Predict Non-Targeted Clearance of Therapeutic Antibodies Using a Human FcRn Dependent in vitro Transcytosis Assay

Predict Non-Targeted Clearance of Therapeutic Antibodies Using a Human FcRn Dependent *in vitro* Transcytosis Assay

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Abstract

The advancement in the protein engineering of therapeutic antibodies has enhanced biologic treatments of antibody-based proteins. The industry is evolving from monoclonal immunoglobulin (IgG) format to IgG antibody-based proteins capable of binding multiple epitopes either on the same or distinct targets. By design, these uniquely structured and functional proteins have the capability to address medical needs that require a multi-targeted approach. There are many challenges these new multispecific IgG-based proteins encounter including understanding what effects the complexities of their structure and function will have not only on actual drug development, but also on their pharmacokinetics (PK) compared to their monoclonal counterparts. Non-targeted biological features that guide the disposition of these multispecific proteins are not fully understood. In vitro assays are one way to predict and evaluate PK parameters and are a great alternative to the animal studies typically used for these types of evaluations, especially in circumstances when the animals do not demonstrate human characteristics. Implementation of a cell-based assay that is designed in such a way to overcome limitations of an in vitro assay approach to PK prediction provides an alternative to expensive, low-throughput, non-translatable in vivo studies.

Non-specific clearance of an antibody-based biotherapeutic can be influenced by several different properties, one of which is the interactions with neonatal Fc receptor (FcRn) which plays an important role in serum immunoglobulin (IgG) homeostasis. The Fc region of the antibody interacts with the Fc receptor and contributes to the recycling of

the antibody back to the cell surface and releasing it into the extracellular fluid (Kamath, 2016). An internally produced Madin-Darby canine kidney (MDCK II) cell line overexpressing huFcRN was used to establish a transcytosis-based assay format used as a surrogate for the *in vitro* recycling process. This method proved capable of predicting non-specific clearance for monoclonal antibodies demonstrated by a correlation between *in vitro* and *in vivo* results (Chung et al., 2019). Will the same hold true for multispecific antibody-based biotherapeutics? Various marketed multispecific antibody therapeutics were tested in the huFcRN transcytosis assay. The goal of this work was to establish correlation between huFcRN cell transcytosis levels and in-house rat clearance data and published human clearance data for these marketed multispecific antibody therapeutics.

The purpose of this work was to assess a huFcRn *in vitro* transcytosis assay as a potential method to quantitatively predict non-targeted clearance of multispecific antibody therapeutics in humans, ultimately supporting drug candidate selection and or optimization. Under the conditions tested and presented in this paper the assay is not suited to provide this type of analysis for multispecific antibodies. There is still work that can be done to optimize the current assay format to better align with the huFcRn recycling process.

Dedication

This thesis is dedicated to my parents, Joseph & Donna, my brothers Joey & Jesse, and my son Tyson for their endless love and support. To my husband Mark, your unwavering patience, love, and constant encouragement allow me to continually chase my dreams, and for that I am forever grateful.

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Chapter I.

Introduction

Small molecule drugs have dominated the pharmaceutical industry since the start of modern-day medicine, serving as leaders of traditional medical treatment. These drug types include aspirin, Benadryl, diphenhydramine, and other pills that we typically have in our medicine cabinets and use regularly. Other traditional small molecule examples include penicillin used to treat many types of bacterial infections and atorvastatin (Lipitor) which is used as a treatment to maintain healthy cholesterol levels (Ngo HX, 2018). The continuous appeal for small molecules developability as treatments can be attributed to a few key features. Their low molecular weight (MW) and uncomplicated chemical structures allow for easily predicted pharmacokinetics (PK) and pharmacodynamics (PD), resulting in simpler dosing protocols. Because most small molecules are extremely stable and orally bioavailable, they make it easy for patients to take appropriately. Lastly, because of their size and structure characteristics, and overall stability, the development of small molecular drugs involves straightforward manufacturing, characterizing, and regulatory procedures (Ngo & Garneau-Tsodikova, 2018).

The pharmaceutical industry has been evolving into an era of biologics. To note, the Food and Drug Administration (FDA) defines a biologic as a generally large, complex molecule. These molecules are manufactured through biotechnology in a living system, i.e., microorganism, plant cell, or animal cell. Compared to small molecules that are synthesized in a laboratory and simple in structure, and typically do not trigger an

immune response; biologics are characteristically more challenging to characterize given their structural complexity and that they are produced in living organisms. These attributes of biologics typically result in sparking an immune response, ultimately making it extremely challenging to predict clinical effects in patients (Makurvet, 2021).

> *"*The Food and Drug Administration (FDA) considers a biologic to be any therapeutic serum, toxin, antitoxin, vaccine, virus, blood, blood component or derivative, allergenic product, analogous product, or derivatives applicable to the prevention, treatment, or cure of injuries or disease of man. "(Raychaudhuri & Smriti K Raychaudhuri, 2009).

Biologics are made from natural sources. The term "biological product" means a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein, or analogous product, or arsphenamine or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment, or cure of a disease or condition of human beings (United States Public Health Services Act 42 USC §262(i)).

Recently, the pharmaceutical field has started unlocking the potential of biomolecules like proteins and nucleic acids as therapies. More specifically, proteins called antibodies which pose unique ability to recognize and bind to very specific biomolecules have been gaining momentum. This rise in antibody medicine over the past couple of decades established their significance as a prominent class of therapeutics. Hundreds of biotherapeutics have been developed and approved for human use across major disease areas including cancers, immune-related diseases, organ transplantation, cardiovascular disease, ophthalmologic disease, infectious disease, and hematological disease. Two monoclonal antibodies have recently been approved for Alzheimer's Disease: Leqembi® and Aduhelm®. These target-based treatments across multiple therapeutic areas have proven to be safe and efficacious, due to the high specificity and

optimal binding affinity traits of antibodies. The features of these novel treatments not only lead to substantial benefit to patients but also exemplify precision tools that can be utilized to interrogate human disease biology (Ghilardi et al., 2020). As of June 2022, over 150 antibody therapies have been approved worldwide with two thirds representing monospecific basic antibodies (Figure 1) (Goulet & Atkins, 2019) and one third representing more complex biological treatments some of which include antibody-drug conjugates, bispecific antibodies, antibody fragments, radio-labeled antibodies, antibody conjugate immunotoxin, immunoconjugates and Fc-Fusion proteins. The advantage of these complex therapies is more precise and successful treatments, with the caveat of increased challenges in their characterization required to ensure safety and efficacy (Lyu et al., 2022).

The use of protein engineering over the last few years has led to the successful construction of BsAbs, resulting in this class of drugs to be one of the fastest growing in the market (Zhang et al., 2021). At the present time, nine bispecific antibodies have been approved worldwide, greater than 180 BsAbs are in preclinical development, and there have been over 50 clinical trials investigating BsAbs. This antibody class has a wide range of applications which include tumor immunotherapy as well as in the treatment of other diseases such as hemophilia A, diabetes, Alzheimer's disease, and ophthalmological diseases (BioPharma PEG, 2023). The ability of BsAbs to target two different epitopes simultaneously, either on the same antigen or on two different antigens, contributes greatly to their therapeutic potential. When circumstances arise for which, a bispecific antibody does not provide a purposeful benefit compared to combining the same two corresponding monoclonal antibodies, the bispecific format is still favored from a

production standpoint; production is simpler, and more cost-effective. Producing a bispecific eliminates the necessity to develop two distinct drugs which would require separate manufacturing processes and the filing of two separate safety data packages. Bispecifics may also enable new mechanisms of action potentially unavailable to monospecific antibodies, a major contributing factor to the success of this format in the pharmaceutical field (Hussain & Ellerman, 2018).

BsAbs and other antibody-based therapeutics exist in many formats and are typically categorized based on the existence or absence of an Fc (fragment crystallizable) domain making the molecule either IgG or non-IgG like (Figure 2) (Spiess et al., 2015). The presence of the Fc fragment employs additional effector functions that could be key components contributing to the mechanism of action and provide key links between both innate and adaptive immunity. The effector functions are induced by the Fc region of the antibody which interacts with both complement protein and specialized Fc-receptors. For the purposes of this study, we will focus on the IgG-like antibody formats. It has been demonstrated that therapeutic antibodies have many benefits which include long half-lives, high selectivity, and tolerance. Despite their success, these antibodies are still plagued by unresolved issues related to their pharmacokinetics (PK) and pharmacodynamics (PD) due to high variabilities, low tissue distributions, poorly defined PK/PD characteristics for novel antibody formats, and high rates of treatment resistance (Tang & Cao, 2021).

The success of novel antibody-based biotherapeutic formats in the clinic is slow-going in comparison to existing monoclonal antibody therapeutics. A few explanations for what elements contribute to the sluggish success may include our partial

understanding of the specifics of their biological mechanism of action, poorly defined exposure-response profiles, inadequate safety margins, strategic industry decisions and immunogenicity, just to name a few. The complex and diverse nature of these proteins, both in terms of their structure and tractability impacts the increase in possibility for uncertainty in their pharmacokinetic and disposition profiles. These characteristics, alongside the previously detailed challenges including unforeseen abnormal PK profiles, necessitate additional experimental protein engineering efforts. Taken together, these factors increase the limitations of BsAb advantages when compared to pharmacologically similar monospecific mAbs (Datta-Mannan, et al., 2021).

Cancer continues to be the second leading cause of death in the United States. Monoclonal antibody strategies against cancer antigens have proven to be successful for only a select few cancer types. Though these antibodies have excellent tolerability profile and efficacy in various cancers, rarely are these antibodies effective as single therapy cancer treatments. These monoclonal antibodies have shortcomings that involve the failure to activate multiple pathways, have limited tumor tissue penetration, and suboptimal engagement to their targets as just some of the downsides to this modality (Fousek & Ahmed 2015). Advancements in protein engineering techniques have allowed for the creation of multispecific (i.e., bi- and tri-specific) antibodies capable of binding several specific targets simultaneously resulting in more precise and efficacious treatments for various diseases, including cancer.

The current focus of biotherapeutics consists of proteins having diverse structures including conjugation to a drug or bi/multispecific in nature versus a monoclonal antibody protein (i.e., Antibody Drug Conjugates (ADCs), Dual Variable Domain IgG

(DVDs), Bi / multispecific etc. The novel bi/multispecific biotherapeutics function by interlocking two or more protein targets by means of well-defined binding areas with or without the chemical pairing to large or small molecules. They have the capability to not only tackle disease complexity but also take advantage of innovative therapeutic systems and evaluate undruggable targets for traditional monospecific biologics (Zhong et al., 2021). As promising as the future potential is for these novel multispecific modalities, their clinical value still must be proven whether used as a single treatment or in combination therapy. Currently, there are 9 FDA-Approved Bispecific antibodies (Table 1) with many multispecific antibodies in an array of different formats being investigated for various disease areas, some of which there is no cure. The multitude of options for multispecific antibody formats enhances their diversity and hopefully allows for the expansion of available biologic treatments for various indications.

Definition of Terms

Adsorption, Distribution, Metabolism & Excretion (ADME): Describes the disposition of a pharmaceutical compound within an organism.

Antibody: A protein made by plasma cells (a type of white blood cell) in response to an antigen (a substance that causes the body to make a specific immune response). Each antibody can bind to only one specific antigen. The purpose of this binding is to help destroy the antigen.

Antibody-Drug Conjugate (ADC): Protein -based drug combining a target-specific binding domain with a cytotoxic domain.

Antibody Fragment: Functional regions of whole immunoglobulin consisting of the antigen binding fragment (Fab) and the class defining fragments (Fc) that do not bind antigen

Antigen Any substance that causes to make an immune response against that substance.

Examples include chemical, bacteria, viruses, or other substances form outside the body:

Bioavailability: The proportion of a drug or other substance which enters the circulation when introduced into the body and so can have an active effect.

Biotherapeutic: A type of treatment that uses substances made from living organisms to treat disease. These substances may occur naturally in the body or may be made in the laboratory.

Biproduct: A product of a chemical reaction or industrial process which is different from the desired product.

Bi-specific Antibody (BsbAb): An antibody that can bind two different antigens at the same time.

Catabolism: The sequences of enzyme-catalyzed reactions by which relatively large molecules in living cells are broken down, or degraded

Endocytosis: The process of engulfing the material from the external environment into the cell.

Endosome: The assorted collection of organelles that function in the sorting and delivery of internalized material from the cell surface and the transport of materials from the Golgi to the lysosome or vacuole.

Neonatal Fc Receptor (FcRn): FcRn functions in preventing IgG degradation and help maintain IgG levels through recycling.

Golgi: A stack of small flat sacs formed by membranes inside the cell's cytoplasm (gel-like fluid). The Golgi body prepares proteins and lipid (fat) molecules for use in other places inside and outside the cell.

Half-life: The time it takes for a drug's active substance in your body to reduce by half, can range from a few hours to a few days. The half-life of drug is related to concentration found in plasma and typically determined using pharmacokinetic parameters clearance and volume of distribution.

Immunoconjugate / Immunotoxin: An agent made up of an immune substance, such as a monoclonal antibody, that is chemically linked to a cell-killing substance such as a toxin, a radioactive molecule, or a drug.

IgG: Immunoglobulin

Immunotherapy: Treatment of disease by activating or suppressing the immune system.

In vitro: A biological process occurring in a laboratory vessel or other controlled experimental environment rather than within a living organism or natural setting.

In-vivo: A biological process occurring within a living organism or natural setting.

Fc-Fusion Protein: Proteins composed of the Fc (fraction crystallizable) domain of IgG genetically linked to a peptide or protein of interest.

Lysosome: A sac-like compartment inside a cell that has enzymes that can break down cellular components that need to be destroyed.

Metabolite: A substance made or used when the body breaks down food, drugs or chemicals, or its own tissue.

Monospecific Antibody: Antibody having specificity to a singular antigen or epitope.

Multispecific: Involving more than one species.

Pharmacokinetics: The activity of drugs in the body over a period, including the processes by which drugs are absorbed, distributed in the body, localized in the tissues, and excreted.

Pre-clinical Drug Development: Refers to the testing of a drug, procedure, or other medical treatment in animals before trials may be carried out in humans. During preclinical drug development, the drug's toxic and pharmacological effects need to be evaluated through in vitro and in vivo laboratory animal testing.

Pinocytosis: The process by which the cell takes in the fluids along with dissolved small molecules. The cell membrane folds and creates small pockets and captures the cellular fluid and dissolved substances.

Radio-labeled Antibody: Monoclonal antibody linked to a radionuclide that can be used as therapeutic and/or diagnostic agents in the management of cancer.

Transcytosis: The vesicular transport of macromolecules from one side of a cell to the other, is a strategy used by multicellular organisms to selectively move material between two environments without altering the unique compositions of those environments

Vacuole: A vacuole is a small membrane-bound cell organelle in animal cells that helps to sequester waste products.

Adsorption, Distribution, Metabolism & Excretion

Vital to both the clinical and commercial success of any therapeutic is understanding the Adsorption, Distribution, Metabolism and Excretion (ADME) profile of the drug candidate and having the capability to optimize the drug candidate's characteristics accordingly. Making the necessary enhancements to the ADME profile would lead to an improved, efficacious drug candidate. To better accomplish this, it is important to understand the unique attributes of the therapeutic protein and how each attribute specifically impacts the ADME processes – how do these attributes affect pharmacokinetics (PK) - and ultimately the clinical function of the therapeutic protein (Jing et al., 2019).

The distinct properties of a drug, whether small or large molecule, influence how the drug molecule is affected by the body. Irrespective of how the drug is administered, its interactions with various enzymes and other proteins, tissues, cells, and their components determine its fate. There are distinct differences between the ADME characteristics of small, less complex, chemical molecule drugs in comparison to characteristics of large molecule drugs. Some of these differences include a longer half-life, a more limited volume of distribution and a longer time to reach peak concentration for biologics. (Small Molecule Vs Biologics | Drug Development Differences, n.d.).

Adsorption (A) describes how a drug enters the body and connects its movement from site of administration to the bloodstream. There are four main routes of administration for drug delivery, and they include 1) ingestion through the digestive track, 2) inhalation through the respiratory system 3) dermal application via the skin or eye and 4) injection through direct administration into the bloodstream or tissues. The

bioavailability, or amount of unchanged drug that has been adsorbed and make it into the central compartment (blood), is heavily influenced by the route of administration. Biotherapeutic proteins are typically administered parenterally; intravenously (IV) subcutaneously (SC) or intramuscularly (IM). Oral dosing is typically not an option for biologic therapeutics as it is for small molecule drugs due to their size, polarity, and propensity of the protein to be degraded by enzymes found in the gastrointestinal tract (Zhao et al, 2012).

A biotherapeutic bypasses the adsorption phase when dosed IV due to direct administration into the bloodstream, thus maximum serum concentration is immediate. The absorption rate is slower for SC dosing, due to the path through the extracellular matrix (ECM) therefor maximum serum concentration tend to fall below the concentration levels attained with IV dosing (Bittner et al., 2018). The decreased rate of adsorption into the bloodstream with SC dosing is affected by the protein's features including molecular structure, weight, size, and charge (Jing et al., 2019).

Distribution (D) refers to the delivery of the drug throughout tissues within the body after it has been absorbed, either via the bloodstream or from cell-to-cell interactions. Understanding where the drug goes, how fast it gets there, and the extent of the distribution helps developers of the drug better understand the drug's efficacy. The volume of distribution can also be attributed to a protein's MW, size charge and structure. Biotherapeutic proteins, such as mAbs, are distributed in the blood plasma and limited interstitial space while larger antibody-based proteins are mostly distributed in the blood. Smaller proteins and protein fragments, i.e., fAbs, tend to have a larger distribution due to their size (Jing et al., 2019). Distribution to the tissue occurs by paracellular or

transcellular movement after IV dosing or by absorption subsequently following parenteral injections. Monoclonal antibodies distribute to tissue paracellularly, though not by passive diffusion, i.e., randomly between cells, but rather by convective transport, distribution occurs with movement down a pressure gradient. Transcellular trafficking for mAbs consists of three different activities: 1) fluid-phase pinocytosis (i.e., proteins are taken up by cells from the fluid space nearby), 2) receptor-mediated endocytosis (primarily through Fcγ receptor binding or because of cell surface binding to certain antigens), and 3) phagocytosis. The primary pathway used by mAbs to penetrate endothelial cells is fluid-phase pinocytosis. Once inside the endothelial cell, mAbs can use the neonatal Fc Receptor (FcRn) recycling pathway to take part in the transcytosis phase. At this stage, mAbs can be relocated in one of two directions, either interstitial space or the vascular region (Zhao et al., 2012).

Following metabolism, the drug needs to be excreted (E), or eliminated, from the body. In the case of biotherapeutics, excretion of proteins with a MW <69kDa are cleared mainly through the kidney. Larger proteins that tend to be restricted to the vascular region, and need eliminating, require one of a couple mechanisms to come into play to transport the protein from the vascular endothelium to the underlying tissue to finally reach degradation. These mechanisms include receptor-mediated and passive (fluid-phase endocytosis) protein transport from the vascular endothelium to the underlying tissue and subsequent intracellular degradation (Hamuro et al., 2012).

In a scenario where there is no TMDD, the majority of IgG- based mAbs exhibit long half-lives, typically ranging from 3-4 weeks. The increase in half-life can be attributed to FcRn-mediated antibody recycling which occurs under acidic conditions.

Under these circumstances, the IgG antibody can enter the endosome through fluid phase pinocytosis. At this stage, the proteins are bound to the FcRN receptor and are not able to be relocated to lysosome for degradation, essentially spared, unlike the unbound, or free antibodies that will be degraded (Zhao, et al., 2012).

Understanding metabolism and clearance aids the drug developer in realizing both the safety potential and the possible toxicity aspects of the drug candidate. This allows for the identification of the possible increase of any dangerous by-product levels and therefore optimization of drug candidate properties accordingly if possible.

These ADME processes describe a drug's overall disposition through pharmacokinetics (PK), i.e., how a drug is processed by a living organism and what is the organism's body doing to the drug. Understanding the uniqueness of biotherapeutic protein's characteristics and how these characteristics affect ADME activities better enable researchers to assess the safety and toxicity of their drug candidate. This knowledge around the possible effects on ADME activities aids in determining a drug candidate's viability and assists in providing targets for future research and development (Small Molecule Vs Biologics | Drug Development Differences, n.d.).

Research Challenges

Prediction of human PK is typically based on data generated from in vivo studies utilizing rodents and non-human primates. Certain drawbacks to this methodology include the usage of animals, the experiments are extremely costly and very low throughput. Often, there is the additional challenge that the animal species used to assess PK properties do not exemplify human properties. These differences include but are not limited to FcRn (neonatal Fc receptor) binding, differences in target-mediated drug

disposition and species-specific immunity (Liu et al., 2021). An alternative to these expensive, low-throughput, non-translatable in vivo studies is the implementation of a cell-based assay that is designed in such a way to overcome limitations of an in vitro assay approach to PK prediction.

The structural complexity of diverse biotherapeutic proteins compared to therapeutic mAbs have the potential to affect their pharmacokinetic (PK) parameters including distribution, metabolism and excretion processes resulting in distinctive pharmacokinetic profiles, often shorter half-lives in comparison to mAbs. The added binding specificities and molecular structure intricacies of BsAbs and other antibody-based therapeutics increase the difficulties in performing standard pharmacokinetic and safety assessments required for new drug development. Data generated from the MDCK II *in vitro* cell-based assay may be used as a surrogate for IgG recycling *in vivo*, enabling the prediction of drug clearance in in vivo PK/PD models and facilitate human dose prediction. The potential to predict drug clearance would greatly impact the acceleration of the BsAb drug candidate through the various stages of the drug development pipeline, from early engineering design to selection of a clinical candidate.

Many strategies to enhance or optimize the efficacy of therapeutic biologics (TBs) by various researchers have been investigated. Modification of protein structure is one strategy that is tried and can entail fusing smaller proteins to another protein section or conjugating an active peptide or chemical to a protein. The purpose behind this strategy is to boost the pharmacologic movement or extend the exposure of the drug in circulation. Another typical strategy entails altering the proteins via conjugation or engineering techniques to enhance the amount of time the protein distributes within the body. Two

different approaches commonly used to accomplish this strategy include those that increase the hydrodynamic volume (i.e., increase in size), and those that exploit neonatal Fc receptor (FcRN)-mediated recycling in conjunction with increasing volume (Tibbitts et al., 2016).

The different strategies utilized to optimize TBs result in distinctive properties rendering ADME assessment more challenging and include 1) large molecular size, 2) complicated structure, 3) surface charge, 4) stability, 5) modifications of the therapeutic protein, 6) species difference in target-binding properties and 7) host immune response to the biologic. The appropriate methods to characterize absorption, distribution, metabolism, and excretion (ADME) of TBs are still not up to par, characterizing the exposure, metabolic or catabolic biotransformation, target-mediated disposition (TMDD), and elimination of TBs in the preclinical phase is much more challenging as compared with performing the characterization of small molecules (Lai & Zhong, 2022). Non-specific clearance is a key PK parameter of therapeutic monoclonal antibodies (mAbs). This PK property is typically assessed in vivo in mice and Cynomolgus (CYNO) monkeys during pre-clinical drug development for promising TBs. The data generated from these in vivo studies are used to produce models for prediction of human dose levels. Presently, in vivo testing is a vital part of safety assessment, and is a regulatory requirement before a drug can progress into clinical trials. The recent development and validation of in vitro assays for initial therapeutic screenings focus on funneling out molecules with a high toxicity potential and in some scenarios, substitute or at least lessen the utilization of certain in vivo assessments (Goh et al., 2015). The surge in focus on the establishment of new in vitro assays throughout the pharmaceutical industry is in

response to the demand for early identification of favorable drug candidates while also abiding by the 3Rs. The 3Rs refer to the collection of ideologies that summarize Replacement, Reduction, and Refinement of the use of animals in research. The 3Rs function as a hardy framework for decreasing animal use and suffering (addressing the harms to animals) and measures to enable superior science and translation (addressing the benefits) (Graham & Prescott, 2015). The main goal of the 3Rs is to alter traditional animal testing practices so that animal experimentation is reduced to a minimum whenever possible. In implementing, the 3Rs attempt to decrease animal distress and suffering during research to a minimum. The 3Rs are supported by many government laws and regulatory systems. (Animal Use Alternatives (3Rs) | National Agriculture Library, n.d.).

The implementation of an in vitro assessment of non-specific clearance in the earliest stages of drug development would reduce research costs, allow for reliable and efficient studies, and help to decrease the number of animals studies. Non-specific clearance can be influenced by several different properties, one of which is the interactions with neonatal Fc receptor (FcRn) which plays an important role in serum immunoglobulin (IgG) homeostasis. FcRn receptor expression is found predominantly in endothelial cells, especially in the large vascular beds of skin and muscle, and to a slightly lesser extent in liver. IgGs are antibodies produced from plasma cells (white blood cells) and are the most common antibody found in blood circulation. The FcRn receptor is responsible for protecting IgG antibodies from breaking down in the vesicles of the endosome. The endosome is the organelle responsible for sorting and delivering internalized material from the cell surface and transporting the materials from Golgi to

lysosome or vacuole within the cell. In doing so, the FcRn salvages internalized IgGs from degradation, resulting in extension of their half-lives in systemic circulation in addition, the processes of cellular uptake via pinocytosis, (the ingestion of liquid into a cell by the budding of small vesicles from the cell membrane) or endocytosis (cells absorb external material by engulfing it with the cell membrane) could also play a role in determining the rate and extent of IgG catabolism.

Madin-Darby canine kidney cells (MDCK) cells can be cloned to specifically express human FcRn under conditions resembling the FcRn-mediated IgG salvage pathway. This novel in vitro assay offers a unique benefit to biopharmaceutical scientists as a time-efficient, cost-effective, and animals paring tool for evaluation of mAb-based drug candidates during lead selection and optimization, and process development. The availability of this in vitro assay to quantitate and predict FcRn mediated PK properties is extremely advantageous in the optimization and even selection of novel biologic drug candidates. The investigative work by Chung and colleagues using an MDCK cell line, stably expressing FcRn, in a transcytosis assay format, demonstrated a correlation between the in vitro test readouts and in vivo clearance in humans for 53 mAbs with diverse structure, function and pharmacological properties. (Chung et al., 2019).

The proven establishment of FcRn in vitro assay for mAbs opened the possibility for the development and optimization of an in vitro assay for a selected group of multispecific antibodies which would prove beneficial in supporting pre-clinical drug development for these unique biotherapeutics. Abbvie scientists generated two separate MDCK II cell lines expressing functionally cloned human and CYNO FcRn. The FcRn-

mediated antibody recycling process in vivo is extremely complex and therefore challenging to mimic the complete cycle of antibody-FcRn interactions *in vitro*.

> "Even though IgG recycling in vivo, which takes place, for example, in vascular endothelial cells, and the transcytotic IgG movement are not identical processes, the transcytotic IgG trafficking in vitro under optimized conditions may be used as a surrogate for IgG recycling in vivo." (Jaramillo et al., 2017)

The team tested a series of monoclonal antibodies, some with binding enhancing mutations, using the FcRn cloned MDCK II cell lines in a transcytosis-based assay as an alternative approach for a recycling-based assay. They successfully demonstrated enhanced transcytosis which correlated well with human and CYNO PK, consistent with both internal data and literature.

Hypothesis

The primary goal of this thesis was to determine if there is a correlation between in *vitro* Human FcRn MDCK II transcytosis data for a multispecific antibody and *in vivo* clearance data. Grounded in literature and internally generated data, a correlation was confirmed between the *in vitro* transcytosis results of marketed mAbs utilizing huFcRn MDCK II cells and available human clearance data from Chung et al. and is shown in Figure 4. The question lies whether this correlation can be recapitulated with multispecific antibody modalities given their complex structure and function.

Implications of Research

The significance of this research is multifaceted. Determining a correlation between small scale *in vitro* assessment of a multispecific therapeutic antibody's transcytosis levels and *in vivo* clearance parameters would allow for the de-risking of

these therapeutics at early stages within the drug discovery phase. This development of a distinct *in vitro* assay specifically for multispecific antibodies to predict non-targeted clearance would be advantageous as this functional assay would play a critical role in the early identification of the antibody's promising characteristics. This would not only reduce cost and labor resources, allowing numerous clinical candidates to move through the pipeline more quickly, but could potentially reduce the number of *in vivo* studies to be performed.

Chapter II.

Materials and Methods

The work performed in support of this study included a combination of cell-based *in vitro* testing and rat *in vivo* assessment utilizing seven bispecific therapeutic antibodies with various biologically engineered structures.

In vitro Serum Stability (IVSS)

The seven bispecific antibodies (Figure 5) to be used for both *in vitro* and *in vivo* testing were evaluated in the IVSS assay to ensure they were stable, i.e., stayed intact with no signs of clipping, in serum prior to being dosed in rats. (Figure 6). Five different structures of IgG like bispecific antibodies were tested in this study, each with a unique architecture; knob-in-hole (KIH), Duobody (Dbody), Fiddler Crab (Fab-Fc x scFc) Crayfish (IgG-scFv) and 2:1 Cross mAb (XmAb).

The KIH is an asymmetric bispecific IgG antibody consisting of an Fc region with two distinct heavy chains and two different Fab arms. The CH3 domains of two distinct heavy chains are mutated to make either a “knob” or “hole” allowing for heterodimerization. The features of the KIH are comparable to normal IgG antibodies including half-life, Fc-mediated effector functions and decreased immunogenicity (*Overview of Knobs-in-Holes Bispecific Antibody - Creative Biolabs*, n.d.)

The duobody approach is based on the Fab arm exchanges with IgG4s. Production of this asymmetric IgG like bispecific antibody requires the individual expression of two

homodimeric antibodies that are purified, mixed under redox conditions, blended together and result in the pairing of a heterodimeric antibody(*Bispecifics*, n.d.).

Fiddler crab is an IgG like bispecific antibody with an asymmetric structure consisting of a modified Fab region with single-chain Fv (scFv) fused to the N-terminus. The Crayfish is a symmetric bispecific IgG like antibody consisting of two scFv chains fused to the C-terminus regions of the heavy chains on the antibody. The Cross mAb is an IgG like asymmetric bispecific antibody with a trivalent structure; bispecific 2 + 1 heterodimeric CrossMabVH-VL with charge pairs in the non-crossed Fab and KiH.

The antibodies were incubated in rat serum at 37°C for one week. Samples were collected at four time points. Biotinylated anti-human IgG was immobilized onto streptavidin cartridge. Enriched biologics were analyzed directly on intact level in LC-MS under denaturing conditions. The IVSS assay is not a quantitative assay. Recovery rates between 75% and 125% (+/- 25%) meet the assay acceptance criteria.

Human-FcRn MDCK II Cells

The team at Abbvie generated an MDCK II cell line that recombinantly expresses functional human FcRn, a heterodimer of FcRn and beta-2 microglobulin (B2M) (Figure 7). Once clone was identified, single cells were sorted from high expressing cells. Subclone selections were chosen based on transcytosis results. For the experimental work detailed in this study, the cells utilized were at passage +11.

Transcytosis Assay

Human FcRn over-expressing MDCK II cells were cultured for a week prior to being seeded for assay. Day one, 100,000 cells per well were seeded in 24-well plates

(Greiner bio-one catalog # 662641) and the inserts were filled with MDCK II cell culture medium (DMEM, 10% HI FBS, 1% MEM NESS & G-418). Seeded plates were maintained for 24 hours at 37 °C, 5 % CO2, 95 % humidity to reach confluence. Trans Epithelial Electrical Resistance (TEER) measurements were measured using World Precision Instruments (WPI) EVOM™ on day two prior to being treated with antibodies. Cells were treated with either 50 or 100µg/mL of antibody, three wells/concentration, 6 wells total for each antibody. Two different concentrations were tested because the initial work performed with mAbs was performed in 96-well plates using 50µg/mL and we were not certain if that concentration would be optimal for 24-well plates. Antibodies include three reference or control mAbs and various bispecific antibodies. Antibody treated cells incubated for 24hours at 37 °C, 5 % CO2, 95 % humidity. Collection of transcytosed samples was performed on day three and a second TEER measurement was taken following sample removal. Transcytosed samples were analyzed using the MesoScale Discovery (MSD) platform to quantify antibody levels (Figure 8).

To determine final transcytosis concentrations for each of the antibody treatments, a concentration value for all 4 readings per well was attained using a four-parameter logistic model for curve fitting using the ten-fold diluted sample for all samples. The regression equation for the calibration curves was then used to back calculate the measured concentrations. To control for outliers within the four replicates per treated well, I used the Interquartile Range in Microsoft Excel.

Trans Epithelial Electrical Resistance (TEER) Analysis

The integrity and confluence of the huFcRn MDCK II monolayers were examined pre- and post-antibody treatment utilizing TEER analysis. Electrical resistance across

each specific cell layer was measured manually with World Precision Instruments (WPI's) EVOM™ Manual TEER (Figure 9). This non-invasive technique allows for the quantitative measurement (OHMs) of the cell monolayer and qualitative measurement of cell confluence via EVOM^{TM} innovative technology which verifies an increase or a plateau in tissue resistance. The system is specifically devised for the non-destructive analysis of epithelial monolayer confluence through the production of a low AC current that prevents electrode metal deposits. Capacitance or membrane voltage do not alter resistance readings. The cells were removed from 37°C / 5% CO2 incubator and equilibrated to room temp in biosafety cabinet for 30 minutes. To perform the measurement the transwell insert was removed from cell culture well, placed in the EVOM^{TM} electrode holder containing cell media, electrode was then placed appropriately into holder, tissue sampling frequency was12.5 Hz. OHMs readings were recorded for the two separate measurements of each cell monolayer.

MesoScale (MSD) Analysis

Transcytosed samples were analyzed for the therapeutic antibodies in a total anti-human MSD (Meso Scale Discovery) assay with electro chemiluminescent detection. In the total assay, total therapeutic antibody was analyzed by employing a donkey-anti human IgG as capture reagent (Jackson Immuno Research Catalog # 709-006-098) and a goat anti human IgG sulfo Tag detection (MSD catalog # R32AJ-1). The specific therapeutic antibody being analyzed was used for the construction of the standard curves.

In the assay, samples were analyzed in the presence of 100% cell culture medium. MSD standard curve fitting, and data evaluation was performed using XLfit4 software

(Version 5.5.0). Calibration curves were plotted from MSD luminescence units versus theoretical standard concentrations. A four-parameter logistic model was used for curve fitting. The regression equation for the calibration curves was then used to back calculate the measured concentrations. Twelve-point standard curves were run for each tested antibody (0.0001-5µg/mL) (Figure 10). The linear range for the tested antibodies was 0.002-1.67 µg/mL, with a lower limit of quantitation (LLOQ) of 0.002 µg/mL. For each therapeutic antibody, 4 samples per treated well were analyzed, each sample was analyzed neat and at 1 to 10 dilutions. Values that were below the quantitation limit were treated as 0 when computing mean concentrations.

Rat Pharmacokinetic (PK) Study and LCMS Analysis

A total of three Sprague Dawley rats were dosed 5mpk IV, 2mL per kg, per therapeutic antibody. Whole blood was collected at the following timepoints (hours); 0.8, 1, 6, 24, 48, 72, 168, 216, 336 and 504 hours post dose. The whole blood was further processed into serum.

For the assay, calibration curves were prepared by spiking the reference standard into commercially obtained rat serum and serially diluting. Unknowns and standards were diluted with PBS containing a stable isotope-labeled mAb as the internal standard. Enrichment was performed on an AssayMap Bravo system (Agilent) using immobilized protein A cartridges (P/N G5496-60000). Tryptic digestion was carried out for 2 h at 60 °C and was quenched with formic acid prior to LCMS analysis. Samples were analyzed on an Exion LC coupled to a SCIEX 7500 MS equipped with a Luna Omega Polar C18 column (Phenomenex). Data analysis was performed in Sciex OS using the MQ4 peak

integration algorithm. A linear regression equation was fitted to the calibration curve with a 1/x2 weighting and was used to calculate unknown sample concentrations. Lower limits of quantitation ranged from 0.5 to 3.4 ug/mL.

Total antibody concentrations were graphed using GraphPad Prism 9.5.0. Noncompartmental analysis (NCA) was performed using Phoenix WinNonlin Software for clearance levels.

Literature Search Human Clearance

A review of literature was performed to determine human clearance levels for the antibody-based therapeutics tested. To note, the bispecific antibodies used for the *in vitro* and *in vivo* assessments are marketed antibodies that were produced internally using patent information. The three monoclonal antibodies (mAb -WT, QL and YTE half-life extension versions) are proprietary monoclonal antibodies and were used as controls for some of the methods tested, human clearance data does not exist for these antibodies (Table 2).

Chapter III.

Results

The results of the data analysis from each of the *in vitro* and *in vivo* experiments are detailed in accordance to the experimental parameters.

In vitro Serum Stability (IVSS)

The IVSS assay is not a quantitative, but a qualitative method used to evaluate a molecule's intactness in the presence of serum. All seven bispecifics assessed in the IVSS assay showed no signs of clipping. KIH-1, Dbody-1 and Fab-Fc x ScF-Fc-1 met the acceptance criteria as their recovery rates were between 75% and 125% (+/- 25%) in rat serum compared to the recovery rates in buffer. Recovery rates refer to the % of intact heavy and light antibody chains. KIH-2A, Dbody-2, IgG scFc-1, and XmAb (2:1)-1 had recoveries between 50-75% when compared to buffer. These results confirmed the intactness of the tested bispecifics in the presence of rat serum, enabling these bispecific antibodies to be dosed in Sprague Dawley rats to assess PK levels from serum matrix at multiple timepoints across a twenty-one-day period (Figure 11).

Transcytosis Assay

The TEER values from pre-and post-treatment demonstrated the integrity of the huFcRn monolayer remained confluent and intact as the average values per well across all treatments were in line within the expected range for MDCK Cell lines. The levels of transcytosis between pre-and post-treatment indicated that the monolayer remained intact and there was minimal disruption throughout treatment (Figure 12).

The final transcytosis concentrations for all antibody treatments are listed in Table 3. The expectation was that the 50µg/mL treatment may not be optimal to allow for differentiation of transcytosis concentrations for the reference control mAbs in a 24 well format. Therefore, a second higher treatment, 100 µg/mL, was also tested. For both treatments, transcytosis results for the tested mAbs were highest for mAb-YTE, followed by mAb-QL and mAb-WT had the lowest levels. These results align with internal historical data demonstrating and increase in transcytosis levels for mAbs with Fc mutations (QL or YTE) in comparison to mAb WT. The 100µg/mL treatment resulted in 0.02% of the mAb-WT being transcytosed, comparable to the range observed by Chung et al. Concentration of antibody treatment influenced transcytosis levels under the conditions tested. The 50µg/mL treatment resulted in a larger difference between the levels of transcytosis between the mAb-WT and mAb-YTE, about 18-fold, versus the 100µg/mL treatment where the fold difference was about 6. These results demonstrate that treatment concentration is a key parameter to consider during study design.

The results for the 50µg/mL treatment showed Dbody-1 having the lowest amount of transcytosis (2.3ng/mL) which was lower than all the mAbs tested at this concentration and KIH-1 showed the highest amount of transcytosis (33.7ng/mL) which was higher than mAb WT tested but less than mAb-QL and mAb-YTE.

In the 100 µg/mL group, Dbody-1 showed the lowest levels of transcytosis (5.8ng/mL) which was lower than all the mAbs tested at this concentration. Duobody-2 had the levels of transcytosis (124.7ng/mL) which was higher than all the mAbs tested at 100 µg/mL: 20.3, 66.6, and 118.1 g/mL for mAb-WT, mAb-QL and mAb-YTE respectively. The differences between Dbody -1 and Dbody-2 are the antigens recognized

by each of the bispecific molecules, neither of the molecules have any Fc mutations for effector function.

HuFcRn Cell Transcytosis and Rat Clearance

Total antibody concentrations were graphed using GraphPad Prism 9.5.0 (Figure13). The concentration-time profiles for all seven bispecific antibodies were approximately linear, consistent with no rodent cross-reactivity. Two antibodies (Fab-Fc-x scFv-1 and IgG-scFv-1) had slightly increased clearance compared to the other antibodies within the tested conditions.

Noncompartmental analysis (NCA) was performed using Phoenix WinNonlin Software to determine rat clearance levels and human clearance levels were confirmed via literature. HuFcRn MDCK II cell transcytosis values for both treatment concentrations were plotted against Rat clearance levels using Pearson's correlation and two-tailed test to calculate the p-value with PRISM Graphpad software (Figure 14). The R^2 values for both 50 and 100µg/ml treatments were 0.038 and 0.044 respectively, while the p values were 0.68 and 0.65 respectively. These data combined indicate there is no significant correlation between huFcRn MDCK II cell transcytosis concentrations and the rat clearance value for the diversified antibodies tested under these conditions. There was a strong linear relationship observed between the KIH-1 and KIH-2A antibodies and their related clearance values for both treatments, R^2 values for both 50 and 100µg/ml treatments was 1.0. To note, depending on the treatment concentration, the linear orientation between the two KIH proteins was different. There were too few pairs to determine any correlation. The KIH-1 and KIH-2A antibodies share similar protein

structure, neither have any Fc mutations, the difference between the molecules are the antigens they recognize.

HuFcRn Cell Transcytosis and Human Clearance

The human clearance levels were confirmed via literature search and were plotted against HuFcRn MDCK II cell transcytosis values for both treatment concentrations using Pearson's correlation and two-tailed test to calculate the p-value with PRISM Graphpad software (Figure 15). The R^2 values for both 50 and 100µg/ml treatments were 0.040 and 0.0140 respectively, while the p values were 0.69 and 0.82 respectively. These data combined indicate there is no significant correlation between huFcRn MDCK II cell transcytosis and the human clearance values for the diversified antibodies tested under these conditions. There was a strong linear relationship observed between the KIH-1 and KIH-2A antibodies and their related clearance values for both treatments, R^2 values for both 50 and 100µg/ml treatments was 1.0. To note, as was observed with the rat clearance data, depending on the treatment concentration, the linear orientation between the two KIH proteins was different for the human clearance and huFcRn transcytosis comparison. There were too few pairs to determine any correlation. The KIH antibody trend aligns with the results observed with the rat clearance versus transcytosis.

Chapter IV.

Discussion

In this study we aspired to determine if a huFcRn *in vitro* assay could be used to quantitatively predict non-targeted clearance for multispecific therapeutic antibodies as had been proven successful with monoclonal antibodies. Antibody-based therapeutics are developed to treat a wide range of ailments including cancer, infectious diseases, neurological disorders, and immune related illnesses. The antibody formats are evolving from symmetrical monoclonal antibodies comprised of two identical heavy and light chains to more complex modalities. These multifaceted structures have very intricate functions affecting PK parameters, in particular, non-targeted clearance.

The experimental work performed in this study focused on seven total multispecific antibodies representing five unique structures (Figure 5). The molecules were analyzed for stability in rat serum prior to dosing the rats for PK analysis. All the antibodies tested showed no signs of clipping, however, four of the seven antibodies; KIH2-A, Dbody-2, IgG-scFv-1 and XmAb (2:1)-1 showed significant loss in recovery in comparison to buffer control after seven days. This drop in recovery could be attributed to possible aggregation in the serum following the 7-day incubation. The linear PK results for the rat study (Figure 13) were consistent for no cross reactivity for all the therapeutic antibodies tested in the twenty-one-day study. Two of the antibodies, Fab-Fc x scFc and IgG ScFv-1, did show faster clearance. One area of focus to consider to better understand this occurrence would be the assessment of non-specific binding. Do the differences in polyspecificity of the unique antibody structures attribute to their faster clearance?

TEER analysis confirmed that the total concentration of antibodies measured from the receiver wells were a result of the antibodies being transcytosed, the huFcRN MDCK II cell monolayer was intact and viable at both the time of treatment and at the time of sample collection. MSD analysis for total transcytosed antibody levels for the reference mAbs trended with previous internal data with mAb-WT having the lowest concentration levels followed by mAb-QL and mAb-YTE respectively for both treatment concentrations. These results aligned with those observed in the publications by Ward et al., in 2015 and Chung et al., in 2018 (Figure 16). The results from both teams also showed an increase in transcytosis levels between WT antibodies and YTE half-life extended antibodies.

Our 100µg/mL treatment huFcRn MDCK II cells resulted in 0.02% of the mAb-WT being transcytosed, similar to the range observed by Chung et al. This alignment in results helps to validate the notion that the lack of correlation is not to do so much with the assay conditions but is most likely antibody specific.

Our internal data demonstrated the significance of treatment concentration. The trend for transcytosis levels for the mAbs was the same for both treatments, however depending on the treatment concentration, the ratio between the mAb-WT and mAb-YTE varied considerably, the lower 50µg/mL treatment having a higher ratio of 18 compared to 6 for the 100 µg/mL treatment. (Table 3).

Analysis of the huFcRn MDCK II Cell transcytosis levels for the seven multispecific therapeutic antibodies plotted with the rat clearance levels showed no significant correlation under the study conditions tested for both treatment concentrations (50 and 100µg/mL) for all seven multispecific antibodies tested, p= 0.67 and 0.65

respectively (Figure 14). The same finding was observed for the analysis of the same huFcRn MDCK II Cell transcytosis levels plotted with the human clearance data. Treatments of 50 and 100µg/mL resulted in p= 0.69 and 0.85 respectively. (Figure 15).

Though correlation between mAbs and FcRn cell transcytosis data had been confirmed externally and internally, this was not recapitulated with multispecifics under the same testing conditions in our study. To note, the structures of all the multispecifics tested in the study incorporated huIgG backbones and therefore recognizable by huFcRn. One thought is steric hindrance, as these multispecific antibodies are larger in size than mAbs, but multispecifics showed equivalent transcytosis levels as the mAb-WT under the conditions tested. Focusing on differences in structure as a factor for lack of correlation requires additional consideration. Is something happening *in vivo* that alters the binding of these multispecifics to FcRn? It was observed that some of these multispecifics showed poor recovery in the IVSS assay and may be possibly due to aggregation which could affect FcRn binding. Another idea is that a subset of a given transcytosed multispecific is somehow physically transformed immediately following transcytosis in such a way that may not necessarily effect their FcRn binding, but effect how they are recognized in the MSD assay, i.e. the binding of either the capture or detecting antibodies used and therefore not quantified.

The concentration levels for all the multispecifics tested were within the same concentration range of the tested mAb-WT, therefore extremely low transcytosis levels would not be the cause for missed correlation. None of the tested multispecifics had any half-life mutations. Upon further scrutiny, a correlation was identified between the KIH antibodies specifically and clearance data for both rat and human. This observation

demonstrates that antibody structure may play a critical role when trying to make any connections between transcytosis and clearance data. To further investigate this theory, a larger number of multispecifics for each specific format would have to be tested/

One limitation for this study was the number of multispecifics in each format available for testing. As demonstrated with the KIH antibodies, having a higher number of antibodies with the knob-in-hole structure may have enabled a correlation to be formulated between *in vitro* transcytosis levels and *in vivo* clearance. Another limitation for this study was the lack of availability of the tested multispecifics with different half-life mutations, i.e., QL or YTE, to increase to FcRn binding. If available, would we have detected the same trend that was observed with mAbs in terms of levels of transcytosis increased with QL or YTE mutation compared to WT.

Future Plans

Future plans include testing these same multispecific therapeutic antibodies in human KI FcRn mice. Two different strains available and work is ongoing to determine which strain is better suited for our project needs. The huFcRn in vivo data could be used to aid in validating the *in vitro* findings.

The cell-based *in vitro* assay can be optimized to be more aligned with actual FcRn recycling activities, including adjustment of pH levels, and measuring antibody concentrations from receiver well, cell monolayer as well as the cell supernatant at different time points to determine amount of antibody that is transcytosed and then recycled.

More in-depth analysis of the actual multispecific antibodies following their *in vitro* cell transcytosis will be investigated. The evaluation will be used to determine if

these molecules are changed in any following transcytosis. A variety of platforms will be utilized to better understand their characteristics following transcytosis including propensity to aggregate, intactness and binding to the antihuman Fc antibodies used in the MSD quantitation assay.

Appendix

Table 1. List of FDA Approved Bispecific Antibodies.

Trade Name	Active Ingredient	Year Approved	Indication
Blincyto	blinatumomab	2014	To treat Philadelphia chromosome-negative relapsed or refractory B cell precursor acute lymphoblastic leukemia
Hemlibra	emacizumab-kxwh	2017	To prevent or reduce the frequency of bleeding episodes in hemophilia A with factor VIII inhibitors
Rybrevant	amivantamab-vmjw	2021	To treat locally advanced or metastatic non-small cell lung cancer with certain mutations
Kimmtrak*	tebentafusp-tebn	2022	To treat a form of unresectable or metastatic uveal melanoma
Vabysmo	faricimab-svoa	2022	To treat neovascular (wet) age-related macular degenerated and diabetic macular edema
Tecvayli	teclistamab-cqyv	2022	To treat relapsed or refractory multiple myeloma
Lunsumio	mosunetuzumab-axgb	2022	To treat relapsed or refractory follicular lymphoma
Epkinly	epcoritamab-bysp	2023	To treat relapsed or refractory diffuse large B-cell lymphoma
Columvi	glofitamab-gxbm	2023	To treat relapsed or refractory diffuse large B-cell lymphoma or large B-cell lymphoma

*Kimmtrak is technically a bispecific molecule, not a bispecific antibody. Like some of the other bispecific antibodies used to treat some cancers, Kimmtrak has one arm using an antibody fragment to bring killer T cells to the tumor. Kimmtrak's other arm is an analogous structure found on T cells, the T cell receptor, instead of an antibody fragment to target a tumor antigen.

(Research, 2024)

Table 2. Published Human Clearance Results

ANTIBODY DRUG ID	CLEARANCE (mL/Day)	WEB LINK TO DATA
KIH-1	240	https://link.springer.com/article/10.1007/s40265-018-0861-2
Dbody-1	360	https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/761210s000lbl.pdf
KIH-2A	1080	https://link.springer.com/article/10.1007/s40265-022-01749-5
Dbody-2	449	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9646474/pdf/40265_2022_Article_1793.pdf
Fab-Fc-x scFc-1	312	https://link.springer.com/article/10.1007/s00280-022-04471-x
IgG-scFv-1	1300	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10694581/
XmAb(2:1)-1	970	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10245362/pdf/40265_2023_Article_1894.pdf

Table 3. Transcytosis Results; 100 and 50µg/mL Treatment huFcRn Cells

Antibody Drug ID	nG/mL Transcytosis (100µG/mL Treatment)	STDEV	nG/mL Transcytosis (50µG/mL Treatment)	STDEV
mAb-WT	20.3	8.3	6.0	0.4
mAb-QL	66.6	7.9	39.9	4.4
mAb-YTE	118.1	13.3	109.3	54.1
KIH-1	8.0	0.5	33.7	44.8
Dbody -1	5.8	1.6	2.3	0.5
KIH-2A	12.5	2.6	5.4	1.0
Dbody -2	124.7	168.0	8.3	2.5
Fab-Fc x scFc-Fc -1	10.6	3.6	6.9	4.7
IgG-scFv - 1	16.8	3.0	31.5	15.7
XMAb (2:1)-1	22.9	6.0	8.3	1.4

Transcytosis results following antibody-based therapeutic treatment (50/100µg/mL) of huFcRn MDCK II cells using MSD platform for analysis of total antibody levels from supernatant bellow the cell monolayer on the transmembrane of the receiver well.

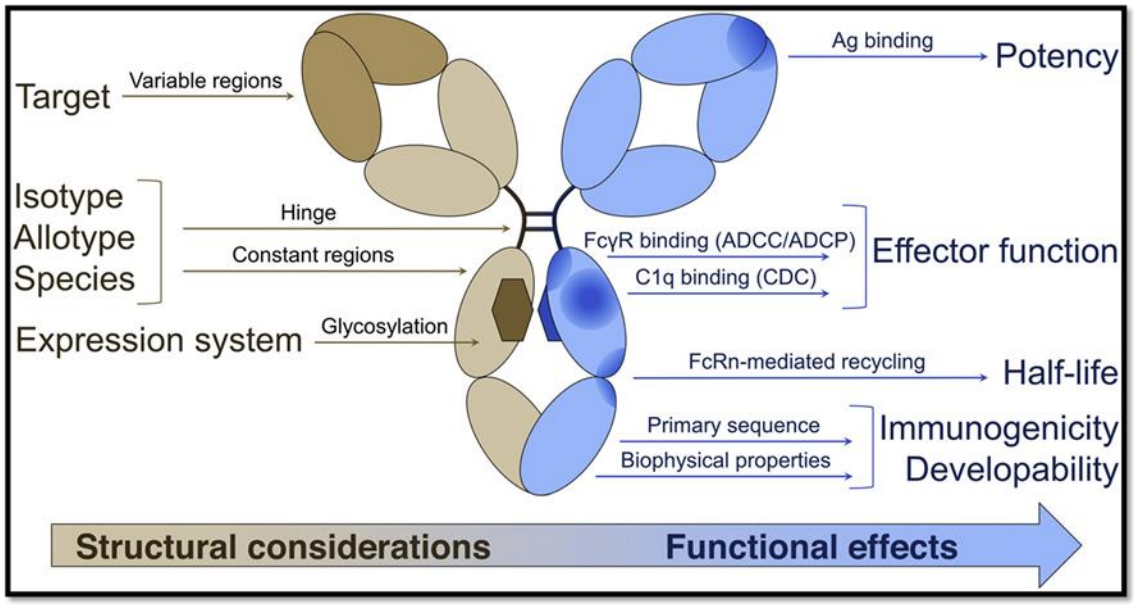


Figure 1. Structural and functional properties of an Antibody

Fundamental structural and functional properties of an antibody to consider when developing an IgG based therapeutic to achieve desired biological and clinical effects (Goulet & Atkins, 2019).

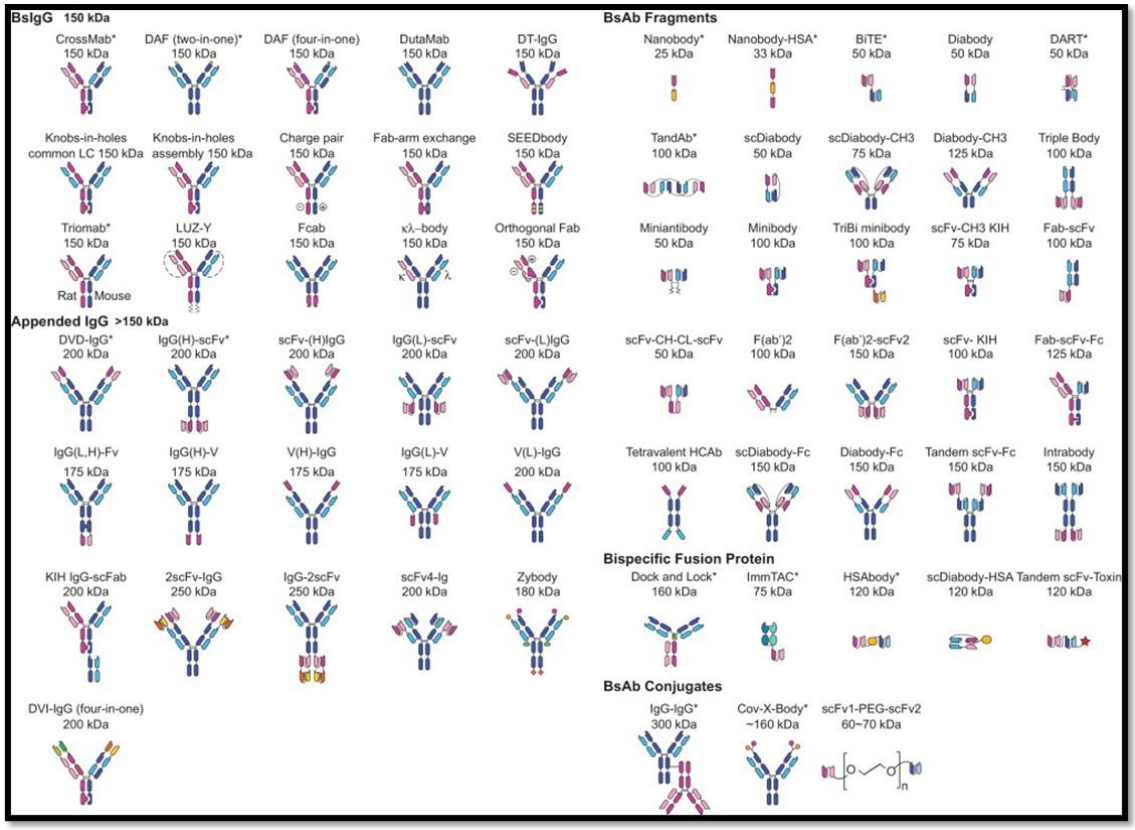


Figure 2. Bispecific Antibody Formats & Other Bispecific Immunotherapeutics

These are examples of the various bispecific antibody formats and additional bispecific immunotherapeutics. The heavy chains are color coded as dark blue, dark pink and dark green; lighter shades of same colors depict the light chains (Spiess et al., 2015).

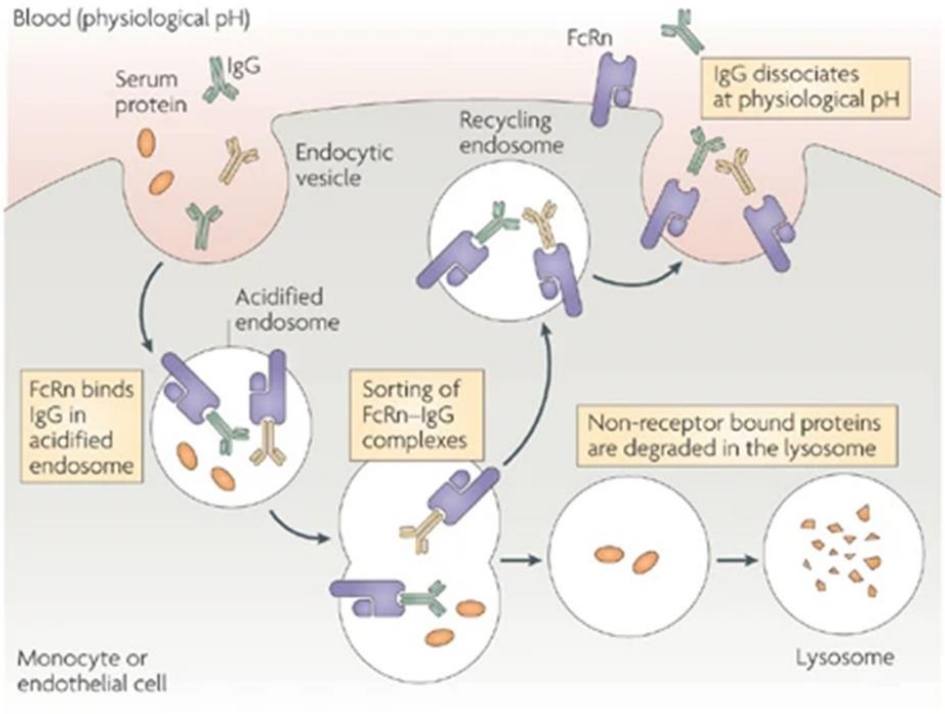


Figure 3. Proposed Role of the Neonatal Fc Receptor (FcRn)

Endothelial cells and circulating monocytes express FcRn, a receptor for IgG. Bound to the receptor, serum IgG is internalized within an acidic endosomal section. The FcRn then recycles the serum IgG back into circulation, prolonging the serum half-life of the protein. If a serum protein does not have a recycling receptor, it is fated for lysosomal degradation (Roopenian & Akilesh, 2007)

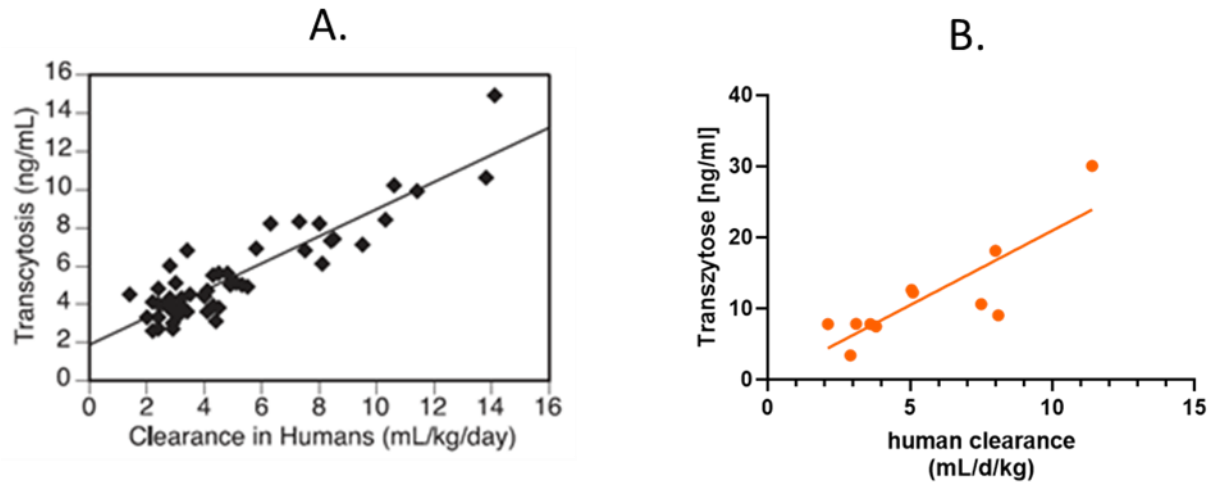


Figure 4. Antibody Transcytosis Results Plotted Against Human Clearance Data

Significant correlation observed between the transcytosis results for marketed monoclonal antibodies in relation to published human clearance data A) Antibody transcytosis results plotted against human clearance data (Chung et al., 2019) B) Internal antibody results plotted against human clearance data (unpublished).

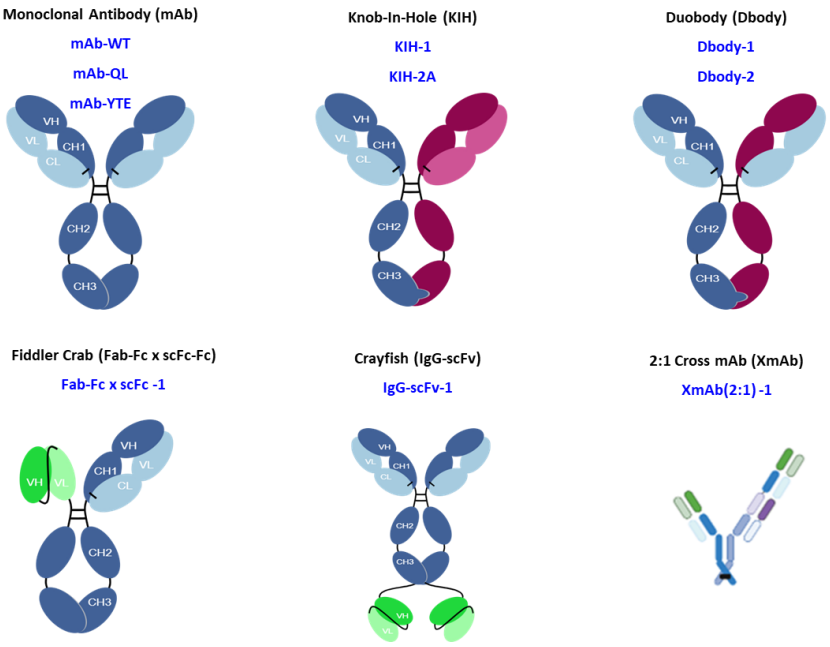


Figure 5. Structural Formats of Tested Antibodies

Images depict the structures and the names (royal blue) of the antibody-based therapeutics tested in various in vitro and in vivo studies throughout this work. mAbs have two identical heavy chains (dark blue) and two identical light chains (light blue). KIHs have two unique heavy chains (dark blue / dark pink) and two unique light chains (light blue / light pink). Dbodys have unique heavy chains (dark blue /dark pink) with identical light chains (light blue). Fab-Fc x scFc-Fc has two unique heavy chains (dark blue/dark blue green) and one light chain (light blue). IgG scFv has two identical heavy chains (dark blue / green) and two identical light chains (light blue). XmAb has two unique heavy chains (H1, H2) and three unique light chains (LC1, LC2, LC3).

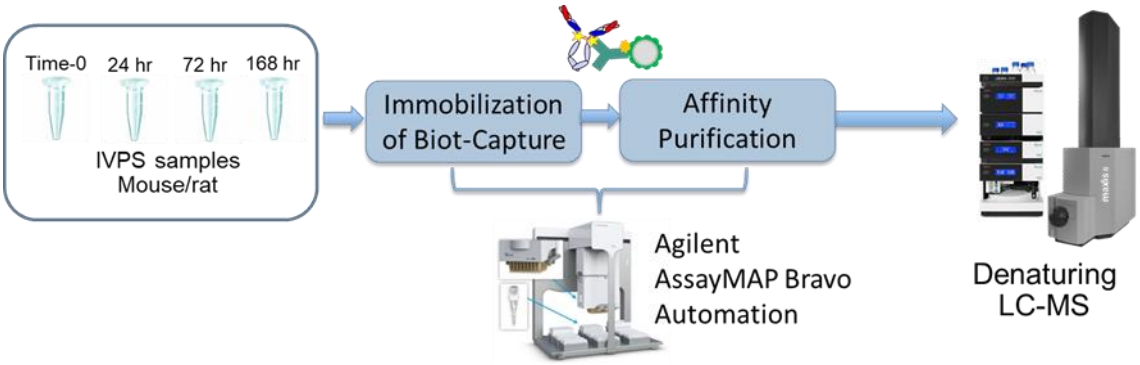


Figure 6. In vitro Serum Stability Assay (IVSS) Workflow

Humanized antibody-based therapeutics were incubated in serum at 37°C for one week. Samples were collected at four timepoints. The in vitro samples were then purified with anti-human IgG, and then analyzed directly on intact level in LC-MS under denaturing conditions.

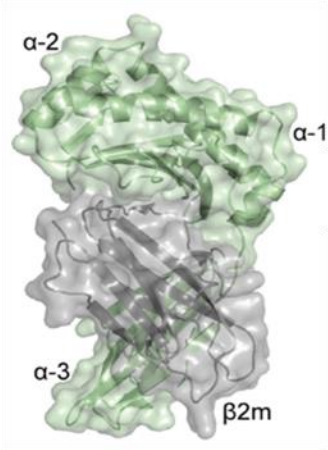


Figure 7. Structure of Functional FcRn

Functional FcRn image with human FcRn heavy chain illustrated in green non-covalently linked to the gray area representing beta-2 microglobulin (Pyzik et al., 2019).

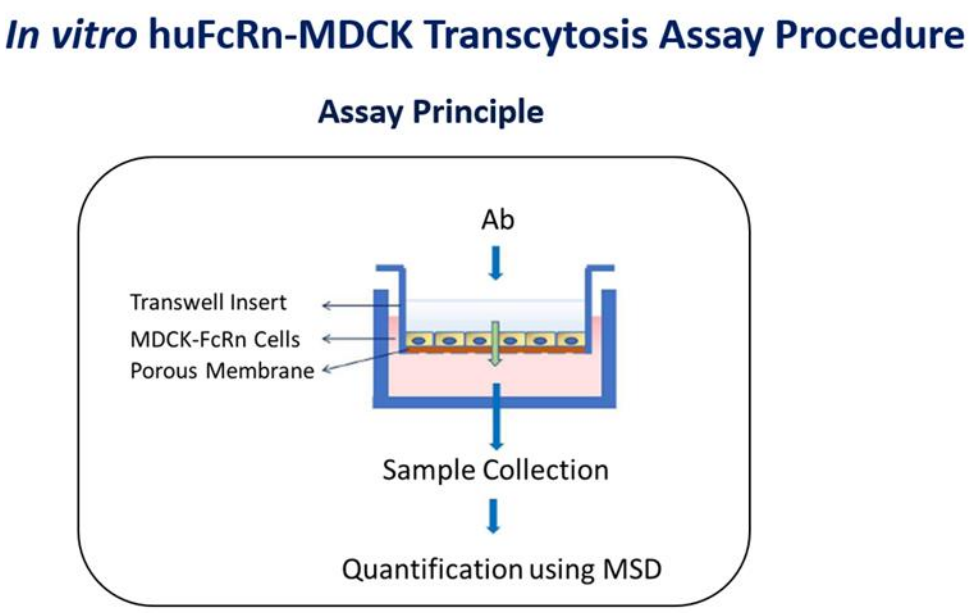


Figure 8. In vitro huFcRn MDCK Transcytosis Procedure

Diagram of the transcytosis assay in which cells are seeded onto transwell insert for culturing, antibody-based therapeutic is added to cell culture media above the transwell. Samples are pulled from receiver well below transmembrane and analyzed for total antibody levels using MSD.

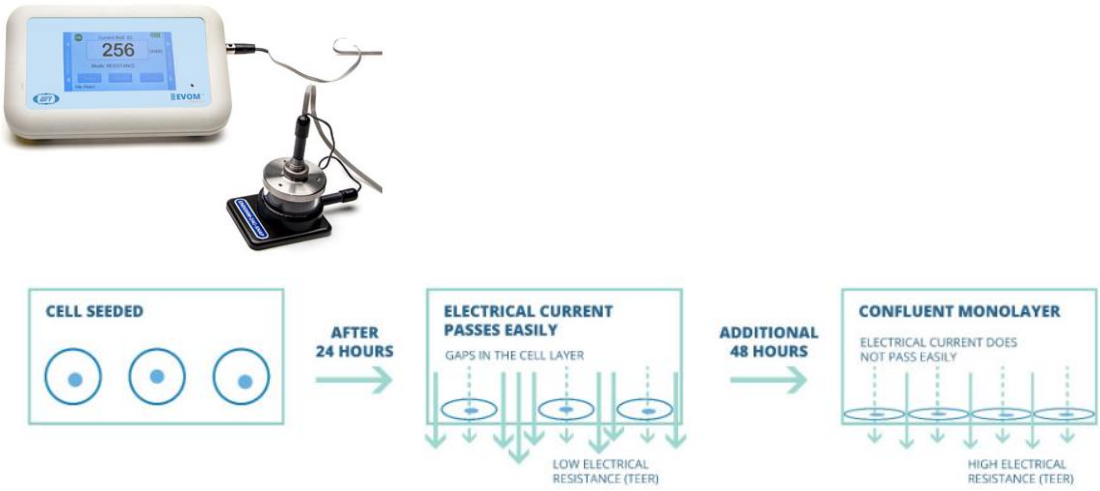


Figure 9. TEER Measurement

The pictorial shows an example of a TEER Measurement tool and depicts how the measurement is acquired. Differences in TEER levels are dependent upon amount of current passing through paracellularly.

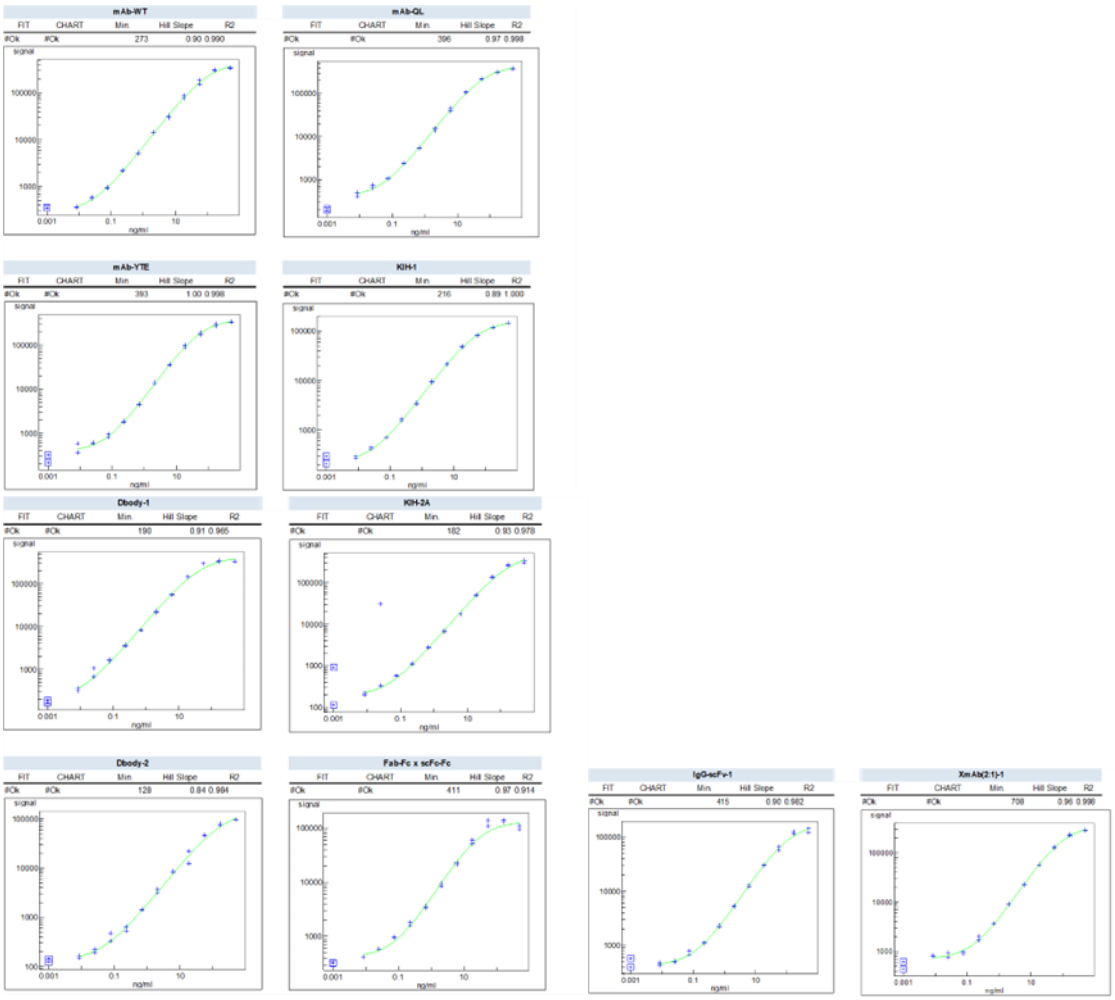
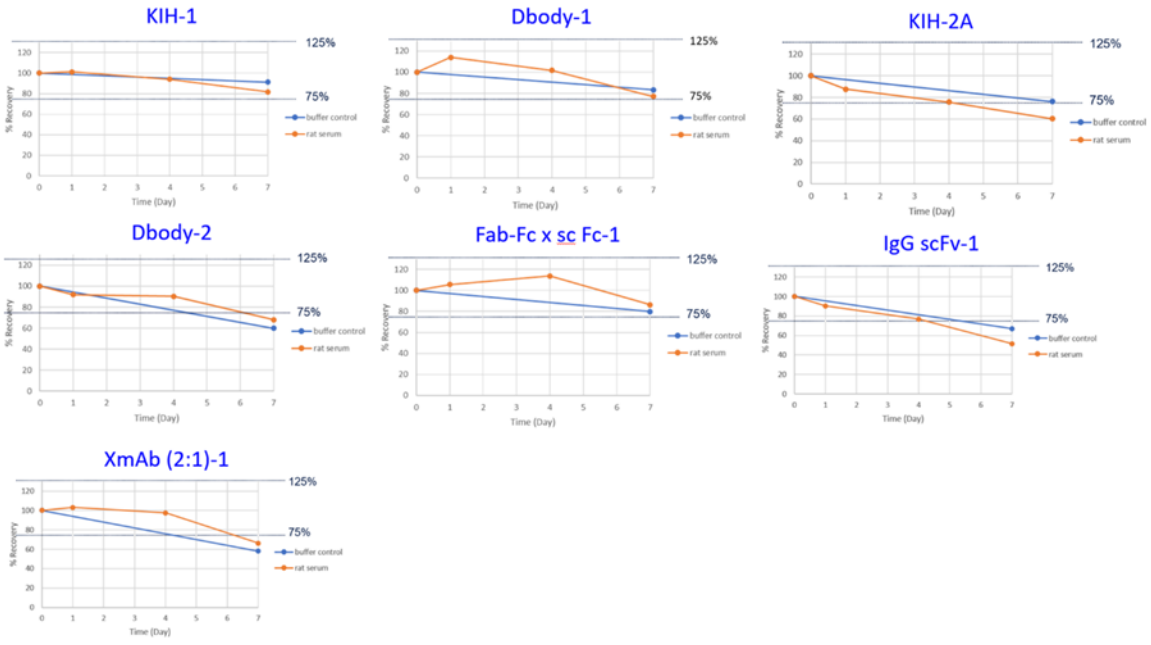


Figure 10. Standard Curves Tested Antibody-Based Therapeutics

Calibration curves were prepared in cell culture media and were plotted from MSD luminescence units versus theoretical standard concentrations. A four-parameter logistic model was used for curve fitting. The regression equation for the calibration curves was then used to back calculate the measured concentrations for each antibody-based therapeutic.



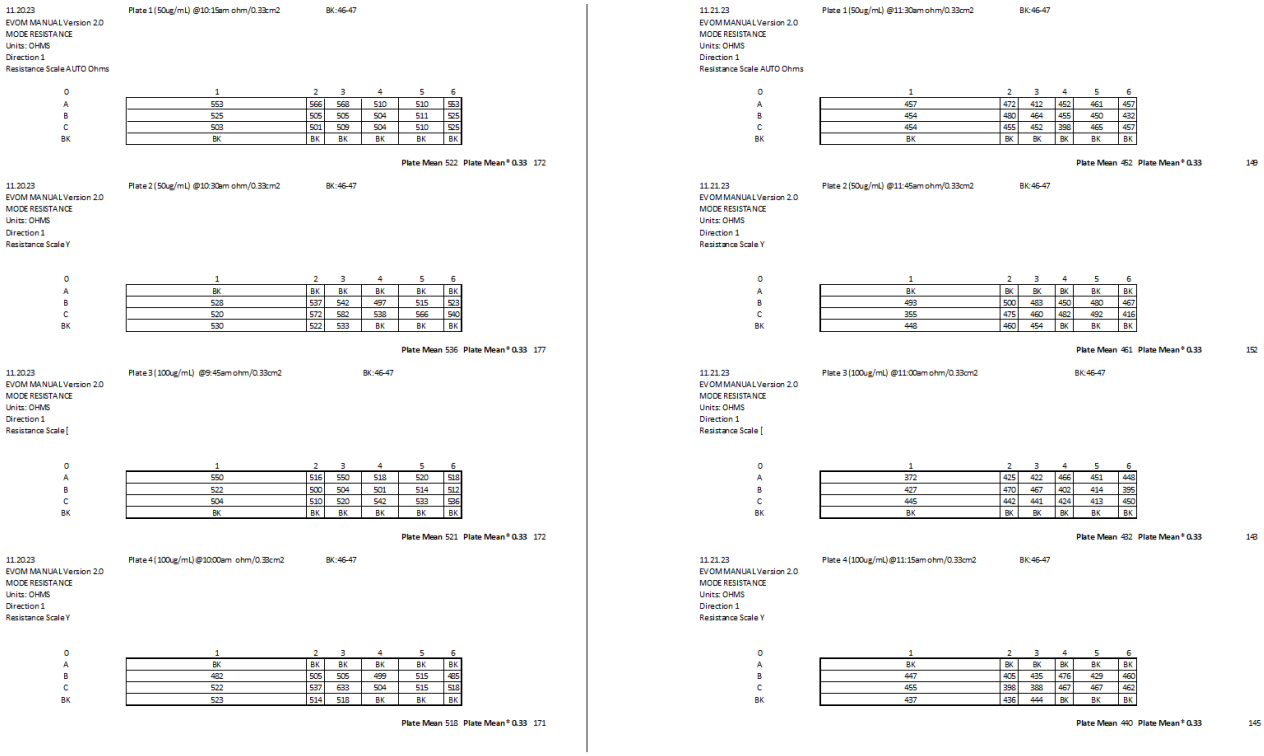
Antibody Drug ID	IVSS
KIH-1	
Dbody-1	
KIH-2A	
Dbody-2	
Fab-Fc x scFc-1	
IgG-scFv-1	
XmAb(2:1)-1	

No clipping; No significant change of % recovery (75%-125%)

No clipping; % recovery is 50%-75%

Figure 11. IVSS Rat Serum Test Results

The IVSS results for each therapeutic antibody tested in both buffer and rat serum were graphed as percent recovery. Green denotes antibodies that were within acceptable percent recovery range, yellow denotes antibodies at or below acceptable recovery range. None of the antibodies evaluated showed evidence of clipping under the conditions tested.



11.20.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale AUTO Ohms
Plate 1 (50ug/mL) @10:15am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	553	566	568	510	510	553
B	525	505	505	504	511	525
C	503	501	509	504	510	525
BK	BK	BK	BK	BK	BK	BK

Plate Mean 522 Plate Mean * 0.33 172

11.20.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale Y
Plate 2 (50ug/mL) @10:30am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	BK	BK	BK	BK	BK	BK
B	528	537	542	497	515	523
C	520	572	582	538	566	540
BK	530	522	533	BK	BK	BK

Plate Mean 536 Plate Mean * 0.33 177

11.20.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale [
Plate 3 (100ug/mL) @9:45am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	550	516	550	518	520	518
B	522	500	504	501	514	512
C	504	510	520	542	533	536
BK	BK	BK	BK	BK	BK	BK

Plate Mean 521 Plate Mean * 0.33 172

11.20.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale Y
Plate 4 (100ug/mL) @10:00am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	BK	BK	BK	BK	BK	BK
B	482	505	505	499	515	485
C	522	537	633	504	515	518
BK	523	514	518	BK	BK	BK

Plate Mean 518 Plate Mean * 0.33 171

11.21.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale AUTO Ohms
Plate 1 (50ug/mL) @11:30am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	457	472	412	452	461	457
B	454	480	464	455	450	432
C	454	455	452	398	465	457
BK	BK	BK	BK	BK	BK	BK

Plate Mean 452 Plate Mean * 0.33 149

11.21.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale Y
Plate 2 (50ug/mL) @11:45am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	BK	BK	BK	BK	BK	BK
B	493	500	483	450	480	467
C	355	475	460	482	492	416
BK	448	460	454	BK	BK	BK

Plate Mean 461 Plate Mean * 0.33 152

11.21.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale [
Plate 3 (100ug/mL) @11:00am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	372	425	422	466	451	448
B	427	470	467	402	414	395
C	445	442	441	424	413	450
BK	BK	BK	BK	BK	BK	BK

Plate Mean 432 Plate Mean * 0.33 143

11.21.23
EVOM MANUAL Version 2.0
MODE RESISTANCE
Units: OHMS
Direction 1
Resistance Scale Y
Plate 4 (100ug/mL) @11:15am ohm/0.33cm2
BK:46-47

0	1	2	3	4	5	6
A	BK	BK	BK	BK	BK	BK
B	447	405	435	476	429	460
C	455	398	388	467	467	462
BK	437	436	444	BK	BK	BK

Plate Mean 440 Plate Mean * 0.33 145

Figure 12. TEER Results

The TEER results for pre-treated and post-treated huFcRn MDCK II cells treated with antibody-based therapeutics.

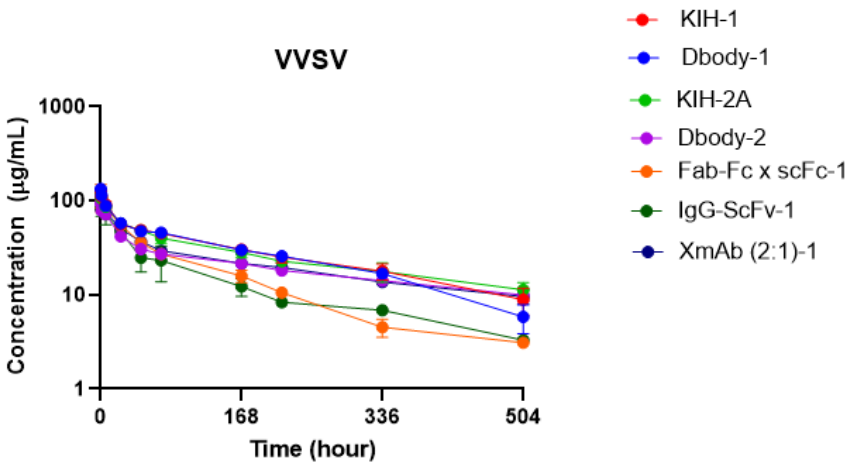


Figure 13. Pharmacokinetic Results for Sprague Dawley Rats

The pharmacokinetic results following 5mg/kg IV dosing of Sprague Dawley rats with antibody-based therapeutics showing total antibody levels measured from serum at each timepoint.

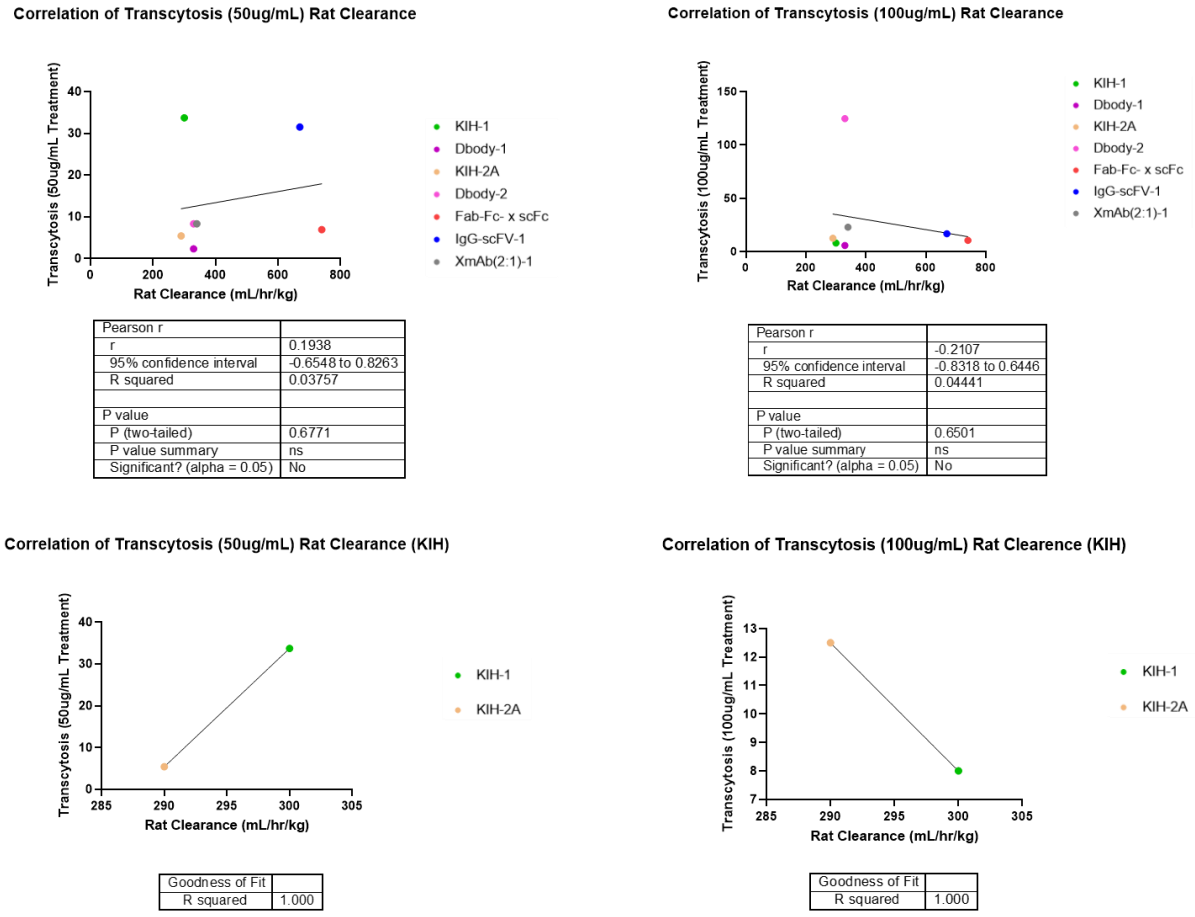


Figure 14. HuFcRn MDCK II Cell Transcytosis versus Rat Clearance

Results for HuFcRn Cell MDCK II transcytosis levels versus rat clearance levels were plotted and Pearson analysis was performed using GraphPad PRISM. Also shown are the linear plots for the KIH antibody transcytosis levels versus correlating rat clearance levels.

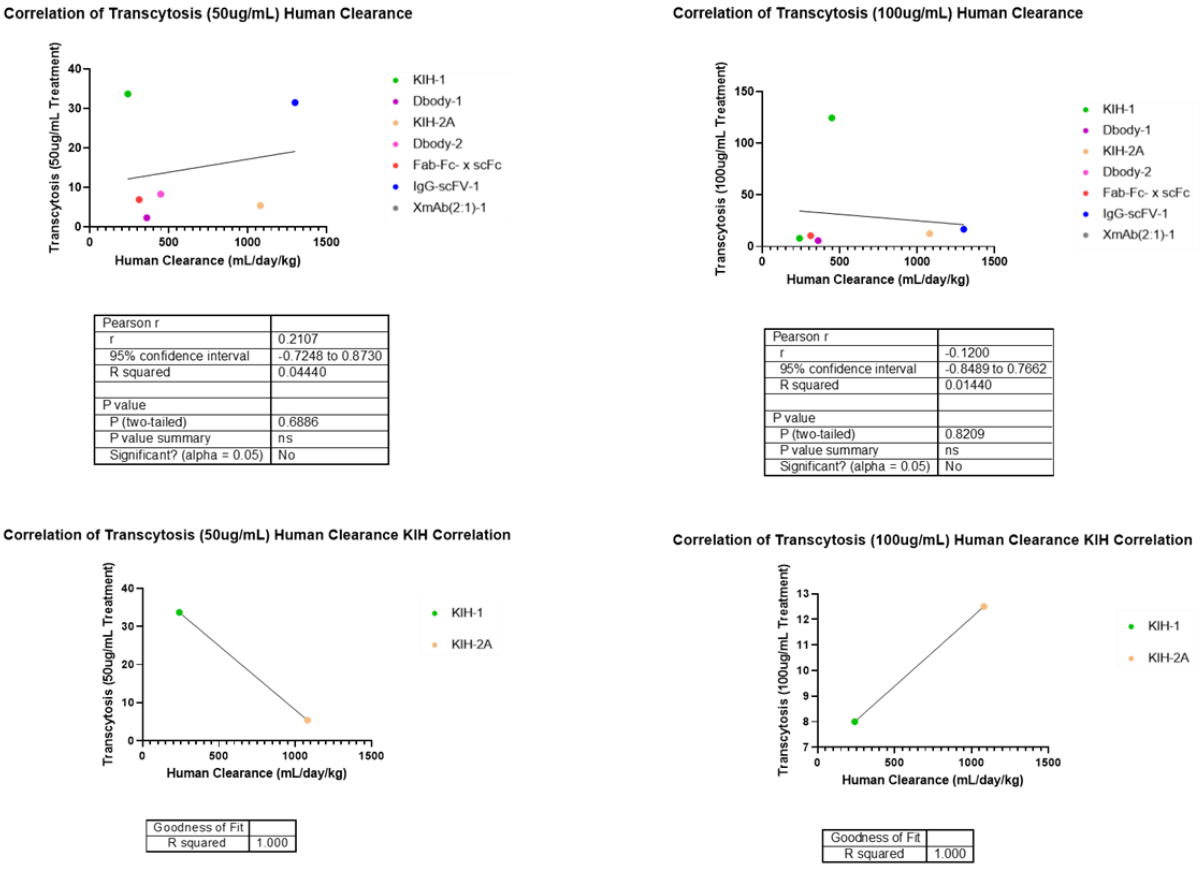


Figure 15. HuFcRn MDCK II Cell Transcytosis versus Human Clearance

Results for HuFcRn Cell MDCK II transcytosis levels versus human clearance levels were plotted and Pearson analysis was performed using GraphPad PRISM. Also shown are the linear plots for the KIH antibody transcytosis levels versus correlating human clearance levels.

Table 1
Properties of half-life extended antibodies.

Mutations	IgG species (isotype)	FcRn species	Fold increase in affinity at pH 6.0[a]	Species/strains for half-life analysis	Fold increase of half-life
T252L/T254S/T256F	Mouse IgG1	Mouse	~3.5	Mice	~1.6
M252Y/S254T/T256E	Human IgG1	Cynomolgus monkey	~9	Cynomolgus monkey	~3.7
	Human IgG1	Human	~11	Human	~3.5
	Human IgG1	Human	~1.9	Human FcRn transgenic mice	~2.7
	Human IgG4	Human	~4.7[b]	Human FcRn transgenic mice	~1.7
M252Y/S254T/T256E/S228P	Human IgG4	Human	~4.7[b]	Human FcRn transgenic mice	~2.9
Y31-M252Y/S254T/T256E	Human IgG1	Human	~25.5	Human FcRn transgenic mice	5
	Human IgG1	Human	~25.5	Cynomolgus monkey	~1.8
T250Q/M428L	Human IgG2	Human/rhesus monkey	~28[c]	Rhesus monkey	~1.8
	Human IgG1	Human/rhesus monkey	~29[c] (human)/ 37[c] (rhesus)	Rhesus monkey	~2.5
	Human IgG4	Human	49[c]	–	–
	Human IgG4	Human	~10.7–115[d]	Cynomolgus monkey	~0.86–2.6

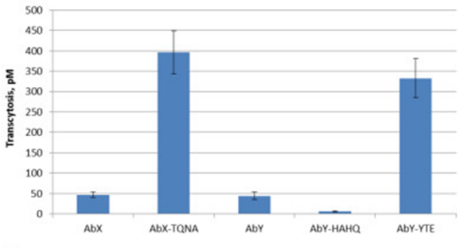


Figure 16.Half-Life Extensions using FcRn Mutations

Table 1 in the figure details the increase in half-life for different mutation sequences (Ward et al., 2015). The bar graph illustrates the effect of Transcytosis of mAbs at varying strengths of FcRn binding affinity. AbX and AbY are humanized IgG1 mAbs; both AbX-TQNA (T309Q/N434A) and AbY-YTE (M252Y/S254 T/T256E) carry mutations that increase binding to FcRn, whereas AbY-HAHQ (H310A/H435Q) carries mutations that decrease binding to FcRn (Chung et al., 2018).

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