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

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List of Abbreviations
ER – estrogen receptor
HER2 – Human epidermal growth factor receptor-2
HR – Hormone receptor
IEX – Ion-exchange chromatography
IHC – Immunohistochemistry
IQR – Interquartile range
LBA – Ligand binding assays
LC – Liquid chromatography
MS – Mass spectrometry
NST – No special type
PBS – Phosphate Buffered Saline
PR – progesterone receptor
SRM – Selected reaction monitoring
TP – Therapeutic protein
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 (LC-MS) 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 5 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 biological 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 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 biological 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 (Gianni et al., 2016; Ishii et al., 2019; Nahta and Esteva, 2007). However, certain patients have been reported to exhibit a poor response (Shang et al., 2022) after being treated with these antibodies (de Melo Gagliato et al., 2016; Nahta and Esteva, 2006; Rimawi et al., 2015). 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 biological samples by making use of the highly specific interaction between an affinity ligand and its target protein (Hulme and Trevethick, 2010). While 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 biological 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, anti-drug antibodies or the nature of the affinity ligand (e.g. monoclonal versus polyclonal antibodies) (Bults et al., 2016; Gao et al., 2018; Zheng et al., 2014). 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 de Merbel, 2019; van den Broek et al., 2013). In comparison to 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 (Merbel, 2019).

The 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, 2006; 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 to 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 (Robinson et al., 2006; Wood et al., 1995) 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). While some studies have analyzed antibodies by middle-down MS (Biacchi et al., 2015; Pan et al., 2016; Sokolowska et al., 2017; Srzentić et al., 2014; Wang et al., 2013), 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 anti-pertuzumab Affimer® reagents (anti-trastuzumab 386_737_C3 and anti-pertuzumab 00557_709213) were produced and supplied by Avacta Life Sciences (Wetherby, U.K.). Gibco Dulbecco’s phosphate-buffered saline (DPBS; 10×; cat. no. 14200-067) was purchased from Thermo Fisher Scientific (Roskilde, Denmark). Human plasma (K$_2$EDTA) was obtained from BiolVT (Burgess Hill, United Kingdom). SoluLyse Reagent for Bacteria (cat. no. L100125) was obtained from Genlantis (San Diego, California, USA). Maleimide Irreversible Thiol-Coupling SepFast MAG 4HF beads (cat. no. 390201-5mL) were purchased from BioToolomics (Durham, United Kingdom). Dimethyl sulfoxide (DMSO; cat. no. 34943-1L), Eppendorf® Deepwell plates, Protein LoBind®, 96 wells (cat. no. 0030504119), Eppendorf® Protein LoBind® tubes (0.5mL – cat. no. 0030108434 and 2mL – cat. no. 0030108132), sodium tetraborate (Na$_2$B$_4$O$_7$; cat. no. 71997-100G), sodium acetate (S2889-250G), acetic acid (A6283-1L), Tween 20 (cat. no. P5927-500mL), 2-Hydroxy-3-morpholinopropanesulfonic acid (MOPSO; cat. no. M8389-100G), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; cat no. H4034-100G), N,N-bis(2-hydroxyethyl)glycine (bicine; cat no. B3876-25G), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO; cat no. C2278-100G), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; cat no. C6070-100G) and hydrochloric acid (HCl ≥37%; cat. no. 30721-1L) 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 (NKI), 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 ASCO-CAP guideline (Wolff et al., 2013). All patients received three to nine cycles of trastuzumab and pertuzumab combined with chemotherapy as neoadjuvant treatment. Both hormone-receptor negative (ER and PR expression < 10%) and positive (ER and/or PR expression ≥ 10%) patients were eligible. Trastuzumab (6mg/kg, with a loading dose of 8mg/kg at the first treatment cycle only) and pertuzumab (420mg, with an 840-mg loading dose at the first treatment cycle only) were administered intravenously every three weeks for one year. Blood withdrawal took place three weeks after the previous dose, before the first 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, 9 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 (MRI) of the breast, cytology or histology of the baseline marked pathological lymph node in the case of clinical lymph node-positive disease. In hormone-receptor (HR)-positive cases, vacuum-assisted core biopsies (VACBS) were also part of the response evaluation after 3 and 6 cycles. Surgical response was locally assessed, and pathological 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 8 patients were prioritized for analysis based on the concentrations of trastuzumab and pertuzumab, respectively, as measured by LC-MS/MS in the selected reaction monitoring (SRM) mode according to Bults et al., (2022). Subjects having a concentration of at least 80μg/mL at various time points 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 (IHC 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 (NST). Five out of the eight patients had a pathologic complete response at surgery after neoadjuvant chemotherapy plus HER2-directed antibodies of varying duration.

Table 1

**In Vitro Stressing of Trastuzumab and Pertuzumab**

Both antibodies were spiked separately at 2mg/mL in PBS (pH 7.4) after which the corresponding samples were aliquoted into 100μL 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 50mM sodium borate, pH 9) and 40μL was then incubated with 12μg of Affimer® reagent (300μg/mL) in coupling buffer overnight at room temperature and 700rpm in the dark. The coupling process was performed separately for the anti-trastuzumab and anti-pertuzumab Affimer® reagents in batch with the coupling mixture (coupling buffer containing 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 50mM cysteine in coupling buffer for 1 hour at room temperature and 700rpm to block unreacted sites. After blocking, the beads were washed with Tris-HCl + 0.5M NaCl, pH 8 followed by 0.1M sodium acetate buffer, pH 4, containing 0.5M 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 100μL of this mixture was added to 40μL anti-trastuzumab Affimer (No. 386_737_C3) functionalized beads in protein Lobind plates and incubated for 1.5 hours at room temperature and 700rpm to capture trastuzumab. The samples were then transferred to another plate containing 40μL anti-pertuzumab Affimer reagent® (No. 00557_709213) functionalized beads and incubated for 1.5 hours at room temperature and 700rpm to capture pertuzumab. After the capture process, beads were washed three times with wash buffer (transferred to fresh protein LoBind plates after the second wash step) and an additional three times with 1xPBS to remove excess Tween-20.

**Elution of Captured TPs**

The beads were transferred to 2mL protein Lobind tubes and incubated with 20μL of 50mM glycine HCl, pH3 at room temperature and 350rpm for 15 minutes. The resulting supernatant was transferred to 0.5mL protein Lobind tubes containing 10μL of 1.5M MOPSO pH 7.5 for neutralization. The elution process was repeated 6 times producing a final volume of 370μL for each sample.
**IEX Analysis**

An Agilent 1200 HPLC system containing a MabPac SCX-10 column (4 × 250 mm, 5μm, 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 (CV) at 0.5 mL/min (62.8 min 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 GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA).

**Bioinformatics and statistical analysis**

LC traces were exported in CSV 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 64GB 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 physiological conditions (Spanov et al., 2021). After three 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 (Spanov et al 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 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 150μg/mL. The spiked antibodies were then captured, eluted and analyzed by IEX and the results were compared to the direct analysis of the unstressed, 1 week- and 2 week-stressed trastuzumab and pertuzumab (40μg) preparations used for spiking. Figure 1 shows the chromatograms of trastuzumab and Figure 2 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 to pertuzumab.

Figure 1

Figure 2

Analysis of Patient Samples

Plasma samples from patients were analyzed at various time points 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. While not identical, profiles at the various time points are rather similar except for day 126 and notably day 210 (Figure 3A), which show mainly the unmodified form of trastuzumab. The profiles for pertuzumab at the different time points are all very similar comprising far fewer charge variants than for trastuzumab (Figure 3B). While the samples were taken 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 8 different patients across two-time ranges (panels A and B: trastuzumab from day 62 to 67 and 155 to 166, respectively; panels C and D: pertuzumab from day 62 to 67 and 155 to 166, respectively). The profiles of both trastuzumab and pertuzumab are
similar across different patients throughout 2 different time ranges. As mentioned earlier, there are again two instances (subject 4 day 67 (Figure 4A) and subject 7 day 166 (Figure 4B) where samples were collected after injecting a new dose of trastuzumab.

Figure 3A

Figure 3B

Figure 4

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 non-linear 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 (COW) of the LC-UV traces interpolated to the same retention time measurement points (Tomasi et al., 2004). Figure S1 (supporting information) shows the raw, interpolated and COW-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 (Ahmad et al., 2011; Suits et al., 2008). Figure 5 shows a heatmap of the similarity scores between all possible pairs of chromatograms following hierarchical clustering (Figure S2, 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 (S4 Day 67 and S7 Day 166; post-dose sampling as explained above).

Figure 5
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 enzyme-linked immunosorbent assay (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), Spanov et al, 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 3 clusters.
Authorship Contributions

Participated in research design: Olaleye O, Spanov B, van de Merbel N and Bischoff R

Conducted experiments: Olaleye O, Spanov B, and Buls P.

Performed data analysis: Olaleye O, Spanov B, Buls P and Horvatovich P

Wrote or contributed to the writing of the manuscript: Olaleye O, Spanov B. van der Voort A, Sonke G, Govorukhina N, Horvatovich P, van de Merbel N and Bischoff R
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No author has an actual or perceived conflict of interest with the contents of this article
Figure Legends

Figure 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, 1 week- and 2 weeks-stressed preparations and D, E and F are from the same preparations after spiking into plasma followed by capture with an anti-trastuzumab Affimer® reagent.

Figure 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, 1 week- and 2 weeks-stressed and D, E and F are from the same preparations after spiking into plasma followed by capture with an anti-pertuzumab Affimer® reagent.

Figure 3: IEX analysis of plasma samples from one patient at 6 time points after enrichment of trastuzumab (panel A) and pertuzumab (panel 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).

Figure 4: IEX analysis of samples from 8 different patients after enrichment of trastuzumab (panels A and B) and pertuzumab (panels C and D) with the Affimer® reagents. Panels A and C show the charge-variant profile of trastuzumab and pertuzumab, between days 62 to 67 and panels B and D show the charge-variant profile of trastuzumab and pertuzumab, respectively, between days 155 to 166.

Figure 5: Heatmaps showing similarity 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 hierarchical clustering. S1-S8 refers to Subjects 1 to 8.
### Table 1: Female Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Participants (n=8)</th>
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<tbody>
<tr>
<td><strong>Age</strong> in years, median (IQR)</td>
<td>54.0 (41-59)</td>
</tr>
<tr>
<td><strong>Menopausal status, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Pre/peri menopausal</td>
<td>5 (62.5%)</td>
</tr>
<tr>
<td>Post</td>
<td>3 (37.5%)</td>
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<tr>
<td><strong>Clinical tumor stage, n (%)</strong></td>
<td></td>
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<tr>
<td>cT0</td>
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</tr>
<tr>
<td>cT1</td>
<td>2 (25%)</td>
</tr>
<tr>
<td>cT2</td>
<td>6 (75%)</td>
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<tr>
<td><strong>Clinical lymph node status, n (%)</strong></td>
<td></td>
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<tr>
<td>Negative</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Positive</td>
<td>4 (50%)</td>
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<tr>
<td><strong>Stage, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>6 (75%)</td>
</tr>
<tr>
<td>III</td>
<td>2 (25%)</td>
</tr>
<tr>
<td><strong>HER2-status, n (%)</strong></td>
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<tr>
<td>3+ at IHC</td>
<td>8 (100%)</td>
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<tr>
<td><strong>ER-status, n (%)</strong></td>
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</tr>
<tr>
<td>Positive (≥ 10%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Negative (&lt;10%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td><strong>PR-status, n (%)</strong></td>
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</tr>
<tr>
<td>Positive (≥ 10%)</td>
<td>2 (25%)</td>
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<tr>
<td>Negative (&lt;10%)</td>
<td>6 (75%)</td>
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<tr>
<td><strong>Tumor grade, n (%)</strong></td>
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<td>Grade 2</td>
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<tr>
<td>Grade 3</td>
<td>7 (87.5%)</td>
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<td><strong>Histology, n (%)</strong></td>
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<td>NST/ductal</td>
<td>8 (100%)</td>
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<tr>
<td><strong>Pathologic outcome, n (%)</strong></td>
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</tr>
<tr>
<td>Pathologic complete response (ypT0/is, N0)</td>
<td>5 (62.5%)</td>
</tr>
<tr>
<td>No Pathologic complete response</td>
<td>3 (37.5%)</td>
</tr>
</tbody>
</table>

**Abbreviations:** IQR = interquartile range; IHC = immunohistochemistry; ER = estrogen receptor; PR = progesterone receptor; NST = no special type
Figure 1

A

B

C

*Unmodified Form

D

E

F
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.