Prediction of thyroid C-cell carcinogenicity after chronic administration of GLP1-R agonists in rodents.

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Abstract

Increased incidence of C-cell carcinogenicity has been observed for glucagon-like-protein-1 receptor (GLP-1r) agonists in rodents. It is suggested that the duration of exposure is an indicator of carcinogenic potential in rodents of the different products on the market. Furthermore, the role of GLP-1-related mechanisms in the induction of C-cell carcinogenicity has gained increased attention by regulatory agencies. This study proposes an integrative pharmacokinetic/pharmacodynamic (PKPD) framework to identify explanatory factors and characterize differences in carcinogenic potential of the GLP-1r agonist products. PK models for four products (exenatide QW (once weekly), exenatide BID (twice daily), liraglutide and lixisenatide) were developed using nonlinear mixed effects modelling. Predicted exposure was subsequently linked to GLP-1r stimulation using *in vitro* GLP-1r potency data. A logistic regression model was then applied to exenatide QW and liraglutide data to assess the relationship between GLP-1r stimulation and thyroid C-cell hyperplasia incidence as pre-neoplastic predictor of a carcinogenic response. The model showed a significant association between predicted GLP-1r stimulation and C-cell hyperplasia after 2 years of treatment. The predictive performance of the model was evaluated using lixisenatide, for which hyperplasia data were accurately described during the validation step. The use of a model-based approach provided insight into the relationship between C-cell hyperplasia and GLP-1r stimulation for all four products, which is not possible with traditional data analysis methods. It can be concluded that both pharmacokinetics (exposure) and pharmacodynamics (potency for GLP-1r) factors determine C-cell hyperplasia incidence in rodents. Our work highlights the pharmacological basis for GLP-1r agonist-induced C-cell carcinogenicity. The concept is promising for application to other drug classes.

Keywords: GLP-1r agonists; C-cell carcinogenicity; pharmacology; PKPD modelling; prediction

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44 **Abbreviations:** AUC: area under curve; BID: twice daily; GLP-1r: glucagon-like-protein-1 receptor; MTC:

45 medullary thyroid carcinoma; PKPD: pharmacokinetics/pharmacodynamics; QW: once weekly

Introduction

Glucagon-like-peptide-1 receptor (GLP-1r) agonists are used as adjunctive therapy to treat type II diabetes. Mimicking the effect of endogenous GLP-1, they improve the balance between insulin and glucagon secretion, lower gastric emptying and reduce appetite (Garber, 2012). The first GLP-1r agonist on the market in 2005 was exenatide (Byetta®, Amylin Pharmaceuticals), which requires twice daily administrations (BID). In 2012, a slow release microsphere formulation of exenatide (Bydureon®, Amylin Pharmaceuticals) was approved, requiring once weekly injection (QW). Just before that, liraglutide (Victoza®, Novo Nordisk) became available to patients with a dosing regimen based on a once daily administration. Subsequently other GLP-1r agonists were approved, inlcuding lixisenatide (Lyxumia®, Sanofi, once daily injection), dulaglutide (Trulicity®, Eli Lilly, once weekly injection) and albiglutide (Tanzeum®, GlaxoSmithKline, once- or biweekly). The latter two are antibody products to ensure a long dosing interval.

A toxicological concern for these GLP-1r agonists is the increased incidence of thyroid C-cell adenoma and carcinoma in rodents (Joffe et al., 2009; Knudsen et al., 2010). Interestingly, this was shown for the long acting liraglutide (Knudsen et al., 2010; Madsen et al., 2012), but not for the short acting exenatide when administered subcutaneously (Knudsen et al., 2010). However, when administered by continuous infusion, exenatide also exhibited carcinogenic potential (Knudsen et al., 2010; Madsen et al., 2012). Based on the aforementioned findings, mechanistic studies were performed to better understand the causal relation between treatment with long acting GLP-1 receptor agonists and thyroid C-cell changes. Given that no carcinogenicity has been observed in GLP-1r knockout mice, these adverse events are likely to be GLP-1r specific (Knudsen et al., 2010; Madsen et al., 2012). Furthermore, plasma calcitonin concentrations were found to increase after sub-chronic treatment with GLP-1r agonists, as well as calcitonin mRNA levels in C-cells (Knudsen et al., 2010). Therefore, a mode of action was proposed for this

carcinogenicity which includes i) stimulation of GLP-1r on the thyroid C-cells; ii) increased production and secretion of calcitonin; iii) C-cell hyperplasia, ultimately leading to C-cell adenomas and carcinomas (Joffe et al., 2009; Knudsen et al., 2010; Rosol, 2013). There is a general believe that the possibility of C-cell hyperplasia progressing to neoplasia is high, although no direct evidence exists to support this statement.

The potential for carcinogenicity represents a serious concern during the development and regulatory approval of medicines. This concern is even greater when differences in the exposure profile seem to play a role in the incidence of events. From a clinical safety perspective, identification of the mechanisms of action underlying these adverse events can provided the basis for predicting the risk of carcinogenicity, rather than relying on empirical evaluation of standard protocols (e.g., 2-year rodent carcinogenicity outcome) (Laan et al., n.d.; Moggs et al., 2016; van der Laan et al., 2016).

In fact, to characterise which factors determine the potential for carcinogenic effects of short and long acting GLP-1r agonists, one needs to take into account both the pharmacokinetic (PK) and pharmacodynamic (PD) processes. The interaction between pharmacokinetic and pharmacodynamic processes ultimately determines the relation between administered dose and carcinogenic effect.

When combined with modelling and simulation concepts, pharmacokinetic-pharmacodynamic data can provide the basis for a parametric approach, which allows not only for an integrated evaluation of the pathophysiological processes and drug effects in a given experimental condition, but also the extrapolation and prediction of the treatment effects across a range of scenarios (Danhof et al., 2005; Sahota et al., 2016, 2014). In addition, using the appropriate model parameterisation, it is possible to distinguish drug-specific processes from those that are specific for the biological system, disentangling disease or species-related effects from drug effects. For example, the processes leading to GLP-1r stimulation are dependent on the pharmacokinetics and the potency of each drug, but the relation between GLP-1r stimulation and C-cell hyperplasia are determined primarily by the downstream effects

of the relevant pathways (i.e., biological system). Assuming selectivity of action for the different compounds, it is possible to integrate the data from different GLP-1r agonists and to develop a generic PKPD framework as a tool to predict the effects of novel compounds with a similar target or mechanism of action.

The primary aim of the current investigation is therefore to develop a PKPD model that enables the identification of the factors that contribute to the differences between the various GLP-1 analogues and products. To that purpose, pharmacokinetic models will be developed to describe systemic exposure. Predicted drug levels will be combined with GLP-1r stimulation data for the development of a PKPD model. Subsequently, the relation between GLP-1r stimulation and C-cell hyperplasia, as a marker of pre-neoplastic response, will be characterised using logistic regression. To ensure appropriate parameterization and generalizability of the model for prospective evaluation of novel compounds, model development will be based on data from liraglutide and exenatide QW. We will then evaluate the predictive performance of the proposed framework for the evaluation of carcinogenicity of GLP-1r agonists using lixisenatide as a paradigm compound. Finally, an overview of the carcinogenic potential relative to the GLP-1r stimulation in rats is provided for four GLP-1r agonists that are currently approved for type 2 diabetes in humans.

Methods

Data

Data was extracted from dossiers that were available in the repository of the Dutch Medicines Evaluation Board. The companies responsible for the Marketing Application (AstraZeneca, Cambridge, England for the two exenatide-containing products; Novo-Nordisk, Bagsvaerd, Denmark, for liraglutide; Sanofi, Paris,

114 France for lixisenatide) agreed with the use of these data for this publication. Table I provides an overview
115 of the studies that were used. Pharmacokinetic data, i.e. plasma drug concentrations were obtained from
116 toxicokinetic studies for exenatide BID (European Medicines Agency, 2006), exenatide QW (European
117 Medicines Agency, 2011), liraglutide (European Medicines Agency, 2009) and lixisenatide (European
118 Medicines Agency, 2012), and data from the first dosing regimen was used for development of the
119 pharmacokinetic models. Pharmacodynamic data were obtained from 2-year carcinogenicity studies for
120 exenatide QW (European Medicines Agency, 2011), liraglutide (European Medicines Agency, 2009) and
121 lixisenatide (European Medicines Agency, 2012). The incidence of thyroid C-cell hyperplasia after 104
122 weeks was used as a pharmacodynamic measure and as a surrogate for the carcinogenic effect. I.e., the
123 occurrence of hyperplasia preceded the observation of tumours, and showed a better dose-response
124 relationship as compared with the adenoma (European Medicines Agency, 2009). Hyperplasia data were
125 categorized in scores 0 (no hyperplasia) and 1 (hyperplasia) to allow quantification of the response. The
126 carcinogenicity studies included preliminary death of animals, which were also evaluated for hyperplasia.
127 The mortality rate was similar among dose groups, and not correlated with carcinogenicity. Since it is
128 unknown whether a negatively scored animal would have developed hyperplasia between the time of
129 death and the end of the study, data from before week 104 were excluded for the purpose of the current
130 analysis.

131 ***PK modelling***

132 PK models describing the time course of the plasma drug concentrations were developed based on a
133 nonlinear mixed effects modelling approach, as implemented in NONMEM® version 7.2 (ICON plc, USA).
134 One- and two-compartment models were tested and compared to describe the pharmacokinetics of the
135 different drugs. Fixed and random effects were included in a stepwise manner, assuming the inter-
136 individual variability around the pharmacokinetic parameters to be log-normally distributed:

$$\theta_i = \theta_{TV} * e^{\eta_i} \quad \text{(Eq. 1)}$$

where θ_{TV} is the typical value for the population, and η_i is a random variable with zero mean and variance ω^2.

A proportional error model described residual error:

$$Y_{ij} = F_{ij} * (1 + \varepsilon_{ij}) \quad \text{(Eq. 2)}$$

where F_{ij} is the predicted drug concentration, and ε_{ij} is a random variable with zero mean and variance ω^2.

The models were evaluated using the following selection criteria: i) significant drop in objective function (> 3.84; $p < 0.05$, $df = 1$); ii) parameter precision; iii) goodness-of-fit; and iv) visual predictive check.

GLP-1r stimulation and C-cell hyperplasia

Linking the PK models to PKPD models describing GLP-1r stimulation: Subsequently, using the post-hoc parameter estimates from the PK modelling step, predicted plasma drug concentrations were linked to a sigmoid E_{max} drug effect model (eq. 3):

$$GLP1r\ stimulation = \frac{E_{max} * C_{drug,plasma}}{EC_{50} + C_{drug,plasma}} \quad \text{(Eq. 3)}$$

As PK data was collected from a separate experiment, population predicted values were used, as imputation of individual drug concentration profiles was not possible. PKPD modelling was based on a hybrid approach in that EC_{50} values were obtained from *in vitro* calcitonin assays of the four GLP-1r agonists in a rat medullary thyroid carcinoma (MTC) 6-23 cell line (table II), assuming this to represent *in vivo* GLP-1r stimulation. This can be justified by the fact mechanistic studies have shown that calcitonin release is a direct consequence of GLP-1r stimulation (Knudsen et al., 2010; Madsen et al., 2012; Rosol,

2013). In addition, the E_{max} was fixed to one, assuming similar intrinsic efficacy for all four GLP-1r agonists, which indeed was observed from *in vitro* assays (Knudsen et al., 2010). As a consequence, the simulated receptor stimulation is a relative measure.

Logistic regression to predict C-cell hyperplasia incidence: Using the integrated PKPD model, the average plasma drug concentration at steady state ($C_{ss,drug,plasma}$) and GLP-1r stimulation at steady state ($R_{ss,GLP\text{-}1r}$) were simulated for each drug following the dosing schemes of the carcinogenicity studies (table I, studies 6 - 8). The $C_{ss,drug,plasma}$ and $R_{ss,GLP\text{-}1r}$ were subsequently tested for association with C-cell hyperplasia incidence at week 104 using logistic regression (eq. 4)

$$Hyperplasia\ incidence\ (\%) = 100\% * \frac{e^{\alpha + \beta * [P]}}{1 + e^{\alpha + \beta * [P]}}, \qquad \text{(Eq. 4)}$$

in which α is the intercept (i.e. the placebo effect) and β is the slope (i.e. the drug effect) of the logistic regression analysis. *P* denotes the predicting variable, either $C_{ss,drug,plasma}$ or $R_{ss,GLP\text{-}1r}$.

The logistic regression was performed for exenatide QW and liraglutide, testing both $C_{ss,drug,plasma}$ and $R_{ss,GLP\text{-}1r}$ as predictor for C-cell hyperplasia incidence at 104 weeks. P-values were calculated for the slope (β) of the regression model to determine whether the influence of the predicting variables were significant. R statistical software version 3.1.1 was used to perform the simulations and logistic regression analyses.

Model validation and simulations: A hybrid model was constructed with $R_{ss,GLP\text{-}1r}$ linked to C-cell hyperplasia, using data from exenatide QW and liraglutide. This model was subsequently validated comparing the predicted with the observed hyperplasia incidence at week 104 after different dose levels of lixisenatide. Finally, with this model, the average incidence on C-cell hyperplasia for all four GLP-1r agonists was simulated for the dosing schemes as depicted in table I (studies 5 – 8).

Results

This study used a model-based approach to integrate data from four GLP-1r agonist products, which is not possible with the traditional protocols for the evaluation of carcinogenicity in preclinical species. Here we show that both pharmacokinetics and pharmacodynamics determine the C-cell hyperplasia incidence, thereby highlighting the pharmacological basis of GLP-1r agonist induced C-cell carcinogenicity.

In the next paragraphs we present the results of the PK model development and subsequently show that GLP-1r stimulation is a good predictor of C-cell hyperplasia at 104 weeks. Then, the predictive performance of the model is shown for C-cell hyperplasia after different dose levels of lixisenatide. Finally, an overview is provided for GLP-1r stimulation and the predicted C-cell hyperplasia incidences for the four products under the simulated study designs.

PK models

Exenatide BID: A one-compartment model with first-order elimination was found to best describe the pharmacokinetics of exenatide BID. Even though exenatide BID was administered subcutaneously, its pharmacokinetic disposition had to be described as an i.v. bolus starting at 0.5 hours (first sampling). This assumption was required because no samples were collected that reflect the absorption phase. (table III).

Exenatide QW: Given that the release of exenatide from the subcutaneously administered microspheres occurs in three phases, in which the fraction of first burst release is negligible, the pharmacokinetic model for exenatide QW included an initial zero order release phase at 0 hours and a second zero order release phase for the remaining drug at an estimated time point of 89 hours (table III). The fraction remaining for the second release phase was determined to be 0.54 by calculating the area under the curve (AUC) of the second peak relative to the total AUC of the average drug concentrations. The duration of the release

phases were 57 and 205 hours, respectively. The bioavailability was fixed to 75% as provided by the assessment report (European Medicines Agency, 2011). A two-compartment model with first order elimination best described the pharmacokinetics of exenatide QW. Inter-individual variability was quantified for clearance and peripheral volume of distribution.

Liraglutide: The time course of liraglutide concentrations in plasma was described by a one-compartment model with first-order elimination (table III). The characterization of first order absorption was allowed, since enough data points were obtained during the absorption phase.

Lixisenatide: Distribution of lixisenatide from the subcutaneous region to plasma was characterised by a zero order process (table III). The bioavailability of the highest dose (2000 µg/kg) was fixed to 3% based on the results described in the assessment report (European Medicines Agency, 2012). In addition, to account for dose non-linearity, the bioavailability of the lower doses was estimated relative to the highest dose.

Performance of the population PK models: In general the population parameters were estimated with sufficient precision(relative standard error (RSE) < 30%). By contrast, parameters describing the inter-individual variability for exenatide QW and the peripheral compartment had a RSE > 50%. Nevertheless, the goodness-of-fit evalutation (figure S1) and the visual predictive checks (figure 1) showed reasonable description of the observed data, with exception of exenatide BID concentrations, which appear to be slightly over-predicted at 0.5 hours (figure 1A, figure S1).

GLP-1r stimulation and C-cell hyperplasia

Relationship between drug exposure and GLP-1r stimulation: To get insight into the role of the pharmacokinetics in the pharmacodynamics of GLP-1r agonists, the short acting exenatide BID was

compared to the long acting liraglutide (figure 2). It is seen that for liraglutide the drug is not totally cleared from the system after 24 hours post dose, leading to drug accumulation following chronic dosing (figure 2C). For exenatide BID, even with twice-daily administration, the drug is fully cleared from the system before the next administration and no accumulation occurs (figure 2A). Consequently, the GLP-1r stimulation, even after a low dose of liraglutide (0.075 mg/kg), is continuously above 75% during the first 48 hours (figure 2D), whereas for exenatide BID it is back to 0% before the next administration (figure 2B).

Logistic regression model development: No statistically significant association or relationship was found between the simulated $C_{SS,drug,plasma}$ and C-cell hyperplasia after treatment with exenatide QW or liraglutide (*p = 0.08 and 0.11;* figure 3A,C). However, their $R_{ss,GLP\text{-}1r}$ estimations were significantly associated with C-cell hyperplasia (*p = 0.04 and 0.01;* figure 3B,D). Furthermore, when combining the $R_{ss,GLP\text{-}1r}$ of both drugs, the association was even more significant (*p < 0.001;* figure 3E*)*. The regression slopes (*β* in eq. 4) for GLP-1r stimulation and C-cell hyperplasia were 1.04, 0.99 and 0.97 for exenatide QW, liraglutide and the combined regression, respectively. Their similarity in the slope estimates suggests that the association between GLP-1r stimulation and C-cell hyperplasia is not compound specific (i.e. it does not depend on which compound is used).

Model evaluation: The predictive performance of the hybrid model (table II) was subsequently evaluated with the experimental data from other studies using lixisenatide, showing reasonable agreement between observed data and model predictions (figure 4). The model appears to slightly under-predict the C-cell hyperplasia incidence in the placebo and highest dose group, but correctly predicts treatment effects for the low and medium dose groups. Furthermore, the model predicts maximal C-cell hyperplasia already

after the lowest dose (47%), whereas the observed data show a slight dose dependent increase (45% - 52%).

Prediction of GLP-1r agonist-induced C-cell hyperplasia: Finally, an integrated overview of the relationship between GLP-1r stimulation and C-cell hyperplasia incidence was derived for all four GLP-1r agonist-containing products following treatment with the same dosing schemes of the carcinogenicity studies of each drug (figure 5). The incidence of C-cell hyperplasia does not exceed 50%, even when the stimulation of the GLP-1 receptor is maximal. A maximum of 50% hyperplasia has also been observed for liraglutide (Knudsen et al., 2010), and the effect appears to be saturable, as indicated by the fact that there was no dose-dependent increase for lixisenatide (figure 4). Also, even after placebo treatment, there is a 25% incidence of hyperplasia. This is substantiated by observations as shown in figures 3 and 4. Sprague-Dawley rats are known to spontaneously develop tumours (Nakazawa et al., 2001). Also, based on the tested dosing schemes, model predictions show that exenatide shows a relatively lower potential for C-cell hyperplasia when compared to liraglutide and lixisenatide. Moreover, for exenatide BID, modelling results predict lower incidence of hyperplasia than exenatide QW. It must be noted that the experimental designs and dosing schemes (table I) used in the simulations do not reflect the human dosing regimens, but rather the typical experimental protocols in rodents. The currently approved dosing regimen in humans results in exposures (based on AUC) that are 5, 23 and 130 higher for exenatide BID (U.S. Food and Drug Administration, 2008), 2.1, 10 and 26 higher for exenatide QW (European Medicines Agency, 2011), 0.5, 2.2 and 7.6 higher for liraglutide (Joffe et al., 2009), and >270 higher for lixisenatide (European Medicines Agency, 2012).

Discussion

In the current investigation we evaluate the feasibility of a PKPD framework in which the carcinogenic potential of GLP-1r agonists with respect to thyroid C-cells is evaluated in an integrative manner. It is based on the assumption that given the mechanism of action of these compounds, carcinogenicity results from GLP-1r stimulatory effects on C-cells. The degree of GLP-1r stimulation is in turn related to the extent and duration of exposure to the individual drugs. Whereas dosing regimen is a critical factor, the use of a PKPD model has shown that both exposure levels and drug potency on GLP-1r are determinants for the C-cell hyperplasia incidence.

This work fits in the renewed interest in the characterisation of the relationship between pharmacological properties and carcinogenicity (Laan et al., n.d.; Moggs et al., 2016; van der Laan et al., 2016) as part of a weight-of-evidence approach in the prediction of carcinogenicity (International Council for Harmonisation, 2016; Sahota et al., 2016, 2014). Similar work has been done to explore the utility of a biomarker-guided approach to predict the long term safety of naproxen in humans on basis of rat studies (Sahota et al., 2015, 2014). The authors showed that, using a model-based approach, biomarker data could be integrated and interspecies differences could be assessed. Indeed, a model-based approach distinguishes the drug specific properties from the biological system-specific properties, thereby providing the basis for interspecies translation (Danhof et al., 2008). Such analysis is not possible by the traditional methods for the evaluation of carcinogenicity, in which experimental data is assessed in a fragmented manner.

In the context of carcinogenicity evaluation, one of limitations of standard experimental protocol designs is the difficulty in establishing exposure-effect relationships in a strictly quantitative manner. These studies typically do not include detailed PK-analysis enabling further evaluation of the impact of inter-individual variability and underlying disease processes. On the other hand, by using a model-based

approach, data from different sources can be combined, enabling the integration of PK and PD data. We studied the carcinogenic potential of the various products with respect to exposure data of four GLP-1r products, potency for the GLP-1r, and the induction of (pre-)neoplastic phenomena, all within one model-based PKPD framework. Such approach is not limited to GLP-1r products, but well applicable to other drug classes. As an example, multiple adrenergic β_2-agonists appear to have a different carcinogenic potential, which may be caused by a distinct pharmacological profile (van der Laan et al., 2016).

It should be highlighted that the GLP-1r stimulation, which is a key component of this framework, is causal to C-cell carcinogenicity in our analysis. In contrast to wild type mice, GLP-1r knockout mice were shown not to develop C-cell hyperplasia after treatment with GLP-1r agonists (Madsen et al., 2012). The knockout mice did not show a calcitonin response after treatment, which implies that this response is a direct consequence of GLP-1r stimulation. Calcitonin has therefore been suggested as a biomarker of GLP-1r agonist induced rodent C-cell carcinogenicity (Joffe et al., 2009). However, to our knowledge a causal relationship between increased calcitonin concentrations and C-cell hyperplasia has not been previously demonstrated. Other factors, such as age (Knudsen et al., 2010; Kurosawa et al., 1988), interfere with the treatment related effect in rats, and calcitonin levels after 7 months of treatment could not be correlated with C-cell carcinogenicity endpoints (Knudsen et al., 2010). Furthermore, both calcitonin synthesis and release are different between healthy C-cells and those with focal hyperplasia, complicating its use as biomarker for C-cell hyperplasia (Rosol, 2013). In addition, the sensitivity of calcitonin reflecting C-cell functionality is not optimal, although provocative testing with pentagastrin, Ca^{2+} or omeprazole has been proposed to handle this problem (Vitale et al., 2002). It is thus questionable whether calcitonin could be used as a causal biomarker for C-cell hyperplasia (Joffe et al., 2009). On the other hand, liraglutide caused upstream activation of mTOR (Madsen et al., 2012), which is associated with several hallmarks of cancer, such as tumorigenesis, cell survival and proliferation (Laplante and Sabatini, 2013). This suggests that GLP-1r stimulation may lead to activation of these hallmarks, which could be causal to C-cell carcinogenicity,

rather than calcitonin itself. In addition to investigating the role of calcitonin in C-cell hyperplasia, further efforts regarding biomarker discovery for GLP-1r induced C-cell carcinogenicity is warranted.

The extrapolation of current findings and clinical implications of GLP-1r agonist carcinogenicity in rodents requires careful consideration of a number of factors. First, a much lower GLP-1r density has been shown in humans as compared to rodent C-cells, suggesting that findings in rodents are not likely to be relevant in the clinic (Körner et al., 2007; Waser et al., 2014). Also, in phase III clinical trials, no signs have been observed indicating C-cell carcinogenicity. Nonetheless, the earliest GLP-1r agonist, exenatide BID, is only on the market since 2005, and the earliest long-acting GLP-1r agonist, liraglutide, is only on the market since 2010. Moreover, histological studies have shown a consistent expression of the GLP-1r in MTC's and hyperplastic C-cells (Gier et al., 2012), suggesting that some patient subgroups may be vulnerable to GLP-1r agonist carcinogenicity. Therefore, a concern cannot be fully excluded (Joffe et al., 2009), and model-based simulations could be utilized to assess the interspecies differences for C-cell hyperplasia, eventually for different patient subgroups as part of the risk mitigation procedure.

We are aware of the limitations of the data available, and therefore we have made some assumptions. First of all, it was assumed that free plasma concentrations were reported, although it was not explicitly stated for every study whether concentrations were corrected for plasma protein binding. Whereas evidence may exist showing that plasma protein binding for these compounds is not restrictive, these data were not available either. This might have affected the interpretation of the results, since only the free drug concentrations exert pharmacological activity. The requirement for free concentrations should therefore be considered on a case-by-case basis. Second, because no information was available on the distribution of free drug to the thyroid, we assumed similar drug concentrations in plasma and thyroid. Drug distribution into tissue is influenced by characteristics of the drug (Danhof et al., 2007), and this assumption might have affected the predictive ability of the model. Third, the EC_{50} values were obtained

from a calcitonin assay (rat MTC 6-23 cell line) assuming that this assay is representative for GLP-1r stimulation. However, the other frequently used cAMP assay showed similar relative potencies among the products with liraglutide and lixisenatide having a 65 – 130 and 0.5 – 1.8 higher potency than exenatide (Knudsen et al., 2010; Schwahn et al., 2013). Moreover, as discussed previously, calcitonin release is a direct consequence of GLP-1r stimulation (Knudsen et al., 2010; Madsen et al., 2012; Rosol, 2013). Fourth, similar maximal effect was observed in the calcitonin and cAMP assays (Knudsen et al., 2010; Schwahn et al., 2013). Therefore, the E_{max} was fixed to 1 and a relative GLP-1r stimulation was associated with C-cell hyperplasia incidence. Fifth, the ratio between the *in vitro* potencies of the products was assumed to be similar to the ratio between the *in vivo* potencies. However, the binding to the receptor, which can be an important determinant of the potency, may be dependent on the characteristics of the drugs (de Witte et al., 2016), and could affect the predictive ability of the model. Sixth, we have assumed that pharmacokinetics and pharmacodynamics do not change over a period of 104 weeks. However, it is known that antidrug antibodies can develop over time for lixisenatide up to 12 months (European Medicines Agency, 2012). Even though bound drugs are unlikely to show pharmacological activity, these antibodies do extend the half-life of the drug in the systemic circulation. This could lead to increased accumulation of the plasma drug concentration, and potentially to a higher steady state drug concentrations and the corresponding GLP-1r stimulation.

Despite these assumptions, we believe that the current model parameterisation is sufficiently robust to support the assessment of compounds acting via GLP-1r system. The quality of the predictions for lixisenatide C-cell hyperplasia incidence (figure 4) indicates that model performance is appropriate. However, we acknowledge that further validation is required for wider use of the model and extrapolation of the findings to other products, e.g. albiglutide and dulaglutide.

In conclusion, this study highlights the pharmacological basis for GLP-1r agonist-induced C-cell carcinogenicity. It is shown that GLP-1r stimulation is a better predictor of C-cell hyperplasia than plasma drug concentrations of exenatide QW and liraglutide. Our analysis indicates that non-linear processes of receptor binding and activation need to be taken into account for accurate prediction of delayed drug effects. It also stresses the role of pharmacological biomarkers for prediction of carcinogenic potential. Both the extent and duration of exposure and the potency of the drug determine the degree of GLP-1r stimulation, and thus the C-cell hyperplasia, indicating that both PK and PD properties contribute to C-cell carcinogenicity. Our work highlights the value of model-based approaches to improve risk assessment and management of drugs with carcinogenic potential in a way that is not possible with traditional methods. The role of regulatory agencies is unique in this regard, because they have access to carcinogenic data from multiple same-in-class products.

Conflicts of interest

The authors have no conflicts of interest to declare.

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457 **Table I. An overview of the studies and data that was used for model development**

	Sample size	Drug	Dosing	Observation times	Observation
			Pharmacokinetic data		
1	420	Byetta *(exenatide BID)*	18, 70, 250 µg/kg, s.c., once daily	0.5, 1, 2, 3, 4, 6, 9, 12 hours	Plasma drug concentration
2	15	Bydureon *(exenatide QW)*	2.4 mg/kg, s.c., biweekly	15 min, 1, 4, 8 hours, 1, 3, 5, 8, 11, 15, 18, 22, 25, 29, 32, 36, 39 days	Plasma drug concentration
3	84	Victoza *(liraglutide)*	0.1, 0.25, 1 mg/kg, s.c., once daily	0, 1, 2, 4, 6, 8, 12, 24 hours	Plasma drug concentration
4	234	Lyxumia *(lixisenatide)*	5, 100, 2000 µg/kg, s.c., twice daily (t=0h; t=8h)	10, 20 min, 1, 3, 8 hours	Plasma drug concentration
			Pharmacodynamic data		
5	195	Byetta *(exenatide BID)*	0, 18, 70, 250, s.c. once daily	104 weeks	C-cell hyperplasia
6	227	Bydureon *(exenatide QW)*	0.3, 1, 3.0 mg/kg, s.c., biweekly	104 weeks	C-cell hyperplasia
7	256	Victoza *(liraglutide)*	0.075, 0.25, 0.75 mg/kg, s.c., once daily	104 weeks	C-cell hyperplasia
8	573	Lyxumia *(lixisenatide)*	0, 40, 200, 1000 µg/kg, s.c. twice daily (t=0h; t=8h)	104 weeks	C-cell hyperplasia

458 BID: twice daily; s.c.: subcutaneous; QW: once weekly

459

Table II. Parameters of the PKPD model describing the GLP-1r stimulation and the C-cell hyperplasia incidence

Parameter	Value (RSE)
E_{max} (%)	100 (fixed)
$EC_{50,exenatide}$ (pM)	55 (fixed)
$EC_{50,liraglutide}$ (pM)	5300 (fixed)
$EC_{50,lixisenatide}$ (pM)	25 (fixed)
α	1.1 (19%)
β	0.97 (30%)

EC_{50} values were obtained from a calcitonin assay in a rat MTC 6-23 cell line (Knudsen et al., 2010)

α: intercept of the regression model; β: slope of the regression model

464 **Table III. Pharmacokinetic parameter estimates for exenatide BID, exenatide QW, liraglutide and lixisenatide**

Parameter	Estimate (RSE%)
Exenatide BID	
$CL_{central}$ (L/h)	0.35 (11%)
$V_{central}$ (L)	0.35 (15%)
IIV	
$CL_{central}$	0.14 (18%)
Exenatide QW	
$CL_{central}$ (L/h)	0.72 (11%)
$V_{central}$ (L)	1.29 (24%)
$V_{peripheral}$ (L)	79.2 (15%)
$Q_{peripheral}$ (L/h)	29.12 (17%)
F	0.75 (FIX)
$Fraction_1$	0.46 (FIX)
$Fraction_2$	0.54 (FIX)
$Duration_1$ (h)	57.32 (15%)
$Duration_2$ (h)	204.57 (12%)
$Release_1$ (h)	0 (FIX)
$Release_2$ (h)	89.24 (2%)
IIV	
$CL_{central}$	0.08 (87%)

$V_{peripheral}$	0.07 (118%)

Liraglutide

$CL_{central}$ (L/h)	0.005 (8%)
$V_{central}$ (L)	0.037 (9%)
k_a (h^{-1})	0.27 (3%)
IIV	
$V_{central}$	0.46 (20%)

Lixisenatide

$CL_{central}$ (L/h)	0.03 (12%)
$V_{central}$ (L)	0.03 (12%)
Duration (h)	0.19 (14%)
$V_{peripheral}$ (L)	0.0058 (109%)
$Q_{peripheral}$ (L/h)	0.0019 (190%)
$F_{dose < 2000\ \mu g/kg}$	0.12 (14%)
$F_{dose = 2000\ \mu g/kg}$	0.03 (FIX)

$CL_{central}$: clearance from the central compartment; Duration: duration of the lixisenatide distribution from subcutaneous to plasma; $Duration_1$: duration of the first exenatide QW release; $Duration_2$: duration of the second exenatide QW release; F: bioavailability; $Fraction_1$: fraction exenatide QW released during the first release; $Fraction_2$: fraction exenatide QW released during the second release; IIV: inter-individual variability; k_a: absorption rate constant; $Q_{peripheral}$: Intercompartmental clearance between central and peripheral compartment; $Release_1$: starting time of the first exenatide QW release; $Release_2$: starting time of the second exenatide QW release; $V_{central}$: central volume of distribution; $V_{peripheral}$: peripheral volume of distribution

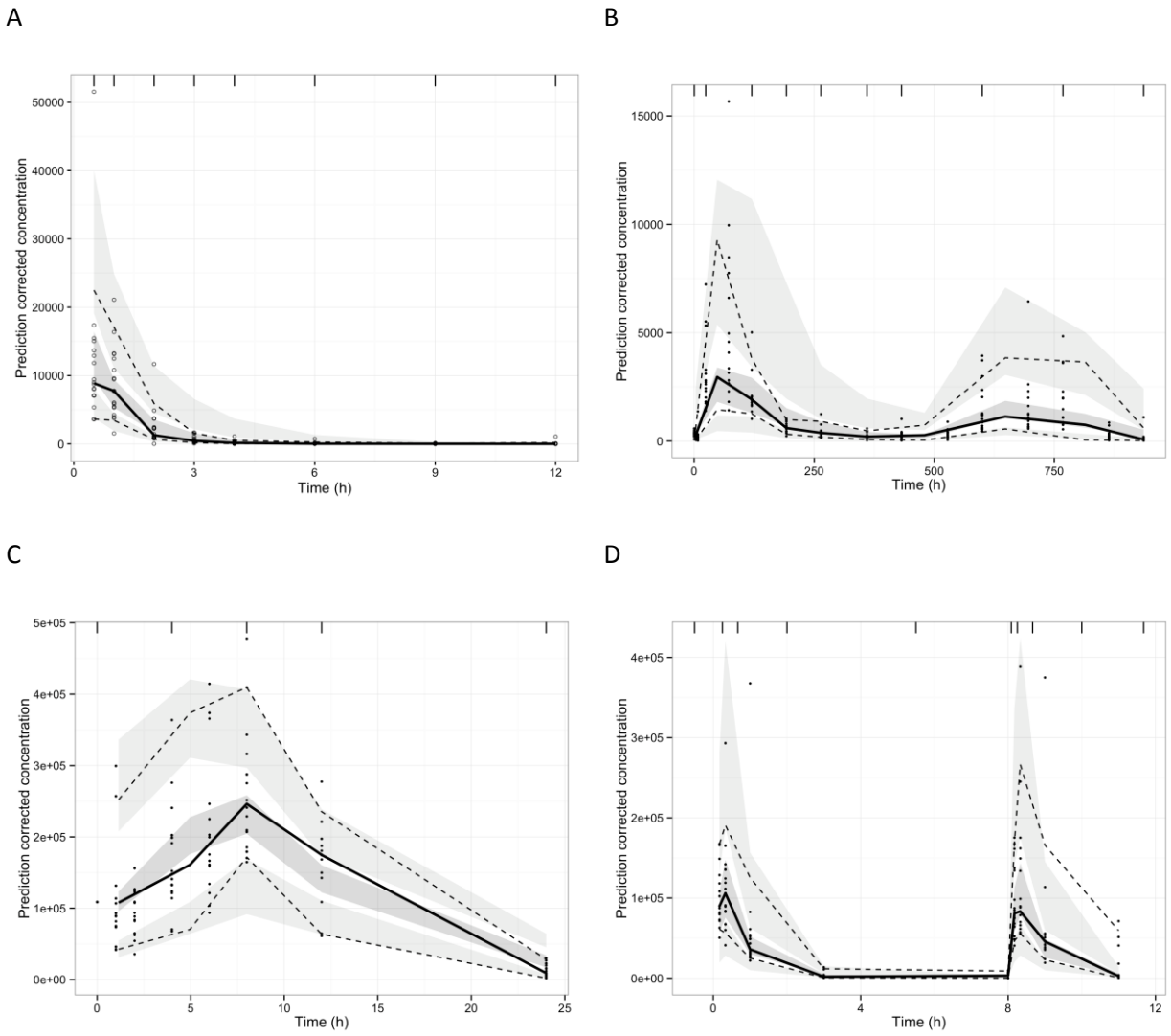
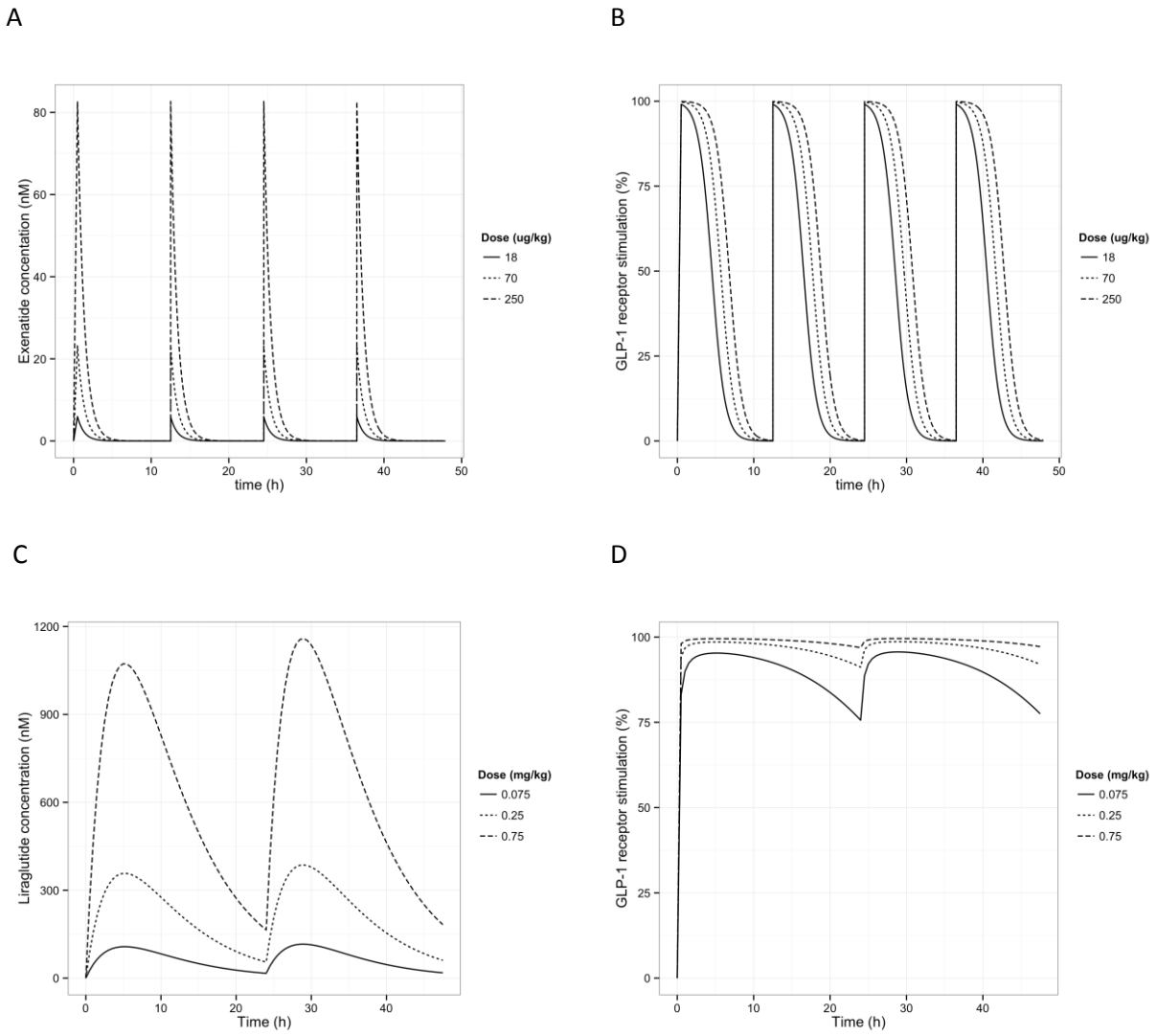


Figure 1. Prediction corrected visual predictive checks for the pharmacokinetic models of exenatide BID (A), exenatide QW (B), liraglutide (C) and lixisenatide (D). Dark grey shades represent the 90% prediction intervals of the median and light grey shades the 90% prediction intervals of the 95% confidence limits. Black solid lines represent the observed medians and black dashed lines the observed 95% confidence limits. Black dots represent the observed data.

478

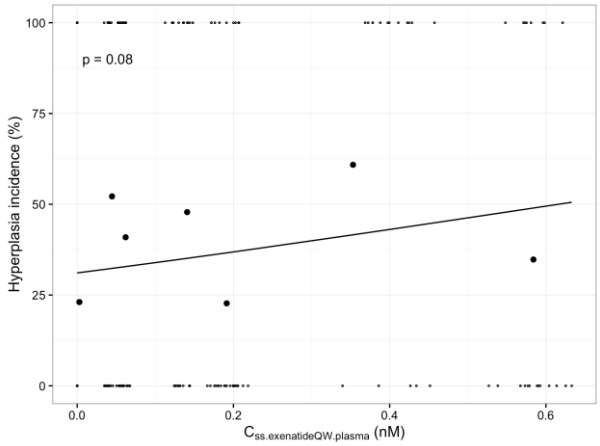


479 **Figure 2. Simulated pharmacokinetics (A) and pharmacodynamics (B) after exenatide BID (A and B) and liraglutide (C and D)**

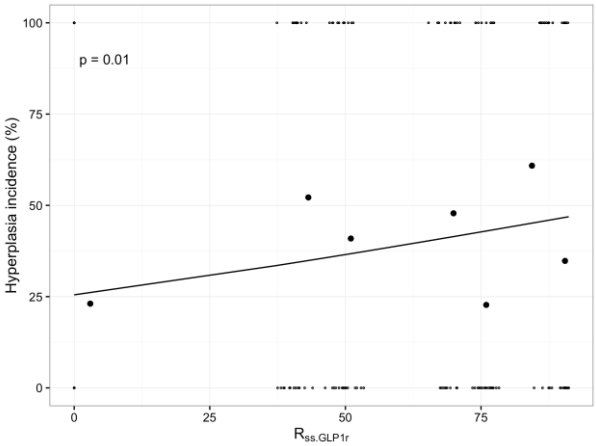
480 **from 0 – 48 hours.**

481

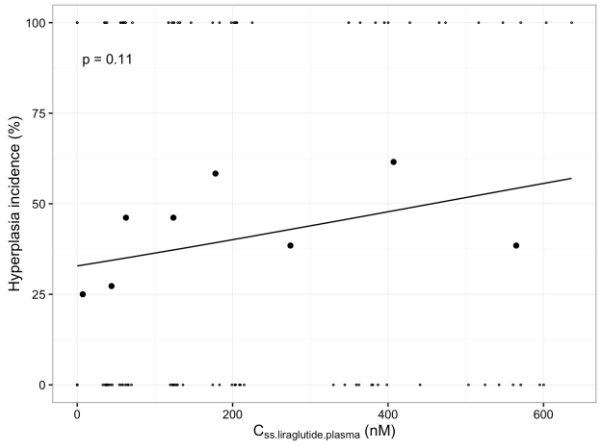
A


p = 0.08
Hyperplasia incidence (%)
100
75
50
25
0
0.0
0.2
0.4
0.6
Css.exenatideQW.plasma (nM)

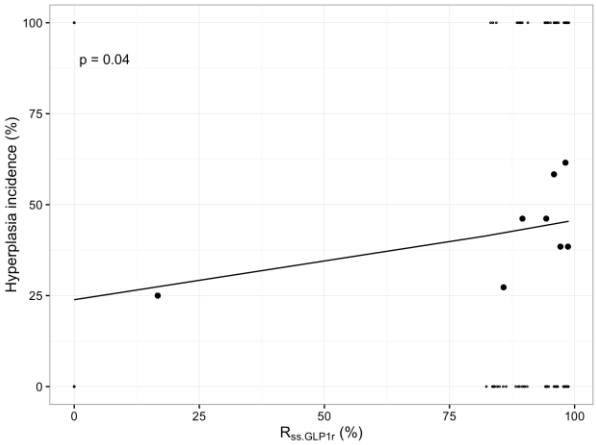
B


p = 0.01
Hyperplasia incidence (%)
100
75
50
25
0
0
25
50
75
Rss.GLP1r

C


p = 0.11
Hyperplasia incidence (%)
100
75
50
25
0
0
200
400
600
Css.liraglutide.plasma (nM)

D


p = 0.04
Hyperplasia incidence (%)
100
75
50
25
0
0
25
50
75
100
Rss.GLP1r (%)

E

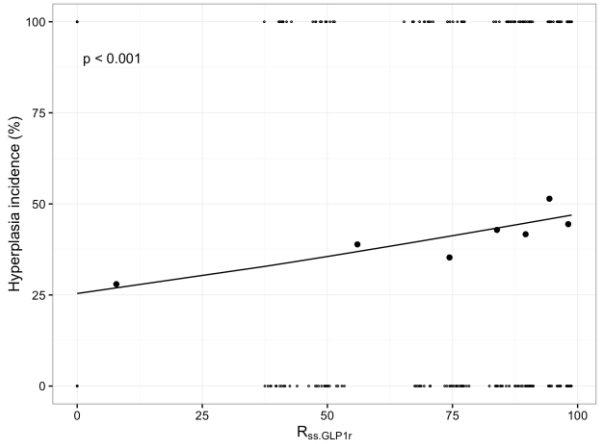
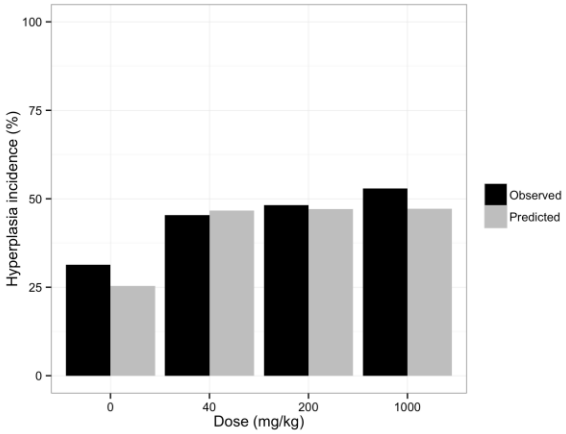

p < 0.001
Hyperplasia incidence (%)
100
75
50
25
0
0
25
50
75
100
Rss.GLP1r

Figure 3. Logistic regression between steady state plasma drug concentration or GLP-1r stimulation and C-cell hyperplasia risk for exenatide QW (A and B), liraglutide (C and D) and both drugs combined (E, only GLP-1r stimulation). The solid line represents the predicted C-cell hyperplasia incidence (%), the small dots the observed hyperplasia incidence (0 = no hyperplasia, 100 = hyperplasia), and the large dots the observed hyperplasia incidence (%). The latter was calculated over 7 bins with equal data density.

488



489

490 **Figure 4. Predicted versus observed hyperplasia incidence (%) after 0, 40, 100 and 1000 µg/kg lixisenatide.**

491

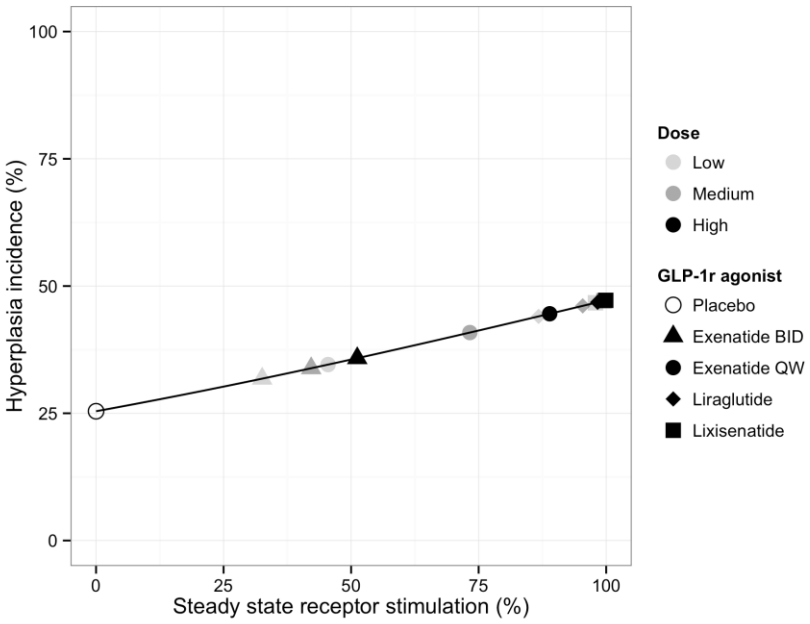


Figure 5. Overview of predicted hyperplasia incidence (%) for placebo and low, medium and high dose of exenatide BID, exenatide QW, liraglutide and lixisenatide as function of steady state GLP-1r stimulation. Simulations were performed according to the dosing schemes in table I (studies 5 - 8).