U.S. FOOD & DRUG ADMINISTRATION

Biosimilar User Fee Act (BsUFA) III Regulatory Science Pilot Program

ANNUAL REPORT

CONTENTS

Check if this report is Progress or Final Report:

☒ **Progress report** ☐ **Final report**

1. REPORT OVERVIEW[1]

Complete table 1 below based on the information provided in the subsequent sections of this report. This table will be used verbatim (i.e., copy/ paste) in any summary materials to evaluate the return on investment of the project.

Table 1: High-level overview of the project objective, aim(s) progress, outcomes, and timelines for communication and regulatory impact (1-2 sentence max per table cell).

Project Title:	**Systematic Analytical Characterization of Innovator and Biosimilar Products with the Focus on Post-translational Modifications**
Investigator:	**Anna Schwendeman**
Organization:	**University of Michigan**
Grant No. (if applicable)	**1 U01FD007763-01-Schwendeman**
Project Objective:	**The project aims to develop novel methodologies to evaluate structural similarities and differences between innovator and multiple biosimilar products and apply them across different protein drug classes. The proposed work will establish the correlation between post-translation modification differences, receptor binding, antibody dependent cellular cytotoxicity, aggregation propensity, immunogenicity and clinical efficacy to aid approvals of biosimilar and interchangeable protein products.**

Specific Aim(s)	Progress	Outcomes	Communication Timeline
1. Examine the range of structural differences across reference and approved biosimilar products of several drug classes (filgrastim, insulin glargine, and multiple mAbs).	Compared multiple insulins (glargine and lispro) and multiple mAbs (Trastuzumabs and Rituximabs)	Minor differences observed, methodologies have been developed	Two manuscripts are being drafted focused on trastuzumabs and rituximabs, targeting Fall 2024 submissions. Insulin data continues to be collected.
2. Identify HOS differences across innovators and approved biosimilar products of several drug classes.	Compared multiple products by IM-MS and CD/near UV methods	Minor differences observed	The data will be potentially added to Aim 1 publications.
3. Compare levels of non-covalent and covalent aggregates and determine how formulation differences, excipient sources and container-closure variations impact protein stability under stress conditions across different classes of innovator and biosimilar products.	The methodologies to detect S-S aggregates by LC-MS/MS were developed	Minor differences between innovator and biosimilar mAbs were observed.	Manuscript was published in 2023 (link to manuscript?)

[1] This section will be used by program for broader research portfolio and regulatory impact analysis by the BsUFA III steering committee.

Specific Aim(s)	Progress	Outcomes	Communication Timeline
4. Examine glycosylation microheterogeneity and its impact on Fc-receptor binding and ADCC across approved biosimilar mAb products for different indications (cancer and IBD).	Multiple lots and versions (reference and different biosimilars) of trastuzumabs and rituximabs were compared.	Minor differences were observed.	Same as Aim 1.
5. Perform interviews with major biosimilar developers to assess and identify technical and regulatory challenges in the development of interchangeable biosimilars.	We have completed multiple rounds of interview with 3 companies and are in the process of establishing CDAs with more companies for additional interviews.	The interviews covered the overall barriers that companies encounter while developing biosimilars and seeking interchangeability approval from FDA. Broadly, the development challenges discussed included the validation of analytical methods, immu-nogenicity problems related to PTM modifications, clinical study requirements, and interchangeability designation.	We intend to summarize the findings as a brief publication upon completion of additional interviews in 2025.

2. PROGRESS SUMMARY

Describe the overall project objective, aims, for this study. These must be the same objective and specific aims from funded spend plan/application. Include milestones and activities with timelines for each aim (What was accomplished under each aim?) (No word max). *Note, text in this section should directly support content in the 'Progress' column in table 1.*

Project Objective:

- This research aims to compare and characterize the differences between biosimilars and originator biological drugs including infliximab (Remicade®, Inflectra®, and Renflexis®), filgrastim (Neupogen® and TPI filgrastim Releuko®), rituximab (Rituxan® and Accellbia®), trastuzumab (Herceptin® and HERtiCAD), and bevacizumab (Avastin® and Avegra®) with the goal of addressing and minimizing barriers to interchangeability approval.

- To establish orthogonal methods for the characterization of a protein's primary sequence, interrogation of higher order structural (HOS) differences, detection of post-translational modifications, interrogation of differences in glycan microheterogeneity and correlation of these differences to Fc-receptor binding and antibody-dependent cellular cytotoxicity (ADCC), and examination of the presence of non-covalent and covalent aggregates.

- To explore methodologies for the detection of differences across multiple product types (insulin, filgrastim, and therapeutic mAbs) and biosimilar versions of the same reference products.

Aim 1: Examine the range of structural differences across reference and approved biosimilar products of several drug classes (filgrastim, insulin glargine, and multiple mAbs).

Procurement of protein/peptide drug products

Upon the announcement of BsUFA grant funding in the Fall of 2022, we have been procuring lots of various proteins. In table 1, we have listed the current proteins, including mAbs and insulins, that we have in house. We procured 3 different lots of each protein so that we could monitor lot-to-lot variability on our various assays.

Table 1. The inventory of insulin products and mAbs

Products	Manufacture	Lot#	Expiration
Insulin			
Insulin lispro			
Humalog®Kwikpen	Eli Lilly & Co	D420814A	08/2024
Humalog®Kwikpen	Eli Lilly & Co	D371847A	01/2024
Humalog®Kwikpen	Eli Lilly & Co	D627054C	01/2026
Admelog®Solostar	Sanofi	3F508A	03/31/2025
Admelog®Solostar	Sanofi	3F5151	06/30/2025
Admelog®Solostar	Sanofi	3F546A	9/30/2025
Lyumjev™Kwikpen®	Eli Lilly & Co	D522060E	07/22/2024
Lyumjev™Kwikpen®	Eli Lilly & Co	D539311D	08/19/2024
Lyumjev™Kwikpen®	Eli Lilly & Co	D650895A	08/25/2023
Insulin glargine			
Lantus®Solostar®	OR from Sanofi	2F8431A	07/31/2024
Lantus®Solostar®	OR from Sanofi	3F8832A	12/31/2024
Lantus®Solostar®	OR from Sanofi	3F9117A	09/30/2025
Basaglar®Kwikpen®	BS from Eli Lilly & Co	D510461D	05/11/2024
Basaglar®Kwikpen®	BS from Eli Lilly & Co	D437809F	09/24/2023
Basaglar®Kwikpen®	BS from Eli Lilly & Co	D637529A	07/17/2025
Semglee®	BS from Mylan	BF21005474	12/2023
Semglee®	BS from Mylan	BF22000063	01/2024
Semglee®	BS from Mylan	BF22002484	06/2024
Rezvoglar™Kwikpen®	BS from Mylan	D540791C	09/02/2024
mAb			
Trastuzumab			
Herceptin®	OR from Genentech	3514960 3576752 3593466	03/2024 06/2024 04/2027
Kanjinti™	BS from Amgen (Developed by Amgen and Allergan)	1141539A 1149306B 1161696	10/2024 04/2025 09/2025
Ogivri®	BS from Mylan (Developed by Mylan and Biocon Biologics)	BF19006696 BF22002939 BF23001860	03/2023 08/2026 02/2027

Products	Manufacture	Lot#	Expiration
Rituximab			
Rituxan®	OR from Genentech (Developed by Biogen Idec)	3491321	08/2024
		3575428	10/2025
		3580141	03/2026
Ruxience™	BS from Pfizer	FC5021	05/2023
		GN4445	04/2024
		GY2269	12/2024
Truxima®	BS from Teva (Developed by Celltrion Inc.)	0L0031	11/2024
		1B1011	01/2025
		1E11001	04/2025
Riabni	BS from Amgen	1128511	07/2023
		1141263	03/2024
		1157031	06/2024

Examine structural features and differences of multiple approved biologics and biosimilars

We characterized the structures of the monoclonal antibodies Rituxan, Herceptin, and their respective biosimilars, as well as the NIST mAb, utilizing intact mass spectrometry (MS) and peptide mapping techniques. Figure 1 illustrates the intact MS spectra for Rituxan, Herceptin, and NIST mAb, representing data collected for each antibody. Intact MS data were acquired from three different lots of each antibody, including both fully glycosylated and de-glycosylated forms of the innovators and biosimilars, with triplicates for the NIST mAb. The intact MS spectra of fully glycosylated antibodies provided N-glycosylation information, whereas those of de-glycosylated antibodies revealed other modifications, such as N-terminal pyroGlu formation (-17.0 Da), C-terminal Lys clipping (-128.2 Da), and Lys-term glycation (+162.2 Da). Data processing was performed using Protein Metrics software (Byos, Intact workflow). As expected, given their approved status, the intact MS showed only slight differences between these products, particularly in glycan identification and post-translational modifications (PTMs). When considering the molecular weight of antibodies, approximately 150 kDa, intact MS data—capable of identifying the distribution of molecular weights—are not the most sensitive for detecting smaller differences in PTMs between biosimilars and innovators. To probe differences between innovators and biosimilars more sensitively, we also conducted peptide mapping.

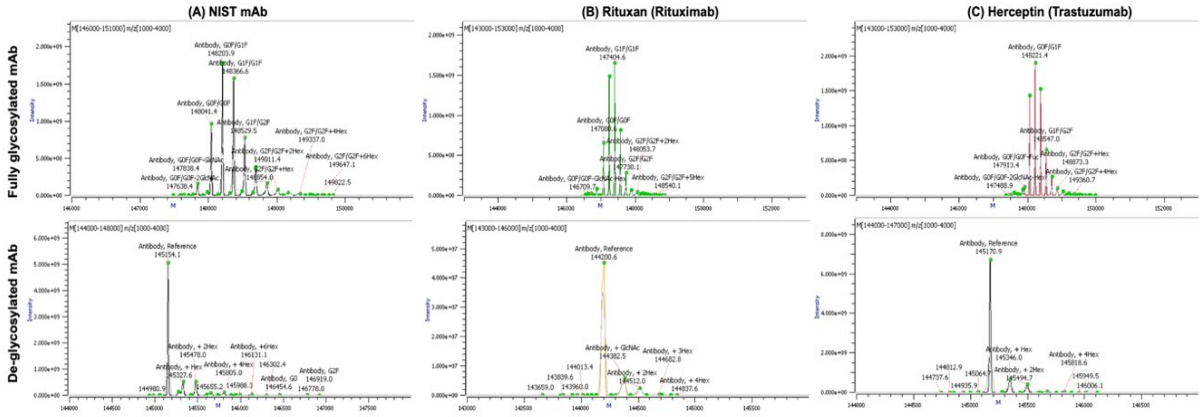


Figure 1 Deconvoluted intact MS spectra for fully glycosylated and de-glycosylated (A)NIST mAb, (B)Rituxan and (C)Herceptin. Shown are the glycans or PTMs identified by Protein Metrics Intact MS workflow.

Figure 2 illustrates sequence coverage maps after protein digestion for NIST mAb, Rituxan (rituximab), Herceptin (trastuzumab) and their respective biosimilars. We conducted assays for NIST mAb, rituximabs, and trastuzumabs, with three lots of each protein processed simultaneously. Overall, sequence coverage ranged from 98% to 100%. This figure highlights our capability to achieve complete sequence coverage and identify structural motifs such as deamidation (+0.9840 Da), oxidation (+15.9949 Da), and ammonia loss (-17.0265 Da), along with pyroGlu formation and Lys clipping, which was also observed in the de-glycosylated intact MS spectra. These data were generated from protein digestion on the AssayMAP Bravo liquid handling robot, followed by data acquisition on a ThermoFisher Orbitrap LC-MS/MS system and processing using Protein Metrics software (Byos, PTM workflow).

Figure 2 LC-MS/MS analysis of Rituximab and its biosimilars after Trypsin and Lys-C digest showing 98-100% sequence coverage and locations of PTMs by Protein Metrics PTM workflow.

Figure 3 presents the results obtained from intact MS and peptide mapping. The percentages were derived from either intensity (intact MS spectra) or XIC area summed isoX normalized (peptide mapping) data.

Although the precise numerical values slightly differed, the overall trends in PTMs were consistent. In trastuzumab, the heavy chain, which contains N-terminal Glu and C-terminal Lys, exhibited 1-2% N-terminal pyro-Glu formation and complete C-terminal Lys clipping. Among rituximabs, Ruxience, a biosimilar of Rituxan, demonstrated greater stability, showing reduced N-terminal Glu formation on the light chain and C-terminal Lys clipping on the heavy chain. This trend was corroborated by the de-glycosylated intact MS, as depicted in Figure 3c.

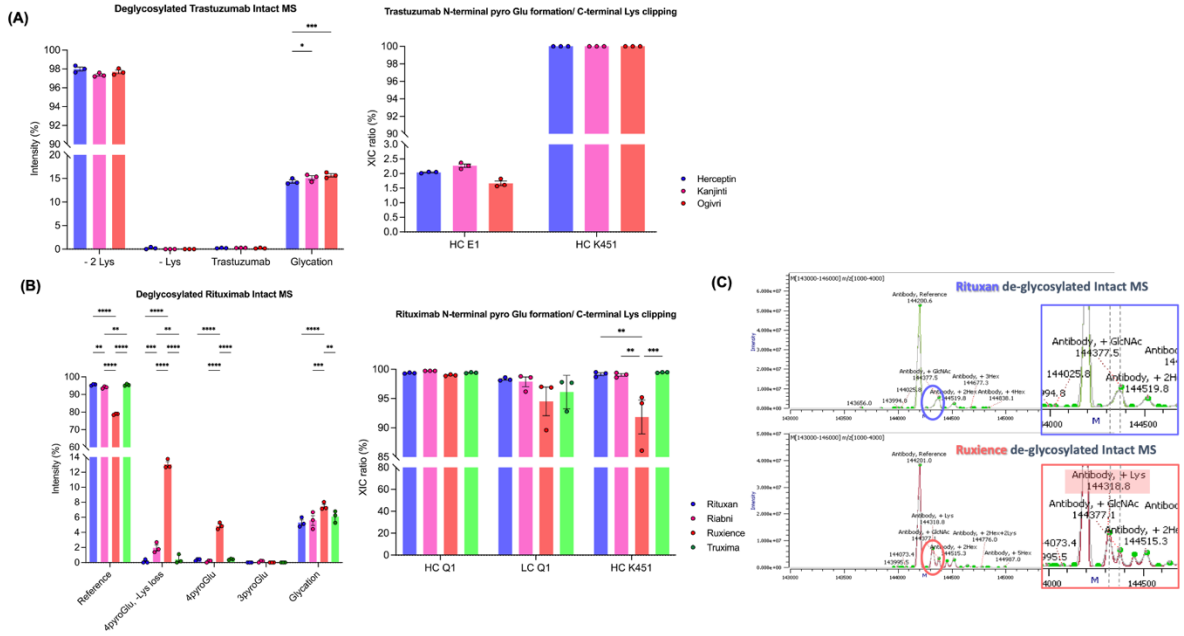


Figure 3 Comparison of PTMs such as N-term PyroGlu formation, Lys clipping, and Glycation acquired from Intact MS and protein digestion assay. (A) Trastuzumab innovator and biosimilars, (B) Rituximab innovator and biosimilar. (C) Intact MS spectra of Rituxan and Ruxience. HC: Heavy Chain; LC: Light Chain. N=3; Error bars are standard deviation.

As for insulin, we used Fourier Transform Infrared (FTIR) spectroscopy to examine its chemical structure. Amide I and amide II bands are primarily caused by carbonyl stretching and NH bending vibrations, respectively. As both carbonyl and NH are involved in hydrogen bonding in protein secondary structures, the position and shape of these bands are highly dependent on the secondary structure. As shown in Figure 4A, FTIR spectra of the insulin products were compared by evaluating the positions and shapes of the amide I (~1650 cm-1) and amide II (~1550 cm-1) bands between 1700 and 1500 cm-1. The presence of the amide I band indicates alpha-helix structures for insulin. Additionally, the intrinsic fluorescence of proteins is caused by three amino acid residues with aromatic side chains: phenylalanine, tyrosine, and tryptophan. Among these, tryptophan plays the most important role due to its excitation and emission spectra having the longest wavelength (near the UV range) and the longest lifetime. Intrinsic fluorescence measurements were performed by inducing excitation at 278 nm, and scanning for emission from 278 to 400 nm. As shown in Figure 4B, the similar peaks at 310 nm suggest the same ratio of aromatic amino acids in both the insulin lispro and insulin glargine products.

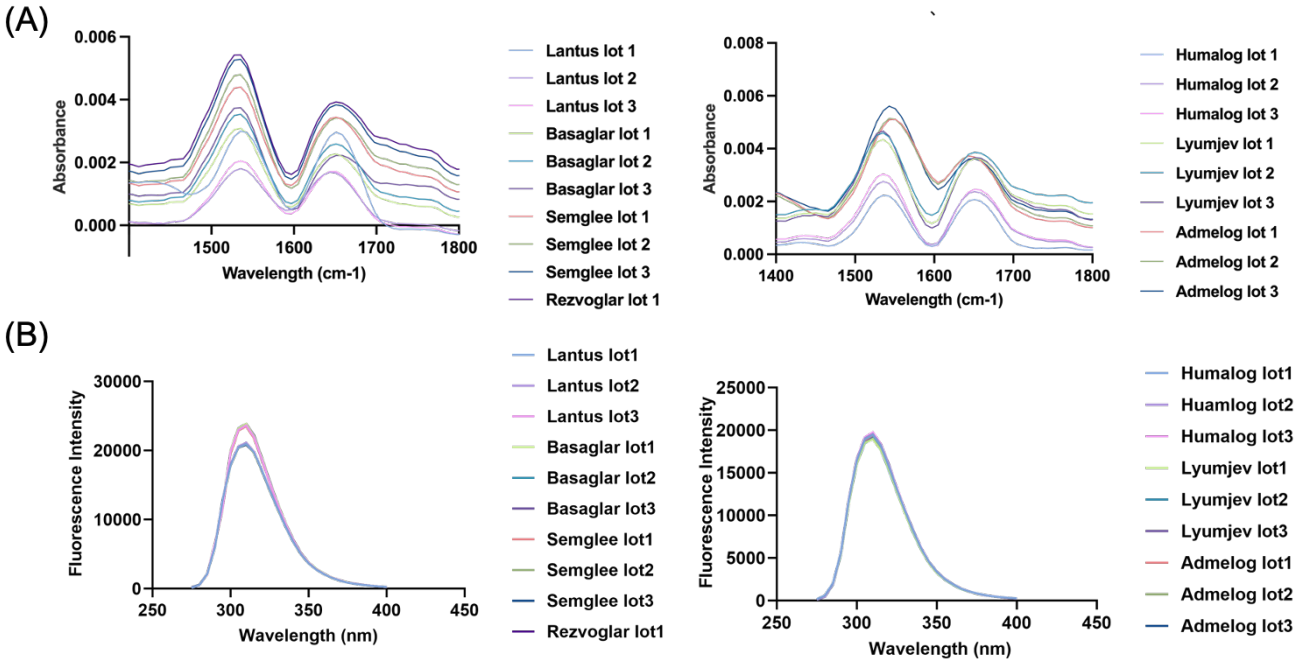


Figure 4. The structure of insulins. (A) FTIR spectrogram. Insulin glargine(left) and insulin lispro(right). (B) Intrinsic fluorescence spectral plots. Insulin glargine(left) and insulin lispro(right).

Aim 2: Identify HOS differences across innovators and approved biosimilar products of several drug classes.

Higher order structure of proteins

In collaboration with Brandon Ruotolo's lab at the University of Michigan, we have run some of our current samples (Table 1) on an ion-mobility mass spectrometer (IM-MS). All of the rituximab and trastuzumab samples were analyzed (Figure 5). IM-MS detected differences in protein unfolding, allowing us to further monitor how differences in storage conditions, excipients, container systems, etc. impact protein stability.

IM-MS detects protein features by quantifying centroid drift times (or CCS) corresponding to a conformation present across multiple collision voltages. CIU50 analysis models the transition region between features and quantifies the midpoint voltage between adjacent features – termed “CIU50”. Comparisons of CIU50 values between different samples can provide insights on protein stability. Root-mean-squared deviation (RMSD) analysis calculates global differences between two CIU fingerprints.

Another benefit of this method is that it requires minimal handling from scientists in order to obtain data. Therefore, its data should be reflective of protein stability changes due to the experimental conditions rather than from human error in handling. IM-MS is a more niche type of MS, which is why collaborating with experts in the Ruotolo lab is beneficial for the project’s progress. They have also agreed to help run additional proteins, including insulin samples.

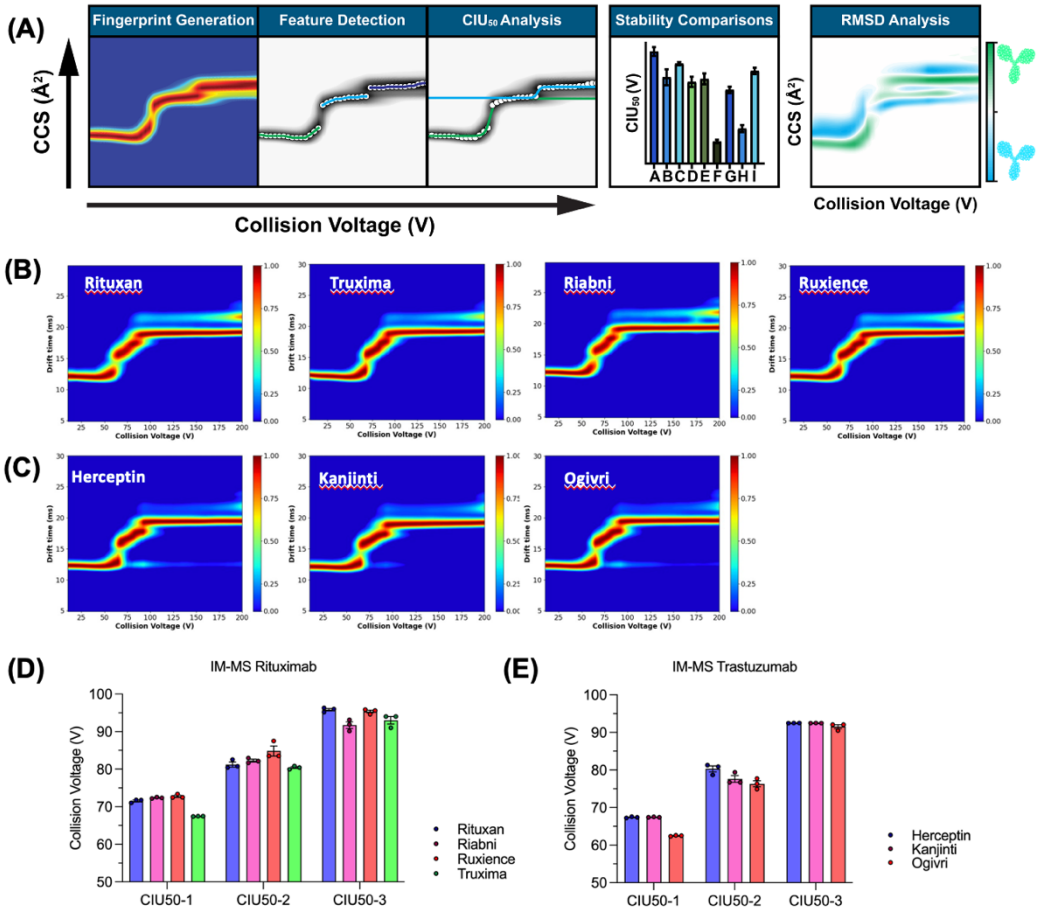


Figure 5 (A) Schematic representation of IM-MS analysis. IM-MS spectra and average CIU fingerprints and standard deviation of innovator and biosimilars of (B) rituximab and (C) trastuzumab. Quantitation of CU50-1, CIU50-2 and CIU50-3 transitions of (D) rituximabs and (E) trastuzumabs. N=3; Error bars are standard deviation.

Circular dichroism (CD) spectra were recorded at room temperature with a scanning speed of 200 nm/min and a bandwidth of 1 nm in a quartz cell with a path length of 0.1 cm. Far-UV spectra were recorded from 190 to 260 nm with samples diluted tenfold. The data were smoothed using the Savitzky-Golay method, with the convolution width set to 10 nm. Two characteristic negative peak maxima at 208 and 222 nm indicate the presence of alpha-helix-rich protein in insulin products, consistent with the FTIR results (Figure 6A). Near-UV spectra were recorded from 250 to 350 nm without sample dilution, showing two similar negative peaks around 255-270 nm and 275-285 nm, indicating the presence of phenylalanine and tyrosine. This result is consistent with our intrinsic

fluorescence findings. Thermal stability measurements were performed using a Nano DSC differential scanning calorimeter (TA Instruments, New Castle, DE). This instrument measures the heat reaction from tertiary structure changes that occur when a biomolecule unfolds or melts. The results demonstrate a high similarity in unfolding temperatures (Tm = 60°C ± 1.5°C), indicating similar thermal stability and conformation for the three insulin glargine products (Figure 6C).

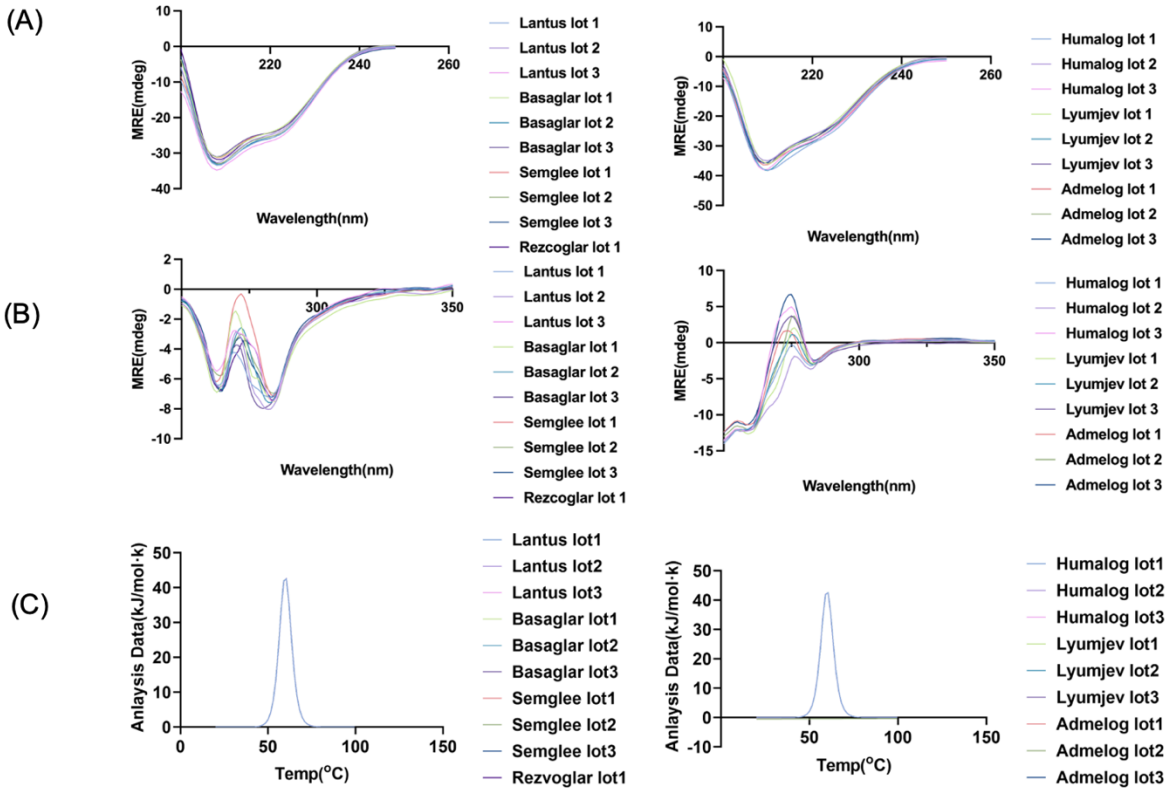


Figure 6. High order structure of insulins. (A)far-UV CD Profiles (B) near-UV CD Profiles (C) Nano DSC profiles. Abbreviations: UV, ultraviolet; CD, circular dichroism; DSC, differential scanning calorimetry.

Aim 3: Compare levels of non-covalent and covalent aggregates and determine how formulation differences, excipient sources and container-closure variations impact protein stability under stress conditions across different classes of innovator and biosimilar products.

Aggregation/degradation of proteins as they relate to structural and environmental features

We have conducted stress studies on Rituxan, Herceptin, and their respective biosimilars to assess the aggregation and degradation characteristics of rituximabs and trastuzumabs under stressed conditions. Antibodies were detected using dual wavelengths of 214 nm and 280 nm. We observed the excipient peak at 214 nm across all antibodies (Figure 7). Consequently, we used the SEC chromatograph at 280 nm to compare

the either aggregation or degradation of proteins. Generally, rituximabs exhibited higher aggregation than trastuzumabs when exposed to heat and shaking over time. For trastuzumabs, there were minimal differences between the innovator and its biosimilars. Among rituximabs, the innovator (Rituxan) and two of the biosimilars (Truxima and Riabni) formed more aggregates when exposed to heat and shaking over time, whereas one biosimilar (Ruxience) formed more fragments under the same conditions (Figure 8, Table 2).

Using SDS-PAGE, we confirmed these aggregation and fragmentation trends (Figure 9). All antibodies except Ruxience showed an aggregation peak around 280-290 kDa, which is expected to be a dimer, whereas Ruxience exhibited a fragmentation peak below the monomer peak of 140-150 kDa.

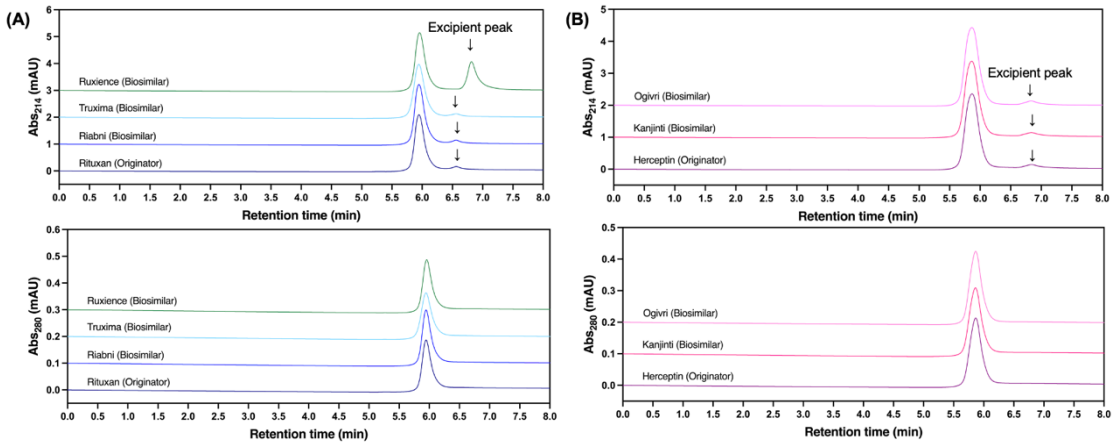


Figure 7 Representative SEC chromatograms at 214 nm and 280 nm for 10 µg of unstressed antibody. (A) Rituximab innovator and biosimilars; (B) Trastuzumab innovator and biosimilars. (N=3)

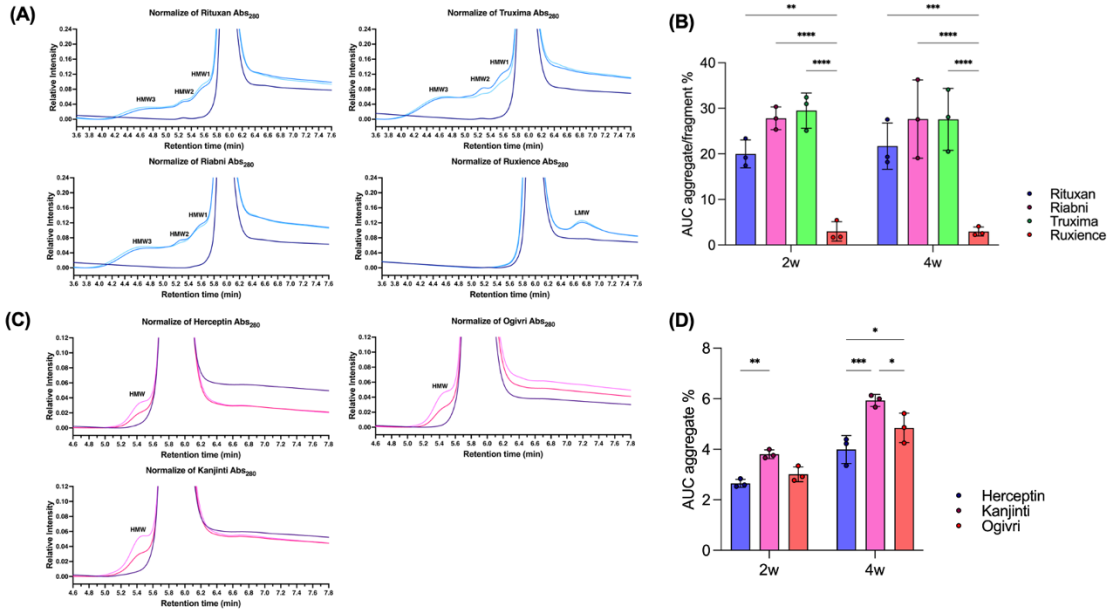


Figure 8 Representative SEC chromatograms at 280 nm for 10 µg of antibody. **(A)** Rituximab innovator and biosimilars at 0 (navy), 2 (blue) and 4 (light blue) weeks; **(C)** Trastuzumab innovator and biosimilars at 0 (purple), 2 (red) and 4 (pink) weeks; SEC data depicted as average % concentration contributions of aggregate, fragment peaks of **(B)** rituximabs and **(D)** trastuzumabs; (N=3, 2-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Stressed samples were shaking at 240 RPM, incubating at 40°C for 2 or 4 weeks.

Table 2. SEC data depicted as average % concentration contributions of monomer, aggregate, fragment peaks (N= 3, mean ± SD). Aggregates and fragments include summations of multiple peaks, where applicable. Stressed samples were shaking at 240 RPM, incubating at 37°C for 2 or 4 weeks. All samples were diluted to 1.5 mg/ml to load 15 µg of antibody on the column. N= 3, mean ± SD

Abs_{280}	2W AUC aggregation %	4W AUC aggregation %
Herceptin	2.653 ± 0.160	3.990 ± 0.554
Kanjinti	3.804 ± 0.173	5.913 ± 0.237
Ogivri	3.010 ± 0.295	4.845 ± 0.582
Rituxan	19.998 ± 3.049	21.702 ± 5.088
Riabni	27.825 ± 2.492	27.645 ± 8.601
Truxima	29.504 ± 3.873	27.58 ± 6.807
Abs_{280}	2W AUC fragment %	4W AUC fragment %
Ruxience	2.978 ± 2.145	2.960 ± 0.980

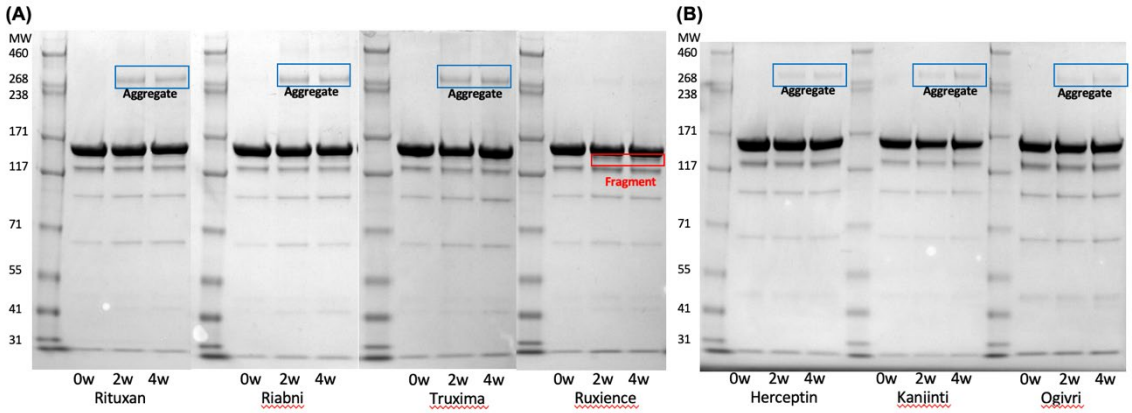


Figure 9 SDS-PAGE gel; **(A)** Rituximab samples and **(B)** trastuzumab samples at each timepoint (0, 2, and 4 weeks) on an Invitrogen NuPAGE 3%–8% Tris-Acetate Gel. Protein samples were diluted to 0.33 mg/ml with water, and 10 µl of the ladder (HiMark™ prestained protein standard) were added into well. Aggregate bands are highlighted in the blue box, while fragment band is highlighted in the red box.

We also previously monitored disulfide shuffling for mAbs under normal and stressed conditions and found no statistically significant differences, likely due to the low abundance of shuffled bonds. We studied disulfide bonds because it has been previously shown that the shuffled disulfide bonds can be a precursor to aggregation. While we did not find statistically significant differences, we did observe a trend where the bevacizumabs, both Avastin and its biosimilar, formed more disulfide bonds over the incubation period compared to the rituximabs, which remained steady in their levels (Figure 10). Although we did not detect significant differences, this confirmed our ability to detect changes in shuffled disulfide bonds using our non-reduced protein digestion, LC-MS/MS data acquisition and Protein Metrics data processing workflow.

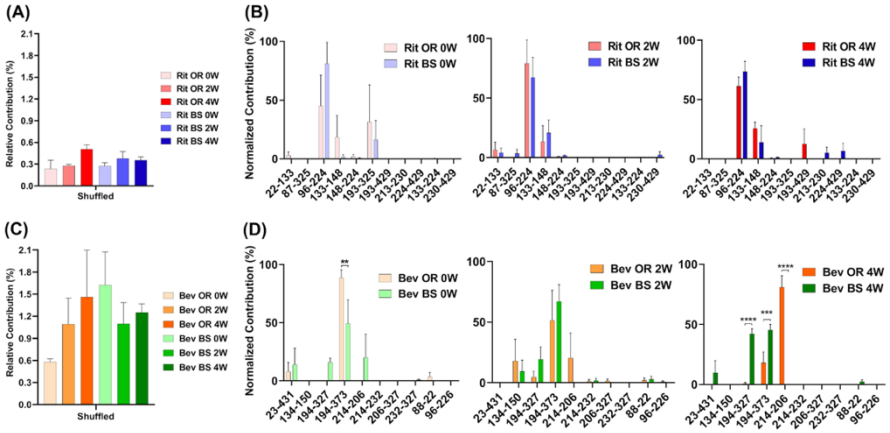


Figure 10 Total shuffled bond contribution relative to the XIC sum of all identified disulfide bonds for **(A)** rituximab originator and biosimilar and **(C)** bevacizumab originator and biosimilar. Prevalence of the shuffled bond locations normalized to the total number of shuffled bonds for **(B)** rituximab originator and biosimilar and **(D)** bevacizumab originator and biosimilar (N= 3, mean ± SD, 2-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

We plan to employ these methods with our current lots of trastuzumabs and rituximabs, as well as future proteins we collect. This would build out our knowledge of IgG1 innovator and biosimilar differences in degradation patterns when exposed to heat and agitation. We would also like to explore excipient sourcing as a variable impacting protein stability and structural modifications given our knowledge that lower quality excipients can impact PTMs and aggregation propensity. To do so, we plan on acquiring excipients from various vendors, removing the excipient of interest through spin filters and spiking in the acquired excipients. Then we can monitor degradation via SEC over time and perform peptide mapping at various stages of the storage period.

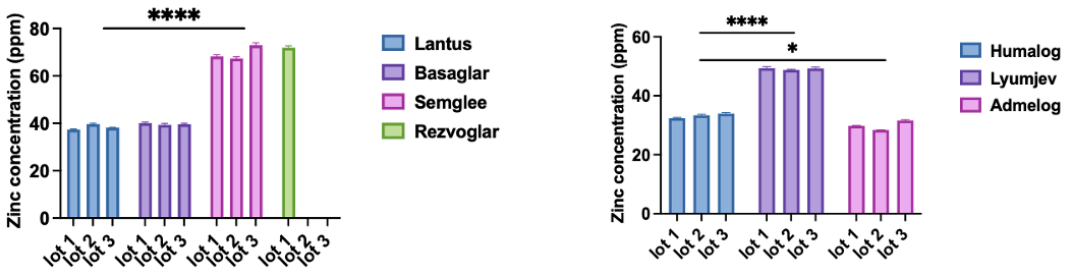
The insulin lispro/glargine concentration was tested using a UPLC method. The aqueous phase consisted of a mixture of 0.1% formic acid and 50 mM ammonium acetate (A), while the organic phase comprised 0.085% formic acid in acetonitrile. The flow rate was set at 0.5 mL/min, and detection was carried out at 214 nm. A 10 µL injection volume was utilized, and the running time was 15 minutes. The column used was the ACQUITY UPLC BEH C18 column, 1.7 µm, 2.1 x 50 mm. The gradient transitioned from 20% to 40% B over 12 minutes. We confirmed that 50 mM ammonium acetate in the aqueous phase can separate A21-desamido from insulin lispro and A0-insulin glargine from insulin glargine, achieving a resolution greater than three in the chromatogram. The results in Figure 11 show that the concentration of insulin lispro and insulin glargine is 100 units/mL, which is consistent with the company reports. Zinc concentration in Semglee and Rezvoglar is higher than in Lantus and Basaglar, while the zinc concentration is higher in Lyumjev than in Admelog and Humalog, indicating potential differences in in vivo stability and zinc-hexamer formation in these products. Further methodology is needed to confirm our hypothesis. Meta-cresol acts as the preservative in the insulin formulations, with a concentration of 2.7 mg/mL in insulin glargine products and 3.0 mg/mL in insulin lispro products.

(A)

Products	Concentration(Units/mL)			Avg	SD
Lantus lot1	100.62	100.74	99.36	100.24	0.76
Lantus lot2	99.12	97.85	99.52	98.83	0.87
Lantus lot3	101.93	101.42	100.57	101.30	0.69
Basaglar lot1	97.66	97.14	96.95	97.25	0.37
Basaglar lot2	100.65	99.14	98.48	99.42	1.11
Basaglar lot3	96.81	96.00	97.61	96.81	0.81
Semglee lot1	98.34	98.54	96.51	97.80	1.11
Semglee lot2	99.87	98.77	99.53	99.39	0.56
Semglee lot3	99.62	99.08	97.98	98.89	0.84
Rezvoglar lot1	98.08	96.52	96.29	96.96	0.97

Product	Concentration(Units/mL)			Avg	SD
Humalog lot1	100.04	103.32	100.32	101.22	1.82
Humalog lot2	98.27	98.31	98.57	98.38	0.16
Humalog lot3	99.14	98.52	99.28	98.98	0.40
Lyumjev lot1	98.21	97.55	97.49	97.75	0.40
Lyumjev lot2	97.76	98.44	98.95	98.38	0.60
Lyumjev lot3	97.81	98.33	98.28	98.14	0.28
Admelog lot1	98.17	97.14	97.37	97.56	0.54
Admelog lot2	97.33	98.72	96.08	97.38	1.32
Admelog lot3	96.44	95.56	96.08	96.02	0.44

(B)



(C)

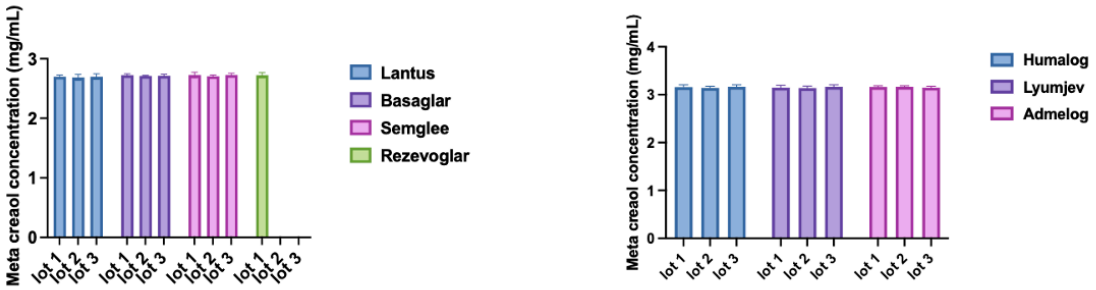


Figure 11. the concentration of insulins and the excipients. (A) the concentration of insulins (B) the zinc concentration in the insulin formulations (C) the concentration of meta-cresol n = 3, ****p < 0.0001, * p < 0.1.

Impurities in the insulin formulations were detected using RP-based and SEC-based analyses. The RP-based method was performed on an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm) operated at 40°C with a Premier UPLC system. Prior to injection, the column was saturated with 80% mobile phase A (0.1% v/v FA in H2O) and 20% mobile phase B (0.1% v/v FA in ACN). The samples were separated using a 15-minute linear gradient from 20% to 60% B at a flow rate of 0.3 mL/min, with detection at 280 nm UV wavelength. The results showed that the impurities in the insulin lispro products were similar (Table 3). However, the percentage of impurities in the insulin glargine products showed differences (Table 4). Further confirmation of the impurities will be carried out using QTOF data processing with Protein Metrics software. The high molecular weight proteins (HMWP) were determined using size exclusion chromatography (SEC) via two different methods. The first analysis was performed on an Xbridge BEH 200 Å SEC column (3.5 µm, 7.8 x 300 mm) thermostated at room temperature. The mobile phase consisted of 650 mL of arginine solution (1 g/L), 200 mL of acetonitrile, and 150 mL of acetic acid. The flow rate was set at 1.0 mL/min, with an injection volume of 100 µL. Detection was carried out at 276 nm, and the run time was 35 minutes. The second analysis followed the USP-reported method, where the mobile phase contained a mixture of acetonitrile, water, and glacial acetic acid (300:400:200), adjusted to pH 3.0. Although the second method showed higher HMWP% detection, there were no significant differences between the insulin lispro and insulin glargine products in HMWP% as determined by one-way ANOVA analysis (Figure 12).

Table 3. The RP-based percent of impurity in the insulin lispro products. n = 3.

Impurities	Humalog (%)			Lyumjev (%)			Admelog (%)		
Lot num	1	2	3	1	2	3	1	2	3
0.59	0.063 ± 0.034		0.04 ± 0.0046	0.02 ± 0.0063	0.01 ± 0.00093	0.01 ± 0.00	0.035 ± 0.018	0.018 ± 0.00061	0.028 ± 0.0031
0.79	0.043 ± 0.006	0.069 ± 0.017	0.033 ± 0.00072	0.03 ± 0.0074	0.02 ± 0.0018	0.027 ± 0.0058	0.026 ± 0.00	0.025 ± 0.0022	0.016 ± 0.0024
0.92	0.115 ± 0.009	0.082 ± 0.004	0.091 ± 0.037	0.14 ± 0.0065	0.11 ± 0.016	0.12 ± 0.021	0.026 ± 0.00	0.019 ± 0.0022	0.020 ± 0.0018
1.07	0.219 ± 0.009	0.187 ± 0.01	0.206 ± 0.0089	0.26 ± 0.0021	0.25 ± 0.0012	0.19 ± 0.10	0.30 ± 0.0028	0.24 ± 0.012	0.19 ± 0.0053
1.13	0.291 ± 0.006	0.261 ± 0.018	0.269 ± 0.014	0.34 ± 0.0017	0.33 ± 0.0065	0.27 ± 0.11	0.41 ± 0.0014	0.36 ± 0.0042	0.26 ± 0.0036
1.20	0.046 ± 0.002	0.054 ± 0.007	0.061 ± 0.00099	0.06 ± 0.0013	0.06 ± 0.0017	0.06 ± 0.00	0.04 ± 0.0042	0.11 ± 0.0022	0.041 ± 0.0018
1.24	0.073 ± 0.007	0.09 ± 0.002	0.084 ± 0.014	0.11 ± 0.003	0.10 ± 0.0032	0.08 ± 0.028	0.16 ± 0.0039	0.07 ± 0.0011	0.091 ± 0.0014

Table 4. The RP-based percent of impurity in the insulin products. n = 3.

Impurities	Lantus (%)			Basaglar (%)			Semglee (%)			Rezvoglar (%)
Lot num	1	2	3	1	2	3	1	2	3	1
0.89				0.03 ± 0.004			0.022 ± 0.096		0.024 ± 0.00099	
1.11	0.066 ± 0.034	0.058 ± 0.0021	0.061 ± 0.046	0.021 ± 0.0049	0.032 ± 0.00	0.031 ± 0.00				
1.16	0.15 ± 0.022	0.073 ± 0.0043	0.067 ± 0.0054	0.096 ± 0.0011	0.042 ± 0.017	0.075 ± 0.0037	0.17 ± 0.001	0.14 ± 0.0022	0.12 ± 0.0019	0.06 ± 0.0032
1.22	0.019 ± 0.0023	0.015 ± 0.00032	0.018 ± 0.0014	0.03 ± 0.002	0.039 ± 0.0035	0.038 ± 0.0033				0.019 ± 0.0033
1.31	0.024 ± 0.0012	0.025 ± 0.0024	0.022 ± 0.0023				0.021 ± 0.0021	0.029 ± 0.0012		
1.35	0.05 ± 0.0024	0.038 ± 0.0015	0.023 ± 0.0074							

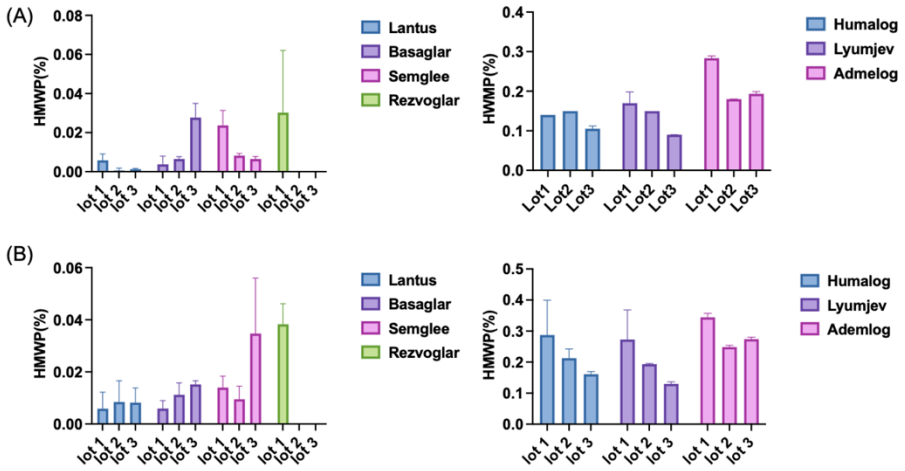


Figure 12. High molecular weight products (HMWP) detection in insulin formulations. (A) HMWP (%) using the patent method. (B) HMWP (%) using the USP method. Abbreviations: USP, United States Pharmacopeia.

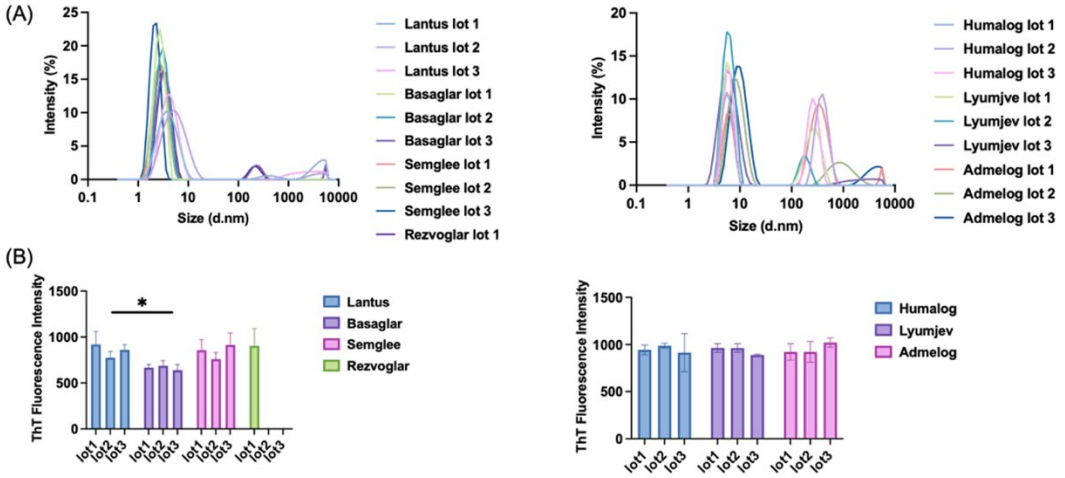


Figure 13. Aggregation detection in insulin products. (A) Physical aggregation by DLS analysis. (B) Amyloid fibrils by ThT analysis. Abbreviation: DLS, Dynamic Light Scattering; ThT, Thioflavin T.

Aim 4: Examine glycosylation microheterogeneity and its impact on Fc-receptor binding and ADCC across approved biosimilar mAb products for different indications (cancer and IBD). In vitro glucose uptake of insulin products.

Comparing glycan analysis methods for multiple mAbs for future "best practice" guidance

We have made great progress in this aim since the start of the funding period in Fall of 2022. As we mentioned in the last year annual report, we had performed five different types of glycan analyses on NIST mAb ("standard"), Rituxan and three biosimilars and Herceptin and two biosimilars. These methods included three released glycan kits analyzed with a fluorescence detector (2AB Express, RapiFluor-MS and Instant-PC), intact MS and protein digestion followed by LC-MS/MS (Table 5). We observed varying levels of sensitivity,

reproducibility, and robustness across the methods. Among the three released glycan kits, Instant-PC had yielded the highest number of fluorescence peaks, and, therefore, the greatest number of unique glycans. We had encountered challenges in confidently identifying some of the peaks due to the absence of a procainamide library in the UNIFI software. We circumvented this issue by downloading the Protein Metrics API. Given its ability to detect the highest number of unique glycans, we conducted released glycan analysis with all three different lots of each antibody using instant-PC, as shown in Figure 14. In addition to released glycan analysis, we also conducted intact MS and protein digestion followed by LC-MS/MS.

Table 5. Comparison of the total number of glycans, broken down by glycan type, detected on NIST mAb via the five methods. N = 3; shown is mean ± standard deviation

Average total # glycan type					
NIST mAb (LC-FLR)	**2AB**	**RF**	**PC**	**Intact**	**Digestion**
Total # unique glycans	16.33 ± 0.58	16.67 ± 0.58	19.00 ± 0.99	4.00 ± 0.00	22.33 ± 1.15
Total # afucosylated	6.33 ± 1.53	2.00 ± 0.00	3.00 ± 0.00	0.00 ± 0.00	8.33 ± 0.58
Total # mannosylated	4.33 ± 0.58	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 1.00
Total # sialylated	2.67 ± 0.58	1.33 ± 0.58	2.00 ± 0.00	0.00 ± 0.00	3.33 ± 0.58
Total # galactosylated	1.67 ± 0.58	4.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 0.00
Total # bisecting GlcNAc	1.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	2.00 ± 0.00

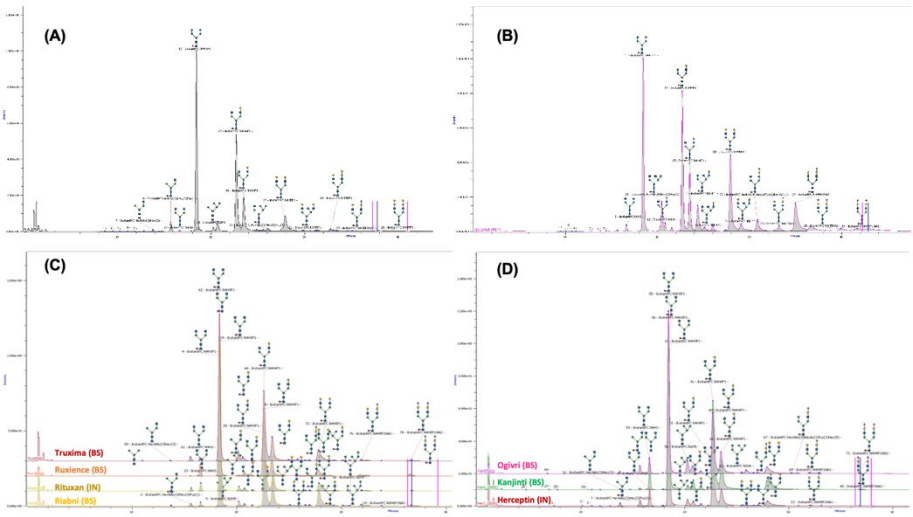


Figure 14 LC plots of released glycan which labeled with instant PC for the **(A)** NIST mAb, **(B)** IgG standard, **(C)** rituximabs and **(D)** trastuzumabs. Glycans were detected using fluorescence and identified using the Protein Metrics released glycan workflow. (Lot N=3)

From previous research, we drafted an initial manuscript using the NIST mAb as our standard to highlight the differences in glycan analysis methods. We used Herceptin and its biosimilars as a case study to show how sensitive methods, such as LC-MS/MS, can detect significant differences in low-level glycans. Notably, we observed that the two trastuzumab biosimilars were more different from each other than from Herceptin. To confirm these previous results, we conducted N-glycosylation analyses with three different lots of antibodies through instant PC, intact MS and peptide mapping. As previously shown, the differences between biosimilars were greater than between the innovator and biosimilars. For example, Ogivri had more high-mannose glycans and sialylated glycans compared to Kanjinti, while Herceptin's high-mannose and sialylated glycan profiles were at an intermediate level between Ogivri and Kanjinti, making them both similar to their reference (Figure 15). Similarly, Truxima had more high-mannose glycans compared to Ruxience, whereas Rituxan's high-mannose glycan profile was at an intermediate level between Truxima and Ruxience. (Figure 15). These trends were consistently observed in both peptide mapping and intact MS analyses.

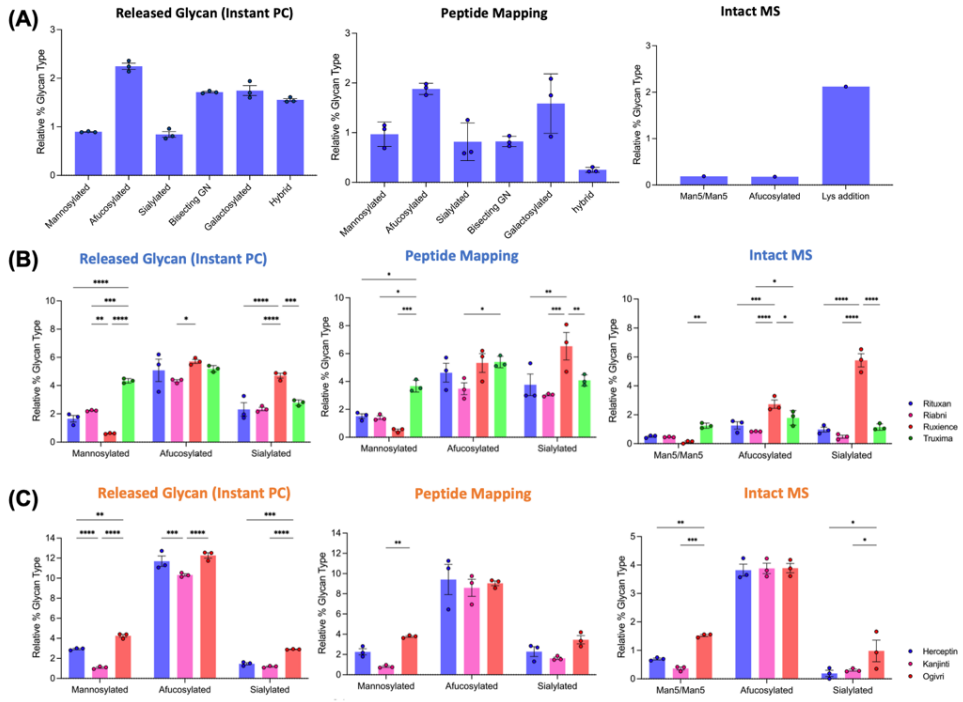


Figure 15 Relative % contribution of each glycan type for the **(A)** NIST mAb, **(B)** rituximabs, and **(C)** trastuzumabs as identified by Released glycan analysis (Instant-PC), LC-MS/MS after protein digestion, and fully glycosylated intact MS. Glycans were identified using the Protein Metrics. N=3; Error bars are standard deviation. *p < 0.05, **p<0.01, ***p<0.001, **** p<0.0001.

A significant improvement in this research was the similarity between peptide mapping data and released glycan data, despite deriving percentages from either AUC normed area % or XIC area summed isoX normalized from released glycan data and peptide mapping data, respectively. Small differences likely resulted from some LC fluorescent peaks of the released glycan containing mixtures of glycans (Figure 16). For instance, the retention time of 20.5 min to 21.3 min in the released glycan LC data of trastuzumabs showed Herceptin containing a mixture of N4H4 and HexNAc(3)Hex(4)Fuc(1) with higher ratio of N4H4. In contrast, Ogivri showed a higher ratio of HexNAc(3)Hex(4)Fuc(1), and Kanjinti showed only N4H4. As it is difficult to separate the mixture peak, this affected our results. Overall, these advancements highlight our ability to comprehensively analyze N-glycan profiles using various methodologies, each offering unique strength in terms of sensitivity and robustness.

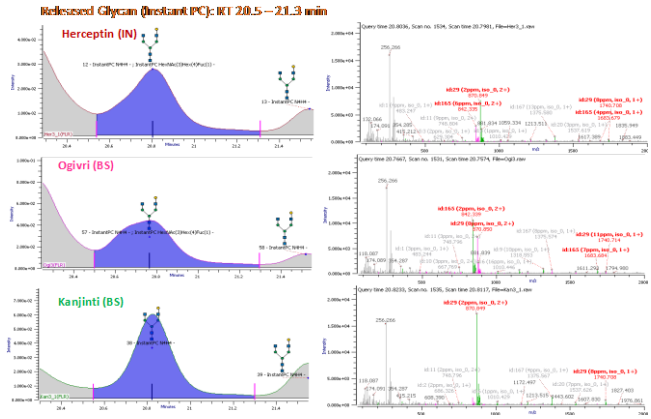


Figure 16 LC fluorescent peak of trastuzumabs containing a mixture of N4H4 and HexNAc(3)Hex(4)Fuc(1) is shown. The retention time is 20.5 – 21.3 min

We also measured the *in vitro* binding and downstream ADCC capacity for Herceptin, Rituxan, and their biosimilars (Figure 17). Kanjinti was found to have a slightly lower IC50 value in the Lumit assay, although no difference was detected in the ADCC assay. Further work is ongoing to determine potential causes for these discrepancies and whether glycosylation may affect other binding parameters such as binding cooperativity, using three different lots.

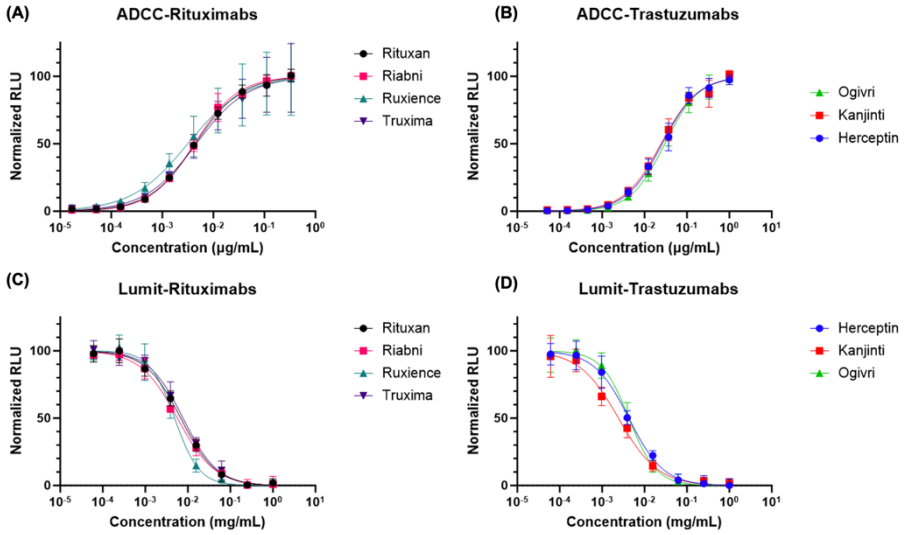


Figure 17 ADCC assays for **(A)** rituximabs and **(B)** trastuzumabs. Fc binding (Lumit) for **(C)** rituximabs and **(D)** trastuzumabs. Depicted are protein concentration vs. normalized relative luminescence unit curves.

These findings piqued our curiosity, and we would like to further explore how biosimilars for the same reference product might be more different than we realized. We plan to collect lots of other marketed biosimilar products for proteins with multiple FDA approved biosimilars including adalimumab, infliximab, and bevacizumab.

Additionally, for our insulin products, we performed glucose uptake assays using two cell lines, 3T3-L1 differentiated adipocytes and HepG2 cells, both known for their sensitivity to glucose uptake. 3T3-L1 cells were treated with MDI and insulin media for 14 days, resulting in adipocyte-like cells. On day 14, the cells were exposed to low glucose media containing insulin lispro or insulin glargine at concentrations ranging from 0 to 100 ng/ml for 24 hours. HepG2 cells were fasted in serum-free media for 2 days before being treated with high glucose media containing insulin lispro or insulin glargine at concentrations ranging from 0 to 3.25 IU/ml for 40 minutes. Glucose uptake was then measured using a commercial kit (Cat No. J1341, Promega), and luminescence intensity was recorded with a plate reader. As shown in Figure 18, there were no significant differences in glucose uptake between insulin lispro and insulin glargine.

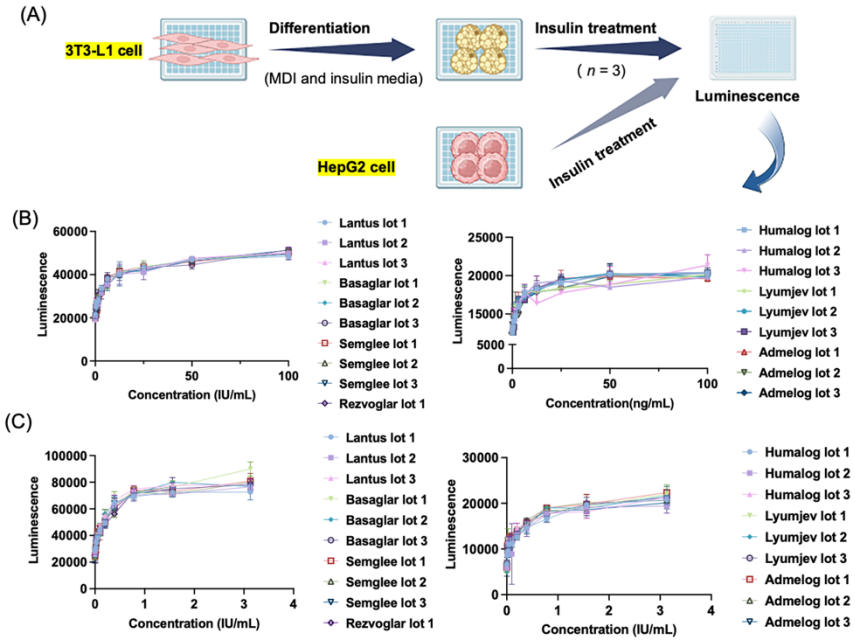


Figure 18. Glucose uptake of insulin. (A) Illustration of glucose uptake in 3T3-L1 differentiated cells and HepG2 cells. (B) Glucose uptake in 3T3-L1 differentiated cells. (C) Glucose uptake in HepG2 cells. *n* = 3.

Aim 5: Perform interviews with major biosimilar developers to assess and identify technical and regulatory challenges in the development of interchangeable biosimilars.

Industry interviews to uncover biosimilar development and approval roadblocks

To uncover the true roadblocks in biosimilar and interchangeable development, multiple rounds of conversations with biosimilar manufacturers, Teva, Viatris and Fresenius Kabi, were held. The goal of these interviews is to assess and identify technical and regulatory challenges that impact development of interchangeable biosimilars. The process involves establishing CDAs with companies that are willing to participate and share their experience, then conducting multiple rounds of interviews- the first interviews are typically unstructured where the interviewees share their development and/or approval hurdles, potential solutions and insights into their biosimilar pipeline. After the initial interview, we conduct follow-up discussions where we dive into specific pain points in more depth. The insights from these interviews are described herein. These outcomes are not inclusive of everything that we heard, but rather focus on common concerns shared across multiple

interviewed parties. Over the next year we plan to conduct additional interviews with more companies. Insights from these interviews will be summarized as a brief publication upon completion of this aim.

These interviews covered the overall barriers that companies encounter while developing biosimilars and seeking interchangeability approval from the FDA. Broadly, the development challenges discussed included the validation of analytical methods, immunogenicity problems related to PTM modifications, clinical study requirements, and interchangeability designation.

The level of immunogenicity in new biological molecules has been decreasing since the advent of humanized antibodies. However, the impact of PTM modifications, including glycosylation, on immunogenicity is not firmly established, and more research is needed to determine if glycans or other factors are responsible for immunogenicity in patients. *In vitro* immunogenicity studies face challenges in correlating with *in vivo* results, therefore, there is a need to validate analytical methods, addressing differences in data outcomes and reproducibility issues. In a similar vein, there is uncertainty with regards to which glycans, and at what level, are problematic in vivo. There is no clear answer on the level of specific glycans that result in reduced in vivo efficacy and/or altered pharmacokinetics.

It is important to establish acceptable differences in biosimilar products, considering that even innovator batches exhibit variability. Careful evaluation and understanding of these factors are necessary to ensure the safety and efficacy of biosimilars. However, it needs to be recognized that orthogonal methods will not yield the same exact results. They should yield similar trends, but the values may differ between methods. That is why creating validated, best practice methods for companies to universally perform is critical.

It is also important to understand why proteins fail during development or during early clinical trials. Such information is not available in the public domain. We propose acquiring lots of “failed” products from multiple biosimilar manufacturers to see if our methods could tease out the main reasons why a biosimilar candidate was abandoned. If we could make this “failure” information known, perhaps companies could address risks earlier on in the development cycle. Ideally, this information would also lead to discussions with multiple stakeholders regarding the establishment of concrete guidelines and acceptance criteria for certain analytical methods.

Furthermore, the need for multiple switches and the differences in regulatory perspectives on interchangeability designation between the FDA and EMA contribute to increased time and complexity in clinical studies for biosimilars. The FDA's requirement for a larger clinical program compared to the EMA leads to higher development costs and fewer attractive business cases. Leveraging data analytics and modeling technologies can provide valuable insights and potentially reduce the need for extensive clinical trials, improving the efficiency of biosimilar development. Where this would be of great value would be for phase 3 trials as they are large and very expensive, sometimes more so than the reference innovator.

Another area of interest for BsUFA work that was discussed, but is perhaps out of our wheelhouse, is the lack of a true cellular/ bodily fluid matrix to test samples in. While in vitro assays are helpful in giving baseline information about a protein therapeutic’s functionality, they are not reflective of what would happen in the presence of cells in their native environment. If there could be a more realistic matrix in which to perform functionality and efficacy assays (antigen binding, ADCC, CDC) without having to conduct animal studies or clinical studies, this could change development decisions earlier in the pipeline.

Lastly, biosimilar companies are now developing new products that require regulatory clarity. These include antibody drug conjugates (ADC), fusion proteins (such as Trulicity), bispecifics, Fabs, nanobodies, and products like Botox. Without clear guidances, it is challenging for companies to streamline their characterization processes. Currently, they often perform more in vitro assays than necessary to mitigate the number of review cycles that the drug might have to undergo if not enough data is presented the first time.

3. RESEARCH OUTCOMES

Describe project specific outcomes since the start of the budget cycle or last report inform or achieve the project objective (500-word max). *Note, text in this section should directly support content in the 'Outcomes' column in table 1.*

In addition, if there is a concern about public dissemination of the research outcomes prior to completion of the project, notify the BsUFA III regulatory science pilot program ***immediately*** to discuss either 1) requesting that this section is redacted from the publicly posted version or 2) only including abstract-level detail.

Overall, we successfully obtained three different lots of each drug, enhancing the statistical reliability of our study. This allowed for systematic analytical characterization of both innovator and biosimilar products, focusing on post-translational modifications.

Aim 1: We investigated structural differences across reference and approved biosimilar products of several drug classes, including insulins (glargine and lispro) and monoclonal antibodies (mAbs) (rituximab and trastuzumab). Utilizing various analytical techniques, such as mass spectrometry (intact MS and peptide mapping) for mAbs and Fourier Transform Infrared (FTIR) spectroscopy for insulins, we identified minor variations between the reference drugs and their biosimilars. This led to the establishment and development of orthogonal methodologies to detect these subtle structural distinctions.

Aim 2: We compared multiple mAbs and insulins using Ion Mobility-MS (IM-MS) and Circular Dichroism/Near UV methods, respectively. Minor differences were observed. IM-MS, a highly sensitive mass spectrometry technique capable of detecting small changes in mAb higher-order structures (HOS), was used in collaboration with Dr. Ruotolo's lab at the University of Michigan, ensuring unbiased results.

Aim 3: We assessed the levels of non-covalent and covalent aggregates or fragments under stress conditions across various innovator and biosimilar products, employing Size-Exclusion Chromatography (SEC) UPLC. Interestingly, Ruxience, a biosimilar of rituximab, formed more fragments rather than aggregates. All other mAb products, innovators and biosimilars, formed aggregates over time. Other than the fragments in Ruxience, only minor differences were observed between innovator and biosimilar mAbs and insulins.

Aim 4: We examined the N-glycosylation microheterogeneity of mAbs using released glycan and peptide mapping analyses, as well as its impact on Fc-receptor binding and Antibody-Dependent Cellular Cytotoxicity (ADCC). Both analytical techniques produced very similar results. Differences in the N-glycosylation profile were more pronounced between biosimilars than between biosimilars and reference innovators (either Herceptin or Rituxan). However, no significant differences were found in ADCC and Fc-receptor binding analyses across the mAbs.

Aim 5: To uncover the true roadblocks in biosimilar and interchangeable development, multiple rounds of conversations with biosimilar manufacturers, namely Teva, Viatris and Fresenius Kabi, were held.

4. REGULATORY IMPACT

Describe project specific regulatory impact. This section should clearly identify and describe how the project will inform or impact biosimilar development or regulation (500-word max).

Our findings from Aim 1 to Aim 4 piqued our curiosity, so we explored how biosimilars for the same reference product might be more different than we initially supposed. We have seen only a few published studies comparing competing biosimilars head-to-head, and believe that performing more experiments in this space could help in determining acceptance criteria and goalposts for some characterization techniques. It could also inform decision-makers on the feasibility of interchangeability between biosimilars of the same reference drug product.

As described in Aim 5, our communication efforts will bridge current gaps between the FDA and industry and will allow for a neutral third party to share both sides' thoughts and concerns. It will also help the agency to adjust BsUFA research priorities and help in the negotiations with industry stakeholders around funding of the next cycle of BsUFA.

Together, these aims are well aligned with the two aims of the BsUFA III Regulatory Science Pilot Program i.e. (1) advancing the development of interchangeable products, and (2) improving the efficiency of biosimilar product development

5. COMMUNICATION AND DISSEMINATION

Describe project specific communication and dissemination for this study. Include citations for any publications, abstracts, talks/speaking events etc. *Note, text in this section should directly support content in the 'Communication Timeline' column in table 1.*

If the contents of Section 3 are either be redacted or written at an abstract-level detail due to concerns about public dissemination of the results and outcomes prior to completion of the project (see Section 3), this section *must* include the plan and timeline for communication of all the results and outcomes of the project (500-word max).

1. Na Y, Kinzer J, Ford M, **Schwendeman A**, Structural comparison of innovator and biosimilar monoclonal antibodies, Rituximab and Trastuzumab, using the mass spectrometry-based methods of post-translational modification analyses, ***GPEN***, Copenhagen, Denmark, Jul. 2024 (Poster).

Monoclonal antibodies (mAbs) play a crucial role in modern healthcare, with biosimilars mimicking their innovator counterparts. However, unlike small molecule drugs, mAbs are produced through recombinant processes, resulting in inherent structural variability due to post-translational modifications (PTMs). This heterogeneity can impact target protein binding via the Fab domain, receptor interaction through the FC domain, and induce protein aggregation-associated immunogenicity. Our study focused on comparing the heterogeneity

of innovator mAbs with their biosimilars, specifically rituximab and trastuzumab pairs. We employed various analytical techniques, including intact mass spectrometry, released glycan analysis, and peptide mapping. Our findings revealed consistent trends across analytical methods, reflecting the orthogonal approach for assessing microheterogeneity. This insight enhances our understanding of acceptable differences across product types.

2. Rivera-Fuentes NA, Vallao M, Erlenbeck A, Armbruster MR, Cicali A, Na Y, Zhao T, Kim M, **Schwendeman A**, Ruotolo BT, Ion mobility-mass spectrometry and collision induced unfolding methods reveal structural differences in stressed biosimilar therapeutic products, ***ASMS***, Anaheim, CA, Jun. 2024 (Poster).

Monoclonal antibody (mAb) biosimilars are designed to reproduce the sequences, efficacies, and safety features of innovator mAb therapeutics. However, biosimilars that utilize different cell lines for large-scale production, and differing formulations to support manufacturing and storage may result in minor changes in mAb higher-order structures (HOS), leading to knock-on changes in therapeutic efficacy and safety that are challenging to detect. Ion mobility-mass spectrometry (IM-MS) coupled to collision induced unfolding (CIU), is a multidimensional technology that enables fast and sensitive protein structure and stability assessment. Here, we explore the ability and mechanistic underpinnings of IM-MS and CIU to probe HOS as well as stability changes across biosimilar and innovator products, including mAb and insulin constructs.

3. Kinzer J. Unraveling the Twin Tales of Biosimilar and Innovator Glycans. ***Protein Metrics Invited Webinar Series.*** Mar 2024. https://proteinmetrics.com/unraveling-the-twin-tales-of-biosimilar-and-innovator-glycans-replay/

N-glycans are omnipresent in the Fc region of monoclonal antibody products. Modifications to a glycan profile can impact a protein's stability/folding, binding affinities, effector functions, clearance rates, and/or immunogenicity. Their characterization is critical to ensuring efficacy and safety in patients. The multitude of glycan characterization methods, each with different levels of sensitivity and reliability, complicates standardization efforts. To tackle this, we've conducted comparative glycan analysis studies, examining various innovator mAbs, their biosimilars, and different characterization techniques for NIST mAb.

4. Zhao T, Shay B, Vander Roest M, Kinzer J, **Schwendeman A**, Analyzing Insulin Glargine Biosimilars: Stability, Degradation, and Structural Characterization, ***AAPS***, Orlando, FL, Oct. 2023 (Poster)

Insulin glargine became the first insulin analogue with a biologic product definition, paving the way for the emergence of biosimilar alternatives such as Semglee (insulin glargine-yfgn) and Rezvoglar (insulin glargine-aglr) to Lantus. However, due to differences in manufacturing processes and pharmaceutical formulations, innovator insulins may undergo distinct chemical modifications compared to their biosimilars. Moreover, insulin glargine is highly sensitive to environmental stresses and is prone to structural changes, unfolding, and aggregation. Even minor alterations that affect the structural integrity of insulin could have profound implications on its biological efficacy, particularly in terms of physiological and pharmacological activities. Therefore, it is crucial to thoroughly understand the potential differences in aggregation propensity among biosimilar versions of insulin glargine. We have chosen Basaglar as a model drug to investigate these implications and established analytical methods for evaluation critical quality attributes. Analytical characterization of Basaglar provides insights into its comparability to innovator insulin glargine, aiding in the assessment of biosimilar insulin products.

5. Kinzer J. Biologics In Vitro Characterization Advancements to Streamline Development and Approval Timelines. ***University of Michigan Thesis Records***. Jul 2023. http://hdl.handle.net/2027.42/178001

Many of the top-selling drug products on the market, with sales in the billions annually, are monoclonal antibodies (mAbs). Due to the success of originator mAb products, it is unsurprising that the overall biologics market is saturated with competition in the form of other originator products approved for similar indications, or, as products lose their exclusivity, in the form of biosimilars. Despite being approved for similar indications,

competitor products can have differences in their structure and function. To determine the extent of these differences and the efficacy and safety implications they might provoke, numerous in vitro and in vivo assays have to be conducted and validated prior to drug approval. Yet, often the methods performed by each company for their drug's approval are disparate. Therefore, to aid in developing universally performed, best-practice methods for biologics development, we have studied characteristics including Fab binding affinity, Fc binding affinity, antibody dependent cellular cytotoxic activity, disulfide shuffling, degradation patterns, and glycosylation profiles of numerous competing originators and originator/biosimilar pairs. From our initial studies with three anti-TNFα mAbs, we sought to not only determine any correlation between higher binding affinity, glycosylation patterns and efficacy, but also to look into the feasibility of repeating these assays with additional lots and drug products for future validation. Similarly, we monitored structural similarities and differences, including disulfide bonds and glycans, for originator and biosimilar mAbs. The results from these experiments were used to identify indicators (i.e. mannosylated glycans, shuffled disulfide bonds) of potentially reduced therapeutic efficacy and/or safety concerns. We also were interested in seeing the extent of variability between originators and biosimilars and between multiple biosimilars of the same reference, as that could have implications for drug interchangeability. By performing a range of structural and bioactivity assays on approved protein therapeutics, we aim to aid in the development and validation of characterization methods for new biologics and biosimilars.

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6. CHALLENGES

Describe project specific challenges for this study. This section should include:

- Changes in approach and reasons for change.
- Actual or anticipated problems or delays and actions or plans to resolve them.
- Changes that have a significant impact on expenditures.
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
- (500-word max).

As part of Aim 5, we have already successfully met with three different biopharmaceutical companies. However, scheduling interviews with various biopharmaceutical companies has proved challenging. Initially, the companies did not bring the appropriate representatives to the first calls. In subsequent meetings, though, they included the correct members, such as regulatory agency representatives or scientists. Therefore, to gather sufficient data and understand the challenges they encounter during the biosimilar approval or interchangeability authorization process, regular interviews (every 3 to 6 months) are necessary instead of one-off interviews.

Additionally, introducing our programs and conveying the purpose of the interviews required significant time to negotiate with many new companies. Fortunately, we have reached agreements with global biopharmaceutical companies like Biocon and Celltrion for future interviews. However, more time is needed for routine interviews and to engage with new companies.

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7. NEXT STEPS

Describe plans or next steps, especially if there are changes from the original proposal (500-word max).

First of all, we plan to draft the manuscript of a methodology paper focused on the optimization study of N-Glycan characterization methods for NIST mAb. (Plan to submit before Aug ’24).

As part of an Aim 1, we are currently analyzing several PTMs including deamidation, oxidation, deoxidation, and acetylation through peptide mapping. We will finalize the analysis of all of the antibodies in house. Following the completion of the PTMs analysis through peptide mapping, we will prepare two application papers that detail an overall structural difference study. These papers will include IM-MS, intact MS, released glycan analysis, and peptide mapping analysis for both innovators and biosimilars of trastuzumab and rituximab. (Plan to submit Sept ’24 – Oct ’24).

So far, as part of Aim 5, we have completed four interviews with 3 different biopharmaceutical companies. However, the current number of interviews is insufficient to generalize the findings and publish an article. Therefore, we plan to conduct additional interviews with more biopharmaceutical companies. We plan to publish an article on our initial insights from these meetings once we have 8-10 companies represented. The manuscript will provide a summary of the interviews conducted thus far as part of Aim 5.

Additionally, we know that biosimilar companies are interested in studying other biologics such as antibody-drug conjugates (ADCs), fusion proteins, nanobodies and fAbs. Of particular interest to our group is Trulicity, a fusion protein for GLP-1 expected to lose patent exclusivity in the next few years. Using our in house Trulicity (Dulaglutide) and Victoza (Liraglutide), we would like to start characterizing them prior to loss of exclusivity.

8. REFERENCES

References used in progress report.

9. ADDITIONAL A: ADDITIONAL MATERIAL

Include any additional material to support the report content (optional).