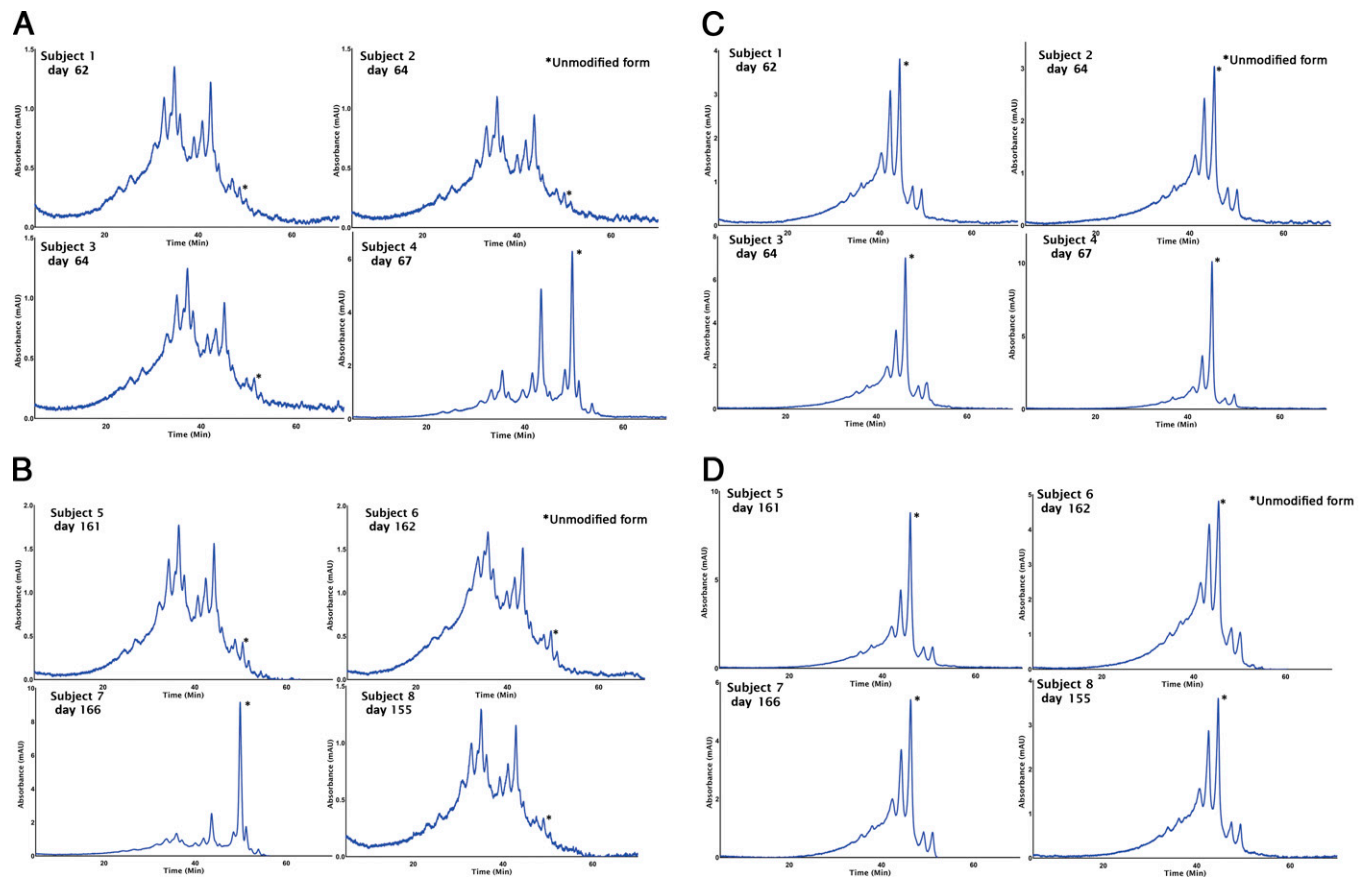
Fig. 4. IEX analysis of samples from eight different patients after enrichment of trastuzumab (A and B) and pertuzumab (C and D) with the Affimer reagents. (A) and (C) show the charge variant profile of trastuzumab and pertuzumab, between days 62 and 67, and (B) and (D) show the charge variant profile of trastuzumab and pertuzumab, respectively, between days 155 and 166

from the same patient, the reason for this discrepancy on days 126 and 210 is likely because these samples were collected directly after injecting a new dose of trastuzumab and before a new dose of pertuzumab. It is important to note that trastuzumab and pertuzumab are dosed separately. Figure 4 shows the results from eight different patients across two time

ranges (panels A and B: trastuzumab from day 62 to 67 and day 155 to 166, respectively; panels C and D: pertuzumab from day 62 to 67 and day 155 to 166, respectively). The profiles of both trastuzumab and pertuzumab are similar across different patients throughout two different time ranges. As mentioned earlier, there are again two instances [subject
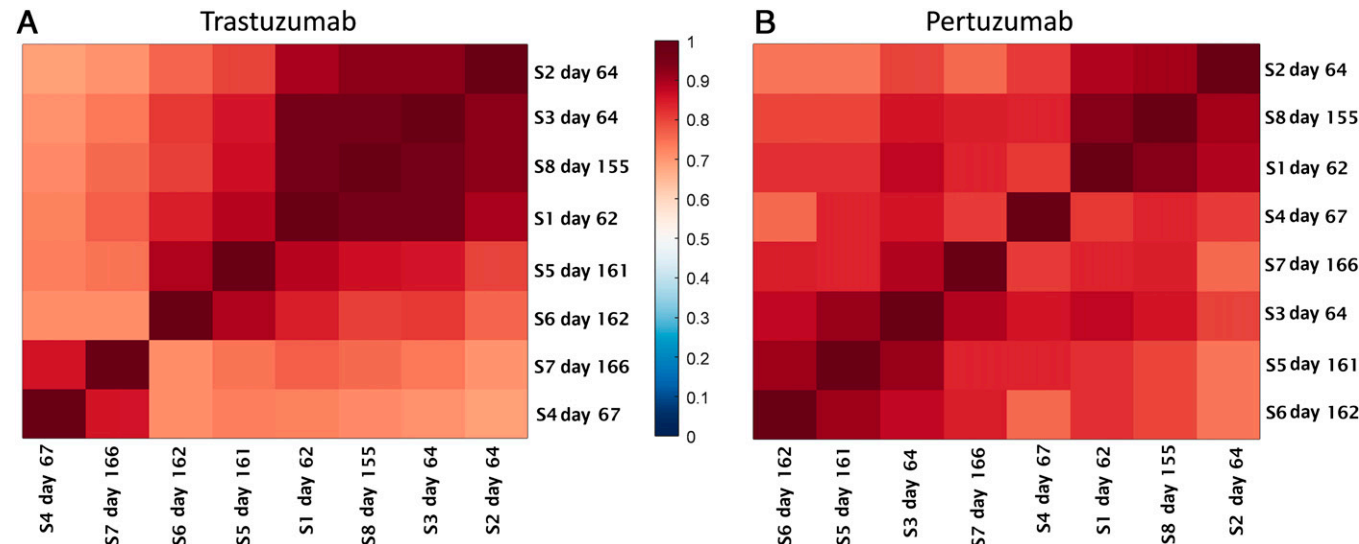

Fig. 5. Heatmaps showing similarity of trastuzumab (A) and pertuzumab (B) LC-UV traces between eight patients. Similarity score has a range between 0 (completely different LC-UV traces) and 1 (identical LC-UV traces). The order of LC-UV traces in rows and columns was arranged after hierarchical clustering. S1–S8, subjects 1–8.

4, day 67 (Fig. 4A) and subject 7, day 166 (Fig. 4B)] where samples were collected after injecting a new dose of trastuzumab.

Similarity between Charge Variant Profiles. Ion-exchange chromatography is sensitive to experimental parameters such as small changes in pH, salt concentration, buffering capacity, and temperature of the chromatographic column. These small variations of experimental parameters often lead to nonlinear retention time shifts, which must be corrected prior to comparing profiles (Mitra et al., 2014; Mitra et al., 2018). Automated alignment was performed using correlation-optimized warping of the liquid chromatrography-ultraviolet (LC-UV) traces interpolated to the same retention time measurement points (Tomasi et al., 2004). Supplemental Figure 1 (supporting information) shows the raw, interpolated, and correlation-optimized warping–aligned LC-UV traces. The aligned chromatograms of trastuzumab and pertuzumab show highly similar, characteristic profiles. LC-UV trace similarity was quantified by measuring the area under the curve of the overlap between two traces divided by the geometric mean of the area under the curve of the two individual traces (Suits et al., 2008; Ahmad et al., 2011). Figure 5 shows a heatmap of the similarity scores between all possible pairs of chromatograms following hierarchical clustering (Supplemental Figure 2, supporting information). The heatmap indicates a high average similarity of more than 0.6 (60% of overlap of peaks between the two traces) between the traces for each TP. The pertuzumab traces show, on average, higher similarity than for trastuzumab. Trastuzumab shows clustering for two patients (subject 4, day 67 and subject 7, day 166; postdose sampling as explained above).

Conclusion

We describe a novel approach that combines affinity enrichment for capturing and IEX for separating charge variants of trastuzumab and pertuzumab from the plasma of breast cancer patients. The Affimer reagents used for the enrichment were initially applied to *in vitro* stressed samples of trastuzumab and pertuzumab that were spiked in plasma, showing that they are able to enrich all of the observed charge variants. It is important that the approach does not introduce a bias with respect to certain variants that may be relevant in terms of clinical efficacy or side effects. For example, it has been shown that a certain modification on trastuzumab resulted in the loss of recognition by anti-idiotypic antibodies, affecting the results of an ELISA (Bults et al., 2016). The ability of the Affimer reagents to bind to the different charge variants is further supported by the binding site mapping study performed by Olaleye et al. (unpublished data) and the fact that the binding sites of the Affimer reagents on trastuzumab and pertuzumab were not affected by any of the observed modifications (Spanov et al., 2021; unpublished data).

The similarity of charge variants observed upon in vitro stressing and in patient samples as well as across samples from different patients indicates that the formation of these variants is most likely due to a chemical process and is not patient specific. This argues against a possible link between the response to therapy and the biotransformation of trastuzumab and pertuzumab as far as concerns charge variants. However, a larger and more systematic study is required, taking additional modifications into account to conclude on this point, since hierarchical clustering of the chromatographic traces showed minor differences, resulting in three clusters.

Authorship Contributions

Participated in research design: Olaleye, Spanov, van de Merbel, Bischoff.
Conducted experiments: Olaleye, Spanov, Bults.
Performed data analysis: Olaleye, Spanov, Bults, Horvatovich.
Wrote or contributed to the writing of the manuscript: Olaleye, Spanov, van der Voort, Sonke, Govorukhina, Horvatovich, van de Merbel, Bischoff.

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Biotransformation of Trastuzumab and Pertuzumab in Breast Cancer Patients assessed by Affinity Enrichment and Ion Exchange Chromatography

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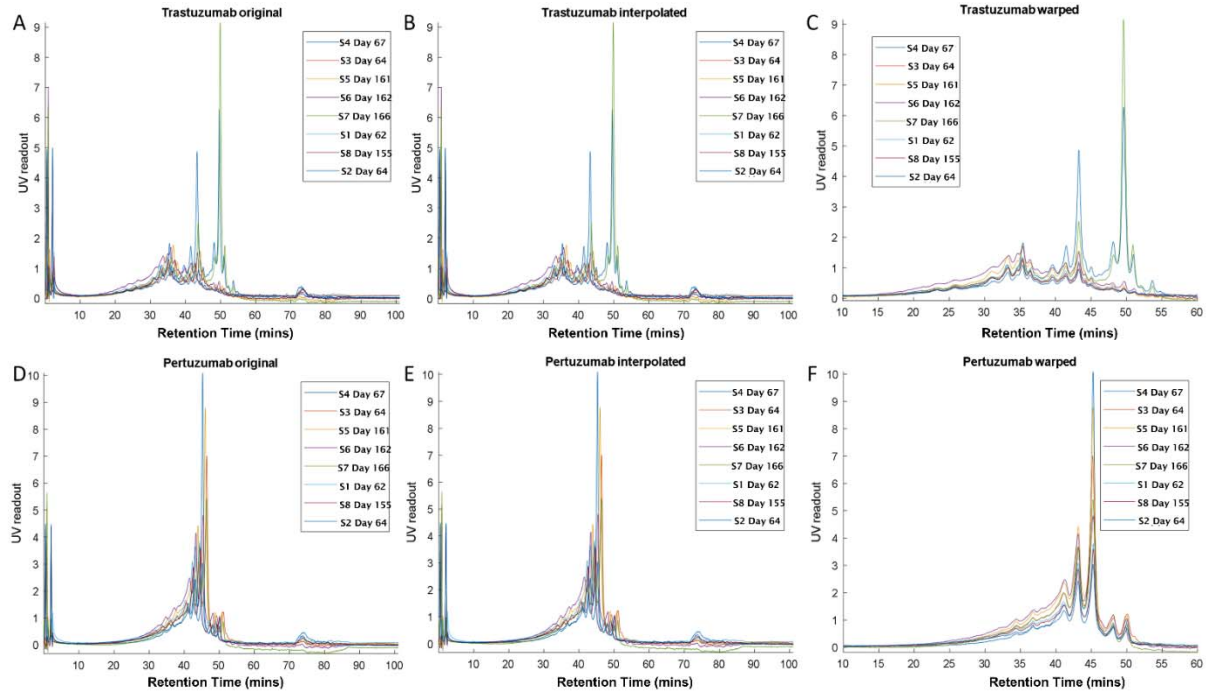
Figure S1: Overlaid LC-UV traces of trastuzumab (plots A, B and C) and pertuzumab (plots D, E and F) extracted from the plasma of 8 patients at different time points. LC-UV traces are shown in original form (A and D), after interpolation (B and E) and after interpolation with time alignment (C and F). The original and interpolated traces are shown between 0-100 minutes, while the interpolated and aligned traces are shown between 10-60 minutes, as this was the range from which the similarity score was calculated. S1-S8 refers to Subjects 1 to 8.
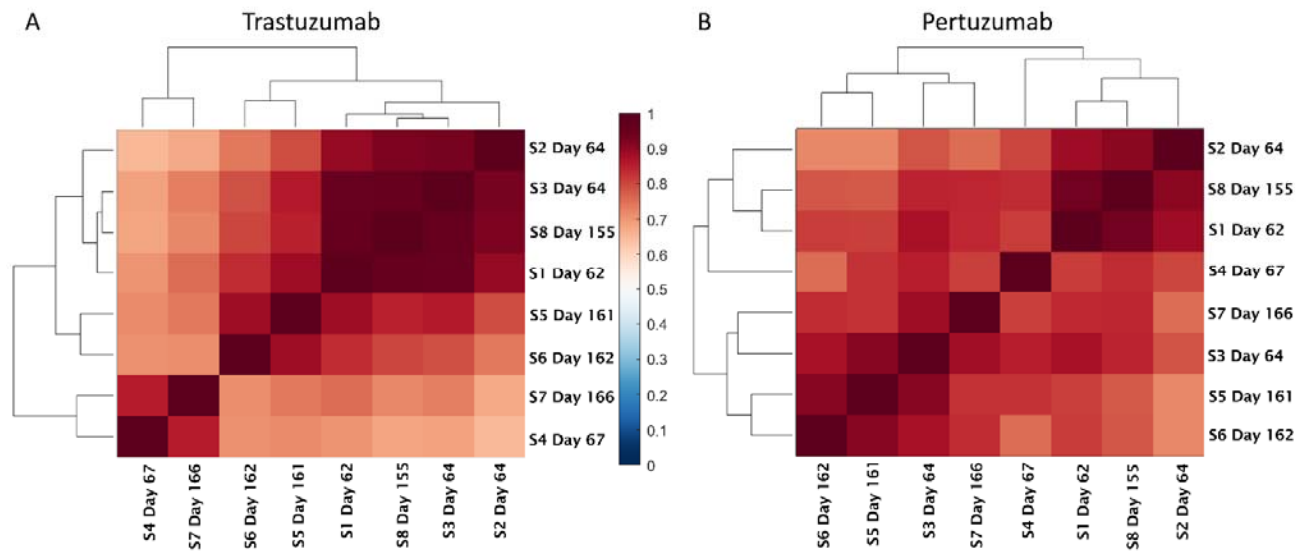
Figure S2: Hierarchical clustering of the heatmaps of Trastuzumab (A) and Pertuzumab (B) LC-UV traces between 8 patients. Similarity score has a range between 0 (completely different LC-UV traces) and 1 (identical LC-UV traces). The order of LC-UV traces in rows and columns was arranged after. S1-S8 refers to Subjects 1 to 8.