DISCUSSION

Insulin was the first therapeutical protein manufactured via recombinant DNA techniques that became commercially available in 1982.[1] It marked the start of the biopharmaceutical era, which saw ever increasing market values and expanding developmental budgets.[1–5] More than 30 years have passed, yet many of the applied pharmacological concepts have changed little over time. An excellent example hereof is the field of bioequivalence research, even though only slightly older than recombinant human insulin.[6]

Bioequivalence is a regulatory concept which entails that a generical drug product ('test product') is therapeutically equivalent to the originator ('reference product') and can be used interchangeably.[6,7] Equivalence generally has to be demonstrated statistically on four endpoints: pharmacokinetic, pharmacodynamic, clinical, and *in vitro* endpoints.[6] Maximum plasma concentration (C_{max}) and area under the plasma concentration-time profile (AUC) are historically used to establish pharmacokinetic bioequivalence. Although various reports have criticised the use of these parameters to compare 'exposure' between two drugs,[8,9] C_{max} and AUC, determined in a non-compartmental analysis (NCA),[10] remain the required parameters by regulatory agencies to allow market authorisation.

With regard to biopharmaceuticals, the term biosimilarity is used, as it understood that the biotechnological manufacturing process cannot create an *exact* copy of the reference product. Instead of equivalence, a high degree of similarity has to be demonstrated on the aforementioned endpoints, and any remaining difference should be clinically insignificant.[11–13] Biopharmaceuticals are more complex than small molecules; their concentration-time profiles being no exception.[14–16] Even though a NCA is inadvertently ill-suited to cover the non-linear elimination pathways of monoclonal antibodies (MABs) and other large biopharmaceuticals, it still remains the gold standard in biosimilarity research.

In ***chapter 1***, two methods are described of how a population pharmacokinetics approach can be used to support the pharmacokinetic biosimilarity claim, and which can perhaps in the future even replace the NCA. Although such a method is not new,[17–19] it has never before been applied before to a MAB. Benefits of a modelled approach over a NCA are that a pharmacokinetic model can accurately describe the non-linear elimination pathways of MABs. Also, a model is not concerned with differences in administered doses. This is especially important for biopharmaceuticals with non-linear pharmacokinetics, since the NCA assumes linearity in its correction. Furthermore, a population pharmacokinetics approach can correct for covariates, and is relatively little affected by missing samples or deviations in sample collection times and administration time or dose.

More importantly, use of a population pharmacokinetic model allows for statistical testing of differences between test and reference products via covariate analysis. This can be done for all model parameters. Because these parameters are related to pharmacokinetic properties, such as absorption and elimination rates, the methods described in *chapter 1* may circumvent the problems[8,9] identified with the NCA in establishing pharmacokinetic biosimilarity. The chapter also discusses how model-based simulations can be used to proof that a therapeutical concentration is reached at the site of action with the test product, which further supports a biosimilarity claim.

However, a prerequisite for applying any modelling approach to pharmacokinetic data is a correct understanding of the mechanisms governing the distribution of a pharmaceutical over the body, which is still incomplete, as demonstrated in the *chapters 2 & 3*. The developed model in *chapter 1* (*figure 1.1*) has a so-called central compartment, to which drug product is added during administration and from which drug product is cleared via a linear and a non-linear process. After completion of intravenous infusion, the addition of drug to the central compartment terminates immediately. Since elimination continues, the maximum concentration in this compartment is theoretically reached at the end of infusion (EOI), assuming a constant volume. In other words, the time to C_{max} (t_{max}) equals the infusion duration, which is not the case for many MABs, including trastuzumab (*table 2.1*).

The fact that t_{max} can occur after EOI seems to have been ignored since the first introduction of large biopharmaceuticals. At best, the 'delayed' t_{max} is reported without further explanation (see *table 2.1*), but more frequently, this parameter is lacking from publications and one has to guess when C_{max} occurred. As an example, in at least five publications[20–24] on bevacizumab, a C_{max} is reported without a t_{max}. In other cases, a too sparse sampling design is chosen to allow the phenomenon to be observed; possibly, because C_{max} is expected at EOI and the slow elimination of many biopharmaceuticals does not necessitate dense sampling in the first hours after EOI. Yet, the finding of an increase in plasma concentration after intravenous administration has ceased is a strong indication that biopharmaceuticals do not always follow current pharmacokinetic theory, which is to a large extent still based on experience with small molecules.

Chapter 2 investigated two closely related, plausible theories to explain the 'delayed' t_{max}. The first hypothesis is that the biopharmaceutical is bound to and released from the vessel wall or taken up and released by endothelial cells, particularly in the presence of a high local concentration at the infusion site. After EOI, the concentration drops, and drug substance is released from the wall or by the cells, causing a rise in plasma concentration and hence a delayed t_{max}. The second hypothesis only applies to cases where infusion lines are flushed (*e.g.* with normal saline) in order to also administer the line content. If biopharmaceuticals can adsorb to the infusion line and desorb when the infusion line is flushed, drug administration actually continues after the anticipated EOI; thus, causing an *apparent* delay in t_{max}.

The performed studies found evidence for binding to endothelial cells. In an artificial vessel covered with endothelial cells, the MABs trastuzumab and bevacizumab were observed to adsorb to the luminal surface, though the pattern differed (*figure 2.1*). This adsorption seemed to be concentration-dependent and easily reversible upon washing the cell-layer with a lower concentration. Binding of tested MABs to the extracellular matrix was also noted. Together with existing knowledge on interaction between proteins and body surfaces,[25,26] these observations point to non-specific binding. Adsorption to endothelial cells and subsequent desorption can therefore theoretically explain a delayed t_{max}.

A lower recovery than expected was sometimes observed in experiments where administration procedures were mimicked with a standard infusion lines. However, the wash-out from the infusion line during flushing did not contain any quantifiable biopharmaceutical, other than what can be predicted based on laminar flow. Thus, flushing of the infusion lines cannot contribute to a delay in t_{max}.

Both the finding of adsorption to endothelium and the possibility of drug loss during infusion are relevant to the clinical pharmacologist. The concept of dose-response requires knowledge of the drug exposure at the site of action at a certain moment in time, and thus knowledge of the *exact* dose administered. Adsorption of biopharmaceuticals to endothelium, of which the delayed t_{max} is only one symptom, results in an uneven distribution over the vascular compartment (*figure 2.4*). In that respect, predicting the exposure at the site of action becomes even more difficult based on a limited number of plasma samples collected from a single vein.

Although the performed studies do not preclude alternative mechanisms to be involved in causing an increase in plasma concentration after EOI, these yet unidentified mechanisms pose the same challenge for the clinical pharmacologist, namely how relevant the measured quantity at the sampling site is in studying drug effects and the relationship between the two (pharmacokinetic-pharmacodynamical model). In any case, the observation that $C_{max} > C_{EOI}$ implies the biopharmaceutical is not evenly distributed over the vascular compartment.

Chapter 3 further adds to the complexity of the pharmacokinetics of some biopharmaceuticals. MABs, and perhaps biopharmaceuticals in general, demonstrate highly variable plasma concentrations over time within the same individual (*figure 3.1*), as opposed to the stability one expects from current theory.[14–16,27,28] This feature may have gone undetected because the collection of multiple samples within an hour is uncustomary for supposedly slowly distributing and eliminated drugs like MABs. A simple solution would be to ascribe the fluctuations to normal (assay) variability. However, for various reasons, such an explanation must be considered unlikely, as argued in this chapter.

Adsorption to the endothelium may not only account for the delay in t_{max}, it may also account for the observed fluctuations in the plasma concentration, if one includes the dynamical state of the endothelium and circulation in the equation. For example, numerous physiological and pathological stimuli have

been identified that can influence the endothelium, including the glycocalyx.[29–33] Such changes to the endothelium may affect the local balance between adsorption and desorption with rises or falls in plasma concentration as manifestations. Additionally, blood flow can be increased or diminished to certain organs, depending on overall body activity, and, in tissues, capillary beds can be opened or closed, depending on local metabolic demands.[34] Thus, the endothelial surface area available for adsorption varies, as do the haemodynamical characteristics and with it the wash-out of adsorbed biopharmaceutical. These and other possible explanations – as discussed in *chapter 3* – are still speculative without support from dedicated studies. Moreover, it remains to be established whether the observation for certain MABs can be extended to all large therapeutical proteins.

If one cannot accurately predict drug concentration at the (desired) site of action in a particular patient at any moment, one cannot instigate rational (effective) pharmacotherapy. This notion undermines the current quest for personalised medicine. For many rheumatological biopharmaceuticals, it remains challenging to accurately predict efficacy and toxicity from animal or *ex vivo* models, or to correlate clinical effects and pharmacokinetic parameters.[35,36] In patient trials, usually, only a few plasma samples are collected for determination of drug concentration and the investigated pharmacokinetic-pharmacodynamic relationship is based on a single parameter, such as through or steady-state concentration. Considering that the presented data indicate that the plasma concentration of MABs can fluctuate significantly within short times, a lack of correlation between clinical effects and standardised pharmacokinetic parameters may be explained.

On the other hand, if one understands the basic factors that together determine local drug concentration, one can fully individualise treatments to maximise efficacy, or at least ensure adequate drug exposure at the site of action. As a hypothetical case, it may – for example – be considered that eating can be beneficial to patients with an intestinal neoplasm, who are being treated with a particular MAB, as alimentation directs blood flow – and hence a therapeutical protein – to the digestive tract. However, the *in vivo* situation is usually far more complicated than predicted by current theory and models, a recurrent theme in this thesis. Therefore, influencing a single factor will probably not revolutionise pharmacotherapy.

Even if we were to fully understand the pharmacokinetics of biopharmaceuticals, clinical effects following biopharmaceutical treatment would sometimes be difficult to predict upfront, especially unwanted, toxic effects.[37–41] For example, certain MABs are also associated with inflammatory reactions, typically upon first administration, which can be severe.[42–44] These reactions are classified as 'adverse immunostimulation' or type α reaction, although the terms are not necessarily interchangeable (see *box 6.1*). The occurrence of a severe, unopposed, systemic inflammatory reaction following administration of a drug is particularly troublesome as these syndromes are life-threatening, similar to sepsis and anaphylaxis.[45–48]

For some MABs, the underlying mechanism of the adverse immunostimulation (AI) has been unravelled,[49–51] but for others, it remains to be elucidated. This lack of insight, combined with the fact that involved immune pathways in the pathogenesis of the AI differ between biopharmaceuticals, does not facilitate the development of a standardised platform which can be used to screen compounds for AI.

Chapter 4 sheds light on one of the lesser understood cases of AI, by employing an *ex vivo* whole blood incubation assay. This study compared individuals who had shown clinical signs of an inflammatory reaction following trastuzumab administration with those who had not. Higher *ex vivo* IL-6 release was observed in the clinical 'responders' compared to 'non-responders', which correlated to maximum body temperature (*figure 4.1*). A similar linear correlation was found between the *ex vivo* TNF-α response and maximum body temperature, but only within the 'responders'. Nonetheless, the magnitude of the differences was too small to serve as a screening tool for (trastuzumab-associated) immunostimulation.

A factor complicating the implementation of novel screening methods for AI is that results vary in different cytokine release assays utilising living human tissues, depending on the exact test conditions and donors included.[52–54] Together with the fact that it is often unknown how *in vitro* cytokine release translates into clinical effects, this makes the interpretation of many assay results difficult, as was also acknowledged by an EMA workgroup.[55]

Not only can inflammatory reactions be induced by the drug substance, they can also arise as a result of (microbial) impurities or contaminants in the drug product. Biopharmaceuticals are usually produced in

cell based platforms, frequently of microbial origin.[56] This process inevitably introduces foreign (non-human) substances in the drug product, substances that can elicit an immune response. Nonetheless, standard toxicological studies are not directed at capturing immune responses toward a pharmaceutical. Surprisingly, even for biopharmaceuticals, despite their inherent risk, dedicated immunotoxicological studies are not required by the regulatory guidelines.[57] Yet, by following the guidelines, potentially dangerous immunostimulation induced by (microbial) impurities in biopharmaceuticals can be missed, as detailed in *chapters 5 & 6*.

The first case (described in *chapter 5*) is that of a recombinant human Apolipoprotein A-I Milano, with code name ETC-216.[58] After it had been administered to healthy volunteers and patients with coronary artery disease, ETC-216 was found to induce AI in the third clinical trial. During subsequent analyses, it was discovered that ETC-216 contained several immunostimulatory host cell proteins (HCPs), one of which was flagellin.[59,60] In the second case (described in *chapter 6*), the AI was already observed in the first clinical trial with a recombinant human plasma protein. Here, the cause was also traced back to HCPs, one of which stimulated toll-like receptor (TLR)4.

In ***chapter 5***, a study is described where an *ex vivo* cytokine release assay was used once more to test ETC-216. The results indicated a strong IL-6 and TNF-α response elicited by ETC-216, but not by the remanufactured product (MDCO-216). The difference was statistically significant in all examined donor populations: healthy volunteers, and patients with stable and acute coronary artery disease. *In vivo* administration of MDCO-216 to healthy volunteers and patients with stable coronary artery disease confirmed the result that the immunostimulatory HCPs were successfully removed.[61]

However, the conclusion that the AI could have been prevented by using this assay is premature. *Table 5.3* clearly shows that the response to a stimulus varies between different populations, and also within populations. Comorbidities and medication use, as well as other patient characteristics, may explain some of the found differences. Because such factors can either increase or decrease an individual's susceptibility to AI, translation of *ex vivo* results to *in vivo* effects is less than straightforward, increasing the complexity of predicting AI during the preclinical phase of development.

An important question to be answered is why the preclinical safety testing had not revealed the immunostimulatory propensity of ETC-216. Can other tests than those commonly applied or required by international guidelines predict AI, especially if caused by impurities of microbial origin? ***Chapter 6*** illustrates the main shortcomings of current practice on the basis of two case histories.

The first shortcoming relates to the sensitivity of the used assays during quality control of the drug substance. Endotoxin is a recognised impurity or contaminant within biopharmaceuticals, yet the commonly employed limulus amoebocyte lysate (LAL) assay failed to detect its presence in one of the cases. Assays for other HCPs only detect a selection of all host cell proteins. Because the identity of the found HCPs remains hidden, these tests generate results of unknown significance. Nonetheless, even if all HCPs are characterised, for many impurities, the clinical effect and hence a safe level is unclear, least of all when dealing with combinations of impurities. This is the second shortcoming: the quantification instead of the qualification of impurities.

More sophisticated test platforms are available, which utilise human immune cells or cells transfected which immune receptors and try to mimic the *in vivo* situation. These platforms can detect a number of untoward reactions that would previously not have been discovered, such as those caused by endotoxin, flagellin, peptidoglycan, and others. However, no laboratory test is fail-safe, as also discussed above. Furthermore, many of the developed test platforms, including the applied *ex vivo* cytokine release assay in *chapters 4 & 5*, are back-translations, starting from an unanticipated clinical finding and trying to reproduce it *in vitro*. They are validated only against known examples of biopharmaceuticals causing AI. Thus, their claims to accurately predict AI still need to be substantiated by new test cases.

Toxicological studies in animals may overcome the first two shortcomings, provided the chosen species' immune system reacts similarly to the impurities as does the human one. Indeed, ETC-216 induced AI in cynomolgus monkeys, but this was missed because sensitive biomarkers (*e.g.* circulating cytokines) were not included and the safety measurements that could have suggested AI (*e.g.* vital parameters, haematology results) were done too infrequently. Moreover, immunotoxicity is neither routinely investigated, nor is it required by the guidelines. Even if dedicated

immunotoxicological studies are performed, the focus is on long-term immunosuppression or enhancement and not on rapidly occurring, transient reactions that characterise most cases of AI.

Awareness of the possibility of AI is another important aspect for early detection and prevention of similar cases in the future. This calls for more openness and the sharing of safety information. Proposals to be implemented in the guidelines – aiming to increase drug safety – can only occur after proper scientifical debate.

Despite decades of experience with biopharmaceuticals, our understanding of many *in vivo* pharmacokinetic and adverse effects is still limited, although this observation should not deter from continuing to perform dedicated studies with this class of drugs. On the contrary, it should inspire to investigate these poorly understood aspects. Clinical trials remain essential, for only such trials allow integration of all complex mechanisms occurring simultaneously in different, complex tissues and thus proof-of-concept. Where it comes to potentially hazardous effects of biopharmaceuticals, such as adverse immunostimulation, all reasonable efforts must be focussed on detecting these before administering the product to humans. Even then, however, human *in vivo* data are required to guide or validate preclinical research.

Sticky proteins, *i.e.* the dynamical binding ('stickiness') of proteins to various (bodily) surfaces, is a concept that can theoretically explain some of the ill-understood pharmacokinetic characteristics of biopharmaceuticals, most notably, the delay in t_{max} after intravenous infusion and the highly variable plasma concentration over time. *Dirty proteins* denotes a concept where therapeutical proteins induce an inflammatory reaction ('adverse immunostimulation') in humans, whose immune system perceives the biopharmaceutical a hostile ('dirtiness'). The active ingredient can serve as the stimulus, as can the co-administered contaminants or impurities.

Although these issues with biopharmaceuticals remain difficult to predict, because the underlying mechanisms are not completely elucidated, knowledge of these concepts – *sticky & dirty proteins* – appears to be indispensable for clinical pharmacologists and physicians primarily involved in patient care.

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