BsUFA annual report

Project (Grant): 1 U01FD007763-01-Schwendeman

1. Overview of funded project objective and aims

1.1. Project objective

This research aims to compare and characterize the differences between biosimilars and originator biological drugs including insulin (Humalog®, Admelog®, Lyumjev®, Lantus®, Basaglar, Semglee®) filgrastim (Neupogen® and TPI filgrastim Releuko®), infliximab (Remicade®, Inflectra®, and Renflexis®), rituximab (Rituxan® and Accellbia®), trastuzumab (Herceptin® and HERtiCAD), and bevacizumab (Avastin® and Avegra®) with the goal of reducing the existing high barriers to interchangeability approval.

To establish orthogonal methods for characterization of 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, therapeutic mAbs, future work: ADCs, Fabs, nanobodies) and biosimilar versions of the same products.

1.2. Project aims

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

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

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.

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).

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

2. Research Progress

2.1. Results and outcomes of the Project (How the results and outcomes of the project inform or achieve the project objective.)

Aim 1a) 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 purchased for this project. We are looking to expand upon our inventory during the rest of the funding period to include more lots of each protein. Ideally, we would procure at least 3-4 lots of each protein so that we can monitor lot-to-lot variability on our various assays. Additionally, we know that there is interest among the biosimilar companies to study 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. It is expected to lose patent exclusivity in the next few years. We anticipate that there will be many biosimilars entering the market given the success of Trulicity, so we would like to start characterizing it prior to its loss of exclusivity. In addition, analytical characterization of ADCs is more complex as drug conjugation might impact the pharmacokinetics and immunogenicity of these products. Thus, we plan to explore methods for characterization of ADC's heterogenicity. Different product categories will be used in experiments conducted in Aims 1-4.

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
Admelog®Solostar	Sanofi	3F508A	03/31/2025
Admelog®Solostar	Sanofi	3F5151	06/30/2025
Lyumjev™Kwikpen®	Eli Lilly & Co	D522060E	07/22/2024
Lyumjev™Kwikpen®	Eli Lilly & Co	D539311D	08/19/2024
Insulin glargine			
Lantus®Solostar®	OR from Sanofi	2F8431A	07/31/2024
Lantus®Solostar®	OR from Sanofi	3F8832A	12/31/2024
Basaglar®Kwikpen®	BS from Eli Lilly & Co	D510461D	05/11/2024
Basaglar®Kwikpen®	BS from Eli Lilly & Co	D437809F	09/24/2023
Semglee®	BS from Mylan	BF21005474	12/2023
Semglee®	BS from Mylan	BF22000063	01/2024
mAb			
Trastuzumab			
Herceptin®	OR from Genentech	3514960	03/2024
		3576752	06/2024
Kanjinti™	BS from Amgen	1141539A	10/31/2024
		1149306B	04/30/2025
Ogivri®	BS from Mylan	BF19006696	03/2023
		BF22002939	08/2026

Products	Manufacture	Lot#	Expiration
Rituximab			
Rituxan®	OR from Genentech	3491321	08/2024
		3575428	10/2025
Ruxience™	BS from Pfizer	FC5021	05/31/2023
		GN4445	04/30/2024
Truxima®	BS from Teva	0L0031	11/2024
		1B1011	01/2025
Riabni™	BS from Amgen	1128511	07/31/2023
		1141263	03/31/2024

Aim 1b) Examine structural features and differences of multiple approved biologics and biosimilars

For the mAbs, namely Rituxan and its biosimilars, Herceptin and its biosimilars, and NIST mAb, we have characterized the antibody structure using intact mass spectrometry (MS). Shown in Figure 1 are some of the intact MS spectra that we have collected for these mAbs. We observed that there are slight differences between these products, for example in the glycan identification. For the most part though, as expected given their approved status, the intact MS showed high similarity between the innovators and biosimilars. Intact MS, while helpful for confirming protein features such as molecular weight, is not the most sensitive at detecting smaller differences in post-translational modifications between biosimilars and innovators. Hence the reason we also performed protein digestion and glycan analysis (Aim 4) to further probe differences between innovators and biosimilars.

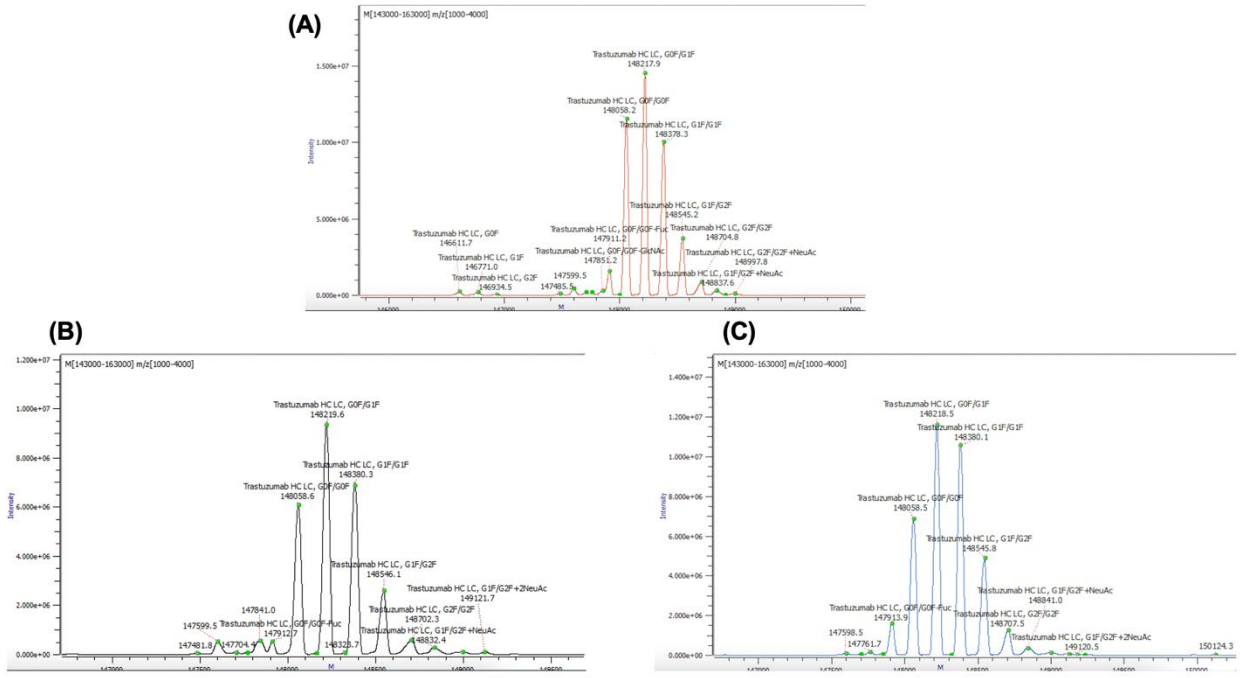


Figure 1. Deconvoluted intact MS spectra for **(A)** Herceptin and two of its biosimilars **(B)** Ogivri and **(C)** Kanjinti. Shown are the glycans identified by Protein Metrics Intact MS workflow.

In Figure 2, we show sequence coverage maps after protein digestion for TPI filgrastim and its reference innovator Neupogen. We included this figure to highlight our capabilities to capture complete sequence coverage and identify the location of structural motifs such as deamidation and oxidation sites (red dots on sequence coverage maps). This type of data can be generated from protein digestion on the AssayMAP Bravo followed by data acquisition on an LC-MS/MS system and data processing using Protein Metrics software. We have performed such workflows for our NIST mAb, rituximabs and trastuzumabs and are planning to perform another round of digestion once we have N=3 lots of each protein so that we can process all lots simultaneously.

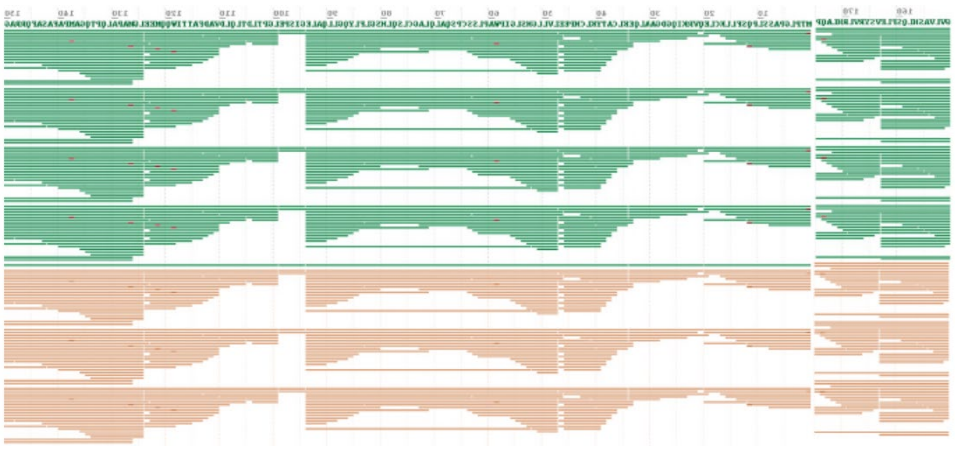


Figure 2. LC-MS/MS analysis of TPI-filgrastim (top 4) and Neupogen (bottom 3) after Glu-C digest showing 100% sequence coverage and locations of oxidations and deamidations (in red dots).

We have created intact MS methods for insulin lispro and insulin glargine by QTOF, which is shown in Figure 3. To process the obtained data, we are currently awaiting an update to our UNIFI software from Waters. These established methods will be applied to determine any differences between the insulin biosimilars.

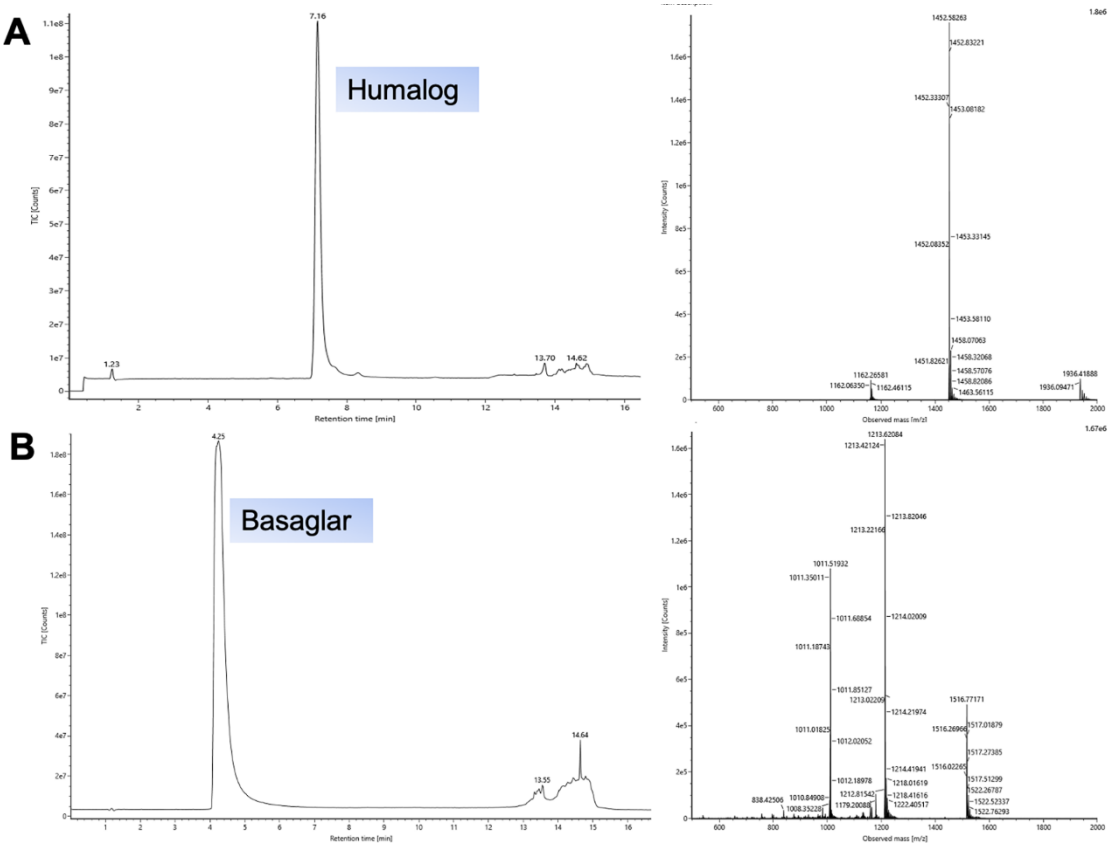


Figure 3. The chromatograms and spectrums of Humalog and Basaglar obtained by QTOF. **(A)** The chromatogram and spectrum of intact Humalog. **(B)** The chromatogram and spectrum of intact Basaglar.

Aim 2) Higher order structure of proteins

We have contacted Dr. Brandon Ruotolo's lab about running some of our current samples (Table 1) on their ion-mobility mass spectrometer (IM-MS). They have agreed to run these samples after our initial project discussion occurring in mid-July. They have also agreed to help run future lots and/or additional proteins. From these experiments we anticipate collecting data similar to that which is shown in Figure 4.

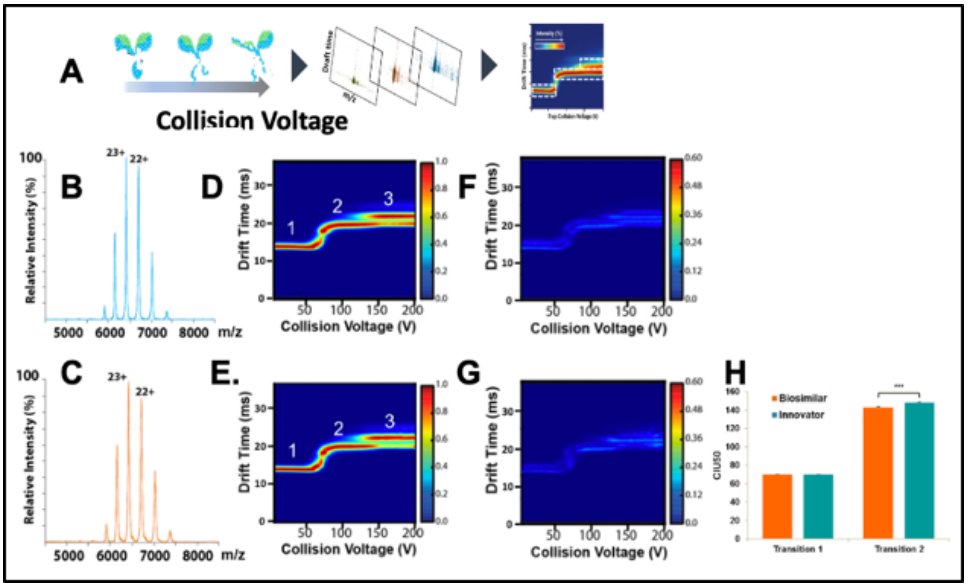


Figure 4. (A) Schematic representation of IM-MS analysis. IM-MS spectra and average CIU fingerprints and standard deviation of innovator **(B, D, F)** and biosimilar **(C, E, G)** rituximab. **(H)** Quantitation of CU50-1 and CIU50-2 transitions between 1: initial compact state, 2,3: unfolded states. CIU50 values of innovator and biosimilar at transition 1 and 2. (n=3, ***p<0.001, student t test)

IM-MS detects differences in protein unfolding, allowing us to monitor how differences in storage conditions, excipients, container systems, etc. impact protein stability. 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.

Furthermore, we investigated the use of intrinsic fluorescence in the ultraviolet spectrum to measure insulin stability in the context of thermal cycling. The characteristic spectrum results from the inclusion of fluorescent amino acid residues and is reduced upon heating and loss of native three-dimensional conformation (Figure 5A). Meta-cresol, a stabilizing excipient found in most injectable insulin formulations, was found to interfere with measurement accuracy and exhibit concentration-based auto-quenching of the fluorescent signal. Ultracentrifuge filtration was used to remove the majority of meta-Cresol from Basaglar and Admelog injectable pens and concentrations of insulin and meta-cresol were confirmed via UPLC (Figure 5B). The meta-cresol-reduced insulin was remeasured and found to yield more consistent results (Figure 5C). This revised methodology will be used to investigate the stability of insulin originators and biosimilars under forced stress conditions.

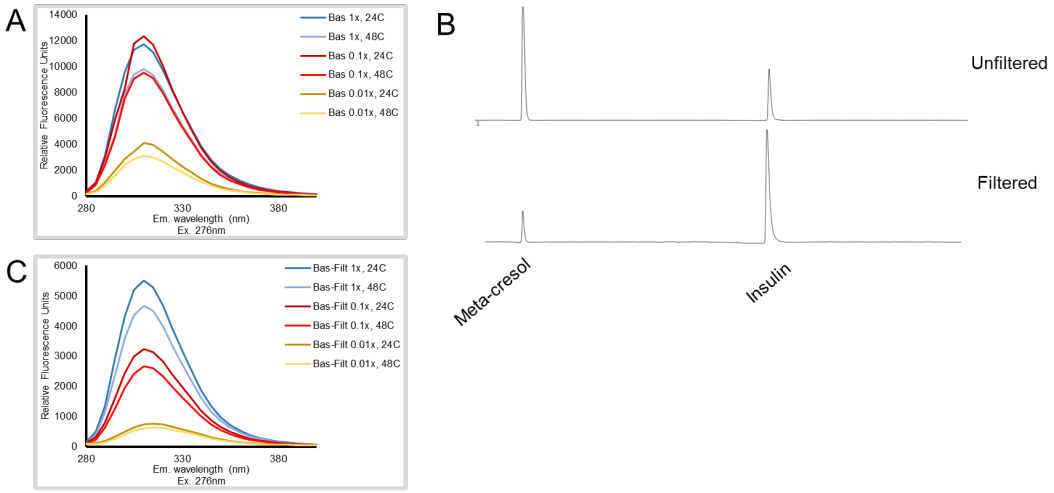


Figure 5. UV fluorescent measurement of Basaglar pre- and post-filtration. **(A)** UV fluorescent spectrum of Basaglar formulation at 1x, 0.1x, and 0.01x dilutions, measured at 24°C and 48°C. Concentration based auto-quenching is evident from overlap of 1x and 0.1x dilutions. **(B)** Representative UPLC chromatograms of unfiltered (top) and filtered (bottom) Basaglar, showing removal of the majority of the meta-cresol. **(C)** UV fluorescent spectrum of filtered Basaglar at 1x, 0.1x, and 0.01x dilutions, showing reduced auto-quenching as a result of removal of meta-cresol.

Aim 3) Aggregation/degradation of proteins as they relate to structural and environmental features

We have performed disulfide shuffling and degradation studies on Rituxan and Avastin and a biosimilar from each. From these studies, we determined that the rituximabs and bevacizumabs degrade differently under stressed conditions. The rituximabs formed more fragments when exposed to heat and shaking over time whereas the bevacizumabs formed more fragments under the same conditions (Figure 6). Between the innovators and biosimilars, there were rather minimal differences. The only significant difference was observed for Avastin and its biosimilar after 2 and 4 weeks under stress conditions (Table 2).

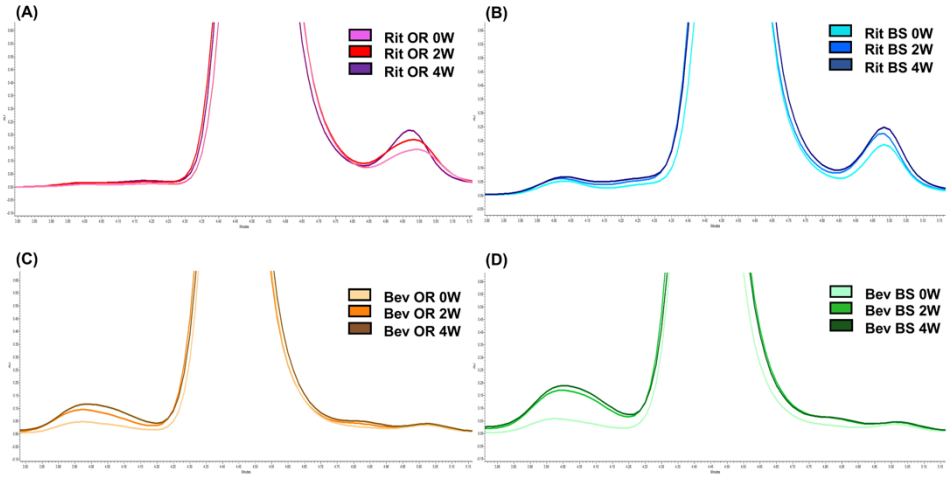


Figure 6. Representative SEC chromatograms at 214 nm for 15 µg of antibody. **(A)** Rituximab OR at 0 (pink), 2 (red) and 4 (purple) weeks;**(B)** Rituximab BS at 0 (light blue), 2 (blue) and 4 (navy) weeks;**(C)** Bevacizumab OR at 0 (peach), 2 (orange), and 4 (brown) weeks;**(D)** Bevacizumab BS at 0 (teal), 2 (green) and 4 (dark green) weeks. Stressed samples were shaking at 240 RPM, incubating at 37°C for 2 or 4 weeks. Chromatograms are zoomed in to depict the increase in aggregates and/or fragments detected in each sample across each timepoint.

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, 2-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*Denotes statistical significance of BS, compared to OR, at same timepoint for the same protein type.

	% Monomer	% Aggregates	% Fragments
Rit OR 0w	92.48 ± 0.26	0.76 ± 0.02	6.76 ± 0.24
Rit OR 2w	91.15 ± 1.33	1.37 ± 0.61	7.49 ± 0.72
Rit OR 4w	91.03 ± 0.26	1.37 ± 0.08	7.61 ± 0.24
Rit BS 0w	90.79 ± 0.01	2.11 ± 0.05**	7.09 ± 0.05
Rit BS 2w	90.51 ± 0.63	1.89 ± 0.58	7.60 ± 0.40
Rit BS 42	89.57 ± 0.50	2.41 ± 0.11*	8.02 ± 0.38
Bev OR 0w	95.34 ± 0.33	2.91 ± 0.39	1.76 ± 0.06
Bev OR 2w	92.43 ± 0.64	5.85 ± 0.78	1.71 ± 0.13
Bev OR 4w	91.14 ± 0.38	7.09 ± 0.37	1.78 ± 0.01
Bev OR 0w	94.78 ± 0.02	3.33 ± 0.06	1.91 ± 0.07
Bev OR 2w	89.88 ± 0.21****	8.38 ± 0.21***	1.74 ± 0.03
Bev OR 4w	87.65 ± 0.53****	10.60 ± 0.52****	1.75 ± 0.01

We also monitored disulfide shuffling for these proteins 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 7). 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.

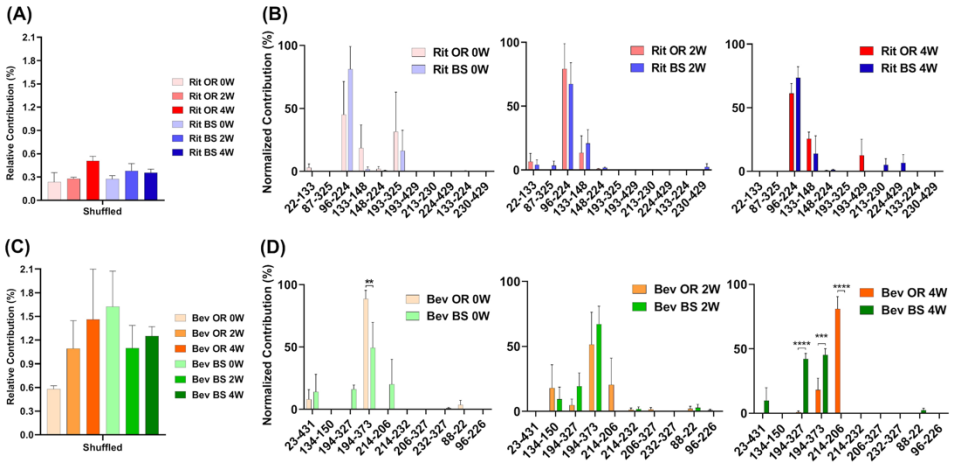


Figure 7. Total shuffled bond contribution relative to the XIC sum of all identified disulfide bonds for **(A)** rituximab originator and biosimilar and **(C)** bevacizumab originator and biosimilar. Prevalence of the shuffled bond locations normalized to the total number of shuffled bonds for **(B)** 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 additional 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 (i.e. polysorbates or trehalose) 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 (such as DetergentOut columns for polysorbates) 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.

For the insulins, we initiated our degradation/aggregation study with insulin lispro, as it was readily accessible to us and allowed us to identify any variations following product stressing. Our methods involve incubating Humalog or Basaglar under shaking/heating conditions followed by the analysis of parameters such as concentration, aggregation, fibrillation, size, and structural dissimilarities (Figure 8).

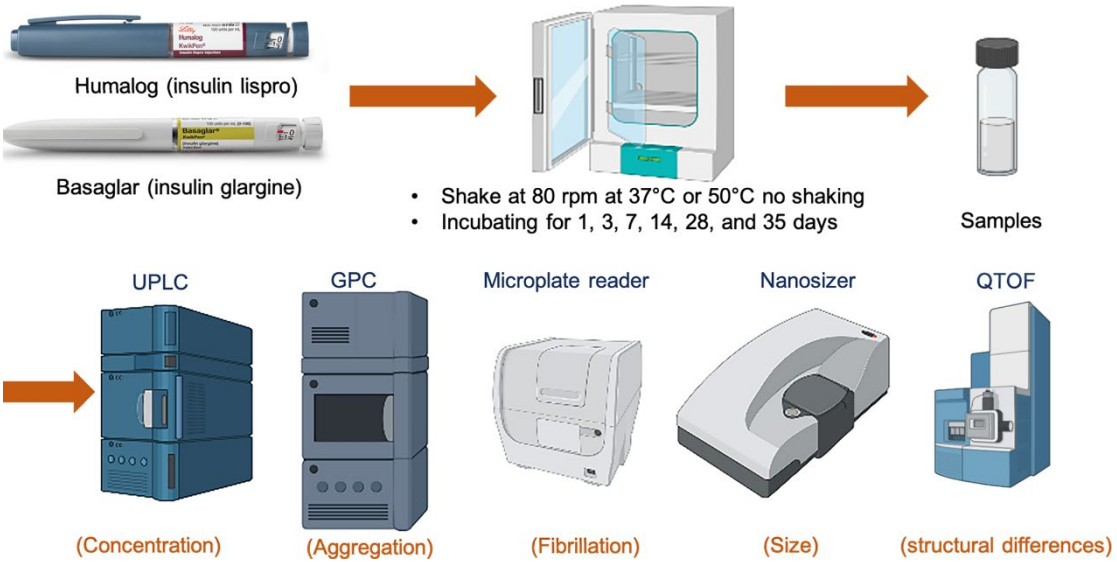


Figure 8. The scheme of insulin lispro and insulin glargine incubating at a stressed condition and then monitoring of changes using different instruments.

Humalog was subjected to incubation at 37°C with shaking at 80 rpm. The concentration of insulin lispro, within a reported range of 95-105 Units/mL, was monitored, with the lower limit indicated by a red dashed line (Figure 9A). After 21 days of incubation, a significant decrease in concentration was observed compared to fresh Humalog (**$P < 0.01$, one-way ANOVA test), with a final concentration of 94.68 ± 0.6 Units/mL. This decrease suggests a time-dependent reduction in Humalog's efficacy. Concurrently, an increase in the peak area for particles with a diameter greater than 100 nm was observed after 14 days of incubation, indicating a potential physical change in the samples (Figure 9B). Analysis of the GPC chromatogram did not reveal any evident changes (Figure 9C). Moreover, the utilization of thioflavin T (ThT) for detecting amyloid fibrils confirmed the formation of amyloid fibrils in Humalog after 14 days of incubation at 37°C (Figure 9D). In addition, the same batch of Humalog was subjected to testing at 50°C. After just three days of incubation, a significant decrease in concentration was observed compared to fresh Humalog, with a concentration of 92.2 ± 2.5 Units/mL (Figure 9E). Furthermore, even after just one day of incubation at 50°C, a decrease in the peak area for particles with a diameter of 5 nm was observed (Figure 9F). GPC chromatogram analysis revealed a decrease in peak area, with the appearance of high molecular weight products (HMWP) at 5 minutes (Figure 9G). Moreover, amyloid fibrils were observed in Humalog after three days of incubation at 50°C (Figure 9H).

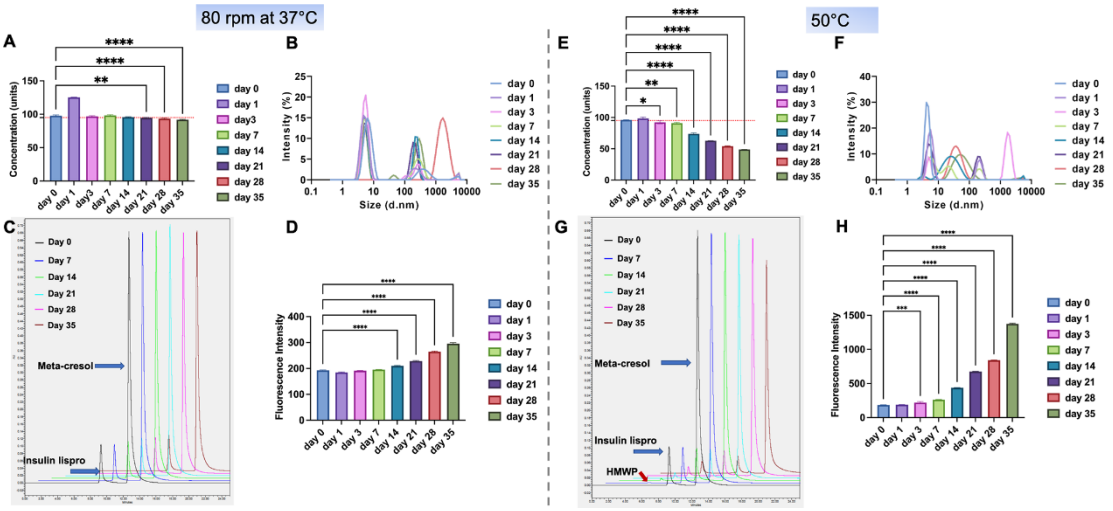


Figure 9. The changes of Humalog under different stressed conditions. A. The concentration of insulin lispro after incubating at 80 rpm at 37°C for different periods. $n = 3$, $^{**}P < 0.01$, $^{****}P < 0.0001$. B. The size of Humalog after incubating at 80 rpm at 37°C for different periods. C. GPC chromatograms of Humalog after incubating at 80 rpm at 37°C for different periods. D. The fluorescence intensity of stressed Humalog at 80 rpm at 37°C detected by thioflavin T. $n = 3$, $^{****}P < 0.0001$. The concentration of insulin lispro after incubating at 50°C for different periods. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. E. The size of Humalog after incubating at 50°C for different periods. C. GPC chromatograms of Humalog after incubating at 50°C for different periods. D. The fluorescence intensity of stressed Humalog at 50°C. $n = 3$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

As we found some changes while stressing Humalog, we thought we might find some similar changes in the insulin glargine injection. Upon subjecting insulin glargine injection to incubation at 37°C and 80 rpm for 35 days, a significant decrease in concentration was observed compared to the fresh sample, although the concentration remained above 95 units/mL throughout the incubation period (Figure 10A). Notably, the size exhibited significant changes on day 35 (Figure 10B). Following incubation at 80 rpm and 37°C for 7 days, the GPC chromatogram displayed the presence of HMWP at approximately 8.5 minutes (Figure 10C). However, no apparent amyloid fibrils were observed in Basaglar after incubation under these conditions.

Furthermore, after being incubated at 50°C for 14 days, the insulin glargine in Basaglar showed a significant decrease in concentration which is below 95 Units/mL indicating the loss of potency (Figure 10E). Notably, the size exhibited significant changes after 21 days of incubation (Figure 10F). The GPC chromatogram analysis revealed the presence of high molecular weight products (HMWP) at around 8.5 minutes after incubation at 50°C (Figure 10G). However, no obvious amyloid fibrils were observed in Basaglar after incubation at 50°C (Figure 10H). After finding some differences in the stressed insulin products, we are going to apply these methods to find the impurities and mutations between insulin biosimilars.

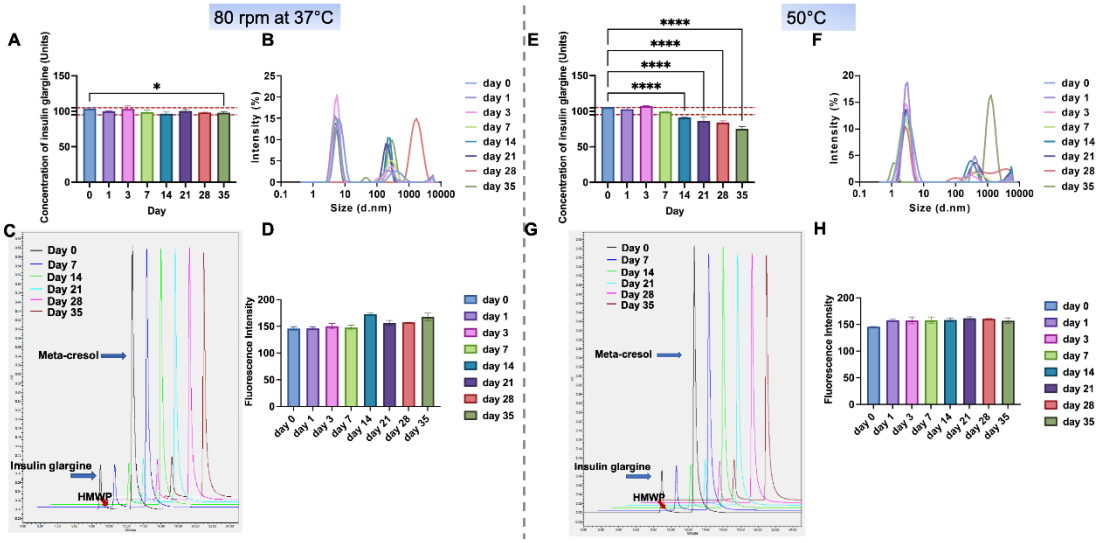


Figure 10. The changes of Basaglar under different stressed conditions. A. The concentration of insulin glargine after incubating at 80 rpm at 37°C for different periods. $n = 3$, $^{*}P < 0.05$. B. The size of Basaglar after incubating at 80 rpm at 37°C for different periods. C. GPC chromatograms of Basaglar after incubating at 80 rpm at 37°C for different periods. D. The fluorescence intensity of stressed Basaglar at 80 rpm at 37°C detected by thioflavin T. $n = 3$. E. The concentration of insulin glargine after incubating at 50°C for different periods. $n = 3$, $^{****}P < 0.0001$. F. The size of Basaglar after incubating at 50°C for different periods. C. GPC chromatograms of Basaglar after incubating at 50°C for different periods. D. The fluorescence intensity of stressed Basaglar at 50°C. $n = 3$.

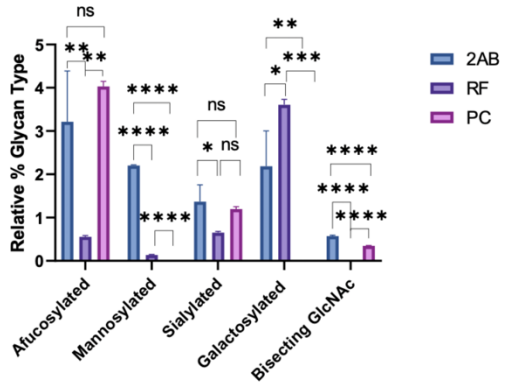
Aim 4) Comparing glycan analysis methods for multiple mAbs for future "best practice" guidances

We have made great progress in this aim since the start of the funding period in the Fall of 2022. We have performed five different types of glycan analyses on NIST mAb ("standard"), Rituxan and 3 of its biosimilars and Herceptin and 2 of its biosimilars. These methods include 3 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. What we found was that, as expected, there were different levels of sensitivity, reproducibility, and robustness across the methods. The protein digestion with LC-MS/MS data acquisition was the most sensitive method, identifying the greatest number of unique glycans for each protein, including identifying lower abundance glycans such as sialic acid or high mannose (Table 3).

Table 3. 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

Across the three released glycan kits, there were also differences in the number of glycans that were able to be readily detected and identified. While InstantPC had the highest number of fluorescence peaks and, therefore, the greatest number of unique glycans, we were unable to confidently identify some of the peaks with their glycan names. This was because the UNIFI software we were using did not have a procainamide library built out yet. We are circumventing this issue by downloading the Protein Metrics API on the Waters computer. Once we have an engineer come download the API, we should be able to reanalyze all three kits simultaneously using the Protein Metrics released glycan workflow. We also observed differences in the relative abundances of low level glycans as measured by these three methods. Figure 11 shows data from NIST mAb, which emphasized the statistically significant differences across the kit methods in identifying lower level glycans.

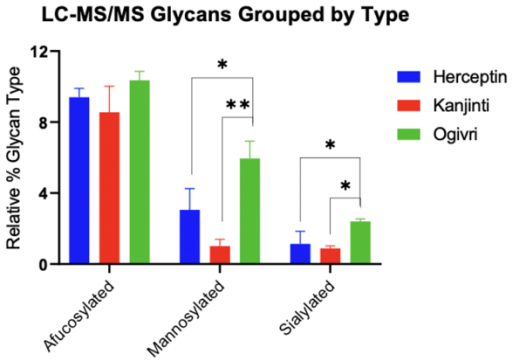


	Relative % Afucosylated	Relative % Mannosylated	Relative % Sialylated	Relative % Galactosylated	Relative % Bisecting GlcNAc
2AB	3.21 ± 1.17	2.20 ± 0.02	1.37 ± 0.38	2.19 ± 0.82	0.57 ± 0.02
RF	0.56 ± 0.03	0.13 ± 0.02	0.65 ± 0.03	3.61 ± 0.13	0.00 ± 0.00
PC	4.03 ± 0.12	0.00 ± 0.00	1.20 ± 0.06	0.00 ± 0.00	0.35 ± 0.01

Figure 11. Relative % contribution of each glycan type for NIST mAb as identified by the three released glycans kits – 2AB express, RapiFluor, and InstantPC. Glycans were detected using fluorescence and identified using the UNIFI released glycan workflow. Note: PC did not have a compatible Dextran ladder to use for UNIFI glycan identification. PC glycan assignment was completed based on a standard with known glycans. N=3; Error bars are standard deviation; *p < 0.05, **p<0.01, ***p<0.001, **** p<0.0001.

From this research, we have drafted an initial manuscript using the NIST mAb as our standard to highlight the glycan method differences. Then we used Herceptin and its biosimilars as a case study to show how sensitive methods, such as LC-MS/MS, are able to detect significant differences in low level glycans. Interestingly, as depicted in Figure 12, we noticed that the two biosimilars were more different from each other than they were from Herceptin. For example, Ogivri had more high mannose glycans compared to

Kanjinti but Herceptin had a high mannose contribution between Ogivri and Kanjinti, thus making them both like their reference (Figure 12). The *in vitro* binding and downstream ADCC capacity was also measured for Herceptin and its biosimilars (Figure 13). Kanjinti was found to have a lower IC50 value in the Lumit assay, though no difference was detected via the ADCC assay. Further work is ongoing to determine potential causes for these discrepancies and if glycosylation may affect other binding parameters such as binding cooperativity.



	Relative % Afucosylated	Relative % Mannosylated	Relative % Sialylated
Herceptin®	9.40 ± 0.50	3.05 ± 1.21	1.14 ± 0.71
Kanjinti®	8.55 ± 1.47	1.01 ± 0.38	0.88 ± 0.14
Ogivri®	10.35 ± 0.51	5.95 ± 0.97	2.40 ± 0.14

Figure 12. Relative % contribution of each glycan type for the three trastuzumabs as identified by LC-MS/MS after protein digestion. Glycans were identified using the Protein Metrics PTM workflow. N=3; Error bars are standard deviation. *p < 0.05, **p<0.01, ***p<0.001, **** p<0.0001.

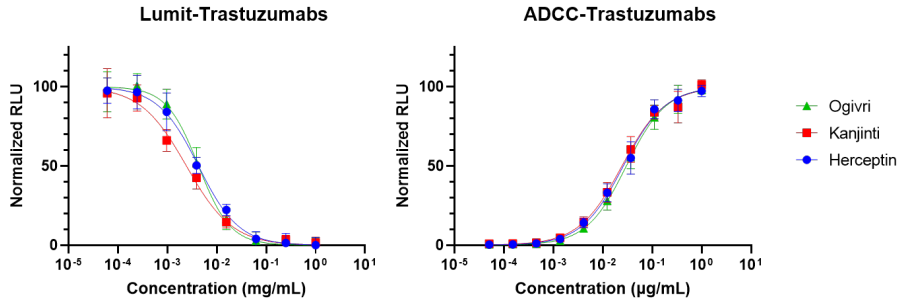


Figure 13. Fc binding (Lumit) and ADCC assays for the three trastuzumabs. Depicted are protein concentration vs. normalized relative luminescence unit curves.

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 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. To do such biosimilar comparison studies, we plan to acquire more lots of the trastuzumab and rituximab biosimilars listed in Table 1. We also plan to collect lots of other marketed biosimilar products for proteins with multiple FDA approved biosimilars including adalimumab, infliximab, and bevacizumab. It is worth noting that here we have only shown the NIST mAb and trastuzumab data as that will be the data presented in our first manuscript. We do have similar data for the rituximabs that we can use for future publications once we have more lots and have homed in on our workflow.

Aim 5) Industry interviews to uncover biosimilar development and approval roadblocks

In this Aim we conducted interviews using a strategy implemented by us on behalf of the Center for Research in Complex Genetics (CRCG); see our website https://www.complexgenerics.org and

https://www.linkedin.com/company/68587354 for more information. The CRCG routinely interviews industry stakeholders, who are also frequently members of the industry committee for GDUFA Research Initiatives, to understand the pressing issues that hinder the development of complex generics and how certain research projects could be initiated to benefit multiple generic companies. We have begun to apply a similar communication strategy with the biosimilar developers. Thus far, we only interviewed two companies, but have additional interviews planned in the fall. These interviews often last 1-2 hours and go over a variety of scientific and regulatory topics. While we often present some overarching topics to start conversations, many times the companies will shift into new topics of discussion organically. These initial interviews covered the overall barriers that companies encounter while developing biosimilars and seeking interchangeability approval from the FDA. Broadly, the development challenges discussed include: 1) validation of analytical methods, 2) interpretation of data obtained by orthogonal methodologies, 3) desire to minimize clinical trial requirements by developing a better understanding of structure/PTM-pharmacokinetics-immunogenicity relationships; 4) clinical studies design, including patient numbers and statistical considerations, 5) interchangeability designation, and 6) emerging products that will become biosimilars in the next decade.

1/2) It is important to establish criteria for acceptable differences in biosimilar products, considering that even innovator batches exhibit variability. Careful evaluation and understanding of biologics' characteristics 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. Some methods provide more in depth details in terms of showing heterogeneity of protein aggregation or PTMs, yet, these methods cannot be used routinely for batch analyses. That is why creating validated, best practice methods for companies to universally perform at various stages of development and manufacturing is critical. Before such methods can be established, there needs to be a more comprehensive and widely shared understanding of the differences between biosimilar/innovator pairs that regulatory bodies will accept.

In a similar vein, 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 examples of "failed" products from multiple biosimilar manufacturers to see if our methods could tease out structural deviations from the innovator in these biosimilar candidates. If we could make this "failed product" information known, perhaps companies could address risks earlier on in the development cycle based on analytical/structural information. Ideally, this information would also lead to discussions with multiple stakeholders regarding the establishment of concrete guidelines and acceptance criteria for certain analytical methods, and, potentially, elimination/reduction of patient size for the clinical end point studies.

3) The level of immunogenicity in new biological molecules has been decreasing since the advent of humanized antibodies. However, the impact of PTM modifications, such as glycosylation, on immunogenicity is not firmly established. More research is needed to determine if glycans or other factors are responsible for immunogenic responses. In a similar vein, there is uncertainty with regards to which glycans, and at what level, are problematic in vivo. Not only can glycans be tied to adverse immunogenic events, but they are also known to negatively impact pharmacokinetics and drug efficacy. Despite this knowledge, there are current gaps in the ability of *in vitro* immunogenicity studies to adequately predict *in vivo* results. Therefore, there is a need to establish validated, reproducible *in vitro* analytical methods that better correlate with *in vivo* results.

4/5) Differences in regulatory perspectives on interchangeability designations 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 interchangeability clinical trials, improving the efficiency of biosimilar development.

6) 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. Many companies are already working on or considering the development of some of these products, thus, some research in these areas and preemptive guidance development would be beneficial.

Another topic that was shared by one company was 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 drug development decisions earlier in the pipeline.

To continue further work on this aim, we have scheduled meetings with two additional companies and are reaching out to others to schedule initial meetings. We also plan on attending the in-person AAM GRx+Biosims conference this fall. During this conference, we would like to schedule more meetings with company representatives present. We plan to publish an article on our initial insights from these meetings once we have 8-10 companies represented.

2.2. The regulatory impact of the results or outcomes of the Project

The main regulatory impact of the results for this ongoing project falls within the FDA goal of "improving the efficiency of biosimilar product development". Specifically, we are focusing on a sub-goal of increasing the accuracy and capability of analytical (structural and functional) and CMC characterization. The main research priorities that result in a regulatory impact were outlined in the BsUFA III regulatory research pilot project roadmap. Our expected contributions to these research priorities are outlined in detail below.

1. Define and standardize approaches for assessing and reporting product quality attributes;

In Aim 1 we focus on obtaining structural characterization data for several product classes such as insulins and mAbs. We are heavily relying on mass spectrometry based methods to examine approved innovator products and their biosimilars. Yet, different mass spectrometry methodologies, equipment, analytical columns, digestion reagents, etc. result in different data. By analyzing the same set of innovator/biosimilar samples by orthogonal methodologies, we gain information about the limitations/capabilities of each method. This research will aid in standardizing the assessment and reporting of product quality attributes.

In Aim 3, we examine the impact of stressing on aggregation via several protein aggregation orthogonal methodologies. Aggregation has been, and will continue to be, examined for two classes of biosimilars, i.e. mAbs and insulins, in our lab. This research will result in publications examining sensitivities and specificities for different aggregation monitoring assays. Such publications may highlight the utility and limitations of applying different methods across several product classes.

2. Similarly, in Aim 4 we describe specifics for measuring the glycosylation of mAbs by a variety of analytical methods. To date, we have studied glycan patterns for several innovator-multiple biosimilars groups, which we plan to expand upon in upcoming months. Through this aim, we will continue to elucidate the differences in various glycan levels measured by orthogonal techniques as well as the capabilities and limitations of different methods/kits. This research could lead to standardization in assessing and reporting glycan microheterogeneity, an important quality attribute known to impact pharmacokinetics and efficacy of some mAbs. Characterize relationships between product quality attributes and clinical outcomes;

From our interactions with industry stakeholders, we have learned that multiple versions of biosimilar product candidates had to be discontinued due to pharmacokinetic differences observed in early-stage clinical PK studies. This later development stage product failure may be tied to the fact that relationships between *in vitro* quality attribute differences and significant *in vivo* PK differences is unclear. While

approved biosimilars are comparable to their reference innovator, there could be statistically significant differences in high-mannose species and afucosylated species levels between two approved biosimilars of the same reference. We have observed such phenomenon in some of our biosimilar pairs (i.e. trastuzumabs, rituximabs), so we plan to conduct animal studies to determine any correlation between the *in vitro* glycan differences and pharmacokinetics of competitor biosimilars. These study results could help in establishing limits for glycosylation differences that impact clinical outcomes. In addition, examination of glycosylation differences across multiple clinically tested and approved biosimilars could guide the development of regulatory acceptance criteria for glycosylation heterogeneity.

3. Improve on and/or develop new analytical technologies;

In Aim 2 of the grant, in collaboration with Dr. Ruotolo's lab, we focus on the development of ion-mobility mass spectrometry methodologies to assess higher-order structures of different mAbs, insulins, ADCs and fusion proteins. Our preliminary data focused on the assessment of mAbs and one of their biosimilars. Next year, we will apply this methodology to innovator/multiple biosimilars groups to examine method sensitivity and define a better way of reporting structural similarities/differences. We will extend the application of IM-MS to ADCs, fusion proteins and insulins after our initial optimization with mAbs.

As there is a general interest in automating protein digestion/mass spectrometry workflows, we are now examining how the use of automatic digestion with an AssayMAP Bravo robot in conjunction with high-throughput glycan analysis compares with data acquired by traditional methodologies. We also have ongoing collaboration with Promega to assess the multiplex Fc-receptor binding kit, Lumit™. As part of this collaboration, we compare the Fc-receptor binding data obtained by these Lumit™ kits with traditional methods like AlphaLISA and SPR.

In addition, in Aim 5 we are conducting interviews with industry stakeholders. This communication will bridge current gaps between the FDA and industry and will allow for a neutral third party to share thoughts and concerns to all stakeholders. Publications arising from these interviews will also highlight some limitations in current development practices (i.e., validation of analytical methodologies, clinical trial requirements, statistical analyses variations) and suggest paths forward. The interviews and related publications will help the agency to adjust BsUFA research priorities and potentially uncover industry concerns with respect to the biosimilar development of upcoming products such as ADCs, fusion proteins, Fabs and nanobodies.

2.3. All public communications of the results and outcomes of the project (publications, abstracts, talks/ speaking events etc.).

Kinzer JL, Halseth TA, Kang J, Kim SY, Kumaran P, Ford M. Saveliev S, Skilton JS, Schwendeman A. Physicochemical characterization and functionality comparison of Humira® (adalimumab), Remicade® (infliximab) and Simponi Aria® (golimumab). *Int J Pharm*, 635: 122646 (2023).

Kinzer JL. Mass Spectrometry Based Characterization of Innovator and Biosimilar Monoclonal Antibodies. *University of Michigan College of Pharmacy Research Forum*. Platform Presentation. 2023.

Kinzer, JL, Halseth, TA, Kang, J, Kumaran, P, Ford, M, Skilton, SJ, Saveliev, S, Nath, N, Schwendeman, AS. Comparability of Glycosylation- Antigen Binding and Fc Effector Function for Three Anti-TNF-α Therapeutics. *AAPS PharmSci 360 Conference*. Poster Presentation. 2022.

3. Problems or challenges faced

1) This is a substantial award - it takes some time to hire personnel, collect multiple lots of products and their biosimilars and establish the research. We will likely request a no-cost extension to accomplish all Aims of the project.

2) It could be helpful to develop a better understanding of how the research conducted in our lab will directly benefit the FDA and industry while satisfying BsUFA III pilot research projects commitments. Some biosimilar development challenges become clear during the interviews we conduct with the industry stakeholders. It would be helpful to align the expectation for our research outcomes with the FDA's expectations for BsUFA III pilot program goals and industry regulatory biosimilar research needs/concerns.

3) There are always some technical issues with respect to method development as we go between different types of products, i.e., mAbs, insulins, fusion proteins and ADCs. This will also feed into the need for a no-cost extension in order to complete our research.

4) There is some variability between reagents, columns, reducing agents, alkylating agents and various equipment/software used to generate and analyze the data. By testing multiple samples and batches across to numerous methods, we will try to address the material-related variability.

5) There are still significant supply chain and personnel issues post COVID-19 pandemic. The delivery of specific reagents and supplies takes unusually long time as well as scheduling instrument maintenance/repairs. We are mitigating these challenges, yet, extra time might be required to complete research experiments.

4. Future plans

4.1. Wet lab:

- Compare FLR glycan data from kits using UNIFI vs. Protein Metrics software
- Finalize assay development to measure stability – UV/CD spectrum, TEM, LC-MS/MS
- Execute stability studies using aforementioned methods
- Optimize and perform assays to detect bioactivity – insulin receptor, Akt phosphorylation
- Develop and apply analytical methods to assess mAb stability, bioactivity under forced stress conditions
- Run additional lots (2 more lots, N=3 per drug per assay) of rituximabs and trastuzumabs through FLR, intact MS and peptide mapping
- Complete Promega bioassays for current and additional rituximab and trastuzumab lots
- Perform additional interviews with biosimilar companies

4.2. Publications:

- Comprehensive review of methodology manuscript
- Insulin biosimilars comparison manuscript
- NIST mAb glycan method optimization and comparison study
- Rituximab/ trastuzumab OR & BS glycan method application study